IMMUNE REGULATION BY MAST CELLS

Jolien Suurmond

Immune regulation by mast cells

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INTRODUCTION

Chapter 1

INTRODUCTION

The immune system protects the body against pathogens by recognizing dangerous invasive organisms and subsequently eliminating them. Different parts of the immune system cooperate to achieve this, including the innate immune system, which is able to recognize pathogens based on repetitive signals associated with danger, and the adaptive immune system, which is able to improve recognition over time and to memorize this recognition (*1*). Mast cells and basophils are immune cells mainly considered as part of the innate immune system, although recent evidence has suggested they might be at the basis of certain adaptive immune responses as well.

MAST CELL DISCOVERY

Mast cells were discovered more than a century ago, by Paul Erlich, who based his discoveries on immunohistochemical studies of "Mastzellen", cells in connective tissue containing granules which stained with basic aniline dyes, and supposedly had a feeding or nutritional function. He used similar techniques to describe granulocytes in blood, and discovered basophils based on their staining with basophilic dyes. In the 1950's, these granules were found to contain histamine, and thus, mast cells and basophils were hypothesized to participate in allergic reactions by releasing their granular contents (*2*).

Mast cell granules contain several mediators besides histamine, including the enzymes tryptase and chymase, discovered in the 1980's. In both human and mouse, these enzymes have a heterogeneous expression pattern, allowing for the identification of two main mast cell subsets in different tissues. In humans, mucosal mast cells express only tryptase (MC_T), whereas skin and submucosal tissue mast cell granules contain both tryptase and chymase (MC_{TC}).

As mast cell granules contain several mediators that could cause wheal-and-flare reactions when administered locally or anaphylactic shock when administered systemically, mast cells were long considered in the context of these allergic reactions. However, the physiological role of mast cells was not clear, and it was questioned why these cells with potent detrimental effects to the host existed without obvious benefits in terms of physiological function. It was not until the last two decades, that our perception of mast cell and basophil function has been broadened to other immune responses as well, and now, these cell types are thought to play a role in various immune responses, ranging from acute to chronic and from innate to adaptive immune responses.

MAST CELL MEDIATORS

Besides releasing their granule content, mast cells can release several other mediators, including membrane-derived lipid mediators, and cytokines and chemokines which are produced *de novo* (Figure 1).

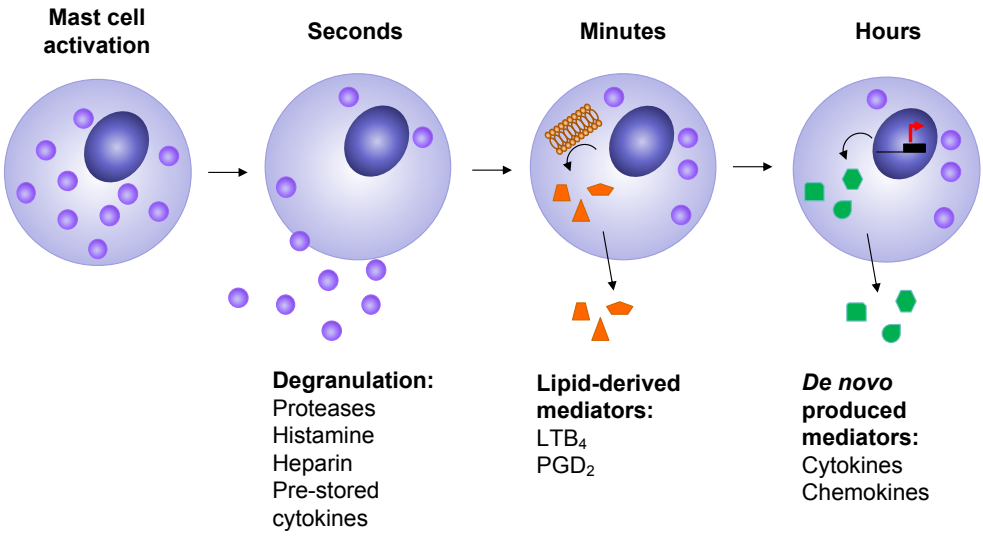

Figure 1: Mediator release by mast cells. Upon activation, mast cells have the ability to degranulate within seconds, thereby releasing pre-formed molecules residing in their granules. Lipid mediators, derived from fatty acids in membranes through arachidonic acid, are synthesized through enzymatic reactions inside the cell and can be released within minutes after activation. De novo transcription and translation leads to secretion of cytokines and chemokines within hours after activation.

GRANULE MEDIATORS

Mast cell granules are structures within the cell which contain inflammatory mediators, which can be released rapidly (within seconds) upon activation of the cell. The granule structure serves mainly two functions: First of all, the granules contents contain very high concentrations of mediators allowing their rapid release into extracellular space. Second, these mediators are often destructive to the intracellular environment (such as proteolytic enzymes), and therefore need to be contained in a separate structure. They are initially produced as inactive precursor proteins (pre-pro-enzymes), after which they are processed through the ER-golgi pathway. Once they enter the granule, they are cleaved into the active protein. This is important, as it allows for immediate proteolytic activity in the affected tissue, and makes the mast cell response so potent.

The granule-resident mediators can be divided into different functional groups. As mentioned, histamine is an important mast cell mediator associated with allergic symptoms. Four different histamine receptors exist, which each lead to different downstream effects, including increased vascular permeability and chemotaxis, each enhancing influx of immune cells into the affected tissue.

Next, proteases such as tryptase, chymase (both serine proteases) and carboxypeptidase (zinc-containing metalloprotease) are the most abundantly expressed proteins in mast cells, constituting 25-50% of the total proteins (*3, 4*). Release of these proteases can lead to pro- and anti-inflammatory effects, depending on the extracellular substrates that are being cleaved. For, example, these proteases can cleave pro-inflammatory cytokines into active forms, or in contrast, degrade pro-inflammatory cytokines or other molecules (*5*). Furthermore, they can degrade toxic substance, such as derived from snake and bee venoms or endogenous toxins released upon injury (see below).

Proteoglycans are another major constituent of granules. These are mainly serglycin proteoglycans that contain heparin and/or chondroitin sulfate chains. They form complexes with mast cell proteases in the granules, which is essential for correct storage and release of these proteases (*6*). The heparin which is released upon degranulation also serves as anti-coagulant.

A fourth type of mediator which has been suggested to be present in granules are cytokines, such as TNF-α (*7*). However, it is not clearly established whether human mast cell granules also contain cytokines, in particular TNF-α.

LIPID-DERIVED MEDIATORS

The second wave of mediators released by mast cells consists of eicosanoids, or arachidonic acid metabolites, which are released within minutes after activation. Arachidonic acid is released from membrane phospholipids by phospholipase A2, and subsequently converted to leukotrienes and prostaglandins by arachidonate 5-lipoxygenase (5-LO) and cyclooxygenases (COX) enzymes respectively. Leukotrienes induce bronchoconstriction, increased vascular permeability and neutrophil chemotaxis, and can induce anaphylactic shock when present in excess. Prostaglandins, and in particular PGD_2 released by mast cells, leads to recruitment of Th2 cells, eosinophils, and basophils, as well as vasodilation and bronchoconstriction.

Together, these early mast cell-derived molecules induce an early inflammatory reaction, allowing other immune cells to enter the tissue.

CYTOKINES AND CHEMOKINES

Upon activation, transcription factors induce *de novo* transcription and production of several molecules, including secretion of cytokines and chemokines. The production and release of these molecules takes several hours, and this response is therefore sometimes referred to as the late phase response, especially in the context of allergic inflammation. Although the release of pre-formed and lipid derived mediators leads to general inflammation, the release of cytokines and chemokines can lead to more versatile functions, as different mast cell triggers can induce the differential production of cytokines, probably through inducing different transcription factors.

Mast cells can produce cytokines affecting many different cell types. For example, several cytokines are most known for their effects on stromal or parenchymal cells. Likewise, the effect of mast cell-derived IL-13 on non-immune cells can lead to parasite expulsion through increased mucous production, epithelial cell turnover, and contraction by intestinal muscle. Mast cell-derived cytokines can also affect various immune cells, both from innate and adaptive immune system, as discussed below. These include IL-3, which can activate T cells, IL-5, which can recruit and activate eosinophils, and GM-CSF, which can affect neutrophil and monocyte expansion and function.

Chemokines produced by mast cells lead to recruitment of different cell types, with neutrophils and other granulocytes as main responder cell types being attracted to the site of injury by mast cell-derived chemokines, such as IL-5, IL-8, MIP1α (CCL3), TNF-α. However, these and other chemokines, such as CCL2/MCP-1, can also attract T cells, monocytes and natural killer cells. This suggests that the mast cell chemokine response can lead to potent influx of a variety of other immune cells.

MAST CELL MEDIATORS AS IMMUNOSUPPRESSORS

In contrast to the pro-inflammatory role that mast cells play during injury and infection, mast cell derived cytokines and granule-derived mediators can also induce regulatory responses. Examples of such cytokines are IL-10 and TGFβ. In particular mast cell-derived IL-10 has been shown to be involved in immune regulation (*8-10*).

In addition, mast cell proteases can cleave proinflammatory cytokines, and thereby limit inflammation. Many cytokines can be cleaved by chymase (at least in the mouse), sometimes leading to inactivation of the cytokines and alarmins, such as IL-3, IL-6, SCF, HSP70 and IL-33 (*5, 11-13*). Therefore, the response of mast cells can modulate the immune system towards anti-inflammatory responses as well.

MAST CELL ACTIVATION

The most well-known receptor for activation of mast cells and basophils is the high affinity receptor for IgE, FcεRI. Due to its high affinity, IgE from the circulation is constantly bound to the cell surface, and upon antigen recognition, this receptor is crosslinked causing the full-blown mast cell response characterized by degranulation and production of lipid derived mediators, cytokines and chemokines. As the IgE is already bound to the receptor, minute amounts of antigen are needed for activation, and activation can occur within seconds after antigen recognition. Therefore, this is a very potent mast cell response.

Several other pathways for mast cell activation have been described. These include Fcγ receptors, complement receptors and innate receptors, such as Toll like receptors. In addition, mast cells can be activated by various cytokines or growth factors, which often influence their proliferation as well.

APPROACHES TO STUDY MAST CELL FUNCTION

Human

Mast cells are not present in blood, and are therefore a difficult cell type to obtain for functional studies. Several mast cell-lines exist, such as LAD-2 and HMC-1, however, each of these have several molecular abnormalities compared to tissue mast cells, including low expression of FcεRI, dysfunction of ckit (receptor for stem cell factor; SCF), and lack of granules. These features constitute a limitation of studies employing these cell lines. Therefore, most studies of mast cell function in the human rely on in vitro expansion of stem cells or tissue mast cells, such as using hematopoietic stem cells from peripheral or cord blood. Although some discrepancies have been described, these have been shown to closely resemble tissue mast cells in most characteristics, such as granule constituents, expression of FcεRI and degranulation.

In vivo approaches in humans include immunohistochemistry or measurement of mast cell specific mediators in serum or other body fluids. Although useful, these studies can merely be used for descriptive studies. Due to the low frequency of mast cells in most tissues, characterization of tissue mast cells in humans is still difficult, but new technologies, such as next generation sequencing, are likely to give us more insight into the exact function of mast cells in different tissues.

Mouse

In the mouse, several in vivo models for mast cell function are being used. The most frequently used is the so-called mast cell knockin model, using the $kit^{W/Wv}$ or $kit^{Wsh/sh}$ mice which display profound mast cell-deficiency. However, the mutation in kit in these mice

affects some other cell types as well. This includes neutropenia observed in $kit^{W/Wv}$ mice and neutrophilia in $kit^{Wsh/sh}$ mice (*14, 15*). Therefore, mast cell reconstitution using mast cells derived from bone marrow of wildtype mice is usually required to confirm that the phenotype is mast cell-specific.

In the last years, several novel mouse strains have been developed which target mast cell molecules, including carboxypeptidase A3 (cpa3), and mast cell proteases (such as Mcpt5) (*16-19*). Several findings using kit mutant mice with knockin mast cells, have been recently challenged using the more specific mast cell deficient mice (*15, 17, 20*). Therefore, these new mast cell deficient mouse models might change the paradigms on the contribution of mast cells to various immune responses.

Differences between mouse and human mast cells

It is important to note that mouse mast cells and human mast cells differ considerably. Their constitution of granules is different, as in humans there is only one chymase gene, whereas in mice, several different chymases can be expressed at the same time (*21*). Their origin and development also differs considerable: in humans the proliferation and differentiation is thought to mainly depend on SCF, whereas in mice, IL-3 alone can induce differentiation and proliferation of mast cells from stem cells (*22*). Also, the expression of activating receptors such as TLR and FcγR might differ between these species.

Importantly, several cytokines which have been shown to be crucial for certain mast cell functions in the mouse (TNF-α and IL-4) were not found in human mast cells (*22, 23*). So, although mouse studies are very important to obtain understanding of mast cell function in vivo, caution needs to be applied for extrapolation of these results to human conditions. As it is difficult and expensive to obtain functional human mast cells, there is a lack of translation of the findings in mice to human disease, and this is therefore an important area of research.

PHYSIOLOGICAL FUNCTIONS OF MAST CELLS

The presence and homology of mast cells between different species suggests that they are essential to our survival. In support of this notion, mast cell-deficient persons have not been identified. Mast cells are located in strategic locations where they can encounter pathogens upon entry of the body. The following paragraphs describe the protective immune responses in which mast cells play an important role.

THE ROLE OF MAST CELLS IN INNATE IMMUNE RESPONSES

Venoms

A physiological role for mast cell degranulation has recently emerged, hypothesizing that such an immune response has evolved as a protective mechanism against venoms which need to be eradicated in a quick manner. Besides direct toxicity of venom contents, venoms can induce toxic endogenous (neuro-) peptides, such as endothelin-1, neurotensin and vasoactive intestinal polypeptide (VIP) (*24-26*). Granule-derived mast cell enzymes have been shown to detoxify or degrade several venom-derived toxins as well as endogenous peptides produced in response to venoms (*26-28*), giving protection to the host (Figure 2).

Venom-induced mast cell degranulation is usually triggered by innate recognition, but their receptors are not known. Nonetheless, as venoms resemble neuropeptides, they might act on mast cells through similar G protein coupled receptors. Besides activation of mast cells via innate receptors, a recent study has demonstrated that IgE memory can also contribute to venom-induced mast cell activation (*29*). In this mouse model, IgE was produced after sensitization with bee venom, which later mediated resistance to a lethal dose of the same venom in a mast cell-dependent manner.

These mast cell responses to venoms are a good example on how immune responses need to be tightly balanced. As described above, both innate and IgE-mediated responses can contribute to protection against these dangerous venoms through release of mast cell proteases. However, uncontrolled mast cell degranulation in allergic individuals can lead to anaphylaxis such as during severe reactions to bee and wasp stings in sensitized individuals.

Parasites

Another type of innate immune response in which mast cells have been shown to play a role is the protection against various parasites, in particular intestinal helminths. These are multicellular pathogens, and can therefore not be controlled or eliminated by traditional immune responses such as phagocytosis or cytotoxicity. Helminth parasites are well-known for their induction of Th2 immune responses (*30*), but they also have several immune evasion strategies, including upregulation of Tregs and anti-inflammatory cytokines such as IL-10 (*31, 32*).

Parasites trigger TLRs and other pattern recognition receptors, generally leading to Th2 responses (*33-37*). Due to their Th2-skewing properties, parasitic infection often promote production of both total and parasite-specific IgE.(*38-40*) In addition, the helminth parasite Schistosoma mansoni has been shown to directly crosslink non-specific IgE/FcεRI in an antigen-independent manner (*41*). The contribution of IgE antibodies or FcεRI to protective

immunity is not completely clear, and seems to depend on the type of parasites and the site of infection (*42-44*).

Mast cells are also thought to contribute to immunity during primary helminth infections in mice (*45, 46*). The mast cell effector molecules during protective responses against parasites are IL-4, IL-13, TNF-α, histamine and mast cell proteases, such as mouse MCP-1, -2 and -6 (Figure 2) (*47-50*). These cytokines play different roles; IL-13 specifically has been suggested to induce goblet cell hyperplasia, causing increased mucus production, leading to trapping of parasites in the mucus as well as enhancing expulsion of the parasites (*51*). Mast cell proteases can enhance intestinal permeability, thereby directly contributing to parasite expulsion (*46, 52*). The other cytokines and proteases are mainly thought to contribute to intestinal inflammation, for example through recruitment of eosinophils (*49*). Although most of these cytokines are also produced by Th2 cells, IL-4 and IL-13 derived from innate cells was shown to be most important for worm clearance during infection with N Brasiliensis (*51*).

Recent data suggest a non-redundant role for basophils in the context of secondary parasite infection. Basophils were shown to be important in the secondary immune response against diverse intestinal helminthes (N. brasiliensis, H. polygyrus, T. muris) and ticks, probably by activation through parasite-specific IgE (*44, 53, 54*).

Together, these observations suggests that whereas mast cells are potent effector cells to prevent parasites from entering the body in a primary infection, basophils may be more potent during IgE-dependent secondary responses against parasites.

Bacteria

The first study to show an important role for mast cells in protection against bacteria originated in 1996, when mast cell-derived TNF-α was shown to confer protection against *E. Coli* (*55*). Several studies have since then shown a protective role of mast cells against various bacteria, including *K. pneumoniae*, *M. pneumoniae*, and *C difficile* (*56-59*). In most of these models, the crucial mechanism of mast cell-mediated protection is recruitment of immune effector cells, mainly neutrophils (Figure 2). Due to their ability to rapidly degranulate, mast cells are thought to be the first cell to initiate an inflammatory response upon bacterial invasion of a tissue. Such an acute response is characterized by increased vascular permeability mediated by histamine and proteases, and recruitment of effector cells by release of lipid-derived mediators (LTB4) and cytokines or chemokines present in granules (TNF) (*7, 60*). This early response is important, as mast cell-deficient mice have a wider spread of bacterial infection, suggesting that mast cells can contain infections to a local tissue, such as lung or skin (*55, 56, 61*).

In addition to their role in acute infection, mast cell may initiate adaptive immune responses to bacteria as well. Mast cell derived TNF was shown to regulate recruitment of T cells to draining lymph nodes during intradermal infection with *E. Coli* (*62*). Furthermore, DCs were recruited to the infected tissue by mast cells, followed by their migration to draining lymph nodes (*63, 64*). Therefore, mast cells may orchestrate T cell activation during bacterial infection by regulating the migration and activation of both T cells and antigen presenting cells.

Different receptors have been shown to contribute to mast cell activation during bacterial infections, including complement receptors (CR1, CR2) and TLR4 (*65-67*). In addition, bacterial toxins may directly activate mast cells through yet unknown receptors (*68*). The exact receptors inducing mast cell degranulation upon bacterial infection are not known, but can consist of a variety of bacterial products and endogenous ligands released upon invasion of the body.

Mast cells are not crucial for protection against all bacteria strains. However, a recent study suggested that this may originate from evasion strategies employed by bacteria to prevent mast cell activation. At least two bacterial strains, Salmonella Typhimurium and Yersinia Pestis, were capable of such mast cell inhibition (*69*).

These studies show that mast cell play a non-redundant role in protection against a variety of bacteria, although some bacteria have developed immune evasion strategies.

Other pathogens

Although fewer studies have been performed evaluating the role of mast cells to viruses and fungi, some studies suggest they can also contribute to protective immunity against these pathogens. For example, mast cells were shown to induce migration and activation of $CD8^+$ T cells and NK cells in the context of viral infections (*70-75*).

Not much is known about the role of mast cells in fungal infections, but the overlapping mechanisms in protection against bacteria, parasites and fungi (such as TLR recognition), suggest that mast cells potentially play a role against fungi as well (*76*).

THE ROLE OF MAST CELLS IN TISSUE HOMEOSTASIS

Mast cells are usually associated with acute responses due to their potent degranulation mechanism; however, they may be an important effector cell in the context of tissue homeostasis. Several findings implicate a role for mast cells in tissue remodeling. Mast cell numbers are often associated with tissue remodeling processes, such as during scarring and fibrosis and pathologic conditions such as bullous pemphigus and scleroderma (*77-79*).

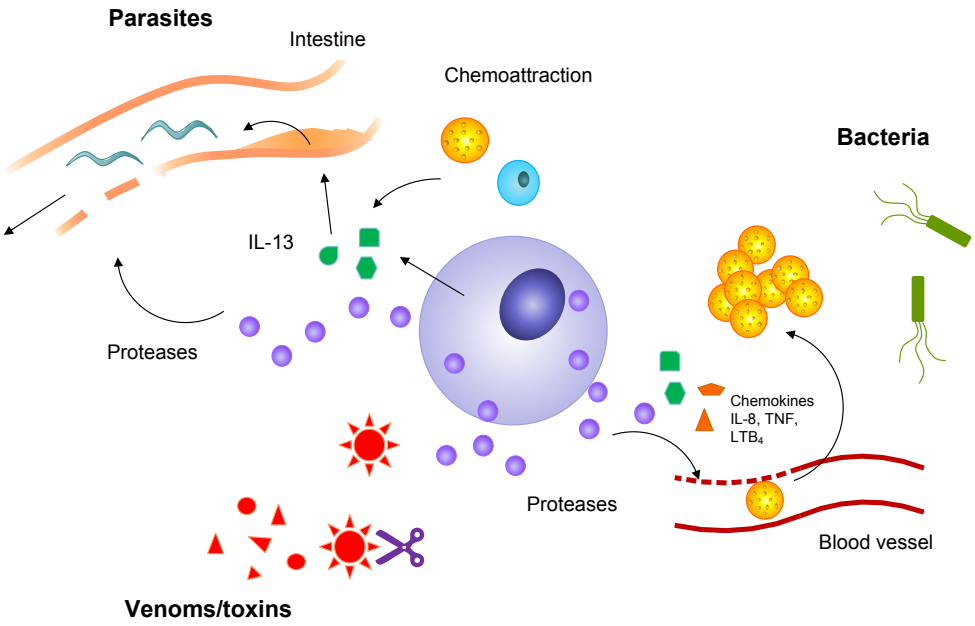

Figure 2: The role of mast cells in protective immunity. Mast cells have been shown to contribute to protection against a variety of pathogens. During parasitic infection, mast cell-derived proteases can lead to intestinal permeability. Cytokines contribute to recruitment of eosinophils and other granulocytes to the site of parasitic infection, and IL-13 specifically can induce the production of mucous; together these processes contribute to expulsion of intestinal parasites. Upon exposure to venoms or toxins, mast cell-derived proteases can degrade or detoxify these molecules, thereby protecting the host. Upon bacterial infection, tissue-resident mast cells are one of the first cell types to respond by releasing proteases, lipid mediators and chemokines. These mast cell-derived molecules increase vascular permeability and recruitment of immune effector cells, including neutrophils.

In addition, mast cells can produce a variety of mediators involved in tissue remodeling, including proteases which can activate several matrix metalloproteinases (MMPs), thereby contributing to breakdown of extracellular matrix proteins (*80, 81*). Mast cells can activate fibroblasts through various growth factors and cytokines and induce TGFβ-dependent collagen production by fibroblasts through a variety of mechanisms (*82-85*). These studies indicate that mast cells can contribute to extracellular matrix turnover by both production and breakdown of extracellular matrix constituents.

Mast cells were also thought to contribute to wound healing and fibrosis in the skin. Mast cells accumulate at the site of skin injury, and some studies using kit-mutant mice showed a functional role for mast cells in fibrosis (*78, 86*). However, some studies using kit-independent mast cell-deficient mice have recently challenged the role of mast cells

in wound healing and fibrosis in the skin (*87*), indicating that more research is needed to understand the role of mast cells in tissue remodeling (*87-90*). Furthermore, most studies have only addressed the role of mast cells during acute wound healing responses, whereas tissue remodeling during chronic inflammatory responses has only been sparsely studied. As mast cell-mediated tissue remodeling could be detrimental in the context of chronic inflammation, this is an important area of research.

THE ROLE OF MAST CELLS IN PATHOGENIC PROCESSES

Although the physiological role of mast cells and basophils is being more acknowledged in recent years, and as it is now generally accepted that these cells play important roles in the first line of defense against a number of pathogens, the route that mediates the recognition of pathogens, is not firmly established. For example, there is a lack of knowledge concerning expression of Toll-like receptors and the specific response that is generated by ligands for these receptors, especially in human mast cells and basophils. Activation through such receptors is important, not only for their role in protective immunity, but also during pathogenic processes, as described below.

MAST CELLS IN ALLERGIC REACTIONS

Mast cells are classically associated with allergic responses, in particular type I hypersensitivity responses, related to IgE. Allergic reactions consist of two phases, the early and late reaction, of which the exact symptoms depend on the location where the body comes in contact with the allergen. The early reaction in the lungs is characterized by bronchoconstriction, edema and mucus production, whereas in the skin it leads to redness, edema and itching. The late phase reaction in all tissues is associated with influx of eosinophils, other granulocytes and lymphocytes. Basophils and mast cells are the main cell types expressing FcεRI, but because mast cells are already present in tissues where allergens are first encountered, and basophils need to be recruited from the blood, mast cells are usually considered as the main cell type in the early phase reaction. Their mediators histamine, proteases, leukotrienes and prostaglandins can directly induce the early allergic symptoms (Figure 3) (*91*). Through their release of cytokines, such as IL-5 and IL-13, mast cells can also contribute to the late phase reaction, in particular by recruiting and activating eosinophils (through IL-5), and inducing mucus production (through IL-13) (*92-95*).

Besides the early and late responses to allergen exposure in sensitized individuals, allergy often leads to chronic inflammation, associated with tissue remodeling. The most well-known example is chronic atopic asthma, which is thought to be driven at least partially

by repeated FcεRI triggering. During chronic asthma, several tissue remodeling processes occur, including fibrosis, matrix deposition, vascular remodeling and mucus secretion. Mast cells are thought to contribute to these processes through their release of proteases (tryptase, chymase), angiogenic factors (VEGF, IL-8), and cytokines (IL-13). However, the role of mast cells in such chronic IgE-mediated responses is not clearly understood (*96*).

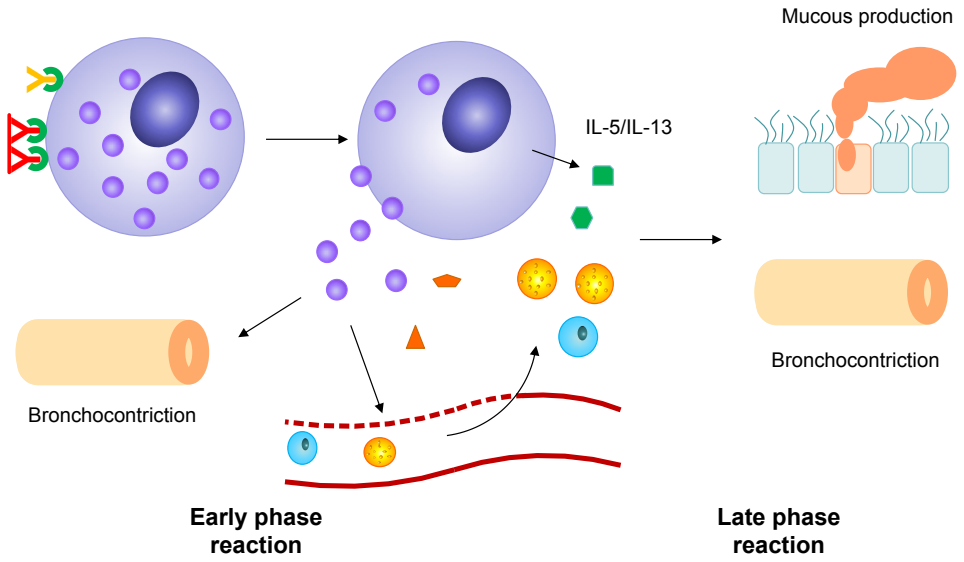

Figure 3: Role of mast cells in allergy. Once specific IgE recognizing allergens (red Y) has been formed, and mast cells have been sensitized, re-exposure to the allergen leads to activation of mast cells, characterized by degranulation, release of lipid mediators and production of cytokines, including type 2 cytokines, IL-5 and IL-13. Degranulation of mast cells during acute allergic reactions in the lung can lead to bronchoconstriction and increased vascular permeability. Production of leukotrienes and chemokines then leads to recruitment of granulocytes and T cells, which further increase allergic symptoms. During the late phase reaction, IL-13 and other mast cell products can increase mucous production and bronchoconstriction in the lung, thereby contributing to further narrowing of the bronchial tubes.

MAST CELLS IN AUTOIMMUNE DISEASE

Mast cells have been correlated to several autoimmune diseases, including T cell-dependent type IV hypersensitivity, and antibody-dependent type II and type III hypersensitivities (*97*). However, in most cases, this association is mainly correlative, depending on the findings of increased mast cell numbers or evidence of mast cell activation in the affected tissues in human disease. For example, increased mast cell numbers and levels of their mediators have been found in the synovium of rheumatoid arthritis patients, blisters of patients with bullous pemphigoid, spinal fluid of multiple

sclerosis patients, salivary glands of Sjögren's syndrome, as well as the skin of scleroderma patients (*98-102*).

Although this provides some suggestion for mast cell involvement, mast cell activation in these tissues is sometimes regarded as merely a consequence of tissue inflammation, rather than playing an important role in the pathogenesis of autoimmune disease. The functional contribution of mast cells to autoimmune disease is difficult to verify in humans, as currently, no specific mast cell inhibitors are available for clinical studies. Therefore, such studies rely on the use of animal models, which in most autoimmune diseases do not fully reflect human disease, but rather, just one component of the disease (*103*). For example, immunization models for arthritis are used to study the initiation of autoreactive T- and B-cell responses, whereas serum transfer models are used to study the effect of autoantibodies and chronic inflammation in arthritis. Next to variable models to study disease, there are now several models for mast cell deficiency, together resulting in seemingly contradictory results on the role pathogenic role of mast cells in autoimmune disease.

The role of mast cells in T cell-dependent autoimmunity has only been studied sparsely. A recent study on collagen induced arthritis showed that mast cell deficient mice display reduced arthritis severity and reduced numbers of collagen-specific Th1 and Th17 cells. Furthermore, mast cell numbers in draining lymph nodes were markedly increased upon immunization in wildtype mice, suggesting a direct role for mast cells in activation of autoreactive T cells (*104*). In contrast, mast cell deficiency in the diabetic NOD mouse, which depends on $CD4^+$ and $CD8^+$ T cells had no effect on disease pathogenesis (*105*). However, it is unclear to which extent the NOD mouse model reflects the autoreactive T cell response that occurs in type 1 diabetes patients (*106*).

In experimental autoimmune encephalitis, contrasting results have been obtained; whereas mast cells have been shown to contribute to autoreactive $CD8^+$ T cell responses, disease severity and recruitment of T cells to the CNS, another study reported no effect of mast cell deficiency on disease severity in the EAE model (*17, 107-109*). As described above, mast cells can contribute to T cell responses in various ways, including T cell activation and recruitment to lymph nodes, so it is conceivable that mast cells indeed play a role in these responses. More research into the function of human mast cells in the priming and activation of T cells is needed to get a better understanding of their capacity to contribute to T cell-dependent autoimmunity.

Autoantibodies

A major effector function thought to contribute to pathogenesis of autoimmune diseases is mediated by autoantibodies. An important group of autoantibodies in

rheumatoid arthritis targets citrullinated proteins (anti-citrullinated protein antibodies; ACPA). These antibodies recognize a variety of proteins or peptides in which the amino acid arginine is modified into citrulline through a posttranslational modification process mediated by Peptidyl Arginine Deiminase (PAD) enzymes. PAD enzymes are normally present inside cells and can be activated by high calcium levels when cells, such as neutrophils, undergo apoptosis, an event readily occurring during inflammation (*110*). PAD enzymes that are transported to the outside of cells can citrullinate extracellular proteins, although intracellular citrullinated proteins can also be released into extracellular space (*111*).

ACPA show a very high specificity for rheumatoid arthritis, and are present in the majority (~70%) of rheumatoid arthritis patients (*112, 113*). When ACPA antibodies are adoptively transferred into mice with a low-level synovial inflammation caused by anti-collagen antibodies, ACPA (reactive with citrullinated fibrinogen or collagen II) could enhance arthritis, implicating their direct involvement in the inflammatory process (*114, 115*).

There is not much evidence for a role of mast cells in the initiation of autoantibody production. In most immunization models, mast cell-deficient mice have similar levels of autoantibodies (*104*). However, mast cells express a variety of Fc receptors and complement receptors, making them an important effector cell type in the propagation of autoantibody-mediated inflammation. In mice, C5aR and Fcγ receptors on mast cells have been shown to play an important role in antibody-mediated arthritis and bullous pemphigoid (*116-119*). In these models, mast cells were shown to play an important role in recruitment of leukocytes, in particular of neutrophils. This is in line with their function observed in the Arthus reaction, where passive transfer of IgG antibodies and antigen lead to a mast cell-dependent neutrophil infiltration of the affected tissue (*120-122*). In contrast, some studies did not observe any effect of mast cell deficiency on autoantibody-mediated tissue inflammation (*17, 104, 123*).

Importantly, the expression of Fcγ receptors differs considerably between mouse and human mast cells (*124-127*), and, as a consequence, not much is known about the capacity of human mast cells to respond to immune complexes, especially in the context of autoimmune disease. Studies on the role of human mast cells in antibody-mediated inflammation may therefore provide more insight into the potential role of mast cells in autoimmune disease. As activation of immune cells through Fcγ receptors is an important effector mechanism of autoantibodies in autoimmune disease, it will be important to understand the capacity of mast cells to respond to Fcγ triggering. In the mouse, activation of mast cells through Fcγ receptors is known to lead to neutrophil

recruitment, however, not much is known about the role of Fcγ receptor activation of mast cells in autoimmune disease in humans.

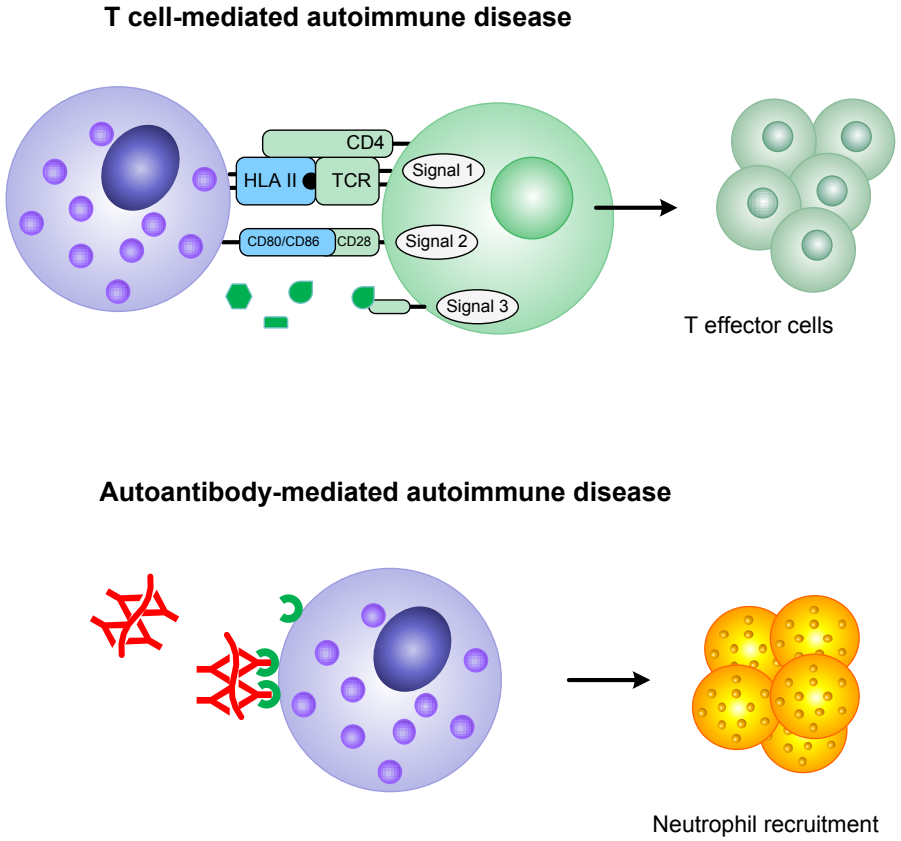

Figure 4: Potential role of mast cells in autoimmune disease. Mast cells have been implicated in T cell responses, therefore they could contribute to T cell-mediated autoimmune disease. Although not much is known about the interaction between mast cells and $CD4^+$ T cells in the human, there are suggestions that mast cells can function as antigen presenting cells, providing the 3 signals crucial for T cell activation: 1) Antigen presentation through HLA class II; 2) Co-stimulation, such as through CD28 or other molecules; 3) Skewing of T helper responses through production of cytokines.

CHRONIC INFLAMMATION

Repeated or continuous activation of the immune system can occur in a variety of conditions, including allergy and autoimmunity. In the case of allergy, after sensitization has occurred, repeated exposure to allergens, such as during yearly pollen season, can quickly induce reactivation of memory T cells and activation of innate immune cells through allergen-specific IgE. In the case of autoimmunity, release of self-antigens can lead to a

sustained activation of autoreactive T and B cells, as well as innate immune cells through antibody effector mechanisms. When the self-antigen or allergen cannot be eliminated, such responses can become chronic. Furthermore, the immune system contains amplification mechanisms to enhance inflammatory responses, and epitope spreading can contribute to further loss of tolerance in autoimmunity and allergy (*128-132*).

Not much is known about what exactly drives the incessant cycle of inflammation during these diseases, but it is likely that a proinflammatory cytokine environment, TLR ligands, antibodies and cellular effector mechanisms all cooperate to maintain chronicity. The danger model, as proposed by Matzinger, hypothesizes that self-antigen in itself is not enough to trigger autoimmunity, but that it needs to be accompanied by danger signals, such as occurring through tissue damage (*133*). In this context, it is interesting that most autoantibodies involved in chronic autoimmune disease have specificities for damage associated molecular patterns (DAMPs) (which I discuss in Chapter 12). This indicates that in autoimmune disease recognition of non-self through adaptive signals (e.g. antibodies) and innate danger signals often coincide, as they can be present even in the same molecule (*134*). In allergy, the danger signals can originate from infection (e.g. during asthma exacerbations) or tissue damage (during tissue remodeling in chronic allergy), although some allergens may also directly activate TLRs similar to self-antigens in autoimmune disease (*135*). Together, recognition of non-self in combination with danger signals can lead to a sustained response when tissue damage leads to additional release of DAMPs recognized by autoantibodies and TLRs (*136*).

The contribution of mast cells in the induction of tissue damage has not been widely studied. Although mast cells are not immediately viewed as a main cell type exhibiting cellular cytotoxicity, one study showed that mast cells can kill opsonized parasites, through release of tryptase (*137*). In addition, some studies have shown that mast cells exhibit cellular cytotoxicity, presumably through releasing their granule constituents such as TNF-α, granzyme B and possibly active caspase-3 (*138-142*). In addition, mast cell granule enzymes granzyme B, tryptase and chymase have been shown to degrade extracellular matrix proteins directly as well as indirectly through activating MMPs (*81, 143, 144*). Together, these processes may contribute to tissue and cellular damage, thereby inducing additional release of DAMPs and self-antigens which can be recognized by autoantibodies.

Furthermore, mast cells may directly release DAMPs upon cell death, for example through the formation of extracellular traps, or through necrosis (*145-148*). Besides direct effects of mast cells on tissue damage, their role in recruitment of neutrophils, activation of T cells and other immune cells can also contribute to inflammatory

responses, local cell death and release of DAMPs and self-antigens, thereby potentially contributing to the continuous amplification of local inflammatory responses.

However, not much is known about mast cell activation pathways in chronic inflammation, including their activation through TLRs, Fcγ receptors, as well as their intrinsic capacities to respond to these triggers in the context of continuous or repeated activation.

THIS THESIS

Although the studies mentioned above reveal a pivotal role for mast cells in a variety of immune responses, their role in autoimmunity has been studied sparsely. The objective of this PhD thesis is to understand mast cell (and basophil) functions and their role in autoimmune disease by focusing on three main aims:

1. To characterize the interaction between innate and Fc receptor triggers on mast cell and basophil function
2. To analyze the interaction between mast cells and $CD4^+$ T cells
3. To understand the function of mast cells in chronic inflammation

INNATE SIGNALS AND FC RECEPTOR TRIGGERING

First, it is important to characterize the specific pathways leading to mast cell activation, especially those pathways that operate in autoimmune diseases. The functional responses of human mast cells to triggering of Fcγ receptors, Toll-like receptors, or cytokines, have only sparsely been scrutinized. Furthermore, it is unknown how different activation pathways cooperate in the context of antibody-mediated responses. Such interactions are relevant for a variety of immune responses, including protective responses against pathogens, allergic reactions, as well as autoimmunity. Therefore, the first part of this thesis focuses on the interaction of these pathways. In Chapter 2 and 3, the activation of human basophils by TLRs and IL-33 was studied, in particular in combination with FcεRI-mediated activation. Furthermore, in these chapters, the effects of basophil activation on T helper cell skewing as well as monocyte activation are described. As basophils have recently emerged as important immunomodulatory cells, these studies will provide insight into their function, in particular in the context of IgE-mediated responses.

In Chapter 4-6, we describe the studies into the effect of the combined activation by innate signals (TLR, IL-33) and Fc receptor triggering in mast cells. In Chapter 4, the

cytokine profile of mast cells upon different TLR ligands in combination with FcεRI triggering is described, providing insight into the specific TLR ligands that could contribute to mast cell responses in allergy. In Chapter 5, I evaluated the activation of mast cells by anti-citrullinated protein antibodies, DAMPs and other TLR ligands. This study therefore aimed to gain understanding of activation pathways that contribute to synovial inflammatory responses in RA patients. In Chapter 6, we aimed to evaluate the role of IL-33 on mast cell activation, and the subsequent mast cell-monocyte interaction.

MAST CELL-T CELL INTERACTIONS

As mast cells are present at strategic locations of the environment/host interface where they can encounter pathogens, they have been implicated in the regulation of adaptive immune responses and the regulation of T cell immunity.

Besides their role in recruiting T cells to lymph nodes during bacterial infection, mast cells themselves were shown to be capable of migrating from the skin to the draining lymph nodes in murine models of contact hypersensitivity and UV radiation (*149, 150*), suggesting they may directly be involved in antigen presentation. Thus far, little information is available on the antigen-presenting capacity of human mast cells. Therefore, in Chapter 7 and 8 we studied whether mast cells possess the required molecular make-up, such as HLA-DR and costimulatory molecules, to activate T-cells through antigen presentation and co-stimulation.

As mast cells produce a variety of cytokines which can act on T cells, they have also been implicated in skewing of specific T cell responses. The effect of human mast cells on skewing of T helper cell responses and the effect of different modes of mast cell activation has not been elucidated. In Chapter 9 we therefore evaluated the effect of mast cells on Th cell responses.

CHRONIC INFLAMMATION

Finally, although mast cells are very potent cells in the context of acute inflammatory responses, only little is known about their function in the context of chronic inflammation. First of all, they are very long-lived (estimated >10 months in rats with a lifespan of 30-36 months) and therefore, can be influenced by inflammatory stimuli that are present during that time (*50, 151*). Further, as they enter a tissue as immature cell, their maturation depends on growth factors and cytokines in the tissue, allowing mast cell plasticity under the influence of local inflammatory conditions (*152, 153*).

However, as mast cells are usually considered in acute responses, not much is known about mast cell function under the influence of prolonged inflammatory stimulation or chronic infections. Therefore, the studies described in Chapter 10 were aimed at understanding mast cell function upon chronic Fc receptor triggering, providing detailed insight into changes in the mast cell transcriptome related to chronic allergy. Chapters 11 provides an overview of the role of mast cells in rheumatic disease, and Chapter 12 and 13 describe how autoantibodies are initiated and how chronic inflammation is propagated in autoimmune disease, also providing a perspective on novel therapeutic targets for the treatment of these diseases.

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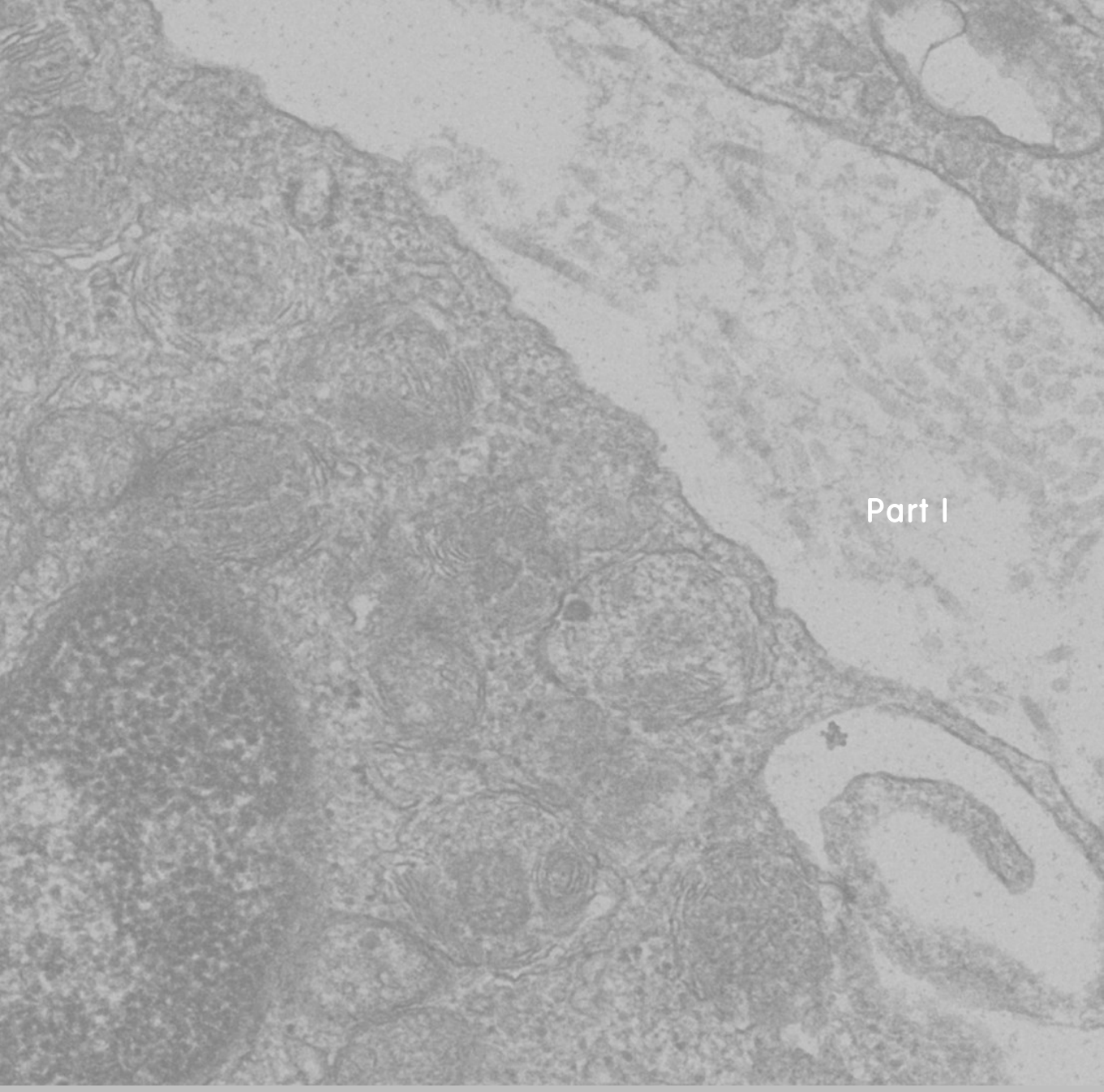

Part I

COMBINED INNATE AND FC RECEPTOR TRIGGERING OF MAST CELLS AND BASOPHILS

ACTIVATION OF HUMAN BASOPHILS BY COMBINED TOLL-LIKE RECEPTOR- AND FCεRI- TRIGGERING CAN PROMOTE TH2 SKEWING OF NAIVE THELPER CELLS

Chapter 2

Jolien Suurmond,[1] Jeroen N Stoop,[1] Aleida M Bakker,[1]
Tom WJ Huizinga,[1] René EM Toes,[1]
Annemie JM Schuerwegh[1,2]

[1]Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; [2]Department of Immunology/Allergology/Rheumatology, University of Antwerp, Antwerp, Belgium

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ABSTRACT

Basophils are mostly known for their involvement in allergic reactions. Recent studies in mice indicate a role for basophils in the induction of adaptive immunity, especially T helper 2 (Th2) responses. Therefore, it would be highly important to understand how basophils respond to pathogen-associated molecules, such as ligands for Toll-Like Receptors (TLRs), and if the basophils could promote Th2 responses via these stimuli. To this end, the activation of basophils via TLRs in combination with activation via IgE was studied, as well as its effect on T helper cell skewing.

Using quantitative PCR, we demonstrated the presence of mRNA for TLRs 1–8 in human basophils. Basophils responded to TLR triggering with differential cytokine production, but not with degranulation. Simultaneous triggering of TLRs and IgE led to synergy in production of IL-4, IL-8, IL-13, and RANTES. Furthermore, the synergistic effects on basophils mediated by IgE and TLR-4 triggering allowed robust Th2 skewing upon activation of naïve human $CD4^+$ T cells.

Our data show that human basophils respond to TLR ligands in synergy with IgE-mediated activation and that the cytokines produced can promote Th2 differentiation. These results indicate a role for basophils in the regulation of T -cell responses in humans.

INTRODUCTION

Basophils are circulating granulocytes that can migrate to tissues when inflammation or other triggers are present locally. Activation of basophils through crosslinking of FcεRI via antigen-specific IgE causes the release of granule contents (histamine, proteoglycans), lipid-derived mediators (leukotrienes), and cytokines. Due to their potent activation via IgE-bound antigens and their production of T helper 2 (Th2) type cytokines such as IL-4 and IL-13, basophils are often considered important innate effector cells in IgE-mediated immune responses. Besides activation through IgE, several other pathways have been described to activate basophils, including activation through complement receptors and Toll-Like Receptors (TLR). Although information about TLR protein expression and the response of basophils to TLR ligands is scarce, human basophils express mRNA of several TLRs, and protein of TLR-2 and -4. However, the functional response of basophils to ligation of most TLRs is unknown (*1-4*).

Basophil responses to TLR ligands may be important, as it is not understood how pathogens and allergens may lead to induction of Th2 responses. As TLR ligation of dendritic cells most often induces type 1 responses through production of IL-12, it is not understood whether

and how Th2 cells can be induced via TLR. IL-4 production is known to be an important factor for induction of Th2 responses (*5*), and therefore, the notion has arisen that Th2 induction would be driven by the combination of DCs that present antigen in the context of MHC class II and accessory cells that provide an early source of IL-4 in the LN (*6*). Additional cytokines able to enhance Th2 responses have been identified, including IL-25, IL-33, and thymic stromal lymphopoietin, which may either act directly on the T cells or change the maturation of APCs to drive Th2 responses (*7*).

Since basophils are a known early source of IL-4 and other cytokines associated with Th2 responses, they have been proposed to play a prominent role in the induction of such responses (*8*). In mice, it was suggested that basophils play a crucial role in responses against Th2-associated helminth parasites and in protease allergens (*9-11*). Basophils were proposed to function as APCs in these studies and were also shown to be present in LNs in models of allergy and helminth infection (*12-14*). However, the function of basophils as APCs during the priming phase of Th2 responses in mice has been challenged by others (*13, 15, 16*), and human basophils could not present antigens to $CD4^+$ T cells (*11, 17, 18*). Therefore, it seems unlikely that human basophils function as APCs. However, by providing an early IL-4 signal that facilitates Th2 skewing after T-cell activation by APCs, basophils could play an important role in skewing of Th2 responses as accessory cells. Indeed, in a mouse model for papain-induced Th2 responses, both DCs and basophils were important for Th2 skewing (*19*).

Since several allergens and helminth parasites can activate TLRs (*20-22*), it would be of high importance to know whether human basophils can be activated via TLR triggering, and if so, which cytokines are released. Until now, little information on these aspects is available for human basophils. Furthermore, as allergic responses and helminth infections may induce IgE responses (*23*), it would be important to know whether these pathways would interact and promote the establishment of a Th2 cytokine milieu. Therefore, in this study, we aimed to understand the activation of basophils by TLR ligands, and to subsequently determine whether this activation could influence Th2 responses.

RESULTS

EXPRESSION OF TLR

To determine the expression of TLR1 to TLR10 and other molecules involved in TLR signaling pathways, a real-time PCR array was performed on mRNA of basophils isolated from peripheral blood of three different donors. Isolated basophils were defined by expression of CD123, FcεRI and CD203c (Fig. 1A). mRNA encoding TLR1 to TLR8 was found

in the basophil populations from all three donors (Fig. 1B). TLR9 and TLR10 mRNA were only detected in one of the three donors, but were readily detected in PBMCs and the monocytic cell-line THP-1 (data not shown).

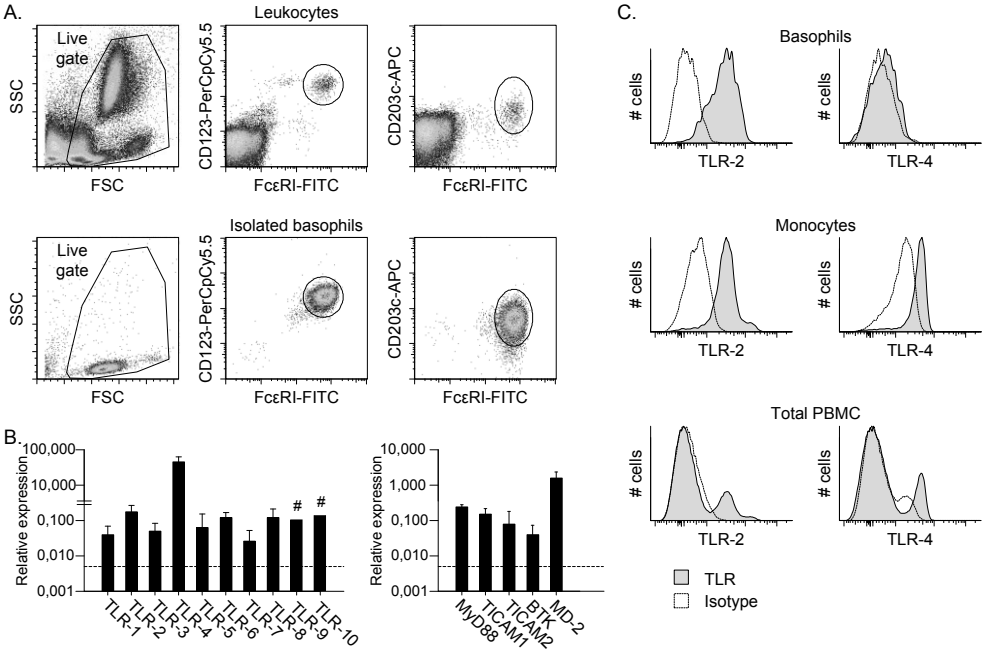

Figure 1. Expression of TLRs by human basophils. (A) Basophils were isolated from peripheral blood leukocytes by negative magnetic isolation. Representative examples of leukocytes before isolation of basophils and the isolated basophil population are shown on the top and bottom, respectively, as analyzed by flow cytometric staining for either CD123 or CD203c with FcεRI. The plots show cells from a live gate based on FSC/SSC characteristics. (B) Total mRNA was isolated from peripheral blood basophils of three different donors. Relative mRNA expression of TLRs (left) and molecules involved in TLR signaling (right), normalized to HPRT1 expression as housekeeping gene, is shown. Data are shown as the mean +SEM pooled from three independent basophil donors. The dotted line indicates the detection limit of the PCR assay. #: mRNA for TLR-9 and -10 was only detected in one out of three donors. (C) Flow cytometric staining of PBMCs for TLR-2 (top) and TLR-4 (bottom). Basophils were gated as shown in (A) and monocytes were gated based on FSC/SSC characteristics. A representative example from three experiments with n = 5 independent donors is shown.

Basophils also expressed mRNA for several molecules associated with TLR signaling, such as MyD88, TICAM1 (TRIF), TICAM2, and BTK and MD-2. Expression of TLRs by basophils was confirmed on the protein level by flow cytometric staining for TLR-2 and TLR-4 (Fig. 1C). Expression of TLR-2 by basophils was comparable to the expression by monocytes.

Expression of TLR-4 by basophils was slightly less than monocytes, but was clearly higher than the isotype control, and higher than other cells in the total PBMC population, confirming the specificity of the staining. These data indicate that human basophils can express TLRs as well as the downstream signaling molecules needed for TLR signal transduction.

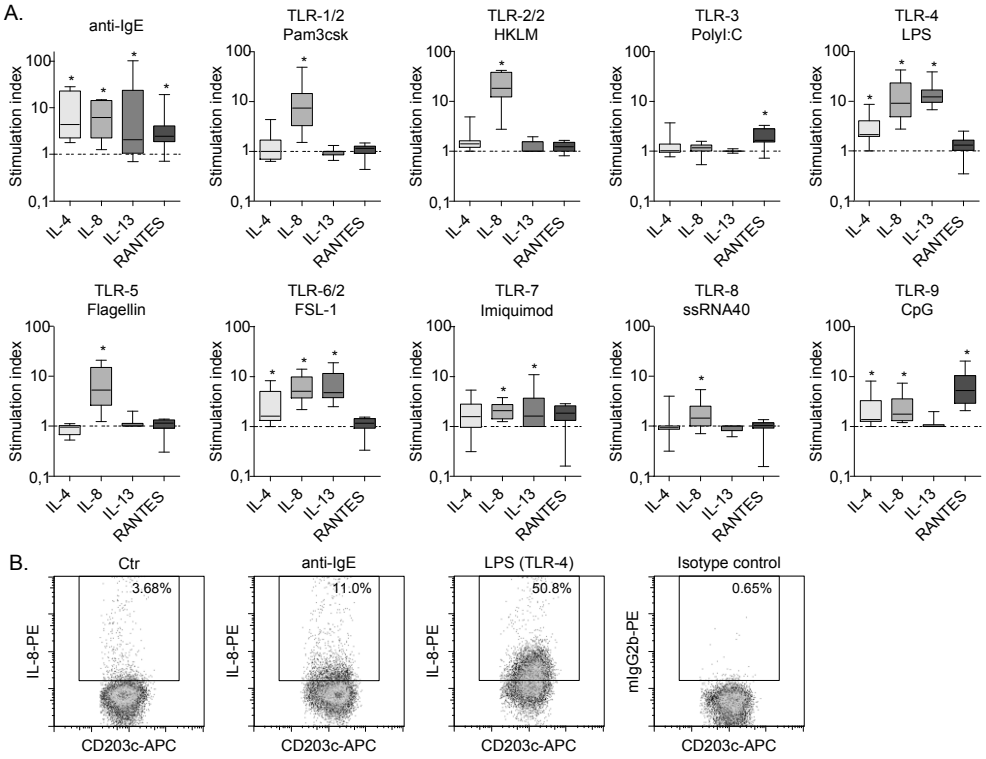

Figure 2. Cytokine production by human basophils in response to TLR ligands. (A) Isolated basophils were stimulated with TLR ligands for 24 h, after which cytokines were measured in supernatant by Luminex assay. Stimulation index was calculated by dividing the amount of cytokine produced after stimulation by the amount of cytokine in the supernatant of unstimulated basophils. Results are shown as a summary of ten experiments performed with basophils from ten different donors. The boxplots show the mean (line), 25–75 percentile (box) and minimum-maximum (whiskers). *$p < 0.05$, increase in stimulation index (above 1), one-sample t-test. (B) Intracellular flow cytometry staining for IL-8 and matching isotype control after overnight stimulation of isolated basophils with anti-IgE and LPS in the presence of brefeldin A. Basophils were gated as $FcεRI^+CD203c^+$ cells, as shown in Fig. 1. A representative example from three experiments with n = 4 independent donors.

CYTOKINE PRODUCTION AFTER BASOPHIL ACTIVATION WITH TLR LIGANDS

To examine whether the mRNA expression of TLRs could be related to functional responses, cytokine production by basophils was measured 24 h after stimulation with TLR ligands. Basophils respond to TLR ligands with production of various cytokines (Fig. 2A, Supplementary Fig. 1 and Supporting Information Table 1 online). Although IL-8 was produced in response to most of the TLR ligands (except TLR-3), production of IL-4 and IL-13 was mainly restricted to ligands for TLR-2/2, -4, and -6/2, and production of RANTES was restricted to the virus-associated TLR ligands PolyI:C (TLR-3) and CpG (TLR-9). These results suggest that the response to TLR ligands was different depending on the TLR that was being triggered. IL-10 production was only observed after ligation of TLR-2 and -4, and had p values >0.005 (not significant after correction for multiple testing). As no ligands are known for TLR-10, the absence of mRNA expression for this TLR could not be confirmed by functional assays. The differences in cytokines produced with different TLR ligands were confirmed by a titration of the TLR ligands (data not shown), indicating that the differential response that was seen was not merely a result of different degrees of activation with the TLR ligands.

Intracellular cytokine staining confirmed that IL-8 production in response to TLR ligands was derived from basophils (Fig. 2B). Altogether, these results indicate that basophils respond to all TLR triggers with cytokine production and interestingly, that basophils produce different cytokines depending on the TLR that is triggered.

DEGRANULATION AND LEUKOTRIENE RELEASE AFTER BASOPHIL ACTIVATION WITH TLR LIGANDS

Besides cytokine production, one of the main effector functions of basophils is mediated via release of granules containing several proinflammatory mediators. Therefore, degranulation of basophils in response to TLR triggering was analyzed by determination of histamine release in supernatant and flow cytometric staining for CD63, which is upregulated on the surface of degranulated basophils (Fig. 3A and B). As expected, basophils released histamine in response to the positive controls anti-IgE and fMLP within 1 h (Fig. 3C). Likewise, CD63 was upregulated on the surface of degranulated basophils.

Although activation of basophils by ligands for TLR-7 and TLR-8 led to small increases in CD63 upregulation and histamine release (TLR-7 only), these responses were low compared with anti-IgE and fMLP and were statistically not significant. No degranulation of basophils was observed following activation by other TLR ligands. Furthermore, no CD63 upregulation was found in response to TLR ligands after 24 h of incubation (data not shown).

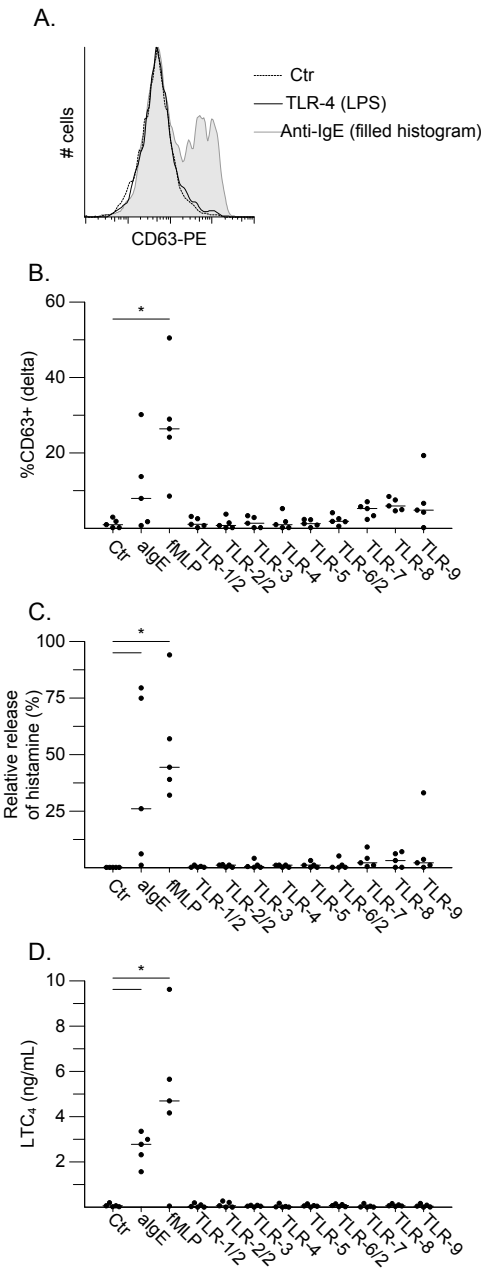

Figure 3. Basophil degranulation and leukotriene production in response to TLR ligands. (A–D) Isolated basophils were stimulated for 1 h with anti-IgE, fMLP, or TLR ligands, after which cells were stained for flow cytometry to determine expression of (A and B) CD63, and supernatant was assayed by ELISA to determine the release of (C) histamine and (D) leukotriene C4. (A) Representative data for CD63 expression in unstimulated basophils (dotted line) or basophils stimulated with anti-IgE (shaded histogram) or LPS (straight line) are shown. Basophils were gated as $CD123^{+}FcεRI^{++}CD203c^{+}$ cells, as shown in Fig. 1. (B) The delta percentage of CD63 that is shown was determined by subtracting the percentage of PE^{+} cells in the isotype control from the percentage of CD63-PE^{+} cells for each indicated ligand. (B–D) *$p<0.05$ compared with unstimulated basophils, repeated measures ANOVA with Bonferroni's posthoc test. Results are shown as a summary of five independent experiments, with the line across the dots representing the median, and each symbol representing an individual donor.

To further examine basophil responses to TLR ligands, we analyzed the release of lipid-derived mediators of basophils by measuring Leukotriene C4 in supernatant. Basophils only responded with leukotriene C4 release after activation with anti-IgE and fMLP and not after activation with TLR ligands (Fig. 3D). Therefore, TLR ligands induce a different response compared with IgE-mediated activation, characterized by production of cytokines and chemokines in the absence of degranulation or release of lipid-derived molecules.

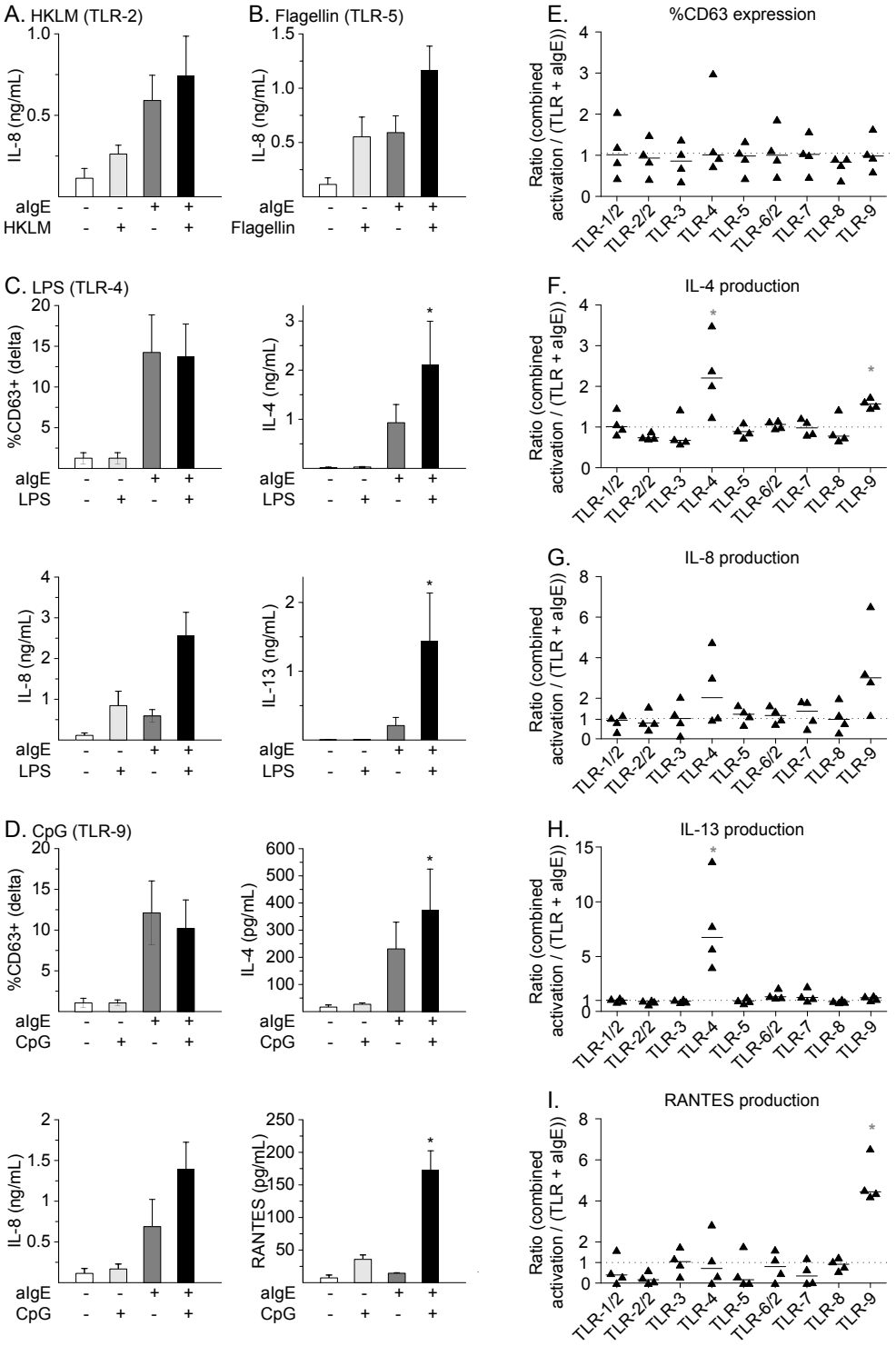
A. HKLM (TLR-2)
IL-8 (ng/mL)
aIgE
HKLM
B. Flagellin (TLR-5)
IL-8 (ng/mL)
aIgE
Flagellin
E. %CD63 expression
Ratio (combined activation / (TLR + aIgE))
TLR-1/2
TLR-2/2
TLR-3
TLR-4
TLR-5
TLR-6/2
TLR-7
TLR-8
TLR-9
C. LPS (TLR-4)
%CD63+ (delta)
aIgE
LPS
IL-4 (ng/mL)
F. IL-4 production
IL-8 (ng/mL)
IL-13 (ng/mL)
G. IL-8 production
D. CpG (TLR-9)
%CD63+ (delta)
CpG
IL-4 (pg/mL)
H. IL-13 production
IL-8 (ng/mL)
RANTES (pg/mL)
I. RANTES production

Figure 4 *(left)*. Synergy in cytokine production after combined TLR- and IgE-mediated stimulation. Basophils were stimulated with TLR ligands alone or in combination with anti-IgE for 24 h, after which degranulation was measured by flow cytometric staining of CD63 and cytokines were measured in supernatant by Luminex assay. (A and B) Examples of additive effects on IL-8 production in response to stimulation with anti-IgE and either TLR-2 or -5 ligands. (C and D) Examples of synergistic effects on cytokine production in response to stimulation with anti-IgE and either TLR-4 or -9 ligands. Percentage of $CD63^+$ basophils on gated $CD123^+FcεRI^{++}CD203c^+$ cells, as shown in Fig. 1. The delta percentage of CD63 that is shown was determined by subtracting the percentage of PE^+ cells in the isotype control from the percentage of CD63-PE^+ cells for each indicated ligand. (A–D) Data are shown as mean + SEM pooled from four independent experiments with basophils from four donors. (E–I) Summaries of CD63 expression and cytokine production for all TLR ligands in combination with anti-IgE are shown as ratio of percentage of $CD63^+$ cells or cytokine production in response to combined triggering divided by the sum of $CD63^+$ cells or cytokine production following stimulation with anti-IgE and TLR ligands separately, with each symbol representing an independent experiment and basophil donor. *$p<0.05$ compared with the additive cytokine production, one-sample t-tests.

SYNERGY BETWEEN TLR- AND FCεRI-MEDIATED ACTIVATION

It has been described that certain cytokines such as IL-3 can enhance the response of basophils upon IgE-mediated stimulation. However, it is not known whether a similar interaction is present between TLR- and IgE-mediated triggering of basophils. Therefore, we next studied the effects of combined TLR- and FcεRI-triggering by combining the different TLR ligands with anti-IgE.

No influence of TLR ligands on IgE-mediated degranulation (CD63 upregulation) was found. A representative example is shown for LPS (TLR-4 ligand) in Figure 4C. Although only additive effects on cytokine production were observed when TLR-1/2, -2/2, -3, -5, -6/2, and -7 ligands were combined with anti-IgE (Fig. 4A and B), combination of ligands for TLR-4 and -9 led to a fivefold increase in IL-4/IL-13 production and IL-4/RANTES production, respectively (Fig. 4C–I), as compared with the combined amounts of cytokines found after separate TLR and FcεRI triggering.

These results indicate that the combined stimulation of basophils with ligands for TLR-4 and -9 and anti-IgE induce a synergy in cytokine production by basophils.

SYNERGY WITH ALLERGEN-MEDIATED ACTIVATION OF BASOPHILS

As simultaneous activation of basophils from allergic individuals through allergen-specific IgE and TLRs could possibly underlie exacerbations of asthma symptoms during pulmonary infection, for example, we next evaluated whether the synergy is also present when basophils of allergic individuals are activated via LPS and allergens.

Although LPS only led to a small increase in degranulation of basophils (Fig. 5A), cytokine production was greatly enhanced when the basophils were stimulated by both LPS and allergen (Fig. 5B), indicating that synergy in cytokine production is also present when TLR triggering is combined with activation via allergen-specific IgE.

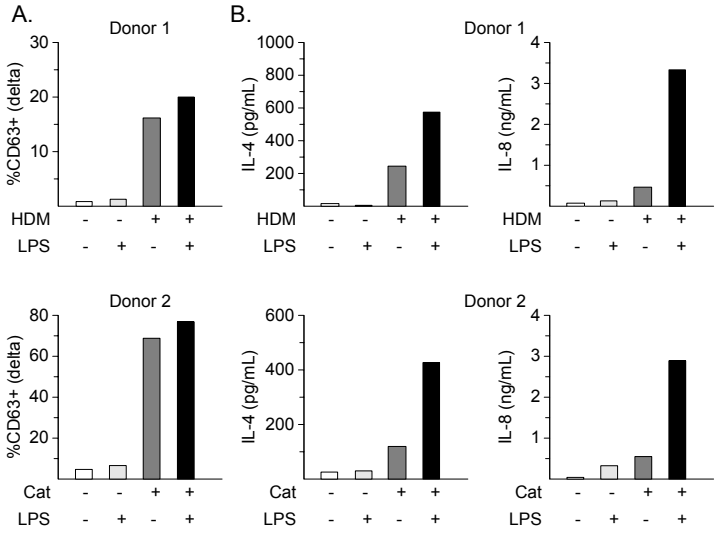

Figure 5. Synergy in cytokine production after combined TLR- and allergen-specific IgE-mediated stimulation. Basophils isolated from two allergic individuals were stimulated with TLR ligands alone or in combination with allergens for 24 h, after which degranulation was measured by CD63 upregulation and cytokines were measured in supernatant by ELISA. (A) CD63 upregulation when allergen-induced stimulation was combined with LPS. Basophils were gated as $CD123^{+}Fc\varepsilon RI^{++}CD203c^{+}$ cells. (B) Production of IL-4 and IL-8 after stimulation of basophils with allergen and LPS. Data are shown as individual experiments with basophils from two donors.

SYNERGY IN BASOPHIL ACTIVATION CAN ENHANCE T HELPER 2 (TH2) RESPONSES VIA IL-4

As basophils respond to combined triggering via TLR-4 and anti-IgE with greatly enhanced cytokine production, we next analyzed the relevance of this synergistic response by evaluating the influence of the cytokine production by basophils on the skewing of naive Th cells. To this end, naive $CD4^{+}$ T cells were stimulated with anti-CD3 and -CD28 in the presence or absence of basophil supernatant for 5 days, after which cytokine production by T cells was analyzed. When T cells were cultured in the presence of supernatant from basophils triggered by the combination of anti-IgE and LPS, the percentage of $IL\text{-}13^{+}$ cells was greatly enhanced (Fig. 6A and B). In contrast, only a relatively small increase in the percentage of $IFN\text{-}\gamma^{+}$ cells was found, indicating that basophils preferentially skewed

toward a Th2 phenotype. To confirm that the intracellular staining of IL-13 reflected enhanced skewing toward Th2 cells, we also measured T -cell cytokines in supernatant (Fig. 6C). Enhanced release of the typical Th2 cytokines IL-4, IL-5, and IL-13 was found when T cells were cultured in the presence of basophil supernatant stimulated with LPS and anti-IgE. Only a relatively slight increase in the production of IFN-γ was found in two out of three donors when supernatant of activated basophils was used. Together, our results indicate that basophils activated by anti-IgE in combination with the TLR-4 ligand LPS can promote the skewing toward Th2 cells.

As IL-4 is able to induce skewing of naive T cells toward Th2 cells and basophils were shown to produce relatively high amounts of IL-4 in response to anti-IgE and LPS, we investigated whether the effects of basophils on Th2 skewing were mediated via IL-4 by using blocking antibodies. Blocking IL-4 in basophil supernatant diminished the percentage of IL-13$^+$ T cells, and the effect of basophil supernatant on Th2 induction was completely reversed (Fig. 6D). These results indicate that synergy in IL-4 production by basophils in response to anti-IgE and LPS can promote the skewing toward Th2 cells.

DISCUSSION

In this study, we extensively analyzed basophil responses to TLR ligands. Although a few studies report cytokine production by basophils in response to TLR-2 and -4 ligands (*2-4*), functional responses toward other TLR ligands have not been reported. Furthermore, the effect of the TLR-4 ligand LPS on basophil activation showed inconsistent results among the different studies. Whereas two studies reported absence of CD11b upregulation and cytokine production (*2, 3*), one study reported production of cytokines in response to LPS (*4*), which is in agreement with our findings. We found that mRNA expression of TLR-4 was relatively high compared with the other TLRs. Furthermore, LPS induced the broadest cytokine production profile among the TLR ligands studied, and we were able to show a large shift in intracellular IL-8 in basophils treated with LPS, suggesting that basophils can indeed respond functionally to the TLR-4 ligand LPS.

By using sensitive multiplex assays, we detected cytokine production by basophils in response to ligands for TLR-1 to -9. There was a very small amount of BDCA-2 positive cells in the isolated basophil fraction in some donors (maximally 0.3%), whereas in other donors, no BDCA-2 positive cells were detected at all (data not shown). However, typical DC cytokines were not detected in response to TLR ligation, suggesting that the cytokine production we observed was not derived from contaminating DCs.

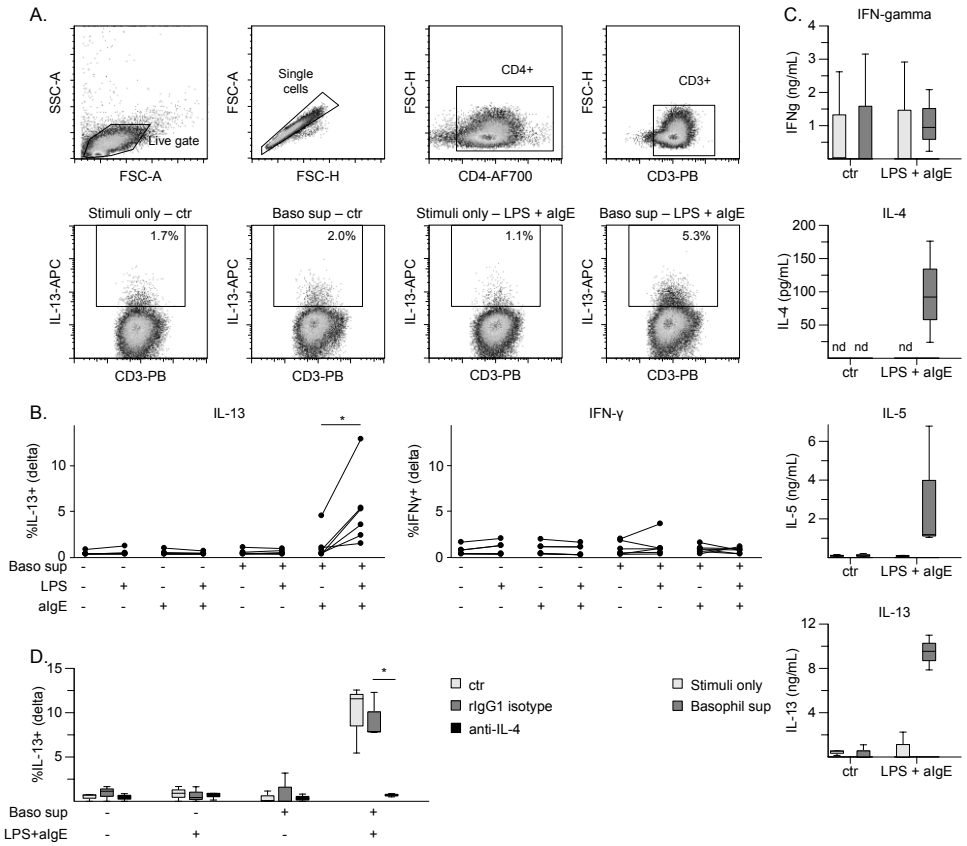

Figure 6. Influence of basophil activation by LPS and anti-IgE on Th-cell skewing. Naive $CD4^+$ T cells were cultured for 5 days in the presence of anti-CD3 and anti-CD28, with or without basophil supernatant, indicated as "basophil sup" or "stimuli only", respectively. T cells were restimulated with PMA/ionomycin to evaluate cytokine production by intracellular staining and by Luminex assay. (A) Representative example from five independent experiments of intracellular flow cytometric staining for IL-13 in the absence or presence of basophil supernatant from unstimulated or LPS- and anti-IgE-stimulated cells. Gating strategy is shown (top). (B) Percentages of $IL\text{-}13^+$ and $IFN\text{-}\gamma^+$ T cells derived from intracellular flow cytometry from five independent experiments performed using supernatants from six independent basophil donors and five independent T-cell donors. The delta percentage of cytokines was calculated by subtracting the percentage of positive cells in the isotype control from the percentage of cytokine-positive cells for each condition. *$p < 0.05$ increase in percentage of $IL\text{-}13^+$ T cells when supernatant from LPS- and anti-IgE-stimulated basophils was compared with that of basophils stimulated with only anti-IgE, paired samples t-test. (C) T-cell cytokine production as measured in supernatant. Data were pooled from three experiments with three independent basophil and T-cell donors. n.d.: not detected. (D) Intracellular staining of IL-13 in cultured naive $CD4^+$ T cells stimulated with basophil supernatant incubated with either an IL-4-blocking antibody or matching isotype control. Summary pooled from three experiments with three independent basophil and T-cell donors. *$p < 0.05$ decrease when basophil supernatant was treated with anti-IL-4 blocking antibody compared with the isotype control, paired samples t-test. The boxplots show the mean (line), 25–75 percentile (box) and minimum-maximum (whiskers).

