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A Unique Hybrid Renal Mononuclear Phagocyte Activation Phenotype in Murine Systemic Lupus Erythematosus Nephritis

Ramalingam Bethunaickan,* Celine C. Berthier,† Meera Ramanujam,* Ranjit Sahu,* Weijia Zhang,‡ Yezou Sun,‡ Erwin P. Bottinger,‡ Lionel Ivashkiv,§ Matthias Kretzler, † and Anne Davidson*

Renal infiltration with mononuclear cells is associated with poor prognosis in systemic lupus erythematosus. A renal macrophage/dendritic cell signature is associated with the onset of nephritis in NZB/W mice, and immune-modulating therapies can reverse this signature and the associated renal damage despite ongoing immune complex deposition. In nephritic NZB/W mice, renal F4/80hi/CD11cint macrophages are located throughout the interstitium, whereas F4/80hi/CD11cbl dendritic cells accumulate in perivascular lymphoid aggregates. We show here that F4/80hi/CD11cint renal macrophages have a Gr1lo/Ly6Clo/VLA4lo/MHCIIhi/CD43lo phenotype different from that described for inflammatory macrophages. At nephritis onset, F4/80hi/CD11cint cells upregulate cell surface CD11b, acquire cathepsin and matrix metalloproteinase activity, and accumulate large numbers of autoantibodies. These changes reverse after the induction of remission. Gene expression analysis of microarrays; SEM, scanning electron microscopy; SLE, systemic lupus erythematosus. Weijia Zhang, ‡ Yezou Sun,‡ Erwin P. Bottinger, ‡ Lionel Ivashkiv,§ Matthias Kretzler, † and Anne Davidson*

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The online version of this article contains supplemental material.

Abbreviations used in this article: GBM, glomerular basement membrane; IRF, IFN regulatory transcription factor; M1, classically activated; M2, alternatively activated; MFI, mean fluorescence intensity; MMP, matrix metalloproteinase; SAM, significance analysis of microarrays; SEM, scanning electron microscopy; SLE, systemic lupus erythematosus.

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environment, and they can present Ag, but they are poor NO producers and are only weakly phagocytic. This has suggested that they are more like dendritic cells (12, 13, 15, 16), and their nomenclature is now a subject of some debate (15). A minor population of mononuclear cells in normal kidneys is CD11bhi/CD62Lhi/Ly6Chi/Gr1lo/iC3b receptor (CCR2)+/F4/80hi/CD11cint/MHCIIhi/CD86hi; during acute renal inflammation or ischemia, this macrophage population increases markedly and secretes proinflammatory cytokines (17). Small populations of CD11bhi/CD11chi/CD49bhi/F4/80lo (CD11chi) cells, which are rare in normal kidneys, appear in large numbers in lymphoid aggregates during nephritis (20) and disappear upon remission induction. These two major populations of infiltrating mononuclear cells are topographically and functionally distinct from each other and are phenotypically different from the proinflammatory Gr1hi macrophages that infiltrate kidneys during acute inflammatory glomerulonephritis or renal ischemia (17). Our data call into question the traditional classification of Kupffer cells as a unique population of mononuclear phagocytes in chronic SLE nephritis that is associated with reversal of proteinuria onset but before the development of terminal renal failure. Young NZB/W F1 mice were sacrificed at 8–16 wk of age. Kidneys also were obtained from 129/SvJ mice in which anti-glomerular basement membrane (GBM) disease was induced 14 d previously exactly as described (21).

In this study, we analyzed the characteristics and function of the major renal mononuclear phagocyte populations from young and nephritic NZB/W mice and from mice in which remission of nephritis was induced with a combination of cyclophosphamide and costimulatory blockade (19). We show that CD11bhi/CD11cint/F4/80hi cells, which are the dominant mononuclear cells in normal renal interstitium, acquire an activated phenotype during active nephritis that reverses upon remission induction. During active SLE nephritis, F4/80hi cells are a major renal source of several proinflammatory cytokines and chemokines (20), but they also secrete molecules associated with tissue protection and repair that in excess may contribute to tissue degradation. In contrast, CD11bhi/CD11cint/F4/80lo (CD11chi) cells, which are rare in normal kidneys, appear in large numbers in lymphoid aggregates during nephritis (20) and disappear upon remission induction. These two major populations of infiltrating mononuclear cells are topographically and functionally distinct from each other and are phenotypically different from the proinflammatory Gr1hi macrophages that infiltrate kidneys during acute inflammatory glomerulonephritis or renal ischemia (17). Our data call into question the traditional classification of Kupffer cells as a unique population of mononuclear phagocytes in chronic SLE nephritis that is associated with reversal of nephritis onset and tissue damage and whose reversal is associated with remission induction.

