Dendritic cells in renal biopsies of patients with ANCA-associated vasculitis

Benjamin Wilde1,2, Pieter van Paassen1, Jan Damoiseaux1, Petra Heerings-Rewinkel1, Henk van Rie1, Oliver Witzke2 and Jan Willem Cohen Tervaert1

1 Division of Clinical and Experimental Immunology, Department of Internal Medicine, University Hospital Maastricht, Maastricht, The Netherlands and 2 Department of Nephrology, University Duisburg-Essen, Essen, Germany

Correspondence and offprint requests to: Jan Willem Cohen Tervaert; E-mail: Secretariaat-IMMUNO@IMMUNO.unimaas.nl

Abstract

Background. Dendritic cells (DCs) maintain immune tolerance and are able to initiate immune responses. Their involvement in ANCA-associated vasculitis (AAV) is unknown. In this study, the participation of DC subsets is investigated in renal biopsies of AAV patients.

Method. A total of 25 patients with biopsy-proven AAV and five healthy controls (HC) with normal renal histology were included. Renal biopsies were stained for mature (CD208), immature (CD209), plasmacytoid (CD303) and Langerhans (CD1a) DC subsets. Furthermore, T-cells were stained using a T-cell marker (CD3). The interstitial cellular infiltrate was graded semi-quantitatively from 0+ (absence of cells) to 3+ (numerous cells). Within the glomeruli, an absolute count was performed for positive cells.

Results. CD208+ and CD209+ cells were found within patients’ glomeruli but not in HC (1 ± 0.3 versus 0.08 ± 0.1 cells/glom; 2 ± 0.3 versus 0.1 ± 0.07 cells/glom). An average of 0.3 ± 0.1 cell/glom expressed CD3 in patients while few cells were found in HC (0.1 ± 0.07 cell/glom). Focal interstitial cellular infiltrates were observed in patients’ biopsies but not in HC. Interstitial infiltration with CD3+ and CD209+ cells was assessed at an average of 1+, but some glomeruli and tubuli were surrounded by CD3+ and CD209+ cells forming clusters. Serial sections revealed that CD209+ cells were present in CD3+ rich areas.

Conclusion. Both mature and immature glomerular DCs are found in renal biopsies of patients with AAV. Immature DCs cluster with T-cells in interstitial infiltrates in these biopsies. Since DCs form aggregates in T-cell areas, we hypothesize that these cells interact with each other and are involved in lymphoid neogenesis.

Keywords: ANCA; AAV; dendritic cells; Wegener’s Granulomatosis

Introduction

ANCA-associated vasculitis (AAV) is a form of small-vessel vasculitis usually presenting with one of three different disease manifestations: Churg–Strauss syndrome (CSS), microscopic polyangiitis (MPA) and Wegener’s granulomatosis (WG). The disease mechanisms and the aetiology are not completely understood [1]. T-cells contribute to disease pathogenesis [2]. So far, it is not known where and how these T-cells are activated by antigen-presenting cells (APCs).

Dendritic cells (DCs), a special subset of APC, initiate and maintain immune responses under physiological circumstances [3]. Immature DCs reside in peripheral tissue waiting to phagocytose and process antigens. When these ‘sentinel’ DCs are facing antigens in the context of a danger signal, they start a lengthy maturation process running through different stages of maturity [4]. Meanwhile, these DCs traffic from peripheral tissue to lymphoid organs where they finally present the antigen to T-cells resulting in activation of these cells. There is evidence that the stage of maturation determines whether immunity or tolerance is induced [5]. In accordance with this model, immature or semi-mature DCs can induce tolerance whereas fully matured DCs have the capability to initiate immune responses [6]. Two distinct markers are known to distinguish mature and immature DCs. CD209 (DC-SIGN) is mainly expressed by immature DCs, whereas CD208 (DC-LAMP) is up-regulated on mature DCs [7–9]. Costimulatory markers, e.g. CD80 provide additional information on the maturity of the DC. DCs expressing costimulatory molecules are considered to be in a maturation process [10]. DCs also play a pivotal role in lymphoid neogenesis that is observed in chronic inflammatory responses. Lymphoid neogenesis means neoformation of tertiary lymphoid tissues within tissues affected by inflammation [11]. This is observed in several autoimmune diseases and during rejection in organ transplantation. The tertiary lymphoid tissue contains all subsets of immune cells including DCs and T-cells [12]. The immune responses seem to be maintained and controlled by these tertiary lymphoid tissues.