We observed different patterns of cytokine production depending on the TLR that was being triggered. Interestingly, the virus-associated TLRs induced different responses than the bacterial-associated TLRs, indicating that basophils can respond in diverse ways to these different types of pathogens. Such different cytokine production profiles have previously been reported for dendritic cells, where different cytokines produced in response to bacterial- or viral -associated TLR ligands were shown to induce different types of immune responses (*24, 25*). Therefore, the different cytokines produced by basophils in response to TLR ligands may also alter the immune response that is elicited upon infection by viruses or parasites.

The synergy we observed when both TLR-4 or -9 and FcεRI were triggered indicates that TLR triggering could enhance IgE-mediated responses, such as in allergy or parasitic infection. The effects of TLR ligands are somewhat comparable to the effects that cytokines have on the priming of basophil responses. For example, IL-3 and IL-33 have both been shown to enhance degranulation and IL-4 and IL-13 production by basophils in response to anti-IgE (*26, 27*). TLR triggering did not influence IgE-mediated degranulation, indicating that the effects of TLR triggering and priming by cytokines on IgE-mediated basophil activation are distinct. In mice, synergy in TNF-α production by mast cells upon TLR and FcεRI triggering has been shown to depend on synergistic activation of protein kinases (*28*). Although TLRs and FcεRI use different molecules for their intracellular signaling (e.g. MyD88 and Syk), they can activate similar transcription factors via mitogen-activated protein kinases, such as p38, Erk, and JNK (*29*). Therefore, the synergy in cytokine production by basophils in our system may have been induced via a similar mechanism.

This synergy in cytokine production of basophils might occur in vivo when both specific IgE and TLR ligands are present, such as during parasitic infections, autoimmune diseases, and allergic reactions. Although parasites can induce specific IgE and activate TLRs through pathogen-associated molecular patterns, chronic allergic inflammation may lead to activation of TLR by damage-associated molecular patterns (*30, 31*). In addition, certain viral and bacterial infections have been associated with asthma exacerbations (*32, 33*), and the presence of endotoxin (TLR-4 ligand) was demonstrated in house dust, and was related to severity of allergic reactions against house dust mite (*21*), Our data indicate that activation of basophils may contribute to these processes by enhanced cytokine production when both specific IgE and TLR ligands are present. The fact that basophil responses to TLR ligands could greatly enhance the IgE-mediated activation suggests that these responses mainly play a role in memory or chronic immune responses, when specific IgE has already been generated. However, since certain allergen and helminth antigens may directly crosslink FcεRI on basophils without the need for specific IgE (*34-36*), these responses may also be enhanced by the presence of TLR ligands at the same time, and TLR activation of

basophils may thus contribute to enhanced cytokine production in primary immune responses against helminths as well.

Our results indicate that TLR ligation of basophils may induce IL-4 production as well as other Th2-like cytokines, and may therefore provide an early IL-4 signal when specific IgE is not (yet) present. As such, basophils may function as accessory cell providing IL-4 signals to naïve T cells in the presence of APCs. Two recent studies reported that human basophils could enhance Th2 and Th17 memory responses, through TCR-independent pathways (*37*). Furthermore, in mice, IL-4-producing basophils were shown to enhance DC-driven antigen-specific induction of Th2 cells in the LN, suggesting that basophils can indeed function as accessory cells to provide the IL-4 signal to naive T cells in vivo (*37*). Our results showed that TLR triggering of human basophils may contribute to the induction or expansion of Th2 responses in a similar way.

In this study, we chose to evaluate the effect of synergistic cytokine production by basophils on T -cell skewing, but similar effects may be observed when evaluating the effect of basophil-derived cytokines on other immune cells or on tissue-resident cells, as, for example, IL-4 and IL-13 have been shown to induce mucus production and parasite expulsion, two important processes in parasite infection and allergic responses (*38, 39*). In addition, release of IL-4 and IL-13 by basophils may induce alternatively activated macrophages, which in turn play a role in tissue homeostasis and repair (*40, 41*). Therefore, synergy in cytokine production by basophils may amplify Th2-type immune responses via T cell-dependent and -independent mechanisms.

In conclusion, we found cytokine production by basophils in response to ligands for TLR-1 to -9. The type of TLR ligand determined the cytokine profile being produced, which suggests that basophils could play different roles in the immune response depending on the pathogens present. Furthermore, increased cytokine production by basophils in response to ligation of TLR and FcεRI at the same time could have important implications for their role in Th2 responses. Altogether these results suggest nonredundant roles for basophils in the immune response against pathogens via TLRs, and for the first time suggest that TLR-activated basophils can enhance Th2 responses.

MATERIALS AND METHODS

CELL ISOLATION

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). For isolation of basophils, total leukocytes were first isolated using HetaSep

(StemCell). Basophils were then isolated by negative magnetic bead isolation using the Basophil Enrichment Kit (StemCell), using the manufacturer's instructions. The purity of isolated basophils was determined by flow cytometric analysis of FcεRI, CD203c, and CD123. The purity of isolated basophils was above 95% in each experiment.

For isolation of $CD4^+$ T cells, naive ($CD45RO^-$) $CD4^+$ T cells were isolated from PBMCs by negative magnetic bead isolation (Miltenyi Biotec). The purity of isolated naive $CD4^+$ T cells was determined as $CD14^-CD3^+CD4^+CD45RA^+CD45RO^-$ cells. The purity of isolated naive $CD4^+$ T cells was above 95% in each experiment.

REAL-TIME POLYMERASE CHAIN REACTION

Basophil lysate was prepared using TRIzol reagent (Invitrogen). RNA isolation was performed using TRIzol following manufacturer's instructions, including removal of proteoglycans using a high-salt buffer. A cDNA library was constructed using the RT^2 first strand kit (SA Biosciences) following manufacturer's instructions.

Expression of TLRs by basophils was assessed by real-time PCR. The Toll-Like Receptor Signaling Pathway PCR Array (SA Biosciences) was performed to assess the expression of the several genes, including the following: TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, BTK, MD-2, MYD88, TICAM1, and TICAM2. Relative expression of these genes was normalized toward expression of the housekeeping gene HPRT1.

BASOPHIL ACTIVATION

Basophils were cultured in Iscove's Modified Dulbecco's Media containing 10% FCS at a cell concentration of 1 × 106 cells/mL in all experiments, unless indicated otherwise. For TLR-mediated activation, basophils were stimulated using 1 μg/mL Pam3csk, 10^8 cells/mL heat killed *Listeria monocytogenes* (HKLM), 10 μg/mL Poly I:C, 10 μg/mL LPS, 1 μg/mL Flagellin, 1 μg/mL FSL-1, 10 μg/mL Imiquimod, 10 μg/mL ssRNA40, and 5 uM CpG (all from InVivoGen). IgE- or fMLP-mediated activation of basophils was induced using 10 μg/mL goat-anti-human IgE (Nordic) or 1 μg/mL fMLP (Sigma), respectively.

For combined activation with TLR ligands and anti-IgE, basophils were stimulated with 0.1 μg/mL Pam3csk, 10^7 cells/mL HKLM, 2.5 μg/mL Poly I:C, 50 ng/mL LPS, 0.2 μg/mL Flagellin, 0.05 μg/mL FSL-1, 0.5 μg/mL Imiquimod, 5 μg/mL ssRNA40, and 5 uM CpG with or without 0.2 μg/mL anti-IgE.

After 24 h, basophil activation was measured using flow cytometry, multiplex assays, and ELISA. For analysis of degranulation by FACS and release of histamine and leukotrienes, basophils were stimulated for 1 h in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM

NaH2PO4, 1.4 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, 100 mM Hepes, and 0.1% BSA in 50% H2O and 50% D2O). To assess the total histamine content of basophils to calculate the percentage of release, basophils were lysed with 1% Triton X-100 in Tyrode's buffer.

For intracellular cytokine staining, basophils were cultured in the presence of 3 µg/mL brefeldin A for 18 h, after which cells were used for flow cytometry.

ALLERGEN-INDUCED BASOPHIL ACTIVATION

Heparinized peripheral blood was obtained from individuals allergic to house-dust mite allergen or cat epithelia. Leukocytes and basophils were isolated as described above. Basophils were incubated with 0.25 U/mL Dermatophagoides pteronissymus (HAL Allergy) or 25 U/mL Epithelia Felis catus (HAL Allergy), respectively, with or without LPS (5 ng/mL). After 24 h, basophil activation was measured using flow cytometry and ELISA.

EFFECT OF BASOPHIL SUPERNATANT ON TH -CELL SKEWING

Basophil supernatant was collected after culture as described above in the presence of anti-IgE and/or LPS. As control, medium with the same concentration of anti-IgE and/or LPS was used. Naive $CD4^+$ T cells were activated using 5 µg/mL plate-bound anti-CD3 (eBioscience; clone: OKT3) and 1 µg/mL soluble anti-CD28 (Sanquin; clone: CLB-CD28/1, 15E8) in the presence of absence of basophil supernatant that was diluted twice in the culture medium.

After 5 days of incubation, T cells were harvested, washed, and split over two wells. One well of each condition was left unstimulated, whereas the other well was restimulated using 50 ng/mL PMA (Sigma) and 500 ng/mL Ionomycin (Sigma). Cells were restimulated for 5 h in the presence of 10 µg/mL brefeldin A (Sigma) for intracellular cytokine staining of IFN-γ and IL-13, or left overnight without brefeldin A to determine cytokine levels in supernatant.

For blocking experiments, basophil supernatant was incubated with 10 µg/mL anti-IL-4 (clone MP4–25D2, eBioscience) or rat IgG1 isotype control (eBRG1, eBioscience), for 1 h at 37°C before adding the supernatant to the T cells.

FLOW CYTOMETRY

The following antibodies were obtained from BD Biosciences: CD123-PerCpCy5.5 (clone 7G3), CD63-PE (H5C6), CD14-PECy7 (M5E2), CD3-Pacific Blue (UCHT1), CD45RA-FITC (L48), CD45RO-PE (UCHL1), IL-8-PE (G265–8), IFN-γ-FITC (25723.11), IFN-γ-PE (4S.B3), IL-13-

allophycocyanin (JES10–6A2), mIgG1-APC (MOPC-21), mIgG2b-FITC (27–35), rIgG1-allophycocyanin (R3-R4), streptavidin-allophycocyanin. The following were obtained from eBioscience: FcεRI-FITC (AER-37), TLR-2-biotin (TL2.1), TLR-4-biotin (HTA125), mIgG1-PE (P3.6.2.8.1), mIgG2b-PE (eBMG2b), mIgG2a-biotin (eBM2a), and the following were obtained from Miltenyi: CD203c-allophycocyanin and -PE (FR3–16A11), BDCA-2-allophycocyanin (AC144), and Biolegend: CD4-AlexaFluor700 (OKT4). For surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0.5% BSA at 4°C for 30 min.

For staining of TLR-2 and -4, cells were incubated for 15 min with 50 ug/mL human IgG (Jackson Immunoresearch) to block Fc receptors, prior to addition of the primary antibodies. After washing, cells were incubated with strep-allophycocyanin at 4°C for 30 min. For intracellular cytokine staining, basophils and T cells were incubated with anti-bodies against surface antigens as described above, after which they were permeabilized using CytoFix CytoPerm Kit (BD Biosciences). After washing, basophils were incubated with antibodies against intracellular cytokines and incubated at 4°C for 30 min.

After washing, cells were suspended in 1% paraformaldehyde until flow cytometric aquisition on an FACS Calibur (BD) or LSR-II (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

CYTOKINE, HISTAMINE, AND LEUKOTRIENE C4 PRODUCTION

Quantitative immunoassays for EGF, Eotaxin, FGF-2, Flt-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1ra, IL-1α, IL-1β, IL-2, sIL-2Rα, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGF-α, TNF-α, TNF-β, and VEGF in basophil culture supernatants were performed using Milliplex assays (Millipore). Quantitative immunoassays for GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-31, IL-33, MIP-3α/CCL20, TNF-α, TNF-β in T -cell culture supernatants were performed using Milliplex assays (Millipore). Additionally, IL-4, IL-8, and IL-13 production was evaluated using ELISA (from Sanquin and eBioscience). Histamine and Leukotriene C4 were analyzed using competitive ELISA kits (Neogen).

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. For detection of TLR mRNA expression, one-sample T-test was performed. For differences between multiple groups, a repeatedmeasures ANOVA was performed, with Bonferroni's posthoc test. Paired-sample T-test was

performed to analyze differences between two groups. Synergy was calculated as a ratio dividing the cytokine production induced by combined TLR and anti-IgE stimulation by the sum of the cytokine production induced by the separate stimuli (value(TLR + a-IgE)/(value(TLR) + value(a-IgE), after which one-sample T-test was performed. Statistical analysis was performed using SPSS PASW 17.0 and GraphPad Prism 4. P values of <0.05 were considered statistically significant.

ACKNOWLEDGEMENTS

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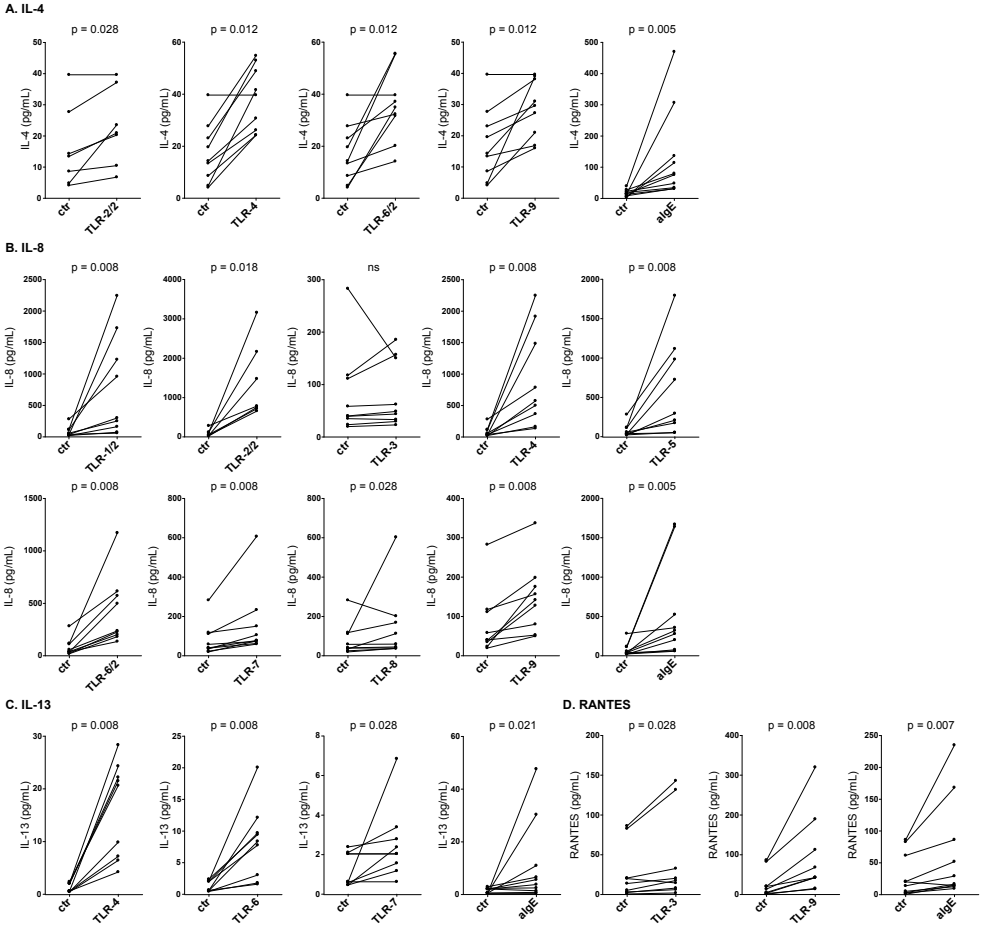
SUPPLEMENTARY FIGURE



Supplementary Figure 1. Cytokine production by human basophils in response to TLR ligands. (A-D) Isolated basophils were stimulated with TLR ligands for 24 hours, after which cytokines were measured in supernatant by Luminex assay. Paired graphs showing cytokine production by basophils left unstimulated (ctr) or stimulated with ligands for the indicated TLR ligands or anti-IgE (aIgE).Results are shown as a summary of 10 experiments performed with basophils from 10 different donors, with each line indicating an individual donor. P values, paired samples T-test.

IgE AND IL-33-MEDIATED TRIGGERING OF HUMAN BASOPHILS INHIBITS TLR4-INDUCED MONOCYTE ACTIVATION

Chapter 3

Felice Rivellese,[1,2] Jolien Suurmond,[1] Amato de Paulis,[2] Gianni Marone,[2] Tom WJ Huizinga,[1] René EM Toes[1]

[1]Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; [2]Department of Translational Medical Sciences and Center for Basic and Clinical Immunology Research (CISI), University of Naples Federico II, Naples, Italy

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ABSTRACT

Basophils are circulating granulocytes, best known as effector cells in allergic reactions. Recent studies in mice suggest that they might also participate in the suppression of chronic inflammation. The aim of this study was to assess the ability of purified human basophils to modulate monocyte responses upon IL-33 and IgE triggering.

Activation of human basophils with IL-33 induced the production of IL-4 and the release of histamine, and enhanced their IgE-mediated activation. In addition, basophils triggered with IL-33 and anti-IgE significantly suppressed the LPS-induced production of the proinflammatory cytokine TNF-α and the upregulation of the costimulatory molecule CD80 by monocytes. These effects were mainly explained by the release of histamine, as they could be inhibited by the histamine receptor 2 antagonist ranitidine, with a smaller contribution of IL-4. In contrast, basophil-derived IL-4 and histamine had opposing effects on the expression of the inhibitory Fc γ receptor IIb and the production of IL-10 by monocytes.

Our data show that basophils can influence monocyte activation and suggest a previously unrecognized role for human basophils in the modulation of monocyte-mediated immune responses, through the balanced secretion of histamine and IL-4.

INTRODUCTION

Basophils are circulating granulocytes, representing less than 1% of peripheral blood leukocytes. They are classically known for their involvement as effector cells in allergic reactions (*1-3*). Because of their paucity and the difficulty in isolating them, there is still relatively little information available on their functions and potential immunomodulatory effects (*4*). Studies in mice suggest a possible role for basophils in the induction of adaptive immunity in the context of Th2 responses, where they have been shown to be the major producers of IL-4 (*5*), although their function as antigen presenting cells and their dispensability in the induction of Th2 responses are still under debate (*6, 7*).

In addition, recent experimental data showed how these rare leukocytes might also exert anti-inflammatory effects in the context of autoimmune and allergic inflammation. In a mouse model of arthritis, basophils have been implicated in the immunosuppressive response mounted upon injection of intravenous immunoglobulin (IVIG) (*8*). Briefly, the authors proposed the following scenario to elucidate IVIG-mediated immune suppression: myeloid-derived cells, in response to the sialylated fraction of IVIG, release IL-33, which, in turn, activates basophils to produce IL-4. Finally, basophil-derived IL-4

induces the upregulation of the inhibitory Fc γ Receptor (FcγRIIb) on inflammatory macrophages, leading to the suppression of antibody-dependent inflammation. Thus, IL-33 was shown to be involved in the suppression of arthritis through its action on basophils. Even though the role of the sialylated fraction of IVIG or basophils in the immunomodulatory effects of IVIG therapy has recently been challenged (*9*), another study using an experimental model of IgE-mediated chronic allergic inflammation, showed that mouse basophils, through IL-4, drive the differentiation of monocytes into anti-inflammatory macrophages, thereby regulating chronic allergic skin inflammation in vivo (*10*).

Human basophils, which can significantly differ from their mouse counterparts (*1, 3, 11*), have been known for a long time to produce IL-4 (*12*), and have been recently shown to induce Th2 skewing of naïve helper T cells in vitro (*13, 14*). On the other hand, the function as antigen presenting cells, still debated in mice, has been ruled-out for human basophils by several groups (*15-17*). In addition to well-known basophil triggers like IgE or IL-3, IL-33 also activates human basophils, inducing the production of several cytokines, including IL-4 (*18-20*).

IL-33 is a member of the IL-1 cytokine family that plays an important role in the induction and effector phases of type 2 immune responses (*21*), suspected to be involved in the pathogenesis of several allergic and autoimmune diseases (*22*), where it can have both protective and deleterious effects in different settings (*23*). For example, in the aforementioned arthritis model (*8*), IL-33 production was critically involved in the suppression of arthritis upon IVIG administration, specifically through its effects on basophils. Since IL-33, as an alarmin (*22*), is released in any inflamed tissue and reaches high levels locally but also in the sera of patients with several allergic and autoimmune conditions, such basophil-driven anti-inflammatory network could be active, independently from exogenous stimuli like IVIG, in any condition in which IL-33 is released upon cell necrosis. Therefore, it would be of invaluable interest to verify whether IL-33 is able to induce anti-inflammatory responses through the activation of human basophils.

To this end, we investigated the interaction of IL-33-stimulated human basophils with monocytes. We found that purified human basophils, triggered via FcεRI ligation and IL-33, suppressed LPS-induced monocyte activation. Unexpectedly, only little of this effect could be attributed to IL-4. Instead, basophil-derived histamine was found to be the main factor involved. Overall, our data show that human basophils are able to influence monocyte activation via the balanced secretion of IL-4 and histamine, and suggest that they play an important role in the regulation of the immune responses.

RESULTS

IL-33 ACTIVATES HUMAN BASOPHILS

To determine basophil activation upon IL-33 triggering, we performed in vitro stimulation of purified human basophils. Basophils were identified by the expression of CD123 (IL-3 receptor), FcεRI (Fig. 1A), and CD203c (Fig. 1B). To exclude contamination with plasmacytoid dendritic cells (pDCs) ($CD123^+$, $BDCA\text{-}2^+$), also known to express low levels of FcεRI (*24*), we verified that the isolated fractions, while positive for the basophil marker CD203c, did not contain $BDCA2^+$ cell (Fig. 1B). IL-4 levels were measured after 24 h of stimulation with different doses of IL-33 or with two classical basophil stimuli, i.e. IgE-mediated cross-linking of the FcεRI and IL-3 (Fig. 1C). In addition, IL-4 production upon combined stimulation of basophils with IL-33 and anti-IgE or IL-3 was evaluated (Fig. 1D). These data show that IL-33 alone induced low but detectable amounts of IL-4, which is in line with previous literature (*20*). Additionally, as shown in Figure 1D, IL-33, at 100 ng/mL, significantly increased IL-4 production upon IgE or IL-3 triggering.

To further study basophil activation, we measured the membrane expression of CD63, as a marker of degranulation (*25*). IL-33, as expected, did not induce degranulation when used alone, but significantly enhanced IgE-mediated degranulation of basophils (Fig. 1E and 1F). In addition, histamine release was evaluated (Fig. 1G). In line with CD63 expression, histamine was released by basophils upon IgE cross-linking. In addition to a spontaneous secretion of histamine in unstimulated basophils, we observed a significant induction of histamine release upon IL-33 triggering. Such histamine release was independent from CD63, which is possibly in line with previous reports of spontaneous and degranulation-independent release of histamine by human basophils (*26, 27*).

These data indicate that IL-33 can activate human basophils, inducing the production of IL-4 and the release of histamine. Furthermore, IL-33 can enhance IgE-mediated basophil degranulation, histamine release and cytokine production.

ACTIVATED HUMAN BASOPHILS MODULATE MONOCYTE ACTIVATION

To evaluate whether human basophils can modulate monocyte activation, monocytes were incubated with supernatants of basophils or control media (containing the stimuli originally used to trigger basophils) and activated with LPS. As shown in Figure 2A, TNF-α production was significantly suppressed when monocytes were conditioned with basophils supernatants. Unstimulated basophils partially inhibited TNF-α production by monocytes, but a significantly stronger inhibition of TNF-α production by monocytes was induced by supernatants of basophils triggered with anti-IgE together with IL-33.

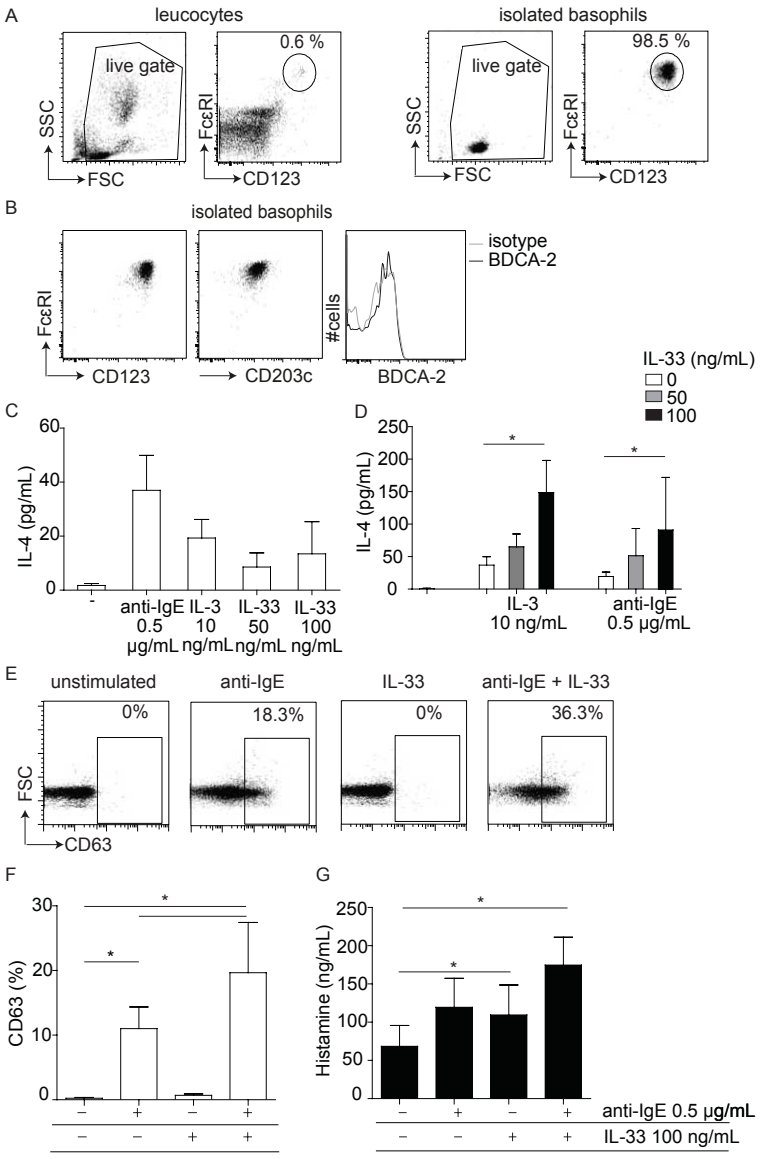


Figure 1. Activation of human basophils. (A) Basophils were isolated from peripheral blood leucocytes of healthy donors by immune-magnetic negative selection. Cells were analyzed by FACS to verify purity. A representative example is shown, with total leucocytes (left), and isolated basophils (right). After gating out debris using FSC/SSC, basophils were identified as $CD123^{+}Fc\varepsilon RI^{++}$ cells. (B) Isolated basophils were analyzed for the expression of CD203c and BDCA2. Total cells from the live gate are shown. (C) Isolated basophils were stimulated with the indicated concentrations of anti-IgE, IL-3, or IL-33 for 24 h and IL-4 production was measured by ELISA in the supernatants after harvesting. (D) IL-4 production after 24 h of incubation of isolated basophils with IL-3 or anti-IgE + indicated concentrations of IL-33. (E, F) Flow cytometric expression of CD63 by isolated basophils after 24 h of stimulation with anti-IgE (0.5 µg/mL), IL-33 (100 ng/mL), or both. A representative example is shown in (E) and the summary of all donors in (F). (G) Histamine release, measured by ELISA, after 24 h of incubation with the indicated stimuli. (A, B, and E) Data shown are representative of five independent experiments performed. (C, D, F, and G) Data are shown as mean + SEM and are pooled from (C, D) three independent experiments (n = 3 basophil donors) or (F, G) five independent experiments (n = 5 donors). * $p < 0.05$ determined by ANOVA with Bonferroni's post-test.

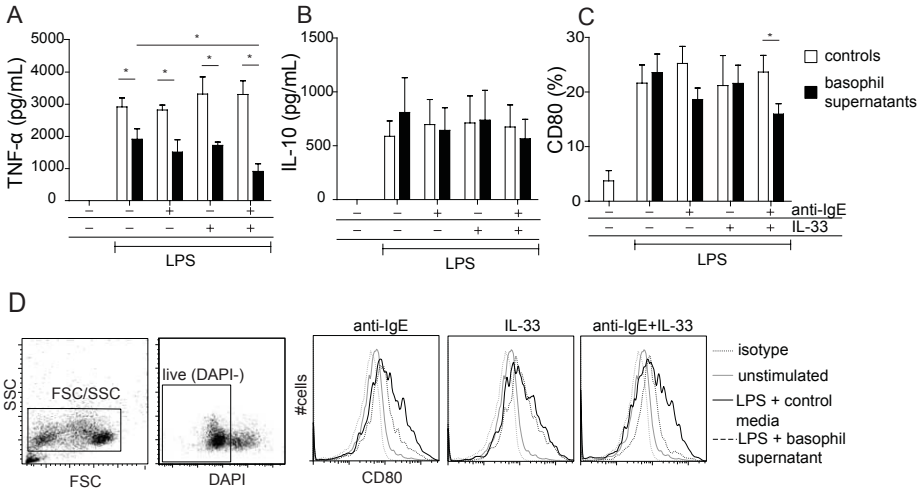


Figure 2. Effects of basophil supernatants on LPS-induced activation of monocytes. (A–C) Monocytes were isolated by positive selection using CD14 immunomagnetic beads, incubated with the supernatants of basophils (black bars) or with control media (white bars) and activated with LPS (5 ng/mL). The stimuli used to activate basophils and also present in the control media are indicated on the x axis. (A) TNF-α and (B) IL-10 were measured by ELISA in the supernatants of LPS-stimulated monocytes after overnight incubation (18 h). (C) Monocyte expression of CD80 was evaluated by (D) flow cytometry after FSC/SSC gating and exclusion of dead cells with DAPI. (A–C) Data are shown as mean + SEM from five independent experiments (n = 5 monocyte donors). (D) Data shown are representative of five independent experiments performed. * $p < 0.05$; for comparison of each basophil supernatant with its own control, Student t-test was performed. For multiple comparisons between basophil supernatants ANOVA with Bonferroni's post-test was performed.

IL-10 production by monocytes was not influenced, indicating that monocytes were still equally able to produce cytokines, and that they were still viable (Fig. 2B). Indeed, the percentages of live monocytes were similar in all conditions, confirming that the observed reduction of TNF-α was not due to impaired survival of monocytes (data not shown), but rather to a suppression of TNF-α production upon exposure to basophil supernatants. To confirm that TNF-α and IL-10 were derived from monocytes, the cytokine levels were also measured in basophil supernatants, and were below detection limit (data not shown). Together, these results indicate that basophils significantly suppress LPS-induced TNF-α, without affecting the production of IL-10, thereby modulating the proinflammatory responses of monocytes.

To further study monocytes activation, we analyzed surface markers by flow cytometry. While the expression of several markers, including activating and inhibitory Fc γ receptors (CD14, HLA-DR, CD16, CD64, CD32a, CD32b), were not significantly influenced (data not

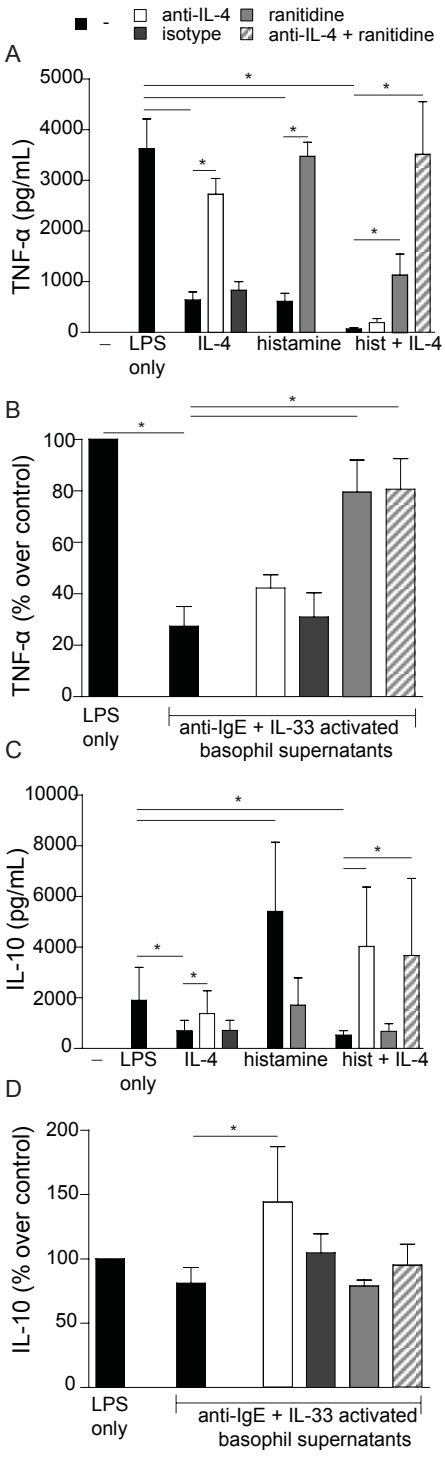
shown), basophils supernatants reduced the percentage of monocytes expressing the costimulatory molecule CD80 upon LPS stimulation (Fig. 2C and D). Although this effect was statistically significant only for the combined activation of basophils with IgE and IL-33, these data further confirm the ability of basophils to modulate monocyte function, by influencing their expression of costimulatory molecules.

Together, these data show that basophil supernatants significantly modulate monocyte proinflammatory activation, by inhibiting the LPS-induced production of TNF-α and upregulation of CD80.

BASOPHIL-DERIVED IL-4 AND HISTAMINE MODULATE CYTOKINE PRODUCTION BY MONOCYTES

We next sought to identify the mechanism by which basophils affected monocyte cytokine production and speculated that IL-4 might play a role in this process. Indeed, IL-4 did inhibit the LPS-elicited TNF-α production by monocytes to a similar extent as activated basophil supernatants (Fig. 3A). Nonetheless, IL-4 blocking in the supernatants of anti-IgE and IL-33 activated basophils only slightly reverted the inhibition of TNF-α (Fig. 3B), indicating that this effect was mainly mediated by other factors. Among other mediators produced by basophils, histamine has been shown to modulate monocytes responses to LPS (*28*). Accordingly, exogenously added histamine could suppress LPS-induced TNF-α production and, when used together with IL-4, the suppression of TNF-α was almost complete, as shown in Figure 3A. Additional experiments (not shown) confirmed that this effect of histamine is specifically mediated by the interaction with histamine 2 receptor (H2R) as it could be inhibited by H2R antagonist ranitidine and not by H1R antagonist cetirizine or H3–4R antagonist thioperamide (at doses from 10–6 to 10–4 M). In line with these observations, preincubation of monocytes with ranitidine significantly prevented the inhibitory effects of activated basophil supernatants, while concomitant blocking of IL-4 in the supernatants showed almost no additional effect (Fig. 3B). These data indicate that basophil-derived histamine is predominantly responsible for suppressing LPS-induced TNF-α production by monocytes.

We next analyzed the influence of exogenous histamine and IL-4 on LPS-induced IL-10 production and, intriguingly, observed opposite effects, as shown in Figure 3C. Consequently, only when IL-4 was blocked in basophil supernatants, the IL-10 inducing effects of histamine could be revealed. The enhanced IL-10 production observed after blocking IL-4 could be reverted by the addition of ranitidine, showing it was mediated by histamine via H2R (Fig. 3D). Together, these data indicate that IL-4 and histamine in basophils supernatants have opposite effects with respect to IL-10 production.



In order to further confirm that basophil derived histamine and IL-4, at the levels found in the supernatants, are relevant mediators involved in the inhibitory effect on TNF-α production, we performed a titration experiment (Supplementary Fig. 1). Both histamine and IL-4 suppressed LPS-induced TNF-α at very low concentrations (as low as 1 × 10−9 M of histamine and 1 pg/mL of IL-4).

Moreover, additional suppression of TNF-α could be obtained by combining histamine and IL-4 at these low concentrations (data not shown). This confirms that low levels of histamine and IL-4 can modulate monocyte responses to LPS triggering.

Figure 3. Basophil-derived mediators and the modulation of LPS-induced cytokine production by monocytes. (A) TNF-α in the supernatants of monocytes conditioned with IL-4 and histamine. Briefly, isolated monocytes were stimulated with LPS (5 ng/mL) with or without IL-4 (10 ng/mL) and/or histamine (10−5 M). Blocking was performed by preincubation with anti-IL4 antibody and matching isotype control or with histamine receptor 2 antagonist ranitidine. (B) Blocking experiments, as described above, were also performed using anti-IgE + IL-33 activated basophil supernatants. TNF-α produced by basophil-conditioned monocytes was compared with the amount produced by monocytes incubated with control media, and is shown as percentage over control. Monocytes stimulated with LPS alone correspond to 100% of TNF-α production. Direct effects of anti-IL-4 and ranitidine on monocytes were excluded by adding them to the respective control media. (C, D) IL-10 concentration was measured in the supernatants, as described in (A, B). (A–D) Data are shown as mean + SEM pooled from five independent experiments (n = 5 monocyte donors). * $p < 0.05$ determined by ANOVA with Bonferroni's post-test.

Altogether, these results show that basophil-derived IL-4 and histamine can differently modulate the response of human monocytes to TLR4-triggering, where opposing effects are observed regarding IL-10-production. In contrast, both molecules, but mainly histamine, contribute to the suppression of LPS-induced TNF-α production by monocytes.

IL-4 AND HISTAMINE SUPPRESS CD80 BUT HAVE OPPOSITE EFFECTS ON THE EXPRESSION OF FCγRIIB BY MONOCYTES

Having established that IL-4 and histamine can have different effects on the modulation of cytokine production by monocytes, we next studied their influence on the expression of monocyte surface markers by flow cytometry. As expected, IL-4 induced the expression of the inhibitory FcγRIIb in monocytes (Fig. 4A). Intriguingly, when histamine was added to IL-4 this effect was lost. Therefore, we speculated that histamine could be responsible for inhibiting the upregulation of FcγRIIb by basophils supernatants. Accordingly, when histamine effects were blocked with ranitidine, basophil supernatants did induce an upregulation of FcγRIIb on monocytes (Fig. 4B).

The upregulation of FcγRIIb was lost when IL-4 present in the basophil supernatant was blocked (data not shown), confirming our hypothesis that basophil-derived histamine masked the FcγRIIb induction by basophil-derived IL-4. In contrast, exogenously added histamine and IL-4 synergized in the inhibition of the LPS-induced CD80-expression on monocytes (Fig. 4C). Accordingly, inhibition of basophil supernatants with ranitidine and anti-IL-4 significantly reverted the inhibitory effect of basophil supernatants on CD80 expression, although the contribution of basophil-derived histamine appeared to be prevalent (Fig. 4D).

Taken together, these results indicate that basophil derived IL-4 and histamine can have combined effects, as shown for the inhibition of the costimulatory molecule CD80, but also contrasting effects. Remarkably, histamine was shown to modulate the ability of basophil-derived IL-4 to upregulate the inhibitory FcγRIIb on monocytes.

BASOPHILS DIRECTLY INHIBIT MONOCYTE ACTIVATION IN COCULTURE

We showed how basophil supernatants inhibit monocyte activation, with a contribution of IL-4 and histamine, two of the main mediators produced by basophils. Nonetheless, the release of mediators by basophils is known to be differentially regulated over time, with an immediate release of high amounts of granule-stored mediators (including histamine) upon IgE cross-linking, followed by an active production of cytokines and other mediators in the later phase of basophil activation.

Therefore, to better reproduce this physiological time course, and to verify possible additional effects dependent on cell-to-cell contact, we performed experiments in which basophils and monocytes were cocultured. LPS-induced TNF-α production by monocytes was inhibited in the presence of basophils (Fig. 5A).

Further activation of basophils with anti-IgE and IL-33 significantly enhanced the inhibition of TNF-α production by monocytes. This effect could be reverted by preincubation of monocytes with ranitidine and only partially by blocking IL-4, again pointing toward a prevalent action of basophil-derived histamine.

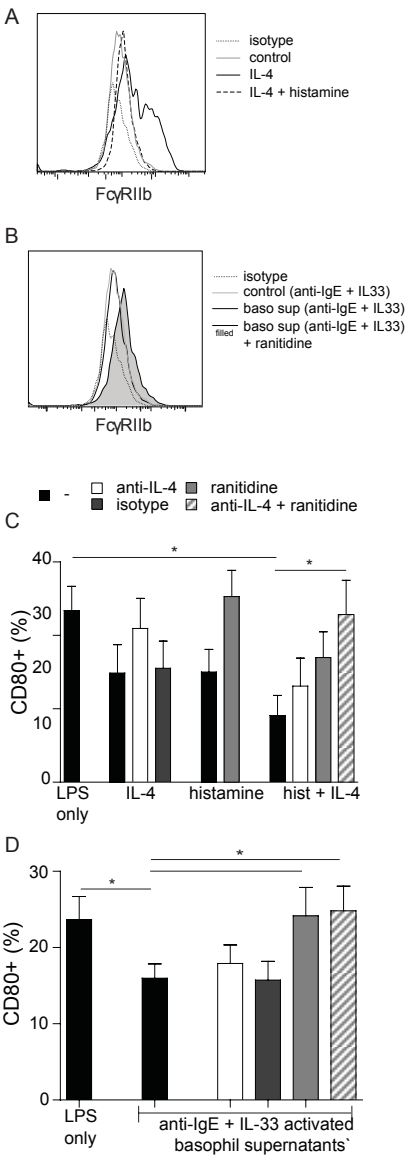


Figure 4. FcγRIIb and CD80 modulation by basophil supernatants. After 18 h of incubation with LPS (5 ng/mL) monocytes were analyzed by flow cytometry for the expression of surface markers. (A) FcγRIIb expressed by monocytes stimulated with rIL-4 (10 ng/mL), histamine (10−5 M), or both. (B) FcγRIIb expressed by monocytes conditioned with anti-IgE + IL-33 triggered basophil supernatants and blocked with H2R antagonist ranitidine. Histograms from a single experiment representative of five independent experiments are shown. (C–D) CD80 expression by monocytes incubated with (C) rIL-4 (10 ng/mL), and histamine (10−5 M) and with (D) anti-IgE + IL-33 triggered basophil supernatants. Blocking was performed by preincubation with anti-IL-4 antibody and matching isotype control or with histamine receptor 2 antagonist ranitidine. $DAPI^+$ cells were gated out as shown in Figure 2D. (C, D) Data are shown as shown as mean+SEM of percentages of $CD80^+$ cells and are pooled from five independent experiments (n = 5 monocyte donors) * $p < 0.05$ determined by ANOVA with Bonferroni's post-test.

Similar to what was observed for supernatants, LPS-induced IL-10 was significantly upregulated only when IL-4 was blocked (Fig. 5B). Control conditions with basophils alone did not produce TNF-α or IL-10, suggesting that the cytokines in the coculture were derived from monocytes (data not shown).Cell viability, as measured by DAPI staining, was comparable in all conditions (data not shown).

Furthermore, activated basophils in coculture with monocytes significantly inhibited LPS-induced CD80 expression with a contribution of both histamine and IL-4 (Fig. 5C and F). On the other hand, the expression of FcγRIIb was not influenced by the presence and activation of basophils, unless histamine effects were blocked by ranitidine (Fig. 5D). These data are similar to the results obtained with supernatants, as we could not observe, for the analyzed parameters, additional or different effects possibly due to cell-to-cell contact.

However, these results indicate that human basophils can influence monocyte responses when cells are being exposed to the proinflammatory stimulus at the same time, further confirming the immunomodulatory properties of basophil-derived soluble mediators.

DISCUSSION

Here, we show that human basophils, in addition to their known effector functions (*29*), can participate in immune regulation, by suppressing monocyte-mediated proinflammatory responses. In particular, purified human basophils, activated via FcεRI ligation and IL-33, inhibited the LPS-induced production of the prototypical proinflammatory cytokine TNF-α and the expression of the costimulatory molecule CD80 by monocytes. In other words, monocyte activation via a strong proinflammatory stimulus such as LPS was dampened when they were exposed to basophil supernatants or cocultured with basophils. Similar results were obtained using basophil supernatants or coculturing basophils and monocytes, suggesting that these effects do not require cell-to-cell contact and are mainly dependent on soluble mediators. Indeed, blocking experiments showed that basophil-derived IL-4 and, most of all, histamine, were responsible for the modulation of monocyte activity.

These data are in line with recent findings in a mouse model of allergic inflammation, in which basophils, via IL-4, were shown to induce the differentiation of inflammatory monocytes into anti-inflammatory macrophages (*10*). Likewise, in a mouse model of arthritis, basophils, by producing IL-4 in response to IL-33, were essential to mediate the immunosuppressive effects of IVIG (*8*).

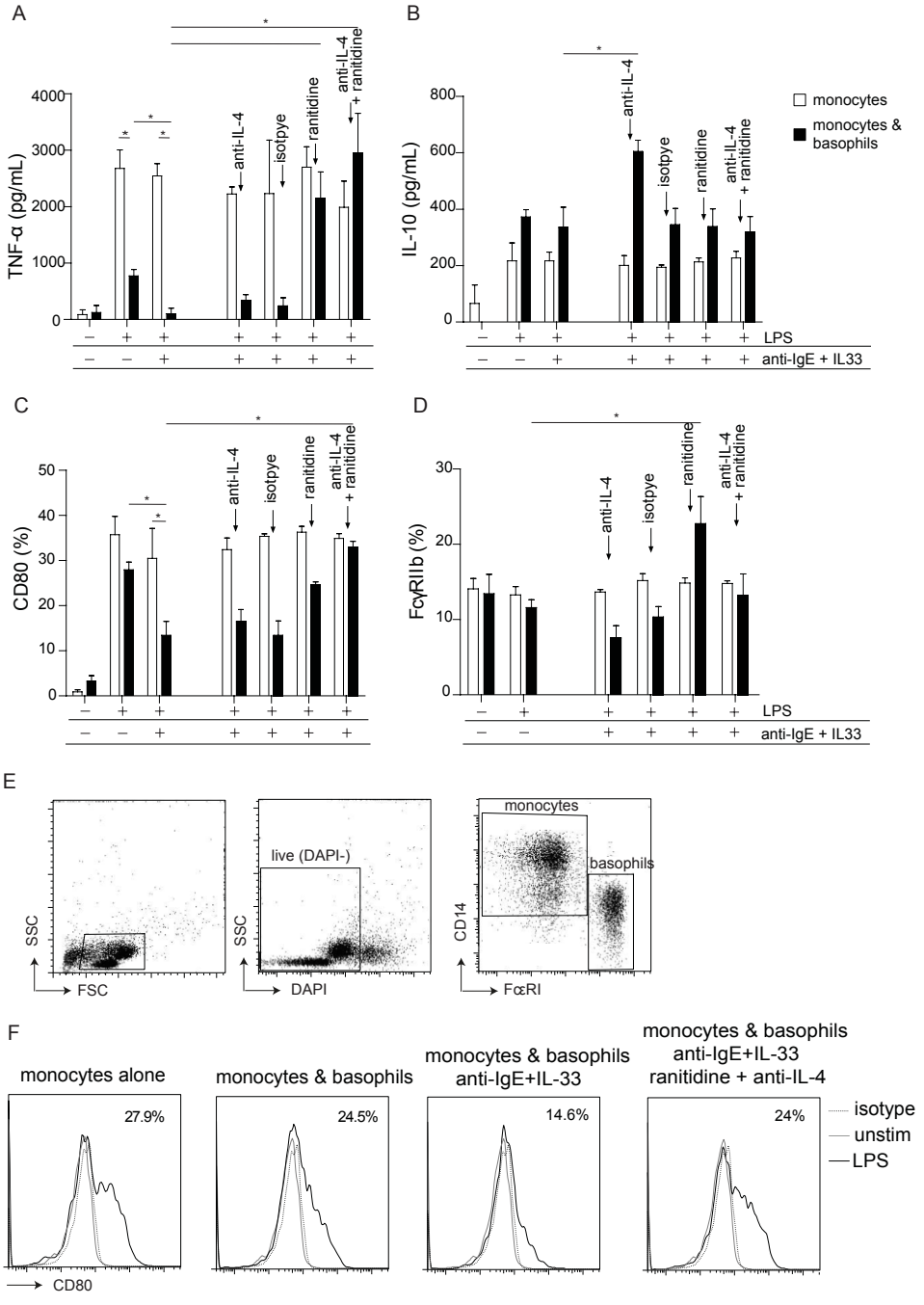

A
TNF-α (pg/mL)
anti-IL-4
isotpye
ranitidine
anti-IL-4 + ranitidine
B
IL-10 (pg/mL)
monocytes
monocytes & basophils
LPS
anti-IgE + IL33
C
CD80 (%)
D
FcγRIIb (%)
E
SSC
FSC
live (DAPI-)
DAPI
monocytes
basophils
CD14
FcεRI
F
monocytes alone
monocytes & basophils
monocytes & basophils anti-IgE+IL-33
monocytes & basophils anti-IgE+IL-33 ranitidine + anti-IL-4
27.9%
24.5%
14.6%
24%
isotype
unstim
LPS
CD80

Figure 5 *(left)*. Monocytes and basophils coculture. Isolated autologous basophils and monocytes were cultured alone or together, in the presence or absence of LPS (5 ng/mL) and anti-IgE (0.5 µg/mL) + IL-33 (100 ng/mL) for 18 h. The concentration of (A) TNF-α and (B) IL-10 in the supernatants of monocytes cocultured with basophils (black bars) or monocytes alone (white bars), followed by the activation with LPS and blocking by preincubation of cells with anti-IL4 antibody, matching isotype control, and ranitidine, was measured by ELISA. The cytokines in conditions with basophils alone were below detection limit and are not shown. (C, D) The percentages of (C) CD80 and (D) FcγRIIb in monocytes and monocytes cocultured with basophils are shown, as described in (A and B). (E) Example of gating strategy. (F) Histograms of CD80 expression by monocytes. Data from one donor representative of three independent experi-ments. (A–D) Data are shown as mean + SEM (n = 3 donors) and are pooled from three independent experiments.* $p < 0.05$; for comparison between two groups, Student t test was performed. For multiple comparisons of coculture conditions, ANOVA was used with Bonferroni's post-test.

We now provide evidence, for the first time, that basophils can mediate similar immunomodulatory effects in human settings. Because of the aforementioned studies in mice (*8, 10*), we expected basophil-derived IL-4 to be the main factor responsible for the anti-inflammatory effects of human basophils. Instead, our results show that histamine, specifically via H2R, is also involved in the modulation of the monocyte response. The interaction of basophil-derived histamine and IL-4 proved to be more complex regarding the modulation of the expression of the inhibitory FcγRIIb and the production of IL-10, which were differently regulated by histamine and IL-4.

In particular, histamine dampened the upregulation of the FcγRIIb by basophil-derived IL-4. A very recent publication offers an intriguing in vivo model to reproduce these findings in humans (*30*). Studying patients with primary immune deficiency, it was found that IVIG therapy suppresses dendritic cell function via IL-33-mediated induction of IL-4 and IL-13. Similar to our in vitro findings with monocytes, the authors did not observe an upregulation of the inhibitory FcγRIIb on dendritic cells, but on the contrary, an inhibition of the activating FcγRIIa. Our data, although not directly related to IVIG treatment, provides additional immunological insights and an alternative mechanism, suggesting that basophil-derived histamine is able to inhibit the IL-4 dependent upregulation of FcγRIIb, without influencing, in the short term, the expression of FcγRIIa. Overall, the involvement of histamine in the immunomodulatory ability of basophils has been apparently underestimated by previous studies, both in mice and humans, and most definitely warrants more attention.

To further show the relevance of histamine-mediated immune modulation, we provide evidence that very low concentrations of histamine still influence LPS-induced cytokine production by monocytes (Supplementary Fig. 1). Accordingly, very low amounts of IL-4 (as low as 1 pg/mL) could further modulate the histamine-induced responses (and vice versa), suggesting a balance between these two mediators in tuning monocyte responses.

The immunomodulatory properties of histamine have been investigated over the past years, showing both pro- and anti-inflammatory effects (*31*). As an example, in line with our observations, histamine via H2R, has been shown to inhibit the cytokine response to microbial products of monocytes (*28, 32*) and dendritic cells (*33*). Differentiation of monocytes into macrophages, on the other hand, induces the preferential expression of H1R over H2R (*34*), leading to increased production of IL-8 upon exposure to histamine (*35*). Therefore, the response to histamine can greatly vary among different cells, depending on expression profile of histamine receptors and among different individuals, possibly depending on genetic variation of histamine receptors (*36*). Our data further underline the relevance of histamine in immune regulation, showing that, among basophil-derived mediators, histamine represents a prominent factor responsible for the modulation of monocyte activation.

As for the intracellular mechanisms involved in the influence of basophil-derived histamine on TLR4 signaling, cAMP, induced upon histamine triggering of H2R (*31*), is known to inhibit NF-kB and therefore TNF-α production (*37*), while enhancing IL-10 (*38*). IL-4, on the other hand, via STAT-6, is able to inhibit both NF-kB activation (*39*) and IL-10 production (*40*). Overall, these intracellular pathways explain the effects on TLR4 signaling we observed when monocytes were exposed simultaneously to basophil-derived histamine and IL-4. While extensively studied independently, very little is known about the combined effects of these mediators on immune cells. In particular, our findings suggest for the first time histamine as an important player, which contribution has been so far underestimated, in the cross-talk between basophils and monocytes.

Although difficult to directly show in a human setting, our data argue for a basophil- and histamine-dependent contribution to immunomodulation in vivo. Plasma levels of histamine in healthy subjects are known to be less than 1 × 10−8 M (*41*). We found that the suppressive effects of histamine on LPS-induced TNF-α production by monocytes were present up to a histamine concentration of 10−9 M. Although other cells are able to secrete histamine, in the peripheral blood the amounts produced by basophils are estimated to be 100× to 1000× higher than the amounts produced by other leukocytes (*42*), suggesting that, even with their relatively low percentage, most of the circulating histamine is likely to

originate from basophils. Basophils can also infiltrate tissues, as shown in several inflammatory conditions (*43, 44*). Upon activation by local stimuli (exogenous and endogenous TLRs ligands, IL-33 etc.) basophil could, potentially, influence monocyte activation locally, as was shown in the previously discussed mouse model of allergic inflammation (*10*).

Unexpectedly, we observed the presence of histamine in basophil cultures in which the basophils remained unstimulated or were stimulated with IL-33 only. Although we can't exclude that histamine release is partially due to in vitro culture conditions, these findings are in line with previous studies showing histamine release by a mechanism defined as "piecemeal'' degranulation (*45*). While it would be tempting to speculate that the described IL-33-induced histamine release by human basophils and subsequent immune regulation are also operative in IgE-independent inflammatory conditions, further studies will be needed to address the relevance of the IgE-independent histamine release by basophils.

In conclusion, our data show that IL-33 significantly enhances IgE-mediated activation of basophils, inducing the production of IL-4 and the release of histamine. These basophil-derived mediators, in turn, were found to modulate monocyte responses, by suppressing their LPS-induced activation. Histamine, in particular, played a central role in basophil-mediated immune modulation. Overall, our observations show that human basophils are able to inhibit monocyte proinflammatory activation and point to a previously unrecognized role for human basophils, through the balanced secretion of IL-4 and histamine, in the regulation of the immune response.

MATERIALS AND METHODS

CELL ISOLATION

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, NL). For isolation of basophils, leukocyte-rich plasma was first prepared with HetaSep (StemCell Technologies) and basophils were then selected by negative magnetic bead isolation using the EasySep™ Human Basophil Enrichment Kit (StemCell Technologies), according to the manufacturers' instructions. The purity of enriched basophils (mean 98%) was determined by flow cytometric analysis of FcεRI, CD123, and CD203c, using BDCA-2 to exclude the presence of contaminating pDC. For monocyte isolation, PBMCs were isolated from buffy coats and $CD14^+$ monocytes were selected using magnetic-labeled anti-CD14 beads (Miltenyi Biotec), according to the manufacturer's instructions (mean purity 96%).

BASOPHIL ACTIVATION

After isolation, basophils were cultured in RPMI-1640 medium containing 10% fetal calf serum, glutamine, penicillin, and streptomycin (Invitrogen) at 37°C in 5% CO2 atmosphere at a concentration of 1 × 106 cells/mL. Basophils were stimulated using 0.5 μg/mL of goat-anti-human IgE (Nordic-MUbio), 100 ng/mL of recombinant human IL-33 (PeproTech), or both. After 24 h, cells were harvested, supernatants were collected and stored at –20°C and cells were used for flow cytometric analysis.

MONOCYTE STIMULATION

After isolation, monocytes were cultured in the same medium under the same conditions as basophils. Monocytes were incubated with basophil supernatants (diluted 1:4 in normal medium) or control media (containing the same stimuli used for basophil activation to exclude their direct effects on monocytes). In parallel, monocytes were incubated with recombinant human IL-4 (PeproTech) at a concentration of 10 ng/mL and histamine (Sigma-Aldrich) at a concentration of 10–5 M. Monocytes were stimulated with LPS from Salmonella typhosa (Sigma-Aldrich) at a concentration of 5 ng/mL. After overnight (18 h) incubation cells were harvested, supernatants collected and stored at –20°C for further analysis and cells were resuspended in PBS with 2 mM EDTA (Sigma-Aldrich) for 15 min on ice. Cells were detached by vigorously pipetting, washed, and resuspended in PBS 0.5% BSA for flow cytometric analysis.

For blocking experiments, supernatants of anti-IgE and IL-33 activated basophils were used. They were preincubated with anti-IL-4 antibody or matching isotype control rat IgG1 (both from eBioscience) at a concentration of 20 μg/mL for 30 min at 37°C in 5% CO2 atmosphere, prior to addition to the monocytes. For inhibition of histamine, monocytes were preincubated for 30 min at 37°C with histamine receptor 2 antagonist ranitidine at a concentration of 10–4 M. For blocking experiments with exogenous histamine in addition to ranitidine, histamine receptor 1 antagonist cetirizine and histamine receptor 3/4 antagonist thioperamide (Sigma-Aldrich) were used, at concentrations from 10–6 to 10–4 M.

BASOPHILS AND MONOCYTES COCULTURE

Isolated autologous basophils and monocytes were cultured alone or together at 1 × 106 cells/mL in a ratio 1:2, in the presence or absence of LPS and anti-IgE/IL-33 at the same concentrations mentioned above, for 16–18 h. Supernatants were collected and stored at –20°C for further analysis, monocytes were detached with PBS 2 mM EDTA as previously indicated and cells were used for flow cytometric analysis.

FLOW CYTOMETRY

For standard surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0.5% BSA at 4ºC for 30 min. After washing, cells were suspended in PBS 0.5% BSA. To exclude dead cells, cells were suspended in PBS/0.5%BSA and just prior to flow cytometric acquisition, 0.2 µM DAPI (Invitrogen) was added.

Cells were acquired on a LSR-II (BD Biosciences) and analysis was performed using FlowJo software (Tree Star, Inc). The following antibodies (clone) were used: CD123-PerCpCy5.5 (clone 7G3), CD63-PE (H5C6), HLA DR-FITC (L243), CD80-PE (L307.4), CD16-FITC (3G8), CD64 PE (10.1), mIgG2b-FITC (27–35), IgG2ak-FITC (X39). IgG2ak-allophycocyanin (MOPC-21) from BD Biosciences; FcεRI-FITC (AER-37), CD14-PerCP-Cy5.5 (61D3), mIgG1-PE (P3.6.2.8.1), mIgG2b-PE (eBMG2b), mIgG2b-PerCP-Cy5.5 (MOPC-21) from eBioscience; BDCA2-PE (AC144) and CD203c (FR 3–16A11) from Miltenyi and CD32-FITC (IV.3) from Stemcell. AlexaFluor488-CD32b (ch2b6-N297Q) and corresponding isotype control (ch4420-N297Q) were generously provided by Macrogenics (*46, 47*).

MEASUREMENT OF CYTOKINES

IL-4 was measured using the IL-4 Human PeliPair™ ELISA kit (Sanquin Reagents), TNF-α and IL-10 were measured using the Human TNF ELISA set (BD Biosciences) and the IL-10 Human PeliPair™ ELISA kit (Sanquin Reagents), respectively. Histamine was measured using the Histamine ELISA kit (Neogen). All kits were used according to manufacturer's instructions.

STATISTICAL ANALYSIS

Data are expressed as mean + SEM. For comparisons of two groups, paired Student's t-test was performed. For comparisons of multiple groups, ANOVA was performed with Bonferroni's post-test to determine statistical significance between each group. The number of independent experiments is indicated in each figure legend and each independent experiment represents a different donor. P-values <0.05 were considered significant and are shown in the figures. Analyses were performed using GraphPad Prism 5 Software (GraphPad Inc Software).

ACKNOWLEDGMENTS

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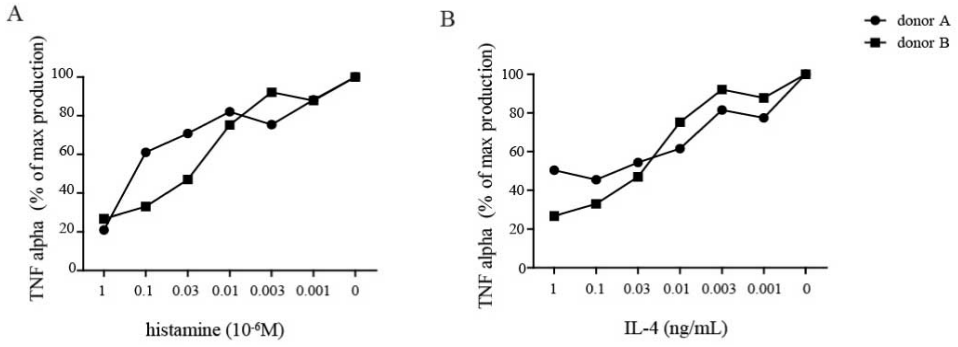
SUPPLEMENTARY FIGURE



Supplementary Figure 1. Titration of IL-4 and histamine effects on LPS-induced activation of monocytes. Isolated monocytes were activated with LPS together with decreasing amount of IL-4 (A) or histamine (B). After over-night incubation, TNF-alpha was measured by ELISA in the supernatants. Results are expressed as percentages of maximal TNF-alpha production. Results from 2 independent experiments (2 donors) are shown.

Differential TLR-induced cytokine production by human mast cells is amplified by FcεRI triggering

Chapter 4

Jolien Suurmond,[1] Annemarie L Dorjée, [1] Edward F Knol,[2]
Tom WJ Huizinga, [1] René EM Toes[1]

[1]Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; [2]Departments of Immunology and Dermatology/ Allergology, University Medical Center Utrecht, Utrecht, The Netherlands

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SUMMARY

Background Mast cells are mainly present in strategic locations, where they may have a role in defence against parasites and bacteria. These pathogens can be recognized by mast cells via Toll-like receptors (TLR). Allergic symptoms are often increased in the presence of pathogens at the site of allergen exposure, but it is unknown which cytokines can mediate such an effect.

Objective To study whether an interaction between IgE- and TLR-mediated activation of human mast cells can contribute to exacerbated inflammatory responses.

Methods Peripheral blood-derived mast cells were stimulated with TLR ligands, in the presence or absence of anti-IgE triggering, after which degranulation was measured using flow cytometry and cytokine production was evaluated by multiplex assays, and ELISA. For evaluation of allergen-specific responses, mast cells were sensitized with serum of allergic individuals or controls, after which they were stimulated using allergens in combination with TLR ligands.

Results Simultaneous triggering of mast cells via IgE and TLR ligands greatly enhanced cytokine production but not IgE-induced degranulation. Different TLR ligands specifically enhanced the differential production of cytokines in conjunction with FcεRI triggering. Importantly, only TLR-4 and TLR-6 were able to induce robust production of IL-13, an important molecule in allergic reactions.

Conclusions & Clinical Relevance These results indicate that the simultaneous presence of pathogen- or danger-associated signals and FcεRI triggering via specific IgE can significantly modify mast cell-mediated allergic reactions via synergistic production of cytokines and inflammatory mediators and provide an explanation of augmented allergic symptoms during infection.

INTRODUCTION

Mast cells are immune cells that reside in all tissues and are most well known for their role in IgE-mediated allergic responses. Mast cells are found at strategic locations where they can encounter pathogens, such as the skin and mucosal surfaces like the gut and the respiratory tract. Mast cells have been well established to play a role in the defence against bacteria, certain parasites (mainly intestinal helminth parasites), and possibly against fungal and viral infections (*1-5*). Recognition of pathogens via Toll-like receptors (TLR) has been shown to contribute to mast cell responses to pathogens (*5*).

In addition to activation via innate receptors in primary responses, IgE that is produced in response to parasites or other pathogens may also play an important role in recognition of pathogens in secondary or chronic infection (*6*) or, as described recently, in the protection against venoms upon second exposure (*7*).

However, although such responses may play an important role in the protection against secondary exposure to pathogens or venoms, these enhanced responses can have detrimental effects in responses when no danger is present, such as is the case in allergic responses (*8*). Viral and bacterial infections have been associated with asthma exacerbations (*9, 10*), and the presence of the TLR-4 ligand endotoxin was demonstrated in house dust and was related to severity of allergic reactions against house dust mite (*11*). Furthermore, allergens have been reported to promote TLR signalling directly (*12*). Therefore, mast cell activation via TLRs may contribute to these processes when triggered at the same time by allergen-specific IgE. Studies using mouse mast cells have shown contrasting data concerning the degranulation and cytokine response upon combined antigen and TLR triggering (*13-15*).

In human mast cells, it has been shown that prolonged pre-treatment with LPS can significantly enhance IgE-mediated mast cell responses (*15*), and it is therefore been hypothesized that TLR triggering of mast cells can contribute to asthma exacerbations. We and others have previously shown that mast cells can differentially secrete cytokines depending on the TLR that is triggered (*16-18*). However, it is unknown whether differential cytokine production is still present upon combined IgE-mediated responses. This is important, as different mast cell mediators have distinct functions in allergic responses.

Therefore, we aimed to characterize human mast cell responses upon combined activation via (allergen-specific) IgE and TLR triggering and to analyse the specific cytokines induced by different TLR ligands.

METHODS

PERIPHERAL BLOOD-DERIVED MAST CELLS

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, the Netherlands). $CD34^+$ hematopoietic stem cells were isolated from mononuclear cells with CD34 microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) and were differentiated into mast cells as described (*16*). The purity of mast cells ranged from 90 to 99%.

MAST CELL ACTIVATION

Cultured mast cells were sensitized with 0.1 µg/mL hybridoma IgE (Diatec Monoclonals, Oslo, Norway) for a minimum of 18 h, after which they were activated with goat anti-human IgE (Nordic-MUbio, Susteren, The Netherlands) to induce IgE-mediated activation. For TLR-mediated activation, mast cells were stimulated using S. aureus Peptidoglycan (Pgn), Poly(I:C), E. Coli K12 lipopolysaccharide (LPS), S. typhimurium flagellin, FSL-1, 10 µg/mL ssRNA40 (all from InVivoGen, San Diego, CA, USA) for 24 h. A titration was performed to determine the optimal concentrations of stimuli, using the following concentrations (high, medium, low): aIgE: 10, 0.5, 0.05 µg/mL; Peptidoglycan: 10, 5, 1 µg/mL; LPS: 10, 0.5, 0.05 µg/mL; Flagellin: 1, 0.1, 0.01 µg/mL; FSL-1: 1, 0.1, 0.01 µg/mL; ssRNA40: 10, 1, 0.1 µg/mL.

For combined activation via IgE and TLRs, IgE-sensitized mast cells were activated with suboptimal and optimal concentrations of anti-IgE (10 and 0.01 µg/mL), Peptidoglycan (10 and 5 µg/mL), LPS (10 and 0.5 µg/mL), flagellin (1 and 0.1 µg/mL), FSL-1 (1 and 0.1 µg/mL) and ssRNA40 (10 and 1 µg/mL).

For allergen-specific activation, mast cells were sensitized for 18 h with serum from house dust mite-allergic individuals and control individuals, diluted 1 : 1 in culture medium. The presence of house dust mite-specific IgE was measured by ImmunoCAP (Phadia Thermo Fisher Scientific, Uppsala, Sweden), with 0.35 UA/mL as cutoff. Mast cells were washed with medium and subsequently activated with 10 µg/mL anti-IgE or 0.25 U/mL house dust mite allergen (Dermatoph. pteronyssinus, HAL Allergy, Leiden, The Netherlands) in the presence or absence of LPS.

FLOW CYTOMETRY

For detection of mast cell degranulation, cells were incubated with fluorochrome-conjugated antibodies recognizing CD117, CD203c and CD63 or matching isotype controls. Cells were taken up in 1% paraformaldehyde until flow cytometric acquisition on a FACS Calibur (BD Biosciences, San Jose, CA, USA). Analysis was performed using FACS Diva software (BD) and FlowJo.

CYTOKINE SECRETION

Quantitative immunoassays for EGF, Eotaxin, FGF-2, Flt-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFNγ, IL-1ra, IL-1α, IL-1β, IL-2, sIL-2Rα, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGF-α, TNF-α, TNF-β and VEGF in culture supernatants were performed using the 42-plex cytokine or custom-made Milliplex assays

(EMD Millipore, Billerica, MA, USA). Additionally, IL-8 and IL-13 production was evaluated using ELISA (eBioscience, San Diego, CA, USA).

STATISTICAL ANALYSIS

Results are expressed as mean + SEM. For comparison of multiple groups, one-Way ANOVA was performed, with Bonferroni's post-test to correct for multiple testing. For comparison of two groups, Student's t-test was performed. Statistical analysis was performed using GraphPad Prism 4 (Graphpad Software, La Jolla, CA, USA). P values of < 0.05 were considered statistically significant.

RESULTS

DIFFERENTIAL CYTOKINE PRODUCTION UPON TITRATION OF TLR LIGANDS

We have previously shown that mast cells functionally express TLR-2, TLR-4, TLR-5, TLR-6, and TLR-8 (*16*). To determine the optimal concentrations of stimuli, the ligands for these TLRs were titrated, after which cytokines were measured in supernatant (Fig. 1). For most cytokines, the highest concentration of TLR ligands was most potent in inducing cytokine production. An exception is IL-8, which was also significantly up-regulated at lower concentrations of several TLR ligands.

Importantly, a differential cytokine profile was observed, with LPS and FSL-1 (TLR-4 and -6 ligands) showing a nearly similar pattern of upregulation of GM-CSF, IL-8 and IL-10, whereas ssRNA (TLR-8 ligand) induced IL-8, MIP-1α and TNF-α. As the optimal concentration of stimuli was different for IL-8 as most other cytokines, we decided to evaluate interaction between TLR- and IgE-mediated activation using both suboptimal and optimal concentrations.

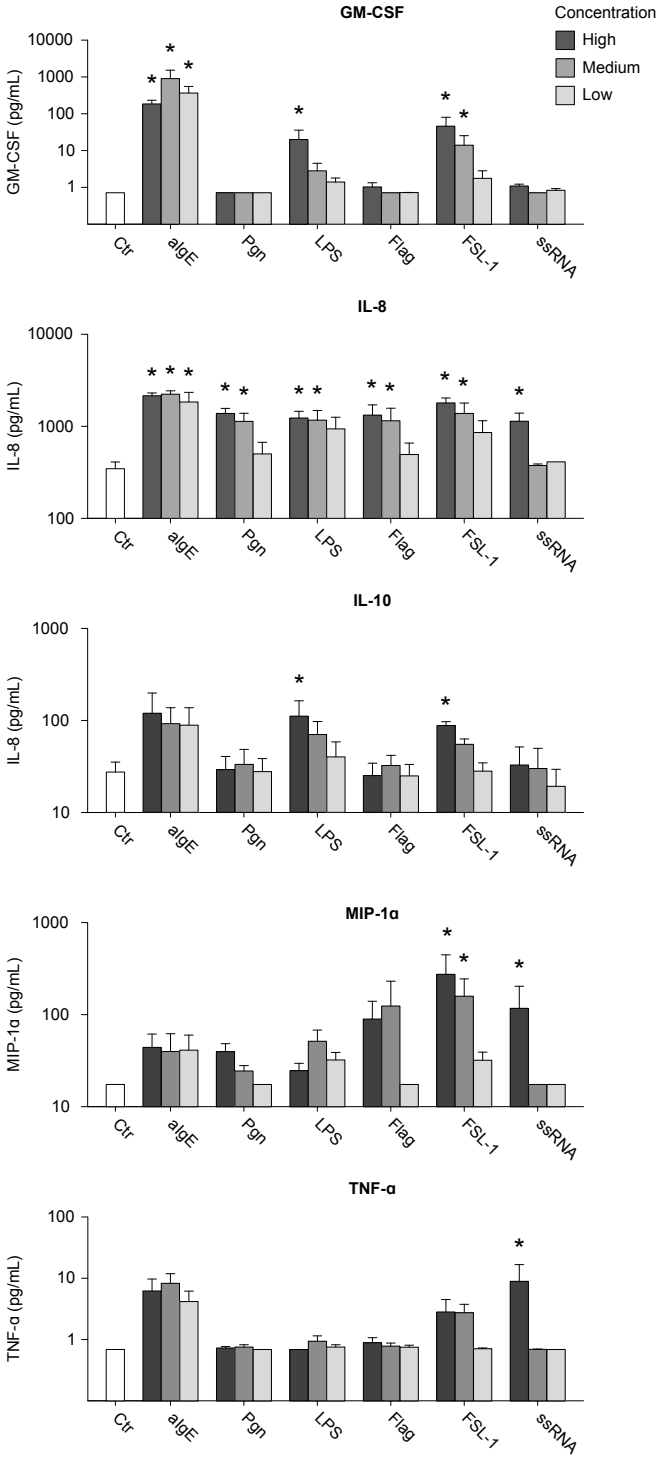

Figure 1. Mast cells were stimulated with control medium (ctr) or different concentrations of anti-IgE, Peptidoglycan (Pgn; TLR-2), LPS (TLR-4), Flagellin (TLR-5), FSL-1 (TLR-6) and ssRNA (TLR-8). The concentrations of stimuli (high, medium, low) used were as follows: aIgE: 10, 0.5, 0.05 µg/mL; Peptidoglycan: 10, 5, 1 µg/mL; LPS: 10, 0.5 and 0.05 µg/mL; Flagellin: 1, 0.1, and 0.01 µg/mL; FSL-1: 1, 0.1, 0.01 µg/mL; ssRNA40: 10, 1 and 0.1 µg/mL. Cytokine production was measured in supernatant after 24 h. Asterisks indicate significantly ($P < 0.05$) increased cytokine production compared to control medium (ctr), using one-way ANOVA with Bonferroni's post-test (n = 3 independent mast cell donors).

DIFFERENTIAL CYTOKINE PRODUCTION IS AMPLIFIED BY COMBINED TLR- AND FCεRI-MEDIATED ACTIVATION

We next evaluated possible interactions between triggering of FcεRI and activation via TLRs. FcεRI was crosslinked using anti-IgE in the presence or absence of TLR ligands for 24 h, after which cytokine production was measured. We observed amplified cytokine responses upon combined stimulation by several TLR ligands and anti-IgE (Figs 2 and 3).

The cytokines were separated based on their pattern of induction by different TLR ligands. The TLR-4 ligand LPS in combination with anti-IgE significantly enhanced the production of GM-CSF, IL-5, IL-8 (at suboptimal concentrations), IL-10 and IL-13, in comparison with anti-IgE alone (Fig. 2). The effect of TLR-6 ligand FSL-1 resembled this pattern, although with a smaller magnitude. Therefore, only IL-8 and IL-13 were significantly enhanced by FSL-1. In contrast to this pattern, TLR-8 ligand ssRNA significantly enhanced the production of several other cytokines, EGF, GRO-α, MIP-1α and TNF-α (Fig. 3).

These results suggest that the response to TLR ligands was different depending on the TLR that was being triggered. Importantly, when comparing the cytokine profiles of TLR ligands without anti-IgE to the profiles in presence of anti-IgE, there is a striking resemblance between the profiles, indicating that enhancement of specific cytokines by FcεRI triggering is defined by specific TLRs. These results indicate that the combination of TLR triggering and anti-IgE synergizes in cytokine production by mast cells, where the exact cytokine profile is defined by the TLR ligand.

SYNERGY BETWEEN ALLERGEN-SPECIFIC IGE-MEDIATED ACTIVATION AND TLR TRIGGERING

To confirm the synergy in production of IL-8 and IL-13 upon triggering with LPS in an antigen-specific system, we next evaluated mast cell activation through allergen-specific IgE. Mast cells were sensitized with serum of house dust mite-(HDM) allergic individuals, and subsequently stimulated with HDM, leading to mast cells degranulation and activation, as shown by upregulation of CD63 and CD203c and release of IL-8 (Figs 4a and b). In contrast, sensitization with serum from non-allergic individuals did not induce HDM-induced mast cell activation.

The presence of LPS did not affect the HDM-induced degranulation (CD63) or upregulation of CD203c (Fig. 4c). Intriguingly however, combining TLR-signals with FcεRI triggering via HDM-specific IgE led to enhanced production of IL-8 and IL-13 (Figs 4d and e). Specifically, IL-13 production increased twofold to eightfold upon combined activation as compared to the cumulative production of each stimulus alone (Figs 4d and e).

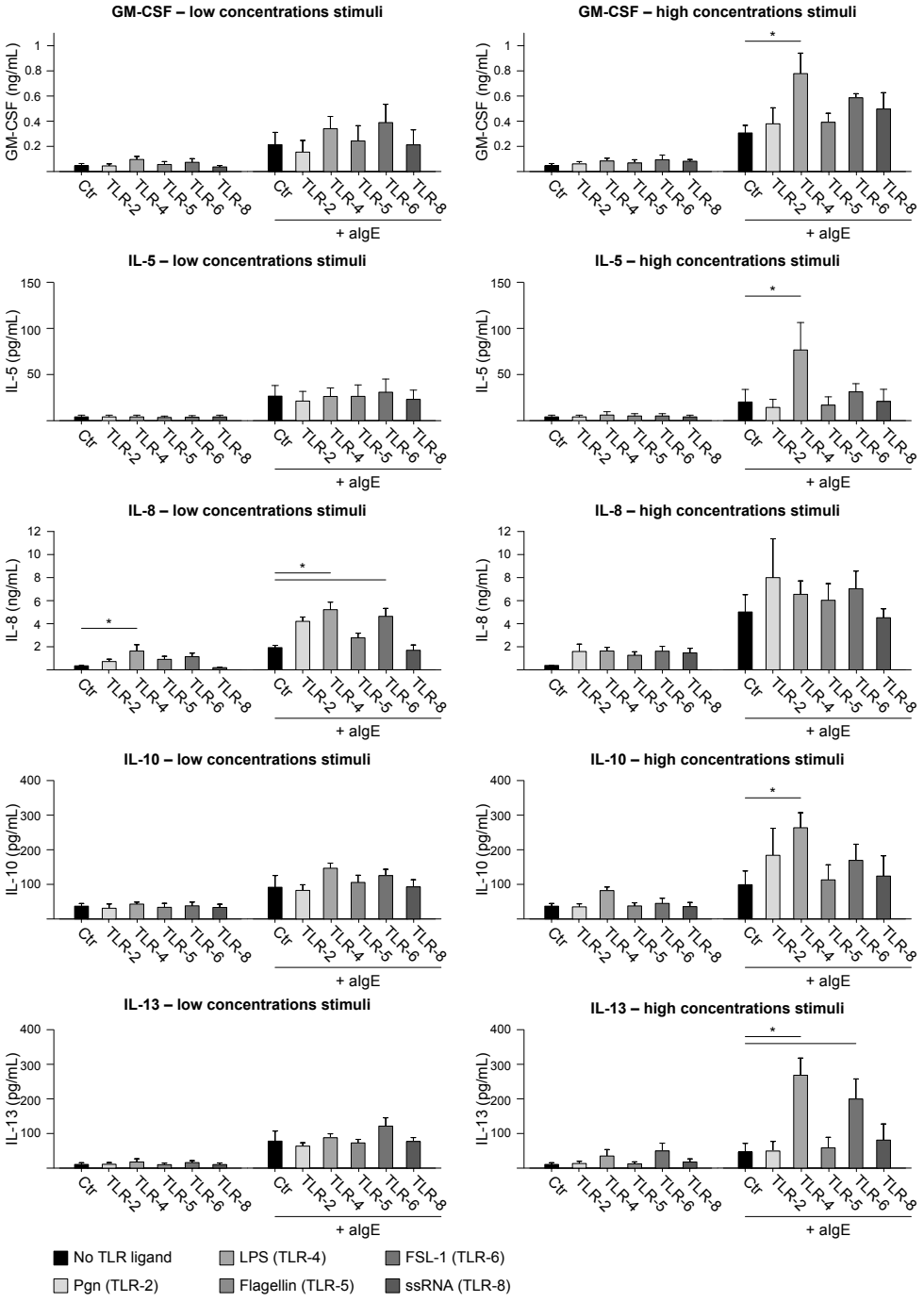

Figure 2. This figure shows the cytokines which are upregulated in response to TLR-4 and TLR-6 ligands. See legend of Figure 3 for experimental details.

