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Structure and function of renal macrophages and dendritic cells from SLE-prone mice

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Abstract

Objective—To characterize renal macrophages and dendritic cells in two murine lupus nephritis models.

Methods—We used a bead based enrichment step followed by cell sorting to isolate populations of interest from young and nephritic mice. Cell morphology was examined by microscopy. Arginase and nitrite production were examined using biochemical assays. Antigen presentation function was determined using mixed lymphocyte reactions. Selected cytokine, chemokine and TLR profiles were examined using quantitative real time PCR.

Results—We identified two populations of macrophages and three populations of dendritic cells in both SLE models. F4/80^{hi} macrophages, that are normal kidney residents and increase during nephritis, do not produce either arginase or nitrite upon cytokine stimulation and acquire a mixed pro and anti-inflammatory functional phenotype during nephritis that resembles the constitutively activated phenotype of gut F4/80^{hi} macrophages. The various cell types differ in their expression of chemokine receptors and TLRs, consistent with variability in their renal location. Resident renal CD103⁺ DCs are the best antigen-presenting cells and can easily be distinguished from CD11c^{hi} myeloid DCs that accumulate in large numbers during nephritis.

Conclusions—Our study highlights the heterogeneity of the macrophage/DC infiltrate in chronic SLE nephritis and provides an initial phenotypic and functional analysis of the different cellular components that can now be used to define the role of each subset in nephritis progression or amelioration. Of note, the dominant macrophage population that accumulates during nephritis has an acquired phenotype that is neither M1 nor M2 and may reflect failure of resolution of inflammation.

Macrophages and dendritic cells (DCs) play a vital role in adaptive immune responses by processing and presenting antigens to T cells in secondary lymphoid tissues. These cells also reside in peripheral tissues where they have both sentinel and tolerogenic roles (1-4). Mononuclear cells are rapidly recruited to peripheral organs upon local injury, and differentiation and expansion may also occur *in situ* (5-6). These cells have a high degree of plasticity, with the same cell being sequentially able to mediate tissue injury and repair

(7-9). Given the pluripotent functions of mononuclear phagocytes it is not surprising that many phenotypic and functional variants have been described (10-14).

Mononuclear phagocyte populations in normal kidneys have been only partially characterized (8, 15-18). The dominant resident population has both macrophage and DC like features and forms a network throughout the interstitium and surrounding glomeruli (19-22). Macrophages that initially enter the kidneys following acute renal injury express Ly6C/Gr1 and secrete pro-inflammatory cytokines typical of classically activated “M1” macrophages (6, 18, 23-24). In contrast, during the repair phase these cells may switch their phenotype to a pro-repair “M2” phenotype (8-9).

CD68⁺ mononuclear phagocyte infiltration in chronic lupus nephritis is associated with a poor prognosis in humans (25-27). Several subtypes of these cells have been identified in lupus nephritis biopsies but their functions and origins are unknown (28-29). Using three different murine SLE nephritis models we have shown expansion and activation of the dominant CD11b⁺/F4/80^{hi} population and strain dependent infiltration with CD11c^{hi} myeloid DCs (20, 22). In the present study we used a bead based method followed by cell sorting to isolate homogeneous populations of renal mononuclear phagocytes that reflect the original cellular distribution. Using this method we identified 5 subsets of renal mononuclear phagocytes, 4 of which were simultaneously isolated from the kidneys of two different strains of lupus prone mice. This approach allowed us to study the structural and functional status of different cell subsets from pre-diseased and nephritic mice that were subjected to similar *in vivo* and *in vitro* conditions. Our data show that the standard inflammatory (M1) vs. anti-inflammatory (M2) paradigm is insufficient to understand the chronic inflammation associated with SLE nephritis.

MATERIALS and METHODS

Laboratory animals

Female NZB/W and male NZW/BXSB mice were followed clinically as previously described (20, 30). We analyzed young mice of 10-12 weeks without serum autoantibodies or proteinuria and diseased mice (>20 weeks for NZW/BXSB and >30 weeks for NZB/W mice) with 300mg/dl proteinuria for >2 weeks. C57BL/6 mice were aged to 24 weeks in our facility. All experiments were approved by the IACUC of the Feinstein Institute for Medical Research.

Isolation of macrophages and DCs

Kidneys were processed as previously described (22) and the cell pellet was resuspended in PEB buffer (0.5% BSA, 2mM EDTA in PBS pH 7.4) containing anti-mouse CD16 (BD Pharmingen San Diego, CA). Anti-CD11c coated MACS beads (Miltenyi Biotech – Bergisch-Gladbach, Germany) 10ul/10⁷ cells were added for 15 minutes followed by anti-CD11b coated beads for 15 minutes. Cells were then stained with anti-F4/80-PE (Invitrogen, Carlsbad, CA), anti-CD11c-FITC (BD Pharmingen) and anti-CD11b-APC (eBiosciences, San Diego, CA) and loaded into the AutoMACS. Separation of the labeled cells was performed using either POSSEL_S or POSSEL_D program.

