Mast cell-mediated immune modulation in experimental Rheumatoid Arthritis and Atherosclerosis

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Magna opera Domini, exquirenda omnibus, qui cupiunt ea.

"Machtig zijn de werken van de Heer, wie ze liefheeft, onderzoekt ze."

Psalm 111 vers 2

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Chapter 1

General Introduction

Introduction

The immune system is constantly challenged by a multitude of environmental agents that attempt to break through anatomical barriers such as the skin and intestinal tract to reach the interior of the human body. In the defense towards these invading pathogenic agents, mammals are equipped with a powerful immune system that comprises non-cellular effector mechanisms as well as immune cells. The immune system can roughly be dived into two parts: the innate and the adaptive arm of immunity. Hallmark of innate immunity is the rapid activation in a "non-specific" manner without the development of immunological memory. Cells of the innate immune system can found in various tissues, especially at sites that are in close proximity to the external environment. Tissue resident dendritic cells and mast cells are the first immune cells to encounter these invading pathogens. Together with other types of immune cells such as natural killer cells, neutrophils and macrophages these cells belong to the innate arm of the immune system. Adaptive immunity takes longer to establish but it is highly specific and very potent and has memory. Key players in adaptive immunity are dendritic cells, T and B cells, responsible for cellular and humoral immunity respectively.

Mast cell biology

The history of mast cell biology starts with Paul Erhlich's thesis in June 1878. In his thesis he describes a cell type that is clearly visible and distinguishable from other cells with his new aniline dye. The "well-fed appearance" of the cell led him to designate these cells as 'Mastzelle' [1]. He described the presence of large cytoplasmic granules inside the cell, which he thought to have a nutritional function. Currently, it is known that these granules contain large amounts of preformed mediators such as proteases, cytokines and other mediators. Mast cells reside in many different tissues throughout the body but predominantly at sites near the body surface, such as the skin, the airways, and the intestinal tract, but also close to the vasculature and joints [2].

Nowadays, mast cells are regarded as critical effector cells in the acute phase of bacterial and viral infections as well as in the immune response towards parasites. Besides the critical role in host defense, mast cells are also implicated in a number immune driven disorders such as rheumatoid arthritis and cardiovascular diseases.

Mast cell development and heterogeneity.

Mast cells originate from multipotent hematopoietic stem cells in the bone marrow. Mast cell progenitors (MCP) circulate as immature precursors derived from the bone marrow via the vascular system into peripheral tissues. In connective or mucosal tissues, the MCPs mature into tissue resident mast cells under influence of several growth factors [3]. The micro-environment is essential for mast cell development. Especially stem cell factor (SCF), produced by stromal cells, is an essential growth, differentiation, proliferation and survival factor for both murine and human mast cells [4,5].

Binding of SCF to its receptor c-Kit (CD117) leads to the activation of its intrinsic kinase activity that controls the transcription of different mast cell-specific genes. All hematopoietic progenitor cells express c-Kit, but downregulate it upon differentiation into all leukocyte lineages except for mast cells, which express c-Kit throughout their lifespan, thus remaining responsive to SCF. Mice with mutations in the c-Kit receptor locus, Kit W/W^{-v} and $KitW^{sh}/W^{sh}$, lack mast cells [4,6].

Other key factors that influence mast cell development and survival are Interleukin (IL)-3, IL-4, IL-5, IL-6, IL-9, IL-10, Interferon γ (IFNγ), Nerve Growth Factor (NGF), Transforming Growth Factor- β (TGF-β), Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and thrombopoietin (TPO) [7]. The lifespan of a mast cell is believed to be relatively long, as radioactively labeled mast cells were still detectable at 84 days after injection of the radioactive marker [8].

The phenotype of the mast cell differs between the various tissues in which they reside. This is mostly due to the micro-environment and the presence or absence of the above-mentioned growth factors at the site were the MCP differentiate into mast cells [7].

Human mast cells can be divided into MC_T (tryptase-positive, chymase-negative) and MC_{TC} (tryptase-positive, chymase-positive) mast cells [9,10]. The MC_{TC} subset can be found in connective tissue such as skin, submucosa, usually in intimate contact with microvascular and neuronal networks. The MC_T subset is mainly found at mucosal and epithelial surfaces within the gut and lung [11]. However, the distribution of the subsets in humans is not as clear as in mice and is altered in diseases such as rheumatoid arthritis were both subsets are present within the synovium [12].

In mice and rats, two major types of mast cells are described; the mucosal (MMC) and connective tissue type (CTMC) mast cell. This distinction is based on the location, cell size, cellular content and staining characteristics. The human MC_T share characteristics with the murine MMC mast cells, while the human MC_{TC} share characteristics with the murine CTMC mast cells [11]. The MMC is located predominantly in the epithelium of the intestinal and respiratory tract. MMCs are smaller and contain fewer granules compared with CTMC, and they express mouse mast cell chymase (mMCP)-1 and -2 but not tryptase [13]. Interestingly, the MMC population, but not the CTMC population, expands upon T cell-dependent responses towards intestinal parasites indicating that MMC are dependent on T cells for their survival [13,14]. The CTMC type can be found throughout the body in various tissues such as the skin and peritoneal cavity, and they express mMCP-4, -5, -6 and carboxypeptidase A. The phenotype of mast cells has been shown to be dynamic, since MMC can differentiate into CTMC but also vice versa [15].

Receptors expressed by mast cells

In order to respond to stimuli such as pathogens, mast cells express a variety of receptors such as Fc-receptors, pattern-recognition receptors and complement receptors.

Fc-receptors

Antibodies are a crucial part of the adaptive immune system. They bind to antigens via the variable part of the Fab fragment leading to the formation of immune complexes. The constant region of the antibody, the Fc part, can bind to C1q and Fc-receptors. The Fc-receptors are widely expressed by many (non)-immune cells, but in particular by innate immune cells. Each immunoglobulin isotype (IgA, IgM, IgE and IgG) binds to a specific Fc-receptor: IgA binds to FcαR, IgM to FcμR, IgE to FcεR and IgG to FcγR [16]. These receptors combine the specificity of adaptive immunity with the powerful effector functions of innate cells. Mast cells express receptors for IgE (FcεRI) and IgG (FcγR), which will be discussed below.

FcεRI receptor

A main characteristic of human and murine mast cells is the expression of the high affinity receptor for immunoglobulin E (IgE), the FcεRI. It is composed of an α-chain, which is responsible for binding IgE, a β-chain, important for the amplification of the intracellular signaling, and a disulphide linked y-chain, needed for the initiation of the intracellular signaling cascade [17]. The FcεRI binds IgE with a very high affinity in the absence of antigens. As a result, mast cells are coated or sensitized with IgE molecules that can bind to specific antigens. To activate mast cells via the FcεRI a certain antigen needs to bind to the IgE molecule and crosslink at least two IgE molecules bond to their receptors. This causes the activation of an internal signaling cascade which includes activation of tyrosine kinases, such as Syk, Lyn, Fyn, and BTK and phosphorylation of numerous adaptor proteins [18,19]. Finally, this cascade leads to cytoskeletal rearrangements resulting in the release of the preformed mediators stored in the granules inside the cells, a process referred to as degranulation. This is the most powerful and fastest way of activating mast cells and within seconds they are able to release their mediators into the environment.

Fcy Receptors

Receptors for the Fc part of IgG antibodies, FcyRs, bind extracellular monomeric IgG's or immune complexes. To date, six FcyRs are described in humans; FcyRI, FcyRIIA, FcyRIIB, FcyRIIC, FcyRIIIA and FcyRIIIB [20] (Table 1). In mice, four different classes of FcyRs have been described; FcyRI, FcyRIIB, FcyRIII and FcyRIV (Table 1). High affinity receptors like FcyRI can bind IgGs with and without antigens, while low affinity receptors like FcyRII/III only bind antibodies that have formed immune complexes. Also the different IgG subclasses (human: IgG1-IgG4, mice: IgG1,2a, 2b, 3) bind with varying affinity and specificity to the different FcyRs [21,22]. Stimulation of FcyRs will trigger an intracellular signaling

pathway that leads to activation and/or inhibitory signals. The signal outcome depends on the intracellular motifs of the receptor. Receptors with an immunoreceptor tyrosine-based activation motif (ITAM) will initiate an activating signaling pathway upon receptor aggregation, while immunoreceptor tyrosine-based inhibition motifs (ITIM) are coupled to inhibitory receptors. Upon activation by immune complexes, ITIM is phosphorylated and initiates the recruitment of inhibitory molecules e.g. SHIP [23]. In general, the FcyRs are activating, with the FcyRIIB receptor as the exception of being an inhibitory receptor [24].

The low affinity receptor FcyRIIA is expressed in cultured and isolated human mast cells [25–27]. Cultured human mast cells also express the inhibitory receptor FcyRIIB [26]. Expression of the high affinity receptor FcyRI can be induced by IFNy on human but not on mouse mast cells [28,29]. Murine mast cells constitutively express FcyRIIB and FcyRIIIA [30]. Since FcyRIII expresses the same subunits as the FcεRI, they can trigger a similar response upon activation [31]. Stimulation of freshly isolated peritoneal mast cells or cultured mast cells via either the FcεRI or FcyRIII results in a comparable β-hexosaminidase activity in the releasate, which is an indicator for degranulation [32]. Stimulation of cultured human mast cells with oxLDL-immune complexes, which activate via FcγR, results in release of histamine and tryptase [33]. These data indicate that both human and murine mast cells can also be activated via their activating FcγRs, which is comparable to IgE mediated activation in terms of released mediators.

Table 1: FcR expressed by human and mouse mast cells.

	Receptor	**Ligand**	**Action**	**Ref.**
Human				
	FcεRI	Monomeric IgE	Activating	
	FcγRI (IFNγ induced)	Monomeric IgG	Activating	[28,29]
	FcγRIIA	IgG Immune complex	Activating	[25–27]
	FcγRIIB	IgG Immune complex	Inhibitory	[26]
Mouse				
	FcεRI	Monomeric IgE	Activating	
	FcγRIIIA	IgG Immune complex	Activating	[30]
	FcγRIIB	IgG Immune complex	Inhibitory	[30]

Pattern-recognition receptors

Cells of the innate immune system detect pathogens via several pattern-recognition receptors (PRRs). These PRRs detect components of microorganisms, known as pathogen associated molecular patterns (PAMPs). Each PRR reacts with specific PAMPs, thereby activating specific signaling pathways, each leading to a specific antipathogenic outcome [34]. Several PPRs have been described, such as the nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectins and Toll-like receptors (TLRs). On mast cells, the TLR family is studied mostly. To date, 10 members of the TLRs have been described in the human genome, and 13 are found in the murine genome [35]. Human mast cells express TLR1-7 and 9-10, whereas murine mast cells express TLR1-4 and 6-9 [36]. In general, stimulation of mast cells with TLR agonists results in the production and secretion of cytokines and chemokines, but not in degranulation. However, some reports show that TLR2, but not TLR4 agonists can induce degranulation of both human and murine mast cells, establishing that stimulation of mast cells via individual TLRs results in a very specific receptor-dependent response [37,38].

Complement receptors

The complement system is a highly efficient part of the innate immune system and is characterized by a biochemical cascade that consists of around 30 plasma proteins. Complement activation via one of the three pathways leads to cleavage of C3 and C5. The splice products C3a and C5a are potent inflammatory proteins. Complement receptors are membrane-bound proteins expressed by many (non)-immune cells [39]. Human and murine mast cells express receptors for complement component C3a (C3aR) and C5a (C5aR) [40,41] and binding of complement factors leads to cellular activation and to the secretion of cytokines (C3aR) or degranulation of mast cells (C5aR) [41].

Mediators released by mast cells

Upon activation, mast cells are able to secrete a wide range of mediators. There are two major ways how mast cells release their mediators into the environment. The first process is the release of pre-formed mediators that are stored inside the granules of the mast cells, which is the process referred to as degranulation. Secondly, mast cells can, upon receptor-mediated activation, start to express, produce and secrete chemokines, lipid mediators and cytokines. The release pathway initiated upon mast cell activation is dependent on the type of triggered surface receptor, e.g. activation via the FcεRI will cause degranulation, whereas TLR stimulation will result in the release of de novo produced mediators.

Degranulation

Mast cell degranulation is the active release of granules, which are filled with a large panel of preformed mediators. Several external stimuli can induce mast cell degranulation, of which IgE crosslinking on the FcεRI by a certain antigen is most commonly known.

However, degranulation can also occur after binding of complement factors like C5a, neuropeptides such as substance P and Neuropeptide Y, or by IgG-immune complexes to their specific receptors. Upon activation mast cells will actively release granules into the extracellular environment, which can have a strong local effect, but can also induce systemic events such as anaphylaxis. A large number of (mast cell specific) mediators can be found inside mast cell granules, which are summarized in table 2.

Many of the mast cell effector functions are closely related to the biological action of the mediators present in the granules. For example, proteases like tryptase, chymase and carboxypeptidase have been implicated in tissue remodeling and recruitment of other immune cells [42,43]. The presence of histamine and serotonin is a key characteristic of mast cell granules. They have a potent effect on vascular permeability and contribute to the symptoms of allergic diseases [44]. In addition to several enzymes, mast cell granules also contain preformed cytokines such as TNFα. To date, murine mast cells but not human mast cells are the only immune cells that have preformed TNFα and therefore are an important source of TNFα during acute phase reactions [45]. Furthermore, mast cell derived TNFα is shown to drive both the hypertrophy of the draining lymph nodes and recruitment T cells to the site of infection [46].

Table 2: Content of mast cell granules.

Mediator	Mediator class	Function	Ref.
Tryptase	Serine proteases	Protective (parasitic infections) or damaging functions (autoimmunity)	[48]
Chymase	Serine proteases	Protective (parasitic infections) or damaging functions (autoimmunity)	[49]
Carboxypeptidase A3 (CPA3)	Metalloproteinase	Degradation of toxins derived from snake venom	[50,51]
Histamine	Vasodilator	Increases vascular permeability	[52]
Serotonin	Neurotransmitter	Role in mast cell mediated signaling to nerve endings	[53]
Dopamine	Neurotransmitter	Role mast cell mediated signaling to nerve endings	[54]
Cathepsin B/C/L/D/E	Proteases	Processing of pro-chymases/tryptases/pro-cpa3 to functional proteases.	[55]
TNFα	Cytokine	Pro-inflammatory activities in acute phases	[45]
IL-4	Cytokine	Mast cell driven T_h2 T cell polarization	[56]
β-Hexosaminidase	Lysosomal enzyme	Degradation of bacterial cell wall peptidoglycan as a bactericidal mechanism	[57,58]

(adapted and modified from Wernersson and Pejler Nat. Rev. Immunol 2014 [47])

Cytokine/chemokine release

Besides the release of the above mentioned mediators stored in the granules, mast cell activation also leads to the de novo production and secretion of many different mediators like lipid mediators as well as a wide range of cytokines and chemokines. Table 3 summarizes the majority of mast cell mediators that can be released upon stimulation. Cytokines that are produced by mast cells can be divided into pro-inflammatory and immunomodulatory. The array of mediators released by mast cells depends on the specific activation pathway, which enables mast cells to initiate and modulate the immune response in a manner appropriate for the pathogen.

Table 3: Mediators produced and secreted by mast cells.

Mediator	Function	Ref.
Lipid-derived		
LTC4, LTB4, PGD2 and PGE2	Recruit effector cells, regulation immune response, promote angiogenesis, edema and bronchoconstriction.	[60–64]
Platelet-activating factor	Activates immune effector cells, enhances angiogenesis and induces physiological inflammation	[64]
Cytokine		
TNFα, IL-1α, IL-1β, IL-6, IL-18, GM-CSF, LIF, IFNα and IFNβ	Induce inflammation	[45,65–69]
IL-3, IL-4, IL-5, IL-9, IL-13, IL-15 and IL-16	Induction of a T helper 2 like immunological phenotype	[70,71]
IL-12 and IFNγ	Induction of a T helper 1 immunological phenotype	[72,73]
IL-10, TGF-β and VEGF	Regulate inflammation and angiogenesis	[74,75]
Chemokine		
CCL2, CCL3, CCL4, CCL5, CCL11 and CCL20	Recruit effector cells like monocytes and DCs and regulate immune responses	[76–80]
CXCL1, CXCL2, CXCL8, CXCL9, CXCL10 and CXCL11	Recruit effector cells like neutrophils, T cells and regulate immune responses	[71,81,82]
Other		
Nitric oxide and superoxide radicals	Bactericidal	[83–85]
Antimicrobial peptides	Bactericidal	[86]

(Adapted and modified from Marschall, Nat. Rev. Immunol. 2004 [59])

Physiological and pathophysiological role of mast cells

In a physiological state, mast cells and basophils act as the first line of defense against parasites such as worms and protozoa. Parasites can establish a long lasting, persistent infection in the host and are very efficient in escaping the immune system. Frequently, parasite infections cannot be controlled by cellular and molecular mechanisms alone. Therefore, an IgE mediated response is elucidated that will activate both basophils and mast cells upon encountering the parasite. The release of the described mediators will

create an environment that allows a quick elimination of the parasite.
Nowadays in most industrialized countries a parasitic infection is rare, while hypersensitivity reactions towards antigens like pollen are common. Most of these responses are IgE mediated and are referred to as type I hypersensitivity reactions. Hallmark of a hypersensitivity reaction or allergy is the production of IgE antibodies towards a harmless antigen. Upon contact with the targeted antigen, this will initiate a mast cell mediated immune response, which is similar to the response upon parasite infection.
Next to its contribution to host defense and allergy, mast cells have also been implicated in many immune driven disorders such as asthma, multiple sclerosis (MS), atherosclerosis and arthritis [87–90]. Asthma is characterized by airway obstruction, hyper responsiveness and inflammation. Most of the asthmatic patients exhibit hypersensitivity towards defined environmental allergens, like house dust mite [91]. As in other hypersensitivity type I reactions, IgE is the main immunoglobulin isotype in asthma [92]. Inhalation of allergens will activate IgE sensitized mast cells and induce the subsequent release of mediators like histamine and lipid mediators, which act as bronchoconstrictors [91]. Cytokines like IL-4, IL-5 and IL-13, produced by mast cells, will induce immunoglobulin class switching of B cells to produce IgE. Blockage of IgE by the monoclonal antibody omalizumab reduces both the response to allergens and airway inflammation in asthmatic patients [93].
Mast cells were first observed over 100 years ago in central nervous system (CNS) lesions of MS patients [94]. The expression of mast cell specific proteases is increased during the chronic phase of MS, as measured by microarray analysis. and elevated levels of tryptase are found in the cerebrospinal fluid of MS patients [95,96]. Hallmark of MS is the loss of the myelin sheath around the neurons and mast cell derived proteases are able to degrade myelin sheath proteins [97], indicative of an active contribution of mast cells to the pathology of MS.
Mast cells have also been implicated in a number of other (autoimmune) diseases, such as systemic lupus erythematosus, osteoarthritis and Sjögren's syndrome [98–100]. Most of the data that connect mast cells to these conditions are obtained from observational studies showing mast cell activation during disease.

Mouse models for mast cell deficiency

Over the past decades, the contribution of mast cells to physiological and pathophysiological processes has been studied in mast cell deficient mouse strains. Three frequently used mast cell deficient mouse strains are the WCB6F1 $Kitl^{Sl}/Kitl^{Sl\text{-}d}$ (Sl/Sld) mice, the WBB6F1-$Kit^{W}Kit^{W\text{-}v}$ (W/W^{v}) mice and the $Kit^{W\text{-}Sh}/Kit^{W\text{-}Sh}$ (sash) mice, which all have defects in the SCF signaling pathway. WCB6F1 $Kitl^{Sl}/Kitl^{Sl\text{-}d}$ (Sl/Sld) mice lack SCF due to a loss of function mutation in the SCF gene [4]. The $Kit^{W}Kit^{W\text{-}v}$ (W/W^{v}) mice have a deletion mutation, resulting in a non-functional Kit-protein lacking the transmembrane domain and is therefore not expressed, and a point mutation in the Kit signaling pathway that markedly decreases the activity of the receptor [101]. $Kit^{W\text{-}Sh}/Kit^{W\text{-}Sh}$ (sash) mice contain

a large genetic inversion affecting the transcriptional regulatory elements upstream of the Kit transcription start site on chromosome five [102]. Bone marrow-derived mast cells from wild-type or specific knockouts can be used to reconstitute the mast cell population in these mouse models, therefore are also referred to as mast cell knockin models.

Because c-Kit is not only expressed by mast cells, mutations of c-Kit affect other cells of hematopoietic and non-immune origin. The W/W^v mice suffer from basal neutropenia, anemia, sterility and lack of melanocytes [6]. $Kit^{W\text{-}Sh}/Kit^{W\text{-}Sh}$ mice are fertile and lack anemia, but suffer from other hematopoietic abnormalities, such as expanded myeloid and megakaryocyte populations [103,104]. Therefore, new models of mast cell deficiency have been developed, which are independent of c-Kit mutations.

Cell type-specific knockout mice can be generated by the use of site-specific recombination systems. For example, the Cre/loxP recombination system has been shown to be very efficient and is used frequently to study individual cell types *in vivo* [105]. This system is based on the ability of the enzyme Cre recombinase (Cre) to catalyze recombination between two DNA recognition sites, i.e. the loxP sites. Cre deletes sequences between these sites resulting in a single LoxP sequence, subsequently leading to depletion of the gene of interest. The expression of Cre is usually under control of a cell specific protein, leading to depletion of cells specifically controlled by that protein. To create mast cell-specific knockouts several proteins/receptors have been used, e.g. FcεRIβ, MCPT5 or Cpa3 [106–108]. To specific establish mast cell deficiency, these Cre mice can be crossed with mice that express the diphtheria toxin (DT) under control of a loxP-flanked stop cassette. Expression of Cre activates the expression of DT resulting in cell death [109]. Another possibility is the crossing of Cre-expressing mice with mice that have a floxed allele of an anti-apoptotic gene e.g. Mcl1 [108].

Recently a new inducible mast cell knockout mouse model was presented, in which mast cells can be selectively depleted. In this the so-called RMB (red mast cell and basophil) mouse, the 3'-UTR of the gene encoding the FcεRI β chain contains the human DT receptor (FcεRIβ-DTR), resulting in depletion of mast cells and basophils upon treatment with DT [106]. At 12 days after the DT injection, basophils are completely repopulated, whereas mast cells remain depleted up to at least 2 months [106]. Therefore, this model can be used to study the effects of mast cell depletion when mice display clinical manifestations of diseases such as arthritis and atherosclerosis, which may provide more insight into the active contribution of mast cells in progression of several diseases.

Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a common autoimmune disorder that affects around 0,5 – 1% of the adult population in industrialized countries [110]. A healthy joint is composed of two bone ends covert by a layer of cartilage, which is essential for distribution of pressure on the bones. Furthermore, the cavity inside the joint is filled with synovial fluid that ensures optimal sliding between the joints and is produced by a single layer of synoviocytes that forms the synovial membrane (Fig. 1a). RA is characterized by persistent inflammation of the synovial membrane (synovitis) in the joints. RA starts with the influx of leukocytes, such as monocytes and neutrophils, into the synovial layer leading to thickening of the membrane. The release of mediators by the leukocytes leads to the destruction of cartilage and bone of the joint, which is the hallmark of RA (Fig. 1b). Although all joints in the body can be affected by RA, it affects most commonly the hands, feet and knees [111]. The prevalence of RA differs between different populations. While a very high RA prevalence is found in native American populations, a very low prevalence is reported in South-East Asian populations [112,113]. The incidence of RA is approximately two times higher in women than in men and the prevalence increases with age [114]. RA is a systemic inflammatory disease, which frequently coincides with symptoms like weight loss, fever, increased cardiovascular risk as well as disorders in the vascular system.

The persistent inflammation causes a significant increase in the mortality, morbidity and disability rate in RA patients [110,115,116]. Genetic as well as environmental risk factors have been described for the development of RA. The most important genetic factor is the HLA class II locus. While the presence of the HLA-DRB1*04 gene strongly predisposes to RA, the presence of the HLA-DRB1*13 allele is protective for development of RA [117,118]. It is reported that these alleles are involved in the development or protection of Anti-Citrullinated Protein Antibodies $(ACPA)^{+}$-RA [119]. Environmental risk factors for RA are alcohol intake, low vitamin D levels, low socioeconomic status and smoking [120], and of these, smoking is the most dominant one, which doubles the risk of developing RA [121]. Patients with RA are treated with disease-modifying anti-rheumatic drugs (DMARDs), which reduce the inflammatory response both locally and systemically, prevent progression of joint destruction and improve the function of the affected joints [122]. DMARDs form a heterogeneous collection of drugs of which the mechanisms of action are not completely understood. Nowadays, the most frequently used DMARD is methotrexate, which can be combined with other similar drugs such as sulfasalazine, hydroxychloroquine and leflunomide [123]. Since the immune system plays a dominant role in RA several biological agents have been developed to target specific components of the immune system, such as anti-TNF (infliximab), CD80/86 blockade (Abatacept), anti-CD20 (Rituximab) and IL-6R blockage (Tocilizumab) [110]. These biologicals have proven to be very effective and lead to a therapeutic improvement for RA patients [110]. Current treatment aims to achieve the lowest possible disease activity and ultimately remission. Nevertheless, RA remains to be a major autoimmune disease leading to (partial) disability and loss of productivity, and

eventually to high costs in healthcare [124].

The immune system in Rheumatoid Arthritis

Despite the fact that RA is a considerable health problem for society, relatively little is known about the exact pathology and etiology of the disease. Unquestionably, the immune system plays a very dominant role in the pathogenesis of RA. Leukocytes such as macrophages, neutrophils, mast cells and lymphocytes accumulate during the progression of RA within the synovial tissue and fluid. The active interplay between the innate and adaptive immune system leads to the development of auto-reactive T cells, production of auto-antibodies by B cells and secretion of a variety of inflammatory mediators by innate cells like macrophages, neutrophils and mast cells [125,126].

Hallmark of autoimmunity is the development of a strong immune response toward self-antigens. RA is characterized by the presence of a variety of antibodies targeting (modified) self-antigens like collagen type II, rheumatoid factor, citrullinated (Anti-Citrullinated Protein Antibodies or ACPA) and carbamylated proteins (anti-CarP) [127,128]. Of these

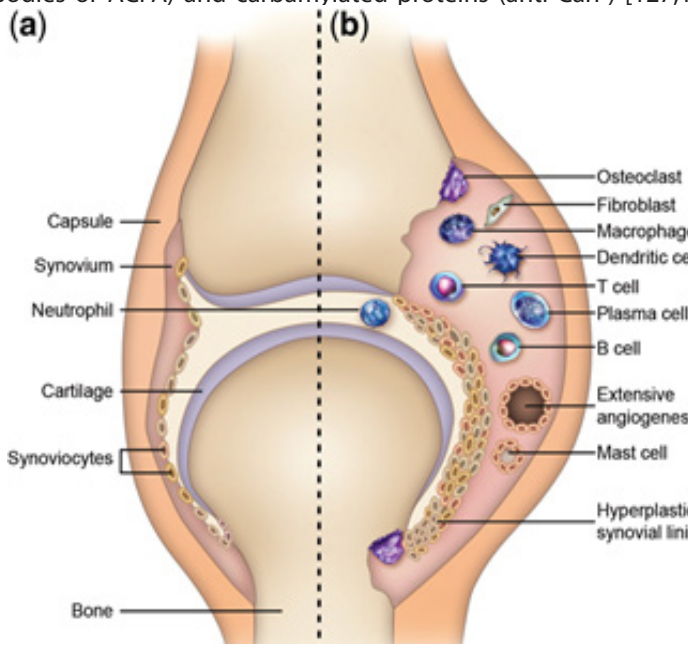

Figure 1. (A) A healthy joint is composed of two adjacent bony ends each covered with a layer of cartilage, separated by a joint space and surrounded by the synovial membrane and joint capsule. (B) Hallmark of Rheumatoid Arthritis (RA) is the inflammatory response of the synovial membrane that is characterized by an influx and local activation of a variety of mononuclear cells, such as T cells, B cells, plasma cells, dendritic cells, macrophages, mast cells, as well as by new vessel formation. Hallmark of RA is bone destruction caused by activated osteoclasts. Bone repair by osteoblasts usually does not occur in active RA. Within the synovial fluid many neutrophils can be found, as well as mediators released by many activated immune cell like neutrophils, plasma cells and mast cells leading to cartilage destruction.

Adapted and modified from Smolen and Steiner Nature Reviews Drug Discovery 2003; 473:488 [121]

antibodies, ACPA are of great interest because they have been shown to be very specific for RA. Only a low frequency of ACPA has been detected in non-RA diseases like systemic lupus erythematosus (5,5%), primary Sjögren's syndrome (13,3%), psoriatic arthritis (9,4%), juvenile idiopathic arthritis (5%) [129–132]. In early RA patients, ACPA can be detected in 50-70% of the cases, which renders ACPA an important clinical biomarker for RA. Target of ACPA are citrullinated proteins, hence their name anti-citrullinated protein antibodies. Citrullination or peptidylarginine deimination is a physiological process catalyzed by a family of enzymes called peptidyl arginine deiminases (PAD-1-4). These enzymes convert the positively charged amino acid arginine to an uncharged amino acid citrulline in the presence of relatively high calcium concentrations [133]. The antigens targeted by ACPA are highly diverse as they show reactivity towards many different citrullinated proteins, such as collagen, vimentin, fibrinogen, enolase, fibronectin, vinculin and histones [134–137]. These citrullinated proteins that have been identified within the synovial compartment and are targets of ACPA. The presence of both a wide array of antigens and high levels of ACPA within the synovial fluid indicates a direct pathogenic role for ACPA in the process of synovial inflammation via e.g. the formation of immune complexes or complement activation [138,139]. Interestingly, ACPA can be detected in the serum for up to 10 years before onset of RA, without any clinical signs of arthritis [140,141].

To date, it is largely unknown how the tolerance of T and B cells is breached in the early phase of RA. Many studies have shown that there is a correlation with the HLA-locus expressed by antigen presenting cells and the development of RA, suggesting a role for T cells in the pathogenesis of RA [119]. Furthermore, high numbers of T cells can be detected in the inflamed synovium and T cells are required in experimental arthritis models [142]. Recently, it was shown that a peptide sequence present in citrullinated vinculin and many microbes, DERAA, can bind to and is presented to T cells via HLA-molecules associated with RA-susceptibility [143]. Nevertheless, direct targeting of T cells by depleting CD4-specific or CD52-specific antibodies has been unsuccessful [144], possibly due to the fact that besides the depletion of pathogenic effector T cells also regulatory T cells (Treg) are depleted. In the rheumatoid synovial joint both T_h1 and T_h17 cells as well as regulatory T cells have been detected [145]. Especially T_h17 cells, producers of IL-17A, have shown to enhance the secretion of inflammatory cytokines by several joint cells like fibroblasts and chondrocytes [146]. Although regulatory T cells have been detected in tissues from RA patients, their functional capacity is described to be limited due to the suppressive effects of TNFα [147].

As mentioned, humoral immunity plays a dominant role in RA and experimental models of arthritis. Throughout the synovium B cells, plasmablasts and plasma cells can be found. Depletion of B cells by the anti-CD20 antibody rituximab has been proven to be effective in RA, as it reduces the level of ACPA antibodies and inflammatory cytokines like IL-6 and TNF, which are amongst others produced by B cells [148].

Innate effector cells, including macrophages, neutrophils, natural killer cells and mast cells

have been implicated in the pathogenesis of RA. Macrophages are central effector cells during synovitis, and they act through the release of a range mediators like cytokines (TNFα, IL-1, IL-6, IL-12, IL-15, IL-18, IL-23), chemokines (MCP-1, IL-8), reactive oxygen intermediates, nitrogen intermediates, matrix degrading enzymes and the expression of MHC class II [149]. These macrophages display an M1-like phenotype and can be activated via many pathways, such as via TLRs, cytokines, immune complexes and lipid mediators. Neutrophils are found in large numbers predominantly within the synovial fluid but also in the pannus region of the inflammation. Upon activation via e.g. immune complexes, they secrete potent effectors of cartilage destruction, such as serine and metalloproteases, but also RANKL and BAFF, which are known to activate osteoclasts and B cells [150]. Over time, mast cells also accumulate within the synovial tissue and produce large numbers of cytokines and chemokines upon activation via one of the many receptors they express. The contribution of mast cells in RA will be discussed in more detail in chapter 2.
To conclude, activated innate effector cells are present in high numbers within the inflamed synovium and are thought to have a great impact on the process of joint destruction. More insight in the contribution of innate immune cells to RA progression could lead to new therapeutic targets that positively modulate the immune response.

Arthritis mouse models
A cornerstone of experimental biomedical research is the use of animal models to explore basic pathophysiological mechanisms. Much of the current knowledge regarding the pathogenesis of rheumatoid arthritis has been obtained using models of experimental arthritis. These models have given much insight into the contribution of the immune system to RA pathology. Nonetheless, none of the available animal models exactly resemble the pathology of human RA, which is the reason that these models are referred to as "arthritis" models instead of RA models. Roughly, the models of experimental arthritis can be divided in either actively (immunization based) – or passively (antibody-infusion based) – induced arthritis.

Collagen induced arthritis (CIA)
This frequently used model for arthritis was discovered in the mid-1970s by Kang et al [151]. In an attempt to raise antibodies towards collagen type II, the authors unexpectedly found that 40% of the immunized rats developed inflammatory arthritis. Subsequent studies have shown that immunization of mice with collagen type II (in the presence of complete Freund's adjuvant) also resulted in the development of arthritis [152]. Since cartilage destruction is largely mediated through autoantibodies against collagen type II, this model resembles rheumatoid arthritis in several aspects [153,154].
The pathogenesis of CIA is rather complex, involving both cellular and humoral immunity. Chronic inflammation in CIA is thought to be mediated by anti-collagen autoantibodies and T_h17 cells. After initiation of the autoreactive response, effector mechanisms include complement and Fc receptor activation, production of IL-1β and TNFα [155–157].

The onset of clinical symptoms occurs around 14 to 21 post immunization, characterized by gradually increasing inflammation of joints in the paws. At the end stage, inflammation becomes less intense and the swelling disappears followed by ankylosis of the affected joints.

Antibody-induced arthritis

The basis for this model are autoantibodies directed to glucose-6-phosphate (GPI), which originate from crossing mice expressing a T cell receptor reacting to self-antigens (KRN-C57BL/6 mice) with autoimmune-prone NOD mice leading to systemic T cell activation towards GPI [158]. Serum of these K/BxN mice can be used to passively induce arthritis in wild-type mice. Anti-GPI antibodies home to distal joints within minutes, where they activate the complement system and subsequently form immune complexes, thereby inducing the development of arthritis. These autoantibodies activate the inflammatory response via complement receptors, Fc receptors and depend on production of TNFα and IL-1. The recipient mice will develop arthritis in 6 to 7 days after injection. However, this is a more transient arthritis that often resolves after 15 to 30 days and repeated injections of serum are required to maintain the disease. Furthermore, it has been established that transfer of GPI antibodies or anti-collagen type II antibodies from K/BxN mice into recipient mice is sufficient to induce disease. The K/BxN mouse model resembles human RA in terms of leukocyte infiltration, synoviocyte proliferation as well as cartilage and bone erosion

Additional arthritis models

Besides the CIA and the K/BxN mouse models of arthritis, a number of other inducible arthritis mouse models have been developed. The models include antigen-induced arthritis, adjuvant-induced arthritis, oil-induced arthritis and proteoglycan-induced arthritis. However, these models are not as frequently used as CIA or K/BxN mice and display a relatively slow onset of RA.

The IL-1β/mBSA induced arthritis model has been published in 1990, but the precise mechanism is to date unknown [159]. The model is based on an intra-articular injection of methylated bovine serum albumin (mBSA) into the knee joint together with a subcutaneous injection of recombinant IL-1β in the rear footpath of the mouse. Additional injections of IL-1β are necessary to fully induce arthritis. This procedure results in an acute arthritis starting 4–7 days after the first injection, which resolves around day 28. Monocytes and neutrophils are present in the affected joints suggesting the involvement of innate immunity in the development of the IL-1β/mBSA induced arthritis, but also T cells seem to contribute to its initiation and progression [160]. Since the arthritis develops rather quickly, this model can be used to study acute inflammatory responses. In addition, this model is not dependent on a certain MCH haplotype such as in the CIA model, therefore it can be used in e.g. C57BL/6 mice.

Several of these mouse models have been used to study the role of mast cells in experimental arthritis. The outcome of these studies are rather contradictory and are discussed in more detail in chapter 2.