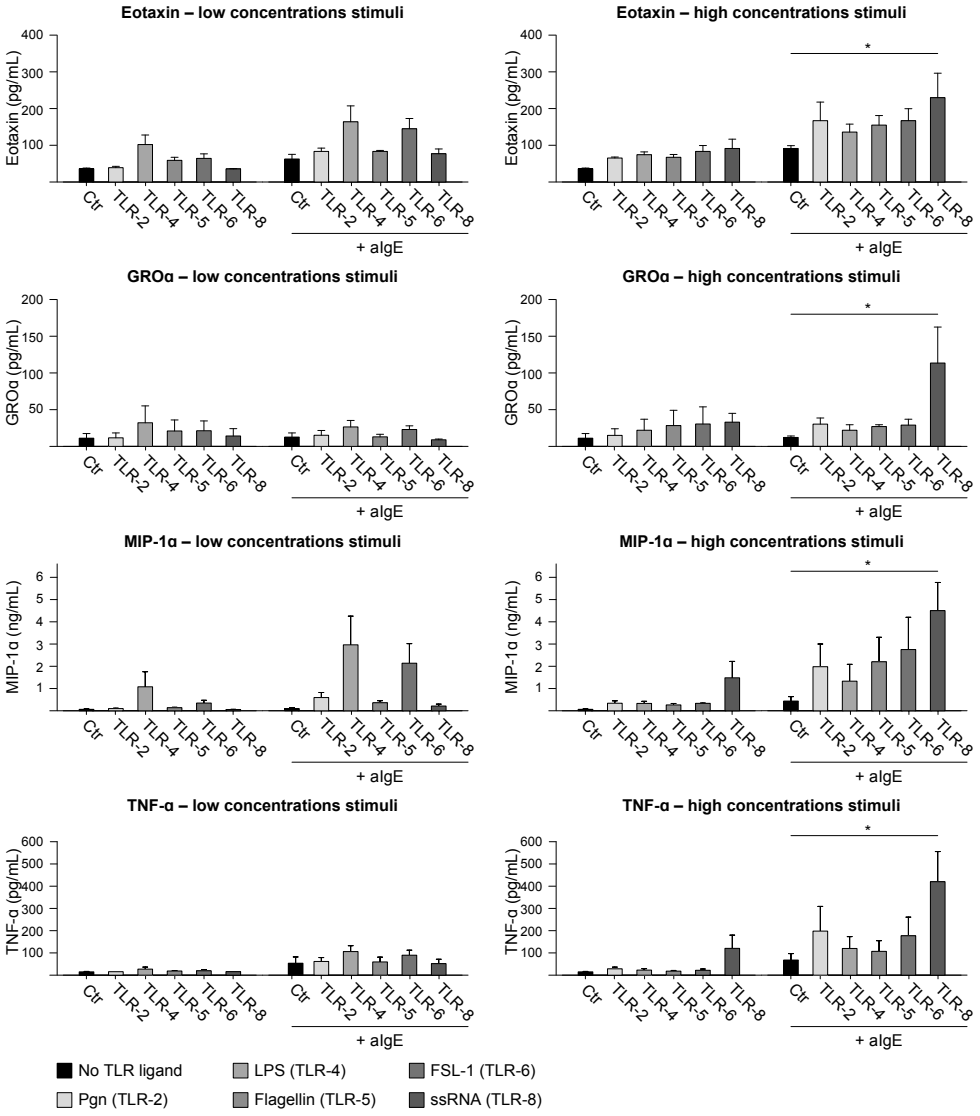

Figure 3. Mast cells were stimulated with Peptidoglycan, LPS, Flagellin, FSL-1 and ssRNA alone or in combination with anti-IgE at low (left) or high (right) concentrations. Low concentrations used were as follows: anti-IgE (0.01 µg/mL), Peptidoglycan (5 µg/mL), LPS (0.5 µg/mL), flagellin (0.1 µg/mL), FSL-1 (0.1 µg/mL) and ssRNA40 (1 µg/mL). High concentrations used were as follows: anti-IgE (10 µg/mL), Peptidoglycan (10 µg/mL), LPS (10 µg/mL), flagellin (1 µg/mL), FSL-1 (1 µg/mL) and ssRNA40 (10 µg/mL). Cytokine production was measured in supernatant after 24 h. Asterisks indicate significantly ($P < 0.05$) increased cytokine production compared with control medium (ctr) or anti-IgE alone, as indicated in the figure, using one-way ANOVA with Bonferroni's post-test. Data shown are from $n = 3$ independent experiments and mast cell donors. This figure shows the cytokines which are upregulated in response to TLR-8 ligand.

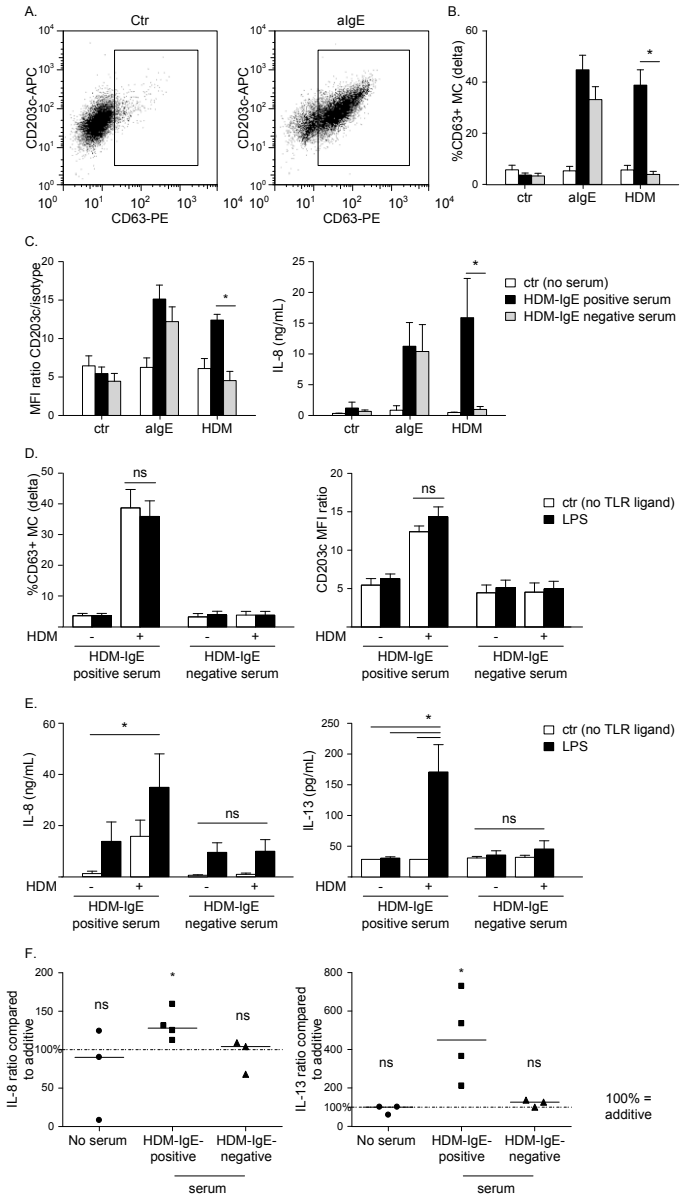

Figure 4. Mast cells were sensitized with serum of house dust mite allergic individuals, after which they were stimulated for 24 h with house dust mite (HDM) allergen or anti-IgE as positive control. (a) Representative flow cytometry plots for CD63 and CD203c, gated on CD117^{+} mast cells. (b,c) Summary of data of CD63 and CD203c surface expression as well as IL-8 production in supernatant. (d) Surface expression of CD63 and CD203c upon combined activation of sensitized mast cells with HDM and LPS. (e) Production of IL-8 and IL-13 after combined activation of sensitized mast cells with HDM and LPS. (f) Relative increase in cytokine production upon combined activation is shown as percentage of cytokine production in response to combined triggering divided by the sum of the cytokine production following stimulation with HDM and LPS separately. Asterisks indicate significantly ($P < 0.05$) increased degranulation (b,c) or cytokine production (c–f) as indicated in the figure, using Student's t-test. Data shown are from n = 3 independent experiments and mast cell donors, using serum from 4 HDM-IgE-positive and 3 HDM-IgE-negative individuals.

Together, these results show that TLR- and IgE-mediated activation of human mast cells can synergistically enhance their activation, leading to enhanced cytokine production by mast cells sensitized with allergen-specific IgE when exposed to allergens and TLR ligands.

DISCUSSION

Our results show that the combination of IgE- and TLR-mediated activation greatly enhances cytokine production by mast cells, but not their degranulation. Whereas mast cell degranulation is important for acute-phase allergic responses, cytokine production is considered to be more important for the, more inflammation related, late-phase responses (*19, 20*). Therefore, our results suggest that the presence of TLR ligands may specifically enhance the late-phase responses in allergy. Our results indicate that the type of cytokine response that is induced depends on the TLR that is being triggered. For example, IL-13 production was only enhanced by ligands for TLR-4 and TLR-6. The presence of endotoxin (TLR-4 ligand) in house dust has been associated with allergic symptoms (*10*), and LPS has been shown to enhance allergic symptoms in a mouse model of house dust mite allergy (*21*). IL-13 is an important cytokine implicated in the induction of several key symptoms in allergy, such as enhancing mucus hypersecretion and airway hyperresponsiveness (*22*). Our results indicate that synergy in IL-13 production by mast cells when they are exposed to LPS and house dust mite could contribute to endotoxin-enhanced allergic responses to house dust mite.

A clear inherent limitation of our study is that we used in vitro-generated mast cells, rather than primary mast cells isolated from tissue. The number of mast cells obtained from tissues is limited, and isolation of mast cells often leads to their activation, making it difficult to perform functional studies evaluating cytokine production (*23*). We and others have shown that in vitro-generated mast cells resemble tissue mast cells in various aspects (*16, 23, 24*). However, we cannot exclude that cytokine production by mast cells in vivo is also influenced by the local tissue environment. We did not observe a response of mast cells to house dust mite extract as measured by CD63-upregulation, or IL-8, respectively, IL-13-release. In contrast, this allergen only induced such a response when mast cells were sensitized with serum from allergic individuals, suggesting that the house dust mite extract did not directly activate mast cells. This is in contrast with studies in the mouse showing that der p 2 present in house dust mite could directly promote TLR signalling (*12*). Our results suggest that the activation of human mast cells by house dust mite allergens through specific IgE is much more potent than the activation through TLR.

Other studies have evaluated TLR-mediated potentiation of IgE-dependent release of TNF-α, for example using mast cell-lines or mouse mast cells. In mouse bone marrow-derived mast cells, a number of cytokines were synergistically enhanced by combined FcεRI and TLR-2 or TLR-4 triggering, but only few cytokines were analysed (*13*). In the human mast cell line LAD2, no TNF-α potentiation was observed when TLR-2 or TLR-4 ligands were combined with anti-IgE (*25*). In our study, we also did not observe significant enhancement

of TNF-α with LPS. Rather, a group of cytokines, including TNF-α, was specifically enhanced with the TLR-8 ligand ssRNA, and another group of cytokines, including IL-5 and IL-13, was enhanced by LPS. Therefore, our results suggest that potentiation of cytokine production depends on the specific TLR that is being triggered, and that analysis of mast cell responses to TLR ligands requires the measurement of multiple cytokines. We provide a technique to sensitize mast cells with allergen-specific IgE using serum of allergic individuals, which allowed us to analyse allergen-specific mast cell responses in a specific manner.

In conclusion, our data show that IgE-mediated activation of human mast cells is synergistically enhanced by TLR ligands in a cytokine-specific and TLR-ligand-specific manner. These data could therefore contribute to further the understanding of exacerbations of allergic reactions during infections.

ACKNOWLEDGEMENTS

We thank J.A. Bakker and A. Grummels (CMIL, Leiden University Medical Center) for measurement of house dust mite-specific IgE in serum. This work was supported by the Dutch Arthritis Foundation, the Dutch Organization for Scientific Research (Vici grants), the Research Foundation Sole Mio, the Leiden Research Foundation (STROL), the Centre for Medical Systems Biology (CMSB) within the framework of the Netherlands Genomics Initiative (NGI), the IMI JU funded project BeTheCure, contract no 115142-2, and European Union (Seventh Framework Programme integrated project Masterswitch; grant Number: 223404).

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TOLL-LIKE RECEPTOR TRIGGERING AUGMENTS ACTIVATION OF HUMAN MAST CELLS BY ANTI-CITRULLINATED PROTEIN ANTIBODIES

Chapter 5

Jolien Suurmond,[1] Felice Rivellese,[1] Annemarie L Dorjée,[1] Aleida M Bakker,[1] Yoann JPC Rombouts,[1,2] Theo Rispens,[3] Gertjan Wolbink,[3] Arnaud Zaldumbide,[4] Rob C Hoeben,[4] Tom WJ Huizinga,[1] René EM Toes[1]

[1]Department of Rheumatology, Leiden University Medical Center (LUMC), Leiden, The Netherlands; [2]Center for Proteomics and Metabolomics, LUMC, Leiden, The Netherlands; [3]Sanquin Research and Landsteiner Laboratorium, Academic Medical Center, Amsterdam, The Netherlands; [4]Department of Molecular Cell Biology, LUMC, Leiden, The Netherlands

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ABSTRACT

Objective Mast cells may play a role in rheumatoid arthritis (RA), but activation of human mast cells in autoimmune settings has been little studied. Toll-like receptors (TLR) and Fcγ receptors (FcγR) are important receptors for cellular activation in the joint, but expression and stimulation of these receptors in human mast cells or the functional interplay between these pathways is poorly understood. Here, we analysed triggering of human mast cells via these receptors in the context of anti-citrullinated protein antibody-positive (ACPA+) RA.

Methods RNA and protein expression of TLRs and FcγR was quantified using PCR and flow cytometry, respectively. Mast cells were stimulated with TLR ligands (including HSP70) combined with IgG immune complexes and IgG-ACPA.

Results Human mast cells expressed TLRs and produced cytokines in response to TLR ligands. Both cultured and synovial mast cells expressed FcγRIIA, and triggering of this receptor by IgG immune complexes synergised with activation by TLR ligands, leading to two- to fivefold increased cytokine levels. Mast cells produced cytokines in response to ACPA immune complexes in a citrulline-specific manner, which synergised in the presence of HSP70.

Conclusions Our data show that synovial mast cells express FcγRIIA and that mast cells can be activated by IgG-ACPA and TLR ligands. Importantly, combined stimulation via TLRs and immune complexes leads to synergy in cytokine production. These findings suggest mast cells are important targets for TLR ligands and immune complexes, and that combined activation of mast cells via these pathways greatly enhances inflammation in synovial tissue of RA patients.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic inflammation of the synovial lining of the joint. Antibodies against citrullinated proteins (ACPA) can be found in the majority of patients with established RA (*1*). These antibodies recognise proteins in which arginine is converted into citrulline through a post-translational modification. ACPA are associated with disease progression (*2*) and their pathogenic potential has been shown in mice (*3, 4*). The exact way by which ACPA contribute to inflammation is not known, but ACPA immune complexes have been shown to allow the activation of monocytes and macrophages (*5, 6*). However, several other immune cells may contribute to ACPA-mediated immune responses in RA.

One of these cell subsets are mast cells, which comprise up to 5% of the synovial cells. Several findings in patients support an active role of mast cells in RA pathogenesis. Mast cell numbers are increased in RA synovium (*7, 8*). Furthermore, several mast cell mediators have been observed in the synovial fluid of RA patients (*8, 9*), and these mediators have been shown to have pathogenic effects on synovial fibroblasts (*10, 11*), suggesting that mast cells may play a role in inflammatory processes in RA. However, the specific pathways leading to mast cell activation in RA have not been shown.

The main biological function of mast cells is considered to be protection against pathogens (*12*). Recognition of pathogens by mast cells via toll-like receptors (TLRs) may lead to inflammatory responses such as recruitment of neutrophils and T cells. TLR ligands can also be released during chronic inflammatory responses, and are thought to contribute to the chronicity of RA (*13-17*). Although studies performed in mice suggest that mast cells can be activated by TLR ligands, there is only limited information on TLR triggering of human mast cells. Some studies have shown expression of TLR-2, -3 and -4 by human mast cells, but the response to other TLRs and the type of response that is induced in mast cells is not clear (*18-22*). It has been suggested that the innate responses of mast cells towards pathogen-derived or endogenous TLR ligands may be enhanced by the presence of specific antibodies, such as those present during a memory response to pathogens, allowing a rapid clearance of the infecting microbe.(*12*) However, in the context of autoimmunity, such an enhanced response can have harmful effects, and may contribute to aberrant and continuous activation of the immune system.

As mast cells are present in RA synovium, a location where endogenous TLR ligands and citrullinated proteins have been found, combined mast cell activation via TLR and ACPA immune complexes could play a role in chronic immune activation in RA. Because the expression of TLRs and the functional response towards TLR ligands in human mast cells is not established, and because it is not known whether mast cells can be activated by ACPA immune complexes, we aimed to thoroughly analyse mast cell responses upon TLR and FcγR triggering as well as the interplay between TLR- and IgG-mediated responses in ACPA+ RA.

METHODS

PERIPHERAL BLOOD-DERIVED MAST CELLS

Buffy coats from healthy volunteers were obtained from a blood bank (Sanquin, The Netherlands). $CD34^+$ haematopoietic stem cells were isolated from peripheral blood mononuclear cells (PBMCs) with CD34 microbeads (Miltenyi Biotec). Isolated stem cells

were differentiated into mast cells as described.(*23*) After 6–8 weeks, the purity of mast cells was determined by flow cytometric analysis of CD117, FcεRI, CD203c and intracellular tryptase. The purity of mast cells ranged from 90% to 99%.

REAL-TIME PCR

One million mast cells per donor were lysed in TRIzol reagent (Invitrogen). RNA was isolated using TRIzol. A cDNA library was constructed using the RT2 First Strand Kit (SA Biosciences). Expression of mRNA for TLRs and the TLR signalling pathway was assessed by real-time PCR, using the Toll–Like Receptor Signaling Pathway PCR Array (SA Biosciences). Expression was normalised to housekeeping gene HPRT1.

MAST CELL ACTIVATION

Cultured mast cells were sensitised with 0.1 µg/mL IgE (Diatec) for 18 h, after which they were activated with 10 µg/mL goat anti-human IgE (Nordic). For TLR-mediated activation, mast cells were stimulated using 1 µg/mL Pam3csk, 10 µg/mL zymosan, 10 µg/mL Staphylococcus aureus peptidoglycan, 10 µg/mL poly(I:C), 0.1–10 µg/mL Escherichia coli K12 lipopolysaccharide (LPS), 1 µg/mL Salmonella typhimurium flagellin, 1 µg/mL FSL-1, 5–10 µg/mL imiquimod, 10 µg/mL ssRNA40 and 5 µM ODN2006 (CpG) (all from InvivoGen) for 24 h.

For FcγR-mediated activation by plate-bound IgG (pb IgG), culture plates (U-bottom, 96 wells) were coated with 10 µg/mL purified human IgG (Jackson ImmunoResearch) in PBS at 37° for 1.5 h (*24*). For activation by soluble immune complexes, human IgG was heat aggregated (HA) at 63°C for 30 min, after which insoluble complexes were removed by centrifugation at 13 000 rpm for 5 min. Mast cells were stimulated using 100 µg/mL HA IgG (*25*).

For blocking studies, mast cells were incubated with 20 µg/mL anti-CD32 (clone IV.3; Stemcell), mIgG2b isotype control (eBioscience), 10 µg/mL anti-TLR-2 (clone TL2.1; eBioscience) or anti-TLR-4 (clone HTA-125; eBioscience) for 1 h at 37° prior to activation, after which cells were diluted 1:1 in culture medium containing the indicated stimuli.

IGG-ACPA MEDIATED MAST CELL ACTIVATION

Biotinylated CCP2 peptide and the arginine control peptide were obtained from Dr J W Drijfhout, Department of Immunohematology and Bloodtransfusion, LUMC, Leiden, The Netherlands. Nunc MaxiSorp plates (VWR) were first coated overnight with 5 µg/mL streptavidin (Sigma). After washing, biotinylated CCP2 and arginine control peptides (1 µg/mL) were coated for 1 h at room temperature, and the coated wells were

subsequently incubated for 1 h at 37°C with serum of ACPA-positive and ACPA-negative RA patients, diluted 50× in PBS/0.1% BSA. After washing, mast cells were added, and were cultured in the presence or absence of 10 µg/mL human HSP70 (Enzo Life Sciences). Alternatively, CCP2- or arginine control-coated plates were incubated with 5 µg/mL ACPA mAb.

The ACPA IgG monoclonal antibody used here was previously cloned by van de Stadt et al (*26*). Briefly, ACPA antibody was produced following transduction with heavy and light chain cDNA expressing lentiviral vectors by HEK 293 T cells (*27*), and antibodies were purified from supernatant using fast protein liquid chromatography (ÄKTA; GE Healthcare) equipped with HiTrap Protein A columns (GE Healthcare) (*28*). Following purification, the quantity of ACPA was assessed by standard total IgG ELISA (Bethyl Laboratories) according to the manufacturer's instructions.

PATIENT SAMPLES

Diagnoses of RA or osteoarthritis (OA) were made according to 1987 and 1986 American College of Rheumatology criteria, respectively (*29, 30*). The presence of total IgG-ACPA and rheumatoid factor (RF) in serum was tested by routine diagnostic ELISA. Synovial tissues were obtained from patients with established RA and OA who had undergone knee or hip replacement surgery. Synovial tissue was digested in 1 mg/mL collagenase IA (Sigma-Aldrich) and 50 µg/mL DNAse (Sigma–Aldrich) at 37°C for 1 h, after which single cell suspensions were obtained using a 70 µm filter. All patients gave written informed consent, and the study was approved by the Leiden University Medical Center human ethics committee.

FLOW CYTOMETRY

A list of antibodies used in this study is shown in online supplementary table S1 (online). For surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0.5% BSA at 4°C for 30 min. Intracellular tryptase staining was performed as described.23 Flow cytometric acquisition was performed on FACSCalibur (BD) and LSR II (BD) flow cytometers. Analysis was performed using FACSDiva (BD) and FlowJo software.

CYTOKINE AND LEUKOTRIENE C4 PRODUCTION

Quantitative immunoassays in culture supernatants were performed using the 42-plex cytokine Milliplex assay (Millipore). Additionally, IL-8 production was evaluated using ELISA (eBioscience). Leukotriene C4 was analysed using a competitive ELISA kit (Neogen).

STATISTICAL ANALYSIS

Results are expressed as mean±SEM. For detection of TLR mRNA expression, the one-sample t test was performed. For differences between multiple groups, one-way ANOVA was performed, with Bonferroni's post hoc test to correct for multiple testing. Synergy was defined as an increase in mast cell activation with the combination of stimuli compared to the additive mast cell response induced by the stimuli separately, determined using the paired-samples t test. p Values of <0.05 were considered statistically significant.

RESULTS

EXPRESSION OF TLR

To determine the expression of TLR1 to -10 and other molecules involved in TLR signalling pathways, real-time PCR was performed on the mRNA of peripheral blood-derived mast cells. Mast cell cultures were defined by expression of CD117, CD203c, FcεRI and intracellular tryptase, indicating that the culture resulted in mature mast cells (see online supplementary figure S1).

mRNA encoding TLR-1 to -9 was found in all three mast cell donors (figure 1A), although expression of TLR-3 and -9 was low compared to the other TLRs. The relative expression levels of TLRs in mast cells were comparable to PBMCs and most often higher than in the monocyte cell line THP-1 (see online supplementary figure S2). Mast cells also expressed mRNA for several molecules associated with TLR signalling, such as MyD88, TICAM1 (TRIF), TICAM2 and BTK (figure 1B).

These data indicate that human mast cells express TLR-1 to -9 as well as the downstream signalling molecules, needed for TLR signal transduction.

MAST CELL ACTIVATION BY TLR LIGANDS

To examine whether the expression of TLRs by mast cells could be related to functional responses, cytokine production in response to TLR ligands was measured. Figure 2A and online supplementary figure S3 show that mast cells respond to TLR ligands with the production of several cytokines. Cytokine responses to ligands for TLR-3 and -9 were low or absent, in line with their low mRNA expression. In contrast, GM-CSF, IL-8 and MIP-1α were produced in response to most TLR ligands, IL-10 and IL-13 were mainly produced in response to ligands for TLR-2, and TNF-α production was mainly restricted to ligands for TLR-2, -4 and -8.

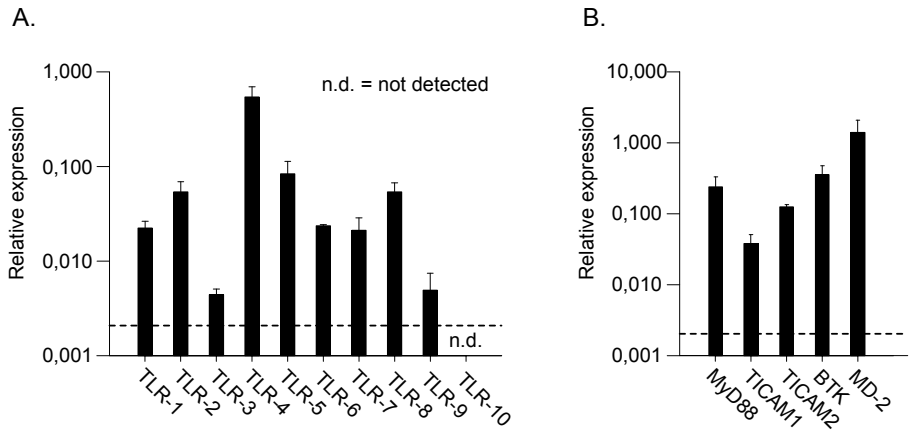

Figure 1. Total mRNA was isolated from independent peripheral blood-derived mast cell cultures of different donors (n=3). Relative mRNA expression of (A) Toll-like receptors (TLRs) and (B) molecules involved in TLR signalling, normalised to HPRT1 expression as housekeeping gene. The dotted line indicates the detection limit. nd, not detected.

Besides cytokine production, one of the main effector functions of mast cells is mediated via granule release and production of lipid-derived mediators. In contrast to stimulation with anti-IgE, these responses were absent when mast cells were activated with TLR ligands (figure 2B–D). Therefore, TLR ligands induce a different response as compared to anti-IgE, characterised by the production of several cytokines in the absence of degranulation or release of lipid-derived molecules. Importantly, different TLR triggers can induce different response modes as exemplified by different cytokines induced by different TLR ligands.

ACTIVATION OF MAST CELLS BY IGG IMMUNE COMPLEXES VIA FCγRII

As the expression of FcγRs by human mast cells and their possible activation via these receptors is not established, we next evaluated the potential of human mast cells to be activated by IgG immune complexes. First, we evaluated expression of the different FcγRs by flow cytometry (figure 3A,B). Mast cells were found to express FcγRIIA (CD32A) but no other FcγR. We next stimulated mast cells with IgG immune complexes, and found that mast cells produced IL-8 in response to plate-bound IgG (figure 3C). Importantly, this response was almost completely inhibited by blocking antibodies to FcγRIIA (figure 3D). In addition, mast cells produced IL-8 in response to soluble immune complexes (HA IgG), and this response was also blocked by anti-FcγRIIA (figure 3E). No degranulation was observed in response to IgG immune complexes (data not shown). Together, these data show that human mast cells are activated by IgG immune complexes, mainly via FcγRIIA.

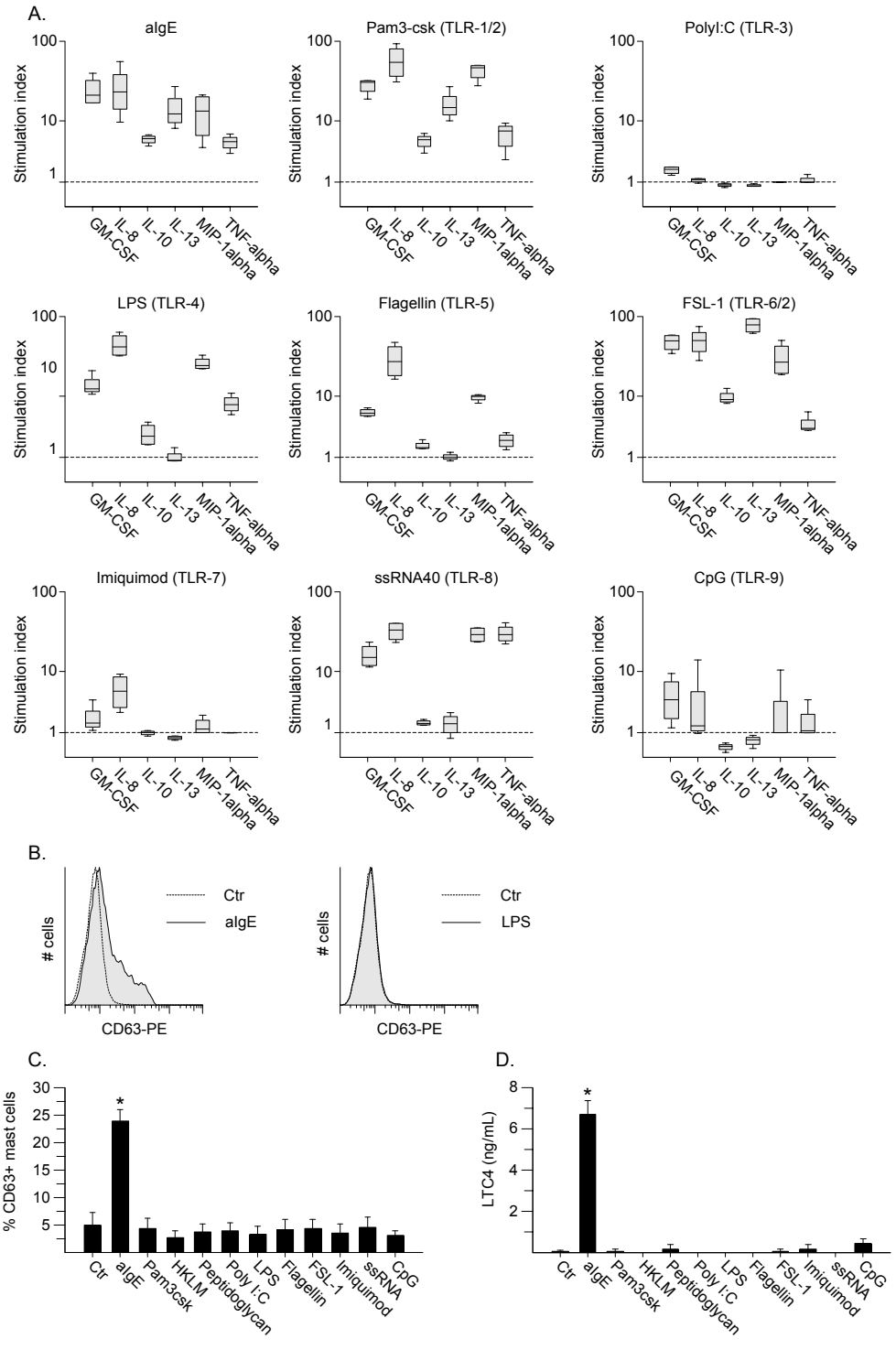
A.
aIgE
Pam3-csk (TLR-1/2)
PolyI:C (TLR-3)
LPS (TLR-4)
Flagellin (TLR-5)
FSL-1 (TLR-6/2)
Imiquimod (TLR-7)
ssRNA40 (TLR-8)
CpG (TLR-9)
Stimulation index
100
10
1
GM-CSF
IL-8
IL-10
IL-13
MIP-1alpha
TNF-alpha
B.
cells
Ctr
aIgE
LPS
CD63-PE
C.
% CD63+ mast cells
30
25
20
15
10
5
*
Ctr
aIgE
Pam3csk
HKLM
Peptidoglycan
Poly I:C
LPS
Flagellin
FSL-1
Imiquimod
ssRNA
CpG
D.
LTC4 (ng/mL)
8
6
4
2
*

Figure 2 *(left)*. (A) Mast cells were stimulated with the indicated Toll-like receptor (TLR) ligands for 24 h after which cytokines were measured in supernatant. The stimulation index was calculated by dividing the amount of cytokine produced after stimulation by the amount of cytokine in the supernatant of unstimulated mast cells. (B–D) Mast cells were stimulated with TLR ligands for 1 h, after which cells were used for flow cytometry to assess degranulation with anti-CD63 (B,C) and supernatant was used for the determination of leukotriene C4 (LTC4) release (D). Data were obtained from two independent experiments performed in duplicate. Asterisks indicate significant increases compared to unstimulated mast cells ($p<0.001$).

SYNERGY BETWEEN TLR- AND FCγR-MEDIATED ACTIVATION

As mast cells responded to triggering of TLR as well as FcγRII, we next evaluated the possible interaction between triggering with IgG immune complexes and TLR ligands (figure 4A,B). No additional effects were observed when TLR-3 or -9 ligands were combined with plate-bound IgG, which is in line with the absence of activation with these ligands. However, combination of other TLR ligands with IgG led to synergy in cytokine production (figure 4B). Similar to the results obtained with plate-bound IgG, IL-8 production by mast cells in response to LPS was greatly enhanced by soluble immune complexes (figure 4C).

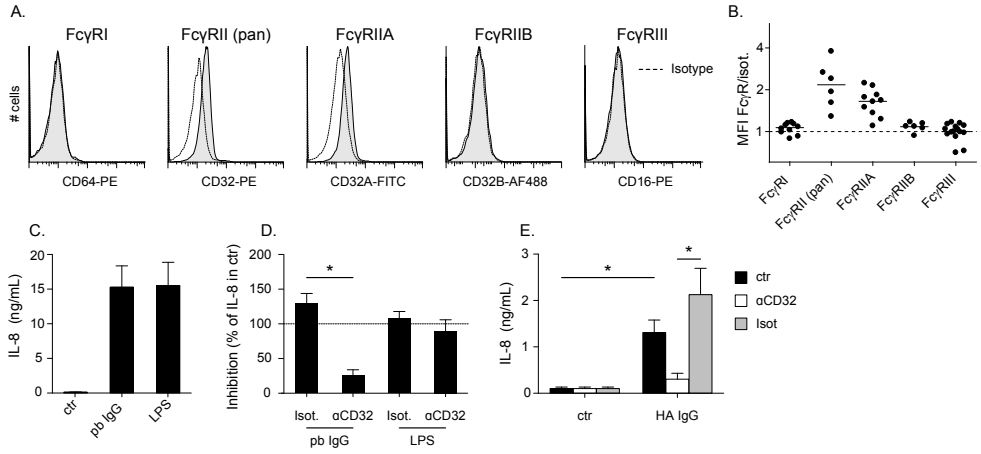

Figure 3. (A,B) Expression of Fcγ receptors (FcγR) by cultured mast cells was analysed by flow cytometry. Representative examples are shown in (A) and a summary of all donors (n=6–15) analysed is shown in (B). (C) IL-8 production by mast cells in response to plate-bound (pb) IgG and lipopolysaccharide (LPS) (n=13). (D) Inhibition of IL-8 production in response to pb IgG or LPS by anti-CD32 or isotype control (n=6), indicated as a percentage of total IL-8 production in stimulated mast cells without blocking antibodies. (E) IL-8 production in response to heat-aggregated IgG (HA IgG), and inhibition by anti-CD32 or isotype control (n=4).

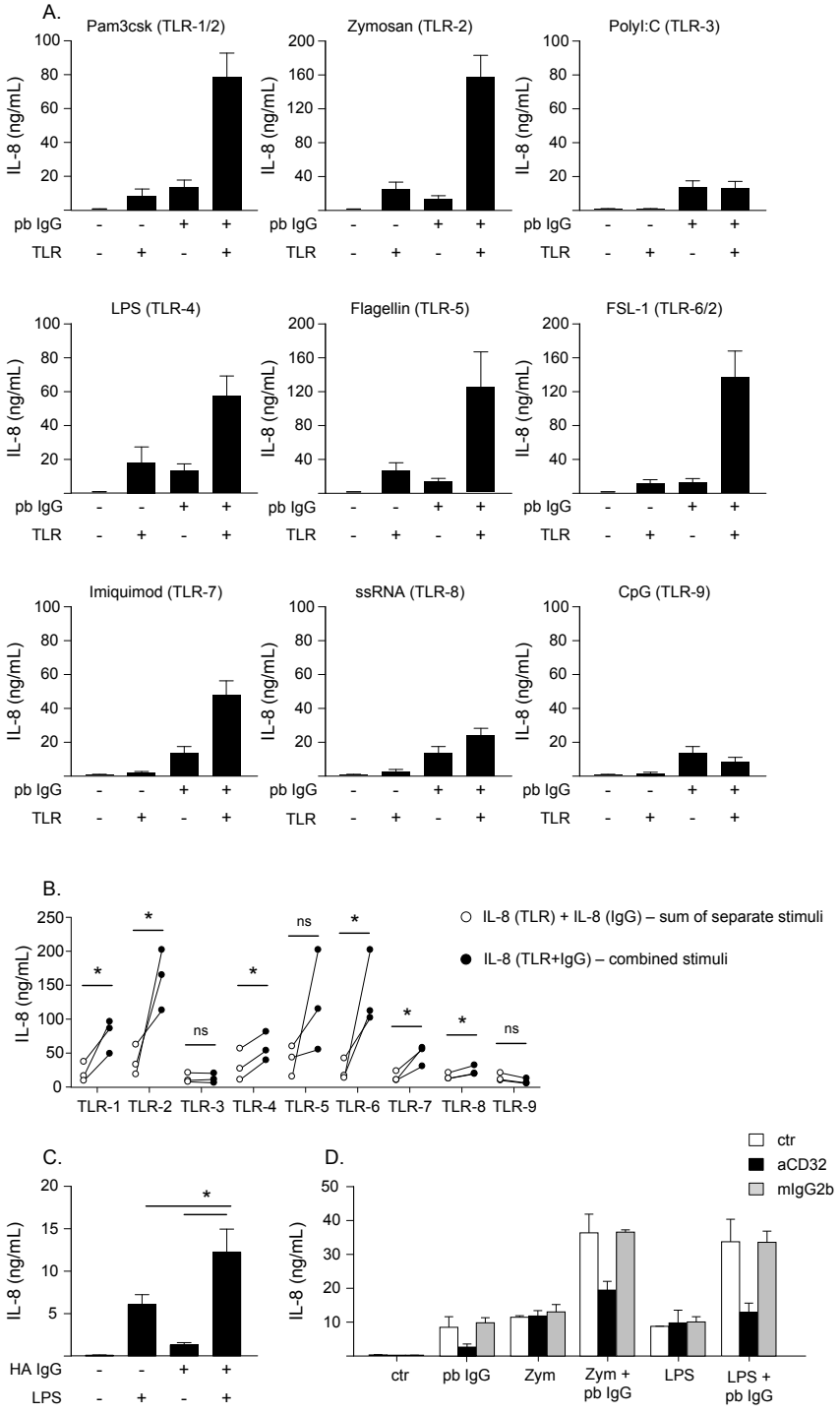
A.
Pam3csk (TLR-1/2)
Zymosan (TLR-2)
PolyI:C (TLR-3)
LPS (TLR-4)
Flagellin (TLR-5)
FSL-1 (TLR-6/2)
Imiquimod (TLR-7)
ssRNA (TLR-8)
CpG (TLR-9)
IL-8 (ng/mL)
pb IgG
TLR
B.
IL-8 (TLR) + IL-8 (IgG) – sum of separate stimuli
IL-8 (TLR+IgG) – combined stimuli
TLR-1 TLR-2 TLR-3 TLR-4 TLR-5 TLR-6 TLR-7 TLR-8 TLR-9
C.
HA IgG
LPS
D.
ctr
aCD32
mIgG2b
ctr
pb IgG
Zym
Zym + pb IgG
LPS
LPS + pb IgG

Figure 4 *(left)*. Mast cells were stimulated with Toll-like receptor (TLR) ligands alone or in combination with plate-bound IgG immune complexes. (A–C) IL-8 production was measured in supernatant after 24 h upon combined triggering with plate-bound (pb) IgG and TLR ligands. (A) Summary graphs showing the mean±SEM of IL-8 in supernatant (n=3). (B) IL-8 production for all TLR ligands in combination with plate-bound IgG is shown (closed symbols) compared to the sum of cytokine levels following stimulation with IgG and TLR ligands separately (open symbols). Each symbol represents an independent donor. Asterisks indicate significantly ($p<0.05$) increased cytokine production when TLR ligands were combined with anti-IgE compared to the cumulative effect of the stimuli alone. (C) IL-8 production in response to heat-aggregated IgG (HA IgG) in combination with lipopolysaccharide (LPS) (n=4). (D) IL-8 production in response to plate-bound IgG in combination with zymosan (zym) and LPS after blocking with anti-CD32 or isotype control (n=2).

Expression of FcγRs was not changed by TLR ligands (data not shown), indicating that the synergistic effect was dependent on FcγRIIA, the only FcγR that was expressed. Indeed, blocking of FcγRIIA led to almost complete inhibition of IL-8 production, resulting in IL-8 production comparable to the TLR ligands alone (figure 4D). These results indicate that combined stimulation of mast cells via TLR and FcγRIIA can markedly enhance inflammatory responses by mast cells.

Expression of FcγR by synovial mast cells

Although mast cells are present in synovial tissue, the number of cells that can be isolated in a non-activated manner does not allow for in vitro mast cell activation for functional studies. Nonetheless, we were able to analyse the expression of FcγR by synovial mast cells by flow cytometry (figure 5). Synovial mast cells from all RA and OA donors were found to express FcγRII. Further analysis in three donors showed that FcγRIIA, and not IIB, is expressed by synovial mast cells. Expression of other FcγRs was very low or absent, except for one donor, in which both FcγRI and FcγRIII were detected. These results confirm the comparability between cultured mast cells and synovial mast cells regarding FcγR expression and indicate that the molecular make-up of synovial mast cells allows them to be activated by IgG immune complexes via FcγRIIA.

Synergy between TLR- and IgG-ACPA-mediated activation of mast cells

We next wished to evaluate whether human mast cells can respond to ACPA immune complexes to confirm the observed synergy between IgG- and TLR-mediated activation in an antigen-specific system.

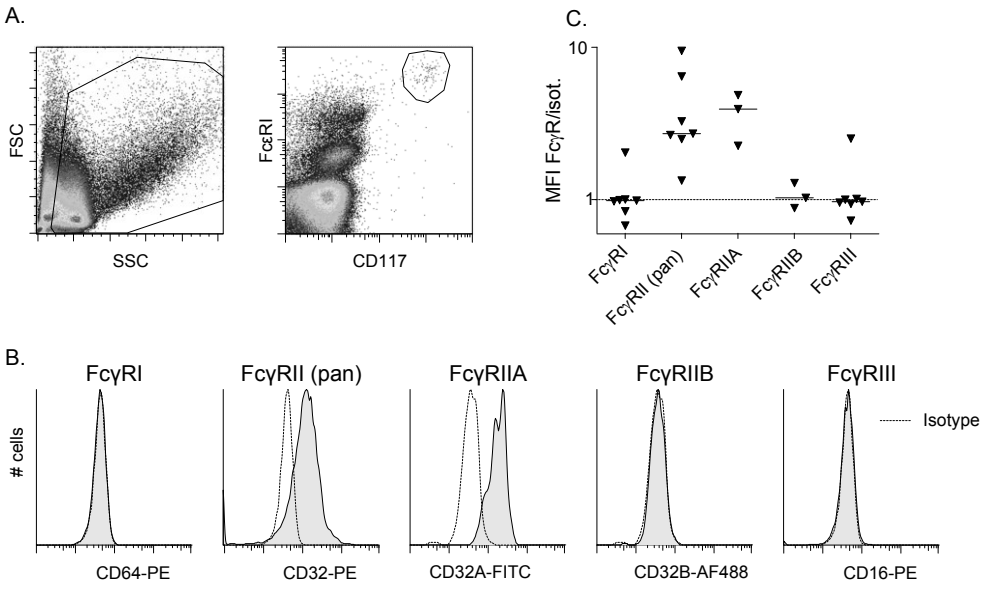

Figure 5. (A) Representative example of mast cell gating is shown. Mast cells were gated as $CD117^{+}IgE^{+}$ or $CD117^{+}Fc\varepsilon RI^{+}$ cells within the live cells as gated based on forward (FSC) and sideward scatter (SSC). Representative examples of Fc receptor staining are shown in (B) and a summary of all patients analysed (n=6 rheumatoid arthritis patients and n=4 osteoarthritis patients) is shown in (C), with each symbol representing an individual patient.

First, mast cells were stimulated with ACPA immune complexes on CCP2-coated plates, resulting in IL-8 production (figure 6A,B). This activation was citrulline dependent, as the arginine control peptide, or the use of serum from ACPA-negative patients, did not induce IL-8 release. Mast cell activation was inhibited by pre-incubating mast cells with an anti-FcγRIIA antibody (figure 6C).

The use of RF-negative serum (figure 6D) or an ACPA monoclonal IgG antibody (figure 6E) also induced IL-8 production, confirming that mast cell activation by ACPA immune complexes is not dependent on the presence of RF. These results show that mast cells can be activated by ACPA immune complexes, and that this is mainly mediated via FcγRIIA.

Subsequently, we evaluated the activation of mast cells by the endogenous TLR ligand HSP70 (figure 6F). To confirm that activation with HSP70 was TLR dependent, mast cells were pre-incubated with blocking antibodies to TLR-2 and -4. As shown in figure 6G, activation of mast cells by HSP70 was inhibited by blocking TLR-4.

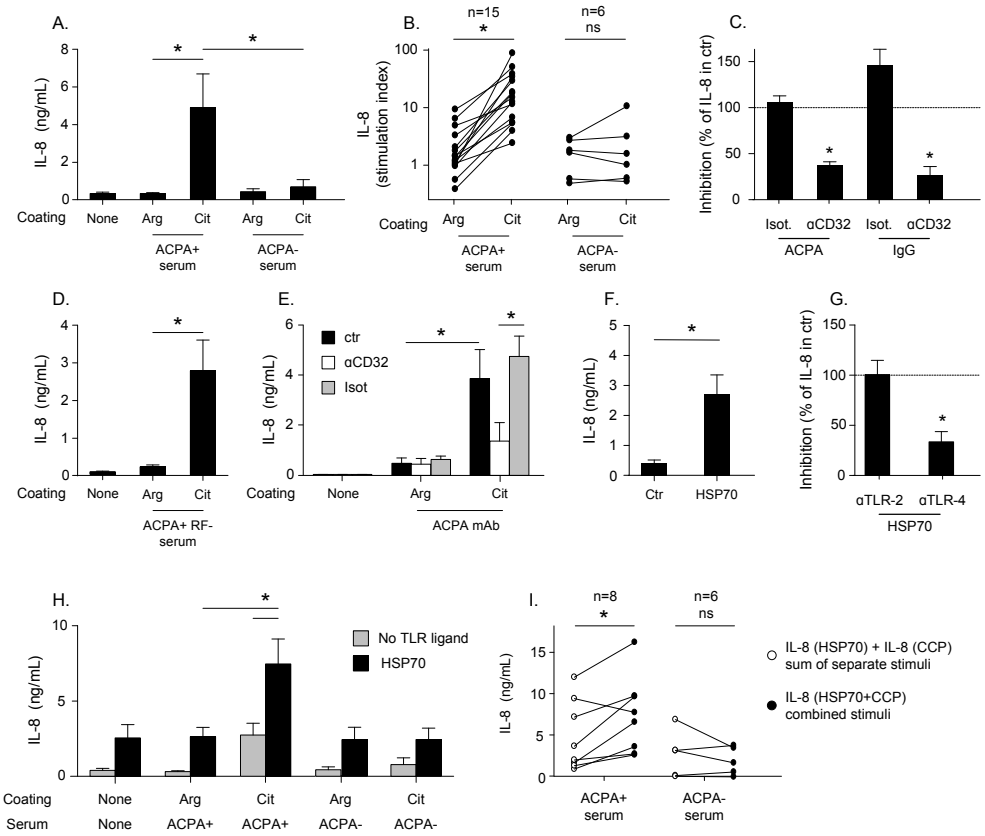

Figure 6. (A, B) Mast cells were stimulated on CCP-coated plates incubated with no serum (ctr) or serum of ACPA+ and ACPA− RA patients, as indicated, for 24 h, after which IL-8 production was measured in supernatant. Results are shown as the summary of all experiments (A) or with each symbol representing an individual patient serum tested (B). (C) Inhibition of IL-8 production by mast cells in response to plate-bound ACPA, IgG or LPS by anti-CD32 antibodies or isotype control, indicated as a percentage of total IL-8 production in stimulated mast cells without blocking antibodies. (D) Mast cells were stimulated on CCP-coated plates incubated with the serum of ACPA+RF− patients. Results are shown as the summary (mean±SEM) of three independent mast cell donors using the serum of two different patients. (E) Mast cells were stimulated on CCP-coated plates incubated with ACPA IgG monoclonal antibody. Results are shown as the summary (mean±SEM) of four independent mast cell donors. (F) IL-8 production by mast cells in response to endogenous TLR ligand HSP70. (G) Inhibition of IL-8 production by mast cells in response to HSP70, by anti-TLR-2 or anti-TLR-4 antibodies, indicated as a percentage of total IL-8 production in stimulated mast cells. (H,I) IL-8 production by mast cells in response to combined triggering using plate-bound ACPA and HSP70 or their separate stimulations. Results are shown as the summary of all independent (n=5) experiments (H) or pairwise (I), with each symbol representing an individual patient serum tested. (I) IL-8 production after combined stimulation with HSP70 and ACPA (closed symbols) compared to the sum of IL-8 following separate stimulation with HSP70 and ACPA (open symbols). Asterisks indicate significant ($p<0.05$) differences between the indicated conditions, using paired samples t test.

Combined activation of mast cells by ACPA immune complexes and HSP70 led to considerable increases in IL-8 production as compared to the single stimulus (figure 6H). This increase was significantly higher than the cumulative production of the separate stimuli when ACPA-positive serum was used, whereas no additional IL-8 production was observed when ACPA-negative serum was combined with HSP70 (figure 6I). Together, these results show enhanced cytokine production by mast cells when exposed to ACPA immune complexes and endogenous TLR ligands.

DISCUSSION

In this study, we show that human mast cells can be activated by TLR triggering as well as FcγR ligation. Only limited information is available on the activation of human mast cells via TLR and FcγR. Here, we have extensively analysed mast cell responses towards ligands for all TLR ligands and found different patterns of cytokine production depending on the TLR that was triggered. Such different cytokine production profiles in response to TLR triggering have previously been reported for dendritic cells and basophils, and are thought to contribute different types of immune responses to different pathogens (*31, 32*). Our results therefore indicate that different cytokines produced by mast cells may further promote these differences.

In addition to the response to pathogen-derived TLR ligands, we show that mast cells also respond to endogenous TLR ligands which are present in RA synovium. Endogenous ligands in synovium have been postulated to induce a positive feedback loop leading to chronic immune activation (*13, 33*). Activation of TLR may be important for several chronic rheumatic diseases, including RA and spondyloarthritis (SpA) (*34*). Since mast cells are relatively abundant in RA and SpA synovium (*7, 8, 35*), they may represent an important cell type involved in TLR-driven chronic inflammation.

Besides activation of mast cells via TLR ligands, we show for the first time that human mast cells can be triggered by ACPA immune complexes in a citrulline-dependent manner. Human mast cells were activated via IgG-ACPA, mediated by FcγRIIA (*36-38*). FcγRI was not expressed by synovial mast cells in the majority of patients, which is also in line with the absence of FcγRI expression by mast cells in other tissues (*38*). As ACPA immune complexes activated mast cells mainly via FcγRIIA and as this receptor is expressed by synovial mast cells from all patients analysed, we consider it most likely that this receptor is responsible for mast cell activation by ACPA immune complexes. A clear limitation of our study is that it is not feasible to perform functional assays using mast cells isolated from synovium. However, although we cannot show that synovial mast cells respond in the same way as

cultured mast cells, the cultured mast cells generally resemble the phenotype of synovial mast cells in their granule composition and in their expression of FcγR.

Importantly, we showed that combination of IgG- and TLR-mediated activation greatly enhanced cytokine production by human mast cells. Synergy between TLR and Fc receptor triggering has been previously described for basophils and dendritic cells (*32, 39*), and likely represents a physiological function of the immune system to mount an enhanced response when antibodies are produced after the first encounter with a pathogen. Synergy in the production of chemokines may dictate the type and extent of immune cells which are attracted to the site of inflammation (*40*), and may therefore also have detrimental effects in the context of autoimmunity. For example, IL-8 is increased in synovial fluid of RA patients and was shown to contribute to 30–60% of neutrophil chemoattraction by synovial fluid (*41*). Therefore, synergistic IL-8 production by mast cells in response to ACPA immune complexes and endogenous TLR ligands may lead to increased neutrophil recruitment, and thus contribute to inflammatory processes in RA.

In conclusion, we found that human mast cells functionally express TLRs and FcγRIIA and that triggering of TLR via endogenous TLR ligands can synergise with IgG-ACPA-mediated activation, greatly increasing cytokine production. These results suggest a role for combined TLR and FcγRIIA triggering of mast cells in the pathogenesis of RA.

FUNDING

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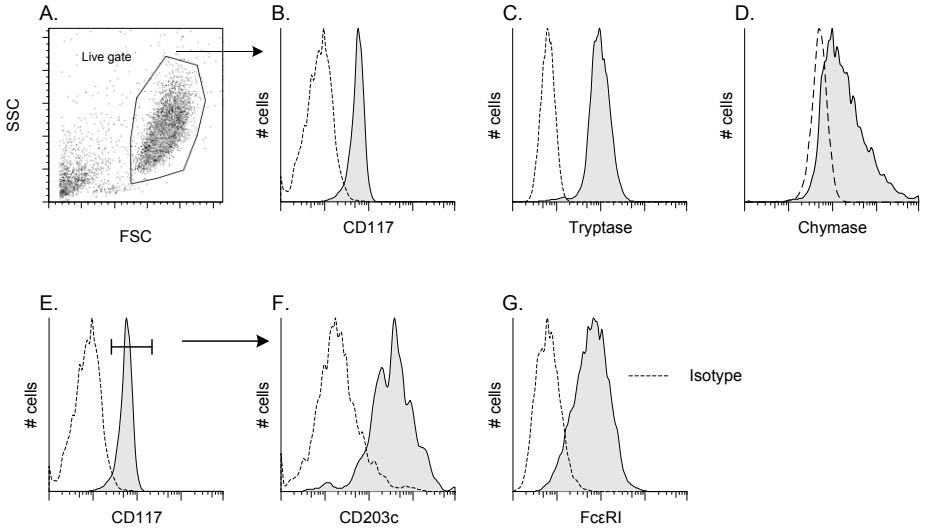
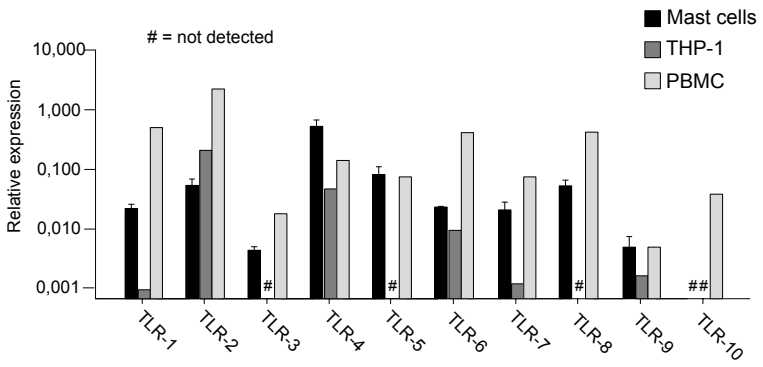
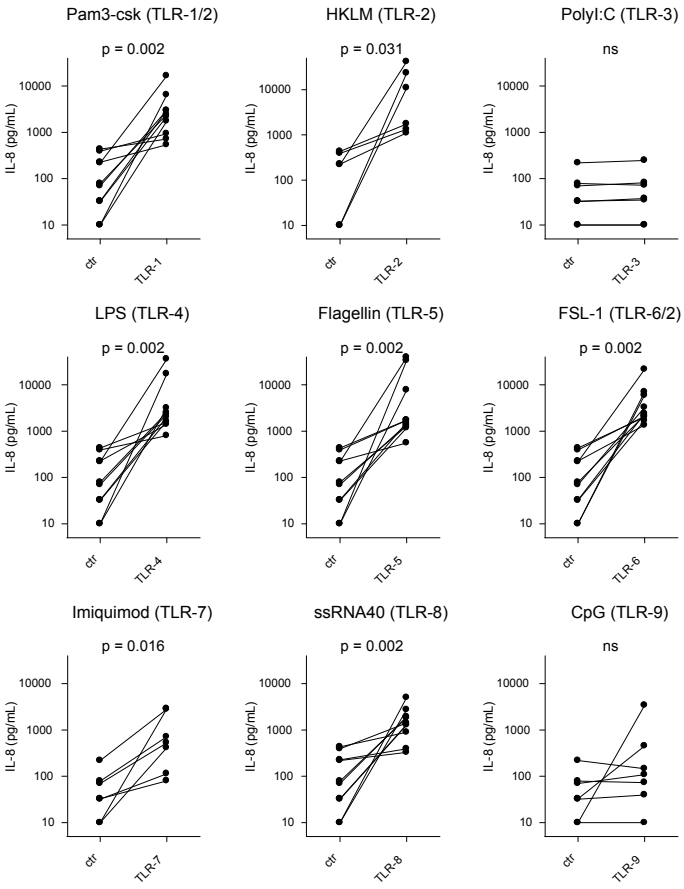
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Characterization of mast cells. Peripheral blood-derived mast cells were cultured from $CD34^+$ stem cells for 6-8 weeks. Mast cells were identified as $CD117^+CD203c^+FcepsilonRI^+Tryptase^+$ cells (>90% pure). (A) Gating of live cells (cell debris excluded based on Forward and Sideward scatter). (B) CD117 expression of total live cells as gated in A. (C,D) Intracellular Tryptase and chymase expression of total live cells. (E) Gating of $CD117^+$ mast cells. (F,G) Expression of CD203c and FcepsilonRI on $CD117^+$ mast cells as gated in E. Representative FACS plots are shown.



Supplementary Figure 2. Total mRNA was isolated from independent peripheral blood-derived mast cell cultures of different donors (n=3) and of the monocytic cellline THP-1 and total peripheral blood mononuclear cells (PBMC). Relative mRNA expression of TLRs was normalized to HPRT1 expression as housekeeping gene.



Supplementary Figure 3. Mast cells were stimulated with the indicated TLR ligands for 24 hours after which production of IL-8 was measured in supernatant. Paired data of n=6 to n=10 donors is shown. P values shown are derived from paired samples T-test

Ability of IL-33- and immune complex-triggered activation of human mast cells to down-regulate monocyte-mediated immune responses

Chapter 6

Felice Rivellese,[1,2,3] Jolien Suurmond,[1] Kim LL Habets,[1] Annemarie L Dorjée,[1] Nandhini Ramamoorthi,[4] Michael J Townsend,[4] Amato de Paulis,[2] Gianni Marone,[2] Tom WJ Huizinga,[1] Costantino Pitzalis,[3] René EM Toes[1]

[1]Leiden University Medical Center, Leiden, The Netherlands; [2]University of Naples Federico II, Naples, Italy; [3]William Harvey Research Institute and Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK; [4]Genentech Research and Early Development, South San Francisco, USA

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ABSTRACT

Objective Mast cells have been implicated in the pathogenesis of rheumatoid arthritis (RA). In particular, their activation by interleukin-33 (IL-33) has been linked to the development of arthritis in animal models. The aim of this study was to evaluate the functional responses of human mast cells to IL-33 in the context of RA.

Methods Human mast cells were stimulated with IL-33 combined with plate-bound IgG or IgG anti–citrullinated protein antibodies (ACPAs), and their effects on monocyte activation were evaluated. Cellular interactions of mast cells in RA synovium were assessed by immunofluorescence analysis, and the expression of messenger RNA (mRNA) for mast cell–specific genes was evaluated in synovial biopsy tissue from patients with early RA who were naive to treatment with disease-modifying antirheumatic drugs.

Results IL-33 induced the up-regulation of Fcγ receptor type IIa and enhanced the activation of mast cells by IgG, including IgG ACPAs, as indicated by the production of CXCL8/IL-8. Intriguingly, mast cell activation triggered with IL-33 and IgG led to the release of mediators such as histamine and IL-10, which inhibited monocyte activation. Synovial mast cells were found in contact with $CD14^+$ monocyte/macrophages. Finally, mRNA levels of mast cell–specific genes were inversely associated with disease severity, and IL-33 mRNA levels showed an inverse correlation with the levels of proinflammatory markers.

Conclusion When human mast cells are activated by IL-33, an immunomodulatory phenotype develops, with human mast cells gaining the ability to suppress monocyte activation via the release of IL-10 and histamine. These findings, together with the presence of synovial mast cell–monocyte interactions and the inverse association between the expression of mast cell genes at the synovial level and disease activity, suggest that these newly described mast cell–mediated inhibitory pathways might have a functional relevance in the pathogenesis of RA.

INTRODUCTION

Mast cells are tissue-resident cells of hematopoietic origin, classically known as effector cells in IgE-mediated inflammation and as mediators of host antimicrobial defenses. In addition, mast cells have been involved in the pathogenesis of many human diseases, including autoimmune disorders (*1*). However, emerging evidence suggests that mast cells might also have immunoregulatory/homeostatic functions (*2*). Therefore, their exact role in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis (RA), is still debated (*3*). In particular, contrasting data have been obtained from animal models of

arthritis (*4-8*), which could be attributable to the aforementioned double-edged functions of mast cells or to the different disease models and methods used to obtain their depletion in vivo (*9*).

In humans, several lines of evidence point toward a contributory role of mast cells in the pathogenesis of RA. First, they are constitutively present in the synovial membrane (*10*) and their numbers are increased in RA (*11, 12*), possibly correlating with parameters of disease activity and progression (*13*). Furthermore, activation of mast cells in rheumatoid synovial tissue has been linked to the production of proinflammatory cytokines (*14*), several mast cell mediators have been observed in the synovial fluid of patients with RA (*15*), and these mediators have been shown to have pathogenic effects on synovial fibroblasts (*16*). Recently, we showed that mast cells are activated by immune complexes containing anti–citrullinated protein antibodies (ACPAs), with Toll-like receptors (TLRs) augmenting the response of mast cells to ACPA-containing immune complexes (*17*). Despite these findings, the interaction of mast cells with other synovial immune cells has never been fully investigated, and their ability to influence immune responses during the course of RA is still uncertain.

Among the multiple factors known to activate mast cells, interleukin-33 (IL-33), a member of the IL-1 cytokine family (*18*), has been shown to enhance the survival, adhesion, and differentiation of these cells, and to induce the production of several proinflammatory cytokines (*19, 20*). Furthermore, IL-33 was found to prime mouse and human mast cells for activation by IgG immune complexes (*21, 22*). In vivo, IL-33 has been implicated in the ability of mast cells to contribute to the development of arthritis in murine experimental models (*23, 24*), and high levels of IL-33 have been found in the serum and synovial fluid of patients with RA and those with other inflammatory arthritides (*25*). In addition to these proinflammatory properties, which are consistent with the described role of IL-33 as an alarmin, recent evidence suggests that this cytokine can also mediate immunoregulatory responses in various settings, by, for example, promoting regulatory T and B cell activity and suppressing monocyte activation through its effects on basophils (*26-31*). Thus, similar to the findings regarding mast cells, the specific functions of IL-33 in the pathogenesis of autoimmune diseases, including arthritis, are still unclear (*32, 33*).

To better understand the roles of IL-33 and mast cells in RA, we examined the effects of IL-33 on human mast cells. We hypothesized a novel scenario in which activation of mast cells triggered by the alarmin IL-33 may play a homeostatic role, by contributing to the resolution of inflammation through a feedback system that leads to down-regulation of monocyte-mediated immune responses in RA synovial tissue.

MATERIALS AND METHODS

PERIPHERAL BLOOD–DERIVED MAST CELLS

Buffy coat cells were obtained from the peripheral blood of healthy volunteers, with samples collected from a blood bank in The Netherlands (Sanquin). $CD34^+$ hematopoietic stem cells were isolated from peripheral blood mononuclear cells (PBMCs) using CD34 microbeads (Miltenyi Biotec). Isolated $CD34^+$ stem cells were differentiated into mast cells using a previously described method (*34*). After 6–8 weeks, the purity of the mast cells was determined by flow cytometry analyses for the expression of CD117 (c-Kit), Ig ε-class–binding Fc receptor type I (FcεRI), and CD203c; purity ranged from 90% to 99%.

MAST CELL ACTIVATION

Plate-bound IgG was used as a model to study FcγR-mediated activation of mast cells (*17, 35*). Briefly, culture plates (96-well or 48-well flat-bottomed plates) were coated with 100 μg/ml of purified human IgG (Jackson ImmunoResearch) in phosphate buffered saline (PBS) for 1.5 hours at 37°C and washed 2 times with PBS. Mast cells were then cultured at a concentration of 1 × 106/ml in RPMI 1640 medium containing 10% fetal calf serum, glutamine, penicillin, and streptomycin (all from Invitrogen) together with 100 ng/ml of stem cell factor (Tebu-Bio), without or with 100 ng/ml of recombinant human IL-33 (PeproTech). After 24 hours, the cells were harvested and supernatants were collected and stored at −20°C until further analysis by flow cytometry.

IgG ACPA-MEDIATED MAST CELL ACTIVATION

Cyclic citrullinated peptide 2 (CCP2) peptides were obtained from Dr. J. W. Drijfhout (Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands). Nunc Maxisorp plates (VWR Scientific Products) were coated with CCP2 peptide or arginine as a control peptide, and then incubated at 37°C for 1 hour with serum from patients with ACPA-positive RA, with the serum samples diluted 50 times in PBS–0.1% bovine serum albumin (BSA). After washing, mast cells were added to the wells and cultured in the presence or absence of 100 ng/ml of recombinant human IL-33. After 24 hours, the cells were harvested, and supernatants were collected and stored at −20°C.

MONOCYTE ISOLATION AND STIMULATION

$CD14^+$ monocytes were isolated from buffy coat PBMCs using magnetic-labeled anti-CD14 beads (Miltenyi Biotec), according to the manufacturer's instructions. Isolated monocytes

(purity >95%) were cultured in the same medium as used for mast cells. Monocytes were incubated with mast cell supernatants (diluted 1:4 in medium) or control medium, and then stimulated with lipopolysaccharide (LPS) from Salmonella typhosa (Sigma-Aldrich) at a concentration of 5 ng/ml. After overnight (18-hour) incubation, the cells were harvested and supernatants were collected and stored at −20°C until further analysis by flow cytometry.

For blocking experiments, supernatants of activated mast cells were preincubated with anti–IL-10 antibody or rat IgG2a as a matched isotype control (BD Biosciences), at 10 μg/ml for 30 minutes at 37°C in an atmosphere of 5% CO2, prior to being incubated with the monocytes. For inhibition of histamine, monocytes were preincubated for 30 minutes at 37°C with the histamine receptor 2 antagonist ranitidine (Sigma) at 10−4M (*28*).

PATIENT SAMPLES

Serum samples were obtained from patients with RA, and the presence of total IgG ACPAs was tested by routine diagnostic enzyme-linked immunosorbent assay (ELISA). For immunofluorescence analysis, synovial tissue was obtained from 3 patients with established ACPA-positive RA (ages 59, 70, and 76 years) who had undergone surgery. For messenger RNA (mRNA) sequencing, mRNA was extracted from synovial tissue samples obtained by ultrasound-guided biopsy from patients with early active RA (<12 months' duration) who were naive to treatment with disease-modifying anti-rheumatic drugs (DMARDs) (n=40), enrolled in the Pathobiology of Early Arthritis Cohort (details at http://www.peac-mrc.mds.qmul.ac.uk) at the Centre for Experimental Medicine and Rheumatology of Queen Mary University (London, UK) (*36*). Additional patient characteristics are listed in Supplementary Table 1 (online). All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA (*37*). Written informed consent was obtained from the patients, and the study was approved by local human ethics committees.

IMMUNOFLUORESCENCE ANALYSIS

Synovial tissue samples were fixed with 4% (weight/volume) formaldehyde (Merck) in PBS and stored in 70% (volume/volume) ethanol. The tissue samples were then embedded in paraffin, in 4-μm sections. Slides were deparaffinized with xylene (Merck), and endogenous peroxidase activity was blocked with 1% hydrogen peroxide (Merck) in methanol for 10 minutes. After antigen retrieval with a Tris–EDTA solution (pH 9; Dako) for 30 minutes at 96°C, slides were stained with monoclonal mouse anti-human antibodies for tryptase (0.2 μg/ml; Millipore) or polyclonal goat anti-human antibodies for tryptase (8 μg/ml; Santa Cruz Biotechnology), in combination with polyclonal goat

anti-human CD14 antibodies (2.5 μg/ml; Abcam) or monoclonal mouse anti-human CD3 (2.8 μg/ml) or CD20 (0.4 μg/ml) antibodies (both from Dako) in PBS–1% BSA for 1 hour. For control sections, matching isotype control antibodies for CD14 (normal goat IgG; Merck) or for CD3 and CD20 (each mouse IgG1; Dako) were used. Detection of staining was performed using donkey anti-mouse/goat Alexa Fluor 568 and donkey anti-goat/mouse Alexa Fluor 488 (each 2 μg/ml; Invitrogen). All slides were mounted with Vectashield Hard Set mounting medium with DAPI (Vector) and visualized using a Zeiss Axio Scope A1 and AxioVision 4.9.1. Stained sections were scored by counting the number of cells in 10 high-power fields (400× magnification), with scoring performed in a blinded manner by 3 observers (FR, KH, and ALD). Interobserver agreement was evaluated using the intraclass correlation coefficient (with a cutoff value of >0.7 to indicate acceptable agreement).

FLOW CYTOMETRY

For flow cytometry staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS–0.5% BSA at 4ºC for 30 minutes. To exclude dead cells, 0.2 μM DAPI (Invitrogen) was added. Flow cytometric acquisition was performed on an LSR-II flow cytometer (BD Biosciences). Analysis was performed using FACSDiva (BD Biosciences) and FlowJo software (Tree Star). The following (clonal) antibodies were used: PerCP–Cy5.5–conjugated CD14 (61D3), fluorescein isothiocyanate (FITC)–conjugated FcεRI (AER-37), phycoerythrin (PE)–conjugated mouse IgG1 (61D3) (all from eBioscience), FITC-conjugated CD16 (3G8), PE-conjugated CD63 (H5C6), PE-conjugated CD64 (10.1), PE-conjugated CD80 (L307.4), FITC-conjugated HLA–DR (L243), FITC-conjugated mouse IgG2b [27-35] (all from BD Biosciences), FITC-conjugated CD32 (IV.3) (StemCell Technologies), and Alexa Fluor 488–conjugated CD32b (ch2b6-N297Q) and a corresponding isotype control (ch4420-N297Q) (Macrogenics) (*38*).

MEASUREMENT OF CYTOKINES

Quantitative immunoassays in mast cell culture supernatants were performed using a 42-plex cytokine Milliplex assay (Millipore). In addition, the following ELISA kits were used: human IL-8 Ready-SET-Go! ELISA kit (eBioscience), human tumor necrosis factor (TNF) ELISA set (BD Biosciences), human IL-10 PeliPair ELISA kit (Sanquin Reagents), and histamine ELISA kit (Neogen).

MESSENGER RNA SEQUENCING

Total RNA from synovial tissue was extracted using a Qiagen RNeasy mini kit in accordance with the manufacturer's protocol, including on-column DNase digestion.

Quality control of samples was done to determine RNA quantity and quality, prior to their processing RNA sequencing. The concentration of total RNA samples was determined using NanoDrop 8000 (Thermo Scientific). The integrity of RNA samples was determined using both a 2100 Bioanalyzer and a 2200 TapeStation (Agilent Technologies). Where available, 1 µg of total RNA was used as an input material for library preparation, using an Illumina TruSeq RNA Sample Preparation kit (version 2).

Generated libraries were amplified with 10 cycles of polymerase chain reaction (PCR). The size of the libraries was confirmed using a 2200 TapeStation and High Sensitivity D1K screen tape (Agilent Technologies), and their concentration was determined using a quantitative PCR–based method with a Library quantification kit (Kapa). The libraries were first multiplexed (5 per lane) and then sequenced on an Illumina HiSeq2500 to generate 50 million paired-end, 75-basepair reads (in synovial samples). For the data analysis, the Genomic Short-read Nucleotide Alignment Program (details available at http://research-pub.gene.com/gmap/) was used to map and assemble transcripts, using the University of California, Santa Cruz human genome reference sequence hg19 and associated transcriptome map (available at http://genome.ucsc.edu/).

STATISTICAL ANALYSIS

Results are expressed as the mean±SEM. For comparison between 2 groups, 2-sample t-tests were performed. For differences between multiple groups, one-way analysis of variance was performed, with the Bonferroni post hoc test to correct for multiple testing. Statistical analysis was performed using GraphPad Prism software (version 5). P values less than 0.05 were considered significant.

RESULTS

MODULATION OF HUMAN MAST CELL ACTIVATION BY IL-33

We investigated the activation of mast cells by stimuli known to be present in human synovium and implicated in the pathogenesis of RA, i.e., IL-33 and IgG. Stimulation of mast cells with plate-bound IgG mainly induced the release of CXCL8/IL-8, while stimulation with IL-33 induced higher amounts of histamine and IL-10 (Figure 1A).

Likewise, only IL-33, and not plate-bound IgG, induced the up-regulation of the mast cell activation marker CD203c. The histamine release induced by IL-33 triggering was not accompanied by an up-regulation of CD63, a marker of mast cell degranulation (Supplementary Figure 1A).

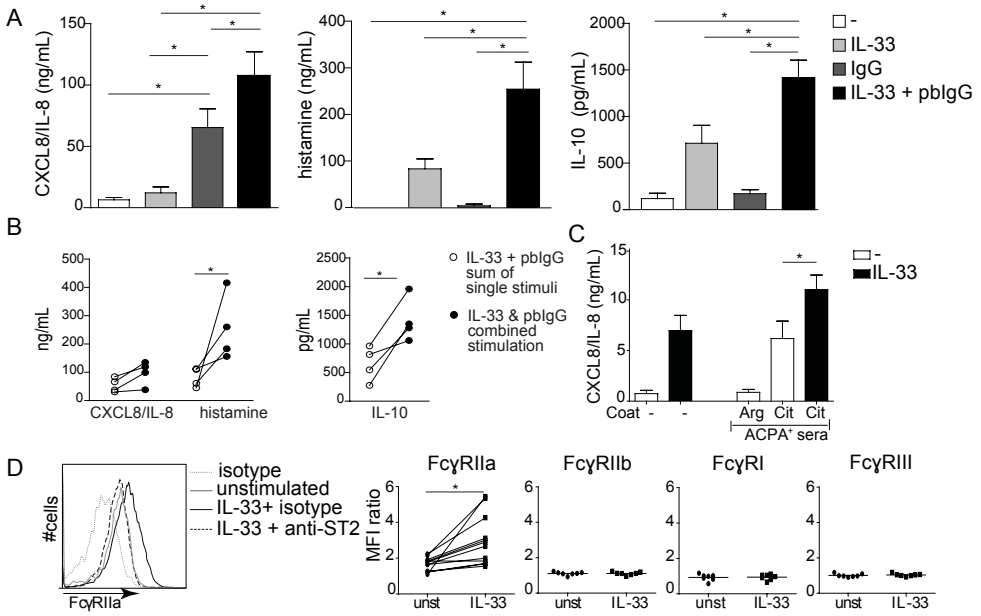

Figure 1. Activation of mast cells via interleukin-33 (IL-33) and immune complexes. Mast cells were left unstimulated (unst) or were activated with IL-33 and/or plate-bound IgG (pbIgG) for 24 hours. A, Levels of CXCL8/IL-8, histamine, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA). Values are the mean±SEM from 4 independent experiments (n=4 donors). B, Synergy was assessed by comparing the sum concentrations of mediators (same as those in A) measured in cell supernatants with single stimuli (IL-33+IgG) and the actual values measured in cell supernatants stimulated simultaneously (IL-33 & IgG). Each symbol joined by a line represents a single mast cell donor (n=4 donors from 4 independent experiments). C, CXCL8/IL-8 levels were measured by ELISA in unstimulated or IL-33–stimulated mast cells after incubation with serum from 3 patients with anti–citrullinated protein antibody (ACPA)–positive rheumatoid arthritis bound to citrullinated (Cit) peptides or arginine (Arg) controls. Values are the mean±SEM results from 6 mast cell donor samples. –= no plate coating. D, Left, Histograms show Fcγ receptor type IIa (FcγRIIa) expression by mast cells left unstimulated or upon stimulation with IL-33 or upon antibody blocking of the IL-33 receptor ST2; matched isotype was used as control. Representative results of 4 independent experiments (n=4 mast cell donors) are shown. Right, Expression of FcγRs was examined in mast cells left unstimulated or stimulated with IL-33. Results are expressed as the ratio of mean fluorescence intensity (MFI) relative to the values for the isotype control; data are from 5 independent experiments. Each symbol represents a single mast cell donor (n=7 or more); horizontal line indicates the mean. ∗=P < 0.05 by analysis of variance with Bonferroni post hoc test for multiple comparisons, and by Student's t-test for comparisons between 2 groups.

Combination of the 2 stimuli led to an increased activation of mast cells, as indicated by the release of significantly higher amounts of CXCL8/IL-8, histamine, and IL-10 when compared to that observed in unstimulated mast cells or mast cells incubated with either stimulus alone (Figure 1A). To distinguish whether the effect was additive or synergistic, we

compared the sum concentration of CXCL8/IL-8, histamine, and IL-10 in cell cultures incubated with each stimulus alone to the actual amounts produced upon combined stimulation with IL-33 and plate-bound IgG (Figure 1B). This comparison showed higher amounts of histamine and IL-10 in cell cultures that received combined stimulation, indicating that IL-33–triggered mediators have a synergistic effect, whereas in the production of CXCL8/IL-8, only an additive effect of each stimulus was observed.

Having found that plate-bound IgG–mediated activation of mast cells is modulated by IL-33, we wondered whether IL-33 would also enhance the activation of mast cells induced by IgG ACPAs. Similar to previous findings from our group (*17*), mast cell activation, evaluated by measurement of CXCL8/IL-8 levels, was observed upon triggering with IgG ACPA immune complexes, which were formed by binding of the serum of patients with ACPA-positive RA to citrullinated peptides (Figure 1C). Importantly, mast cells were not activated by ACPA-positive serum incubated with the arginine control (Figure 1C) or by serum from ACPA-negative patients (results not shown). In addition, IL-33 significantly enhanced the ACPA-induced production of CXCL8/IL-8.

We next sought to identify the mechanism by which IL-33 enhances mast cell activation, and hypothesized that IL-33 modulates the expression of FcγRs on mast cells. Indeed, IL-33 induced a significant up-regulation of the activating FcγRIIa (Figure 1D). This effect was mediated by the IL-33 receptor ST2, as demonstrated by the finding that FcγRIIa expression could be blocked by anti-ST2 antibodies (Figure 1D). Importantly, we did not detect any notable changes in the expression of the other FcγR types by cultured human mast cells, and none of them were influenced by IL-33 stimulation (Figure 1D).

Taken together, these results indicate that IL-33, via its receptor ST2, induces the up-regulation of FcγRIIa, which thereby enhances the activation of mast cells upon triggering with plate-bound IgG, as well as IgG ACPAs, as demonstrated by the increased production of CXCL8/IL-8. More specifically, IL-33 induces IgG-activated mast cells to release histamine and IL-10, but not CXCL8/IL-8, in a synergistic manner.

SKEWING BY IL-33 TOWARD A TH2/IMMUNOMODULATORY PHENOTYPE IN MAST CELLS

To further support the notion that IL-33 is able to modulate mast cell activation by IgG, we performed a multiplex assay on mast cell supernatants. Consistent with the findings in previous literature [20-22], mast cells triggered with IL-33 and plate-bound IgG secreted a wide range of mediators. When comparing the 2 stimuli, we found that plate-bound IgG induced higher amounts of classic proinflammatory mediators, whereas IL-33 induced higher levels of Th2 and immunomodulatory cytokines such as IL-5, IL-10, and IL-13 (see Supplementary Figure 1B). Interestingly, using the combination of IL-33 and

plate-bound IgG to trigger mast cell activation induced higher levels of these cytokines when compared to stimulation with IL-33 alone. In contrast, no additional effects of IL-33 (i.e., no augmentation of the effects of IgG) on the production of proinflammatory mediators such as TNF or FLT-3 ligand could be observed (Figure 2A). In addition, consistent with the data presented in Figure 1B, the combined stimulation showed a synergistic effect that was only present for those mediators whose production was induced by IL-33 (Figures 2A and B).

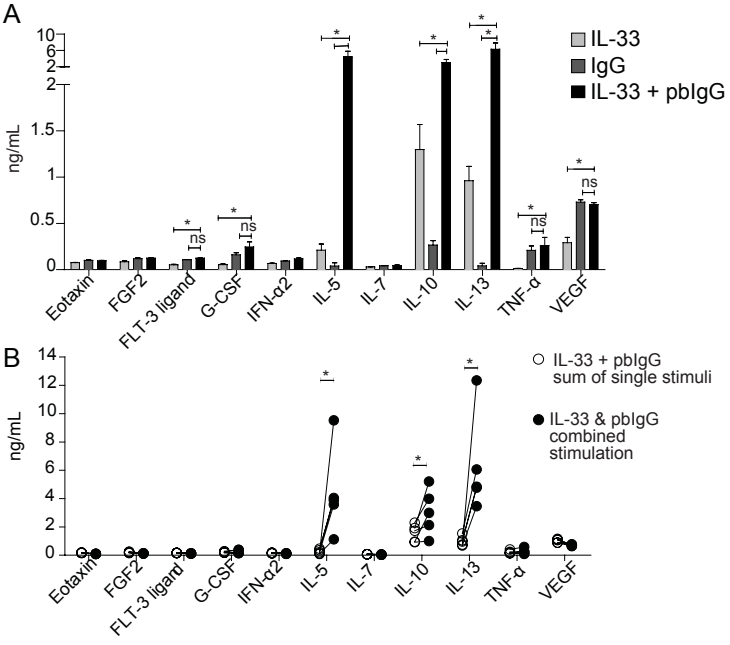

Figure 2. Stimulation of mast cells with IL-33 skews the cells toward an immunomodulatory phenotype. A, Production of various mediators in cell cultures was compared between single stimulations and combined stimulations with IL-33 and plate-bound IgG. Values are the mean±SEM from 3 independent experiments in 3 mast cell donor samples, as measured by multiplex assay. B, Synergy of the single or combined stimulations was assessed as described in Figure 1B. Symbols joined by lines represent individual donors. Only mediators exhibiting significantly increased levels upon stimulation of mast cells with either IL-33 or plate-bound IgG are shown. ∗=P < 0.05 by analysis of variance with Bonferroni post hoc test in A and by Student's t-test in B. NS=not significant; FGF2=fibroblast growth factor 2; G-CSF=granulocyte colony-stimulating factor; IFN-α2=interferon-α2; TNF=tumor necrosis factor; VEGF=vascular endothelial growth factor (see Figure 1 for other definitions).

