T-cell mediated immunity in Wegener's granulomatosis

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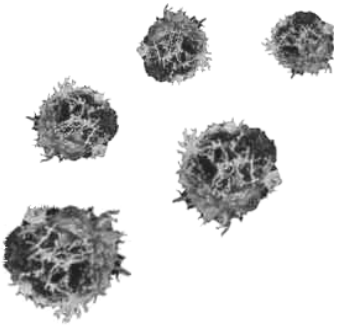
CHAPTER 8

Summary, general discussion and future perspectives



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Introduction

Although the pathogenetic mechanisms involved in Wegener's granulomatosis (WG) are not completely understood, considerable evidence support the concepts that activated T-cells play an important role in disease expression. It is, however, not clear which subsets of T-cells are involved in the aberrant (auto)immune response in WG.

In order to understand aberrant T-cell responses in WG, it is mandatory first to consider normal T-cell homeostasis. T-cell precursors arise in the bone marrow and migrate into the thymus. In the thymus, these cells enter a selection process and learn how to recognize antigens and to discriminate between self- and nonself antigens. Only around 5% of T-cells survive the selection process and leave the thymus as natural regulatory T-cells (nT_{Reg} cells) or conventional naïve T-cells (single positive CD4 or CD8 T-cells). The nT_{Reg} cells enter the circulation and control auto-reactive responses in a cell contact-dependent way. Conventional naïve T-cells migrate towards secondary lymphoid organs as lymph nodes via the high endothelial venules (HEV). This migration is guided by the expression of the lymph node homing receptor CCR7 on naïve T-cells, which is required for passage across HEV in the lymph node vasculature. Upon antigenic stimulation, naïve T-cells express the activation marker CD69 and undergo expansion and differentiation into different T-cell subsets (Th1, Th2, Th17, cytotoxic T-cells, and induced T_{Reg} cells) which are characterized by the production of distinct cytokines and effector functions. This diversification in functional subsets of T-cells is dependent on the balance of cytokines locally present at the time of their induction, and is more obvious for $CD4^+$T-cells compared to $CD8^+$T-cells. It is well known that IL12 promotes commitment to the Th1 lineage that secretes IFN-γ and TNF-α, thereby inducing cell-mediated immunity to combat intracellular pathogens[1;2]. Under the influence of IL4, T-cells can differentiate into the Th2 lineage that secretes IL4, IL5, IL10 and IL13, thereby providing help for B-cells and inducing humoral immunity which is essential for the elimination of extracellular pathogens[2;3]. In addition, the combination of TGFβ and IL6 induce the development of Th17 cells that secrete IL17 and IL21, which play an important role in host defence against extracellular pathogens that are not efficiently cleared by Th1- and Th2 responses[4;5]. Moreover, TGFβ, in the

absence of IL6, induces the development of a subtype of regulatory T-cells termed induced T_{Reg} cells (iT_{Reg} cells) that secrete IL10 and TGFβ, which facilitate the suppression of autoimmune responses via cytokine-dependent mechanisms[4].

In addition to the role of cytokines, the strength of antigenic stimulation is a major factor in determining T-cell differentiation. Cells receiving insufficient stimulation remain nonfit and die by neglect, whereas cells receiving excessive stimulation die by activation induced cell death (AICD). Only the fittest cells survive and enter the effector pool[6;7]. Following expansion of effector T-cells and elimination of pathogens, a contraction phase ensues where apoptosis of effector cells ensures a return to homeostasis, whereas a fraction of antigen-specific T-cells survives and differentiates into memory T-cells. Based on the strength of the antigenic signal, two types of memory T-cells develop, which are defined by the expression of the lymph node homing chemokine receptor CCR7. Naïve T-cells receiving a relatively weak stimulus will develop preferentially into $CCR7^+$ central memory T-cells (T_{CM}) that predominantly home to lymph nodes, whereas priming by a strong stimulus results in differentiation towards $CCR7^-$ effector memory T-cells (T_{EM}) that fail to migrate to lymph nodes but have acquired the capacity to home to nonlymphoid tissues[8].

Determination of the phenotype of these various subpopulations in the peripheral blood of WG-patients may give insights into T-cell homeostasis in this disease. Perturbation in this homeostasis and decrease in one of the subpopulations during active disease may indicate a selective migration of these particular cells from the peripheral blood into the tissues where they may contribute to granuloma formation and vascular damage.

