Immune regulation in IgA nephropathy

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Chapter 3

Immuno-histological analysis of dendritic cells in nasal biopsies of IgA Nephropathy patients

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Summary

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide. Intranasal vaccination of patients with IgAN has shown mucosal and systemic IgA hyporesponsiveness. Here we investigated whether this IgA hyporesponse in IgAN patients can be explained by reduced numbers or altered subset distribution of dendritic cells (DCs) in nasal mucosa.

Eighteen IgAN patients and 18 healthy volunteers were recruited for this study. Nasal biopsies were taken, after local anaesthesia, from the lower edge of the inferior turbinate. Staining for different subsets of DCs was performed, using specific monoclonal antibodies. To detect myeloid dendritic cells we used CD1a, DC-SIGN and BDCA-1 as a marker and for plasmacytoid DCs we used BDCA-2. DC-cell numbers in the epithelium and in lamina propria were counted separately and expressed as positively stained cells per mm^2.

Both myeloid and plasmacytoid DC could be demonstrated in nasal biopsies. Quantification showed that IgAN patients contained significantly more DC-SIGN positive cells in the lamina propria compared to controls. In addition in IgAN patients we observed more CD1a positive cells in the epithelium. No differences in BDCA-1 and BDCA-2 positive cells were found between patients and controls. The number of positively stained cells in the epithelial layer correlated strongly with the number of positively stained cells in the lamina propria.

Patients with IgAN have higher numbers of CD1a positive cells in the epithelial layer and more DC-SIGN positive cells in the lamina propria. Therefore the earlier observed IgA hyporesponsiveness in IgAN patients after mucosal vaccination cannot be explained by lower numbers of nasal DCs.

Introduction

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide [1-3]. The disease is characterized by depositions of IgA1 in the mesangium of the kidney [4]. Recurrence of IgAN after renal transplantation is very common, indicating that next to renal factors, specific characteristics of IgA itself (quantitative and/or qualitatively) play a role in the pathogenesis [5]. IgA serum titers are increased in 50% of the patients, but the fact that other diseases with higher serum IgA titers, like HIV infection, are not associated with IgA depositions in the kidney, shows that higher serum levels of IgA are not the only cause of IgAN [6;7]. Alternatively, qualitative alterations in IgA such as differences in glycosylation, are thought to contribute to disease pathogenesis [8;9].

Although the pathogenesis of the disease is not clear, there are reasons to believe that immune responses at mucosal surfaces play an important role. This idea stems from the observation that an upper respiratory tract infection in about 40% of IgAN patients leads to aggravation of their complaints in the form of increased blood levels in urine (synpharyngitic hematuria) [10]. It has been proposed that tonsils are responsible for the increased IgA levels in the serum [11]. Retrospective studies suggest that tonsillectomy is correlated with a favourable clinical course of the disease [12]. Several immunization studies, using different antigens and different routes of administration have shown conflicting results [13;14]. Simultaneous mucosal and systemic vaccination in IgAN patients with two neoantigens lead to an impaired IgA response upon the mucosal challenge, whereas no differences were seen after systemic vaccination [15;16].

Given the role of dendritic cells (DCs) as professional antigen presenting cells involved in the initiation of immune responses and antibody formation, there is some suggestion that aberrant DC function contributes to IgAN. Not only do they link innate and adaptive immunity, but DCs might also be decisive via the interaction with T and B cells for the induction of effector immune responses or tolerance [12;17;18]. DCs have a direct effect on B cells and can induce isotype switching and enhance immunoglobulin

production [19;21]. In the presence of CD40 activated naïve B cells, DC can skew isotype switching towards IgA [19]. Recently we have shown in an in vitro model that DC of IgAN patients induce less IgA production by naïve B cells compared to DC from control persons [22].

Different subsets of DCs have been described, based on phenotypic characteristics. There are two major subsets of DCs, namely myeloid DCs and plasmacytoid DCs. The myeloid DCs are found at two sites in tissues: Langerhans cells, which express the surface marker langerin and CD1a are located mainly in the epithelial layer, and interstitial DCs, expressing the C-type lectin DC-SIGN and/or the blood dendritic cell antigen 1 (BDCA-1). Plasmacytoid DCs are characterized by expression of BDCA-2 and /or CD123 [23-25]. These two different subsets of DCs have different functions, that are only partially characterized, and might be dependent on the activation status and the localisation of the DC [2;26]. In addition interactions between subsets of DCs and cells that are locally present, may determine the outcome of an immune response. The effect of the mucosal microenvironment on DC response, and thereby on IgA production is still unclear.