So far, it has not been shown whether and which subsets of DCs take part in inflammatory processes in AAV. This study assesses the participation of DC subsets in inflammation due to AAV in kidney biopsies.
Material and methods

Patients
A total of 25 consecutive patients with AAV presenting with biopsy-proven renal involvement from 2003 until 2006 were included into this study (21 males and 4 females, mean age 58 ± 3 years, Table 1). Diagnoses were made in accordance to the Chapel Hill criteria as well as in accordance to the American College of Rheumatology criteria [13,14]. Parameters such as serum creatinine, proteinuria and ANCA-titres were obtained at the time of the biopsy (Table 1). Five biopsies with normal histology and normal electron microscopy findings served as negative controls.

Frozen sections
Renal tissue was snap-frozen in cold (−80°C) isopentane and stored at −80°C. Serial cryostate (Adamas, Leersum, The Netherlands) sections were cut at 4µm and applied to a coated glass slide.

Immunohistochemistry
Slides were immediately fixed in cold (−20°C) acetone for 10 min after cutting and then air-dried followed by neutralization of endogenous peroxidase with 0.3% H2O2 at room temperature (RT). Each of the slides was incubated with one of the following primary antibodies: anti-CD1a (1:20); anti-CD209 (1:20) (Abcam, Cambridge, UK); anti-CD3 (1:50), anti-CD68 (1:50) and anti-CD19 (1:500) (DAKO, Heverlee, Belgium); anti-CD80, clone 37711, mouse IgG1, R & D Systems, Wiesbaden, Germany). Incubation with the second primary antibody (anti-CD209, clone DCN46, mouse IgG2b, Dianova, Hamburg, Germany). Incubation with the second primary antibody (anti-CD80, clone 37711, mouse IgG1, R & D Systems, Wiesbaden, Germany) and staining with the second secondary antibody (goat Cy3-conjugated, subclass specific anti-mouse IgG2b, Dianova, Hamburg, Germany). Incubation with the second primary antibody (anti-CD80, clone 37711, mouse IgG1, R & D Systems, Wiesbaden, Germany) and staining with the second secondary antibody (goat Cy3-conjugated, subclass specific anti-Mouse IgG1, Dianova) was followed. Several washing steps were inserted in between. Appropriate, subclass-specific isotype controls were used.

Evaluation of renal specimen
Renal sections were examined, and labelled cells were counted three times to reduce counting errors. Results were confirmed in a double-blinded fashion by one of our group (PvP). Glomeruli within the sections were counted and expressed as glomeruli per biopsy. The labelled cells within glomeruli were counted and are shown as cells/glomerular cross section. If the Bowman’s capsule was destructed extensively so that the glomerular compartment could not be delineated anymore or if the location of positive cells was not with certainty within the borders of the Bowman’s capsule, these cells were not considered as residing within the glomeruli.

