Immune checkpoint pathways in the ageing immune system and their relation to vasculitides

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Chapter 1

General Introduction and outline of the thesis
AGING OF THE IMMUNE SYSTEM

The world’s population aged 60 years and older is expected to increase from 900 million in 2015 to a total of 2 billion by 2050 (1). The increase of life expectancy around the world poses a major challenge for healthcare services as unfortunately, the health of the elderly population is not well maintained. Most of the health problems experienced by aged individuals are associated with chronic conditions due to frailty and progressive deterioration of immune function with age (2–4).

Aging is characterized by multiple changes in the immune system. As a result there is a progressive reduction of its ability to mount effective humoral and cellular responses against threats, contributing to a higher risk of infection, cancer, and autoimmune diseases in the elderly (5,6). This age-related decline in immune functions is commonly referred to as immunosenescence (5–8) which is a multifactorial process influenced by both intrinsic (genetic) and extrinsic factors. Immunosenescence affects both branches of the immune system, the innate immune system, consisting of neutrophils, monocytes, natural killer (NK), and dendritic cells (DC) as well as the adaptive immune system, comprising B and T lymphocytes (7) (Figure 1).

AGEING-ASSOCIATED CHANGES OF THE IMMUNE SYSTEM

The age-associated decline in immune function contributes to increased susceptibility for infectious and autoimmune diseases. As time passes by, immune cells undergo a series of changes including impaired signaling and overall aberrant effector functions leading to an overall deterioration of immune function (9). Ageing associated changes of immune cells have a strong impact on vaccination efficacy, lead to a diminished resistance to infections and are associated with a state of chronic low grade inflammation referred to as inflammaging (9–12). Inflammation is a major contributor to the pathogenesis of several age-associated diseases such as metabolic disorders (13), type 2 diabetes (14), Alzheimer’s disease (15) and rheumatoid arthritis (16,17) among others. There is an intricate link between immunosenescence and inflammation, as these two mutually influence each other and synergistically contribute to the development of a variety of detrimental states. Hence, a better understanding of the molecular and cellular mechanisms underlying age-related inflammation and immunosenescence could aid the development of better strategies for disease prevention and quality of life improvement of the elderly population.
General introduction and outline of the thesis

Impact of ageing on immune checkpoint molecules

Persistent exposure to antigens can lead to a state of functional impairment of the immune system termed exhaustion. Immune exhaustion typically refers to dysfunctional T cells characterized by poor effector function, sustained expression of inhibitory receptors and a transcriptional state different from that of functional effector or memory T cells (26,28). Exhaustion is co-regulated by a variety of cell surface inhibitory receptors such as Cytotoxic T lymphocyte-associated protein 4 (CTLA-4), Programmed Death-1 (PD-1), lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin mucin 3 (TIM-3) (28,29). Of note, several studies show that exhaustion can be reversed by reinvigoration of immune cells through immune checkpoint (IC) therapy such as CTLA-4 and PD-1 blockade (30–33). Although IC are involved in T cell exhaustion it is important to consider that expression of IC is not limited to exhausted cells and that these IC molecules are of vital importance in the regulation of a normal immune response (Table 1).
Moreover, the expression of a certain IC molecule on exhausted cells does not indicate that the IC molecule is the cause of their exhaustion or that it critically contributes to their functional impairment (34,35).

A recently discovered B7 family member is the B7-H5 molecule V-domain-containing Ig suppressor of T-cell activation (VISTA), also referred to as PD-1 homolog (PD-1H), platelet receptor Gi24 and, Differentiation of Embryonic Stem Cells 1 (Dies1). For practical purposes, in this thesis, the B7-H5 molecule will be referred to as VISTA from here on. VISTA is a 55-65 kD type I immunoglobulin membrane protein with the extracellular domain homologous to PD-L1. VISTA is highly expressed on myeloid cells and to a lesser extent on T cells and tumor-infiltrating lymphocytes (36). VISTA can act both as ligand and as receptor on both APCs and T cells to inhibit T cell activation, proliferation and cytokine production (i.e. IL-2, IFN-γ) (36–38). Interestingly, in 2019, Wang et. al. identified a novel ligand for VISTA, V-Set and Immunoglobulin domain containing 3 (VSIG-3) and demonstrated that the VSIG-3/VISTA co-inhibitory pathway was able to inhibit human T-cell proliferation and cytokine production (39). Of particular importance to this thesis, it has been demonstrated that VISTA-deficient mice develop an age-related pro-inflammatory phenotype characterized by spontaneous T-cell activation and enhanced T-cell-mediated immune responses to neoantigens. Moreover, when interbred with 2D2 T-cell receptor transgenic mice that are susceptible to the development of autoimmune encephalomyelitis, increased disease incidence and severity was observed (40) further attesting to the impact of VISTA on suppression of inflammatory T cell responses.
General introduction and outline of the thesis

