In Situ B Cell-Mediated Immune Responses and Tubulointerstitial Inflammation in Human Lupus Nephritis


J Immunol 2011; 186:1849-1860; Prepublished online 27 December 2010; doi: 10.4049/jimmunol.1001983
http://www.jimmunol.org/content/186/3/1849

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/12/27/jimmunol.1001983.DC1

Why The JI?
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References
This article cites 85 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/186/3/1849.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2011 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
In Situ B Cell-Mediated Immune Responses and Tubulointerstitial Inflammation in Human Lupus Nephritis


The most prevalent severe manifestation of systemic lupus erythematosus is nephritis, which is characterized by immune complex deposition, inflammation, and scarring in glomeruli and the tubulointerstitium. Numerous studies indicated that glomerulonephritis results from a systemic break in B cell tolerance, resulting in the local deposition of immune complexes containing Abs reactive with ubiquitous self-Ags. However, the pathogenesis of systemic lupus erythematosus tubulointerstitial disease is not known. In this article, we demonstrate that in more than half of a cohort of 68 lupus nephritis biopsies, the tubulointerstitial infiltrate was organized into well-circumscribed T:B cell aggregates or germinal centers (GCs) containing follicular dendritic cells. Sampling of the in situ-expressed Ig repertoire revealed that both histological patterns were associated with intrarenal B cell clonal expansion and ongoing somatic hypermutation. However, in the GC histology, the proliferating cells were CD138+CD20low/− tubular basement membrane immune complexes. These data implicate tertiary lymphoid neogenesis in the pathogenesis of lupus tubulointerstitial disease.


The most prevalent severe manifestation of systemic lupus erythematosus (SLE) is nephritis, which is characterized by immune complex deposition, inflammation, and scarring in glomeruli and the tubulointerstitium. Numerous studies indicated that glomerulonephritis results from a systemic break in B cell tolerance, resulting in the local deposition of immune complexes containing Abs reactive with ubiquitous self-Ags. However, the pathogenesis of systemic lupus erythematosus tubulointerstitial disease is not known. In this article, we demonstrate that in more than half of a cohort of 68 lupus nephritis biopsies, the tubulointerstitial infiltrate was organized into well-circumscribed T:B cell aggregates or germinal centers (GCs) containing follicular dendritic cells. Sampling of the in situ-expressed Ig repertoire revealed that both histological patterns were associated with intrarenal B cell clonal expansion and ongoing somatic hypermutation. However, in the GC histology, the proliferating cells were CD138+CD20low/− tubular basement membrane immune complexes. These data implicate tertiary lymphoid neogenesis in the pathogenesis of lupus tubulointerstitial disease.


The most prevalent severe manifestation of systemic lupus erythematosus (SLE) is nephritis, which is characterized by immune complex deposition, inflammation, and scarring in glomeruli and the tubulointerstitium. Numerous studies indicated that glomerulonephritis results from a systemic break in B cell tolerance, resulting in the local deposition of immune complexes containing Abs reactive with ubiquitous self-Ags. However, the pathogenesis of systemic lupus erythematosus tubulointerstitial disease is not known. In this article, we demonstrate that in more than half of a cohort of 68 lupus nephritis biopsies, the tubulointerstitial infiltrate was organized into well-circumscribed T:B cell aggregates or germinal centers (GCs) containing follicular dendritic cells. Sampling of the in situ-expressed Ig repertoire revealed that both histological patterns were associated with intrarenal B cell clonal expansion and ongoing somatic hypermutation. However, in the GC histology, the proliferating cells were CD138+CD20low/− tubular basement membrane immune complexes. These data implicate tertiary lymphoid neogenesis in the pathogenesis of lupus tubulointerstitial disease.


The most prevalent severe manifestation of systemic lupus erythematosus (SLE) is nephritis, which is characterized by immune complex deposition, inflammation, and scarring in glomeruli and the tubulointerstitium. Numerous studies indicated that glomerulonephritis results from a systemic break in B cell tolerance, resulting in the local deposition of immune complexes containing Abs reactive with ubiquitous self-Ags. However, the pathogenesis of systemic lupus erythematosus tubulointerstitial disease is not known. In this article, we demonstrate that in more than half of a cohort of 68 lupus nephritis biopsies, the tubulointerstitial infiltrate was organized into well-circumscribed T:B cell aggregates or germinal centers (GCs) containing follicular dendritic cells. Sampling of the in situ-expressed Ig repertoire revealed that both histological patterns were associated with intrarenal B cell clonal expansion and ongoing somatic hypermutation. However, in the GC histology, the proliferating cells were CD138+CD20low/− tubular basement membrane immune complexes. These data implicate tertiary lymphoid neogenesis in the pathogenesis of lupus tubulointerstitial disease.
and are required to locally maintain activated T cells (28). Several reports noted that infiltrating T cells are a prominent feature of LN (29–33), whereas the presence of B cells was recently noted (34–36). However, the significance of these lymphocyte populations in the interstitial infiltrate was unclear.

