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*J Immunol* 2006; 177:8258-8265; doi: 10.4049/jimmunol.177.11.8258
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Role for Nephritogenic T Cells in Lupus Glomerulonephritis: Progression to Renal Failure Is Accompanied by T Cell Activation and Expansion in Regional Lymph Nodes

Harini Bagavant, Umesh S. Deshmukh, Hongyang Wang, Timothy Ly, and Shu Man Fu

Autoactive T cells are critical in the initiation and maintenance of autoantibody responses that are a hallmark of systemic lupus erythematosus. However, the direct contribution of T cells in end-organ disease like lupus glomerulonephritis (GN) is poorly understood. In this study, we investigated the role of T cells in progression of lupus GN in NZM2328 mice, a murine model of spontaneous systemic lupus erythematosus. At 26 wk of age, NZM2328 female mice showed glomerular immune complex deposits and acute proliferative GN. This was associated with up-regulation of MHC class II and the detection of T cells and CD11c⁺ dendritic cells in the glomeruli. The regional lymph nodes (LN) showed preferential activation of T cells and an oligoclonal T cell response with skewed expansion of certain Vβ families. This suggests an Ag-driven response occurring in the regional LN of nephritic mice during acute GN. In contrast, male NZM2328 mice developed glomerular immune complexes and acute GN, but rarely progressed to fatal chronic GN. Significantly, male kidneys at 40 wk of age did not have detectable dendritic cells and T cells in the glomeruli. Thus, glomerular immune complex deposition initiates an immune response against renal Ags in the regional LN, leading to T cell recruitment into the kidney during acute proliferative GN. This T cell activation and infiltration are influenced by gender-dependent end-organ factors and may determine the progression of acute GN to chronic GN and renal failure. *The Journal of Immunology, 2006, 177: 8258–8265.*

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by the presence of autoantibody responses to cellular Ags (1). In humans, SLE affects multiple organ systems, including the kidney, skin, lung, brain, and heart. The critical role of T cells in autoimmune responses in lupus mice is well established (2–4). Autoactive Th cells are required for the generation of IgG autoantibodies associated with SLE. However, the role of T cells in the pathogenesis of end-organ disease is unclear (5). Renal involvement in SLE is an example of immune complex glomerulonephritis (GN). Deposition of immune complexes in the glomeruli and the ensuing inflammatory response are critical mediators of lupus GN. We have used New Zealand mixed (NZM) 2328 mice, a murine model of spontaneous SLE, to investigate the pathogenesis of lupus GN (6). Male and female NZM2328 mice spontaneously develop serum autoantibodies to dsDNA and nuclear Ags, glomerular immune complex deposits, and lupus GN. Similar to human SLE, NZM2328 females develop fatal renal failure (7). Genotypic and phenotypic characterization of NZM2328 mice has shown that autoantibody responses (specifically anti-dsDNA and anti-nuclear Abs) and GN are regulated by distinct genetic intervals. NZM2328 congenic mouse strain, NZM2328.C57Lc4, fails to generate anti-dsDNA Abs, but develops renal immune complexes and dies of lupus GN, thereby dissociating anti-dsDNA Abs and renal disease (8). Non-dsDNA Ags, including nucleosomes, laminin, α-actinin, and others, have been identified as nephritogenic target Ags (9–11). However, we and others (12–14) have shown that deposition of immune complexes per se is not sufficient to induce progressive GN and renal failure. There is some evidence for the role of autoreactive T cells as well as the end-organ response in dictating the outcome of disease. Clinical studies have shown a significant correlation between prognosis and severity of T cell infiltration (15).

The mapping of lupus susceptibility genes in NZM2328 also identified distinct genetic intervals associated with acute proliferative GN from proteinuria and chronic GN (7). Thus, the progression of acute proliferative GN (characterized by immune complex deposition, mesangial expansion, and increased glomerular cellularity) to chronic GN (with glomerulosclerosis, fibrosis, tubular atrophy, and interstitial inflammation) is determined by different genetic factors and possibly distinct pathogenetic processes. In addition, neonatal thymectomy in NZM2328 mice resulted in accelerated renal immune complex deposition and acute proliferative GN by 20 wk in both male and female mice (16). However, at 30 wk, only female mice developed chronic GN, whereas males did not. Thus, acute proliferative GN may or may not progress to chronic GN and renal failure. This is also supported by clinical studies on the natural history of lupus GN (17). In the present study, we have investigated the role of T cells in progression of lupus GN. The results suggest that activation of T cells in kidney draining lymph nodes (LN) and their infiltration in the glomeruli may play an important role in determining the progression to renal failure.
Materials and Methods

