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.

References

1. Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. The Lancet. 2010;376:1094–108.
2. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat. Immunol. 2011;12:204–12.
3. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CMM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. Annu. Rev. Immunol. 2005;23:749–86.
4. Nigrovic PA, Lee DM. Synovial mast cells: role in acute and chronic arthritis. Immunol. Rev. 2007;217:19–37.
5. Crisp AJ, Chapman CM, Kirkham SE, Schiller AL, Krane SM. Articular mastocytosis in rheumatoid arthritis. Arthritis Rheum. 1984;27:845–51.
6. Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. Nat. Protoc. 2007;2:1269–75.
7. Dahdah A, Gautier G, Attout T, Fiore F, Lebourdais E, Msallam R, et al. Mast cells aggravate sepsis by inhibiting peritoneal macrophage phagocytosis. J. Clin. Invest. 2014;124:4577–89.
8. Willemze A, Trouw LA, Toes REM, Huizinga TWJ. The influence of ACPA status and characteristics on the course of RA. Nat. Rev. Rheumatol. 2012;8:144–52.
9. Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ. B Cells and Humoral Immunity in Atherosclerosis. Circ.

Res. 2014;114:1743–56.

10. van der Helm-van Mil AHM, Verpoort KN, Breedveld FC, Toes REM, Huizinga TWJ. Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. Arthritis Res. Ther. 2005;7:R949–58.
11. Vossenaar ER, Zendman AJW, van Venrooij WJ, Pruijn GJM. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. BioEssays. 2003;25:1106–18.
12. Giles JT, Fert-Bober J, Park JK, Bingham CO, Andrade F, Fox-Talbot K, et al. Myocardial citrullination in rheumatoid arthritis: a correlative histopathologic study. Arthritis Res. Ther. 2012;14:R39.
13. Fert-Bober J, Giles JT, Holewinski RJ, Kirk JA, Uhrigshardt H, Crowgey EL, et al. Citrullination of myofilament proteins in heart failure. Cardiovasc. Res. 2015;cvv185.
14. Sokolove J, Sharpe O, Brennan M, Lahey LJ, Kao AH, Krishnan E, et al. Citrullination within the atherosclerotic plaque: A potential target for the anti-citrullinated protein antibody response in rheumatoid arthritis. Arthritis Rheum. 2013;65:1719–24.
15. Willems S, Vink A, Bot I, Quax PHA, de Borst GJ, de Vries J-PPM, et al. Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. Eur. Heart J. 2013;34:3699–706.
16. Jönsson F, Daëron M. Mast cells and company. Front. Immunol. 2012;3:16.
17. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat. Rev. Immunol. 2014;14:478–94.
18. Kovanen PT. Mast cells: multipotent local effector cells in atherothrombosis. Immunol. Rev. 2007;217:105–22.
19. Heikkilä HM, Lätti S, Leskinen MJ, Hakala JK, Kovanen PT, Lindstedt KA. Activated mast cells induce endothelial cell apoptosis by a combined action of chymase and tumor necrosis factor-alpha. Arterioscler. Thromb. Vasc. Biol. 2008;28:309–14.
20. Sun J, Sukhova GK, Wolters PJ, Yang M, Kitamoto S, Libby P, et al. Mast cells promote atherosclerosis by releasing proinflammatory cytokines. Nat. Med. 2007;13:719–24.
21. Van Doornum S, McColl G, Wicks IP. Accelerated atherosclerosis: An extraarticular feature of rheumatoid arthritis? Arthritis Rheum. 2002;46:862–73.
22. Giles JT, Post WS, Blumenthal RS, Polak J, Petri M, Gelber AC, et al. Longitudinal predictors of progression of carotid atherosclerosis in rheumatoid arthritis. Arthritis Rheum. 2011;63:3216–25.
23. Semb AG, Rollefstad S, Provan SA, Kvien TK, Stranden E, Olsen IC, et al. Carotid plaque characteristics and disease activity in rheumatoid arthritis. J. Rheumatol. 2013;40:359–68.
24. Mäki-Petäjä KM, Hall FC, Booth AD, Wallace SML, Yasmin, Bearcroft PWP, et al. Rheumatoid Arthritis Is Associated With Increased Aortic Pulse-Wave Velocity, Which Is Reduced by Anti–Tumor Necrosis Factor-α Therapy. Circulation. 2006;114:1185–92.
25. Westlake SL, Colebatch AN, Baird J, Kiely P, Quinn M, Choy E, et al. The effect of methotrexate on cardiovascular disease in patients with rheumatoid arthritis: a systematic literature review. Rheumatol. Oxf. Engl. 2010;49:295–307.
26. Micha R, Imamura F, Wyler von Ballmoos M, Solomon DH, Hernán MA, Ridker PM, et al. Systematic review and meta-analysis of methotrexate use and risk of cardiovascular disease. Am. J. Cardiol. 2011;108:1362–70.

