Cardiovascular, Pulmonary and Renal Pathology

The Contribution of B Cells to Renal Interstitial Inflammation

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Local B-cell infiltrates play a role in tissue fibrosis, neolymphangiogenesis, and renal allograft survival. We sought to characterize the B-cell infiltrates, factors involved in B-cell recruitment, and lymphangiogenesis in renal interstitial injury (ie, acute and chronic interstitial nephritis and chronic IgA nephropathy). CD20-positive B cells formed a prominent part of the interstitial infiltrating cells. Together with CD3-positive T cells, the CD20-positive B cells formed larger nodular structures. CD10-positive pre-B cells were rare, and the majority were mature CD27-positive B cells. Proliferating B cells were detected within nodular infiltrates. The level of mRNA expression of the chemokine CXCL13 was increased and correlated with CD20 mRNA in the tubulointerstitial space. CXCL13 protein was predominantly found at sites of nodular infiltrates, in association with CXCR5-positive B cells. Furthermore, sites of chronic interstitial inflammation were associated with a high number of lymphatic vessels. B-cell infiltrates form a prominent part of the interstitial infiltrates both in primary interstitial lesions and in IgA nephropathy. CXCR5-positive B cells might be recruited via the chemokine CXCL13 and seem to contribute to the formation of intrarenal lymphoid follicle-like structures. These might represent an intrarenal immune system. (*Am J Pathol* 2007, 170:457–468; DOI: 10.2353/ajpath.2007.060554)

During chronic kidney diseases an inflammatory process occurs within the tubulointerstitium, which finally results in fibrosis.¹ The severity of interstitial leukocyte accumula-

tion, monocytes/macrophages, and T lymphocytes, is associated with renal function at the time of biopsy.^{2–5} Because B cells are considered to be important mostly in lymph nodes, spleen, and in humoral immune responses, little attention has been paid to their potential role as intrarenal infiltrating cells.

Several new aspects of B-cell function have surfaced. These include the release of proinflammatory cytokines and chemokines, antigen presentation, T-cell activation, a role in tissue fibrosis, neolymphangiogenesis (ie, *de novo* formation of lymphatic vessels), and ectopic lymphogenesis; ie, formation of tertiary lymphatic organs in inflamed tissues.^{6–8} Furthermore, therapeutic studies targeting B cells via anti-CD20 antibodies have renewed interest in B-cell biology during chronic diseases.

At sites of chronic inflammation, ectopic formation of lymphoid follicle-like aggregates containing B cells has been described, eg, in autoimmune diseases such as thyroiditis and rheumatoid arthritis, as well as during renal allograft rejection.^{7,9,10} A contribution of B cells to the formation of lymphoid-like structures has been proposed.^{6,11} The accumulation of B cells could be mediated by chemokines.^{12,13} CXCR5 is a chemokine receptor expressed by B cells, which binds the chemokine CXCL13.¹⁴ CXCR5 and the corresponding ligand CXCL13 play a role in B-cell migration to secondary lymphoid organs, and in lymphoid organogenesis.^{15,16} Furthermore, high expression of CXCL13 has been demonstrated in synovial tissue with large B-cell aggregates, suggesting a potential role of CXCL13 for B-cell accumulation.^{17,18}

Previously, the relative percentage of B cells in the renal interstitium of chronic kidney diseases was considered to be low.^{19–22} In contrast, a prominent accumulation of CD20-positive B cells has recently been described in membranous nephropathy.²³ Furthermore, in renal allografts a detrimental role for CD20-positive B cells has

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Table 1. Clinical Features of Study Patients

	Gender m/f	Mean age and range in years	Mean serum-creatinine and range in mg/dl	Mean proteinuria (g/24 hours)
Acute interstitial nephritis	5/5	64 (12 to 76)	4.9 (1.1 to 21.5)	0.5 (0.3 to 11.5)
Chronic interstitial nephritis	14/15	42 (9 to 84)	4.2 (2 to 11.8)	1.0 (0.1 to 15.5)
IgA nephropathy	11/7	37 (18 to 74)	3.6 (1.1 to 10)	2.5 (0.2 to 5.5)

m, male; f, female.

been postulated because they were associated with increased graft loss.²⁴

Here, we describe that CD20-positive B cells form a prominent part of interstitial infiltrates in both primary interstitial disease as well as in secondary involvement during primary IgA nephropathy. The B-cell infiltrate is associated with increased local expression of the chemokine CXCL13 and the corresponding receptor CXCR5 on B cells. Furthermore, T- and B-cell infiltrates form lymphoid-like nodular structures, which are surrounded by newly formed lymphatic vessels in these chronic diseases. These data invite speculations about a role of these intra-renal B-cell-rich lymphoid follicle-like structures in a local immune response in chronic renal diseases.