In this article, we demonstrate that in moderate or severe TI the inflammatory infiltrates are usually organized into structures reminiscent of those observed in secondary lymphoid organs. Most commonly, aggregates of T and B cells containing plasmablasts were observed. However, in some renal biopsies there were germinal center (GC)-like structures containing well-organized follicular dendritic cell (FDC) networks and centroblasts. Both lymphoid structures were functional, because they were associated with in situ B cell clonal expansion and somatic hypermutation. These findings implicate organ-intrinsic adaptive-immune responses in the pathogenesis of lupus TI.

Materials and Methods

Patients and renal biopsies

The University of Chicago Medical Center Institutional Review Board approved this study. We reviewed the pathology files at the University of Chicago Medical Center for inpatient renal biopsies consistent with LN between 2001 and 2007. Among this group, 68 subjects had biopsies containing sufficient material for analysis (six or more glomeruli and a length ≥0.5 cm) and that did not display Class I or VI nephritis, as defined by the 2003 International Society of Nephrology/Renal Pathology Society revised LN classification criteria (37, and who, on review of records, fulfilled American College of Rheumatology revised criteria for the classification of SLE (38). Each diagnostic biopsy sample consisted of at least three tissue cores that were predominantly divided for light microscopy, with smaller portions submitted for immunofluorescence and electron microscopy. Using the National Institutes of Health system, the activity and chronicity indices were scored at the time of renal biopsy by one of two renal pathologists (A.C., S.M.M.) (39). Control normal renal tissue was obtained from autopsy studies. The clinical charts were reviewed to collect pertinent clinical data, including age, gender, disease activity and chronicity indices were scored at the time of renal biopsy by a full-house (IgG, IgA, IgM, C3, C1q) pattern, which is characteristic of LN. Standard procedures for electron microscopy were applied to evaluate the renal biopsies using a Philips CM10 electron microscope.

In situ immune responses in lupus nephritis

In this article, we demonstrate that in moderate or severe TI the interstitial infiltrate was categorized into three patterns: diffuse (28), and appropriate HRP-conjugated secondary Abs. Isotype controls for each mAb were run concomitantly to confirm the absence of nonspecific staining. Laser capture microdissection (LCM) using the Arcturus Pixcell II (Molecular Devices, Sunnyvale, CA) platform and Capture HS LCM caps (Molecular Devices) was performed to isolate CD38- or Ki-67–staining cells. The pulse power was 70 mW, size spot diameter was 15 μm, pulse duration was 5 ms, and target voltage was 170 mW. Extraction reservoirs were placed directly on the HS LCM caps.

Synthesis of cDNA

Ten microliters of lysis buffer (0.5× PBS, 10 mM DTT, 20 RNAsin [Promega, Madison, WI], and 1 U Prime RNase Inhibitor [Eppendorf, Hamburg, Germany]) was aliquoted directly into the extraction reservoir of the LCM cap. Caps were immediately placed on dry ice and stored at −70°C. cDNA was synthesized within the extraction reservoir using 25 μL RT-PCR buffer [375 ng random hexamer primer (pd(N)6, Amersham Pharmacia Biotech), 2 mM 2-deoxyadenosine 5'-triphosphates, 1× Coral Load PCR buffer [Qiagen, Valencia, CA], 1.25 U AmpliTaq Gold polymerase [Qiagen], and 8 μL cDNA (or first-round PCR product)].

Amplification of Ig H and L chains

Ig γ, Igδ, and Igκ rearrangements were amplified by two rounds of nested PCR in a 40-μL volume (50 nM primers, 1 mM 2-deoxyadenosine 5'-triphosphates, 1× Coral Load PCR buffer [Qiagen, Valencia, CA], 1.25 U HotStarPlus Taq polymerase [Qiagen], and 8 μL cDNA [or first-round PCR product]).