Summary

In **chapter 2,** we reviewed the literature on the dysbalances in T-cell phenotypes and pathogenic roles of $CD4^+$T-cells, especially $CD4^+$ effector memory T-cells, in the induction and expression of various autoimmune diseases including ANCA-associated vasculitis. In **chapter 3**, we examined the distribution of peripheral naive and memory $CD4^+$- and $CD8^+$T-cells in WG-patients in remission and during active disease, in order to investigate

the phenotype of T-cells involved in the setting of vascular injury in WG. We observed a persistent expansion of $CD4^+T_{EM}$ cells with a reciprocal decrease in naïve $CD4^+$T-cells in WG-patients during remission, whereas no differences were found in the distribution of $CD8^+$T-cell subpopulations. The skewing towards $CD4^+T_{EM}$ cells provides an important hint for the presence of a strong and persistent antigenic trigger in WG-patients, as generation of T_{EM} cells requires both a strong and persistent immunological stimulus. Interestingly, our cross-sectional and follow-up data demonstrated that WG-patients with active disease exhibited a significant decrease in circulating $CD4^+T_{EM}$ cells compared with WG-patients in remission. For the interpretation of these results, two possibilities have been taken into consideration. *The first possibility* is that a decrease in the $CD4^+T_{EM}$-pool in active disease occurred due to migration of those cells into sites of inflammation. *The second possibility* is that $CD4^+T_{EM}$ cells are regulatory T-cells, which might operate by maintaining remission when increased, whereas a decrease in this cell populations occur during or preceding a relapse.

The first possibility has been explored in **chapter 4**. Since infiltrating T-cells could contribute to renal inflammatory lesions of WG-patients with renal involvement, these cells were expected to appear in the urinary sediment. We have analysed the phenotype of $CD4^+$T-cells in the urinary sediment and in the peripheral blood of patients with ANCA-associated vasculitis (AAV) with and without renal involvement, both during remission and disease relapse. We demonstrated that the majority of $CD4^+$T-cells in the urine of AAV-patients belong to the T_{EM} population. Most interestingly, we observed an increase in urine $CD4^+T_{EM}$ cells in concordance with a decrease in circulating $CD4^+T_{EM}$ cells in WG-patients with active renal involvement compared to patients in remission and to patients with active non-renal disease. The number of these cells clearly decreased in the urine following treatment, and their percentages in the urine were negatively correlated to their percentages in the peripheral blood. Therefore, we state that $CD4^+T_{EM}$ cells migrate towards inflammatory sites and act as effector cells in tissue injury and disease expression in WG.

To evaluate the second possibility, we determined the expression of *FoxP3* (T_{Reg} cell marker) in $CD4^+T_{EM}$ cells from WG-patients (**chapter 3**). Interestingly, the expanded $CD4^+T_{EM}$ cells from WG-patients in remission

had a non-regulatory phenotype ($FoxP3^{-}CD4^{+}T_{EM}$), whereas no difference was observed in the percentages of regulatory $CD4^{+}T_{EM}$ cells ($FoxP3^{+}CD4^{+}T_{EM}$) between WG-patients in remission and WG-patients with active disease. These data indicate that expansion of $CD4^{+}T_{EM}$ cells is not owing to an enrichment of T_{Reg} cells, and raises an obvious question why relapses occur in WG if both patients in remission and patients with active disease have similar percentages of T_{Reg} cells. It has been suggested that abnormalities in T_{Reg} cell numbers and/or function are involved in the pathogenesis of several autoimmune diseases (as reviewed in **chapter 5**). Therefore, in **chapter 6** we questioned whether a similar abnormality in T_{Reg} cells might occur in patients with WG. We found that T_{Reg} cells ($FoxP3^{+}CD25^{High}CD4^{+}$T-cells) exhibiting a memory phenotype ($CD45RO^{+}$) were significantly increased in the peripheral blood of WG-patients as compared with healthy controls. The increase in T_{Reg} cells in WG-patients did not appear to be due to the effects of immunosuppressive treatment. Next, we evaluated *in vitro* the suppression capacity of T_{Reg} cells purified from WG-patients in comparison to age- and sex-matched healthy controls. Indeed, we observed an impaired suppressor function of T_{Reg} cells from WG-patients, and we demonstrated that lack of suppression was not attributed to either altered survival of T_{Reg} cells or to a resistance of responder cells towards inhibition. As *FoxP3*-expression is also observed in human activated non-regulatory $CD4^{+}$T-cells[9-11], it is possible that the *FoxP3*-expressing $CD4^{+}$T-cells in WG-patients are rather activated T-cells than T_{Reg} cells.

