Renal Interstitial Infiltration and Tertiary Lymphoid Organ Neogenesis in IgA Nephropathy

Guangchang Pei, Rui Zeng, Min Han, Panli Liao, Xuan Zhou, Yueqiang Li, Ying Zhang, Ping Liu, Chunxiu Zhang, XiaoCheng Liu, Ying Yao, and Gang Xu

Summary
Background and objectives Previous studies have identified inflammatory features that enable the prediction of renal outcome of IgA nephropathy (IgAN); however, validation of these findings is still needed. This prospective study was performed to determine the characteristics of renal interstitial infiltration and tertiary lymphoid organ (TLO) neogenesis in a cohort of Chinese patients with IgAN.

Design, setting, participants, & measurements Adult patients with IgAN were recruited into this study from June 2009 to June 2010. Inflammatory cells in renal biopsy tissues were detected by immunohistochemistry and immunofluorescence. Correlations between the density of interstitial inflammatory cells, grades of TLOs, and clinicopathologic features were evaluated. Of 152 eligible patients, 72 (47%) were successfully followed-up by telephone at 30 months after renal biopsy. Twelve patients were classified as the severe group and 60 patients were classified as the stable group, according to the progression of serum creatinine levels during the follow-up period. A comparison of the severity of interstitial infiltration and the frequency of TLO neogenesis between the two groups was performed.

Results The accumulation of interstitial inflammatory cells was correlated with decreased renal function, heavy proteinuria, and severe glomerular, interstitial, and arterial lesions in patients with IgAN. TLOs, identified as nodular inflammatory infiltrates containing organized DC-SIGN+, CD4+, CD8+, and CD20+ cells, were observed in 37.5% of patients. Patients with high-grade TLOs exhibited a high percentage of mesangial hypercellularity and crescents as well as severe interstitial and arterial lesions. Patients in the severe group exhibited more severe interstitial infiltration and a higher percentage of TLO neogenesis (83.3% versus 33.3%; P=0.001) compared with patients in the stable group.

Conclusions As contributors to an active local inflammatory response, the severity of interstitial infiltration and the frequency of TLO neogenesis are correlated with glomerular, interstitial, and arterial lesions as well as IgAN progression.


Introduction
IgA nephropathy (IgAN), which features IgA deposition in the glomerular mesangium, is the most common form of primary GN worldwide (1,2). The average renal survival rates at 5 and 10 years are 85.1% and 77.1%, respectively (3). Assessing the prognosis is challenging but extremely important. Previous studies have identified that clinical features such as severe proteinuria, arterial hypertension, and elevated serum creatinine are predictors of IgAN (4–7). Histologic features are also recognized as important prognostic factors in IgAN (7). According to the Oxford IgAN classification, mesangial hypercellularity (M), segmental glomerulosclerosis (S), endocapillary hypercellularity (E), and tubular atrophy/interstitial fibrosis (T) are histologic predictors of IgAN prognosis independent of the clinical features (8,9). The MEST scoring system has recently been confirmed in several independent populations and is proven to be a valuable tool for prognostic purposes (10,11). However, other risk factors associated with MEST variables should also be included.

Interstitial inflammatory infiltration is another prominent pathologic feature associated with IgAN. Inflammatory cells present in the renal interstitium include monocytes/macrophages, dendritic cells, and T and B lymphocytes (12–16). CD68+ macrophage accumulation and CD3+ or CD8+ lymphocyte infiltration are suggested to predict IgAN progression (12,13,15). DC-SIGN+ cells, a special subset of dendritic cells that is essential for dendritic cell–induced T cell proliferation, have also been found in the kidney of different types of human GN (14). However, there is no detailed research on DC-SIGN+ cells in IgAN. Interstitial B lymphocyte infiltration has also been reported (16). However, there are few studies of all cellular combinations in patients with IgAN.

