**CHAPTER 3**

# Are monuclear cells predominant actors of endothelial damage in vasculitis?

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## Introduction

Antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) constitute a group of disorders characterized by autoimmune necrotizing inflammation of small blood vessels, which leads to systemic organ damage [1]. This group of systemic vasculitides includes Granulomatosis with poliangiitis (GPA), microscopic polyangiitis (MPA), and Churg-Strauss syndrome (CSS). These disorders are predominantly associated with the presence of circulating ANCA that are directed against proteins in cytoplasmic granules of neutrophils. ANCA with specificity for proteinase-3 (PR3-ANCA) are associated particularly with GPA, whereas ANCA with specificity for myeloperoxidase (MPO-ANCA) are predominant in MPA and to a lesser degree in CSS [2]. Although it remains unknown how these conditions develop, it has been postulated that ANCA in vivo bind to surface expressed autoantigens (PR3 or MPO) on pre-activated (primed) neutrophils, which enhances neutrophil degranulation and the release of toxic products that cause endothelial damage ultimately leading to necrotizing vasculitis.

In vivo experimental studies have clearly demonstrated that MPO-ANCA are pathogenic factors [3,4]. An immunopathogenic role for MPO-ANCA has also been strongly suggested by the occurrences of neonatal MPA in a child born to a mother with a history of MPO-ANCA-associated pulmonary-renal syndrome [5]. In contrast to MPO-ANCA, in vivo evidence is limited for a direct vasculitic pathogenicity of PR3-ANCA. More recently, Little et al. [6] injected PR3-ANCA containing human IgG into mice with a chimeric human-mouse immune system including human neutrophils. These mice developed glomerulonephritis (in a minority paucimune crescentic) and, in a few, pulmonary capillaritis, but granulomatous inflammation, characteristic for human PR3-ANCA GPA, was not observed [6]. It has been demonstrated that $CD4^+$ T-cells are the key player in the generation of granulomatous response.

For instance, CD4-deficient mice did not generate typical mononuclear granulomatous lesions following *Mycobacterium tuberculosis* infection [7]. In humans, the extent of granuloma formation was correlated with peripheral CD4 T-cells counts in HIV patients with mycobacterial infection [8,9]. This suggests a primary role of cell-mediated immunity in initializing granuloma formation.

## Role of CD4 $T_{EM}$ cells and their effector cytokines (IL-17 and IL-21) in ANCA-associated vasculitides

In AAV, neutrophil-mediated tissue damage has been considered an important part of disease pathogenesis. However, several observations support a key role of T-cells in disease manifestations as well. The important role of $CD4^+$ T-cells in the expression of crescentic glomerulonephritis (CG) has been demonstrated by Ruth et al. [10]. They induced experimental anti-MPO-associated CG by immunizing C57BL/6 mice with human MPO followed by subsequent challenge with anti-glomerular basement membrane antibodies (anti-GBM). Mice depleted of $CD4^+$ T-cells at the time of administration of antimouse GBM developed significantly less glomerular crescent formation and less cell influx when compared with control mice. These data provide convincing evidence that $CD4^+$ T-cells are crucial in granuloma formation and glomerulonephritis. Studies in AAV-patients also support this notion. It has been shown that MPO-induced proliferation of peripheral blood mononuclear cells from MPA-patients was completely lost after the depletion of $CD4^+$ T-cells, but not after depletion of $CD8^+$ T-cells [11]. In addition, IgG subclass distribution of ANCA (IgG1 and IgG4) implies isotype switching of ANCA for which $CD4^+$ T-helper cells are required [12].

In line with this observation, an altered phenotype of $CD4^+$ T-cells has been found in AAV-patients. An expanded population of $CD4^+$ T-cells lacking