Based on the described hyporesponsiveness upon mucosal challenge of IgAN patients, and the central role of DCs in initiating immune responses, and our observation that DC from patients with IgAN induce less production of IgA by B cells, we hypothesised that IgAN patients in addition might have reduced numbers of DCs or that IgAN patients may have an altered composition of certain DC subsets at mucosal sites. To answer this question, nasal biopsies of 18 IgAN patients and 18 control persons were stained for specific DC subset markers and the results analysed with respect to DC cell numbers and subset composition.

Materials and methods

Selection of IgAN patients and control persons

Patients with biopsy proven IgAN were selected for entry in this study. Control persons in this study are persons with no underlying immunological

disease, who visited the ear nose throat department for a surgical nasal septum correction and were asked for permission for a nasal biopsy during this operation. Neither patients nor controls were using immunosuppressive drugs at the time of the study nor in the three months before biopsies were taken. No infections or macroscopic hematuria were present at the time of the study.

Mean age of the patients was 51 years (range 23-71) versus 39 years (range 10-59) of the controls (n.s.). The male: female ratio was 78% versus 22% in the IgAN patient group and 72% versus 28% in the control group. Renal function was stable in most of the patients. Mean creatinine clearance was 78 ml/min (range 24-136). Mean proteinuria was 1.3 gram per litre (range 0-4.7). Neither the IgAN patients nor the control persons had an infection of the upper respiratory tract at the time of biopsy.

The study was approved by the ethical committee of the Leiden University Medical Centre. All individuals gave informed consent.

Nasal biopsies

One nasal biopsy was taken from each person. All biopsies were taken by the same investigator. Local anaesthesia was achieved by applying a cotton wool carrier with 50-100 mg of cocaine and 3 drops of adrenaline (1:1000) under the inferior turbinate, without touching the biopsy site. Biopsy specimens were taken from the lower edge of the inferior turbinate by using a Gerritsma forceps [27] and embedded in Tissue-Tek II OCT compound. The biopsies were immediately frozen and stored at -150 ^{o}C.

Immunohistochemical staining

Each tissue specimen was cut into 6µm-thick sections. All stainings were developed with the immunoalkaline phosphatase method, as previously described [28]. All cryosections that were accepted for evaluation had an undamaged basal membrane of at least 1 mm length and a surface of the lamina propria of at least 1 mm^2. The mean length of the basal membrane was 3.8 mm and the mean surface of the epithelial layer was 3.0 mm^2. Multiple fields were counted and the number of cells is expressed as positively stained cells per mm^2. As a negative control we replaced the primary antibody by

mouse IgG. Different subsets of DCs were stained using specific monoclonal antibodies. Myeloid dendritic cells were stained using mouse monoclonal antibodies directed to CD1a (Leu 6, Becton Dickinson, San Jose CA), anti DC-SIGN (CD209, DC-SIGN 1 R&D) or BDCA-1 (clone AD5-8E7; Miltenyi Biotec). Plasmacytoid DCs were stained with monoclonal antibodies directed to BDCA-2 (clone AC144; Miltenyi Biotec).

Biopsies were coded and counted for positively stained cells, independently by two investigators for each antibody, as has been described previously [28]. Positively stained cell numbers in the epithelium and in the subepithelial lamina propria, 100 μm deep along the basal membrane, were counted, using a light microscope (Zeiss, Jena, Germany) with an eye piece graticulate at a magnitude of 400x and were expressed as positively stained cells per mm^2.

Double staining

For double staining of BDCA-1 or BDCA-2 with DC-SIGN, acetone fixed sections were incubated with 0.1% H_2O_2 and 0.1% NaN_3 in PBS for 30 min at room temperature (RT) to block endogenous peroxidase activity. The slides were blocked with PBS containing 1% PBS and 5% heat inactivated normal human serum (NHS) for 45 minutes at RT and subsequently incubated with antibodies directed against BDCA-1 or BDCA-2 in PBS with 1% BSA and 1% NHS overnight at RT. Antibody binding was detected using a HRP labeled goat-anti-mouse antibody (Dako, Glostrup, Denmark) in PBS with 1% BSA and 1% NHS for 60 min at RT. Next, the slides were blocked again with PBS containing 1% PBS and 5% NHS for 45 minutes followed by an overnight incubation with antibody against DC-SIGN in PBS with 1% BSA and 1% NHS at RT. The presence of DC-SIGN antibody was detected by incubation with a rabbit-anti-mouse IgG2b specific antibody (Jackson, Suffolk, UK) in PBS with 1% BSA and 1% NHS at RT followed by detection with a biotinylated goat-anti-rabbit antibody (Dako, Glostrup, Denmark) in PBS with 1% BSA and 1% NHS and amplified with a streptavidin biotin complex containing alkaline phosphatase (Dako, Glostrup, Denmark). Eventually, development with Tyramide-FITC in tyramide buffer (NEN™ Life Science Products)

for 30 min at RT was used to detect the presence of BDCA-1 or BDCA-2 positive cells. For detection of DC-SIGN positive cells, Fast Red TR salt and naphtol tablets (both from Sigma-Aldrich, Zwijndrecht, The Netherlands) were dissolved according to manufacturers instructions. For development, equal amounts of Fast Red TR and naphtol solutions were mixed and added to sections for 15 minutes at RT. Slides were mounted with mowiol.