Furthermore, an additional cell count was performed semi-quantitatively and done separately for peri-glomerular and peri-tubular interstitial localization. A score ranging from 0 (0 = absence of mononuclear cell infiltration, 1 = few mononuclear cell infiltration, 2 = moderate mononuclear cell infiltration) to 3 (3 = severe mononuclear cell infiltration) was used for assessing severity of cell infiltration. Depending on the size of the biopsy, 8–16 high power fields were evaluated and each scored. Finally, the mean score for each renal specimen was calculated out of this data. Serial sections were used to reveal colocalizations.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>MPO (N = 10)</th>
<th>PR3 (N = 15)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>61 (range 34–79)</td>
<td>64 (range 21–74)</td>
</tr>
<tr>
<td>Sex</td>
<td>8M/2F</td>
<td>13M/2F</td>
</tr>
<tr>
<td>Scrat creat/µmol/l</td>
<td>325 (range 108–674)</td>
<td>210 (range 80–826)</td>
</tr>
<tr>
<td>Uprot/creat g/mol</td>
<td>100 (range 17–808)</td>
<td>101 (range 7–677)</td>
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</table>

Statistical analysis
All data were analysed with Statistica Version 7.1. (StatSoft Inc, Tulsa, USA). Non-parametric Mann–Whitney U-test was used to compare cell counts between two groups. Spearman’s rank correlation was performed to reveal correlations between mononuclear cell infiltration and clinical data. A P-value < 0.05 was considered as significant.

Results
Patients’ biopsies contained an average of 12 ± 4 (range 5–26) glomeruli, whereas 11 ± 3 (range 9–15) glomeruli were found in healthy controls (HC). Focal interstitial cellular infiltrates were found in all patients’ biopsies. These infiltrates were localized around the glomeruli and in the peri-tubular interstitium. In both cases, the infiltrate was strongly positive for CD209 suggesting the presence of immature DCs (Figure 1A and B). Only few cells expressing markers for other DC subsets (CD208, CD1a or CD303) were detectable (Figure 2). CD209+ cells were found to form clusters around the glomeruli and in the peri-tubular interstitial compartment (Figure 1A and B).

The peri-glomerular CD209+ infiltrates were semi-quantitatively scored with 0.8 ± 0.5; a stronger presence of CD209+ cells was apparent in peri-tubular interstitial areas (1 ± 0.4) (Figure 1B, Table 2). These cells were forming aggregates in most cases but diffuse infiltrates were observed, too.

Next, stainings for other cell subsets were performed to evaluate colocalization with DCs. T-cells (CD3+) were localized within areas containing CD209+ DCs (Figure 3A and B, Table 2). T-cells (CD3+) were distributed around the glomeruli and present in the peri-tubular interstitial compartment (0.6 ± 0.4 and 0.9 ± 0.4). B-cells (CD19+) were scarcely present in only four renal AAV specimens.

As revealed by serial sections, CD68+ expression was found to be present in CD209+ rich areas around the glomeruli as well as in the peri-tubular interstitial compartment (0.7 ± 0.3 and 1 ± 0.3).

To evaluate the activation state of DCs, CD209+ aggregates were assessed for CD80 expression using serial sections. CD80 expression was demonstrated in some CD209+ areas around the glomeruli and in the peri-tubular interstitium (0.4 ± 0.5 and 0.4 ± 0.4) (Figures 3C and 4). This was confirmed by double immunofluorescence staining (Figure 5).

Furthermore, immunostaining revealed CD209+ and CD208+ DCs within patients’ glomeruli; a mean of 2.5 ± 1.8 cells/glom expressed CD209 whereas CD208 was present on 0.8 ± 0.8 cell/glom (Table 2). Macrophages (CD68+) were present within the glomeruli, as well (2.7 ± 1.7 cells/glom). T-cells were rarely detected (0.4 ± 0.5 cell/glom). B-cells were not found within glomeruli.
Dendritic cells in AAV

Fig. 1. DC subsets in ANCA-associated glomerulonephritis. Representative sections from one renal biopsy at a magnification of ×200 and ×400 are shown. Immature DCs were stained with CD209 (DC-SIGN). Immature DCs were abundantly present around the glomerulus (A) and in the interstitial compartment (B), partly forming aggregates.