Atherosclerosis

Cardiovascular diseases (CVD), such as coronary heart disease and cerebrovascular disease, are the leading cause of death worldwide [161]. Environmental risk factors for CVD are a high-fat diet, smoking, sedentary lifestyle, stress, hypertension, [162]. Atherosclerosis, which is the main underlying cause of CVD, can be considered as a chronic, systemic, lipid-driven autoimmune-like disease that affects the large- and medium-sized arteries. Originally, it was thought that atherosclerosis was the result of passive accumulation of lipids in the wall of the blood vessels. Over time, this lesion will expand and eventually occlude blood vessel, which will trigger clinical symptoms of ischemia. However, it is now widely accepted that atherosclerosis is, besides lipid-driven, also a chronic inflammatory condition were both the innate and adaptive arm of immunity contribute significantly to the initiation and progression of the atherosclerotic plaque [163]. Current therapeutic options are the use of lipid lowering drugs like statins and anti-hypertensive drugs. Often these drugs are combined with recommendations to change lifestyle such as a reduction in dietary (cholesterol) intake, to quit smoking and to increase physical exercise. However, statins are not always effective and the recommended changes in lifestyle are often ignored. This underscores the importance of new therapeutic targets that are able to modulate the initiation and progression of atherosclerosis or even induce regression of the atherosclerotic plaque.

Pathology and etiology of atherosclerosis

Early lesion development: Endothelial dysfunction

In physiological conditions, the innermost layer of the artery is responsible for regulating the vascular tone and has an anti-coagulant and anti-inflammatory function (Fig. 2a). In response to damage as induced by hypertension, hypercholesterolemia or smoking, the endothelium of the artery becomes dysfunctional, as indicated by increased expression of pro-inflammatory cytokines and cellular adhesion molecules such as VCAM-1 [164]. This is accompanied by an increased permeability of the endothelium, which allows an influx of inflammatory leukocytes and lipids into the vessel wall. The early phase of atherosclerosis is characterized by the accumulation of low-density lipoprotein (LDL) and monocytes in the sub-endothelial layer. Here, the LDL undergoes modification such as lipolysis, proteolysis and oxidation [165]. Of these LDL modifications, the oxidized form of LDL or oxidized LDL (oxLDL) is believed to be a major auto-antigen in atherogenesis [166]. The microenvironment inside the early lesion induces maturation of monocytes to inflammatory macrophages, which will secrete inflammatory cytokines like TNFα and IL-6 [167]. Moreover, macrophages express scavenger receptors that enables them to take up

oxidation specific molecules such as oxLDL and cellular debris [168]. As a result cholesterol esters accumulate within the cell. This transforms the macrophage into a lipid rich 'foam cell' because of the lipid droplets that provide the cell a foamy appearance. In this initial phase the lesion is referred to as early lesion or fatty streak, which can either disappear or progress to an advanced atherosclerotic lesion (Fig. 2b) [169].

Lesion progression and destabilization

Under the influence of cytokines and growth factors secreted by local macrophages and foam cells, smooth muscle cells (SMCs) migrate from the media into the intimal layer of

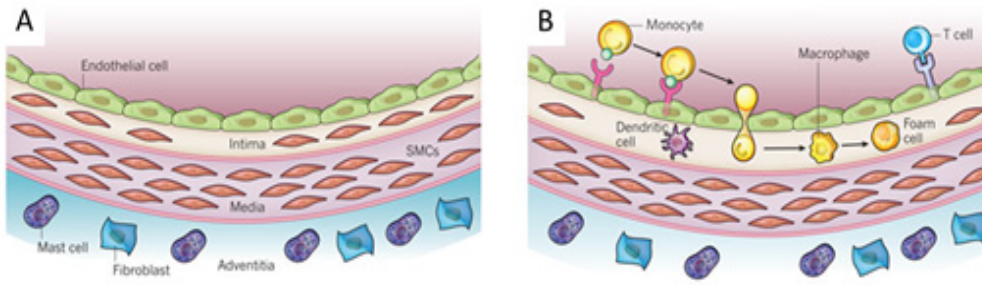

Figure 2. (A) A healthy artery is composed of multiple layers, which are from inner to outer layer the endothelial, intima, media and adventitia. (B) Increased endothelial permeability enables LDL to cross into the vessel wall where it is quickly modified into immunogenic oxidized LDL. Furthermore, endothelial activation leads to upregulation of cellular adhesion molecules on the surface of endothelial cells, which causes adhesion and migration of immune cells like monocytes and T cells. The inflammatory milieu causes differentiation of monocytes into macrophages who turn into foam cells, which accumulate and form a 'fatty streak'.
Adapted and modified from Libby et al. Nature 2011; 473:317. [170]

the vessel. In the intima they start to produce collagen and other extra cellular matrix components, which results in the formation of a fibrous cap (Fig. 3a). Other inflammatory cells like T_h1 cells, dendritic cells and mast cells infiltrate the lesion and cytokines, IFNγ and IL-1β, produced by these cells may further enhance the foam cell formation [171–173]. A combination of relative hypoxia, the inflammatory milieu, increased oxidative stress and excessive protease activity in the plaque will cause apoptosis of lipid loaded macrophages and foam cells. This leads to the deposition of lipids within the plaque and causes the formation of a necrotic core underneath the fibrous cap. Neovascularization takes place in the lesion, which upon leakiness may result in intraplaque hemorrhage and accumulation of even more inflammatory cells [174].

The composition of the atherosclerotic lesion is essential for maintaining lesion stability. Changes in the morphology of a lesion can negatively influence plaque stability resulting in an unfavorable clinical outcome. An unstable lesion is characterized by a large necrotic core, that is covered by a thin fibrous cap. Fibrous cap erosion is caused by smooth muscle cell apoptosis and collagen degradation, which is mediated by inflammatory cells e.g. macrophages that secrete matrix metalloproteinases [175]. Other mediators secreted from immune cells can also contribute to the degradation of lesion components. For example, IgE mediated mast cell activation results in the secretion of many proteases like chymase that inhibit expression and growth of collagen and induces apoptosis of SMCs [176,177]. At a certain point this thinning of the fibrous cap causes rupture of the plaque, exposing its thrombogenic content to the blood, resulting in acute thrombosis and potentially an acute cardiovascular event (Fig 3b).

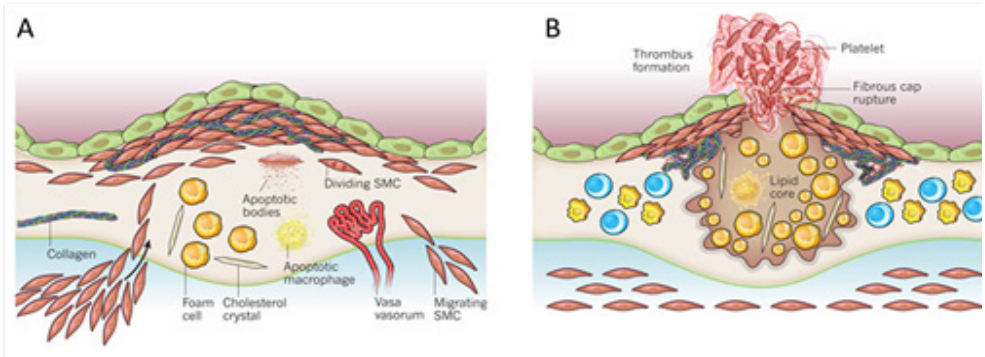

Figure 3. Both foam cell formation and smooth muscle proliferation cause a thickening of the vessel and the formation of a fibrous cap that covers a necrotic core (A). As the plaque enlarges, it causes narrowing of the lumen but also thinning of the fibrous cap. Finally, the plaque ruptures, which can lead to thrombosis and clinical events (B).
Adapted and modified from Libby et al. Nature 2011; 473:317. [170]

Mouse models of atherosclerosis

Atherosclerosis is a complex multifactorial disease were both dyslipidemia and immunity interact to induce an atherogenic response. The use of laboratory animals is crucial to evaluate the complex cell-cell interaction in atherosclerosis. The mouse has become the most commonly used animal for biomedical research due to ability of genetic modification. Nonetheless, mice are highly resistant to atherosclerosis and C57BL/6 mice only develop small fatty streak lesions when put on a high fat and high cholesterol diet for a long period [178]. Mice with deficiencies in the lipid metabolism have been created to induce lesion development. In atherosclerosis studies, apoE KO, apoE*3-Leiden transgenic and LDLr knockout mice as well apoE/LDLr double knockout mice are frequently used [179–182]. In mice, apolipoprotein E and the LDL receptor are essential in the clearance of chylomicrons and VLDL from the circulation. Therefore mice deficient in apoE and LDLr or both have increased levels of cholesterol and triglyceride-rich lipoproteins when placed on a high fat and cholesterol diet, which results in lesion development regions with shear stress like the aortic root.

Recently, a new murine atherosclerosis models has been proposed, which is based on Adenovirus mediated overexpression of Proprotein convertase subtilisin kexin 9 (PCSK9) [183]. Overexpression of PCSK9 resulted in elevated plasma total cholesterol and LDL, which is nearly identical to that of LDLR knockout mice. Likewise, mice injected with this PCSK9-encoding virus developed atherosclerosis, which was comparable with LDLr knockout mice based on lipid profile and histological analysis of the aortic root [184].

The immune system in atherosclerosis

In combination with dyslipidemia, the immune system plays an essential role in the initiation, progression and destabilization of the atherosclerotic plaque. Cells of both arms of the immune system are involved in the process of atherogenesis [185].

Monocytes and macrophages

In the early stages of atherosclerosis monocytes are recruited to the arterial wall under influence of chemokines CCL2 (MCP-1) ligands for CCR2 and CXCR3 and CCL7 [186,187]. Both in mice and in humans, different populations of monocytes have been described. In general, circulating murine monocytes ($CD11b^{+}CD115^{+}F4/80^{low}Ly6G^{-}$) can be differentiated on basis of the expression of Ly6C. Monocytes that are Ly6Chi are comparable with the human classical monocytes ($CD14^{+}CD16^{-}$) based on gene expression profiles, while the $Ly6C^{-}$ monocytes share properties with the human non-classical monocytes ($CD14^{dim}CD16^{+}$). Of these two subsets, the $Ly6C^{hi}$ subset of monocytes infiltrates the vessel wall [188]. In the intima, the monocytes differentiate into macrophages in the presence of macrophage colony-stimulating factor, which is produced by local cells like endothelial and smooth muscle cells. The macrophage plays a dominant role in all phases of atherosclerosis and outnumbers all other immune cells. Via scavenger receptors such as SR-A1 and CD36,

macrophages take up modified lipoproteins such as oxLDL and cellular debris, which are digested in lysosomes [189]. The accumulation of lipids in the macrophages will activate the pro-inflammatory signaling pathway resulting in the secretion of pro-inflammatory cytokines like IL-6 and TNFα. Also, local endogenous ligands like HSP60 or oxLDL, which bind to TLRs, induce cytokine production and accelerate foam cell formation [190]. In advanced atherosclerotic lesions, the macrophages are unable to efflux the absorbed cholesterol, which results in apoptosis of the cell and expansion of the necrotic core.

Neutrophils

Neutrophils are the most abundant cell type in the circulation and upon activation they release various mediators like MMPs that can influence plaque stability. They have been detected in early atherosclerotic lesions of apoE$^{-/-}$ mice, but also in human carotid atherosclerotic plaques [191,192]. Via chemotactic molecules (C5a, C3a, fMLP) and chemokines (IL-8) they are recruited into peripheral tissues. Interestingly, it has been shown that systemic IgE-mediated mast cell activation in mice leads to the recruitment of neutrophils into the atherosclerotic lesion [193]. However, the role of neutrophils in atherosclerosis is not completely confirmed yet, which is probably due to the short life span of the cell.

Mast cells

In physiological conditions, mast cells are located around the blood vessels and during atherogenesis their amount increases with the highest number in rupture prone plaques [194,195]. Analysis of human plaques obtained after carotid endarterectomy showed that intraplaque mast cell number correlated with atherosclerotic plaque progression and micro vessel density, but also with the incidence of future cardiovascular events [196]. The causality between mast cells and plaque progression and destabilization is shown in a study where systemic mast cell activation led to increased plaque growth, which was inhibited by administration of the mast cell stabilizer cromolyn [197]. Inhibition of chymase by a chemical inhibitor resulted in reduced lesion size and increased stability in apoE$^{-/-}$ mice [198]. Atherosclerosis-related stimuli like Substance P, C5a, neuropeptide Y, oxLDL-immune complexes and endogenous TLR ligands have been shown to activate mast cells [33,190,199–201]. These activation pathways often result in the secretion of proatherogenic cytokines such as TNFα, IL-6 and IL-8. Combined, these data clearly establish that mast cells actively contribute to atherosclerosis by the recruitment of leukocytes like neutrophils, by the induction of intraplaque apoptosis and to destabilization of the plaque via the release of proteases.

Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells that are required for the stimulation and differentiation of naïve T cells and the development of antigen specific T cell-mediated immune responses. In atherosclerosis, DCs are responsible for the initiation of an adaptive immune response; they take up antigens, e.g. oxLDL, and present them in secondary lymph nodes to naïve T cells [185]. During the progression of atherosclerosis the number of DCs increases in apoE$^{-/-}$ mice [202]. Modulation of the immune response by both oxLDL-pulsed mature DCs and oxLDL-induced apoptotic DCs resulted in a decrease in lesion development [203,204].

T cells

In the lymphoid organs DCs present antigens via MHCII to the T cell receptor (TCR) on naive CD4^{+} T cells. For optimal T cell activation two additional signals are required from the DC: co-stimulation and the secretion of cytokines. Co-stimulation via molecules like CD80/86 will activate the T cell and the presence of cytokines secreted by the DC will skew the T cell towards a certain subset. Key T cells subsets in atherosclerosis are T_h1, T_h2, T_h17 and Tregs. T_h1 T cells are the predominant type of CD4^{+} T cells in human and murine atherosclerosis they secrete a range of proatherogenic cytokines like IFNγ, TNFα, IL-2 and IL-12 [205,206]. Especially IFNγ, a hallmark cytokine of T_h1 T cells, influences lesion progression and destabilization both by accelerating the ongoing inflammatory response through macrophage activation and inhibiting the production of collagen by smooth muscle cells [207]. LDLr$^{-/-}$ mice also deficient for IFNγ develop smaller atherosclerotic lesions in the aortic arch and descending aorta compared to control mice [208]. T_h2 T cells are present in low numbers in the atherosclerotic lesion and they produce cytokines like IL-4, IL-5, IL-10 and IL-13 [206]. These cytokines influence the maturation of B cells into antibody producing plasma cells and downregulate the production of IFNγ thereby inhibiting T_h1 responses. The role of T_h2 T cells is rather controversial: on one hand IL-4 deficiency reduces atherosclerosis, while on the other hand the T_h2 cytokines IL-5 and IL-13 have been shown to be important for the activation of atheroprotective B-1 B cells, which produce athero-protective IgM antibodies [209,210]. Another potent inflammatory CD4^{+} T cell subset is the T_h17 T cell, which produces large amounts of IL-17, IL-21 and IL-22. Key cytokines in T_h17 T cell biology are IL-6 and TGF-β for induction, IL-21 for the proliferation and IL-23 for the maintenance of T_h17 T cells [211]. Although T_h17 T cells have been implicated in many other immune-driven disorders, their role in atherosclerosis is still under debate. Blockade of IL-17A in apoE$^{-/-}$ mice and IL-17A$^{-/-}$ apoE$^{-/-}$ mice showed reduced lesion development compared to control mice [212,213]. However, other studies showed that IL-17 deficiency had either no effect or resulted in a significant increase in lesion size [214,215]. The main function of regulatory T cells (Tregs) is the regulation of immune responses via the suppression of immune cell proliferation and cytokine production. In mice, Tregs express surface molecules CD4 and CD25, and the transcription

factor Forkhead box protein P3 (FoxP3). Furthermore, Tregs secrete large amounts of anti-inflammatory IL-10 and TGF-β, which is beneficial for dampening inflammation in atherosclerosis. Similarly, depletion of $CD4^+FoxP3^+$ cells in $apoE^{-/-}$ mice results in increased lesion formation [216].
$CD8^+$ T cells recognize antigens via the MHC class I molecule, which is expressed on all nucleated cells. Upon activation cytotoxic $CD8^+$ T cells secrete the cytotoxin perforin and granzymes that will induce apoptosis of the targeted cell. Furthermore, activated $CD8^+$ T cells secrete large amounts of the proatherogenic IFNγ. $CD8^+$ T cells are present in both human and murine atherosclerotic lesions but their role is still under debate [211,217,218].

B cells
Next to a powerful innate and cellular immune response in atherosclerosis, there is also a humoral response. B cells and plasma cells are key players in this response and produce antibodies towards modified self-antigens, such as oxLDL [210,219]. Both in human and murine serum samples IgG antibodies have been detected towards oxLDL, of which the amount correlates with the severity of the disease [219]. In mice, several B cell subsets have been identified; B1, B2 and B10 cells. B1 cells are known to produce natural IgM antibodies independent of T cell help. In atherosclerosis, these B1 cell produce oxLDL-specific IgM that is protective since it prevents foam cell formation and other inflammatory reactions towards oxLDL [220]. B2 B cells are the conventional B cells that are able to produce high titers of immunoglobulins reactive against several antigens like modified lipoproteins, which accelerate the immune response in atherosclerosis [210]. Depletion of B2 cells, but not B1 cells with an CD20 monoclonal antibody in atherosclerosis-prone $apoE^{-/-}$ and $LDLr^{-/-}$ mice, resulted in a significant reduction of atherosclerosis [221,222], indicating that B2 cells are atherogenic whereas B1 cells are atheroprotective in atherosclerosis. Of interest are B10 B cells that are able to produce IL-10 upon stimulation. A study that created chimeric $LDLr^{-/-}$ mice with a B cell specific deficiency in IL-10 showed that B cell derived IL-10 does not alter atherosclerosis in mice [223], but more research is needed to unravel the role of this B cell subset.

CVD risk in RA patients
Since the introduction of immune targeting therapies in combination with DMARDs, the therapeutic efficiency in RA treatment has significantly increased [224]. Despite this important therapeutic progress, RA is still associated with elevated mortality rates, which are mainly caused by cardiovascular diseases like acute myocardial infarction, cerebrovascular accidents and congestive heart failure [225]. RA patients have accelerated progression of subclinical atherosclerosis compared to healthy age-matched controls that may precede the mentioned clinical events [226,227]. Analysis of carotid plaques in active RA patients showed a more unstable, rupture-prone plaque phenotype [228]. This atherosclerosis-prone phenotype in RA patients can only be partly be explained by

traditional risk factors like dyslipidemia, smoking, diabetes mellitus, hypertension and increased BMI [229].

The main common characteristic in both RA and atherosclerosis is the persistent systemic inflammation and immune dysregulation, which leads to synovial inflammation and destabilization of atherosclerotic lesions. In fact, both diseases share many inflammatory pathways like acute phase cytokines (TNFα, IL-6 and IL-1β) and the production of disease associated autoantibodies such as ACPA or anti-oxLDL-IgGs, which are implicated in the pathogenesis of both RA and atherosclerosis [219,230–232].

Presence or absence of ACPA not only influences the clinical progression and response to treatment, it also affects the extra-articular diseases like the cardiovascular risk in RA patients. Even though both ACPA negative and ACPA positive RA patients have a comparable clinical manifestation in the early phases of RA, the sero-positive patient group is associated with a more progressive disease in the established phase of RA. Furthermore, ACPA positivity is also associated with an increased risk in cardiovascular diseases like ischemic heart disease in RA patients [233]. ACPA may influence plaque progression and destabilization in RA patients, as it is known that ACPAs are able to recognize different citrullinated proteins and are cross-reactive [234], while it is also reported that citrullinated proteins are present within the atherosclerotic lesions as well as PAD3 enzymes that drive the citrullination [235–237]. Additional research should focus on the precise mechanisms how dysregulated (immune) pathways in RA contribute to the accelerated atherogenesis in RA patients.

Aim of thesis

Rheumatoid arthritis and atherosclerosis are disorders affecting a large proportion of the world population. Although not completely understood, it is well accepted that the immune system plays a dominant role in the pathology and etiology of both diseases. As members of the innate immunity, mast cells are strategically located at surfaces that are in close contact with the external environment. Therefore they are one the first immune cells that respond to invading pathogens by the release of (preformed) mediators. Mast cells can also be found around blood vessels and in the joint in the synovial layer. Here, they can influence the micro-environment by the release of immune regulatory mediators that influence other local (immune) cells.

This thesis aims to obtain more insight in the role of mast cells in the immune driven disorders rheumatoid arthritis and atherosclerosis, as well as the potential contribution of mast cell activators like immunoglobulins to these diseases. The role of mast cells in rheumatic diseases is reviewed in **chapter 2**. Here we summarize the current physiological and pathophysiological role of mast cells in human arthritis and in mouse models of arthritis. Like in human RA, mouse models of arthritis are composed of a pre-clinical and a clinical phase of arthritis. In both phases it is thought that mast cells could play a role. In **chapter 3** we took advantage of the mast cell inducible knockout mouse model to deplete

mast cells in either the pre-clinical or clinical phase of collagen induced arthritis. Depletion of mast cells in the pre-clinical phase, but not the clinical phase, significantly reduced the clinical score of the mice. Furthermore, the T cell phenotype in mast cell depleted mice show a marked reduction in arthritogenic T_h17 T cells and an increase in protective $FoxP3^+$ T cells, which coincided with a altered cytokine response towards collagen. Despite the fact that ACPA is highly specific for RA, we were able to detect ACPA in two cohorts of non-RA cardiovascular patients. As described in **chapter 4** we determined the CCP3 reactivity of sera from three cardiovascular cohorts (AtheroExpress, Mission and Circulating Cells). We found that a small proportion of non-RA cardiovascular patients were positive for CCP3. Clinical analysis showed a correlation with long-term mortality and CCP3 positivity in the MISSION! cohort. Mast cells are implicated in both in human atherosclerotic lesion and in mouse models of atherosclerosis. There are a number of endogenous ligands described that could activate mast cells in the atherosclerotic plaque. In the study described in **chapter 5** we aimed to find a correlation between either the number of mast cells or their activation status and circulating serum immunoglobulins. We were unable to detect a significant correlation with serum immunoglobulin levels and plaque characteristics, indicating that other (endogenous) ligands, besides immunoglobulins, might also activate mast cells in the atherosclerotic lesion. The study in **chapter 6** presents a new mouse model is characterized to study the role of mast cell in atherosclerotic lesion development. We depleted mast cells before the induction of atherosclerotic lesions in this RMB-$apoE^{-/-}$ mouse model and detected a significant reduction in lesion size compared to mast cell competent mice. Furthermore, the mast cell depleted lesions were characterized by an increased collagen content and a reduced necrotic core size, suggesting that absence of mast cells in the early phases of atherosclerosis increases plaque stability. The involvement of mast cells in lesion progression is described in **chapter 7**. Using RMB-$LDLr^{-/-}$ mice we studied the effect of mast cell depletion on established lesions. While depletion of mast cells had no effect on lesion size, the phenotype of the plaque significantly changed towards a more stable plaque. We observed a reduced total macrophage area and an increased collagen content in lesions in mast cell depleted mice. Further analysis of circulating blood leukocytes showed a significant reduction in inflammatory monocytes and in serum we detected reduced levels of pro-atherogenic cytokines. Finally, all the results described in this thesis and future perspectives are summarized and discussed in **chapter 8**.

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Chapter 2

Mast cells in rheumatic disease

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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 (Gabriel, 2001). 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 (Gyorgy et al., 2006). 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 (Baeten et al., 2001; Makrygiannakis et al., 2006). 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 (Klareskog et al., 2008).

ACPA show a very high specificity for rheumatoid arthritis, and are present in the majority (~70%) of rheumatoid arthritis patients (Nishimura et al., 2007; Schellekens et al., 2000). 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 (Rantapaa-Dahlqvist et al., 2003; Ronnelid et al., 2005). 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 (Huizinga et al., 2005; Klareskog et al., 2006; Pedersen et al., 2007; van der Helm-van Mil et al., 2006). 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 (Meyer et al., 2003; Ronnelid et al., 2005). 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 (Kuhn et al., 2006; Uysal et al., 2009).

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 (Shi et al., 2011).

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 (Gondokaryono et al., 2007; Martin et al., 2003; Midwood et al., 2009; Pullerits et al., 2003; Taniguchi et al., 2003).

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 TLR4 (Sokolove et al., 2011). Therefore, chronic inflammation is often related to release or generation of TLR ligands, leading to a self-amplifying inflammatory loop (Fig. 1).

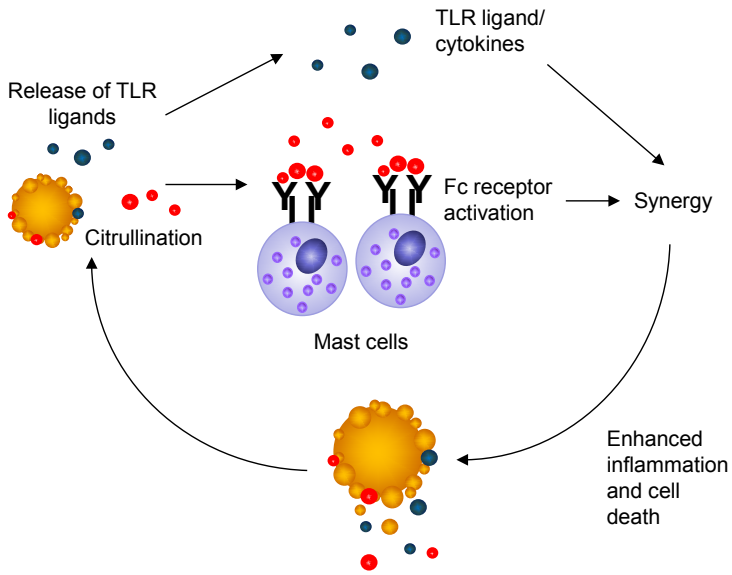

Figure 1. Damage associated molecular patterns (DAMPs), cytokines and citrullinated proteins are all implicated in rheumatoid arthritis pathogenesis and are released upon inflammation, in particular in association with cell death. Both have been shown to activate mast cells: citrullinated proteins can form immune complexes with ACPA autoantibodies, and activate mast cells through Fc γ receptors; DAMPs can activate mast cells through Toll Like Receptors (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 ligands and citrullinated proteins.

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 (van der Helm-van Mil et al., 2006).

However, T cells themselves may also exert pathogenic effects, for example through their production of cytokines. Initially, T_h1 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, T_h17 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 (Benedetti and Miossec, 2014).

Cytokines & 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 TNFα and IL-6 (Smolen et al., 2007). 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 (Sayed et al., 2008). 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

(Crisp et al., 1984; Malone et al., 1986). 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, (Olsson et al., 2001) 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 (MC_T) and tryptase-chymase double-positive cells (MC_{TC}). Whereas normal synovium mainly contains MC_{TC} cells, early inflammation in rheumatoid arthritis is associated with a selective expansion of MC_T, followed by increases of MC_{TC} in established or chronic disease (Gotis-Graham and McNeil, 1997; McNeil and Gotis-Graham, 2000; Olsson et al., 2001). These changes are often correlated with clinical characteristics; MC_T numbers in early disease associate with inflammation, whereas the MC_{TC} 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 (Buckley et al., 1997; Frewin et al., 1986; Lavery and Lisse, 1994; Malone et al., 1986). Furthermore, mast cells have been reported to be the main IL-17-positive cells in the inflammatory joint of rheumatoid arthritis and spondyloarthropathy patients (Hueber et al., 2010). 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 (Verpoort et al., 2006).

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 (Trouw et al., 2009). 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 (Nigrovic et al., 2010). 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, (Benhamou et al., 1990; Fang et al., 2013; Latour et al., 1992) the receptor involved in arthritis induced by anti-collagen autoantibodies (Díaz de Ståhl et al., 2002). Human mast cells have been shown to express FcγRIIA, whereas there is some controversy regarding expression of FcγRI (Jonsson et al., 2012; Lee et al., 2013; Suurmond et al., 2014a). We have recently shown that human cultured mast cells could be activated by ACPA immune complexes in a citrulline-dependent manner (Suurmond et al., 2014a). 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 (Kulka et al., 2004; Matsushima et al., 2004; McCurdy et al., 2003; Varadaradjalou et al., 2003).
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 TLR2, TLR4 and endosomal TLRs which sense nucleic acids (Midwood et al., 2009; Piccinini and Midwood, 2010).
We have recently shown that human mast cells indeed respond to HSP70, an endogenous ligand for TLR4, which is present in rheumatoid arthritis synovium (Suurmond et al., 2014a). Another endogenous TLR ligand, the extra domain A of fibronectin, can induce joint inflammation in mice in a mast cell- and TLR4-dependent manner, (Gondokaryono et al., 2007) suggesting that this pathway of mast cell activation can contribute to pathogenic responses in RA.

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 (Junttila et al., 2013). IL-33 has been shown to enhance arthritis in a mast cell-dependent manner, (Xu et al., 2008) 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 (Gaudenzio et al., 2013; Kambayashi et al., 2009; Suurmond et al., 2013). Besides antigen presentation, mast cell-derived cytokines can also induce T cell activation (Nakae et al., 2005). 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 (Baram et al., 2001). Whereas regulatory T cells can inhibit mast cell activation, the effect of T cells involved in rheumatoid arthritis, such as T_h17 cells, is not known (Gri et al., 2008; Kashyap et al., 2008). 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 T_h1 and T_h17 cytokine secretion (Schubert et al., 2014).

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 (Suurmond et al., 2014a; Suurmond et al., 2014b; Vogelpoel et al., 2014). 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 (Gebhardt et al., 2002; Lorentz et al., 2005; Ochi et al., 2000; Rivellese et al., 2014). 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 (Kashiwakura et al., 2013). 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 (Suurmond et al., 2014a). 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 (Abraham and St John, 2010). 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.

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 (Abraham and Malaviya, 1997). 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 (Malaviya et al., 1996). 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 (Chen et al., 2001; Koch et al., 1991; Suurmond et al., 2014a). In mice, mast cell-derived TNFα and leukotriene B4 can both mediate neutrophil recruitment as well (Biedermann et al., 2000; Nigrovic et al., 2007; Zhang et al., 1995; Zhang et al., 1992). In addition, histamine can increase vascular permeability, thereby augmenting neutrophil recruitment (Fig. 2A) (Binstadt et al., 2006).

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 (Lafyatis et al., 1989).

Synovial fibroblasts can be activated by multiple pathways, including TLR activation, and cytokines (Pierer et al., 2004). Cytokines implicated in this process are TNFα, IL-1, and IL-17 (Granet et al., 2004; Hot et al., 2012). 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 (Sawamukai et al., 2010; Zenmyo et al., 1995).

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 (Xu et al., 2008).

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, TNFα and GM-CSF, but can also be mediated by mast cell granule-derived mediators such as heparin, tryptase and chymase (Fig. 2C) (Azizkhan et al., 1980; Blair et al., 1997; Muramatsu et al., 2000; Paleolog, 2002). 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 (Ribatti, 2013; Wulff and Wilgus, 2013). 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.

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) (Tolboom et al., 2002). 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 (Van Wart and Birkedal-Hansen, 1990). 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 (Gruber et al., 1988; Magarinos et al., 2013). 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 (Nakamura et al., 1996; Seitz et al., 2013).

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

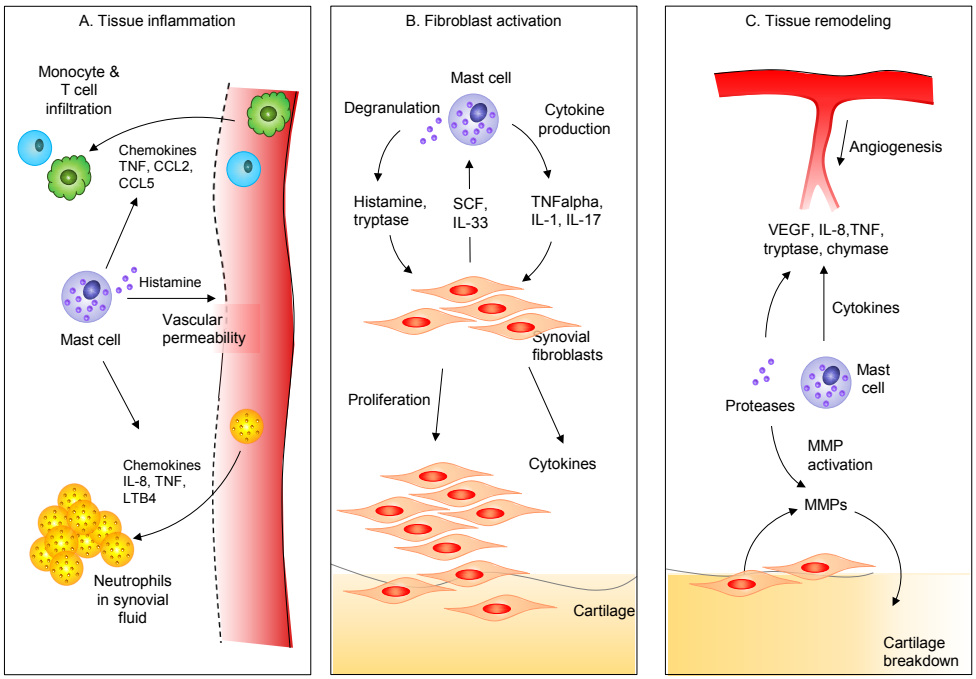

Figure 2. Mast cell contribution to pathogenic processes in rheumatoid arthritis.
(A) Activated mast cells can amplify tissue inflammation through several mechanisms. They increase vascular permeability through release of histamine, leading to increased recruitment of immune cells. In particular, neutrophils 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 TNFα, 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.

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, $Kit^{W}Kit^{W\text{-}v}$ 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 (Lee et al., 2002). 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, $Kit^{W\text{-}Sh}/Kit^{W\text{-}Sh}$ 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 (Zhou et al., 2007). In addition, $Kit^{W}Kit^{W\text{-}v}$ mice had normal arthritis development in the collagen induced arthritis model (Pitman et al., 2011). Unlike neutropenic $Kit^{W}Kit^{W\text{-}v}$ mice, $Kit^{W-sh}/^{W-sh}$ mice have a baseline pro-inflammatory phenotype, including neutrophilia (Michel et al., 2013; Nigrovic et al., 2008). Therefore, these confounding results have sometimes been attributed to the neutrophilia in $Kit^{W-sh}/^{W-sh}$ mice, which renders them insensitive to mast cell-mediated neutrophil recruitment, a critical event in early arthritis development (Brown and Hatfield, 2012).

Of the mast cell deficient mouse models independent of kit, two models have been used to study arthritis. In one study, the $Cpa3^{Cre/+}$ 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 (Feyerabend et al., 2011). 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, (Schubert et al., 2014) 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 (Magnusson et al., 2009). 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) (McNeil et al., 2008; Shin et al., 2009). 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 (Heger et al., 2014).
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.

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 (Cox, 1967). 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 (Kobayashi et al., 1999). In addition, it was shown that intra-articular treatment of cromolyn or salbutamol prevented angiogenesis, pannus formation and joint destruction in mice.(Kneilling et al., 2007) Recently however, the specificity of cromolyn and the sensitivity of different types of mast cells to cromolyn in mice is under debate (Oka et al., 2012). Also, the specificity of salbutamol can be questioned since it has also inhibits the secretion of pro-inflammatory cytokines by macrophages and T cells (Kneilling et al., 2007). Therefore, development of mast cell-specific therapeutics is needed to establish the exact role of mast cells in rheumatoid arthritis.

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

Mouse strain	Deficiency	Arthritis model	Outcome	References
Mast cell deficiency				
$Kit^{W}Kit^{W-v}$ (W/Wv)	Mast cell deficient through SCF-receptor mutation	K/BxN	Mast cell deficient mice resistant to develop arthritis. Restored with systemic or local engraftment of BMMCs	Kneilling et al., 2007; Lee et al., 2002
		CIA	No effect of mast cell deficiency	Pitman et al., 2011
Kit^{W-Sh}/Kit^{W-Sh}	Mast cell deficient through defects in SCF	α-collagen type II antibody transfer	No effect of mast cell deficiency	Zhou et al., 2007
Cpa3-Cre (cre-master)	Mast cell deficient through Cre-mediated toxicity	K/BxN	No effect of mast cell deficiency	Feyerabend et al., 2011
Mcpt5-Cre iDTR	Mast cell deficient upon injection of DT (only connective tissue-like MC)	K/BxN	No effect of mast cell deficiency	(Schubert et al., 2014)
		CIA	Reduced arthritis in mast cell deficient mice	
Mast cell protease-deficiency				
Chymase	$mMCP4^{-/-}$	CIA	Reduced arthritis	Magnusson et al., 2009
Tryptase Heparin complexes	$mMCP6^{-/-}$ $mMCP7^{-/-}$	K/BxN	Reduced arthritis	Shin et al., 2009
	$NDST\text{-}2^{-/-}$ & Combinations	mBSA/IL-1β	Reduced arthritis	McNeil et al., 2008
Mast cell-conditional knockout				
A20-deficiency	Mcpt5-Cre A20Fl/Fl	CIA	Exacerbated arthritis	Heger et al., 2014
Pharmacological mast cell inhibition				
Cromolyn	(not mast cell specific)	CIA	Reduced arthritis	Kneilling et al., 2007; Kobayashi et al., 1999

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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Chapter 3

Mast cell depletion in the pre-clinical phase of collagen induced arthritis reduces clinical outcome by lowering the inflammatory cytokine profile.