Materials and Methods

Mice

NZB/NZW F1 females were purchased from The Jackson Laboratory (Bar Harbor, ME). Urine was tested weekly for proteinuria by dipstick (Fisher Scientific, Pittsburgh, PA). After fixed proteinuria appeared, a single dose of cyclophosphamide and six doses of CTLA4 Ig and anti-CD154 Ab were administered as described previously (19). Mice were sacrificed 3–4 wk after entering complete remission (≤30 mg/dl proteinuria on at least two occasions 7 d apart). Nephritic mice were sacrificed 2–6 wk after proteinuria onset but before the development of terminal renal failure. Young NZB/W F1 mice were sacrificed at 8–16 wk of age. Kidneys also were obtained from 129/SvJ mice in which anti-glomerular basement membrane (GBM) disease was induced 14 d previously exactly as described (21).

Flow cytometry and cell sorting

Single-cell suspensions were prepared from perfused kidneys and stained with Abs to CD11c, CD11b, CD4, CD8, B220, PDCA, VLA4, MHCII, CD80, CD86, CD43, CD44, CD62L, Gr1, Ly6C (BD Pharmingen), and F4/80 (Caltag-Invitrogen, Carlsbad CA), as described previously (20, 22). CD11bhi/CD11cint and CD11bhi/CD11cint/F4/80hi cells were isolated using a BD FACSAria Ilu cell sorter. A dump gate for CD49b, CD4, CD8, CD5, and B220 was used to exclude NK cells and lymphocytes, and DAPI was used to exclude dead cells. Isolated cells were >90% pure. Cytospin preparations of isolated cells were stained with Wright-Giemsa stain.

The proteolytic activities of cathepsins (B, L, and S) and matrix metalloproteinases (MMPs; MMP2, MMP3, MMP9, and MMP13) were measured in vivo using activatable fluorescent sensors ProSense 680 and MMPSense 680 (Visen Medical, Bedford, MA; Ref. 23). A total of 5 nmol of probe in 100 μl sterile PBS was injected i.v. 24 h before sacrifice, and kidney cells were stained with Abs to CD11b, CD11c, and F4/80 as above. F4/80hi cells from nephritic and young mice were sorted into ProSenseB and ProSenseG populations and subjected to real-time PCR for IL-10, iNOS, Mincle, MIP-14, and TIMP-1. Results were normalized to β-actin.

Electron microscopy studies

For transmission electron microscopy studies, sorted renal cell pellets were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 2% aqueous uranyl acetate. For transmission electron microscopy studies, sorted renal cell pellets were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 2% aqueous uranyl acetate, dehydrated with ethanol, and embedded in LX-112 resin (LADD Research Industries, Burlington VT). Ultrathin sections were cut on a Reichert Ultracut UCT, stained with uranyl acetate followed by lead nitrate, and examined in a Tecnai T12 transmission electron microscope (FEI, Hillsboro, OR). Micrographs were captured on a Gatan Science 626 macrograph. The proteolytic activities of cathepsins (B, L, and S) and matrix metalloproteinases (MMPs; MMP2, MMP3, MMP9, and MMP13) were measured in vivo using activatable fluorescent sensors ProSense 680 and MMPSense 680 (Visen Medical, Bedford, MA; Ref. 23). A total of 5 nmol of probe in 100 μl sterile PBS was injected i.v. 24 h before sacrifice, and kidney cells were stained with Abs to CD11b, CD11c, and F4/80 as above. F4/80hi cells from nephritic and young mice were sorted into ProSenseB and ProSenseG populations and subjected to real-time PCR for IL-10, iNOS, Mincle, MIP-14, and TIMP-1. Results were normalized to β-actin.

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FIGURE 1. Flow cytometric analysis of renal mononuclear phagocytes. The gating strategy for CD11bhi cells is shown in Supplemental Fig. 1. A, Gating strategy for the F4/80hi (right), F4/80lo (lower left), and CD11cint (upper left) subpopulations of CD11bhi cells. B, The increase in the percentage of CD11bhi cells as a percentage of live cells in the kidneys of nephritic mice is accounted for mainly by an increase in the F4/80hi population and an influx of CD11cint cells. There is an increase in lymphocyte infiltration (percentage of cells in the dump gate) in nephritic mice. Cellular infiltration resolves upon remission induction (young and remission mice compared with nephritic mice; six to eight mice per group; *p < 0.002, **p < 0.001, ***p < 0.001). C, CD43 staining of the CD11cint (black) and F4/80hi (gray) populations from young and nephritic mice. Light gray is an isotype control for CD43. D, VLA4 and MHCII staining of the CD11cint (black) and F4/80hi (gray) populations from young and nephritic mice. Light gray is an isotype control for VLA4. E, Gr1 staining shows a small population of neutrophils in nephritic mice (arrow). Plots are representative of at least five mice per group. Experiments were repeated more than three times.
citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

For scanning electron microscopy (SEM) studies, sorted cell pellets were quick-fixed in 1% osmium tetroxide, 0.1 M sodium cacodylate, 0.2 M sucrose, and 5 mM MgCl2 (pH 4) (SEM buffer) for 10 s, followed by 2.5% glutaraldehyde in SEM buffer. Secondary fixation was performed with 1% osmium tetroxide in SEM buffer for 30 min, and pellets were dehydrated with ethanol. Pellets then were subjected to critical point dry using liquid carbon dioxide in a Tousimis Samdri 795 critical point drier (Rockville, MD) followed by sputter coat with gold–palladium in a Denton Vacuum Desk II sputter coater (Cherry Hill, NJ). Samples were examined on a JEOL JSM6400 scanning electron microscope (Peabody, MA), using an accelerating voltage of 10 kV.

