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block the transport of proteins from the Golgi system to the cell surface or extracellular environment (Chapter 2 and unpublished observations).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.

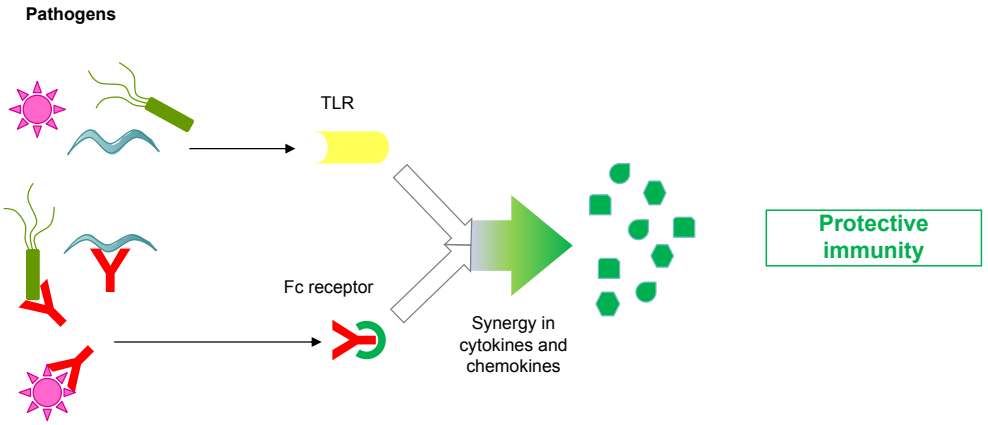

Figure 1. Synergy between TLR and Fc receptors can contribute to protective immunity against pathogens. Pathogens such as bacteria, parasites and viruses can be recognized by antibodies during secondary responses. These antibodies can trigger mast cell activation. When PAMPs from the pathogens trigger 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.

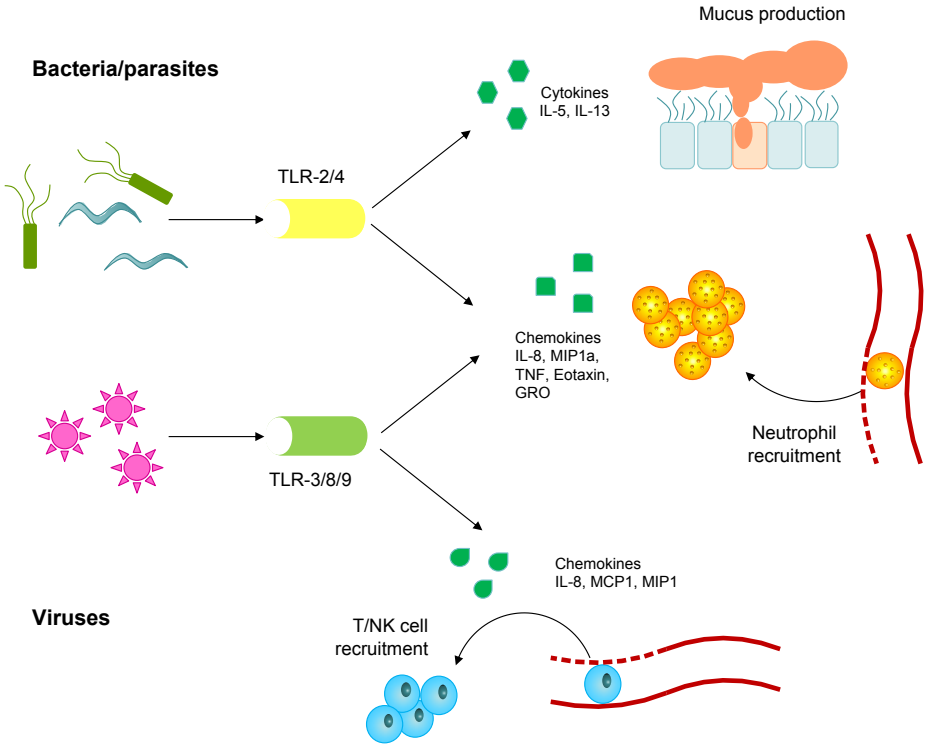

Figure 2. Pathogen-specific responses mediated through triggering of specific TLRs. The type of pathogen can fine-tune the response mode of mast cells. Bacterial and parasitic TLR ligands, through production of specific cytokines in response to triggering of TLR-2 and -4, can induce mucus production 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*).