Materials and Methods

Study Population

This study used archival renal biopsies from patients with primary acute interstitial nephritis ($n = 10$), chronic interstitial nephritis ($n = 29$), and IgA nephropathy ($n = 18$) (Table 1). The diagnosis was based on light microscopy, immunohistochemistry, and electron microscopy. Criteria for the diagnosis of chronic interstitial nephritis were the presence of an interstitial infiltrate, in combination with interstitial fibrosis, without significant glomerular lesions (by light and electron microscopy), and the absence of significant glomerular immune deposits. The diagnosis of acute interstitial nephritis was based on the presence of an interstitial infiltrate (commonly with tubulitis) and interstitial edema in the absence of significant glomerular lesions on light and electron microscopy, associated with rapid decline of renal function. IgA nephropathy was diagnosed when widening and/or hypercellularity of the mesangium in combination with IgA immune deposits in the mesangium were present. Excluded from the series were patients with lupus erythematoses, vasculitis, infectious interstitial nephritis (eg, leptospirosis, Hantaan nephritis), nephrotoxic renal failure, and other forms of glomerulonephritis. Five donor allograft biopsies taken before implantation served as normal controls. The results of the morphological evaluation are illustrated in Table 2. Biopsies were

randomly chosen according to the availability of biopsy material remaining after diagnostic evaluation.

Tubulitis was quantified in analogy to the Banff classification as 0 to 3 (with 1: one to four infiltrating leukocytes; 2: 5 to 10 infiltrating leukocytes; 3: >10 infiltrating leukocytes per tubular cross-section). Interstitial fibrosis was quantified as the area involved in interstitial fibrosis (grade 1: <1/3 of the biopsy; grade 2: > 1/3 but <2/3; grade 3: >2/3). The severity of interstitial fibrosis was graded from 0 to 3 (semiquantitatively mild to severe). Severity and involved area were multiplied and illustrated as a score (Table 2). Immunohistochemistry was routinely performed for IgG, IgA, IgM, C3, and C1q in all biopsies. The glomerular immune deposits were scored semiquantitatively and described in the text.

For mRNA quantification, parts of human renal biopsies, performed for diagnostic purposes, were obtained according to the local ethical committees' directives and samples processed according to the protocol of the European Renal cDNA Bank/Kroener-Fresenius Biopsy Bank (ERCB/KFB).²⁵ Glomerular and tubulointerstitial samples were manually microdissected and RNA-isolated. Histology reports and clinical data were stored anonymously. Included were RNAs extracted from 51 biopsies: acute interstitial nephritis ($n = 22$), chronic interstitial nephritis ($n = 4$), IgA nephropathy ($n = 15$), and pretransplant controls ($n = 10$). The biopsies used for mRNA evaluation were from a different patient cohort than those used for the histomorphology because insufficient material remained after the histological studies.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded renal biopsies similar to that as described previously.^{5,26} In brief, tissue sections were dewaxed and rehydrated. Incubation in 3% hydrogen peroxide was used to block endogenous peroxidases. An autoclave oven was used for antigen retrieval. An avidin/biotin blocking kit (Vector, Burlingame, CA) was used to block endogenous biotin. Incubation with the primary antibody for 1 hour was followed by incubation with biotinylated secondary reagents (Vector) and the ABC

Table 2. Pathological Features of the Study Population (Mean, SEM)

	% Glomerulosclerosis	Interstitial sclerosis score	Tubulitis score	Tubular atrophy score
Acute interstitial nephritis	9	4 (±0.7)	2.1 (±0.23)	0.9 (±0.2)
Chronic interstitial nephritis	32	7.5 (±0.4) [†]	1.2 (±0.12) [†]	2.2 (±0.13) [†]
IgA nephropathy	53	6.6 (±0.6) [*]	1.2 (±0.16) [†]	1.8 (±0.2)

* $P < 0.05$, [†] $P < 0.01$, [‡] $P < 0.001$ versus acute interstitial nephritis.

Figure 1. Immunohistochemistry on sections from human tonsils (**A–E, J, K**) and an allograft nephrectomy (**F–I**) performed with a monoclonal antibody against CD20 (**A, D, G**), CD3 (**C, E**), podoplanin (D2-40, **H**), CXCL13 (**J**), CXCR5 (**K**), or with the corresponding isotype IgG controls (**B, F, I**). **D** and **E** illustrate double immunofluorescence on a human tonsil with CD20 (green, **D**) and CD3 (red, **E**), with a cell separation of the two markers. Original magnifications: $\times 100$ (**A–C, J, K**); $\times 200$ (**F–I**).

reagent (Vector). 3',3'-Diaminobenzidine (Sigma, Taufkirchen, Germany) metal enhancement (resulting in a black color product) was used as a detection system.