Fig. 2. Enumeration of DC subsets in patients’ renal specimens (tubulo-interstitium). DC subsets were quantified as described in the Material and methods section. Immature DCs were abundantly found (1 ± 0.4) whereas other DC subsets were present in small numbers (mature DCs: 0.1 ± 0.2 Langhans DCs: 0.0 ± 0.1 Plasmacytoid DCs: 0.1 ± 0.1).

The amount of immature DCs showed no significant correlation with clinical parameters such as creatinine, proteinuria, PR3-ANCA or MPO-ANCA-levels.

Kidney biopsies from HC were lacking T-cells (CD3+), and specific DC subsets (CD1a, CD208+). Very few interstitial cells expressing CD68, CD80, CD303 or CD209 were detected.

HC showed no macrophages or DCs within glomeruli.

Discussion

This study emphasises the participation of DC subsets in the inflammatory process of AAV. Previous studies in other diseases such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and giant cell arteritis already referred to the relevance of DCs in autoimmunity [15–18].

Numerous immature CD209+ DCs forming aggregates were present in the renal specimen in our study. Woltman et al. recently demonstrated the presence of immature CD209+ DCs in healthy kidneys as observed in our HC biopsies [19]. However, DC aggregates as in our AAV patients were neither present in our HC biopsies nor in the healthy kidneys examined by Woltman et al. [19].

Recently, Segerer et al. reported a relative absence of CD209+ DCs from glomeruli in patients with different types of renal diseases [20]. This is in contrast to our findings since we found CD209+ DCs in glomeruli in diseased patients. Segerer et al. studied renal biopsies from eight patients with necrotizing GN (NGN). The diagnosis in these patients was not further specified [20]. NGN may be associated with AAV, anti-GBM disease, subacute bacterial endocarditis, Henoch–Schönlein purpura and other disease entities [21]. Thus, a more heterogenous patient population that is different from ours might have contributed to the varying observations. Moreover, focal destruction of the Bowman’s capsule common in AAV might have facilitated the influx of DC’s from the interstitium in our study. The CD209 molecule promotes interaction with T-cells as well as neutrophils and confers the potential to modify immune responses [8,22,23]. Segerer et al. reported co-staining of CD209 and CD68 in the interstitium, but not in glomeruli suggesting that DCs infiltrate the interstitium and macrophages the glomeruli. In AAV, however, we found that DCs infiltrated not only the interstitium but also glomeruli.

### Table 2.

<table>
<thead>
<tr>
<th>Cell type/Location</th>
<th>Tubulo-interstitium (score)</th>
<th>Peri-glomerular compartment (score)</th>
<th>Within glomerulus (cells/glom)</th>
</tr>
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<tbody>
<tr>
<td>Immature DC</td>
<td>1.0 ± 0.4</td>
<td>0.8 ± 0.5</td>
<td>2.5 ± 1.8</td>
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<tr>
<td>Mature DC</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.2</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1.0 ± 0.3</td>
<td>0.7 ± 0.3</td>
<td>2.7 ± 1.7</td>
</tr>
<tr>
<td>Activation marker</td>
<td>0.4 ± 0.5</td>
<td>0.4 ± 0.4</td>
<td>1.6 ± 2</td>
</tr>
<tr>
<td>T-cells</td>
<td>0.9 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td>Langhans DC</td>
<td>0.04 ± 0.08</td>
<td>0.05 ± 0.07</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>Plasmacytoid DC</td>
<td>0.1 ± 0.1</td>
<td>0.06 ± 0.1</td>
<td>0.3 ± 0.5</td>
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Woltman et al. recently demonstrated the presence of immature CD209+ DCs in healthy kidneys as observed in our...
An influx of immature DCs into the peripheral tissue is caused under inflammatory conditions by secretion of chemoattractants [24,25]. After migration into the peripheral tissue, these immature DCs capture and process antigen that is released due to inflammation. Subsequently, the antigen is presented in the lymph node leading to modulation of the immune response. As shown by other authors before, this is a physiological phenomenon [24,26]. This might explain the presence of immature DCs in AAV to some extent.

