Ageing of innate immunity in health and vasculitic diseases

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CHAPTER 7

Summary, discussion and future perspectives

SUMMARY OF FINDINGS IN THIS THESIS

Age-related alterations of the immune system may contribute to the development of autoimmune diseases, including anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AAV), giant cell arteritis (GCA) and polymyalgia rheumatica (PMR). This thesis aimed to explore phenotypical and functional changes in the innate arm of the immune system during the ageing process in healthy individuals as well as in ageing-related systemic vasculitides such as GCA/PMR and in an experimental mouse model of MPO-ANCA-associated vasculitis. In **chapter 2**, the effect of age and inflammageing on the severity of disease in the anti-MPO IgG/LPS induced glomerulonephritis model was investigated. We found that increased production of pro-inflammatory cytokines in conjunction with increased numbers of activated innate immune cells and ageing-specific changes in the kidney aggravated severity of anti-MPO IgG/LPS mediated glomerulonephritis. Next, we studied changes in innate immunity in the process of ageing in a cross-sectional study comparing healthy young and elderly individuals. More specifically, in **chapter 3**, we investigated whether ageing affects the expression of pattern recognition receptors (PRRs) and cytokine responses to a set of well-defined PRR ligands. We found that most PRR-mediated responses were similar when comparing young and old individuals. Interestingly, we revealed an age-dependent reduction of AIM2 expression and activation which was associated with reduced cytokine responses to the cytosolic DNA mimic poly(dA:dT) in healthy elderly individuals which may negatively impact responses to double-stranded DNA viruses with age. In **chapter 4**, we studied the effects of ageing on monocyte subsets distribution and their cytokine (pro- and anti-inflammatory) responses to defined TLR ligands. We found that levels of CD16+ monocytes (both intermediate and non-classical monocytes) increased with age and produced more pro-inflammatory cytokines. In **chapter 5** and **6**, we further investigated the role of these CD16+ monocytes in GCA and PMR patients. In **chapter 5**, we revealed an altered distribution of monocyte subsets in GCA and PMR patients. Also, in temporal arteries of GCA patients, we found that the majority of the macrophages in transmural infiltrates expressed CD16 and CX3CR1, but lacked CCR2, thereby resembling the phenotype of the non-classical monocytes in the blood. Our findings suggest that non-classical monocytes may thus be the precursors of tissue macrophages in GCA guided by the CX3CR1/CX3CL1 chemokine axis. In **chapter 6**, we reported that PMR patients with low CD16+ monocyte counts are more prone to relapse. This finding may support the hypothesis that CD16+ monocytes contribute to inflammation by migrating to PMR tissue.

IMPLICATIONS OF FINDINGS AND MAIN QUESTIONS REMAINING

Why is the anti-MPO model more severe in aged mice?

In chapter 2, we showed that aged mice develop more severe clinical and pathological disease upon induction of anti-MPO IgG/LPS mediated glomerulonephritis. This finding is in accordance with increased AAV severity in aged humans [1-3]. We concluded that this may be attributed to age-related changes of the immune system as well as in the kidney itself. To further dissect the exact contribution of the ageing immune system and/or the ageing of organs, more studies are required. For instance, transplanting hematopoetic stem cells (HSC from the bone marrow) of old mice to young mice, and vice versa, followed by induction of anti-MPO/LPS mediated glomerulonephritis could reveal if aged HSC or the senescent microenvironment are critical contributors to disease severity. Likewise, by transplanting kidneys from old to young mice, and vice versa, the influence of the ageing kidney to the severity of the disease could be determined. In addition, we demonstrated a reduction in the expression of the inflammation/senescence related biomarker klotho in the anti-MPO IgG/LPS mediated glomerulonephritis model, which was more pronounced in aged mice. These observations warrant further studies into the role of klotho in AAV. A correct understanding of the regulatory role of klotho may lead to the design of novel therapeutic approaches to dampen inflammatory and ageing features of AAV and may provide more insight into the inflammation/ageing complex in general. Improved knowledge of the mechanisms that aggravate disease in AAV may inspire further research for treatment alternatives.