The anti-human CD20 antibody (clone L26; DakoCytomation, Dako Deutschland, Hamburg, Germany), the anti-CD3 antibody (clone CD3-12, rat anti-human; Serotec,

Figure 2. Immunohistochemistry performed on sections from biopsies with acute interstitial nephritis (**A–G**), or a pretransplant biopsy as control (**H**), with a monoclonal antibody against CD20 (**A, C, E, G, H**) and on consecutive section against CD3 (**B, D, F**). CD20-positive infiltrating B cells were found in diffuse infiltrates (**A**), in small cellular aggregates (**C**), and in larger nodular formations (**E**). CD3-positive T cells commonly infiltrated the tubular epithelium (arrows in **F**). A single CD20-positive B cell in a peritubular capillary is illustrated in the control (**H**, arrow). Original magnifications, $\times 200$.

Oxford, UK), and the anti-podoplanin antibody (D2-40; Signet Laboratories, Dedham, MA) were used on consecutive sections.^{5,26} D2-40 has been described as a reliable marker for lymphatic endothelium in paraffin-embedded tissue and binds to human podoplanin.^{27,28} As negative controls the primary antibody was replaced by isotype-matched irrelevant immunoglobulins on tissue sections from human tonsils and renal allografts. Triple immunohistochemistry for podoplanin, MIB1, and either CD3 or CD20 was performed as previously described.⁹

B-cell infiltrates were further characterized by staining for CD10 (clone 65C6; NeoMarkers, Fremont, CA), a marker preferentially expressed by immature B cells (pre-B cells). Double immunofluorescence for CD20/CD27 (clone 137B4; Dianova, Hamburg, Germany) was performed on selected biopsies because CD27 is mainly expressed by memory B cells. Immunohistochemistry of CXCL13 (anti-human CXCL13; R&D Systems, Wiesbaden, Germany) was performed as described.²⁹ CXCR5 was localized using a monoclonal rat anti-CXCR5 anti-

body (a gift from Elisabeth Kremmer, GSF, Munich, Germany).³⁰

Establishment of the Staining Procedures

Sections from human tonsils and allograft nephrectomies were used to establish the staining procedures (Figure 1). The anti-CD20 antibody produced a very reliable staining pattern of B-cell areas (Figure 1A). No positive color product was found in tissue sections exposed to isotype IgG controls (Figure 1, B and F). As expected the staining pattern was different for CD3-positive T cells (Figure 1C), and double immunofluorescence demonstrated separation of CD20-positive B cells and CD3-positive T cells (Figure 1, D and E). Podoplanin as a lymphatic endothelial marker was stained with the antibody D2-40, which produces a reliable staining pattern after heat-based antigen retrieval in archival tissue (Figure 1H). Larger lymphatic vessels were present in the allograft nephrectomy in association with arteries. Smaller lymphatic vessels were found in association with interstitial infiltrates (Figure 1H). Isotype controls were negative (Figure 1I). CXCL13 was expressed within follicles, and CXCR5-positive B cells were present in consecutive sections (Figure 1, J and K).

Digital Image Analysis

Morphometric analysis was performed on 15 consecutive high-power fields ($\times 400$) by the use of Qwin software (Leica, Bensheim, Germany). The area of positive signal was measured for CD3- and CD20-positive cells by an observer blinded to the diagnosis and expressed as fraction of the area of the high-power field. Biopsies were only included when positive cells could be clearly identified. Figures represent means (bars indicate SEM). For the comparison of means the nonparametric Mann-Whitney *U*-test was used. $P < 0.05$ was considered to be statistically significant.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR was performed on a TaqMan ABI 7700 sequence detection system (Applied Biosystems, Darmstadt, Germany) using heat-activated *TaqDNA* polymerase (Amplitaq Gold; Applied Biosystems, Darmstadt, Germany). Quantification of the given templates was performed according to the standard curve method. Serial dilutions of standard cDNA from a human nephrectomy were included in all PCR runs and served as standard curve. Commercially available predeveloped TaqMan reagents were used for the target genes (CD20, CXCL13, CXCR5, interleukin-6, transforming growth factor- $\beta 1$) and three endogenous control genes (18S rRNA, cyclophilin, GAPDH; Applied Biosystems). The normalization to any of the three reference (housekeeping) genes gave comparable results. The data shown in the text and figures are normalized to 18S rRNA. All measurements were

Figure 3. Immunohistochemistry performed on sections from biopsies with chronic interstitial nephritis with a monoclonal antibody against CD20 (**A, C, E, G, H**) and on consecutive section against CD3 (**B, D, F**). Original magnifications, $\times 200$.

performed in duplicates. Controls consisting of bidistilled H_2O were negative in all runs.

