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

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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

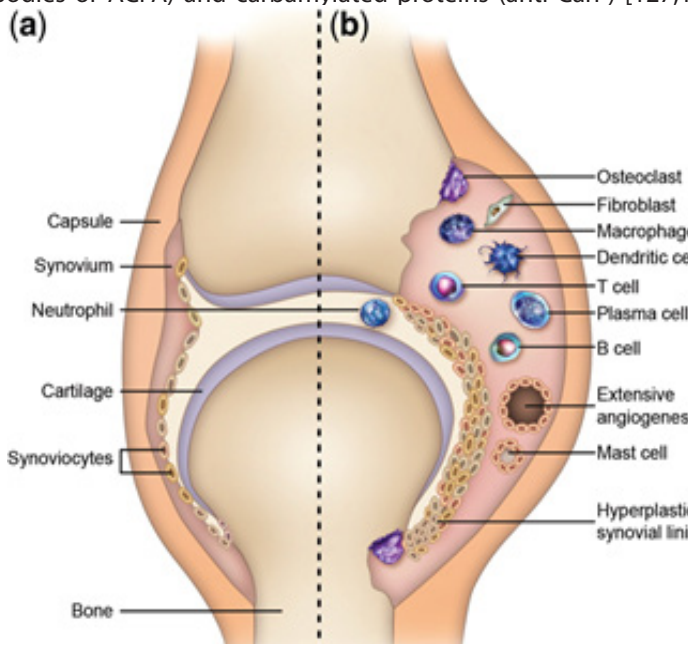

Figure 1. (A) A healthy joint is composed of two adjacent bony ends each covered with a layer of cartilage, separated by a joint space and surrounded by the synovial membrane and joint capsule. (B) Hallmark of Rheumatoid Arthritis (RA) is the inflammatory response of the synovial membrane that is characterized 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

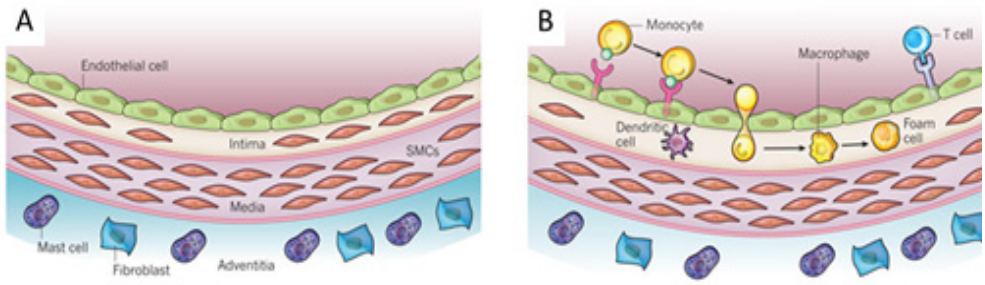

Figure 2. (A) A healthy artery is composed of multiple layers, which are from inner to outer layer the endothelial, intima, media and adventitia. (B) Increased endothelial permeability enables LDL to cross into the vessel wall where it is quickly modified into immunogenic oxidized LDL. Furthermore, endothelial 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).

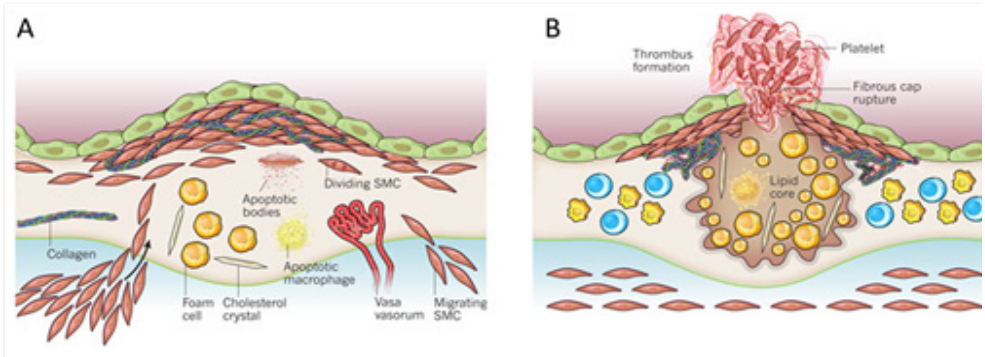

Figure 3. Both foam cell formation and smooth muscle proliferation cause a thickening of the vessel and the formation of a fibrous cap that covers a necrotic core (A). As the plaque enlarges, it causes narrowing of the lumen but also thinning of the fibrous cap. Finally, the plaque ruptures, which can 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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