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Abstract

Background

Rheumatoid arthritis (RA) is a multifactorial autoimmune disease, which is characterized by inflammation of synovial joints leading to the destruction of cartilage and bone. Infiltrating mast cells can be found within the inflamed synovial tissue, however their role in disease pathogenesis is unclear. Therefore we have studied the role of mast cells during different phases of experimental arthritis.

Methods

We induced Collagen-Induced Arthritis (CIA), the most frequently used animal model of arthritis, in an inducible mast cell knock-out mouse and determined the effect of mast cell depletion on the development and severity of arthritis.

Results

Depletion of mast cells in established arthritis did not affect clinical outcome. However, depletion of mast cells during the pre-clinical phase resulted in a significant reduction in arthritis. This reduction coincided with a decrease in circulating $CD4^+$ T cells and inflammatory monocytes but not in the collagen-specific-antibody levels. Mast cell depletion resulted in reduced levels of IL-6 and IL-17 in serum. Furthermore, stimulation of splenocytes from mast cell depleted mice with collagen type II resulted in reduced levels of IL-17 and enhanced production of IL-10.

Conclusions

Here we show that mast cells contribute to the pre-clinical phase of CIA. Depletion of mast cells before disease onset resulted in an altered collagen specific T cell and cytokine response. These data may suggest that mast cells play a role in the regulation of the adaptive immune response during the development of arthritis.

Introduction

Rheumatoid arthritis (RA) is characterized by progressive inflammation of the synovial joints that leads to the breakdown of cartilage and bone, eventually resulting in malformation of hands and feet, thereby reducing the quality of life for the patient [1]. In the western world, RA affects around 0.5 – 1% of the general population [2]. The etiology and pathology of RA are not completely understood and environmental and genetic factors are thought to play a role in disease pathogenesis [3,4]. Various types of immune cells, such as macrophages, B cells, T cells and mast cells have been described to contribute to the initiation and progression of joint destruction [5]. Mast cells are potent innate immune effector cells and accumulate in the synovium during RA progression. Over time, mast cells can account for up to 5% of all nucleated cells within the inflamed synovial tissue [6,7]. Mast cells express a wide range of surface-receptors that allow them to be activated by different ligands, such as IgE, cytokines, (endogenous) TLR ligands and IgG-immune-complexes [8]. Many of these ligands have been detected within the inflamed synovial tissue of RA patients. Depending on the activation route, mast cells can release a wide range of preformed mediators such as chymase, tryptase and histamine and can also release cytokines and chemokines [9]. The precise role of mast cells in the pathogenesis of RA is unknown, but activation of synovial mast cells could potentially contribute to the further progression of joint destruction either by the recruitment of leukocytes such as neutrophils and monocytes but could also facilitate the breakdown of cartilage in the joint by activating osteoclasts via release of mediators like histamine [10,11]. To date several mouse studies have been conducted to study the role of mast cells in experimental arthritis. Different results were obtained in these studies, which could potentially have been caused by the choice of mast cell deficient mouse strain or the method of arthritis induction [12–15]. However, most of these studies were performed in arthritis models based on the infusion of autoreactive antibodies such as with the K/BxN model. The pathogenesis of K/BxN model is based on the transfer of serum containing anti-glucose-6-phosphate (GPI) antibodies obtained from K/BxN mice. Infused anti-GPI antibodies in recipient mice will home to distal joints were they form immune complexes, which activate an inflammatory response via complement receptors, Fc receptors and is future depended on production of TNFα and IL-1. Adaptive immune cells such as T cells are reported not to be required for disease induction in this model [16,17]. Nonetheless, T cells are thought to play a major role in RA, therefore we studied mast cells in the collagen induced arthritis (CIA) model where T cells contribute significantly to the initiation of the pathogenic immune response [18,19]. For example, in a study conducted by Jansen et.al. $CD4^+$ T cells were depleted in CIA mice using either abatacept or a $CD4^+$ depleting antibody. This depletion resulted in a significant reduction of collagen specific antibodies, which coincided with a lower disease activity [20]. This study confirms the importance of T cells in the early phases of CIA in the establishment of a strong humoral immune response towards collagen type II. CIA has many similarities with RA, like cartilage degradation,

fibrin deposition, mononuclear infiltration, synovial cell hyperplasia, pannus formation, periosteal bone formation and eventual ankyloses of one or more joints [18]. Comparable to human RA, CIA is composed of a preclinical (prodromal) and a clinical phase. The preclinical phase is defined as the period following the initial immunization with collagen and preceding the onset of clinical symptoms, and could be considered as a model for the prodromal stage of RA in which the underlying auto-immune response is already present but there is no visible manifestation of clinical symptoms. The clinical phase of CIA is characterised by an irreversible destruction of synovial joints. Recently, it was reported that depletion of mast cells in MCPT5-Cre-iDTR mice, before the first immunization with collagen type II (CII), could reduce the clinical outcome by altering the T cell subsets in the draining lymph nodes [21].

Although the role of mast cells in the initial onset of the pathogenic immune response is of great scientific importance, information about mast cells in later stages of disease, might be more helpful for potential therapeutic intervention. Therefore the aim of this current study is to further dissect the role of mast cells in arthritis by depletion of them during these later phases of the disease.

To this end, we made use of the red-mast cell basophil (RMB) mouse, which is a novel mast cell inducible knock-in mouse strain based on the transgenic expression of the simian diphtheria toxin receptor (DTR). Normally, mice are resistant to diphtheria toxin (DT) but cells who express the DTR will become highly sensitive to DT, which will cause apoptosis after challenges with DT. In the RMB mice, the DTR is expressed under control of the promoter from the β-chain of the high affinity receptor for IgE (FcεRI) [22]. In mice, mast cells and basophils express a high affinity receptor for IgE (FcεRI) composed of one alpha, one beta, and two gamma chains, which is essential for cell surface expression [23]. Although it has been reported that other immune cells such as dendritic cells and monocytes can express the FcεRI these cells lack the β-chain of the receptor [24,25]. In summary, only basophils and mast cells express the β-subunit, which allows a cell specific ablation in this current mouse model [22,23,26,27]. As reported previously, basophils are depleted only for a short period of time (<12 days) and the depletion of mast cells lasts at least above 6 months in the peritoneal cavity [22]. In this study, we employed the RMB mouse to determine the contribution of mast cells to the pre-clinical and clinical phase of arthritis by depleting mast cells during these stages.

Material and Methods

Mice

All animal work was performed conform national guidelines and experiments were approved by the animal welfare committee of the Leiden University Medical Centre.

The red mast cell and basophil mice (RMB or B6; B6.Ms4a2^{tm1Mal} mice) [22] were backcrossed for one generation with wild-type DBA/1 mice (Harlan BV, The Netherlands) in order to obtain mice that are highly susceptible for collagen induced arthritis and in which FcεRIβ expressing cells can be depleted by injection of DT.

Collagen Induced Arthritis and Collagen Antibody Induced Arthritis

Collagen Induced Arthritis (CIA) was induced in 8-10 week old male RMB-DBA/1 mice by injection in the tail base with 100 µg of bovine collagen type II (CII) (2mg/mL) (Chondrex Inc. (US) emulsified in Complete Freund's Adjuvant (CFA) (1 mg/mL; Difco (US)). On day 21 the mice received a subcutaneous boost with 100 µg CII in incomplete Freund's adjuvant (Difco) [28].

Collagen antibody induced arthritis was induced by intravenous injection of 1 mg anti-collagen antibodies (Athrogen 5 Clone 2 Chondrex (US)) intravenously on day 0 [29]. A clinical score was assigned based on a scoring protocol in which each swollen or red phalanx was given 0.5 point and 1 point per toe. A red or swollen knuckle was given 1 point, a red or swollen footpad was given 1 point and a swollen ankle and/or wrist were given 5 points. The maximum score for each paw was 15 points, resulting in a maximum possible score of 60 points per mouse. For the immunoglobulin levels, mice were bled before immunization, on day 21, and at the end of follow up. Blood was centrifuged and serum was harvested and stored at −20°C until use.

In vivo depletion of FcεRIβ$^+$ cells

To systemically deplete all FcεRIβ$^+$ cells mice were injected i.p. for three times with a one day interval with 1 µg Diphtheria Toxin (DT) (DT Unnicked, *C. diphtheria* (Cat #322326), CalBiochem (US), (40 ng/g bodyweight).

To deplete mast cells and basophils in the clinical phase of arthritis, mice received either DT or PBS upon clinical manifestation of arthritis. The mice were divided over two groups with a similar clinical score at the day of injection. Mast cells and basophils were depleted in one group by *i.p.* DT injection, while the control group received *i.p.* injections with PBS.

To deplete mast cells in the pre-clinical phase of arthritis, mice were injected with either DT or PBS starting 7 days after the first immunization. Efficiency of depletion was measured by FACS analysis for circulating basophils (CD49b$^+$/FcεRI$^+$/IgE$^+$) three days after last DT injection. At sacrifice mast cells in the joint were visualized by staining with a napthol AS-D chloroacetate easterase staining kit (CAE) (Cat# 91C-1KT, Sigma-Aldrich, Germany). For a schematic overview of the arthritis experiment, see supplemental figure 1.

Histology

Hind legs of arthritic mice were harvested at end of the study. Tissues were fixed in 4% formalin and decalcified in PBS containing 10% EDTA for 14 days before embedding into paraffin. 5 µm thick sections were cut and either a toluene blue staining or an enzymatic staining (CAE), was performed to quantify the amount of mast cells.

To analyse the joint inflammation, sections were stained with haematoxylin and eosin (H&E). Histopathological changes were scored using the following parameters; 0: No inflammation, 1: hyperplasia of the synovial layer, infiltration of leukocytes into the joint 2: Pannus formation 3: Destruction of cartilage 4: Destruction of bone and extensive infiltrates. The sample treatment protocol was withheld from the evaluators to prevent bias.

Flow cytometry

At sacrifice, blood was obtained in EDTA tubes and erythrocytes were removed using a specific erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3).

Blood leukocytes were stained extracellularly to determine a) monocytes (NK1.1$^-$/Ly6G$^-$/CD11b^{hi}), inflammatory monocytes (NK1.1$^-$/Ly6G$^-$/CD11b^{hi}/Ly6C^{hi}/CCR2$^+$) and neutrophils (NK1.1$^-$/Ly6G^{hi}/CD11b^{hi}), b) basophils (CD3$^-$/

$CD4^-/CD19^-/CD8^-/CD49b^+/IgE^+/CD117^-$), c) T cells ($CD3^+/CD4^+$) and d) B cells ($CD19^+/B220^+$). The antibodies used (eBiosciences, US) are summarized in table 1. Flow cytometry analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (Becton Dickinson, US).

Table 1: Antibody panels used for flow cytometry analysis.

Staining	FITC	PE	PerCP	APC	e-Fluor-450
A.	NK1.1 (Clone: PK136)	Ly6G (Clone: 1A8)	Ly6C (Clone: HK1.4)	CCR2 (Clone: 475301)	CD11b (Clone: M1/70)
B.	IgE (Clone: R35-72)	IgE (Clone: R35-72)	CD3/4/19/8 (dump channel)	CD49b (Clone: HMa2)	n/a
C.	CD44 (Clone: IM7)	CCR7 (Clone: 4B12)	CD8α (Clone: 53-6.7)	CD62L (Clone: MEL-14)	CD4 (Clone: GK1.5)
D.	IgM (Clone: II/41)	CD45RA (Clone: RA3-6B2)	CD19 (Clone: eBio1D3)	IgD (Clone: 11-26c)	CD5 (Clone: 53-7.3)

Stimulation of splenocytes

At sacrifice, a single cell suspension was prepared from the spleen by using a 70 µm cell strainer (Falcon, US). Erythrocytes were removed using a specific erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3).

Regulatory T cell numbers were determined by staining extracellular with eFluor-450 conjugated rat anti mouse CD4. Next, cells were fixed and permeabilized according to supplier's protocol (eBiosciences). Subsequently, cells were stained with APC conjugated rat anti-mouse/human FoxP3 or corresponding isotype as a control (eBioscience).

To determine inflammatory T_h17 T cell phenotype in the spleen, 400.000 splenocytes/well were cultured in 96 well round-bottom plates (Greiner Bio-One, The Netherlands) and stimulated with anti-CD3 and anti-CD28 (2 µg/mL each, eBioscience) in complete IMDM, supplemented with 10% heat inactivated fetal calf serum, 100 u/mL penicillin/streptomycin, 2 mM L-Glutamine (PAA, Austria) and 20 mM β-mercaptoethanol (Sigma-Aldrich). After 1 hour, brefeldin A (Sigma-Aldrich) was added up to a concentration of 10 µg/mL to inhibit secretion of the cytokines. After an additional 4 hours of incubation, cells were washed twice with FACS Buffer (PBS, 1% BSA, 2mM EDTA) and stained for T cell surface markers.

Cells were first stained with eFluor-450 conjugated rat anti mouse CD4. Next, cells were fixed and permeabilized according to supplier's protocol (eBiosciences), followed by intracellular staining with PE conjugated rat anti-mouse IL-17 or corresponding isotype as a control (eBioscience).

For the determination of the cytokine profile, splenocytes were cultured at 300.000 cells/well in triplicate and stimulated with either αCD3/28 (2 µg/mL each) or 50 µg/mL collagen type II for 96 hours, while unstimulated cells served as controls. Subsequently, the supernatant was collected for cytokine analysis.

Serum immunoglobulin detection and cytokine levels

Total IgG1, IgG2a, IgG2c and IgE serum levels were determined by ELISA according to manufacturer's manual (Bethyl, US). Collagen specific IgG1, IgG2a, IgG2c and IgE were determined using an in-house ELISA procedure. In short, bovine collagen was coated overnight at a concentration of 10 µg/mL in carbonate-bicarbonate buffer

(pH9,6) on NUNC Maxisorp plates. Plates were washed with PBS/0,05% Tween20, and blocked with PBS/10% milk for 2 hours. After washing, the plates were incubated with 1/8000 diluted serum in PBS/1% BSA/ 0,05% Tween20 for 18 hours at 4C°. The different Igs were detected using an HRP-conjugated goat anti-mouse Ig antibody (Southern Biotec, US) diluted in PBS/1% BSA/ 0,05% Tween20. HRP enzyme activity was visualized using ABTS. As a standard, serial dilutions of a pooled serum sample from mice with CIA were used. Cytokine levels were determined using a commercially available ELISA kit (BD: IL-6, TNF, IFNγ, IL-10) and eBiosciences (IL-17A). All cytokine ELISA's were performed according to manufacturer's protocol.

Statistical analysis

Data are expressed as mean ± SEM. All data presented was tested with the Shapiro-Wilk test for normal distribution. An unpaired two-tailed Student T-test was used to compare normally distributed data between two groups of animals. Data of two groups with more than one variable were analysed by two-way ANOVA followed by Bonferroni post hoc test.

Clinical scores of mice were compared by calculating the area under the curve (AUC) of the clinical score from each mouse overtime followed by an unpaired two-tailed Student T-test. Statistical analysis was performed using Prism (Graphpad). Probability values of $P<0.05$ were considered significant.

Results

FcεRIβ⁺ cell depletion in established arthritis does not reduce clinical score or delay disease progression

Arthritis is characterized by a constant activation and recruitment of immune cells into the joint leading to the destruction of cartilage and bone. Mast cells accumulate in the inflamed joint and could therefore actively contribute to the disease progression in CIA. To investigate the contribution of mast cells to the progression of established CIA in RMB-DBA/1 mice, FcεRIβ⁺ cells were depleted in the effector phase of the disease (Suppl. Fig 1a.). As shown in figure 1a, depletion of FcεRIβ⁺ cells after clinical manifestation of CIA had no effect on the clinical score. In both groups we detected similar levels of specific immunoglobulins towards collagen type II (CII) in serum (Fig. 1b). Mast cells were present in the joints of saline treated mice, whereas mast cell were completely absent in DT treated mice (Fig. 1c). Further analysis of the different blood leukocyte populations by flow cytometry showed no differences in percentages basophils, neutrophils, (inflammatory)-monocytes, CD4⁺ T cells and B cells (Fig. 1d). Taken together, these data indicate that depletion of FcεRI⁺ cells after the onset of CIA did not affect progression of CIA.

To further study the role of mast cells in the effector phase of arthritis we used the collagen antibody-induced arthritis (CAIA) model in RMB-DBA/1 mice [29]. Unlike the CIA model, this model does not require an active adaptive immune response towards collagen type II. The CAIA model depends on the injected pathogenic anti-collagen antibodies and resembles the effector phase of collagen induced arthritis after the adaptive immune response has developed.

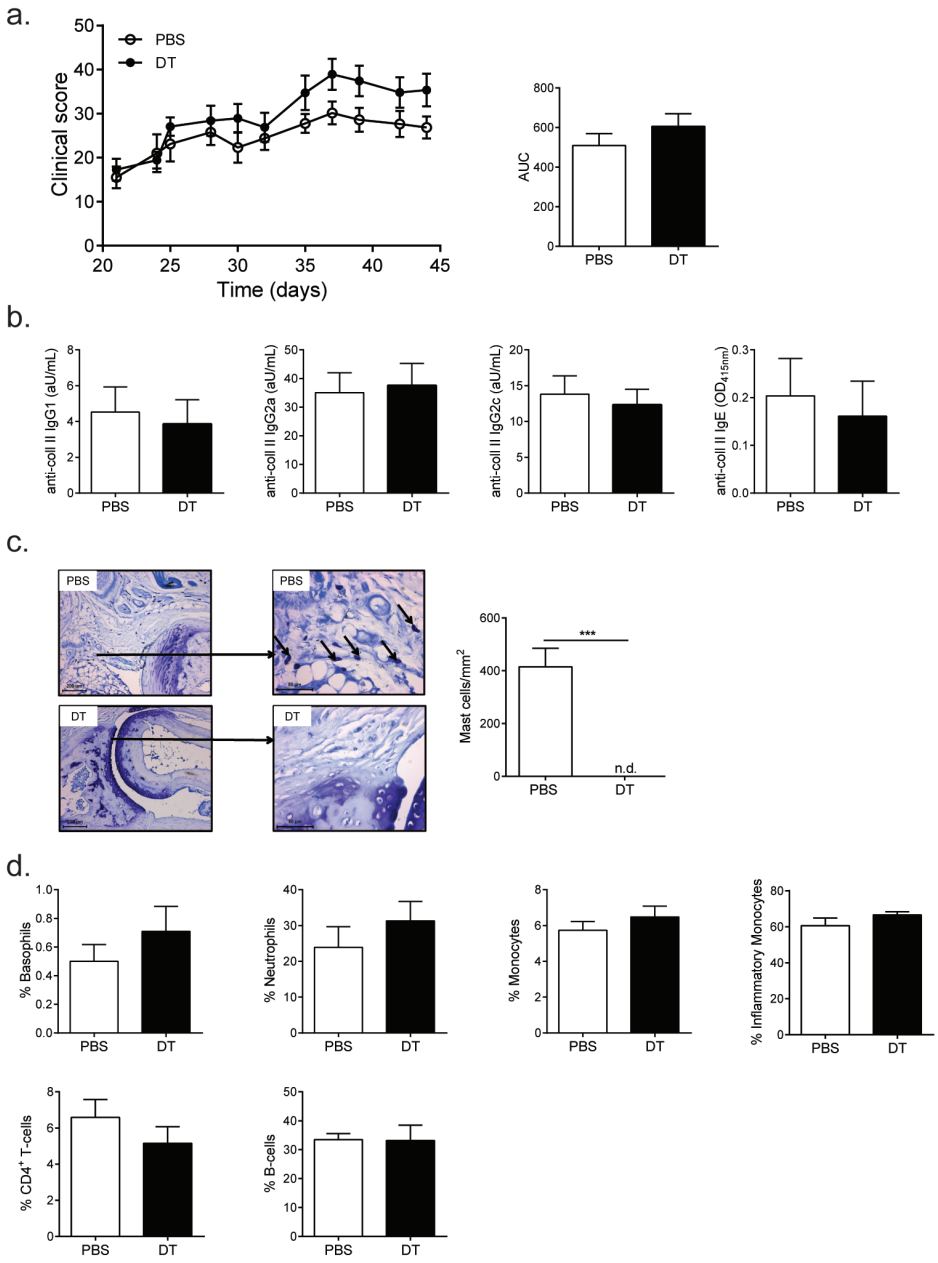

Figure 1. Depletion of FcεRIβ^{+}-cells in clinical phase of CIA does not influence clinical outcome in RMB-DBA/1 mice.

(A) Progression of CIA was monitored by clinical scoring of RMB-DBA/1 mice injected with either PBS or DT (n=15/group). (B) Serum levels of IgG1, IgG2a, IgG2c and IgE isotype antibodies directed against collagen type II were quantified in serum from PBS or DT injected RMB-DBA/1 mice (n=15/group). (C) Mast cell numbers were determined in ankle joints of PBS and DT treated mice (n=15/group). Arrows show mast cells in the joint. (D) FACS analysis for common peripheral leucocytes in both groups (***P<0.001).

A representative example of 2 independent experiments is depicted.

Mast cell deficient or competent RMB-DBA/1 mice were *i.v.* injected with anti-collagen antibodies and scored daily for arthritis development (suppl. Fig. 1b). We did not observe a significant difference in clinical score between both groups (Fig. 2a). At sacrifice, a total mast cell depletion was confirmed in the joints of DT treated mice (Fig. 2b). Flow cytometry analysis of the blood compartment showed no significant differences in blood leukocytes subsets (basophils, neutrophils, monocytes, inflammatory monocytes, T cells and B cells) as depicted in Figure 2c. These findings suggest that mast cells do not play a role in experimental arthritis once arthritis and a fully developed and the anti-collagen type II antibody response has been developed.

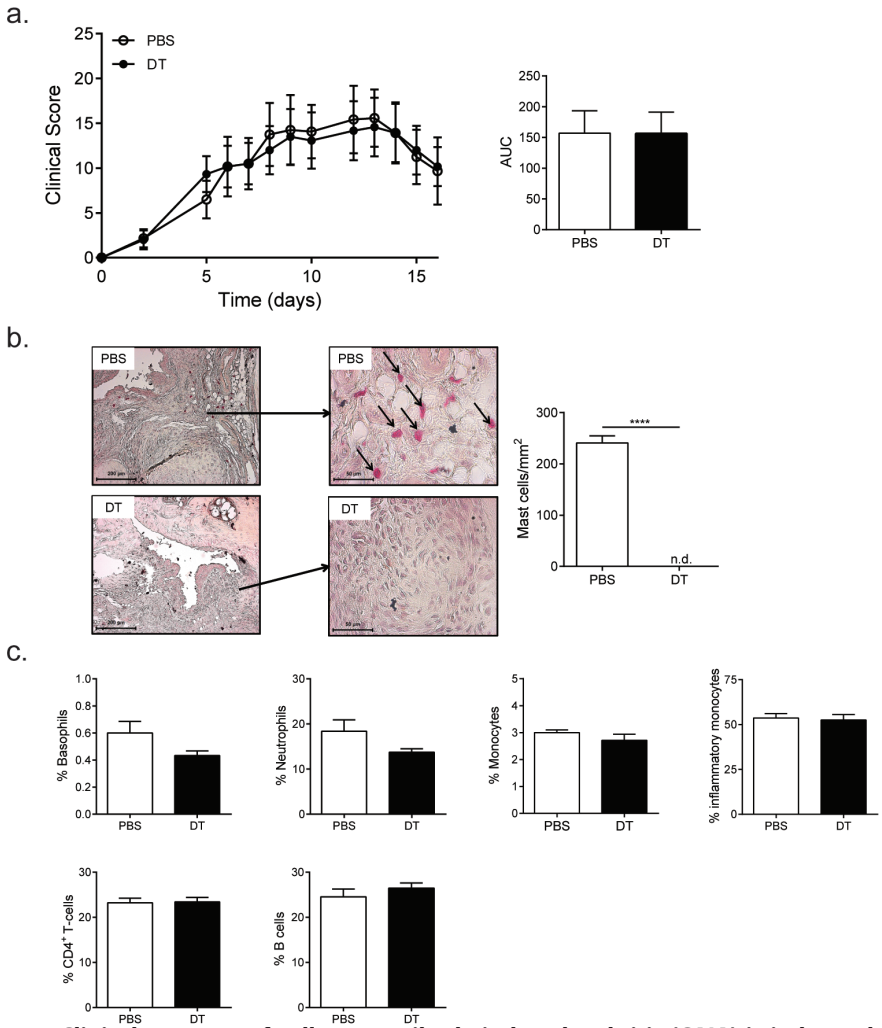

Figure 2. Clinical outcome of collagen antibody induced arthritis (CAIA) is independent of FcεRIβ⁺-cells.

(A) Progression of CAIA induced arthritis in both mast cell competent and depleted RMB-DBA/1 mice (n=6/group). (B) Mast cell numbers in the ankle joint of PBS and DT injected RMB-DBA/1 mice (n=6/group). (C) FACS analysis for common peripheral leucocytes in both groups (n=6/group).

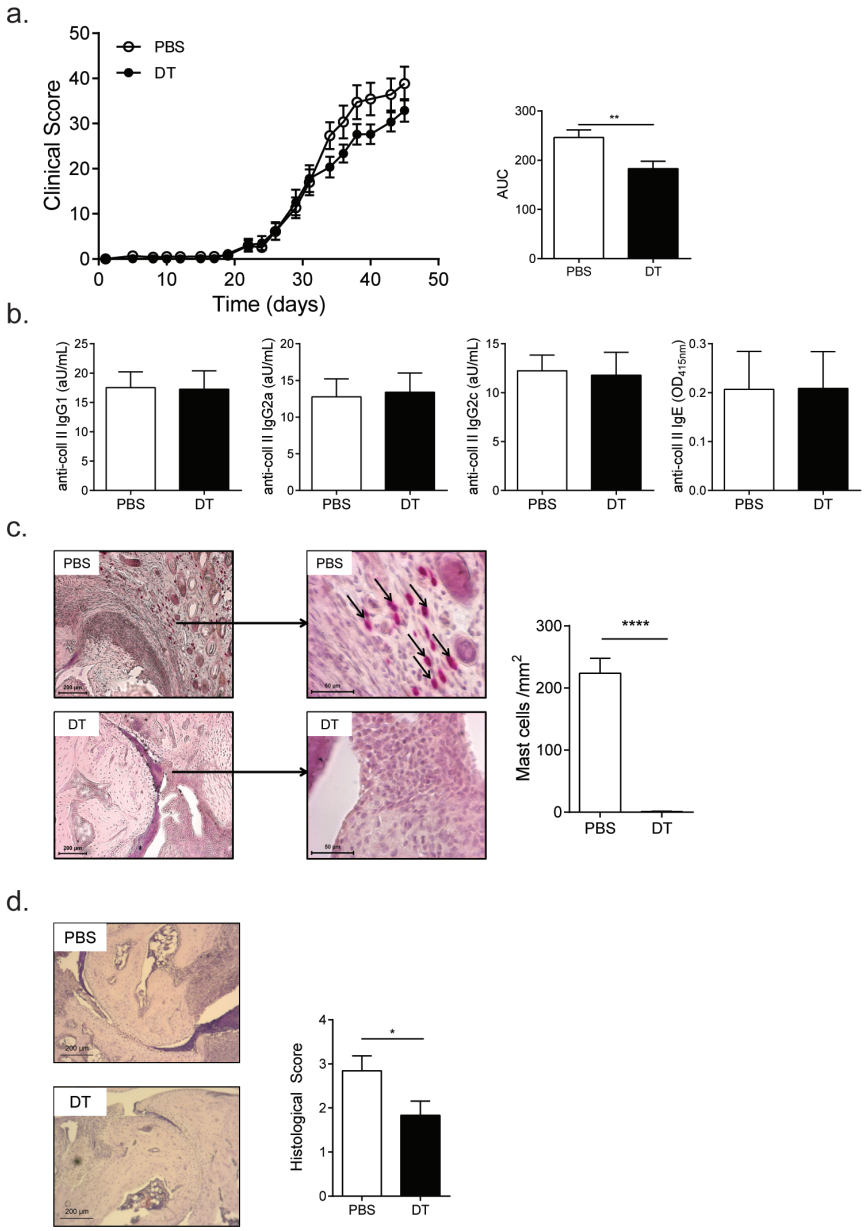

Figure 3. Absence of FcεRIβ^{+}-cells during preclinical phase of CIA reduces clinical outcome of arthritis in effector phase of disease.

(A) Progression of CIA was monitored by clinical scoring of RMB-DBA/1 mice, which have been injected with either PBS or DT in the preclinical phase of CIA. (n=15/group). (B) Serum levels of IgG1, IgG2a, IgG2c and IgE isotype antibodies directed against collagen type II were quantified in serum from PBS or DT injected RMB-DBA/1 mice (n=15/group). (C) Mast cell numbers were determined in ankle joints of PBS and DT treated mice (n=6/group). Arrows show mast cells in the joint. (D) Histological score of joint inflammation in the ankle joint of PBS and DT treated mice (n=15/group). Representative H&E stained sections of ankles obtained from PBS and DT treated RMB-DBA/1 mice. *P<0.05. A representative example of 2 independent experiments is depicted.

Absence of mast cells in the pre-clinical phase of CIA reduces clinical outcome

Mast cells can secrete various mediators that can regulate the immune response. Therefore mast cells could potentially influence the clinical course of arthritis by regulating collagen-specific B and T cell response required for the development of disease. The CII-specific response starts to develop directly after the first immunization, but mice typically do not develop arthritis until after the booster injection 3 weeks later. To investigate whether mast cells can play an immunoregulatory role during this phase of disease, we depleted FcεRIβ$^+$ cells in RMB-DBA/1 mice 7 days after the first immunization as schematically shown in supplementary figure 1c.

Absence of FcεRIβ$^+$ cells in the pre-clinical phase of CIA resulted in significantly lower clinical score (AUC: PBS 246±16 vs. DT 183±15, p=0.0085) (Fig. 3a). Depletion of FcεRIβ$^+$ cells did not affect anti-collagen type II antibody titers (Fig. 3b). Histological analysis showed a complete depletion of mast cells in the joints of DT treated mice (Fig. 3c), whereas in PBS treated mice mast cells were present in the affected joints. Further analysis of the joint inflammation in both groups showed a significant reduction in histological score in FcεRIβ$^+$ cell depleted mice (Fig. 3d).

At the end of follow up (day 45), we detected reduced serum levels of the inflammatory cytokines IL-6 (PBS 510±52 vs. DT 339±46 pg/mL, p=0.02) and IL-17 (PBS 521±75 vs. DT 326±50 pg/mL, p=0.04) and elevated anti-inflammatory IL-10 (PBS 158±9 vs. DT 212±18 pg/mL, p=0.02) in the serum of FcεRIβ$^+$ cell depleted mice compared to FcεRIβ$^+$ cell competent mice (Fig. 4a). We did not observe any differences of TNFα levels in the serum (Fig. 4a) and expression levels of TNFα within the inguinal lymph node (data not shown). We performed flow cytometry analysis on the blood compartment for circulating basophils, monocytes, neutrophils, T cells and B cells. Since the mice received the last DT injection more than 12 days before sacrifice, we detected a complete repopulation of basophils in DT treated mice (Fig. 4b). Peripheral blood neutrophil and total monocytes (Fig. 4b) were not different between groups. Nonetheless we did observe a reduction inflammatory monocytes (CD11b$^+$/Ly6G$^-$/Ly6C^{hi}/CCR2$^+$) in FcεRIβ$^+$ cell depleted mice (Fig. 4b). Furthermore, CD4$^+$ T cells in FcεRIβ$^+$ cell depleted mice was decreased with 22% (Fig. 4b), while the percentage of CD8$^+$ T cells (Fig. 4b) and B cells (Fig. 4b) was not affected.

To further investigate the phenotype of the circulating CD4$^+$ T cells, we stimulated splenocytes with α-CD3/28, followed by intracellular flow cytometry staining for different T cell subsets. The balance between T_h17 and regulatory T cells is thought to influence arthritis severity in mice (25,26). As shown in figure 5a,b depletion of FcεRIβ$^+$ cells influenced T cell cytokine production, as it resulted in decreased IL-17 producing T cells (PBS 1,02±0.15 vs. DT 0,67±0,04 %, p=0.02) and an increase in FoxP3$^+$ regulatory T cells (PBS 6,50±0,27 vs. DT 7,80±0,34 %, p=0.01) (Fig 5a,b) compared to non-depleted mice. To study the antigen-specific response towards collagen type II, splenocytes were stimulated with CII and cytokine production was analysed by ELISA. Mast cell depletion resulted in

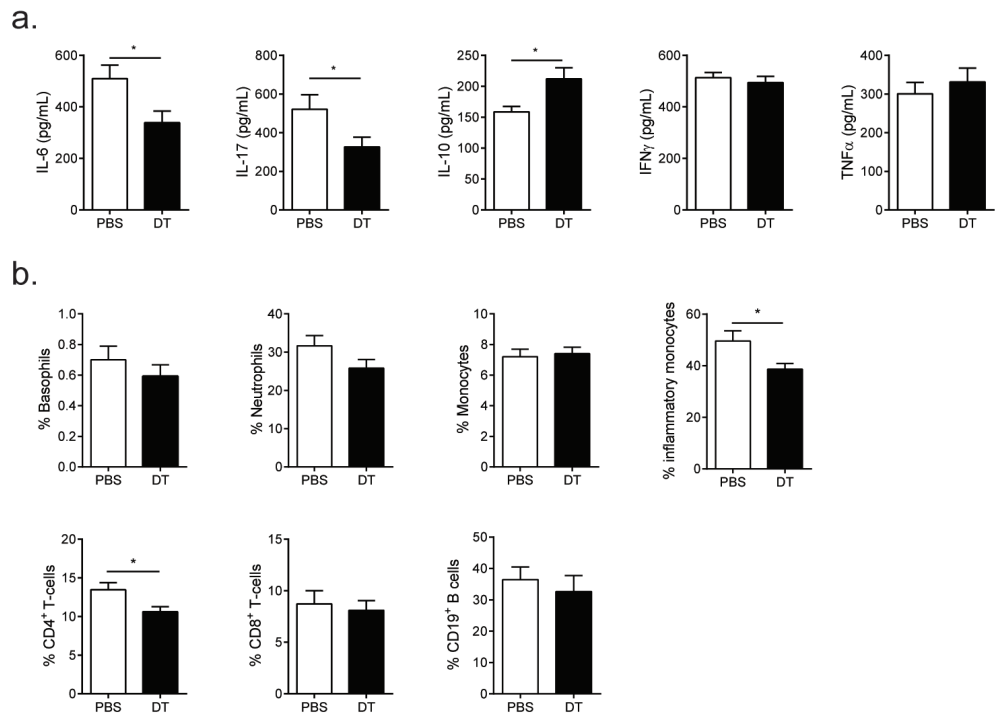

Figure 4. Preclinical FcεRIβ$^+$-cell depletion influences systemic cytokine profile and peripheral leucocytes subsets.
(A) Serum levels of IL-6, IL-17, IFNγ and IL-10 were quantified in serum of PBS and DT treated RMB-DBA/1 mice.(n=15/group) (B) FACS analysis of the blood compartment for peripheral leucocytes (n=15/group). (**P<0,01 ***P<0.001). A representative example of 2 independent experiments is depicted.

an increase in collagen specific IL-10 production (PBS 875±225 vs. DT 1912±96 pg/mL, p=0.002) and a decrease in collagen specific IL-17 production (PBS 301±73 vs. DT 121±33 pg/mL, p=0.03) (Fig 5c). This change in the T cell cytokine response was in line with the intracellular cytokine staining results for the CD4$^+$ T cells.

As DT-injections can lead to side effects, we performed additional control experiments in which wild-type mice (C57Bl/6-DBA/1) were treated with either PBS or DT in a similar experimental set-up as described in supplementary Figure 1c. We did not observe any effects of DT on clinical score (Suppl. Fig 2a), serum cytokine profile (Suppl. Fig 2b). and blood leukocyte subsets (Suppl. Fig 2c) in wild-type control mice. Aspecific stimulation of splenocytes resulted in an increase in IL-17 and TNFα, but no differences in CII specific changes in cytokine profile in DT treated compared to PBS treated wild type mice (Suppl. Fig. 2d). Intracellular analysis for T_h17 and FoxP3$^+$ T cells showed also no differences (Suppl. Fig. 2e).

Taken together these data suggests a regulatory role for mast cells in the early stages of CIA, when the immune response is established that precedes the onset of clinical symptoms.