BrdU incorporation

Mice were loaded i.p. with 1 mg BrdU (Sigma-Aldrich, St. Louis, MO), followed by feeding with water containing 0.8 mg/ml BrdU for up to 60 d. Groups of four mice were sacrificed at intervals, and kidney cells were analyzed by flow cytometry as above, with the addition of anti-BrdU Ab (BD Pharmingen) according to the manufacturer’s instructions. Similar experiments were performed in mice fed with BrdU for 7 or 15 d after nephritis onset. To determine whether remission was associated with the migration of the cells out of the kidneys, we began BrdU feeding 48 h after the initiation of the remission induction regimen and sacrificed mice 21 and 40 d later. In alternate experiments, we fed mice with BrdU for >60 d from the time of the remission until relapse. BrdU then was stopped, and the mice were sacrificed 21 d later.

Labeling of peripheral blood monocytes with fluorescent beads

A total of 100 μl per mouse of a 1:10 dilution of yellow-green fluorescent 1.0-μm latex particles (Polysciences, Warrington, PA) was injected i.v. Peripheral blood and organs were harvested 3 d later and subjected to flow cytometry and immunohistochemical analysis. In alternate experiments, isolated kidney cells were exposed in vitro to latex beads for 30 or 45 min and analyzed by flow cytometry.

RNA amplification, labeling, and GeneChip array hybridization

We performed Affymetrix microarray analyses of cDNA obtained from sorted NZB/W F4/80hi cells from six young kidneys, seven nephritic kidneys, and four kidneys harvested 3–4 wk after the induction of complete remission. RNA was synthesized from sorted cell populations using a PicoPure RNA Isolation kit (Arcturus Molecular Device Corporation, Sunnyvale, CA). The quality of total RNA was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and verified on a NanoRNA chip (Agilent, Santa Clara, CA).

FIGURE 2. Analysis of renal mononuclear phagocyte turnover. Mice were fed BrdU from days 2 to 40 after remission induction (B–F) or for >60 d after remission induction followed by BrdU withdrawal at relapse (G). B, C, F, and G represent cells gated on CD11b and F4/80hi. A, BrdU uptake over time in NZB/W mice (four mice per group). B and C, Downregulation of CD11b expression 21 d after remission (C) compared with the nephritic control (B) occurs in both the BrdU+ (newly arrived) and BrdU− (resident) population of F4/80hi cells. D, Twenty-one days after remission, all of the CD11ch cells (two shaded histograms) are BrdU+, whereas many F4/80hi cells (two black histograms) are still BrdU−. E, Twenty-one days after remission, CD11b+ cells in the spleens are nearly all BrdU+, compared with only a small proportion of B cells and T cells. F, Forty days after remission induction, one third of F4/80hi cells are still BrdU−. G, Twenty-one days after relapse, upregulation of CD11b is evident in both the BrdU+ (resident) and the BrdU− (newly arrived) population of F4/80hi cells. Data for B–G are representative of four mice per group. Experiments were repeated once.

FIGURE 3. In vivo labeling of PBMCs 3 d after the administration of latex beads. A and B, Uptake of fluorescent beads occurs in the peripheral blood CD11bhi/F4/80hi/Gr1hi population. C and D, Bead-laden cells are restricted to the F4/80hi population of renal CD11bhi cells. E–G, No beads are detected in the renal node (E) or thymus (F), but there is some trafficking to CD11bhi cells in the spleen (G). H, Immunofluorescence analysis of kidneys shows beads within F4/80hi cells (original magnification ×10 and ×40). Data are representative of five mice per group. Experiments were repeated once.
A total of 30 ng of total RNA was amplified and labeled with biotin (Ovation Biotin system; NuGEN Technologies, San Carlos, CA), fragmented, and hybridized to Affymetrix Mouse Genome 430 Plus 2.0 GeneChip arrays (Santa Clara, CA). Arrays were washed, stained, and scanned by GeneChip Scanner 3000 7G according to the Affymetrix Expression Analysis Technical Manual.

CEL files were processed using the GenePattern analysis pipeline (http://www.genepattern.com). Normalization was performed using the robust multichip average method and version 10 of the Mouse Entrez Gene custom CDF annotation from Brain Array (http://brainarray.mbni.med.umich.edu/Brainarray/default.asp). The resulting data were log2-transformed. Of the 16,539 mouse genes represented on the Affymetrix GeneChip, 14,415 were expressed above the 27 poly-A Affymetrix control expression baseline and used for further analyses. Unpaired significance analysis of microarrays (SAM) was performed for each statistical comparison between the relevant groups (prenephritic versus nephritic F4/80hi cells, nephritic versus remission F4/80hi cells, and prenephritic versus remission F4/80hi cells). Genes regulated between two groups with a q value (false discovery rate) < 5% were considered significant, and gene lists of interest were uploaded for literature-based pathway analyses on Genomatix BiblioSphere Pathway Edition software (http://www.genomatix.de).