Statistical analysis

Statistical analysis was performed, using the Mann-Whitney test. The Spearman correlation coefficient was calculated to determine the correlation between the different subsets of DCs. P values of <0.05 were considered significant.

Results

Demonstration of DC subsets in nasal biopsies

We collected nasal biopsies from 18 IgAN patients and 18 control persons. Biopsies were analysed by hematoxylin eosin (HE) staining to check the quality of the tissue and confirm the presence of an epithelial layer (Figure 1A).

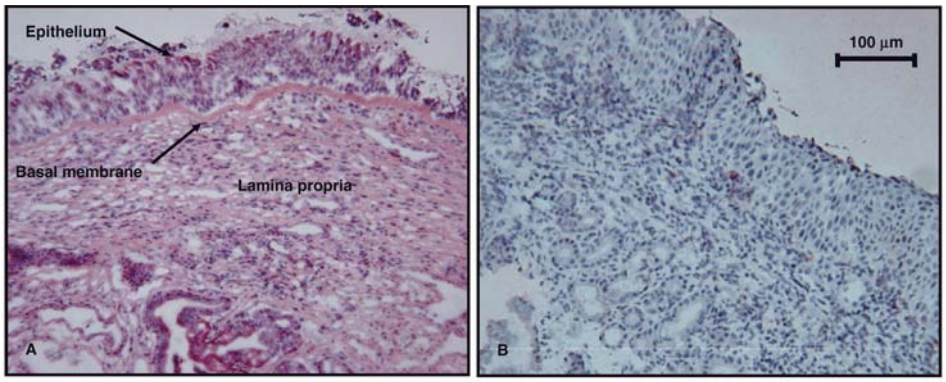

Figure 1. HE staining and negative control of nasal mucosa. Light microscopic section of nasal mucosa, stained with hematoxylin eosin at a magnification of 100x (A). The outer epithelial layer is divided from the lamina propria by the basal membrane. Negative control of a biopsy stained for BDCA-1 (B).