The defect in T_{Reg} function may underlie loss of self-tolerance and may result in an inappropriate Th1/Th17/Th2 response. To this end, in **chapter 7,** we evaluated the distribution of circulating Th1, Th2, and Th17 cells in WG-patients, and we analysed the cytokine pattern of PR3-activated $CD4^{+}$T-cells. Antigen specific T-cell responses were determined by detecting de novo expression of the early activation marker CD69 and the intracellular cytokine expression of IFNγ, IL17, and IL4 as markers for Th1-, Th17-, and Th2-cells, respectively, following antigenic stimulation *in vitro*. We demonstrated a skewing towards Th17 and Th2 cells following *in vitro* stimulation of peripheral blood from WG-patients in remission. Most interestingly, increase in PR3-specific Th17 cells was exclusively observed in ANCA-positive WG-patients. This may indicate a role of Th17 cells in

induction of the humoral autoimmune response in this disease. In support of this, a study in patients with lupus nephritis revealed a direct role of IL17 in overproduction of autoantibodies[12]. On the other hand, IL17 may participate indirectly in the pathogenesis of WG by priming of neutrophils resulting in translocation of PR3 to their surface. In addition, IL17 is capable to attract and recruit neutrophils to the site of inflammation.

Taking all these observations into account, future perspectives for an active therapy for WG could be built on depletion of circulating $CD4^+T_{EM}$ cells, manipulation of T_{Reg} cell function, and neutralization of IL17.

General discussion and future perspectives

In this thesis, we demonstrated that $CD4^+T_{EM}$ cells are expanded in the peripheral blood of WG-patients in remission and that the number of these cells reduces during active renal disease and appear in the urine, which is consistent with increased migration of these cells to sites of inflammation once disease activity occurs. This suggests that $CD4^+T_{EM}$ cells contribute to tissue injury and granuloma formation in this disease.

A growing body of evidence implicates $CD4^+$T-cells as a major factor in the generation of a mononuclear granulomatous response, which supports our findings. In CD4-deficient mice, delayed and poorly organized granuloma formation was observed following *Mycobacterium tuberculosis* infection[13;14]. In HIV-infected patients, depletion of $CD4^+$T-cells leads to defective granuloma formation in pulmonary reactivity to *Mycobacterium tuberculosis*[15]. In WG-patients with active disease, $CD4^+$T-cells with a memory phenotype are enriched in granulomatous lesions and broncho-alveolar lavage fluid[16;17]. Most interestingly, Ruth *et al*. have recently demonstrated a key role for $CD4^+$T-cells in the expression of crescentic glomerulonephritis[18]. They induced experimental autoimmune anti-MPO associated glomerulonephritis by immunizing C57BL/6 mice with human MPO and administration of anti-mouse GBM to induce cell influx into glomeruli. Mice depleted of $CD4^+$T-cells at the time of administration of anti-mouse GBM developed significantly less glomerular crescent formation and cell influx when compared to control mice. In contrast, B-cell-deficient mice

developed similar severe crescentic glomerulonephritis with accumulation of effector cells as control mice. These findings suggest that $CD4^+$T-cell mediated immunity is a major effector pathway of injury in vasculitis/glomerulonephritis. In patients with ANCA-associated "pauci-immune" glomerulonephritis, T-cells expressing a memory phenotype (CD45RO) were prominently present in glomeruli[19]. This finding corresponds well with our results presented in **chapter 4** where a remarkable increase of $CD4^+T_{EM}$ cells was observed in the urinary sediment of WG-patients with active renal involvement compared to patients in remission and patients with active non-renal disease. These cells clearly decreased or disappeared from the urine during remission, which reflects their participation in renal injury. Indeed, the capability of $CD4^+T_{EM}$ cells to infiltrate and destroy microvessels has been recently reported by Shiao *et al.*[20]. They found that human $CD4^+T_{EM}$ cells transferred into a SCID chimera model bearing a human skin graft, infiltrated this graft and destroyed human microvessel endothelial cells in the graft. Thus, the appearance of $CD4^+T_{EM}$ cells in the urine of WG-patients confirms their involvement in renal injury and disease development. Detection of $CD4^+T_{EM}$ cells in the urine using flow cytometry technique could provide a new approach for assessing disease severity in WG-patients with renal involvement. This procedure has advantage over the renal biopsy in that it can be repeated as often as necessary and thus facilitates monitoring of the progression and regression of renal lesions over time.