Mice

NZM2328 and NZM2328.C57Le1 mice used in this study were bred in the Animal Facility at the Department of Comparative Medicine, University of Virginia. C57L/J mice were obtained from The Jackson Laboratory. Animal housing and procedures have been approved by the Institutional Care and Use Committee and are in accordance with National Institutes of Health guidelines. Mice were sacrificed at different ages, and tissues were harvested, including axillary and inguinal LN, kidney draining LN, and kidneys. Each kidney was cut transversely into pieces of 3- to 5-mm thickness and processed either by fixing in 10% phosphate-buffered formalin for histopathology, or in 4% paraformaldehyde, followed by sucrose for immunostaining of infiltrating cells, or snap frozen in liquid nitrogen for immune complex deposit staining and RNA isolation. Mice were perfused with saline for analysis of kidney-infiltrating cells by flow cytometry and RNA isolation studies. Mice were anesthetized with tribromoethanol (0.5 ml i.p.), and abdominal and thoracic cavity opened. The tip of the left ventricle was punctured with a needle connected to i.v. infusion set, and the tip of the right atrium was cut to allow blood to flow out. The mice were infused with 10–15 ml of saline over 3–5 min. At this time, the abdominal organs, including liver and kidney, were pale and blood vessels were clear. Saline-perfused kidneys were harvested and processed, as described above.

Histopathology

Kidneys were collected from mice at the time of sacrifice, and one piece was fixed in 10% neutral buffered formalin. The tissues were then processed for paraffin embedding. Sections (5 μm) were cut and stained with H&E and evaluated for pathological changes. Each kidney was evaluated for severity of acute and chronic GN, as previously described (7, 8, 16). Acute GN was graded on a scale of 0–4, assigned for extent of cellular proliferation, with 1 showing mild, focal mesangial involvement; 2 and 3 for severity of acute and chronic GN, as previously described (7, 8, 16). In 4% paraformaldehyde, followed by sucrose for immunostaining of infiltrating cells, or snap frozen in liquid nitrogen for immune complex deposit staining and RNA isolation. Mice were perfused with saline for analysis of kidney-infiltrating cells by flow cytometry and RNA isolation studies. Mice were anesthetized with tribromoethanol (0.5 ml i.p.), and abdominal and thoracic cavity opened. The tip of the left ventricle was punctured with a needle connected to i.v. infusion set, and the tip of the right atrium was cut to allow blood to flow out. The mice were infused with 10–15 ml of saline over 3–5 min. At this time, the abdominal organs, including liver and kidney, were pale and blood vessels were clear. Saline-perfused kidneys were harvested and processed, as described above.

Direct immunofluorescence

Detection of IgG immune complex and complement C3 deposits in kidney was studied by direct immunofluorescence, as previously described (12). Kidneys were frozen in liquid nitrogen and embedded in OCT compound, and 5-μm sections were cut in a cryostat. The sections were fixed in cold acetone, washed in PBS, and blocked with normal goat serum (1/50 dilution in 3% BSA in PBS) for 20 min at room temperature. The slides were then incubated with either goat anti-mouse IgG (1/50 dilution; Southern Biotechnology Associates) or anti-C3 complement (1/100 dilution; Cappel Laboratories) conjugated to FITC for 45 min. The slides were washed in PBS, mounted in Vectashield (Vector Laboratories), and observed under the microscope. Intensity of glomerular staining was scored on a scale of 0–4, with 0 indicating no staining and 4 indicating maximum.

Immunostaining for infiltrating cells

Kidneys were fixed in 4% paraformaldehyde, followed by 30% sucrose in PBS, and embedded in OCT compound, and 5-μm sections were cut in a cryostat for staining of immune infiltrating cells. Serial sections were stained with biotin-conjugated Abs for macrophages (F4/80), dendritic cells (CD11c, clone HL3), MHC class II (M5/114,15.2), T cells (anti-CD5, clone 53-7.3 and anti-Thy-1.2, clone 53-2.1), and B cells (B220, clone RA3-6B2). The tyramide-biotin system (PerkinElmer Laboratories) was used for detection, according to the manufacturer’s directions. The images were captured with a fluorescence microscope at ×20 magnification. To colocalize CD11c+ and MHC II+ cells infiltrating the glomeruli, kidney sections were stained first with FITC-labeled Ab to MHC class II (M5/114,15.2), followed by a goat anti-FITC Ab and a FITC-labeled anti-goat IgG to amplify the signal. The sections were then stained with biotinylated anti-CD11c Ab and detected by the tyramide-biotin system. Using the same protocol, kidney sections were also stained for B and T cells using FITC conjugated Ab to B cells (B220) and biotinylated anti-CD5 Ab. Confocal images were captured using Zeiss confocal microscope and Zeiss LSM image software.