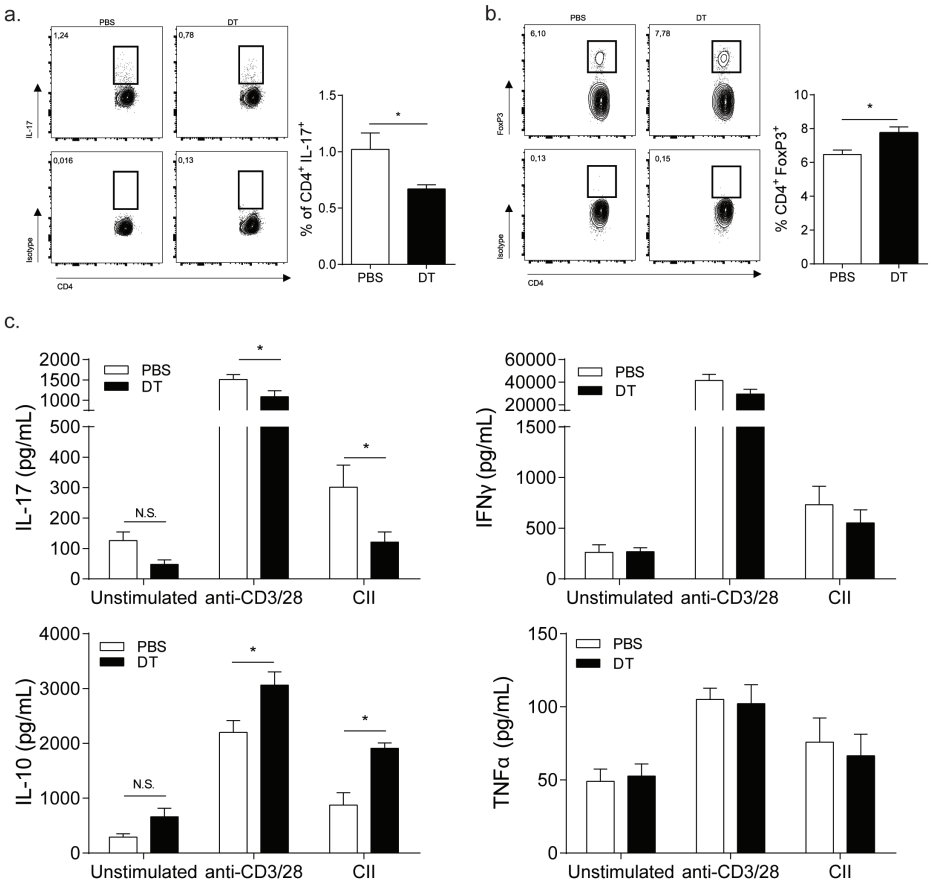

Figure 5. Altered CD4⁺T-cell phenotype in spleen and enhanced anti-inflammatory response towards collagen type II of splenocytes from FcεRIβ⁺-cell depleted mice.
(A) Splenocytes from PBS and DT injected RMB-DBA/1 mice were stained intracellular for IL-17 after stimulation with anti-CD3/28 (n=15/group). (B) Splenocytes from PBS and DT injected RMB-DBA/1 mice were stained intracellular for FoxP3. (n=15/group). (C) Cytokine release of splenocytes from PBS or DT injected RMB-DBA/1 mice after re-stimulation with either αCD3/28 or collagen type II (n=15/group). (*P<0.05). A representative example of 2 independent experiments is depicted.

Discussion

Mast cells are well known for their contribution to allergies and hypersensitivity [32]. They have also been implicated in autoimmune diseases such as RA. Increased mast cell numbers in the synovium were observed, as well as elevated levels of mast cell activation markers, such as tryptase and chymase, in synovial fluid [33].

Both in RA as well as in experimental arthritis models the possible pathogenic role of mast cells is still under debate. Several studies have been performed in mice with mutations in the gene encoding for the c-Kit receptor causing mast cell deficiency. C-kit signaling however is not only essential in mast cell development and survival but also affects many other hematopoietic lineages like stem cells, innate lymphoid cells, neutrophils and non-

hematopoietic cells such as melanocytes and germ cells [34]. To circumvent the side-effects of cKit mutations, mice have been generated with a normal Kit signalling pathway, in which mast cell deficiency is more selective. Induction of arthritis by K/BxN serum transfer in for example Cpa3-cre mice induced clinical arthritis, which was comparable to mast cell competent mice [15]. Similarly, pharmacological stabilization of mast cells in the clinical phase of CIA with the drug Nedocromil was unable to reduce the clinical score of DBA/1 mice compared to placebo treated mice [35]. In contrast, it was shown that the mast cell inducible knock-out Mcpt5-cre iDTr mouse developed reduced levels of collagen induced arthritis, when mast cells were depleted before induction of arthritis (i.e. before the first immunization with CII) [21]. This depletion reduced both the number of immune cells in the draining lymph nodes and the amount of secreted inflammatory cytokines in response to collagen II. DT treatment of these Mcpt5-Cre iDTR mice results however in a reduction in the number of connective tissue type mast cells (CTMC) only , not that of mucosal mast cells (MMC) [36]. Interestingly, it is reported that mast cells in the inflamed synovium express less Mcpt5 compared to perivascular mast cells [37], indicating a micro-environmental regulation of the mast cell phenotype inside the synovium.

In the current study, we sought to investigate the contribution of mast cells to the different stages of collagen induced arthritis, when the first immunization was done in a mast cell competent mouse to exclude the possibility that the absence of mast cells affected the immunization efficiency. We have crossed the RMB mouse on a C57BL/6 background [22] with the DBA/1 mouse, thus generating the RMB-DBA/1 mouse, in which mast cells can be selectively depleted while being highly susceptible to the induction of CIA. We observed an >90% incidence of CIA in these RMB-DBA/1 mice, which is comparable to the incidence in homozygous DBA/1 mice [20,28], rendering this a valuable mouse model to study mast cells in CIA. Using this model, we were able to deplete fully FcεRIβ^{+} cells (mast cells and basophils) at any phase of disease. Activated mast cells secrete a wide range of proteases and lipid mediators, but also of a number of cytokines and chemokines, such as IL-6, IL-8 and CCL2. These cytokines are described to influence both the adaptive immune response and attraction of leukocytes to the side of inflammation [38]. Activated basophils are well-known for their capacity to secrete cytokines such as IL-4, IL-13,which influence the T cell skewing towards a T_h2 response [39]. The repopulation kinetics of mast cells and basophils after the last DT injection differs. While a complete recovery of basophils is observed within 12 days after the last DT injection, mast cells depletion lasts for the entire duration of the experiment. Therefore, it is highly likely that the majority of the observed effects in this study are due to mast cell depletion rather than the absence of basophils. Nonetheless, we cannot exclude that basophils do contribute to the immune response in this relatively short period.

Our data suggest that mast cells are involved during the initiation of arthritis and that their role is limited after the first appearance of clinical symptoms, at least in the CIA model. During the preclinical phase of CIA, we and other have detected mast cell specific

activators like collagen specific IgE antibodies [40]. IgE-mediated activation leads to the degranulation of mast cells resulting in the release of immune modulating mediators. Furthermore, a peak of degranulated (activated) mast cells in the knee and digits was recently shown around the booster injection in the CIA model [35]. This may suggests that mast cells contribute to the early development of an immune response in experimental arthritis. For example, it has been shown that mast cells can contribute to T cell priming through the release of TNFα in the draining lymph nodes leading to expansion of the tissue [41]. Our ex vivo experiments indicated that mast cell depletion in the preclinical phase resulted in an altered T cell skewing, as we detected a marked reduction in IL-17 and an increase in IL-10 production by splenocytes of mast cell depleted mice after stimulation with collagen type II.

IL-6 is a key cytokine for the development and maintenance of T_h17 cells in mice [42]. This cytokine can be produced by various innate immune cells including mast cells. IgE mediated mast cell activation results in high secretion of both IL-6, which is a potent promoter of T_h17-cell induction, and TNFα, which can drive the hypertrophy of the draining lymph nodes and the recruitment of naïve $CD4^+$ T cells into the lymph node [41,43]. In the current study, we detected collagen type II specific IgE antibodies, indicating the presence of a CIA specific mast cell activator. Mast cell derived cytokines such as IL-6 may influence T cell skewing or other cellular interactions in the lymph node. The importance of IL-6 in CIA has been demonstrated by blocking IL-6, which reduces the severity of arthritis [44]. Clinical trials in human RA with anti-IL6R (Tocilizumab) have demonstrated that blockade of IL-6 has therapeutic efficacy in (early) RA patients [45]. In this study we observed lower levels of serum IL-6 in mast cell depleted mice. This reduction of IL-6 coincided with an altered T cell skewing towards a more anti-inflammatory T-cell phenotype.

T_h17 cells have been implicated to play a role in CIA by driving arthritis progression through e.g. osteoclast activation [44,46]. Furthermore, it was shown that anti-IL-17 treatment significantly reduced arthritis development and severity in mice [46,47]. Also in human RA, IL-17 and IL-17^+ cells have been reported to contribute to RA progression. For example, elevated levels of IL-17A can be detected in serum and synovial fluid of RA patients [48], and IL-17^+ cells can be present in synovial tissue from RA patients. Interestingly, the most abundant IL-17^+ cell type in RA synovium were mast cells [49]. Flow cytometry analysis of stimulated splenocytes from mast cell depleted mice showed a decrease in $CD4^+$ IL-17^+ T cells and an increase in regulatory $CD4^+$ $FoxP3^+$ T cells. Furthermore, we detected elevated levels of IL-10 in the supernatant of splenocytes after stimulation with collagen II. In CIA, the protective role of IL-10 has been previously been shown by both systemic treatment of IL-10 and in mice deficient for IL-10 [50–52].

Whether mast cells play a role in the established phase of RA is not known. However, it is known that they can represent an abundant cell type in the inflamed synovium [6,7]. Likewise, it has been shown that the auto immune response coinciding with sero-positive RA, represents features of an active ongoing immune response [53]. This could also

include the mast cell which might play a role in the modulation of this response either in the inflamed synovial tissue or in the draining lymph node by the secretion of cytokines [54]. Whether mast cells also mediate other effects in RA is not known. However, the presence of mast cells in both human RA and mouse experimental arthritis suggests a contributing part. Although CIA shows many similarities with human RA it also differs in terms of progression of arthritis. Since human RA is less progressive and shows also flares of arthritis it could be that mast cells here do play a role.

The role of mast cells has also been investigated in other models of auto-immune diseases, such as experimental autoimmune encephalomyelitis (EAE). Similar to the results obtained from experimental arthritis studies, also data from EAE studies vary depending on the mast cell -deficient mouse strain used [55]. As circulating IL-6 and IL-17 levels were reduced in this study, it would be of interest to determine mast cell dependent effects on EAE with our RMB mouse, since it has been shown that IL-6 and IL-17 are important in EAE development [56]. Taken together, the selective absence of mast cells can have different consequences in different diseases, depending on the time of mast cell depletion, the mouse strain used and/or the experimental conditions used. As disease manifestation varies between individual patients, it is conceivable that the contribution of mast cells to disease development can vary between individuals, between disease stages as well as between different diseases.

Conclusions

In conclusion, we show that depletion of mast cells during the initiation of experimental arthritis decreases disease severity, while depletion of mast cells in established disease had no effect. Depletion of mast cells in the pre-clinical phase of CIA is associated with a more anti-inflammatory T cell response, suggesting that mast cells could play a role in the regulation of the adaptive immune response in early arthritis.

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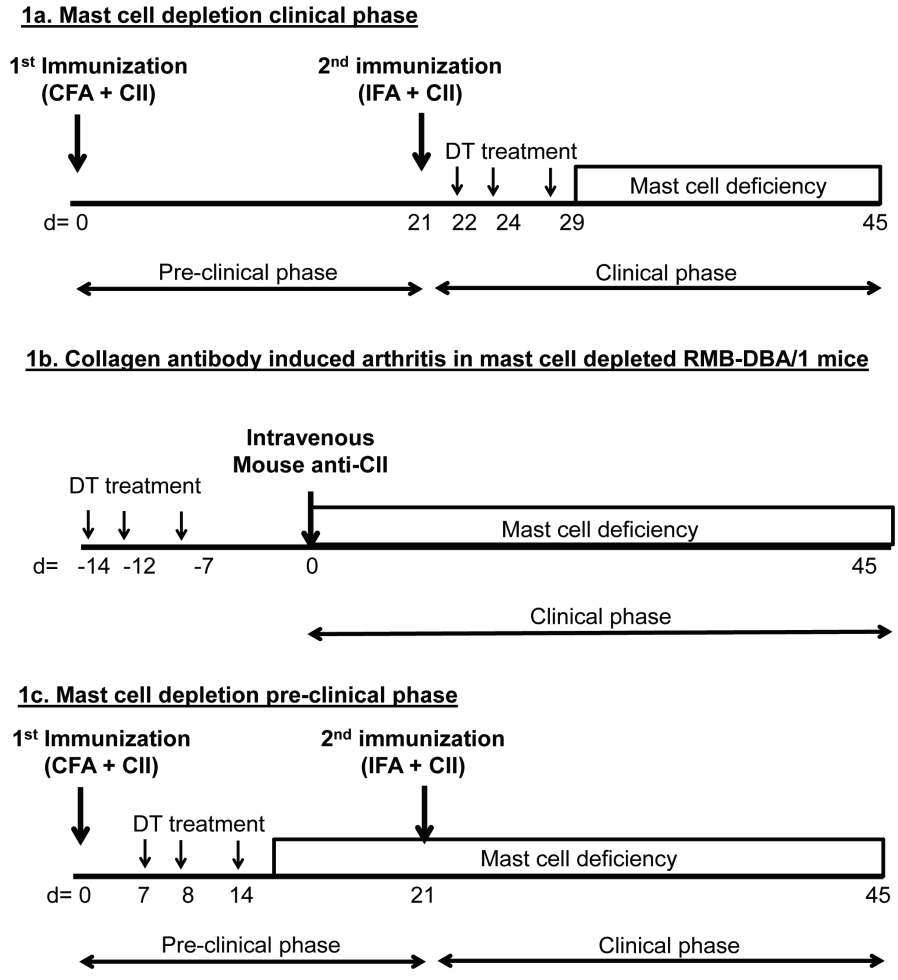
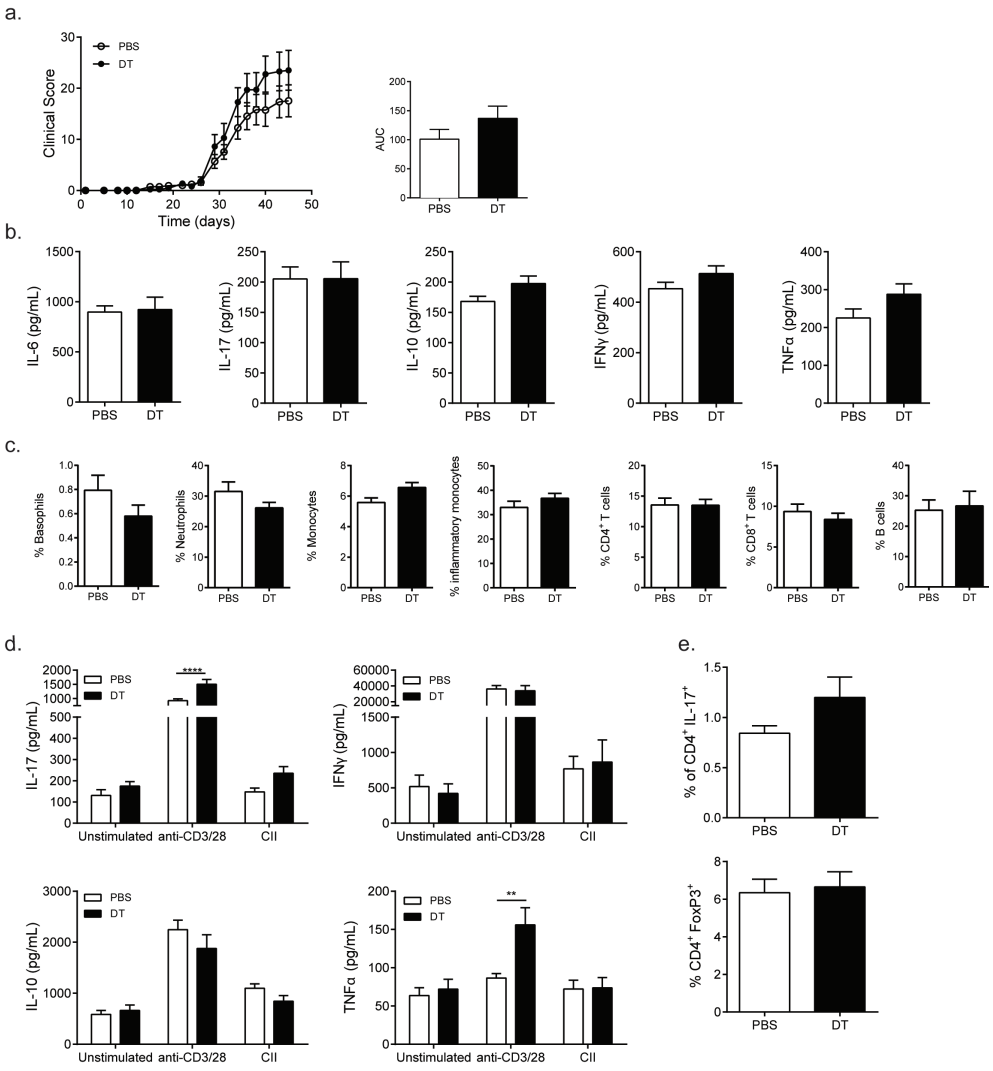
Supplementary figures



CFA:	Complete Freund's adjuvant
CII:	Bovine collagen type II
IFA:	Incomplete Freund's adjuvant
DT:	Diphtheria Toxin

Supplementary figure 1. Study outline of performed arthritis experiments in RMB-DBA/1 mice.
(A) Mast cell depletion in clinical phase of CIA. (B) Collagen antibody induced arthritis in mast cell depleted RMB-DBA/1 mice. (C) Mast cell depletion in pre-clinical phase. (CFA: Complete Freund's Adjuvant, CII: Collagen type II, IFA: incomplete Freund's Adjuvant, DT: diphtheria toxin)



Supplementary figure 2. DT treatment in wild-type control animals.
(A) Progression of CIA was monitored by clinical scoring of C57Bl/6-DBA/1 mice injected with either PBS or DT. (B) Serum levels of IL-6, IL-17, IFNγ and IL-10 were quantified in serum of PBS and DT treated C57Bl/6-DBA/1 mice. (C) FACS analysis of the blood compartment for peripheral leucocytes in PBS and DT treated C57Bl/6-DBA/1 mice. (D) Cytokine release of splenocytes from PBS or DT injected C57Bl/6-DBA/1 mice after re-stimulation with either αCD3/28 or collagen type II or unstimulated medium control (n=15/group). (E) Splenocytes from PBS and DT injected C57Bl/6-DBA/1 mice were stained intracellular for IL-17 after stimulation with anti-CD3/28 (n=15/group). Splenocytes from PBS and DT injected C57Bl/6-DBA/1 mice were stained intracellular for FoxP3. (**P<0,01, ****P<0,001) All graphs n=15/group).

Chapter 4

Presence of anti-citrullinated protein antibodies in sera of non-rheumatic cardiovascular patients is associated with long-term mortality.

Manuscript submitted

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Abstract:

Objective:
Cardiovascular (CV) mortality is higher in patients with rheumatoid arthritis (RA), in particular when anti-citrullinated protein antibodies (ACPA) are present. Recently, ACPA have also been described in CV patients without RA.
In this study, we aimed to confirm the presence of ACPA in the serum of non-rheumatic CV patients. In addition, we aimed to assess the relation between ACPA with clinical parameters, plaque phenotype and long-term mortality.
Methods
Sera of CV patients who were included into either the AtheroExpress, Circulating Cells or MISSION! study were analyzed for presence of ACPA. After exclusion of patients with known rheumatic diseases, we analyzed the associations between ACPA positivity and clinical characteristics in all three cohorts. Furthermore, we aimed to associate ACPA with either vulnerable plaque characteristics in the AtheroExpress cohort or long-term mortality in the MISSION! study.
Results
Compared to sera from healthy controls, we detected a significantly higher percentage of ACPA positivity in the sera of patients in all three study cohorts. Clinical analysis of these ACPA negative and positive CV patients showed that presence of ACPA did not associate with general characteristics such as lipid profile, BMI or other known risk factors. Plaque phenotype did not differ between ACPA negative and positive CV patients in the AtheroExpress study. However, we observed an increased cumulative cardiac mortality in ACPA positive CV patients compared to ACPA negative CV patients in the MISSION! cohort. Corrected for age, ACPA positivity was independently associated with long-term mortality.
Conclusion
This study confirms that ACPA is present in a subpopulation of non-rheumatic CV patients. Clinical analysis showed that long-term mortality in STEMI patients without RA was independently associated with the presence of ACPA. Therefore, ACPA in patients without RA might act as a cardiovascular factor.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of the synovial tissue, which affects around 1% of the world population. The persistent synovitis leads to the breakdown of cartilage and bone that ultimately results in joint disability [1]. The generation of autoantibodies is a key characteristic of the majority of RA patients and especially anti-citrullinated protein antibodies (ACPA) are frequently found in the sera and synovial fluid of RA patients [2]. Citrullination or peptidylarginine deimination is a physiological process catalyzed by a family of enzymes called peptidyl arginine deiminases (PAD-1-4) [3]. These enzymes convert the positively charged amino acid arginine to an uncharged amino acid citrulline in the presence of relatively high calcium concentrations [3]. Clinically, ACPA is an important marker for a more progressive RA compared to ACPA$^-$ negative RA patients [4]. Additionally, ACPA positivity in RA patients is associated with a higher cardiovascular mortality [5].

The major underlying pathology of many acute cardiovascular diseases is the process of atherosclerosis. Atherosclerosis is the sub-endothelial accumulation of lipids and inflammatory cells in the arterial wall, resulting in the development an atherosclerotic plaque, generally composed of a lipid core covered by a fibrous cap. Thrombosis after rupture or erosion of such a fibrous cap can lead to occlusive vascular diseases such as myocardial infarction or stroke [6]. Citrullination of proteins is not restricted to the synovial tissue and is reported to also occur in other tissues, for example within the myocardium and inside the atherosclerotic lesion [7, 8]. In addition, PAD enzymes have been detected in high levels in atherosclerotic lesions of non-RA patients with cardiovascular disease [8]. Low levels of circulating ACPA have been reported in a small proportion of patients with acute cardiovascular syndromes in the absence of rheumatic diseases [9]. Formation of immune complexes composed of ACPA and citrullinated proteins can activate the complement cascade as well as Fc-receptor mediated activation of local leukocytes, thereby fuelling the ongoing inflammatory response. Therefore, the presence of ACPA in cardiovascular patients could potentially contribute to atherosclerotic lesion growth in the vessel wall, however up to date a causal relation between ACPA and cardiovascular diseases such as atherosclerosis has not been established.

In the current study, we aimed to determine whether ACPA is detectable in CV patients without reported RA. In addition, we aimed to associate ACPA positivity with clinical characteristics, plaque phenotype and long-term mortality in these patient cohorts.

Methods

Study population and design

Athero-Express

A total of 135 patients of the Athero-Express were included in this study. The Athero-express biobank involves patients that underwent carotid endarterectomy (CEA) in two Dutch teaching hospitals in Utrecht and Nieuwegein, the Netherlands [10]. The criteria to perform carotid endarterectomy were based on the recommendations by the Asymptomatic Carotid Atherosclerosis Study (ACAS study) for asymptomatic patients and the North American Symptomatic Carotid Endarterectomy Trial and the European Carotid Surgery Trial (NASCET study) for symptomatic patients [11–15]. The local medical ethical boards of both participating hospitals approved this study. The participating patients signed a written informed consent prior to inclusion. The patient's baseline characteristics and medical history were obtained via questionnaires and the patient medical records.

Circulating Cells

The study cohort consists of a total of 443 patients, these patients form a subgroup of the Centre for Translational Molecular Medicine (CTMM) – Circulating Cells study cohort [16]. In brief, Circulating Cells is a multi-centre study in which CAD patients scheduled for coronary angiography were included. Exclusion criteria were age <18 years, inability to give informed consent, suspected drug or alcohol abuse, serious concomitant disease and serious recent infectious disease in the last 6 weeks or suspected elevated state of the immune system, and non-cooperativeness. The Circulating Cells protocol was approved by the review board or ethics committee of each participating centre. Nine months after inclusion, patients were contacted and any major adverse cardiovascular events (MACE) that occurred in the preceding months were recorded. MACE is the primary endpoint of this study and is defined as cardiovascular disease related death, myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting and cardiovascular accident.

Mission

Data for this study were used from 300 patients with ST-segment elevation myocardial infarction (STEMI), who were initially included in the MISSION! Intervention Trial. STEMI was defined as ongoing chest pain (>30 minutes), accompanied with ST-elevation (≥0.2 mV in ≥2 leads in V1-V3 or ≥0.1 mV in other leads) or presumed new left bundle branch block (LBBB) and a typical rise and fall of cardiac biomarkers. In case of out-of-hospital cardiac arrest, only patients with return of spontaneous circulation at the moment of arrival at the catheterization laboratory were included. Patients with prior myocardial infarction (n=11) or prior revascularization (n=6) were excluded. In addition, patients with evidence for rheumatic disease (n=8) were excluded for analysis. The MISSION! Intervention Trial was conducted from February 2004 to October 2006. Clinical and angiographic results in patients with STEMI treated with either Bare Metal Stents (BMS) or Sirolimus Eluting Stents (SES) during primary percutaneous coronary intervention (PCI) was evaluated [17].

Information on all-cause mortality was obtained from the Dutch Municipality Records registry. Cause of death was retrieved from general practitioners. Clinical follow-up data was collected during the 30 days, 3, 6 and

12 months outpatient clinic visits. Follow-up data on serious adverse events including myocardial infarction, revascularisation and stroke was obtained by telephone interviews at 2, 5 and 10 years after admission. The study protocol was approved by the institutional ethical committee. Written informed consent was obtained from all patients before enrolment in the study. In the current study, baseline blood samples derived from the patients before primary PCI were used for anti–citrullinated protein antibodies (ACPA) determination.

SYNTAX Scoring

To assess the complexity of coronary artery disease, coronary angiographic primary PCI images were used to calculate the SYNTAX score [18]. This was performed by an experienced interventional cardiologist.

Anti–citrullinated protein antibodies measurement

ACPA positivity was determined based on reactivity of sera against a third generation cyclic citrullinated peptide (CCP3) in a commercially available ELISA system (Quanta LiteTM CCP3.1 IgG/IgA Cat# 704550, INOVA Diagnostics Inc., US). According to supplier's manual, values above 20 aU/mL were considered CCP3.1 positive.

Statistical analysis

Normally distributed continuous variables were reported as mean and standard deviation, and compared with Student's t-test. Skewed distributed continuous variables were reported as median and interquartile range, and compared with Mann-Whitney U test. Categorical variables were reported as number and percentage, and compared with Pearson's chi-square test. Event-free survival was analyzed with Kaplan-Meijer estimates and compared between groups with the log-rank test. Cox regression was performed to assess the association between the ACPA positivity and primary and secondary outcome measures. All statistical tests were performed with SPSS software (Version 22.0, SPSS IBM Corp., Armonk, New York). P-values <0.05 assessed by two-sided tests were considered to be statistically significant.

Results

Presence of ACPA was determined by screening sera, obtained from CV patients included in the AtheroExpress, Circulating Cells and MISSION! cohorts, for reactivity towards a cyclic citrullinated peptide (CCP3).

Baseline characteristics

The baseline characteristics of patients included in the AtheroExpress are summarized in table 1. In brief, the cohort has a mean age of 67 and a male prevalence (71%), which reflects a relatively typical population of patients with vascular occlusive diseases. The majority of patients was symptomatic (74%) as illustrated by the incidence of amaurosis fugax, a TIA or a stroke, was hypertensive (86%) and used statins (69%).

Table 1: Baseline patient characteristics of AtheroExpress

Characteristic	**Median (±SD)**
Age, mean years (SD)	67 (9)
BMI, mean kg/m2 (SD)	27 (4)
	n (%) of patients
Male	96/135 (71%)
Smoker	55/134 (41%)
Diabetes mellitus	25/135 (19%)
Statin use	93/135 (69%)
Hypertension	116/135 (86%)
Hypersensitive	27/132 (20%)
History VI	4/135 (40%)
History MI	30/134 (22%)
Clinical presentation	
Asymptomatic	35/135 (26%)
Symptomatic	100/135 (74%)
Amaurosis fugax	22/135 (16%)
TIA	51/135 (38%)
Stroke	27/135 (20%)

The circulating cells cohort consist of a total of 443 patients with a mean age of 63 and a male prevalence of 71%. The majority of the patients were diagnosed with stable coronary artery disease (CAD) (81%), whereas the remaining patients suffered from unstable CAD (non-ST elevation myocardial infarction/unstable angina) (19%). Table 2 summarizes the baseline characteristics of this patient cohort.

Table 2: Baseline patient characteristics of Circulating Cells

Characteristic	**Median (±SD)**
Age, mean years (SD)	63 (10)
BMI, mean kg/m2 (SD)	27 (4)
Men (%)	314/443 (71%)
	Median (IQR)
SBP (mm Hg)	136 (122-145)
DBP (mm Hg)	78 (70-85)
Glucose (mmol/L)	6,55 (5,40-6,90)
HB (mmol/L)	8,70 (8,30-9,20)
LDL-C (mmol/L)	2,51 (1,87-3,01)
HDL-C (mmol/L)	1,08 (1,24-0,89)
Triglycerides (mmol/L)	1,57 (0,99-1,90)
hsCRP (ng/mL)	7919 (1685-7235)
WBC (x1000 cells/µL)	7,3 (5,9-8,5)
	n (%) of patients
Current smoker	97/443 (22%)
Diabetes Mellitus	20/443 (20%)
Hypertension	281/443 (64%)
Beta-Blocker	336/443 (76%)
Ca-antagonist	130/443 (29%)
Aspirin	369/443 (83%)
Vitamin K antagonist	47/443 (11%)
Statin	351/443 (79%)

The MISSION! cohort consists of a total of 275 patients with a mean age of 59 and a male prevalence of 76%. Baseline characteristics are summarized in table 3.

Table 3: Baseline patient characteristics of Circulating Cells

Characteristic		**n= 246**
Patient characteristics		
Age, mean (SD), y		58,9 (11,5)
Woman		65 (275)
Body mass index, mean (SD), kg/m2		26,6 (4,5)
Risk factors		
Treated hypertension		76 (28)
Diabetes		25 (9)
Treated hyperlipidemia		48 (17)
Current smoker		152 (55)
Family history of CVD		122 (44)
Clinical characteristics		
Out of hospital cardiac arrest		7 (3)
Cardiogenic shock		5 (2)
Anterior infarction		145 (53)
Infarct size, median (IQR), g/m2		9,1 (4,4 – 15,7)
Troponine max, median (IQR)		5.8 (2,4 – 10)
CK max, median (IQR)		1985 (966 – 3633)
Troponine max (log)		0,62 (0,55)
CK max (log)		3,3 (0,5)
Number of vessels diseased (>50%)	1	154 (56)
	2	107 (39)
	3	12 (4)
Complete revascularization		275 (100)
Culprit vessel	RCA	82 (30)
	LAD	150 (55)
	LCX	43 (16)
Proximal lesion		138 (50)
TIMI flow before intervention 0		189 (69)
Drug-eluting stent		135 (49)
Irreversible ischemia myoview		198 (72)
Irreversible ischemia myoview		107 (39)
Syntax score before pPCI (IQR)		14,5 (8,8 – 20,4)
Syntax score after pPCI (IQR)		8,9 (6,7 – 13,2)
CRP, median (IQR)		0,5 (0,0 – 6,3)
CRP (log)		4,6 (5,5)
CRP >3mg/L		108 (39)
Clinical endpoints		
Re-infarct		36 (13)
Cardiac death		13 (5)
Death		35 (13)
Re-infarct or death		65 (24)

CCP3-reactivity in the sera of CV patients

CCP3-positivity in the tested cohorts as well as a group of age matched healthy controls is depicted in figure 1. We observed that 1,9% (3/160) of the healthy controls, 8,1 % (11/135) of the Athero- Express patients, 5,3% (23/430) of the Circulating Cells patients and 10,0% (30/292) of the Mission! patient cohort showed reactivity for the CCP3 peptide. Statistical analysis showed that the percentages of CCP3 reactive antibodies in all the cohorts are significantly elevated compared to healthy controls.

Clinical characteristics

AtheroExpress

Next, we aimed to correlate ACPA positivity with clinical observations within the AtheroExpress database. We did not observe associations between ACPA positivity and any of the following plaque characteristics: fat deposition, collagen, smooth muscle cells, macrophages, microvessel density. In addition, clinical presentation (asymptomatic (n=35) or symptomatic (n=100) patients) was not associated with ACPA positivity. Furthermore, analysis of the symptomatic patients did not show any association with clinical presentation (data not shown).

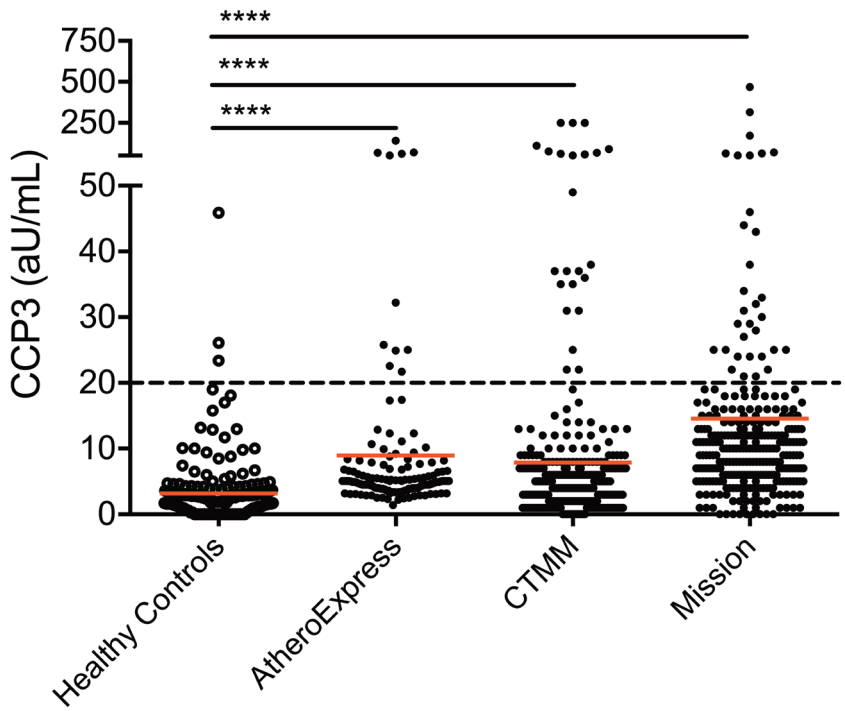

Figure 1. Presence of ACPA by determination of CCP3 reactive antibodies in the sera of age-matched healthy controls and three cohorts of cardiovascular patients. Dashed line indicates the cut-off value, which was set on 20 aU/mL. **** P=0.0001

Circulating Cells

Clinical analysis of ACPA positivity in the circulating cells cohort showed no significant differences in clinical characteristics between CCP3 negative and positive patients as shown in table 3. However, the majority of the CCP3 positive patients were diagnosed with stable CAD (90%).

Table 4: Characteristics of CCP3 negative and positive CV patients in the Circulating Cells cohort

Characteristic	CCP3 Negative	CCP3 Positive	P value
Age, mean years (SD)	62 (10)	67 (10)	0,174
BMI, mean kg/m2 (SD)	26,9 (4,4)	26 (3)	0,453
Men	301/421 (71%)	13/22 (59%)	0,212
SBP (mm Hg) (IQR)	134 (121-145)	143 (128-158)	0,174
DBP (mm Hg) (IQR)	80 (70-85)	77 (73-82)	0,944
Glucose (mmol/L) (IQR)	6,0 (5,4-6,9)	6,0 (5,6-6,7)	0,687
HB (mmol/L) (IQR)	887 (8,3-9,2)	8,6 (8,4-9,1)	0,742
LDL-C (mmol/L) (IQR)	2,39 (1,88-3,02)	2,20 (1,81-2,63)	0,350
HDL-C (mmol/L) (IQR)	1,04 (0,89-,1,23)	1,03 (0,94-1,37)	0,730
Triglycerides (mmol/L) (IQR)	1,32 (1,00-1,91)	1,05 (0,84-1,62)	0,080
hsCRP (ng/mL) (IQR)	2954 (1672-7281)	3997 (2033-7134)	0,381
WBC (x1000 cells/µL) (IQR)	7,1 (5,9-8,5)	7,3 (6,2-8,9)	0,549
Current smoker	92/421 (22%)	5/22 (23%)	0,928
Diabetes Mellitus	86/421 (21%)	4/22 (18%)	0,790
Hypertension	265/421 (65%)	16/22 (73%)	0,360
Beta-Blocker	317/421 (75%)	19/22 (86%)	0,237
Ca-antagonist	123/421 (29%)	7/22 (32%)	0,794
Aspirin	353/421 (84%)	16/22 (73%)	0,173
Vitamin K antagonist	44/421 (10%)	3/22 (14%)	0,636
Statin	334/421 (79%)	17/22 (77%)	0,816

MISSION!