the costimulatory molecule CD28 was observed in peripheral blood and in granulomatous lesions of patients with GPA [13,14]. These $CD28^{-}CD4^{+}$ T-cells display upregulation of the T-cell differentiation marker CD57 and show intracytoplasmic perforin expression indicating cytotoxic potential of these cells [13]. A more detailed analysis of T cells showed that expansion of $CD4^{+}$ T-cells in GPA occurred within the $CD4^{+}$ effector memory population ($T_{EM}$) characterized by being positive for CD45RO and negative for the lymphoid homing receptor CCR7 [15]. The generation of these $CD4^{+}$ $T_{EM}$ cells needs a strong and persistent trigger [16], which suggests that T-cells in GPA are in a persistent state of an ongoing immunological trigger, also during remission. Defect in regulatory T-cell function, found in GPA-patients, may also contribute to the expansion of $CD4^{+}$ $T_{EM}$ cells [17,18]. Surprisingly, these $CD4^{+}$ $T_{EM}$ cells are decreased in number during relapsing disease [15,19]. They are supposed to migrate then into lesional tissues. In accordance, infiltrating T-cells in lung lesions and glomeruli were shown to consist mainly of $CD4^{+}$ T-cells with a memory phenotype [14,20,21]. Indeed, our cross-sectional and follow-up studies confirmed migration of $CD4^{+}$ $T_{EM}$ cells during active renal disease into the diseased organs [19]. We observed a remarkable increase in $CD4^{+}$ $T_{EM}$ cells in the urinary sediment with a concomitant decrease of circulating $CD4^{+}$ $T_{EM}$ cells of GPA-patients with active renal involvement [19]. These urinary $CD4^{+}$ $T_{EM}$ cells decreased or disappeared from the urine during remission, which might reflect their role in renal injury. In line with this, Wilde et al. demonstrated an expansion in a specific subset of circulating $T_{EM}$ cells in GPA expressing CD134 and reported $CD134^{+}$ $T_{EM}$ cells in active lesions, which support their migration to inflamed sites [22]. Importantly, CD134 costimulation was shown to program $CD4^{+}$ T-cells to express lytic molecules and to perform cytotoxic function [23]. This may indicate that these $T_{EM}$ cells have a major role in tissue injury in AAV. Furthermore, Ordonez et al. found that AAV-patients exhibit an expanded $CD45RC^{Low}$ $CD4^{+}$ T-cell population that is a source of IL-17 [24].

Over the past few years, Th17 cells have challenged the classical Th1/Th2 paradigm, and have been implicated in a growing number of autoimmune and inflammatory diseases [25]. It has been reported that IL-17 enhances the production of autoantibodies, and induces CXC chemokine release and expression of adhesion molecules responsible for the recruitment of neutrophils to the site of inflammation [26-28]. This cytokine also promotes the production and release of IL1-β and TNF-α by macrophages [29], which are essential for triggering the translocation of PR3 on the surface of neutrophils. Thus, IL-17 is likely involved in the recruitment of neutrophils and other immune cells to the site of inflammation, which contribute to granuloma formation and also to create conditions for ANCA-induced neutrophil-dependent endothelial cell lysis. In GPA patients, skewing toward Th17 cells and increased serum IL-17A as well as increased MPO and PR3 specific Th17 cells were reported.

The most convincing experimental evidence that Th17 cells contribute to the pathophysiology of AAV comes from a recent study by Gan et al. [30]. They studied the effect of IL-17A, the key Th17 effector cytokine, on the development of necrotizing glomerulonephritis (NG) mediated by anti-MPO autoimmunity in IL-17A deficient mice in comparison with C57BL/6 wild-type mice. Both mice were immunized with MPO and developed cellular and humoral autoimmune responses to MPO. Glomerular injury in those mice was induced by injecting a low dose of heterologous anti-GBM, which triggered NG by recruiting neutrophils to glomeruli. They found that MPO-immunized C57BL/6 wild-type mice showed significant glomerular injury, whereas the glomeruli in IL-17-deficient mice were nearly completely protected due to diminished neutrophil accumulation and MPO deposition. This suggests a crucial role of IL-17A in renal tissue injury. Besides IL-17, Th17 cells can produce IL-21, a cytokine that is produced primarily by T follicular helper ($T_{FH}$) cells and is required for B-cell class switching and antibody production, and which induces differentiation of B-cells towards plasma cells by