Table 1. Immune checkpoint molecules and functions (41–43)

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Expressed on</th>
<th>Ligand/Receptor</th>
<th>Function</th>
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</table>
| CD28 (CD28/B7 family) | Resting T cells | CD80/CD86 on APCs | • Delivers a co-stimulatory signal  
• Lowers TCR activation threshold  
• Promotes T-cell proliferation, survival, cytokine production, T-cell dependent B cell functions |
| ICOS/CD278 (CD28/B7 family) | Activated T cells, T follicular helper cells | ICOSL/CD275 on APCs (DCs, macrophages) and B cells | • Induces proliferation  
• Supports formation of follicular helper T cells through the induction of the transcription factor Bcl6 and is critical for germinal center formation. |
| CD40 (TNF receptor family) | APCs and B cells | CD40L/CD154 on activated T cells | • Stimulates cytokine secretion of B cells with subsequent T cell activation (44) |
| CTLA-4/CD152 (CD28/B7 family) | Resting/Activated T cells | CD80/CD86 on APCs (Binds with higher affinity than CD28) | • Delivers a co-inhibitory signal  
• Increases TCR activation thresholds  
• Upregulates indoleamine 2,3-dioxygenase (disabling T lymphocytes to proliferate due to tryptophan shortage) |
| PD-1/CD279 (CD28/B7 family) | T cells, B cells, DCs, monocytes, NK T cells, exhausted cells and Tregs | PD-L1/CD274 on APCs, B cells and T cells. PD-L2/CD273 on APCs. PD-L1 also binds to CD80 | • Delivers an inhibitory signal  
• Inhibits cell survival factor Bcl-xL |
| VISTA/ B7-H5/ PD-1H/Gi24/ Dies1 (CD28/B7 family) | Highly expressed on hematopoietic cells. Low expression on T cells and Tregs | VSIG-3 on activated T cells proposed by Wang et. al. in 2019 (39). | • Inhibits T-cell proliferation, cytokine production and expression of the T cell activation markers CD44 and CD69 (38)  
• Behaves both as a ligand and receptor to suppress T-cell activation (38,45,46). |

APC, antigen presenting cell; CTLA-4, cytotoxic T lymphocyte antigen-4; DC, dendritic cell; NK, natural killer; PD-1, programmed death-1; PD-L, programmed death ligand; TCR, T-cell receptor; Th, T helper; Treg, regulatory T cell.
AGING-ASSOCIATED AUTOIMMUNE RESPONSES

While aging is linked to decreased immune responses, there is also evidence for the appearance of age-related development of auto-immune diseases such as vasculitis (47,48). The mechanism of developing autoimmunity during aging is not clear. However, one possible explanation is that, in an attempt to maintain effective immunity against infections and cancer, immune cells undergo several phenotypic and functional alterations (i.e acquisition of innate-like receptors by senescent cells) to be able to mediate rapid effector functions. Unfortunately, such a process may carry an increased risk of autoimmune and inflammatory diseases in the elderly (6). Another major factor associated with the development of age-related autoimmunity is the increased prevalence of autoantibodies including rheumatoid factor and antinuclear antibodies upon aging (6,47–50).

In addition, the aging process is characterized by altered intercellular communication, genomic instability, stem cell exhaustion and cellular senescence (51). An important contributor to the above mentioned hallmarks of aging is inflammaging. Inflammaging in particular could be an important contributor to the development of T-cell driven autoimmune diseases in the elderly. This is because inflammaging likely reflects a shift from an anti-inflammatory state to a pro-inflammatory state where pro-inflammatory Th cells predominate over anti-inflammatory regulatory T cells in the CD4+ T cell compartment of older individuals (52,53). Likewise, aged monocytes and neutrophils contribute to inflammaging by a functional shift towards a pro-inflammatory phenotype and overall decreased function (54,55).

VASCULITIS

Vasculitis is defined as an inflammatory process in which the vessel wall is the primary site of inflammation. Vasculitis can affect any type of vessel ranging from capillaries, venules and arterioles to veins and arteries. Based on the size of vessels involved and specific clinical and pathological features, the most recent and currently most used classification of systemic vasculitides was proposed at the 2012 International Chapel Hill Consensus Conference (Table 3) (56). As mentioned before, the aging process entails considerable changes in the immune system which may facilitate the induction of some of these vasculitides. Vasculitides such as antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) and Giant Cell Arteritis (GCA) in which abnormalities in adaptive and innate immunity play a central role in their pathogenesis, are predominantly diseases of the elderly.
Table 3. Names of vasculitides adopted by the 2012 International Chapel Hill Consensus Conference on the Nomenclature of Vasculitides (56)