The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through accession number GSE27045.

Real-time PCR

Verification of selected differentially expressed genes was performed using Custom TaqMan Expression Arrays (Applied Biosystems) as per the manufacturer’s instructions. Five internal controls identified by the microarray analysis were tested, and the data were normalized to the mean of the three

FIGURE 4. Morphology of renal mononuclear phagocytes by Wright-Giemsa staining and SEM. A, Gating strategy for the sorting of F4/80hi (dashed), CD11chi (gray), and F4/80lo (black) populations (see also Supplemental Fig. 1). B, The F4/80lo population consists of round macrophage-like cells and some neutrophils (data not shown). C and D, There is an increase in volume and marked vacuolization of the F4/80hi population in nephritic mice (D) compared with that of young mice (C). E, The CD11chi population has a veiled morphology characteristic of dendritic cells. Original magnification ×100 for Wright-Giemsa stains and ×750 and ×10,000 for SEM. E–H, Representative transmission electron microscopy visualization of F4/80hi cells from young (F) and nephritic mice (G, H) show multiple vacuoles in the nephritic mice, many of which have double membranes (inset from H). Data are representative of three to four mice per group. Experiments were repeated once.

FIGURE 5. Functional studies of renal mononuclear phagocytes. A, In vivo cathepsin labeling of young F4/80hi cells (green), nephritic F4/80hi cells (red), and nephritic CD11cim cells (blue). CD11b+ lymphocytes are shown in gray. B, MFI values for cathepsin activity (**p < 0.02, *p < 0.01). C, In vivo MMP activity of young F4/80hi cells (green), nephritic F4/80hi cells (red), and nephritic CD11cim cells (blue). CD11b+ lymphocytes are shown in gray. D, MFI values for MMP labeling (**p < 0.05). E, In vitro phagocytosis assay shows that the majority of bead uptake is in the renal F4/80hi population (red) compared with the CD11cim population (green). Beads were incubated with renal cells for 45 min in this experiment. F, RNA from sorted F4/80hi ProSensehi (gray, young; black, nephritic) and ProSenseim (white, nephritic) cells was analyzed by real-time PCR. Data are representative of at least four mice per group. Experiments were repeated twice.
most stable control genes (Gus-B, Oaz1, and β-actin) and then to the mean of the young controls. To identify significantly differentially expressed genes between groups, SAM (version 2.1) (24) was carried out. Genes regulated between two groups with a q value (false discovery rate) <5% were considered significant.

**Results**

**Remission induction using cyclophosphamide and costimulatory blockade**

We have reported previously that the disappearance of proteinuria after remission induction therapy is associated with decreased glomerular damage and interstitial inflammation scores by light microscopy and with markedly increased longevity (19, 20). Remission of nephritis in the group of mice studied here was characterized by a disappearance of proteinuria from ≥300 to ≤30 mg/dl on repeated measurements over a 2–4 wk period, whereas untreated mice remained proteinuric. Blood urea nitrogen levels remained within the normal range in the treated mice (22.0 ± 8.1 versus 22.8 ± 2.6 in young mice; p = NS), whereas they increased in controls (49.6 ± 25.0; p < 0.001 versus both young and remission mice). A decrease in interstitial lymphocytic infiltration of the kidneys evaluated by the quantification of renal cells expressing B220, CD5, CD4, CD8, or CD49b also was observed in the remission mice compared with that in the controls (Fig. 1B, Supplemental Fig. 1).

