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Ageing of innate immunity in health and vasculitic diseases

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CHAPTER 1

General introduction
and outline of the thesis

AGEING OF THE IMMUNE SYSTEM

Ageing has profound effects on all cells and tissues of a living organism, including those of the immune system [1]. Age-related alterations of the immune system, termed immunosenescence, have a strong clinical impact and may underlie increased susceptibility to infections, chronic diseases and impaired responses to vaccination in the elderly [2-4]. Ageing applies to all cells of the immune system and thus affects both innate and adaptive immune responses. Immunosenescence may have a profound impact on development and progression of age-associated diseases including specific systemic vasculitides such as anti-neutrophil cytoplasmic autoantibody associated small vessel vasculitis (AAV) and giant cell arteritis (GCA). In this introduction, the main age-related alterations in innate and adaptive immune components and responses will be briefly reviewed. In addition, age-associated vasculitides, AAV and GCA will be introduced.

AGEING AND ADAPTIVE IMMUNITY

Adaptive immune responses decline with age as evidenced by decreased delayed type hypersensitivity responses (e.g. tuberculin skin test) in elderly people. The main characteristic of adaptive immunity is the ability to deal with pathogens/insults in an antigen-specific manner and to develop antigen-specific memory cells to deal more efficiently with pathogens/insults upon recall exposure (second contact with the same antigen). The effects of ageing on the adaptive immune system have been reviewed recently by Frasca et al [5] and are summarized in Figure 1. In brief, ageing of the adaptive immune system is characterized by a reduced output of naive B and T cells and by a proportional increase in memory B and T cells as a result of the growing number of environmental challenges as we age. Due to a reduction in bone marrow output of progenitor cells (common lymphocyte precursors) and thymic involution in young adulthood, the production of naïve B and T cells gradually declines with age whereas the number of antigen-experienced memory and, in particular, effector T cells increases [6, 7]. As a result, the diversity of the TCR repertoire decreases with ageing. Of note, also the effector T cell proliferative capacity becomes more limited, as a result of shortened telomeres. However, aged effector T cells show an increased resistance to programmed cell death [8, 9]. Taken together, reduced diversity and functionality of the T cell repertoire with ageing likely underlies reduced capability to mediate effective immune responses against new antigens in the elderly. Thymic involution also leads to a decreased output of naïve regulatory T cells (Tregs) which may contribute to development of age-related autoimmune diseases [10]. Conversely, numbers of the induced Treg (iTreg) appear to increase with age leading to increased Treg/Teffector ratios which may add to

the reduced effector responses seen with ageing [11]. Similar to the T cell pool, the aged peripheral B cell pool fills up with antigen-experienced memory cells at the expense of a concurrent replacement of naive B cells, which also limits diversity of the B cell repertoire [12]. Moreover, ageing-related failure of negative selection and loss of B cell function will contribute to a decrease of antibody affinity and an increase of autoreactive antibodies [8, 13-15].

AGEING AND INNATE IMMUNITY

Whilst adaptive immune responses decline with age, the basal activity of the innate immune system appears to increase with age. This chronic, low grade inflammation (termed inflamm-ageing) seen with ageing is evidenced by increased serum levels of inflammatory cytokines (e.g. Interleukin (IL)-1β, IL-6, IL-8 and Tumor necrosis factor (TNF-α)) and acute phase proteins. In aged subjects, alterations in production of inflammatory mediators might be caused by pre-existing conditions such as autoimmune or degenerative diseases, cancer, frailty, or other factors. Alternatively, the accumulation of these cytokines and acute phase proteins may create a pro-inflammatory environment that might accelerate the development of autoimmune diseases [1].

Cumulative evidence indicates that ageing exerts significant effects on all cells of the innate immune system [16]. These ageing-associated changes impact both numbers and functions of multiple innate immune cell types as will be briefly discussed in the next paragraphs (Figure 1).