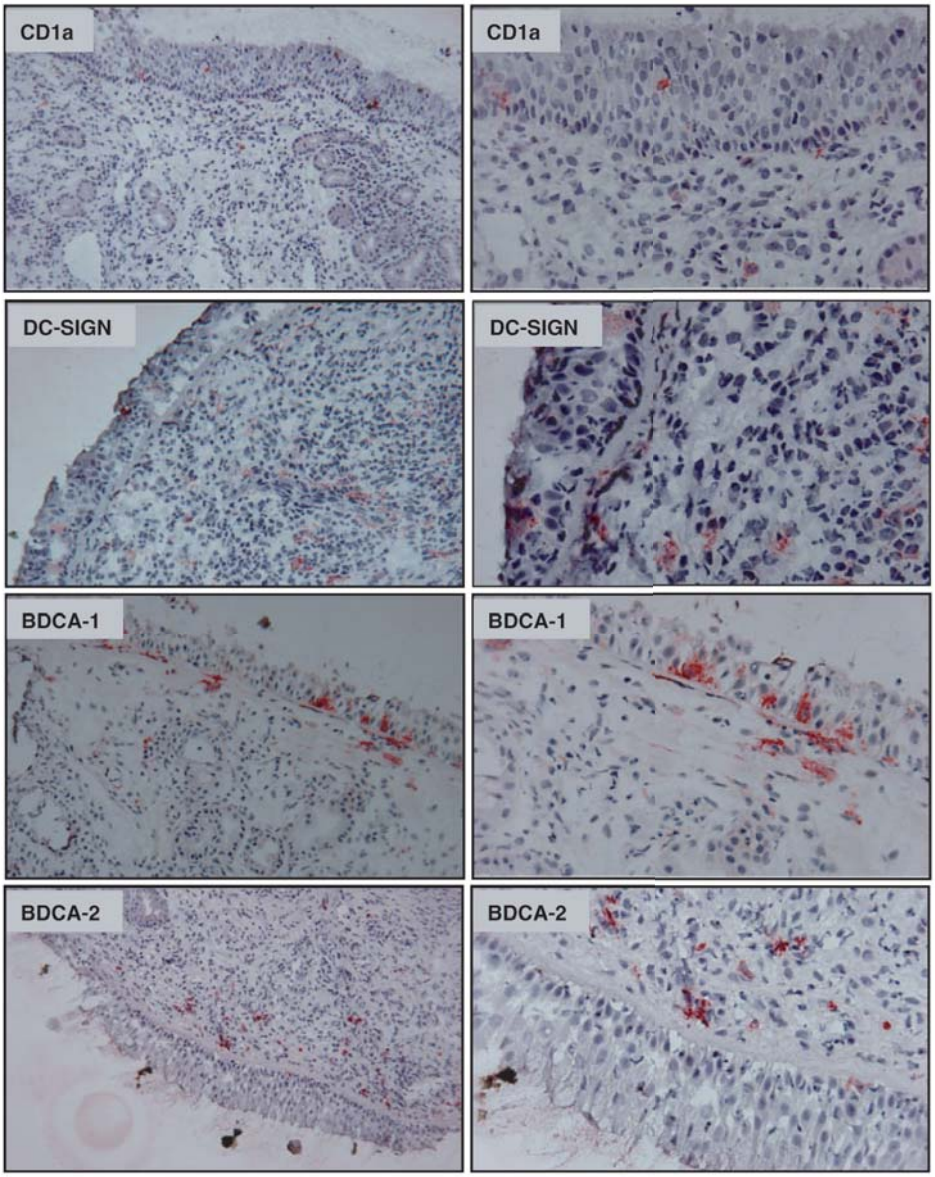

Figure 2. Expression of DC-SIGN, CD1a, BDCA-1 and BDCA-2 in nasal mucosa. Cryosections of nasal mucosa were stained for expression of DC-SIGN, CD1a, BDCA-1 and BDCA-2 as described in *material and methods*. The left panel shows cryosections at an original magnification of 100x and the right panel at a magnification of 250x. CD1a positive cells are more frequently located in the epithelial layer. DC-SIGN positive cells and BDCA-2 positive cell are mainly located in the lamina propria. Shown are representative pictures of IgAN patients.

Cryosections were specifically analysed for the presence of DCs using specific monoclonal antibodies. To identify myeloid DCs, cryosections were stained for CD1a, DC-SIGN and BDCA-1. To quantify plasmacytoid DCs, cryosections were stained for BDCA-2. After staining, the number of cells was counted and related to the surface tissue area and compared to the negative control stainings (Figure 1B).

Positive cells are coloured red and were counted by two investigators independently. In all cryosections an epithelial layer, with a variable thickness was visible. The epithelial layer is separated from the lamina propria by the basal membrane. In the lamina propria, glands and vessels are visible, which were excluded from the analysed surface. In both the epithelial layer and the lamina propria DCs are present. Pictures of representative cryosections with an original magnification of 100x (left panel) and 250x (right panel) are shown in figure 2.

Quantification of myeloid dendritic cells in nasal biopsies

Different subsets of myeloid DCs were identified, using CD1a, DC-SIGN and BDCA-1 as markers. Biopsies were stained for BDCA-1, which is a broadly expressed marker for myeloid DCs. Compared to CD1a and DC-SIGN the absolute number of positively stained cells was highest for BDCA-1. No differences in BDCA-1 positively stained cells between epithelial layer and lamina propria were present. The number of positively stained cells did not differ between the control group and the IgAN patients (Figure 3).

The number of DC-SIGN positive cells was higher in the lamina propria than in the epithelial layer. Moreover the number of DC-SIGN positive cells in the lamina propria was significantly higher in IgAN patients than in the control group (median 71.8 vs. 41.6 $p=0.022$). In contrast no significant differences were found in the number of DC-SIGN positive cells in the epithelium between the two groups (Figure 3).

In both the control group and in the IgAN patient group the number of CD1a positive cells was higher in the epithelial layer than in the lamina propria. The number of CD1a positive cells in the epithelial layer was significantly higher in IgAN patients than in control persons (median 72.8 vs

20.2, p=0.009). In contrast the number of CD1a positive cells in the lamina propria did not differ between the IgAN patient and the control group (Figure 3). We also looked at the actual ratio numbers of DC between the different compartments. No significant differences in tissue distribution of positively stained cells was present between the two groups with respect to the different myeloid markers (data not shown).