Sequence analysis

PCR products were cloned into the pCR4-TOPO TA vector (Invitrogen), and plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen). Multiple bacterial clones were randomly selected for sequencing to ensure proper sampling. The V-QUEST program from the international ImMunoGeneTics (IMGT) information system (http://imgt.cines.fr/IMGT_vquest/vquest/) was used to identify the V(D)J germline, as well as the CDRs and framework regions (FRWs). Nucleotide and amino acid mutations in the V-region were identified by alignment with the closest corresponding germline using IMGT. For the CDRs, the closest corresponding germline V regions and mutations were amplified and sequenced to confirm that the observed mutations did not represent allelic polymorphisms. Multiple nucleotide changes in a single codon were scored as a single replacement mutation.
were defined by similar CDR3 regions, as identified by the junction analysis software provided by IMGT. Sequences that were out of frame or contained mutations that resulted in a nonproductive sequence were excluded from analysis (~15% of all sequences; data not shown). For tabulating clonal frequency, a clone was counted if it was obtained from separate LCM picks or if it differed by three or more nucleotides from other cloned sequences within the same LCM “pick”. This latter criterion was used to exclude clones that might appear different as a result of PCR error. Ag-driven selection was calculated using the IAVA applet from Losso et al. (45) (http://www-stat.stanford.edu/immunoglobulin/). Genealogical trees showing the relationships between plasma cells were constructed by analysis of the pattern of somatic mutations.

Results
To examine the possible pathogenic significance of B cells in LN, we identified 68 patients, with SLE diagnosed by a rheumatologist, who had undergone diagnostic renal biopsies for presumed LN (C. Hsieh et al., submitted for publication). The average age of this cohort was 31 y, 85% were female, and 81% were African American. At the time of biopsy, the average duration of disease was 36 mo, the median creatinine was 1.0 mg/dl, and 81% had detectable Abs to dsDNA. Twenty-five percent had only low-dose oral prednisone prior to renal biopsy (≤20 mg/dl). Three of the biopsies were International Society of Nephrology/Renal Pathology Society class II, 22 were class III, 33 were class IV, and 10 were class V. Tissue sections from each biopsy were examined first by immunohistochemistry with Abs to CD45, CD3, CD4, CD20, and CD138.

Ectopic lymphoid structures in LN
Three distinct patterns of B cell infiltration were evident (Fig. 1). In 48% of biopsies (33/68), the predominant pattern was one of diffuse and scattered lymphocytic (CD45+) infiltration, with varying degrees of coinfiltration with CD20+ B lymphocytes or CD138+ plasma cells. Plasma cells and B cells were invariably excluded from glomeruli with only rare circulating CD20+ B cells in glomerular capillaries. In 46% of cases (31/68), there were well-circumscribed aggregates of CD20+ B cells or CD138+ plasma cells with CD3+ T cells in tubulointerstitium. Most of these T cells expressed CD4 (data not shown). Finally, in 6% of biopsies (4/68), structures consistent with GCs were observed. In these structures, CD20+ B cells preferentially occupied the central zone, whereas CD3+CD4+ T cells tended to occur peripherally. Staining with the dendritic cell marker CD21 revealed a central dense reticular network characteristic of FDCs. CD138+ plasma cells were rare within the central FDC network but common in the surrounding areas. The spatial organizations of B cells, T cells, FDCs, and tingible body macrophages were all consistent with the observed histological structures being bona fide GCs.

Chemokines induce and maintain the spatial organization of immunocytes within secondary lymphoid organs (46). Therefore, we examined whether some of these same chemokines were present in SLE renal biopsies (Fig. 2). When biopsies with T:B aggregates or GC phenotype were stained, CXCL12 (46) and BAFF (47) were observed in almost all samples tested with prevalences of 95% (18 of 19 biopsies) and 100% (4 of 4 biopsies), respectively. Several other chemokines were also commonly expressed. Approximately 70% (12/17) of biopsies with the T:B aggregate or GC phenotypes had detectable staining for CXCL13 (35), 50% (8/16) had detectable staining for CCL21, and 42% (8/19) had detectable staining for CXCL10. These chemokines were not detectable in normal renal tissue (data not shown).

Interestingly, there was little chemokine staining in renal biopsies with a diffuse histological phenotype (n = 10; data not shown). There were no clear differences between the T:B aggregates and GC phenotypes, except that the GC phenotype was associated with more intense chemokine staining. These data suggested that similar factors may organize and maintain the T:B and GC-like patterns.
histological phenotypes. In contrast, different mechanisms may mediate the diffuse accumulation of plasma cells and B cells in LN.

To begin to determine whether the observed histological structures were functional (48), we determined whether they were associated with in situ lymphocyte proliferation. Therefore, biopsies manifesting the diffuse, T:B aggregate and GC patterns were stained with Abs specific for the proliferative marker Ki-67. As demonstrated in Fig. 3, the centers of the GC structures contained numerous small proliferating cells. Ki-67+ cells were also commonly observed in T:B aggregates (14 of 22 biopsies; 64%). In contrast, biopsies with a diffuse histology infrequently had Ki-67+ cells (2 of 11 biopsies; 18%). When present, there was usually no more than one Ki-67+ cell per high-power field. Ki-67+ tubular epithelial cells were infrequently observed in biopsies from all three histological groups (data not shown).