Impact of ageing on monocytes

Monocytes originate from myeloid stem cell progenitors and differentiate into macrophages and dendritic cells (DC). Three main monocyte subsets can be classified by distinct transcriptional and functional characteristics: classical monocytes (CD14^{bright}CD16-), intermediate monocytes (CD14^{bright}CD16+) and non-classical monocytes (CD14^{dim}CD16+) [17] (Figure 2). Classical monocytes form the majority of monocytes in the peripheral blood and display a high antimicrobial capability owing to their potent capacity for phagocytosis and their enhanced production of antimicrobial proteins. CD16+ monocytes (i.e. intermediate and non-classical monocytes) display inflammatory characteristics by producing pro-inflammatory cytokines upon activation and show features of antigen presenting cells [18]. Monocytes can migrate to peripheral tissues to give rise to macrophages and dendritic cells. The migratory properties of monocyte subsets are differentially regulated based on their chemokine receptors expression profiles [19]. Classical monocytes show a markedly higher CCR2 expression but low CX3CR1 expression, whereas non-classical monocytes show high CX3CR1 expression but no CCR2
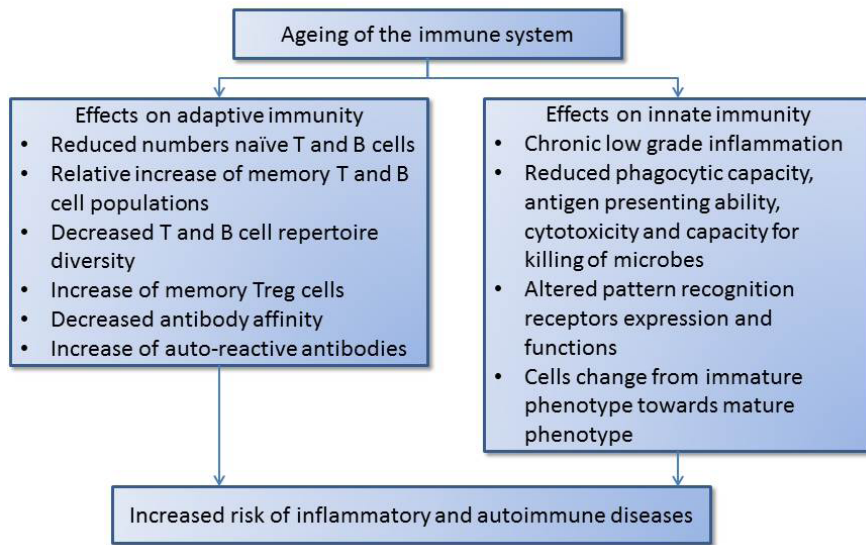
Figure 1. Major changes of the immune system with ageing. Ageing has profound effects on both innate and adaptive arms of the immune system. Treg = T regulatory cell.

expression and intermediate monocytes show low levels of CCR2, but high levels of both CX3CR1 and CCR5. Transcriptional profiling suggests that monocytes expressing CD16 (both intermediate and non-classical subsets) exhibit a phenotype resembling dendritic cells (DCs) and macrophages [20], plus they exhibit an increased capacity to adhere to endothelial cells and thereby more readily migrate across the endothelium [21, 22]. In light of information derived from murine studies, the majority of experts favor a theory for the development of human monocyte subsets whereby classical monocytes leave the bone marrow and enter the peripheral circulation. Moreover, a developmental relationship is assumed for classical monocytes to develop into intermediate monocytes, some of which could finally develop into non-classical monocytes [23].

Absolute numbers of monocytes in elderly individuals were found unchanged compared to young subjects in two studies [24, 25]. Yet, another study with smaller sample sizes reported on increased numbers of monocytes in elderly when compared to young individuals [26]. In line with this notion, increasing age is associated with a shift within monocyte subsets. Proportions and numbers of CD16+ monocytes are increased while proportions of the classical monocytes are decreased in aged subjects [27]. Moreover, non-classical monocytes demonstrate shorter telomeres and express the senescence-associated marker β-galactosidase, suggesting that this subset indeed represents a more aged phenotype [21].