**NZB/W kidneys contain several distinct mononuclear phagocyte populations**

Renal CD11b<sup>+</sup> and CD11c<sup>+</sup> mononuclear phagocytes in the kidneys of young, nephritic, and remission mice were analyzed by flow cytometry using the gating strategy shown in Supplemental Fig. 1. The major (F4/80<sup>hi</sup>) population in young mice had a phenotype similar to that of renal dendritic cells, namely, CD11b<sup>hi</sup>/CD11c<sup>lo</sup>/F4/80<sup>hi</sup>/Gr1<sup>lo</sup>/VLA4<sup>lo</sup>/MHCII<sup>lo</sup>/CD43<sup>lo</sup>/CD62L<sup>lo</sup> (17). A smaller population of cells (F4/80<sup>lo</sup>) was CD11b<sup>hi</sup>/CD11c<sup>lo</sup>/F4/80<sup>lo</sup>/Gr1<sup>hi</sup>. A third, infrequent population (CD11c<sup>hi</sup>) was CD11b<sup>hi</sup>/CD11c<sup>lo</sup>/F4/80<sup>lo</sup>/Gr1<sup>lo</sup>/MHCII<sup>lo</sup>/CD43<sup>lo</sup>/CD62L<sup>lo</sup> (Fig. 1). A fourth population, comprising <2% of all of the cells in the lymphocyte/monocyte gate, was CD11b<sup>hi</sup>/CD11c<sup>lo</sup>/F4/80<sup>lo</sup>/Gr1<sup>lo</sup> and may correspond to the CD103<sup>+</sup> population found in other tissues (25). Finally, <0.5% of all of the cells in the lymphocyte/monocyte gate had a plasmacytoid dendritic cell phenotype (B220<sup>+</sup>/PDCA<sup>lo</sup>).
The latter two populations did not increase in frequency during the disease course and were not studied further. CD11b<sup>hi</sup> cells increased by 3- to 4-fold in nephritic compared with young kidneys (Fig. 1A, 1B). The F4/80<sup>hi</sup> subset increased by 2- to 3-fold and upregulated the surface expression of CD11b, F4/80, Ox40L, and CD80 (20). These cells were also MHCII<sup>hi</sup>/VLA4<sup>hi</sup>/CD43<sup>hi</sup> (Fig. 1C, 1D) The CD11c<sup>hi</sup> subset underwent 10-fold expansion (Fig. 1A, 1B) and was MHCII<sup>hi</sup>/VLA4<sup>hi</sup>/CD43<sup>hi</sup> (Fig. 1C, 1D). The F4/80<sup>hi</sup> subset expanded 3-fold during nephritis but remained a minor population (Fig. 1A, 1B). Few neutrophils (CD11b<sup>hi</sup>/F4/80<sup>−</sup>/Gr1<sup>hi</sup>) were detected either before or during nephritis (Fig. 1E). The renal CD11b<sup>hi</sup> population of mice in complete remission resembled that of young prenephritic mice (Fig. 1A, 1B). Importantly, we could not identify the CD11b<sup>hi</sup>/CD11c<sup>hi</sup>/F4/80<sup>hi</sup>/VLA4<sup>hi</sup>/Ly6C<sup>hi</sup> population reported by others in acute models of renal inflammation, including anti-GBM disease (Supplemental Fig. 1A, 1H). Conversely, the CD11c<sup>hi</sup> population that we identified in nephritic NZB/W mice was not increased in frequency in mice with anti-GBM disease (data not shown).

**Cell turnover rates of the major mononuclear phagocyte populations**

Using BrdU incorporation, we determined that the CD11c<sup>hi</sup> population had a renal half-life of 6.5 d in young mice. In contrast, the F4/80<sup>hi</sup> population had a half-life of 16 d (Fig. 2A); this did not increase in nephritic mice. To analyze the fate of CD11b<sup>hi</sup> cells during remission, we administered BrdU starting 48 h after the initiation of remission induction. At day 21, 64.6 ± 8.7% of the F4/80<sup>hi</sup> population was still BrdU<sup>+</sup> (Fig. 2C, 2D), indicating that the cells had not proliferated or been replaced by newly formed cells. In contrast, splenic CD11b<sup>hi</sup> cells were nearly all BrdU<sup>+</sup> (Fig. 2E). Even 40 d after remission induction, 32.7 ± 6.7% of the renal F4/80<sup>hi</sup> population remained BrdU<sup>+</sup> (Fig. 2F). Both BrdU<sup>+</sup> and BrdU<sup>−</sup> populations in remission kidneys downregulated CD11b expression (mean fluorescence intensity [MFI] 41,900 ± 15,338 in remission mice versus 74,860 ± 16,222 in nephritic controls; p < 0.04, compare Fig. 2B with Fig. 2C, 2F). In contrast, at day 21, all of the remaining CD11c<sup>hi</sup> cells were BrdU<sup>+</sup>, confirming the rapid turnover of this population (Fig. 2D). In an alternate experiment, we administered BrdU for >60 d to mice in remission and stopped it when the mice relapsed. Twenty-one days later, all of the CD11c<sup>hi</sup> cells were BrdU<sup>+</sup> (data not shown), indicating that they had been replaced, whereas 34.1 ± 11.7% of F4/80<sup>hi</sup> cells were still BrdU<sup>+</sup> (Fig. 2G), indicating that they had been present at the time of relapse. Both BrdU<sup>+</sup> and BrdU<sup>−</sup> populations had upregulated CD11b expression (MFI in relapsed mice 72,900 ± 8661, p = NS, compared with nephritic controls; compare Fig. 2G with Fig. 2C, 2F). In sum, these data suggest that the activation of F4/80<sup>hi</sup> cells, as assessed by the upregulation of CD11b, occurs in situ and that this activation reverses upon remission induction. In contrast, the renal CD11c<sup>hi</sup> population turns over rapidly and rapidly clears from the kidneys upon remission induction.

**Origins of renal F4/80<sup>hi</sup> macrophages**

We used latex beads to label PBMCs in nephritic NZB/W mice. Beads were endocytosed into peripheral Gr1<sup>lo</sup> cells (Fig. 3A, 3B) and 3 d later were detected only within the renal F4/80<sup>hi</sup> population (Fig. 3C, 3D, 3H). No beads were detected in renal lymph nodes (Fig. 3E) or thymus (Fig. 3F), although a small number was detected in the spleen (Fig. 3G). Thus, peripheral blood Gr1<sup>lo</sup> monocytes are the precursors of the renal F4/80<sup>hi</sup> population. CD11b expression was higher on renal cells than that on matched peripheral blood cells (data not shown), which is consistent with our conclusion that the activation of this cell population occurs in situ in the kidneys.

