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Costimulatory blockade with CTLA4Ig and anti-CD40L along with a single dose of cyclophosphamide induces remission of systemic lupus erythematosus nephritis in NZB/W F1 mice. To understand the mechanisms for remission and for impending relapse, we examined the expression profiles of 61 inflammatory molecules in the perfused kidneys of treated mice and untreated mice at different stages of disease. Further studies using flow cytometry and immunohistochemistry allowed us to determine the cellular origins of several key markers. We show that only a limited set of inflammatory mediators is expressed in the kidney following glomerular immune complex deposition but before the onset of proteinuria. Formation of a lymphoid aggregate in the renal pelvis precedes the invasion of the kidney by inflammatory cells. Regulatory molecules are expressed early in the disease process and during remission but do not prevent the inevitable progression of active inflammation. Onset of proliferative glomerulonephritis and proteinuria is associated with activation of the renal endothelium, expression of chemokines that mediate glomerular cell infiltration, and infiltration by activated dendritic cells and macrophages that migrate to different topographical areas of the kidney but express a similar profile of inflammatory cytokines. Increasing interstitial infiltration by macrophages and progressive tubular damage, manifested by production of lipocalin-2, occur later in the disease process. Studies of treated mice identify a type II (M2b)-activated macrophage as a marker of remission induction and impending relapse and suggest that therapy for systemic lupus erythematosus nephritis should include strategies that prevent both activation of monocytes and their migration to the kidney. The Journal of Immunology, 2008, 180: 1938–1947.

Systemic lupus erythematosus (SLE) nephritis is characterized by immune complex-mediated glomerular and tubulointerstitial inflammation, leading to chronic renal insufficiency in up to 30% of affected patients. Maintenance of disease remission after treatment of a renal flare remains a challenging clinical problem (1). Recently, it has become possible to study the mechanisms involved in induction of complete remission of nephritis in NZB/W mice. The combination of a single dose of cyclophosphamide administered along with six doses of CTLA4Ig and six doses of anti-CD154 (triple therapy) induces prompt reversal of proteinuria in NZB/W mice with established nephritis (2). Although immune complexes and complement persist in the glomeruli, histologic changes in the glomeruli reverse and there is a decrease in expression of several chemokines with efflux or death of renal inflammatory cells (2).

To further understand how inflammatory cells migrate to and from the inflamed NZB/W kidney during active disease and remission, we undertook targeted real-time PCR analysis of 61 inflammatory molecules in the kidneys of NZB/W F1 at various disease stages. Our results show that expression of inflammatory mediators follows the deposition of immune complexes in the glomeruli but that distinct subsets of genes are up-regulated at sequential stages of disease. Our findings yield insight into the progressive inflammatory process in SLE nephritis and identify an activated type II macrophage population as a key marker of proteinuria onset and disease remission.

Materials and Methods

Animals

NZB/NZW F1 females were purchased from The Jackson Laboratory. Urine was tested weekly for proteinuria by dipstick (Multistick; Fisher Scientific). Once fixed proteinuria of ≥300 mg/dl on two occasions 24-h apart appeared, a single dose of 50 mg/kg cyclophosphamide and six doses of 100 μg of CTLA4Ig and 250 μg of anti-CD154 were administered as previously described (2). Sixteen treated mice that entered remission (≥30 mg/dl proteinuria on at least two occasions 3 days apart) were sacrificed at intervals starting 3 wk after treatment. Proteinuria status was confirmed by overnight collection of urine in metabolic cages before sacrifice. Control groups of untreated NZB/W F1 mice were sacrificed at the following ages: 6 wk (5 mice), 16 wk (5 mice), 23 wk without any proteinuria (23w NP: 10 mice, 5 with and 5 without high-titer IgG anti-DNA Abs), 23 wk with new onset proteinuria >300 mg/dl (23w P: 6 mice), and 36–40 wk with established proteinuria >300 mg/dl (36–40w: 12 mice).