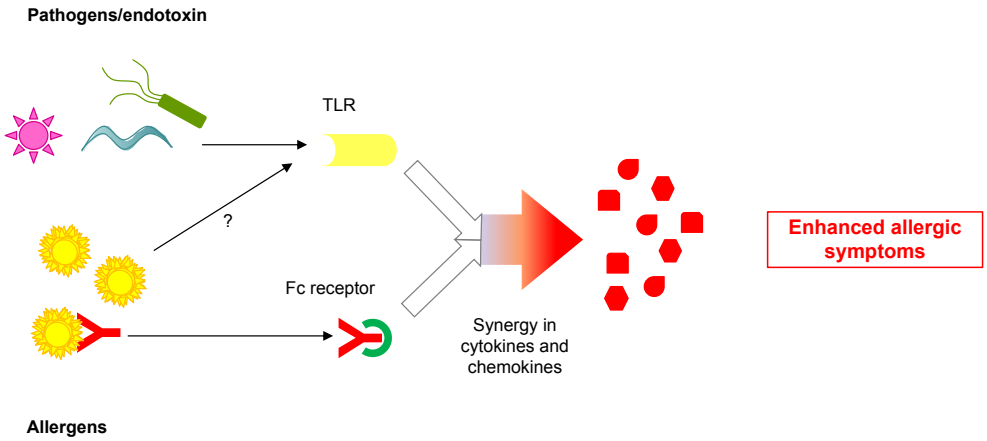

Figure 3. Enhancement of allergic responses through triggering of TLR by pathogens or allergens. Allergic exacerbations are associated with the presence of viral or bacterial infections. In addition, some allergens have been suggested to directly trigger TLRs. When TLR ligands derived from these pathogens 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.

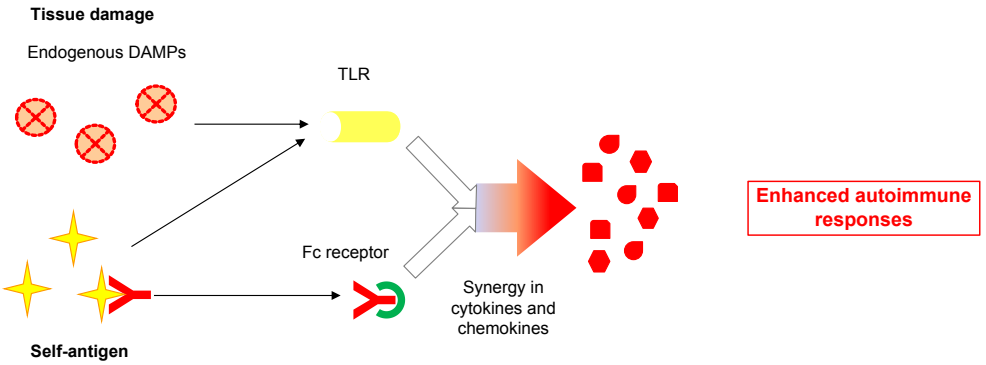

Figure 4. Enhancement of autoimmune responses through triggering of TLR and autoantibodies. Autoimmune disease is often associated with the presence of endogenous DAMPs, released as a consequence of tissue damage. Self-antigens against which autoantibodies are produced are also often recognized by TLRs. 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*).