Several lines of evidence confirm the central role of T_{EM} cells in the pathogenesis of autoimmune diseases. First, autoantigen-specific T-cells in patients with diabetes mellitus type-1[21] and patients with multiple sclerosis are T_{EM} cells[22]. Second, T_{EM} cells are the predominant cells in the synovium of patients with rheumatoid arthritis [21;23], in the skin lesions of patients with psoriasis [23;24], and in multiple sclerosis inflammatory brain infiltrates[25]. Third, experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis, was induced by transfer of myelin-specific T_{EM} cells into naïve recipients[26]. Last, colitis has been induced in SCID mice by adoptive transfer of colitogenic lamina propria $CD4^+T_{EM}$ cells obtained from inflamed mucosa of colitic SCID mice[27;28]. According to the aforementioned evidence and our own findings, we believe that $CD4^+T_{EM}$ cells act as a key trigger of disease expression and relapse in WG. It is possible that endothelial cells,

during active WG, express ligands for $CD4^+T_{EM}$ cells that augment their trans-endothelial migration and effector function. Indeed, the kidney endothelium from WG-patients with active disease express the major histocompatibility complex class I chain-related molecule A (MICA)[29]. This molecule provides a costimulatory signal to $CD4^+T_{EM}$ cells via interaction with NKG2D, a member of the killer immunoglobulin-like receptor family that mediates their cytotoxic function. It has been shown that $NKG2D^+CD4^+$T-cells can kill targets via NKG2D-MICA interaction[30]. Therefore, it is possible that the same mechanism contributes to renal injury and disease progression in WG-patients. Accordingly, selective targeting of $CD4^+T_{EM}$ cells without impairing other parts of cellular immunity might have value in the treatment of WG. Recent studies revealed the presence of high levels of the voltage-gated Kv1.3 K^+ channel on activated T_{EM} cells. Inhibition of the Kv1.3 channel effectively decreases cytokine production and suppresses proliferation of autoantigen-specific T_{EM} clones from patients with rheumatoid arthritis and patients with type-1 diabetes mellitus[23]. In addition, blockade of the Kv1.3 channel effectively prevented autoimmune disease in experimental autoimmune encephalomyelitis (EAE) and suppressed delayed-type hypersensitivity (DTH) in rats[26;31]. Therefore, selectively targeting of $CD4^+T_{EM}$ cells using Kv1.3-blockers may hold therapeutic promise for WG.

Since T_{Reg} cells inhibit the proliferation of both naïve and memory T-cells[32] and reduce transendothelial migration and recruitment of T-cells[33], a defect in suppressive function of T_{Reg} cells that we observed in WG-patients can sustain the persistent expansion and migration of $CD4^+T_{EM}$ cells towards inflammatory sites, thereby contributing to granuloma formation in this disease. Although malfunction of T_{Reg} cells may favor pathogenic responses in several autoimmune diseases, the etiology of this malfunction still remains unresolved and needs further study. Future studies will have to demonstrate whether pharmacological manipulation of T_{Reg} cell function is a promising therapeutic approach for WG and other autoimmune diseases.