Quantitative real-time PCR (qPCR)

After perfusion with 60 ml of cold sterile saline, kidneys were immediately removed into TRIzol (Invitrogen Life Technologies) and total RNA was prepared. Quality of the RNA was verified on a NanoRNA chip (Agilent). Five micrograms of RNA was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen Life Technologies). Amplification was performed in
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pooled Abs to CD4, CD8, F4/80, CD11b, CD11c, CD5, CD49b, and B220. CD19-positive B cells (n = 14), CD4-positive T cells (n = 10) CD11b/CD11c<sup>high</sup> dendritic cells (n = 4), and CD11b/CD11c<sup>low</sup> or CD11b/F4/80<sup>high</sup> macrophages (n = 10) were then isolated from kidneys of nephritic mice using a FACS Aria (BD Biosciences). Isolated cells were >90% pure. RNA was synthesized from the isolated cell populations using a picopure RNA isolation kit (Arcturus Molecular Device) and qPCR was performed using primers specific for CXCR5, IL-1<sub>beta</sub>, BAFF, TNF-alpha, CCL13, and CXCR5 as above.

**Histologic and immunohistochemical analysis of kidneys**

H&E sections of kidneys were scored by a single observer (M.M.) blinded to the treatment group as previously described (4). In brief, glomerular and interstitial disease were scored separately for each kidney using a semiquantitative scale from 0 to 1 (absent) to 3 to 4 (severe). Immunohistochemistry was performed using Abs for IgG (Southern Biotechnology Associates), CD4, CD8, B220, CD62L, CD11c, CD138, F4/80, IL-17 (all from BD Pharmingen), and Foxp3 (eBioscience). Slides were counterstained with 4',6-diamidino-2-phenylindole (Molecular Probes and Invitrogen Life Technologies) and images were captured using a digital charge-coupled device camera system connected to a Zeiss microscope.

**Statistical analysis**

The TIGR MultiExperiment Viewer (TMEV) software package was used for statistical analysis of qPCR data. The average of the raw data for each sample (Ct value) was normalized to the internal control (housekeeping gene β-actin). Normalized expression data were log 2-transformed and scaled to the expression value for a single 6-wk-old mouse given an arbitrary value of 1 (0 by log scale). Cluster analyses show these values and are scaled from −2 or −3 (green) to 3 (red). Statistical analysis between experimental groups was performed using specialized implementations of ANOVA with the t test statistic and permutations available in the TMEV package. Unsupervised hierarchical clustering with bootstrap procedures of samples based on gene expression data was performed using Euclidean metrics with average or complete linkage and visualized using TMEV. To graphically display the results of the statistical analyses, data were scaled to the mean of the 10 young control mice. Data shown in the cluster analyses are for all significant genes. For simplicity, data shown in the graphs represent genes that were significantly different both by statistical analysis of microarray (SAM) and t test analysis and that had >2-fold increased expression over the mean of young controls.

**Results**

**Histologic analysis of kidneys**

Six groups of mice were used in this study: 1) 10 mice 6–16 wk old (6–16w); 2) 10 mice 23 wk old without proteinuria (23w NP); 3) 6 mice 23 wk old with new onset proteinuria >300 mg/dl (23w P) 4) 10 mice 36–40 wk old with established proteinuria >2 wk (36–40w); 5) 7 mice sacrificed 3–4 wk after beginning remission induction therapy and with complete resolution of proteinuria (3–4w post); and 6) 9 mice sacrificed ≥5 wk after remission induction therapy and with complete resolution of proteinuria confirmed on multiple occasions (≥5w post). Kidneys were scored separately for glomerulonephritis and interstitial inflammation using a semiquantitative score from 0 to 4 rated from early or focal to severe as previously described (4). Glomerular inflammation was absent or mild in mice younger than 16 wk of age and in 23-wk nonproteinuric mice. Proliferative glomerulonephritis appeared at the onset of proteinuria (p < 0.001 23w P vs 23w NP). Values are normalized to the mean of 6- to 16-wk mice given a value of 1. Data are shown as log 2. Values of p for the up-regulated genes are p < 0.01 (*) or p < 0.001 (**) as shown.