Flow cytometry

Frequencies of T and B cells infiltrating the kidney were studied by flow cytometry. Mice were perfused with saline, and kidneys were harvested, minced, and treated with 1.2 mg/ml bovine collagenase type II (Worthington Biochemical) for 20 min at 37°C with intermittent shaking to obtain single-cell suspensions. The cells were filtered and stained with Abs to CD3 and B220. Stained cells were acquired using a BD FACS scan and analyzed using Flow Jo software (Tree Star). To study frequencies of recently activated T cells, LN cells were harvested from mice at different ages. Single-cell suspensions were made, and three-color staining was conducted with fluorochrome-conjugated Abs to CD4 (H129.19), CD8 (53-6.71), and CD69 (H1.2F3), and their isotype controls (BD Pharmingen). Cells were acquired, and gates were set for CD4-PE-Cy5- and CD8 FITC-positive cells. Frequencies of CD69-PE-positive cells in each gate were determined.

Multiplex RT-PCR for cytokine and chemokine expression in the kidney

Mice were perfused with saline, and kidneys frozen in liquid nitrogen were used for isolation of RNA using RNAqueous kit (Ambion). Ten micrograms of total RNA was used for preparation of cDNA (Cloned AMV First-Strand and cDNA Synthesis kit; Invitrogen Life Technologies), and 2–5 μl was used for each multiplex PCR (Maxim Biotech). The expected amplification sizes are as follows: for mouse inflammatory cytokines genes (GM-CSF (200 bp), TGF-β (249 bp), IL-1α (294 bp), TNF-α (351 bp), IL-6 (453 bp), and for Th1/Th2 cytokine genes (IL-13 (201 bp), IL-12 (237 bp), IFN-γ (284 bp), IL-5 (325 bp), IL-4 (371 bp), IL-2 (436 bp), and IL-10 (538 bp)). Primers for amplification of GAPDH as housekeeping control gene were used. The PCR conditions were as per manufacturer’s instructions, and PCR products were separated on a 2% agarose gel and stained with ethidium bromide for visualization. Densitometric analysis of the bands was conducted using ImageQuant 5.2 software (Molecular Dynamics).

Spectratyping of CDR3 of the TCRs

LN and kidneys from mice perfused with saline were used. Peripheral (axillary and inguinal) LN and regional (kidney) LN were studied. RNA isolation and cDNA preparation were as described above. PCR amplification was conducted using 23 Vβ primers and 1 fluorescent Cβ primer, as described by Paulsson et al. (18). The PCR products were analyzed on an automated high resolving PAGE system in an Applied Biosystems model 3100 DNA genetic analyzer. Data were analyzed using Genotyper 2.0 software program.

Statistical analysis

Statistical analyses were done by Student’s t test using GraphPad Prism 3.0 software (GraphPad).

Results

Kinetics of renal immune complexes, acute and chronic GN in NZM2328 mice

Female NZM2328 mice were sacrificed at different ages. The histopathological changes of GN and presence of renal immune complex deposits were studied, as shown in Fig. 1. Compared with a normal glomerulus (Fig. 1A), mesangial expansion with an increase in glomerular size and cellularity was seen in mice with acute GN (Fig. 1B). All of the photographs in Fig. 1 were taken at the same magnification. The inflammatory changes were predominantly restricted to the glomeruli and periglomerular regions. Little or no inflammation was seen in the interstitial areas, and the tubules were normal. Some mice showed inflammatory foci around the larger blood vessels in the renal medulla (data not shown). Chronic GN was characterized by the glomerular sclerosis, loss of peripheral capillary loops, and Bowman’s space around the glomerular tuft (Fig. 1C). There was extensive interstitial inflammation, accumulation of proteinaceous casts in the tubules, and tubular atrophy. Female mice did not have detectable immune complex deposits at 11 wk (Fig. 1D). At 26 wk, most of the mice had IgG immune complexes and C3 complement deposited in the glomeruli (Fig. 1, E and F). The kinetics of renal disease in NZM2328 female mice studied at 11, 19, 26, 32–34, and 36–40
wk is depicted in Fig. 2. Renal immune complexes of the IgG isotype were rare at 19 wk (Fig. 2A) (16). By 26 wk, most mice had brightly staining IgG and C3 deposits in the glomeruli and histological changes of acute proliferative GN (Fig. 2B). Changes of acute GN and inflammatory cell infiltration were typically associated with glomerular immune complex deposits. At 36–40 wk, females had renal immune complex deposits, severe acute proliferative GN, as well as chronic GN (Fig. 2C). Our earlier studies have shown an excellent correlation between histopathologic changes of chronic GN and severe proteinuria, a clinical indicator of impaired renal function (7).