A number of studies confirmed that monocyte function is compromised with age (Figure 2). Firstly, the phagocytic capacity of monocytes and macrophages is impaired with age [28, 29]. Secondly, their ability to process antigens and present peptides to T cells is also hampered by ageing evidenced by decreased levels of Major histocompatibility complex (MHC) class II molecules [30, 31]. Thirdly, spontaneous production of pro-inflammatory cytokines such as IL-6 is increased in the elderly individuals [32]. Finally, the expression and function of pattern recognition receptors (PRR) which recognize pathogen associated molecular
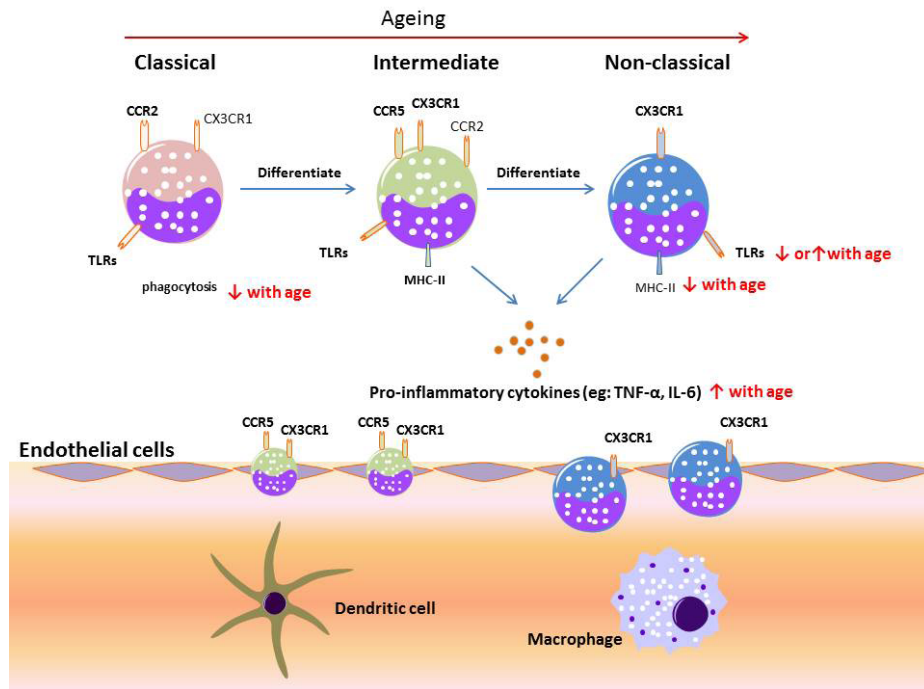

Figure 2. The potential functional roles of monocyte subsets and their alterations upon ageing. Most experts accept that monocytes leave the bone marrow as classical monocytes (CD14^{bright}CD16-), which have high antimicrobial capability owing to their potent capacity for phagocytosis and their enhanced production of antimicrobial proteins. Their phagocytosis capacity is compromised with age. Classical monocytes can differentiate into intermediate (CD14^{bright}CD16+) monocytes and further differentiate into non-classical monocytes (CD14^{dim}CD16+) in the circulation. Numbers of CD16+ monocytes (intermediate and non-classical monocytes) accumulate upon ageing. CD16+ monocytes' antigen presentation capacity is down-modulated evidenced by decreased expression of MHC-II molecules. Toll-like receptors (TLRs) expressions on the monocytes are also modulated by ageing. Moreover, CD16+ monocytes produce high levels of inflammatory mediators (such as TNF and IL-1β) and their production increases with age. Chemokine receptors CX3CR1 mediate adhesion of intermediate and non-classical monocytes to facilitate endothelial migration, renewing the macrophage and dendritic cell pool in the tissue.

patterns (PAMPs) is also altered by ageing. Several studies have analyzed expression of PRR and PRR cytokine responses and how they are affected by age. Most studies mainly focused on analysis of Toll-like receptors (TLRs) expression and cytokine responses. It has been reported that monocyte subsets in the elderly individuals demonstrate a reduced capacity to produce IL-6 and TNF-α upon TLR1/TLR2 stimulation [33]. In addition, IL-6 synthesis in response to a TLR7/8 ligand was also diminished in monocytes from older adults [33]. Conversely, an elevated TLR5 expression and Mitogen-activated protein kinases (MAPK) signaling was reported resulting in higher IL-8 production by monocytes of older adults [34]. These changes in specific TLRs expression levels and responses to specific ligands may underlie impaired responses to specific classes of pathogens contributing to the increased rate of infections in elderly individuals. Alternatively, some pathogens sensed by specific TLRs may elicit higher inflammatory responses in elderly and contribute to inflammageing [35]. Last but not least, the function of monocyte-derived cell types, such as macrophages and DCs are also compromised with age. For instance, dysregulation of TLR3 responses following infection of aged human macrophages with West Nile Virus was reported [36]. Likewise, defects in cytokine production by DC upon stimulation of multiple TLRs in elderly individuals has been observed [37]. Thus, alterations of monocyte function with ageing may well contribute to the imbalance of inflammatory and anti-inflammatory responses in elderly individuals.