Taken together, these findings indicate that IL-33 is not simply enhancing mast cell activation by IgG, but is actually able to fine-tune the responses of mast cells, by

inducing the production of a specific set of Th2-associated and immunomodulatory mediators.

INTERACTION OF MAST CELLS WITH MONOCYTES IN RA SYNOVIUM

To better understand the possible consequences of mast cell activation at the synovial level in RA, we investigated the occurrence of cellular interactions between mast cells and other immune cells ex vivo, by performing immunofluorescence staining for mast cells reactive with tryptase, as well as staining for the monocyte/macrophage marker CD14, the B cell marker CD19, or the T cell marker CD3, in tissue sections from the synovium of patients with RA. We found numerous tryptase-positive mast cells scattered in the synovium of patients with RA. Interestingly, tryptase-positive cells showed clear cell-to-cell interaction with $CD14^+$ cells, $CD3^+$ cells, and $CD20^+$ cells (Figures 3A–C).

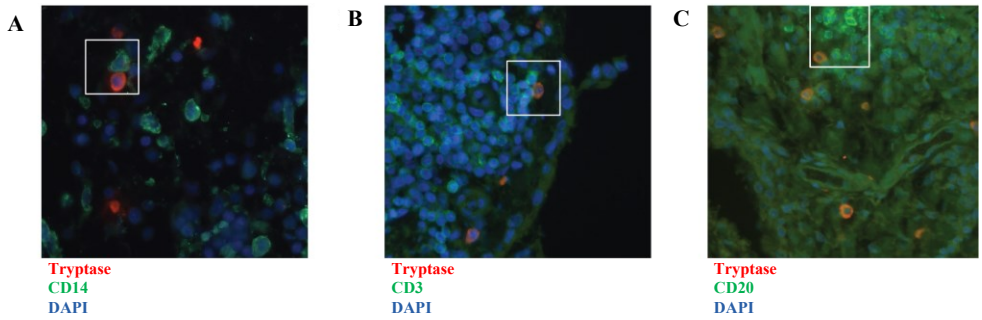

Figure 3. Mast cell interaction with immune cells in rheumatoid arthritis (RA) synovial tissue. RA synovial tissue was analyzed by immunofluorescence staining, showing tryptase-positive mast cells (red) closely interacting with other immune cells (green), including $CD14^+$ cells (A), $CD3^+$ T cells (B), and $CD20^+$ B cells (C). Nuclear staining with DAPI (blue) is also shown. Representative images from 1 of 3 donors are shown. Original magnification ×40. Boxed areas highlight the cellular interactions.

To quantify these interactions, we counted the number of tryptase-positive cells in close contact with each cell type. As shown in Table 1, a substantial proportion of mast cells (mean 19.5%) was found to be in contact with $CD14^+$ cells in the synovial tissue of 3 patients with RA. Similarly, the proportion of total $CD14^+$ cells interacting with mast cells was considerably higher than the proportion of B and T cells interacting with mast cells.

These results show that mast cells in the synovial tissue of patients with RA are in close contact with immune cells. In particular, a substantial number of synovial mast cells are localized in proximity to synovial $CD14^+$ cells.

Table 1. Interactions between mast cells (MCs) and other immune cells in RA synovia.

	RA 1	RA 2	RA 3	Mean*
Mast cells	31,3	11,7	30,7	24,6
Close to $CD14^+$	6,7 (21,4%)	1,7 (14,6%)	6 (19,6%)	4,8 (19,5%)
Mast cells	18,7	24	26,3	23
Close to $CD20^+$	1,7 (9.1%)	3 (12,5%)	0,3 (1,1%)	1,7 (7.3%)
Mast cells	27,3	25,6	38,3	30,4
Close to $CD3^+$	3,3 (12,1%)	1,7 (6,6%)	2 (5,2%)	2,3 (7,6%)
$CD14^+$ cells	80,7	7,0	125,3	71
Close to mast cells	8,3 (10,3%)	0,3 (4,3%)	11,0 (8,8%)	6,5 (9,2%)
$CD20^+$ cells	49,6	45,0	240,3	111,6
Close to mast cells	1 (2,0%)	0,3 (0,7%)	2,3 (1%)	1,2 (1,1%)
$CD3^+$ cells	358,6	0	14,7	124,4
Close to mast cells	6,7 (1,9%)	0 (0 %)	0 (0 %)	2,2 (1,8%)

Means of the number of cells counted in 10 high power fields by 3 independent and blind observers. RA = Rheumatoid arthritis, n=3. *Mean of the three patients.

MODULATION OF MONOCYTE RESPONSES BY IL-33- AND IgG-ACTIVATED MAST CELLS

Since a substantial proportion of mast cells were located near $CD14^+$ cells in the RA synovium (Figure 3 and Table 1), we next examined whether IL-33–primed mast cell supernatants were able to influence the activation of $CD14^+$ cells. To this end, LPS was used to boost the proinflammatory activation of $CD14^+$ monocytes, acting as a model for TLR-4–induced activation of monocytes. Monocyte responses were evaluated in the presence or absence of mast cell supernatants. We used TNF production as the response marker because mast cells produce only minimal amounts of TNF compared to LPS-stimulated monocytes. As shown in Figure 4A, in the presence of supernatants of mast cells, the TLR-4–mediated TNF production by monocytes was inhibited. Activation of mast cells with IL-33 and plate-bound IgG significantly enhanced the inhibition of TNF production without affecting monocyte survival (as measured by DAPI staining) (results not shown). This effect was dependent, in part, on IL-10, since we found that TNF production could be partially inhibited by anti–IL-10 blocking antibodies (Figure 4B).

In addition, blocking of histamine receptor 2 with ranitidine also partially reverted the induced production of TNF by monocytes. When both histamine and IL-10 were blocked, the ability of monocytes to produce TNF was retained. Importantly, the possibility that IL-33 may have exerted direct effects on monocytes was excluded, since no effect on the release of cytokines by monocytes was observed after adding this cytokine to the control medium.

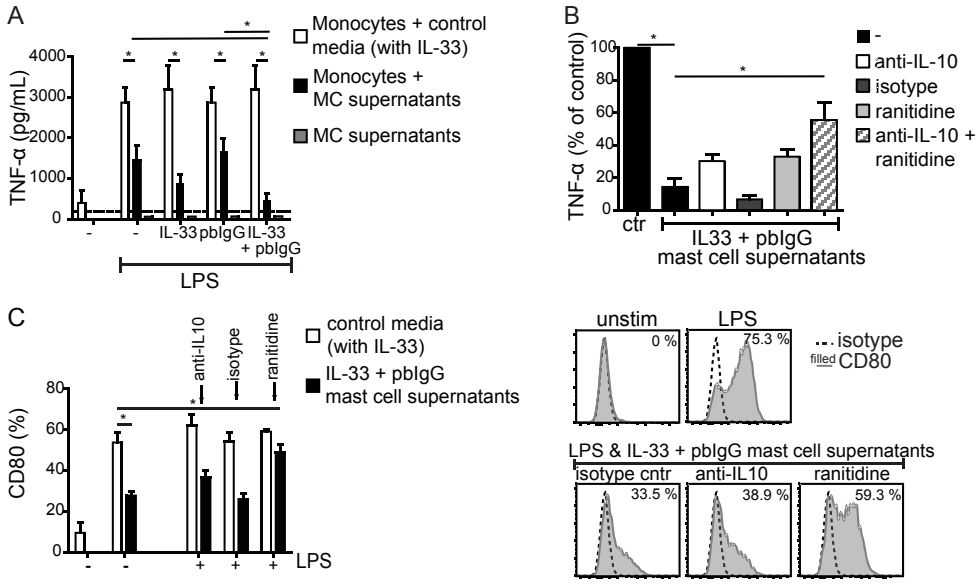

Figure 4. Mast cells (MCs) modulate monocyte activation. A, Monocytes were incubated either with control medium containing the same stimuli as used for mast cells (i.e., interleukin-33 [IL-33]) or with mast cell supernatants, followed by triggering with lipopolysaccharide (LPS). Levels of tumor necrosis factor (TNF) in cell cultures stimulated with IL-33 and/or plate-bound IgG (pbIgG) were measured by enzyme-linked immunosorbent assay after 18 hours. TNF levels in mast cell supernatants were below the predetermined level of detection (dotted horizontal line). B, Monocytes were incubated with the supernatants of activated mast cells and anti–IL-10 blocking antibody, correspondent isotype control, the histamine receptor 2 antagonist ranitidine, or the latter two combined. Levels of LPS-induced TNF are shown as a percentage of the values in control (ctr) medium, which was set at 100%. Results in A and B are the mean±SEM in samples from 6 monocyte donors and 12 mast cell donors in 5 independent experiments. C, Monocytes were left unstimulated (unstim) or were triggered with LPS after incubation with mast cell supernatants (IL-33+plate-bound IgG) or control medium with IL-33 alone, together with anti–IL-10 blocking antibody, isotype control, or ranitidine. Expression of CD80 by monocytes was analyzed by flow cytometry (right; representative results shown) and quantified as the mean±SEM percentage expression in samples from 6 monocyte donors and 8 mast cell donors in 4 independent experiments (left). ∗=$P < 0.05$ by Student's t-test for comparison between 2 groups, and by analysis of variance with Bonferroni post hoc test for multiple comparisons.

Membrane markers of monocyte activation were also evaluated, and the influence of mast cell mediators was assessed with blocking experiments. Interestingly, the LPS-induced expression of the costimulatory molecule CD80 was reduced by mast cell supernatants (Figure 4C). This effect was mainly dependent on histamine, since the down-regulated expression of CD80 could be reversed by incubation with the histamine receptor 2 antagonist ranitidine.

Taken together, these findings indicate that activation of mast cells triggered by IL-33 inhibits the proinflammatory responses of monocytes, as shown by the suppression of TNF production and CD80 expression. This effect is presumably mediated through the release of IL-10 and histamine.

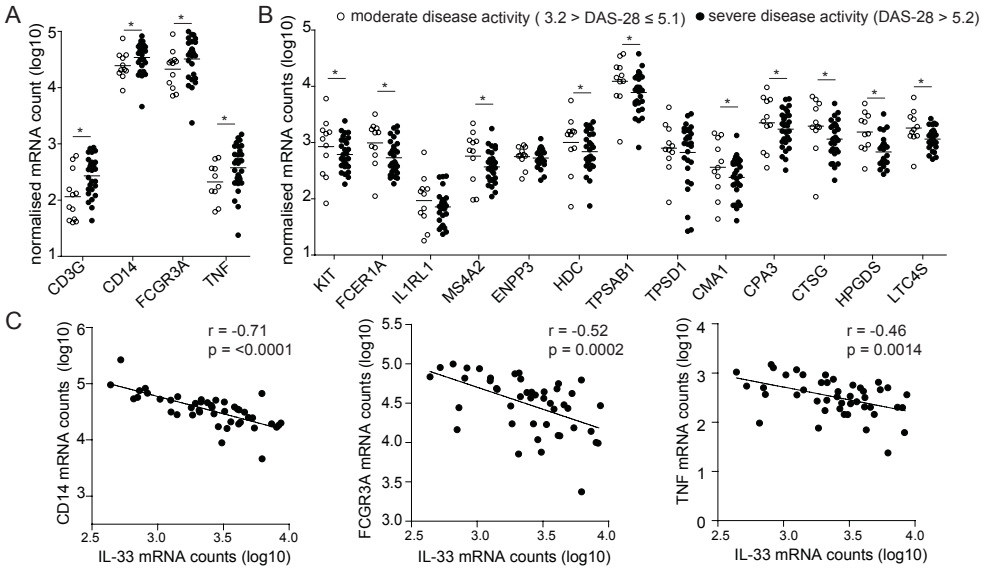

Figure 5. Gene expression in the synovial tissue of patients with early rheumatoid arthritis. A and B, Levels of mRNA for immune cell markers and tumor necrosis factor (TNF) (A) and for mast cell–specific genes (B) in patients with moderate disease activity (defined as a Disease Activity Score in 28 joints [DAS28] 3.2 to ≤5.1) and those with severe disease activity (DAS28 >5.2). Symbols represent individual patients; horizontal lines indicate the mean. ∗ = P < 0.05 by Student's t-test. C, Correlation of interleukin-33 (IL-33) mRNA levels with levels of CD14, Fcγ receptor type IIa (FCGR3A), and TNF mRNA. Each symbol represents an individual patient (n = 40), with the regression line and the Pearson's correlation coefficient. CD3G = CD3 antigen, γ subunit; CD14 = monocyte differentiation antigen CD14; FCGR3A = Fc fragment of IgG, low affinity IIIa (receptor for CD16); KIT = c-Kit hardy-zuckerman 4 feline sarcoma viral oncogene homolog (stem cell factor receptor); FCER1A = Fc fragment of IgE, high affinity I receptor for α subunit; IL1RL1 = interleukin-1 receptor–like 1 (ST2/IL-33 receptor); MS4A2 = membrane-spanning 4 domains, subfamily a, member 2 (FcεRIβ); ENPP3 = ectonucleotide pyrophosphatase/phosphodiesterase 3; HDC = histidine decarboxylase; TPSAB1 = tryptase, α/β-1; TPSD1 = tryptase, δ-1; CMA1 = chymase 1; CPA3 = carboxypeptidase A3, mast cell; CTSG = cathepsin G; HPGDS = prostaglandin d2 synthase, hematopoietic; LTC4S = leukotriene c4 synthase.

INVERSE ASSOCIATION BETWEEN SYNOVIAL EXPRESSION OF MAST CELL-RELATED GENES AND DISEASE ACTIVITY IN EARLY RA

To investigate whether the mast cell–mediated immunomodulatory/homeostatic functions observed in vitro might have a functional relevance in patients with RA, we analyzed the expression of mRNA extracted from the synovial biopsy tissue of patients with early RA (<12 months' duration) who were naive to DMARD therapy. As expected, the levels of mRNA for immune cell markers (e.g., CD3, CD14, and CD16) and TNF were significantly higher in patients with severe disease activity (measured as the Disease Activity Score in 28 joints [DAS28] (*39*), with severe disease activity defined as a DAS28 of >5.2) than in patients with moderate disease activity (defined as a DAS28 of 3.2 to ≤5.1) (Figure 5A). In contrast, the opposite observation was made for the mRNA expression levels of mast cell–specific genes, selected on the basis of a recent study describing highly specific genes for human mast cells (*40*).

Using the mRNA levels of these genes as a proxy for the presence of mast cells, we observed that most of the mast cell–related genes (such as c-Kit, α/β1-tryptase, and chymase, among others) displayed a significantly lower expression in patients with severe disease activity compared to patients with moderate disease activity (Figure 5B).

These findings are consistent with the observations made in our in vitro studies, as they suggest that mast cells, unlike other immune cells, are not associated with a more severe clinical phenotype. Finally, the levels of IL-33 mRNA showed an inverse correlation with the levels of proinflammatory markers, such as CD14, FcγRIIIa, and TNF (Figure 5C). Supplementary Table 2 (online) provides the raw RNA sequence data on the analyzed genes in the 40 patients with early RA.

Taken together, these findings further support our hypothesis that IL-33–mediated activation of mast cells has an immunomodulatory/homeostatic role in RA and could potentially influence the level of disease severity in patients with RA.

DISCUSSION

Persistency of inflammation is the hallmark of RA. In particular, an imbalance between pro- and antiinflammatory signals is presumed to be the basis of the chronic inflammation observed in the course of RA. Among the many cells and pathways potentially involved in this process, we have now identified IL-33–mediated mast cell activation as a new mechanism leading to the down-regulation of immune responses in the context of RA. To examine the involvement of mast cells in the inflammatory response in RA, we investigated

their activation by plate-bound immune complexes (plate-bound IgG) and found that activation of mast cells can be modulated by IL-33, a cytokine previously implicated in the ability of mast cells to contribute to experimental arthritis (*23*). It has been suggested that IL-33 induces the accumulation of proinflammatory cytokine mRNA in murine mast cells (*21*), whereas no clear mechanisms were proposed to explain the combined effects of IL-33 and immune complexes on human mast cells (*22*). Our data show that IL-33, via its receptor ST2, induces the expression of the activating receptor for the Fc fragment of IgG (FcγRIIa) in human mast cells. This, together with the observations of additional intracellular interactions, might explain the enhancement of IgG-mediated activation of mast cells in the presence of IL-33.

We recently showed that mast cells can be activated by immune complexes formed by binding of ACPAs from the sera of patients with RA to citrullinated antigens, a response that can be augmented by the triggering effects of TLRs (*17*). In the present study, we extended these findings by showing that IL-33 augments the activation of mast cells induced by IgG ACPAs. Overall, these observations suggest that mast cells, activated by IL-33 and IgG, both of which are known to be present in the synovial compartment, might contribute to the inflammatory response in RA, thereby supporting the longstanding hypothesis that mast cells play a deleterious role in RA. By further exploring the influence of IL-33 on mast cell activation, we found that IL-33 induces human mast cells to release immunomodulatory mediators, such as IL-10 and histamine, as well as other cytokines associated with type 2 immune responses, such as IL-5 and IL-13. The release of these IL-33–induced mediators was further enhanced by IgG triggering. In particular, the combined stimulation with IL-33 and IgG showed a synergistic effect that, intriguingly, was present for IL-33–induced mediators (such as IL-5, IL-10, IL-13, and histamine) and absent for mediators induced by IgG (such as CXCL8/IL-8). These findings are important because they indicate that IL-33 is able to prime mast cells toward a Th2/immunomodulatory phenotype, a phenotype that becomes more prominent following the concomitant activation of mast cells by IgG.

Since IL-33 is found in the synovial fluid of patients with RA, we hypothesized that the effects of IL-33 on mast cells might be relevant in modulating the immune responses in RA, by, for example, influencing the behavior of other synovial immune cells. Immunostaining of the RA synovial tissue demonstrated that human synovial mast cells were in close proximity to $CD14^+$ monocyte/macrophages, $CD3^+$ T cells, and $CD20^+$ B cells. In particular, synovial mast cells were most commonly found in proximity to $CD14^+$ monocyte/macrophages. Without implying that such interactions are specific for RA, these findings could nevertheless indicate that mast cell–monocyte interactions at the synovial level are frequent and might, therefore, have functional consequences.

To validate the hypothesis that IL-33 triggering of mast cell activation may exert immunomodulatory effects, we explored the ability of these cells to influence monocyte responses. Mast cells activated with IL-33 and plate-bound IgG were able to dampen the activation of monocytes, inhibiting both the production of the prototypic proinflammatory cytokine TNF and the up-regulation of the costimulatory molecule CD80. These effects were mediated, at least in part, by the release of IL-10 and histamine from mast cells. Among other mediators with known immunomodulatory functions, IL-4 was recently shown to be responsible for mast cell–mediated inhibition of peritoneal macrophage phagocytosis in a mouse model of sepsis (*41*). However, we consider the involvement of mast cell–derived IL-4 in our in vitro system to be unlikely, since we could not detect this cytokine in the supernatants of stimulated mast cells. In our experiments, monocytes were stimulated with bacterial LPS, acting via TLR-4, a commonly used model to study monocyte activation. Independent of the effects of LPS, several endogenous stimuli, ligands of TLR-4, have been implicated in the pathogenesis of RA (*42*), suggesting that the inhibition of TLR-4–mediated activation of monocytes by mast cells might be relevant in the context of RA.

Although several studies explored the ability of mast cells to influence both innate (*41, 43*) and adaptive (*44, 45*) immune responses, the direct cross-talk between human mast cells and monocyte/macrophages has never been investigated. This study is the first to show that mast cells and $CD14^+$ monocyte/macrophages can interact at the synovial level. Furthermore, mast cells triggered with stimuli known to be present in the inflamed synovium and classically considered to be proinflammatory (i.e., IL-33 and IgG) surprisingly gain the ability to suppress the activation of monocytes.

Taken together, these findings might help to understand the contrasting observations obtained in animal models of arthritis, in which, upon mast cell depletion, their pro- or antiinflammatory functions might be revealed or balanced, depending on the specific experimental conditions. Moreover, considering the heterogeneity of RA, the role of human mast cells might be even more complex and possibly multifaceted (*2*). Most of the evidence points toward a deleterious role for mast cells in patients with RA, with some reports proposing the mast cell c-Kit receptor as a therapeutic target (*46-49*). However, these studies used relatively nonspecific inhibitors (multitargeted tyrosine kinase inhibitors), which could have effects on other receptors on different cells (*50*). Similarly, the alarmin IL-33 has been proposed as a promising treatment target for RA (*51*), even though its involvement in the pathogenesis of RA has not been clearly established (*33*). Our results indicate that mast cells, in addition to their well-known proinflammatory functions, are also able to mediate regulatory/homeostatic responses, in particular when exposed to IL-33.

To confirm the relevance of the latter hypothesis, we studied a cohort of patients with early (duration <12 months) RA who were naive to DMARD therapy. Interestingly, although the presence of many types of immune cells, as determined by cell-specific gene expression, was associated with high disease activity, the presence of mast cells displayed an inverse association with disease severity. At the same time, IL-33 mRNA levels were inversely correlated with the levels of proinflammatory markers, such as CD14, CD16 (FcγRIIIA), and TNF. These observations make it tempting to speculate that mast cells, in particular when triggered by IL-33, could function in an immunomodulatory manner in the synovial tissue of patients with RA. Thus, the findings provide a rationale for future studies aimed at unravelling the complex influence of mast cells on disease activity in different stages of RA.

In conclusion, the results of this study indicate that mast cells, finely balancing between their well-known proinflammatory functions and their IL-33–triggered anti-inflammatory functions as observed herein, might play a previously unrecognized role as immunomodulatory/homeostatic cells in the pathogenesis of RA.

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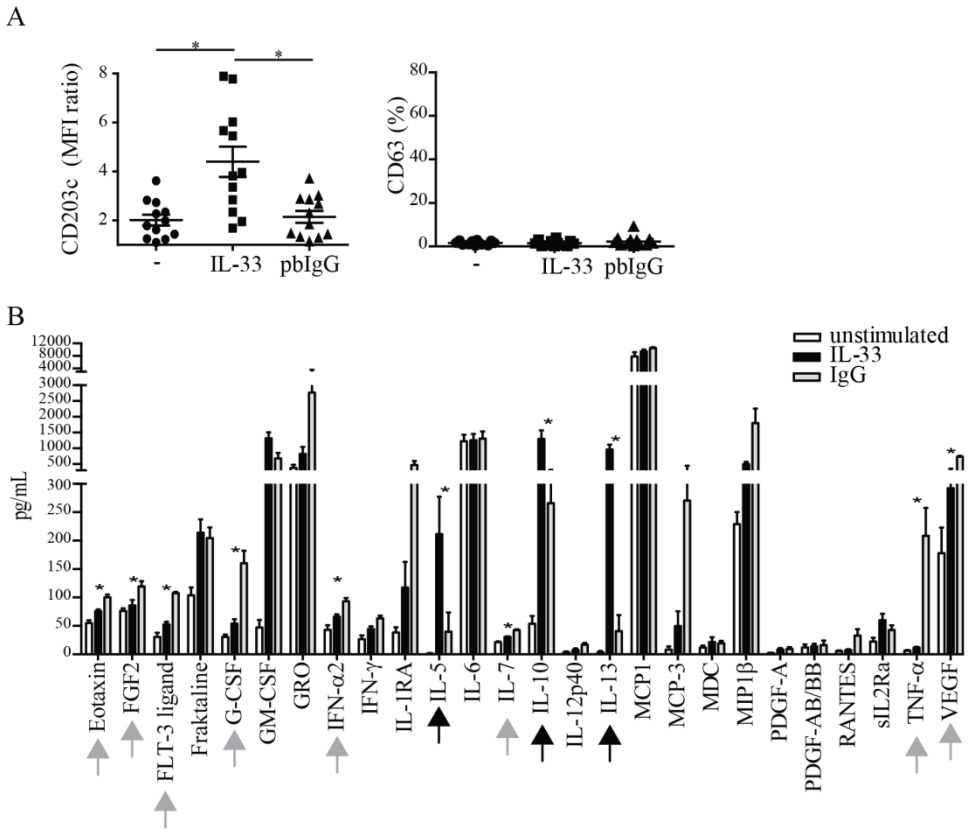
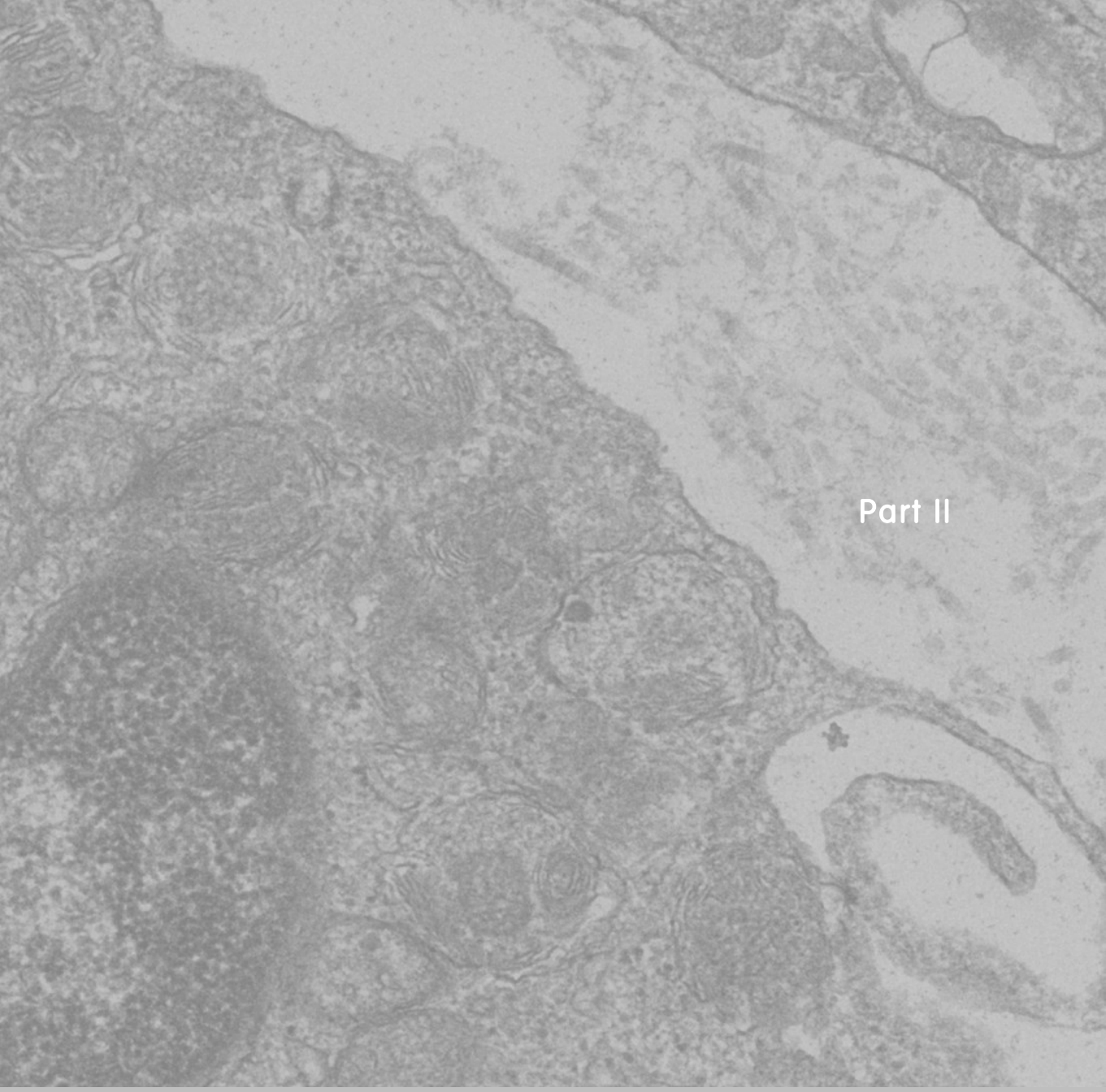
SUPPLEMENTARY FIGURE



Supplementary Figure 1. FACS and multiplex analyses of IL-33 and pbIgG-triggered mast cells. Mast cells were triggered with IL-33 or plate bound IgG for 24h. A, Expression of CD203c (left) as Median Fluorescence Intensity (MFI) ratio to isotype, and CD63 (right), as % of positive cells. Each symbol represent a mast cell donor (n=12) from 5 independent experiments. *p<0.05 determined by ANOVA with Bonferroni's post-test (for multiple comparisons) B, Of the 42 mediators included in the panel, the ones above minimal detection levels are shown**. Levels of each mediators in IL-33 and IgG-triggered mast cells were compared, black arrows indicate mediators which levels were significantly higher in IL-33-triggered mast cells, grey arrows mediators which levels were significantly higher in IgG-triggered mast cells. n=3 mast cells donors, 3 independent experiments. *p 0.05 determined by Student's t test, comparing IL-33 and IgG-triggered mast cells for each mediator. **As IL-8 levels were over the detection range, this cytokine is not shown (IL-8 levels were already measured by ELISA, as shown in Figure 1).



Part II

Interactions between mast cells and $CD4^+$ T cells

COMMUNICATION BETWEEN HUMAN MAST CELLS AND CD4$^+$ T CELLS THROUGH ANTIGEN-DEPENDENT INTERACTIONS

Chapter 7

Jolien Suurmond,[1,†] Jurgen van Heemst,[1,†] Jacqueline van Heiningen,[1] Annemarie L Dorjée,[1] Marco W Schilham,[2] Feddo B van der Beek,[3] Tom WJ Huizinga,[1] Annemie JM Schuerwegh,[1,4] René EM Toes[1]

[1]Department of Rheumatology, Leiden University Medical Center (LUMC), Leiden, The Netherlands; [2]Department of Pediatrics, LUMC, Leiden, The Netherlands; [3]Department of Otorhinolaryngology, LUMC, Leiden, The Netherlands; [4]Department of Immunology/ Allergology/Rheumatology, University of Antwerp, Antwerp, Belgium

[†]These authors contributed equally to this work.

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ABSTRACT

Mast cells (MCs) are immune cells residing in tissues where pathogens are first encountered. It has been indicated that MCs might also be involved in setting the outcome of T-cell responses. However, little is known about the capacity of human MCs to express MHC class II and/or to capture and present antigens to $CD4^+$ T cells.

To study the T-cell stimulatory potential of human MCs, $CD34^+$ stem cell derived MCs were generated. These cells expressed HLA-DR when stimulated with IFN-γ, and, importantly, presented peptide and protein for activation of antigen-specific $CD4^+$ T cells. The interplay between MC and T cell led to increased HLA-DR expression on MCs. MCs were present in close proximity to T cells in tonsil and expressed HLA-DR and CD80, indicating their ability to present antigens to $CD4^+$ T cells in T-cell areas of human LNs.

Our data show that MCs can present native antigens to human $CD4^+$ T cells and that HLA-DR expressing MCs are present in tonsil tissue, indicating that human MCs can directly activate T cells and provide a rationale to study the potential of MCs to prime and/or skew human T-cell responses.

INTRODUCTION

Mast cells (MCs) are most well known because of their role in IgE-mediated immune responses as they express the high-affinity FcεRI. MCs are present at strategic locations of the environment/host interface where they can encounter pathogens. Therefore, they have been implicated in the regulation of adaptive immune responses as well as in the regulation of T-cell immunity. Secretion of TNF by skin MCs was shown to be essential for LN hypertrophy and T-cell recruitment during bacterial infection (*1*).

Furthermore, MCs were shown to be capable of migrating from the skin to the draining LNs in murine models of contact hypersensitivity and UV radiation (*2, 3*). In the draining LNs, the secretion of chemokines by MCs has been implicated in the recruitment of T cells (*2, 4*). These studies, therefore, indicate that MCs can be involved in the regulation of T-cell trafficking toward lymphoid organs, suggesting that MCs may have a role in the induction of primary and/or local memory T-cell responses. Indeed, anti-CD3-stimulated T cells displayed enhanced proliferation and cytokine secretion in the presence of MCs, indicating that MCs are capable of enhancing T-cell responses (*5-7*).

A first direct demonstration that MCs can also present antigens to T cells came from mouse studies showing that OVA-specific TCR-transgenic $CD8^+$ T cells can be activated by MCs via

MHC class I (*8, 9*). Likewise, using OVA-specific TCR-transgenic $CD4^+$ T cells, it was demonstrated in vitro that mouse MCs can also activate MHC class II restricted T cells when loaded with OVA peptide, but were less efficient in presenting OVA protein (*10, 11*).

Thus far, little information is available on the antigen-presenting capacity of human MCs. Human MCs have also been shown to express MHC class II, especially after incubation with IFN-γ, which suggests their capacity to present antigen to $CD4^+$ T cells (*12-14*). Indeed, activation of T-cell hybridomas with superantigens by these cells has been reported (*14*). However, T-cell activation by superantigen does not require the presentation of antigenic peptides by MHC molecules or the processing of protein via endogenous pathways. Human MCs have been reported to be present in the interfollicular area in tonsil (*7*), indicating that they may be involved in the activation of T-cell responses in lymphoid organs. However, it is unknown whether these MCs possess the required molecular make-up, such as HLA-DR and costimulatory molecules, to present antigen and activate T cells in lymphoid organs.

As it is unclear whether human MCs are able to process and present native antigen to $CD4^+$ T cells, we aimed to study the interaction between these cells. Our data show that human MCs can present both peptide and entire protein antigens to $CD4^+$ T cells, using two different antigen-specific $CD4^+$ T-cell responses as read-out. Interaction of $CD4^+$ T cells with MCs led to the enhanced expression of HLA class II molecules on MCs via release of IFN-γ by T cells. In addition, we show that human MCs, analyzed ex vivo from tonsil, are present in T-cell areas where they express HLA-DR and CD80. Together, these data indicate that antigen-specific MC–T cell interactions could contribute to the outcome of immune responses in the human.

RESULTS

HUMAN PERIPHERAL BLOOD DERIVED MC EXPRESS HLA-DR WHEN STIMULATED WITH IFN-γ

Human MCs were generated from $CD34^+$ stem cells. Typical flow cytometry plots identifying MCs by combined expression of CD117, CD203c (Ectoenzyme E-NPP3), FcεRI, and intracellular tryptase are shown in Figure 1A, indicating that the 6-week culture results in mature ($CD117^+$ $tryptase^+$) MCs. No expression of the DC markers DC-SIGN and CD1c (BDCA-1) was found on our cultured MCs (data not shown). Only low, but detectable, levels of HLA-DR were detected on resting MCs (Fig. 1B and C).

Because it was previously shown for MC lines that IFN-γ could induce expression of HLA class II, we evaluated whether this could lead to upregulation of HLA-DR on our cultured

MCs as well. Incubation with 50 ng/mL IFN-γ for 24 h induced upregulation of HLA-DR (Fig. 1B and C). No induction of expression of HLA class II was found after stimulation with TLR ligands, anti-IgE, or recombinant IL-4 (data not shown). These results indicate that in the presence of IFN-γ, MCs can acquire the expression of HLA class II, and possibly the ability to present antigen to $CD4^+$ T cells.

Of the costimulatory molecules tested (CD80, CD86, ICOSL, OX40L, CD40L, and CD40), we found expression of CD80 but not CD86 or any of the other markers, as assessed by flow cytometry (Fig. 1D and E). Together, these data indicate that human MCs can express molecules required for stimulation of $CD4^+$ T cells.

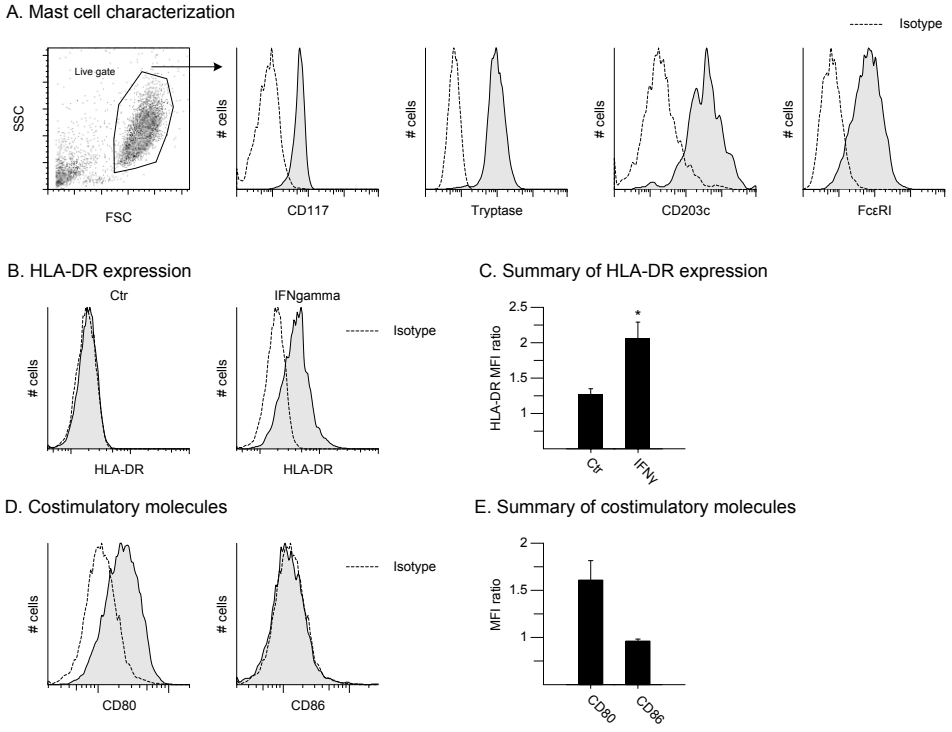


Figure 1. Expression of HLA-class II and costimulatory molecules by human peripheral blood derived mast cells (MCs). (A) Representative flow cytometric plots of MC characterization after 6–8 weeks differentiation showing expression of CD117, intracellular tryptase, FcεRI, and CD203c. Histograms show gated live cells based on forward scatter (FSC) and sidescatter (SSC) characteristics as shown in the first panel. (B, C) MCs were cultured in the presence of 50 ng/mL IFN-γ for 24 h, after which expression of HLA-DR was assessed. (D, E) Expression of CD80 and CD86 in resting MCs. (B, D) Representative examples of MCs gated as $CD117^+$ $CD203c^+$. (C, E) Summaries (mean +SEM) of independent MC cultures (n = 3). Expression (MFI ratio) is indicated as MFI of the marker of interest (e.g. HLA-DR) divided by the MFI of the matching isotype control. Asterisk in (C) indicates significant ($p < 0.05$) increase in IFN-γ-treated cells compared with ctr MCs, using Student's t-test.

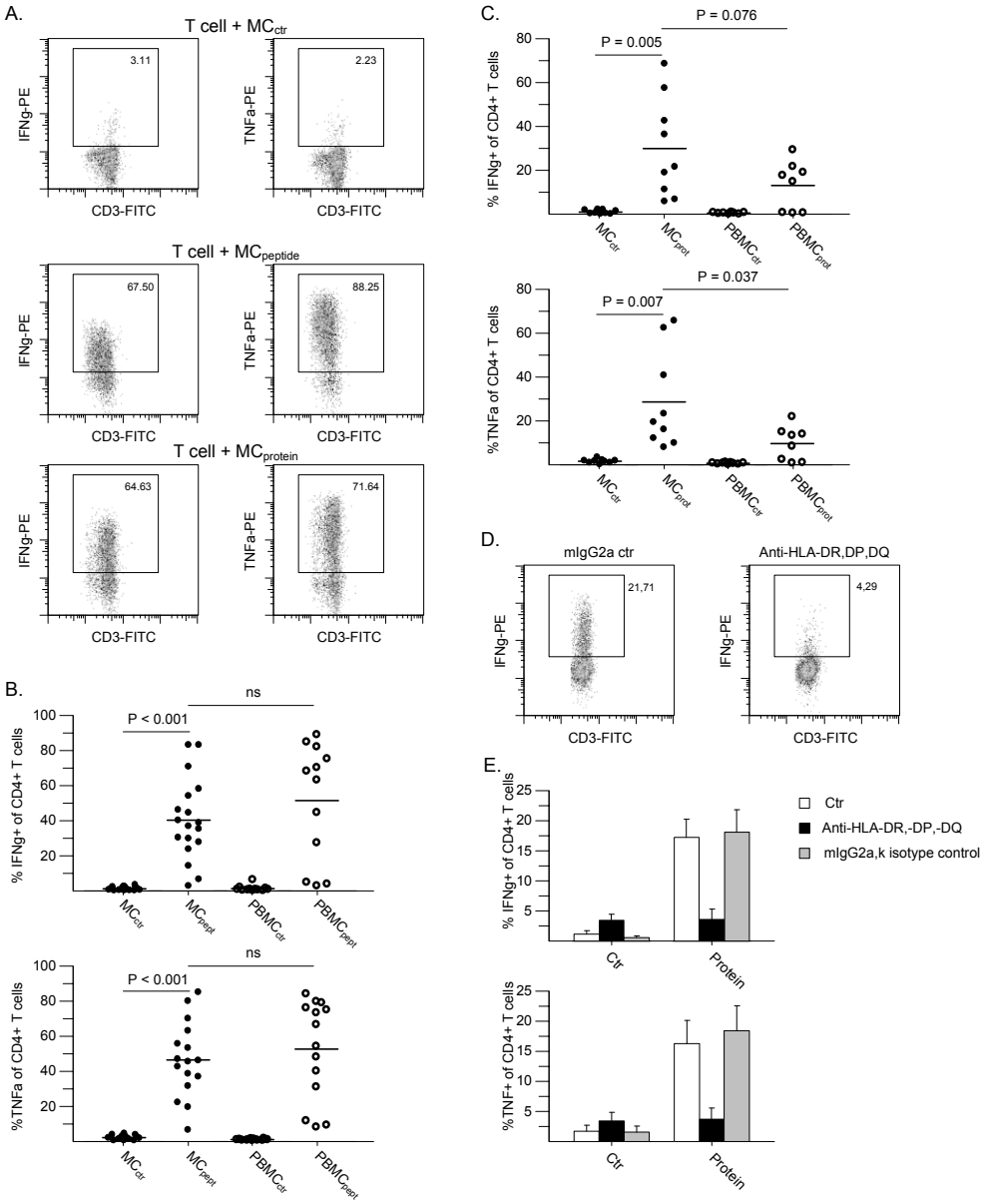


Figure 2. Antigen presentation by human mast cells (MCs) leads to activation of adenovirus-specific T cells. (A) Representative flow cytometric plots of TNF-α and IFN-γ production by gated $CD3^{+}CD4^{+}$ T cells after overnight coculture with MCs preincubated with medium (ctr), adenovirus-specific peptide or protein, in the presence of brefeldin A. (B, C) Summary of T-cell activation after preincubation of MCs with peptide and protein. Each dot shows an independent MC culture or PBMCs donor (n = 18 and n = 9 for peptide and protein, respectively). p values comparing the indicated conditions were calculated using Student's t-test. (D, E) Cytokine production by T cells after preincubation of MCs with blocking HLA class II antibodies (n = 3). (E) Data are shown as mean +SEM pooled from (n = 3) independent MC cultures.

HUMAN MC CAN PRESENT PEPTIDE AND PROTEIN TO CD4$^+$ T CELLS

To evaluate the potential of human MCs to present antigen to CD4$^+$ T cells, MCs were preincubated with an adenovirus-derived peptide or hexon IID protein. Subsequently, MCs were cocultured with an adenovirus-specific T-cell clone recognizing the particular peptide. As shown by intracellular cytokine staining, MCs preincubated with peptide, in contrast to control MCs, were capable of inducing robust cytokine production by the T cells (Fig. 2A and B). The capacity of MCs to induce T-cell responses with the peptide was comparable to that of PBMCs. Importantly, MCs preincubated with the hexon IID protein were also capable of activating T cells, indicating the capacity of MCs to take up, process, and present native protein antigen to CD4$^+$ T cells (Fig. 2A and C).

Presentation of protein antigens by MCs appeared rather efficient, as 5 μg/mL protein, equivalent to 225 nM, was still able to activate adenovirus-specific T cells. Furthermore, although there was no difference between the T-cell responses induced by MCs compared with PBMCs when they were incubated with peptide, MCs were more efficient than PBMCs in inducing T-cell responses with the protein antigen (Fig. 2C). Similar results were obtained when T-cell-derived cytokine levels in supernatant were measured by ELISA (data not shown).

To confirm that these T-cell responses were HLA class II dependent, MCs were next incubated with blocking HLA class II or isotype control antibodies. CD4$^+$ T-cell responses induced by MCs preincubated with hexon IID protein were almost completely inhibited by HLA class II blocking antibodies, compared with the respective isotype control (Fig. 2D and E), further confirming that the MCs stimulated the T cells via HLA class II.

Although the data presented above indicate that human MCs can present protein antigens to T cells, they do not show whether polyclonal T cells can be activated by MCs. To determine whether polyclonal T cells, rather than cloned T cells potentially harboring a high-avidity TCR to the peptide–MHC complex, are also activated by MCs, we next generated CD4$^+$ bulk T cells expanded for 1 week with pooled tetanus toxoid, tuberculin, and Candida albicans. Subsequently, autologous MCs were incubated with these recall antigens and used as APCs for polyclonal bulk T cells. MCs were capable of activating autologous polyclonal CD4$^+$ T cells, confirming their T-cell-activating capacity (Fig. 3). The response by CD4$^+$ T cells that was induced by MCs was comparable to or better than our positive control, autologous adherent PBMCs.

Together, these results indicate that human MCs are capable of taking up and processing native proteins for presentation to CD4$^+$ T cells in the context of HLA class II.

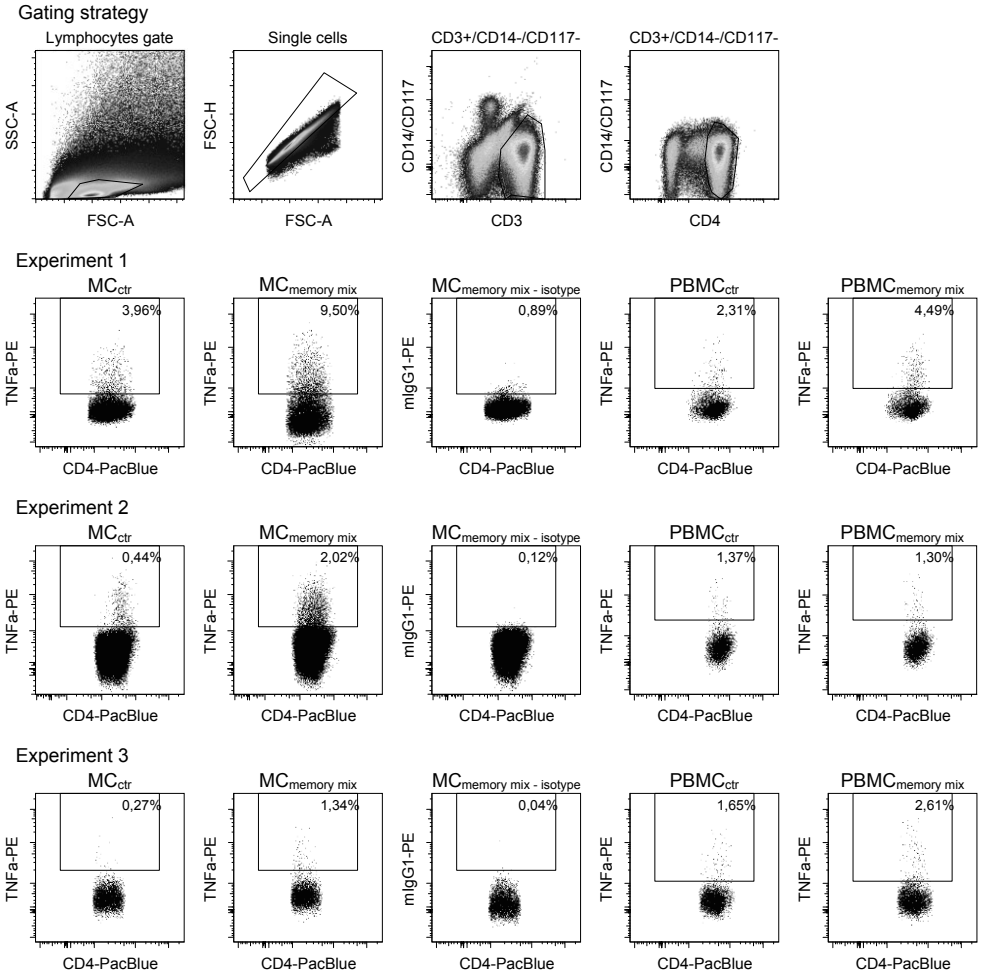


Figure 3. Antigen presentation by human mast cells (MCs) to autologous bulk $CD4^+$ T cells. Polyclonal $CD4^+$ T-cell bulks were generated by activation of PBMCs with the recall antigens tetanus toxoid, tuberculin purified protein derivative, and Candida albicans for 7 days. Autologous MCs or adherent PBMCs preincubated with medium (ctr) or these recall antigens (memory mix) were cultured overnight with the bulk T cells in the presence of brefeldin A. Flow cytometric plots of TNF-α production by T cells in three independent experiments are shown. T cells were gated based on FSC/SSC characteristics, CD14/CD117 negativity, $CD4^+$, and $CD3^+$ as shown in top panel.

CD4$^+$ T CELLS CAN MODULATE MC VIA IFN-γ

As the interaction between APCs and T cells can be bidirectional, the potential of CD4$^+$ T cells to modulate MC phenotype was analyzed. To this end, MCs preincubated with peptide were cocultured with adenovirus-specific CD4$^+$ T cells to evaluate potential effects of T-cell activation on the MCs. As shown in Figure 4A and B, the expression of HLA-DR by MCs was enhanced when T cells were activated by MCs preincubated with peptide. MCs cultured without T cells or MCs not preincubated with peptide did not show an increase in HLA-DR expression. Upregulation of HLA-DR was inhibited by blocking IFN-γ, ranging from 14 to 100% inhibition as compared with the isotype control (Fig. 4C and D).

These data indicate that the effect of activated T cells responsible for HLA class II upregulation on MCs was mainly mediated via IFN-γ. Together, these results show that MCs not only activate T cells, but also CD4$^+$ T cells that can modulate MCs by upregulation of HLA class II, indicating a bidirectional interplay between MC and T cell.

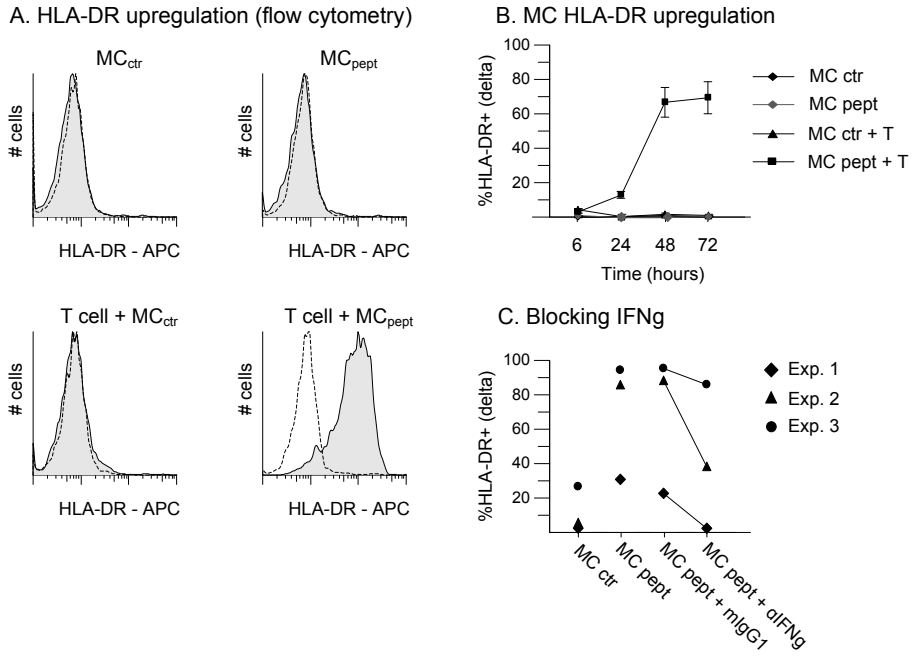
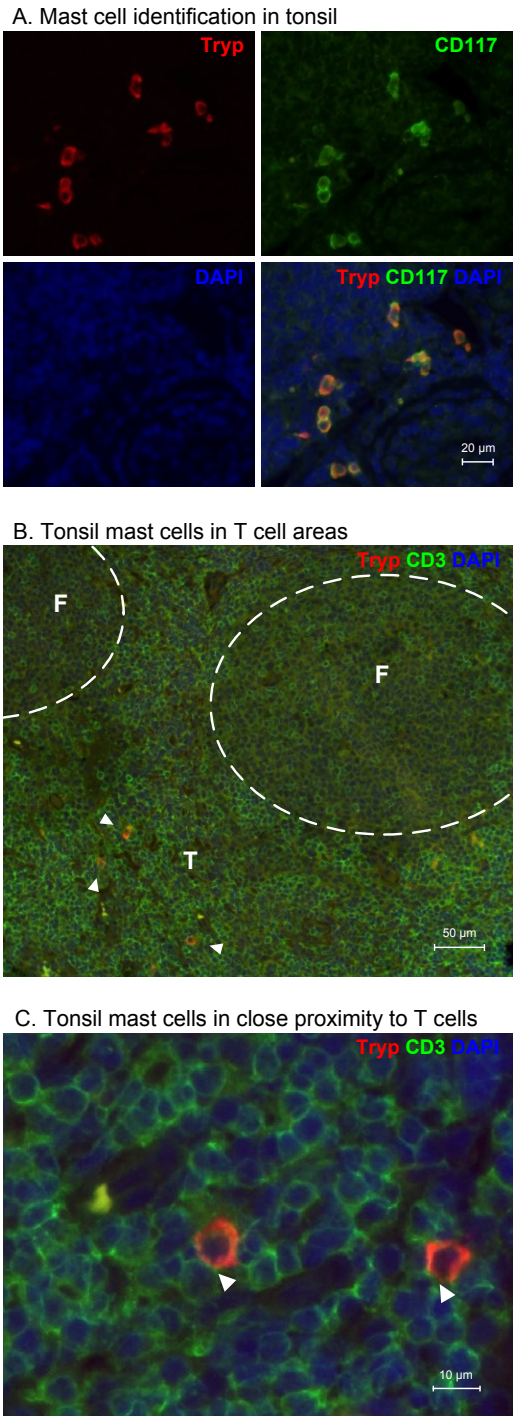


Figure 4. Induction of HLA class II on mast cells (MCs) through IFN-γ production by CD4$^+$ T cells. (A, B) Representative flow cytometric plots and summary of HLA-DR expression by MCs preincubated with medium (ctr) or adenovirus-specific peptide (pept), after coculture with or without adenovirus-specific CD4$^+$ T cells for the indicated time periods (n = 2). (C, D) Representative flow cytometric plots and summary of HLA-DR expression by MCs, after coculture for 48 h with adenovirus-specific CD4$^+$ T cells in the presence of isotype control antibodies (mIgG1) or blocking antibodies to IFN-γ (n = 3). MCs are gated as CD203c$^+$ cells as shown in Figure 1. Data are shown as mean ± SEM pooled from (n = 3) independent MC cultures.



HUMAN TONSILS CONTAIN HLA-DR$^+$ MC

To evaluate whether HLA-DR$^+$ MCs can also be found in vivo in T-cell areas of lymphoid organs, tonsil sections were stained for MCs. MCs were identified as CD117$^+$ tryptase$^+$ double-positive cells and were found in all (n = 6) tissues analyzed (Fig. 5A). All tryptase$^+$ cells expressed CD117, whereas also some CD117$^+$ tryptase- cells could be identified, indicating that CD117 alone cannot uniquely define tonsillar MCs. Tryptase$^+$ MCs were most often found in T-cell areas characterized by abundant expression of CD3, and were commonly observed in close proximity to CD3$^+$ T cells (Fig. 5B and C). These results indicate that MCs are present in T-cell areas of lymphoid organs and at locations allowing T cell–MC interactions.

Figure 5. Mast cells (MCs) are present in T-cell areas of human tonsil. (A) MCs in tonsils are characterized as tryptase (tryp)$^+$ CD117$^+$ double-positive cells. (B, C) Double staining of tryptase and CD3. (B) MCs are mainly found in T-cell areas of the tonsil. "F" indicates the B-cell follicles and "T" indicates the T-cell area, defined by expression of CD3. Arrowheads show Tryp$^+$ MCs present in the T-cell area. (C) MCs are often found in close proximity to T cells. Arrowheads show examples of Tryp$^+$ MCs in close contact with CD3$^+$ T cells. Original magnifications: 20× (A, B) and 40× (C). (A–C) Images are representative examples of fluorescent stainings of tonsils from six independent donors.

Nonetheless, as evaluated by immunofluorescent staining of tonsil sections, we could not detect HLA-DR on these tryptase$^+$ MCs (data not shown). As HLA-DR expression might be relatively low on tonsillar MCs, the detection of its expression might be obscured by the high HLA expression of other APCs that are abundantly present in tonsil. Therefore, we also analyzed MCs in tonsil cell suspensions using a more sensitive flow cytometric technique with antibodies recognizing CD117, CD203c, FcεRI, HLA-DR, and costimulatory molecules (Fig. 6). MCs were identified by their expression of high levels of CD117 and were further characterized by expression of the MC markers CD203c and FcεRI (Fig. 6A and B), and absence of the DC markers DC-SIGN and CD1c (data not shown). HLA-DR expression was found on MCs of all six donors analyzed, both when analyzed as percentage or mean fluorescence (Fig. 6C and D). The relative immunofluorescence was approximately tenfold lower than on other HLA-DR$^+$ cells in the tonsil, which is in line with the difficulty to detect HLA-DR on MCs in a convincing manner by staining of tonsil sections.

The expression of costimulatory molecules by tonsillar MCs was also evaluated (Fig. 6E and F). Although only low levels of CD86 were found, all four donors analyzed showed significant expression of CD80 compared with the isotype control, confirming the data obtained from MCs cultured from blood (Fig. 1C).

Together, these results show that MCs expressing HLA-DR and costimulatory molecules are present in human tonsil, and that the majority of these MCs are located in close proximity to T cells, arguing for MC–T cell interaction in human LNs.

DISCUSSION

In this article, we show for the first time that cultured human MCs are capable of presenting native protein antigens to CD4$^+$ T cells. The interaction between MCs and CD4$^+$ T cells modulated the MC phenotype, indicating that both cell types can influence each other in an antigen-dependent manner. Furthermore, the interaction between these cell types may be physiologically relevant as the presence of MCs in proximity to T cells was also shown in lymphoid organs.

A clear inherent limitation of our study is that we are unable to show the ability of MCs to present antigen in vivo. Likewise, there were some differences between the cultured MCs and their in vivo counterparts. Whereas the cultured MCs readily expressed HLA-DR after coculture with the T cells, the tonsil-resident MCs already expressed HLA-DR when evaluated directly ex vivo. This difference might be explained by the exposure of MCs to IFN-γ either in the tonsil or in the periphery by either T cells or innate IFN-γ-producing cells such as NK cells.

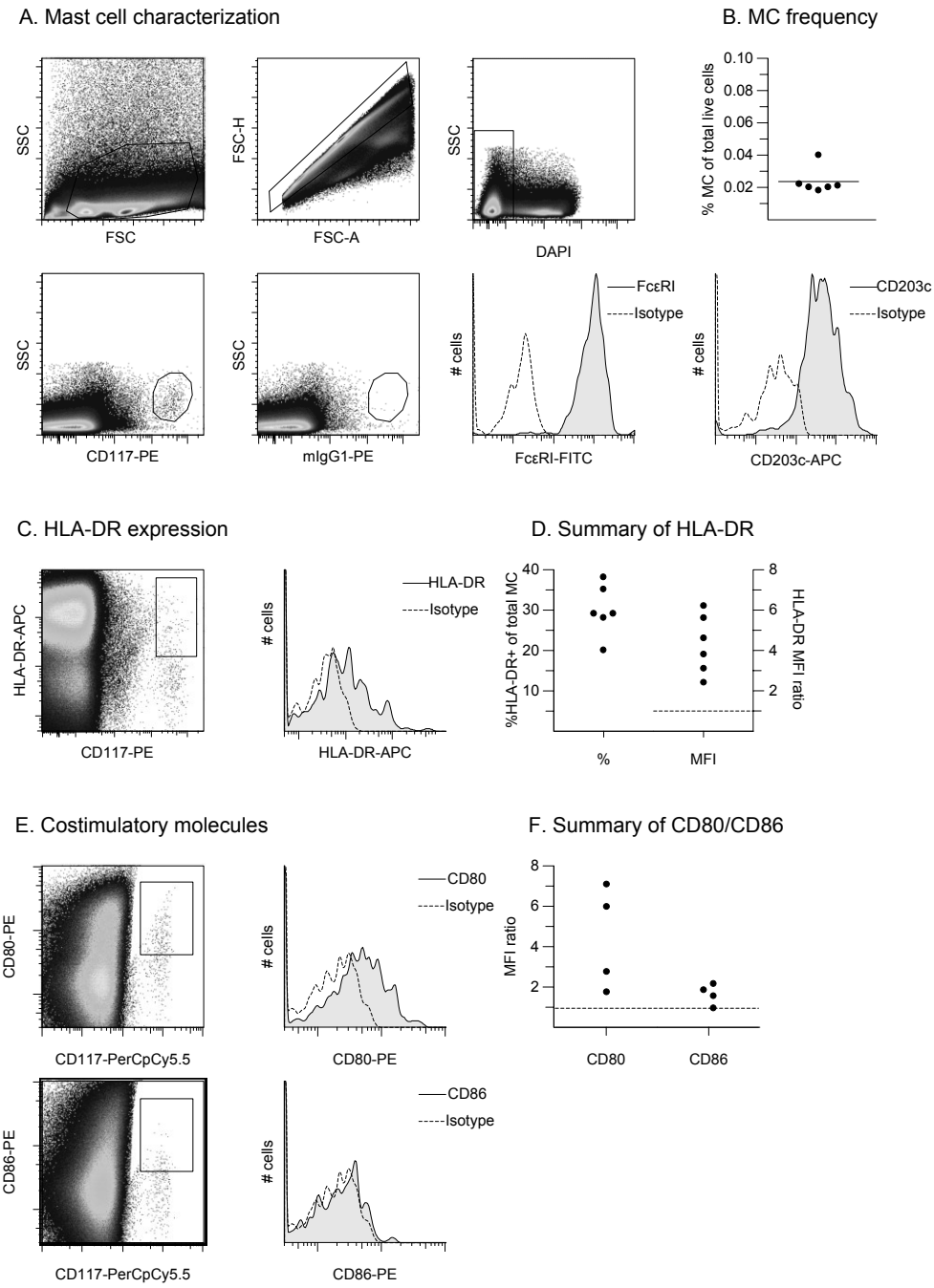

A. Mast cell characterization
B. MC frequency
SSC
FSC
FSC-H
FSC-A
SSC
DAPI
% MC of total live cells
0.10
0.08
0.06
0.04
0.02
SSC
CD117-PE
SSC
mIgG1-PE
cells
FcεRI
Isotype
FcεRI-FITC
cells
CD203c
Isotype
CD203c-APC
C. HLA-DR expression
D. Summary of HLA-DR
HLA-DR-APC
CD117-PE
cells
HLA-DR
Isotype
HLA-DR-APC
%HLA-DR+ of total MC
40
30
20
10
HLA-DR MFI ratio
8
6
4
2
%
MFI
E. Costimulatory molecules
F. Summary of CD80/CD86
CD80-PE
CD117-PerCpCy5.5
cells
CD80
Isotype
CD80-PE
MFI ratio
8
6
4
2
CD80
CD86
CD86-PE
CD117-PerCpCy5.5
cells
CD86
Isotype
CD86-PE

Figure 6 *(left)*. Mast cells (MCs) in tonsil express HLA-DR and CD80. (A, B) MC characterization in total tonsil cell suspensions by CD117, FcεRI, and CD203c (n = 6). Live cells were gated based on FSC/SSC characteristics and DAPI negativity and their expression of CD117 or isotype control are shown in the dotplots. Histograms show expression of FcεRI and CD203c by gated CD117hi cells as shown in the dotplots. (C, D) Expression of HLA-DR by MCs characterized as in (A) (n = 6). (E, F) Expression of CD80 and CD86 by MCs characterized as in (A) (n = 4). Expression (MFI ratio) in (D) and (F) is indicated as MFI of the marker of interest (e.g. HLA-DR and CD80) divided by the MFI of the matching isotype control. Dotted lines in (D) and (F) indicate absence of expression of the indicated marker compared with the isotype control (MFI ratio = 1). Data shown are representative of tonsils from four to six independent donors.

Nonetheless, as the phenotype of MCs for the other markers evaluated (CD117, tryptase and CD80) was comparable, we believe that the cultured MCs are a good representation of their counterparts in vivo, and that the biological effects observed in vitro are conceivably present in vivo as well.

The presentation of antigen to $CD4^+$ T cells by human MCs may serve to stimulate T cells in diverse locations, such as lymphoid organs and peripheral tissues. As MCs can produce a different set of cytokines than other APCs (*15-17*), our findings could potentially have important implications for the skewing of T helper cells and maybe even for the outcome of naïve T-cell priming in LNs. Since both cultured and tonsil-resident MCs express HLA class II and CD80, their potential in stimulating naïve $CD4^+$ T cells cannot be excluded. Skin MCs have been shown to migrate from infected/allergic areas to draining LNs in the mouse (*2, 3*). Nonetheless, mouse MCs, although able to activate memory T cells, were not able to prime naïve $CD4^+$ T cells, probably due to the lack of costimulatory molecules (*10, 11*).

As MCs only comprised approximately 0.02–0.04% of total tonsil cells as shown in Fig. 6, and as their expression of HLA class II molecules was much lower than that of other professional APCs such as DCs, we do not consider it likely that MCs play a crucial role in priming of T cells in primary immune responses. In contrast, we consider it more likely that they might be more important in activation of secondary T cells in either LN or peripheral tissue. Especially, the latter is an intriguing possibility as MCs are present at several strategic locations where pathogens are first encountered. It could be very beneficial to the host to swiftly activate effector memory T cells that are known to reside in tissues (*18*), by MCs that are well known for their immediate and strong actions upon activation.

Furthermore, MCs could produce several cytokines upon activation through activation via IgE and TLR, which may have an impact on T-cell activation or skewing. In addition, $CD4^+$ T cells could also influence MCs, e.g. by inhibiting or modulating their reactivity. As murine

MCs were shown to specifically enhance activation of $CD4^+$ Treg cells through MHC class II (*10*), such T cell–MC interactions could also function to inhibit MC responses, such as observed in a murine model of anaphylaxis (*19*). The expression of MHC class II by MCs depended on the production of IFN-γ by T cells in this study, showing the ability of T cells to influence MC phenotype. Therefore, interaction between MCs and T cells might direct the responsiveness of both cell types, allowing for optimal control of both cell populations.

We have not shown how the MCs have taken up antigen for presentation to T cells in our study. However, we observed no enhancement of antigen presentation when protein antigens were targeted to the FcεRI on the MCs via hapten-specific IgE and hapten-coupled protein (data not shown). The lack of enhancement of presentation by Fc-receptor-targeted antigens by human MCs contrasts findings obtained in mice, as two studies reported enhanced antigen presentation by Fc-receptor-mediated uptake (*11, 20*). Nonetheless, another study using murine MCs indicated decreased antigen presentation when antigen was routed to IgE/FcεRI, possibly because antigens were protected from proteolytic degradation and subsequent presentation as peptide in MHC (*10*). Further research into this aspect is required, but our data indicate that Fc-receptor-mediated uptake and presentation of Ig(E)-bound antigen by human MCs, if present, is likely to be less efficient as compared with the ability of DCs to take up and present immune-complexed antigens (*21*).

In conclusion, our results show that cultured human MCs can function as APCs, by uptake and processing of protein, with subsequent presentation to $CD4^+$ T cells via HLA class II. Furthermore, expression of HLA class II and CD80 is found on MCs isolated from human tonsil, where they are found in close proximity to T cells. In addition, the interaction between MCs and $CD4^+$ T cells led to changes in the MC phenotype in an antigen-specific manner, indicating an intimate interaction between these two distinguished cell types.

MATERIALS AND METHODS

MAST CELLS

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). PBMCs were isolated using a standard Ficoll procedure, after which $CD34^+$ hematopoietic stem cells were isolated with CD34 microbeads (Miltenyi Biotec). Isolated $CD34^+$ stem cells were differentiated into MCs using serum-free medium (StemPro 34 + supplement, Gibco) with 30 ng/mL IL-3, 100 ng/mL IL-6, and 100 ng/mL stem cell factor (SCF) at 50 000 cells/mL as described (*22, 23*). Half of the medium was replaced weekly with serum-free medium containing 100 ng/mL IL-6 and 100 ng/mL

SCF. All recombinant cytokines were obtained from Peprotech. After 6–8 weeks, the purity of MCs was determined by flow cytometric analysis of CD117 (c-kit), FcεRI and CD203c, and intracellular tryptase. The purity of MCs ranged from 90 to 99%.

CD4$^+$ T-CELL CLONE

An adenovirus-specific CD4$^+$ T-cell clone (M2.11) was used to determine antigen presentation by MCs (*24*). This clone recognizes a peptide derived from hexon protein II, in the context of HLA-DRB1*03:01. MCs from blood of HLA-DRB1*03:01 positive donors were generated as described above. MCs were incubated with 5 μg/mL peptide for 24 h, or 1–50 μg/mL protein (22.2 kDa polypeptide fragment D from hexon protein II (*24*)) for 6–72 h, before coculture with the CD4$^+$ T cells. Before coculture with the T-cell clone, MCs were thoroughly washed to remove all soluble protein or peptide. Subsequently, the MCs were added to the CD4$^+$ T cells in a 1:1 ratio in RPMI with 10% human serum and 100 ng/mL SCF at 200 000 cells/mL. After 1 h, 5 μg/mL brefeldin A was added and incubated overnight. To determine the effects of activation of the T-cell clone on the MCs, the cells were cultured without brefeldin A for the indicated timepoints.

Blocking antibodies and isotype controls for HLA class II (BD Pharmingen, clone Tü39, 10 μg/mL) were added to the MCs, and incubated for 1 h at 37°C before the MCs were added to the T cells. Blocking antibodies remained present during the coculture. Blocking antibodies and isotype controls for IFN-γ (eBioscience, clone NIB42, 20 μg/mL) were added to the cocultures directly.

CD4$^+$ T-CELL BULK RESPONSES AGAINST RECALL ANTIGENS

Polyclonal CD4$^+$ T-cell bulks were generated by activation of PBMCs with the recall antigens tetanus toxoid at 0.75 Lf/mL (Netherlands Vaccine Institute), tuberculin purified protein derivative (0.5 μg/mL) (Netherlands Vaccine Institute), and C. albicans 0.005% (HAL allergy) in IMDM/5% human serum for 7 days, after which antigen presentation by autologous MCs was evaluated. MCs or autologous PBMCs were incubated with the recall antigens mentioned above for 16 h, before coculture with autologous bulk CD4$^+$ T cells. After this incubation, the cells were thoroughly washed to remove all soluble protein. Autologous PBMCs were used as positive control and were allowed to adhere to the plate at 37°C for 2 h, after which nonadherent cells were removed. Subsequently, the CD4$^+$ T cells were added to the APCs in IMDM/5% human serum at 2 × 106 cells/mL. After 1 h, 10 μg/mL brefeldin A was added and incubated overnight.

TONSILS

Human tonsils were obtained after tonsillectomy. A portion of these tissues were fixed with 4% formaldehyde in PBS, stored in 70% ethanol, and embedded in paraffin for immunofluorescent staining of sections. The remaining part of the tonsils was washed with RPMI with 10% FCS and cut into small pieces. Subsequently, the tonsils were dispersed through a 70 µm filter to get single-cell suspensions. Cells were frozen until flow cytometric staining and acquisition. This study was performed in accordance with the declaration of Helsinki and local ethical guidelines.

IMMUNOFLUORESCENCE

Tonsil tissues were treated according to the method described by Schuerwegh et al. to deparaffinize and retrieve antigens with EDTA (*25*). Slides were incubated with 8 µg/mL goat anti-human tryptase, 26.5 µg/mL rabbit anti-human CD117, 60 µg/mL rabbit anti-human CD3, or matching isotype controls for 1 h at room temperature. After washing, slides were stained with 2 µg/mL donkey α-goat AF 568 and 2 µg/mL donkey α-rabbit AF 488 for 1 h at room temperature. After washing, slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei. Slides were analyzed on a Zeiss Axio ScopeA1 microscope.

FLOW CYTOMETRY

For detection of HLA-DR and costimulatory molecules expression by cultured MCs, cells were incubated with fluorochrome-conjugated antibodies recognizing CD117, CD203c, HLA-DR, CD80, and CD86 or matching isotype controls. Intracellular staining of tryptase was performed as described (*26*).

For detection of expression of HLA-DR and costimulatory molecules on tonsil MCs, cells were incubated with fluorochrome-conjugated antibodies recognizing CD117, CD203c, FcεRI, HLA-DR, CD86, and CD80 or matching isotype controls for each of these antibodies. Just prior to flow cytometric acquisition, 0.2 µM DAPI was added for exclusion of dead cells.

For intracellular cytokine staining by $CD4^+$ T cells, the cells were incubated with fluorochrome-conjugated antibodies recognizing CD3 and CD4, after which they were permeabilized using CytoFix CytoPerm Kit (BD Biosciences). To gate out any remaining monocytes in the $CD4^+$ T-cell bulks, antibodies to CD14 were added as well. After washing, cells were incubated with PE-labeled anti-IFN-γ, TNF-α, or matching isotype control. Cells were taken up in 1% paraformaldehyde until flow cytometric acquisition. Flow cytometry was performed on FACS Calibur (BD) or LSR II (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical analysis was performed using SPSS PASW 17.0 and GraphPad Prism 4. For differences between two groups, Student's t-tests were performed. p values of <0.05 were considered statistically significant.

ACKNOWLEDGEMENTS

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HUMAN MAST CELLS CO-STIMULATE T CELLS THROUGH A CD28-INDEPENDENT INTERACTION

Chapter 8

Jolien Suurmond, Annemarie L Dorjée, Tom WJ Huizinga, René EM Toes

Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

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ABSTRACT

Mast cells are innate immune cells usually residing in peripheral tissues, where they are likely to activate T-cell responses. Similar to other myeloid immune cells, mast cells can function as antigen presenting cells. However, little is known about the capacity of human mast cells to co-stimulate CD4+ T cells.

Here, we studied the T cell-stimulatory potential of human mast cells. Peripheral blood-derived mast cells were generated and co-cultured with isolated CD4+ T cells. In the presence of T-cell receptor triggering using anti-CD3, mast cells promoted strong proliferation of T cells, which was 2-5 fold stronger than the "T-cell promoting capacity" of monocytes. The interplay between mast cells and T cells was dependent on cell-cell contact, suggesting that co-stimulatory molecules on the mast cell surface are responsible for the effect. However, in contrast to monocytes, the T cell co-stimulation by mast cells was independent of the classical co-stimulatory molecule CD28, or that of OX40L, ICOSL, or LIGHT.

Our data show that mast cells can co-stimulate human CD4+ T cells to induce strong T cell proliferation, but that therapies aiming at disrupting the interaction of CD28 and B7 molecules do not inhibit mast cell-mediated T-cell activation.

INTRODUCTION

Mast cells are innate immune cells derived from myeloid progenitors. They have been originally described as one of the effector cells in allergic IgE-mediated responses. More recently, their role in immune regulation, and specifically in regulation of adaptive immune responses, has been recognized.(*1*) For example, we and others have shown that mast cells can function as antigen presenting cells through cognate interactions with $CD4^+$ T cells both in human and mouse.(*2-4*) $CD4^+$ T cell activation in this context was dependent on recognition of specific antigens in the context of MHC class II. Activation and skewing of T cells generally requires the presence of 3 signals, consisting of antigen presentation through MHC, co-stimulation and specific cytokine signals.(*5*) Co-stimulation is the interaction of membrane-bound receptors on T cells and antigen presenting cells that enhance signals through the T cell receptor, and that is necessary to induce full T cell activation, proliferation and survival. Classical co-stimulation consists of CD28-mediated signaling by B7 family members on antigen presenting cells, although different molecules, such as members of the TNF receptor superfamily or signaling

lymphocyte activation molecule (SLAM) family are known to regulate the balance between T cell death and survival as well.(*6*)

We have previously shown the presence of mast cells in T cell areas of lymphoid organs,(*2*) as well as their colocalization in synovial tissue,(*7*) suggesting that mast cells could modulate T cell responses. Although mast cells have been shown to function as antigen presenting cells, their capacity to provide co-stimulatory signals to T-cells has been studied sparsely. In the mouse, TCR-stimulated T-cells displayed enhanced proliferation and cytokine secretion in the presence of mast cells, indicating that mast cells are capable of enhancing T-cell responses.(*8, 9*) This effect in the mouse was directly mediated through TNF production by the mast cells. However, several important differences have been shown between mouse and human mast cells, one of which being the low production of TNF by human mast cells in contrast to their mouse counterparts.(*10*)

Co-stimulation pathways are therapeutic targets, for example in autoimmune diseases. CTLA4-Ig, which targets the CD28-dependent interaction between T cells and antigen-presenting cells through binding to B7 molecules on antigen presenting cells, is used as treatment for rheumatoid arthritis.(*11*) Recently, mast cells were shown to play an important role in T cell-dependent inflammation in a mouse model of arthritis, suggesting that mast cells may directly regulate T cell responses in this disease.(*12*) Therefore, it is important to understand if mast cells can regulate T cell activation through co-stimulation, and if CTLA4-Ig can regulate this interaction.

Our data show that human mast cells can induce robust proliferation responses of $CD4^+$ T-cells in the presence of T cell receptor stimulation. This interaction depends on cell-cell contact, but is independent of CD28. Together, these data indicate that mast cell-T cell interactions contribute to the outcome of adaptive immune responses in humans, and that these interactions are not targeted by "conventional" co-stimulation blockade.

RESULTS

$CD4^+$ T CELL ACTIVATION AND PROLIFERATION IS GREATLY ENHANCED BY MAST CELLS

To evaluate the co-stimulatory function of mast cells, isolated $CD4^+$ T cells were activated with anti-CD3 in the presence or absence of human peripheral blood-derived mast cells. T cell activation was measured by evaluating the expression of activation markers CD69 and CD25 (Figure 1A-C). To gate out mast cells ($CD117^+$) from the co-culture, T cells were gated as $CD3^+CD117^-$ cells (Figure 1A).

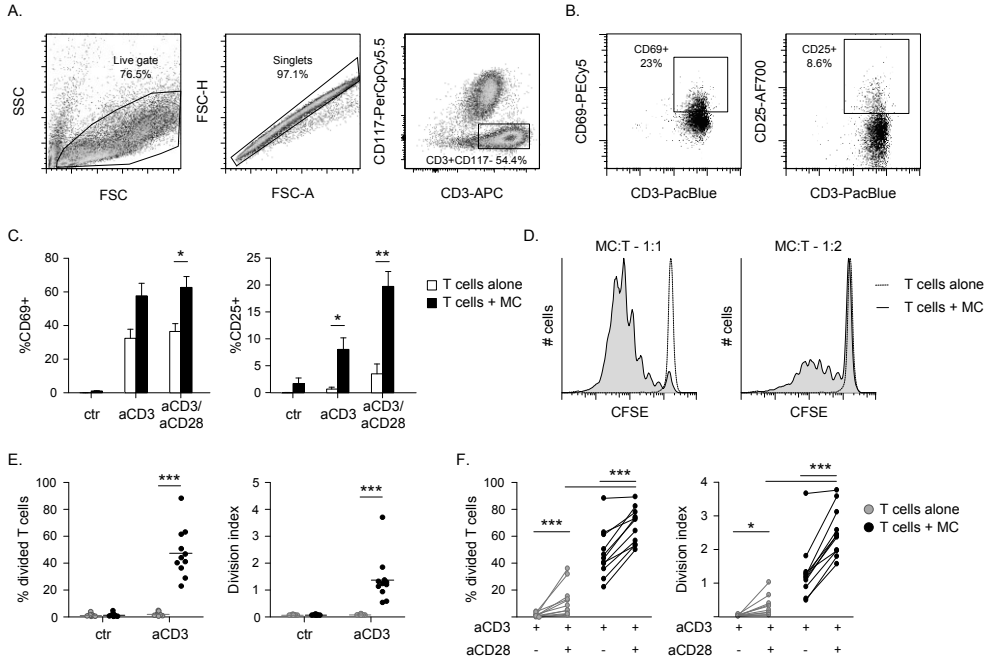


Figure 1 *(left)*: CD4$^+$ T cell co-stimulation by mast cells. A-C) Isolated human CD4$^+$ T cells were co-cultured with autologous peripheral blood-derived mast cells for 24 hours, in the presence or absence of anti-CD3 and anti-CD28, after which they were stained for activation markers CD69 and CD25. T cells were gated as CD3$^+$CD117$^-$ cells as shown in A. Representative flow cytometry plots of T cells in co-culture with mast cells and in the presence of anti-CD3 (B) and summary (C) of 3 independent experiments are shown. D-F) Isolated CD4$^+$ T cells were CFSE-labeled and co-cultured with autologous mast cells for 5 days, in the presence or absence of anti-CD3 and anti-CD28, after which they were stained for flow cytometry. T cells were gated as CD3$^+$CD117$^-$ cells as shown in panel A. Representative flow cytometry plots for CFSE (D) and summary of 11 independent experiments performed in duplicate with anti-CD3 (E) or anti-CD3 and anti-CD28 (F) are shown. G) Isolated total and naïve CD4$^+$ T cells were CFSE-labeled and co-cultured with autologous mast cells for 24 hours, in the presence or absence of anti-CD3 and anti-CD28, after which they were stained for flow cytometry. T cells were gated as CD3$^+$CD117$^-$ cells as shown in A. Representative example of 2 experiments performed in duplicate is shown. The percentage of divided T cells is depicted as % of the input frequency, indicating how many cells from the original cell population have divided. Division index represents the average number of cell divisions that a cell in the original population has undergone. Results are expressed as mean±SEM (C,G), or as individual dots where each dot/line indicates an independent experiment (E,F). Asterisks indicate statistically significant difference (*p<0.05; **p<0.01; ***p<0.005) using Students T-test.

Anti-CD3-stimulation led to an upregulation of these markers on T-cells. However, the upregulation, in particular that of CD25, was much more pronounced when T cells were co-cultured with mast cells and anti-CD3, suggesting that mast cells can enhance T-cell activation in the presence of a TCR stimulus.