Male NZM2328 mice developed acute GN as well as renal immune complexes of IgG and C3 (Fig. 1, G–I). Similar to the female kidney, the immune complexes were first predominantly in the mesangial regions and later extended to the peripheral loops with increasing disease severity. However, males had a lower mean severity of acute GN and did not develop chronic GN at 40 wk. At 44–46 wk, 4 of 12 mice developed chronic GN. The kinetics of lupus GN in the male mice is shown in Fig. 2, D–F.

Identification and quantitation of kidney-infiltrating cells

There is a significant increase in the numbers of T and B cells infiltrating the kidney at 26 wk during acute GN, before the onset of irreversible renal failure. Female NZM2328 mice at 8 and 26 wk were perfused with saline, and kidneys were harvested. Single-cell suspensions were made from one kidney, and the cells were stained with Ab to CD3 and B220 and analyzed by flow cytometry. As shown in Fig. 3A, there was a significant increase in CD3+ and B220+ cells in the kidneys at 26 wk of age. In the H&E-stained sections, the glomerular size and numbers of nuclei per glomerulus in 10 glomeruli per kidney were estimated to give a quantitative assessment of the histopathology (Fig. 3, B and C).

To localize the distribution of inflammatory cells in the kidney, serial sections of paraformaldehyde-fixed kidneys from 11- and 26-wk female NZM2328 mice were stained with Ab to T cells (anti-CD5 Ab or anti-Thy1.2 Ab), B cells (B220 Ab), macrophages (F4/80 Ab), dendritic cells (CD11c Ab), and MHC class II
Kidney from a young nonnephritic female showed few inflammatory cells in the interstitial regions (Fig. 4, top panel). Nephritic kidneys had increased numbers of inflammatory cells throughout the kidney specifically in the perivascular regions (data not shown). The distribution of these cells in the renal cortex was unique (Fig. 4, middle panel). F4/80-positive macrophages were restricted to the interstitial regions surrounding glomeruli and tubules. T cells and CD11c⁺ dendritic cells were detected infiltrating the glomeruli. This was accompanied by up-regulation of MHCII expression. CD5⁺ and Thy1.2⁺ cells in the cortex did not stain with B220 Ab. In addition, flow cytometric analysis of spleen and kidney LN from nephritic NZM2328 female mice showed that the B220-negative CD5⁺ lymphocytes were CD3⁺ T cells (data not shown). B220⁺ B cells were rarely found in the renal cortex, although clusters of B cells were seen mostly in the medullary regions (middle panel).

Colocalization of CD11c dendritic cells and MHC II expression was done by confocal microscopy (Fig. 5). In addition to CD11c dendritic cells, other MHC II-expressing cells were also seen in the glomeruli. This intraglomerular invasion of CD11c dendritic cells along with other MHC class II-expressing cells and T cells in nephritic glomeruli may be important in presentation of glomerular Ags and progression to chronic GN.

In male mice at 40 wk, staining of immune cell infiltrates showed few CD11c⁺ cells and T cells in the interstitial regions of the renal cortex with some MHC II expression (Fig. 4, bottom panel). Significantly, these cells were not seen inside glomeruli. F4/80 macrophages were seen in the cortex surrounding the glomeruli. Distribution of inflammatory cells in 44- to 46-wk-old males with severe GN was similar to female mice at 26 wk.