Beside the key role of T_{Reg} cells in the manifestation of autoimmune diseases, a recent breakthrough has revealed a new target that is represented by the finding that Th17 cells are the main subset capable of inducing autoimmunity. It was assumed, until recently, that multiple sclerosis

and its mouse model, that is experimental autoimmune encephalomyelitis (EAE), are caused by IFNγ-producing Th1 responses[34]. However, mice deficient in either the Th1-polarizing cytokine IL12, IFNγ, or the IFN-receptor, show increased susceptibility to EAE[35-37], whereas induction of disease was blocked in mice deficient in IL17 or in the Th17 polarizing cytokine IL23[37;38]. Similarly, blocking IL17 in rodent models of arthritis protects against inflammation and bone destruction, whereas administration of IL17 exacerbates disease[39]. In addition, increased expression of IL17 in humans has been observed in a growing list of autoimmune and inflammatory diseases[40-44]. These data suggests a role for Th17 cells, rather than Th1 cells, in the pathogenesis of autoimmune inflammatory diseases. Consistent with these findings, we found a significant increase in the percentages of Th17 cells in *in vitro* stimulated peripheral blood from WG-patients as compared to healthy controls, whereas no difference was observed in the percentages of Th1 cells. Most importantly, we found a relative increase in PR3-specific Th17 cells, rather than Th1 cells, in ANCA-positive WG-patients in comparison to ANCA-negative WG-patients and controls. This suggests involvement of Th17 cells in the process of autoantibody production in WG. The role of IL17 in overproduction of anti-dsDNA autoantibodies has been investigated in patients with lupus nephritis[45]. More recently, Hsu *et al*. reported that IL17 promotes the formation of autoantibody-producing germinal centers in autoimmune BXD2 mice[46]. This suggests a pathogenic potential of Th17 cells in WG comparable to other autoimmune diseases. Apart from induction of autoantibody production, IL17 is capable of priming neutrophils and promoting their migration. It has also been shown that IL17 promotes the release of IL1β and TNFα from macrophages[47]. These proinflammatory cytokines prime resting neutrophils resulting in translocation of proteinase 3 (PR3) on their cell surface[48] and induce upregulation of adhesion molecules on neutrophils as well as on the vascular endothelial cells[49;50], which creates conditions for ANCA-induced neutrophil-dependent endothelial cell lysis. In addition, IL17 induces CXC chemokine release from human bronchial epithelial cells that specifically attract neutrophils to the site of inflammation in association with increased activity of elastase and myeloperoxidase[51;52]. Thus, increased Th17 response in WG-patients may contribute to neutrophil

recruitment and granuloma formation. Interestingly, induction of PR3-expression on neutrophils by IL17, together with the breakdown of T_{Reg}-mediated self-tolerance mechanisms, may trigger an autoreactive T-cell response against PR3 ending up in PR3-ANCA production in WG-patients. In this way, Th17 cells trigger both innate and adaptive arms of the immune response in WG. This finding will lead to a better understanding of the pathogenesis of WG, and generate new concepts with therapeutic implications.

Different subpopulations of T-cells have distinct functions and their interplay is crucial in eliminating microbes. Imbalances in immune homeostasis due to disturbed distribution of these T-cell subpopulations can be damaging to the host. In WG, the trigger that leads to an imbalance in T-cell homeostasis remains to be defined. It is generally accepted that the initial trigger of pathogenic immune responses in WG takes place in the respiratory tract, since the involvement of the respiratory tract frequently is the initial manifestation of this disease. Chronic nasal carriage of *Staphylococcus aureus* has been reported to be a strong risk factor for relapse in WG and to be associated with ANCA positivity[53]. This exogenous trigger can elicit an inflammatory response resulting in PR3-release from neutrophil granulocytes. Due to a dysfunction of T_{Reg} cells in WG-patients, naïve $CD4^+$T-cells may undergo repeated PR3 autoantigenic stimulation and differentiate into pathogenic $CD4^+T_{EM}$ cells that migrate quickly to sites of inflammation and contribute to tissue damage. In addition, PR3 induces activation of Th17 cells that release IL17. This cytokine enhances the production of proinflammatory mediators and aggravates priming of neutrophils. Moreover, IL17 may promote germinal center responses and induce the production of ANCA autoantibodies. ANCA mediated neutrophil activation also triggers reactive oxygen radical production and causes degranulation and release of the proteolytic enzymes that damage the endothelial cells ending up in vasculitis.

Collectively, the dual limbs of adaptive immunity, cellular and humoral, as well as the innate immunity are all involved in the pathogenesis of WG. This revised view on the pathophysiology of vasculitis may lead to the identification of possible new targets for therapeutic intervention.

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