**The three major renal mononuclear phagocyte populations have different morphologies**

Cells were sorted into the subpopulations shown in Fig. 4A. Isolated F4/80<sup>hi</sup> cells were round, with large nuclei and little cytoplasm, which is consistent with a macrophage phenotype (Fig. 4B). In young and remission mice, F4/80<sup>hi</sup> cells were large and round with kidney-shaped nuclei and small dendrites (Fig. 4C). In nephritic mice, the F4/80<sup>hi</sup> cells became swollen and contained multiple double-membrane vacuoles characteristic of autophagic vacuoles (Fig. 4D, 4G, 4H). Oil Red O stain was negative for lipid (data not shown). CD11c<sup>hi</sup> cells were large with many dendrites (Fig. 4E) and had a veiled morphology by electron microscopy consistent with a myeloid dendritic cell phenotype (Fig. 4E).

**Renal mononuclear phagocyte subsets have different functional properties**

The F4/80<sup>hi</sup> population in nephritic mice had a significant increase in both cathepsin and MMP activity; no increase in either ca-
Affymetrix microarray analyses were performed using cDNA from sorted NZB/W F4/80<sup>hi</sup> cells. We found 694 genes differentially expressed in nephritic compared with young kidneys (q value <0.01, fold change ≥1.5 and ≤0.7 for the upregulated and downregulated genes, respectively) (Supplemental Table I) and 794 genes differentially expressed in remission compared with nephritic kidneys (Supplemental Table II). A total of 378 genes overlapped between the two comparisons and thus were biomarkers of nephritis activity (Supplemental Table III). Only 12 genes were differentially regulated between young and remission kidneys. We examined the 378 coregulated genes in more detail using the natural language processing tool (Genomatix Bibliosphere) (26, 27). Transcriptional network analysis integrating these 378 differentially regulated mRNAs with literature mining (PubMed database) and automated promoter analysis highlighted ITGAM, Stat3, TIMP-1, IL-10, and CCL2 among important regulatory nodes (Fig. 6, Supplemental Table IV).

We chose a panel of 75 genes with varying degrees of upregulation at nephritis onset and a set of control genes for confirmatory studies using quantitative real-time PCR. Six additional genes specific for the M1/M2 macrophage genotype but without significant differences by microarray also were included. Using cutoff values of 2-fold overexpression and a q value <0.05, 49/75 genes were significantly different between young and nephritic mice, and 50/75 were significantly different between nephritic

Table I. Top 10 canonical and signal transduction pathways (Genomatix literature mining) as assessed by Genomatix Pathway System software

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<td>2 IL-12-mediated signaling events (63)</td>
<td>1.83 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>9</td>
<td>2.86</td>
</tr>
<tr>
<td>3 FOXM1 transcription factor network (42)</td>
<td>2.41 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>7</td>
<td>1.90</td>
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<tr>
<td>4 Fibrilosis pathway (16)</td>
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<td>4</td>
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<td>5 Inhibition of matrix metalloproteinases (9)</td>
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<tr>
<td>6 Aurora B signaling (41)</td>
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<td>1.86</td>
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<tr>
<td>7 Costimulatory signal during T cell activation (20)</td>
<td>1.11 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<td>0.91</td>
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<td>8 Phospholipase C6 in phospholipid associated cell signaling (4)</td>
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<td>6</td>
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<td>10 Basic mechanism of action of ppara pparb(d) and pparg and effects on gene expression (5)</td>
<td>1.86 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<td>8 IL-6 (IFN, β2) (581)</td>
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<td>From the 794 genes regulated in macrophages from remission compared with nephritic kidneys</td>
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<td>2 Cell division cycle 2 (G&lt;sub&gt;i&lt;/sub&gt; to S and G&lt;sub&gt;2&lt;/sub&gt; to M) (216)</td>
<td>1.18 × 10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>41</td>
<td>13.72</td>
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<td>13.79</td>
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<td>33</td>
<td>13.41</td>
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mice and treated mice. There was 94% overlap between these two gene sets (Fig. 7, Supplemental Table V).

Pathway analysis using the Genomatix Pathway System (28) was performed on the 694- and 794-gene lists. The top 10 canonical pathways and signal transduction pathways (Genomatix literature mining) are displayed in Table I. Prothrombotic and tissue degradation pathways were among the most significantly regulated canonical pathways in nephritic compared with young kidney macrophages, and IL-10 was the top significant signal transduction pathway. A total of 28 genes of this pathway were altered in the macrophages from remission compared with nephritic kidneys. The top functions identified using Ingenuity Pathway Analysis software included inflammatory response, cell-to-cell signaling and interaction, hematological system development and function, and immune cell trafficking.