Expression of proinflammatory and Th1-type cytokines in NZM2328 female kidneys

Kidneys of three to five mice at 8, 11, 26, and 40 wk were perfused with saline and used for analysis of chemokines and cytokines by multiplex PCR. A representative picture from one of two experiments with the results of inflammatory cytokine (top panel) and Th1/Th2 cytokine (bottom panel) gene expression is shown (Fig. 6A). A densitometric analysis of PCR products in the top and bottom panels was done, and band intensities are presented as ratio with the control GAPDH band intensity (Fig. 6B). Inflammatory cytokines were significantly higher in NZM2328 mice at 26 and 40 wk compared with 40-wk-old nonlupus control C57L/J females.

![FIGURE 3. Acute proliferative GN at 26 wk is accompanied by increased infiltration of T and B cells in the kidney. Saline-perfused kidneys from 8- and 26-wk-old mice were treated with collagenase, and single-cell suspensions obtained from one kidney were stained with fluorescein-conjugated anti-CD3 and anti-B220 Ab and analyzed by flow cytometry to show frequencies of infiltrating T and B cells. A, Each data point represents one mouse. The opposite kidney was fixed in 10% formalin and processed for histopathology. A quantitative assessment of disease was done by measuring cross sectional diameter of glomeruli (B) and numbers of nuclei in each glomerulus (C). Each data point (B and C) represents a mean of 10 glomeruli/mouse studied.](http://www.jimmunol.org/)

![FIGURE 4. Immunostaining of inflammatory cells, F4/80⁺ macrophages, CD11c⁺ dendritic cells, MHC II up-regulation, and CD5⁺ T cells in renal cortex of prenephritic 11-wk-old (top panel), 26-wk-old NZM2328 female (middle panel), and 40-wk-old NZM2328 male (bottom panel). Glomeruli (g) are indicated. Kidneys in 26-wk-old female mice had increased inflammatory cells in the renal cortex with F4/80⁺ macrophages in the interstitial regions. In addition, CD11c⁺, MHC II⁺, and CD5⁺ cells were seen infiltrating the glomeruli. B220⁺ B cell clusters were seen in medullary regions of 26-wk-old females, but rarely in the cortical regions (middle panel). Histopathology by H&E staining and IgG immune complex deposits in the 26-wk-old female kidney are also shown. Interstitial regions in the 11-wk-old female and 40-wk-old male renal cortex show few CD11c⁺ dendritic, T cells, and MHC II expression around glomeruli (g) and tubules (t). Female NZM2328 at 11 wk and male mice at 40 wk do not show infiltration into the glomeruli.](http://www.jimmunol.org/)
(TNF-α \( p < 0.0005 \), IL-1β \( p < 0.03 \), TGF-β \( p < 0.003 \), and GM-CSF \( p < 0.04 \)), as well as prenephritic 8-wk-old NZM2328 females \( p < 0.014 \). At 40 wk, one of three mice also showed increased expression of IL-6. A multiplex PCR using cytokines related to Th1 or Th2 cell differentiation (Fig. 6A, bottom panel) showed IL-12 as the major cytokine elevated in diseased mice at 26 wk \( p < 0.03 \) and at 40 wk \( p < 0.02 \) compared with prenephritic mice at 8 wk. The ratios for IL-12 shown are significantly \( p < 0.04 \) higher in NZM2328 kidneys at 26 and 40 wk compared with 40-wk-old C57L/J kidneys. Thus, early inflammatory changes were associated with elevated IL-12 at 26 wk in some females and a Th1 bias in the kidney. Furthermore, two of three NZM2328 mice showed IFN-γ expression at 40 wk, supporting the Th1 bias. Th2 cytokines (IL-10, IL-4, IL-5, and IL-13) were not detected.

**Increased frequency of recently activated CD4+ T cells in regional LN**

The results presented led to the hypothesis that Ag-specific T cell activation and infiltration into the kidney may be critical in the development of chronic GN. Because the Ag recognized by these T cells is unknown, an alternative approach was to study activation of T and B cells in the regional kidney LN compared with peripheral (axillary and inguinal) LN. Kidney and peripheral LN were harvested from NZM2328 females and males at different ages. Single-cell suspensions were made and stained by fluorochrome-conjugated Abs to CD4 and CD8 T cell subsets and CD69, and analyzed by flow cytometry to study frequencies of recently activated (CD69+⁷) CD4+ and CD8+⁷ cells. Female mice showed an enrichment of activated CD4+⁷ T cells in the kidney LN compared with the peripheral LN (Fig. 7A). This preferential activation in the kidney draining LN was first statistically significant at 26 wk of age. This period corresponds with the kinetics of Ab deposition and the onset of acute GN in NZM2328 females. Such activation was not seen in CD8+⁷ T cells. NZM2328.C57Lc1 congenic mouse strain has a NZM2328 genetic background, except on a chromosome 1 segment that regulates susceptibility to GN and has been replaced with a segment from nonlupus C57L/J (8). The NZM2328.C57Lc1 mice are protected from spontaneous SLE, do not develop autoantibodies or GN, and were used as age-matched control nonnephritic mice. Increased frequencies of CD69+⁷CD4+⁷ T cells in progression of lupus GN