The positive fraction was further fractionated by cell sorting as shown in Figure 1 and the purity of the populations was evaluated by post sort analysis. Populations were further characterized by staining with anti-mouse CD103-PerCP-Cy5.5, BTLA-PE, 33D1-biotin, F4/80-Pacific blue (Biolegend, San Diego, CA), CD8 α -PerCP, MHCII-AF700, CD14-PerCP-Cy5.5 (eBiosciences), CD43-PE, CD62L-PE, MHCI-FITC (BD Pharmingen), DEC205-PE (Miltenyi Biotech) and VLA4-FITC (Southern Biotech, Birmingham, Alabama) (20, 22).

Wright Giemsa staining of purified cells

10⁴ sorted cells were used for cytospin (600rpm). Slides were fixed in ice-cold methanol and stained according to manufacturers' instructions (Volu-Sol. Inc. Salt Lake City, UT).

Phase contrast microscopy to study motility of sorted populations

Isolated cell populations were cultured in DMEM/10% FCS with 10ng/ml M-CSF (macrophages) or GM-CSF (DCs) for 24-48 hours. The morphology and movement of the cells was monitored using a ZEISS microscope for 5 minutes in time lapse mode of 5-15 seconds.

Phagocytosis

Renal cell suspensions stained as above, were resuspended in DMEM with 10% FCS and incubated with 1 μ m fluorescent beads (YG 18860, Polysciences Inc., Warrington, PA) for 30 minutes at 37°C. Control incubations were carried out at 4°C. Cells were washed, and analyzed by flow cytometry.

Assay for iNOS and arginase activity

Sorted F4/80^{hi} cells were cultured with M-CSF (10ng/ml) and either IFN γ (10ng/ml) (R&D Systems, Minneapolis, MN) and LPS (100ng/ml) or IL4 alone (10ng/ml) for 48 hours. Bone marrow cells were cultured for 5 days in 10% DMEM with M-CSF and then treated with IL-4 or LPS +/- IFN- γ for 48 hours. The supernatant was assayed for nitrites using the Griess Reagent kit (Invitrogen).

Arginase activity was measured in bone marrow cells and in the F4/80^{hi} population as previously described (31). 50 μ l of 9.5mM MnCl₂ in 50mM Tris-HCl (pH 7.5) was incubated at 55°C for 10 min with 50ul of sample lysed in 0.1% Triton-x-100 with protease inhibitors, (Roche Diagnostics, Mannheim, Germany). 25 μ l of 0.5M arginine (pH 9.7) was added and incubated at 37°C for 60 min. 200ul of acid (H₂SO₄:H₃PO₄:H₂O, 1:3:7) was added to 50ul of the reaction mixture followed by 25ul of α -isonitrosopropiophenone (Sigma-Aldrich, St. Louis, MO) and the samples heated for 45 minutes at 95°C. The samples were cooled for 10 minutes in the dark and the amount of urea formed was calculated using the absorbance at 540 nm and a urea standard curve as reference.

Antigen presenting function

Sorted populations were cultured for 5 days at a ratio of 1:4 with CFSE-labeled splenic CD4⁺ T cells isolated from allogeneic donors (BALB/c for NZW/BXSB and C57BL/6 for NZB/W mice) using magnetic beads (Miltenyi Biotech). Harvested cells were gated on CD4

and the proliferative index measured using FlowJo software. Splenic CD11c cells cultured with allogeneic spleen cells were used as a positive control. The proliferation index of each sorted population was normalized to the value obtained for the CD11b^{hi}/F4/80^{lo} population of each sample to yield a relative proliferation index (RPI) for each experiment.

Gene expression studies

Real-time PCR was performed on total mRNA from isolated cells as previously described (20, 32). Single F4/80^{hi} cells from nephritic NZB/W mice were sorted into 96 well plates containing 4 µl of lysis buffer and snap frozen on dry ice. cDNA was synthesized and qPCR performed as previously described (33) using primers for beta-actin to test for the presence of cells. cDNA(s) in positive wells were tested in triplicate for CCL2, IL-10, IL1RN (20), CFB (forward, CTCGAACCTGCAGATCCAC, reverse TCAAAGTCCTGCGGTCGT) and TIMP-1 (forward: GCAAAGAGCTTTCTCAAAGACC, reverse: AGGGATAGATAAACAGGGAAACACT). The PCR program was: 5 min at 95°C; 45 cycles of 10 sec at 95°C, 10 sec at 55°C, and 10 sec at 72°C; followed by melting analysis.