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All mice sacrificed 3–4 wk after remission induction had complete histologic and clinical remission defined as a glomerular score ≤2 and an interstitial score ≤1. Mice sacrificed 5–14 wk after treatment had no proteinuria but were histologically similar to 23-wk-old mice with new onset proteinuria, indicating that they had impending disease relapse (Fig. 1).

Early expression of inflammatory markers in the kidneys of prenephritic NZB/W mice

There was no glomerular Ab deposition (Fig. 2A) and no difference in the expression of any of the inflammatory markers in the kidneys between 6- and 16-wk-old mice and these two groups were pooled and used as controls for all subsequent analyses (6–16 wk). Although the kidneys of 23-wk-old nonproteinuric mice appeared histologically normal, faint staining of the glomeruli with anti-IgG was observed in all of the mice in this group regardless of levels of anti-dsDNA Abs in the serum (Fig. 2B). Expression of a limited set of inflammatory markers was increased in the kidneys of these mice compared with mice ≤16 wk of age (Fig. 2D). These early markers included the chemokine CXCL13 (BLC) and its receptor CXCR5, CCL20 (MIP-3α) and its receptor CCR6, and CCR8 whose ligand CCL1 is expressed by monocytes and T cells. L-selectin (CD62L) was also increased, indicating migration of L-selectin-positive lymphocytes into the kidney, as was GlcNAc2 (CHST4), a marker for high endothelial venules. The negative regulatory molecules PD-1 and Foxp3 were also increased in the prenephritic kidneys. H&E staining of the kidneys of prenephritic mice revealed small collections of lymphocytes in the perihilar region but no cellular infiltration of the renal parenchyma and no glomerular or interstitial damage (see Fig. 1). Foxp3 (see Fig. 7) and L-selectin staining (data not shown) were confined to this perihilar region.

Expression of inflammatory markers associated with proteinuria onset and disease progression

Bright staining with anti-IgG was observed in the glomeruli of proteinuric 23-wk-old mice (Fig. 2C). Of 61 markers, 15 were up-regulated in the kidneys of these mice compared with young or nonproteinuric controls. Several cytokines and chemokines were...
up-regulated in the kidneys of these mice compared with age-matched nonproteinuric mice (23w NP). The five cytokines were IL-1, TNF-α, IL-6, BAFF, and IL-12. The up-regulated chemokines were CCL3 (MIP-1α), and CCL9 (MIP-1γ) as well as the CCR2 receptor. In addition, up-regulation of endothelial activation markers VCAM-1, E-selectin, and P-selectin was noted (Fig. 3). When mice with new onset proteinuria (23w P) were compared with 6- to 16-wk-old mice, CCL2 (MCP-1), CCL5 (RANTES), CCR5, and CXCR3 were also found to be up-regulated (data not shown).

We next determined which inflammatory markers were increased over the entire course of disease compared with young mice. A comparison of all four untreated groups using ANOVA showed that expression of 52 of 61 inflammatory markers analyzed increased over time as disease progressed to the terminal stages (Table I). Only nine genes (IL-4, IL-12, IL-17, PDL1, CX3CL1, CCL11, CCL21, and CXCL12) were not significantly up-regulated across the four untreated groups during the disease course. Some of these genes (IL-4, PDL1, CCL21, CXCL12) were not expressed at any time during the disease course, whereas others were variably expressed in individual mice (e.g., IL-12 and IL-17 were expressed at high levels in three of eight 36- to 40-wk mice tested but were not expressed in any mice at earlier disease stages).

To further determine whether the expression of inflammatory markers in the kidneys corresponded with disease stage, we performed two-dimensional cluster analysis of the data from the four untreated groups. This analysis showed that the inflammatory kidney phenotype corresponded with the presence or absence of proteinuria (Fig. 4) and with the glomerular (but not interstitial) score. Two clearly distinguishable clusters accurately identified mice with and without proteinuria; all of the proteinuric mice had glo-}