Impact of ageing on neutrophils

Neutrophils are the most abundant leukocytes in the peripheral blood compartment. Typically, they are the first immune cells recruited to the site of infection by chemokines and products released from microorganisms [38]. Studies have demonstrated that age does not modify the total number of circulating neutrophils [16]. However, the neutrophil's response to survival components such as GM-CSF in elderly individuals is reduced following infection [39]. Research spanning two decades has shown that many aspects of neutrophil function such as phagocytic capacity, synthesis of reactive oxygen species (ROS), chemotactic activity and migration accuracy are compromised in elderly [40-44]. As a consequence, the efficiency of neutrophils in the removal of microbes is decreased in the elderly individuals. Thus, reduced neutrophil function with ageing affects the first line response to infection in elderly individuals and may inadvertently cause tissue damage [44].

Impact of ageing on natural killer (NK) cells

NK cells are innate cytotoxic lymphocytes that play an important role in host defense against certain malignancies and viral infections [45]. It appears that there is an age-related increase in the overall numbers of circulating NK cells in humans [46, 47]. At the same time, a phenotypical change of NK cells in elderly

individuals from immature CD56^{bright} NK cells toward mature CD56^{dim} NK cells has been reported [48]. This may lead to a decreased production of chemokines in response to IL-2 or IL-12 in NK cells from elderly individuals as chemokines are mainly produced by CD56^{bright} NK cells [49]. Although it was reported that there is an age-associated defect in NK cytotoxicity based on per cell analysis [50], overall NK cell cytotoxicity is largely maintained in healthy elderly individuals which may be due to the increase in total circulating NK cells [42]. As NK cells interact with monocytes promoting inflammation and also induce DC maturation, age-related changes of NK cell function are likely to contribute to dysregulation of other cells as well [51, 52]. Further studies are needed to unravel the consequences of aged NK cell function on other cellular traits and functions in the immune system.

VASCULITIS

Vasculitis is an inflammatory process targeting the vessel wall as the primary site of inflammation. The vasculitis process may affect vessels of any type or any size including capillaries, venules, arterioles, veins and arteries. The most recent classification of systemic vasculitides as proposed by the 2012 International Chapel Hill Consensus Conference is based on the size of vessels involved and specific clinical and pathological features (Table 1) [53]. Notably, some of these vasculitides, such as anti-neutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) and giant cell arteritis (GCA) primarily affect elderly individuals. Evidence has accumulated that abnormalities in adaptive immunity as well as innate immunity play a critical role in initiation and the perpetuation of AAV and GCA.

ANCA–associated vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a life-threatening, small vessel vasculitis that is characterized by the presence of pathogenic autoantibodies against the neutrophil and monocyte lysosomal enzymes proteinase-3 (PR3) or myeloperoxidase (MPO). AAV can manifest in any organ of the body, but most frequently affects the upper and lower respiratory tract and kidneys [54]. AAV is predominantly a disease of the elderly. The incidence of AAV increases with age, peaking in those aged 65 to 74 years, and age is a predictor of disease outcome as older patients usually have a poorer prognosis. Animal models of AAV have provided important insights into the pathogenesis of AAV [55]. Based on clinical, in vitro and in vivo experimental observations, the pathogenesis of acute AAV is thought to involve a series of sequential inflammatory events that eventually cause vessel injury [56]. In brief, ANCAs and pro-inflammatory stimuli, most likely of infectious origin, synergize to cause a destructive inflammatory process [56]. A central event in this process is ANCA-

Table 1. Names of vasculitides adopted by the 2012 International Chapel Hill Consensus Conference on the Nomenclature of Vasculitides [53]

Large vessel vasculitis (LVV)