Quantification of plasmacytoid dendritic cells in nasal biopsies

Plasmacytoid DCs were stained using BDCA-2 as a surface marker. The number of positively stained cells in the lamina propria was higher than in the epithelial layer. No differences were found in the number of BDCA-2 positively stained cells between the IgAN patients and the control persons, neither in the epithelial layer, nor in the lamina propria (Figure 4). With respect to BDCA-2, no difference in actual ratio of numbers of DC between different sites was present between the IgAN patients and the controls. The number of plasmacytoid DC in these nasal biopsies was lower than the number of myeloid DC.

Correlations between the number of positively stained cells

To determine whether CD1a, DC-SIGN, BDCA-1 and BDCA-2 positively stained cells in the epithelial layer are correlated with the number of positively stained cells in the lamina propria, we calculated Spearman correlation coefficients. For all stainings the number of positively stained cells in the epithelial layer appeared to be correlated to the number positively stained cells in the subepithelial lamina propria. The correlation coefficient varied from 0.49 to 0.69 with p values of maximal 0.0017 (Figure 5). In both the control group and in the IgAN patients this correlation was found.

We also investigated the correlation between the different subsets of DCs. In the epithelial layer there was a correlation between $CD1a^{+}$ cells and $BDCA\text{-}1^{+}$ cells and a correlation between $DC\text{-}SIGN^{+}$ and $BDCA\text{-}1^{+}$. In the lamina propria no correlation between CD1a and BDCA-1 was found. Furthermore a correlation between epithelial BDCA-1 and epithelial BDCA-2 was present and between epithelial BDCA-1 and lamina propria BDCA-2 . (Table 1).

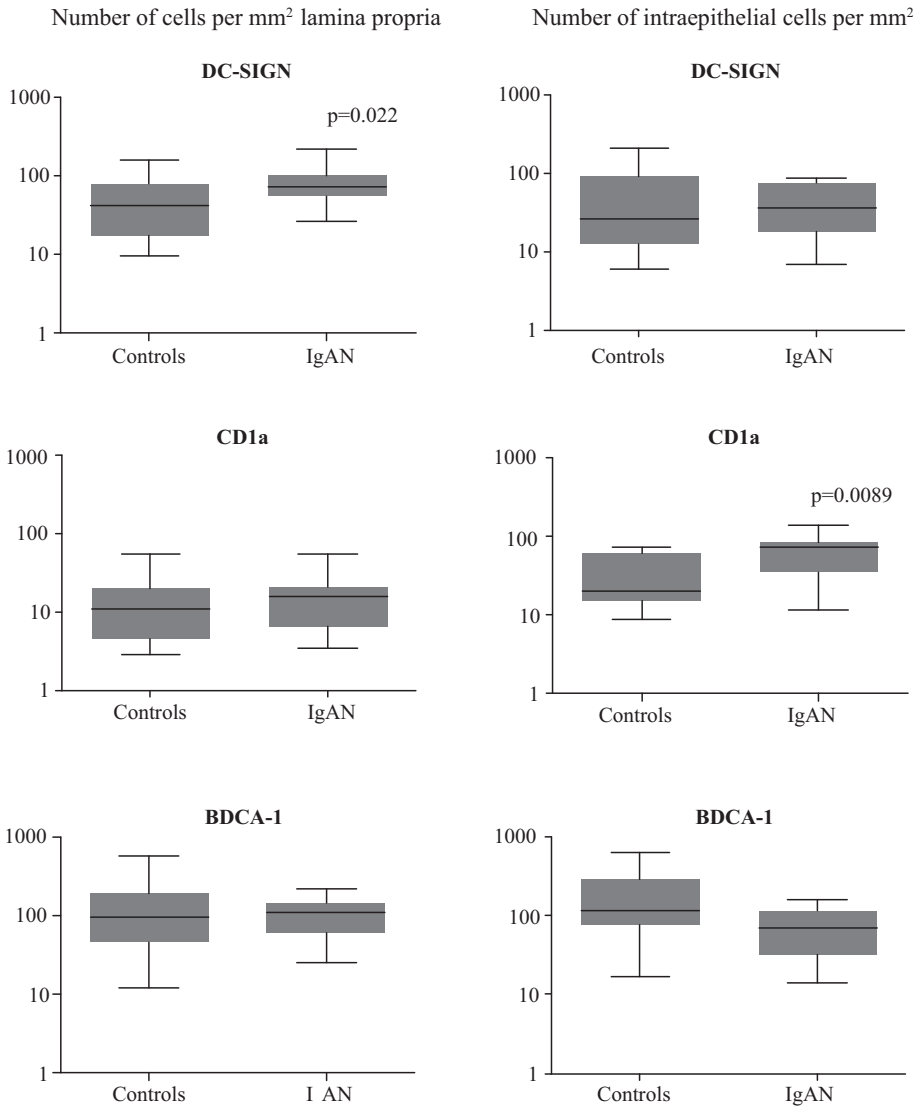

Figure 3. Number of different subsets of myeloid DCs in nasal mucosa of controls and IgAN patients. Cryosections of nasal biopsies from control persons and IgAN patients were stained for DC-SIGN, CD1a and BDCA-1 respectively. Positive cells in the epithelial layer (right panel) and in the lamina propria (left panel) were counted as described in materials and methods section. Bars represent median ± interquartile range and the smallest and greatest values in the distribution. Statistics were performed using the Mann-Whitney test. p values of <0.05 were considered significant.