The gene expression studies revealed a mixed phenotype with the expression of proinflammatory and anti-inflammatory genes as well as genes involved in tissue repair. We have confirmed the increased protein expression of CD11b and MMP14 previously (20, 29). To confirm increased protein expression of CXCL13, IL-10, and IKKε, we performed immunohistochemistry, flow cytometry, and Western blot analysis (Fig. 8). To determine if the presence of cathepsin activity could distinguish populations of F4/80hi cells with different gene expression profiles, we sorted ProSensehi and ProSenselo subsets from nephritic mice and ProSensehi cells from young mice and performed real-time PCR for the expression of IL-10 (regulatory), IKKε, Timp-1, and MMP14 (repair). Cells from young mice had low expression of all of the markers, whereas increased expression of all of the markers was observed in both ProSensehi and ProSenselo populations from nephritic mice with no difference in the expression between the two populations (Fig. 5F). In accord with these data, we were unable to distinguish F4/80hi subpopulations on the basis of intracellular IKKε staining (Fig. 8E).

Discussion

Renal mononuclear phagocyte infiltration is associated with poor disease outcome in SLE nephritis (1). In this study of NZB/W lupus-prone mice, we show that there is an expansion of two major populations of mononuclear phagocytes in nephritic kidneys. The renal F4/80hi population is the major population in normal and prenephritic kidneys and has a CD11chi/CD11cint/F4/80hi/Gr1lo/Ly6Cint/VLA4hi/MHCIIhi/CD80hi/CD43hi/CD62Llo phenotype. This population is derived from circulating Gr1lo monocytes that migrate into nephritic kidneys where they become activated in situ and form a cuff around the glomeruli and around the edges of large interstitial inflammatory infiltrates (20). Nephritic NZB/W kidneys additionally accumulate a population of CD11bhi/CD11cint/F4/80hi/MHCIIhi/Gr1lo/VLA4hi/CD43hi/CD62Llo myeloid dendritic cells that are located only within lymphoid aggregates (20) and disappear upon remission induction. Differences in the location of dendritic cells and macrophages similarly have been observed in human SLE kidney biopsies (14). Notably, the CD11bhi/Ly6Cint/VLA4hi/CD43hi/CD62Lhi inflammatory macrophage population (17) that is found in most models of acute renal inflammation remains a minor population in NZB/W mice and in two other murine SLE models that we have studied (30, 31).

The two major renal mononuclear phagocyte populations are functionally distinct. The F4/80hi population is phagocytic and acquires both MMP and cathepsin activity at nephritis onset. The cells also increase their volume due to the accumulation of autophagocytic vacuoles. Intracellular degradation of cytoplasmic proteins and organelles by autophagy is required for type I IFN responses to viral infections (32) but is also a physiologic response to inflammation or cellular stress and can be induced in human macrophages in vitro by exposure to growth factors and IL-10 (33). Although this process conserves energy, protects the cell from death, and helps to clear apoptotic bodies (34), it may enhance the presentation of nuclear and cytosolic Ags (35, 36) and facilitate inflammation (37). In contrast, the CD11chi population is phagocytically weakly phagocytic, downregulates the expression of MHC class II, and does not acquire MMP activity or accumulate autophagocytic vesicles. Downregulation of MHC class II has been observed in dendritic cells exposed to hypoxia (38) or activated by cytokines, including IL-10 (39).

To further understand why infiltrating F4/80hi cells are associated with tissue damage, we performed gene expression analysis on isolated cell populations from young, nephritic, and remission mice. One goal of our gene expression analysis was to gain insight into the mechanisms by which renal F4/80hi macrophages contribute to the pathogenesis of lupus nephritis by determining whether they exhibit features of classical or alternative activation. In addition to immune complexes and TLR ligands that promote an M2b phenotype, lupus nephritic macrophages are exposed to opposing signals from TNF-α and tissue damage, and we hypothesized that they may exhibit a mixed or potentially novel

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**FIGURE 8.** Protein expression of analytes of interest. A and B, CXCL13 staining was observed in periglomerular infiltrates of nephritic mice (A) but not young controls (B). Original magnification ×10. C, Periglomerular F4/80hi macrophages were present only in nephritic mice (same mouse as A). Original magnification ×10. D, Intracellular IKKε staining was significantly increased in F4/80hi cells of nephritic mice (blue) compared with young mice (red) and isotype controls (purple). E, IKKε staining was uniform in both ProSensehi and ProSenselo populations from nephritic mice. F and G, Western blot analysis for IL-10 in sorted F4/80hi renal cells from young NZB/W mice (lane 1), mice with new onset proteinuria (lane 2), and mice with established proteinuria (lanes 3 and 4). Red bands show the actin control. H, IL-10/actin ratio.
phenotype. Our data demonstrate that extensive changes in gene expression occur at proteinuria onset and reverse with remission induction. Surprisingly, we found that the expression profile has features that are consistent with a regulatory phenotype (7, 40), including the expression of IL-10, PPAR-γ, antiapoptotic genes, cytokine inhibitors such as IL-1RA and IL-18 binding protein, effector cytokine receptors such as Mer kinase and transglutaminase 2, and anti-inflammatory receptors such as the PGE2 receptor. Induction of SOCS3 is associated with a failure to upregulate IL-6 and TNF-α. There is an increase in CD11b, an integrin required for cell activation, adhesion, and phagocytosis but whose engagement also can mediate negative feedback on the TLR pathways and induce the production of IL-10 (11, 41).

