GENERAL INTRODUCTION AND OUTLINE OF THE THESIS
The number of elderly individuals is expected to rise substantially in the coming years, both in developed and less-developed countries. Besides improved birth control, a dramatic increase in life expectancy is responsible for this rapid aging of the global population [1]. Although the increase in life span is an important biomedical and social success, it also poses a substantial burden on the health care systems, as the increased life span does not keep pace with an increase in health span [1]. Indeed, aging is an important risk factor for infections, cardiovascular disease, cancer and autoimmunity. Strategies to improve human health span and to minimize morbidity during the last phase of life will therefore be a key challenge in the decades to come.
Aging of the immune system

Aging has a profound impact on all organ systems of the human body, including the immune system [2]. Current evidence indicates that aging-related changes of the immune system contribute to the enhanced risks for infections, cancer, autoimmunity and cardiovascular diseases in the elderly [3-8]. In particular the balance between inflammatory and anti-inflammatory immune responses seems to be important to preserve health in the elderly [9]. This balance, however, may become disturbed in the elderly, as both the innate and adaptive arms of the immune system are affected by aging.

Aging-associated changes of the innate immune system

Neutrophils, monocytes/macrophages, dendritic cells and natural killer cells are all part of the innate immune system. The innate immune system not only represents the first-line of defence against microbes and malignant cells, but also plays an important role in the development of autoimmune diseases [10]. Although innate immune cells are largely retained in the elderly, their functions change substantially with age [11]. Unfortunately, harmful functions of the innate immune system become more prominent in the elderly [12], whereas its anti-microbial functions deteriorate [13]. For example, neutrophils in aged individuals lose their potential to migrate efficiently towards microbes. Inefficiently migrating neutrophils in aged subjects promote tissue damage and systemic inflammation on their way via premature secretion of their granular content [14]. In addition, monocytes/macrophages of aged individuals produce more pro-inflammatory cytokines than those of young individuals [15]. Overall, an aging-associated increase in chronic, low-grade inflammation, termed ‘inflammaging’ [16], seems to contribute to the development of autoimmune diseases in the elderly [7,16], while directly compromising anti-microbial immunity [13].

Aging-associated changes of the adaptive immune system

The adaptive immune system, which is comprised of T and B cells, provides highly effective, antigen-specific immunity against microbes and malignant cells. An important characteristic of the adaptive immune system is the development of antigen-specific memory upon the first exposure to antigens. Such memory develops as naive T or B cells differentiate into memory and effector cells. Human adults have typically acquired memory against a wide variety of pathogens. However, the development of immune responses and immunological memory upon novel antigenic challenges, seems to occur less efficiently in the elderly, as indicated by poor vaccine responses [17]. In contrast, inappropriate
activation and differentiation of auto-reactive T and B cells seems to increase with age, as suggested by increased prevalence of auto-antibodies and T-cell driven autoimmune diseases in the elderly [18,19].

**Impact of aging on naive T cell homeostasis**

T cells provide help (i.e. CD4+ T cells) to other immune cells or directly kill (i.e. CD8+ T cells) virally-infected host cells and cancer cells. As T cells require activation via their specific T cell receptor, a large and diverse naive T cell repertoire is required to deal with a wide variety of microbial and cancer antigens [20]. Current data indicate that the naive T cell repertoire is created early in life, when substantial thymic output is present [21]. After the second decade of life, however, thymic involution occurs and thymic output hardly contributes to the maintenance of the naive T cell repertoire. Instead, homeostatic proliferation and long-term survival of existing naive T cells is required to maintain the naive T cell repertoire later in life [21]. Animal studies indicate that peripheral maintenance of naive T cells requires low affinity recognition of self-peptide/major histocompatibility complexes and homeostatic cytokines such as interleukin-7 (IL-7) [22]. Indeed, IL-7 also promotes the homeostatic proliferation of naive T cells in humans, as indicated by therapeutic trials with IL-7 [23]. Other homeostatic cytokines, such as interleukin-2 (IL-2) and interleukin-15 (IL-15) are currently thought less important for the homeostasis of naive T cells [22]. In addition, aging-associated loss of CD31 on naive T cells is considered evidence that human naive T cells require T cell receptor engagement for their survival [24]. This line of reasoning is further supported by recent data showing that clonal expansion of the naive T cell repertoire occurs in aged humans [25]. Intriguingly, naive CD4+ T cells are much better maintained in elderly humans than naive CD8+ T cells [26]. Detailed mechanistic explanations for this difference are currently lacking. A recent study indicated that naive CD4+ T cell numbers in humans are predominantly influenced by (unknown) genetic factors, whereas naive CD8+ T cell numbers are mostly determined by environmental factors, such as infections [27]. Investigating the mechanisms that influence the maintenance of naive T cells will help us understand why elderly individuals are susceptible to infections and cancer on one hand, and autoimmunity on the other hand.