CCP3 positivity did not associate with clinical parameters within the MISSION! as shown in table 2.

Table 5: Characteristics of CCP3 negative and positive CV patients in the MISSION! cohort

Characteristic		CCP3 Negative	CCP3 Positive	P value
		n= 246	N = 29	
Patient characteristics				
Age, mean (SD), y		58,4 (11,6)	62,7 (10,4)	0,056
Woman		55 (22)	10 (34)	0,146

Characteristic		CCP3 Negative	CCP3 Positive	P value
Body mass index, mean (SD), kg/m2		26,5 (4,1)	27,0 (5,5)	0,586
Risk factors				
Treated hypertension		70 (28)	6 (21)	0,376
Diabetes		23 (9)	2 (7)	0,664
Treated hyperlipidemia		44 (18)	4 (14)	0,583
Current smoker		136 (55)	16 (55)	0,991
Family history of CVD		108 (44)	14 (48)	0,667
Clinical characteristics				
Out of hospital cardiac arrest		7 (3)	0 (0)	0,355
Cardiogenic shock		5 (2)	0 (0)	0,445
Anterior infarction		128 (55)	17 (63)	0,413
Infarct size, median (IQR), g/m2		8,6 (4,4-15,4)	13,1 (4,7-18,7)	0,250
Troponine max, median (IQR)		5,5 (2,4-9,5)	8,6 (2,4-14,1)	0,126
CK max, median (IQR)		1948 (957-3512)	2297 (1045-4657)	0,258
Troponine max (log)		0,60 (0,55)	0,78 (0,52)	0,129
CK max (log)		3,2 (0,5)	3,3 (0,5)	0,264
Number of vessels diseased (>50%)	1	141 (58)	13 (45)	0,183
	2	92 (38)	15 (52)	0,144
	3	11 (5)	1 (3)	0,792
Complete revascularization		244 (100)	29 (100)	~
Culprit vessel	RCA	74 (30)	8 (28)	0,781
	LAD	133 (54)	17 (59)	0,641
	LCX	39 (16)	4 (14)	0,773
Proximal lesion		126 (51)	12 (41)	0,316
TIMI flow before intervention 0		169 (69)	20 (71)	0,814
Drug-eluting stent		124 (50)	11 (38)	0,204
Irreversible ischemia myoview		178 (78)	20 (80)	0,824
Irreversible ischemia myoview		96 (43)	11 (46)	0,780
Syntax score before pPCI (IQR)		14,5 (9,0-20,5)	14,3 (7,3-19,9)	0,418
Syntax score after pPCI (IQR)		9,0 (7,0-13,0)	8,0 (4,3-14,5)	0,389
CRP, median (IQR)		0,0 (0,0-6,0)	5,0 (0,0-9,0)	0,122
CRP (log)		(-)4,8 (5,5)	(-)3,1 (5,4)	0,124
CRP >3mg/L		92 (43)	15 (60)	0,110
Clinical endpoints				
Re-infarct		32 (13)	4 (14)	0,906
Cardiac death		9 (4)	4 (14)	0,031
Death		27 (11)	8 (28)	0,011
Re-infarct or death		54 (22)	11 (38)	0,055

Using the MISSION! cohort we were able to investigate whether CCP3 positivity correlated with long term mortality, which is of interest, as it is known from RA patient studies that ACPA positivity increases the overall mortality rate. As shown in figure 2 there was an association between ACPA positivity and mortality rate after 10 years of follow-up. One possibility could be that the atherosclerotic lesion complexity, location and number of affected coronary arteries is dependent on ACPA levels. These parameters are used to calculate the SYNTAX score. We determined the SYNTAX score of all included patients in the MISSION! cohort. We did not observe a correlation between ACPA levels and SYNTAX score (data not shown).

Discussion

The pathogenesis of RA is characterized by the development of different autoreactive antibodies such as rheumatoid factor (RF), anti-carbamylated proteins antibodies (a-CarP) and anti-citrullinated protein antibodies (ACPA) [2, 19, 20]. Especially ACPA is an important clinical biomarker, which is strongly associated with a more progressive disease course, response to treatment and degree of joint destruction [2]. In addition, the presence of ACPA in RA patients is associated with increased incidence of cardiovascular disease and mortality [21]. Products of persistent inflammation in RA, e.g. cytokines and

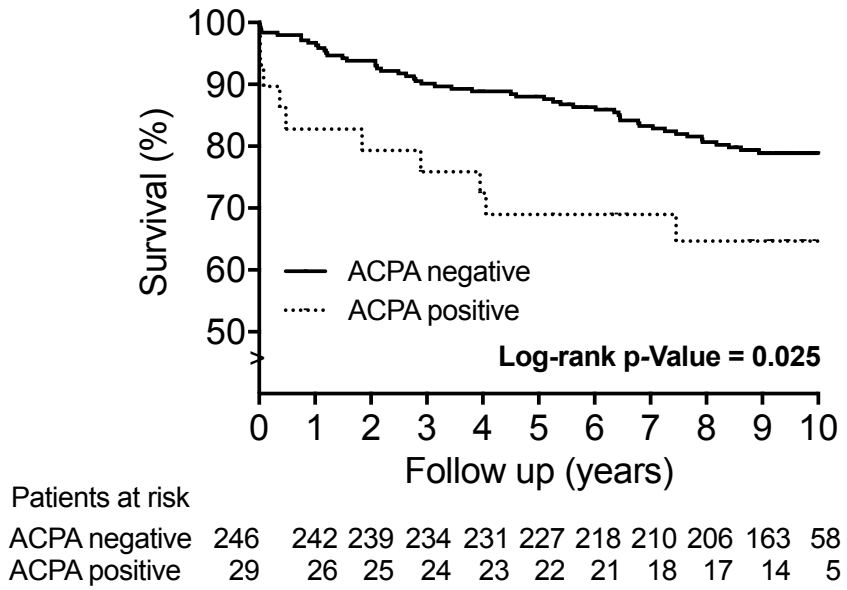

Figure 2. Increased cumulative cardiac mortality was observed in ACPA positive patients compared to ACPA negative patients. Corrected for age, ACPA positivity was independently associated with long-term mortality [HR 2.4 (CI 1.1-5.4) p-Value= 0.025].

antibodies, are thought to directly influence the progression of atherosclerosis [22]. Both RA and atherosclerosis share similar immunological pathways and therefore, presence of ACPA may also affect the vascular inflammation. For example, innate immune cells (macrophages, neutrophils and mast cells) with ACPA bound to their Fcγ receptors can get further activated upon interaction of cell-surface bound ACPA with citrullinated antigens [23–25]. To date, several studies have shown the presence of citrullinated proteins inside atherosclerotic lesions and within the myocardial tissue of RA as well as non-RA patients [7, 26]. In addition, enzymes that drive the citrullination process, PAD enzymes, are also known to be present inside the plaque [8]. Taken together, ACPA mediated activation of local intraplaque immune cells could lead to the release of pro-inflammatory mediators such as cytokines, chemokines and proteases. In turn, this will further enhance the lipid accumulation and destabilization of the atherosclerotic lesion.

In this study, we showed that ACPA can be detected in the serum of a subpopulation of CV patients, who had no history of rheumatic diseases. We screened three cohorts; AtheroExpress, Circulating Cells and MISSION! for the presence of ACPA. In all three tested cohorts we found that a significant proportion (5-10%) of the CV patients showed reactivity towards citrullinated peptides (CCP3) compared to 1% positivity in healthy age-matched controls. This observation is in line with a previous study, which reported presence of ACPA in 10,4% of the cases in a cohort of coronary heart disease patients compared to 3,8% in controls [9]. These data indicate that an humoral immune response towards citrullinated proteins can not only develop in RA, but also in a chronic systemic inflammatory diseases such as atherosclerosis.

In contrast to RA, the pathogenic contribution of autoreactive antibodies in cardiovascular diseases is still a subject of debate. On one hand, IgM producing B1 B cells have shown to be atheroprotective, while on the other hand IgG producing B2 B cells are reported to aggravate atherosclerosis [27]. Next to antibodies, B cells produce a variety of mediators and are able to present antigens to T cells. This underscores that B cells and their products can significantly contribute to the pathogenesis of atherosclerosis and RA. Indeed, B cell depleting therapies, such as the use of rituximab, in RA have been shown to be effective and these studies have also shown beneficial effects on the progression of atherosclerosis. Treatment of RA patients with rituximab for a period of 4 months showed an improvement in lipid profiles and a reduction in carotid intima- media thickness [28]. In addition, improvements in endothelial function as determined by flow- mediated vasodilation and in systemic inflammation as indicated by serum levels of hsCRP were reported in another clinical study, in which RA patients were treated with rituximab [29]. These studies underline a significant role for B cells in vascular inflammation in active RA patients, which is mediated by the secretion of cytokines and antigen presentation and/or the production of auto- reactive antibodies.

Association analyses of ACPA positivity in cardiovascular patients with parameters such as lipid profile, inflammatory markers and medication use did not reveal any associations in

all three analyzed cohorts. We compared plaque phenotype of ACPA positive CV patients of the AtheroExpress cohort, which did not reveal any differences in vulnerable plaque characteristics between ACPA positive and negative patients. In RA, the presence of ACPA is associated with increased mortality [30, 31]. Also in non-rheumatic cardiovascular patients of the MISSION! cohort we observed a significant association between ACPA positivity and long term mortality. We did not investigate the causal mechanism in this current study but one possibility could be the accelerated atherosclerosis in ACPA positive patients. We further analyzed this by characterizing the complexity of the coronary arteries disease by SYNTAX scoring the patients in the MISSION! cohort. However, this analysis did not reveal an association between SYNTAX score and ACPA positivity. Further research could focus on investigating the systemic inflammatory status of these patients by determining circulating proatherogenic mediators. In addition, the intraplaque citrullination could be different between APCA negative and positive patients, which could also influence the plaque stability.

There are several limitations to our study. The reported APCA titers in the presented cohorts are relatively low compared to the levels in established RA patients. This could indicate that there is a difference in the development of ACPA producing B cells and/or the inflammatory milieu in cardiovascular versus RA patients. Further research should focus on the comparison of cardiovascular ACPA with RA ACPA in terms of glycosylation, fine specificity and avidity as well as isotype class. In addition, we used a commercially available kit to determine ACPA levels. Although this assay is a highly sensitive and reproducible method, we were unable to control for citrulline specificity by including an arginine control. Finally, although we excluded all patients with known rheumatic diseases, we cannot exclude that these ACPA positive CV patients will develop symptoms of RA in the future.

In conclusion, here we confirm the presence of ACPA in serum of a subpopulation of CV patients. Analysis showed an association between ACPA positivity and long-term mortality. Further research should focus on characterizing the phenotype of cardiovascular ACPA compared to rheumatic ACPA in terms of isotype, glycosylation and affinity towards citrullinated proteins.

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Chapter 5

Circulating immunoglobulins are not associated with intraplaque mast cell number and other vulnerable plaque characteristics in patients with carotid artery stenosis

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Abstract

Background

Recently, we have shown that intraplaque mast cell numbers are associated with atherosclerotic plaque vulnerability and with future cardiovascular events, which renders inhibition of mast cell activation of interest for future therapeutic interventions. However, the endogenous triggers that activate mast cells during the progression and destabilization of atherosclerotic lesions remain unidentified. Mast cells can be activated by immunoglobulins and in the present study, we aimed to establish whether specific immunoglobulins in plasma of patients scheduled for carotid endarterectomy were related to (activated) intraplaque mast cell numbers and plasma tryptase levels. In addition, the levels were related to other vulnerable plaque characteristics and baseline clinical data.

Methods and results

OxLDL-IgG, total IgG and total IgE levels were measured in 135 patients who underwent carotid endarterectomy. No associations were observed between the tested plasma immunoglobulin levels and total mast cell numbers in atherosclerotic plaques. Furthermore, no associations were found between IgG levels and the following plaque characteristics: lipid core size, degree of calcification, number of macrophages or smooth muscle cells, amount of collagen and number of microvessels. Interestingly, statin use was negatively associated with plasma IgE and oxLDL-IgG levels.

Conclusions

In patients suffering from carotid artery disease, total IgE, total IgG and oxLDL-IgG levels do not associate with plaque mast cell numbers or other vulnerable plaque histopathological characteristics. This study thus does not provide evidence that the immunoglobulins tested in our cohort play a role in intraplaque mast cell activation or grade of atherosclerosis.

Introduction

The incidence of atherosclerotic disease is increasing by the aging population and the life style in the Western world. The mast cell, a prominent inflammatory cell type and a major effector cell in allergy and asthma, has been shown to accumulate both in the rupture-prone shoulder region of human atheromas (1,2) and in the perivascular tissue during atherosclerotic lesion progression (3). Recently, we have shown that intraplaque mast cell numbers are associated with plaque vulnerability and interestingly, with future cardiovascular events (4). In that study, mast cells numbers associated with vulnerable plaque characteristics such as lipid core size, intraplaque haemorrhage, microvessel density and inflammatory cell accumulation, suggesting that mast cells actively contribute to atherosclerotic plaque progression and destabilization. Inhibition of mast cell activation may therefore be of interest for future therapeutic interventions. However, the mechanism of mast cell activation during the development of atherosclerosis remains up to date unresolved. Previously, we and others have established that mast cells in the vessel wall can be activated by for example neuropeptides (5), complement factors (6) and lipid mediators (7) in animal models of atherosclerosis. Furthermore, the mast cell expresses the high-affinity IgE receptor (FcεR1) and the IgG receptor (FcγR) (8,9). Mast cells can be activated via IgE mediated crosslinking of the FcεR, after which mast cells release granules into the surrounding area. IgE levels have been shown to be elevated in patients with unstable angina pectoris (10) and intriguingly, also higher in dyslipidemic men as compared to control subjects (11). Furthermore, Lappalainen et al demonstrated that specific oxLDL-IgG immune complexes were able to induce mast cell activation (12). Circulating specific IgE and IgG antibodies or lipid-immunoglobulin immune complexes, which exert their effects through the FcεR and FcγRs, are known to play a role in several immune responses (9) and may thus also be involved in mast cell activation within the atherosclerotic plaque, thereby affecting plaque stability. Based on these observations, we hypothesize that circulating immunoglobulins may be involved in or be reflective of mast cell activation and thereby accelerate the destabilization of the atherosclerotic plaque. This study was designed to assess the presence of associations between immunoglobulin expression and mast cell numbers in plaques from patients with carotid stenosis. Hence, we assessed total and ox-LDL specific IgG and total IgE plasma levels and related their numbers to several mast cell parameters and established vulnerable plaque characteristics. In additions, immunoglobulin levels were related to clinical characteristics.

Materials and Methods

Study population and design

A total of 135 patients of the Athero-Express were included in this study. The Athero-express biobank involves patients that underwent carotid endarterectomy (CEA) in two Dutch teaching hospitals in Utrecht and Nieuwegein the Netherlands (13). The criteria to perform carotid endarterectomy were based on the recommendations by the Asymptomatic Carotid Atherosclerosis Study (ACAS study) for asymptomatic patients and the North American Symptomatic Carotid Endarterectomy Trial and the European Carotid Surgery Trial (NASCET study) for symptomatic patients (14-18). Patients were operated between March 2002 and August 2008 of which intraplaque mast cell numbers were available (4). In that study, patients were selected who remained healthy and patients who suffered from an event during follow-up in a 2:1 ratio. Of 135 patients blood plasma samples were available. Total mast cell numbers and baseline characteristics did not differ between the patients of which plasma was and was not available. The local medical ethical boards of both participating hospitals approved this study. The participating patients signed a written informed consent prior to inclusion. The patient's baseline characteristics and medical history were obtained via questionnaires and the patient medical records.

Materials

The carotid plaques used in this study were processed as described previously (13). In short, after surgical dissection the plaque was cut into segments of 5 mm. The segment with the largest plaque area was fixed in formalin and embedded in paraffin for histology. The two adjacent sections were frozen in liquid nitrogen and used for protein isolation. In addition, blood was drawn prior to CEA procedure and plasma was stored at -80 °C.

Quantification of immunoglobulin and MC tryptase levels in patient plasma

Plasma total IgE and IgG levels were measured using a human IgG and IgE ELISA according to manufacturer's protocol (Bethyl Laboratories, Montgomery, TX). Plasma IgG-oxLDL levels were measured by coating cupper oxidized human LDL in PBS (pH=9,0) on MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) overnight at 4˚C. Diluted Samples and standards (Biomedica, Wien) were added and incubated for 2 hours at 37˚C. Supernatants were discarded and plates were washed thoroughly. Anti-human IgG-HRP (Bethyl Laboratories, Montgomery, TX) was added as detection antibody for 1 hour at 37˚C. Bound oxLDL-IgG was visualized by using 2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma). Colour was measured at an optical density of 415 nm using a conventional ELISA reader. Between each ELISA step plates were washed with PBS containing 0.05% Tween20. MC tryptase levels were determined in plasma samples using an ImmunoCAP® 250 tryptase assay (Phadia AB, Uppsala, Sweden)

Immunohistochemistry

Sections were stained for mast cell tryptase (mast cells), CD68 (macrophages), smooth muscle cells (alpha actin), and CD34 (endothelial cells) as previously described (4). Total mast cell numbers were determined by counting all (degranulating) mast cells present in a plaque cross-section at x40 magnification (4). A degranulating mast cell was defined by a group of mast cell tryptase positive extracellular granules in close proximity of each other or in close proximity of a mast cell. The total plaque area (mm^2) was measured using the analySIS 2.8 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) to determine the distribution density of mast cells expressed as numbers of mast cells/mm^2. Image-analyzing software was used to determine positive macrophage and smooth muscle cell staining expressed as a percentage of covered plaque area (13). Microvessels were counted in three hot-spots and were expressed as average microvessel density per hotspots (19). Collagen content (picrosirius red) was scored semi-quantitatively. The size of the extracellular lipid core (atheroma) was assessed by the H&E and picrosirius red stain (13).

Statistics and data analysis

IBM SPSS statistics version 20 was used for all analyses (IBM corporation, Armonk, NY, USA). Immunoglobulin levels are not normally distributed; non-parametrical testing was used to determine differences. The Mann-Whitney U test was used to study immunoglobulin levels as a continuous variable for all risk factors. The Spearman correlation coefficient was calculated to assess associations between immunoglobulin levels and all continuous variables in this study. Differences were considered significant with a p-value of below 0.05.

Results

Baseline patient characteristics

Total IgE, total IgG and oxLDL-IgG plasma levels were measured in a total of 135 patients that underwent carotid endarterectomy. Baseline clinical characteristics of the 135 patients are provided in Table 1. The studied patient population with a mean age of 67 and a male prevalence (71%) reflects a relatively typical population of patients with vascular occlusive diseases. The majority of patients was symptomatic (74%) as illustrated by the incidence of amaurosis fugax, a TIA or a stroke, was hypertensive (86%) and used statins (69%).

Table 1. Baseline characteristics of patients in relation to immunoglobulin plasma levels

		Total IgG	p-value	oxLDL-IgG	p-value	Total IgE	p-value
Age, mean years (sd)	67 (9)	r = -0.082	0.347	r = 0.031	0.724	r = 0.097	0.265
BMI, mean kg/m2 (sd)	27 (4)	r = -0.115	0.19	r = 0.001	0.995	r = -0.126	0.15
Gender							
Male	96/135 (71%)	16.8 [11.7-23.5]	0.694	336 [239-499]	0.074	131.1 [57.5-317.4]	0.169
Female	39/135 (29%)	17.2 [12.6-23.1]		282 [233-358]		76.7 [31.4-293.4]	
Current smoker							
Yes	55/134 (41%)	16.6 [10.3-23.3]	0.635	303 [252-481]	0.427	173.4 [65.5-449.8]	0.032
No	79/134 (59%)	17.1 [12.3-23.2]		306 [219-502]		91.5 [41.5-203.0]	
Diabetes mellitus							
Yes	25/135 (19%)	15.4 [12.1-22.9]	0.63	288 [241-391]	0.554	79.8 [41.9-288.5]	0.329
No	110/135 (81%)	17.0 [12.3-23.7]		320 [238-489]		125.0 [57.3-312.1]	
Statin Use							
Yes	93/135 (69%)	17.1 [12.3-22.8]	0.72	288 [224-406]	0.004	97.5 [43.1-276.8]	0.012
No	42/135 (31%)	16.8 [23.3-25.0]		399 [282-584]		157.3 [75.2-545.4]	

Table 1. Baseline characteristics of patients in relation to immunoglobulin plasma levels. (continued)

		Total IgG	p-value	oxLDL-IgG	p-value	Total IgE	p-value
Hypertension							
Yes	116/135 (86%)	17.2 [12.5-23.2]	0.633	302 [233-480]	0.207	113.0 [49.1-304.8]	0.265
No	19/135 (14%)	15.0 [10.4-26.0]		341 [260-537]		183.3 [68.8-594.0]	
Hypersensitive							
Yes	27/132 (20%)	17.1 [12.6-22.2]	0.906	305 [233-412]	0.539	148.1 [44.6-449.8]	0.401
No	105/132 (80%)	16.6 [11.6-23.9]		303 [237-489]		115.6 [50.5-304.7]	
History VI							
Yes	54/135 (40%)	18.5 [11.9-23.5]	0.39	297 [216-483]	0.391	86.2 [47.7-274.4]	0.239
No	81/135 (60%)	15.4 [11.9-23.5]		306 [254-484]		135.4 [54.2-358.9]	
History MI							
Yes	30/134 (22%)	19.0 [12.7-22.7]	0.673	321 [222-428]	0.62	85.6 [51.7-282.9]	0.601
No	104/134 (78%)	16.5 [12.2-23.5]		304 [239-508]		129.7 [49.1-310.8]	
Clinical presentation							
Asymptomatic	35/135 (26%)	15.8 [12.3-23.2]	0.419*	314 [239-505]	0.377*	113.0 [47.0-304.9]	0.431*
Symptomatic	100/135 (74%)	18.1 [12.1-24.1]		293 [224-399]		144.3 [68.8-356.7]	
Amaurosis fugax	22/135 (16%)	16.6 [12.5-24.9]		296 [240-424]		120.9 [46.3-327.8]	
TIA	51/135 (38%)	16.2 [11.5-23.2]		300 [223-512]		106.3 [32.9-281.6]	
Stroke	27/135 (20%)	15.4 [12.3-22.7]		352 [257-515]		135.4 [66.6-319.9]	

*Data are presented as No. (%) and median [IQR] unless otherwise indicated; r = Spearman's rank correlation coefficient; sd = standard deviation; IQR = interquartile range; BMI = body mass index; TIA = transient ischemic attack; *p-value represents statistical analysis for asymptomatic patients versus symptomatic patients (composed of amaurosis fugax, TIA an stroke).*

Plasma immunoglobulin levels and mast cell parameters

A correlation with borderline significance was found between total IgE and oxLDL-IgG (r=0.169, p=0.051). Total IgG did not correlate with either total IgE or oxLDL-IgG. No association was found between total IgE, total IgG and oxLDL-IgG plasma levels and total mast cell numbers or total degranulating mast cells (Table 2). In addition we did not observe any association between the three immunoglobulins and tryptase plasma levels.

Table 2. Immunoglobulin plasma levels with respect to mast cell parameters

	Total IgG	p-value	oxLDL-IgG	p-value	Total IgE	p-value
Total mast cells	r = -0.038	0.664	r = 0.137	0.114	r = -0.038	0.664
Mast cells/mm2	r = -0.104	0.230	r = 0.115	0.186	r = -0.014	0.872
Degranulating mast cells/mm2	r = -0.074	0.519	r = 0.008	0.946	r = -0.076	0.506
Plasma tryptase	r = -0.076	0.378	r = 0.040	0.643	r = 0.064	0.457

Data are presented as Spearman's rank correlation coefficient (r)

Plasma immunoglobulin levels and vulnerable plaque characteristics

As depicted in Figure 1, no consistent associations were found between immunoglobulin levels and measures of vulnerable plaque phenotype. There was no association between immunoglobulin levels and any of the following plaque characteristics: fat deposition, collagen, smooth muscle cells, macrophages, microvessel density.

Plasma immunoglobulin levels and clinically relevant characteristics

Table 1 provides an overview of the associations between the three immunoglobulin expression levels and relevant clinical characteristics. A negative association was observed between statin use and total IgE (97.5 [43.1-276.8] vs. 157.3 [75.2-545.4] ng/mL, p=0.012) and oxLDL-IgG (288 [224-406] vs. 399 [282-584] mU/mL, p=0.004). Higher total IgE levels were observed in smokers compared to non-smokers (91.8 [62.3-145.0] vs. 76.7 [47.1-111.2] ng/mL, p=0.002). Clinical presentation was not associated immunoglobulin levels: no differences were observed in expression levels for any of the immunoglobulins between asymptomatic patients (n=35) and symptomatic (n=100) patients. Furthermore, we did not detect any differences in any of the circulating immunoglobulin levels between the subgroups of symptomatic patients (i.e. patients suffering from amaurosis fugax, TIA or stroke). Additionally, no association was found between immunoglobulin levels and the delay between surgery and presentation of symptom*s*.

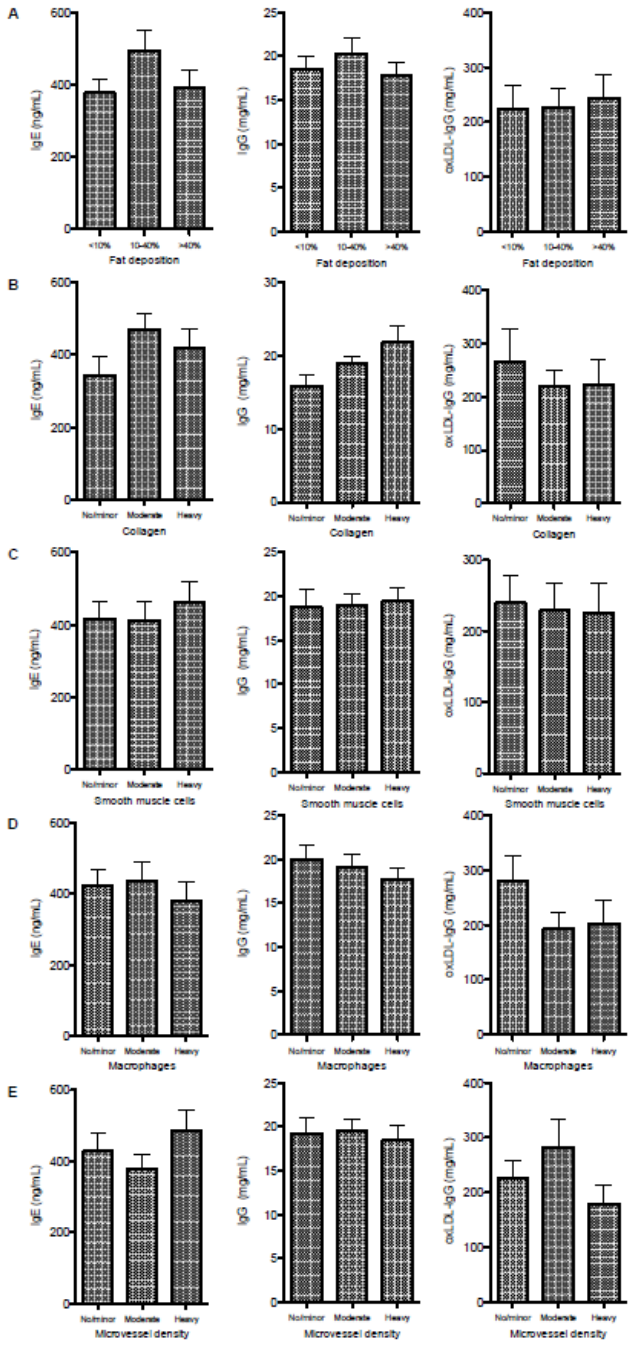

Figure 1. No association were found between IgE (left graphs), IgG (middle graphs) and oxLDL-IgG (right graphs) levels and any of the plaque characteristics: fat deposition, collagen, smooth muscle cells, macrophages and microvessel density.

Discussion

Progression of an atherosclerotic plaque is often characterized by measures of plaque vulnerability. A vulnerable plaque is prone to rupture resulting in severe cardiovascular complications. Experimental studies have shown that mast cell activation results in progression and destabilization of the atherosclerotic plaque (20,21). In addition, in human plaques, mast cells correlated with vulnerable plaque characteristics and appeared associated with future combined cardiovascular events (4). It has been suggested that activated mast cells induce intraplaque neoangiogenesis thereby making the plaque more susceptible for rupture. For therapeutic intervention, identification of the endogenous mast cell activators during the progression and destabilization is of great value. In allergy and asthma, IgE is the main trigger for mast cell degranulation via crosslinking of the FcεR, while specific IgG immune complexes can exert mast cell activation resulting in cytokine release via binding to various Fcγ receptors. Previous studies have demonstrated that plasma IgE levels may be linked to the presence of cardiovascular diseases (11) and mice lacking the FcεR displayed reduced atherogenesis (22). Furthermore, oxLDL specific IgG molecules that can form immune complexes with oxLDL have been detected in human and rabbit atherosclerotic plaques (23) and these immune complexes were able to induce TNFα and IL-8 release from human mast cells (12). Taken together, these data suggest that IgE and specific IgGs may be important mast cell activators in cardiovascular disease.

It is however still unknown whether circulating immonuglobulins are capable of activating mast cells in human atherosclerotic plaques. Here we show that circulating IgG, IgE and oxLDL-IgG are not associated with mast cell determinants in a patient cohort with severe carotid stenosis that underwent an endarterectomy. No association was observed between any of the three immunoglobulins measured in this study and total plaque mast cell numbers or mast cell tryptase plasma levels. In addition, we did not observe any correlation with intraplaque degranulating mast cell numbers. Our results do not provide supportive evidence that increased immunoglobulin levels induce activation of mast cells in advanced human atherosclerotic plaques.

Previously, IgE was shown an independent marker for cardiovascular disease in men (24). Therefore we explored the possibility of an association between the three immunoglobulins had any future adverse events in patients with established cardiovascular disease. We did not find any association between immunoglobulin levels and future cardiovascular events, however, we were underpowered for analysing risk prediction for the immunoglobulins in our cohort. Nevertheless, no association was found with histological markers of plaque vulnerability, one of the most important determinants for future cardiovascular complications.

Serum oxLDL specific IgG antibodies have previously been linked to the presence and destabilization of the atherosclerotic plaque (25). As mentioned above, specific IgGs have been observed within the atherosclerotic plaque. In the current study, we did not detect any correlation between plasma oxLDL-IgG levels and mast cell activation or plaque phe-

notype. However, systemic oxLDL-IgG levels may not reflect the local oxLDL-IgG immune complexes that may actually activate the mast cell within the atherosclerotic plaque. Histological analysis of immune complexes, and with that also local IgE content, and colocalization with mast cells within the plaque may provide more information on the mechanisms of mast cell activation in atherosclerosis.

Interestingly, lower IgE and oxLDL-IgG levels were observed in patients that used statins. When we differentiated between the patients on statins and the patients not on statins it does not affect the outcome of the associations observed between immunoglobulin levels and mast cell parameters or plaque vulnerability (data not shown). The inhibitory effects of statin use on oxLDL-IgG antibody levels have been previously described (25), however reduced levels of IgE after statin treatment has to our knowledge not been reported before in patients with atherosclerosis. These data thus identify a novel effect of statin treatment, in addition to lipid-lowering, possibly by affecting antibody production of B cells.

The results of our study may be limited by the relative small number of patients. We have previously established that plasma tryptase levels of these patients correlate with plaque mast cell number and activation status, thus being reflective of mast cell activation. However, using these patients we were unable to detect a relation between any of the measured circulating immunoglobulin levels and intraplaque mast cell numbers or its activation, which may be improved by using a larger patient cohort. In addition, we measured plasma immunoglobulin levels at a single time-point that is at time of carotid endarterectomy, which is most reflective of the mast cell status within the plaque at that time point. Measuring plasma immunoglobulin levels at multiple time-points, for example at follow-up, may provide more information on the variation in circulating immunoglobulin levels together with disease progression.

In conclusion, no associations were found for total IgE, total IgG and oxLDL-IgG and the presence of total mast cell numbers or the number of degranulating mast cells in atherosclerotic plaques. Furthermore, the immunoglobulins were not related to most of the established characteristics of the rupture-prone atherosclerotic plaques. Taken together, this study does not provide supportive evidence that the three investigated circulating immunoglobulins activate mast cells during the progression of atherosclerotic disease. We can however not exclude that the mast cells may be activated by other specific immunoglobulins, or that local factors within the vessel wall are more predominant determinants of mast cell activation in the atherosclerotic plaque. Future research on the local environmental specific IgE and IgG levels within the plaque may thus shed more light on mechanism of mast cell activation in atherosclerosis.

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Chapter 6

ApoE deficient inducible mast cell knockout mice: A new model to study the role of mast cells in atherosclerosis.

Manuscript in preparation

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Abstract

Objective

Atherosclerosis can be considered as a lipid-driven chronic inflammatory disease of the large- and medium sized arteries. Innate mast cells accumulate in the atherosclerotic lesion during its development and can take up to 6% of all nucleated cells in rupture prone lesions. In the past years, a number of experimental atherosclerosis studies have been performed in mice to unravel the contribution of mast cells and their mediators to atherogenesis. Frequently, mice have been used with alterations in the stem cell signaling pathway like the $Kit^{W\text{-}sh/W\text{-}sh}$ mice, which results in mast cell deficiency. However, it became evident that $Kit^{W\text{-}sh/W\text{-}sh}$ mice suffer from bystander defects such as neutrophilia. To study the development of atherosclerosis without these side effects, we used a mast cell inducible knockout mouse, the Red Mast cell Basophil (RMB) mouse, in which mast cell deficiency is independent of stem cell signaling.

Methods and Results

RMB mice were crossed with apoE deficient mice to obtain RMB-$apoE^{-/-}$ mice. In these mice, mast cells were depleted by intraperitoneal Diphtheria Toxin (DT) injection or PBS injection as a control. Directly after mast cell depletion, mice were placed on a western type diet to induce lesion development. Two weeks after the start of the diet, semi-constrictive collars were placed around the carotids followed by 4 additional weeks of WTD. At sacrifice, we did not detect any differences in body weight, total cholesterol levels or total leukocyte counts between mast cell depleted and competent groups. Histological analysis of the carotids showed a consistent depletion of mast cells after DT treatment, which coincided with a reduction in both lesion size and necrotic core area and an increased collagen content. Furthermore, we detected reduced levels of circulating IL-6 and TNFα in mast cell depleted mice.

Conclusions

In this current study we confirmed in a novel mouse model that mast cells contribute to the initiation phase of atherosclerotic lesion development.

Introduction

Mast cells are innate immune cells which are located throughout the body in peripheral tissues as skin, gut and within the vascular system [1]. Close to the external environment they are one of the first immune cells able to respond to invading pathogens. Mast cells are equipped with a variety of receptors that enables them to react within seconds to a number of stimuli. IgE-mediated activation is by far the most powerful activation pathway of mast cells, which results in the release of preformed mediators such as tryptase, chymase and histamine stored within the granules of the cell. The acute release of mediators is followed by the secretion of newly synthesized mediators including chemokines and cytokines [2]. The role of mast cells in allergy and hypersensitivity is well known, but more recent, also the contribution of mast cells to immune driven disorders such as rheumatoid arthritis and atherosclerosis has been identified [3–5].

Mast cells accumulate within atherosclerotic lesions at sites of plaque rupture, where they can be up to 6% of all nucleated cells [6]. The lesion contains a number of different stimuli such as endogenous toll like receptor (TLR) ligands and immune complexes that could potentially activate mast cells. For example, oxLDL-immune complexes are able to activate mast cells via FcγRs to release tumor necrosis factor (TNFα), interleukin 8 (IL-8) and monocyte chemotactic protein 1 (MCP-1), which may enhance the inflammatory response within the lesion [7]. Upon activation, mast cells can release proteases like chymase and tryptase that are able to degrade collagen as well as HDL and induce apoptosis of smooth muscle cells, which will induce and accelerate lesion destabilization [8–10]. Likewise, systemic mast cell activation leads to plaque growth and destabilization, which can be rescued by the administration of the mast cell stabilizer cromolyn [11]. Moreover, atherosclerosis-prone mice deficient for mast cells ($LDLr^{-/-}Kit^{W\text{-}sh/W\text{-}sh}$) develop smaller and more stable lesions compared to control $LDLr^{-/-}$ mice [12]. Although these studies demonstrate that mast cells contribute to atherosclerotic lesion development and destabilization, the use of $Kit^{W\text{-}sh/W\text{-}sh}$ mice is limited due to the fact that these mice with a defect in c-kit signaling suffer from several complications such as neutrophilia and expansion of splenic myeloid-derived suppressor cells [13–15]. To circumvent these undesired effects, mice have been developed in which mast cell deficiency is induced independently of SCF signaling, for example the carboxypeptidase A(Cpa3)-Cre; $Mcl\text{-}1^{fl/fl}$ mice, the mast cell protease 5 (Mcpt5)-$Cre^{+}iDTR^{+}$ mice and the Red-Mast cell-Basophil (RMB) mice [16–19]. Cpa3-Cre; $Mcl\text{-}1^{fl/fl}$ mice express Cre recombinase under control of Cpa3 promoter, which is combined with a floxed allele of the antiapoptotic factor. This combination results in deficiency in mast cells and a marked reduction in basophils. Recently, mice have been developed were mast cells can be depleted upon injection with diphtheria toxin (DT). Examples are the Mcpt5-$Cre^{+}iDTR^{+}$ mice and RMB mice that co-express the simian DT receptor together with proteins highly expressed by mast cells, which are for these two mouse models Mcpt5 and FcεRIβ, respectively. Administration of DT to these mice will causes apoptosis of all cells that express the DT receptor.