To confirm the enhanced activation of T cells after co-culture with mast cells, we next analysed T cell proliferation by a CSFE-dilution assay (Figure 1D,E). As expected, in the absence of mast cells or other forms of co-stimulation, virtually no T cell proliferation was observed in response to anti-CD3 alone. In line with the requirement of co-stimulation to trigger full T cell activation, anti-CD28 signalling significantly enhanced T cell proliferation (Figure 1F). Intriguingly, an even more pronounced T-cell activation was observed in the presence of mast cells.

Although CD28-signalling further enhanced T-cell proliferation in the presence of mast cells, the contribution of mast cells did not require the concomitant presence of a CD28-dependent signal. However, the effect of mast cells was dependent on T cell receptor triggering, as no effect of mast cells was observed in the absence of anti-CD3 (Figure 1E).

CO-STIMULATION BY MAST CELLS IS CELL-CONTACT DEPENDENT

As co-stimulatory molecules on professional antigen presenting cells, such as dendritic cells, can be upregulated after their activation, the effect of stimulation of mast cells was evaluated. Mast cells were activated using anti-IgE and LPS, two stimuli that induce a robust activation of mast cells and result in degranulation and cytokine production.(*13*) As shown in Figure 2A-B, the activation of mast cells had no effect on T cell proliferation, indicating that resting mast cells already have the capacity to induce T cell proliferation.

Importantly, the co-stimulatory capacity of mast cells was not dependent on crosslinking via FcγR (Supplementary Figure 1). Whereas IL-8 production by mast cells was significantly reduced in the presence of blocking FcγR antibodies (anti-CD32A), no effect on T cell proliferation was observed. Together these results indicate that co-stimulation by mast cells is independent of activation, by TLR ligands or Fc receptor crosslinking.

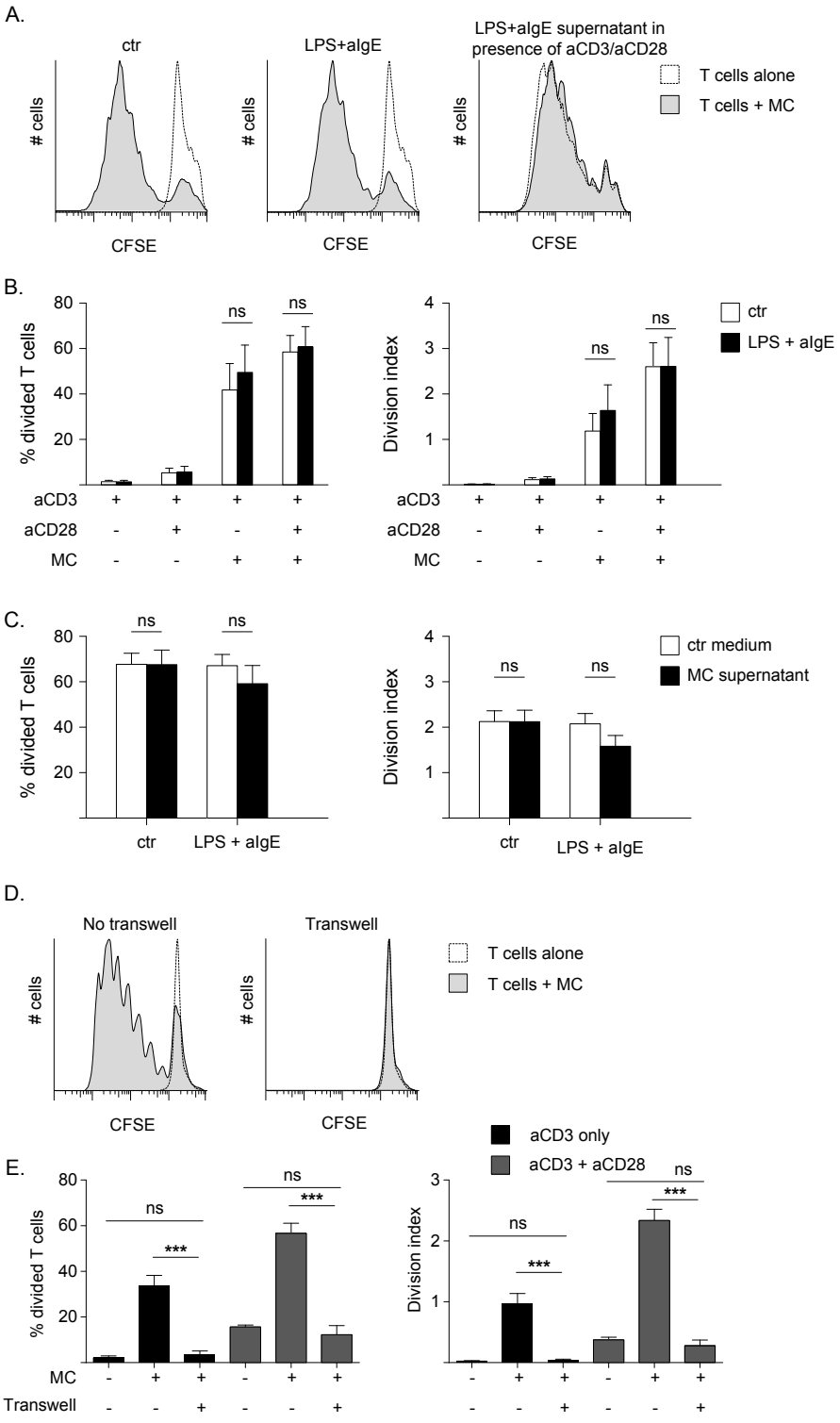

A.
ctr
LPS+aIgE
LPS+aIgE supernatant in presence of aCD3/aCD28
cells
CFSE
T cells alone
T cells + MC
B.
% divided T cells
Division index
ns
ctr
LPS + aIgE
aCD3
aCD28
MC
C.
ctr medium
MC supernatant
D.
No transwell
Transwell
E.
aCD3 only
aCD3 + aCD28
***
Transwell

Figure 2 *(left)*: $CD4^+$ T cell co-stimulation by mast cells is cell-contact dependent. A-C) Isolated human $CD4^+$ T cells were CFSE-labeled and co-cultured with autologous mast cells or mast cell supernatant for 5 days, in the presence of anti-CD3 and/or anti-CD28, after which they were stained for flow cytometry. T cells were gated as $CD3^+CD117^-$ cells as shown in Figure 1A. Mast cells were left unstimulated (ctr) or were stimulated with LPS + aIgE. Summary of 3 independent experiments is shown. A) Representative flow cytometry plots of T cell proliferation in the presence of unstimulated mast cells and anti-CD3 (left), mast cells stimulated with LPS + anti-IgE in the presence of anti-CD3 (middle), or mast cell supernatant in presence of anti-CD3 and anti-CD28 (right). B) T cell/mast cell coculture. Summary of 3 independent experiments is shown. C) T cell culture in the presence of mast cell supernatant. Summary of 6 independent experiments is shown. D,E) Isolated $CD4^+$ T cells were CFSE-labeled and co-cultured in transwell plates with autologous mast cells for 5 days, in the presence of anti-CD3 and/or anti-CD28, after which they were stained for flow cytometry. Mast cells were cultured in the same well as the T cells, or were separated by a transwell membrane (+). D) Representative flow cytometry plots of T cells cultured in presence of anti-CD3 and in the presence of mast cells either together in the same well (left) or separated by a transwell (right). E) Summary of 3 independent experiments is shown. The percentage of divided T cells is depicted as % of the input frequency, indicating how many cells from the original cell population have divided. Division index represents the average number of cell divisions that a cell in the original population has undergone. Results are expressed as mean±SEM. Ns indicates no significant difference was found using students T-test (B,C). Asterisks indicate statistically significant difference (***$p<0.005$) using ANOVA with Bonferroni's post-hoc test (E).

To analyse whether cell-cell contact or mast cell-derived soluble mediators were responsible for the co-stimulatory effects, we next cultured T cells in the presence of mast cell supernatant. However, the presence supernatant from mast cell cultures did not result in enhanced proliferation, but if anything, the opposite (Figure 2A,C). To directly address the requirement for cell contact between mast cells and T cells, transwell experiments were performed. The effect of mast cells on T cell proliferation was absent when mast cells were separated from T cells by the transwell membrane (Figure 2D-E). These results indicate that the co-stimulatory effect mediated by mast cells requires cell-cell-contact, and is not dependent on soluble mediators secreted by mast cells.

$CD4^+$ T CELL PROLIFERATION INDUCED BY MC COMPARED TO MONOCYTES

To compare the co-stimulatory capacity of mast cells to other cells known to effectively co-stimulate T-cells, we compared the T cell proliferation induced by mast cells to that induced by total PBMCs (Figure 3A). At a similar ratio of PBMCs/mast cells to T cells, mast cells displayed a considerable higher capacity to induce T cell proliferation in the presence of anti-CD3 as compared to PBMCs.

The superior co-stimulatory ability of mast cells compared to PBMCs was also observed in titration experiments as lower number of mast cells were required to achieve a similar T-cell proliferation (Figure 3B). Next, we also wished to compare the co-stimulatory capacity of mast cells to isolated $CD14^+$ monocytes. As shown in Figure 3C-D, mast cells were also better in supporting T cell proliferation than monocytes, further confirming the potent co-stimulatory ability of mast cells.

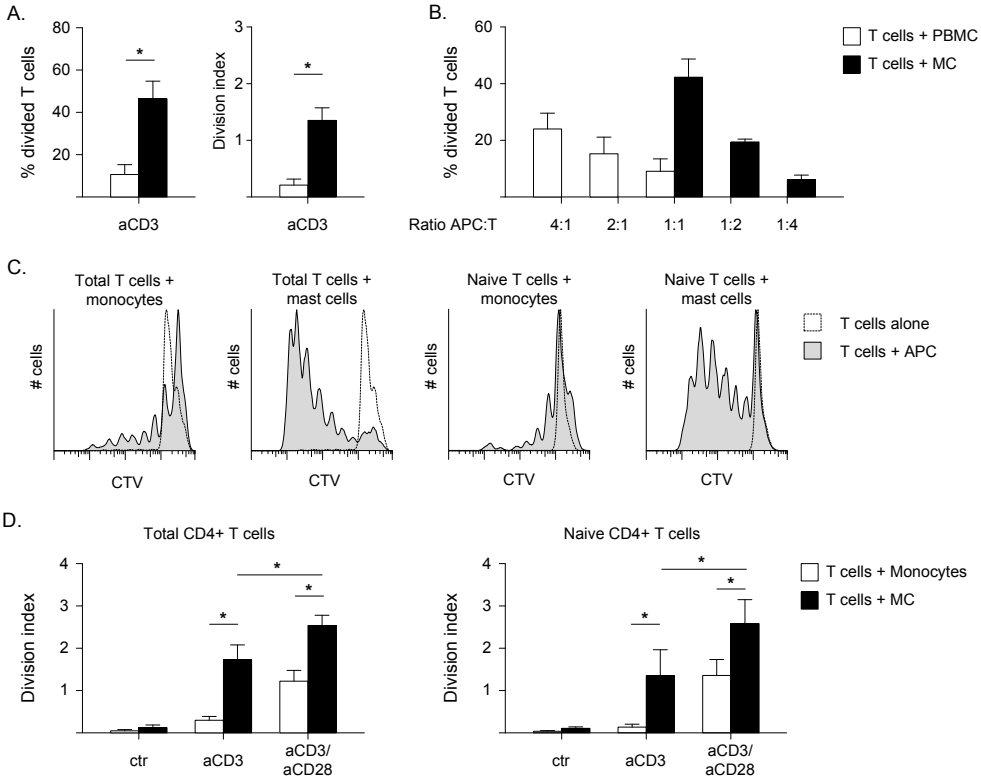


Figure 3: Mast cell co-stimulation compared to monocytes. A,B) Isolated human $CD4^+$ T cells were CFSE-labeled and co-cultured with autologous mast cells or PBMCs for 5 days, in the presence of anti-CD3, after which they were stained for flow cytometry. T cells were gated as $CD3^+CD117^-CD14^-$ cells as shown in Figure 1A. Ratios of co-stimulatory cells (APC) to T cells was 1:1 in 3 independent experiments (A) or titrated ranging from 4:1 – 1:1 for PBMCs and from 1:1 – 1:4 for mast cells in 2 independent experiments (B). C,D) Isolated total and naïve $CD4^+$ T cells were Cell Trace Violet (CTV) labeled and co-cultured with monocytes or mast cells for 5 days, in the presence or absence of anti-CD3 and CD28, after which they were stained for flow cytometry. C) Representative flow cytometry plots of CTV staining of T cells cultured in the presence of anti-CD3, gated as in Figure 1A. D) Summary of 3 independent experiments is shown. Asterisks indicate statistically significant ($p<0.05$) differences obtained using paired-samples T test (A), or Two-way ANOVA with Bonferroni's posthoc test (D).

As the co-stimulatory requirements of T cells differ between memory and naïve $CD4^+$ T cells, with naïve T cells being more dependent on a co-stimulatory signal,(*14*) we also evaluated the contribution of mast cells to proliferation of naïve $CD4^+$ T cells. As depicted in Ffigure 3C-D, mast cells were fully capable of inducing robust proliferation of naïve T cells in the presence of only anti-CD3, in contrast to monocytes which only induced minimal T cell proliferation. These results therefore indicate that mast cells are potent inducers of-stimulation of $CD4^+$ T cells in the presence of a TCR stimulus, also when the T-cells have a naïve phenotype.

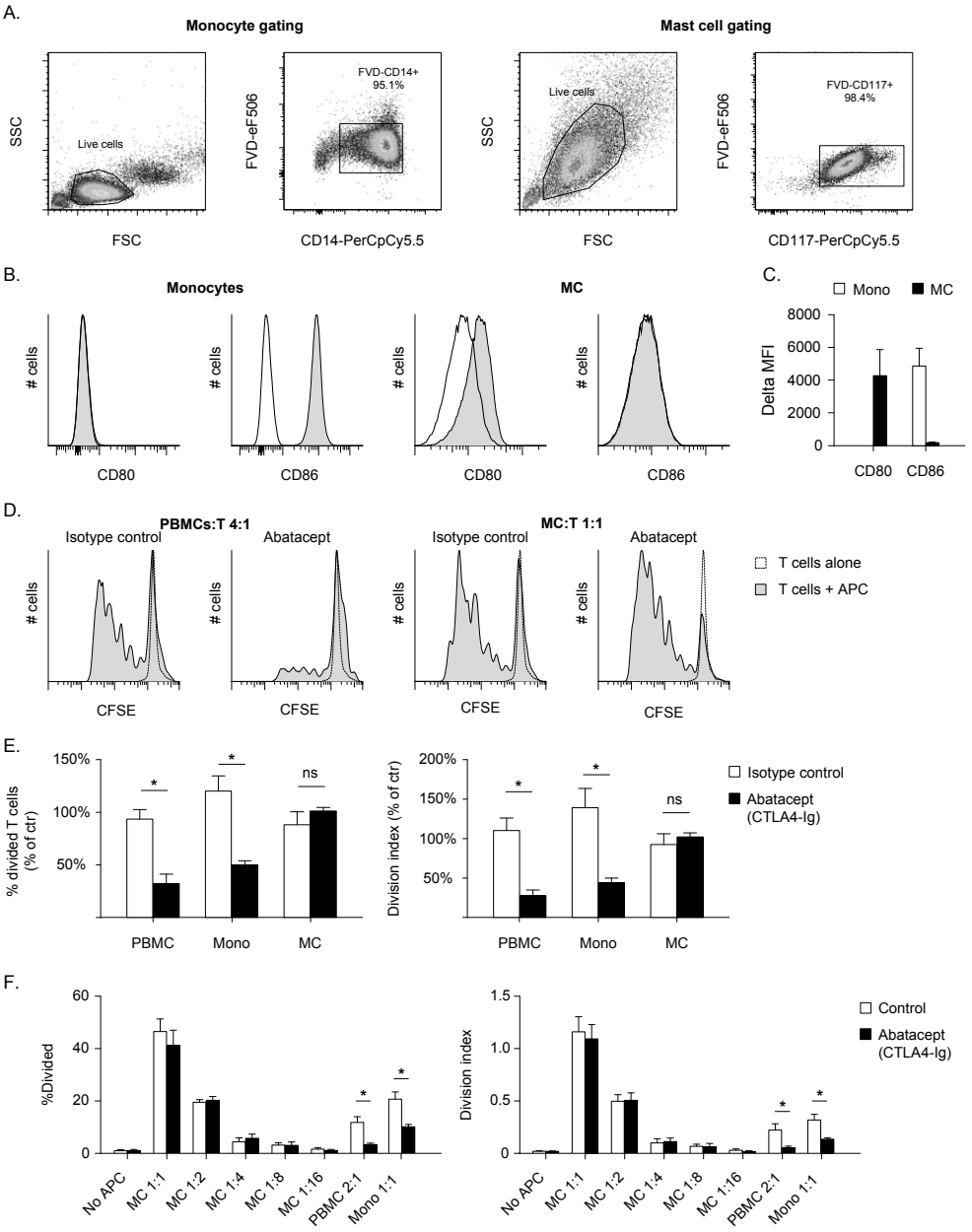
$CD4^+$ T CELL PROLIFERATION INDUCED BY MC DOES NOT DEPEND ON CLASSICAL CO-STIMULATION VIA CD28

We and others have shown previously that mast cells express CD80, which is known to interact with CD28 on T cells to induce co-stimulation.(*2, 4*) Therefore, it has been postulated that this molecule could be responsible for a co-stimulatory signal coming from mast cells. We first compared the expression levels of CD80 and CD86 on mast cells and monocytes (Fig 4A-C). Similar to our previous findings, human mast cells express CD80 but no CD86. In comparison, we observed that resting monocytes express CD86, but no CD80. However, we have to note that the high level of autofluorescence in mast cells (due to their granularity) makes it difficult to directly compare expression levels between the two cell types.

To analyse the contribution of this pathway to T cell co-stimulation by mast cells, we incubated mast cells, PBMCs or monocytes with CTLA4-Ig (Abatacept) or isotype control, prior to co-culture with the T cells. CTLA4-Ig remained present throughout the culture period. Whereas CTLA-4Ig significantly decreased T cell proliferation when T-cells were cultured with PBMCs or isolated monocytes, no effect of CTLA4-Ig was observed when mast cells were used as co-stimulatory cell (Fig 4D,E). Likewise, dilution of mast cells to achieve lower mast cell:T cell ratios to mimic the co-stimulatory capacity of PBMCs and monocytes did not lead to a detectable effect on T-cell proliferation supported by mast cells (Fig 4F).

Our results indicate that co-stimulation by mast cells is independent of CD28, in contrast to the ability of PBMCs and monocytes to co-stimulate T-cells.

We next evaluated whether other known co-stimulatory molecules were responsible for $CD4^+$ T cell activation by mast cells. Based on published microarray databases and RNA sequencing results, we identified the following potential T cell co-stimulatory molecules expressed by human mast cells that can be blocked by neutralizing antibodies: OX40L (TNFSF4), ICOSL (B7H2), and LIGHT (TNFSF14).(*15, 16*)



As shown in Figure 5, co-stimulation by mast cells was not inhibited using blocking antibodies against OX40L, ICOSL, or LIGHT, suggesting that the activation of $CD4^+$ T cells by mast cells was not mediated via these receptor/ligand systems and that other, possibly unknown, receptors are responsible for T cell co-stimulation by human mast cells.

Figure 4 *(left)*: Mast cell-mediated co-stimulation is independent of CD28A-C) Cultured mast cells and isolated monocytes were stained for CD80 and CD86 and analysed by flow cytometry. A) Debris and doublets were excluded based on forward and sideward scatter (FSC/SSC). Live monocytes were gated as $CD14^{+}FVD^{-}$ (fixable viability dye eFluor506). Live mast cells were gated as $CD117^{+}FVD^{-}$ cells. B) Representative examples of CD80 and CD86 expression by monocytes and mast cells. C) Summary expression of CD80 and CD86 by monocytes and mast cells. Delta-MFI was calculated by subtracting the MFI of isotype control from the MFI of CD80 or CD86 staining. D-F) Isolated $CD4^{+}$ T cells were CFSE-labeled and co-cultured autologous mast cells, monocytes or PBMCs for 5 days, after which they were stained for flow cytometry. Prior to co-culture, co-stimulatory cells were treated with control medium, isotype control IgG, or Abatacept (CTLA4-Ig), and these treatments remained present during the co-culture. D) Representative flow cytometry plots of CFSE staining of T cells cultured in the presence of anti-CD3, gated as in Figure 1A. T cells cultured with PBMCs as APC in a ratio 1:4 (left) and T cells cultured with mast cells as APC in a ratio 1:1 (right). E,F) Summary of 5 independent experiments using n=5 independent mast cell donors, n=3 independent PBMC donors and n=2 independent monocyte donors. E) T cell proliferation was normalized to control-medium treated PBMCs, monocytes or mast cells (T+APC ctr: 100%). F) T cell proliferation at different mast cell:T cell ratios as indicated in the axis titles. The percentage of divided T cells is the precursor frequency, indicating how many cells from the original cell population have divided. Division index is the average number of cell divisions that a cell in the original population has undergone. Results are expressed as mean±SEM. Asterisks indicate statistically significant difference (*$p<0.05$) using Students T-test. Ns indicates no significant difference was found using students T-test (E).

DISCUSSION

In this study we showed that mast cells can mediate T cell co-stimulation through a CD28-independent pathway. Although we analyzed a possible role for OX40L, ICOSL, and LIGHT, we have not identified the molecular mode of action explaining the ability of human mast cells to provide co-stimulation to T-cells. We also analyzed the contribution of FcγR crosslinking of mast cells by anti-CD3. Mast cells were activated by anti-CD3 antibodies in an FcγRIIA-dependent manner, but blocking of this receptor did not affect T cell proliferation induced by mast cells. Therefore, this activation of mast cells is unlikely to contribute to the T cell activation we observed.

We hypothesize that classical members of the B7 and TNF receptor families are unlikely to account for the observed co-stimulation by mast cells. For example, some of the molecular interactions that are described to be involved in priming of naïve $CD4^{+}$ T cells, HVEM-LIGHT, CD27-CD70 are either not expressed by mast cells, or were not inhibited by the blocking antibodies we used. However, there are many other members of the TNF receptor family and SLAM family, for which their role in co-stimulation of $CD4^{+}$ T cells is currently unknown.(*6*)

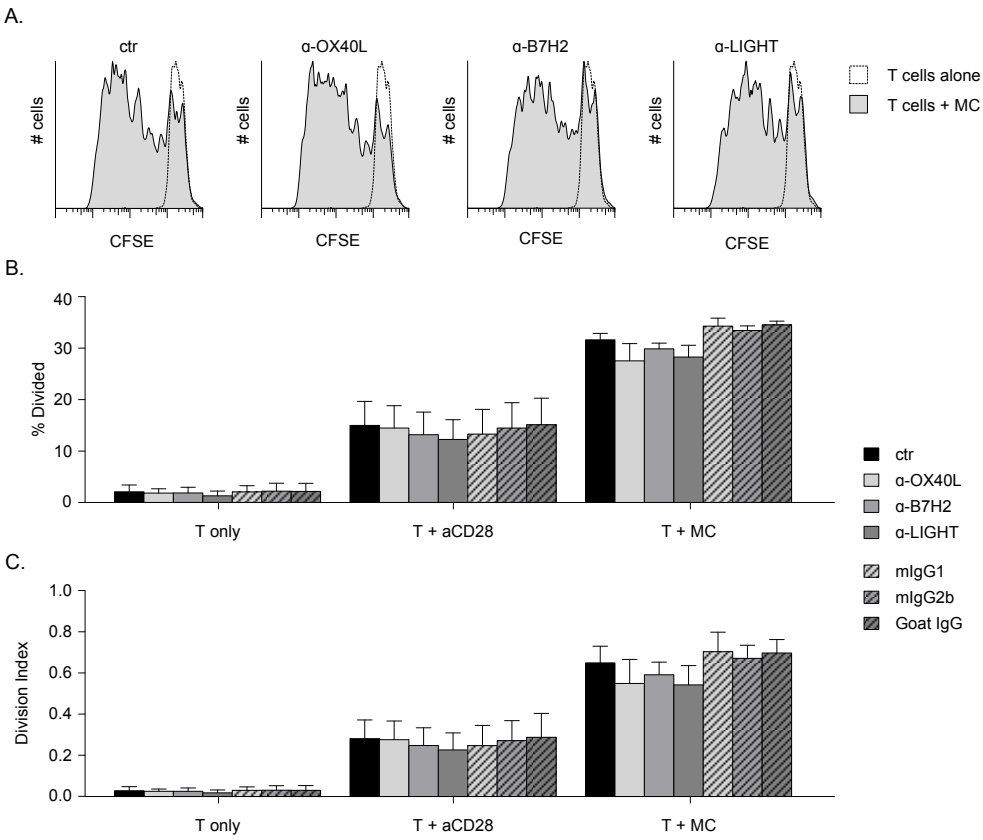


Figure 5: Blocking co-stimulatory molecules in the mast cell-T cell interaction. Isolated human $CD4^+$ T cells were labeled with cell trace violet (CTV) and co-cultured with mast cells for 4-5 days, in the presence of anti-CD3, after which they were stained for flow cytometry. Live T cells were gated as $CD3^+CD117^-CD14^-$ cells as shown in Figure 1A. As positive control, T cells were stimulated in the presence of anti-CD3 and anti-CD28 (T+aCD28). Prior to co-culture, mast cells were treated with control medium, isotype control mIgG1 (control for OX40L), mIgG2b (control for B7H2), goat IgG (control for LIGHT) or anti-OX40L, anti-B7H2 (ICOSL), anti-LIGHT, and these antibodies remained present during the co-culture. A) Representative flow cytometry plots of cell trace violet (CTV) staining. B,C) Summary of 3 independent experiments is shown. The percentage of divided T cells is the precursor frequency, indicating how many T cells from the original cell population have divided. Division index is the average number of cell divisions that a T cell in the original population has undergone. Results are expressed as mean±SEM. No statistically significant differences between blocking antibodies and their matching isotype control antibodies were found using students T-test.

Therefore, it remains to be shown which of these, or other unknown receptors, is responsible for co-stimulation by human mast cells. Other studies have shown T cell proliferation by activated mast cells was partially mediated by TNF (mouse mast cells) and OX40L/OX40 interactions (both mouse and human).(*8, 9, 17*) However, T cell activation

through TNF and OX40L/OX40 depend on mast cell activation, which is required for production or expression of these molecules.(*8, 9*) Furthermore, T cell activation by these molecules usually only occurs in pre-activated or effector T cells. (*18, 19*) As we observed strong T cell activation in the absence of mast cell stimulation, and activation of naïve T cells by mast cells was as strong as that of total $CD4^+$ T cells, we propose that these pathways are unlikely to account for the full co-stimulatory signal provided by mast cells, as also shown by transwell experiments and inhibition of OX40L.

Because a redundancy of co-stimulatory molecules might be present with respect to the ability of human mast cells to provide co-stimulatory signals to T-cells, it might be difficult to identify the pathway that is responsible for the observed effect.(*20*) However, know-how on the molecular details underlying the effects observed is likely of relevance as these effects may have important implications for therapies aiming to block co-stimulatory pathways such as employed in autoimmune diseases. Whereas CTLA4-Ig is being successfully used as treatment for a variety of autoimmune diseases, it does not completely inhibit T cell activation, suggesting that CD28-independent pathways may contribute to autoreactive T cell responses as well.(*11, 21-23*) Our results suggest that this treatment may not inhibit mast cell-mediated, and possibly other forms of T cell activation. Indeed, several studies point to an important contribution of CD28-independent T cell activation in autoimmune disease.(*24, 25*) Therefore, our results suggest that CD28-independent inhibitors of T cell activation may prove beneficial effects against CD28-independent autoimmune T cell responses such as those supported by mast cells and possibly other cells.

MATERIALS AND METHODS

MAST CELL CULTURE

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). PBMCs were isolated using a standard Ficoll procedure, after which $CD34^+$ hematopoietic stem cells were isolated with CD34 microbeads (Miltenyi Biotec). Isolated $CD34^+$ stem cells were differentiated into mast cells using serum-free medium (StemPro 34 + supplement, Gibco) with 30 ng/mL IL-3, 100 ng/mL IL-6 and 100 ng/mL Stem Cell Factor (SCF) at 50.000 cells/mL as described.(*26*) Half of the medium was replaced weekly with serum-free medium containing 100 ng/mL IL-6 and 100 ng/mL SCF. All recombinant cytokines were obtained from Peprotech. After 6-8 weeks, the purity of mast cells was determined by flow cytometric analysis of CD117 (c-kit), FcεRI and CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99%.

T CELL ACTIVATION

For isolation of T cells, autologous CD45RO-negative (naïve) or total $CD4^+$ T cells were isolated from PBMCs by negative magnetic bead isolation (Miltenyi Biotec). The purity of isolated T cells was above 95% in each experiment. As positive controls, total PBMCs or isolated $CD14^+$ monocytes (Miltenyi Biotec) were used to stimulate T cells.

Isolated T cells were labeled with Cell Trace CFSE or Cell Trace Violet (both from Invitrogen) to measure cell proliferation. T cells were activated in the presence or absence of 5 µg/mL platebound anti-CD3 (eBioscience; clone: OKT3) and/or 1 µg/mL soluble anti-CD28 (Sanquin; clone: CLB-CD28/1, 15E8) and were cultured in the presence or absence of mast cells in the indicated ratios. Activation of T cells was measured after 24h by flow cytometry for CD25 and CD69. Proliferation and cytokine production were measured after 4-5 days of incubation. To this end, T cells were harvested, washed, and restimulated using 50 ng/mL PMA (Sigma) and 500 ng/mL Ionomycin (Sigma). Cells were restimulated for 5 hours in the presence of 10 µg/mL brefeldin A (Sigma) for intracellular cytokine staining.

For mast cell activation, cells were sensitized with 0.1 µg/mL hybridoma IgE (Diatec) for a minimum of 18 hours, after which non-bound IgE was washed away. Mast cells were activated with 10 ug/mL goat anti-human IgE (Nordic) and 1 ug/mL E. Coli K12 lipopolysaccharide (LPS; InVivoGen).

For blocking experiments, mast cells, PBMCs or monocytes were incubated with 100 µg/mL CTLA4-Ig (Abatacept, Bristol-Myers Squibb), 100 µg/mL human IgG isotype control, 10 µg/mL anti-OX40L (MAB10541-100, R&D Systems), 10 µg/mL anti-B7-H2/ICOSL (MAB165-100, R&D Systems), 0.1 µg/mL anti-LIGHT/TNFSF14 (AF664-SP, R&D Systems), 10 µg/mL anti-CD32A (IV.3, StemCell), 10 µg/mL mouse IgG1 isotype control (16-4714, eBioscience), 10 µg/mL mouse IgG2b isotype control (16-4732, eBioscience), 0.1 µg/mL polyclonal goat IgG isotype control (AB-108-C, R&D systems), for 30 minutes at 37 ºC prior to co-culture with the T cells.

To analyse the requirement of cell-cell contact, transwell plates with 0.4 uM-pore membrane (Corning) were used.

FLOW CYTOMETRY

Antibodies to CD3, CD14, CD117, CD25 and CD69 were obtained from BD Biosciences. For surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0,5% BSA at 4 ºC for 30 min. To exclude dead cells fixable viability dye eFluor506 (eBioscience) was added during incubation of surface antibodies. After washing, cells were suspended in 1% paraformaldehyde until flow cytometric aquisition on a FACS Calibur (BD)

or LSR-II (BD). Analysis was performed using FACS Diva (BD) and FlowJo software. Cell proliferation was analysed as described.(*27*) The % divided cells was defined as the probability that a cell has divided at least once from the original population. The division index was defined as the average number of cell divisions that a cell in the original population has undergone.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 4. For differences between two groups, student's T tests were performed. For differences between more than two groups, One-way ANOVA with Bonferroni's posthoc test was performed. P values of <0.05 were considered statistically significant.

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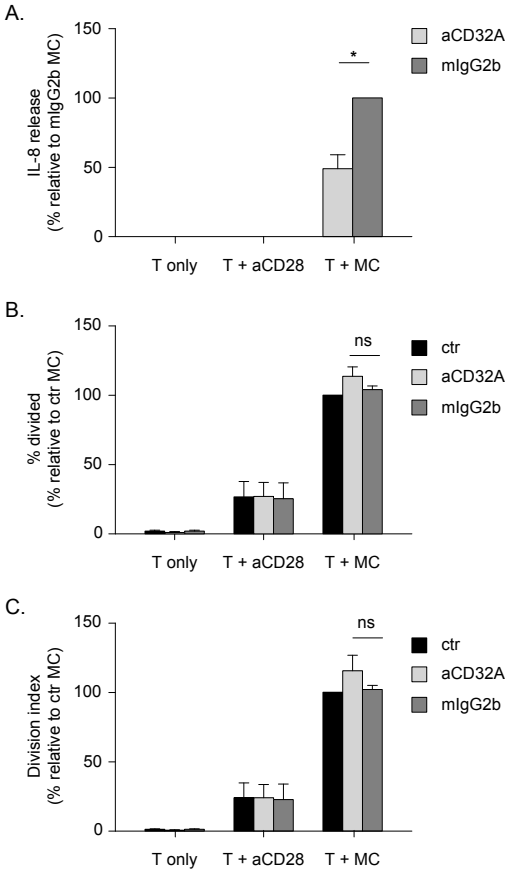
SUPPLEMENTARY FIGURE



Supplementary Figure 1: Blocking FcγR on mast cells. Isolated human $CD4^+$ T cells were labeled with cell trace violet (CTV) and cocultured with autologous mast cells for 5 days, in the presence of anti-CD3, after which they were stained for flow cytometry. T cells were gated as $CD3^+CD117^-CD14^-$ cells as shown in Figure 1A of the manuscript. Prior to coculture, mast cells were treated with control medium, isotype control mIgG2b, or anti-CD32A, and these antibodies remained present during the coculture. A) Release of IL-8 in presence of mIgG2b or anti-CD32A was measured by ELISA. No IL-8 was detected in the absence of mast cells. IL-8 production was normalized to mIgG2b control (100%). Asterisk indicates statistically significant difference calculated using Two-way ANOVA and Bonferroni's posthoc test. B,C) T cell proliferation in presence of mIgG2b or anti-CD32A. T cell proliferation was normalized to control-medium treated mast cells (T+MC ctr: 100%). Ns indicates no statistically significant differences were found using Two-way ANOVA and Bonferroni's posthoc test. Results are expressed as mean±SEM of 3 independent experiments.

TH17 EXPANSION BY HUMAN MAST CELLS IS DRIVEN BY INFLAMMASOME-INDEPENDENT IL-1β

Chapter 9

Jolien Suurmond, Kim LL Habets, Annemarie L Dorjée,
Tom WJ Huizinga, René EM Toes

Department of Rheumatology, Leiden University Medical Center, Leiden,
The Netherlands

Submitted

ABSTRACT

Mast cells (MC) are most well-known for their role in innate immune responses. However, MC are increasingly recognized as important regulators of adaptive immune responses, especially in setting the outcome of T cell responses.

In this study we determined the effect of MC on cytokine production by naive and memory human T helper cells. $CD4^+$ T cells were cultured with MC supernatant or control medium, after which cytokine production by T cells was determined. Supernatant of activated MC specifically increased the number of IL-17 producing T cells. This enhancement of Th17 cells was not observed in the naive $CD4^+$ T cell population suggesting MC affected memory Th cells. The effect of MC was inhibited for approximately 80% by blocking antibodies to IL-1β and the recombinant IL-1 receptor antagonist anakinra. Importantly, secretion of active IL-1β by mast cells was independent of caspase activity indicating Th17 expansion by mast cells occurred through inflammasome-independent IL-1β.

Together, these studies reveal a role for human MC in setting the outcome of T cell responses through release of caspase-independent IL-1β, and provide evidence for a novel contribution of MC in boosting the Th17-axis in mucosal immune responses.

INTRODUCTION

Recent research has suggested an important role for mast cells in $CD4^+$ T cell responses. We and others have shown that human mast cells can function as antigen presenting cells, by taking up and processing antigens and subsequent activation of $CD4^+$ T cells via HLA class II molecules.(*1-3*) Although the precise contribution of mast cells in T-cell activation is still controversial, mast cells often colocalize with $CD4^+$ T cells, both in lymphoid organs as well as peripheral tissues.(*2, 4*) Therefore, it is conceivable that mast cells can interact with $CD4^+$ T cells and drive skewing of specific T helper cell subsets, through their secreted cytokines and other mediators.

Due to their role in allergy, mast cells are often associated with Th2 responses, but recent data suggest they may also contribute to Th17 responses, for example through an indirect effect on dendritic cells, or through an OX40-mediated crosstalk with regulatory T cells (Tregs) as shown in mice.(*5, 6*) IL-9 and IL-33, two cytokines which can modulate mast cell responses, have been related to Th17-mediated pathology in mice.(*7, 8*) Furthermore, several mast cell derived cytokines have been implicated in Th17 skewing, including IL-6, TGFβ, and IL-1β.(*9*)

Importantly, mast cells are predominantly present at mucosal surfaces which are important sites for Th17 activation and immunity. Although Th17 cell activation at mucosal barriers is important in driving protection against bacteria, aberrant Th17 responses can also contribute to pathogenic processes, such as neutrophilic infiltration or airway remodeling in asthma.(*10-12*)

Although studies performed in mice point to a role of mast cells in Th17 cell biology, little is known of the contribution of mast cells to Th17 responses in humans, also because the cytokines produced by MCs can differ considerably between mice and man.(*13, 14*) Understanding of the pathways that contribute to activation and maintenance of memory Th17 cells in humans is particularly important for inflammatory diseases, as Th17 memory cells are thought to be the driver of chronic inflammation.(*15*)

Therefore, we aimed to evaluate the influence of mast cells on Thelper cell responses. Here, we show that activated mast cells are capable of expanding Th17 cells. Studies investigating the mode of action revealed that this occurred through a synergistic release of IL-1β upon combined TLR/FcεRI receptor triggering. Secretion of active IL-1β by human mast cells upon stimulation was independent of caspase activation, suggesting a novel mechanism of inflammasome-independent IL-1β release by mast cells. As allergic responses in mucosal tissue are often associated with both Th17 responses and involvement of mast cells, these results provide a link between inflammasome-independent IL-1β production and pathogenic Th17 responses.

MATERIALS AND METHODS

MAST CELL CULTURE

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). PBMCs were isolated using a standard Ficoll procedure, after which $CD34^+$ hematopoietic stem cells were isolated with CD34 microbeads (Miltenyi Biotec). Isolated $CD34^+$ stem cells were differentiated into mast cells using serum-free medium (StemPro 34 + supplement, Gibco) with 30 ng/mL IL-3, 100 ng/mL IL-6 and 100 ng/mL Stem Cell Factor (SCF) at 50.000 cells/mL as described.(*16*) Half of the medium was replaced weekly with serum-free medium containing 100 ng/mL IL-6 and 100 ng/mL SCF. All recombinant cytokines were obtained from Peprotech. After 6-8 weeks, the purity of mast cells was determined by flow cytometric analysis of CD117 (c-kit), FcεRI and CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99% (Supplementary Figure 1).

MAST CELL ACTIVATION

Mast cells were sensitized using 0,1 ug/mL hybridoma IgE (Diatec, clone HE-1) for a minimum of 18 hours. Mast cells were thoroughly washed to remove the soluble IgE, after which IgE was crosslinked using 10 ug/mL polyclonal goat anti-human IgE (Nordic). Activation of TLR was achieved using 1 ug/mL LPS (Invivogen). Activation of Fc-gamma receptors was achieved by using platebound IgG as described before.(*17*) Mast cells were activated in StemPro medium + supplement in the presence of 100 ng/mL SCF for 24 hours, after which supernatant was harvested.

For inhibition of caspase, 20 uM Z-VAD-FMK (Sigma) was added to mast cells, 15 minutes prior to their activation, and the drug remained present throughout the 24 hour stimulation. The levels of lactate dehydrogenease (LDH) was analysed in mast cell supernatant (BioAssay Systems) as a measure of cell death.

$CD4^+$ T CELL SKEWING

For isolation of $CD4^+$ T cells, naive (CD45RO-negative) or total $CD4^+$ T cells were isolated from PBMCs by negative magnetic bead isolation (Miltenyi Biotec). The purity of isolated naive $CD4^+$ T cells was determined as $CD14^-CD3^+CD4^+CD45RA^+CD45RO^-$ cells, and the purity of total $CD4^+$ T cells was determined as $CD14^-CD3^+CD4^+$. The purity of isolated naive or total $CD4^+$ T cells was above 95% in each experiment.

Naive or total $CD4^+$ T cells were activated using 5 µg/mL platebound anti-CD3 (eBioscience; clone: OKT3) and 1 µg/mL soluble anti-CD28 (Sanquin; clone: CLB-CD28/1, 15E8) in the presence or absence of mast cell supernatant which was diluted 1:1 in IMDM/10% FCS. After 5 days of incubation, T cells were harvested, washed, and restimulated using 50 ng/mL PMA (Sigma) and 500 ng/mL Ionomycin (Sigma).

Cells were restimulated for 5 hours in the presence of 10 µg/mL brefeldin A (Sigma) for intracellular cytokine staining. For analysis of proliferation, isolated $CD4^+$ T cells were labeled with Cell Trace Violet (Invitrogen), after which they were cultured as mentioned above.

For blocking experiments, mast cell supernatant was incubated with 20 µg/mL mIgG1 isotype control (clone P3.6.2.8.1, eBioscience), IL-1RA (anakinra; kindly provided by dr. M Schilham), anti-IL1α (Bio-Techne, Minneapolis, MN), anti-IL-1β (clone CRM56, eBioscience), anti-IL-6R (Tocilizumab), or anti-TNF-α (Etanercept), for 1 hour at 37 ºC before adding the supernatant to the T cells.

IL-1β SECRETION AND ACTIVITY

The amount of IL-1β in mast cell supernatant was quantified using ELISA (Biolegend). The amount of IL-1α in mast cell supernatant was quantified using Luminex assays (Millipore). The IL-1 activity was measured by incubating the EL4.NOB-1 cell-line with mast cell supernatant or control medium as described,(*18*) after which secretion of mouse IL-2 by this cell-line was measured using ELISA (eBioscience). The NOB-1 cell-line was kindly provided by prof. Leo Joosten, Radboud University, Nijmegen, The Netherlands.

FLOW CYTOMETRY

The following antibodies were obtained from BD Biosciences: CD3-AlexaFLuor700 (clone UCHT1), CD4-APC (clone SK3), IL-10-PE (clone JES3-9D7), IL-13-APC (clone JES10-6A2), IFNγ-FITC (clone 25723.11), TNF-α-AlexaFluor488 (clone MAb11), mIgG1-FITC (clone X40), rIgG1-PE (clone R3-R4), mIgG1-AlexaFluor488 (clone MOPC-21); from eBioscience: IL-17A-AlexaFluor647 and -eFluor660 (clone eBio64CAP17), mIgG1-eF660 (clone P3.6.2.8.1) , mIgG1-PE (clone P3.6.2.8.1); and from R&D systems: IL-22-PE (clone 142928).

For surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0,5% BSA at 4 ºC for 30 min. For discrimination between live and dead cells, cells were incubated with Fixable Viability Dye eFluor506 (eBioscience), prior to fixation and permeabilization. For intracellular cytokine staining, T cells were permeabilized using CytoFix CytoPerm Kit (BD Biosciences). After washing, cells were incubated with antibodies against intracellular cytokines and incubated at 4 ºC for 30 min. After washing, cells were suspended in 1% paraformaldehyde until flow cytometric aquisition on a FACS Calibur (BD), LSR-II (BD), or Fortessa (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

Cell proliferation was analysed as described.(*19*) Briefly, the % divided cells was defined as the probability that a cell has divided at least once from the original population. The division index was defined as the average number of cell divisions that a cell in the original population has undergone.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical analysis was performed using SPSS PASW 17.0 and GraphPad Prism 4. For differences between two groups, student's T tests were performed. ANOVA with Bonferroni posthoc tests were used to analyse differences between multiple groups. P values of <0.05 were considered statistically significant.

RESULTS

ACTIVATED HUMAN MAST CELLS INDUCE TH17 EXPANSION

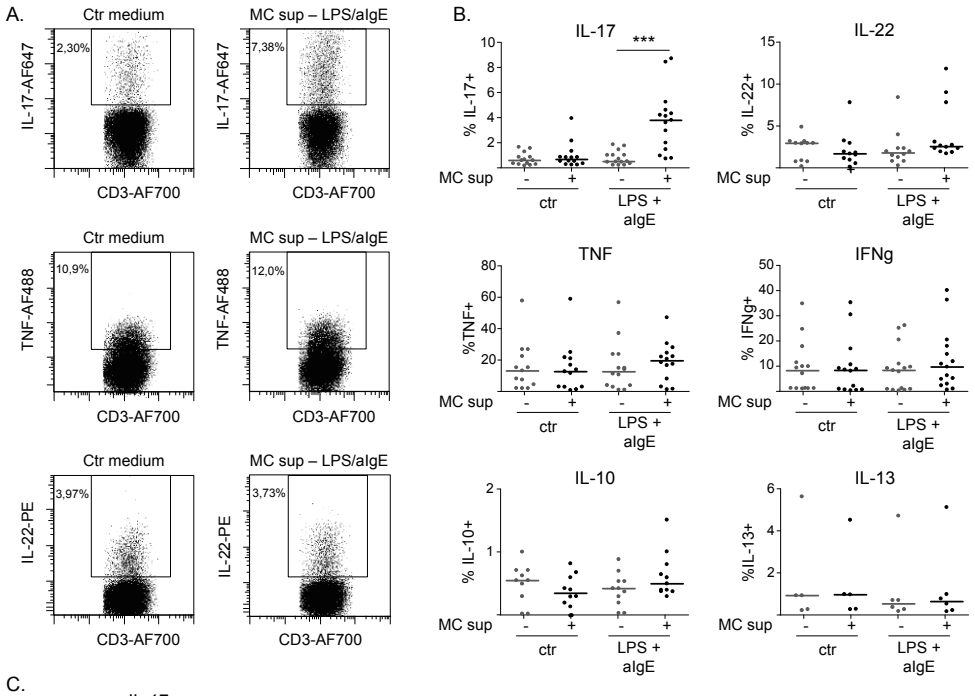
To evaluate the effect of mast cells on cytokine production by Th cells, isolated total $CD4^+$ T cells were incubated with supernatant obtained from mast cell cultures stimulated via TLR-4 and FcεRI. Medium containing the same concentrations of stimuli were used as control. After 5 days, cytokine production by T cells was analysed by intracellular flow cytometry upon restimulation with PMA/ionomycin (Figure 1A,B). Live $CD3^+$ T cells were gated as shown in Supplementary Figure 2. Of the analysed cytokines produced by responding T-cells, mast cells most significantly enhanced Th17 responses, characterized by a two- to eight-fold expansion of IL-17^+ cells (p=0.0001). Although enhancements of other Th cytokines like IL-22 and TNF-α were observed when T cells were activated in the presence of supernatant from some mast cell donors, these responses were not present in all T cell and mast cell donors and were therefore not statistically significant.

As enhancement of IL-17 responses could result from expansion of memory T cells or from *de novo* induction from naive Th cells, we next analysed the capacity of mast cell supernatant to skew IL-17 cytokine production from naive Th cells. Naive Th cells require specific cytokines for their differentiation into functional cytokine-producing Th cells. In contrast to the ability of mast cell supernatant to enhance Th17 responses from total Th cells, mast cells were not capable of inducing IL-17^+ T cells from naive Th cells (Figure 1C).

Together, these results indicate that activated mast cells can secrete substances that allow the increase of IL-17 production from T cell populations through an effect on memory Th cells. As only an increase in IL-17 producing T cells was noted, these results indicate a specific effect on IL-17 production but not on the production of other cytokines.

EXPANSION OF TH17 CELLS BY MAST CELLS IS INDEPENDENT OF PROLIFERATION

To address whether enhancement of Th17 responses by mast cells was driven by specific expansion of Th17 cells, we next analysed whether IL-17 producing T cells had undergone accelerated proliferation. To this end, cells were labeled with Cell Trace Violet fluorescent dye, and stimulated for 5 days with anti-CD3 and anti-CD28 in the presence of mast cell supernatant. At day 5, the proliferation history of IL-17, IFNγ, and TNF-producing cells was analysed. As shown in Figure 2, IL-17-producing cells had not undergone a more extensive proliferation compared to control, non-IL-17 producing T cells or IL-17-producing cells not exposed to supernatant of activated mast cells.



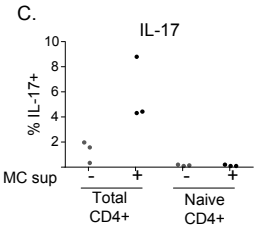


Figure 1. Supernatant of activated mast cells enhances Th17 responses. Mast cells were left untreated, or stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Isolated $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence of mast cell supernatant, or control medium containing the same stimuli. After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of BFA for 5 hours, to analyse their cytokine profile. A) Representative plots for intracellular cytokines IL-17, TNF-α, and IL-22. Live T cells were gated as shown in Supplementary Figure 2. B) Summary of 12 independent experiments, with each dot indicating a different mast cell supernatant. Asterisks indicate significant increase in the percentage of $IL\text{-}17^+$ T cells with supernatant from activated mast cells, as analysed using paired t-test (***p=0,0001). C) Summary of 3 independent experiments analyzing IL-17 production using intracellular cytokine staining of isolated total and naïve $CD4^+$ T cells, each treated with supernatant from a different mast cell donor.

Similar results were obtained when T cells producing other cytokines were analysed, although a small decrease in proliferation of IFNγ- and TNF-producing cells was noted in some donors (Figure 2C,D). Therefore, we conclude that the increase in Th17 cells by mast cells is independent of proliferation.

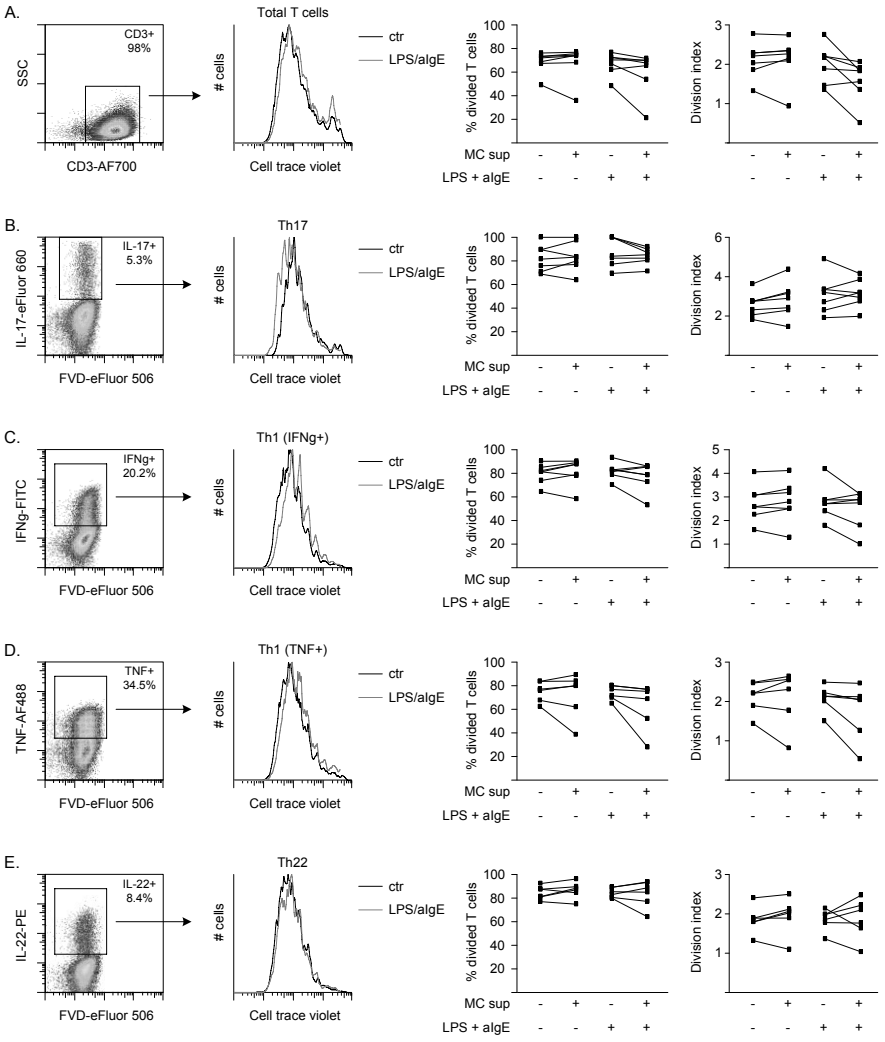


Figure 2: Proliferation of T helper cell subsets after culture with mast cell supernatant. Mast cells were left untreated, or stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Isolated $CD4^+$ T cells were labeled with Cell Trace Violet and activated with anti-CD3 and anti-CD28 in the presence of mast cell supernatant, or control medium containing the same stimuli. After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of brefeldin A for 5 hours, to allow the analysis of proliferation of different T helper cell subsets based on their cytokine profile. A-E) Representative flow cytometry plots of Cell Trace Violet as measure of proliferation (histograms) in T helper cell subsets based on cytokine staining, as shown in the plots on the left. Live T cells were gated as shown in Supplementary Figure 2. Summary of proliferation from 5 independent experiments is shown on the right, where each dot and line represents a different mast cell supernatant. The percentage of divided T cells is depicted as % of the input frequency, indicating how many cells from the original cell population have divided. Division index represents the average number of cell divisions that a cell in the original population has undergone. No significant differences were observed using paired samples t-test ($p<0,05$).

CHARACTERIZATION OF MAST CELL-INDUCED TH17 CELLS

Previous studies have identified different types of human Th17 cells with distinct effector function and differentiation requirements. Whereas memory Th17 cells have been shown to express IL-17 and TNF, other Th17 cell subsets co-express IFNγ, IL-22 or IL-10. Moreover, a substantial plasticity between these subsets has been reported, depending on the cytokine environment they are in.(*20-23*) To determine which of these subsets were preferentially expanded by mast cells, IL-17 staining of responding T cell cultures was combined with staining for TNF-α and IL-22 or IFNγ and IL-10 (Figure 3). Mast cell supernatants mainly enhanced the proportion of IL-17 single-positive cells, as well as the proportion of TNF-α/IL-17 double-positive T cells. Only a small minority of IL-17 producing cells produced IFNγ or IL-10.

Together, these results further confirm a specific effect on IL-17 production and suggest that mast cell-induced IL-17 producing T cells represent classical Th17 cells producing IL-17 and TNF, in the absence of other cytokines.

MAST CELLS PRODUCE ACTIVE IL-1β UPON TLR/FCεRI TRIGGERING

To better understand the mechanism responsible for strengthening of the Th17 axis by mast cells, we next wished to determine the role of cytokines secreted by mast cells in this process. Several cytokines (IL-1, IL-6, IL-23, TNF-α) have been described to enhance human memory Th17 responses. (*9*) As human mast cells are known to produce IL-1β, we first evaluated whether IL-1β could contribute to Th17 response enhancement, by using recombinant IL-1β (Fig 4A). As also noted by others, culturing T cells with recombinant IL-1β resulted in enhanced Th17 responses.(*21*) This effect could by blocked by the IL-1-receptor antagonist, anakinra, confirming the IL-1 receptor-dependency (Fig 4B).

We further wished to analyse whether mast cell supernatant contained IL-1β. As shown in Fig 4C, we observed increased production of IL-1β, but not IL-1α, in supernatant of activated mast cells, as detected by ELISA. To further confirm the presence of IL-1 in mast cell supernatant, the IL-1 activity was measured using the cell-line NOB-1, which produces mouse IL-2 in response to active IL-1. This cell-line responded to supernatant of activated mast cells, characterized by release of mouse IL-2 (Fig 4D). Importantly, treatment of the mast cell supernatant with inhibitors to IL-1α and IL-1β revealed a predominant inhibition of IL-1 activity by anti-IL-1β, suggesting that the majority of active IL-1 in mast cell supernatant is IL-1β (Fig 4D).

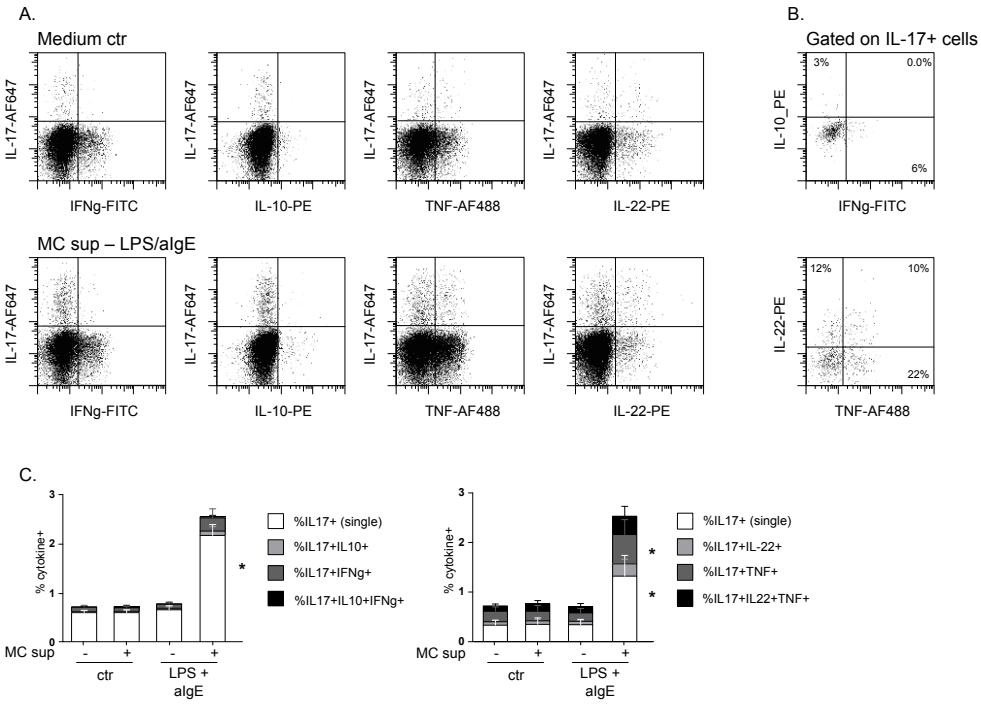


Figure 3. Characterization of Th17 cells induced by mast cells. Mast cells were left untreated, or were stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Isolated $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence of mast cell supernatant, or control medium containing the same stimuli. After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of brefeldin A for 5 hours, to analyse their cytokine production. A) Representative flow cytometry dot plots of combined staining for IL-17 with IFN-gamma, IL-10, TNF-α, IL-22. Live T cells were gated as shown in Supplementary Figure 2. B) Representative flow cytometry plots of IFN-gamma and IL-10, respectively TNF-α and IL-22 production by a T cell population, gated on IL-17^+ T cells, is shown. C) Summary of 7 independent experiments using mast cell supernatant from 9 different mast cell donors and T cells from 7 independent donors, showing the percentage of Th17 cell subsets defined by co-expression of other cytokines. Results are shown as mean +/- SEM. Asterisks indicate significant increase in the percentage of IL-17^+ T cell subset with supernatant from activated mast cells, analysed using two-way ANOVA and Bonferroni post hoc test (*p<0,001).

We next wished to analyse whether Th17 expansion was specific to FcεRI crosslinking or whether also other modes of mast cell activation induced IL-1 release and possibly a Th17-skewing phenotype. Therefore, we obtained supernatant of mast cells activated with IgG immune complexes (platebound) and LPS. We have previously shown that activation of mast cells through FcεRI or FcγRIIA in combination with LPS leads to a synergy in cytokine production.(*17, 24*)

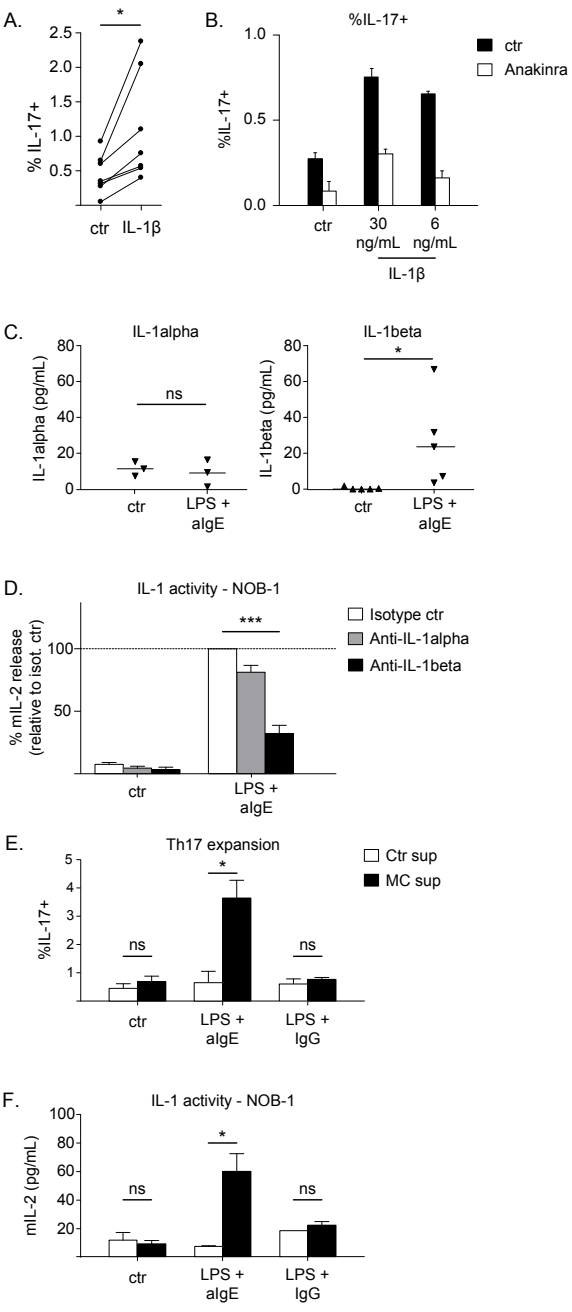


Figure 4. Mast cells produce active IL-1β upone TLR/FcεRI triggering. A,B) Isolated $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence or absence of recombinant IL-1β and with or without anakinra (IL-1 receptor antagonist). After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of BFA for 5 hours, to analyse their cytokine profile. A) Summary of 7 independent experiments. B) Representative example of blocking experiment using anakinra. Mean +/- SEM is shown. C) Mast cells were left untreated, or stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Levels of IL-1α and IL-1β in mast cell supernatant were measured using Luminex and ELISA, respectively. Summary of 3-5 different mast cell donors is shown. D) Mast cells were treated as in C). NOB-1 cells were cultured in the presence of mast cell supernatant, in combination of anti-IL-1α, anti-IL-1β or isotype control antibodies for 24 hours, after which the secretion of mouse IL-2 by NOB-1 cells was measured using ELISA. Percentage mIL-2 release that is shown was calculated relative to the amount of mouse IL-2 released by NOB-1 cells when treated with supernatant from mast cells activated with LPS + anti-IgE in presence of isotype control. Mean +/- SEM from 4 independent mast cell donors is shown. E) Mast cells were left untreated, stimulated with LPS and anti-IgE, or stimulated with LPS and platebound IgG immune complexes. T cells were cultured as in A) in the presence or absence of mast cell supernatant. Mean +/- SEM from 3 independent mast cell donors is shown. F) Mast cells were treated as described in E). NOB-1 cells were cultured in the presence of mast cell supernatant as described in D). Mean +/- SEM from 4 independent mast cell donors is shown. Asterisks indicates significant difference ($p<0.05$), using students T-test (A, C, E, and F) or Two-way ANOVA with Bonferroni posthoc test (D).

Interestingly, mast cell activation via FcγRIIA in combination with LPS did not induce increased Th17 responses as these were only observed when mast cells were activated with anti-IgE and LPS (Fig 4E). This correlated with IL-1 activity in mast cell supernatant, as this was only increased by stimulation through anti-IgE and LPS, and not by IgG immune complexes and LPS (Fig 4F). Together, these results indicate that mast cells secrete active IL-1β, specifically in response to anti-IgE in combination with LPS.

IL-1β RELEASE BY MAST CELLS IS INFLAMMASOME-INDEPENDENT

IL-1β is a cytokine that is initially produced as inactive pro-IL-1β, and therefore needs to be cleaved to generate the active molecule prior to secretion. The most well-known pathway leading to cleavage of IL-1β is the inflammasome-caspase-1 pathway. The specific pathways leading to IL-1β cleavage and secretion by mast cells are not known. Therefore, we analysed whether secretion of active IL-1β by mast cells was caspase-dependent, using a pan-caspase inhibitor (Z-VAD). The monocyte cell-line THP-1 was used as positive control. These cells secrete active IL-1β upon activation with LPS, due to inflammasome activation by endogenous ATP.(*25*) As depicted in Figure 5, secretion of active IL-1β by the monocyte cell-line THP-1 was almost completely reversed using Z-VAD (Fig 5A,B; left). In contrast, no inhibition of IL-1 activity was observed using this inhibitor in mast cells (Fig 5A,B; right).

Cell death can also lead to cleavage of pro-IL1β by mast cells, through aspecific release of pro-IL1β and chymase followed by extracellular cleavage. Therefore, LDH levels were quantified in mast cell supernatant as a measure of cell death. No significant increase in LDH levels were observed upon mast cell stimulation (Fig 5C), suggesting that the inflammasome-independent cleavage of IL-1β by mast cells is not due to cell death.

MAST CELLS INDUCE TH17 RESPONSES THROUGH INFLAMMASOME-INDEPENDENT IL-1β

To determine the contribution of IL-1β secreted by mast cells on Th17 responses, we next evaluated the effect of neutralizing IL-1β in mast cell supernatant, by using IL-1 receptor antagonist IL-1RA (anakinra) as well as a blocking antibody to IL-1β (Fig 6A-B). Both IL-1RA and anti-IL-1β almost completely reversed the expansion of the Th17 response induced by supernatant from activated mast cells. This effect was specific for Th17 responses, as no effect of these inhibitors on TNF production by T cells was observed (Fig 6B, right panel).

We next compared the blocking of IL-1β to that of potential other mast cell-derived cytokines described to enhance Th17 responses, IL-6 and TNF-α (Fig 6C). Whereas anti-IL-1β again led to a significant reduction in the number of Th17 cells, no effect was

observed with anti-IL-6R. Blocking of TNF-α led to an increase in the number of Th17 cells, indicating that TNF was not involved in the skewing towards Th17 responses by mast cells. As we observed that IL-1β release by mast cells was inflammasome-independent, we wished to confirm that Th17 responses induced by mast cell supernatant were not affected by caspase-inhibition. Indeed, Th17 expansion by mast cells was not affected by the pan-caspase inhibitor Z-VAD (Fig 6D).

Together, these results indicate that human mast cells can drive enhancement of Th17 responses through secretion of inflammasome-independent IL-1β.

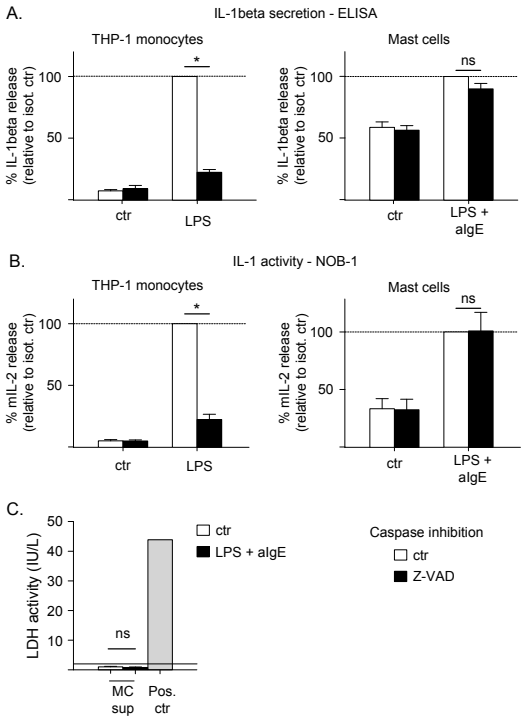


Figure 5 IL-1β release by mast cells is inflammasome-independent. A-B) THP-1 cells and mast cells were left untreated, or stimulated with LPS or LPS + anti-IgE, respectively. Cells were treated with Z-VAD 15 minutes prior to activation. Supernatant was collected after 24 hours. Secretion of IL-1β was measured using ELISA (A). NOB-1 cells were cultured in the presence of cell supernatant, or control medium containing the same stimuli for 24 hours, after which their secretion of mouse IL-2 was measured using ELISA (B). Representative example of THP-1 cell-line is shown (left), and a summary (mean +/- SEM) of 4 different mast cell donors (right). C) Analysis of LDH activity in mast cell supernatant. Mean +/- SEM from 14 independent mast cell donors is shown. Asterisks indicate statistically significant ($p<0.05$) differences analysed using student's T-test. ns indicates no significant differences were found

DISCUSSION

In this study, we show that human mast cells can enhance Th17 responses from memory $CD4^+$ T cells. This is in line with a recent study showing increased IL-22 production by T cells when mast cells presented bacterial antigens.(*2*) Together, these results therefore suggest that human mast cells can contribute significantly to the Th17/Th22 axis.

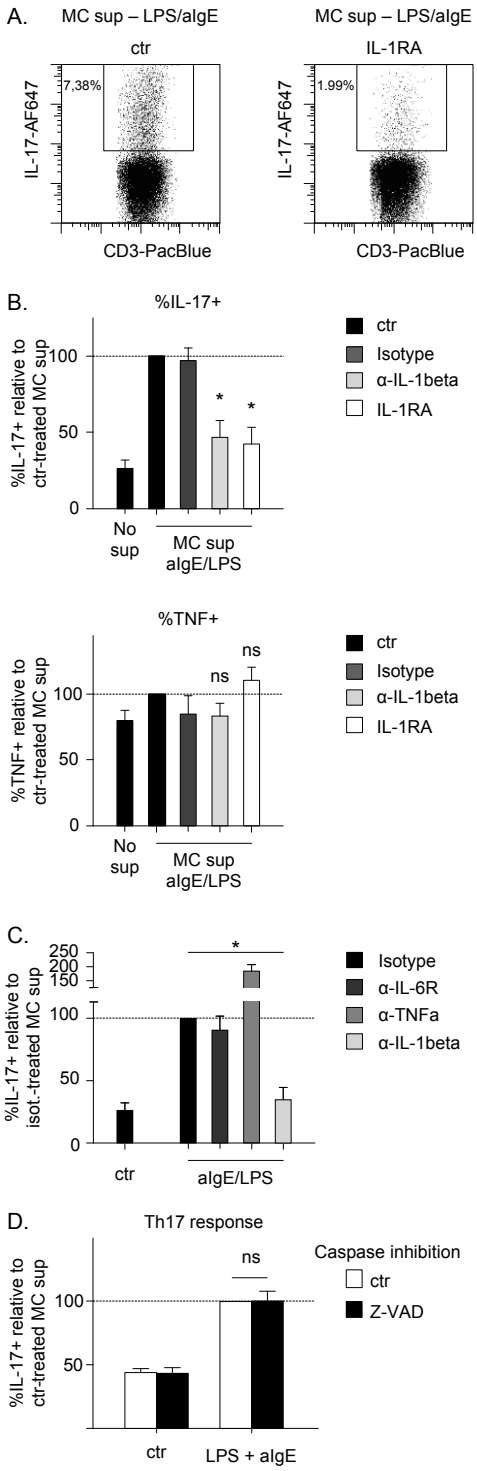


Figure 6. Mast cells induce Th17 responses through inflammasome-independent IL-1β. A-D) Mast cells were left untreated, or stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Isolated $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence of mast cell supernatant or control medium containing the same stimuli. After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of brefeldin A for 5 hours, to analyse their cytokine profile. A-C) Blocking of cytokines in mast cell supernatant was achieved using IL-1RA or blocking antibodies against IL-1β, IL-6R, or TNF-α. Mast cell supernatant or control medium was incubated with these inhibitors prior to addition of the supernatant to the isolated T cells. A) Representative flow cytometry plots of IL-17 production Live T cells were gated as shown in Supplementary Figure 2. B) Summary of inhibition of IL-17 (top) and TNF-α (bottom) in T cells in response to mast cell supernatant untreated (ctr), or treated with anakinra, anti-IL-1β, or isotype control. Mean +/- SEM from 6 independent experiments is shown. C) Summary of inhibition of IL-17 in T cells in response to mast cell supernatant treated with isotype control, anti-IL-6R, anti-TNF-α, or anti-IL-1β. Mean +/- SEM from 3 independent experiments is shown. D) Mast cells were treated with Z-VAD, starting 15 minutes prior to their activation.. Mean +/- SEM from 5 independent experiments is shown. Asterisks indicate statistically significant ($p<0.05$) differences analysed using student's T-test (B) or Two-way ANOVA with Bonferroni posthoc test (C). Ns indicates no significant differences were found, analysed using Two-way ANOVA with Bonferroni posthoc test (D). Percentage $cytokine^+$ T cells that is shown was calculated relative to the % of $cytokine^+$ T cells when treated with supernatant from mast cells activated with LPS + anti-IgE in the presence of isotype control (B, C), or in absence of caspase inhibition (D).

Our results suggest that Th17 expansion by mast cells was induced by IL-1β. IL-1β is a cytokine that is produced in an inactive 31-kDa molecule residing in the cytoplasm, and needs to be cleaved to generate the active 17 kDa molecule, that can be secreted. The most well-known pathway leading to cleavage of IL-1β is the caspase-1 pathway, which is activated upon inflammasome activation. Although inflammasome activation in mast cells can lead to secretion of IL-1β,(*26, 27*) in vivo IL-1β release during sterile inflammation has been shown to be at least partly independent of caspase-1, and mast cell- and neutrophil proteases have been suggested to contribute to secretion of active IL-1β.(*28-32*)

Our results confirm and extend these data, by showing that IL-1β release by human mast cells in response to TLR/FcεRI receptor triggering is caspase-independent as well as that such IL-1-release can lead to boosting of Th17 responses.

A likely candidate for cleavage of pro-IL-1β by mast cells is chymase, one of the granule proteases in mast cells, which has been shown to cleave pro-IL-1β in vitro into an active form of 18kd.(*31*) This would be in line with our observation that IL-1β release by mast cells depends on their degranulation through FcεRI crosslinking. However, as the amounts of IL-1β released in this study were relatively low, we were unable to visualize the mast cell-derived IL-1β by western blot or intracellular staining to analyse the mechanism of cleavage of IL-1β by mast cells. IL-1β is known to be active at extremely low concentrations (<1 pg/mL),(*33*) thereby complicating such analyses.

Our results suggest a novel role of mast cells in driving Th17 responses directly through release of IL-1β in a caspase-independent manner. This is of particular interest in the context of mucosal immunity. Whereas mast cells have been shown to play beneficial roles in protection against bacteria at mucosal surfaces, they also contribute significantly to allergic responses at these same locations.(*34, 35*) Our results suggest that Th17 expansion by mast cells was dependent on FcεRI triggering. Interestingly, allergic responses which are classically considered to be driven by Th2 cells, are now increasingly recognized as also having a Th17 component.(*36*) Our results therefore provide a direct link between IgE-mediated mast cell activation and expansion of Th17 cells in the context of allergic responses.

Together, these studies reveal a role of human mast cells in setting the outcome of T cell responses through the release of inflammasome-independent IL-1β, and suggest a novel contribution of these cells in boosting of immune responses in mucosal barriers by specifically targeting the Th17-axis.

ACKNOWLEDGEMENTS

We thank prof. C. Dinarello for his useful suggestions and critical appraisal of this study. This work was supported by the Dutch Arthritis Foundation, the Dutch Organization for Scientific Research (Vici grant), the Research Foundation Sole Mio, the Leiden Research Foundation (STROL), the Centre for Medical Systems Biology (CMSB) within the framework of the Netherlands Genomics Initiative (NGI), the IMI JU funded project BeTheCure, contract no 115142-2, and European Union (Seventh Framework Programme integrated project Masterswitch; grant Number: 223404).

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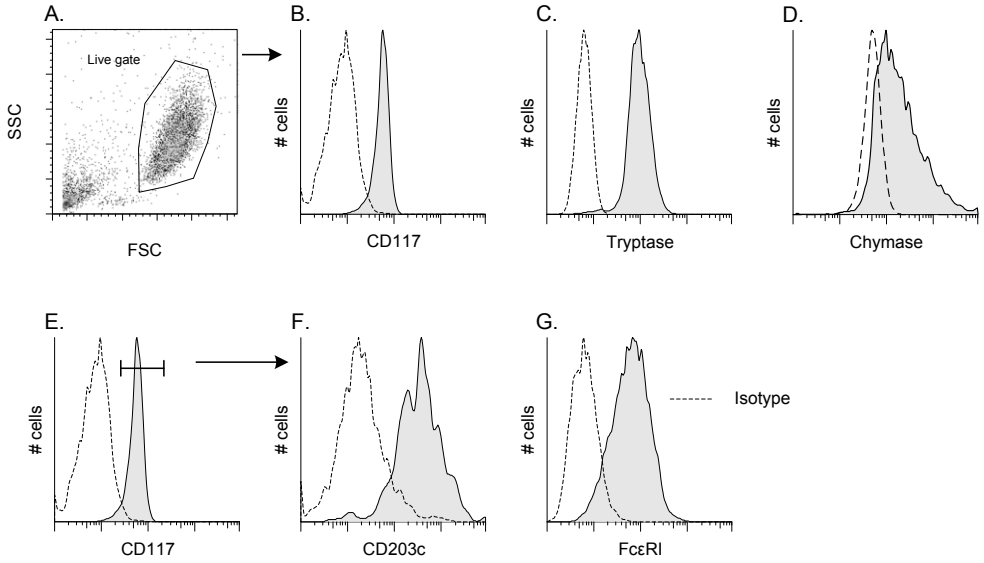
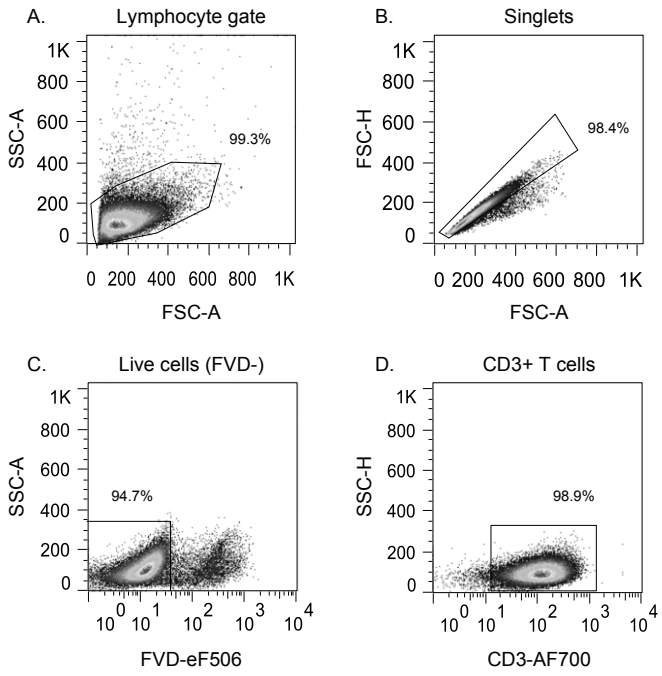
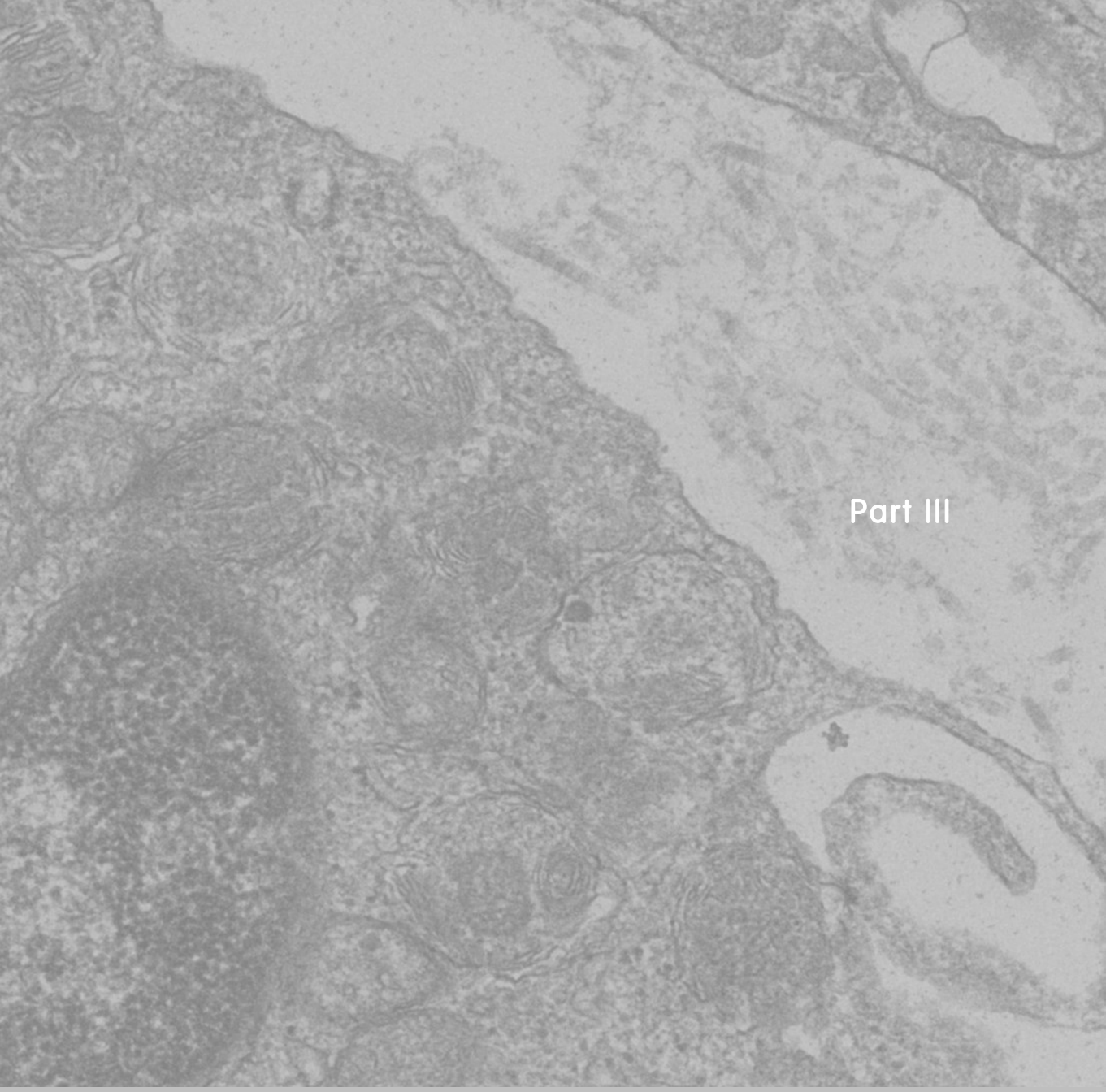
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Characterization of mast cells. Peripheral blood-derived mast cells were cultured from CD34$^+$ stem cells for 6-8 weeks. Mast cells were identified as CD117$^+$ CD203c$^+$ FcεRI$^+$ Tryptase$^+$ cells (>90% pure). A) Gating of live cells (cell debris excluded based on Forward and Sideward scatter). B) CD117 expression of total live cells as gated in A. C) Intracellular Tryptase expression of total live cells. D) Gating of CD117$^+$ mast cells. E,F). Expression of CD203c and FcεRI on CD117$^+$ mast cells as gated in D. Representative FACS plots are shown.