To determine whether the observed proliferating cells were lymphocytes, we performed two-color immunohistochemistry with Abs specific for Ki-67 and either CD20 or CD3. Ki-67+CD4+ positive cells were infrequently observed in all three histological patterns (data not shown). In contrast, numerous Ki-67-CD20+ cells were observed in the GC structures (Fig. 2). Ki-67-CD20+ cells were also observed in the T:B aggregates, but they were a minor fraction of the Ki-67+ cells (<10%). Most Ki-67+ cells were CD20low or CD20+. However, most of these Ki-67+ cells in the T:B aggregates expressed CD138 and, therefore, were plasmablasts.

In situ lymphocyte organization correlated with the extent of TI and specific pathological features (Fig. 4). Severe interstitial inflammation (>25% of the interstitial infiltrated by inflammatory cells) was more likely to be found in biopsies with the T:B aggregate (29 of 31 biopsies) and GC (4 of 4 biopsies) patterns compared with 16 of 33 biopsies with the diffuse pattern (p = 0.00002, Fisher exact test) (Fig. 4A). Detectable tubular basement membrane immune complexes (TBMCs) (immunofluorescence) were also infrequent in biopsies manifesting a diffuse pattern (6/33) (Fig. 4B, 4C) but were a usual feature of biopsies with T:B aggregates or GCs (21/33) (Fig. 4F). This difference was highly significant (p = 0.00014). Electron microscopic analysis of the biopsy in Fig. 4D, which had a T:B aggregate histological pattern, revealed that the TBMCs resided within the tubular basement membrane (TBM), characteristic of lupus interstitial nephritis (Fig. 4E). Additional immunofluorescence and electron microscopy images demonstrating TBMCs are provided in Supplemental Fig. 2.

Characterization of in situ Ig repertoire

The presence of lymphoid-like structures and aggregates of proliferating plasmablasts in LN suggested that in situ Ag-driven clonal expansion and somatic hypermutation were occurring. To test this directly, we used LCM coupled to RT-PCR and sequencing to characterize the in situ H and L chain repertoire in renal biopsies from LN patients. We analyzed nine patients. On anti-CD38 Ab stained fresh-frozen sections we used LCM to sample a GC from one LN patient (patient A) and T:B aggregates from four other LN patients (patients B–E). For comparison, we sampled the expressed Ig repertoire in three LN patients with a diffuse histology (patients F–H) and one nonlupus patient with idiopathic interstitial nephritis (patient I). We also stained sections with anti-Ki67 Abs and sampled a cluster of proliferating cells in a LN patient with T:B aggregates on biopsy (patient J).

Ig clonal restriction and Ag-driven somatic mutation within the intrarenal GC

Patient A was a 27-year-old white female with a 7-mo history of SLE who had been treated with low-dose methotrexate and prednisone. The dsDNA Ab titer at the time of biopsy was 1:2560, and anti-Sm Ab was negative. LCM was used to sample 12 separate areas within a GC and another 12 from the surrounding tubulointerstitium. Each LCM pick sampled one to eight visible CD38+ cells. The distribution and frequency of the most commonly expressed Ig genes identified from the GC or surrounding parenchyma are provided in Fig. 5. A total of 26 γ, 41 λ, and 8 κ distinct sequences were identified. Because only a few κ sequences were isolated, they were excluded from further analysis.
Of the 26 distinct expressed γ V sequences identified, 12 were cloned from the GC, and 14 were cloned from the surrounding tubulointerstitium. Among the 12 GC γ sequences, four (33%) arose from a single rearrangement (VH3-9*01D3-10*01JH3*02). This rearrangement was also the predominant expressed H chain detected in the parenchyma surrounding the GC (Fig. 5). Another three (25%) of the GC γ V sequences identified arose from another single rearrangement (VH3-23*01D3-22*01JH3*02). These data indicated that more than half (58%) of the γ-chain repertoire within the GC arose from two unique recombination events.

All of the VH3-9*01D3-10*01JH3*02-encoded sequences were heavily mutated compared with reported germline segments, with all containing a similar core of coding and noncoding mutations (Fig. 6A). It was possible that some of the apparent mutations in VH3-9*01D3-10*01JH3*02 represented an unreported allelic polymorphic form of VH3-9. However, these mutations were not observed when the corresponding genomic VH3-9 segment from patient A was amplified from peripheral blood and sequenced (data not shown).

Comparison of the different VH3-9*01D3-10*01JH3*02-expressed sequences isolated from the GC and the surrounding parenchyma suggested that some B cells had sequentially acquired Ig mutations during clonal expansion. The relative genealogy of these mutations can be demonstrated in a clonal tree (Fig. 6B). The accumulation of such hierarchical γ mutations, as well as the fact that they were identified in closely adjacent B cells, is consistent with in situ clonal expansion and somatic hypermutation (49).