Results

Interstitial Nephritis

Biopsies from patients with acute, primary interstitial nephritis ($n = 10$, Figure 2) and from patients with chronic, primary interstitial nephritis ($n = 29$, Figure 3) were evaluated for CD20-positive B cells and CD3-positive T cells. The clinical information about the study population is summarized in Table 1 and the morphological evaluation in Table 2. Patients with acute interstitial nephritis dem-

onstrated less glomerulosclerosis and less interstitial fibrosis as expected, but more tubulitis than patients with chronic interstitial nephritis (for details about the morphological evaluation, see Materials and Methods). Glomerular IgG or IgA deposits were not detectable; minor deposits of IgM in the mesangial area were present in 5 of 10 biopsies.

In chronic interstitial nephritis the biopsies contained more fibrosis, less tubulitis, and more tubular atrophy than in acute interstitial nephritis. Glomerular IgG was not detectable, minor deposits of IgA in the mesangium were described in three biopsies (but attributable to the degree considered insignificant), and minor mesangial IgM deposits were common (14 of 29). Five donor allograft biopsies taken before implantation served as normal controls. Only a small number of CD20-positive cells, scattered through the tubulointerstitium, were detectable in histologically normal tissue from donor kidneys (Figure 2H).

CD20-positive B cells formed a prominent part of the interstitial infiltrate in both acute and chronic interstitial nephritis (Figures 2 and 3). The CD20-positive B-cell infiltrates could be assigned to three general patterns of distribution, albeit with considerable overlap (Table 3). Six of 10 biopsies with acute interstitial nephritis and 14 of 29 biopsies with chronic interstitial nephritis demonstrated diffuse interstitial infiltrates of CD20-positive cells as single or double layers of cells between the tubuli (Figure 2A, Figure 3A). Larger nodular aggregates were present in 2 of 10 biopsies with acute interstitial nephritis and in 8 of 29 biopsies with chronic interstitial nephritis (Figure 2, E and F; and Figure 3, E–G). Two of 10 biopsies with acute and 7 of 29 with chronic interstitial nephritis contained aggregates of CD20-positive cells, smaller than the nodular structures, but larger than the diffuse infiltrates (Figure 2, C and D; and Figure 3, C and D). These patterns are not exclusive because biopsies with nodular aggregates also contain smaller CD20-positive B-cell aggregates and/or diffuse infiltrates. Whereas T cells commonly infiltrated the tubular epithelium in acute interstitial nephritis (Figure 2F, arrows), CD20-positive cells were only occasionally found within the tubular epithelium. Both cell types were rare in glomeruli and, if present, localized to glomerular capillaries. There was no correlation between these three patterns of cell infiltrations and the serum creatinine level at the time of biopsy.

The three groups defined by the main distribution pattern did not demonstrate differences in the area of CD3-positive T cells or area of CD20-positive B cells. The overall area of CD3-positive T cells and CD20-positive B cells were comparable (Figure 4A). In general, CD3-positive T-cell infiltrates occurred in parallel with CD20-

Table 3. Distribution of the Patterns of the CD20-Positive Cell Infiltrates in the Biopsies

Interstitial CD20-positive infiltrates	Diffuse infiltrates	Small aggregates	Nodular infiltrates
Acute interstitial nephritis	6/10	2/10	2/10
Chronic interstitial nephritis	14/29	7/29	8/29
IgA nephropathy	7/18	7/18	4/18

Figure 4. A: Quantification of the infiltrates by digital morphometry, determined as the percentage of area staining positive (bars show SEM). **B:** Quantification of podoplanin-positive lymphatic vessels per high-power field (bars show SEM). a.Int., acute interstitial nephritis; c.Int., chronic interstitial nephritis; IgA N., IgA nephropathy.

positive B cells. Because B cells might be involved in neolymphangiogenesis, lymphatic vessels were stained by immunohistochemistry for D2-40, an antibody against podoplanin as a marker for lymphatic endothelium. As expected, larger lymphatic vessels were found in association with arteries (Figure 5A).^{9,31} There was a clear difference in the pattern of podoplanin-positive vessels in acute versus chronic interstitial nephritis (Figures 4B and 5). In acute interstitial nephritis, the number of lymphatic vessels was small, and predominantly found along larger arteries, as is the case in the normal kidney (Figure 5). Some lymphatic vessels were filled with inflammatory cells (Figure 5B). In contrast, in primary chronic interstitial nephritis, a high number of small lymphatic vessels was present throughout the cortex, and these were not associated with arteries. A morphological association was found between these small lymphatic vessels within the cortex and areas of inflammation because podoplanin-positive vessels were rarely found in areas of well-preserved renal tissue. The number of podoplanin-positive vessels was significantly higher in chronic, primary interstitial nephritis compared with acute interstitial nephritis (Figure 4B).