However, there is some evidence that these physiological mechanisms or associated pathways may be altered in autoimmune disease such as AAV [12,25]. Bjerkeli et al. recently reported that serum levels of the chemokine CX3CL1 are elevated in patients with WG [27]. This chemokine enhances the recruitment of DCs to the peripheral tissue [28,29]. Moreover, Zhou et al. detected genetic alterations of the chemokine receptor 5 (CCR5) in patients suffering from WG [30]. This receptor and its ligands are crucial for DCs leaving vessels and entering peripheral tissue as receptor blocking abrogates migratory influx [31]. Thus, the large amounts of immature DCs located in kidney biopsies of AAV patients could be the result of an increased influx with these DCs evoked by altered migration pathways. An increased migration of DCs could lead to an intensified
presentation of self-antigens in the draining lymph node thus sustaining or facilitating the inflammatory response [32].

The increased presence of DCs might also be caused by a reduced exit to lymphoid organs as has been demonstrated for giant cell arteritis [16]. Krupa et al. revealed that DCs located in inflamed vessels were trapped and thus not able to leave the peripheral tissue. Maturation and immune modulation took place in the peripheral tissue [16]. However, whether the DCs located in AAV remain in the kidney or are on their way to enter the lymph nodes cannot be ascertained from our study.

The immature DCs colocalized with T-cells. Since DCs belong to the professional APC, an immune interaction between T-cells and DCs is likely. Therefore, the immune response in the kidney itself might show some features of lymphoid neogenesis as communication between T-cells and DCs normally happens in lymphoid organs but rarely in peripheral tissue [11].

However, typical tertiary lymphoid organs (TLO) in the context of lymphoid neogenesis were not present as B-cell follicles were missing. Because of a chance of sampling error due to the small size of the biopsy as compared to the size of the kidney, the presence of B-cell follicles and thus typical tertiary lymphoid tissue cannot be excluded with certainty.

It has to be pointed out that the DCs colocalizing with T-cells were immature. Immature DCs in general are able to induce tolerance and normally lack costimulators [3,4,33–35]. Therefore, antigen presentation by immature DCs to T-cells results in anergy or induction of regulatory T-cells [4,33,35]. Thus, the DCs present in the renal specimen might have a dampening function on the inflammation in the kidney.

However, some immature DCs in our study expressed CD80 that is widely known as a costimulatory molecule [37]. Csornok et al. showed the WG autoantigen PR3 to up-regulate CD80 and to induce maturation of DCs. These DCs were capable of stimulating T-cells [38]. Indeed PR3-positive cells such as monocytes and neutrophils are present in the interstitium and might be activated by ANCA resulting in local release of PR3 as reported by Brouwer et al. [39]. Subsequently, PR3 and the presence of PR3-positive cells could promote CD80 expression on the DCs found in our renal specimen, thus driving the inflammatory response.

Moreover, a proinflammatory role for immature DCs was recently shown by Mizoguchi et al. recently. In this study, immature DCs were predominant key players regarding granuloma formation and cytokine production in experimental models of intestinal inflammation [40]. These DCs were a source for IL-23 that induced proinflammatory Th17 T-cells. This novel T-cell subset is of major importance for autoimmune inflammation and could have a role in AAV [41–44]. The immature DCs present in kidney biopsies of patients with AAV may facilitate the inflammatory process by inducing Th17 T-cells and promoting granuloma formation.

Taken together, DCs may play a pathophysiological role in inflammation related to AAV. It is likely that these DCs modulate T-cell immune responses locally, thus showing some features of lymphoid neogenesis. Whether the inflammatory response is dampened or facilitated by this DC—T-cell interaction remains unknown. Further studies are needed to clarify these disease mechanisms.

Conflict of interest statement. None declared.

References
