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

Anthony Chang, Scott G. Henderson, Daniel Brandt, Ni Liu, Ritesh Guttikonda, Christine Hsieh, Natasha Kaverina, Tammy O. Utset, Shane M. Meehan, Richard J. Quigg, Eric Meffre and Marcus R. Clark

*J Immunol* published online 27 December 2010
http://www.jimmunol.org/content/early/2010/12/24/jimmunol.1001983

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

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
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/− plasmablasts in T:B aggregates. The presence of GCs or T:B aggregates was strongly associated with tubular basement membrane immune complexes. These data implicate tertiary lymphoid neogenesis in the pathogenesis of lupus tubulointerstitial inflammation. *The Journal of Immunology, 2011, 186: 000−000.*

The clinical manifestations of systemic lupus erythematosus (SLE) are myriad and range from mild arthralgias and skin rashes to life-threatening nephritis (1). Patients with focal or diffuse proliferative lupus nephritis (LN) have a more rapidly progressive disease and require more aggressive treatment than do those with mesangial proliferative or membranous LN (2). Significant morbidity and mortality are consequences of the nephritis and the cytotoxic therapeutic regimens used to treat it (3).

Pathologically, LN is characterized by immune complex deposition and inflammation in glomeruli and the tubulointerstitium that, if left untreated, can result in scarring and irreversible organ failure. Of the pathological manifestations of LN, glomerulonephritis (GN) is the best studied and the feature most often replicated in murine models of autoimmune disease (4–6). GN is also the renal manifestation most clearly related to the central pathogenic feature of SLE: systemic loss of B cell tolerance (1, 7, 8).

The presence of serum anti-dsDNA Abs identifies lupus patients at increased risk for GN, whereas increasing titers of anti-dsDNA Abs often herald renal flares in individual SLE patients (1). Anti-dsDNA Abs have been isolated from SLE renal biopsies (9, 10), and infusion of some murine and human anti-dsDNA Abs can induce GN in mice (11, 12). Furthermore, recent immunoelectron microscopic studies directly demonstrated the presence of anti-dsDNA Ab-containing immune complexes in diseased glomeruli (13, 14). These observations led to the hypothesis that anti-dsDNA IgG Abs play a central role in the pathogenesis of lupus GN.

However, tubulointerstitial inflammation (TI) is also a common feature of LN (15−17) (C. Hsieh, A. Chang, R. Guttikonda, D. Brandt, T. O. Utset, and M.R. Clark, submitted for publication). On renal biopsy, the presence and degree of TI identify those patients with LN who are at risk for progression to renal failure (15, 16) (C. Hsieh et al., submitted for publication). In contrast, the National Institutes of Health activity index, which primarily assesses glomerular inflammation, does not correlate with prognosis (15, 18, 19). Furthermore, the presence of tubulointerstitial scarring on renal biopsy is more predictive of subsequent renal failure than is glomerular scarring (15, 16) (C. Hsieh et al., submitted for publication). TI can occur independently of GN (20, 21), and TI severity does not correlate with titers of anti-dsDNA Abs (C. Hsieh et al., submitted for publication). These data indicate that TI is an important manifestation of LN that might arise from different pathogenic mechanisms from those implicated in GN.

Organ-specific inflammation is a defining feature of many autoimmune diseases, including Hashimoto’s thyroiditis (22), rheumatoid arthritis (RA) (23), Sjögren’s syndrome (24), and multiple sclerosis (25). In these diseases, infiltrating lymphocytes are often highly organized and resemble lymphoid structures found in secondary lymphoid organs during a normal immune response. This feature is referred to as tertiary lymphoid neogenesis (TLN). B cells within these lymphoid structures secrete autoantibodies...
(26, 27) 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 manifestations, medication history, and serological parameters. Standard procedures were used to process formalin-fixed, paraffin-embedded 2-μm tissue sections for evaluation by light microscopy. For each biopsy, five distributed H&E stains and three periodic acid-Schiff stains were performed.