Tertiary lymphoid organs (TLOs) are actually nodular inflammatory infiltrates containing organized dendritic
cells, B and T lymphocytes, and other cellular components in chronically inflamed nonlymphoid tissues or organs (17–19). TLOs have also been identified in CKD, including IgAN (16,20,21). Heller et al. reported that proliferating and memory B cells were present within TLOs and chemokine CXCL13 might contribute to TLO neogenesis by recruiting CXCR5+ Bcells to injured kidneys (16). TLOs support the production of autoantibodies and predict poor outcomes of chronic rejection (22,23). However, the precise characteristics of TLOs have not been fully defined in a cohort of patients with IgAN.

In this prospective study, interstitial CD68+, DC-SIGN+, CD4+, CD8+, and CD20+ cells and the cellular components of TLOs were examined by immunohistologic staining in renal biopsy samples of patients with IgAN. The main interest focused on the correlations between severity of inflammatory cell infiltration, frequency of TLO neogenesis, and clinicopathologic features.

### Materials and Methods

#### Patients

This study complied with the Declaration of Helsinki and was approved by the Committee on Research Ethics of the Huazhong University of Science and Technology, Tongji Hospital. From June 2009 to June 2010, 205 adult patients with IgAN were recruited to this study according to the scheme presented in Figure 1. All patients met the diagnostic criteria for IgAN, which were published by the Oxford classification working group (9). Patients with hepatitis B virus infection as well as individuals who had received glucocorticoids or immunosuppressant treatment before renal biopsy were excluded (Figure 1).

#### Histologic Evaluation

Paraffin sections for light microscopy were stained with hematoxylin and eosin, periodic acid–Schiff, Masson's...
trichrome, and periodic acid–silver methenamine methods. Direct immunofluorescence for IgA, IgG, IgM, C3, C1q, and folate receptor α was performed in frozen sections. Histopathologic evaluation was independently performed according to the Oxford classification (8,9) by M.H. and Y.L. who were not aware of the clinical data.

**Immunohistochemistry and Immunofluorescence**

Detection of CD68 (Long Island Biotech, Shanghai, China), DC-SIGN (Santa Cruz Biotechnology, Dallas, TX), CD4, CD8, CD20, CD21, CD138, D2–40 (Maxim-bio, Fuzhou, China), CXCL12, CXCRI4, CXCL13 (Bioss-bio, Beijing, China), CXCR5, CCL21, and CCR7 (Abcam, Cambridge, UK) was performed on paraffin sections using a streptavidin-peroxidase kit (ZSGB-bio, Beijing, China) according to the manufacturer’s instructions. Antibody reactions were visualized by using diaminobenzidine (DAKO, Tokyo, Japan). For immunofluorescence double staining, primary antibodies against CD3, CD45RO, CD20 (Maxim-bio), CD27 (Abcam), IgG, and IgM (ZSGB-bio) and Alexa Fluor 488 or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used. Colocalization was analyzed by confocal laser scanning microscopy.

**Quantitative Analyses of Inflammatory Cells and Definition of TLOs**

The numbers of each subset of interstitial inflammatory cells were counted under five equivalent high-power

---

**Figure 2. Different subsets of inflammatory cells in the renal interstitium.** (A–F) Inflammatory cells around Bowman’s capsule of glomeruli. (G–L) Inflammatory cells between tubules. A and G were stained with PAS. Cells stained by immunohistochemistry were as follows: CD68+ (a macrophage marker) (B and H), DC-SIGN+ (marked a subset of dendritic cells) (C and I), CD4+ (a T helper cell marker) (D and J), CD8+ (a cytotoxic T cell marker) (E and K), and CD20+ (a B lymphocyte marker) (F and L). Antibody reactions (dark brown) were visualized by DAB. DAB, diaminobenzidine; PAS, periodic acid–Schiff. Bar, 200 μm.
To evaluate the presence of TLOs, we initially defined nodular inflammatory infiltrates (including small cellular aggregates and larger follicular-like structures) in the renal interstitium on periodic acid–Schiff-stained sections as TLO candidates, which were then examined in serial sections stained with hematoxylin and eosin, Masson’s trichrome, and periodic acid–silver methenamine. TLOs were finally identified after immunohistochemical staining confirmed that the structures contained organized DC-SIGN+, CD4+, CD8+, and CD20+ cells. The frequency of TLO neogenesis in patients with IgAN was analyzed by a simple grading system comprising three grades: without TLOs (without TLOs under 10 equivalent HPFs), grade 1 (with 1 tertiary lymphoid organ under 10 equivalent HPFs), and grade 2 (with ≥2 TLOs under 10 equivalent HPFs).