Supplementary Figure 2. T cell gating strategy. Live $CD3^+$ T cells were gated as follows: A) A lymphocyte gate was used based on Forward scatter (FSC) and Side scatter (SSC). B) Single cells were selected based on FSC-area (A) and FSC-height (H). C) Live cells were gated based on negativity for Fixable Viability Dye (FVD) eFluor 506. D) T cells were gated based on CD3 expression.



Part III

Antibody-mediated chronic inflammation in allergy and autoimmunity

REPEATED FCεRI TRIGGERING REVEALS MODIFIED MAST CELL FUNCTION RELATED TO CHRONIC ALLERGIC RESPONSES IN TISSUE

Chapter 10

Jolien Suurmond,[1] Kim LL Habets,[1] Zuotian Tatum,[1,2]
Joris J Schonkeren,[1] Peter AC 't Hoen,[2] Tom WJ Huizinga,[1]
Jeroen FJ Laros,[2] René EM Toes,[1] Fina A Kurreeman[1]

[1]Department of Rheumatology, Leiden University Medical Center (LUMC), Leiden, The Netherlands; [2]Department of Human Genetics, LUMC, Leiden, The Netherlands

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ABSTRACT

Background Activation of mast cells through FcεRI plays an important role in acute allergic reactions. However, little is known about the function of mast cells in chronic allergic inflammation, or the impact of repeated FcεRI triggering occurring in such responses.

Objective We aimed to identify changes in mast cell function after repeated FcεRI triggering, and to correlate these changes to chronic allergic responses in tissue.

Methods Human cord blood-derived mast cells were treated for two weeks with anti-IgE. The function of naive or treated mast cells was analysed by RNA sequencing, quantitative RT-PCR, flow cytometry, and functional assays. Protein secretion was measured using ELISA and multiplex assays.

Results We observed several changes in mast cell function after repeated anti-IgE triggering. Whereas the acute response was dampened, we identified 289 genes significantly upregulated after repeated anti-IgE. Most of these genes (84%) were not upregulated after a single anti-IgE stimulus indicating a significantly different response mode, characterized by increased antigen presentation, response to bacteria, and chemotaxis. Changes in the mast cell function were related to changes in expression of transcription factors RXRA and BATF and others. Importantly, we found a substantial overlap between the genes upregulated after repeated anti-IgE triggering with genes upregulated in chronic allergic tissues, in particular chronic rhinosinusitis.

Conclusion Our study provides evidence for intrinsic modulation of mast cell function upon repeated FcεRI-mediated activation. The overlap with gene expression in tissues is suggestive of a direct link between repeated IgE-mediated activation of mast cells and chronic allergy.

KEY MESSAGES

Repeated activation of mast cells through FcεRI leads to a modulation of their function, related to changes in gene expression during chronic allergy

Chronic activation of mast cells such as occurring in chronic allergy may therefore contribute to changes in tissue homeostasis

INTRODUCTION

Mast cells play an important role in allergic responses upon activation through the high-affinity FcεRI. Their rapid degranulation and release of lipid-derived mediators leads to an immediate response, characterized by increased vascular permeability. This response can also lead to several tissue-specific phenomena, such as contraction of airway smooth muscle cells and mucus secretion in the airways, or anaphylaxis in case a systemic response is triggered (*1, 2*). Acute symptoms can occur within minutes of exposure to the associated allergens, and are thought to be induced through release of histamine, prostaglandins and leukotrienes by mast cells. During late phase responses, mast cell-derived cytokines and chemokines (IL-5, IL-13) also contribute to the inflammatory reactions observed in allergy, in particular through their effects on leukocyte infiltration and activation (*3*).

However, allergic disease is often associated with repeated exposure to allergens which is thought to lead to chronic inflammation and changes in tissue homeostasis. In the lungs, these may lead to increased contractability of the smooth muscles, leading to obstruction of the bronchia, increased mucus production, and airway wall remodeling (*1*). In the skin, atopic dermatitis is characterized by fibrotic lesions, containing thickened dermis and epidermis, and accumulation of lymphocytes and mast cells (*4, 5*). Chronic rhinosinusitis (CRS) is characterized by inflammation of the upper airways. CRS is frequently divided into two groups based on the presence of nasal polyps. CRS with nasal polyps is associated with nasal obstruction and olfactory loss and is characterized by eosinophilia and Th2-related inflammation (*6, 7*).

Importantly, these diseases are all associated with repeated antigen exposure and triggering through IgE. Whereas mast cells and IgE antibodies play a crucial role in acute symptoms of an allergic reaction, their role in chronic inflammation and tissue remodeling is less well-defined (*8*). Chronic allergic responses in tissue are the result of a complex interplay between different immune cells (mast cells, eosinophils, macrophages, dendritic cells, lymphocytes) and stromal cells. Mast cells can secrete several mediators that have the potential to influence tissue remodeling, such as FGF-2, VEGF and amphiregulin (*9, 10*).

In some mouse models of chronic asthma, mast cells are dispensable for induction of eosinophilia or airway hyperresponsiveness. These models are thought to be driven by the Th2-derived cytokines IL-4 and IL-5 (*11-14*). However, in other mouse models of chronic asthma, induced through repeated antigen exposure after initial sensitization, mast cells and activation through FcεRI are shown to be crucial for several hallmarks of chronic asthma, such as tissue remodeling, goblet hyperplasia and leukocyte infiltration (*15-17*). Mast cells have also been shown to play a crucial role in chronic dermatitis in mice (*5, 18*).

However, the exact changes in mast cells that occur after repeated antigen exposure are not well understood, especially in the context of human disease. Although mast cells alone are unlikely to provide all the signals leading to tissue remodeling, appreciation of the mechanisms contributing to their involvement in chronic inflammation can enhance our understanding of these diseases and thereby allow us to design novel therapeutics aimed at the long-term changes induced in tissues.

As tissue-resident mast cells are long-lived cells (estimated up to months or years) (*19*), we hypothesized that mast cells will be exposed repeatedly to antigen, and that this may lead to intrinsic changes in mast cell function and/or biology relevant to chronic allergy. Studies using several pre-clinical (asthma) animal models have offered significant progress in our understanding of the role and function mast cell function in (chronic) allergy. Nonetheless, still little is known on cell-intrinsic changes induced upon repeated antigen exposure of mast cells in general and human mast cells in particular.

In this study, we modelled repeated antigen exposure of human mast cells in vitro, and show that mast cells respond differently after repeated- compared to acute FcεRI activation. We captured these changes in mast cell function using full RNA sequencing, and identified several novel genes specifically upregulated after repeated activation. Furthermore, the expression pattern of these genes related to changes in gene expression also observed in tissue during chronic atopic diseases, together indicating that repeated antigen exposure of mast cells triggers a response mode that may directly contribute to tissue remodeling and inflammation.

METHODS

EXPERIMENTAL SETUP

Cord blood-derived mast cells were obtained as described in Supplementary Methods. The purity of mast cells was determined by flow cytometric analysis of CD117 (c-kit), FcεRI, CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99% (Supplementary Fig 1 A-D). Cultured mast cells were sensitized with 1 µg/mL hybridoma IgE (Diatec) for a minimum of 24 hours before each stimulation. Mast cells were cultured at $1x10^6$ cells/mL.

For repeated anti-IgE stimulations, mast cells were stimulated once weekly with 0.2 µg/mL goat-anti-human IgE (Nordic). After two weeks, mast cells were counted using Trypan Blue, and cultured again at $1x10^6$ cells/mL. Mast cells were stimulated with control medium or 1 µg/mL anti-IgE for 6 hours for RNA analysis, or with 0.01-10

µg/mL anti-IgE for 1, 6 and 24 hours for analysis of protein expression and secretion. For supernatant transfer experiments, sensitized mast cells were stimulated with 0.2 µg/mL goat-anti-human IgE (Nordic) or control medium. After 24h, supernatant was harvested. Non-sensitized mast cells were cultured in this supernatant for two weeks. Supernatant was replaced after 1 week. At the end of two weeks, mast cells were sensitized and activated as described above for collection of RNA, and analysis of protein expression and cytokine secretion.

RNA isolation, sequencing, qPCR, measurements of protein expression measurements and comparisons to publicly available gene expression data were performed as described in Supplementary methods, and samples processed for RNA-seq are shown in Online Repository Table E2.

STATISTICAL ANALYSIS

For comparison of naïve and treated mast cells, paired-samples T-test was performed. For differences between multiple groups, repeated measures ANOVA was performed, with Bonferroni's post-hoc test to correct for multiple testing. To compare frequencies of gene overlap, Fisher's Exact test was used. P values of <0.05 were considered statistically significant (except for the sequencing analysis, where significance was defined as described above). Statistical analysis was performed using GraphPad Prism 5. Venn diagrams were created using BioVenn (*20*).

RESULTS

REPEATED FCεRI TRIGGERING OF MAST CELLS

We set up a model for repeated FcεRI triggering of mast cells to mimic repeated IgE-mediated activation such as occurring during chronic allergic responses (Fig 1A). Human cord blood-derived mast cells were stimulated for two weeks with 0.2 µg/mL anti-IgE (Repeated αIgE) or control medium (Naive). We chose to stimulate the cells weekly, as more frequent stimulations with anti-IgE led to decreased FcεRI expression, resulting from prior stimulations (data not shown). The first stimulation led to a moderate level of degranulation of mast cells as well as cytokine production (Fig 1B,C). Twenty-four hours prior to each stimulation, mast cells were sensitized with human IgE. At the end of the two weeks, mast cells were either left untreated (N and R) or stimulated with anti-IgE (N+αIgE and R+αIgE).

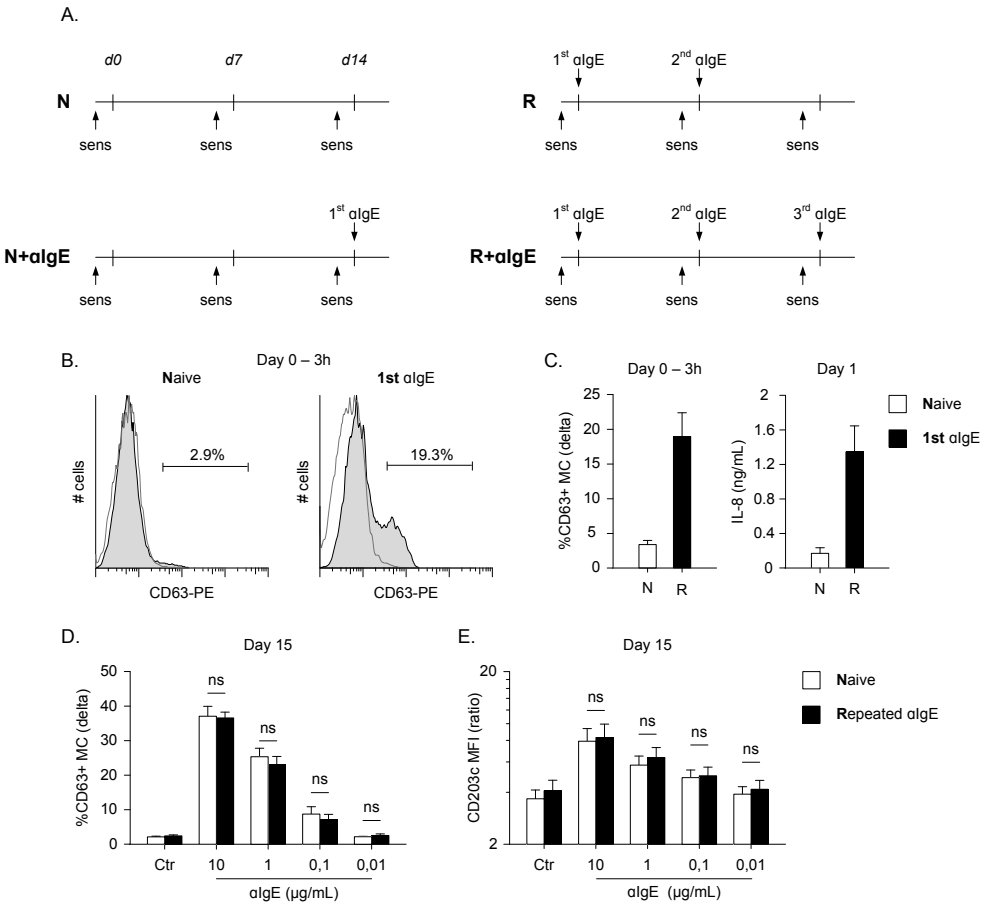

Figure 1. Repeated mast cell stimulation through FcεRI. A) Experimental setup of repeated stimulation of mast cells. Human cord blood-derived mast cells were stimulated for two weeks with 0.2 μg/mL anti-IgE (Repeated αIgE) or control medium (Naive). Twenty-four hours prior to each stimulation, mast cells were sensitized with human IgE. At the end of the two weeks, mast cells were either left untreated (N and R) or stimulated with anti-IgE (N+αIgE and R+αIgE). B) Representative flow cytometry plots for CD63 of naive mast cells (N) or mast cells 3 hours after their first anti-IgE stimulation (R), showing their degranulation in response to the first anti-IgE on day 0. C) Summary of surface CD63 expression as in B) and IL-8 secretion 24 hours after the first anti-IgE stimulation or control medium. D, and E) Surface CD63 and CD203c expression in response to 24 hours of anti-IgE stimulation, in naive mast cells or mast cells exposed to two weeks of stimulation (Repeated αIgE). CD63 staining as in C, and CD203c MFI ratio calculated as in E. Data are represented as mean ± SEM from n=3 (C) or n=6 (D, and E) independent experiments. Ns indicates that no significant differences were found between naive mast cells (N) and cells exposed for two weeks to anti-IgE (R), using paired T-test.

No difference in cell number, viability, maturation, or morphology was observed between the different treatments (Supplementary Fig 1 E-I). After two weeks, mast cells were stimulated with anti-IgE to evaluate changes in mast cell function when they had been previously exposed to anti-IgE. No difference in degranulation (CD63 upregulation) or upregulation of activation marker CD203c was observed after repeated activation through FcεRI (Fig 1D, E).These results show that in our in vitro model, mast cells maintained the intrinsic capacity to respond to FcεRI triggering after repeated activation through this receptor, allowing the study of downstream functional changes in these cells after (repeated) stimulation of the FcεRI-receptor.

DISTINCT RESPONSES TO FCεRI TRIGGERING UPON REPEATED EXPOSURE

To capture the complete mast cell transcriptome after two weeks of stimulation through FcεRI, we performed deep sequencing on RNA from three independent experiments. We isolated RNA 6 hours after stimulation with control medium or anti-IgE at the end of the two weeks treatment. Quality controls are described in the Supplementary Methods, and results are shown in Online Repository Table E2 and Supplementary Figure 2.

To analyse the response of mast cells after repeated anti-IgE stimulation, we first selected those genes that are upregulated by single anti-IgE stimulation in naïve mast cells (Fig 2A, Online Repository Table E4A) (*21*). Expression of 1919 genes (449 genes >2-fold) was significantly upregulated after 6 hours of single stimulation with anti-IgE. The average upregulation of these genes was significantly dampened in mast cells which were exposed repeatedly to anti-IgE for 2 weeks (R+αIgE) (Fig 2B,C). Of these 449 genes, 22 showed a significant dampening of their expression after repeated stimulation (Online Repository Table E5A, E6A, and Fig 2D). CSF2 and CCL4, cytokines/chemokines known to be secreted by mast cells, displayed the most pronounced dampened responses. We validated these findings by qPCR and measured secreted proteins GM-CSF (CSF2) and MIP-1β (CCL4) in mast cell supernatant obtained after 24h (Fig 2E-J) confirming this dampened response at the protein level in both GM-CSF and CCL4 (albeit a non-significant trend for CCL4). These results suggest a reduced responsiveness to FcεRI triggering by mast cells after repeated stimulation. In support of this notion, the expression of those genes that were downregulated by a single anti-IgE stimulation, was less downregulated after repeated stimulation (Supplementary Fig 3, Online Repository Table E4B), suggesting an overall dampening of mast cell transcriptional responses to FcεRI triggering after repeated stimulation.

We did not observe changes in expression of most genes associated with arachidonic acid-derived products (Fig 2K and Online Repository Table E7). In contrast to the decreased production of cytokines we observed, degranulation and release of lipid mediators LTC_4 and

PGD_2 were not affected by repeated FcεRI triggering (Fig 1H and Fig 2L,M). These results suggest that repeated FcεRI triggering affected the *de novo* transcription of FcεRI-responsive genes, but not initial FcεRI signaling leading to degranulation and release of lipid mediators.

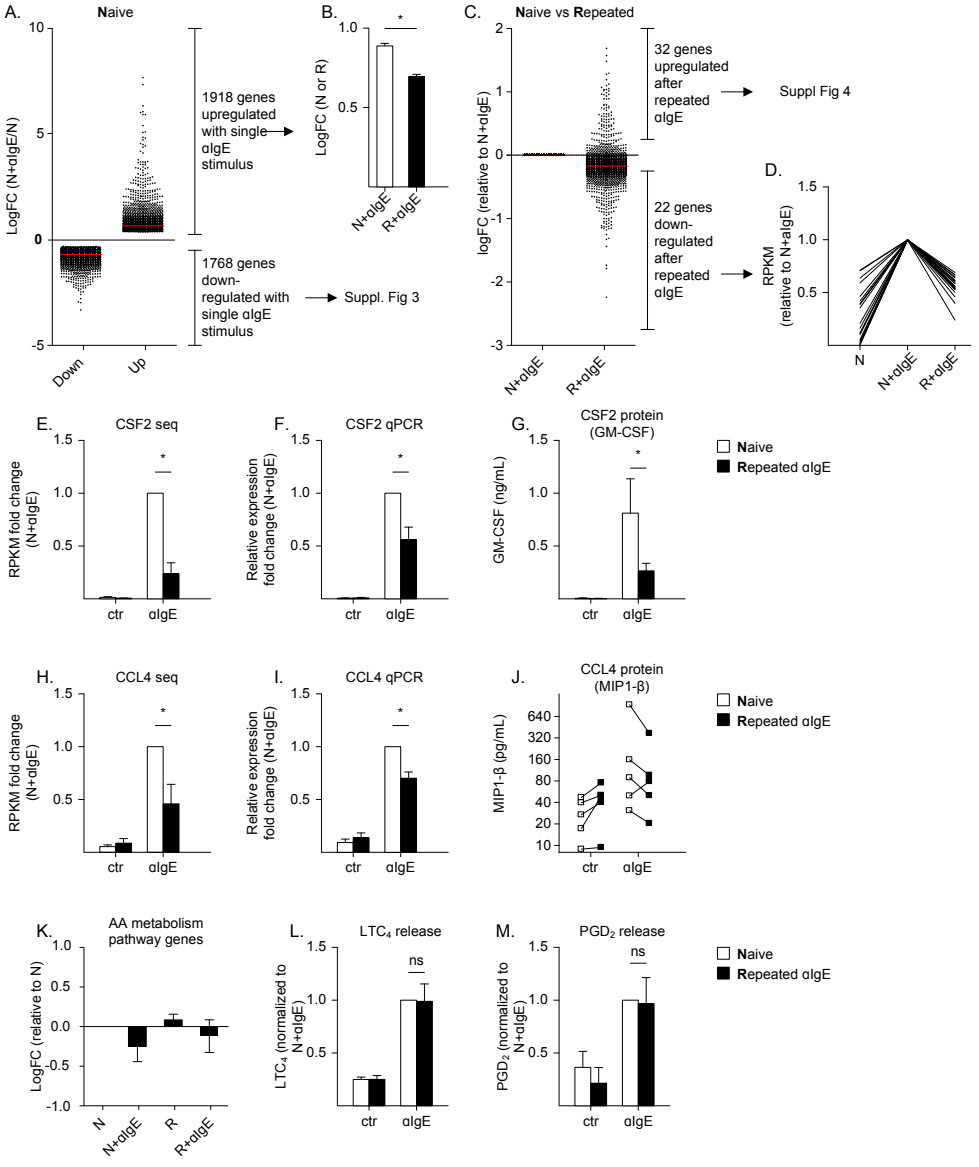

Figure 2 *(left)*. Dampened genes after repeated anti-IgE. A) Gene expression obtained using RNA sequencing in response to a single 6 hour anti-IgE stimulation (N+αIgE), compared to control mast cells (N). Log fold change (LogFC) relative to Naive (N) mast cells of the differentially expressed genes is shown. B) Genes which were significantly upregulated after a single anti-IgE were selected as shown in A), and compared to their upregulation after repeated stimulation (R+αIgE). LogFC was calculated compared to unstimulated mast cells at the end of the two week stimulation (N+αIgE/N and R+αIgE/R). C) Genes which were significantly upregulated after a single anti-IgE were selected as shown in A), where each dot indicates a different gene. Those genes significantly upregulated (top) and tolerized (bottom) after repeated anti-IgE (R+αIgE) compared to a single anti-IgE (N+αIgE) stimulation are indicated. Data are shown as fold change relative to N+αIgE. D) Genes which are significantly downregulated after repeated anti-IgE, compared to single anti-IgE using differential expression analysis. Each line represents a single gene, and data is shown as relative RPKM normalized to single anti-IgE stimulation (N+αIgE). E, and H) Gene expression of CSF2 and CCL4, obtained using RNA sequencing. Data is shown as RPKM fold change relative to single anti-IgE stimulation (N+αIgE). F, and I) Gene expression of CSF2 and CCL4, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to single anti-IgE stimulation (N+αIgE). G, and J) Protein levels of CSF2 (GM-CSF) and CCL4 (MIP-1β) in supernatant, 24 hours after stimulation with anti-IgE of naive mast cells or mast cells which were exposed to anti-IgE for two weeks. K) Expression of genes in the Arachidonic Acid (AA) metabolism pathway, obtained using RNA sequencing in response to a single 6 hour anti-IgE stimulation (N+αIgE), compared to control mast cells (N). Log fold change (LogFC) relative to Naive (N) mast cells of the differentially expressed genes is shown. L,M) Protein levels of LTC4 and PGD2 in supernatant, 1 hour after stimulation with anti-IgE of naive mast cells or mast cells which were exposed to anti-IgE for two weeks. Data are represented as mean ± SEM from n=3 (B, E, H, and K) or n=5 (F, G, I, and J) or n=6 (L and M) independent experiments. Asterisks indicate significant differences obtained using paired T-test $p<0.05$ (B), differential expression analysis FDR<0.05 (C, E, and H) or repeated measures ANOVA with Bonferroni posthoc test $p<0.05$ (F, G, I, L, and M).

Despite this dampening of the acute response of mast cells on the global level, we observed that 32 FcεRI-responsive genes displayed increased upregulation of their expression after repeated stimulation with anti-IgE (Fig 2C, Supplementary Fig 4A, Table E5B, Online Repository Table E6B). The upregulation of the top three significant genes, TMEM45B, EMR3 and CH25H was confirmed using qPCR (Supplementary Fig 4B-G).

Together, these results show that repeated anti-IgE triggering alters the mast cell response, characterized by overall dampening of most FcεRI-responsive genes, as well as by upregulation of a particular set of genes.

To capture whether there were functional differences between the genes which were dampened compared to those that showed further upregulation, we analysed GO biological pathways represented by these genes (Online Repository Table E8).

Dampened responses were assigned to pathways involved in leukocyte proliferation and activation (6 out of 22 genes), whereas upregulated genes participated mainly in pathways involved in the response to bacteria (5 out of 32 genes), response to wounding (6 out of 32 genes), and chemotaxis (3 out of 32 genes).

Together, these results show that mast cell function can be modulated by repeated FcεRI triggering resulting in a different response mode as compared to acute stimulations.

DE NOVO UPREGULATION UPON REPEATED FCεRI TRIGGERING

To capture any other changes occurring in the mast cell transcriptome after repeated stimulation with anti-IgE, irrespective of their responsiveness to single anti-IgE stimulation, we performed differential expression analysis on the total gene set. Two hundred eighty nine (289) genes were significantly upregulated after repeated anti-IgE stimulation, of which 243 genes did not respond to single anti-IgE stimulation (Online Repository Table E9). Upregulation of most of these genes did not increase further by 6 hours of the additional 3rd anti-IgE stimulation (R+αIgE), consistent with a persistent upregulated expression (Fig 3A,B). The 20 most strongly upregulated genes are shown in Online Repository Table E5C. Upregulation of TREM2, OLFM4 and CCL18 were confirmed using qPCR (Fig 3C-H).

To verify the upregulation of the most highly upregulated gene CCL18 at the protein level, the secretion of CCL18 was measured in supernatant. Whereas secretion of CCL18 secretion was not detected upon acute anti-IgE activation of naive mast cells stimulated for 6 or 24 hours, we observed a significant release of CCL18 after 2 weeks of stimulation through FcεRI (Fig 3I,J), confirming the sustained secretion of CCL18 in supernatant after repeated stimulation with anti-IgE.

As a control for the specificity of anti-IgE stimulation, we have stimulated mast cells with IgG immune complexes at the end of the two weeks culture with anti-IgE. In contrast to restimulation with anti-IgE, cytokine production upon stimulation with platebound IgG immunecomplexes was not affected or was even increased (CCL4) when mast cells had been treated for two weeks with anti-IgE (Supplementary Fig 5A-C). Furthermore, stimulation of mast cells through FcγRIIA for two weeks did not affect gene expression of CSF2, CCL4 and CCL18 (Supplementary Fig 5 D-F).

These data further confirm the specificity of FcεRI stimulation, and suggest that the changes in mast cell function as observed after repeated stimulation are FcεRI-intrinsic.

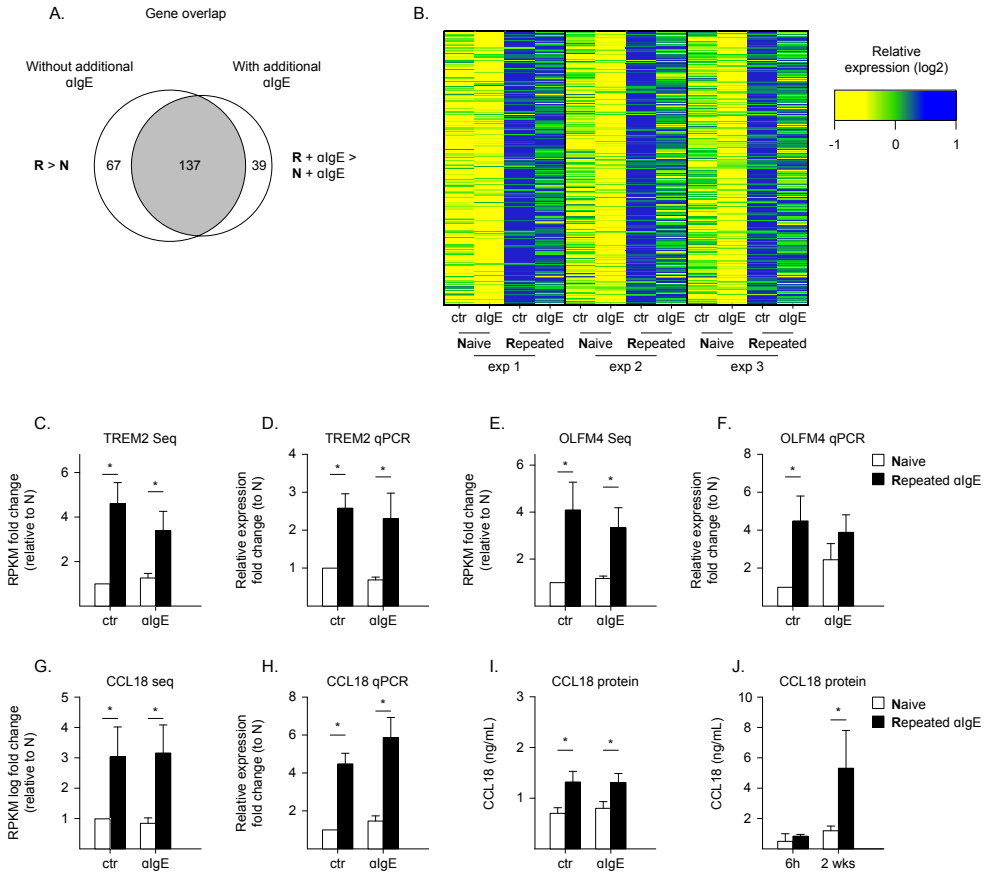

Figure 3. Persistent upregulation of genes after repeated anti-IgE. A) Overlap of genes upregulated after repeated anti-IgE, without an additional stimulation (R>N) or with an additional stimulation (R+αIgE>N+αIgE), obtained using differential expression analysis. B) Heatmap showing relative expression of all genes in A. Each row represents a single gene. The relative expression in 3 independent experiments is shown. C, E, and G) Gene expression of TREM2, OLFM4, and CCL18, obtained using RNA sequencing. Data is shown as RPKM fold change relative to naïve mast cells (N). D, F, and H) Gene expression of TREM2, OLFM4, and CCL18, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to naïve mast cells (N). I) Protein levels of CCL18 in supernatant, 24 hours after stimulation with anti-IgE of naive mast cells or mast cells which were exposed to anti-IgE for two weeks. J) Protein levels of CCL18 in supernatant, 6 hours after stimulation with anti-IgE of naive mast cells (left), or 2 weeks after stimulation with repeated anti-IgE or control medium (right). Data are represented as mean ± SEM from n=3 (C, E, G, and J) or n=5 (D,F, and H,) or n=4 (I) independent experiments. Asterisks indicate significant differences obtained using differential expression analysis FDR<0.05 (C, E, and G), repeated measures ANOVA with Bonferroni posthoc test $p<0.05$ (D,F, H, and I), or paired T-test $p<0.05$ (J). N = naive mast cells; N+αIgE = a single 6 hours stimulation of naïve mast cells with anti-IgE; R = repeated anti-IgE stimulation for two weeks; R+αIgE = 6 hours stimulation with anti-IgE after repeated anti-IgE stimulation.

MODULATION OF MAST CELL FUNCTION REVEALED THROUGH PATHWAY ANALYSIS

To confirm that the genes upregulated after repeated FcεRI triggering were not found after single anti-IgE stimulation, we compared them to those genes upregulated after a single anti-IgE stimulus in the CAGE study (*22*) (Supplementary Fig 6A,B). Only a small proportion of these genes overlapped (less than 10%), confirming that the repeated stimulation through FcεRI leads to changes in mast cell function, characterized by upregulation of novel genes. To analyse whether upregulated expression of these novel genes after repeated FcεRI stimulation is related to global functional changes, we performed pathway enrichment analysis of these genes, using GO term biological pathways in DAVID (Online Repository Table E10) (*23*).

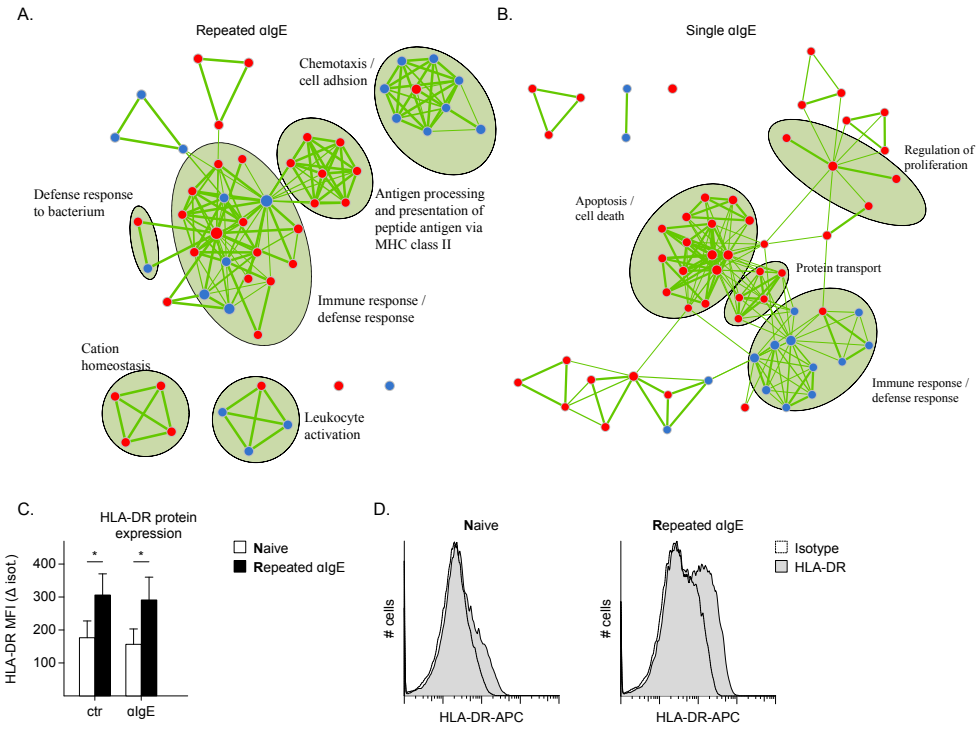

Figure 4. Overlap in genes and enriched pathways between repeated and single stimulation of mast cells. A, and B) Interaction network of GO terms enriched in genes upregulated after repeated anti-IgE (A) or single anti-IgE (B) stimulation. Node size reflects the significant/number of genes, and thickness of the lines indicates the connectivity between two GO terms. Pathways that are unique for either of the gene sets are highlighted in red. C, and D) Protein expression of HLA-DR, obtained using flow cytometry. Data is shown as delta MFI of HLA-DR compared to isotype control. Data are represented as mean ± SEM from n=5 (C) independent experiments. Asterisks indicate significant differences obtained using repeated measures ANOVA with Bonferroni posthoc test $p<0.05$ (C).

Although a substantial number of pathways overlapped, the majority (60-70%) of enriched pathways was uniquely enriched after repeated stimulation compared to single stimulation (Supplementary Fig 6 C,D). Biological pathways were then clustered using Cytoscape and DAVID, a clustering analysis based on the overlap of genes in closely related biological pathways. (Fig. 4A,B, Online Repository Table E10) (*24, 25*). The most prominent pathway clusters after repeated FcεRI triggering were "antigen processing and presentation", "response to bacteria", and "chemotaxis". To confirm the upregulation of key genes involved in antigen presentation, we analysed the expression of HLA class II genes by qPCR, confirming the sequencing results (Supplementary Fig 7). Furthermore, protein expression of HLA-DR, analysed using flow cytometry showed significantly increased cell surface expression of HLA class II (Fig 4C,D).

Since we observed significant pathways related to "response to bacteria" upon repeated triggering, we next sought out to confirm this enhanced response to bacteria in our in-vitro setup, using LPS to stimulate TLR-4 (Supplementary Fig 8A-C, Online Repository Table E11). A significant enhancement in upregulation of LPS-responsive genes was observed. Furthermore, the RNA expression and protein secretion of LPS-induced cytokines IL-8 and CCL4 were also significantly enhanced after repeated activation through FcεRI (Supplementary Fig 8D-I).

Together, these results show that mast cell function after repeated anti-IgE exposure is modulated, and enriched for a variety of pathways including antigen processing and presentation, response to bacteria, and chemotaxis.

MODULATION OF MAST CELL TRANSCRIPTOME RELATED TO CHANGES IN TRANSCRIPTION FACTORS RXRA AND BATF

To analyse whether autocrine signaling of factors released by mast cells themselves could contribute to the changes in gene expression, we studied whether supernatant from activated mast cells could induce similar gene expression profiles as direct stimulation. Downregulation of CSF2 and CCL4 and upregulation of EMR3 were only induced by direct stimulation (Fig 5A-C). In contrast, upregulation of TMEM45B, TREM2, and CCL18 were also induced by supernatant transfer suggesting a role for autocrine signaling in the upregulation of these genes (Fig 5D-F). qPCR data for CSF2, CCL4 and CCL18 were confirmed on the protein level by ELISA (data not shown).

These results suggest that the modulation of expression of some genes can be explained through autocrine signaling, although the expression of other genes is directly affected by repeated FcεRI-signaling.

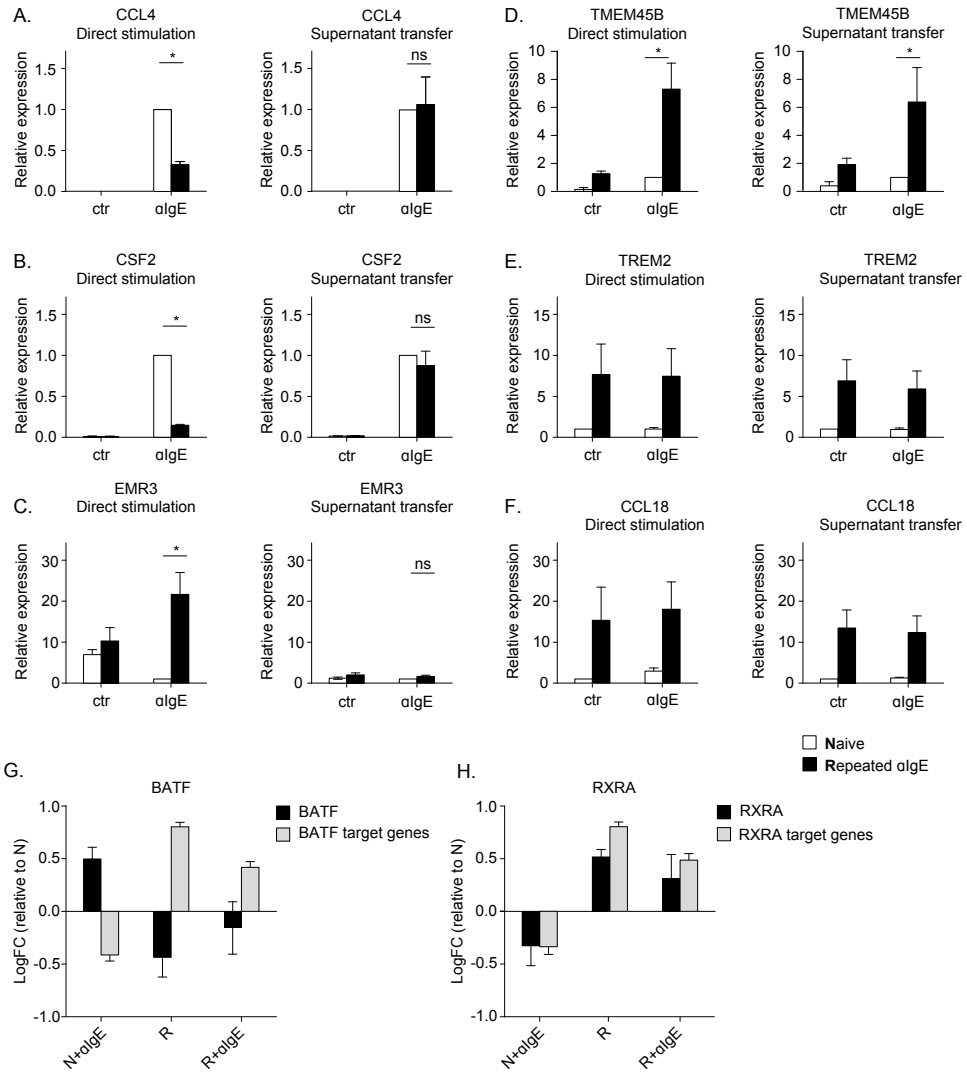

Figure 5. Autocrine signaling and transcription factors. A-F) Mast cells were stimulated for two weeks with 0.2 µg/mL anti-IgE (Repeated αIgE) or control medium (Naive) through direct stimulation (left) or using supernatant from activated mast cells obtained 24h after activation (right). Gene expression of CCL4, CSF2, EMR3, TMEM45B, TREM2, and CCL18, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to cells stimulated for 6h with anti-IgE (CCL4, CSF2, EMR3, TMEM45B) or to naïve mast cells (TREM2, CCL18). G-H) RNA expression analysis of transcription factors BATF (A) and RXRA (B) and their target genes as identified by Cscan enrichment analysis. Expression of these genes in mast cells exposed to repeated anti-IgE stimulation is shown as fold change relative to naive mast cells. Data in are represented as mean ± SEM from n=4 independent experiments (A-F) or from n=3 independent experiments (G-H). Asterisks indicate significant differences obtained using or repeated measures ANOVA with Bonferroni posthoc test $p<0.05$.

To analyse whether transcription factors are involved in the regulation of transcription after repeated activation of mast cells through FcεRI, we first analysed the presence of transcription factors in our differentially expressed genes using DAVID (Online Repository Table E12). We observed upregulated expression of BHLHE41, CEBPA, CEBPD, CEBPE, KLF2, MAFB, NFE2, RXRA, STAT4, and downregulated expression of BATF and NR4A1. In order to identify whether target genes of these transcription factors were significantly enriched in the differentially expressed genes after repeated FcεRI triggering, we used the analysis tools Cscan and Pscan to assesses which transcription factor binding sites are significantly overrepresented, providing thus hints on which transcription factors could be common regulators of these genes (Online Repository Table E13) (*26, 27*).

Significantly enrichment for binding sites or predicted binding affinity to CEBPA, RXRA, BATF was observed in at least two of the analysis tools used. Interestingly, one of the transcription factors that showed decreased expression after repeated FcεRI stimulation, BATF, is known to be a transcriptional repressor (*28*). Indeed, when we analysed the gene expression profiles of target genes for these transcription factors as derived from Cscan analysis, there was an inverse relationship between expression of BATF and its target genes (n=73), whereas there was a positive relationship between RXRA (transcriptional activator) with its target genes (n=60) (Figure 5G,H and Online Repository Table E14). Together, these two transcription factors were found to bind the regulatory regions of 101 of the 218 de novo upregulated genes which were analysed in Cscan. Because Pscan does not use experiment-derived data, and CEBPA was only present in the Pscan analysis tool, we could not study the expression of CEBPA target genes.

Together, these results indicate that changes in expression of transcription factors RXRA, BATF and possibly others, including CEBPA, may contribute to the modified RNA expression profile after repeated FcεRI stimulation.

NOVEL GENES UPON REPEATED FCεRI TRIGGERING ARE IMPLICATED IN CHRONIC ATOPIC TISSUES

As some of our most highly upregulated genes, such as CCL18, have been implicated in chronic atopic conditions (atopic nasal polyps, atopic asthma, and atopic dermatitis), we next analysed the overlap of our genes with genes upregulated in tissue of these chronic atopic conditions, identified using microarrays or RNA-seq (Online Repository Table E15). We found a striking overlap with genes associated with atopic allergies, in particular with tissue from nasal polyps from atopic rhinosinusitis patients (Fig 6A,B and Supplementary Fig 9A,B).

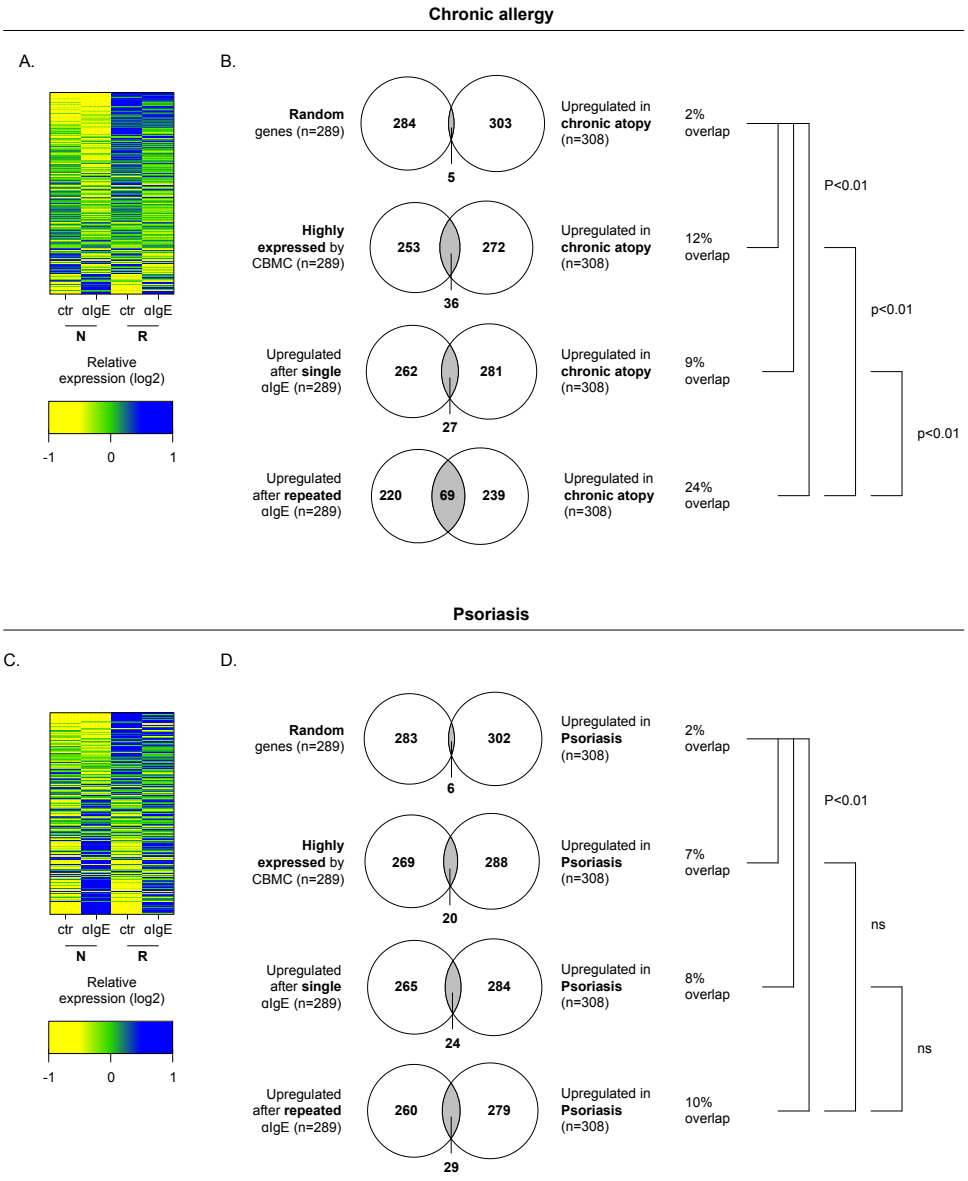

Figure 6. Association of upregulated genes with chronic allergy. A, C) Heatmap showing relative expression of genes upregulated in atopic allergy patients or psoriasis patients in mast cells exposed to repeated anti-IgE stimulation. B, D) Overlap of genes upregulated in atopic allergy patients or psoriasis patients with random genes (top), genes most highly expression by mast cells (2nd row), genes upregulated in mast cells after a single anti-IgE stimulus (3rd row), or genes upregulated in mast cells after repeated anti-IgE (bottom). Data are represented as mean ± SEM from n=3 (A and C) independent experiments. Overlap was calculated as percentage of the mast cell or random gene list. Asterisks indicate significant differences obtained using paired T-test $p<0.05$ (A and C), or Fisher's exact test $p<0.05$ (B and D).

Sixty-nine out of 308 genes upregulated in chronic allergy were significantly upregulated upon repeated stimulation of mast cells through FcεRI, representing 24% of the upregulated genes after repeated stimulation (Fig 6B), a percentage much higher than that with random genes (max 2%), the genes most highly expressed by mast cells (12%), or the genes upregulated with a single anti-IgE stimulus (9%). The overlap with two other atopic diseases was less pronounced, but for atopic dermatitis still more than obtained with random genes (Supplementary Fig 9C,D).

As control, the same analysis was performed with genes upregulated in psoriasis (Fig 6C,D). Whereas psoriasis genes were related to genes highly expressed by mast cells, possibly reflecting the increased number of mast cells in psoriasis tissue, the overlap did not increase when analyzing genes upregulated after repeated stimulation. These results suggest that chronic IgE-mediated exposure of mast cells may contribute to changes in tissue observed in chronic atopic conditions.

DISCUSSION

In this study, we aimed to characterize mast cell-intrinsic changes after repeated exposure to antigen, such as occurring in chronic allergy. We have used cultured human cord blood-derived mast cells which remain viable and stable in phenotype over several weeks, and showed that several changes in mast cells occur after repeated stimulation with anti-IgE. Obviously, in vitro stimulation is unlikely to capture all the changes that occur in vivo after repeated antigen exposure, as these are the result of interactions between a variety of cell types difficult to mimic in vitro. However, we hypothesized that in addition to those changes, cell-intrinsic changes as a consequence of repeated activation are likely to occur, and we were able to capture these changes using RNA sequencing.

To our knowledge, this is the first study to evaluate the effect of repeated FcεRI stimulation on human mast cells. Importantly, a high proportion of upregulated genes after repeated FcεRI triggering were related to changes in RNA expression in tissue of chronic allergic disorders, in particular chronic rhinosinusitis with nasal polyps. Our most highly upregulated gene CCL18 is highly linked to allergy, and was found to be expressed by mast cells in nasal polyps (*29*). Whereas some studies were able to show increased CCL18 mRNA expression by mast cells after a single anti-IgE stimulation, most studies failed to show secretion of CCL18 protein in response to acute anti-IgE stimulation (*30-32*). Our data indicate that robust expression and secretion of CCL18 in response to anti-IgE requires repeated or prolonged activation, and may explain how

expression of CCL18 in mast cells is induced in chronic allergic tissue. Besides recruitment of Th2 cells through upregulation of chemokines, several genes involved in T cell activation or antigen presentation to T cells were also upregulated in mast cells after repeated FcεRI triggering. Although the function of mast cells as antigen presenting cells is controversial (*33*), we and others have previously shown that human mast cells can function as antigen presenting cells through surface expression of HLA class II, and that tissue mast cells express HLA class II and costimulatory molecules (*34, 35*). Therefore, the upregulation of HLA class II observed after repeated FcεRI triggering suggests that repeated exposure to antigen may enhance this function of mast cells, thereby further contributing to T cell activation in chronic allergic tissue.

The GO-term "response to bacteria" was also highly upregulated after repeated stimulation of mast cells. Several studies have shown that combined stimulation of FcεRI and TLR can induce a synergistic cytokine response in mast cells and basophils, suggesting that this pathway could contribute to allergic exacerbations in the presence of pathogens (*36-39*). We now show that the response of mast cells to TLR-4 triggering is also enhanced after repeated stimulation through FcεRI, indicating that antigen exposure of mast cells can lead to long-term changes in mast cells associated with increased responsiveness to bacteria or LPS, thereby potentially contributing to allergic exacerbations.

These longterm changes in response mode of mast cells are likely regulated by transcription factors. Significant enrichment for target genes of CEBPA, RXRA, BATF were observed, suggesting that these may contribute to the modified RNA expression profile after repeated FcεRI stimulation. CEBPA is a well-known transcription factor involved in myeloid differentiation and is known to antagonize mast cell fate at the progenitor stage (*40, 41*). Likewise, retinoic acid, which can act through RXRA in mast cells, was shown to reduce mast cell differentiation during the progenitor stage, but did not affect mast cell lineage stability after full maturation (*42*). The function of these transcription factors has not been studied widely in mature mast cells. Interestingly, one study showed that overexpression of CEBPA in murine mast cells increased their response to bacterial stimulation (*43*), effects that we observed in mast cells after repeated FcεRI triggering as well. Our results therefore warrant further investigation of the transcriptional regulation of the function of mature mast cells in the context of chronic inflammation.

Whereas several immune-related pathways were upregulated, we observed an overall dampening of the FcεRI-triggered acute mast cell response after repeated triggering through FcεRI. Although mast cell degranulation was not affected, the expression of

many genes was reduced. Similar observations have also been made in the macrophage response to repeated LPS stimulation (*44*), where the gene-specific effects have been named tolerized genes (for genes that display a reduced expression) or non-tolerizable (for genes that display an unaltered expression).

Although we have not studied the mechanism of the dampened response observed in mast cells repeatedly exposed to FcεRI-triggering, it is interesting that several inhibitory receptors which can dampen FcεRI-induced mast cell activation were upregulated, including LAIR1, LILRB2, LILRB3, and VSTM1, providing a possible explanation for the dampened mast cell responses observed (*45, 46*). Interestingly, several inhibitory receptors are also upregulated in tissue from allergic patients (Online Repository Table E16), suggesting that dampening of inflammatory responses through upregulation of inhibitory receptors might represent an attempt to maintain tissue homeostasis (*47*).

In conclusion, our study provides a thorough analysis of mast cell-intrinsic changes after repeated FcεRI triggering. These changes were associated with an increase in release of chemotactic factors, including CCL18, increased antigen presentation to T cells, and increased response to bacterium, showing that the mast cell response modes are modulated after repeated antigen exposure. Importantly, these changes underlie several pathogenic mechanisms in chronic allergy, and were associated with changes in tissue of chronic allergic conditions. Therefore, our study may provide insight into the mechanisms of mast cell involvement in chronic allergic responses.

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SUPPLEMENTARY METHODS

CORD BLOOD-DERIVED MAST CELLS

Heparinized cord blood was obtained through the department of Obstetrics of the Leiden University Medical Center (Leiden, The Netherlands), and was performed in accordance with the Declaration of Helsinki. Mononuclear cells were isolated using a standard Ficoll procedure, after which $CD34^+$ hematopoietic stem cells were isolated with CD34 microbeads (Miltenyi Biotec). Isolated $CD34^+$ stem cells were differentiated into mast cells using serum-free medium (StemPro 34 + supplement, Gibco) with 5 ng/mL IL-3, 100 ng/mL IL-6 and 100 ng/mL Stem Cell Factor (SCF) at 50,000 cells/mL as described (48, 49). Half of the medium was replaced weekly with serum-free medium containing 100 ng/mL IL-6 and 100 ng/mL SCF. In addition, 50 ng/mL SCF was added once weekly. When cell number exceeded $1x10^6$/mL, additional medium was added instead of replacing the medium.

All recombinant cytokines were obtained from Peprotech. After six weeks, mast cells were incubated with the same medium containing 10% FCS for one week to induce maturation, and were maintained in this medium throughout the experiment. The purity of mast cells was determined by flow cytometric analysis of CD117 (c-kit), FcεRI, CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99% (Supplementary Fig 1A-D). Morphology of mast cells was analysed using cytospins stained with Toluidine blue.

RNA ISOLATION

RNA of four samples (donor 1) was isolated from pelleted Mast Cells cultures with a standard Trizol protocol, modified for samples with high proteoglycan content (according to manufacturers' instructions, Life Technologies). However, increased RNA yields were observed if RNA was isolated with the mirVana miRNA Isolation Kit (Ambion). As a result, RNA from all other donors were isolated with mirVana isolation kit.

cDNA SYNTHESIS AND QPCR

150 to 500ng RNA was used for cDNA synthesis with SuperScript® III Reverse Transcriptase (Life Technologies) according to manufacturer's standard instructions in a total volume of 20µl. cDNA samples were diluted 1:18 in distilled water.

The primer sequences, primer concentrations, and Melting Temperatures used for qPCR are shown in Online Repository Table E1. All qPCR reactions were performed with 4µl SensiFAST™ SYBR® No-ROX (Bioline), and 3µl diluted cDNA. qPCRs were performed in a Biorad CFX-384 with the following protocol: 2 min 95,0°C; 45 cycles of 5s 95,0°C, 10s Tm, 25s 72°C; followed by 10s 95,0°C; 7: Melt Curve 65,0°C to 95,0°C: Increment 0,5°C/5s. Relative expression was calculated with ddCq method normalized to housekeeping gene RPL5. Similar results were obtained when normalization was done using B2M as housekeeping gene.

RNA-SEQ

Four RNA samples from donor 1 were subjected for RNA sequencing with Illumina HiSeq 2000 at local facility LGTC (paired end, strand specific, 2x100bp). For the second and third donor, sixteen samples were sequenced with Illumina HiSeq 2000 at BGI, China (2x 90bp). Sequence libraries were prepared based on Illumina TruSeq protocol modified by the sequence center.

In brief, RNA samples were DNAse treated and mRNA was selected with oligo-dT beads. mRNA was fragmented by heat incubation. Standard 1st strand synthesis was performed with Superscript III (ThermoFisher, Life Technologies) and T nucleotides and samples were subjected to RNAseH treatment. Second strand synthesis with U nucleotides was performed and dsDNA fragments were End repaired, 3'phosphorylated and dA-tailed. Adapters were ligated and second strand was removed by USER digest. Libraries were pre-amplified and sequenced on Illumina Hiseq.

RNA-SEQ QUANTIFICATION

Analysis of RNASeq data was done using the in-house pipeline Gentrap (version 0.4.3; https://git.lumc.nl/rig-framework/gentrap). In particular, adapters were detected with FastQC (version 0.10.1) and removed by cutadapt (version 1.1) if the overlap is greater than 12 base pairs. Reads were trimmed with sickle (version 1.2) using the quality threshold of 33 and minimum length after trimming greater than or equal to 50. Only cleaned and trimmed reads were used for downstream analysis. GSNAP (version 2014-5-15) was used to align high quality reads to the human reference genome (version

GCA_000001405.15_GRCh38, without alternative loci) (50). Alignment quality metrics were collected using Picard (http://picard.sourceforge.net version 1.86).

A minimum of 18 million reads per sample were obtained, and at least 15 million reads remained after quality control (Online Repository Table E2). Greater than 94% of cleaned reads were mapped to the GRC38reference genome. Exon annotation was based on the RefSeq transcript annotation downloaded from UCSC genome browser. As expected for polyA$^+$ RNA, >70% of mapped reads aligned to annotated exons.

Sample to sample spearman correlation coefficient (r) ranged from 0.90 to 0.96 between independent experiments and from 0.97-0.99 between technical replicates (Supplementary Fig 2 A). We observed a good correlation of gene expression in unstimulated samples with the expression of genes in skin MC expanded in vitro or analysed directly ex vivo as present in public databases(22) (R^2 = 0.76 and 0.70 respectively; Supplementary Fig 2 B). Furthermore, genes differentially expressed in anti-IgE stimulated mast cells as compared to naïve mast cells largely overlapped with those found in the CAGE study with skin-derived mast cells and those previously reported to be expressed by peripheral blood-derived mast cells (Supplementary Fig 2 C) (51).

Gene expression (number of reads per gene) was counted by htseq-count (HTSeq version 0.6.1, stranded = no, mode = intersection-nonempty, minimum alignment quality = 10) using RefSeq annotation including non-protein coding regions (accessed at 2014-09-25) (52). Due to overlapping annotations in RefSeq, genes with no unique features were excluded. As a result, 25,109 genes were used for RPKM (Reads per Kilobase per Million) calculations using EdgeR.

DIFFERENTIAL EXPRESSION

Genes with RPKM >3 in at least one sample for each donor (9,955 genes) were used in differential expression analysis to reduce noise from genes with very low expression levels. To identify pairwise differentially expressed genes, we used the generalized linear model (GLM) implemented in the EdgeR package (version 3.4.2) (21). In particular, both the treatment conditions and donor pairing were included in the GLM design to account for all systematic sources of variation. The Cox-Reid profile-adjusted likelihood (CR) method was used to estimate gene-wise dispersion based on the give GLM design. After fitting to the GLM design, the GLM likelihood ratio test was performed to detect differentially expressed genes. The P-value for multiple testing was adjusted using the Benjamini-Hochberg correction with a false discovery rate (FDR) of 0.05.

GO TERM ENRICHMENT AND VISUALIZATION

To understand the biological functions of gene sets, we performed GO term enrichment analysis using DAVID.(23, 53) Enriched GO terms were then imported into the Cytoscape (version 3.10) plugin EnrichmentMap (version 2.0) (24, 25). Terms with FDR < 0.01 were visualized. The threshold for overlapping terms was set at 0.6-0.7.

TRANSCRIPTION FACTOR ANALYSIS

From the list of differentially expressed genes, transcription factors were identified by the GO term "transcription factor activity" using DAVID. In order to identify whether target genes of these transcription factors were significantly enriched in the differentially expressed genes after repeated FcεRI triggering, we used the analysis tools Cscan and Pscan, identifying binding sites of transcription factors (Cscan) or predicted binding affinity (Pscan) to promoter regions of the gene lists from mast cells (26, 27, 54, 55). Target genes of transcription factors for which binding has been shown in Chip-Seq experiments were generated using Cscan.

MEASUREMENTS OF PROTEIN EXPRESSION

For flow cytometry, cells were incubated with fluorochrome-conjugated antibodies for CD117, IgE, FcεRI, CD63, CD203c, and HLA-DR diluted in PBS 0,5% BSA at 4 ºC for 30 min. Intracellular staining for tryptase and chymase was performed as described (49). Flow cytometric acquisition was performed on FACS Calibur (BD) and Fortessa (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

Cytokines in culture supernatants were measured using Milliplex assays (Millipore) and ELISA for IL-8 (eBioscience), and CCL18 (R&D). Lipid mediators leukotriene C_4 (LTC_4) and Prostaglandin D_2 (PGD_2) were analysed by competitive ELISA (Neogen and Cayman Chemical respectively).

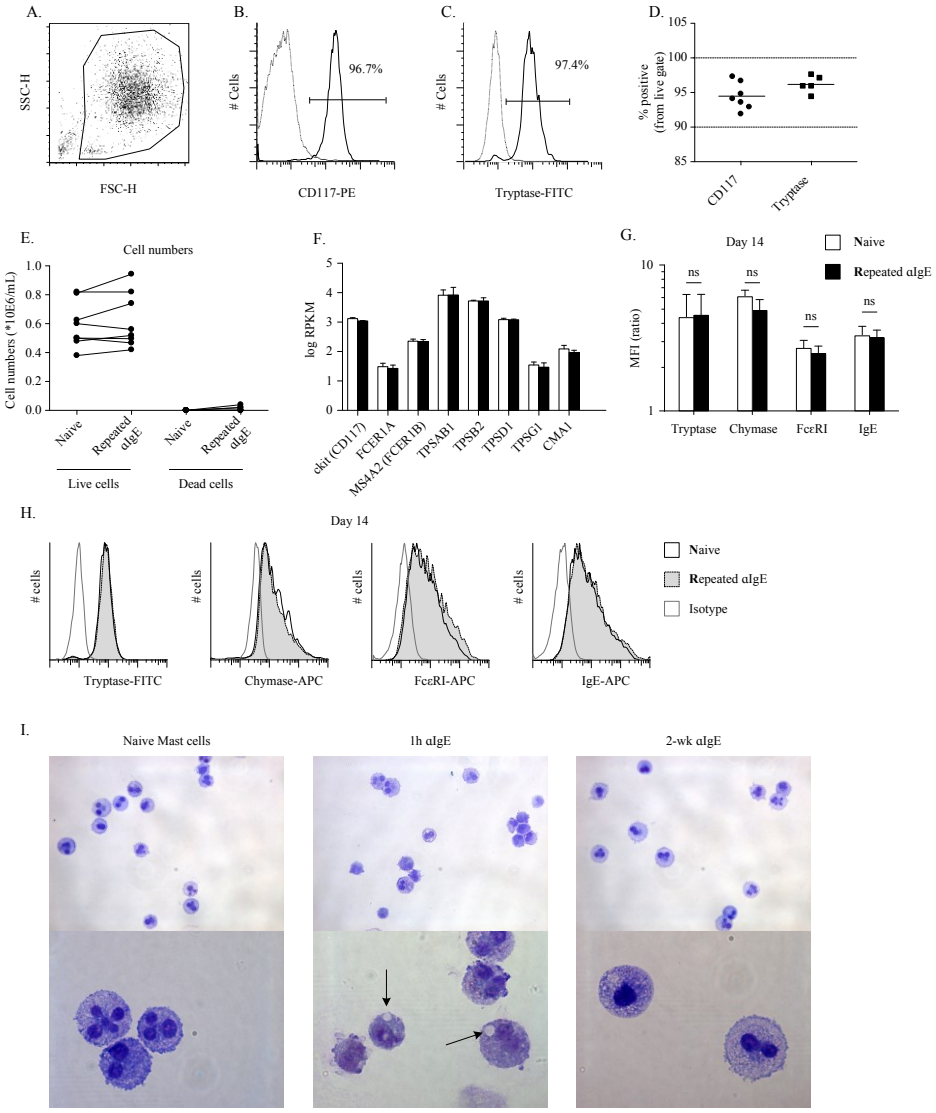
PUBLICLY AVAILABLE GENE EXPRESSION DATA

For comparison of gene expression in mast cells to other studies, public available RNA sequencing data was obtained from the FANTOM5 project deep cap analysis gene expression (CAGE) sequencing of skin-derived mast cells (22). Microarray data was obtained from a study of peripheral blood-derived mast cells (51).

For comparison to gene expression in atopic tissues, we searched Pubmed for microarray or RNA sequencing studies of tissue from patients with any chronic atopic or allergic disease (Online Repository Table E3) (56-62). Only those studies which examined whole tissue (as opposed to e,g, epithelial cells) were selected. Furthermore, the selection criteria

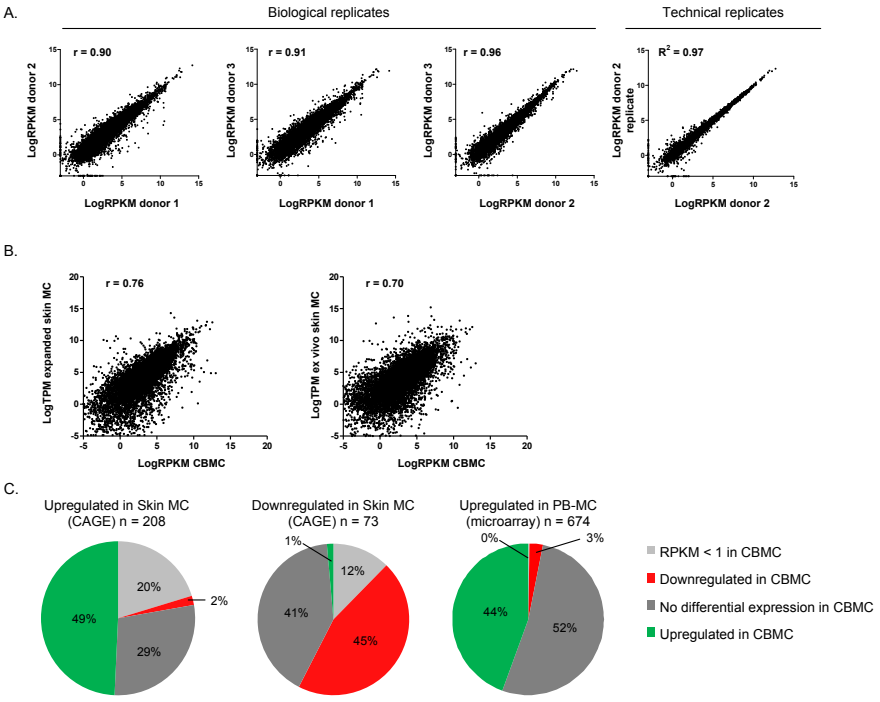
included the requirement for studies that had included patients with confirmed atopy or allergy. Expression data from psoriasis patients was used as control (63). A list of inhibitory receptors was based on known expression by mast cells and additional genes derived from the literature (Online Repository Table E16) (64).

SUPPLEMENTARY FIGURES



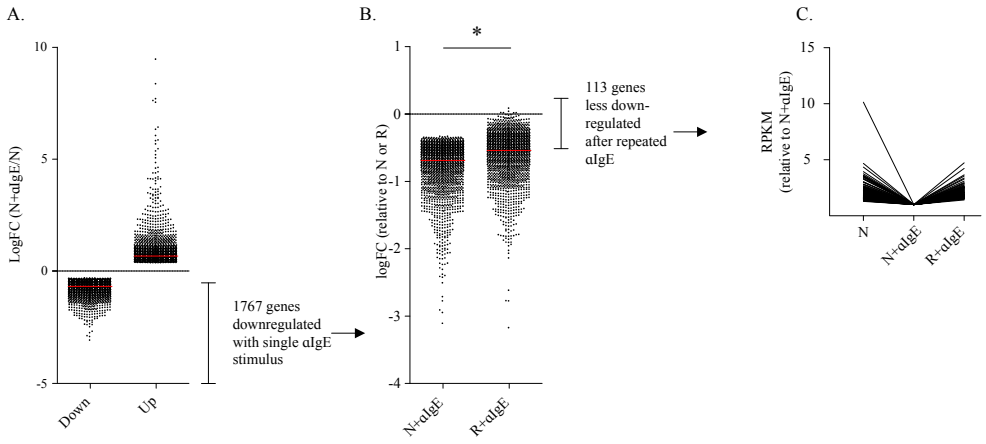
Supplementary Figure 1. Mast cell characteristics during culture. A-D) Gating strategy and purity of mast cells based on flow cytometry analysis of CD117 and tryptase.

A) Gating of live cells (excluding debris) based on forward scatter (FSC) and side scatter (SSC). B,C) Expression of CD117 and intracellular tryptase in live cells, gated as in A. Dotted line indicates isotype control. D) Summary of the percentage of CD117 and tryptase positive cells within the live gate in n=7 (CD117) or n=5 (tryptase) mast cell donors. E) Cell numbers after two-week culture of cord blood derived mast cells. Mast cells were cultured for two weeks with control medium (Naive) or anti-IgE (Repeated αIgE). The starting cell number was 1*10E6/mL medium. Numbers of live and dead cells at the end of two weeks stimulation were counted using Trypan Blue. Ns = no significant differences observed when comparing Naive mast cells versus anti-IgE-treated mast cells, analysed using paired samples T-test. N = 8 independent experiments with different mast cell donors. F) Transcripts for mast cell maturation markers after two weeks of FcεRI stimulation. Mast cells were cultured as described in E. Gene expression was obtained using RNA from 3 independent experiments performed with different mast cell donors. Results are shown as mean +/- SEM. RPKM = Reads Per Kilobase per Million. G,H) Intracellular tryptase and chymase expression and cell surface FcεRI expression and IgE binding at the end of the two weeks in naive mast cells (N) or mast cells stimulated for two weeks with anti-IgE (R). Representative flow cytometry plots (H) and a summary (G) are shown. MFI ratio is calculated as a ratio of the median fluorescence intensity (MFI) of staining divided by the MFI of the isotype control for each individual sample.I) Cytospins of mast cells were stained with Toluidine blue. Representative images of naive mast cells, mast cells stimulated for 1h with anti-IgE, and mast cells stimulated for 2 weeks with anti-IgE, Magnification: 20x (top) and 100x (bottom). Arrows indicate sites of degranulation.

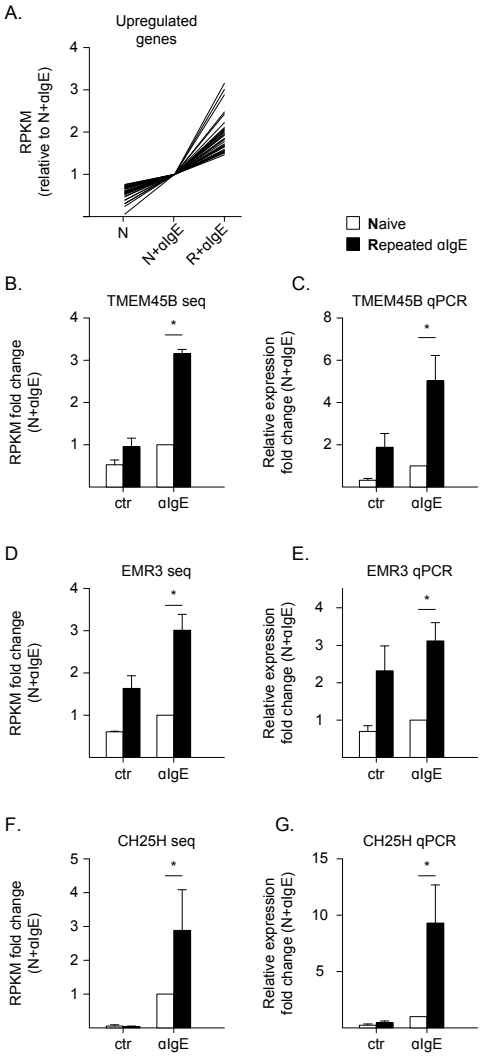


Supplementary Figure 2. Comparison of RNA sequencing results in our study with public data on mast cell transcriptome.

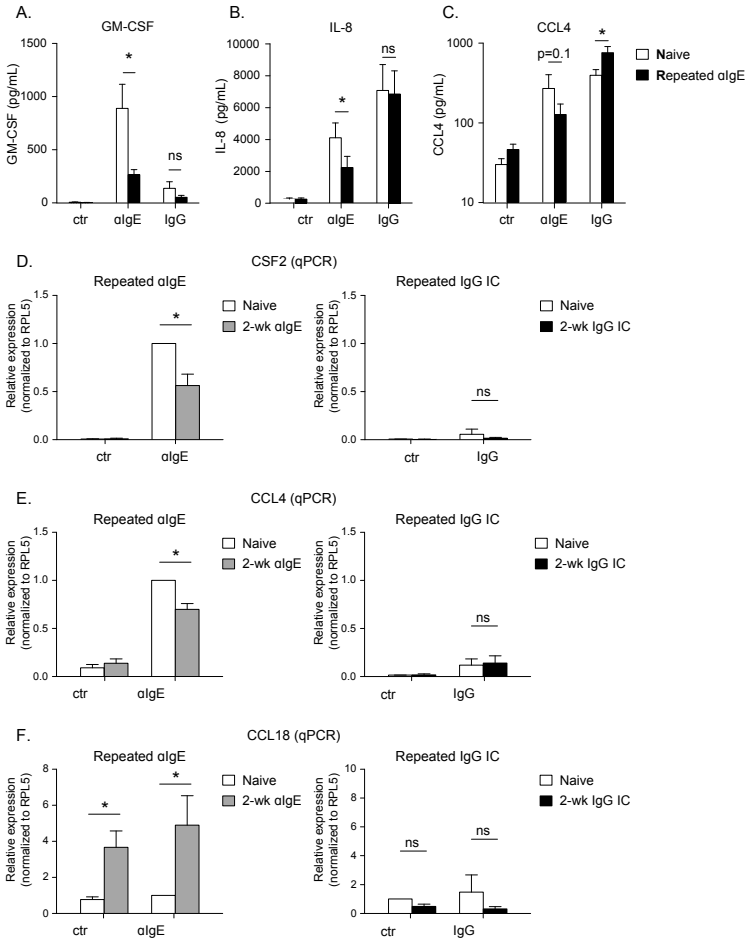
A) Spearman correlation of expression values (logRPKM) in biological or technical replicates. Each dot indicates an individual gene. B) Spearman correlation of expression values obtained in our study (CBMC) compared to skinMC expanded in vitro or analysed directly ex vivo, obtained through publicly available data from CAGE sequencing. Each dot indicates an individual gene. C) Overlap of differentially expressed genes upon single anti-IgE stimulation in our study, with publicly available data from CAGE sequencing (left, middle), or microarray (right). Upregulated genes in skin MC (left), downregulated genes in skin MC (middle), or upregulated genes in PB-MC (right). The number indicated above the piechart is the number of genes differentially expressed in those studies. CAGE: cap analysis gene expression; CBMC: cord blood-derived mast cells; MC: mast cells; PB-MC: peripheral blood-derived mast cells; RPKM: reads per kilobase per million; TPM: transcripts per million.



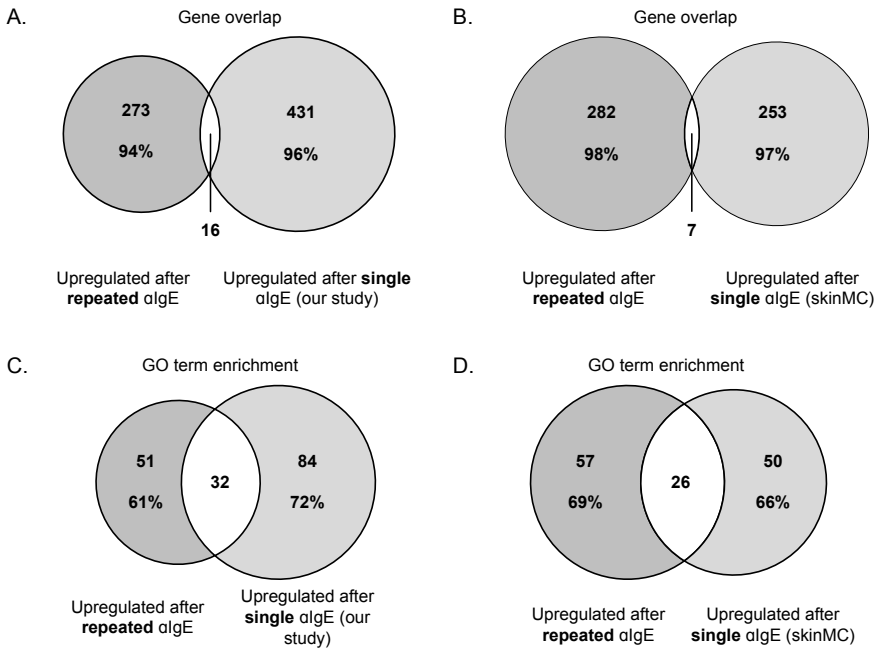
Supplementary Figure 3. Dampened downregulation after repeated stimulation. A) Gene expression obtained using RNA sequencing in response to a single 6 hour anti-IgE stimulation (N+αIgE), compared to control mast cells (N). Log fold change (LogFC) relative to Naive (N) mast cells of the differentially expressed genes is shown. B) Genes which were significantly downregulated after a single anti-IgE were selected as shown in A), where each dot indicates a different gene. 113 genes had significantly less downregulation after repeated stimulation. C) Genes which had significantly dampened downregulation after repeated anti-IgE, compared to single anti-IgE using differential expression analysis. Each line represents a single gene, and data is shown as relative RPKM normalized to single anti-IgE stimulation (N+αIgE). Asterisks indicate significant differences obtained using paired T-test $p<0.05$ (B).N = naive mast cells; N+αIgE = a single 6 hours stimulation of naïve mast cells with anti-IgE; R = repeated anti-IgE stimulation for two weeks; R+αIgE = 6 hours stimulation with anti-IgE after repeated anti-IgE stimulation.



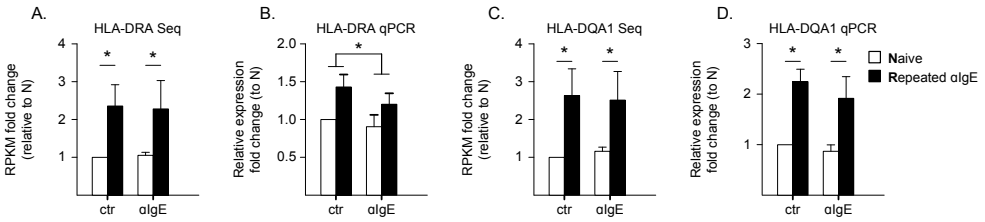
Supplementary Figure 4. Upregulation of FcεRI-responsive genes after repeated anti-IgE. A) Genes which are significantly upregulated after repeated anti-IgE, compared to single anti-IgE using differential expression analysis. Each line represents a single gene, and data is shown as relative RPKM normalized to single anti-IgE stimulation (N+αIgE). B, D, and F) Gene expression of TMEM45B, EMR3, and CH25H, obtained using RNA sequencing. Data is shown as RPKM fold change relative to single anti-IgE stimulation (N+αIgE). C, E, and G) Gene expression of TMEM45B, EMR3, and CH25H, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to single anti-IgE stimulation (N+αIgE). Data are represented as mean ± SEM from n=3 (B, D, and F) or n=5 (C, E, and G) independent experiments. Asterisks indicate significant differences obtained using differential expression analysis FDR<0.05 (B, D, and F) or repeated measures ANOVA with Bonferroni posthoc test p<0.05 (C, E, and G). N = naive mast cells; N+αIgE = a single 6 hours stimulation of naïve mast cells with anti-IgE; R = repeated anti-IgE stimulation for two weeks; R+αIgE = 6 hours stimulation with anti-IgE after repeated anti-IgE stimulation.