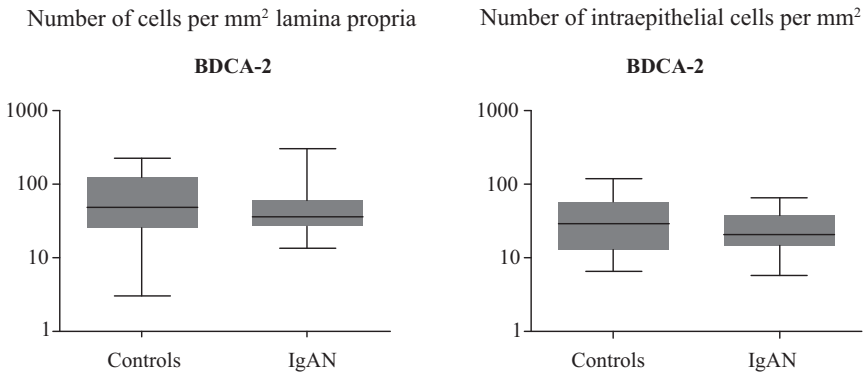

Figure 4. Number of plasmacytoid DCs in nasal mucosa of controls and IgAN patients. Cryosections of nasal biopsies from control persons and IgAN patients were stained for BDCA-2, a marker for plasmacytoid DCs. Positive cells in the epithelial layer and in the lamina propria were counted as described in materials and methods sections. Bars represent median ± interquartile range and the smallest and greatest values in the distribution. Statistics were performed using the Mann-Whitney test.

Table 1. Correlation of different subsets of DCs in nasal mucosa. Nasal mucosa was stained for the presence of different subsets of DCs. Spearman correlation coefficients were determined to calculate statistical relations between the different DC molecules. p values of <0.05 were considered significant.

	DC-SIGN epithelium	CD1a epithelium	BDCA-1 epithelium	BDCA-2 epithelium
DC-SIGN-E	x	n.s		
DC-SIGN-LP	r=0.69 ***	n.s	n.s.	n.s.
CD1a-E	n.s	x		
CD1a-LP	n.s	r=0.49 ***	n.s.	n.s.
BDCA-1-E	r=0.50 ***	r=0.39 *	x	
BDCA-1-LP	n.s	r=0.38*	r=0.60 ***	n.s.
BDCA-2-E	n.s	n.s	r=0.38 *	x
BDCA-2-LP	n.s	n.s	r=0.50 **	r=0.55 ***