Statistical Analyses
Statistical analyses were performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL). Nonparametric statistics were used to analyze between-group differences and relationships. Correlations were assessed using the Spearman’s rank correlation test for two continuous variables and the Mann–Whitney U test when median comparisons were made. Rate comparisons were performed by chi-squared tests. All statistical tests were two sided.

Results
Patients’ Baseline Features
According to the selection scheme, 152 patients were enrolled. A total of 72 patients were followed-up by telephone 30 months after renal biopsy. The clinical characteristics of these patients are presented in Table 1.

Different Subsets of Interstitial Inflammatory Cells
In our renal biopsy specimens of IgAN, CD68+, DC-SIGN+, CD4+, CD8+, and CD20+ cells were detected in the serial sections and were found in 100%, 96.7%, 100%, 100%, and 96.7% of patients, respectively. CD68+, DC-SIGN+, CD4+, CD8+, and CD20+ cells were found around the Bowman’s capsule and between tubules. CD68+ and DC-SIGN+ cells were scattered throughout the renal interstitium. Interstitial CD4+, CD8+, and CD20+ cells could be analogously assigned to three patterns of distribution: a diffuse pattern, small cellular aggregates, and larger follicular-like structures.

Association of Interstitial Inflammatory Cells with Clinicopathologic Features
Correlations between interstitial infiltration and clinical features were analyzed by Spearman’s analysis (Table 2). The density of CD68+, DC-SIGN+, CD4+, CD8+, and CD20+ cells was significantly associated with serum creatinine and proteinuria levels. Among the different subsets,
Figure 3. | Inflammatory cells, lymphatic vessels, chemokines, and their receptors within TLOs. (A) Representative PAS-stained renal TLOs. Cells stained by immunohistochemistry were as follows: CD68 (B), DC-SIGN (C), CD4 (D), CD8 (E), CD20 (F), CD21 (a follicular dendritic cell marker) (G), CD138 (a plasma cell marker) (H), D2–40 (labeled lymphatic vessels) (I), CXCL13 (J), CXCL12 (K), CCL21 (L), CXCR5 (M), CXCR4 (N), and CCR7 (O). Antibody reactions (dark brown) were visualized using DAB. CXCL13/CXCR5, CXCL12/CXCR4, and CCL21/CCR7 indicate the chemokines and corresponding receptors that are most likely involved in TLO neogenesis (17). Organized inflammatory cells in TLOs are shown in A–H. Arrows in G suggest follicular dendritic cells in the center of the TLOs, arrows in H indicate CD138⁺ plasma cells in TLOs, and arrows in I indicate D2–40⁺ lymphatic vessels that surround or disperse within the TLOs. All panels depict serial sections from the same patient. DAB, diaminobenzidine; PAS, periodic acid-Schiff; TLO, tertiary lymphoid organ. Bar, 100 μm.
the density of DC-SIGN+ cells were the most strongly correlated with serum creatinine levels ($r=0.42, P<0.001$), and the density of CD68+ cells were the most strongly correlated with proteinuria levels ($r=0.44, P<0.001$). The density of DC-SIGN+ cells and CD20+ cells had good correlation with serum uric acid levels ($r=0.24, P<0.01$; and $r=0.27, P<0.01$, respectively). The density of CD4+ and CD8+ cells was associated with the severity of erythrocyturia ($r=0.24, P<0.01$; and $r=0.27, P<0.01$, respectively) (Table 2).