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** Intraglomerular infiltration of CD11c⁺ (A) and MHC II (B) cells in 26-wk-old NZM2328 female mouse kidney. C, Superimposition of phase-contrast image of glomerulus with CD11c⁺ (red) and MHC II⁺ (green) cells shows colocalization (yellow) of the two markers and is indicated by arrows. Additional intraglomerular MHC II-expressing cells are also seen. MHC II-positive cells and CD11c dendritic cells are also detected surrounding the glomeruli.

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

**Figure 6.** A, Expression of inflammatory chemokines and cytokines in NZM2328 female kidneys at 8, 11, 26, and 40 wk of age using multiplex RT-PCR. NZM2328 mice were perfused with saline at different ages, kidneys were harvested, and RNA was isolated. RT-PCR was conducted, and the products were separated on a 2% agarose gel and stained with ethidium bromide for the expression of inflammatory cytokines (top panel) and Th1/Th2 cytokines (bottom panel). Kidneys from nonlupus mice C57L/J females at 40 wk were used as controls. B, Intensities of RT-PCR products were detected by densitometric scanning of the gels in A. Data were analyzed using Image Quant 5.2 software (Molecular Dynamics), and the results are expressed as cytokine/GAPDH.
T cells were not seen in NZM2328.C57Lc1 female mice. In the NZM2328 mice, increased frequency of CD69⁺CD4⁺T cells in kidney draining LN persisted with the progression of disease. At 37–40 wk, NZM2328 females developed chronic GN with interstitial infiltration and a corresponding increase in recently activated (CD69⁺) cells in the kidney draining LN compared with the peripheral LN. Preferential activation is not seen in nonnephritic NZM2328.C57Lc1 (Lc1) mice or in CD8⁺T cells. A, Preferential activation of CD4⁺T cells is also seen in male NZM2328 kidney LN at 26 wk, albeit at frequencies lower than female. Similar activation is not seen in control NZM2328.C57Lc1 males at 24 wk or in CD8⁺T cells (data not shown). **, p < 0.03 by Student’s t test comparing kidney LN and peripheral LN.

**FIGURE 7.** Frequency of recently activated (CD69⁺) T cells in kidney draining LN (■) compared with peripheral (axillary and inguinal) LN (□) in female and male NZM2328 mice. LN cells were harvested and stained with anti-CD4 PE CyChrome5, CD8 FITC, and CD69 PE, and analyzed by three-color flow cytometry. Each bar, Mean ± SEM of four to six mice per group. A, Female mice at 26 wk have increased frequency of CD69⁺CD4⁺ T cells in the kidney draining LN compared with the peripheral LN. Preferential activation is not seen in nonnephritic NZM2328.C57Lc1 (Lc1) mice or in CD8⁺ T cells. B, Preferential activation of CD4⁺T cells is also seen in male NZM2328 kidney LN at 26 wk, albeit at frequencies lower than female. Similar activation is not seen in control NZM2328.C57Lc1 males at 24 wk or in CD8⁺T cells (data not shown). **, p < 0.03 by Student’s t test comparing kidney LN and peripheral LN.