<table>
<thead>
<tr>
<th>Large vessel vasculitis (LVV)</th>
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<tbody>
<tr>
<td>Takayasu arteritis (TAK)</td>
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<tr>
<td>Giant Cell Arteritis (GCA)</td>
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<tr>
<th>Medium vessel vasculitis (MVV)</th>
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<tr>
<td>Polyarteritis nodosa (PAN)</td>
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<td>Kawasaki disease (KD)</td>
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<tr>
<th>Small vessel vasculitis (SVV)</th>
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<tbody>
<tr>
<td>Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)</td>
</tr>
<tr>
<td>Microscopic polyangiitis (MPA)</td>
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<tr>
<td>Granulomatosis with polyangiitis (Wegener's) (GPA)</td>
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<tr>
<td>Eosinophilic granulomatosis with polyangiitis (Churg-Strauss) (EGPA)</td>
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<th>Immune complex SVV</th>
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<tr>
<td>Anti–glomerular basement membrane (anti-GBM) disease</td>
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<tr>
<td>Cryoglobulinemic vasculitis (CV)</td>
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<tr>
<td>IgA vasculitis (Henoch-Schönlein) (IgAV)</td>
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<tr>
<td>Hypocomplementemic urticarial vasculitis (HUV) (anti-C1q vasculitis)</td>
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<th>Variable vessel vasculitis (VVV)</th>
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<tr>
<td>Behçet's disease (BD)</td>
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<td>Cogan's syndrome (CS)</td>
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<th>Single-organ vasculitis (SOV)</th>
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<tr>
<td>Cutaneous leukocytoclastic angiitis</td>
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<td>Cutaneous arteritis</td>
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<tr>
<td>Primary central nervous system vasculitis</td>
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<tr>
<td>Isolated aortitis</td>
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<td>Others</td>
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<tr>
<th>Vasculitis associated with systemic disease</th>
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<tr>
<td>Lupus vasculitis</td>
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<tr>
<td>Rheumatoid vasculitis</td>
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<tr>
<td>Sarcoïd vasculitis</td>
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<td>Others</td>
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<tr>
<th>Vasculitis associated with probable etiology</th>
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<tr>
<td>Hepatitis C virus–associated cryoglobulinemic vasculitis</td>
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<tr>
<td>Hepatitis B virus–associated vasculitis</td>
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<tr>
<td>Syphilis-associated aortitis</td>
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<tr>
<td>Drug-associated immune complex vasculitis</td>
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<tr>
<td>Drug-associated ANCA-associated vasculitis</td>
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<tr>
<td>Cancer-associated vasculitis</td>
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<td>Others</td>
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ANCA-associated vasculitis

The anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) comprise a group of systemic autoimmune diseases characterized by chronic and systemic inflammation of small vessels. This life-threatening condition bears as a hallmark the presence of pathogenic autoantibodies against the neutrophil and monocyte lysosomal enzymes proteinase-3 (PR3) or myeloperoxidase (MPO). Within the group of AAV, three disorders with similar clinical and histopathological features can be distinguished: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA) (56,57). For this thesis we will focus on GPA. The incidence of AAV increases with age, with a peak-age of onset around 64-75 years (58). Of note, age is a predictor of AAV outcome characterized by poorer prognosis in older patients. The common clinical manifestations of AAV are necrotizing inflammation of small- to medium-sized blood vessels with no or little deposits of immunoglobulins or complement in the vessel wall (57). Most frequently it affects the upper and lower respiratory tract and kidneys, but disease manifestations may occur in any organ of the body (59). While the AAV pathogenesis is not yet fully understood, there is a series of sequential inflammatory steps by which ANCA-mediated neutrophil activation leads to vascular inflammation: First, adhesion molecules are upregulated on vascular endothelial cells within a pre-existing pro-inflammatory environment. Pro-inflammatory cytokines such as TNFα prime neutrophils resulting in translocation of PR3 and MPO to the cell surface, increasing their accessibility for the circulating ANCA. These pre-activated neutrophils roll over and firmly adhere to the activated endothelial cells and, upon ANCA binding, become fully activated. Neutrophil activation results in degranulation of proteolytic enzymes and production of reactive oxygen species (ROS) that are injurious to the endothelium eventually leading to vasculitis (59–64).