We also evaluated the relationship between interstitial infiltration and pathologic features. The density of CD68+ cells, DC-SIGN+, CD4+, CD8+, and CD20+ cells was significantly associated with the severity of tubular atrophy/interstitial fibrosis, arterial wall thickening, and presence of arterial hyalinosis (Table 2). Among the different subsets, the density of CD68+ cells most strongly correlated with the severity of tubular atrophy/interstitial fibrosis ($r=0.62, P<0.001$). The density of CD4+ cells most strongly correlated with the presence of crescents ($r=0.25, P<0.001$). The density of CD8+ cells most strongly correlated with the severity of arterial wall thickening ($r=0.42, P<0.001$). The density of CD20+ cells most strongly correlated with the presence of arterial hyalinosis ($r=0.35, P<0.001$) (Table 2). The density of DC-SIGN+ and CD4+ cells was significantly correlated with the presence of mesangial hypercellularity ($r=0.27, P<0.01$; and $r=0.23, P<0.01$, respectively)

Figure 4. | T and B lymphocytes and plasma cells within tertiary lymphoid organs. Double immunofluorescence was performed for CD3 (green) (A), CD45RO (red) (B), double-positive (yellow) (C), CD27 (green) (D), CD20 (red) (E), double-positive (yellow) (F), IgG (green) (G), CD138 (red) (H), double-positive (yellow) (I), IgM (green) (J), CD138 (red) (K), and double-positive (yellow) (L) cells. CD45RO and CD27 are markers of memory T cells and memory B cells, respectively. G–L suggest that plasma cells have the ability to produce antibodies. Bar, 100 μm.
In addition, the density of all cellular combinations was frequently found adjacent to the injured glomerulus and were also detected within the TLOs. CD20+ B cells (Figure 3F) and CD21+ follicular dendritic cells were also detected within the TLOs, suggesting that the plasma cells within the TLOs were capable of producing Ig.

The frequency of TLO neogenesis was also assessed with clinical parameters at the time of the renal biopsy. As shown in Table 3, patients with high-grade TLOs showed a significant increase of CD3+ cells in the most cellular area of the TLOs, corresponding receptors CXCR5, CXCR4, and CCR7 were also detected within the TLOs. CD45RO is a marker of memory T cells and CD27 is expressed on memory B cells (26,27). In this study, many of the CD3+ cells within the TLOs were colocalized with the corresponding receptors CXCR5, CXCR4, and CCR7, indicating that these T lymphocytes were activated memory T cells (Figure 4, A–C). In addition, the majority of CD20+ cells expressed CD27, suggesting that these B lymphocytes were mature memory B cells (Figure 3, D–F). IgG+ CD138+ cells (Figure 3, G–I) and IgM+ CD138+ cells (Figure 3, J–L) were also discovered within the TLOs, suggesting that the plasma cells within the TLOs were capable of producing Ig.

The frequency of TLO neogenesis was also assessed with clinical parameters at the time of the renal biopsy. As shown in Table 3, patients with high-grade TLOs showed a significant increase of CD3+ cells in the most cellular area of the TLOs, corresponding receptors CXCR5, CXCR4, and CCR7 were also detected within the TLOs. CD45RO is a marker of memory T cells and CD27 is expressed on memory B cells (26,27). In this study, many of the CD3+ cells within the TLOs were colocalized with the corresponding receptors CXCR5, CXCR4, and CCR7, indicating that these T lymphocytes were activated memory T cells (Figure 4, A–C). In addition, the majority of CD20+ cells expressed CD27, suggesting that these B lymphocytes were mature memory B cells (Figure 3, D–F). IgG+ CD138+ cells (Figure 3, G–I) and IgM+ CD138+ cells (Figure 3, J–L) were also discovered within the TLOs, suggesting that the plasma cells within the TLOs were capable of producing Ig.

### Table 3. Clinical and demographic characteristics in patients with different grades of tertiary lymphoid organs at the time of biopsy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Tertiary Lymphoid Organ Grade</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (n=93)</td>
<td>1 (n=33)</td>
</tr>
<tr>
<td>Male sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age≥50 (yr)</td>
<td>7.5</td>
<td>21.2</td>
</tr>
<tr>
<td>Serum creatinine≥1.2 (mg/dl)</td>
<td>20.5</td>
<td>36.4</td>
</tr>
<tr>
<td>Serum uric acid≥7.0 (mg/dl)</td>
<td>16.5</td>
<td>34.5</td>
</tr>
<tr>
<td>Proteinuria≥2.0 (g/24 h)</td>
<td>31.2</td>
<td>50.0</td>
</tr>
<tr>
<td>Erythrocyturia≥3+</td>
<td>50.5</td>
<td>42.4</td>
</tr>
<tr>
<td>BP grade≥2b</td>
<td>5.4</td>
<td>15.6</td>
</tr>
<tr>
<td>Presence of crescentsa</td>
<td>12.9</td>
<td>28.6</td>
</tr>
<tr>
<td>Presence of arterial wall thickening</td>
<td>6.4</td>
<td>21.2</td>
</tr>
</tbody>
</table>