**Impact of aging on T helper cells and regulatory T cells**

When naive T cells recognize their specific antigen in the presence of co-stimulatory signals (i.e. CD28 engagement by CD80 or CD86), these cells undergo activation, proliferation and eventually differentiation into memory and effector T cells. Naive CD4+ T cells can differentiate into distinct subsets of T helper (Th) cells, such as interferon-γ (IFN-γ) producing Th1 cells, interleukin-4 (IL-4) producing Th2 cells and interleukin-17 (IL-17) producing Th17 cells [28]. This polarization process highly depends on the presence of polarizing cytokines during the activation of naive CD4+ T cells (Figure 1). In addition, accumulating
Introduction and thesis outline

Figure 1. Development of distinct T cell lineages in humans. The effect of polarizing cytokines on the development of distinct CD4+ T cell populations is shown.

Evidence indicates that substantial plasticity between these Th subsets exists [29]. For instance, Th17 cells can differentiate into Th1 cells when exposed to Th1 polarizing cytokines [30]. Although Th cells are important in the host defence against different types of microbes, inappropriate activity of these cells may contribute to a break of tolerance and ultimately autoimmune diseases (Th1, Th2, Th17) and allergies (Th2) [28]. Various studies have shown that Th1
and Th2 cells are retained with age [31,32]. However, conflicting data have been reported with regard to Th17 cells, as both an aging-associated increase and decrease of Th17 cells has been reported [33,34].

Regulatory T (Treg) cells represent another specialized population of CD4+ T cells (Figure 1). Treg cells suppress the activation, proliferation and effector functions of other immune cells [35]. As such, Treg cells represent an important constraint on immune responses and inflammation [36]. Currently, two types of regulatory T cells are recognised: thymus-derived (natural) Treg cells and peripherally-derived (induced) Treg cells [37]. Unfortunately, markers distinguishing these two populations of Treg cells are so far lacking. The most important Treg cell marker FOXP3 is expressed by both thymus-derived Treg cells and peripherally-derived Treg cells [35]. Ample evidence indicates that Treg cells increase with age [38,39]. Although an increase in Treg cells could be an important adaptation against ‘inflammaging’ in the elderly, data from animal studies indicate that this adaption develops at the expense of antimicrobial and antitumor responses [40-42]. So far, only limited data support this latter notion in humans [43].

**Impact of aging on cytotoxic CD8+ T cells**

CD8+ T cells are critical in the host defence against viruses and cancer. Upon activation, naive CD8+ T cells differentiate into effector-memory CD8+ T cells, which directly kill target cells through the release of perforin and granzymes. Furthermore, these effector-memory CD8+ T cells produce substantial amounts of pro-inflammatory cytokines such as INF-γ and TNF-α [44]. For many years, aging-associated expansion of differentiated CD8+ T cells was thought to directly compromise the naive T cell pool by progressively taking more and more ‘immunological space’ from naive T cells. Recent studies, however, strongly argue against this notion, as absolute numbers of effector-memory CD8+ T cells do not expand during adulthood [26,45]. However, latent infection with cytomegalovirus (CMV) is associated with an absolute increase in effector-memory CD8+ T cells. These virus-specific effector-memory CD8+ T cell may comprise a substantial portion of the circulating CD8+ T cell pool in order to restrain CMV reactivation [46,47].