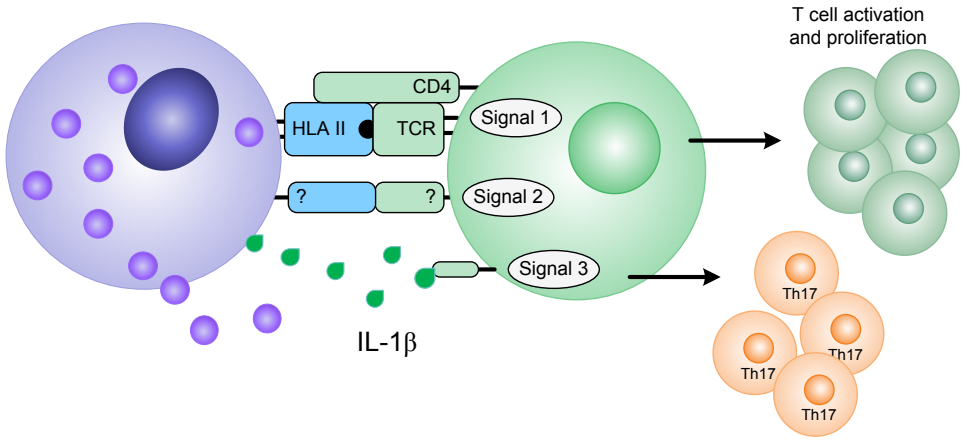

Figure 5. Mast cells interactions with $CD4^+$ T cells lead to T cell activation and Th17 expansion. The results of this thesis show that human mast cells can directly activate T cells by providing all 3 signals required for T cell effector function: 1) Antigen presentation through HLA class II; 2) Co-stimulation, 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.

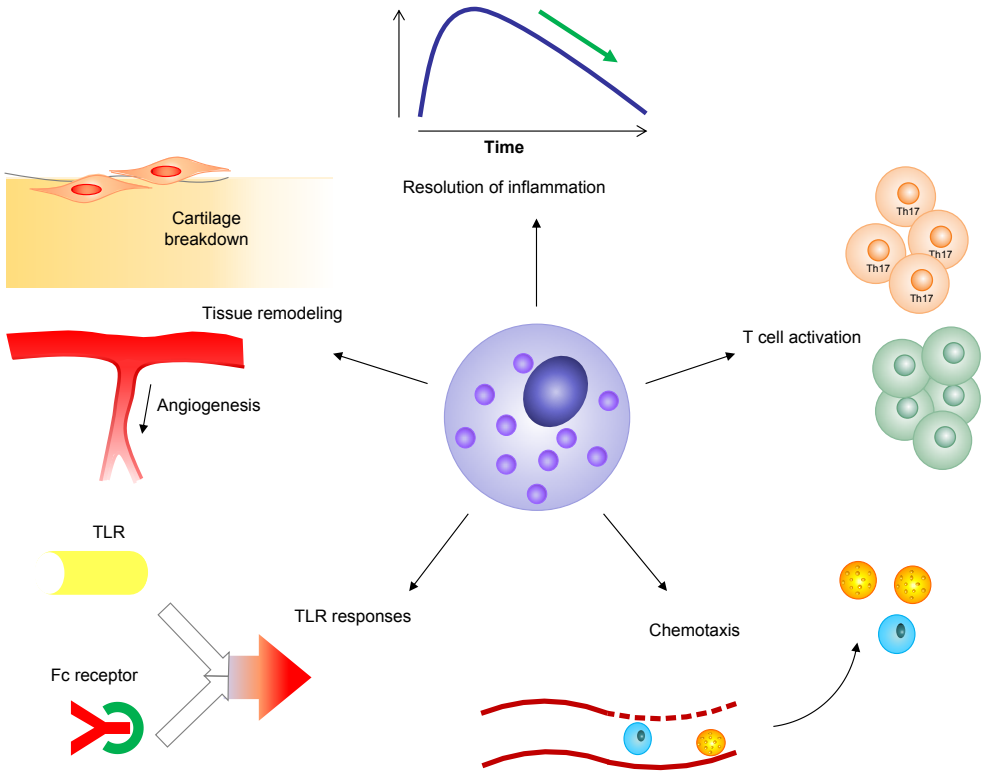

Figure 6. Potential roles of mast cells in chronic antibody-mediated inflammation. Upon repeated Fc receptor triggering, we observed changes in several processes related to chronic inflammation: 1) upregulation of inhibitory receptors, potentially contributing to resolution of inflammation; 2) enhanced 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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