To examine whether Ag was selecting for particular somatic mutations within the GC, we analyzed the type and frequency of mutations in the VH3-9*01D3-10*01JH3*02-encoded V regions (containing CDR1 and CDR2). In the absence of antigenic selection, replacement (R) and silent (S) mutations occur randomly in the CDRs and FWRs. However, if there is Ag-driven selection, R mutations in the CDRs will be overrepresented (50), whereas R mutations in the FWRs will be underrepresented. We used the multinomial method of Lossos et al. (45) to determine the probability that B cells expressing VH3-9*01D3-10*01JH3*02-encoded V regions had undergone Ag-driven selection (Fig. 6C). R mutations were overrepresented (R/S = 8.1; \( p = 0.028-0.035 \)) in the CDRs and underrepresented in the FWRs (12.8 to 13.7; \( p = 0.018-0.041 \)).

A similar picture was obtained when the λ-chain GC repertoire was analyzed. Of the seven sequences obtained from the GC (Fig. 5), two were from one unique recombination (VL2-8*01JL2*01) (Fig. 7A), and two were from another unique recombination (VL1-44*01JL3*02), indicating that more than half (57%) of the detectable GC λ clones arose from one of two unique recom-
bination events. Both of these rearrangements were also found in the surrounding parenchyma (Fig. 5).

Alignment of the different VL2-8*01JL2*01-derived sequences with predicted germline sequences indicated that all had accumulated somatic mutations (Fig. 7A). However, the overall number of mutations was less than that observed in the clonally expanded GC sequences (Fig. 6A). All three mutations observed in CDR1 and CDR2 encoded amino acid replacements, again suggesting selection by Ag. Sequencing of a different, nonselected VL2-8*01 containing lV region from patient A confirmed that the observed mutations did not represent allelic variation (data not shown). Assembly of the different VL2-8*01JL2*01 sequences into a clonal tree revealed a mutational hierarchy suggestive of ongoing clonal expansion and somatic hypermutation (Fig. 7B). In the most related VL2-8*01JL2*01 sequences (Al4b,c), the distribution of R and S mutations in the CDR (R/S = 3:0; p = 0.021) was consistent with Ag-driven selection (Fig. 7C). A similar trend was seen in the FWRs (R/S = 3:1; p = 0.155).

Clonal selection in the tubulointerstitium surrounding the GC

Analysis of the surrounding parenchyma provided further evidence of clonal selection. Of the 34 distinct l sequences identified in the tubulointerstitium, four arose from a single VL2-14*01JL2*01 rearrangement, and three arose from a single VL1-47*01JL2*01 rearrangement. In addition, four distinct interstitial l sequences arose from two genomic rearrangements identified in GC-expressed sequences (Fig. 5). Therefore, 32% (11/34) of the identified expressed l sequences were derived from four rearrangement events. Alignment and further analysis of the VL2-14*01JL2*01-related clones with the predicted corresponding germline segments

![Figure 6](http://www.jimmunol.org/)

**Figure 6.** Evidence of in situ clonal expansion and Ag-selected somatic hypermutation in a intrarenal GC. **A,** Nucleotide sequences of VH3-9*01 D3-10*01 JH3*02 amplified transcripts were aligned with germline. Identical nucleotides are indicated by dashes. Dots indicate unmutated intervening portions of the V regions. Numbers refer to IMGT amino acid positions. Sequences isolated from the GC are marked with an asterisk. **B,** Genealogical relationships of sequences are illustrated in a clonal tree with a predicted germline clone at the top. C, R/S ratios of clones in FWRs and CDRs (including the FWR 5’ to CDR1, which is not shown in A). The p values represent the probability that the observed mutations arose from random chance and had not been selected for affinity to Ag.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>FWRs</th>
<th>CDRs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R/S</td>
<td>p</td>
</tr>
<tr>
<td>Ahla*</td>
<td>12/7</td>
<td>0.028</td>
</tr>
<tr>
<td>Ahlb*</td>
<td>12/7</td>
<td>0.028</td>
</tr>
<tr>
<td>Ahlc*</td>
<td>12/7</td>
<td>0.028</td>
</tr>
<tr>
<td>Ahld*</td>
<td>13/7</td>
<td>0.041</td>
</tr>
<tr>
<td>Ahle*</td>
<td>13/7</td>
<td>0.041</td>
</tr>
<tr>
<td>Ahlf*</td>
<td>13/7</td>
<td>0.041</td>
</tr>
<tr>
<td>Ahlg*</td>
<td>12/8</td>
<td>0.018</td>
</tr>
</tbody>
</table>
revealed evidence of sequential somatic hypermutation and Ag-driven clonal selection (Supplemental Fig. 3).