Oligoclonal expansion of Ag-specific T cells

Preferential expansion of Vβ families as an indication of an Ag-specific response could not be demonstrated by staining of the kidney LN cells with a panel of Vβ-specific Abs. Therefore, spectratyping of CDR3 from different LN was conducted for identification of a skewed oligoclonal response. Twenty-three different Vβ primers from Vβ1-Vβ20 and one Cβ primer were used for PCR amplification. The PCR product for each Vβ family showed four to seven distinct peaks (spectratypes) reflecting size differences in the CDR3. Within a family, the height of each peak reflects the frequency of that particular TCR spectratype in the starting material. In a polyclonal T cell population, the frequencies of the different TCR spectratypes follow a Gaussian distribution (19). An increased frequency of any particular CDR3 length reflected a change in the normal distribution is indicative of an oligoclonal expansion. The primers for Vβ19 failed to amplify a PCR product from NZM2328 mice (spleen and LN), suggesting a deletion of this family (data not shown). Peripheral LN cells were studied in individual mice (n = 3/group) at 11 and 40 wk of age. A representative mouse is shown in Fig. 8. Cells were pooled from three mice/group for analysis of kidney LN. Most of the Vβ families were identical in the peripheral and kidney LN (representative families Vβ1, 2, 8.2, and 14 are shown in Fig. 8). These TCR spectratypes show the typical Gaussian distribution suggestive of a polyclonal expansion. CDR3 distributions in several Vβ families (Vβ1, 2, 5.1, 16, 17, and 18) in kidney LN at 40 wk were different from the LN of mice at 11 wk and peripheral LN of the same mice at 40 wk. The most significant change was seen in the Vβ3 family showing a single dominant spectratype indicating oligoclonal expansion of this family. This experiment was repeated with kidney draining LN from another cohort of three mice at 40 wk of age studied individually. In the second cohort, one of three mice showed a single dominant Vβ3 peak. The other two mice showed only two dominant peaks, demonstrating a skewed distribution and an oligoclonal expansion in the Vβ3 family. As shown in the figure, Vβ3.1, 5.2, 16, 17, and 18 families do not show any single dominant clonal expansion. However, the individual peaks of these families in the kidney LN and kidney at 40 wk show a different relative frequency compared with the normal distribution in the 11-wk-old peripheral LN, indicating an Ag-driven T cell expansion. Significantly, the oligoclonal expansion in the kidney draining LN was also reflected in the TCR analyses of kidneys from the same mice (Fig. 8, bottom panel). These signify T cell

**FIGURE 8.** Spectratyping of CDR3 of TCRs from peripheral LN (top panel) and kidney LN (middle panel) in 11- and 40-wk-old NZM2328 females. Bottom panel, TCR spectratypes from kidneys pooled from the same 40-wk-old NZM2328 females. RNA was isolated from peripheral LN (individually), and kidney LN and kidney (pools of three mice), and RT-PCR was conducted, as described in Materials and Methods. The PCR products were analyzed using Applied Biosystems model 3100 DNA genetic analyzer and Genetyper 2.0 software. Left panel, Representative Vβ families (Vβ3, 1, 2, 8.2, 14) that have a normal distribution of TCR spectratypes and are identical in all the groups. Right panel, Vβ (Vβ3, 5.1, 5.2, 16, 17, 18) families with skewed repertoires in the kidney LN and kidneys of 40-wk mice suggestive of Ag-specific expansion.
activation and expansion driven by renal Ags. The finding of skewed repertoires of multiple Vβ families also implies autoreactive T cell responses against multiple autoantigens or epitopes in the kidney.

Discussion
Lupus GN is a prototype immune complex disease of the kidney. The progression of renal disease is dependent on a variety of pathogenetic processes through FcR-mediated inflammation (13) complement system (20, 21), local expression of inflammatory chemokines and cytokines (14, 22, 23), and molecules affecting innate immune responses (TLRs) (24, 25). Clinical reports show a correlation between interstitial T cell infiltration and poor renal function. The present study shows that glomerular immune complex deposits are accompanied by changes of acute proliferative GN and production of proinflammatory cytokines in the kidney. This is associated with T cell activation in the regional LN. Entry of activated APCs and T cells into the glomeruli occurs in a Th1-promoting cytokine environment and is critical for onset of chronic GN and progressive loss of renal function. This is influenced by the local milieu in the kidney and by gender-dependent factors.

In NZM2328 mice, glomerular immune complex deposits are followed by expression of inflammatory cytokines and chemokines. These changes are followed by inflammatory cell recruitment into the kidney. Similar findings have also been reported in other models of lupus GN (22, 23). The recruitment of T cells has been interpreted to be suggestive of bystander activation and potentially the initiation of a non-Ag-specific immune response. However, our study shows the presence of MHC class II as well as CD11c expression within the glomeruli. The expansion of certain Vβ families in the draining LN and in the kidney adds credence to the hypothesis that this is indicative of an Ag-specific T cell response driven by glomerular or immune complex Ags. This T cell response may play an important role in the progression of lupus nephritis from acute to chronic GN. Schiffer et al. (26) have reported that treatment of proteinuric NZB/W F1 mice with CTLA4-Ig along with cyclophosphamide can protect from fatal renal failure without affecting glomerular immune complex deposits or anti-DNA Ab-producing B cells. Thus, blockade of molecules important for T cell costimulation can prevent disease progression. Their results are consistent with our hypothesis. In addition, the protected mice showed reduced accumulation of CD11c+ dendritic cells, along with decreased expression of CXCL13, a dendritic cell chemokine and potent attractant of T and B cells. Thus, the CD11c+ cells, likely to be dendritic cells, play an important role in progression of renal disease.