Standard immunohistochemistry was performed on serial paraffin tissue sections using mAbs to CD3 (LanBrew, Fremont, CA), CD20 (DAKO), CD45 (DAKO), MUM1 (DAKO), and CD138 (DAKO) as primary reagents and appropriate HRP-conjugated secondary Abs. Isotype controls for each primary reagent, using the matched secondary reagent, are provided in Supplemental Fig. 1. mAbs were retrieved by boiling slides for 20 min under pressure in 1 mM EDTA (pH 8). Double stains were done using the EnVision G/2 Doublestain System (DAKO). The number of positively staining cells was counted without knowledge of the clinical data by one renal pathologist (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 manifestations, medication history, and serological parameters. Standard procedures were used to process formalin-fixed, paraffin-embedded 2-μm tissue sections for evaluation by light microscopy. For each biopsy, five distributed H&E stains and three periodic acid-Schiff stains were performed.

Standard procedures for direct immunofluorescence microscopy were used with all slides, including the following Ags: IgG, IgA, IgM, C3, C1q, fibrinogen, k and λ L chains, and albumin (DAKO, Carpinteria, CA). The intensity of immunofluorescence staining was semiquantitatively scored on a scale of 0 to 4+. All biopsies were stained with CD21 (DAKO) and Ki-67 (Labvision), and others were stained with CD4 (Labvision), CD8 (Labvision), CXCL10 (R&D Systems, Minneapolis, MN), FDC (R&D Systems), CXCL13 (R&D Systems), and BAFF (Alexis Biochemicals, San Diego, CA). The interstitial infiltrate was categorized into three patterns: diffuse and scattered, T:B cell aggregates, and ectopic GC. An arbitrary cut-off of SLE and no significant immunosuppressive therapy prior to renal biopsy. Patient I was a 28-year-old male with hypertension and anabolic steroid use who had acute kidney injury, hyperbilirubinemia, and no evidence of SLE. Eight-micrometer-thick frozen tissue sections were placed on positively charged slides and immunostained for CD38 (DAKO) or Ki-67, as previously described (40). Briefly, cryosections were fixed in acetone at −20°C for 5 min, washed with PBS on ice for 30 s, treated with 0.1% H2O2 for 30 s, and incubated in PBS with 10% normal goat serum for 2 min. Tissue sections were incubated with anti-CD38 or anti-Ki67 Abs for 10 min, rinsed in PBS, incubated with HRP-conjugated horse anti-mouse Abs and 5% goat serum in PBS for 5 min, rinsed in PBS, and incubated in 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA) for 5 min. All incubation steps were performed with the glass slide on an ice pack. Finally, the sections were dehydrated in graded alcohols (95 and 100% EOH) for 30 s each and cleared with two rinses of xylene for 3 min each. 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 (pdN6), Amersham Pharmacia Biotech], 2 mM 2′-deoxynucleoside 5′-triphosphates-Mix (Promega), 10 mM DTT, 5% v/v Nonidet P-40, 10 U RNAsin, 15 U Prime RNase Inhibitor, and 125 U Superscript II reverse transcriptase (Invitrogen)]. The cap was incubated for 60 min at 37°C. RT-PCR reactions were collected by centrifugation at 8000 rpm for 60 s into a 500-μl collection tube and heat inactivated at 70°C for 15 min.

Amplification of Ig H and L chains

γ, ιg, and ιp rearrangements were amplified by two rounds of nested PCR in a 40-μl volume (50 mM primers, 1 mM 2′-deoxynucleoside 5′-triphosphates, 1× Coral Load PCR buffer [Qiagen, Valencia, CA], 1.25 U HotstartPlus Taq polymerase [Qiagen], and 8 μl cDNA [first-round PCR product]). The IgG-specific primers consisted of mixtures complementary to conserved leader and framework regions from H and L chain variable regions and conserved 3′ constant L chain regions (41–43) and the 3′ C region (44). It should be noted that the originally published primer sequences 3′SalI-JH1/2 and 3′SalI-JH4/5 each had a base pair deletion. The correct sequences are 3′SalI-JH1/2 5′-TGCGAAGTCGACGTGAAGACAGTGGCAAGCAG-3′ and 3′SalI-JH4/5 5′-TGGCAAGTCCGAGCTGGAGGAGACAGTGGCAAGCAG-3′. Targeted sequences were amplified using 50 cycles of PCR under the following conditions: 94°C for 10 s, 53°C for 30 s, 72°C for 30 s (γ/ιg) or 60°C (ιp) for 30 s, 72°C for 55 s, with a final extension cycle of 72°C for 7 min.