Comparison of the repertoire between the GC and surrounding tubulointerstitium revealed important interrelationships and significant differences. As described above, the repertoire of expressed γ and ι Ig chains in the GC was well represented in the parenchyma. However, several clonally expanded and/or prevalent parenchymal Ig chains were absent from the GC (Fig. 5). These observations suggested that the GC can contribute to the parenchymal repertoire but that the parenchyma does not necessarily contribute to the GC repertoire.

In situ Ig expression in T:B aggregates

Patient B was a 40-year-old African American female with a 4-mo history of SLE treated with 20 mg/d of oral prednisone. The anti-Sm Ab was positive, and the anti-dsDNA Ab titer prior to biopsy was 1:320. LCM was used to obtain 28 independent samples from two different T:B aggregates. A total of 68 distinct γ, 27 ι, and 31 κ sequences was identified. Within the γ population, 10 rearrangements were observed more than once (24 of 68 sequences, for an overall clonality of 35%). The most common rearrangement was observed four times (4/68; VH1-3*01 D4-23-*01 JH4*02). Alignment of these cDNA fragments with the predicted germline sequence revealed that the identified sequences had undergone extensive somatic hypermutation (Fig. 8A). These clones could also be assembled into a simple clonal tree, suggesting ongoing somatic hypermutation (Fig. 8B).

C

<table>
<thead>
<tr>
<th>Sequence</th>
<th>FWRs</th>
<th>CDRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al4a</td>
<td>5/2</td>
<td>0.212</td>
</tr>
<tr>
<td>Al4b</td>
<td>3/1</td>
<td>0.155</td>
</tr>
<tr>
<td>Al4c</td>
<td>3/1</td>
<td>0.155</td>
</tr>
</tbody>
</table>

FIGURE 7. Analysis of a γ chain selected in the GC also revealed evidence of Ag-selected somatic hypermutation. A, Nucleotide sequences of VL2-8*01 JL2-01 amplified transcripts were aligned with germline, as in Fig. 6. B, Genealogical relationships of sequences are illustrated in a clonal tree with a predicted germline clone at the top. The black circle indicates a proposed intermediate clone. C, R/S ratios of clones in FWRs and CDRs. The p values were calculated as in Fig. 6.

In the GC histology, 17 of 41 distinct ι-chains used VL2-14*01, and 10 used VL1-44*01. For the four patients with a T:B aggregate histology, 33 of 94 were VL2-14*01, and 19 were VL1-44*01. Overall, these two V regions were found in ∼55% of identified ι-chains. In the GC and T:B aggregate histologies, the VL2-14*01 and VL1-44*01 segments were primarily found in expanded clonal populations. These results indicated that the overall frequency of specific expressed ι variable segments was similar between the GC and T:B histological patterns.

To determine whether the observed restricted-expressed repertoires were a specific feature of the GC and T:B aggregates, we sampled the expressed Ig repertoire in three LN patients who had diffuse B cell infiltration on biopsy (Supplemental Table I; patients F–H). From patient F, we cloned and compared 37 distinct Ig H chains. Only two arose from the same rearrangement. In the second patient (patient G), of 37 sequences, 3 arose from one rearrangement, 3 arose from another rearrangement, and 2 arose from a third rearrangement. In patient H, of 31 sequences, 3 arose from one rearrangement, and there were two examples where the same rearrangement was observed twice. A similar degree of clonality
was observed in a nonlupus patient (patient I) who had idiopathic acute interstitial nephritis with diffuse B cell infiltration (Supplemental Table I). In that patient, we identified 34 unique sequences that arose from 29 unique recombination events. Four recombinations were observed twice, whereas none was observed three or more times. Therefore, a modest degree of clonal restriction can be observed in biopsies with a diffuse pattern of B cell infiltration. However, this degree of clonality does not seem to be a specific feature of lupus interstitial nephritis.

In general, we observed more clonal restriction in biopsies with more organized lymphocytic infiltrates. However, there were exceptions; one biopsy with T:B aggregates had a similar degree of clonality (patient E) as that observed in one of the biopsies manifesting a diffuse pattern of B cell infiltration. However, this degree of clonality does not seem to be a specific feature of lupus interstitial nephritis.

In general, we observed more clonal restriction in biopsies with more organized lymphocytic infiltrates. However, there were exceptions; one biopsy with T:B aggregates had a similar degree of clonality (patient E) as that observed in one of the biopsies manifesting a diffuse pattern of B cell infiltration. However, this degree of clonality does not seem to be a specific feature of lupus interstitial nephritis.