27. Daïen CI, Duny Y, Barnetche T, Daurès J-P, Combe B, Morel J. Effect of TNF inhibitors on lipid profile in rheumatoid arthritis: a systematic review with meta-analysis. Ann. Rheum. Dis. 2012;71:862–8.
28. van Sijl AM, Peters MJL, Knol DL, de Vet RHC, Sattar N, Dijkmans BAC, et al. The effect of TNF-alpha blocking therapy on lipid levels in rheumatoid arthritis: a meta-analysis. Semin. Arthritis Rheum. 2011;41:393–400.
29. Barnabe C, Martin B-J, Ghali WA. Systematic review and meta-analysis: anti-tumor necrosis factor α therapy and cardiovascular events in rheumatoid arthritis. Arthritis Care Res. 2011;63:522–9.
30. Choy E, Ganeshalingam K, Semb AG, Szekanecz Z, Nurmohamed M. Cardiovascular risk in rheumatoid arthritis: recent advances in the understanding of the pivotal role of inflammation, risk predictors and the impact of treatment. Rheumatology. 2014;53:2143–54.
31. Toms TE, Panoulas VF, Douglas KMJ, Griffiths H, Sattar N, Smith JP, et al. Statin use in rheumatoid arthritis in relation to actual cardiovascular risk: evidence for substantial undertreatment of lipid-associated cardiovascular risk? Ann. Rheum. Dis. 2010;69:683–8.
32. Hahn BH, Grossman J, Chen W, McMahon M. The pathogenesis of atherosclerosis in autoimmune rheumatic diseases: Roles of inflammation and dyslipidemia. J. Autoimmun. 2007;28:69–75.
33. Danninger K, Hoppe UC, Pieringer H. Do statins reduce the cardiovascular risk in patients with rheumatoid arthritis? Int. J. Rheum. Dis. 2014;17:606–11.
34. McCarey DW, McInnes IB, Madhok R, Hampson R, Scherbakova O, Ford I, et al. Trial of Atorvastatin in Rheumatoid Arthritis (TARA): double-blind, randomised placebo-controlled trial. The Lancet. 2004;363:2015–21.
35. Schoenfeld SR, Lu L, Rai SK, Seeger JD, Zhang Y, Choi HK. Statin use and mortality in rheumatoid arthritis: a general population-based cohort study. Ann. Rheum. Dis. 2015;annrheumdis – 2015–207714.
36. Kneilling M, Hültner L, Pichler BJ, Mailhammer R, Morawietz L, Solomon S, et al. Targeted mast cell silencing protects against joint destruction and angiogenesis in experimental arthritis in mice. Arthritis Rheum. 2007;56:1806–16.
37. Kobayashi Y, Shirahase H, Zhao HF, Kakizoe E, Okunishi H. [Effects of orally available prodrug of cromoglycic acid on collagen-induced arthritis mice]. Nihon Yakurigaku Zasshi Folia Pharmacol. Jpn. 1999;114 Suppl 1:154P – 158P.
38. Shi G. Treatment and prevention of cardiovascular disease using mast cell stabilizers [Internet]. 2008 [cited 2015 Nov 25]. Available from: http://www.google.com/patents/US20080027111
39. Ridker PM, Danielson E, Fonseca FAH, Genest J, Gotto AM, Kastelein JJP, et al. Rosuvastatin to Prevent Vascular Events in Men and Women with Elevated C-Reactive Protein. N. Engl. J. Med. 2008;359:2195–207.
40. Ridker PM, Thuren T, Zalewski A, Libby P. Interleukin-1β inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). Am. Heart J. 2011;162:597–605.
41. Dhimolea E. Canakinumab. mAbs. 2010;2:3–13.