Takayasu arteritis (TAK)

Giant cell arteritis (GCA)

Medium vessel vasculitis (MVV)

Polyarteritis nodosa (PAN)

Kawasaki disease (KD)

Small vessel vasculitis (SVV)

Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV)

Microscopic polyangiitis (MPA)

Granulomatosis with polyangiitis (Wegener's) (GPA)

Eosinophilic granulomatosis with polyangiitis (Churg-Strauss) (EGPA)

Immune complex SVV

Anti-glomerular basement membrane (anti-GBM) disease

Cryoglobulinemic vasculitis (CV)

IgA vasculitis (Henoch-Schönlein) (IgAV)

Hypocomplementemic urticarial vasculitis (HUV) (anti-C1q vasculitis)

Variable vessel vasculitis (VVV)

Behcet's disease (BD)

Cogan's syndrome (CS)

Single-organ vasculitis (SOV)

Cutaneous leukocytoclastic angiitis

Cutaneous arteritis

Primary central nervous system vasculitis Isolated aortitis

Others

Vasculitis associated with systemic disease

Lupus vasculitis

Rheumatoid vasculitis

Sarcoid vasculitis

Others

Vasculitis associated with probable etiology

Hepatitis C virus–associated cryoglobulinemic vasculitis

Hepatitis B virus–associated vasculitis

Syphilis-associated aortitis

Drug-associated immune complex vasculitis

Drug-associated ANCA-associated vasculitis

Cancer-associated vasculitis

Others

mediated activation of neutrophils [57]. Full-blown ANCA-mediated neutrophil activation is dependent on priming with pro-inflammatory stimuli that causes translocation of ANCA antigens to the cell surface [58]. Following interaction of the F(ab)$_2$ portion of ANCAs with ANCA antigens on the cell surface, and binding of the Fc portion to Fc receptors, neutrophils become activated releasing reactive oxygen species and proteolytic enzymes [59, 60]. Importantly, ANCAs also increase neutrophil adherence to endothelial monolayers [61] and co-incubation of ANCA-activated neutrophils and endothelial cells results in endothelial cell lysis in vitro [62]. In addition to neutrophils, it has been shown that monocytes can be activated by ANCA as well, thereby contributing to an inflammatory environment [63, 64]. It is conceivable that the age-related alterations in immune cell distribution and function, especially inflamm-ageing, may well contribute to the severity of AAV in elderly individuals.

Giant cell arteritis (GCA) and Polymyalgia rheumatica (PMR)

Giant cell arteritis (GCA) is a systemic large vessel vasculitis which predominantly affects large and medium-sized arteries, with a predilection for the cranial branches of the aorta. Due to luminal occlusion, GCA results in a series of classic symptoms, such as headache, blindness, and stroke [65]. Polymyalgia rheumatica (PMR) is a closely related inflammatory disease characterized by systemic inflammation and pain and stiffness of both shoulders and hips [66]. GCA and PMR are known to frequently co-occur, as 50% of GCA patients also have PMR. The immunopathogenesis of GCA and PMR is complex and not yet well understood. Of note, it has been proposed that age-related abnormalities in both innate and adaptive immunity create a state of chronic inflammation and impair immune responses against pathogens, thereby rendering a subset of elderly individuals more vulnerable to GCA and PMR development. Several pathogenetic mechanisms in GCA and PMR may provide clues to this hypothesis. Firstly, ageing may shift the balance of anti-inflammation towards pro-inflammation, as seen by increased levels of the ageing-associated pro-inflammatory cytokine IL-6 in GCA and PMR [67]. Secondly, ageing may affect DC migration [68]. Indeed, misdirected DC populations in GCA lesions were found to display abnormal chemokine receptor expressions [69]. Thirdly, aged (CD16+ monocytes) monocytes may contribute to the Th1 and Th17 bias [70] as these T helper cells are expanded both in the GCA artery lesion and GCA peripheral blood [71, 72]. Fourthly, aged monocytes may be precursors of tissue destructive macrophages in vascular lesions in GCA. Lastly, dysfunction of monocytes during ageing such as decreased TLR7 responses [33] are also found in active GCA/PMR patients [73]. Further studies are needed to precisely define how immunosenescence may contribute to GCA and PMR development in the aged population.