In the current study we used the RMB mouse model to study the role of mast cells in atherosclerosis. In this mouse model, the repopulation kinetics of basophils and mast cells after depletion with DT is shown to different. Basophils are depleted relatively shortly (<12 days), whereas systemic mast cell depletion is maintained for up to 2 months after the last DT injection [19]. We crossed these RMB mice with apoE$^{-/-}$ mice to create an inducible mast cell knockout on an atherosclerotic-prone background, creating the RMB-apoE$^{-/-}$. In this study we used this mouse model to characterize the contribution e of the mast cell to atherosclerotic lesion initiation.

Material & Methods

Mice

All animal work was performed conform the guidelines from the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Leiden University Animal Welfare committee. The red mast cell and basophil mouse strain (RMB mice, official name: B6.Ms4a2tm1Mal) was provided by the laboratory of dr. P. Launay INSERM U1149, Paris, France [19]. These mice express the simian diphtheria toxin receptor under control of the promoter of the FcεRIβ gene. Injection of diphtheria toxin induces apoptosis of all FcεRIβ expressing cells, which are in mice only basophils and mast cells [20]. The RMB mice were crossed with apoE$^{-/-}$ mice to obtain atherosclerosis-prone RMB-apoE$^{-/-}$ mice.

To systemically deplete all FcεRIβ^{+} cells, RMB-apoE$^{-/-}$ mice were injected i.p. on day 1, 3 and 8 with 25 ng/g bodyweight DT (Diphtheria Toxin Unnicked, C. diphtheriae (Cat #322326, CalBiochem)). Two weeks before the start of the experiment, mice received either DT injections as described above to deplete FcεRIβ^{+} cells or PBS as a control. The efficiency of DT treatment to deplete mast cells was determined by both FACS analysis for circulating basophils (CD49b^{+}/FcεRI^{+}/IgE+) three days after last DT injection and by a mast cell staining in the carotids and hearts (CAE, Sigma) at end of the study.

At week 0 mice were fed a Western type diet (0.25% cholesterol and 15% cacao butter, SDS, Sussex, UK) for total 6 weeks. Two weeks after the start of Western type diet (WTD), perivascular collars were placed around both left and right carotid artery, which causes low shear stress and disturbed blood flow at the proximal side of the collar that results in endothelial activation, increased endothelial adhesion molecule expression and atherosclerotic lesion formation [21]. In short, mice were anaesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, The Netherlands), fentanyl citrate and fluanisone (1.26 mg/kg and 2 mg/kg respectively, Janssen Animal Health, Sauderton, UK) and a semi-constrictive collar was placed.

At sacrifice, mice were anaesthetized as described above and sacrificed via orbital exsanguination. Fixation through the left cardiac chamber was performed with phosphate-buffered saline (PBS). Subsequently, both common carotid arteries and the heart were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, UK) for histological analysis. Serum was obtained for determination of cytokine and immunoglobulin levels. Splenocytes, blood and heart lymph nodes were isolated for FACS analysis of leukocytes.

Histology and morphometry

Carotid arteries and hearts were embedded in Tissue-Tek® O.C.T.™ compound (Sakura BV, The Netherlands) after which cryosections were prepared. Section of the carotid arteries were stained with hematoxylin-eosin (H&E) to

determine lesion size and necrotic core area (in μm^2) at the side of maximal stenosis. Lesions in the aortic root were quantified in Oil Red-O-stained sections. Mean lesion area (in μm^2) was calculated from 6 serial sections, starting at the appearance of the tricuspid valves.

Collagen content was determined by staining with picrosirius red and an enzymatic staining (CAE, Sigma-Aldrich, Germany) was used for the visualization of mast cells. Perivascular mast cells were counted manually by a blinded independent operator. The necrotic core size was defined as the a-cellular, debris-rich plaque area as percentage of the total plaque area. The atherosclerotic lesion areas and histological stainings of both carotids and aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging analysis system (Leica Ltd, UK).

Flow cytometry

At sacrifice, blood was obtained in EDTA tubes and white blood cell (WBC), red blood cell (RBC) and platelets (PLT) were determined using an automated Sysmex XT-2000iV veterinary haematology analyzer (Sysmex orporation, Kobe, Japan). For flow cytometry analysis, erythrocytes were removed using a specific erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3).

Blood leukocytes were stained extracellular with four different stains to determine a) monocytes ($NK1.1^-$/$Ly6G^-$/$CD11b^{hi}$), inflammatory monocytes ($NK1.1^-$/$Ly6G^-$/$CD11b^{hi}$/$Ly6C^{hi}$/$CCR2^+$) and neutrophils ($NK1.1^-$/$Ly6G^{hi}$/$CD11b^{hi}$), b) basophils ($CD3^-$/$CD4^-$/$CD19^-$/$CD8^-$/$CD49b^+$/IgE^+/$CD117^-$), c) T cells ($CD3^+$/$CD4^+$), d) B-cells ($CD19^+$/$B220^+$). The different used antibodies are summarized in table 1. Antibodies were obtained from (eBiosciences, US). Flow cytometry analysis was performed on the FACSCantoII and obtained data was analyzed using FACSDiva software (BD Biosciences, US).

Table 1: Antibody panels used for flow cytometry analysis

Staining	FITC	PE	PerCP	APC	e-Fluor-450
A.	NK1.1 (Clone: PK136)	Ly6G (Clone: 1A8)	Ly6C (Clone: HK1.4)	CCR2 (Clone: 475301)	CD11b (Clone: M1/70)
B.	IgE (Clone: R35-72)	IgE (Clone: R35-72)	CD3/4/19/8 (dump channel)	CD49b (Clone: HMa2)	n/a
C.	CD44 (Clone: IM7)	CCR7 (Clone: 4B12)	CD8α (Clone: 53-6.7)	CD62L (Clone: MEL-14)	CD4 (Clone: GK1.5)
D.	IgM (Clone: II/41)	CD45RA (Clone: RA3-6B2)	CD19 (Clone: eBio1D3)	IgD (Clone: 11-26c)	CD5 (Clone: 53-7.3)

Serum analysis

Serum cytokine and immunoglobulin levels were determined using a commercially available ELISA kit (BD, US, Bethyl laboratories, US). All procedures were according to manufacturer's protocol.

Serum concentrations of total cholesterol was determined by enzymatic colorimetric assay according to protocol of supplier (Roche Diagnostics, US). Precipath (Standardized serum Roche Diagnostics, US) was used as internal standard.

Statistical analysis

Data are expressed as mean ±SEM. A 2-tailed Student's t-test was used to compare individual groups in the in vivo studies. A level of $P<0.05$ was considered significant.

Results

No effect of FcεRIβ^{+}-cell depletion on body weight, total cholesterol and leukocyte counts in RMB-apoE$^{-/-}$ mice

A schematic overview of the current study is depicted in figure 1a. In short, FcεRIβ^{+} cells were depleted in RMB-apoE$^{-/-}$ mice before induction of atherosclerosis by western type diet feeding. DT efficiency was determined by flow cytometry analysis for circulating blood basophils. At 3 days after the last DT injection, we observed a population of basophils in PBS treated mice and absence of basophils in the DT treated group (Fig. 1b). Subsequently, mice were fed a western type diet for a total of 6 weeks, two weeks after start of diet mice received perivascular collars to accelerate lesion development. At sacrifice, we did not observe weight differences between the PBS and the DT treated group (Fig. 1c). Total serum cholesterol level (Fig. 1d), total leukocyte and red blood cell numbers, as well as platelet counts (Fig. 1e) did not differ between the mast cell depleted and control treated group.

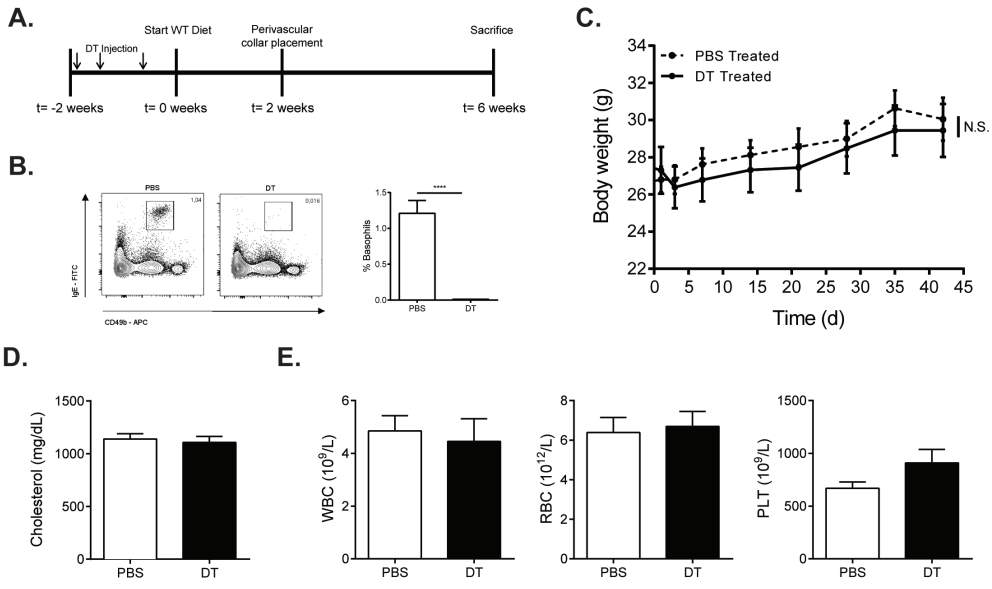

Figure 1. The effect of FcεRIβ^{+}-cell depletion on body weight, total cholesterol and leukocyte counts in RMB-apoE$^{-/-}$ mice

(A) Flow-chart of the in vivo experimental set-up. (B) Gating stratagy of blood basophils and summary of all PBS and DT treated mice. (C) Body weight was monitored during the experiment in PBS- or DT-treated RMB-apoE$^{-/-}$ (n=15/group). (D) Total serum cholesterol levels were determine in PBS- or DT treated RMB-apoE$^{-/-}$ mice at the end of the experiment (n=15/group). (E) Total white blood cell (WBC), red blood cell count (RBC) and platelet count was determined in PBS or DT-treated RMB-apoE$^{-/-}$ mice at sacrifice (n=15/group). ***P<0.001.

Depletion of FcεRIβ⁺ cells reduces lesion size and increases lesion stability in carotid arteries.
Atherosclerotic lesion size and composition in the carotid arteries was analyzed after 4 weeks of lesion development. Mast cells are the only tissue resident cells that express the β-chain of the FcεRI [20]. DT treated RMB-apoE$^{-/-}$ mice showed a complete absence of mast cells, while mast cells were present in PBS-treated RMB-apoE$^{-/-}$ mice (Fig. 2a). Quantification of the atherosclerotic lesion size in the carotid artery showed that depletion of FcεRIβ^{+} cells led to a 28% reduction in mean lesion size compared to PBS-treated mice (DT: 78.6 ± 7.9*10^{3} μm^{2} versus PBS: 109.1 ± 8.3*10^{3} μm^{2}, p=0.02, Fig. 2b). Additionally, we analyzed the collagen content using a Sirius Red staining and the percentage of necrotic area as a measure for plaque stability. Depletion of FcεRIβ^{+} cells resulted in an increased collagen content in the lesions of DT-treated mice compared to PBS-treated animals (DT: 10.7 ± 1.1% versus PBS: 4.7 ± 0.7%, p=0.0003, figure 2c) and a reduction in the relative necrotic core area (DT: 32.3 ± 4.3% versus PBS: 44.9 ± 4.3%, p=0.02, Fig. 2d).

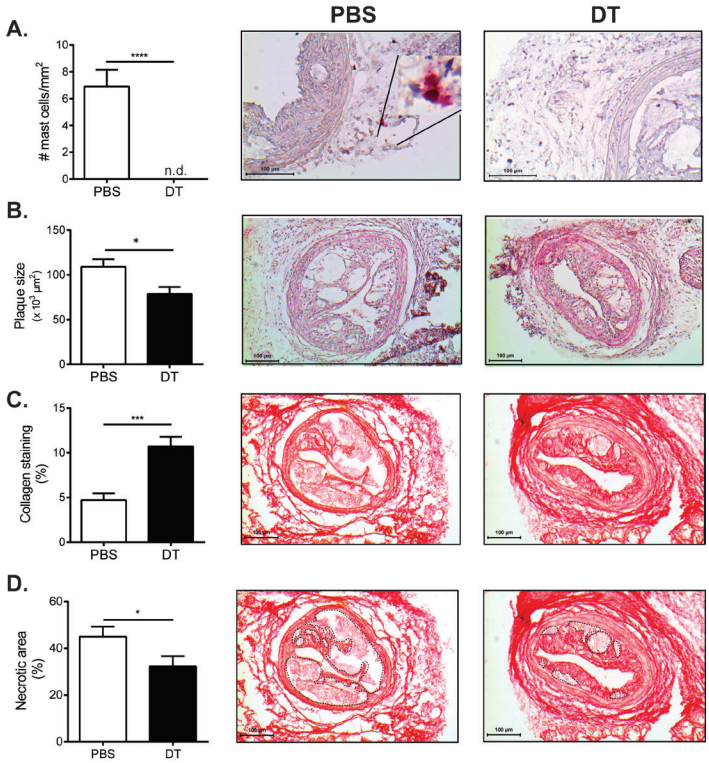

Figure 2. Depletion of FcεRIβ^{+} cells reduces lesion size and increases lesion stability in carotid arteries.
(A) An efficient depletion of perivascular mast cells was observed in DT-, but not in PBS treated RMB-apoE$^{-/-}$ mice (n=15/group). Magnification insert shows mast cells. (B) DT treatment resulted in a reduction in lesion size compared to non-depleted mice (n=15/group). The depletion of FcεRIβ^{+}-cells prior to induction of lesion development coincided with an increase in collagen content (C) and a reduction in necrotic core area (D) compared to non-mast cell-depleted mice (n=15/group). (D: dotted lines indicate necrotic core). *P<0.05, ***P<0.001.

FcεRIβ⁺ cell depletion does not influence lesion size but increases collagen content in aortic root.

Similarly as in the carotid arteries, we observed a complete depletion of perivascular mast cells in the aortic root of DT-treated mice (Fig. 3a). Analysis of the lesion size in the aortic root of PBS and DT treated mice did not reveal any difference in Oil-Red-O positive area (DT: 4.7 ± 0.5*10^5 µm^2 versus PBS: 4.1 ± 0.2*10^5 µm^2, p=NS, Fig. 3b). The collagen content in the lesions of FcεRIβ⁺ cell depleted mice was increased compared to mast cell competent mice (DT: 16.6 ± 1.6% versus PBS: 9.1 ± 1.6% p=0.006, Figure 3c). We observed a trend towards a reduction in necrotic core area in FcεRIβ⁺ cell depleted mice (Fig. 3d).

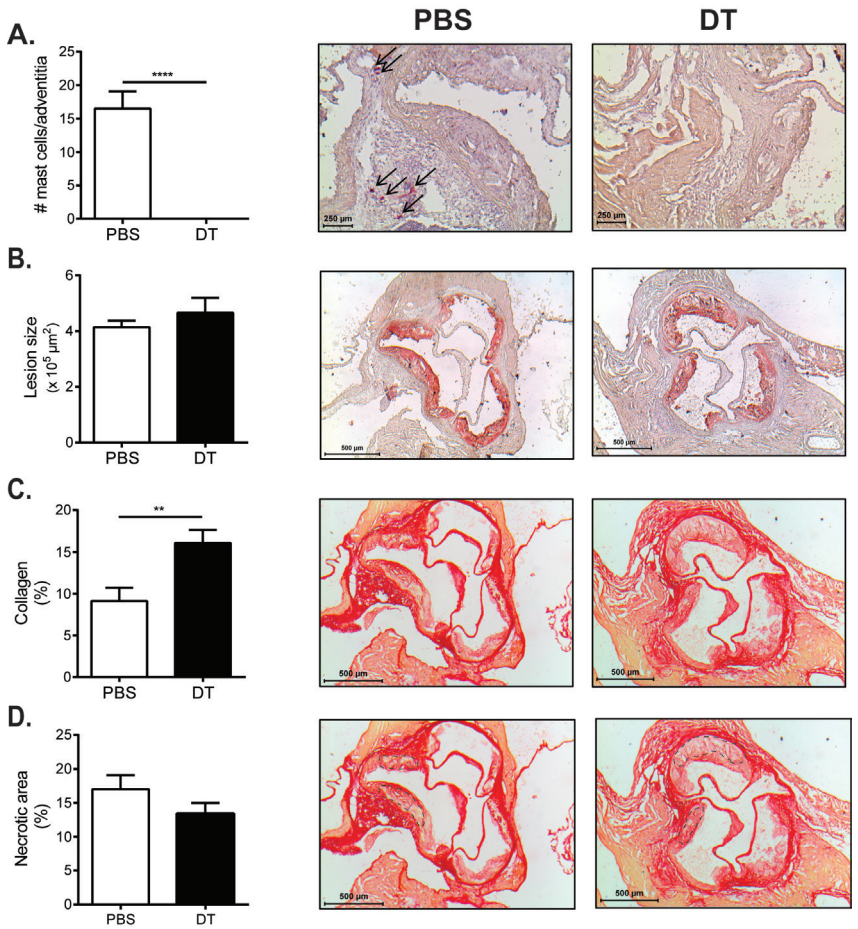

Figure 3. FcεRIβ⁺ cell depletion does not influence lesion size but increases collagen content in aortic root.

(A) An efficient depletion of adventitial mast cells was observed in DT injected RMB-apoE$^{-/-}$ mice (n=15/group) Arrows indicate mast cells. (B) Despite the efficient FcεRIβ⁺-cell depletion, no effect was observed on lesion size between both groups. (C) Collagen content was significantly increased whereas the necrotic core area. (D) was slightly decreased in FcεRIβ⁺-cell deleted mice compared to non-depleted mice. **P<0.01 ****P<0.0001.

At sacrifice, flow cytometry was performed on the WBC population to determine the numbers of circulating innate immune cells such as basophils, neutrophils, (inflammatory) monocytes (Fig. 4a) and adaptive immune cells such as $CD4^+$ T cells, $CD8^+$ T cells and B cells (Fig. 4b). As depicted, we did not detect any difference in the analyzed leukocyte subsets between PBS and DT treated mice.

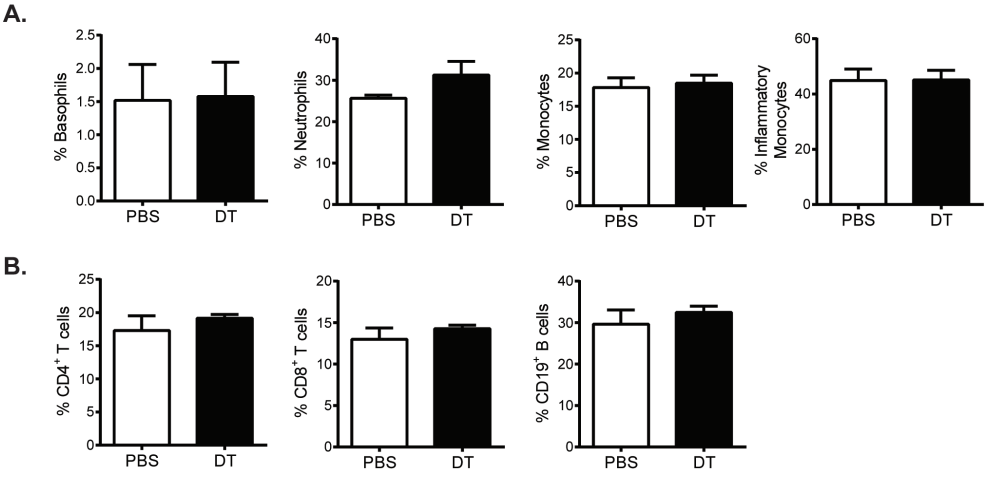

Figure 4. Depletion of FcεRIβ$^+$ cells had no effect on circulating leukocytes in the blood.
(A) Circulating innate basophils ($CD49b^+/IgE^+/CD3^-/CD19^-$), Neutrophils (CD11b/Ly6G), Monocytes ($CD11b^{high}/Ly6G^-/NK1.1^-$), Inflammatory monocytes ($CD11b^{high}/CCR2^+/Ly6C^+/Ly6G^-/NK1.1^-$) and (B) adaptive $CD4^+$ T cells, $CD8^+$ T cells and B-cells ($CD19^+$) in both PBS and DT treated RMB-$apoE^{-/-}$ mice (n=15/group).

Depletion of FcεRIβ⁺ cells decreases levels of pro-atherogenic cytokines and increases total IgE levels.

In the serum we determined pro-atherogenic mediators such as IL-6, TNFα and MCP-1 and as well as total IgG and IgE levels. Circulating IL-6 levels were reduced upon depletion of mast cells (DT: 218.9 ± 22.7 pg/mL versus PBS: 409.2 ± 40.8 pg/mL, p=0.006), and also TNFα levels were reduced (DT: 69.0 ± 5.9 pg/mL versus PBS: 91.9 ± 6.9 pg/mL, p=0.02). No differences in circulating MCP-1 were detected between groups. Total IgG levels in serum did not differ between FcεRIβ⁺ cell competent and FcεRIβ⁺ cell depleted mice. However, we did observe an elevated concentration of total IgE in the serum of FcεRIβ⁺ cell depletedmice (DT: 1024.0 ± 28.5 pg/mL versus PBS: 840.8 ± 80.2 pg/mL, p=0.04).

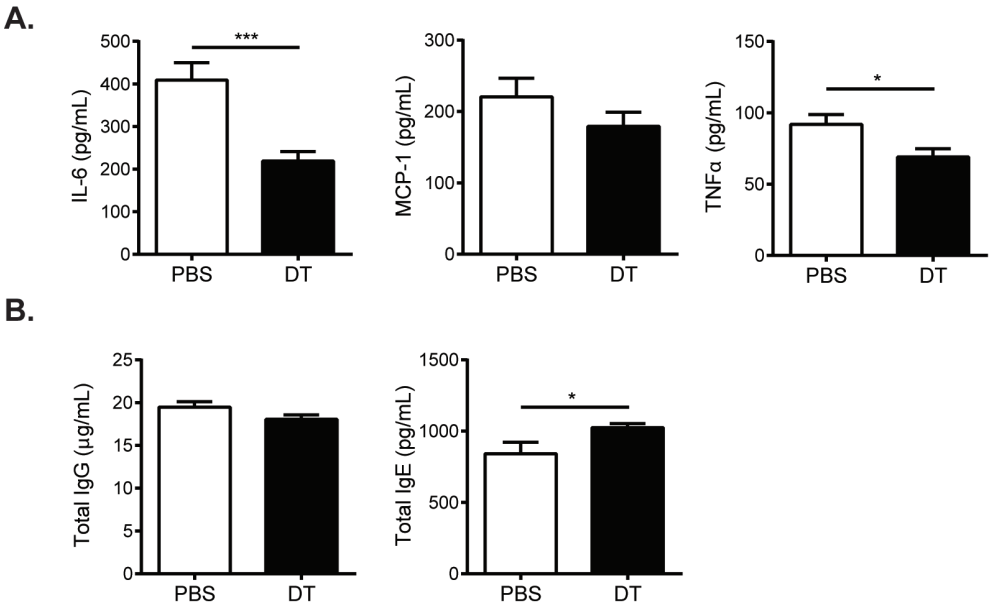

Figure 5. Depletion of FcεRIβ⁺ cells decreases levels of pro-atherogenic cytokines and increases total IgE levels.

(A) Serum levels of IL-6, TNFα and MCP-1 were quantified in serum of PBS and DT treated RMB-apoE$^{-/-}$ mice (n=15/group). (B) Serum levels of total immunoglobulin G (IgG) and immunoglobulin E (IgE) (n=15/group). *P<0.05, ***P<0.001.

Discussion

Mast cells are tissue resident cells located at sites that are in close proximity with the external world like the skin or within the gut [1]. Furthermore, mast cells are located around blood vessels and can influence vascular homeostasis by the release of several mediators, such as proteases, chemokines and cytokines. [4]. Already 60 years ago, mast cells have been implicated in cardiovascular diseases among which atherosclerosis [22]. Many experimental studies have been performed to unravel the contribution of the mast cell and its mediators to plaque initiation, progression and destabilization [11, 12, 23–25]. However, these studies were generally performed in mice with mutations in the stem cell factor signaling (SCF) pathway, like the $Kit^{W\text{-}sh/W\text{-}sh}$ mice, in which a chromosomal inversion causes a disruption of the c-kit (SCF receptor) gene thereby blocking kit expression [13]. Since mast cells need SCF signaling for their maturation, proliferation and survival, these mice lack mast cells [26]. However, it became evident that SCF is also essential for other (non)-immune cells and, as a consequence, these mice suffer from side-effects such as neutrophilia [15]. In the current study we used the recently generated red-mast cell basophil (RMB) mouse strain, which is independent of c-kit [19]. These mice express the DT receptor under control of the promoter of the FcεRIβ-gene, which is expressed exclusively by basophils and mast cells. Nonetheless, there is a marked difference in the repopulation kinetics reported of these cells after depletion.

At sacrifice, we confirmed this difference in repopulation kinetics as blood basophils were completely repopulated while a complete depletion of tissue resident mast cells was still observed in DT treated animals at 6 weeks after depletion of $FcεRIβ^+$ cells. Moreover, the contribution of basophils to atherogenesis is still largely unknown. Basophils have up to now not been demonstrated to be present within the atherosclerotic lesion, which could be caused by their limited lifespan due to early apoptosis outside the bloodstream [27]. Furthermore, we started the $FcεRIβ^+$ cell depletion 14 days before start of western-type diet, and thus the induction of atherosclerotic lesion development, and 4 weeks before perivascular collar placement around the carotids. Therefore, we can conclude that the observed effects of $FcεRIβ^+$ cell depletion on atherogenesis in this study are most likely due to the depletion of tissue-resident mast cells rather than the relative short absence of circulating basophils.

A stable atherosclerotic plaque is characterized by the presence of a relatively small necrotic core, which is covered by a thick layer of smooth muscle cells that produce extracellular matrix molecules such as collagen. In the current study depletion of mast cells had no effect on body weight, total cholesterol and total leukocyte count as it was comparable to PBS control mice. However, we did observe that mast cell depletion resulted in a significant reduction in lesion size and an increase in collagen content in both carotid artery and aortic root lesions. Furthermore, the necrotic core area was significantly reduced in the carotid arteries and a similar trend was observed in the aortic root in mast cell depleted mice. These observations are in line with a previous study that showed that

in LDLr$^{-/-}$Kit$^{W\text{-}sh/W\text{-}sh}$ mice lesions are smaller with a higher collagen content after 12 weeks of diet [12]. Mast cell derived IL-6 and IFNγ have been identified to be the key mediators in lesion development that study. In addition, we have previously shown that inhibition of chymase, one of the mast cell specific proteases, was able to reduce plaque progression in apoE$^{-/-}$ mice, which coincided with enhanced collagen content and decreased necrotic core size [28]

In our current study we detected a reduction in serum IL-6 in mast cell depleted mice. IL-6 promotes endothelial dysfunction, SMC proliferation and migration as well as recruitment and activation of inflammatory cells, thereby accelerating vascular inflammation [29]. Additionally, it was previously demonstrated that IL-6 affects the expression of scavenger receptors SR-A and CD36, which are involved in the uptake of oxLDL and thus promotes the formation of foam cells, a hallmark of early atherosclerotic lesion formation [30]. However, in vivo data discussing IL-6 in atherogenesis are rather contradictory. On one hand it was shown that administration of recombinant IL-6 to apoE$^{-/-}$ mice results in accelerated atherosclerosis, which coincided with an increase in inflammatory cytokines [31]. On the other hand, apoE$^{-/-}$ mice deficient for IL-6 developed larger lesions compared to apoE$^{-/-}$ controls. These studies indicate that IL-6 has both pro- and anti-inflammatory functions in atherosclerosis and that these functions are tightly balanced, which is essential for the immune response outcome during lesion development.

Furthermore, TNFα is an important acute phase cytokine that is implicated in many autoimmune disease such as rheumatoid arthritis and also in atherosclerosis. TNFα is expressed predominantly by activated macrophages but also by many other local (immune) cells like foam cells, smooth muscle cells and mast cells [32–34]. Since macrophages and mast cells have either transmembrane bound TNFα or preformed TNFα in their granules these cells are able to rapidly release TNFα upon stimulation [34–36]. Therefore, mast cells can be a source of TNFα in acute phase reactions [34]. TNFα stimulates both macrophages and smooth muscle cells to synthesize matrix proteases resulting in degradation of the fibrous cap [37, 38]. Moreover, TNFα is capable of decreasing the suppressive capacity of Tregs [39] and mast cell derived TNFα was shown to drive both the hypertrophy of the draining lymph nodes and recruitment T cells to the site of infection [40]. In our study, the depletion of mast cells coincided with a significant reduction in serum TNFα. Sun et al. described that mast cell derived TNFα did not affect atherogenesis [12]. However, TNFα$^{-/-}$ bone marrow derived mast cells used in that particular study produced relatively higher amounts of both IL-6 and IFNγ, i.e. 50 and 30% respectively, compared to wild-type bone marrow derived mast cells. Therefore it cannot be excluded that the observed effects on lesion size are due to enhanced secretion of these proatherogenic cytokines. Furthermore, it has been shown that mice deficient for both apoE$^{-/-}$ and TNFα developed a marked reduction in lesion size, which indicates an active involvement of TNFα in atherogenesis [41].

Taken together, this study establishes that absence of mast cell before the induction of

atherosclerosis is beneficial for both lesion growth and stability as well as for the systemic inflammatory milieu. Our data are correspond with previous experimental atherosclerosis studies demonstrating a critical role for mast cells in atherogenesis, without effects on other hematopoietic cells and shows that the RMB-apoE$^{-/-}$ mouse model can be of great value to study the role of mast cells in atherosclerosis.

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Chapter 7

Depletion of mast cells in established atherosclerosis increases plaque stability and reduces systemic inflammation in RMB-LDLr$^{-/-}$ mice

Manuscript in preparation

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Abstract

Objective

Mast cells are potent immune effector cells that are known to contribute to the initiation of atherosclerosis, and are implicated in the destabilization of atherosclerotic lesions matrix degradation via the release of proteases. Furthermore, mast cells can influence T cell skewing via the release of cytokines and cell-cell contact. To study the effects of mast cells on progression of atherosclerosis we used an inducible mast cell knockout mouse, the red mast cell basophil (RMB) mouse, that lacks the LDLr (RMB-LDLr$^{-/-}$). In this novel mouse model mast cells can be depleted after the development of atherosclerotic lesions, which enables us to investigate its effects on atherosclerotic lesion progression.

Methods and Results

We induced atherosclerosis in RMB-LDLr$^{-/-}$ mice by western type diet feeding for 10 weeks. After 10 weeks, a baseline group was sacrificed, while the other groups received either DT to deplete all FcεRIβ^{+} cells or PBS as a control. After successful depletion of the mast cells, mice received diet feeding for an additional 6 weeks. No differences were observed in atherosclerotic lesion size between the groups, but a significant increase in collagen content and a reduction in macrophage content and necrotic core area was observed in mast cell depleted mice compared to non-depleted mice. Furthermore, mast cell depletion coincided with a marked reduction in serum IL-6 levels and an increase in IL-10 levels compared to mast cell competent mice. In addition, an increase in regulatory T cells was observed in both the spleen and the heart lymph nodes of mast cell depleted mice.

Conclusions

Mast cell depletion in the progression phase of atherosclerosis improved the lesion stability and reduced systemic inflammation. Besides the well-established role of mast cells in the initial phases of lesion development, this study shows that mast cells also actively contribute to the progression and destabilization of atherosclerosis plaques.

Introduction

Atherosclerosis is a chronic lipid-driven inflammatory disease of the large- and medium sized arteries that involves complex immunologic mechanisms such as accelerated inflammation and reduced tolerance [1]. Next to its involvement in early atherogenesis, the immune system actively contributes to the progression and destabilization of an atherosclerotic plaque. Factors released by activated inflammatory leukocytes such as neutrophils, lipid-laden macrophages, T cells and mast cells result in the loss of matrix components such as collagen and apoptosis of smooth muscle cells, which accelerate the process of plaque destabilization [2]. Frequently, the process of plaque destabilization induces plaque rupture, which leads to acute atherothrombotic occlusion and clinical manifestations such as myocardial infarction and stroke [3]. Activated immune cells, among which mast cells, accumulate in the shoulder region of advanced atherosclerotic lesions, which can trigger matrix degradation by the release of specific proteases [4]. Especially mediators such as tryptase, chymase and histamine that are stored inside the granules of the mast cells are major contributors to plaque destabilization [5]. Circulating tryptase and chymase levels are increased in serum of cardiovascular patients suffering from acute myocardial infarction or unstable angina pectoris compared to patients with stable angina pectoris or healthy controls [6, 7].

To date, a number of experimental atherosclerosis studies have been performed to unravel the contribution of mast cells to atherogenesis and plaque destabilization [8–13]. The mast cell deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mouse strain has been crossed with LDL receptor (LDLr) or ApoE deficient mice and these mice develop smaller and more stable lesions compared to mast cell competent LDLr or ApoE deficient mice [9, 12]. Likewise, systemic mast cell activation by dinitrophenyl (DNP) or compound 48/80 in murine experimental atherosclerosis results in enhanced plaque growth accompanied by features of plaque destabilization such as an increase in intraplaque hemorrhage, macrophage apoptosis and serum tryptase activity, which could be prevented by the mast cell stabilizing drug cromolyn [8, 10]. Specific inhibition of chymase by the use of a protease inhibitor in murine experimental atherosclerosis improves the lesion stability as shown by an increase in collagen content and a decrease in necrotic core area [11]. Although these studies establish the contribution of mast cells to atherogenesis, these studies were performed in $Kit^{W\text{-}sh/W\text{-}sh}$ mice, which are constitutively mast cell deficient and this limits the possibility to study the specific contribution of mast cells to more advanced stages of atherosclerosis. Patients with unstable angina usually have advanced unstable lesions, which underlines the importance to study immune cells such as mast cells in advanced atherosclerotic plaques.

To that aim, we obtained a novel mouse model, the red-basophil-mast cell (RMB) mouse, in which we can study mast cell biology in vivo. In these RMB mice we can efficiently deplete FcεRIβ^{+}-cells (i.e. mast cells and basophils) by injection with diphtheria toxin (DT). Basophils are quite short depleted (<12 days), while a systemic depletion of mast cells

is maintained for up to 6 months after the last DT injection [14]. In the current study we induced atherosclerosis in RMB-LDLr$^{-/-}$ mice by western type diet feeding. After lesion induction, the mice received DT to deplete all FcεRIβ^{+}-cells followed by an additional period of diet feeding. At sacrifice, we analyzed lesion size and composition within the aortic root and analyzed systemic parameters such as circulating cytokine levels and blood leukocyte numbers, thereby establishing that mast cells actively contribute to plaque destabilization during the progressive phase of atherosclerosis.

Materials & Methods

Mice

All animal work was performed conform the guidelines from the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Leiden University Animal Ethics committee. The red mast cell and basophil mouse strain (RMB mice, official name: B6.Ms4a2^{tm1Mal}) was provided by the laboratory of dr. P. Launay, INSERM U1149, Paris, France [14]. These mice express the simian diphtheria toxin receptor under control of the promoter of the FcεRIβ gene. Injection of diphtheria toxin will therefore induce apoptosis of all FcεRIβ expressing cells (mast cells and basophils). RMB mice were crossed with LDLr$^{-/-}$ mice (The Jackson Laboratory, USA) to obtain atherosclerosis-prone RMB-LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were included in the study as a control for DT-mediated side effects [15, 16].