IgA Nephropathy

A total of 18 biopsies with IgA nephropathy were studied (Figure 6 and Tables 1 and 2). The morphological pattern of glomerular injury was categorized according to the subclassification scheme proposed by M. Haas.³² Three

Figure 5. Immunohistochemistry was performed for CD20 (C, E) and podoplanin (A, B, D, F) on renal biopsies with acute interstitial nephritis (B), chronic interstitial nephritis (A, C, D), and IgA nephropathy (E, F). Normal lymphatic vessels surrounding an artery are illustrated in A (arrows). B shows a lymphatic vessel filled with inflammatory cells. C and D are consecutive sections from a chronic interstitial nephritis with a prominent accumulation of CD20-positive cells in association with small lymphatic vessels (F). Original magnifications: $\times 100$ (A); $\times 200$ (B-F).

cases displayed only minor glomerular changes (subclass 1); in eight cases, we observed a FSGS-like pattern (subclass 2), one case each had either focal (subclass 3) or diffuse proliferative lesions (subclass 4), and in five cases, prominent chronic changes with $>50\%$ glomerular scars dominated (subclass 5). As expected IgA nephropathy was the disease with the highest level of global glomerulosclerosis (Table 2). The interstitial lesions were similar to that of biopsies with chronic interstitial nephritis. By definition, IgA deposits were found in the mesangial area, concomitant IgM (15 of 18), and C3 (14 of 18) were present.

Similar to primary interstitial diseases, a considerable number of CD20-positive cells was found within the interstitium. As in primary interstitial diseases, the three patterns of CD20-positive infiltrates were also present in secondary interstitial injury during IgA nephropathy. The percentage of the three distribution patterns was also comparable with the primary interstitial diseases (Figure 6, Table 3). Both CD20-positive B cells and CD3-positive T cells were rarely found in glomerular tufts in IgA nephropathy. The interstitial areas of CD20- and CD3-positive cells were not statistically different in chronic, primary interstitial diseases and secondary interstitial involvement in IgA nephropathy (Figure 4A). There was no apparent association between the subclasses of IgA nephropathy according to Haas³² and the number of B cells.

Figure 6. Immunohistochemistry performed on sections from biopsies with IgA nephropathy with a monoclonal antibody against CD20 (A, C, E, G) and against CD3 (B, D, F). Original magnifications, $\times 200$.

Similar to chronic, primary interstitial nephritis, a prominent number of podoplanin-positive vessels was found in secondary interstitial involvement of IgA nephropathy (Figure 5F). In addition to the expected lymphatic vessels in proximity to larger arteries, a high number of small podoplanin-positive vessels was present in areas involved by inflammation, interstitial fibrosis, or around sclerosed glomeruli. The pattern of podoplanin-positive vessels was more focal in IgA nephropathy—as were the infiltrates and fibrotic areas—compared with the picture in chronic, primary interstitial nephritis. The number of podoplanin-positive interstitial lymphatic vessels was comparable with that in chronic, primary interstitial nephritis, but was again significantly higher in IgA nephropathy as compared with acute interstitial nephritis (Figure 4B).

Characterization of B-Cell Infiltrates

To further characterize the interstitial infiltrates, we performed triple immunohistochemistry for podoplanin-positive lymphatic endothelium (Figure 7, brown), MIB1 for proliferating cells (Figure 7, red), and for either CD3-

positive T cells or CD20-positive B cells (Figure 7, blue) in selected biopsies. The triple stain immunohistochemistry allowed a clear localization of proliferating cells within the infiltrates, and also an overlay of T- and B-cell areas within the infiltrates on consecutive sections (illustrated in Figure 7, C, F, and I). In biopsies with acute interstitial nephritis, lymphatic vessels were commonly found to be filled with inflammatory cells (Figure 7, A–C). These cells were predominantly CD3-positive T cells (Figure 7A), but also a low number of CD20-positive B cells were found within these vessels (Figure 7B).