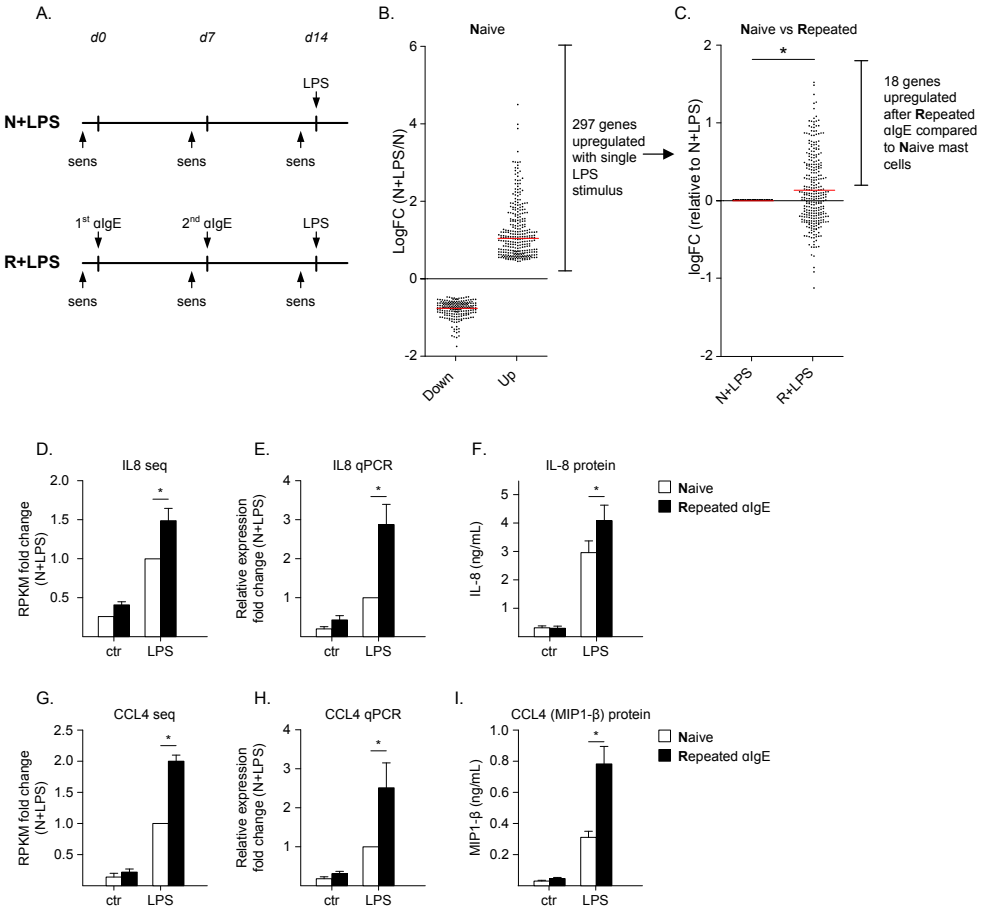
Supplementary Figure 5. Difference between stimulation with anti-IgE and IgG immune complexes. A-C) Mast cells were sensitized and stimulated with anti-IgE as described in the manuscript. Mast cells were re-stimulated at day 14 with 1 µg/mL anti-IgE or platebound IgG immune complexes. After 24 hours, mast cells supernatant was harvested, and cytokines were measured using ELISA (IL-8) or multiplex assays. Mean +/- SEM of pooled data from 3 independent experiments with different mast cell donors, each performed in duplicate. Asterisks indicate significant ($p<0.05$) differences between naïve mast cells and those treated for two weeks with anti-IgE, analysed using paired samples T-test. Ns = not significant. D-F) Mast cells were stimulated for 2 weeks with anti-IgE (left) or with IgG immune complexes (right). At day 14, mast cells were re-stimulated for 6 hours with anti-IgE or IgG immune complexes respectively, after which RNA expression was analysed by qPCR as described in the manuscript. Mean +/- SEM of pooled data from 2 independent experiments with different mast cell donors each performed in duplicate. Data is shown as relative expression normalized to household keeping gene RPL5 as a fold change to a single aIgE stimulation (N+aIgE). Asterisks indicate significant ($p<0.05$) differences between naïve mast cells and those treated for two weeks with anti-IgE, analysed using Two-way ANOVA. No significant differences (ns) were observed in expression of these genes after two-week stimulation with IgG immune complexes.



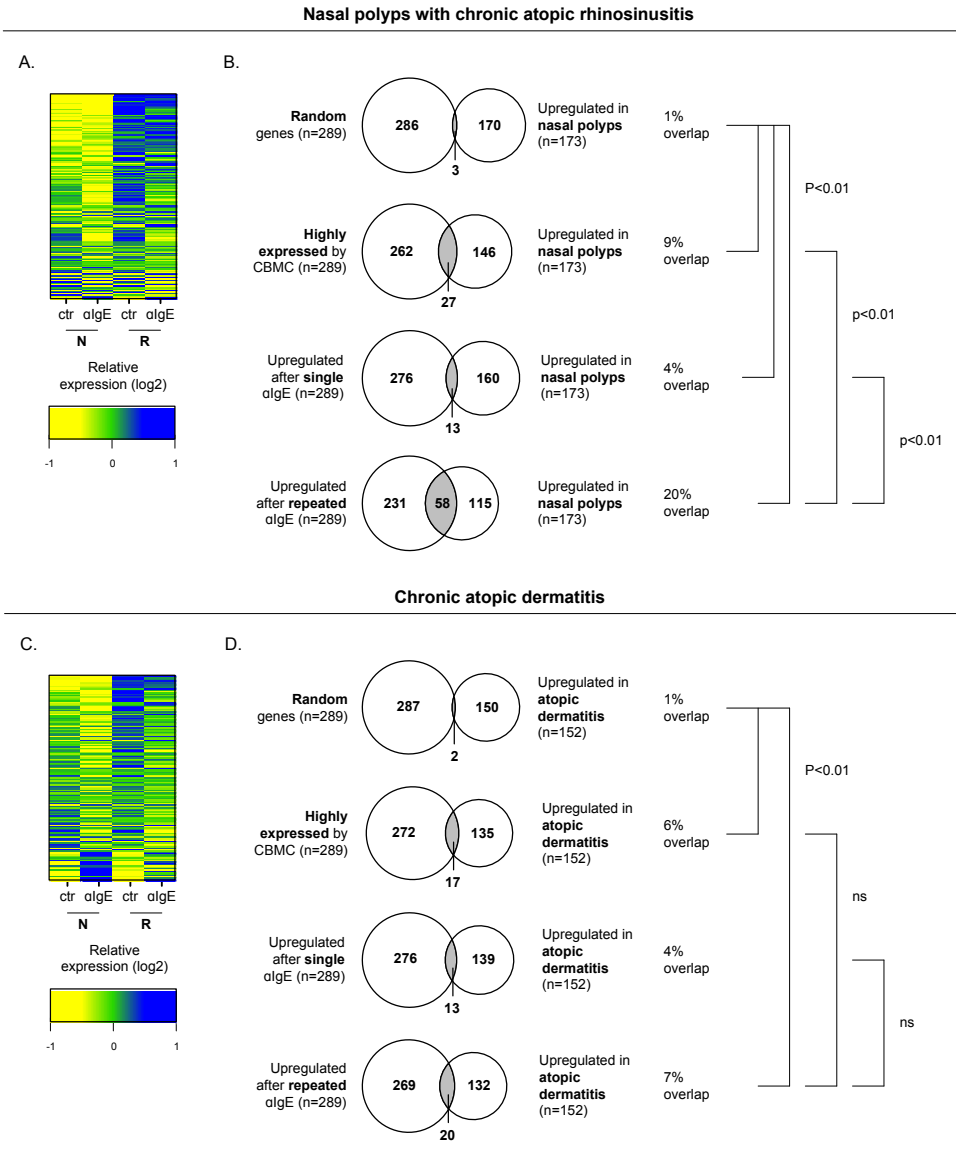
Supplementary Figure 6. Overlap in genes and enriched pathways between repeated and single stimulation of mast cells. A-B) Overlap of genes upregulated after repeated anti-IgE and genes upregulated after a single anti-IgE stimulation identified using differential expression analysis in this study (A) or in skin-derived mast cells from CAGE sequencing (B). C-D) Overlap of enriched GO terms after repeated anti-IgE and genes upregulated after a single anti-IgE stimulation identified using differential expression analysis in this study (C) or in skin-derived mast cells from CAGE sequencing (D)



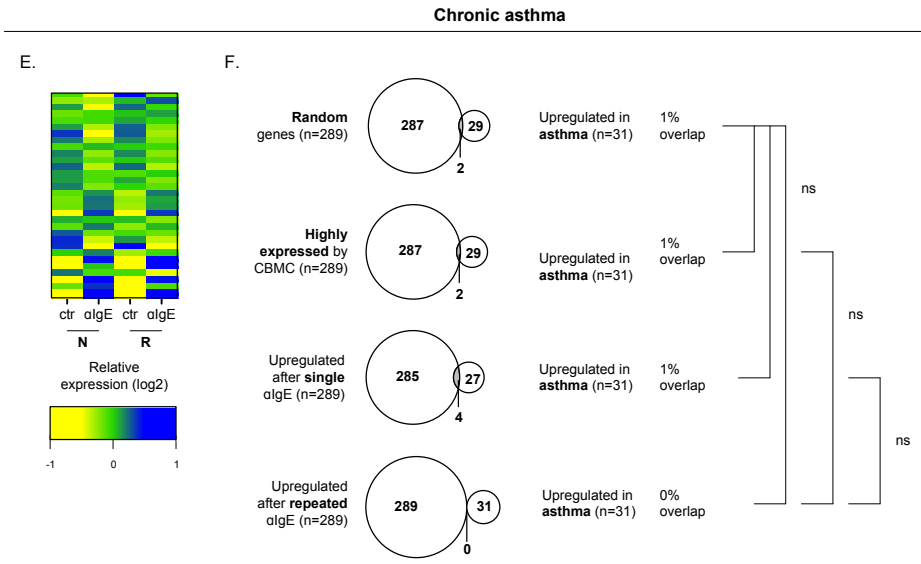
Supplementary Figure 7. HLA-class II RNA expression by qPCR. A, and C) Gene expression of HLA-DRA and HLA-DQA1, obtained using RNA sequencing. Data is shown as RPKM fold change relative to naïve mast cells (N). B, and D) Gene expression of HLA-DRA and HLA-DQA1, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to naïve mast cells (N). Data are represented as mean ± SEM from n=3 (A and C) or n=5 (B and D) independent experiments. Asterisks indicate significant differences obtained using differential expression analysis FDR<0.05 (A and C), or repeated measures ANOVA (B) with Bonferroni posthoc test p<0.05 (D).



Supplementary Figure 8. Response to LPS is enhanced after repeated activation. A) Experimental setup of repeated stimulation of mast cells. Human cord blood-derived mast cells were stimulated for two weeks with 0.2 µg/mL anti-IgE (Repeated αIgE) or control medium (Naive). At the end of the two weeks, mast cells were stimulated with LPS (N+LPS and R+LPS). B) Gene expression obtained using RNA sequencing in response to a single 6 hour LPS stimulation (N+LPS), compared to Naive mast cells (N). Log fold change (LogFC) relative to Naive (N) mast cells of the differentially expressed genes is shown. C) Genes which were significantly upregulated after LPS stimulation were selected as shown in B), where each dot indicates a different gene. Data are shown as fold change relative to N+LPS. D, and G) Gene expression of IL8 and CCL4, obtained using RNA sequencing, 6 hours after stimulation with LPS. Data is shown as RPKM fold change relative to LPS stimulation in naive mast cells (N+LPS). E, and H) Gene expression of IL8 and CCL4, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change LPS stimulation in naïve mast cells (N+LPS). F, and I) Protein levels of IL-8 and CCL4 (MIP-1β) in supernatant, 24 hours after stimulation with LPS of naive mast cells or mast cells which were exposed to anti-IgE for two weeks. Data are represented as mean ± SEM from n=3 (D, and G) or n=5 (E, F, H, and I) independent experiments. Asterisks indicate significant differences obtained using differential expression analysis FDR<0.05 (D and G) or repeated measures ANOVA with Bonferroni posthoc test p<0.05 (E, F, H, and I).



Supplementary Figure 9. Association of upregulated genes with chronic allergic diseases. A, C, E) Genes upregulated in tissue of atopic rhinosinusitis with nasal polyps (A), atopic dermatitis (C) or atopic asthma (E) patients were selected from public databases. Relative expression of these genes in mast cells exposed to repeated anti-IgE stimulation is shown as heatmap. B, D, F) Overlap of genes upregulated in tissue of atopic rhinosinusitis with nasal polyps (B), atopic dermatitis (D) or atopic asthma (F) patients with random genes (top), genes highly expressed by mast cells (second row), genes upregulated in mast cells after a single anti-IgE stimulus (third row), or genes upregulated in mast cells after repeated anti-IgE (bottom).



Supplementary Figure 9 *(cont.)*. Overlap was calculated as percentage of the mast cell or random gene list. P values indicated were derived from Fisher's exact test after correcting for multiple testing ($p<0.01$). The overlap with random genes was analysed 5 times with separate lists of random genes, and the highest overlap obtained in these five tests was used for comparison in this figure.

MAST CELLS IN RHEUMATIC DISEASE

Chapter 11

Jolien Suurmond,[1,†] Daniël van der Velden, [1,2,†]
Johan Kuiper,[2] Ilze Bot, [2] René EM Toes[1]

[1]Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; [2]Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden, The Netherlands

[†]These authors contributed equally to this work.

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ABSTRACT

Rheumatoid Arthritis is a chronic autoimmune disease with a complex disease pathogenesis leading to inflammation and destruction of synovial tissue in the joint. Several molecules lead to activation of immune pathways, including autoantibodies, Toll-Like Receptor ligands and cytokines. These pathways can cooperate to create the pro-inflammatory environment that results in tissue destruction. Each of these pathways can activate mast cells, inducing the release of a variety of inflammatory mediators, and in combination can markedly enhance mast cell responses.

Mast cell-derived cytokines, chemokines, and proteases have the potential to induce recruitment of other leukocytes able to evoke tissue remodeling or destruction. Likewise, mast cells can secrete a plethora of factors that can contribute to tissue remodeling and fibroblast activation. Although the functional role of mast cells in arthritis pathogenesis in mice is not yet elucidated, the increased numbers of mast cells and mast cell-specific mediators in synovial tissue of rheumatoid arthritis patients suggest that mast cell activation in rheumatoid arthritis may contribute to its pathogenesis.

PATHOGENIC PATHWAYS IN RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a systemic autoimmune disease characterized by chronic inflammation of the synovial lining of the joint, and is one of the most common autoimmune diseases affecting approximately 1% of the general population (*1*). Synovitis, inflammation of the synovial tissue, is mediated through leukocyte infiltration of the tissue, and leads to hyperplasia of fibroblast-like synoviocytes and tissue remodeling. Likewise, synovitis can induce cartilage destruction and bone erosion, ultimately leading to destruction of the joint. Clinically, synovitis induces pain and swelling of the involved joints, and the tissue destruction evoked can lead to disabilities if left untreated.

It is currently believed that different cells of the immune system play a role in the pathogenesis of rheumatoid arthritis. However, the exact cause of rheumatoid arthritis is not known. Genetic risk factors (such as HLA) underlying disease susceptibility are often involved in T- and B-cell responses and the presence of activated B cells and T cells in the inflamed synovium of rheumatoid arthritis patients indicate that adaptive immunity plays a prominent role. Furthermore, the presence of autoantibodies in the majority of patients points towards an important role for B cells in rheumatoid arthritis. However, besides the role of adaptive immune cells in initiation of autoreactive responses, innate immune cells are thought to play an important role during the effector phase by sustaining inflammation.

Treatment is usually aimed at lowering disease activity via immunosuppression, which can be achieved in various ways including through the interference with B cell-mediated immunity, co-stimulatory pathways, and inhibition of proinflammatory cytokines, suggesting that these pathways play an important role in disease pathogenesis.

AUTOANTIBODIES

A major effector function thought to contribute to pathogenesis in rheumatoid arthritis is mediated by autoantibodies. The classical autoantibody system associated with rheumatoid arthritis is rheumatoid factor, which recognizes the Fc portion of IgG. However, rheumatoid factor is not specific for rheumatoid arthritis patients, as it is also produced in a number of other inflammatory conditions, therefore its role in disease pathogenesis is often questioned.

An important group of autoantibodies in rheumatoid arthritis targets modified proteins, with anti-citrullinated protein antibodies (ACPA) being the most well-characterized. These antibodies recognize a variety of proteins or peptides in which the amino acid arginine is modified into a citrulline through a posttranslational modification process mediated by Peptidyl Arginine Deiminase (PAD) enzymes. PAD enzymes are normally present inside cells and can be activated by high calcium levels when cells, such as neutrophils, undergo apoptosis, an event readily occurring during inflammation (*2*). PAD enzymes that are transported to the outside of cells can citrullinate the extracellular matrix and in doing so can create targets for ACPA. Citrullinated proteins can be found in a variety of inflamed tissues, including the synovial tissue of rheumatoid arthritis patients (*3, 4*). ACPA can recognize many citrullinated proteins such as vimentin, filaggrin, and fibrinogen. Because fibrinogen and vimentin are also present in the extracellular matrix of the synovium, these proteins are often considered as important target antigens for ACPA (*5*).

ACPA show a very high specificity for rheumatoid arthritis, and are present in the majority (~70%) of rheumatoid arthritis patients (*6, 7*). Since their discovery ACPA are mainly used as diagnostic marker. However, it is now becoming increasingly clear that ACPA might also play a functional role in the pathology of rheumatoid arthritis. Several observations underlie this notion. ACPA can be observed already years before the onset of symptoms, and rarely develop after onset of clinical manifestation of rheumatoid arthritis (*8, 9*). The latter indicates that it is not likely that ACPA are a consequence of the inflammation present in rheumatoid arthritis patients. ACPA+ and ACPA- patients differ considerably with respect to the underlying genetic and environmental risk factors, suggesting that rheumatoid arthritis consists of two different disease entities: ACPA+ and ACPA- rheumatoid arthritis (*10-13*). Furthermore,

ACPA+ and ACPA- rheumatoid arthritis patients have a different disease course with ACPA+ patients having a more progressive disease, characterized by increased radiological joint damage and worse disease activity scores (*9, 14*). These findings suggest that ACPA contribute to disease pathogenesis. When ACPA antibodies are adoptively transferred into mice with a low-level synovial inflammation caused by anti-collagen antibodies, ACPA (reactive with citrullinated fibrinogen or collagen II) could enhance arthritis, implicating their direct involvement in the inflammatory process (*15, 16*).

Other autoantibodies present in rheumatoid arthritis patients include antibodies directed against carbamylated proteins, or anti-Carbamylated Protein Antibodies (anti-CarP), another autoantibody directed towards modified proteins. Like ACPA, Anti-CarP are present before disease onset and associate with disease severity in (ACPA-negative) rheumatoid arthritis patients, and could potentially contribute to disease pathogenesis (*17*).

TOLL LIKE RECEPTOR LIGANDS

Toll like receptor (TLR) activation is another important pathway for immune activation in rheumatoid arthritis. Although TLR are particularly known for their role in protection against pathogens, through their recognition of pathogen associated molecular patterns, endogenous ligands have been reported to trigger these receptors as well. Such endogenous ligands are present in conditions of stress or tissue damage, and often are intracellular molecules that can be either passively or actively released upon cell death. As rheumatoid arthritis, like other inflammatory conditions, is related to tissue destruction, cell death and the associated presence of endogenous TLR ligands is a common feature in synovium of patients. Several examples have been described of damage associated endogenous TLR ligands present in synovium, including HMGB1, heat shock proteins, tenascin c, and fibronectin (*18-22*). These endogenous ligands are thought to contribute to the chronicity of inflammation, as they can activate TLRs, inducing an inflammatory response, further tissue and cellular damage, and thereby the sustained release of damage associated TLR ligands.

Next to damage-associated TLR ligands, cell death can also lead to release of PAD enzymes into the extracellular environment, leading to generation of citrullinated proteins, including fibrinogen. Citrullinated fibrinogen, one of the antigens recognized by ACPA, was shown to trigger TLR-4 (*23*). Therefore, chronic inflammation is often related to release or generation of TLR ligands, leading to a self-amplifying inflammatory loop (Fig. 1).

T HELPER CELLS

The strong genetic association of the HLA region with disease susceptibility suggests the involvement of T helper cells in the etiology of rheumatoid arthritis. The association to HLA-DR alleles is not completely understood, but is specifically related to the ACPA response and could therefore be attributed to the helper function of T cells by which they can drive autoantibody responses by B cells (*13*). However, T cells themselves may also exert pathogenic effects, for example through their production of cytokines. Initially, Th1 cells, producing IFNγ and TNFα were thought to drive the immune response in rheumatoid arthritis. Since discovery of a wide variety of T helper cell subsets, Th17 cells (producing IL-17) have been proposed as the most relevant subset of T cells in relation to arthritis, although their putative role in- or contribution to the pathogenesis of rheumatoid arthritis in humans is unclear (*24*).

CYTOKINES AND CHEMOKINES: INFLAMMATORY MEDIATORS

The importance of proinflammatory cytokines in the pathogenesis of rheumatoid arthritis is well established. The development of biologic agents that target various immune mediators has dramatically improved the patient prognosis in the past decades, and most of these biologicals target cytokines or cytokine receptors. Established and approved therapies for rheumatoid arthritis block cytokine responses to TNF and IL-6 (*25*). Cytokines are produced in response to immune cell activation, and can activate cells in an autocrine, paracrine or systemic manner, leading to gene transcription of other cytokines, MMPs and other proinflammatory molecules. Thereby they contribute to the self-amplifying loop of immune activation. The cytokines mentioned above have a variety of target cells and functions, thereby able to trigger tissue inflammation, cartilage destruction, bone erosion and angiogenesis.

ACTIVATION OF MAST CELLS IN RHEUMATOID ARTHRITIS

The mast cell is a potent immune cell from the myeloid lineage and is well-known for its granules containing inflammatory mediators which can be rapidly released upon activation. Mast cells reside at interfaces with the external environment, where they act as first line of defense against invading pathogens, such as parasites and bacteria. In addition, mast cells play an important role in allergic diseases (*26*). As there is overlap in the mechanisms involved in hypersensitivity in allergy and autoimmune diseases, a role for mast cells in autoimmune disease has long been postulated. Several clinical findings support an active role of mast cells in rheumatoid arthritis pathogenesis, and suggest that mast cells are activated in the synovium of rheumatoid arthritis patients.

MAST CELL HYPERPLASIA IN SYNOVIUM

It has been shown that increased numbers of mast cells are present in synovium of rheumatoid arthritis patients, with numbers up to 5% of the total cell number in synovium (*27, 28*). Increased mast cell numbers, or so-called mast cell hyperplasia, is a hallmark of multiple autoimmune diseases. Growth factors and cytokines in synovial tissue, such as stem cell factor, the critical growth factor for mast cell survival, as well as IL-3 and IL-4 are present in synovial tissue of rheumatoid arthritis patients. These mediators can induce proliferation of mast cells, whereas in addition, stem cell factor and TGFβ have been shown to induce recruitment of mast cells (*29*), suggesting that the accumulation of mast cells in synovium may be the consequence of an ongoing inflammatory response mediating mast cell expansion through increased recruitment and proliferation.

In addition to the accumulation of mast cells, it has been reported that the proportion of different mast cell subsets is changed in the synovium of rheumatoid arthritis patients. Two main subsets of mast cells exist based on the expression of proteases, divided in tryptase-only positive cells (MCT) and tryptase–chymase double-positive cells (MCTC). Whereas normal synovium mainly contains MCTC cells, early inflammation in rheumatoid arthritis is associated with a selective expansion of MCT, followed by increases of MCTC in established or chronic disease (*29-31*). These changes are often correlated with clinical characteristics; MCT numbers in early disease associate with inflammation, whereas the MCTC numbers in chronic disease associate with tissue remodeling features, which may underlie active involvement of both subsets in different pathological processes.

MAST CELL MEDIATORS IN SYNOVIAL TISSUE OR FLUID

Mast cells produce a range of mediators, through three major pathways of secretion. First of all, they are characterized by presence of intracellular granules, containing preformed mediators such as histamine, proteases, proteoglycans, and heparin, which are rapidly released upon degranulation. Certain activation pathways can induce the release of lipid-derived mediators, produced from arachidonic acid, such as leukotrienes and prostaglandins. Finally, mast cell activation induces gene transcription, leading to de novo synthesis of cytokines, chemokines and growth factors, which can be released within several hours of activation.

Although most of de novo-produced cytokines are not mast cell-specific, several preformed granule proteins are more or less specifically expressed by mast cells, including the mast cell specific proteases tryptase and chymase. Both histamine and

tryptase are elevated in synovial fluid of rheumatoid arthritis patients likely reflecting local mast cell activation (*28, 32-34*). Furthermore, mast cells have been reported to be the main IL-17-positive cells in the inflammatory joint of rheumatoid arthritis and spondyloarthropathy patients (*35*). As discussed below, several of these mediators can contribute significantly to inflammation in the joint.

MAST CELL ACTIVATION PATHWAYS IN RHEUMATOID ARTHRITIS

Mast cells are most well-known because of their role in IgE-mediated immune responses as they express the high affinity FcεRI, and therefore have originally mainly been considered for their role in allergic diseases. However, the importance of mast cells in IgE-independent responses has been appreciated in the last decades, and has led to increased understanding of mast cell function in a variety of immune responses, including autoimmune disease.

Mast cell activation by autoantibodies

Depending on their specific isotype, antibodies can exert immune activation by binding to cellular Fc receptors and activation of complement. Because various isotypes of ACPA (IgG, IgA, IgM) have been previously demonstrated, ACPA are, in principle, able to activate the immune system via both pathways (*36*). The potential of ACPA to activate complement has been shown in vitro. ACPA bound to immobilized antigen activated the complement system, via both the classical and alternative pathways (*37*). These pathways can activate mast cells, for examples through the cleavage product C5a. It has been shown in mice that C5aR activation of synovial mast cells is essential for the induction of arthritis (*38*). However, in humans, it is not clear whether this pathway contributes to autoantibody-mediated mast cell activation.

Besides indirect activation of immune cells via complement activation, autoantibodies can also directly activate cells upon crosslinking of Fc receptors, in particular Fcγ receptors (binding IgG), Fcε receptors (IgE), and Fcα receptors (IgA). As ACPA are mainly present as IgM and IgG isotypes, the binding of IgG-ACPA to Fcγ receptors is thought to play a major role in autoantibody-mediated pathogenesis. Activating Fc receptors are predominantly expressed by myeloid immune cells, including mast cells. In mice, certain mast cell subsets, including synovial mast cells, express the activating FcγRIIIa (*39-41*), the receptor involved in arthritis induced by anti-collagen autoantibodies (*42*). Human mast cells have been shown to express FcγRIIA, whereas there is some controversy regarding expression of FcγRI (*43-45*). We have recently shown that human cultured mast cells could be activated by ACPA immune complexes in a citrulline-dependent manner (*45*). This activation was mediated through crosslinking FcγRIIA. As this receptor was expressed by synovial mast cells from all

patients analysed, we propose that this receptor is a major player in autoantibody-mediated mast cell activation.

Mast cell activation by Toll like receptor ligands

Toll like receptors (TLRs) are expressed by a variety of immune cells, and are considered to act as sentinels of the immune system. As mast cells are thought to play an important role in protection against pathogens, their expression of TLRs has been studied in different cell subsets and species. Although some variation is present in expression of these receptors, mast cells generally express a wide variety of TLRs, and triggering of TLR by pathogen associated molecular patterns induces activation of mast cells (*46-49*). Importantly, mast cells also express those TLRs that are thought to mediate responses to endogenous ligands released in inflammatory conditions. The main receptors involved in such responses are TLR-2, TLR-4 and endosomal TLRs which sense nucleic acids (*20, 50*).

We have recently shown that human mast cells indeed respond to HSP70, an endogenous ligand for TLR-4, which is present in rheumatoid arthritis synovium (*45*). Another endogenous TLR ligand, the extra domain A of fibronectin, can induce joint inflammation in mice in a mast cell- and TLR4-dependent manner (*18*), suggesting that this pathway of mast cell activation can contribute to pathogenic responses in rheumatoid arthritis.

Mast cell activation by cytokines

As described above, several cytokines or growth factors are involved in survival and expansion of mast cells in synovium. In addition, cytokines can activate mast cells directly. Such cytokines include IL-3, IL-4, IL-5, and IL-33, each of which are increased in synovial tissue or fluid of rheumatoid arthritis patients. However, stimulation of mast cells with cytokines alone usually mediates mainly proliferation with only a low level of activation. Importantly, the cytokine environment can play an important role in priming of mast cell responses to other triggers (*51*). IL-33 has been shown to enhance arthritis in a mast cell-dependent manner (*52*), suggesting that activation or priming of mast cells by cytokines can significantly alter inflammatory responses in the joint.

Mast cell-T cell interactions

The interaction between mast cells and T helper cells has been explored in recent years. In both human and mouse, mast cells have been shown to present antigens to $CD4^+$ T cells, thereby enhancing T cell responses with the possibility of skewing specific T helper subsets as well (*53-55*). Besides antigen presentation, mast cell-derived cytokines can also induce T cell activation (*56*).

Although we have recently shown that the interaction between T helper cells and mast cells does not only activate T cells, but can also change mast cell phenotype, the exact influence of T cells on mast cell function has been studied sparsely (*57*). Whereas regulatory T cells can inhibit mast cell activation, the effect of T cells involved in rheumatoid arthritis, such as Th17 cells, is not known (*58, 59*). However, these cell types are likely to interact and it is tempting to speculate that such an interaction contributes to pathogenesis of rheumatoid arthritis. A recent study indeed suggested that mast cells can regulate T cell responses in an arthritis mouse model, by inducing $CD4^+$ T cell expansion and Th1 and Th17 cytokine secretion (*60*).

CHRONIC INFLAMMATION MEDIATED BY A COMPLEX INTERPLAY OF MULTIPLE PATHWAYS

As rheumatoid arthritis is characterized by the activation of multiple immune pathways, these pathways are likely to interact. For example, it has been shown for different types of myeloid cells that activation through TLRs synergizes with triggering of Fc receptors (*45, 61, 62*). As mast cells can be activated by different cytokines, several studies have investigated the interaction between cytokine- and FcεRI-mediated activation. These studies have shown increased degranulation and cytokine production when mast cells are exposed to combined triggers of e.g. IL-3, IL-4 and IL-33 with FcεRI crosslinking (*63-66*). Whereas these studies are important for understanding of the role of cytokines in allergic responses, Fcγ receptors, as compared to FcεRI, are probably more important for mast cell activation in rheumatoid arthritis.

In this context, IL-33 was shown to enhance immune complex mediated mast cell responses through Fc γ receptors (*67*). In addition, we have studied the interaction of Toll Like receptor triggering on Fcγ receptor mediated mast cell activation, and shown that this greatly enhanced cytokine production by human mast cells (*45*). Importantly, we also showed this interaction was present in an antigen-specific system using ACPA autoantibodies and endogenous TLR ligands present in synovium.

Such a synergy between TLR or cytokines and Fc receptor responsiveness likely represents a physiological function of the immune system to mount an enhanced response when antibodies are produced after the first encounter of a pathogen (*68*). Whereas this is conceivably highly beneficial when a pathogen needs to be eliminated, such responses in an autoimmune setting can drive chronic inflammation, because it can lead to further release of modified self-antigens and TLR ligands (Fig. 1). Therefore, synergy in mast cell responses may contribute to chronicity of rheumatoid arthritis.

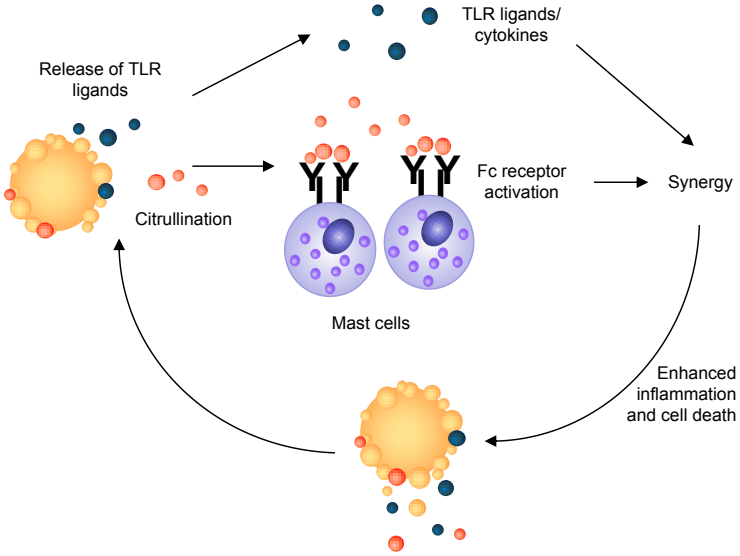

Figure 1. Chronic inflammation in rheumatoid arthritis is amplified by mast cells. Damage associated Toll Like Receptor (TLR) ligands, cytokines and citrullinated proteins are all implicated in rheumatoid arthritis pathogenesis and are released upon inflammation, in particular in association with cell death. Both have been shown to activate mast cells: citrullinated proteins can form immune complexes with ACPA autoantibodies, and activate mast cells through Fcγ receptors; endogenous ligands can activate mast cells through TLRs; various cytokines can activate mast cells. In the environment of the inflamed joint, all of these triggers are present at the same time, and together lead to synergy in mast cell activation. This synergy leads to enhanced tissue inflammation, in particular neutrophil influx, leading to cell death in the tissue. This cell death can lead to an amplification loop by generating more endogenous TLR ligands and citrullinated proteins.

MAST CELL EFFECTOR FUNCTIONS IN RHEUMATOID ARTHRITIS

Mast cells are well-known for their potent and quick effector functions, such as present during allergic reactions. However, as tissue-resident cells, their physiological role is thought to be protection against pathogens, as well as to contribute to wound healing (*69*). Therefore, it is not surprising that they also contribute to these processes during autoimmune responses.

MAST CELL-MEDIATED TISSUE INFLAMMATION

During certain bacterial infections, mast cells can orchestrate a local inflammatory response by rapidly increasing vascular permeability and releasing chemokines. Thereby they contribute to the recruitment of neutrophils and other immune cells, ultimately resulting in amplification of the local inflammatory response (*70*). Rheumatoid arthritis is

also characterized by accumulation of immune cells. Whereas the synovial lining mainly contains monocytes/macrophages and T cells, synovial fluid is the site to which neutrophils are recruited. In humans, it has been shown that neutrophil chemoattraction to the synovial fluid is mainly mediated by IL-8, a cytokine produced (although not exclusively) by mast cells in response to ACPA autoantibodies and TLR ligands (*45, 71, 72*). In mice, mast cell-derived TNF and leukotriene B4 can both mediate neutrophil recruitment as well (*73-76*). In addition, histamine can increase vascular permeability, thereby augmenting neutrophil recruitment (Fig. 2A) (*77*). These and other mast cell-derived chemokines can also induce recruitment of T cells and monocytes, although evidence indicating that this also occurs in the context of autoimmunity is scarce. Growth factors for neutrophils and macrophages, such as GM-CSF and G-CSF are also produced by mast cells, suggesting that besides inducing cellular infiltration, mast cells may also contribute to survival of these cell subsets.

CROSSTALK BETWEEN SYNOVIAL FIBROBLASTS AND MAST CELLS

An important consequence of the chronic tissue inflammation present in rheumatoid arthritis is activation of synovial fibroblasts, also called fibroblast-like synoviocytes, the main stromal cell type of the synovium. Activation of synoviocytes in rheumatoid arthritis leads to their proliferation and reduced apoptosis, secretion of cytokines and chemokines and invasiveness, whereby synoviocytes invade the underlying cartilage/collagen tissue (*78*) Synovial fibroblasts can be activated by multiple pathways, including TLR activation, and cytokines (*79*). Cytokines implicated in this process are TNFα, IL-1, and IL-17 (*80, 81*). Mast cells can produce each of these cytokines, thereby potentially contributing to activation of synovial fibroblasts (Fig. 2B). In addition, other mast cell mediators, such as histamine and tryptase have been shown to induce activation and inhibition of apoptosis in synovial fibroblasts cells as well (*82, 83*) Likewise, interaction between synovial fibroblasts can also lead to bi-directional crosstalk, whereby fibroblasts recruit and activate mast cells, for example through stem cell factor and IL-33 (*52*)

TISSUE REMODELING SUSTAINED BY MAST CELLS

Tissue inflammation and activation of fibroblasts goes hand-in-hand with various tissue remodeling processes, characterized by angiogenesis, breakdown of cartilage and bone erosion. Angiogenesis occurs mainly in the synovial lining of the joint, where rapid-growing fibroblasts and infiltrating immune cells require increased amounts of nutrients and oxygen supplied through the blood. Angiogenesis is mediated by growth factors such as VEGF and FGF, and angiogenic cytokines such as IL-8, TNF and GM-CSF, but can also be mediated by mast cell granule-derived mediators such as heparin, tryptase and chymase

(Fig. 2C) (*84-87*). Mast cells are often found in close proximity to blood vessels, and their numbers are often associated with angiogenesis, especially in the context of tumors and wound healing (*88, 89*). Although no functional data are available on the direct role of mast cells in synovial angiogenesis, their secretion profile suggests that they may contribute this process.

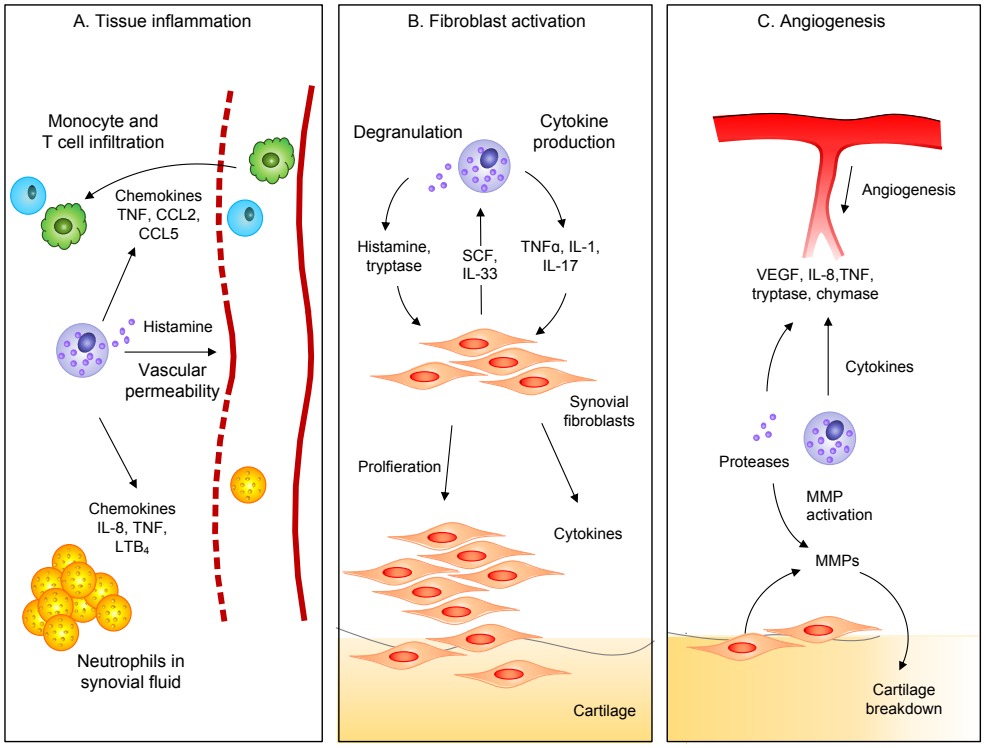

Figure 2. Mast cell contribution to pathogenic processes in rheumatoid arthritis. (A) Activated mast cells can amplify tissue inflammation through several mechanisms. They increase vascular permeability through release of histamine, leading to increased recruitment of immune cells. In particular, neutrophils are recruited into synovial fluid by chemokines such as IL-8, TNFα, and leukotrienes, whereas monocytes and T cells are recruited to the synovial tissue through chemokines such as TNF, CCL2, and CCL5. (B) Mast cells in synovium have a bidirectional interaction with fibroblasts, whereby fibroblasts can activate mast cells through growth factors and cytokines (SCF, IL-33), and activated mast cells in turn can activate synovial fibroblasts. Mast cell degranulation can induce proliferation of fibroblasts by histamine and tryptase, and cytokine production by mast cells (TNFα, IL-1, IL-17) can led to activation of synovial fibroblasts. Together, this crosstalk can induce fibroblasts invading into the underlying cartilage tissue. (C) Mast cell-derived cytokines and proteases can contribute to increased angiogenesis,a process required for the increased metabolic demand in inflamed tissue. Furthermore, various mast cell proteases can lead to extracellular MMP cleavage, leading to their activation, a crucial process in the breakdown of cartilage.

The two main destructive processes in rheumatoid arthritis are cartilage breakdown and bone erosion. Synovial fibroblasts, next to chondrocytes have been implicated in cartilage breakdown. Both cell types secrete matrix degrading enzymes such as matrix metalloproteinases (MMPs) (*90*). These enzymes can break down extracellular matrix proteins such as collagen, aggrecan and fibrinogen.

An important feature of MMPs is their secretion as inactive pro-enzymes which need to be cleaved by other MMPs or other proteinases to become activated (*91*). As this cleavage occurs in the extracellular space, the proteases required for cleavage can be derived from different cellular origins. In this respect, mast cell tryptase could play a prominent role as it is known for its ability to activate MMPs (*92, 93*). In doing so, mast cells can contribute to loss of cartilage through activation of MMPs via secretion of tryptase (Fig. 2C).

Osteoclast activation is the main mechanism leading to bone erosions. Although mast cells are not known to release RANKL, a major factor involved in osteoclast activation, mast cells may contribute to setting the balance in bone homeostasis. For example, patients with mastocytosis (systemic mast cell hyperplasia) exhibit features of accelerated bone turnover, possibly through a direct effect of histamine on osteoclasts (*94, 95*).

In summary, mast cells can secrete a variety of mediators which are implicated in many of the basic pathogenic hallmarks of rheumatoid arthritis.

MOUSE MODELS FOR ARTHRITIS AND MAST CELL INVOLVEMENT

ARTHRITIS MOUSE MODELS

Insight in the contribution of mast cells to pathogenesis of rheumatoid arthritis has also been obtained using models of experimental arthritis.

The first study to show an important role for mast cells in arthritis was performed in mice deficient in kit signaling, KitWKitW-v mice. In this study, experimental arthritis, induced by K/BxN serum transfer, was completely abolished in the absence of mast cells. Transfer of cultured bone marrow derived wild-type mast cells to mast cell deficient mice restored the incidence of arthritis after K/BxN serum transfer, indicating a direct effector function of mast cells in the development of arthritis (*96*). The critical role of mast cells for development of arthritis in this model has boosted the recognition of mast cells as a non-redundant cell in the development of autoimmune disease.

However, the findings from this study have been recently challenged in different models (Table 1). First of all, KitW-Sh/KitW-Sh mice, another mast cell deficient mouse due to defect kit signaling, were able to develop arthritis after passive transfer of anti-collagen type II antibodies (*97*). In addition, KitWKitW-v mice had normal arthritis development in the collagen induced arthritis model (*98*). Unlike neutropenic KitWKitW-v mice, KitW−sh/W−sh mice have a baseline pro-inflammatory phenotype, including neutrophilia (*99, 100*). Therefore, these confounding results have sometimes been attributed to the neutrophilia in KitW−sh/W−sh mice, which renders them insensitive to mast cell-mediated neutrophil recruitment, a critical event in early arthritis development (*101*).

Of the mast cell deficient mouse models independent of kit, two models have been used to study arthritis. In one study, the Cpa3Cre/+ mice, which are mast cell deficient, were fully susceptible to the induction of serum-induced arthritis and clinical scores, histology and gene expression analysis were comparable to wild-type mice (*102*). Therefore, it was concluded that the role of mast cells in arthritis is limited. Whereas mast cell deficiency using Mcpt5-Cre iDTR mice did not affect serum-induced arthritis either, these mice experienced reduced arthritis upon immunization with collagen (*60*), suggesting that further research is needed to increase our understanding of these discrepancies.

Despite these contradictory findings using mice with a complete mast cell deficiency, additional evidence for mast cell-mediated pathogenesis in arthritis comes from studies using mice deficient in mast cell-specific proteases, such as chymase or tryptase. Mice deficient in mMCP4, the homologue of human chymase, develop less severe arthritis upon collagen induced arthritis (*103*). Mice which are deficient in either tryptase mMCP6 and/or -7, especially in combination with heparin-deficiency, display a reduced severity of adjuvant-induced arthritis and K/BxN induced arthritis (for mMCP6 deficiency) (*104, 105*). In addition, mast cell-specific (Mcpt5-Cre-mediated) deficiency in A20, a regulatory molecule, leads to increased mast cell activation, thereby exacerbating collagen induced arthritis (*106*).

As most of these mouse models contain a single deficiency in a mast cell-specific mediator, and are therefore not associated with any other defects such as the kit mutant mice, these studies provide compelling evidence for mast cell involvement in arthritis, despite the contrasting data obtained with mast cell deficient mouse models. Therefore, more research is needed to increase our understanding of the role of mast cells in rheumatoid arthritis.

Table 1. Overview of experimental arthritis in MC-deficient or MC protease-deficient mice.

Mouse strain	Deficiency	Arthritis model	Outcome	Refs
Mast cell deficiency				
$Kit^{W}Kit^{W-v}$ (W/W^{v})	SCF-receptor mutation	K/BxN	Mast cell deficient mice resistant to develop arthritis.	96, 109
		CIA	No effect of mast cell deficiency	98
Kit^{W-Sh}/Kit^{W-Sh}	SCF-receptor mutation	α-collagen type II antibody transfer	No effect of mast cell deficiency	97
Cpa3-Cre (cre-master)	Cre-mediated tocxicity	K/BxN	No effect of mast cell deficiency	102
Mast cell protease-deficiency				
mMCP4-/-	Chymase	CIA	Reduced arthritis	103
mMCP6-/-, mMCP7-/-, NDST-2-/-	Tryptase/ heparin complexes	K/BxN	Reduced arthritis	105
		mBSA/IL-1β	Reduced arthritis	104
Mast cell-conditional knockout				
Mcpt5Cre A20Fl/Fl	A20	CIA	Exacerbated arthritis	106
Pharmacological mast cell inhibition				
Cromolyn	(not mast cell specific)	CIA	Reduced arthritis	109

mBSA: methylated bovine serum albumin; CIA: collagen-induced arthritis; K/BxN: SCF: stem cell factor.

PHARMACOLOGICAL INHIBITION OF MAST CELLS

As several lines of evidence suggest a role for mast cells in rheumatoid arthritis, intervention with mast cell activation could potentially form novel therapies. The drug cromolyn is clinically used as a treatment for asthma patients. The exact mechanism of cromolyn in not completely understood, but it is described to prevent the release of mast cell specific mediators like histamine from rat peritoneal cells (*107*). Cromolyn is described as a mast cell stabilizing agent and is used frequently in mouse studies. The effect of cromolyn as a prolactive on CIA was investigated in DBA/1 mice. A lower clinical score and radiographic score were observed compared to non-treated mice, when cromolyn was administered when first symptoms of clinical arthritis became evident (*108*). In addition, it was shown that intra-articular treatment of cromolyn or salbutamol prevented angiogenesis, pannus formation and joint destruction in mice (*109*).

Recently however, the specificity of cromolyn and the sensitivity of different types of mast cells to cromolyn in mice is under debate (*110*). Also, the specificity of salbutamol can be questioned since it has also inhibits the secretion of pro-inflammatory cytokines by macrophages and T cells (*109*). Therefore, development of mast cell-specific therapeutics is needed to establish the exact role of mast cells in rheumatoid arthritis.

CONCLUSIONS

Rheumatoid arthritis is a complex autoimmune disease caused by environmental and genetic interactions leading to a chronic activation of many (immune) cells in the synovial tissue. The pathology of rheumatoid arthritis involves multiple activation pathways and interactions between a variety of cell types with arthritogenic functions leading to the progression of joint destruction.

Mast cells can also be found in rheumatoid arthritis tissue, which indicates a possible role for this potent cell in the disease pathology. Many in vivo arthritis studies in mice have aimed to clarify the precise role of mast cells. However, since mouse models do not fully reflect the disease process and as some models for mast cell deficiency have additional non-mast cell defects, it is difficult to assess the specific role of mast cells on disease pathogenesis in vivo.

Nevertheless, mast cells have the capacity to respond to a wide range of activating ligands in synovium and their effector functions likely reflect their potential role in pathogenesis of rheumatoid arthritis.

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AUTOANTIBODIES IN SYSTEMIC AUTOIMMUNE DISEASES: SPECIFICITY AND PATHOGENICITY

Chapter 12

Jolien Suurmond, Betty Diamond

Center for Autoimmune and Musculoskeletal Diseases,
Feinstein Institute for Medical Research, Manhasset, NY, USA

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ABSTRACT

In this Review we focus on the initiation of autoantibody production and autoantibody pathogenicity, with a special emphasis on the targeted antigens. Release of intracellular antigens due to excessive cell death or to ineffective clearance of apoptotic debris, modification of self-antigens during inflammatory responses, and molecular mimicry contribute to the initiation of autoantibody production.

We hypothesize that those autoreactive B cells that survive and produce pathogenic autoantibodies have specificity for self-antigens that are TLR ligands. Such B cells experience both B cell receptor (BCR) activation and TLR engagement, leading to an escape from tolerance. Moreover, the autoantibodies they produce form immune complexes that can activate myeloid cells and thereby establish the proinflammatory milieu that further negates tolerance mechanisms of both B and T cells.

INTRODUCTION

The first autoantibodies were discovered in the late 1940s, when both antinuclear antibodies (ANAs) and rheumatoid factors (RFs) were described as serum factors that could bind nuclear antigens and immunoglobulins, respectively (*1, 2*). ANA and RF were recognized as diagnostic features of systemic lupus erythematosus (SLE) or RA, respectively, and as contributors to disease pathogenesis. It is now becoming increasingly clear that autoantibodies play a pivotal role in the pathogenesis of many diseases and that autoantibodies mediate both systemic inflammation and tissue injury (*3*).

Here we focus mainly on the autoantibodies associated with the autoimmune diseases SLE and RA, and we discuss the recent developments on the generation of autoreactivity and the contribution of these antibodies to disease pathogenesis. We propose a model in which autoreactive B cells escape from tolerance because they bind TLR ligands and undergo clonal expansion because of continuous exposure to antigen. Furthermore, the generation of immune complexes containing TLR ligands leads to systemic inflammation through the activation of innate immune cells. This systemic inflammation is part of the disease process and further impairs tolerance mechanisms. While we cite data from studies of SLE and RA to support this paradigm, we believe the model has broader applications.

CENTRAL VERSUS PERIPHERAL TOLERANCE DEFECTS

During B cell development in the BM, autoreactivity is prevented through receptor editing, apoptosis, and induction of anergy in B cells expressing an autoreactive B cell

receptor (BCR). When immature B cells express surface IgM, recognition of a self-antigen in the BM can induce these processes. Transitional B cells emerging from the BM continue to mature in the spleen, where additional tolerance mechanisms are in place. The exact mechanisms in this peripheral compartment are not fully understood, but they require ligand recognition by the BCR, similar to the tolerance checkpoints in the BM (*4*).

After this stage, mature naive B cells can be activated upon antigen recognition, allowing them to enter the germinal center (GC). The GC is a site of rapid clonal expansion of B cells, affinity maturation, class switching, and differentiation to memory B cells or plasma cells. A unique feature of GC-matured B cells is that they have undergone extensive somatic mutation of the antibody genes. As somatic hypermutation can give rise to de novo autoreactivity as well as enhance affinity of existing autoreactive B cells, additional tolerance checkpoints in the post-GC compartment have been suggested to effectively prevent autoreactivity in memory B cells and plasma cells, through either apoptosis or receptor editing (*5, 6*).

The ability to sequence and clone Ig genes from individual B cells has opened the opportunity to study these tolerance checkpoints in health and disease. The results from such studies using B cells from healthy individuals suggest that a high number of autoreactive B cells is generated in the BM and that sequential tolerance checkpoints lead to a gradual decrease in autoreactivity as the B cells mature (*7*).

The presence of autoreactive B cells in healthy individuals indicates that central tolerance is not sufficient to remove all autoreactive B cells with self-targeting BCRs. Indeed, it is well-established that both the B1 subset and marginal zone B subset contain high numbers of autoreactive B cells, and in animal models B cells from these subsets can give rise to pathogenic autoreactivity (*8-10*). Although defects in early B cell tolerance have been found in patients with autoimmune disease (*11, 12*), current data suggest that most of their autoreactive B cells are derived from non-autoreactive precursors. Autoantibodies derived from memory B cells in systemic autoimmune disease are often class-switched and highly somatically mutated, which suggests that they have been involved in GC reactions (*13-19*). Memory B cells making anti-DNA antibodies and anticitrullinated protein antibodies (ACPAs) in patients with autoimmune disease are thought to be derived from non-autoreactive precursors because back-mutation of their somatic mutations to germline sequences removes their autoreactivity (*17, 18*). Furthermore, although naive polyreactive B cells can recognize citrullinated proteins or DNA and therefore could give rise to ACPAs and anti-DNA–producing plasma cells, the affinity of ACPAs and anti-DNA antibodies from naive

B cells is greatly reduced compared with antibodies in patient serum, suggesting that these naive B cells have undergone affinity maturation before developing into plasma cells (*11, 18*). While sequence analyses of autoantibodies have thus far only been performed for a limited number of patients and access to plasma cells is limited by the unavailability of BM samples, there is consistent evidence that many autoreactive B cells in autoimmune disease mature in the GC.

Taken together, these data suggest that defects in central and early peripheral tolerance checkpoints are present in autoimmune disease patients, but the defects in early tolerance checkpoints do not necessarily lead to the generation of pathogenic autoantibodies. As studies point to an important role of somatic hypermutation during GC reactions as a source of autoreactivity, post-GC checkpoints are likely defective in patients with autoimmune disease, but our understanding of these checkpoints is limited.

T CELL HELP FOR AUTOANTIBODY PRODUCTION

Development of high-affinity antibodies requires cognate B cell/T cell interactions to initiate and sustain the GC. Somatically mutated autoantibodies in autoimmune disease are thought to be derived through T cell–dependent pathways, especially because the presence of autoantibodies is often correlated with the presence of specific HLA alleles (*20, 21*). However, at present little is known about the specificity of T helper cells that are able to stimulate autoantibody-producing B cells.

The most direct pathway for T cell helper function is for T cells to recognize the same antigen as the B cells. In RA, one of the genetic risk HLA alleles, HLA-DRB1*0401, has recently been associated with presentation of citrullinated peptides, allowing for activation of citrulline-specific T cells (*22*). These T cells may engage in direct antigen-specific interactions with ACPA-producing B cells when citrullinated antigens are internalized through BCR-dependent recognition. However, the T cell specificities involved in the activation of other autoreactive B cells, such as DNA-reactive B cells, are not known. Some studies suggest that the epitopes for B cells and T cells do not necessarily need to be the same. For example, B cells can internalize multimolecular complexes through BCR-mediated recognition and can then process peptides from different proteins present in the complex. This internalization of multimolecular complexes clearly enables processing of nucleoproteins capable of binding DNA in lupus-prone mice and SLE patients (*23-26*).

T cell help for autoantibody-producing B cells can also originate from T cell responses to foreign antigens through molecular mimicry between viral or bacterial antigens and

self-antigens (*27-30*). Activation of non-autoreactive T cells by foreign antigens can lead to activation of B cells that recognize the foreign antigen but also cross-react with a self-antigen (*31*). It is hypothesized that such activation can lead to selection and maturation of high-affinity self-reactive B cells.

In addition to the typical GC response, class switching and somatic hypermutation can also occur outside of the classical GCs. In several autoimmune diseases, ectopic lymphoid structures can develop, usually in the target organ, such as the synovial tissue in RA or the kidney in SLE. These structures closely resemble the GC with the presence of B/T lymphoid clusters that include T follicular helper cells and follicular dendritic cells. Ectopic lymphoid organs are often associated with the production of high levels of autoantibodies, suggesting that local activation of these cells can contribute to T cell–dependent B cell differentiation, as in the GC (*32, 33*).

TARGET ANTIGENS FOR AUTOANTIBODIES

Autoantibodies can be directed against a variety of molecules, such as nucleic acids, lipids, and proteins, and these target antigens can be located in the nucleus or cytoplasm, on the cell surface, or in the extracellular milieu. When analyzing autoantibodies in the most common systemic autoimmune diseases, it is striking that most of them target ubiquitously expressed intracellular molecules (Table 1).

Among these, ANAs, a group of heterogeneous antibodies targeting multiple distinct nuclear components, are the most common. The molecules recognized by ANAs are normally present in the nucleus and therefore are not accessible to antibodies or B cells. It is only in conditions of cell death that nuclear contents are released into the extracellular environment.

RA can be considered an exception, as the autoantibodies in RA do not generally target intracellular molecules. The classical example is RF, which recognizes the Fc portion of IgG, an abundant protein in serum. Another important group of autoantibodies in RA targets modified proteins, with ACPAs being the best characterized. ACPAs show a very high specificity in clinical diagnostics for RA and are present in the majority of patients with RA. These antibodies recognize a variety of proteins such as fibrinogen or fibrin, vimentin, type II collagen, α-enolase, and histones that have undergone a posttranslational modification in which citrulline is substituted for arginine (*34, 35*). Most of these antigenic citrullinated antigens are extracellular proteins, although intracellular citrullinated proteins have also been identified as targets of ACPAs. To date, it is unknown whether intracellular or extracellular citrullinated antigens are the main driver of the ACPA response.

Table 1. Autoantibody recognition in systemic autoimmune disease.

Antigen location	Antibody	Antigen	Disease	PRR
Nuclear	Anti-Ro (SS-a)	Ro-RNP	SLE, SS	TLR7
	Anti-La (SS-b)	La antigen	SLE, SS	TLR7
	Anti-Sm	Small nuclear RNP	SLE	TLR7
	Anti-dsDNA	dsDNA	SLE	TLR9
	Anti-histone	Histones	SLE	TLR2 and TLR4
	Anti-Scl-70	Topoisomerase I	SSc	
	Anti-centromere	Centromere	SSc	
Cytoplasmic/ mitochondrial	ANCA	Myeloperoxidase (p-ANCA) and proteinase 3 (c-ANCA)	Vasculitis, WG	
	ACA	Cardiolipin	APS, SLE	NLRP3
Modified proteins	ACPA	Citrullinated proteins	RA	TLR4
	Anti-Carp	Carbamylated proteins	RA	
Extracellular	RF	RF (IgG)	RA	
	Lupus anticoagulant	Phospholipids	APS	TLR4?
	α3 Chain of basement membrane collagen (type IV collagen)	α3 Chain of basement membrane collagen (type IV collagen)	GPS	

ACA: anti-cardiolipin antibodies; ACPA: anti-citrullinated protein antibodies; ANCA: anti-neutrophil cytoplasm antibodies; APS: anti-phopholipid syndrome; GPS: Goodpasture's syndrome; PRR: pattern recognition receptor; RA: rheumatoid arthritis; RF: rheumatoid factor; RNP: ribonucleoproteins; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; SSc: systemic sclerosis; TLR: toll-like receptor; WG: Wegener's granulomatosus.

B CELL PROPAGATION IN RESPONSE TO SELF-ANTIGEN OR FOREIGN ANTIGEN

The intracellular location of many autoantibody targets raises the question of how these antigens can drive clonal expansion, a process that requires the recognition of antigens through the BCR, which occurs in the extracellular space.

Two main hypotheses exist that can explain how autoantibodies arise that recognize intracellular self-antigens (Figure 1A). The first theory proposes that apoptosis plays an important role as a source of nuclear and cytoplasmic self-antigens. During this process of programmed cell death, apoptotic blebs appear on the cell surface and microparticles can be released from the cell, both of which contain several cytoplasmic and nuclear components. These include several of the intracellular antigens recognized by autoantibodies, such as histones, dsDNA, RNA, ribonucleoproteins (RNPs), and myeloperoxidase and proteinase 3 in neutrophils (*36-39*).

Furthermore, the process of apoptosis induces several modifications in the nucleus, including histone citrullination and acetylation, which can be recognized by autoantibodies in RA and SLE, respectively (*40, 41*). Apoptotic blebs are normally

quickly engulfed by phagocytes to prevent immune activation (*42*); however, a failure to do this, due either to a genetic defect in one of the apoptosis clearance pathways (as often seen in autoimmune disease) or to excessive release of these blebs due to inflammation, allows for the sustained presence of these self-antigens in the extracellular environment (*43*).

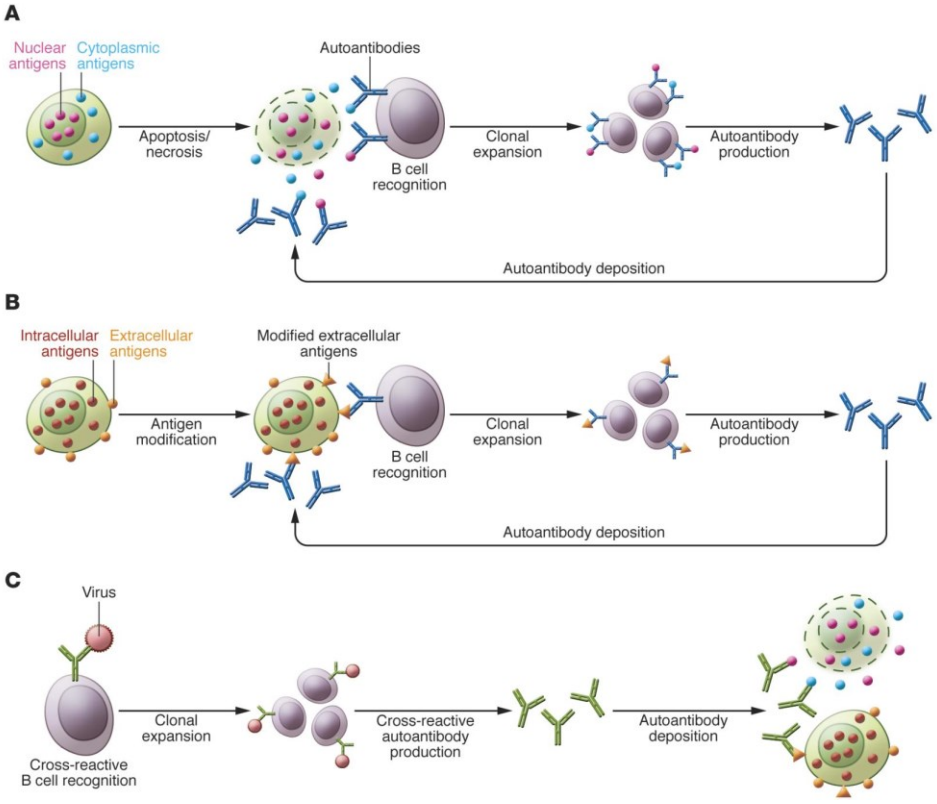

Figure 1. Mechanisms for autoantibody production: apoptosis, antigen modification, and cross-reactivity. Three models can explain the recognition of intracellular antigens by autoantibodies. (A) Cell death through apoptosis or necrosis leads to extracellular exposure of intracellular self-antigens through release of intracellular contents into the extracellular environment, formation of apoptosis blebs, or NETosis. If clearance mechanisms are insufficient, there may be recognition of these antigens by B cells and autoantibody production. (B) Modification of self-antigen generates neoantigens, to which B cells have not been tolerized. (C) Autoantibody production arises from responses to foreign antigens, which cross-react with self-antigens.

Another pathway for propagation of autoantibody responses toward intracellular antigens is through cross-reactivity with extracellular antigens. An example of such cross-reactivity is anti-dsDNA antibodies in SLE, which have been shown to recognize antigens in the glomerular basement membrane, where these autoantibodies can deposit (*44-47*). While the binding of some anti-dsDNA antibodies to glomeruli depends on the presence of nucleosomes containing DNA on the glomerular membrane, the contribution of cross-reactivity to renal pathology has also been demonstrated (*48, 49*).

In RA, ACPAs can target both intracellular and extracellular citrullinated antigens that arise during an inflammatory response (Figure 1B) (*34, 35, 50*). It has not been formally shown, but it is likely that the citrulline epitopes on different antigens form the basis for cross-reactivity between intra- and extracellular antigens. In cardiomyopathy, autoantibodies targeting cardiac myosin, an intracellular antigen, have been shown to cross-react with the β-adrenergic receptor, thereby inducing stimulation of cardiomyocytes. These findings suggest that cross-reactivity between intra- and extracellular antigens can drive clonal expansion.

Similarly, cross-reactivity between foreign antigens and self-antigens can drive the expansion of autoreactive B cells (Figure 1C). Several autoantibodies exhibit such cross-reactivity: dsDNA autoantibodies cross-react with pneumococcal bacteria and Epstein-Barr virus (*29, 45*) and anti-neutrophil cytoplasm antibodies (ANCAs) recognize the bacterial adhesin FimH (*28*). However, this cross-reactivity between foreign antigens and intracellular self-antigens still requires the release of intracellular antigens through apoptosis or cross-reactivity to surface antigens in order for autoantibodies to bind and exert their pathogenic activity. We speculate that B cells with BCRs that recognize only intracellular antigens will propagate less well than those that cross-react with more ubiquitous cell surface or extracellular antigens. Therefore, the basis for clonal expansion of many autoreactive B cells can be found in their cross-reactivity.

TLR RECOGNITION AND ITS ROLE IN ESCAPE FROM TOLERANCE

Many antigens recognized by autoantibodies are able to trigger pattern recognition receptor (PRRs), including TLRs and Nod-Like receptors (NLRs). These receptors are listed in Table 1 and include DNA, RNA, citrullinated fibrinogen, cardiac myosin, cardiolipin, and oxidated phospholipids (*51-56*).

B cells express several TLRs for nucleic acids, including TLR7 and TLR9. Engagement of these TLRs activates B cells. Notably, many antigens recognized by autoantibodies are ligands for B cell TLRs (Table 1), suggesting a role for TLR recognition in the breakdown

of tolerance against these antigens. Indeed, mice deficient in TLR7, TLR9, or the TLR-associated signaling adaptor MyD88 are partially protected against development of ANAs and lupus-like symptoms (*57-60*). This protection derives from the effects that TLR ligation has on reducing B cell tolerance.

Combined BCR and TLR ligation by nuclear antigens has been shown to induce a synergistic signaling response, mediated by colocalization of TLR9 and antigen internalized through BCR (*61-63*). Thus, self-antigens able to activate endosomal TLRs and BCRs will induce robust activation in B cells. Some studies suggest that TLR activation of immature B cells can dysregulate central tolerance pathways (*64-66*). We hypothesize that TLR ligation might also explain the failure of peripheral tolerance in autoimmune disease. Several studies show increased TLR expression and function in activated or memory B cells compared with immature B cells, allowing for robust activation through TLR in the GC (*67-70*). As tolerance in the GC is thought to be driven by a survival advantage through affinity-driven selection, combined BCR/TLR ligation in the GC may facilitate positive selection and escape from tolerance.

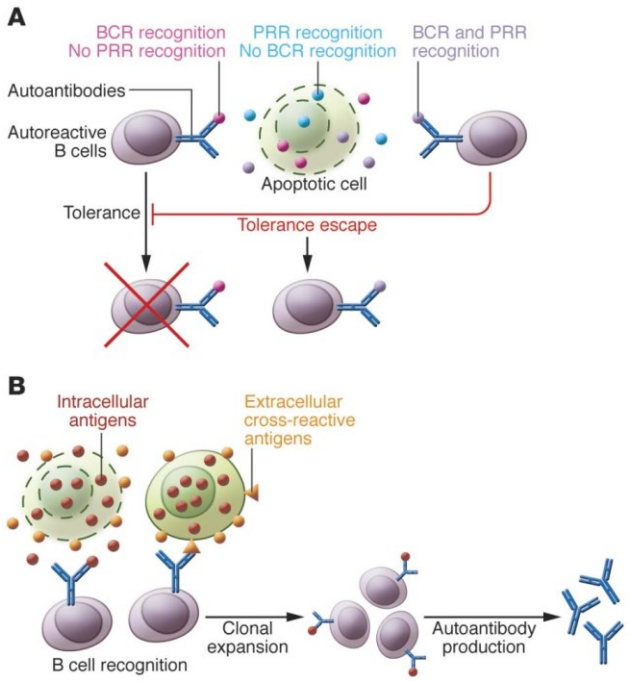

Figure 2. Proposed role of PRR activation and cross-reactivity in expansion of autoreactive B cells and their effector functions. (A) Self-antigens that bind PRRs can activate B cells. We hypothesize that the combined recognition of self-antigens by BCRs and PRRs is required for tolerance escape in autoreactive B cells. (B) B cells require recognition of antigen through their BCR for clonal expansion. As most autoantibodies recognize intracellular self-antigens, cross-reactivity to cell surface or extracellular molecules enhances clonal expansion and differentiation into memory and plasma cells.

TLR ENGAGEMENT AND T CELL–INDEPENDENT B CELL ACTIVATION

Increased levels of the cytokine B cell–activating factor of the tumor necrosis factor family (BAFF) and TLR activation have been implicated in T cell–independent activation of autoreactive B cells (*60, 71*). In vitro, increased TLR activation and BAFF levels have been shown to trigger activation-induced cytidine deaminase expression, which also induces somatic hypermutation and class switching in naive B cells (*72*). Another study showed that neutrophil-derived cytokines including BAFF can induce somatic hypermutation and class switching in marginal zone B cells (*73*). Furthermore, several studies showed that TLR activation can directly instigate IgG class switching and T cell–independent plasma cell differentiation, thereby possibly circumventing the requirement for T cell help in the peripheral compartment (*57, 74, 75*).

It is not clear whether these T cell–independent B cell activation pathways are sufficient to induce the levels of somatic hypermutation that are observed in autoreactive IgG^+ memory B cells. Importantly, TLR activation and T cell help might interact to induce somatic mutation. TLR activation of B cells can enhance antigen presentation and increase surface expression of co-stimulatory molecules, which will facilitate non–antigen-specific interaction with T cells (*76, 77*). Therefore, TLR activation can enhance somatic mutations in T cell–dependent or –independent B cell responses.

Together, these data support an important role for TLR activation in the development of autoantibody responses, especially when activated in combination with BCRs. B cells with BCR specificity for a TLR ligand may be enabled to escape tolerance mechanisms. If such a BCR binds a ubiquitous antigen, such as a cell membrane or extracellular antigen, clonal expansion occurs, with affinity maturation and differentiation to a plasma cell (Figure 2, A and B).

PATHOGENICITY OF AUTOANTIBODIES

The pathogenicity of autoantibodies has been extensively debated. Proof of pathogenicity is derived from the ability of autoantibodies to induce cellular damage or immune activation in vitro or to passively transfer autoimmune disease in animal models, as well as the effectiveness of B cell–targeting therapies. Although such evidence is not established in all autoimmune diseases, the most common systemic autoantibodies, such as ANAs and ACPAs, are considered pathogenic.

Pathogenicity has been most thoroughly studied in mouse models with lupus-like disease. Several studies have shown that passive transfer of anti-DNA antibodies or expression of autoreactive transgenic BCRs can cause inflammation in the kidneys,

although not all antibodies have equal capacity to do so (*78, 79*). In contrast, only limited evidence of the pathogenicity of ACPAs or RF is present in mouse models of arthritis. This may be due to the absence of these autoantibodies in most mouse models; however, when ACPAs are adoptively transferred into mice with low-level inflammation caused by anti-collagen antibodies, ACPAs enhances arthritis, directly implicating them in the inflammatory process in the joint (*80, 81*).

AUTOANTIBODY EFFECTOR FUNCTIONS

The effector functions that are employed by autoantibodies resemble those of antibodies in general and are therefore largely dependent on their isotype. Specific autoantibody isotypes are present in different diseases, with IgA antibodies present in antiphospholipid syndrome and IgE autoantibodies present in SLE (*82-84*). IgM and IgG autoantibodies are the most abundant. IgM autoantibodies are also present in healthy individuals and are therefore often considered to be less pathogenic (*85*). IgM is a potent activator of the complement component 1, q subcomponent (C1q), which has recently been shown to inhibit TLR activation by acting as a ligand for the inhibitory leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), suggesting that IgM might inhibit immune effector responses (*86*). In support of this model are experiments that showed that an inability to secrete IgM in B cells leads to IgG autoantibody production in mice (*87*).

A major pathway for immune activation and tissue damage for systemic autoantibodies is through formation of immune complexes. In situ immune complexes form when antigen is present at specific sites within tissues. Different monoclonal anti-DNA antibodies have distinct patterns of renal deposition, depending on their specificity and cross-reactivity (*45*). Although RA is associated with the presence of circulating immune complexes, the disease is mainly limited to the joints. This is thought to arise in part from preferential binding of antibodies to citrullinated proteins present in the synovium (*88*).

Immune complexes formed in situ can activate the complement system and Fc receptors, leading to tissue inflammation and damage. Complement activation by autoantibodies has been shown to occur through both classical and alternative pathways (*89, 90*) and can lead to direct cell lysis and damage as well as recruitment of leukocytes to further enhance inflammatory responses. Complement-mediated cell lysis has also been shown to serve as a source of antigen, such as DNA, RNP, and citrullinated proteins (*91*).

Activating Fc receptors are expressed by a variety of cells, particularly by immune cells of the myeloid lineage. Autoantibodies have been shown to activate these immune cells through FcγR-dependent pathways (*92-94*). In vivo, FcγR-deficient mice often show decreased autoimmunity (*95, 96*). The capacity of IgG to activate Fc receptors contributes to their increased pathogenicity compared with IgM autoantibodies. However, IgM-RF was recently shown to enhance FcγR-dependent activation by ACPAs, demonstrating that isotypes other than IgG can contribute to pathogenesis (*97*).

CLINICAL EVIDENCE FOR AUTOANTIBODY PATHOGENESIS

Most autoantibodies are present years before disease onset and thus are not merely a consequence of inflammation (*98-100*). Furthermore, for ACPAs, there is evidence for epitope spreading, affinity maturation, isotype switching, and glycosylation changes before clinical symptoms start. Each of these changes is associated with greater pathogenic potential (*101-105*).

In addition to the changes in the intrinsic properties of autoantibodies before disease onset, the target organ can also become susceptible to autoantibody deposition or binding. This is in accordance with the multiple-hit theory of autoimmunity, in which genetic and environmental factors induce an initial break in tolerance leading to production of autoantibodies. A "second hit" is needed for the antibody to exert pathogenic effects and to sustain the inflammatory cascade of the autoimmune response. This phenomenon is perhaps most clearly illustrated in myositis, in which the autoantibodies bind regenerating myocytes and thus sustain a cycle of injury and regeneration (*106*).

CHRONIC INFLAMMATION

As stated above, many autoantibodies target ubiquitously expressed intracellular molecules. For antibodies to bind these targets, these antigens need to be released to the extracellular environment, where antibodies or immune complexes are deposited. As the self-antigens are ubiquitously present, autoantibody effector mechanisms fail to eliminate the antigen, causing inflammatory injury to tissues that leads to additional release of these intracellular antigens. This process causes a vicious cycle wherein each disease flare can initiate further autoantibody production and tissue injury (*107-109*).

Furthermore, combined TLR- and Fc receptor–mediated activation of myeloid cells often leads to an enhanced inflammatory response that is thought to contribute to the chronicity of inflammation (*51, 94, 110, 111*). Therefore, those self-antigens that can

most potently activate B cells because they are TLR ligands may also potently enhance pathogenic inflammatory responses (Figure 3).

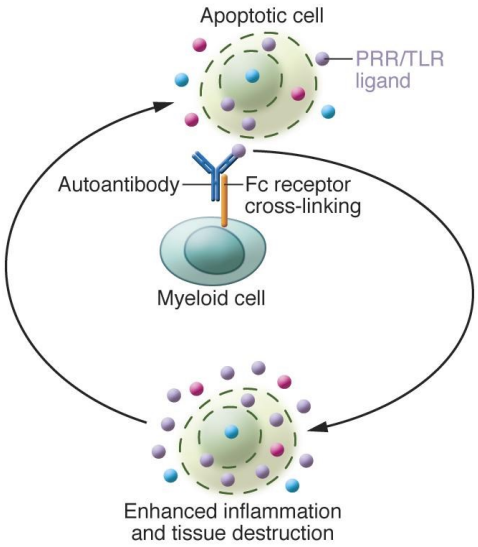

Figure 3. The role of PRR activation by self-antigens in autoantibody pathogenesis. During autoimmune responses, inflammation can lead to cell death and release of endogenous PRR or TLR ligands. When recognized by autoantibodies, these ligands can activate myeloid immune cells through both Fc receptors and PRRs, leading to an enhanced inflammatory response. This response, in turn, can lead to tissue destruction and release of ligands for PRRs and autoantibodies, which further amplifies the chronic inflammatory response.

CONCLUSIONS

A break in tolerance against self-antigens or modified self-antigens can lead to the production of autoantibodies. Such a breach in tolerance can originate from a lack of clearance of apoptotic debris, inflammation-mediated modification of self-antigen, or cross-reactivity between foreign and self-antigens. Some autoantibodies cross-react between an intracellular antigen that is a TLR ligand and cell surface or extracellular antigen, allowing for continued B cell activation and autoantibody-mediated injury.

After initiation of autoantibody production, inflammation may cause release of intracellular or modified self-antigens, leading to antibody effector function and instigation of clinical symptoms as well as to the propagation of the autoreactive B cell clones. These pathogenic processes are mainly mediated by IgG autoantibodies, although other isotypes may contribute. TLRs play an important role during both initiation and pathogenesis of autoantibody responses, as they can recognize several self-antigens such as alarmins. TLR activation can enhance the initiation of autoantibody responses and can synergize with Fc-mediated effector functions. We speculate that those autoreactive B cells that can escape tolerance and expand and

differentiate in response to antigen have BCRs that bind antigens that associate with or are TLR ligands. Reactivity with abundant cell surface or extracellular antigens permits the clonal expansion and survival of these B cells once inflammation has subsided. Furthermore, this cross-reactivity may contribute to their pathogenicity.

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THERAPEUTICS TO BLOCK AUTOANTIBODY INITIATION AND PROPAGATION IN SYSTEMIC LUPUS ERYTHEMATOSUS AND RHEUMATOID ARTHRITIS

Chapter 13

Jolien Suurmond, Yong Rui Zou, Sun Jung Kim,
Betty Diamond

Center for Autoimmune and Musculoskeletal Diseases,
Feinstein Institute for Medical Research, Manhasset, NY, USA

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ABSTRACT

Most current therapies for the autoimmune diseases systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), as well as many of the drugs in the therapeutic pipeline, reduce the autoimmune inflammatory process but lead to a general immunosuppression. The goal of the next generation of therapies should be to reduce autoimmunity while at the same time maintaining immunocompetence. We propose three approaches for accomplishing this goal: (i) modulate antigen presentation to the adaptive immune system, (ii) alter B cell selection in the germinal center and (iii) use decoy antigens to prevent the formation of proinflammatory immune complexes.

These approaches are based on recent advances in the field: We now appreciate the role of dendritic cell function in autoimmune disease and the importance of citrullinated proteins as neoantigens in RA. There is also new recognition that most pathogenic autoantibodies are produced by B cells that have matured within the germinal center and that immune complexes in both diseases contain ligands for toll like receptors. We propose that treatments that target these newly revealed aspects of RA and SLE will decrease systemic inflammation without immunocompromising patients.

INTRODUCTION

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are autoimmune diseases that affect approximately 0.1 and 1 % of the population, respectively. Because individuals in early to mid adulthood are affected by these diseases, their associated morbidity constitutes a considerable economic burden and diminishes quality of life over decades. Moreover, both diseases are associated with early death. Although the prognosis for these diseases has improved substantially over the past decades, overall outcome remains inadequate. Even while on a therapeutic regimen, most patients continue to be symptomatic, and current treatments produce undesirable immunosuppression. For these reasons, there is a number of novel therapeutics under development. Hopefully, some of these will demonstrate dramatic benefit and low toxicity. Here, we propose concepts for therapy that we believe are closely aligned with disease pathogenesis.

In considering the next generation of therapeutic regimens, it is important to consider the full spectrum of therapeutic goals. Certainly, we need to protect against irreversible tissue damage, primarily in the kidney and brain in SLE, and in joints in RA. We also need to prevent the accelerated atherosclerosis that occurs in both SLE and RA and which is believed to be consequent to an ongoing systemic inflammation, which remains active

despite current treatments. We also need to maintain immunocompetence while suppressing autoreactivity and systemic inflammation. Ultimately, an ideal therapy would effect sufficient immunomodulation that patients experienced a prolonged disease-free, drug-free existence, despite their genetic predisposition to disease.

One important therapeutic question is: When do we intervene? In principle, it would be best to intervene at a preclinical stage of disease when there is evidence of abnormal immune activation, but no clinical disease and no detectable target organ injury. This would obviate the need to understand the nature of tissue injury and the contribution of tissue-resident cells to organ damage. Preventive strategies for RA are currently being tested in a clinical trial but they may necessitate treating individuals who may never develop disease with potentially toxic agents. For example, first degree relatives of autoimmune patients can exhibit elevated titers of disease-associated autoantibodies or aberrant cytokine profiles, but most will never develop disease. Individuals with anti-citrullinated protein antibodies (ACPA) who present with painful joints (arthralgia) or undifferentiated arthritis, however, have a high risk of RA. For these people, immunomodulation might prevent full-blown disease.

Treatment after the onset of clinical symptoms needs to ameliorate disease by inhibiting tissue injury, reducing active inflammation and maintaining prolonged immune inactivity in lymphoid tissue. Strategies designed to reduce organ damage would differ considerably from treatments that induce immune quiescence. Moreover, their use would be intermittent, at times of tissue inflammation but not during the prolonged periods of systemic inflammation that are thought to contribute to accelerated cardiovascular disease. In contrast, therapeutics that decreases ongoing activation of autoreactive cells when target organ inflammatory is under control could reduce systemic inflammation without suppressing immunity against pathogens, and prevent acute exacerbations and further organ damage.

To successfully modulate immune responses in SLE and RA, we must understand what cellular interactions or molecular pathways are aberrantly regulated in these diseases. Gene expression analyses based on genome wide association studies (GWAS) suggest that dendritic cell (DC) and B cell intrinsic pathways may be dysregulated in SLE, and T cell intrinsic pathways dysregulated in RA (1). Nonetheless, each disease is promoted by an aberrant repertoire of adaptive immune cells, triggered, we propose, by altered antigen presentation and abnormal B cell selection in the germinal center (GC). Disease activity is sustained, in part, by the pro-inflammatory properties of the disease-specific immune complexes. We suggest that these pathways of disease initiation and propagation will be the targets of next generation therapies.

Should we explore antigen-specific therapeutic interventions or more global immune modulation? The answer may depend on the disease. In SLE, there are diverse autoantibodies and the T cell autospecificities have not been clearly defined. Thus, we may not yet have adequate knowledge of the pathogenic T and B cells in this disease, and targeting cells specific for one or two antigens may not be sufficient. Thus, we favor a global immuno-modulatory therapeutic approach for SLE. In RA, it may be time to explore antigen-specific therapy targeted to citrullinated proteins. Although ACPA are present in only 70% of patients, they clearly contribute to systemic inflammation and to tissue damage.

INITIATION OF AUTOIMMUNITY

DCs are key controllers of immune responses that present both self and foreign antigens to T cells in either a tolerogenic or immunogenic fashion, thereby delicately balancing immune activation and tolerance (2). As SLE and RA are both associated with autoantibodies produced through T cell – B cell collaboration, the DC is thought to play a major role in the initiation of such responses. The regulatory function of DCs is affected by environmental cues such as the microbiome, prior pathogen exposure and cytokines. Thus, mature DCs are stimulated by pattern recognition receptors to upregulate co-stimulatory molecule expression and drive effector T cell responses against pathogens, while immature DCs or DCs exposed to apoptotic cells normally induce T cell anergy or promote regulatory T cells (Treg) differentiation through secretion of cytokines such as TGF and IL-10 (3, 4). Therefore, the activation and differentiation pathways of autoreactive T cells depend on the exact nature of DC activation and presentation of self peptides. Treatments that inhibit DC activation or change the peptides presented by HLA class II molecules could represent new avenues to prevent the initiation of autoimmunity.