Ageing is associated with low-grade systemic inflammation as evidenced by increased plasma TNF-α and IL-6 levels, and this may create a pro-inflammatory environment (inflammageing) that might accelerate the development of autoimmune diseases [4-6]. However, this notion was not verified in this experimental mouse model as we did not detect elevation of these cytokines at baseline. This may be due to detection limits of the cytokine assays and should be re-examined when more sensitive assays become available. However, at baseline, counts of white blood cells and neutrophils were higher in aged mice compared to young mice. Thus, changes in immune cell composition and distribution may be regarded as a pre-existing pro-inflammatory environment contributing to higher sensitivity to autoimmune disease development. Of note, ageing has also been shown to exacerbate cytokine production in response to an acute inflammatory insult [7, 8]. Indeed, we observed increased pro-inflammatory cytokine expression both at the systemic and renal level in aged mice after disease induction. This may be due to elevated activation of monocytes and macrophages in aged mice compared to young mice. The triggers

involved in induction of age-related changes in the immune system are likely shaped by both endogenous and environmental stimuli (e.g. microbiome) sensed by PRRs [9].

Does ageing per se affect PRR responses ?

In **chapter 3**, we investigated age-related differences in whole blood cell cytokine responses to a broad range of PRR agonists engaging different classes of PRRs (TLRs, CLRs, NLRs, RLRs and AIM2). Ageing-dependent alterations of PRR expression and functions are controversial and discrepant results have been reported in the literature [10, 11]. It is likely that several covariates may have impacted the study results. Differences in experimental protocols, such as cell-enrichment methods, origin and dose of stimuli, cytokine assays (ELISA or flow cytometry), as well as characteristics of participants (e.g. gender, race, microbiome and health status) can possibly contribute to these divergent results. In our study we chose to investigate whole-blood cytokine responses as this is a relatively simple assay which closely resembles the natural physiological environment, and thereby preventing undue stimulation of cells through extra manipulation. Our data revealed that healthy ageing *per se* is not necessarily associated with remodeling of innate immune responses as the majority of PRR ligands tested in this study yielded a similar response in young and old healthy participants. The appreciation of whole blood cell assays for immune monitoring with minimal sample handling has grown. Also, the standardization of these whole blood assays for defining human variability in immune responses has high priority. Recently, Duffy et al developed a string of whole-blood, syringe-based assay systems for reproducibly assessing human innate and/or adaptive immune responses to complex stimuli. These novel, whole blood assays were designed to eliminate pre-analytical errors, such as risk of contamination (e.g. endotoxin) and effects of sample handling [12-14]. We fully support this development and plea for more standardized methods to evaluate comprehensively PRR responses in humans and to assess the boundaries of natural variability. Once established, standardized protocols should be disseminated for wider use, to assess more precisely ageing-associated effects on immune responses.

Nevertheless, the major finding of **chapter 3** is an age-dependent reduction of AIM2 expression and activation which may explain reduced cytokine responses to the cytosolic DNA mimic poly(dA:dT) in healthy elderly individuals. It remains to be established if reduced AIM2 responses may account for reduced vaccination efficacy in elderly individuals. To this end, dedicated trials investigating elderly with high and low AIM2 expression for (double stranded DNA) virus vaccine efficacy are required.

Is there a role for aged monocytes in the pathogenesis of GCA/PMR?

Although macrophages are critical to vascular inflammation in GCA, scarce data is available on (local) macrophage heterogeneity and their precursors. As both GCA/PMR are ageing-associated disorders, we hypothesized that CD16+ monocytes,

which increase during ageing, play a role in the immunopathogenesis of GCA/PMR. CD16+ monocytes (both intermediate and non-classical monocytes) are the more mature cells compared to the classical monocytes [15], and display inflammatory characteristics by producing pro-inflammatory cytokines upon activation [16]. In **chapter 4**, we did find higher production of cytokines by CD16+ monocytes and confirmed earlier findings that numbers of these CD16+ monocytes increase with ageing [17]. In **chapter 5**, we revealed an altered distribution of monocyte subsets in GCA and PMR patients. Increased proportions of intermediate and non-classical monocytes have been documented in many inflammatory diseases [16, 18], which is in line with their pro-inflammatory profile. In contrast, our study in newly-diagnosed GCA and PMR patients showed a remarkable, proportional decrease of non-classical monocytes. This may be explained by 1) enhanced apoptosis, 2) blunted differentiation towards non-classical monocytes, or 3) selective migration to the (inflamed) tissues.