![Image](http://www.jimmunol.org/)
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1, 2lowCD62LlowCD43high phenotype typical of M2 monocytes (Fig. 6, F and G). There was a small population of Ly6ChighGr1high cells in the peripheral blood; these were CD62LhighCD43int and CD11b−CD11c− (data not shown), typical of M1 monocytes. In the kidneys, the CD11b-positive cells were also predominantly Ly6low and Gr1low but they had down-regulated expression of CD43 (Fig. 6, F and G). The renal CD11b-positive cells were mostly F4/80low macrophages but there was also a smaller population of CD11b+CD4/80+CD11c+ dendritic cells (Table I and Fig. 6I). In contrast, analysis of CD11b-positive cells in the spleens showed only a small population of F4/80+ cells and a larger population of CD11c+ dendritic cells (Fig. 6, H and J). Further analysis of the renal F4/80-positive population showed up-regulation of both CD80 and CD86 compared with F4/80-positive spleen cells (Fig. 6I).

We next compared the renal CD11b population from nephritic mice with that of resident CD11b-positive cells from young mice. CD11b-positive cells from nephritic mice had significantly higher expression of F4/80 (Fig. 7), CD11b, and Ox-40L (Fig. 6, K and L). Only very small numbers of CD11b-positive cells were found in the spleens and peripheral blood of young mice. To determine the location of inflammatory cells in the kidneys, immunohistochemical staining was performed for B220, CD138, CD4, CD8, F4/80, and CD11c. Large peritubular collections first started to appear at the age of 23 wk and these consisted of B cells, T cells, and CD11c-positive dendritic cells with a “cap” of F4/80-positive macrophages (Fig. 7)). With the onset of proteinuria, large numbers of strongly positive F4/80 interstitial macrophages appeared in the renal parenchyma and in and around the glomeruli (Fig. 7, A–C) but not within the lymphoid aggregates (arrow, Fig. 7A). With further progression of disease, large disorganized perivascular and periglomerular collections of mixed B cells, T cells, and dendritic cells appeared within the kidney parenchyma, again surrounded by several layers of macrophages (Fig. 7, D–F). Small numbers of CD4 T cells were also observed in the interstitium and around glomeruli (Fig. 7E). In contrast, B cells, CD8 T cells, and dendritic cells rarely invaded the interstitium but were confined to mixed inflammatory infiltrates around blood vessels and occasionally around glomeruli (Fig. 7, D and F). Large numbers of plasma cells were also found in the peritubular and periglomerular infiltrates (Fig. 7, L and M). Upon remission induction, parenchymal infiltrates resolved, leaving just the initial peritubular collections of cells (Fig. 7G). Although L-selectin and Foxp3 were expressed early in the

A large population of CD11bhigh cells was present both in the kidneys and in the peripheral blood of sick mice. In the peripheral blood, these cells were predominantly of the Ly6ClowGr-
disease process mRNA expression of these markers did not decrease after remission induction. Immunohistochemistry revealed that both L-selectin (Fig. 7, N and O) and CD4+ Foxp3-expressing cells (Fig. 7, H–K) were still present in the perihilar collections of remission kidneys, consistent with the qPCR data. Immunohistochemistry also confirmed expression of IL-17 in lymphocytic aggregates in only a few of the older mice (data not shown).

As shown in Fig. 5, remission induction was associated with decreased expression of only a small set of inflammatory mediators whose expression increased again during impending relapse. To determine which cells were making IL-1, BAFF, TNF-α, IL-10, CXCR5, and CXCL13, we performed cell sorting from kidneys of mice with established proteinuria followed by qPCR. We first established that lymphoid cells were making the markers of interest by performing qPCR on separated lymphoid cells and intrinsic renal cells. Fig. 8 shows that BAFF, IL-1, and CXCL13 were made by macrophages and dendritic cells, whereas IL-10 was made by CD4 T cells and dendritic cells with less production by macrophages and dendritic cells, whereas IL-10 was made by all lymphoid cells except B cells as well as by non-lymphoid cells. (Fig. 8). We also showed that CCL20 and lipoxatin-2 were made by intrinsic renal cells and that CCL5 was made predominantly by lymphoid cells but the exact cellular origins of these three markers were not further examined (data not shown).

Our findings identify activated macrophages and dendritic cells as the source of critical markers that herald proteinuria onset, remission, and impending relapse.