MODULATING DC ACTIVATION.

A variety of receptors, including pattern recognition receptors, complement receptors, and Fc receptors can modulate activation of DCs by self antigen (5, 6). DCs express several inhibitory receptors, including LAIR1, a recently identified receptor for the complement component C1q (7). C1q deficiency is a monogenic risk factor for SLE development (8), and low levels of C1q correlate with increased disease activity (9). C1q prevents autoreactivity in at least two ways. It acts as an opsonin of apoptotic cells to facilitate clearance of cell debris, and it regulates DC maturation and cytokine production (10, 11). C1q blocks DC differentiation from monocytes through LAIR1 (7). Monocyte-derived DCs are

immunogenic; after stimulation, they express high levels of co-stimulatory molecules and secrete proinflammatory cytokines, which determine T effector cell phenotype (12). Monocytes from SLE patients show accelerated differentiation (13). C1q also inhibits activation of conventional DCs (cDCs) and plasmacytoid DCs (pDCs) by preventing the engagement of endosomal toll like receptors (TLRs)by immune complexes that contain TLR ligands (14).

Thus, C1q and C1q-like agonists could be potent therapeutics to prevent activation of monocytes, cDCs and pDCs, thereby reducing the proinflammatory, proimmunogenic milieu in autoimmune patients. Additional strategies to modulate activation of DCs may emerge through studies of the microbiome and pathogen-induced immune deviation (15-17), but the data are not yet sufficient to enable the design of therapeutics.

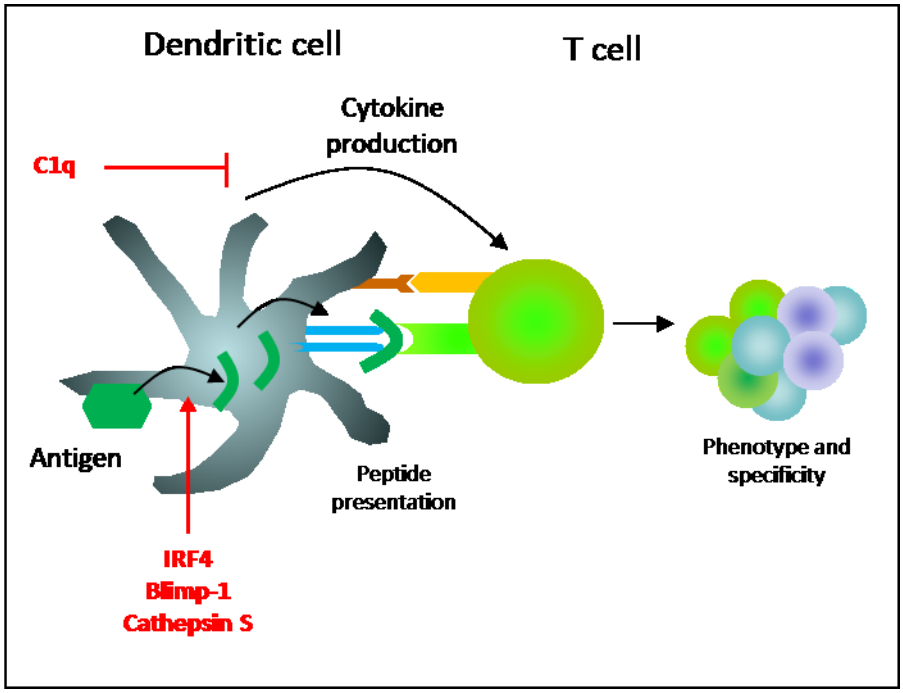

Figure 1. Targets for next-generation therapeutics: DC–T cell interactions. C1q or other ligands for inhibitory receptors on DCs can modulate DC activation by blocking the up-regulation of costimulatory molecules and cytokine production (top red arrow), modulating T cell phenotype and activation. Altering the levels or activity of IRF4, Blimp-1, and cathepsin S (bottom red arrow) could change the peptides presented by DCs through HLA, modulating T cell specificity.

DC ANTIGEN PRESENTATION (MODULATING T CELL REPERTOIRE).

SLE and especially RA have a strong association with certain HLA-DR risk alleles, suggesting that presentation of particular antigens to CD4+ T cells are crucial components of the initiation of autoreactive responses (18). Transcription factors IRF4 and Blimp-1 and the lysosomal protease cathepsin S normally participate in the process of antigen presentation (19). Cathepsin S degrades the MHC class II invariant chain to allow peptide loading to the MHC II molecules in antigen presenting cells (20, 21). Cathepsin S also degrades endo-lysosomal antigens, shaping the spectrum of peptides presented on DCs (22, 23). As cathepsin S has both positive and negative effects on T cell selection in the thymus and the periphery, its abundance needs to be tightly controlled (24). For example, too much cathepsin S in the thymus can destroy immunodominant epitopes of self antigens, leading to escape of autoreactive T cells from negative selection (25). Low Blimp-1 levels in DCs, as occurs in individuals with the SLE Blimp-1 risk allele, can lead to elevated cathepsin S (26). A therapeutic agent that diminishes autoantigen presentation and autoreactive T cell differentiation by modulating levels of Blimp-1, IRF4 or cathepsin S could be an effective strategy against autoimmunity that would not result in global immunosuppression. This approach could be effective even without knowing the self peptides that drive autoreactive T cells in SLE.

Antigen presentation of specific peptides not only initiates autoreactive T cell responses; it also contributes to the production of autoantibodies. This role in autoantibody generation is evidenced by the fact that patients with ACPA+ and ACPA- RA tend to have different HLA class II risk alleles, and recently citrulline-specific effector T cells were shown to be present in those individuals carrying the HLA risk allele associated with ACPA+ RA (27, 28). (Citrulline-specific T cells are also present in healthy individuals, but there these CD4+ T cells exhibit a Treg phenotype. In RA patients, citrulline-specific T cells have a proinflammatory phenotype, suggesting that additional genetic or environmental risk factors allow these T cells to differentiate to effector cells in the periphery where inflammation induces the citrullination of proteins.

Numerous intracellular and extracellular proteins undergo post translational citrullination, with extracellular proteins undergoing citullination primarily in an inflammatory milieu. The exact citrullinated epitopes that activate T cells in RA has only recently begun to be revealed. More knowledge on the processing and presentation of citrullinated antigens in the thymus and periphery is needed before we can design ways to modulate the T cell repertoire in RA patients by inhibiting presentation of citrullinated antigens to CD4+ T cells. Even now, however, we might consider decreasing the formation of citrullinated proteins by interfering with the expression or function of the peptidylarginine deiminases (PAD) that catalyze citrulline formation, especially PAD4, which is found in inflammatory synovium.

PROPAGATION OF AUTOIMMUNITY

Although autoreactive B cells can be directly activated by self-antigens, B cell survival and the generation of memory B cells and long-lived plasma cells in the GC are promoted by proinflammatory cytokines from immunogenic DCs and require help from T follicular help (Tfh) cells (29). Autoantibody-containing immune complexes feed forward to inflammatory responses by activating complement receptors and Fc receptors on DCs and other innate immune cells (30). Furthermore, immune complexes deposited on follicular DCs could serve as a self-antigen reservoir that sustains autoreactive GC reactions (31). Interventions that can break down this detrimental feedback mechanism will be a part of the future therapeutic armamentarium for SLE and RA.

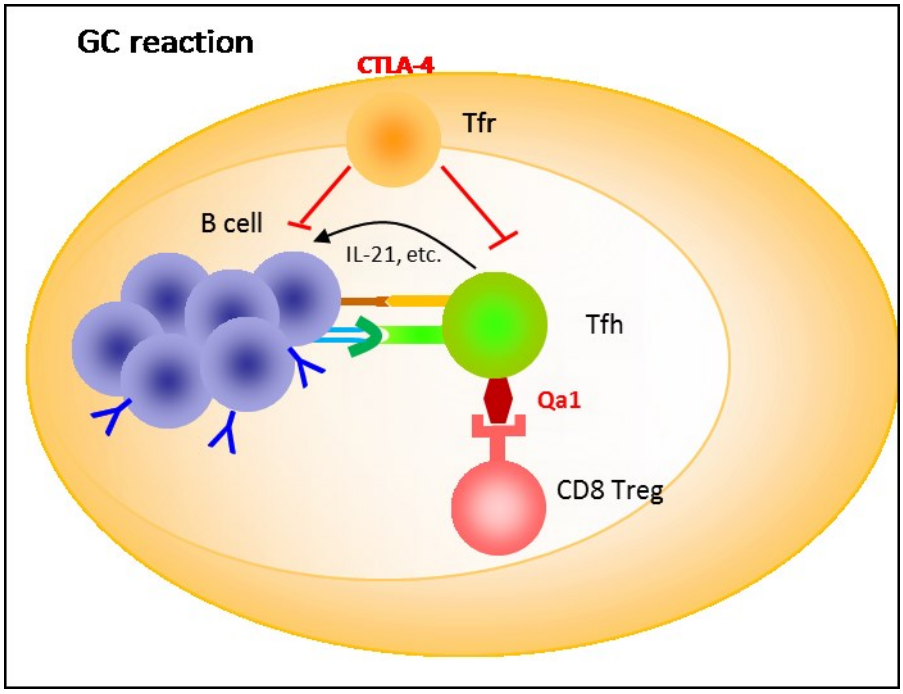

Figure 2. Targets for next-generation therapeutics: GC reactions. The interaction of follicular T helper cells and B cells in the GC leads to propagation of autoimmunity. Enhancing the function of Treg cells in the germinal center may diminish this process. Qa1-restricted $CD8^+$ Treg cells (red cell) and CTLA-4 expressing T follicular regulatory cells can inhibit these GC reactions.

In the GC, antigen-activated B cells undergo random somatic hypermutations in immunoglobulin genes that can cause an increase or a decrease of affinity of B cell receptors. The rate of hypermutation and cell division is proportionally increased with the amount of antigen captured and presented by GC B cells to Tfh cells (32). Thus, B cells

expressing higher affinity B cell receptors are selected to expand and differentiate into memory B cells or long-lived plasma cells. B cells can also gain de novo self-reactivity through the process of hypermutation. These autoreactive B cells normally undergo negative selection, a result of the lack of cognate Tfh cells. But in both SLE and RA, somatic mutations contribute significantly to the generation of autoreactive memory B cells, suggesting that they interacted with Tfh cells in a GC response (33, 34).

Autoreactive B and T cells are normally suppressed by regulatory cells. The regulatory cell population is highly heterogeneous, including Foxp3+ CD4 Treg, Qa-1 restricted CD8 Treg cells and IL-10- and IL-35-producing regulatory B cells (35-37). Even the CD4 Tregs are comprised of functionally diverse subsets that selectively regulate Th1, Th2 or Th17 responses (38, 39). Therefore, the enhancement of a specific subset of regulatory cells in SLE or RA patients may suppress certain GC reactions without globally affecting immunity. For example, Qa-1 is specifically expressed by Tfh cells, and Qa-1 restricted CD8 Tregs inhibit spontaneous GC reactions by targeting autoreactive Tfh cells (40). Reduced numbers and dysfunctional CD4 Tregs have been reported in SLE and RA patients (41, 42).

Whether other regulatory cells are perturbed in SLE or RA patients has not been well studied. More effort is required to develop agents that boost a specific population of regulatory cells and maintain their function in chronic inflammatory condition in SLE and RA patients. One specific Treg subset found in GCs --- T follicular regulatory cells (Tfr) --- inhibit Tfh differentiation and GC B cell responses; their inhibition of Tfh requires expression of CTLA-4 (43, 44). CTLA-4-Ig fusion proteins (abatacept), which block CD28 binding to CD80 and CD86 and thus preventing second signal for T cell activation, have been successfully used to treat RA (45). Abatacept has not been able to prevent flares in SLE patients; howeverthis may be because SLE patients already have increased serum levels of soluble CTLA-4. Moreover, a major B cell tolerance checkpoint is in the GC where a specifically targeted CTLA-4 agonist may boost Tfr function and suppress autoreactivity in GC B cell responses in SLE.

NEUTRALIZATION OF AUTOANTIBODIES

Because autoantibodies are thought to be critical for the pathogenesis of SLE and RA through their generation of immune complexes, another way to neutralize the proinflammatory effects of those complexes without affecting the immunocompetence of patients would be the use of decoy antigens.

In SLE, anti-DNA antibodies contribute to kidney disease and neurotoxicity by through their cross-reactivity to glomeruli and neurons. Moreover, the DNA-containing immune

complexes can activate TLRs and thereby in turn activate myeloid cells and permit DNA-reactive B cells to escape tolerance mechanisms (46). We have developed a small molecule peptidomimetic that prevents the anti-DNA antibody from binding to target organs and from activating TLR 9 (47, 48). Such mimetics are ideal agents to treat autoimmunity as they specifically suppress autoantibody-mediated tissue injury and autoantibody-mediated systemic inflammation, without inhibiting immunity against pathogens.

In RA, citrullination is a central event in the pathogenesis of ACPA+ RA, contributing to both T- and B-cell activation, and induction of chronic inflammation through formation of ACPA immune complexes, which can activate Fc receptors and TLR4 (49, 50).

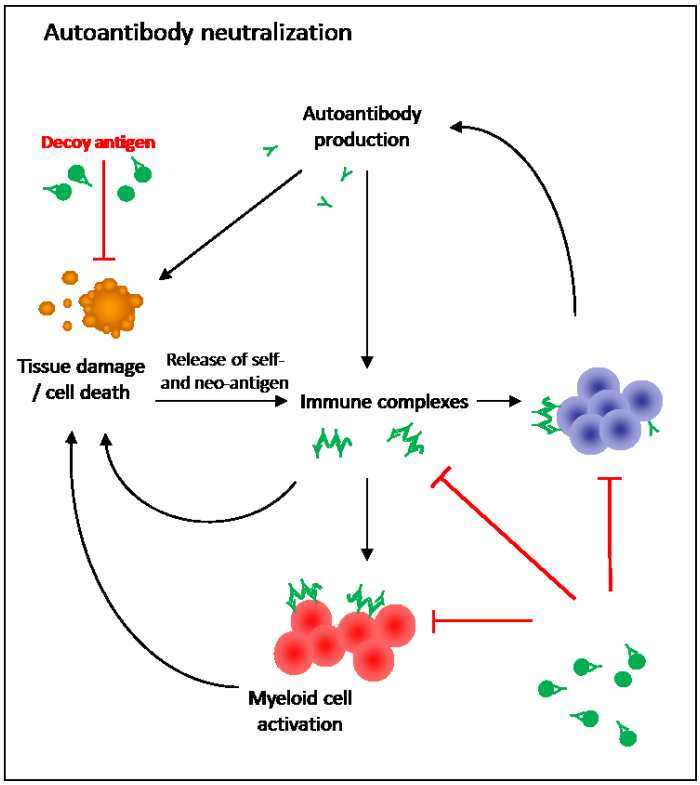

Figure 3. Targets for next-generation therapeutics: Autoantibody neutralization. Autoantibodies induce a chronic cycle of events leading to cell and tissue damage, either through direct effects on tissue or through formation of immune complexes. Decoy antigens can inhibit each of these processes by reducing antibody binding in tissues, inhibiting formation of immune complexes, and interfering with B cell activation.

The use of decoy antigens for citrullinated peptides in RA could reduce activation of myeloid cells, thereby directly reducing inflammatory responses in the joint. Activation of myeloid cells by ACPA can lead to cell death and release of PAD enzymes or citrullinated proteins in the joint (51, 52). This drives an incessant cycle of antigen recognition, immune activation, and further release of PAD enzymes and creation of citrullinated antigens in the joint. Decoy antigens mightn block the formation of inflammatory ACPA immune complexes, thereby preventing myeloid cell activation. In addition, ACPA-producing

plasmablasts are continuously generated in the joint, suggesting that B cells are activated to undergo terminal differentiation to plasma cells by joint-localized citrullinated antigens. Decoy antigens could compete with citrullinated antigens for binding to the B cell receptor; by inhibiting crosslinking of the B cell receptor, these decoys could block B cell activation as well as prevent generation of autoantibody producing plasma cells.

Citrullinated proteins also occur in atherosclerotic plaques (53). Therefore, prevention of ACPA binding to citrullinated targets may reduce the atherosclerotic complications of chronic inflammation in RA patients, in addition to decreasing acute inflammatory responses in the joint.

PRECISION MEDICINE

In both SLE and RA, patients vary in the exact constellation of signs and symptoms that they present, likely reflecting differences in underlying genetic risk and pathogenic pathways. Therefore, the approaches presented here should be applied in a patient-specific manner. For example, modulation of DC activation and antigen presentation may be most useful in SLE patients with a Blimp-1 risk allele, and modulation of the processing of citrullinated antigens in RA may be most effective in those harboring the HLA shared epitope alleles.

As more is learned of autoimmune disease pathogenesis, our ability to modulate the immune response will become more refined. The approaches suggested here represent a starting point. Modulation of antigen presentation and the GC response will interfere with key events in the induction of autoimmunity. Employing decoy antigens can lessen the proinflammatory milieu. Agents that can be used in these strategies are available and deserve serious consideration.

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Summary & Discussion

Chapter 14

AIMS

This thesis focuses on the understanding of mast cell (and basophil) functions, with a special emphasis on the role of mast cells in autoimmune disease. Here, I will discuss the findings of this thesis, based on the specific aims:

1. To characterize the interaction between innate and Fc receptor triggers on mast cell and basophil function
2. To analyze the interaction between mast cells and $CD4^+$ T cells
3. To understand the function of mast cells in chronic inflammation

INTERACTION BETWEEN INNATE & ADAPTIVE IMMUNITY

In the immune system, mast cells are considered to be involved in both innate and adaptive immunity. Their rapid degranulation makes them one of the first cells to respond in innate immune responses, whereas their expression of Fc receptors makes them important effector cells in antibody-mediated adaptive responses. However, these responses are often considered separately, and not much is known about the interaction between innate and adaptive immune triggers, which are often present, as discussed below.

In the first part of this thesis, I aimed at understanding the interaction between triggers of the innate immune system in combination with Fc receptor triggering. In chapters 2, 4, and 5, I showed that interactions of both FcεRI and FcγRIIA with TLR ligands induced synergy in mast cell and basophil activation. This interaction was characterized by a marked increase in cytokine secretion, without affecting Fc receptor-mediated degranulation.

Although the magnitude of the response was largely increased when TLR ligands and Fc receptor triggers were present at the same time, the specificity of the response remained tightly regulated. In particular, the cytokine profile was highly dependent on the TLR ligand that was present, in both basophils and mast cells.

Although we did not investigate the mechanism of synergy, some findings suggest that the enhanced cytokine production was mediated through interaction of the TLR and Fc receptor signaling pathways. The synergy remained present when mast cells or basophils were treated with brefeldin A to block the transport of proteins from the Golgi system to the cell surface or extracellular environment (Chapter 2 and unpublished observations).

This suggests that the synergy did not depend on upregulation of surface receptors or secreted mediators.

In human monocytes and dendritic cells, combined triggering of FcγRIIA and TLR was shown to induce synergy in transcription levels of several cytokines, but the exact signaling pathways leading to the synergy are not known (*1, 2*). A study in murine mast cells investigated the phosphorylation events downstream of TLR and Fc receptors upon combined triggering of these receptors and showed that activation of the JNK kinase pathway was enhanced upon combined triggering of these receptors (*3*). Many of the upstream molecules of the JNK kinase pathway induced by TLR activation (JNK1, MKK4, TAK1, IRAK1, IRAK2, MyD88, TRAM, and TRIF)(*4*) or Fc receptor triggering (BTK, Syk) are expressed by human mast cells, at least at the mRNA level (Chapter 5 and unpublished observations). Therefore, a similar synergy in JNK kinase activation upon activation through TLR and Fc receptors may be underlying the synergy we observed.

As discussed below, the synergy in mast cell activation upon combined triggering of TLR and Fc receptors has important implications for both protective immunity as well as hypersensitivity reactions, such as during allergy or autoimmunity.

TLR AND FC RECEPTOR ACTIVATION IN RESPONSES TO PATHOGENS

The synergy observed upon TLR and Fc receptor triggering reflects a memory response, which is usually characterized by a shorter response time and an enhanced magnitude of the immune response after specific antibodies have been generated (*5*). After primary responses to pathogens, antibodies and T cells can contribute to increased resistance to secondary or chronic infections, and it is this enhanced responsiveness of the immune system that forms the basis for vaccination (Figure 1).

As discussed in the introduction of this thesis, mast cells have been shown to play an important role in the immune response against a variety of pathogens, including bacteria, parasites and viruses. However, only few studies have evaluated the role of mast cells in recall responses against pathogens, when specific antibodies have been formed.

In the case of bacteria, IgG antibodies are most often generated after primary infection. IgG immune complex mediated reactions upon passive antibody transfer in the mouse are dependent on mast cells, in particular through FcγRIII (*6*). The effect of mast cells in such reactions is mainly to recruit neutrophils, eosinophils and other immune cells to the site of antigen exposure. Eosinophil recruitment in a model of passive cutaneous anaphylaxis was augmented by pretreatment with LPS, suggesting that enhanced mast cell responses in presence of TLR ligands and immune complexes may lead to enhanced

protective responses against bacteria (*7*). In addition, neutrophil recruitment upon Helicobacter infection in vaccinated mice was largely reduced in mast cell-deficient animals (*8, 9*). The findings of this thesis are in line with these observations, showing that the production of several cytokines and chemokines, known to recruit and activate neutrophils and eosinophils, were markedly enhanced in the presence of combined TLR and Fc receptor activation.

In the case of parasites, most evidences suggest a dominant role for IgE in protection, although other IgA and IgG isotypes have been postulated to play a protective role as well (*10-12*). In mice, protective immunity, such as induced via vaccination, is IgE-dependent (*13, 14*), and in humans, levels of parasite-specific IgE are correlated with resistance to parasitic infection (*15-18*). Interestingly, a recent study showed that opsonized parasites could be directly killed in vitro by human mast cell-derived tryptase, through the formation of a so-called degranulation synapse (*19*).

In addition to specific antibodies able to enhance mast cell responses during recall immunity, IgE bound to FcεRI can be crosslinked non-specifically by an S mansoni egg antigen and HIV antigen gp120 (*20, 21*). Therefore, the synergy observed between TLR signaling and FcεRI crosslinking may also contribute to innate responses to these pathogens.

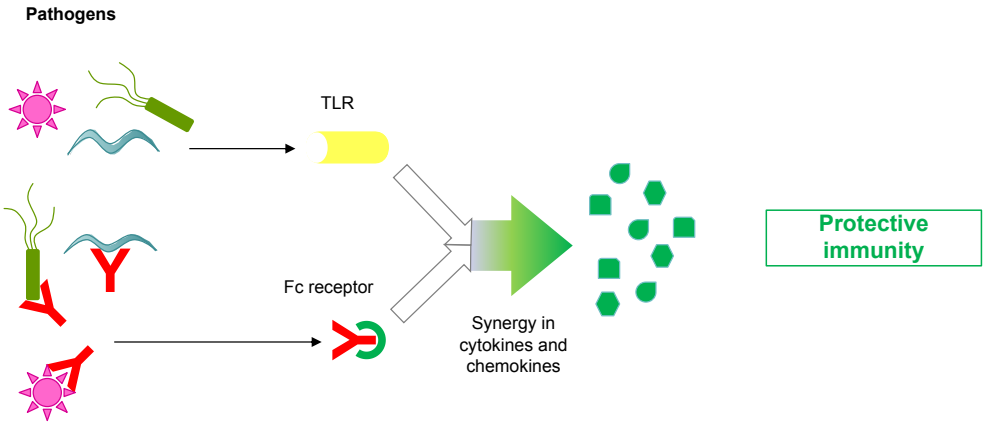

Figure 1. Synergy between TLR and Fc receptors can contribute to protective immunity against pathogens. Pathogens such as bacteria, parasites and viruses can be recognized by antibodies during secondary responses. These antibodies can trigger mast cell activation. When PAMPs from the pathogens trigger TLR activation, production of cytokines and chemokines by mast cells synergizes, thereby contributing to protective immunity.

Pathogen-specific responses

Importantly, we observed cytokine-specific responses, depending on the TLR that was triggered (Chapters 4 and 5), suggesting that the type of TLR-trigger (which typically depends on the type of pathogen encountered) can fine-tune the response mode of mast cells (Figure 2). There is a remarkable resemblance of the cytokines that were induced by specific TLRs in mast cells with their putative protective role for particular pathogens in vivo. For example, TLR-2 and -4 are mostly known for their involvement in bacterial and parasitic infection (*22-25*). Ligands for these TLRs in mast cells induced a cytokine profile characterized by production of GM-CSF, IL-5, IL-13, and MIP-1α. These cytokines may contribute to anti-bacterial immunity; IL-8 and MIP-1α are particularly known to induce recruitment and activation of neutrophils, an important mechanism for the first line of defense against extracellular bacteria (*26-28*). IL-5 and IL-13, in turn, are potent contributors to the clearance of parasites, through induction of eosinophil activation, mucus production and worm expulsion (*29-31*).

Some of the cytokines involved in mast cell-mediated protection against bacteria in mice were not observed upon triggering of bacteria-associated TLRs in human mast cells. These include TNF-α, CXCL1, CXCL2, IL-4 and IL-6 (*8, 27, 32, 33*). However, for several of these cytokines produced by murine mast cells, their production by their human counterparts is not commonly observed (*34*). Furthermore, the functions of these cytokines in bacterial infections largely overlap with the cytokines we observed, suggesting that human mast cells may serve a similar function be it through production of different cytokines.

In contrast, ligands which are associated with viruses, induced a different cytokine profile, which is more associated with recruitment and activation of T cells and NK cells, such as through production of MCP-1, MIP-1β (Chapter 4 and unpublished observations). Recruitment of T cells and NK cells by mast cells has been shown to contribute to anti-viral immunity in mice (*35-37*). Other cytokines produced by mast cells in response to the TLR-8 ligand ssRNA (Eotaxin, GRO-α, TNF-α) are associated to recruitment and activation of eosinophils and neutrophils. Although this process may not be generally appreciated in the context of viral infection, several studies now suggest that eosinophils can play an important role in viral infection, especially during pulmonary infections (*38-40*). Eosinophils can secrete RNAses and cationic granule products, which are known to degrade single stranded RNA (TLR-8 ligand) (*41-43*). Neutrophils can also contribute to anti-viral immunity, for example through secretion of antiviral peptides, formation of NETs, and phagocytosis of infected cells (*44-46*). These results therefore suggest that mast cell-derived cytokines in response to viral TLR ligands may contribute to antiviral immunity through their effects of both innate and adaptive immune cells.

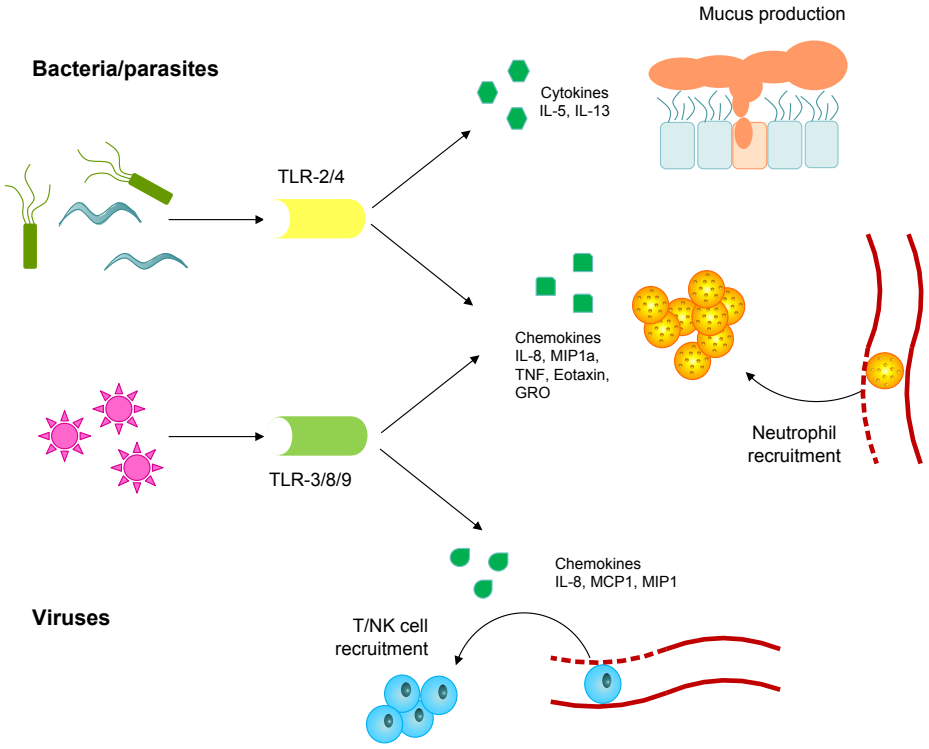

Figure 2. Pathogen-specific responses mediated through triggering of specific TLRs. The type of pathogen can fine-tune the response mode of mast cells. Bacterial and parasitic TLR ligands, through production of specific cytokines in response to triggering of TLR-2 and -4, can induce mucus production and recruitment of granulocytes to the site of infection. In contrast, mast cell-derived cytokines in response to viral TLR ligands are associated with recruitment of T cells and NK cells.

Differential responses determined by the type of TLR have been previously described for different types of dendritic cells and monocytes (*47-49*), suggesting a model where triggering of different TLRs in multiple cell types shape the immune response against different pathogens. In Chapter 2, I described a similar phenomenon for basophils, where the TLRs associated with bacteria and parasites were able to induce type 2 cytokines, IL-4 and IL-13, whereas the TLRs associated with viruses induced secretion of RANTES, suggesting that both mast cells and basophils may contribute to skewing of the immune system to pathogen-specific responses. The specific role of mast cells and basophils seems to be the skewing of type 2 immunity and recruitment of neutrophils and eosinophils, thereby complementing protective responses induced by DCs and other cell types. The further enhancement of these TLR-specific responses by mast cells and basophils in the presence of Fc receptor triggering, suggests that mast cells and basophils may be potent enhancers of pathogen-directed immunity during memory responses.

TLR AND FC RECEPTOR ACTIVATION IN ALLERGY

Besides the role of TLR ligation in pathogen-specific immunity, a role for TLRs has been implicated in allergy as well. For example, viral and bacterial infections have been associated with asthma exacerbations (*50, 51*). Furthermore, chronic allergy may lead to secretion of endogenous TLR ligands (*52*), and some allergens have been shown to contain TLR ligands (*53, 54*). The results described in this thesis suggest that both basophils and mast cell allergic responses are significantly enhanced in the presence of TLR ligands.

Allergy is usually hallmarked by two important processes. The sensitization phase takes place first, where Th2 and IgE responses against the allergen are initiated. During an allergic reaction, the immune system is triggered by re-exposure to the allergen. This effector phase consists of an FcεRI-dependent acute reaction, and a more prolonged reaction (late-phase reaction) caused by cytokines and inflammatory infiltrates.

TLR ligands during allergic sensitization

During the sensitization phase, allergen-specific Th2 responses are primed. Although dendritic cells are required as antigen presenting cells, they usually do not produce IL-4, a cytokine that is necessary for Th2 priming (*55*). It is unclear which cell type provides these early innate type 2 cytokines, but basophils may contribute to this process, in addition to innate lymphoid cells or naïve T cells (*56, 57*). Indeed, basophils were shown to enhance Th2 responses upon house dust mite inhalation in mice (*58*).

The effect of TLR ligands, in particular endotoxin, has been studied in the context of allergic sensitization, although contradicting results have been obtained (*59*). Several studies show that exposure to endotoxin or pathogens (such as in rural areas) is associated with a lower prevalence of allergy (*60-62*). Furthermore, some studies in mice showed that exposure to LPS can reduce allergic sensitization (*63*), presumably by inducing Th1 responses as a consequence of IL-12 production by dendritic cells in response to LPS (*59, 64, 65*). In contrast, one study showed that a low level of endotoxin is required to mount a robust Th2 response against allergens, and several studies showed that TLR-4 is required for allergic sensitization (*58, 66, 67*).

As I showed in this thesis, TLR ligands were able to induce IL-4 production by basophils, thereby providing a link between innate responses and Th2 immunity such as required for allergic sensitization. TLR ligands alone only led to a low level of IL-4, which in itself was not sufficient to induce Th2 skewing. However, TLR ligands together with protease allergens may be able to induce a more robust IL-4 production by basophils (*68*), thereby potentially contributing to allergic sensitization.

TLR ligands during the effector phase of allergic reactions

In contrast to contradicting studies on the role of TLR ligation during allergic sensitization, much more is known about the enhancement of allergic responses by pathogens during the effector phase. Many studies suggest that pulmonary infections, with either virus or bacteria, are associated with asthma exacerbations (*50, 51*), In allergic individuals, challenges with combined endotoxin and allergen induced a synergy in neutrophil and eosinophil recruitment to the nasal tissue, or increased wheal and flare reactions in the skin, depending on the tissue where the challenge took place (*69, 70*), In experimental asthma in rodents, LPS enhanced eosinophilic airway inflammation (*66, 71*). The latter was dependent on TLR-4 expression by mast cells.

Interestingly, we observed a significant enhancement of cytokine production by mast cells upon combined TLR-4 and FcεRI triggering, in particular those cytokines that are known for their role in recruitment and activation of neutrophils and eosinophils (*71-73*). Our results further suggest that basophils are an important source of IL-4 when triggered through TLRs and FcεRI, in line with findings in both human and mouse that basophils are the main source of IL-4 during viral infections and allergen challenge (*74-76*).

Therefore, synergy between TLRs and FcεRI triggering may significantly contribute to allergic reactions, mainly through their actions on basophils and mast cells during the effector phase of allergic responses (Figure 3).

TLR AND FC RECEPTOR ACTIVATION IN AUTOIMMUNITY

As described in chapter 12, many autoantibodies have specificity for TLR ligands, and we propose that this plays a role in the initiation of autoantibody responses, as triggering of TLRs in B cells is thought to mediate tolerance escape. Furthermore, the tissue damage that is associated with chronic inflammation in autoimmune disease often leads to release of endogenous TLR ligands, so-called DAMPs (*77-81*).

Many studies have suggested a role for TLR signaling in the initiation of autoimmune responses, either through its effect on autoantibody production, or through enhancing autoreactive Th cell responses (*82-89*). Knockout animals for TLRs or MyD88 have often reduced autoreactive T cell responses, and such responses are aggravated when immunization is done in the presence of TLR agonists or pathogens (*90*). In vivo, a few studies suggest that TLR ligands can enhance inflammatory responses mediated by autoantibodies. TLR-4 signaling was shown to contribute significantly to thrombosis mediated by anti-phospholipid antibodies (*91*). The use of a TLR-4 antagonist after induction of collagen-induced arthritis led to a reduction in arthritis, suggesting a

contribution of TLR-4 to the inflammatory response mediated by Th cells and autoantibodies (*92*). In addition, both TLR-2 and TLR-4 deficiency led to reduced arthritis symptoms in passive serum-transfer induced arthritis (K/BxN) (*93, 94*).

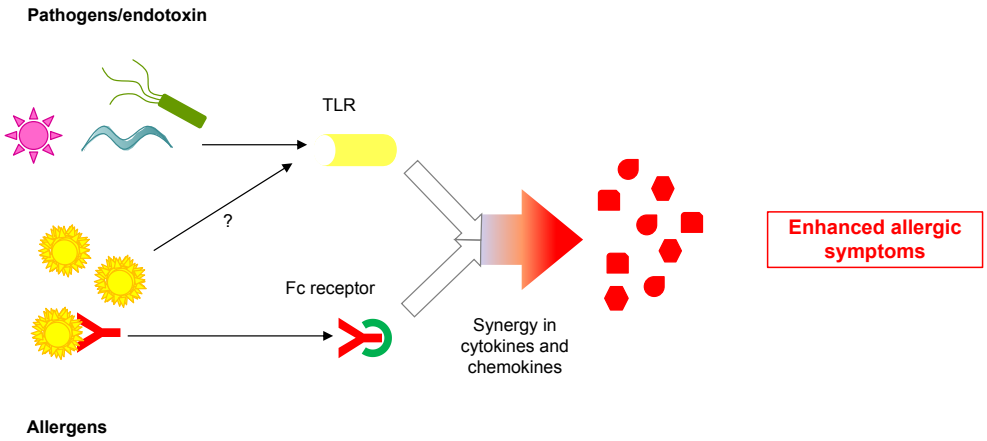

Figure 3. Enhancement of allergic responses through triggering of TLR by pathogens or allergens. Allergic exacerbations are associated with the presence of viral or bacterial infections. In addition, some allergens have been suggested to directly trigger TLRs. When TLR ligands derived from these pathogens or allergens are present at the same time as the allergen to which mast cells and basophils are sensitized, synergy in the production of cytokines and chemokines occurs, thereby leading to enhancement of allergic symptoms.

We proposed in chapter 11-13, that TLR ligation may contribute to autoantibody-induced chronic inflammation, through their synergistic action on myeloid cells, including mast cells. Although the functional role of mast cells during autoantibody-mediated autoimmune disease is not yet clear, we were able to show for the first time that human mast cells can be activated by anti-citrullinated protein antibodies. The synergy observed when the activation by autoantibodies was combined with TLR ligands present in synovium of RA patients suggests that mast cells can significantly contribute to inflammatory responses in RA (Figure 4).

IL-33 AS IMMUNOMODULATORY CYTOKINE DURING ANTIBODY-MEDIATED RESPONSES

Whereas TLR-mediated activation of mast cells and basophils can generally lead to enhanced inflammatory responses, IL-33 is associated with modulatory effects, in particular through their interaction with monocytes. IL-33 specifically enhanced the production of type 2 cytokines (IL-5, IL-13, IL-10) induced by IgG immune complexes in mast cells. In basophils, IL-33 enhanced IL-4 and histamine release induced by FcεRI triggering. This led to dampened TNF-α production by monocytes in response to LPS.

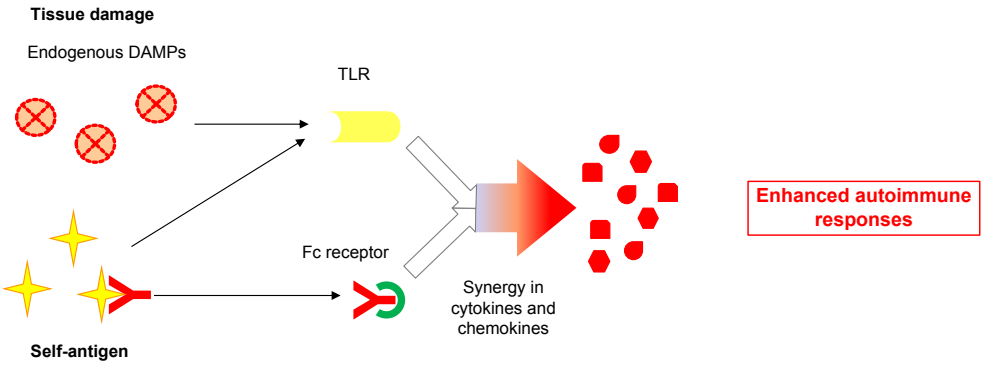

Figure 4. Enhancement of autoimmune responses through triggering of TLR and autoantibodies. Autoimmune disease is often associated with the presence of endogenous DAMPs, released as a consequence of tissue damage. Self-antigens against which autoantibodies are produced are also often recognized by TLRs. Combined activation of mast cells through TLR and Fc receptors during autoimmune responses can enhance their cytokine production, thereby leading to enhanced inflammation.

The reason why IL-33 may have dual roles in allergy and autoimmune disease may lie in its ability to specifically drive the release of type 2 cytokines while inhibiting TNF-α by monocytes. As described above, allergy is driven largely by type 2 cytokines, IL-4, IL-5 and IL-13.

Therefore, enhancement of the production of these cytokines by basophils and mast cells may contribute to allergic symptoms. In particular mast cell-derived IL-13 has been shown to contribute to airway hyperresponsiveness in response to IL-33, although IL-13 derived from innate lymphoid cells is likely representing an additional important source of IL-13 as well (*95-98*). Although studies in mice confirm the regulatory action of basophils on the monocyte/macrophage lineage (*99, 100*), the strong enhancement of type 2 cytokines by IL-33 directly on basophils and mast cells may dominate these immunomodulatory effects, thereby enhancing allergic inflammation.

In autoimmunity, different effects have been observed with IL-33. Several studies showed a reduction in autoreactive responses, for example through reducing autoreactive Th17 responses or through induction of alternatively activated macrophages (*101-104*). In contrast, IL-33 was shown to exacerbate disease in most, but not all, mouse models of arthritis (*105-109*). Interestingly, IVIg was shown to upregulate IL-33, and, via the release of IL-4 by basophils, induced alternatively activated macrophages, thereby reducing inflammation in a mouse model of arthritis (*110, 111*).

The results of this thesis suggest that IL-33 can have both pro-inflammatory and anti-inflammatory actions through mast cells and basophils, by enhancing Th2 immune responses and at the same time reducing monocyte-mediated inflammation. This dual role may explain why contrasting results are obtained on the role of IL-33 in autoimmune disease.

T CELL INTERACTIONS

The second part of this thesis focused on the interaction between mast cells and $CD4^+$ T cells. I showed that human mast cells can contribute to T cell activation, through antigen presentation, co-stimulation, and expansion of Th17 cells (Chapters 7-9).

The antigen presenting capacity of mast cells is an area of debate (*112*). Several recent studies, including the results of this thesis suggest that human mast cells can function as antigen presenting cells and can provide co-stimulation to T cells (Figure 5) (*113, 114*). In mice, antigen presentation by mast cells can induce activation of memory Th cells, but not, or only poorly, of naïve $CD4^+$ T cells, suggesting that mast cells are probably mostly involved in activation of memory $CD4^+$ T cell responses (*115*). The results of this thesis support this hypothesis. First of all, although HLA class II expressing mast cells were present in tonsil, their frequency and expression levels of HLA class II are low, in particular compared to that of professional antigen presenting cells, such as dendritic cells. Furthermore, we found that human mast cells do not produce any of the cytokines required for skewing of naïve $CD4^+$ T cells into classical Th cell subsets, such as IL-4, IL-12, or IL-23 (*116*), in line with their inability to induce skewing of naïve Th cells (Chapter 9). In some studies, protein antigen processing and presentation by mast cells was more efficient when the protein was complexed with IgG or IgE antibodies, suggesting enhanced antigen presentation during memory responses (*117-119*). Together, these findings suggests that mast cells probably do not represent a major cell population involved in the priming of naïve $CD4^+$ T cells in lymphoid organs.

Although the contribution of antigen presentation by mast cells has not been directly addressed in vivo, a number of studies have studied the role of mast cells in modulating T cell responses. First of all, many studies have shown that mast cells can direct the lymph node hypertrophy and migration and activation of both T cells and dendritic cells, for example through releasing exosomes containing TNF (in mice) (*120-124*). This process occurs for example in response to infection, but may also play a role in allergic reactions (*125-130*).

In contrast to these studies suggesting an activating role of mast cells in T cell responses, several studies have also suggested a role for mast cells in perpetuation of Treg responses (*124, 131, 132*). This effect is most often mediated through mast cell-derived IL-10 (*133-136*). Interestingly, a vast number of studies have shown that Tregs can also regulate mast cell-mediated responses, such as during anaphylaxis, suggesting a bidirectional crosstalk between T cells and mast cells in peripheral tissues (*137-141*). Not much is known about the effect of conventional T cells on mast cell function, although our studies indicated that T cells can modulate mast cell phenotype inducing upregulation of HLA class II.

In addition to their role as accessory cell in priming of $CD4^+$ T cell responses by dendritic cells, and their role in activating Treg cells, mast cells may play a role in the promotion of proinflammatory Th cell responses. This has been studied in particular in the context of autoimmunity. In EAE, a mouse model of multiple sclerosis, mast cells were required for activation of adoptively transferred T cells (*142*). In line with this, both total and collagen-specific Th17 cells were reduced in mast cell deficient mice upon induction of collagen-induced arthritis (*143*). What determines this balance between promoting proinflammatory Th cells or Treg cells is not clear, but it seems to be influenced mostly by the inflammatory context; e.g. infection versus tolerance (*144*). In agreement with this, one study showed that mast cells can convert Treg into Th17 cells during autoimmune disease (EAE) in mice, suggesting that mast cells can alter T cell phenotype when tolerance breaks (*145*).

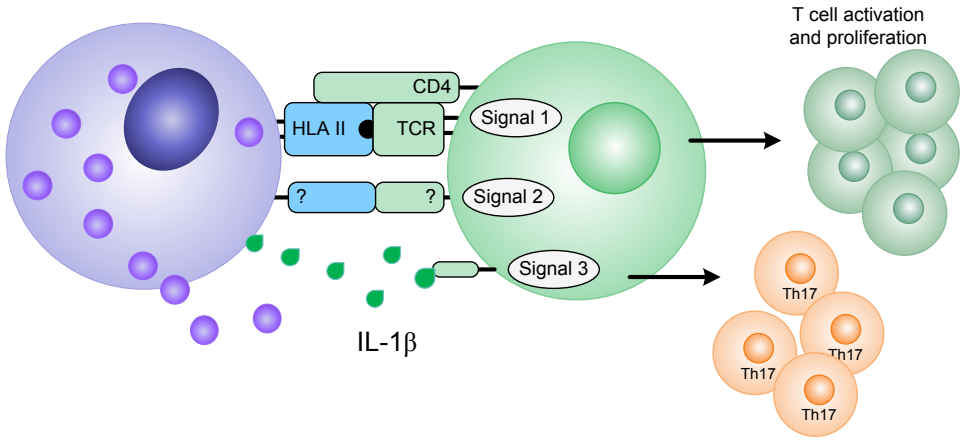

Figure 5. Mast cells interactions with $CD4^+$ T cells lead to T cell activation and Th17 expansion. The results of this thesis show that human mast cells can directly activate T cells by providing all 3 signals required for T cell effector function: 1) Antigen presentation through HLA class II; 2) Co-stimulation, through a CD28-independent mechanism; 3) Expansion of Th17 cells through inflammasome-independent IL-1β.

Importantly, our study showed that mast cells could specifically expand Th17 cells when they were activated through TLR or Fc receptors. Both chronic allergy and autoimmunity are often associated with Th17 cell responses, which are thought to contribute to disease, through neutrophil recruitment as well as tissue-specific effects (*146-153*). Our findings of Th17 cell expansion upon Fc receptor triggering of mast cells provides a potential link between antibody-mediated inflammation and pathogenic Th17 responses. These results together suggest that human mast cells have the complete molecular makeup to induce robust T cell activation, and when activated can drive the expansion of Th17 cells.

CHRONIC INFLAMMATION

Although the role of specific receptors such as TLRs and Fc receptors in chronic inflammation become increasingly known, there is a limited understanding of the behavior of immune cells during chronic inflammation. In chapter 10, we attempted to gain understanding of the function of mast cells in chronic Fc receptor mediated responses. We hypothesized that repeated activation through FcεRI could lead to mast cell-intrinsic changes reflecting what happens during chronic Fc receptor activation in tissue. We observed several changes in mast cell phenotype and function, which were correlated to gene expression in chronic allergy. These changes modified mast cell function, by increasing their expression of molecules involved in antigen processing and presentation, increasing their responsiveness to TLR stimulation, and increased expression and production of chemokines (Figure 6).

CHEMOTAXIS

Although some of the secreted molecules involved in acute IgE-mediated mast cell responses were downregulated, mast cells remained fully able to degranulate, and had increased expression of several chemokines, including CCL18 (Table 1). The chemokines that were upregulated are mostly involved in recruitment of T cells and granulocytes. As infiltration of these cell types can lead to local inflammation, epitope spreading and tissue remodeling, this suggests that mast cells, by retaining or even increasing their chemotactic capacity, may contribute to chronic inflammation by sustaining local leukocyte infiltration.

Interestingly, CCL18, the most highly upregulated gene, is specifically upregulated in chronic inflammatory conditions, such as different forms of chronic allergy and chronic autoimmune diseases (*154-156*). There is no mouse homologue of this chemokine, therefore its role in vivo has been difficult to establish. CCL18 has been mainly shown to

play a role in chemotaxis, in particular that of naïve T cells and memory Th2 cells (*157-160*).

These results suggest that the chemotactic function of mast cells changes during chronic inflammatory stimulation, and can induce several pro-inflammatory effects including persistent recruitment of granulocytes and T cells.

Table 1. Chemokines specifically upregulated after repeated stimulation of mast cells through FcεRI

Gene	Receptor	Cell types recruited
CCL5	CCR1, CCR3, CCR5	T cells, eosinophils, basophils
CCL7	CCR1, CCR2, CCR3	Monocytes
CCL18	CCR8	T cells (Th2)
CCL24	CCR3	Eosinophils, resting T cells
CXCL1	CXCR2>CXCR1	Neutrophils
CXCL2	CXCR2	Granulocytes
CXCL5	CXCR2	Neutrophils
CCL5	CCR1, CCR3, CCR5	T cells, eosinophils, basophils
CCL7	CCR1, CCR2, CCR3	Monocytes
CCL18	CCR8	T cells (Th2)
CCL24	CCR3	Eosinophils, resting T cells

Data obtained from gene expression analysis as shown in Chapter 10; information about chemokine receptors and recruited cell types were obtained from Zlotnik et al. (*161*)

T CELL ACTIVATION

After repeated Fc receptor triggering of mast cells, we also observed enhanced expression of several genes involved in antigen processing and presentation. As described in the part about mast cell-T cell interactions, we showed that human mast cells can function as antigen presenting cells, and that these results therefore may have important implications for the capacity of mast cells to activate $CD4^+$ T cells. $CD4^+$ T cells play an important role in chronic inflammation, for example through driving the production of allergen-specific antibodies or autoantibodies (*162-165*).

Although not much is known about the exact antigen processing pathways in mast cells, repeated triggering through Fc receptors induced upregulated expression of HLA class II molecules, costimulatory molecules (e.g. CD86), and cathepsins S. Therefore, our results suggest that mast cells may have increased capacity for antigen presentation during chronic Fc receptor-mediated inflammation, thereby potentially contributing to T cell activation in the local tissue environment.

TLR RESPONSES

Another important function of mast cells that was enhanced after repeated stimulation through Fc receptors was their response to bacteria. This enhancement was characterized by upregulated expression of several TLRs, and we were also able to show increased cytokine production in response to LPS, a prototype TLR-4 ligand.

Activation of TLRs is thought to initiate a positive feedback loop of inflammation through inducing tissue and cellular damage thereby leading to sustained release of endogenous TLR ligands (*80, 81*). Interestingly, those TLRs most well-known for their involvement in responses to DAMPs, TLR-2 and -4, were upregulated in mast cells after repeated Fc receptor triggering (*166*). Macrophages in synovium of RA patients were also found to exhibit increased expression of TLR-2 and -4, suggesting that upregulation of TLRs may be a common mechanism in different myeloid cell types during chronic inflammation (*167*).

Given the importance of TLRs in the response to endogenous DAMPs, these findings suggests that TLR responses may be enhanced during chronic antibody-mediated responses and that this enhanced TLR responsiveness by mast cells may contribute to this positive feedback loop during chronic inflammation.

TISSUE REMODELING

Chronic inflammation is often characterized by tissue remodeling, leading to long-term, irreversible changes in tissue homeostasis. Possibly the most striking example is rheumatoid arthritis, where loss of bone and cartilage are characteristics of such tissue remodeling.

One of the biological pathways significantly upregulated in mast cells after repeated Fc receptor triggering was wound healing, a process closely related to tissue remodeling, and involving several different processes including coagulation, inflammation, angiogenesis, and tissue regeneration. We observed upregulated gene expression of several tissue-remodeling enzymes and extracellular structural proteins (MMP25, osteopontin, and possibly PADI4) allowing for a direct influence of mast cells on tissue homeostasis (*168-171*).

In addition to these direct effects, mast cells have been shown to have indirect effects as well, such as through activation of stromal cells, and cleavage of pro-MMP enzymes secreted by other cell types (*172-174*). For example, CCL18, although most well-known for its chemotactic functions, has also been shown to induce fibroblast activation and collagen production (*175*). In addition, besides its role in bone metabolism, osteopontin

is associated with a variety of effects associated with tissue repair and fibrosis, presumably through its effect on fibroblasts, epithelial cells and macrophages (*176-179*).

Importantly, although we used an in vitro model of mast cell activation, several changes observed were linked to gene expression in chronic inflammatory conditions. For example, both CCL18, osteopontin are highly upregulated in asthma and allergy, and were associated with markers of tissue remodeling in several chronic inflammatory diseases (*180-189*). These molecules were also found to be expressed by mast cells in such chronic inflammatory diseases when analysed ex vivo (*180, 190*). Therefore, our results show that mast cells upregulate several molecules involved in tissue remodeling after repeated Fc receptor triggering, suggesting that mast cells can significantly contribute to chronic inflammation through modulation of tissue homeostasis.

RESOLUTION OF INFLAMMATION

Inflammatory responses are tightly regulated to prevent tissue damage. When the inflammatory stimulus is eliminated, a resolution phase is initiated, characterized by phagocytosis of apoptotic neutrophils, the production of pro-resolving lipid mediators and anti-inflammatory molecules, as well as removal of tissue debris (*191-193*).

Repeated stimulation of mast cells led to several changes that could potentially contribute to resolution of inflammation. First of all, we observed a dampening of the acute mast cell responses, such as several cytokines and chemokines. Furthermore, the gene expression of several regulatory molecules was upregulated. These include a number of inhibitory receptors (LAIR1, LILRB2, LILRB3, and VSTM1). These and other ITIM motif-bearing inhibitory receptors have been shown to potently inhibit mast cell responses through FcεRI (*194-198*). Therefore, upregulation of these receptors may contribute to dampening of mast cell responses. In addition, heme oxygenase-1 (HO-1, HMOX1), a molecule with anti-inflammatory capacities, was upregulated in mast cells after repeated activation. HO-1 is most well-known as a stress-induced molecule that can suppress activation of myeloid cells (*199-201*). Not much is known about the role of HO-1 in mast cells, but molecules that can upregulate expression of HO-1 diminished mast cell activation, suggesting an immunoregulatory role of HO-1 in mast cells as well (*202, 203*).

Together, these results suggest that in addition to enhanced proinflammatory responses, mast cells also initiate an anti-inflammatory program, characterized by upregulation of immunoregulatory molecules, which could contribute to tissue protection. However, our results also show, that when antigen cannot be removed, therefore leading to repeated or continuous stimulation of mast cells through Fc receptors, proinflammatory effects may dominate over these anti-inflammatory mechanisms.

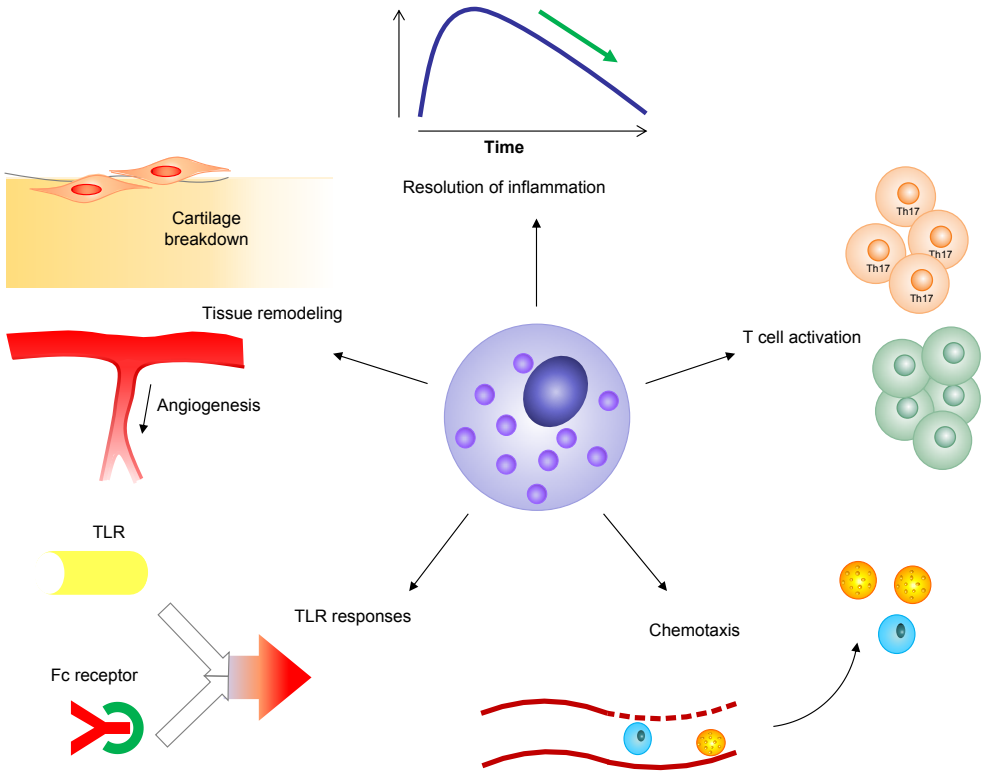

Figure 6. Potential roles of mast cells in chronic antibody-mediated inflammation. Upon repeated Fc receptor triggering, we observed changes in several processes related to chronic inflammation: 1) upregulation of inhibitory receptors, potentially contributing to resolution of inflammation; 2) enhanced antigen processing and presentation through HLA class II; 3) changes in production of chemokines; 4) enhanced TLR responses; 5) upregulation of tissue-remodeling enzymes.

IMPLICATIONS FOR AUTOIMMUNE DISEASE

Autoimmune diseases are characterized by immune responses during which the inflammatory stimulus (self-antigen) cannot be eliminated, leading to chronic inflammation. Our results suggest that mast cells can significantly contribute to chronic inflammation after repeated Fc receptor triggering, through changes in chemotaxis, enhancing T cell activation, upregulating their responsiveness to DAMPs, and upregulation of molecules involved in tissue remodeling. As we only studied FcεRI, it is unknown whether similar processes are upregulated upon repeated triggering of FcγRs in mast cells, which would be highly important in the context of autoimmunity. However, the mast cell response to triggering through FcεRI and FcγRIIA overlap considerably, suggesting similar responses may occur upon repeated stimulation of mast cells with IgG immune complexes (*19*). Interestingly, the chemokine CCL18, which was highly

upregulated in mast cells after repeated FcεRI stimulation, has also been found in increased levels of expression in synovium of RA patients, suggesting that this molecule may be upregulated in mast cells during chronic autoimmune disease as well (*204-206*). Even more interesting, synovial fluid levels of CCL18 were associated with levels of RF, one of the autoantibodies in RA, suggesting that repeated Fc receptor activation as observed in mast cells can present a novel pathway for secretion of CCL18 in response to autoantibodies (*207*). Other molecules upregulated in mast cells after repeated Fc receptor triggering (such as osteopontin) are also associated with autoimmune disease and autoantibodies (*208, 209*). Therefore, these studies may provide more insight into the role of Fc receptor activation during chronic autoimmune disease.

IMPLICATIONS FOR THERAPY OF CHRONIC INFLAMMATORY DISEASES

In chapter 13, we postulated several novel therapeutic approaches for the treatment of autoimmune diseases. As several pathogenic mechanisms overlap between autoimmune disease and chronic allergy, similar strategies may be employed for both types of diseases. Here, I will discuss the implications of these therapies for mast cell-mediated responses during chronic inflammation.

We described therapeutic targets that could influence the interaction between dendritic cells and T cells. Whereas dendritic cells are often viewed as the most potent antigen presenting cells, the second part of this thesis showed that mast cells can also function as antigen presenting cells and can specifically enhance Th17 responses, an effect of mast cells that was also shown in vivo in an mouse arthritis model (*143*). Furthermore, several molecules involved in antigen processing and presentation had increased gene expression in our in vitro model of chronic inflammation, suggesting that chronic inflammatory responses could further enhance T cell activation by mast cells. Therefore, strategies to modulate APC-T cell interactions could inhibit T cell activation by mast cells as well.

First of all, we proposed to modulate activation of antigen presenting cells (dendritic cells and monocytes) by C1q or C1q-like agonists through its effect on LAIR1. In monocytes and dendritic cells, C1q has been shown to inhibit cytokine secretion and maturation, thereby potentially decreasing T cell activation or skewing by cytokines (*210, 211*). Human mast cells also express LAIR1, but the effect of C1q and the functional consequences of ligation of LAIR1 in mast cells has not been studied (*212*). However, it is conceivable that LAIR1 could modulate mast cell function in a similar manner as that of monocytes and dendritic cells, allowing for inhibition of mast cell activation through LAIR1 as therapeutic targets. As mast cell activation was shown to specifically enhance

Th17 cell expansion, inhibition of mast cells using C1q has the potential to modulate chronic inflammatory diseases through its effect on Th17 responses.

Another therapeutic target that we identified to influence T cell activation in autoimmune disease was through inhibition of the presentation of self-antigens to autoreactive T cells by modulation of the levels of Blimp-1, IRF4 or cathepsin S in dendritic cells. Although the expression or function of Blimp-1 or IRF4 in mast cells is not established, we observed increased gene expression of cathepsin S in mast cells after repeated stimulation through Fc receptors. Cathepsin S in mast cells has been shown to be involved in processing of granule proteases, but its role in antigen processing has not been studied (*213*). However, given its central role in antigen processing in other cell types, inhibitors of cathepsin S may potentially modulate antigen processing by mast cells during chronic inflammatory conditions.

This thesis further shows that co-stimulation of T cells by mast cells is B7/CD28-independent. Although blockade of CD28 co-stimulation by CTLA4-Ig (Abatacept) is an effective treatment for autoimmune diseases, inhibition of T cell activation with this therapy is not complete (*214-217*). Furthermore, memory Th17 cells, an important T cell subset in various chronic inflammatory diseases, have been shown to be resistant to inhibition by CTLA-4-Ig, and a recent study indeed showed that treatment of RA patients with CTLA-4-Ig most potently inhibited Th1 responses and had no effect on Th17 cells (*218, 219*). This is interesting as we showed that mast cells specifically enhance Th17 responses, and that T cell co-stimulation by mast cells was independent of B7/CD28. Therefore, blocking the interaction of antigen presenting cells and Th17 cells requires additional blockade besides B7/CD28, and inhibiting the interaction between mast cells and $CD4^+$ T cells could potentially contribute to reducing Th17 cells. More research into the exact pathways that mediate T cell co-stimulation by mast cells may further contribute to inhibition of T cell activation in chronic inflammatory conditions.

Another therapeutic strategy that could affect mast cell-mediated responses is the use of decoy antigens. We proposed in chapter 13 that the use of decoy antigens can reduce the chronicity of inflammatory responses by neutralization of autoantibodies. As reviewed in chapter 12, autoantibodies often target ubiquitously expressed intracellular molecules, which need to be released to the extracellular environment, to allow for binding by autoantibodies. This process can initiate inflammatory responses leading to tissue damage. Inflammation is sustained as the tissue damage can lead to additional release of self-antigen and DAMPs, which further activate myeloid cells and B cells, thereby creating a vicious cycle of tissue damage and autoantibody production. Decoy antigens can bind autoreactive B cell receptors and secreted antibodies, and should be

designed in such as way that they do not trigger TLRs. They can thereby block activation of B cells as well as myeloid cells, and can inhibit tissue damage employed by immune complexes.

Although this strategy would not target mast cells specifically, they are an important target cell type in chronic inflammatory responses, as mast cells express only activating Fc receptors (in most tissues), and as they can release inflammatory cytokines as well as tissue modifying enzymes. We showed in this thesis that combined triggering through Fc receptors and TLR in mast cells can greatly enhance their cytokine release. Furthermore, repeated Fc receptor triggering augmented their responsiveness to TLR ligands. Therefore, blocking of Fc receptor activation in mast cells using decoy antigens has the potential to greatly reduce mast cell activation, also indirectly by reducing their response to DAMPs. Furthermore, repeated Fc receptor triggering led to a modulation of the mast cell response mode, characterized by upregulation of the expression of many genes involved in chronic inflammation, and it is likely that such effects are present in other cell types as well. Therefore, blocking Fc receptor activation might prevent the long-term effects of chronic activation on a cellular level, thereby breaking the vicious cycle of antibody-mediated inflammation.

In conclusion, in this thesis I showed that mast cells can significantly contribute to chronic inflammation through their activation by Fc receptors and TLRs, as well as their interaction with $CD4^+$ T cells, thereby increasing our understanding of their role in allergy and autoimmunity and providing several therapeutic targets to prevent mast cell-mediated immune responses.

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NEDERLANDSE SAMENVATTING

Chapter 15

NEDERLANDSE SAMENVATTING

Mestcellen zijn onderdeel van het afweersysteem, en zijn het meest bekend vanwege de rol die ze spelen in allergie. Tijdens een acute allergische reactie kunnen mestcellen degranuleren, het proces waarbij ze hun granules, vol met stoffen die een afweerreactie kunnen induceren, binnen enkele seconden kunnen uitscheiden. Het is deze functie van mestcellen dat maakt dat ze een beruchte reputatie hebben. Echter, er is nog weinig bekend over de functie van mestcellen buiten de degranulatie om. In de laatste jaren is het steeds meer duidelijk geworden dat mestcellen ook een rol spelen in andere afweerreacties, zoals tijdens chronische allergie en auto-immuunziekten. In dit proefschrift hebben we de functie van mestcellen tijdens deze reacties onderzocht.

ACTIVATIE VAN MESTCELLEN VIA HET AANGEBOREN EN AANGELEERDE AFWEERSYSTEEM

Toll like receptoren (TLR) zijn moleculen die aanwezig zijn in immuuncellen en werken als een sensor voor de aanwezigheid van pathogenen zoals bacteriën en virussen. Als een TLR wordt geactiveerd kan dit een afweerreactie opwekken. In het eerste deel van dit proefschrift hebben we de functie onderzocht van TLR in mestcellen en een ander celtype dat betrokken is bij allergie, de basofiel. In Hoofdstuk 2, 4, en 5 laten we zien dat zowel mestcellen als basofielen TLR tot expressie brengen, en dat stimulatie van deze receptoren tot activatie van mestcellen en basofielen leidt.

Activatie van TLR door pathogenen is een aangeboren eigenschap van het afweersysteem. Wanneer iemand echter herhaaldelijk geïnfecteerd raakt met hetzelfde pathogeen leidt dit normaal gesproken tot een snellere en betere afweerreactie, doordat het aangeleerde afweersysteem een specifieke reactie voor dat pathogeen heeft opgebouwd. Deze bescherming bestaat onder andere uit de productie van antilichamen. Antilichamen kunnen via stimulatie van Fc receptoren ervoor zorgen dat cellen van het afweersysteem het pathogeen direct kunnen herkennen en daardoor een snelle afweerreactie kunnen initieren om het pathogeen uit te schakelen. Mestcellen brengen verschillende Fc receptoren tot expressie, zoals FcεRI, de receptor voor IgE antilichamen, en FcγRIIA, een receptor voor IgG antilichamen. In Hoofdstuk 2, 4, en 5 vroegen wij ons daarom af of de afweerreactie van mestcellen en basofielen verandert als TLR en Fc receptoren tegelijkertijd geactiveerd worden. We hebben laten zien dat de activatie van mestcellen en basofielen sterk verhoogd is onder deze omstandigheden. De mestcellen en basofielen produceerden veel grotere

hoeveelheden cytokines en chemokines. Deze signaalstoffen staan bekend om hun functie om andere immuuncellen te recruteren en activeren en zo een afweerreactie in gang te zetten. Deze synergie in productie van cytokines kan hierdoor bijdragen aan een betere bescherming tegen pathogenen.

Er is echter ook een andere kant aan dit mechanisme. Zowel TLR als antilichamen spelen namelijk ook een rol bij allergie en auto-immuunziekten. Allergie wordt meestal veroorzaakt door IgE antilichamen die een allergeen herkennen. Allergenen zijn eiwitten die overgevoeligheid kunnen veroorzaken. Als een allergisch persoon wordt blootgesteld aan het allergeen leidt dit tot degranulatie van mestcellen en basofielen en wordt zo een allergische reactie in gang gezet. Infectie kan een allergische reactie verergeren, maar het mechanisme waardoor dit gebeurt is niet bekend. In Hoofdstuk 2 en 4 laten we zien dat de activatie van mestcellen en basofielen door TLR en allergeen leidt tot een versterkte activatie en daardoor kan bijdragen aan verhoogde allergische reacties tijdens infecties.

Naast het verhogen van allergische reacties, spelen TLR en antilichamen ook een rol bij auto-immuunziekten zoals reumatoïde artritis (RA). In RA patiënten worden vaak antilichamen gevonden die lichaamseigen eiwitten kunnen herkennen. De antilichamen die gecitrullineerde eiwitten (ACPA) herkennen hebben een sterke associatie met de ontwikkeling van RA. Daarnaast leidt de chronische ontstekingsreactie bij auto-immuunziekten tot uitscheiding van endogene moleculen die TLR kunnen activeren. Over de activatie van mestcellen tijdens auto-immuunziekten is nog niet veel bekend. Daarom hebben we in Hoofdstuk 5 eerst bestudeerd of mestcellen ook geactiveerd kunnen raken door auto-antilichamen. We hebben laten zien dat mestcellen in het onstoken gewricht FcγRIIA tot expressie brengen, en dat mestcellen geactiveerd kunnen worden door ACPA als ze gecitrullineerd antigeen herkennen. In de aanwezigheid van endogene TLR liganden wordt de activatie van mestcellen door ACPA versterkt en wij veronderstellen daarom dat het effect van zowel auto-antilichamen alsmede TLR op de mestcellen kan bijdragen aan chronische ontsteking bij auto-immuunziekten.

Dat de interactie tussen het aangeboren en aangeleerde afweersysteem ook een regulerend effect op het afweersysteem kan hebben laten we zien in Hoofdstuk 3 en 6. Hier bestudeerden we het effect van het cytokine IL-33 op mestcel- en basofiel-activatie. IL-33 is een cytokine dat gerelateerd is met activatie en inhibitie van het afweersysteem, en in verhoogde hoeveelheden voorkomt bij zowel allergie als RA. We

hebben het effect bestudeerd van activatie van mestcellen en basofielen door IL-33 in combinatie met het activeren van FcεRI en FcγRIIA. Dit leidt bij beide celtypes to activatie en productie van cytokines en andere moleculen. Echter, deze uitgescheiden cytokines zorgden voor inhibitie van de activatie van monocyten, en ander type immuuncel. Dit laat zien dat de activatie van het afweersysteem complex is en dat de interactie tussen het aangeboren en aangeleerde afweersysteem tot zowel activatie als inhibitie kan leiden.

We kunnen op basis van deze studies concluderen dat de activatie van mestcellen en basofielen via receptoren van het aangeboren afweersysteem versterkt wordt door receptoren van het aangeleerde afweersysteem. De mestcellen en basofielen kunnen zo bijdragen aan ontstekingsreacties bij allergie en autoafweerziekten, waarbij de specifieke afweerreactie die wordt geinitieerd vooral bepaald wordt door de receptoren van het aangeboren afweersysteem.

MESTCELLEN SPELEN EEN ROL IN ACTIVATIE VAN T CELLEN EN EXPANSIE VAN TH17 CELLEN

T cellen zijn cellen van het aangeleerde afweersysteem. Deze cellen kunnen peptiden herkennen van lichaamsvreemde eiwitten door middel van hun T cel receptor. $CD4^+$ T cellen herkennen alleen peptiden als ze gepresenteerd worden door HLA klasse II moleculen, die tot expressie komen op zogenaamde antigeen presenterende cellen. Wanneer $CD4^+$ T cellen geactiveerd worden gaan ze cytokines produceren en vindt celdeling plaats. De cytokines die $CD4^+$ T cellen produceren kunnen andere cellen van het afweersysteem reguleren en zo bepalen welke specifieke afweerreactie plaatsvindt. De herkenning van peptide door HLA klasse II noemen we ook wel signaal 1. Daarnaast hebben T cellen nog andere signalen nodig; co-stimulatie noemen we signaal 2. Hierbij is er een interactie tussen receptoren op de T cel met costimulatoire moleculen op de antigeen presenterende cel. Deze interactie verhoogd de signaaltransductie van de T cel receptor, en is benodigd voor volledige T cel activatie. Signaal 3 vindt plaats door middel van specifieke cytokines die worden geproduceerd door antigeen presenterende cellen. Deze cytokines kunnen vervolgens de specifieke cytokine respons van de T cellen reguleren, dit wordt het “skewen” of “sturen” van T cellen genoemd.

Over de interactie tussen mestcellen en T cellen is, met name in de mens, nog niet veel bekend. In Hoofdstuk 7 en 8 hebben we eerst onderzocht of mestcellen ook kunnen functioneren als antigeen presenterende cellen. We hebben in Hoofdstuk 7 laten zien

dat mestcellen HLA klasse II tot expressie brengen en peptide en eiwit kunnen presenteren aan $CD4^+$ T cellen (signaal 1). In Hoofdstuk 8 hebben we onderzocht of de mestcellen ook co-stimulatie kunnen geven aan $CD4^+$ T cellen. Met deze studie hebben we laten zien dat mestcellen een sterk co-stimulatie signaal aan de T cellen kunnen geven, en dat deze co-stimulatie niet door de klassieke costimulatie via CD28 wordt gemedieerd.

In Hoofdstuk 9 hebben we onderzocht of mestcellen ook een effect hebben op het skewen of sturen van T cellen. Activatie van mestcellen leidt tot specifieke expansie van Th17 cellen, T cellen die het cytokine IL-17 produceren. We hebben laten zien dat het cytokine IL-1beta geproduceerd door mestcellen voornamelijk verantwoordelijk was voor dit effect.

Deze studies laten zien dat mestcellen een rol kunnen spelen in het initiëren van aangeleerde afweerreacties, door activatie van T cellen en expansie van Th17 cellen. Th17 cellen spelen een rol bij de bescherming tegen extracellulaire pathogenen zoals bacteriën en schimmels, and onze resultaten suggereren daarom dat mestcel activatie kan bijdragen tot betere bescherming tegen zulke pathogenen. De Th17 cellen worden echter ook geassocieerd met auto-immuunziekten en sommige allergiën, en zo kan T cel activatie door mestcellen ook schadelijke effecten hebben.

DE ROL VAN MESTCELLEN TIJDENS CHRONISCHE ONTSTEKING

Chronische ontsteking speelt een belangrijke rol bij verschillende chronische ziektes, zoals chronisch allergiën en auto-immuunziekten. In Hoofdstuk 11 en 12 beschrijven we hoe de samenwerking tussen TLR en antilichamen kunnen bijdragen aan de chroniciteit van auto-immuunziekten zoals RA en Systemische lupus erythematosus (SLE). De antilichamen die een rol spelen in auto-immuunziekten kunnen namelijk vaak antigenen herkennen die tegelijkertijd TLR kunnen activeren. Zoals we in deel 1 van dit proefschrift hebben laten zien kan dit ertoe leiden dat mestcellen, alsmede andere immuuncellen, sterk geactiveerd worden door de combinatie van TLR liganden en antilichamen tijdens auto-immuunziekten.

Hoewel er steeds meer duidelijk wordt over de verschillende receptoren die een rol spelen bij chronisch ontsteking, is er nog weinig bekend over de veranderingen die immuuncellen ondergaan in de context van een chronische activatie van het afweersysteem. In Hoofdstuk 10 hebben we daarom onderzocht hoe de functie van mestcellen verandert als er een herhaaldelijke activatie plaatsvindt via FcεRI. Onze

hypothese was dat herhaaldelijke activatie via FcεRI zou kunnen leiden tot intrinsieke veranderingen in de mestcel die reflecteren wat er gebeurt tijdens chronisch ontsteking. De resultaten van deze studie lieten zien dat er verscheidene veranderingen in het phenotype en de functie van mestcellen plaatsvonden, en dat deze veranderingen waren gerelateerd aan veranderingen in weefsel van patiënten met chronische allergie. Dit bevestigt onze hypothese en suggereert dat herhaaldelijke activatie van het afweersysteem via Fc receptoren kan bijdragen aan veranderingen in weefsel tijdens chronische ontsteking.

CONCLUSIES EN KLINISCHE IMPLICATIES

In Hoofdstuk 13 beschrijven we hoe we kunnen komen tot een nieuwe therapeutische aanpak van auto-immuunziekten. Het doel van nieuwe therapeutische strategiën zou moeten liggen in het aanpakken van de chronische activatie van het afweersysteem zonder daarbij het totale afweersysteem te onderdrukken en daarbij het risico op infecties te verhogen. Auto-immuunziekten zijn getypeerd door een samenspel van vele verschillende immuuncellen en vereisen daarom een aanpak van verschillende onderdelen van het afweersysteem. Hoewel de studies in dit proefschrift met name verricht zijn met mestcellen en basofielen, zijn meerdere van deze processen ook gevonden in andere cellen van het myeloide afweersysteem en zijn de mechanismes dus breder toepasbaar.

We hebben de volgende strategiën geidentificeerd die kunnen bijdragen aan de ontwikkeling van nieuwe behandelingen voor auto-immuunziekten: 1) modulatie van antigeen presentatie aan cellen van het aangeleerde afweersysteem; 2) veranderen van de B cel selectie in de germinal centers; en 3) het gebruik van decoy antigeen om te de formatie van pro-inflammatoire immuuncomplexen te voorkomen. De resultaten van dit proefschrift suggereren dat met name de eerste en derde strategie de bijdrage van mestcellen aan chronische ontsteking kunnen verminderen.

Samenvattend, in dit proefschrift hebben we laten zien dat mestcellen een significante bijdrage kunnen leveren aan chronische ontsteking door middel van activatie via Fc receptoren en TLR, alsmede door hun interactie met $CD4^+$ T cellen. Deze studies dragen daarom bij aan ons begrip van de rol van mestcellen in allergie en auto-immuunziekten en kunnen bijdragen aan de ontwikkeling van nieuwe behandelstrategiën voor deze ziekten.

15

Addendum

LIST OF PUBLICATIONS

1. **Suurmond J**, Habets KL, Tatum Z, Schonkeren JJ, 't Hoen PA, Huizinga TW, Laros JF, Toes RE, Kurreeman FA. Repeated FcεRI triggering reveals modified mast cell function related to chronic allergic responses in tissue. J Allergy Clin Immunol 2016, in press.

2. **Suurmond J**, Dorjée AL, Huizinga TW, Toes RE. Human mast cells co-stimulate T cells through a CD28-independent interaction. Eur J Immunol. 2016, in press.

3. Rivellese F, **Suurmond J**, Habets K, Dorjée AL, Ramamoorthi N, Townsend MJ, de Paulis A, Marone G, Huizinga TW, Pitzalis C, Toes RE. Ability of Interleukin-33- and Immune Complex-Triggered Activation of Human Mast Cells to Down-Regulate Monocyte-Mediated Immune Responses. Arthritis Rheumatol. 2015 Sep;67(9):2343-53.

4. **Suurmond J**, Diamond B. Autoantibodies in systemic autoimmune diseases: specificity and pathogenicity. J Clin Invest. 2015 Jun;125(6):2194-202.

5. **Suurmond J**, van der Velden D, Kuiper J, Bot I, Toes RE. Mast cells in rheumatic disease. Eur J Pharmacol. 2015 May 2. pii: S0014-2999(15)00396-9.

6. **Suurmond J**, Dorjée AL, Knol EF, Huizinga TW, Toes RE. Differential TLR-induced cytokine production by human mast cells is amplified by FcεRI triggering. Clin Exp Allergy. 2015 Apr;45(4):788-96.

7. **Suurmond J**, Zou YR, Kim SJ, Diamond B. Therapeutics to block autoantibody initiation and propagation in systemic lupus erythematosus and rheumatoid arthritis. Sci Transl Med. 2015 Mar 25;7(280):280ps5.

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9. **Suurmond J**, Rivellese F, Dorjée AL, Bakker AM, Rombouts YJ, Rispens T, Wolbink G, Zaldumbide A, Hoeben RC, Huizinga TW, Toes RE. Toll-like receptor triggering augments activation of human mast cells by anti-citrullinated protein antibodies. Ann Rheum Dis. 2015 Oct;74(10):1915-23

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12. Buddingh EP, Schilham MW, Ruslan SE, Berghuis D, Szuhai K, Suurmond J, Taminiau AH, Gelderblom H, Egeler RM, Serra M, Hogendoorn PC, Lankester AC. Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells. Cancer Immunol Immunother. 2011 Apr;60(4):575-86.

CURRICULUM VITAE

Jolien Suurmond werd op 12 oktober 1985 geboren in Gouda. In 2003 slaagde zij voor het VWO aan de GSR Rotterdam. Zij begon datzelfde jaar aan de opleiding Biomedische Wetenschappen aan de Universiteit Leiden. Tijdens haar bachelor deed ze onderzoek op de afdeling Intensive Care in het Leids Universitair Medisch Centrum, onder begeleiding van dr. Koos Burggraaf.

Na het behalen van het bachelor diploma in 2007 vervolgde Jolien haar opleiding met de master Biomedische Wetenschappen aan de Universiteit Leiden. Als onderdeel van de masteropleiding nam ze deel aan een uitwisselingsprogramma met het Karolinska Institute in Stockholm, Zweden en volgde ze verschillende cursussen in klinisch en epidemiologisch onderzoek als onderdeel van het Erasmus Summer Programme aan het Netherlands Institute for Health Sciences in Rotterdam. In 2008 deed ze onderzoek naar de rol van het immuunsysteem bij osteosarcoma en Ewing sarcoma op de afdeling Kindergeneeskunde onder begeleiding van dr. Arjan Lankester en dr. Marco Schilham, waarvoor zij in 2009 de LUMC Student Research Award ontving. Vervolgens sloot zij haar masteropleiding af met een onderzoek naar een nieuw geneesmiddel voor diabetes bij het Centre for Human Drug Research (CHDR) onder begeleiding van prof. Koos Burggraaf. Ze studeerde cum laude af voor haar master Biomedische Wetenschappen in 2009.

Vanaf oktober 2009 tot april 2014 werkte zij als promovendus op de afdeling Reumatologie aan het onderzoek beschreven in dit proefschrift, onder begeleiding van prof. Rene Toes en prof Tom Huizinga.

Momenteel werkt Jolien als postdoc aan het Feinstein Institute for Medical Research onder begeleiding van prof. Betty Diamond. Met behulp van een fellowship van Mallinckrodt Pharmaceuticals onderzoekt zij de rol van FcγRIIB in autoreactieve B cellen.

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