To study the effects of mast cell depletion on established atherosclerosis (Figure 1a), RMB-LDLr$^{-/-}$ mice (7-10 weeks old) were fed a Western type diet (0.25% cholesterol and 15% cacao butter, SDS, Sussex, UK) for 10 weeks to induce atherosclerosis. At week 10, a subset of mice was sacrificed as baseline control, while the remaining mice received either DT injections to deplete FcεRIβ^{+} cells or PBS as a control. To systemically deplete all FcεRIβ^{+} cells, mice were injected i.p. for three times every other day with 25 ng/g bodyweight DT (Diphtheria Toxin Unnicked, C. diphtheriae (Cat #322326), CalBiochem, US). Efficiency of DT treatment was determined by both FACS analysis for circulating basophils (CD49b^{+}/FcεRI^{+}/IgE^{+}) three days after the last DT injection and by a mast cell staining (chloro-acetate esterase (CAE), Sigma-Aldrich, US) of the aortic root at the end of the study. At sacrifice, mice were anaesthetized and sacrificed via orbital exsanguination. Fixation through the left cardiac chamber was performed with phosphate-buffered saline (PBS). Subsequently, the heart was excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, UK) for 18 hours at 4°C for histological analysis. Serum was collected to determine circulating cytokine levels. Splenocytes, EDTA-blood and heart lymph nodes were isolated for flow cytometry analysis of common leukocyte populations.

Histology and morphometry

After fixation, hearts were embedded in Tissue-Tek® O.C.T.™ compound (Sakura BV, The Netherlands) after which 10 µm cryosections were prepared using a Leica CM3050S cryostat and stained with Oil-Red-O (Sigma-Aldrich, US).

Oil-Red-O-stained sections were used to determine lesion size (in µm^2) in the aortic root. Mean lesion area (in µm^2) was calculated from 6 consecutive sections, starting at the appearance of the tricuspid valves. A CAE staining was performed to visualize mast cells, which were in a blinded manner counted manually by an independent operator. Collagen content was determined in corresponding sections by staining with Masson's

Trichrome staining (Sigma-Aldrich, US). Macrophages were visualized with an immunohistochemical staining using a primary rat anti-mouse macrophages/monocytes antibody (Clone moma-2, isotype IgG2b, 1:1000, Cat# MCA519G, AbD Serotec, Germany), while a goat anti-rat alkaline phosphatase (1:500, Cat# A8438 Sigma Aldrich, US) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates.

The atherosclerotic lesion areas and histological stainings of the aortic root cryosections were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging analysis system (Leica Ltd, UK). The percentage of collagen and macrophages in the lesions was determined by dividing the collagen- or MOMA-2 positive area by the total lesion surface area. The necrotic core size was defined as the a-cellular, debris-rich plaque area and represented as percentage of the total plaque area and measured in collagen stained sections.

Serum analysis

Serum concentrations of total cholesterol was determined by enzymatic colorimetric assay according to protocol of supplier (Roche Diagnostics, Switzerland). Precipath (Standardized serum Roche Diagnostics, US) was used as internal standard. Cytokine levels in serum were determined using a commercially available ELISA kit (Becton Dickinson, US). All procedures were according to manufacturer's protocol.

Flow cytometry

At sacrifice, blood was obtained in EDTA tubes and erythrocytes were removed using a specific erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3).

Blood leukocytes were stained extracellularly to determine a) monocytes (NK1.1$^-$/Ly6G$^-$/CD11b^{hi}), inflammatory monocytes (NK1.1$^-$/Ly6G$^-$/CD11b^{hi}/Ly6C^{hi}/CCR2$^+$) and neutrophils (NK1.1$^-$/Ly6G^{hi}/CD11b^{hi}), b) basophils (CD3$^-$/CD4$^-$/CD19$^-$/CD8$^-$/CD49b$^+$/IgE$^+$/CD117$^-$), c) T cells (CD3$^+$/CD4$^+$) and d) B cells (CD19$^+$/B220$^+$). The antibodies used (eBiosciences, US) are summarized in table 1. Flow cytometry analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (Becton Dickinson, US).

Table 1: Antibody panels used for flow cytometry analysis

Staining	FITC	PE	PerCP	APC	e-Fluor-450
A.	NK1.1 (Clone: PK136)	Ly6G (Clone: 1A8)	Ly6C (Clone: HK1.4)	CCR2 (Clone: 475301)	CD11b (Clone: M1/70)
B.	IgE (Clone: R35-72)	IgE (Clone: R35-72)	CD3/4/19/8 (dump channel)	CD49b (Clone: HMa2)	n/a
C.	CD44 (Clone: IM7)	CCR7 (Clone: 4B12)	CD8α (Clone: 53-6.7)	CD62L (Clone: MEL-14)	CD4 (Clone: GK1.5)
D.	IgM (Clone: II/41)	CD45RA (Clone: RA3-6B2)	CD19 (Clone: eBio1D3)	IgD (Clone: 11-26c)	CD5 (Clone: 53-7.3)

Stimulation of splenocytes and heart lymph nodes

At sacrifice, single cell suspensions were prepared from the spleen and heart lymph nodes by using a 70 µm cell strainer (Falcon, US). Erythrocytes were removed using a specific erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM

$NaHCO_3$, 0.1 mM EDTA, pH 7.3).

Regulatory T cell numbers were determined by staining with with eFluor-450 conjugated rat anti mouse CD4. Next, cells were fixed and permeabilized according to supplier's protocol (eBiosciences, US). Subsequently, cells were stained with APC conjugated rat anti-mouse/human FoxP3 or corresponding isotype as a control (eBioscience, US).

To determine inflammatory T cell phenotypes in spleen or lymph nodes, 400.000 cells/well were cultured in 96 well round-bottom plates (Greiner Bio-One, The Netherlands) and stimulated with anti-CD3 and anti-CD28 (2 µg/mL each, eBioscience, US) in complete IMDM, supplemented with 10% hiFCS, 100 u/mL penicillin/streptomycin, 2 mM L-Glutamine (PAA, Austria) and 20 mM β-mercaptoethanol (Sigma-Aldrich, US). After 1 hour, brefeldin A (Sigma-Aldrich, US) was added up to a concentration of 10 µg/mL to inhibit protein transport to the outside of the cell. After an additional 4 hours of culture, cells were washed twice with FACS Buffer (PBS, 1% BSA, 2mM EDTA) and stained for T cell surface markers.

Cells were first stained with eFluor-450 conjugated rat anti mouse CD4. Next, cells were fixed and permeabilized according to supplier's protocol (eBiosciences, The Netherlands), followed by intracellular staining with FITC conjugated rat anti-mouse IFNγ, PE conjugated rat anti-mouse IL-17 and APC conjugated rat anti-mouse IL-10 or corresponding isotypes as a control (eBioscience, The Netherlands). Flow cytometry analysis was performed as described above.

Statistical analysis

Values are expressed as mean ± SEM. Data of three groups were analyzed using a one-way ANOVA. Statistical analysis was performed using Graphpad Prism. Probability values of $P<0.05$ were considered significant.

Results

Body weight, total cholesterol and mast cell depletion

To study the contribution of mast cells to plaque progression, we induced atherosclerosis in RMB-LDLr$^{-/-}$ mice by feeding a western-type diet for 10 weeks, after which we depleted FcεRIβ^{+}-cells using DT (Fig. 1a). Three days after the last DT injection, we performed FACS analysis on blood samples for basophils as a control for depletion efficiency of DT. We detected basophils in both the PBS treated RMB-LDLr$^{-/-}$, whereas basophils were completely absent in the DT treated RMB-LDLr$^{-/-}$ group (Fig. 1b). After depletion of FcεRIβ^{+}-cells, all mice received an additional diet feeding for 6 weeks. At sacrifice, we observed a difference in body weight gain between the DT and PBS treated mice (Suppl. Fig. 1a), caused by the DT treatment as the DT treated LDLr$^{-/-}$ controls showed a similar weight curve as DT treated RMB-LDLr$^{-/-}$ mice. Further analysis showed no differences in total plasma cholesterol levels (Suppl. Fig. 1b) and total lesion size in the aortic root between DT treated LDLr$^{-/-}$ controls or RMB-LDLr$^{-/-}$ mice (Suppl. Fig. 1c). In addition, DT treatment did not result in differences in total leukocyte count and percentage of different circulating leukocytes (Suppl. Fig. 1d). Mast cells were detected in the aortic root of PBS treated RMB-LDLr$^{-/-}$, while significant depletion of mast cells was observed in DT treated mice (Fig. 1d). We also observed a complete depletion of mast cells in the skin upon DT treatment of RMB-LDLr$^{-/-}$ mice (data not shown).

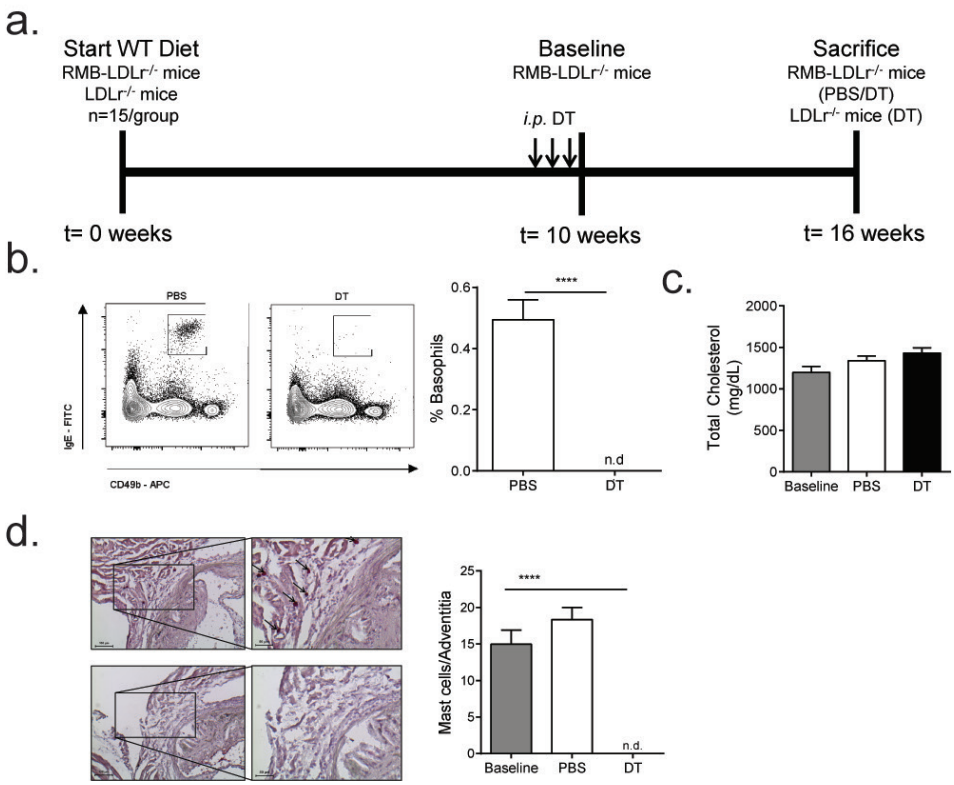

Figure 1. Study outline and basic characteristics in RMB-LDLr$^{-/-}$ and LDLr$^{-/-}$ mice.
(A) Flow-chart of the in vivo experimental set-up. (B) Representative FACS plots of basophil gating in PBS and DT treated mice and summary of all mice. (C) Serum total cholesterol levels from all groups (n=15/group). (D) Perivascular mast cell count in the aortic root of PBS or DT injected RMB-LDLr$^{-/-}$ and LDLr$^{-/-}$ mice (n=15/group). Arrows in magnification point to perivascular mast cells.
**P<0.01 ****P<0.0001.

Histological analysis of the aortic root

Next, we performed histological analysis of the aortic root to determine atherosclerotic lesion size and composition. Quantification of the lesion size in Oil-Red-O stained sections showed that the mean lesion size increased in both PBS and DT treated RMB-LDLr$^{-/-}$ mice, with 82% and 118% respectively, compared to baseline values (Fig. 2a). We did not detect any differences in mean lesion size between PBS and DT treated mice as depicted in figure 2a. The collagen content was significantly elevated in FcεRIβ^{+}-cell depleted mice compared to both the baseline value (DT: 25.4 ± 1.2 % versus Baseline: 13.5 ± 1.0 %, $p < 0.0001$) and the value in PBS treated mice (DT: 25.4 ± 1.2 % versus PBS: 15.6 ± 1.2 %, $p < 0.0001$, Fig. 2b). Furthermore, we observed a significant reduction in macrophage positive area (DT: 22.2 ± 1.1 % versus PBS: 29.7 ± 1.8 %, $p < 0.001$) and percentage of necrotic core area (DT: 14.5 ± 4.6 % versus PBS: 28.2 ± 9.7 %, $p < 0.001$) in FcεRIβ^{+}-cell depleted RMB-LDLr$^{-/-}$ mice compared to non FcεRIβ^{+}-cell depleted RMB-LDLr$^{-/-}$ mice (Fig. 2c-d).

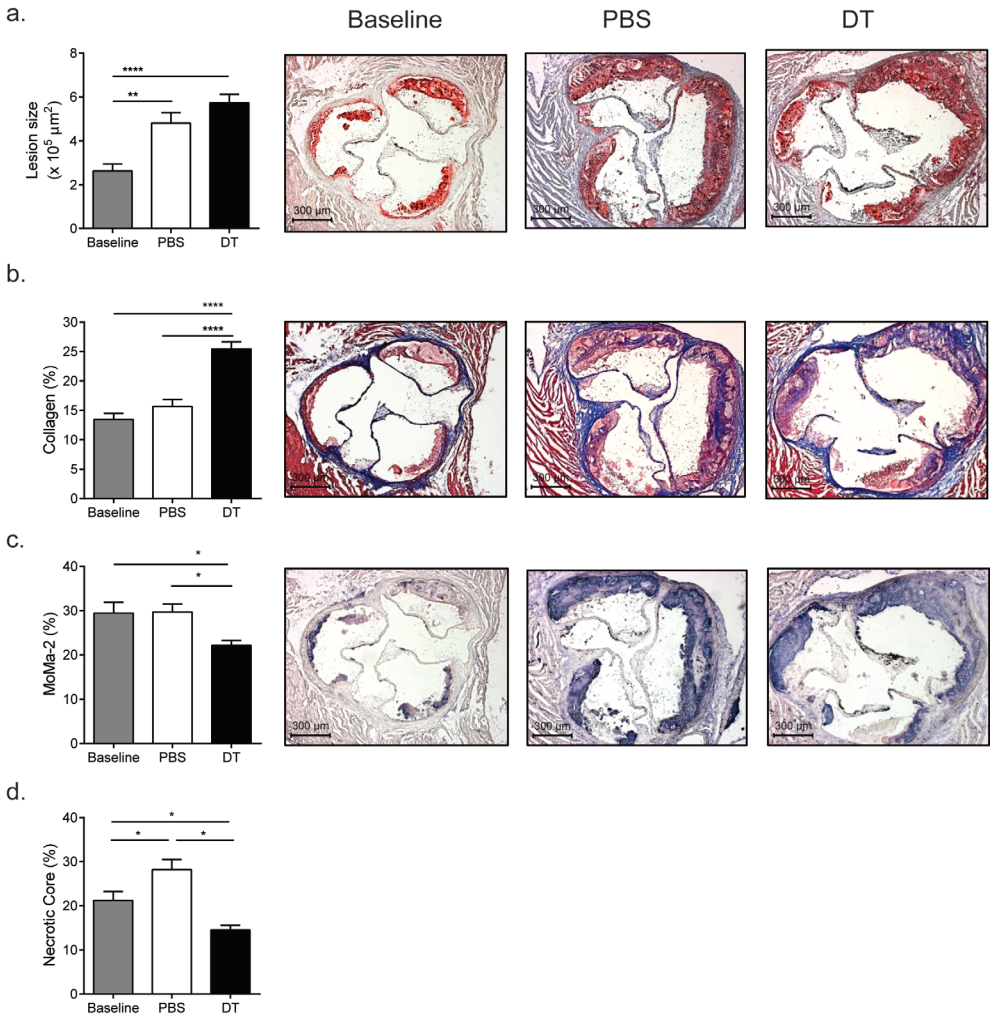

Figure 2. Depletion of FcεRIβ^{+} cells in RMB-LDLr$^{-/-}$ mice does not influence plaque progression but increases lesion stability in the aortic root.
(A) Depletion of FcεRIβ^{+} cells did not influenced the plaque size in DT injected RMB-LDLr$^{-/-}$ mice compared to PBS treated. Compared to non-depleted mice, depletion of FcεRIβ^{+}-cells during lesion progression coincided with an increase in collagen content as determined with Masson's Trichrome staining (B) as well as a reduction in macrophage content as determined by MOMA-2 staining (C) and a reduction in necrotic core (D) as percentage total lesion size (n=15/group). Representative cross-sections stained with Oil-Red-O and haematoxylin, Masson's Trichrome staining and MOMA-2 are shown. **P<0.01, ****P<0.0001.

Systemic cytokine profile in RMB-LDLr$^{-/-}$ mice

We analyzed the serum for proatherogenic cytokines such as IL-6, MCP-1, TNFα, IFNγ, IL-17 and the atheroprotective cytokine IL-10. As depicted in figure 3, no differences were detected in TNFα, IFNγ and IL-17 levels. Interestingly, we observed a significant increase in the chemokine MCP-1 level in the non-FcεRIβ^{+}-cell depleted mice compared to baseline values which was not observed in the FcεRIβ^{+}-cell depleted group. Furthermore, a significant increase in serum IL-10 was detected in FcεRIβ^{+}-cell depleted mice compared to baseline values (DT: 512.2 ± 51.3 versus Baseline: 275.7 ± 19.6 pg/mL, p=0.0005) and non-depleted mice (DT: 512.2 ± 51.3 versus PBS: 357.4 ± 40.2 pg/mL, p=0.02) (Fig. 3). In addition, we observed a marked reduction in circulating IL-6 in FcεRIβ^{+}-cell depleted mice compared to baseline values (DT: 124.5 ± 13.8 versus Baseline: 234.3 ± 12.8 pg/mL, p=0.0005) and non-depleted mice (DT: 124.5 ± 13.8 versus PBS 210.7 ± 20.3 pg/mL, p=0.005) (Fig. 3). DT injection in LDLr$^{-/-}$ mice did not influence cytokine levels (Suppl. Fig 1e).

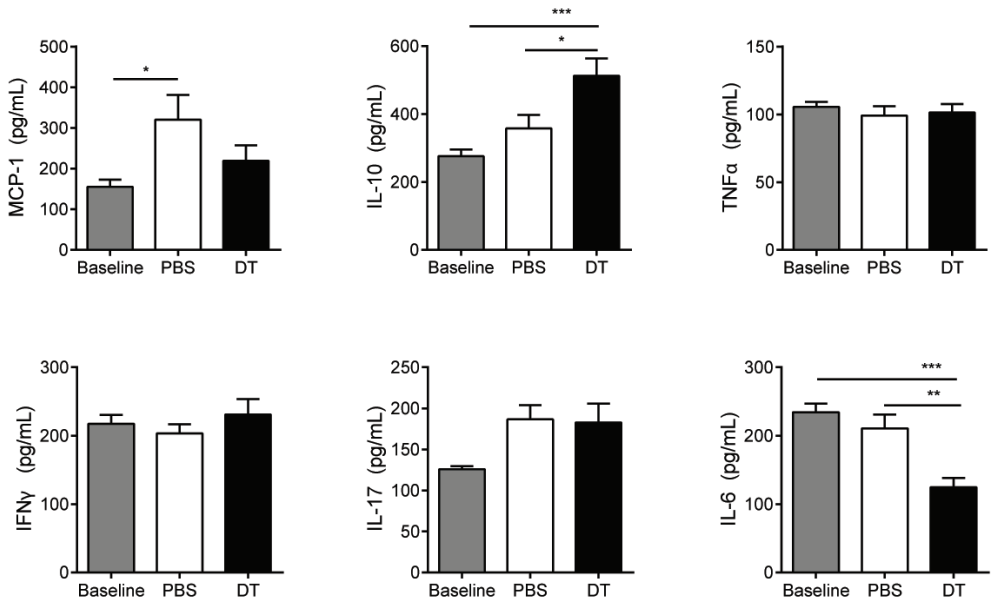

Figure 3. Increased levels of atheroprotective IL-10 and decreased levels of atherogenic MCP-1 and IL-6 after depletion of FcεRIβ^{+} cells in RMB-LDLr$^{-/-}$ mice.
Serum levels of IL-6, MCP-1, TNFα, IFNγ, IL-17 and IL-10 were quantified in serum of all groups (n=15/group). *P<0.05, ***P<0.001.

Flow cytometry analysis

At sacrifice, flow cytometry analysis of the blood for leukocyte populations showed a complete repopulation of basophils in DT treated RMB-LDLr$^{-/-}$ mice (Fig. 4). Although we did not observe a difference in the percentage of total monocyte levels between groups, the percentage of inflammatory monocytes were significantly reduced in DT treated mice. No differences were observed between groups for other leukocyte subsets such as neutrophils, T cells and B cells.

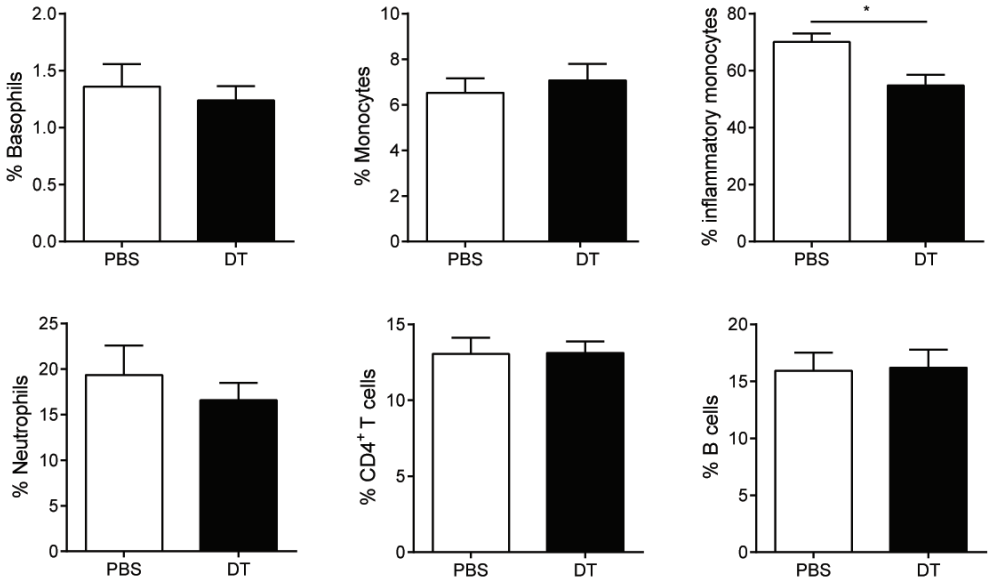

Figure 4. Flow cytometry analysis of circulating blood leukocytes showed a reduction in inflammatory monocytes in FcεRIβ^{+} cell depleted mice.

No differences were detected in circulating blood basophils, monocytes, neutrophils, CD4^{+} T cells and B cells. The percentage of inflammatory monocytes in FcεRIβ^{+}-cell depleted mice was reduced compared to non-depleted mice. *P<0.05.

In both the spleen and the lymph nodes draining from the heart we performed flow cytometry analysis to determine the percentages of T cell subsets. As depicted in figure 5a, we did not observe any difference in total $CD4^+$ T cells or proatherogenic phenotypes such as IL-17^+ (T_h17) and $IFN\gamma^+$ $CD4^+$ (T_h1) T cells in the both spleen and heart lymph nodes. Atheroprotective regulatory T cells were measured in spleen and heart lymph node. Analysis showed a significant increase in $CD4^+$/$FoxP3^+$ regulatory T cell levels in both the spleen (DT 10.0 ± 0.4 % versus PBS 7.6 ± 0.3 %, p=0.0002) and heart lymph nodes (DT: 9.9 ± 0.7 % versus PBS: 7.8 ± 0.5 %, p=0.02) of FcεRIβ$^+$-cell depleted mice compared to non-depleted mice (Fig. 5b). In addition, we did not observe any differences in T cell subsets in DT treated $LDLr^{-/-}$ mice (Suppl. Fig. 1f, g). Figure 5c shows representative FACS plots of the FoxP3 staining within the $CD4^+$ population of the heart lymph nodes.

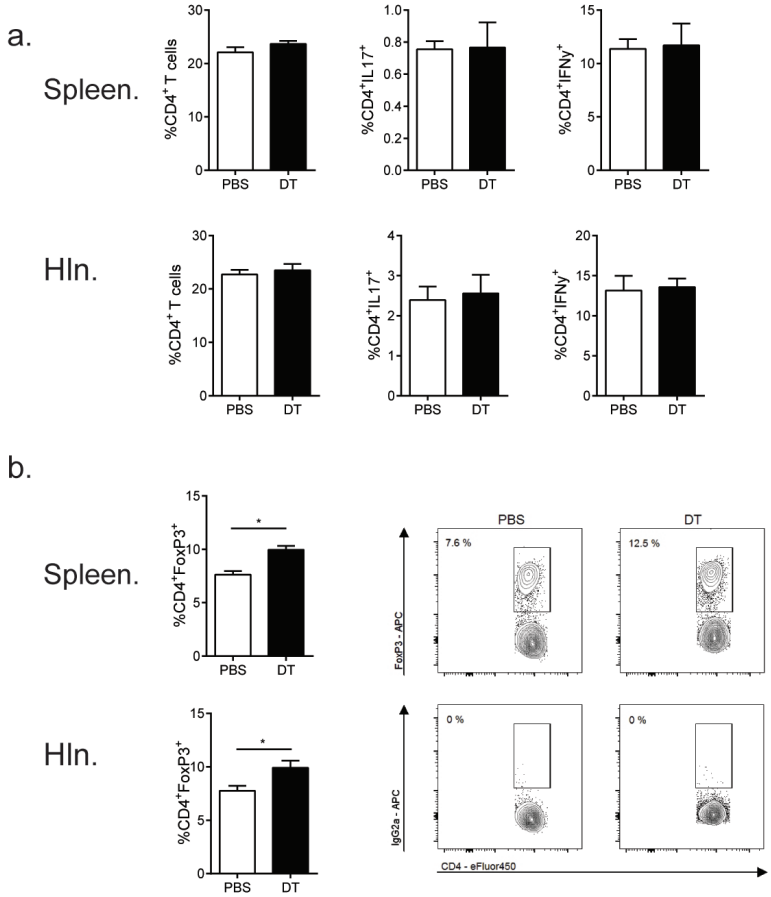

Figure 5. T cell phenotype in spleen and heart lymph nodes showed an increase in regulatory T cells in FcεRIβ$^+$ cells depleted mice.
(A) Analysis of both stimulated splenocytes and heart lymph node cells showed no differences in atherogenic T cell phenotypes. (B) A slight but significant increase in regulatory T cell was detected in splenocytes and heart lymph node cells of depleted mice compared to non-depleted mice. (C) Representative FACS plots are shown for all groups. *P<0.05.

Discussion

The major underlying cause of an acute cardiovascular syndrome (ACS) is the rupture of an unstable atherosclerotic lesion, which leads to ischemia in the distal tissue resulting in clinical symptoms such as a myocardial infarction or a stroke. Plaque destabilization is an important phase that precedes plaque rupture and mast cells can actively contribute to that process by the release of proteases such as tryptase, chymase and matrix metalloproteinases (MMPs), which induce apoptosis of collagen producing smooth muscle cells and degrade the extracellular matrix of the lesion. Indeed, in a number of studies elevated levels of the mast cell specific proteases tryptase and chymase have been reported in the serum of ACS patients compared to non-ACS patients and have been shown to correlate with plaque vulnerability and clinical outcome [17–19]. Additionally, in a recent study by Willems et al. the number of intraplaque mast cells was determined in lesions obtained from patients undergoing carotid endarterectomy. The number of lesional mast cells and serum tryptase levels was significantly higher in patients that had a secondary cardiovascular event compared to patients that remained asymptomatic during follow-up of the study [20]. These data indicate that mast cell numbers in the lesion are associated with future cardiovascular events and one suggest that mast cells indeed contribute to lesion destabilization that can lead to plaque rupture. To further study the effects of mast cells in the progressive phase of atherosclerosis, we made use of a mast cell inducible knockout mouse model on an atherosclerotic prone background (RMB-LDLr$^{-/-}$ mouse). This model enables us to study the contributions of mast cells beyond their role in the initial phases of atherosclerosis.

Our current study shows that absence of mast cells during lesion progression did not affect lesion size, but did result in an increased plaque stability and a reduction in the systemic inflammatory response. Our data are in line with a recent study from Wang et al., where pharmacological stabilization of mast cells in established atherosclerosis in LDLr$^{-/-}$ mice by the use of the drug cromolyn results in a reduced inflammatory plaque phenotype [13]. Similar to our data, mast cell stabilization reduces plaque macrophage content, but unlike their study we did not observe a reduction in plaque size and plasma cholesterol levels in mast cell depleted compared to non-depleted mice. One explanation could be the specificity of cromolyn in mice, which is subject of debate [21]. Cromolyn has been reported to interact with ubiquitous proteins such as G-protein-coupled receptor 35 (GPR35), which are highly expressed in different (non)-immune cells and tissues as in the colon, spleen, monocytes, T cells, neutrophils and dendritic cells [22, 23]. Therefore, one could speculate that cromolyn is able to influence more cellular processes besides the stabilizing effect on mast cells, which could have been contributed to the observed effects on lesion size in cromolyn treated mice [24]. Nonetheless, it can be concluded that mast cell depletion or stabilization increases lesion stability, which can be caused by a reduced release of mediators that induce plaque destabilization. For example, in the atherosclerotic lesion the majority of mast cells expresses the extracellular matrix

degrading enzyme chymase [25]. Activation of mast cells leads to the release of mediators such as chymase, which results in the breakdown of collagen, degradation of HDL and in apoptosis of smooth muscle cells [26].

Mast cells secrete chemokines and cytokines that can attract other immune cells into the lesion and MCP-1 is an important chemokine in the recruitment of inflammatory monocytes into the atherosclerotic lesion [27, 28]. Compared to baseline values, PBS treated RMB-LDLr$^{-/-}$ mice but not mast cell depleted RMB-LDLr$^{-/-}$ mice showed a significant increase in serum MCP-1 concentrations after six weeks of additional diet. Furthermore, we detected a significant reduction in circulating inflammatory monocytes in mast cell depleted RMB-LDLr$^{-/-}$ mice. Together with the reduction of the chemokine MCP-1 levels this could this explain, to a certain extent, the reduction in macrophage positive area observed in mast cell depleted mice.

Pathogenic (T_h1 and T_h17) and protective regulatory T cells (Tregs) are tightly balanced during immune responses. This balance is controlled by cytokines, e.g. IL-12, IL-4 and IL-6, and cell-cell interactions via co-stimulatory molecules like CD80/86, ICOSL, CD40 and OX40L, which are expressed by antigen-presenting cells such as dendritic cells. Mast cells are efficient effector immune cells that upon activation by various ligands actively secrete potent mediators, such as IL-8, TNFα and IL-6, and on top of that also express co-stimulatory molecules such as CD80/86 and OX40L that are able to directly interact with T cells thereby enhancing the proliferation and function of T cells [29–33]. In our current study we were unable to detect any differences in atherogenic T cell phenotypes such as T_h1 and T_h17 T cells between the groups. This could be due to the fact that in established lesions the adaptive immune response has already fully developed and therefore mast cell depletion is unable to significantly alter this established atherogenic T cell phenotypes. In contrast, mast cell depletion coincided with an increase in Tregs in both the spleen and the draining heart lymph nodes and elevated serum levels of IL-10. Tregs are essential in the dampening of immune responses in inflammatory conditions during atherosclerosis by the secretion of anti-inflammatory TGFβ and IL-10 [34]. Mast cells have been shown to directly inhibit Tregs functions via the co-stimulatory molecule OX40-OX40L, the release of histamine and secretion of IL-6 [33, 35, 36].

The acute phase cytokine IL-6 is a key cytokine in the skewing of naïve T cells towards T_h17 cells and inhibition of Treg development [37–39]. Under inflammatory conditions, IL-6 producing mast cells co-localize with Tregs and T_h17 cells in the draining lymph node of immunized mice in an experimental model of multiple sclerosis and appear to play an important role in the promotion of T_h17 cell differentiation and inhibiting Treg suppression [35]. Especially the interaction of the co-stimulatory molecules OX40L and OX40 between mast cells and Tregs, respectively, has been shown to block the function of Tregs and promote T_h17 cell differentiation [35]. T_h17 cells have been implicated in the pathogenesis of murine and human atherosclerosis [40]. In our current study, we observed a reduction of IL-6 and an increase in IL-10 in the serum of mast cell depleted mice compared to

non-depleted mice. Most likely, this reduction in IL-6 positively influenced the T cell homeostasis in favor of IL-10 producing Tregs.

In conclusion, here we show that a systemic mast cell depletion in RMB-LDLr$^{-/-}$ mice with established atherosclerotic lesions locally affects plaque phenotype but also systemically alters cytokine profile and T cell phenotype. Together with observation that intraplaque mast cell numbers correlate with clinical outcome, these data indicate that mast cell stabilization in cardiovascular patients with unstable atherosclerotic lesions could be beneficial in preventing lesion destabilization.

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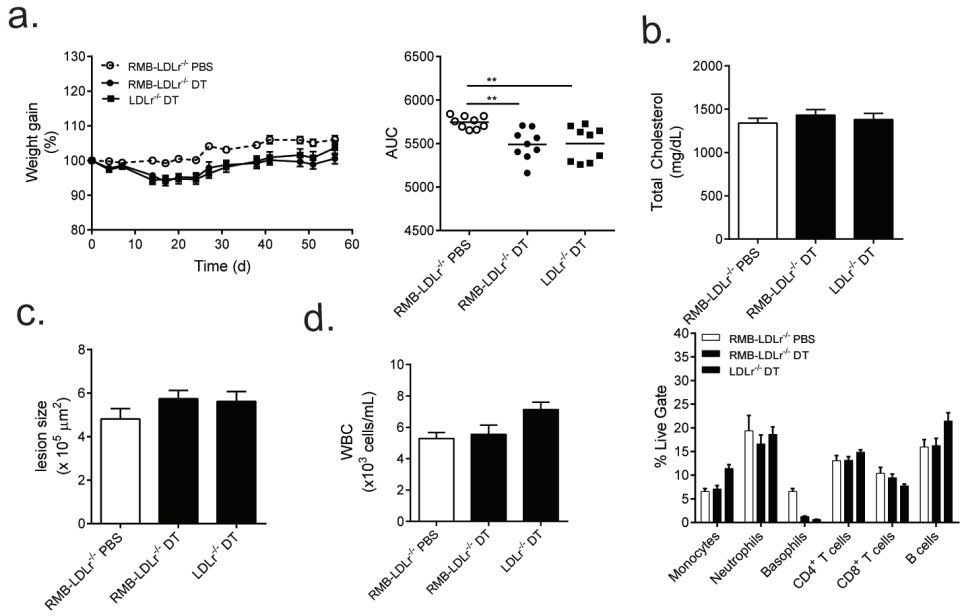
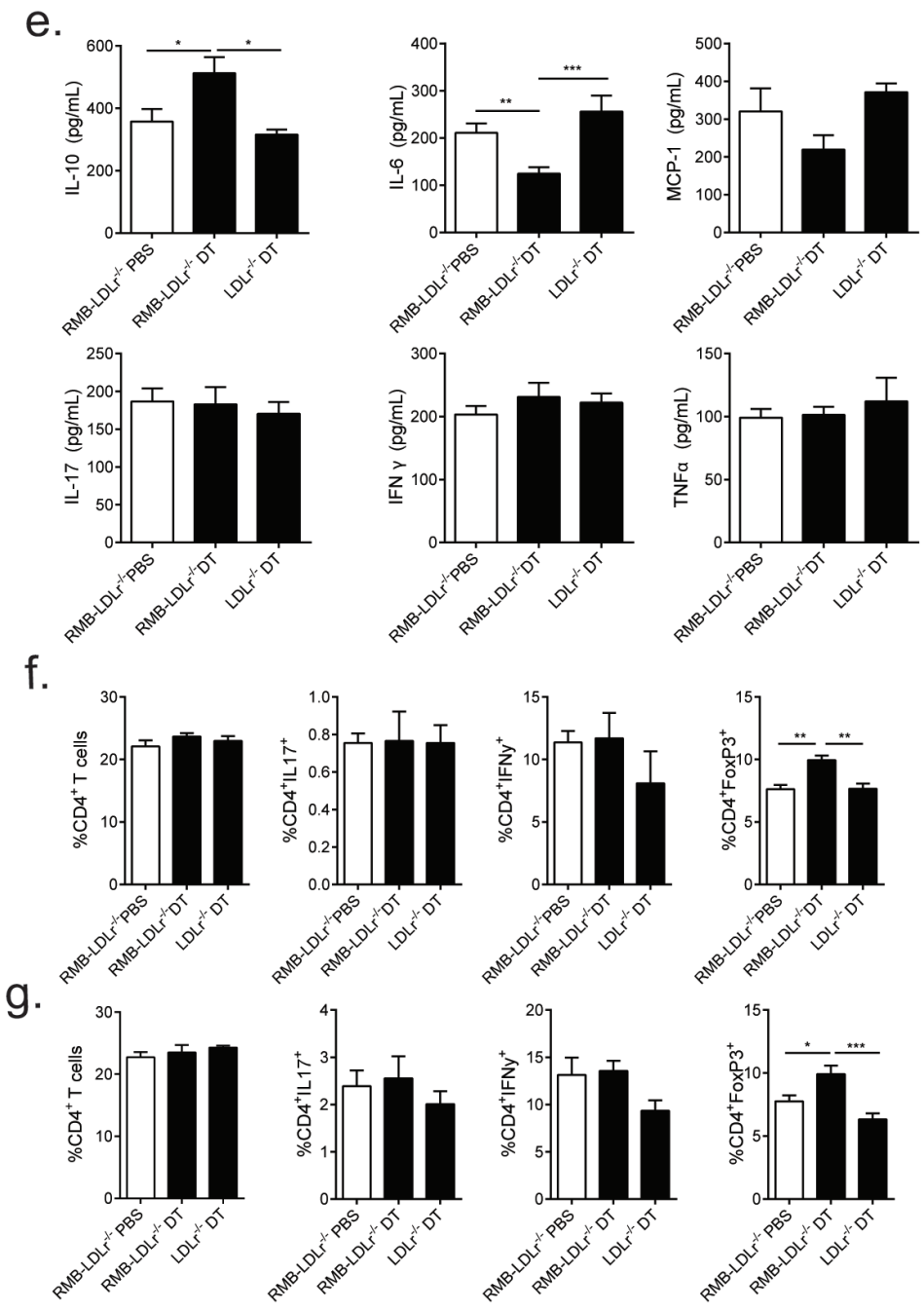
Supplemental data





Supplemental figure 1: DT treatment of LDLr$^{-/-}$ mice as control for DT off-target effects
(A) Body weight of RMB-LDLr$^{-/-}$ and LDLr$^{-/-}$ mice injected with either PBS or DT (n=15/group). Total plasma cholesterol levels (B), total lesion size (C), total WBC and leukocyte subsets (D), cytokine levels (E), T cell subsets in hLN (F) and T cell subsets in spleen (G) of PBS and DT treated RMB-LDLr$^{-/-}$ and DT treated LDLr$^{-/-}$ mice (n=15/group). *P<0.05, **P<0.01, ***P<0.001.