We speculate that the third option, that non-classical monocytes migrate to the sites of inflammation in GCA and PMR, is most likely as we identified transmural macrophage accumulation in GCA temporal artery lesions resembling the phenotype of the non-classical monocytes in the blood ($CD16^{+}CX3CR1^{+}CCR2^{-}$). However, there is no solid kinetic evidence for this statement yet. To further verify this, ex vivo cultured arteries from patients with giant cell arteritis in an autologous co-culture with different monocyte subsets should be investigated [19]. Also, in vivo animal studies applying intravital two-photon and epifluorescence microscopy may provide visualization of the monocytes subsets crawling along and adhering to the vessel wall [20]. Further investigation of these findings in a relevant animal model would be required. Unfortunately, there is no established experimental animal model available for GCA/PMR.

We demonstrated that strong local expression of CX3CL1 in the GCA temporal artery is consistent with massive expression of CX3CR1, suggesting that tissue migration by non-classical monocytes is guided by the CX3CR1-CX3CL1 chemokine axis in GCA. Indeed, previous studies also demonstrated that non-classical monocytes showed an increased capacity to adhere to endothelial cells by virtue of the adhesion-related CX3CR1 which binds to the membrane-bound form of fractalkine (CX3CL1) expressed by endothelial cells [21, 22]. A number of different cell types, for instance endothelial cells and smooth muscle cells, can produce chemokines [23]. Studies by the group of Maria Cid [19] demonstrated that production of chemokines by vessel smooth muscle cells (VSMCs) facilitates recruitment of macrophages, as well as the capacity of VSMCs for producing CX3CL1 [24], highlighting the important role of VSMCs in recruitment and activation of macrophages. The exact mechanisms triggering the migration of CD16+ monocytes are complicated and need further study. Furthermore, as we found that CD16+

macrophages accumulated in the inflamed temporal artery tissue of GCA patients, further studies are required to establish whether these cells are tissue destructive, which signals regulate their functional commitment, how long they survive, and how they contribute to the different phases and perpetuation of the disease.

How do we picture the role of aged monocytes in GCA/PMR pathogenesis?

Thus, on the basis of previous and our own findings we propose a role for non-classical monocytes in the pathogenesis of GCA vascular damage (Figure 1). Non-classical monocytes may enter the vascular wall via the vasa vasorum driven by a gradient of chemokines (via the CX3CR1-membrane bound CX3CL1) and by endothelial adhesion. Non-classical monocytes differentiate into macrophages in the vascular tissue promoting inflammation via secretion of pro-inflammatory cytokines and promoting vascular damage by secretion of matrix metalloproteinases (MMPs) [25]. Dendritic cells are able to recruit T cells and to skew these towards Th1 and Th17 cells [26]. Conversely, Th1 cells producing IFN-γ can facilitate the recruitment of monocytes/macrophages [27]. If and how tissue macrophages contribute to the skewing of Th1 and Th17 cells still needs to be established.

Aged monocytes as a predictor of relapse in GCA/PMR?

In **chapter 6**, we assessed the utility of CD16 monocyte counts as a potential biomarker for disease relapse in PMR. We found low CD16+ monocytes counts to predict time to relapse in PMR. Clearly, these results require validation in other, independent cohorts. Interestingly, low CD16+ monocyte counts were not predictive of a relapse in GCA patients, suggesting differences in GCA and PMR pathogenesis. The pathogenesis of PMR in the inflamed tissue may be more dependent on CD16+ monocytes migrating to the tissue. As there is scarce knowledge on the cells in the inflamed synovium of PMR patients, ultrasound guided biopsies for synovial tissue sampling in PMR patients may help to understand the immune phenotype at the site of inflammation. In addition, positron emission tomography (PET) imageing using [18F] fleorodeoxyglucose (FDG) as well as ultrasmall superparamagnetic particles of iron oxide (USPIO) has been applied to identify macrophages in the tissue [28, 29]. Therefore, application of these imaging modalities may be useful to further pinpoint the role of CD16+ monocytes/macrophages in PMR.