* p<0.05 E=epithelium

** p< 0.01 LP=Lamina Propria

*** p<0.002

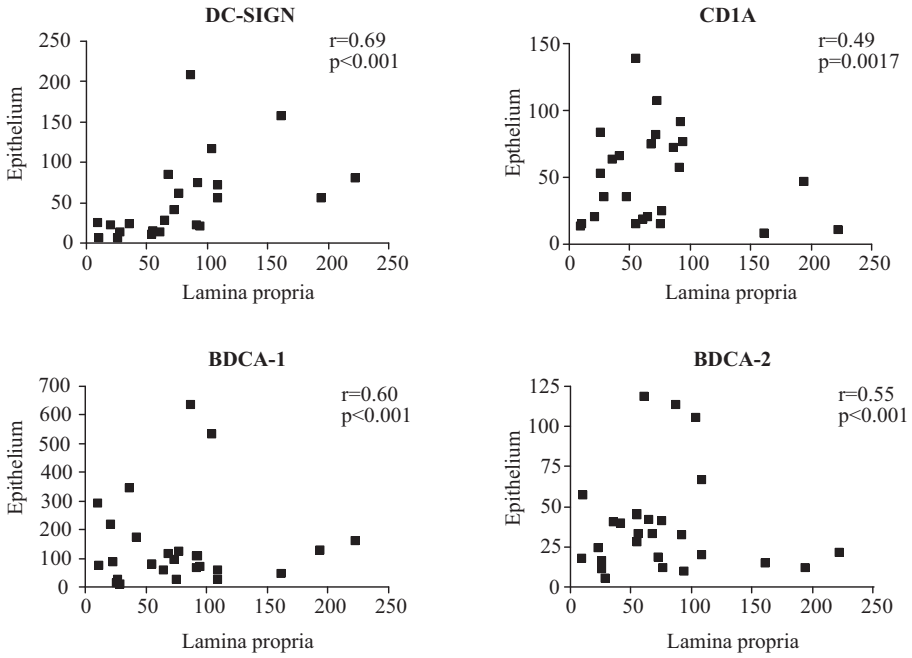

Figure 5. Correlation between DC-SIGN$^+$, CD1a$^+$, BDCA-1$^+$ and BDCA-2$^+$ cells in epithelial layer and lamina propria. Cryosections of nasal biopsies were stained for DC-SIGN, CD1a, BDCA-1 and BDCA-2, as described in materials and methods section. The number of positively stained cells in the lamina propria and epithelial layer are plotted. Spearman correlation coefficients were determined to calculate statistical relations. Filled squares represent DC counts of an individual biopsie.

Double staining for DC-SIGN and BDCA-1 or BDCA-2

To further investigate the correlations between the different markers of DCs, we performed double stainings for DC-SIGN and BDCA-1 or BDCA-2. When staining for two myeloid DC markers, DC-SIGN and BDCA-1 we found a substantial proportion of double positive cells. However also single positive cells were observed (Figure 6A). When double staining for DC-SIGN and BDCA-2 was investigated, we found no overlap in staining (Figure 6B). This is compatible with the fact that these two markers are expressed on DC subsets proposed to be derived from different lineages (myeloid versus lymphoid respectively).

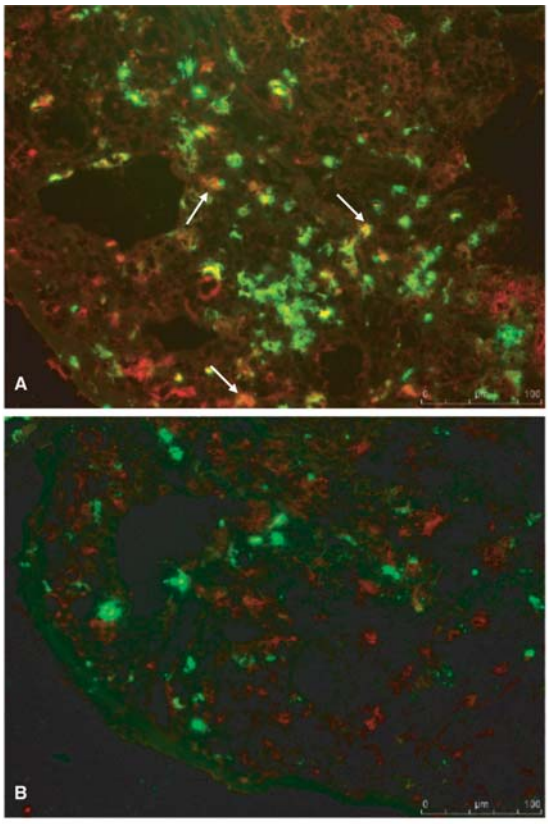

Figure 6. **Nasal biopsies contain DC-SIGN+ BDCA-1+ cells.** Cryosections of nasal biopsies were stained for DC-SIGN (green) and BDCA-1 (red) (A) or BDCA-2 (red) (B) as described in materials and methods sections. White arrows indicate DC-SIGN+ BDCA-1+ cells. Original magnifications were x200.

Discussion

In the present study we investigated the nasal mucosa of IgAN patients for the presence of different subsets and numbers of DCs, as previous experiments suggested an aberrant immunological response in these patients. After mucosal vaccination with a neoantigen, the specific IgA immune respons was hampered in IgAN patients in comparison with controls, whereas no

differences were found in the antigen specific IgG and IgM responses [15]. In the same study no differences were found in immunoglobulin production after simultaneous systemic vaccination with another neoantigen Keyhole Limpet Hemocyanin, (KLH). Therefore we wanted to address the question whether IgAN patients have reduced numbers of DCs in the nasal mucosa, or have a different composition of specific subsets of DCs. To this extent, nasal biopsies were taken from IgAN patients and controls and were stained for the presence of different subsets of DCs. Our results indicate that IgAN patients did not have reduced numbers of DCs, and even showed higher numbers of intra-epithelial $CD1a^+$ cells and DC-$SIGN^+$ cells in the lamina propria.