Discussion

The inflamed kidneys of patients with SLE and NZB/W F1 mice contain many lymphocytes around glomeruli, blood vessels, and in the interstitium (9–11). Our goal was to understand the mechanisms of lymphocytic invasion of the kidneys in SLE nephritis, resolution of nephritis induced by a combination cyclophosphamide and costimulatory blockade and, relapse following cessation of therapy.

Changes in gene expression in the kidney became apparent before either glomerular or interstitial inflammation could be detected by light microscopy and were associated with early immune complex deposition in the glomeruli and formation of a disorganized collection of lymphocytes in the perihilar region. Of the 17 chemokines tested, only CCL20 and CXCL13 and their monomalous receptors were up-regulated at this time. CCL20, a homoeostatic chemokine in gut and skin, is also expressed on inflamed endothelial cells (12). CCL20 has been found in the lymph nodes and spinal cords of mice during the sensitization phase of experimental autoimmune encephalomyelitis and may thus play an important role in the early stages of autoimmune inflammation (13). The CCL20 receptor CCR6 is expressed on memory T cells, monocytes, and follicular and memory B cells (14) all of which were found in the perihilar infiltrates.

CXCL13 is found in ectopic lymphoid tissues in other inflammatory diseases and has been associated with focal B cell infiltrates in some diseases (15, 16) and with accumulation of CD4-positive T cells in others (17). The CXCL13 receptor CXCR5 was expressed predominantly by B cells in the NZB/W kidney. CXCL13 has been reported to derive from CD11b/CD11c myeloid dendritic cells in the NZB/W kidney (18) but our studies show that it is expressed both by CD11b/CD11c<sup>hi</sup> dendritic cells and CD11b/CD11c<sup>lo</sup> macrophages. Although CXCL13 expression is regulated by lymphotoxin α1β2 in secondary lymphoid organs (19), our data suggest that this is not the means by which it is up-regulated in the lupus kidney. Up-regulation of CXCL13 by peripheral blood monocytes of NZB/W mice can be induced by exposure to IL-1 and TNF-α (20).

Cells expressing regulatory molecules including Foxp3 also appeared in the kidney early in the course of nephritis. Recently, it was reported that Foxp3-expressing T cells appear in the CNS.
early in the course of experimental autoimmune encephalomyelitis and remain in the CNS during convalescence, similar to what we have observed here (21, 22). However, presence of these cells was not sufficient to prevent nephritis onset perhaps because activated effector cells become resistant to suppression by regulatory T cells as a result of exposure to IL-6 and TNF-α (22), both of which are highly expressed in the nephritic lupus kidney. PD-1, a CTLA4-like molecule expressed on activated B and T cells, was also expressed early in the disease course. In autoimmune diabetes, the interaction of PDL1, but not PDL2, on pancreatic cells with PD-1 on lymphocytes mediates inhibition of inflammation in the early phases of disease (23). In the NZB/W kidney however only increased expression of PDL1, a receptor expressed predominantly on activated macrophages and dendritic cells (24) was detected.

Significant increases in the expression of several renal chemokines, endothelial activation markers, and monocyte-derived chemokines were observed concomitant with the onset of proteinuria. Four chemokines, CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), and CCL9 (MIP-1y), were overexpressed at the onset of proteinuria. These chemokines have previously been identified as crucial mediators of SLE nephritis in other mouse models and in humans (25, 26) and are produced both by intrinsic renal cells and by infiltrating lymphocytes, CCR5, the receptor for CCL3, and CCL5 and CCR2, the receptors for CCL2, were also up-regulated at this time. These receptors have previously been identified on glomerular-infiltrating cells in both murine and human SLE nephritis (25, 27, 28). Interestingly, CCR1, a receptor that can interact with both CCL3 and CCL9, was up-regulated only in established disease. This is consistent with studies showing that CCR1 is involved mainly in interstitial infiltration that occurs in the later stages of disease (29).