After establishing the pathogenic role of CD16+ monocytes/macrophage in GCA/PMR, specific therapies targeting these monocytes may be investigated for clinical benefit in GCA and PMR. CD16+ monocyte/macrophage–targeted interventions may be directed at reducing monocyte/macrophage recruitment/retention and suppressing their proinflammatory capabilities [25].

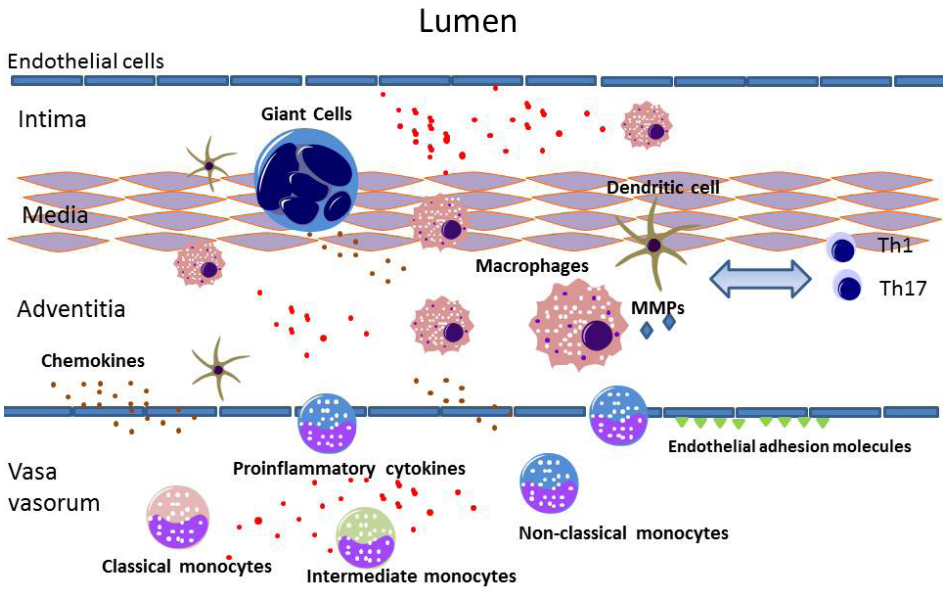

Figure 1. Schematic representation of monocytes/macrophage involvement in the pathogenesis of giant cell arteritis. Monocytes (most likely non-classical monocytes) enter the vascular wall from vasa vasorum driven by the attraction of chemokines and endothelial adhesion molecules. Monocytes differentiate into macrophages in the vascular tissue promoting inflammation of the vascular wall via secretion of proinflammatory cytokines and promoting vascular damage by secretion of matrix metalloproteinases (MMPs). Dendritic cells are activated and release chemokines that recruit Th1 and Th17 cells and macrophages. Macrophages may promote expansion of Th17 cells and conversely, Th1 cells producing IFN-γ can facilitate the recruitment of macrophages. If and how macrophages contribute to the skewing of Th1 and Th17 cells still needs to be established.
● Chemokines; ♦ MMPs; ● Proinflammatory cytokines; ▼ Endothelial adhesion molecules.

CONCLUSION

Understanding alterations in the innate arm of the immune system during the ageing process may help to understand the development of ageing-associated autoimmune diseases. This thesis described how certain aspects of innate immunity are modulated with age and may contribute to the pathogenesis of ageing-related vasculitis. In particularly, the role of CD16+ monocytes, which increase with age, in GCA and PMR has been implicated. More insight into the mechanisms underlying vasculitides which occur more frequently in aged persons is required for designing more rationalized, steroid-sparing, treatment options for patients with GCA/PMR.

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