Giant Cell Arteritis (GCA)

Giant cell arteritis (GCA) is thought to be an immune-mediated inflammatory syndrome which affects the elderly population with women being 3 times more susceptible than men (65). Histopathologically, GCA is characterized by granulomatous inflammation within the layers of the medium- and large-sized lamina elastica containing vessels. The disease does not manifest before the age of 50, having a mean age at onset of 70 years (66); and annual incidence continues to increase up to the eighth decade in life (67). The immunopathogenesis of GCA is complex but mainly driven by 3 different cell types: dendritic cells (DCs), T cells and macrophages (68–70). In the development of GCA, four different phases can be distinguished:
Phase I is characterized by a loss of tolerance and activation of resident DCs of the adventitia by an unknown danger signal. Phase II starts with the recruitment, activation and polarization of CD4 T cells. The presence of pro-inflammatory cytokines such as IL-12, IL-18, IL-23, IL-6 and IL-1β polarizes CD4+ T cells toward Th1 and Th17 cells. In the third phase of the immunopathological model of GCA, due to the strong infiltration of Th1 and Th17 cells and the consequential production of IFN-γ and IL-17 respectively, vascular smooth muscle cells (VSMC) produce several chemokines (CCL2, CXCL9, CXCL10 and CXCL11) that trigger the recruitment of other immune cells (monocytes, Th1 and CD8 T cells). This phase is characterized by differentiation of the recruited monocytes into macrophages and additional IFN-γ production by Th1 and CD8 T cells supporting the chronic Th1 driven inflammatory response observed in GCA. The fourth and last phase, comprises the vascular remodeling processes in which IFN-γ activated macrophages of the media merge into multinucleated giant cells located at the destructed internal lamina elastica producing growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) that promote neovascularization and induce the migration and proliferation of VSMC respectively. The outcome is vascular occlusion and ischemic symptoms triggered by intimal hyperplasia (67,71–74). The combination of multiple dysfunctional immune cells together with the destructed vessel wall has been reported to drive this pathogenic process, which supports the conclusion that multiple etiologic agents must be involved in the induction of GCA (71), therefore characterizing it as having a chronic course.

**AIM AND OUTLINE OF THIS THESIS**

The elderly population is more prone to suffer from chronic immune mediated diseases due to the age-associated changes that the immune system experiences throughout life. This thesis aimed to study changes regarding IC molecule expression on the surface of immune cells during the ageing process in the healthy population. In addition, we aimed to study the contribution of altered checkpoint expression to the pathogenesis of age-related vasculitides such as AAV and GCA.

Information concerning expression and kinetics of IC molecules on immune cells is limited and may well be affected by age and gender. To gain more insight into the impact of age and gender on the expression and kinetics of IC molecules, in chapter 2, expression levels of IC (i.e. VISTA, PD-1, CD40L, ICOS and CTLA-4) on circulating immune cells in fresh blood samples from healthy young and old donors were analyzed and compared between men and women. Furthermore, the kinetics of IC expression on circulating T cells was analyzed after stimulation *in vitro* and
compared between the different age and sex groups.

**In chapter 3**, after reviewing the existing literature, we argue that changes due to age in expression and function of IC molecules lead to an unstable immune system, making it more prone to tolerance failure and autoimmune vasculitis development. Our argument is strengthened by a case report from our hospital describing a melanoma patient treated with IC inhibitors who subsequently developed GCA.

V-domain-containing Ig suppressor of T-cell activation (VISTA) is a recently discovered negative immune checkpoint of the B7 family expressed by myeloid cells and T cells which upon ligation suppresses T cell activation. In mice, VISTA deficiency has been demonstrated to induce an age-related pro-inflammatory phenotype characterized by spontaneous T-cell activation, thereby rendering these mice more prone to develop autoimmunity when interbred onto an autoimmune-susceptible background. Intrigued by these findings, we aimed to investigate the role of VISTA in the immune mediated age-related vasculitides GPA and GCA. In **chapter 4** we investigated the expression of VISTA on leukocytes of GPA patients in remission in comparison to healthy controls. In **chapter 5**, in an effort to understand the possible added contribution of IC pathways to the dysregulation of CD4+ T cells in GCA, we investigated the expression of VISTA and other IC molecules on circulating monocytes and CD4+ T cells of GCA patients. In addition, we assessed IC expression at the vascular site in GCA and non-GCA biopsies and determined the effect of VISTA-Ig engagement on CD4+ subset lineage differentiation *in vitro*.

Throughout the studies presented in this thesis we assessed at least 12 different parameters on a single-cell level by fluorescence-based flow cytometry to analyze the expression of different IC molecules on the surface of a variety of immune cells. This resulted in complex high-dimensional datasets which were at times difficult to analyze by traditional methods. New and better analysis and visualization methods are necessary to improve the accessibility of high-dimensional datasets. Therefore, in **chapter 6**, we show the analysis of data using the t-distributed stochastic neighbor embedding (t-SNE) algorithm for visualization of high-dimensional datasets. While manual gating will continue to aid the analysis of data, these new computational tools will enable scientists to better analyze complex cytometry data and deepen our understanding of complex cellular systems and their interactions.

Finally, in **chapter 7**, we summarize and discuss the implications of the findings presented in this thesis and recommendations for future research are provided.
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General introduction and outline of the thesis


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