Nodular aggregates consisted of a CD20-positive B-cell core containing proliferating B cells (Figure 7F). The majority of proliferating cells within the infiltrates were CD20-positive B cells because the CD3-positive T cells did not stain for the proliferation marker (Figure 7, E and F; arrowhead). Overall, the number of proliferating cells in the center of the follicular structures was low. The periphery of the nodular infiltrates contained mostly CD3-positive T cells, whereas the CD20-positive B cells predominated in the central area of the lymphoid follicle-like structures (Figure 7, D and E). Podoplanin-positive lymphatic vessels surrounded the nodular infiltrates (Figure 7E).

To further characterize the B cells involved in renal inflammation, we performed immunohistochemistry for CD10, a marker for immature B cells (Figure 8C).^{33,34} In renal biopsies, CD10-positive infiltrating B cells were barely detectable (Figure 8C). However, CD10 was expressed by podocytes and proximal tubular epithelial cells, consistent with previous reports (Figure 8C).³⁵ CD27 is expressed by memory B cells.³⁶ Double labeling for CD27 and CD20 demonstrated that the majority of CD20-positive cells expressed CD27, suggesting that most of the CD20-positive cells are mature memory B cells (Figure 8D).

To look for potential mechanisms involved in B-cell recruitment, we determined the mRNA levels of CXCL13 and CXCR5 by quantitative real-time RT-PCR and localized them by immunohistochemistry in selected biopsies (Figures 1, 8, and 9). The chemokine CXCL13 is involved in B-cell organization of secondary lymphoid follicles³⁷ and may be involved in the formation of follicular infiltrates in a mouse model of lupus nephritis³⁸ and in human renal allografts with acute rejection.²⁹ CXCL13 mRNA expression was increased in the tubulointerstitial compartment, particularly in acute interstitial nephritis (Figure 9B). The mRNA expression of CXCL13 correlated significantly with that of its receptor CXCR5 (Spearman $r = 0.42$, 95% confidence interval: 0.11 to 0.65, $P = 0.0081$) and with that of the CD20 B-cell marker (Spearman $r = 0.69$, 95% confidence interval: 0.46 to 0.83, $P < 0.0001$).

By immunohistochemistry, CXCR5 was not expressed by intrinsic renal cells or in areas of well-preserved tissue. CXCR5-positive cells were only found in areas of larger aggregates of CD20-positive B cells, with an overlap of CXCR5 and CD20 on consecutive sections (Figure 8). CD3-positive T cells were negative for CXCR5 (Figure 8B). Within the nodular aggregates, positive staining for CXCL13 could be demonstrated (Figure 8, G and H).

Figure 7. Triple immunohistochemistry for podoplanin (brown), MIB1 (red), and either CD20 (blue; **A, D, G**) or CD3 (blue; **B, E, H**) performed on renal biopsies with acute interstitial nephritis (**A, B**), chronic interstitial nephritis (**D, E**), or IgA nephropathy (**G, H**). Schematic overlay of the triple staining for CD20-positive B cells and CD3-positive T cells is illustrated in **C, F**, and **I**. Please note the proliferating B cells (**arrowhead**) in the center of the follicle, surrounded by T cells and lymphatic vessels (**F, arrows**). Original magnifications: ×200 (**A, B, G, H**); ×100 (**D, E**).

Figure 8. Immunohistochemistry for CXCL13 (A, G), CXCR5 (B, H), and CD10 (C) performed on renal biopsies with IgA nephropathy. Double immunofluorescence was performed for CD27 (D) or PCNA (E) and CD20 (F, I). A–F: A nodular infiltrate of CD20-positive cells (F), some of which are proliferating (E, arrows) is localized close to a glomerulus. CXCR5-positive cells (B) and CXCL13 expression (A) are associated with this B-cell infiltrate. No CD10-positive B cells are detectable in the infiltrate (C). G–I: B-cell infiltrate consisting of CXCR5-positive (H) or CD20-positive cells (I) in an area positive for CXCL13 staining (G).

Thus nodular aggregates contain the chemokine ligand CXCL13 and its corresponding receptor CXCR5 on CD20-positive B cells (Figure 8, A and B). Consistent with the morphological data, there was a prominent expression of mRNA for CD20 in the tubulointerstitium of all three disease entities (Figure 9A). The three diseases did not differ in the expression of CD20 mRNA, but the expression was higher in all three than in the controls (Figure 9A). There was no correlation between the levels of CD20 mRNA or the areas of CD20-positive cells and serum creatinine at the time of biopsy.