synergizing with B-cell activating factor (BAFF) [31,32]. We have recently demonstrated that IL-21 producing $T_{FH}$ were significantly increased in peripheral blood of GPA-patients [33]. The role of IL-21 in vasculitis was previously suggested by Chen et al. [34]. In their study, mice deficient in interferon regulatory factor-4, a protein that inhibits IL-17A production, rapidly developed large-vessel vasculitis and showed increased IL-21 synthesis in addition to increased IL-17A production. Moreover, a role of IL-21 in recruitment of Th17 cells to inflamed tissues has been reported by Caruso et. al. [35] by showing that IL-21 induces gut epithelial cells to secrete macrophage inflammatory protein-3α (MIP-3α), a chemokine that mediates Th17 cell homing to the skin, joints, and mucosal tissues. Given that endothelial cells are known to produce MIP-3α, it is possible that IL-21 in GPA-patients enhances the migration and accumulation of Th17 cells into the vascular wall resulting in inflammation. Besides, IL-21 was shown to enhance granzyme B expression and increase perforin-mediated cytotoxicity by human $CD8^+$ T-cells and NK cells [36-38]. It is therefore conceivable that IL-21, together with IL-17, can contribute to vessel injury and disease progression in GPA-patients.

Taken together, activated $CD4^+$ $T_{EM}$ cells and their effector cytokines (IL-17 and IL-21) are believed to be inducers of tissue injury, and serve as effector cells in the pathogenesis of AAV.

## How and when do $CD4^+$ $T_{EM}$ cells attack endothelial cells in ANCA-associated vasculitis

Over the past decades, considerable research effort has been directed toward investigating and elucidating the pathogenic role of T-cells in endothelial injury in AAV. According to the aforementioned findings, we hypothesize that $CD4^+$ $T_{EM}$ cells act as a key trigger of disease expression

and relapse and are an important player in endothelial injury in AAV. So the question arises: How can $CD4^+$ $T_{EM}$ cells attack endothelial cells and when?

At the functional level, $CD4^+$ $T_{EM}$ cells shown to mimic NK cells by their cytotoxicity and surface expression of the NKG2D molecule [39]. NKG2D is an activating C-type lectin-like homodimeric receptor, which differs from other NKG2 members as it apparently lacks an antagonist and substitutes for CD28-mediated costimulatory signalling in $CD28^-$ $T_{EM}$ cells [40]. One of the NKG2D-ligands is the major histocompatibility complex class-I chain-related molecule A (MICA), which is usually absent on normal cells, but expressed upon cellular injury and stress on target cells such as fibroblasts, epithelial cells, and endothelial cells [41]. The expression of MICA on the surface of endothelial cells makes this polymorphic molecule a possible target in vasculitis. It has been shown that $NKG2D^+CD4^+$ T-cells can kill target cells that express MICA via NKG2D-MICA interaction [42]. Many clinical studies have shown that that the presence of MICA on kidney or heart transplant samples after transplantation is associated with acute or chronic allograft rejection [43-46]. Importantly, NKG2D was anomalously expressed and preferentially detected on circulating $CD4^+$ $T_{EM}$ cells in GPA-patients [47]. It has been reported that IL-15 is the major inducer of $NKG2D^+CD4^+$ T-cells expansion in GPA [48]. In addition, MICA is upregulated in peritubular endothelium and glomerular epithelial cells in AAV-patients during active renal disease. Strikingly, Capraru and colleagues have shown that both NKG2D and MICA are expressed in granulomatous lesions in GPA, but not in disease controls [49]. Therefore, it is likely that killing mechanisms via NKG2D-MICA interaction contribute to vessel injury and disease progression in AAV-patients.

Based on aforementioned finding, we can postulate that expanded population of $CD4^+$ $T_{EM}$ cells, resulting from persistent activation of Th-cells by PR3 or MPO, upregulate their NKG2D protein and migrate to the peripheral

blood, and remain in the circulation during remission. When the disease becomes active, MICA protein will be upregulated on several vascular endothelial cells, especially in the kidney, which attracts $T_{EM}$ cells to the inflammatory areas. The MICA protein on the target cells can bind to NKG2D on $T_{EM}$ cells, which in turn enhances their cytotoxic function, that is killing target cells in a perforin and granzyme dependent way which results in vasculitis. Accordingly, selective targeting of $NKG2D^{+}CD4^{+}T_{EM}$ or inhibiting MICA-expression without impairing other parts of cellular immunity might have value in the treatment of AAV.

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