**Impact of aging on B cells**

Historically, B cells were seen as precursor cells for antibody-producing plasma cells. This function of B cells is decreased in the elderly, as indicated by a poor ability to raise humoral immune responses upon vaccination [17]. During the last decade, awareness has grown for B cell functions not directly related to their antibody producing capacity. B cells present antigens to T cells and can modulate immune responses via secretion of pro-inflammatory (IL-6, TNF-α) and anti-inflammatory (IL-10, TGF-β) cytokines (Figure 2) [48]. Currently, a strong debate is taking place on whether production of these cytokines defines distinct lineages of B cells (i.e. effector B cells and regulatory B cells) or just
represents a temporary ability that B cells acquire during particular stages of differentiation. Enhanced production of pro-inflammatory cytokines by B cells has been linked to development of multiple sclerosis and rheumatoid arthritis (RA) [49,50], while defective IL-10 production has been observed in systemic lupus erythematosus [51]. Interestingly, a recent study indicates that the in vitro capacity of B cells to differentiate into IL-10 producing B cells declines with age [19]. This decreased ability to produce IL-10 is associated with a steady increase in serum autoantibody levels in the elderly. So far, it is unclear whether this decrease in IL-10 production only represents an in vitro phenomenon, or that aging affects the actual number of IL-10 producing B cells in vivo. Furthermore, it remains to be elucidated whether or not aging affects the capacity of B cells to produce pro-inflammatory cytokines.

**Aging-associated autoimmunity**

Aging is associated with development of autoimmunity [18]. The prevalence of autoantibodies, such as rheumatoid factors and anti-nuclear antibodies, increases with age [52,53]. Common autoimmune diseases, such as giant cell arteritis (GCA), polymyalgia rheumatica (PMR) and RA primarily affect the elderly. Interestingly, these aging-associated autoimmune diseases not only share a predilection for elderly individuals [54,55], but also a genetic background. HLA-DR4 is the strongest genetic risk factor for both GCA and RA. In addition, HLA-DR4 has been identified as a risk for PMR in some studies. Notably, most of the association between HLA-DR4 and these diseases can be explained by sequences
encoding for amino-acids at the peptide binding grooves of HLA-DR4, which is
important for antigen presentation to CD4+ T cells [56-58]. In accordance with
this association, CD4+ T cells play an important role in the pathogenesis of
GCA and RA [54,55]. Some evidence indicates that T cells may also contribute
to the development of PMR [59,60].

Several processes could explain the development of T-cell driven autoimmune
diseases in the elderly. Firstly, it is possible that the involuted thymus still
produces very small amounts of new T cells that are highly auto-reactive.
Indeed, recent evidence indicates that thymic involution is associated with
failing negative selection [61]. Secondly, it is possible that auto-reactive T cells
causing aging-associated autoimmune diseases are actually produced early in
life and maintained until these diseases are eventually triggered by unknown
factors. Mechanisms promoting the lifelong maintenance of these T cells would
therefore be critical for the development of autoimmune diseases later in life.
Thirdly, it is possible that aging shifts the balance from anti-inflammatory
immune responses towards pro-inflammatory responses. More specifically, pro-
inflammatory Th cells may predominate over anti-inflammatory Treg cells in the
CD4+ T cell compartment of elderly individuals. In addition, this imbalance may
perhaps also be present among other leukocyte populations, such as the B cell
compartment, as outline above.