Discussion

The main findings in this study were that CD20-positive B cells form a prominent part of the interstitial infiltrate in primary interstitial diseases, as well as in secondary interstitial involvement of a primary glomerular disease, ie, IgA nephropathy. Most B cells were mature according to the absence of CD10 (pre-B cells) and the presence of

CD27 as a B-memory cell marker. An intriguing observation was the formation of lymphoid follicle-like structures in chronic tubulointerstitial inflammation of either primary or secondary interstitial involvement, consisting of a core of B cells (some of which are proliferating), surrounded by T cells and neolymphatics. In the nodular infiltrates, expression of the chemokine CXCL13 was associated with accumulation of B cells expressing the corresponding receptor CXCR5, arguing for CXCL13 involvement in the B-cell recruitment.

In the characterization of the leukocyte subsets in human glomerulonephritis by Hooke and colleagues,¹⁹ B cells (characterized by PHM 14) were rarely seen within glomeruli (consistent with our results), and compromised only 8% of the interstitial infiltrating cells in IgA nephropathy and 12% in interstitial nephritis. Although the number of B cells was found to be relatively low compared with macrophages and T cells (the main interstitial leukocyte populations), there was a significant correlation between B cells and the degree of renal function.¹⁹ In another

Figure 9. Expression of mRNAs for CD20 (A), CXCL13 (B), and CXCR5 (C) in the tubulointerstitial compartment of renal biopsies. Expression was normalized to 18S rRNA as housekeeping gene (bars show SEM). a.Int., acute interstitial nephritis; c.Int., chronic interstitial nephritis; IgA N., IgA nephropathy; ** $P < 0.01$, *** $P < 0.001$).

study in four patients with acute interstitial nephritis, three with chronic interstitial nephritis, and seven with glomerulonephritis, less than 20% of the interstitial infiltrate were found to be B cells.²² The prominent accumulation of CD20-positive B cells that we noted is consistent with a recent publication that demonstrated CD20-positive B cells in membranous nephropathy, as compared with minimal change disease and normal controls.²³ Some of the variations between the studies might be attributable to the different markers used, and different degrees of chronicity in the small number of patients studied. We used CD20 as a marker that is expressed by B cells from the pre-B cell to the preplasma cell stage.⁸ To describe further the stage of B-cell maturation, we used the marker CD10 and CD27. CD10 is expressed by pre-B cells, and CD27, by mature memory B cells. CD10-positive pre-B cells were rarely detectable in the infiltrates. This indicates that the vast majority of CD20-positive B cells are mature cells. Consistent with this interpretation, CD27

was expressed by the majority of CD20-positive B cells indicating that most of these cells are memory B cells.

The B-cell infiltrates could be assigned to three main patterns of distribution within the interstitium, as also described in secondary interstitial injury in membranous nephropathy²³: a diffuse pattern, small cellular aggregates, and larger follicular structures. We were unable to define clinical or morphological features distinguishing these different B-cell infiltrates and believe that these patterns most likely present a continuum, from diffuse infiltrates developing into nodular lymphoid follicles. It may be of interest that a similar pattern of B-cell accumulation has been described for the development of secondary lymphoid organs.³⁹

Outside the kidney formation of lymphoid follicle-like structures has been linked to chronic inflammatory diseases such as rheumatoid arthritis,⁷ thyroiditis,⁸ Sjögren's disease,⁴⁰ and in idiopathic pulmonary fibrosis.⁴¹ In the kidney, nodular infiltrates containing B cells have been described in renal allografts and in membranous nephropathy.^{9,23} We now observe similar nodular infiltrates resembling lymphoid follicles in the interstitium in IgA nephropathy, and in primary forms of interstitial nephritis. The percentage of patients with nodular aggregates did not differ between primary glomerular, primary interstitial diseases, and renal allografts. Therefore, the formation of interstitial B-cell aggregates might be a common response during interstitial injury.²³

Using triple immunohistochemistry, including the proliferation marker Ki-67 or double immunofluorescence for CD20 and PCNA, we were able to demonstrate proliferation of B cells within follicles. This is consistent with a germinal center-like reaction, at least in some of the nodular infiltrates. Similar findings were described in rheumatoid arthritis and allograft rejection, whereas a recent report on idiopathic pulmonary fibrosis did not find proliferating lymphocytes.⁴¹ Thus nodular infiltrates may differ according to the underlying disease and the type of tissue involved. In a detailed analysis of nodular inflammatory cells in renal allografts, these were shown to contain B and T cells, dendritic cells, and to be associated with lymphatic neoangiogenesis.⁹ Consistent with these observations the present study demonstrates B cells, T cells, and lymphatics in both chronic interstitial nephritis and in interstitial lesions of IgA nephropathy.⁹ This picture resembles tertiary lymphoid organ development observed in autoimmune diseases,¹⁰ which have been postulated to locally perpetuate autoimmune reactions and support autoantigenic epitope spreading.⁴² In renal allografts, the accumulation of CD20-positive B cells in the tubulointerstitium was associated with steroid-resistant acute allograft rejection and with graft loss.²⁴ This may relate to the two-way interaction between B cells and T cells. B cells activate T cells via antigen presentation, and T cells provide help to B cells through cell surface antigens and cytokine production.⁸ However, follicle-like structures also contain regulatory T cells (personal, unpublished observation) so that the possibility of local inhibitory immunomodulation has to be considered as well. At present, we do not have follow-up biopsies or