Clinical challenges in treatment and prognosis of GCA/PMR

Corticosteroid therapy is currently the first choice treatment option for GCA and PMR, but long-term corticosteroid therapy is associated with severe side effects [74]. Mixed efficacy of methotrexate has been observed in PMR patients [75]. The role of glucocorticosteroid-sparing agents, such as leflunomide (LEF) and biological therapies such as IL-6 receptor blocking therapy (Tocilizumab) are subject of current studies [76]. An improved understanding of the immunopathogenesis of GCA and PMR may eventually lead to highly needed alternative treatment options for GCA and PMR patients. Identifying prognostic biomarkers will be important to develop therapeutic strategies based on relapse risk. For clinical prognosis of GCA and PMR, traditional biomarkers such as erythrocyte sedimentation rate (ESR) and C-Reactive Protein (CRP) are not disease-specific and lack accuracy for predicting disease relapses [77-79]. Thus, there is a great need for a disease-specific biomarker for GCA and PMR.

AIM AND OUTLINE OF THIS THESIS

Ageing is associated with a dysregulated immune and inflammatory response which likely contributes to the increased incidence of chronic immune mediated diseases in elderly individuals. This thesis aimed to explore numerical, phenotypical and functional changes in the innate arm of the immune system during the ageing process in healthy as well as in age-related vasculitides such as ANCA-associated vasculitis and GCA/PMR. We hypothesized that immunosenescence contributes to the development of ageing-associated vasculitic diseases. More specifically, we hypothesized that age-related changes in the immune system affect severity of experimental ANCA-associated vasculitis and that aged monocytes in particular contribute to the immunopathogenesis of GCA/PMR. New insights may lead to improved care and treatment options for ANCA-associated vasculitis and GCA/PMR patients.

In **chapter 2**, we aimed to determine the effect of age and inflammageing on the development of ANCA–associated vasculitis. Applying a mouse model of anti-MPO-mediated glomerulonephritis based on passive transfer of mouse anti-mouse MPO antibodies and systemic administration of lipopolysaccharide as a pro-inflammatory stimulus, we hypothesized that aged mice (18 months old) develop more severe clinical and pathological disease compared to young mice (3 months old). In addition, we investigated whether age-related changes in the immune system and/or kidney affected the severity of anti-MPO IgG/LPS induced glomerulonephritis.

Innate immune cells are able to recognize PAMPs of microorganisms such as bacteria, parasites, fungi and viruses [80]. Sensing of PAMPs by the innate immune system is provided by several PRRs. In **chapter 3**, we investigated whether ageing affects cytokine responses to a wide range of well-defined PRR ligands, such

as ligands for TLRs, C-type lectin receptors (CLRs), NOD-like receptors (NLRs), retinoic-acid-inducible gene-I like receptors (RLRs) and the cytosolic DNA sensor absent in melanoma 2 (AIM2). Also, we investigated the expression of PRRs on innate immune cells in young versus old individuals.

Of the innate immune cells, monocytes are considered key players of inflammation and pathogen challenge. In **chapter 4**, we investigated how ageing affects classical monocytes (CD14^bright^CD16-), intermediate monocytes (CD14^bright^CD16+) and non-classical monocytes (CD14^dim^CD16+) subsets and their cytokine (pro- and anti-inflammatory) responses to defined TLR receptor ligands.

Tissue migration of monocytes into the vessel wall contributes to the onset and progression of GCA. We hypothesised that ageing-related, pro-inflammatory CD16+ monocyte subsets contribute to the pathogenesis of GCA and PMR. In **chapter 5**, we therefore assessed the distribution of the three different monocyte subsets in GCA and PMR patients when compared to healthy controls. Next, we investigated their traits in the vascular wall of the temporal artery in GCA patients and studied expression of defined chemokine receptors and chemokines likely to be involved in migration of monocytes into the vascular wall in GCA. Lastly, in **chapter 6** we investigated whether numbers of circulating CD16+ monocytes can be of use as a prognostic bio-marker for relapse in GCA or PMR patients.

Finally, in **chapter 7**, the results and implications of the studies described in this thesis are discussed and recommendations for future research are given.

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