DCs as professional antigen presenting cells are present in the epithelial layer and form a continuous network [29]. DCs can migrate and after antigen uptake and processing can induce immune responses. DCs have direct effects on many different cells. It is well known that DC can activate naïve T cells, which subsequently can induce B cell responses. However also direct effects of DC on B cells are described, leading to isotype switching and enhanced immunoglobulin production [19;30;31]. These effects are dependent on CD40-CD40L interaction [32]. Recently it has been reported that DC can also induce Ig production by B cells in a CD40 independent way, through activation of BAFF and APRIL [33;34].

In the current study we investigated the number of nasal DCs of IgAN patients and a control group. Our hypothesis, that the number of nasal DCs in the IgAN patients was lower than the number of nasal DCs in the control group, appeared not to be true. As a matter of fact the number of CD1a positive cells in the nasal epithelial layer and the number of DC-SIGN positive cells in the nasal lamina propria were even significantly higher in the biopsies of the IgAN patients. This means that the formerly observed IgA hyporespons after nasal vaccination can not be explained by a reduced number of antigen presenting cells.

The different subsets of DCs in human are not fully functionally characterized and so far most knowledge about their function is from *in vitro* experiments. Dubois et al. have described an increase of IgA production via stimulation of naive B cells by CD34-derived interstitial DC, but no effect

on IgA production after stimulation with Langerhans cells which are derived from a CD1a positive precursor [31]. This suggests higher IgA production when DC-SIGN positive and BDCA-1 positive DCs are present. IgAN patients appeared to be hyporesponders after mucosal vaccination. Whether this hyporesponse is related to the higher numbers of CD1a positive DCs in the epithelial layer remains speculative. An alternative explanation for the mucosal hyporesponsiveness of IgAN patients, could be that DC are functioning well, but that there are defects in the B or T cells. Recently it was shown that $CD4^+$ T cells of patients with IgAN show an aberrant expression of homing receptors, potentially contributing to altered mucosal immune responses [3].

In vivo the type of DC that captures and presents an antigen to T cells is probably decisive whether a processed antigen gives rise to an immune response or is tolerated. In the pathogenesis of pulmonary diseases like asthma and chronic obstructive pulmonary disease DCs play a central role [36]. As DCs in the upper respiratory tract are in contact with many antigens each day, a dysfunction of this complex system might contribute to the pathogenesis of diseases in which the mucosa is involved, such as IgAN. The additional effect of local tissues on the function or phenotype of DCs *in vivo* is also unknown. It has recently been described by Allam et al. that mucosal myeloid $CD1a^+$ DCs differ from their skin counterparts by the expression of the high affinity receptor for immunoglobulin E [37].

Recently we have studied the capacity of monocyte-derived DCs from IgAN patients and control persons to induce IgA production in an *in vitro* model. We found a reduced capacity of DCs from IgAN patients to increase IgA production by naïve B cells, when DCs were cultured in the presence of IL-10 and CD40L [22]. Whether the increased number of CD1a and DC-SIGN positive DCs in the nasal biopsies of IgAN patients are a kind of compensation for the reduced functional capacity to increase IgA production is only speculative. It would be interesting to study functional activities of mucosal DC of IgAN patients. However techniques and availability of tissue are presently not sufficient to perform proper functional analysis. An alternative way to get more information on DC function could be further phenotypic analysis of tissue DC, preferably during antigen challenge.

In our study we found for all different stainings a strong correlation between the number of positively stained cells in the epithelial layer and in the lamina propria. We also calculated the correlation between different markers and found in the epithelial layer a strong correlation between $CD1a^+$ and $BDCA\text{-}1^+$ and a correlation between $DC\text{-}SIGN^+$ and $BDCA\text{-}1^+$ DCs. To further investigate this correlation we performed double staining for DC-SIGN and BDCA-1 and showed that there were double positive cells. This result is comparable with an analysis of DC subsets in human kidney in which we showed that many cells were double positive for DC-SIGN and BDCA-1 [38]. In cell suspensions of human pulmonary tissue, many $CD1a^+$ DC were also positive for BDCA-1 [36]. A weaker correlation was also present between BDCA-1 and BDCA-2 in the epithelial layer. Based on these observations we conclude that the correlations in DC staining we observed in our nasal biopsies are explained partially by double staining of the same cells and partially by local circumstances that result in the attraction of multiple DC subsets.

In conclusion the number of DCs in the nasal mucosa is not reduced in IgAN patients. We have shown that the number of CD1a positive epithelial DCs and the number of DC-SIGN positive cell in the lamina propria of IgAN patients in nasal biopsies is even significantly higher in comparison with control persons. Taken together with the observed IgA hyporesponse after mucosal vaccination with a neoantigen we therefore believe that reduced hyporesponsiveness of IgAN-DC may be the most probable cause for a mucosal hyporesponse in IgAN.

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