follow-up clinical information and cannot comment on the prognostic value of these follicle-like infiltrates.

Little is known about the role of B cells in the process of renal interstitial fibrosis. In a model of CCl₄-induced liver fibrosis approximately half of the infiltrating cells in the liver were found to be B cells.⁴³ Eliminating B cells significantly improved liver injury with decreased deposition of collagen.⁴³ This improvement was not T-cell- or immunoglobulin-dependent.⁴³ B cells might be involved in interstitial injury through the release of cytokines and chemokines, as antigen-presenting cells, via the augmentation of T-cell responses and through neolymphogenesis.^{7,9}

Neolymphangiogenesis has been described as a prominent finding in the remnant kidney model⁴⁴ and in human allografts.⁹ Our studies now show that both chronic primary interstitial nephritis and secondary interstitial involvement in IgA nephropathy resulted in significantly higher numbers of lymphatic vessels in the interstitium. Furthermore, neolymphatics tended to cluster around the aggregates of inflammatory cells resembling the organization in secondary lymphoid organs. In contrast in acute interstitial nephritis (a disease entity in which biopsies are usually taken early in the disease process), a low number of lymphatic vessels was present in association with arteries, comparable with findings described for the normal kidney.⁹ These data indicate that a chronic type of inflammation is required to initiate neolymphangiogenesis with formation of lymphatic vessels under the influence of mononuclear cell-derived growth factors, predominantly of the VEGF isoforms.^{45,46} Hypothetically, B cells may also play a role in the latter process because B cells contribute to neolymphangiogenesis in lymph nodes.⁶

Local expression of chemokines contributes to the recruitment of inflammatory cells during inflammation, as well as during leukocyte recirculation and lymphoid organogenesis.³⁷ Thus the chemokine CXCL13 and the corresponding receptor CXCR5 contribute to the positioning of CD3-negative/CD4-positive precursor and mature B cells in lymphoid tissue.¹⁶ Mice lacking CXCL13 or CXCR5 are severely deficient in peripheral lymph nodes and Peyer's patches development, including B-cell organization.^{15,47} In this context, our observations are of interest showing increased mRNA expression of CXCL13 in the tubulointerstitial space of renal biopsies with interstitial B-cell infiltrates containing CXCR5-positive cells. Furthermore, the localization of enhanced CXCL13 immunoreactivity to the nodular infiltrates and that of CXCR5 to B cells in the infiltrates point toward a role of CXCL13-CXCR5 for B-cell recruitment into the lymphoid follicle-like structures, similar to their role in secondary lymphoid organ generation.³⁷ A role for CXCL13 in B-cell infiltration has also been postulated in Sjögren's syndrome and allograft rejection.^{29,40}

The formation of lymphoid follicle-like accumulations of T cells, B cells, and probably macrophages and dendritic cells⁹ and their association with neolymphatic vessels in chronic renal interstitial lesions of various primary etiologies invite speculations about their role in the progression of interstitial disease and fibrosis. Because essentially all elements for a local immune response are present within the follicles, these might constitute a local immune sys-

tem in chronic renal disease. At present, it remains unclear if this represents a detrimental or perhaps even beneficial development for the progression of renal diseases, or perhaps either one, depending on the stage of the disease process. The latter possibility should not be dismissed off hand. Should future observations and experimental studies point toward a predominantly detrimental role of infiltrating B cells in chronic renal diseases, targeting these cells for therapeutic purposes may potentially represent an additional strategy in slowing the progression of renal diseases. Initial studies using this approach have been performed for membranous nephropathy, and renal involvement of lupus erythematoses, and mixed cryoglobulinemia.^{48–50} However, the primary target in these diseases would be the antibody-producing B cells, and less likely infiltrating B cells in the kidney. The observation of renal B-cell infiltrates in primary and secondary interstitial disease and the formation of lymphoid follicle-like structures argue in favor of a systematic approach investigating their functional role in the progression of renal disease.

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