Data are presented as percentages. Comparisons between groups were performed by chi-squared tests. RBC, red blood cell; HPF, high-power cortical field; SBP, systolic BP; DBP, diastolic BP.

aThe severity of erythrocyturia was defined as follows: 3+ RBC/HPF as 3+; and 4+ RBC/HPF as 4+.

bBP was graded in three groups: 0, SBP < 140 mmHg and DBP < 90 mmHg; 1, SBP ≥ 140 mmHg or DBP ≥ 90 mmHg; and 2, SBP ≥ 160 mmHg or DBP ≥ 100 mmHg.

cPathologic features were scored using the Oxford classification (8,9). M1 was defined by the presence of >3 cells in the most cellular area, but not adjacent to the vascular stalk, or >50% of the glomeruli. S1 was defined by the presence of tuft adhesion and segmental sclerosis but not global sclerosis. E1 was defined by the presence of an increased number of cells within the capillary lumen, causing narrowing. The percentage of a cortical area damaged by interstitial fibrosis or tubular atrophy was defined as T0 (<25%); T1 (26%–50%), or T2 (>50%).

dThe severity of global glomerulosclerosis and arterial wall thickening was divided into three groups: normal, mild, and marked.

eCrescents and arterial hyalinosis were scored as present or absent.

(Table 2). The association of interstitial CD4+, CD8+, and CD20+ cell density with the presence of segmental glomerulosclerosis was also significant (r=0.33, P<0.001; r=0.32, P<0.001; and r=0.25, P<0.01, respectively) (Table 2). In addition, the density of all cellular combinations was also significantly correlated (Table 2).

**TLOs in the Kidney of Patients with IgAN**

Small cellular aggregates and larger follicular-like structures defined as TLOs were found in 37.5% of patients. TLOs were frequently found adjacent to the injured glomerulus and arteries or beneath the renal capsule (21). We found that the CD20+ B cells (Figure 3F) and CD21+ follicular dendritic cells (Figure 3G) were distributed in the center of the TLOs, the periphery of which contained clustered CD4+ cells, CD8+ cells, scattered DC-SIGN+ cells, and CD138+ plasma cells (Figure 3, A–I). CD68+ cells surrounded the TLOs, but were rarely in the TLOs (Figure 3B). DC40+ lymphatic vessels surrounded or were dispersed within the TLOs (Figure 3I).

Local production of chemokines is a critical event in TLO neogenesis (17). CXCL13 was reported to recruit CXCR5+ B cells, CXCL12 was reported to recruit CXCR4+ T cells, and CCL21 was reported to recruit CCR7+ dendritic cells and T cells, respectively (17,19). As shown in Figure 3, chemokines CXCL13, CXCL12, and CCL21 and the corresponding receptors CXCR5, CXCR4, and CCR7 were also detected within the TLOs.

CD45RO is a marker of memory T cells and CD27 is expressed on memory B cells (26,27). In this study, many of the CD3+ cells within the TLOs were colocalized with CD45RO, indicating that these T lymphocytes were activated memory T cells (Figure 4, A–C). In addition, the majority of CD20+ cells expressed CD27, suggesting that these B lymphocytes were mature memory B cells (Figure 3, D–F). IgG+ CD138+ cells (Figure 3, G–I) and IgM+ CD138+ cells (Figure 3, J–L) were also discovered within the TLOs, suggesting that the plasma cells within the TLOs were capable of producing Ig.
Patients in the severe group also exhibited severe interstitial infiltration of CD68+, DC-SIGN+, CD4+, CD8+, and CD20+ cells as well as a high percentage of TLO neogenesis at the time of biopsy compared with patients in the stable group (83.3% versus 33.3%; P<0.001) (Table 4).