Discussion

Interstitial inflammation is a prominent feature of human LN that, independently of glomerular involvement, identifies patients at risk for subsequent renal failure (15, 16) (C. Hsieh et al., submitted for publication). In this study, we demonstrated that, in more than half of our patient cohort, the interstitial infiltrate was organized into lymphoid-like structures competent to select for B cells expressing a highly restricted Ig repertoire. The presence of lymphoid-like structures strongly correlated with detectable TBMICs. These observations suggested that in LN, GCs and T:B aggregates select for cells that locally secrete pathogenic Abs in the tubulointerstitium.

The two histological patterns, GC and T:B aggregates, seemed to reflect different underlying states of B cell selection. Many aspects of clonal selection in the intrarenal GC were typical of those observed in GCs residing in secondary lymphoid structures (51–53). As has been reported for rodent (54) and human (55) GCs, only a few clones accounted for a majority of the sampled repertoires, with unique mutations observed in some clones (Fig. 9A). These differences, which allowed construction of a clonal tree, suggest ongoing somatic hypermutation (Fig. 9B). Interestingly, analysis of the R/S distribution (Fig. 9C) in the CDR1- and CDR2-containing regions revealed no evidence that these mutations had undergone Ag selection. A similar pattern of mutations was observed in those expressed clones arising from the VH4-34*01D1-1*01JH4*02 recombination (data not shown).

FIGURE 8. Clonal selection in a T:B aggregate. A, Nucleotide sequences of VH1-3*01D4-23*01JH4*02 amplified transcripts were aligned with predicted germline sequences as in Fig. 6. B, Genealogical relationships of sequences are illustrated in a clonal tree with predicted germline clone at top. C, R/S ratios of clones in FWRs and CDRs. The p values were calculated as in Fig. 6.
Furthermore, most, if not all, of the observed predominant clones had undergone somatic hypermutation. However, analysis of the frequency and distribution of mutations in nonselected (singly occurring) GC-expressed Ig chains indicated that most had not undergone Ag selection (data not shown). This latter observation is consistent with elegant in vivo imaging studies demonstrating that GCs are open structures that allow B cells to enter freely and scan for Ag (52, 56, 57). Our results are also very similar to those obtained when human lymph nodes from normal volunteers were characterized (55).

In contrast to the proliferating centroblasts observed in the GCs, plasmablasts predominated in those patients with T:B aggregation.

---

**FIGURE 9.** Clonal selection in a Ki-67+ foci. A. Nucleotide sequences of VH4-34*01Dλ3-18*01JH4*02 amplified transcripts were aligned with predicted germline sequences as in Fig. 6. B. Genealogical relationships of sequences are illustrated in a clonal tree with predicted germline clone at top. C. R/S ratios of clones in FWRs and CDRs. The p values were calculated as in Fig. 6.

---

...
gates. These foci of T cells and plasmablasts are reminiscent of the extrafollicular B cell responses that were recently described in some murine models of autoimmunity (58). In MRL/Mpýtpþ mice and in MRL/Mpýtpþ mice expressing AM14 (a rheumatoid factor Ab), the production of autoantibodies in secondary lymphoid organs preferentially occurs in aggregates of plasmablasts residing outside follicles (59–61). Selection and somatic hypermutation might occur in these sites through T-dependent and TLR-dependent mechanisms (47, 62). However, to our knowledge, we are the first to demonstrate in humans the existence of functional extrafollicular plasmablast aggregates in an organ targeted by autoimmune disease. Furthermore, our data indicated that such plasmablast foci are a usual feature of LN complicated by severe TI.

Direct sampling of the proliferating cells in a patient with T:B aggregates revealed a degree of clonal restriction similar to that observed in an intrarenal GC. These data suggested that Ki-67 expression directly identifies cells undergoing in situ selection. The Ki-67+ cells occurred within areas of T:B aggregates, but not all T:B aggregates contained Ki-67+ cells. This relative discordance may explain the variable clonality observed when aggregates, identified by CD38 staining, were sampled for in situ Ig expression. These data suggested that there might not be tight correlations between the immunohistological characteristics of lymphocyte aggregates and their function in selection.

In the T:B aggregates, we observed selection for germine-encoded and highly mutated Abs. Most Abs that were mutated appeared to have undergone Ag-driven selection. A notable exception was the expressed IgH cloned from the foci of Ki-67+ cells. However, current methods of determining Ag-driven selection cannot assess antigenic pressure on the CD3R because several processes determine diversity in this region. Therefore, if antigenic specificity is determined primarily by the CD3R, then selective processes determine diversity in this region. Thus, if antigenic specificity is determined primarily by the CD3R, then selection might not be apparent for the other regions. This possibility might be applicable to VH4-34*01D5-18*01JH4*02, because the CD3R contains three arginines that are known to confer DNA binding (63).