The cytokine expression profile at the onset of proteinuria, namely, IL-1, IL-10, TNF-α, IL-6, and BAFF, was suggestive of infiltration of the kidney with activated macrophages and dendritic cells. Examination of sorted cell populations from nephritic kidneys showed that macrophages and dendritic cells were the main source of BAFF, IL-1, and CXCL13 and that IL-10 was made by both CD4 T cells and dendritic cells. BAFF is induced on myeloid cells by TLR ligation and is a survival factor for most B cells. It has also been suggested that activated macrophages express BAFF receptors and can stimulate their own survival via BAFF in an autocrine fashion (30). This may help explain why BAFF blockade can induce remission in nephritic NZB/W mice (3). IL-1, derived from infiltrating monocytes, is a pathogenic cytokine in experimental immune complex glomerulonephritis and induces TNF-α production by intrinsic renal cells (31). Sorting experiments showed that TNF-α derived from multiple cell types including intrinsic renal cells but was regulated concomitantly with macrophage and dendritic cell-derived cytokines, confirming its relationship with IL-1 expression. Although systemic TNF-α protects against initiation of SLE (32), local TNF-α production can promote nephritis once immune complex deposition has occurred (33). IL-10 is also pathogenic in the NZB/W model (34) perhaps because it loses its anti-inflammatory properties in the setting of chronic inflammation (35). Blockade of IL-1, IL-10, and TNF-α can all ameliorate nephritis in mice and humans (36–38).

Established proteinuria and increasing renal damage were associated with extensive spreading of the inflammatory response, creating a local chemokine and cytokine “storm.” Interestingly, few T cell-derived cytokines were detected in the kidneys until the late stages of disease. In particular, IL-17 was found in none of the mice with new onset proteinuria and in less than half of the aged mice. This is in contrast to other autoimmune inflammatory diseases such as multiple sclerosis and rheumatoid arthritis in which IL-17 is the predominant inflammatory cytokine made by CD4 T cells (39). Similarly, ICOS, a marker of activated and memory T cells, appeared relatively late in the disease course.

Because there have not previously been murine models of remission of lupus nephritis, little is known about the mechanisms for remission and subsequent relapse of this disease. In experimental immune complex disease, spontaneous remission is associated with complete disappearance of inflammatory markers from the kidney (40); however, in our model there was only partial resolution of the inflammatory phenotype. Disease remission was associated with significant down-regulation of TNF-α, IL-1, BAFF, CCL5, IL-10, and Ox-40L, nearly are all expressed either entirely or in part by activated renal macrophages or dendritic cells. Histologic analysis confirmed disappearance of both F4/80high and CD11c high cells from the kidneys early in the remission process (2); whether these cells died in situ following therapy or migrated out of the kidney remains to be determined. Histologic disease relapse was associated with reexpression of most of these markers, indicating return of activated monocytes to the kidney. Studies in human SLE have similarly shown that clinical and outcome parameters correlate with the degree of glomerular and tubular macrophage infiltration (41).

In nephritic mice, the renal dendritic cell and macrophage populations were segregated topologically: dendritic cells were confined to foci of inflammatory cells surrounding blood vessels and some glomeruli, whereas macrophages were found surrounding the lymphoid aggregates, throughout the interstitium and surrounding area, and occasionally within glomeruli. We show here that the activated renal macrophages were phenotypically different both from peripheral blood monocytes and from the resident macrophages found in young NZB/W kidneys. Renal macrophages in the inflamed kidneys, like those in the peripheral blood, were Ly6C low but they had acquired a high-level expression of F4/80 and increased expression of CD11b, Ox-40L, CD80, and CD86, confirming an activated phenotype. Renal macrophages also lost expression of CD43, a transmembrane glycoprotein that may have a role in cell adhesion. Finally, these macrophages produced cytokines that can amplify the inflammatory response, leading to irreversible renal damage. Thus, the renal macrophages in the NZB/W kidney appear to be most like type II-activated macrophages (also known as M2b macrophages) that can be induced by ligation of Fc receptors and by exposure to TLRs (42, 43).

In summary, our studies show that there is an ordered progression of the inflammatory process SLE nephritis that is recapitulated during relapse after remission induction therapy. We suggest that therapy for SLE nephritis may require an initial combination regimen that depletes activated effector cells followed by maintenance therapy that targets renal endothelial activation, activation of monocytes, and their migration to the kidney.

Disclosures
The authors have no financial conflict of interest.

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