Giant cell arteritis and polymyalgia rheumatica

GCA is a systemic vasculitis affecting large and medium-sized arteries [55].
Besides general symptoms of systemic inflammation (such as weight loss and
fever), GCA patients can develop localized symptoms related to arterial stenosis
caused by arterial wall inflammation and remodelling: e.g. headache, blindness,
jaw claudication and stroke. PMR is a closely related rheumatic disease that
frequently co-occurs with GCA [55]. PMR is associated with synovitis, enthesitis
and bursitis. PMR patients typically develop pain and stiffness of the shoulders,
hips and back. An important role for dendritic cells, macrophages and T cells
has been reported in GCA and PMR [62]. Although cytokine production by B cells
is implicated in the development of various autoimmune diseases, little is known
about the contribution of B cells to the pathogenesis of GCA and PMR. Furthermore,
traditional biomarkers for GCA and PMR, i.e. the erythrocyte sedimentation rate
(ESR) and C-reactive protein (CRP) are not always increased during active disease
in GCA and PMR patients at diagnosis [63,64]. Novel biomarkers for active GCA
and PMR are therefore awaited. Candidate biomarkers for GCA and PMR would
not only include pro-inflammatory cytokines in the serum, but also the activation
state of circulating T cells. Although tremendous progress has been made in the
discovery of targeted treatments in other autoimmune diseases, corticosteroids
have remained the only validated treatment for GCA and PMR since the 1950s
[55]. Unfortunately, corticosteroids are associated with substantial side effects in
elderly patients [65]. Thus, identification of novel targets for treatment in GCA and PMR patients is needed.

**Aim and outline of the thesis**

Age is an important risk factor for the development of autoimmune diseases in the elderly [18]. This thesis explores aging-associated changes of the adaptive immune system and aims to elucidate why aged subjects are susceptible to autoimmune disease. Aging-associated autoimmune diseases, such as GCA, PMR and RA are considered T cell driven diseases [54,62]. As thymic output dramatically drops early in life [21], we postulated that the prolonged maintenance of the naive T cell repertoire, associated with ageing, is a critical factor in the development of autoimmune diseases in the elderly.

In **chapter 2** important risk factors for aging-associated autoimmunity, i.e. HLA-DR4 positivity and female gender were studied in relation to maintenance of the naive CD4+ T cell repertoire in aged individuals. In addition, we investigated the differential impact of aging and latent CMV infection on distinct CD4+ and CD8+ T cell differentiation subsets.

**Chapter 3** provides a mechanistic explanation why the naive CD4+ T cell repertoire is significantly better retained in the elderly than the naive CD8+ T cell repertoire. More specifically, low affinity T cell receptor stimulation and interleukin-2 are identified as factors promoting the maintenance of naive CD4+ T cells, but not naive CD8+ T cells.

**Chapter 4** describes the impact of age and CMV on the human T cell receptor repertoire. To that end, economic statistics were introduced to measure inequality in TCR-Vβ distribution among well-defined CD4+ and CD8+ T cell differentiation subsets. Furthermore, we delineated the effects of vaccination on the naive CD4+ and CD8+ T cell repertoire.

Although a well-maintained balance between pro-inflammatory Th cells and anti-inflammatory Treg cells may be critical to preserve health in the elderly [66], comprehensive studies investigating the impact of aging on this balance are currently lacking. **Chapter 5** therefore elucidates how aging affects the balance between Th cells and Treg cells, and whether this balance may influence vaccine responses in the elderly.

As accumulating evidence indicates that B cells modulate immune responses via secretion of cytokines [48], **chapter 6** studies the impact of aging on the potential of B cells to produce pro-inflammatory or anti-inflammatory cytokines. In addition, B cell cytokine production is studied in relation to the presence of autoantibodies in the elderly.

So far, B cells have received little attention in studies on GCA and PMR. In **chapter 7**, we describe for the first time profound disturbances of B cell homeostasis in GCA and PMR patients. Furthermore, the distribution of pro-inflammatory and anti-inflammatory B cells is explored in these patients.
Chapters 8 and 9 of this thesis are dedicated to identifying novel biomarkers for disease activity in GCA and PMR patients. In chapter 8, we study the expression of activation, proliferation and differentiation markers by T cells of GCA and PMR patients. In addition, we explored the ability of these markers to detect active GCA and PMR. Chapter 9 explores the diagnostic accuracy of 26 distinct serum proteins as biomarkers for active disease in GCA and PMR patients.

Finally, chapter 10 provides a summary and discussion of the findings in this thesis.

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