Chapter 8

General summary and perspectives

Introduction

The immune system is a highly specialized component of the human body, which is essential in the host defense against invading pathogens. Complex interactions between cellular and non-cellular components of the innate and adaptive immunity result in efficient clearance of these pathogens and the development of a lifelong immunological memory towards antigens. These potent immune reactions are tightly balanced by regulatory immune cells that induce tolerance towards harmless antigens and dampen the ongoing immune response by the secretion of regulatory mediators. Imbalance of these activating and regulatory pathways can result in either unresponsiveness or hypersensitivity of the immune system towards a certain antigen. Nowadays, there is a high prevalence of hypersensitive immune reactions, e.g. allergies and immune driven disorders. Rheumatoid arthritis (RA) and atherosclerosis are considered as such immune driven disorders with high frequencies in the industrialized world [1,2]. Environmental factors such as smoking, sedentary life style, stress and a high fat diet as well as genetic risk factors have been described to be major risk factors for both diseases. Although the pathology and etiology of RA and atherosclerosis are not completely understood yet, it is nowadays widely accepted that the immune system plays an important role in the continuous process of joint destruction and vascular occlusion. Central in the pathogenesis of both diseases is the loss of tolerance towards harmless self-antigens and modified self-antigens such as citrullinated proteins or oxidized lipoproteins. This will lead to activation of local immune cells, thereby initiating an inflammatory immune response. Insufficient dampening of this early immune response results in an adaptive immune response, which is composed of T helper cells and antibody-producing B cells. These cells actively secrete mediators such as cytokines and immunoglobulins, which will further activate and recruit other immune cells like macrophages, osteoclasts, neutrophils and mast cells into the inflamed tissue. Mast cells are innate immune cells located in tissues, which are in close proximity to the external world such as skin, gut and lung [3]. Mast cells have been reported to be located inside the joints below the synovial membrane and within vascular tissues. Mast cell activation in RA and atherosclerosis could amplify the ongoing immune response, which actively contributes to the breakdown of cartilage and bone or the occlusion of a blood vessel. In this thesis, the contribution of mast cells to experimental arthritis and atherosclerosis was studied.

Mast cells in (experimental) arthritis

Mast cells are, besides known for their function in host defense responses, implicated in hypersensitivity reactions such as allergies and asthma, but also in the pathogenesis of other immune driven disorders. In the past decades, a number of studies have shown that mast cells accumulate in the synovial tissue and can take up to 6% of all nucleated cells [4,5]. In addition, mast cell specific proteases such as tryptase have been detected in the synovial fluid of RA patients. These data suggest that activated mast cells can affect the ongoing immune response, leading to more persistent joint destruction.

In **Chapter 2** we review the current literature describing mast cells and their potential interactions with other immune cells in rheumatic diseases. This review also summarizes the existing data describing a number of potential ligands present in human RA as well as murine experimental models that can activate mast cells locally in the joint.

The pathogenesis of RA can roughly be divided into a pre-clinical and clinical phase. The pre-clinical phase is defined as the phase were the patient has developed subclinical characteristics of RA, i.e. the generation of autoantibodies and mild symptoms of joint swelling. In the clinical phase, an RA patient displays a high disease activity score (DAS28) will show the presence of autoantibodies in the serum and synovial fluid, and suffers from active breakdown of cartilage and bone in the joints. Collagen induced arthritis (CIA) is a mouse arthritis model that shares several clinical, serological and immunological characteristics with human RA [6]. Comparable to human RA, CIA can also be divided into a pre-clinical and clinical phase and in **Chapter 3** we sought to investigate the contribution of mast cells to these phases of collagen induced arthritis in the inducible mast cell knockout mouse, the red-mast cell basophil mouse (RMB) [7]. Our results show that depletion of mast cells in the pre-clinical phase of collagen induced arthritis, but not the clinical phase, significantly reduced the clinical disease score. We confirmed the independence of mast cells in clinical phase of arthritis by showing similar results in a mouse model of antibody induced arthritis. This indicates that mast cells predominantly influence the early pre-clinical phase of experimental arthritis that precedes the clinical manifestations. Pre-clinical depletion of mast cells resulted in a reduced inflammatory cytokine profile of splenocytes after re-stimulation with collagen type II. Arthritogenic cytokines such as IL-17 and IL-6 were significantly reduced, while protective IL-10 was elevated in mast cell depleted mice. The anti-inflammatory cytokine profile coincided with a decrease in inflammatory T_h17 T cell and an increase in regulatory $FoxP3^+$ T cells. Taken together, this study shows that the role of mast cells is limited once clinical manifestation of arthritis have manifested and that mast cells are modulating the early immune response in experimental arthritis.

Although mast cell depletion in clinically evident arthritis in mice was unable to reduce the clinical score, we cannot exclude that mast cells in human RA do contribute to the progression of the disease, and further research should aim to identify RA specific mast cell activators.

Mast cell activation via immunoglobulins

The majority of RA and cardiovascular patients develops a strong humoral response, which is characterized by high titers of immunoglobulins that target (modified) self-antigens such as collagen, citrullinated proteins or modified lipoproteins [8,9]. In a high number of RA patients, anti-citrullinated protein antibodies (ACPAs) can be detected and serve as a biomarker for a more progressive RA phenotype compared to seronegative RA patients [10]. Citrullination or deimination is a post-translational conformation of an arginine residue within a protein to a citrulline by peptidylarginine deiminases (PADs) [11]. PAD enzymes are present in a variety of cell types, including macrophages and neutrophils. Upon necrosis of these cells, PAD enzymes are released in the environment, which cause the citrullination of proteins in the extracellular matrix. ACPAs target these proteins and can thereby accelerate the process of joint destruction. Recently, it has been shown that citrullination can also occur outside the joint in for example the myocardium and within atherosclerotic lesions [12–14]. Like in RA, in the atherosclerotic plaque macrophages express PAD enzymes, which can drive the local citrullination. In **Chapter 4** we tested whether ACPAs were present in the sera of cardiovascular patients without RA diagnosis. Intriguingly, we found that in cardiovascular cohorts "MISSION!" and "Circulating Cells", a significant proportion of non-RA patients displayed reactivity towards CCP3, and were thus positive for ACPAs. Further clinical analysis of the data showed that the survival rate of ACPA-positive CVD patients within the MISSION cohort is significantly lower compared to ACPA negative CVD patients. Taken together, these preliminary data show that the presence of ACPA in non-RA cardiovascular patients may be of predictive value for future cardiovascular events.

In the sera of cardiovascular patients immunoglobulins recognizing modified lipoproteins such as oxidized LDL (oxLDL) can be detected. Recently, intraplaque mast cell numbers were shown to be associated with atherosclerotic plaque progression and with the incidence of future cardiovascular events [15]. Hallmark of mast cells is the expression of various receptors that can bind a number of ligands present inside the plaque, which may result in mast cell activation. For example, activating immunoglobulin receptors such as the FcεR (IgE) and the FcγR (IgG) are present on both human and murine mast cells [16]. In **Chapter 5** we determined whether specific immunoglobulins in sera of patients scheduled for carotid endarterectomy were related to intraplaque mast cell numbers and plasma tryptase levels. Total IgG, total IgE and oxLDL-IgG were determined the sera of 135 patients. In this study, we did not observe any associations between the measured immunoglobulin levels and mast cell numbers inside the atherosclerotic lesion or other plaque characteristics like lipid core size, degree of calcification, number of macrophages or smooth muscle cells, amount of collagen and number of microvessels. These data indicate that mast cell activation inside the lesion may occur via other receptors than immunoglobulin receptors or that systemic immunoglobulin levels do not reflect localintraplaque mast cell activation status.

Mast cells in experimental atherosclerosis

As mentioned before, mast cells contain many proteases pre-stored inside their granules, which can be released within seconds upon stimulation [17]. Proteases such as tryptase, chymase and matrix metalloproteinases can reduce lesion stability by the breakdown of collagen fibers of the fibrous cap, by degradation of HDL but also by inducing apoptosis of smooth muscle and endothelial cells [18,19]. Furthermore, mast cells can accelerate lesion growth by the recruitment of leukocytes into the lesion by the secretion of cytokines and chemokines. The contribution of mast cells to atherosclerosis has up to now mainly been studied in mast cell deficient mice, which limits the opportunities to study mast cells beyond the initial phase. Furthermore, these mast cell deficient mice such as the $Kit^{W\text{-}sh/W\text{-}sh}$ mice also suffer from side-effects due to the mutation in c-Kit signaling. Therefore, we obtained a novel mouse model, which is inducible knockout for mast cells independent of c-Kit signaling alterations. This model enables us to study mast cells also in the progressive phase of atherosclerosis. We characterize this novel mouse model in **Chapter 6** by first depleting all mast cells and subsequently inducing atherosclerosis by western-type diet feeding and carotid artery collar placement. This study confirmed previous data, showing that mast cells contribute to the initial phase of lesion development. In line with the study from Sun et al. we observed a reduction in lesion size and increased lesion stability as demonstrated by an increase in collagen staining and a reduction in necrotic core size upon mast cell depletion [20]. Furthermore, we observed a reduction in the inflammatory cytokines IL-6 and TNFα in plasma, which may be indicative of a reduction in the inflammatory response.

In **Chapter 7** we aimed to further study mast cell-dependent effects in the progressive phase of atherosclerosis. To this aim, we first induced advanced lesions in RMB-$LDLr^{-/-}$ mice by western type diet feeding for 10 weeks. Next, we depleted mast cells and continued western-type diet feeding for 6 weeks. Histological analysis of the aortic root showed that depletion of mast cells was unable to affect the lesion size but did improve lesion stability. We observed elevated levels of collagen and a significant decrease in both macrophage and necrotic core area in mast cell depleted mice. In addition to the histological phenotype, mast cell depletion also coincided with a reduction in the systemic inflammatory response. Serum analysis revealed a reduction in the atherogenic cytokines IL-6 and MCP-1, and an increase in the atheroprotective IL-10. In addition, we performed flow cytometry analysis for T cell phenotypes in the draining lymph node of the heart and in the spleen. Mast cell depletion was unable to alter pathogenic T_h1 and T_h17 phenotypes but did result in a marked increase in regulatory $FoxP3^+$ T cells. Together with the reported clinical observations in cardiovascular patients, this study confirms that mast cells have a major impact on the plaque stability and is able to modulate the systemic cytokine profile thereby influencing T cell skewing.

Further perspectives

The pathogenesis of RA and atherosclerosis is characterized by an active immune involvement, which amplifies the process of joint destruction and vascular occlusion. Furthermore, both diseases share similar immunological pathways such as development of autoantibodies and infiltration of leukocytes in the affected tissues. Compared to healthy non-RA individuals, there is an increased risk for RA patients to develop cardiovascular diseases, which cannot be explained by traditional risk factors alone [21]. For example, RA is often accompanied by accelerated progression of atherosclerosis and a more unstable rupture-prone plaque phenotype compared to RA negative subjects [22,23]. In addition, vascular inflammation is increased in active RA patients compared to healthy controls, which can improved by anti-inflammatory therapy, e.g. anti-TNFα. [24]. Currently, RA and cardiovascular patients are treated with drugs that dampen the ongoing immune response. In RA, DMARDs often in combination with biologicals such as anti-TNFα are highly effective in slowing down the progression of joint destruction and frequently induce remission of the inflammatory response in the joint. In addition, this treatment regimen can have beneficial effects on the inflammatory response in the vasculature system. A reported meta-analysis of observational studies showed that methotrexate use, but not use of other DMARDs, in RA patients is associated with a 21% reduction in CVD risk compared to patient that do not use methotrexate [25,26]. Although anti-TNF was shown to affect lipid levels (increase in HDL, triglycerides and total cholesterol), there was a significantly reduction in CVD risk in RA patients [27–30]. Cardiovascular patients are usually treated with hydroxymethylglutaryl-coenzyme A reductase inhibitors, or 'statins', to lower circulating cholesterol levels. The majority of RA patients is reported to have dyslipidemia and in active disease there is a more atherogenic LDL:HDL ratio [31,32]. Statin treatment has been shown to be beneficial in RA patients, probably due to both its lipid-lowering as its anti-inflammatory effects [33]. In the TARA (Trial of Atorvastatin in Rheumatoid Arthritis) trial, disease activity score and lipid profile were reported to decline significantly in the statin-treated group compared to the placebo-treated RA patients [34]. Recently, another study by Schoenfeld et.al. showed that statin use in RA patients was associated with a 21% lower risk of all-cause mortality [35]. However, despite the fact that the use of statins has been shown to be effective in RA and CVD patients, a number of patients do not respond to the medication or have serious side effects, which renders new therapeutic targets highly necessary.

In this thesis, we showed that mast cells contribute to the early phase of experimental arthritis. To date however, it is unknown if mast cell stabilization in active human RA is beneficial as a therapeutic treatment despite the high number of mast cells in the synovium and the many reported mast cell activation pathways in RA. In experimental arthritis, mast cell stabilization by cromolyn was shown to be effective in mice as measured by improvement of the radiographic score and histological analysis [36,37]. Taken together, mast cell targeting therapy in RA could be of interest as a new additional treatment next

to the use of DMARDs and biologicals.

Comparable to RA, the development of atherosclerotic lesions is also composed of different phases and many studies have implicated mast cells in these phases. Our data also indicate that mast cell stabilization may have therapeutic potential in patients with cardiovascular diseases. At present, one patent has been issued for the use of cromolyn in cardiovascular patients as a novel treatment [38]. However, no study results are reported yet. Recently, the JUPITER trial demonstrated that statin therapy can significantly reduce the risk of a future heart attack or a stroke in patients with low levels of LDL-cholesterol but who have increased vascular risk due to high levels of c-reactive protein (CRP), which is a biomarker of inflammation [39]. Two large clinical placebo controlled trials (CIRT and CANTOS) are now ongoing to investigate whether inhibition of the inflammatory response can reduce CVD risk. In the CIRT trial, CVD patients are treated with a low dose methotrexate, also prescribed to treat RA patients, to study the effect of inhibition of inflammation on vascular event rates. The CANTOS trial targets the inflammatory IL-1β in cardiovascular patients by use of neutralizing canakinumab, which already has shown to improve clinical outcome in RA patients [40,41]. In the near future, the outcome of these trials will address whether anti-inflammatory medication is able to reduce the incidence of cardiovascular events among patients who remain at risk due to a persistent inflammatory response.

In conclusion, the research performed in this thesis shows that mast cells have immune modulating functions in experimental models of immune driven disorders and future research will establish whether mast cell stabilization in auto-immune diseases is of therapeutic interest.

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Chapter 9

Nederlandse samenvatting

Introductie

Het immuunsysteem is een essentieel onderdeel van het menselijk lichaam in de verdediging tegen pathogene micro-organismen. Het immuunsysteem kan grofweg worden opgedeeld in twee onderdelen: het aangeboren ("innate") en het specifieke, verworven ("adaptive") immuunsysteem. Kenmerkend voor een aangeboren afweerreactie is een snelle reactie van zowel cellulaire als niet-cellulaire componenten gericht tegen een pathogeen. Verworven immuniteit is vaak een tragere, maar hoog specifieke reactie op een infectie, die resulteert in een levenslange bescherming.

Mestcellen zijn onderdeel van het innate immuunsysteem en zijn strategisch gelegen in verschillende weefsels, die in contact staan met de buitenwereld. Evolutionair gezien zijn mestcellen essentieel in de afweerreactie tegen parasieten zoals wormen en protozoa. De interactie van mestcellen met deze pathogenen zorgt ervoor dat binnen enkele seconden voorgevormde mediatoren zoals signaalstoffen en enzymen worden uitgescheiden in het omliggende weefsel. Dit proces staat bekend als degranulatie, en is een fenomeen dat bij mestcellen, basofielen en neutrofielen voorkomt. Door deze activatie worden andere typen ontstekingscellen aangetrokken naar de plaats van infectie, wat bijdraagt aan een snelle eliminatie van de pathogeen.

Echter, in de huidige westerse samenleving zijn parasitaire infecties zeldzaam en worden mestcellen vaker in verband gebracht met overgevoeligheidsreacties zoals allergie en astma. Onderzoek van de laatste jaren heeft duidelijk gemaakt dat mestcellen ook een rol kunnen spelen in de pathogenese van ontstekingsziektes zoals reumatoïde artritis en aderverkalking. In deze ziektes reageren mestcellen zeer snel op (endogene) stoffen met het uitscheiden van signaalstoffen en enzymen. Hierdoor hebben mestcellen nu een negatieve reputatie zowel in allergieën en auto-immuniteiten. Het onderzoek naar de effecten van remming van de activiteit van de mestcel in deze ziektes kan mogelijk tot een nieuwe therapeutische benadering kunnen komen. Om het effect van één celtype te bestuderen in complexe ziektes zoals reumatoïde artritis of atherosclerose zijn dierexperimentele modellen van belang. Deze modellen maken het mogelijk om specifiek één bepaalde cel te verwijderen, en zo het effect hiervan op het ziekteverloop te bestuderen.

In dit proefschrift hebben wij de invloed van mestcellen in dierexperimentele modellen voor reumatoïde artritis en aderverkalking bestudeerd met een focus op de rol van mestcellen in de (sub)-klinische fases van het ziekteproces, waarin er een actieve immuunrespons ontwikkeld was. Ook hebben wij de aanwezigheid van verschillende soorten antistoffen in het serum van cardiovasculaire patiënten onderzocht. Wij hebben de niveaus van antilichamen gecorreleerd aan klinische observaties zoals body mass index (BMI), lipidenprofiel, klinische diagnose van vaatlijden, samenstelling van de atherosclerotische plaque en de uitkomst van de ziekte.

Mestcellen en (experimentele) reumatoïde artritis

Reumatoïde artritis (RA) is de meest voorkomende vorm van gewrichtsontsteking (artritis) en heeft een prevalentie van 0,5 – 1% van de wereldbevolking en naarmate de leeftijd hoger wordt hebben meer mensen RA. Het ziekteproces wordt gekenmerkt door de chronische ontsteking van met name de kleine hand- en voetgewrichten. De ontsporing van het immuunsysteem speelt een belangrijke rol in het proces van gewrichtsdestructie, en de huidige behandelmethoden zijn sterk gericht op het remmen van de chronische ontsteking en het remmen van specifieke inflammatoire eiwitten. Dankzij het op de markt verschijnen van verschillende zogenaamde biologicals zoals tumor necrosis factor (TNF)-blokkers is het mogelijk geworden in veel patiënten een verlaging van ziekteactiviteit of zelfs remissie te bereiken.

In het reumatische gewricht, in het synovium en in het synoviale vocht zijn een aantal typen ontstekingscellen aanwezig. Grote aantallen geactiveerde neutrofielen, macrofagen, T cellen en antistoffen producerende plasmacellen dragen bij aan de activatie van osteoclasten, die actief kraakbeen en botweefsel afbreken. Naast deze ontstekingscellen accumuleren ook mestcellen in het synoviale weefsel, en ze kunnen ongeveer 5% van alle cellen in het reumatische gewricht vormen. In het synoviale vocht van RA-patiënten zijn ook hoge concentraties van door mestcellen geproduceerde enzymen gevonden zoals chymase en tryptase. Deze enzymen kunnen direct zorgen voor gewrichtsafbraak, maar ook via de activatie van osteoclasten kunnen mestcellen bijdragen aan de pathogenese. Bovendien kunnen mestcellen grote hoeveelheden interleukines en chemokines uitscheiden, die niet alleen de infiltratie van nieuwe afweercellen in het gewricht kunnen bewerkstelligen, maar ook systemisch de cellulaire adaptieve afweerreactie kunnen moduleren. Dit maakt duidelijk dat de mestcel een belangrijke speler zou kunnen zijn in de pathogenese van RA.

De chronische ontsteking in RA is een belangrijke schakel in het proces van kraakbeen en bot afbraak. **Hoofdstuk 2** geeft een overzicht van de huidige literatuur over de rol van mestcellen in het proces van gewrichtsafbraak in RA en de huidige kennis opgedaan in dierexperimentele modellen van RA. In dit hoofdstuk is samengevat dat in RA endogene liganden aanwezig zijn die o.a. mestcellen kunnen activeren via Toll-Like Receptoren en receptoren voor IgG antistoffen (FcγR). Ondanks het feit dat er meer mestcellen aanwezig zijn in het ontstoken synoviale weefsel, is de bijdrage van mestcellen aan de pathogenese van RA nog onduidelijk. De afgelopen decennia zijn er door verschillende onderzoeksgroepen studies gedaan om de rol van mestcellen in RA te onderzoeken. Echter, door het gebruik van zowel verschillende artritismodellen als verschillende muismodellen voor mestcel deficiënte is er nog geen eenduidig beeld of mestcellen bijdragen aan reumatoïde artritis. In experimentele artritis modellen die gebaseerd zijn

op het toedienen van pathogene antistoffen lijkt het effect van mestcellen marginaal. Deze modellen worden gekarakteriseerd door de afwezigheid van een adaptieve immuun response en zijn volledig gebaseerd op lokale complement activatie in het gewricht. Een artritis model waarin wel een sterke adaptieve reactie wordt ontwikkeld is het zogenaamde "collagen-induced-arthritis (CIA)" model, dat qua ziekteverloop vergelijkbaar is met humane RA. Wanneer in mestcel deficiënte muizen CIA wordt geïnduceerd, is de klinische score en ontstekingsreactie lager dan in wildtype dieren. Dit duidt erop dat mestcellen een bijdrage leveren aan de pathogenese van artritis via modulatie van de adaptieve immuun response.

De pathogenese van RA wordt gekenmerkt door een preklinische fase en een klinische fase. In de preklinische fase wordt de patiënt met een genetisch risico blootgesteld aan omgevingsfactoren en dit kan leiden tot een subklinische immuunreactie in de gewrichten. In de klinische fase van RA worden de gewrichtszwellingen zichtbaar en circuleren er ook RA specifieke antistoffen zoals reumafactor (RF) en anti-citrullinated protein antilichamen (ACPA). Het hierboven genoemde CIA model voor experimentele artritis wordt eveneens gekenmerkt door een preklinische en een klinische fase en mestcellen kunnen in beide fases mogelijk bijdragen aan het ziekteproces. Omdat de eerder genoemde dierexperimenten uitgevoerd zijn in mestcel-deficiënte muizen, is het onmogelijk de bijdrage van mestcellen in de latere fases van artritis te onderscheiden van een initieel effect. Met behulp van een nieuw muismodel, waarin het mogelijk is mestcellen te depleteren, hebben we in **hoofdstuk 3** gekeken naar het effect van mestcel depletie tijdens beide fases van artritis. Het verwijderen van mestcellen in de preklinische fase van artritis heeft een positief effect op de uitkomst van RA, terwijl het verwijderen van mestcellen in de klinische fase van artritis geen significant effect heeft op de artritis. Mestcel depletie in de initiële fase van RA leidt tot een lagere klinische score van artritis, wat overeenkomt met een verminderde schade in de gewrichten van de enkels. Immunologische analyse van de dieren laat zien dat afwezigheid van mestcellen samenvalt met een kleinere populatie pathogene Th17 cellen en een grotere populatie immuun-dempende, regulatoire T cellen. In het plasma is de concentratie van de inflammatoire interleukines (IL)-6 en IL-17 verlaagd in vergelijking tot mestcel competente dieren. In dit hoofdstuk hebben wij laten zien dat de rol van mestcellen in de klinische fase van CIA gelimiteerd is, maar dat mestcellen in de preklinische fase een bijdrage leveren aan de ontwikkeling van de artritis via hun effect of de pathogene, adaptieve immuunrespons.

Circulerende immunoglobulines in cardiovasculaire patiënten en associaties met klinische parameters

Naast de chronische ontsteking van gewrichten hebben RA patiënten ook vaak systemische klachten zoals algehele malaise, koorts, Sjögren syndroom en aanwezigheid van zogenaamde reumatische noduli: knobbels ontstekingsweefsel, die vaak in de buurt van de gewrichten te vinden zijn. Het meest karakteristieke extra-articulaire effect in het ziekteverloop van RA is een tot op heden niet begrepen verhoogd risico op cardiovasculair lijden. Onderzoek heeft aangetoond, dat RA patiënten een verhoogd risico hebben voortijdig te sterven aan een hartaanval of beroerte. Atherosclerose is vaak de onderliggende pathologie van deze cardiovasculaire syndromen. Het proces wordt gekenmerkt door een lipiden-gemedieerde inflammatie van de vaatwand op plekken met een hoge 'shear stress'. De ontsteking zorgt voor de ophoping van vetten en ontstekingscellen in de vaatwand, de atherosclerotische plaque of laesie genoemd, wat resulteert in een lokale vernauwing. Veel kenmerkende immunologische aspecten van RA komen overeen met het proces van atherosclerose, zoals de ontwikkeling van auto-antilichamen gericht tegen gemodificeerde endogene eiwitten. De meerderheid van RA patiënten ontwikkelt een sterke antilichaamrespons tegen bijvoorbeeld gecitrullineerde eiwitten. Klinisch onderzoek heeft aangetoond dat er een relatie bestaat tussen de aanwezigheid van reuma specifieke antistoffen zoals reumafactor of "anti-citrullinated protein" antilichamen (ACPA) en de ziekte uitkomst en verloop in RA patiënten. Met name ACPA is in de kliniek een belangrijke klinische biomarker voor de effectiviteit van de behandeling, het bereiken van remissie en verhoogde mortaliteit. Recentelijk is aangetoond dat gecitrullineerde eiwitten ook aanwezig zijn in het myocardium en in de atherosclerotische plaque van zowel RA als niet-RA patiënten, en dat ACPA mogelijk pathogene effecten kan bewerkstelligen buiten het gewricht. In **hoofdstuk 4** beschrijven wij dat ACPA gedetecteerd kan worden in het serum van een deel van een cohort cardiovasculaire patiënten in afwezigheid van reumatische klachten. Met behulp van een test voor de detectie van ACPA door middel van cyclic citrullinated peptides (CCP3) vonden wij reactiviteit tegen dit peptide in 6 – 11% van de patiënten. Klinische analyses lieten echter geen correlatie zien tussen bekende cardiovasculaire risicofactoren zoals het lipidenprofiel, BMI of inflammatoire markers en ACPA-positieve cardiovasculaire patiënten. Wel vonden wij een associatie tussen ACPA positiviteit en lange-termijn mortaliteit over 10 jaar na inclusie. De aanwezigheid van ACPA correleerde significant met sterfte. In deze studie laten wij zien dat cardiovasculaire patiënten zonder reumatische (voor)geschiedenis een antistof reactie kunnen ontwikkelen tegen gecitrullineerde eiwitten, die bijdraagt aan voortijdige sterfte.

Circulerende antistoffen kunnen een negatieve invloed hebben op de ontsteking in een gewricht of vaatwand door antigenen te binden en zo immuuncomplexen te vormen. Deze immuuncomplexen binden vervolgens aan zogenaamde Fc-receptoren op ontstekingscellen. Na binding van een immuuncomplex aan deze Fc-receptoren volgt de activatie van de cel, en zal de ontsteking verder worden versterkt door de secretie van signaaleiwitten door de ontstekingscel. Ook mestcellen brengen deze receptoren tot expressie, en binding van een ligand aan deze receptoren vormt ook een zeer potente manier van mestcel activatie. Eerder werk heeft aangetoond dat het aantal mestcellen in een atherosclerotische plaque voorspellend is voor toekomstig cardiovasculair lijden. In **hoofdstuk 5** onderzochten wij of in patiënten met hart en vaatziekten circulerende immunoglobulines correleerden met het fenotype van de plaque, zoals de grootte van de necrotische kern, de mate van calcificatie, het aantal macrofagen en spiercellen, hoeveelheid collageen en het aantal bloedvaatjes. Verder hebben we de antilichaam niveaus gekoppeld aan klinische kenmerken van de patiënt, zoals body mass index (BMI), lipidenprofiel, wel of niet roker, bloeddruk en de mate van cardiovasculair lijden (symptomatisch of asymptomatisch). Wij vonden geen associatie tussen de types immunoglobulines (IgG, IgE en oxLDL-IgG) en genoemde parameters. Mogelijk zijn andere liganden verantwoordelijk voor de activatie van mestcellen in de atherosclerotische plaque, of verschilt de lokale concentratie van immunoglobulines met de circulerende concentratie.

Mestcellen in experimentele atherosclerose

De vorming van atherosclerotische plaques begint al op adolescente leeftijd en ontwikkelt zich asymptomatisch gedurende een groot deel van het leven. Het immuunsysteem speelt in de fase van de groei van de atherosclerotische plaque een belangrijke rol en draagt bij aan de destabilisatie van de plaque. De destabilisatie van de laesie als gevolg van vermindering van de hoeveelheid extracellulaire matrix en celdood is een belangrijk proces, dat vaak een scheuring van de plaque (ruptuur) tot gevolg heeft. Door een ruptuur komt de inhoud van de plaque in de bloedbaan, wat kan leiden tot de vorming van een stolsel, dat ischemie veroorzaakt in het onderliggende weefsel, en kan leiden tot een beroerte of hartaanval. De huidige therapie voor cardiovasculaire patiënten is gebaseerd op het verlagen van het LDL-cholesterol door het gebruik van cholesterolverlagende geneesmiddelen (statines) en door het operatief verwijderen van plaques. Omdat het immuunsysteem een significante rol speelt in het proces van atherogenese, kan de modulatie van de immuunresponse nieuwe therapeutische aangrijpingspunten opleveren. Naast leukocyten zoals macrofagen, zijn ook mestcellen waargenomen in de atherosclerotische laesies, en de hoogste aantallen mestcellen zijn gevonden in instabiele en geruptureerde plaques. Ook mestcel specifieke mediatoren zijn verhoogd aanwezig in het serum van cardiovasculaire patiënten.

Dierexperimenteel onderzoek heeft aangetoond dat systemische activatie van mestcellen resulteert in plaquegroei en destabilisatie. Muizen, die deficiënt zijn voor mestcellen, ontwikkelen kleinere plaques in vergelijking met mestcel competente muizen. Deze studies hebben aangetoond dat mestcellen in de vroege fase van atherosclerose een belangrijke rol spelen. In **hoofdstuk 6** karakteriseerden wij een nieuw muismodel, waarin het mogelijk is mestcellen te verwijderen of depleteren op elk gewenst moment door het toedienen van het eiwit difterie toxine. Dit muismodel is gekruist met een muis, die atherosclerose ontwikkelt, waardoor het mogelijk is om mestcellen te depleteren in latere fases van atherosclerose. In deze studie hebben wij na depletie van mestcellen atherosclerose geïnduceerd door het voeren van een hoog vet dieet en het operatief plaatsen van een collar om de halsslagaders. Analyse van de plaques liet zien dat depletie van mestcellen leidde tot kleinere en stabielere laesies. In het bloed vonden we een daling van het aantal inflammatoire monocyten, en een reductie van de pro-inflammatoire eiwitten IL-6 en MCP-1 in mestcel gedepleteerde muizen. Deze data sluiten goed aan bij eerdere studies in mestcel deficiënte muizen, waar ook kleinere, stabielere laesies werden gevonden. Het voordeel van het huidige muismodel is dat wij nu in staat zijn om de rol van mestcellen in latere fases van atherosclerose te onderzoeken.

Mestcellen kunnen door de uitscheiding van mediatoren zoals histamine, chymase, tryptase en cytokines ook bijdragen aan de progressie van atherosclerose. Deze mediatoren beïnvloeden verschillende processen en induceren bijvoorbeeld de dood van spiercellen en de afbraak van extracellulaire matrix zoals collageen, wat kan resulteren in plaque destabilisatie. De studie beschreven in **hoofdstuk 7** laat het effect zien van mestcel depletie op reeds ontwikkelde atherosclerotische plaques. Analyse van de atherosclerotische plaques toonde aan dat depletie van de mestcellen niet de grootte maar wel de stabiliteit van de laesie gunstig beïnvloedde. De plaquestabiliteit was significant verbeterd: een verhoogd collageengehalte, een kleinere necrotische kern en minder macrofagen in mestcel gedepleteerde muizen ten opzichte van competente muizen. Mestcel depletie leidde tot een toename in het aantal regulatoire T cellen in de milt en drainerende lymfeknopen van het hart. In het plasma van mestcel gedepleteerde muizen vonden we een significante daling in het niveau van het pro-atherogene cytokine IL-6 en een toename van IL-10. Samenvattend laat deze studie zien dat mestcellen in belangrijke mate bijdragen aan de destabilisatie van de plaque, maar ook direct of indirect bijdragen aan de inductie van regulatoire T cellen.

Concluderend, dit proefschrift laat zien dat mestcellen in experimentele modellen voor reumatoïde artritis en atherosclerose significant bijdragen aan de pathogenese van deze ziektes. De depletie van mestcellen heeft zowel op lokaal als systemisch niveau een positief effect op het ziekteproces. Daarnaast hebben wij aangetoond dat bepaalde circulerende antilichamen voorspellend zijn voor het voortijdige sterfte aan hart- en vaatziekten. Deze studies suggereren dat de remming van mestcelactivatie in chronische immuun-gemedieerde ziektes wellicht een nieuw therapeutische aangrijpingspunt kan zijn.

Curriculum Vitae

Daniël van der Velden werd op 20 juni 1984 geboren te Utrecht. In 2004 begon hij de studie Chemistry en Life Sciences aan de Hogeschool Utrecht, waar hij in 2005 zijn propedeuse behaalde. Tijdens de opleiding liep hij stage bij verschillende instellingen en bedrijven, waaronder de vakgroep Cellular Protein Chemistry van de Universiteit Utrecht onder leiding van prof. dr. Ineke Braakman. Na een eindstage bij Crucell Holland B.V. onder begeleiding van dr. David Zuijdgeest, behaalde hij het ingenieursdiploma in juni 2008.

In september 2008 begon hij aan de masteropleiding Biofarmaceutische Wetenschappen aan de Universiteit Leiden. Hij heeft stages gelopen bij de vakgroep Drug Delivery Technology onder begeleiding van dr. Myrra G. Carstens bij het Leiden Academic Centre for Drug Research (LACDR) in Leiden en de afdeling Immunologie onder begeleiding van dr. Junda M. Kel en dr. Mathilde J.H. Girard-Madoux in het Erasmus MC in Rotterdam. Hij behaalde zijn masterdiploma in november 2011, waarna hij bij de afdeling Biofarmacie van het LACDR en de afdeling Reumatologie van het LUMC begonnen is aan zijn promotieonderzoek beschreven in dit proefschrift, onder leiding van prof. dr. Johan Kuiper, prof. dr. René E.M. Toes en dr. Ilze Bot.

Momenteel werkt Daniël als docent Life Sciences bij het Instute of Life Sciences and Chemistry van de Hogeschool Utrecht.

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