Regardless of apparent differences in B cell populations usually selected in each histological type, GC and T:B histological patterns were strongly associated with TBMCs. This suggests that both lymphoid structures select for cells secreting Abs that form in situ immune complexes with locally available Ags. It is possible that the selecting Ags are renal specific, because such Abs have been detected in the peripheral serum of patients with LN (64). However, it is not clear whether these autoantibodies are produced in situ in the kidney or in conventional lymphoid structures.

Our studies focused on in situ Ab secretion; however, it is likely that resident B cells are also contributing to local inflammation by presenting MHC class II-restricted Ags to neighboring T cells (65). The importance of B cells for maintaining systemic pathogenic T cells was demonstrated in MRL/Mpýtpþ mice (66–68) and in SLE patients treated with rituximab (69, 70). B cells are also required for maintaining T cell infiltrates in the synovium of patients with RA (28). The close proximity of T and B cells in intrarenal GCs and T:B aggregates, as well as the attendant clonal expansion of B cells expressing isotype-switched Abs, suggest that these B cells are productively interacting with coresident T cells.

It is unclear whether the available murine models of SLE fully mimic the in situ adaptive-immune responses associated with human lupus interstitial nephritis. Lymphocytic infiltrates in the kidneys of NZB/NZW and MRL/Mpýtpþ mice contain B cells and/or plasma cells, and the Abs that they express display a broad repertoire of specificities (71–74). However, it is not known whether murine models manifest functional intrarenal GCs or TB aggregates. Rather, available evidence suggests that the NZB/NZW and MRL/Mpýtpþ mice have diffuse or perivascular intrarenal lymphocytic infiltrations. The in situ organization of B and T cells into lymphoid-like structures could be a unique feature of human LN.

We observed a high frequency of L chains containing V2-14*01 and V1-44*01 in GC and T:B aggregates. This could reflect a requirement for these segments to encode certain autoactive specificities (clonal convergence). However, V2-14*01 and V1-44*01 are highly represented in the mature B cell peripheral repertoire of normal individuals and patients with SLE (75). Therefore, the high frequencies noted in this study may reflect the repertoire of precursor B cell populations.

The different histological patterns of involvement in LN were reminiscent of those observed in the synovial tissue of RA patients (24, 76, 77). In both cases, histological features ranged from diffuse lymphocyte infiltration to fully formed GCs. Sampling the Ig repertoire in RA synovial GCs (78, 79) and total RA synovium (80, 81) also revealed evidence of clonal restriction and Ag-driven somatic hypermutation. However, unlike human LN, TLN in RA has not been related to specific, prognostically important pathological features (C. Hsieh et al., submitted for publication).

Dense B cell aggregates are present in half of allograft biopsies, and occasional GCs (in 11% of biopsies) may be associated with Ab-mediated rejection (82). TLN is a usual feature in kidneys that have been removed for terminal rejection (83). In contrast, the frequency and significance of TLN in other immune-mediated renal diseases are less clear. Prominent B cell aggregates were reported in IgA and membranous nephropathy (84, 85). However, GCs have not been reported in either nephropathy, and the presence of lymphoid aggregates has not been associated with specific pathological features. A GC was reported in 1 of 16 reported cases of anti-neutrophil cytoplasmic Ab-associated nephritis (34), whereas we did not observe any GCs in 32 cases that we examined (86). These observations suggested that TLN might be much more common in LN than in some other autoimmune nephritides.

In our study, about half of LN renal biopsies had tubulointerstitial infiltrates that were organized into higher-order lymphoid structures. However, this is probably an underestimation of the true prevalence of GCs and T:B aggregates in LN. This is because a diagnostic renal biopsy represents only a small fraction of the entire kidney, so sampling error always remains an important consideration. Even with this limitation, our data clearly demonstrated that the detection of lymphoid structures on diagnostic biopsy is highly predictive of specific pathological features.

Based on our studies, we propose that human LN arises from at least two distinct pathogenic processes. The deposition of proinflammatory immune complexes in glomeruli likely arises from a breach in systemic tolerance. In contrast, our observations demonstrated that interstitial nephritis is associated with in situ tolerance diatheses. The relative importance of each immunological process is unclear. Furthermore, any interdependence between the two processes is not known. However, observations that interstitial nephritis determines renal survivorship (15, 16) (C. Hsieh et al., submitted for publication) indicate that intrinsic immunological processes contribute to disease severity. Identification of the in situ Ags and factors promoting local B cell selection and expansion in the interstitium should yield important biomarkers and could lead to novel therapeutic strategies in LN.

Acknowledgments
We thank Dr. Martin Weigert for critical evaluation of this work. We also thank Dr. Maria Tretiakova for expert assistance with LCM, Dr. Linda Wagner-Weiner for identifying patients, and Sarah Powers for careful reading of the manuscript.
Disclosures
The authors have no financial conflicts of interest.

References