Discussion

Interstitial infiltration, one common but special feature in CKD, was recently confirmed to play an initiative role in regulating renal lesions (28). In this prospective study, we investigated the density of CD68+, DC-SIGN+, CD4+, CD8+, and CD20+ cells and the grades of TLOs and evaluated their association with clinicopathologic features in renal biopsy tissues of patients with IgAN.

Among the pathologic variables, the density of interstitial inflammatory cells showed a strong correlation with the severity of interstitial and arterial lesions (Table 2). We also observed that the density of inflammatory cells was...
significantly correlated with the glomerular lesions (Table 2). In a mouse model, renal interstitial dendritic cells were found to contribute to GN. They captured glomerular antigens and presented them to T helper cells, and the latter recruited and activated cytotoxic T cells thereby driven renal lesions (28). In other words, different cellular subsets cooperate with each other locally to accelerate renal inflammation and injury of the entire kidney. On the basis of the strong correlations of different subsets of inflammatory cells in this study (Table 2), we believed that interstitial inflammatory cells contributed to IgAN progression.

The severity of interstitial infiltration has been used for prognostic purposes (12,13,15). Myllymäki et al. reported that severe interstitial CD3+ infiltration indicated poor outcomes of IgAN, according to a retrospective study (12). In our prospective study, we found that patients in the severe group exhibited more interstitial CD68+, DC-SIGN+, CD4+, CD8+, and CD20+ cell infiltration compared with patients in the stable group (Table 4). Because CD3 is a common T cell marker of both T helper cells (CD4+ T cells) and cytotoxic T cells (CD8+ T cells), our results were consistent with those of Myllymäki et al. (12).

TLOs provide a special space in which the “cooperation” of inflammatory cells occurs in an organized manner in situ in nonlymphoid tissues or organs. TLOs, similar to secondary lymphoid organs, generate effector and memory T cells that lead to allograft rejection and are considered as a fast track for autoimmunity (23,29).

Heller et al. reported that TLOs in IgAN contained B cells, T cells, and lymphatic vessels (16). In our IgAN renal sections, we further observed the presence of DC-SIGN+ dendritic cells, follicular dendritic cells, and plasma cells in TLOs (Figure 3). We also found that part of T and B lymphocytes within TLOs showed a memory phenotype and plasma cells within the TLOs secreted IgG or IgM (Figure 4). TLOs contained all of the essential elements for a local immune response and thus might play a role in IgAN progression (23,29,30). Kelly et al. revealed that renal TLO neogenesis was associated with matrix accumulation and loss of renal function in a mouse model (31). In our study, we found that high-grade TLOs were significantly associated with an elevated level of serum creatinine and severe glomerular, interstitial, and arterial lesions. In addition, patients in the severe group exhibited a high percentage of TLO neogenesis compared with patients in the stable group (Table 3).

This study has several limitations. The follow-up period was not long enough and the cohort with integrated outcome data was not large enough. Additional features such as the mRNA levels of related inflammatory cytokines and chemokines were not measured. Furthermore, whether the TLOs in IgAN kidneys functioned detrimentally as they did in allograft rejection are still not clear. Additional large prospective multicenter and molecular marker–based clinical trials and animal experiments are warranted to validate our results.

Taken together, our findings indicate that in addition to clinical and pathologic variables, the severity of interstitial infiltration and the frequency of TLO neogenesis also indicate the severity of renal lesions and are correlated with IgAN progression. A detailed understanding of the processes leading to TLO neogenesis and the definition of its pathophysiologic role may provide new rationales for the development of therapeutics specifically targeting this process.

Acknowledgments
This work was supported by the National Natural Sciences Foundation of China (Grants 81270770, 81170686, 81270771, 81100485, and 81370798), under the auspices of Scientific Research of Major Projects by the Ministry of Education of China (JYBZZ201201, Grant 2011-313-311028).

Disclosures
None.

References


Received: January 31, 2013 Accepted: September 9, 2013

G.P. and R.Z. contributed equally to this work.

Published online ahead of print. Publication date available at www.cjasn.org.