Sequence analysis

PCR products were cloned into the pCR4-TOPO TA vector (Invitrogen), and plasmid DNA was purified using a 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 (FWRs). Nucleotide and amino acid mutations in the V-region were identified by alignment with the closest corresponding framework regions (FWRs). Nucleotide and amino acid mutations in the V-region were identified by alignment with the closest corresponding framework regions (FWRs). Related clones did not represent allelic polymorphisms. Multiple nucleotide changes in a single exon were scored as a single replacement mutation. Related clones did not represent allelic polymorphisms. Multiple nucleotide changes in a single exon would be scored as a single replacement mutation. Related clones did not represent allelic polymorphisms. Multiple nucleotide changes in a single exon would be 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 JAVA applet from Lossos 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/d). 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 co-infiltration 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...
FIGURE 2. Chemokine expression in lymphoid neogenesis. Strong BAFF immunohistochemical staining was observed in T:B cell aggregates (A; original magnification ×400) and in GCs (B; original magnification ×200). In T:B aggregates, CXCL12 (C) and CXCL13 (D) was often present, whereas CXCL10 (E) and CCL21 (F) were found in fewer cases and often with less staining intensity. C–F, Original magnification ×400.

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 interstitium 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 (TBMCIs) (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. 4B). 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 TBMCIs resided within the tubular basement membrane (TBM), characteristic of lupus interstitial nephritis (Fig. 4E). Additional immunofluorescence and electron microscopy images demonstrating TBMCIs 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 γV sequences identified, 12 were cloned from the GC, and 14 were cloned from the surrounding tubulointerstitium. Among the 12 GC γV 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 γV 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-
Combination 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 CDRI and CDRII 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 CDRI (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 lV 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 lV sequences arose from two genomic rearrangements identified in GC-expressed sequences (Fig. 5). Therefore, 32% (11/34) of the identified expressed lV 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/)
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 λ Ig chains in the GC was well represented in the parenchyma. However, several clonally expanded and/or prevalent parenchymal λ 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 γ2, 27 λ, 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).

Analysis of the distribution of mutations indicated selection for replacement mutations in the CDRs and for silent mutations in the FWRs (Fig. 8C). These findings are consistent with Ag-driven clonal selection.

There was also evidence of clonality when the corresponding L chain sequences were examined. Of the 27 distinct λ sequences, four rearrangements were found more than once, with one observed three times (VL1-40*01 JL3*02), for an overall clonality of 13/27 or 48%. The frequency and distribution of mutations in the VL1-40*01 JL3*02 clones were consistent with extensive somatic hypermutation and Ag-driven clonal selection (Supplemental Fig. 4). There was also evidence of clonality in the 31 κ sequences, with an overall clonality of 11/31 or 35% (data not shown).

Clonality was observed in the Ig repertoire expressed in the three other T:B aggregate biopsies that were sampled (patients C–E). A summary of these results is provided in Supplemental Table I. Clonally related sequences from three of the four biopsies were heavily somatically mutated (Fig. 5, data not shown). However, in one T:B aggregate (patient C), the observed clonally selected κ-chains (9 of 37 distinct sequences) were very similar to the corresponding germline sequences (Supplemental Fig. 5). These results indicated that T:B aggregates are associated with moderate clonal restriction. Ongoing somatic hypermutation was observed but was not an invariant feature of the Ig chains being selected in the T:B aggregates.

In the GC histology, 17 of 41 distinct λ-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 λ-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 λ 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.
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 aggregates.

**FIGURE 9.** Clonal selection in a Ki-67+ focus. **A.** Nucleotide sequences of VH4-34*01 Ds-18*01 JH4*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 extracellular B cell responses that were recently described in some murine models of autoimmunity (58). In MRL/Mp*+/− mice and in MRL/Mp*+/− 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 extracellular plasmablast aggregates in an organ targeted by an autoimmune disease. Furthermore, our data indicated that such plasmablast foci are a usual feature of LN complicated by severe T1.

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 germline-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 CDR3 because several processes determine diversity in this region. Therefore, if antigenic specificity is determined primarily by the CDR3, then selection might not be apparent for the other regions. This possibility might be applicable to VH4-34*01D5-18-*01JH4*02, because the CDR3 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 TBMICS. 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/Mp*+/− 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/Mp*+/− 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 T:B aggregates. Rather, available evidence suggests that the NZB/NZW and MRL/Mp*+/− 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 autoreactive 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 underestimate 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 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