The expression profile also displays a proinflammatory phenotype. Proinflammatory genes include Trem-1, an ITAM-containing cell surface molecule that amplifies TLR-mediated responses (42), activating Fc receptors and formyl peptidyl receptors, C-type lectins associated with tissue injury such as Mincle (43) and oxidized low-density lipoprotein receptor 1 (44, 45), chemokines such as CCL2, CCL5, CCL7, and CCL8, and costimulatory molecules such as CD80 and CD40. We also detected the increased expression of C3, complement factor B, and properdin, suggesting a role for the activated F4/80hi population in the amplification of the alternative complement pathway (46); this, together with the increased production of coagulation factors, could propagate endothelial damage and thrombosis. The cells also upregulate CXCL13, a chemokine that can be induced by IL-10 as well as by IL-1 and TNF-α (11, 47, 48) and that helps to orchestrate lymphoid neogenesis in inflamed tissues. Finally, we observed the expression of MMPs and cathepsin proteases that are associated with tissue repair during the resolution of acute inflammation but could mediate tissue degradation if chronically expressed.

Many of the proinflammatory genes expressed by activated renal macrophages in nephritic NZB/W kidneys are induced in human macrophages by TNF-α (L. Ivashkiv, unpublished observations) but not by the ITAM cross-linking that induces a regulatory macrophage phenotype. TNF-α, made by multiple cell types in inflamed kidneys including nonlymphoid renal cells (20), is pathogenic in the effector phase of SLE nephritis (49). One gene induced by TNF-α and highly upregulated in F4/80hi cells from nephritic kidneys is IKKe (IKKi), a kinase that enhances the induction of a subset of NF-kB-regulated genes and phosphorylates the IFN regulatory transcription factors (IRFs) IRF-3 and IRF-7, major inducers of type I IFNs. IKKe phosphorylates STAT1 and enhances the formation of the ISGF3 transcriptional complex (50) that helps to mediate some of the inhibitory and antiproliferative effects of type I IFNs (51, 52). IKKe is dispensable for acute inflammatory responses (53). In contrast, its expression is markedly upregulated in F4/80hi macrophages derived from chronically inflamed adipose tissue of mice fed a high-fat diet, and it is essential for maintaining low-grade inflammation and propagating obesity in these mice (53). IKKe-deficient mice are less sensitive to arthritis induction in a passive transfer model; the inhibition of type I IFNs and IKKe is therapeutic in this disease (54). These observations suggest a role for IKKe in chronic inflammation.

We have confirmed the increased protein expression of CD11b, CD80, CD86, MMP2, MMP14, IKKe, CXCL13, and IL-10 and the presence of functional cathepsin and MMP activity in the F4/80hi macrophage population of nephritic mice in this and previous studies (Figs. 5, 8, Refs. 20, 29). Further work will be needed to confirm the protein expression of other genes in the expression profile and to determine whether the macrophages express the genes comprising the hybrid profile simultaneously, there are different subsets expressing different aspects of the profile, or the mixed phenotype reflects an evolution of one type of macrophage from another.

Our data, in sum, point to a unique activation profile of macrophages and dendritic cells in SLE kidneys quite different from that found in acute renal inflammation or ischemia. We surmise that this phenotype results from chronic exposure of SLE kidney macrophages to immune complexes, cytokines such as TNF-α, TLR signals, fibrinogen, dead and dying cells, hypoxia, and other danger signals. The resultant mixed phenotype is associated with chronic and progressive renal injury. The M1-like characteristics of kidney macrophages likely contribute to the local inflammatory process, but it is less clear whether the M2 components of the phenotype are protective or pathogenic. It is possible that the expression of M2 genes that are used to resolve inflammation or repair tissues in other settings “inadvertently” contributes to pathogenesis by promoting excessive tissue remodeling and linked proliferation.

Another striking finding is that pathway analysis identified central roles for genes with allelic polymorphisms that have been linked to SLE, including Il10, Igam, Ptpn22, Atg5, and Irf7. The pathogenic role of IL-10–secreting macrophages in chronic inflammation has been suggested by our previous studies in NZB/W mice with proliferative glomerulonephritis accelerated by type I IFNs. In these studies, the depletion of IL-10–producing phagocytic renal macrophages prevented ongoing crescent formation and renal damage (29). IL-10 also may augment mesangial proliferation in inflamed kidneys (29, 55); IL-10 antagonism is therapeutic in NZB/W mice (56), and a small clinical study of anti–IL-10 Ab in humans showed salutary effects (57). It will be worthwhile in future studies to examine the function of the other molecules identified in genetic studies, specifically in macrophages and dendritic cells.

There has been much recent interest in manipulating macrophage programming and migration in acute renal diseases. Therapeutic targeting of key molecules identified here in the murine models should help to define the function of the major renal mononuclear phagocyte cell types found in SLE kidneys. An enhanced understanding of macrophage and dendritic cell phenotypes and function in patients with SLE nephritis will be needed to appropriately target these cell populations in humans.

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Disclosures

The authors have no financial conflicts of interest.

References