Onset of T cell infiltration in the glomeruli corresponds to an increased frequency of recently activated T cells in the regional LN. These changes occur before extensive interstitial inflammation that accompanies chronic lupus GN. The onset of chronic GN at 40 wk is also associated with an oligoclonal expansion of T cells in the regional LN, indicative of a local Ag-specific T cell response. These data suggest an important role for local factors in activation of a nephritogenic immune response. The cytokines detected in the kidney in this model suggest that the pathogenic T cell response has a Th1 bias. It remains to be determined whether oligoclonal expansion of T cells occurs first in the draining LN or the kidney. The finding that skewed TCR Vβ3 spectratypes are detected at both sites suggests that the Ag-specific cells are first activated in the LN and then migrate to the kidney, where they may undergo further expansion. However, the hypothesis that pathogenic T cell activation may occur in the kidney itself cannot be completely excluded. Ag-specific activation of T cells by CD11c+ dendritic cells in the CNS is a critical pathogenic factor and has been reported in the murine models of experimental autoimmune encephalomyelitis (27–30).

Indeed, T cells have been indirectly implicated as important mediators of organ damage in lupus GN. MRL lpr/lpr mice lacking B cells fail to develop lupus GN (31). However, in another study, MRL lpr/lpr mice that lack secreted Iggs, but express a transgenic surface Ab against a hapten on B cells developed a unique type of glomerulosclerosis and interstitial nephritis. Therefore, in MRL lpr/lpr mice, B cells may play an important role as APCs, and thereby T cells as the pathogenic cells in lupus GN (32). Clonal expansions of multiple Vβ families seen in the kidney LN and not peripheral LN further emphasize a kidney Ag-driven pathogenic T cell response associated with progression of lupus GN. A clinical study from SLE patients comparing T cells infiltrating skin lesions and kidney with peripheral blood repertoire showed a preferential oligoclonal expansion of T cells infiltrating the kidney and not skin (33). Murine models provide additional evidence supporting a kidney-reactive T cell immune response. Intrathymic injection of kidney cells and not splenocytes protected MRL lpr/lpr mice from fatal lupus GN (34). Implantation of OVA in the glomeruli could target entry of an OVA-specific T cell line into the glomerulus (35). In lupus, the pathogenic T cells may recognize glomerular proteins or glomerular immune complex Ags.

Male mice develop renal immune complex deposits and a modest regional T cell activation. This is associated with acute GN and rare chronic GN. Thus, despite serum autoantibody responses, renal immune complex deposits, acute proliferative GN, and moderate regional T cell activation, NZM2328 male mice do not show glomerular T cell and CD11c+ cell infiltration and rarely progress to chronic GN and fatal renal failure. These data suggest a role for intrinsic, gender-dependent, end-organ differences in dictating progression of lupus GN. Hormonal factors have been demonstrated to influence T cell proliferation and immune responses (36). Susceptibility to renal disease is also governed by the end-organ despite comparable immune responses (37, 38). Estrogen and estrogen receptor signaling regulate gene expression in the kidney (39). Indeed, mice deficient in estrogen receptor α develop lupus-like GN (40). Thus, the response of the kidneys to immune complex deposition in male and female mice may determine the activation of pathogenic T cells, influx of inflammatory cells, and progression of lupus GN to renal failure. The resistance of male NZM2328 mice to renal failure, but not immune complex deposition or acute proliferative GN, emphasizes the role of the end-organ in disease susceptibility. Our results suggest that these end-organ factors are influenced by gender.

Thus, glomerular immune complex deposition and acute proliferative GN are not sufficient for development of chronic GN and renal failure. Initiation as well as progression of lupus GN are dictated by multiple distinct processes. The investigation into factors contributing to progression of disease will offer the multiple checkpoints for therapeutic intervention.

Acknowledgments
We thank Yan Ge for assistance with the Applied Biosystems DNA genetic analyzer; Amy Morris for tissue sectioning; and the Cell Science Core, Center for Research in Reproduction, University of Virginia, for tissue processing and staining services.

Disclosures
The authors have no financial conflict of interest.
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