RESULTS

Enrichment of mononuclear phagocyte sub-populations from kidneys

We identified 5 populations of mononuclear phagocytes in NZB/W (Figure 1) and NZW/BXSB (Figure S1) kidneys. The first is a population of plasmacytoid DCs (CD11b⁻/CD11c^{lo-int}/F4/80⁻/B220⁺/PDCA^{hi}) that comprises 0.5-1.0% of cells in the lymphocyte monocyte gate (Figure S2A). These cells did not increase in frequency during disease progression and were not studied further. The remaining cells could be distinguished based on expression of the surface markers CD11c, CD11b and F4/80 (Figure 1A-F, S1). A summary of phenotypic markers is presented in Table 1.

CD11b^{hi}/F4/80⁻/Gr1^{hi}/Ly6G^{hi}/Ly6C^{hi} neutrophils constituted <1.5% of all kidney cells (Figure S2B-G) and did not increase during disease; these were excluded during sorting by the lymphocyte monocyte gate. Importantly, CD11b^{hi}/F4/80^{lo-int}/Gr1^{hi} inflammatory macrophages were not found in the kidneys, although these cells were readily detected using the same gating strategy in mice with nephrotoxic nephritis (22) and are reportedly present in the kidneys of mice that develop SLE due to FcRIIB deficiency (34).

Nephritic mice had a 4-5 fold increase in renal CD11b⁺ cells compared with young mice or 24w old C57BL/6 mice (Figure 1G, S1). Using autoMACS purification CD11b⁺ and CD11c⁺ cells were significantly enriched from kidney cell suspensions, with a greater degree of enrichment from the kidneys of young mice that had fewer of these cells (Figure 1H). The enrichment did not alter the intrinsic ratios of the different CD11b^{hi} sub-populations (Figure 1I). Analysis of the flow-through confirmed depletion of most of the desired cells (not shown).

Cell morphology

Homogeneous sub-populations with >95% purity were obtained by cell sorting (Figures 2A-E). Morphology of the isolated populations is shown in Figures 2F-J. We identified two

populations of CD11c^{hi} DCs. The first (population i in Figure 1) is CD11b^{lo-int}/F4/80^{lo}/CD11c^{hi} and does not increase by >2 fold in frequency during nephritis in either strain. This population has thick dendrites and a homogeneous cytoplasm (Figure 2F) and is CD103^{hi}/CD8⁻ (Figure 2K, L); it will subsequently be referred to as CD103⁺ DCs.

The second DC population is CD11b^{hi}/F4/80^{lo}/CD11c^{hi} (population iii in Figure 1) and has a kidney shaped nucleus, small cytoplasmic vesicles and multiple long thin dendrites (Figure 2G). These cells have a veiled morphology by electron microscopy consistent with myeloid DCs (22). They increase markedly during nephritis (Figure 1G) and are located either in lymphoid aggregates in NZB/W mice or within glomeruli in NZW/BXSB mice (20, 35). In young mice these are all MHCII^{hi}, but in nephritic mice, they can be divided into MHCII^{hi} and MHCII^{lo} subsets with the majority being MHCII^{lo}. In NZB/W mice, the MHCII^{lo} population also has moderately lower expression of CD11b (MFI in MHCII^{hi} 22.7 +/- 4.4 x 10³ vs. 15.8 +/- 6.0 x 10³ in MHCII^{lo}; n = 9, p <0.05 – Figure S2H-L).

The CD103⁺ population is readily distinguished from the CD11b^{hi}/CD11c^{hi} myeloid DC population by higher expression of BTLA and DEC205 and lower expression of CD43 and CD14 (Figure 2M-P). In young mice these cells express lower MHCII and higher MHCI than CD11b^{hi}/F4/80^{lo}/CD11c^{hi} myeloid dendritic cells (Table 1) but they do not downregulate MHCII during active nephritis.

We identified two populations of macrophage like cells. The dominant CD11b^{hi}/F4/80^{hi}/CD11c^{lo-int} population (population iv in Figure 1) is found in the kidneys but not in the bone marrow, spleen, lungs (Figure S1) or liver (not shown). These cells form a network throughout the interstitium and surrounding glomeruli (20) as previously described for CX3CR1⁺ resident renal macrophages (21). In young mice these cells have a uniformly stained nucleus and small blunt dendritic processes (Figure 2H) but at proteinuria onset they increase in number and accumulate large cytoplasmic autophagocytic vesicles ((22), Figure 2I). These cells will hereafter be referred to as F4/80^{hi} cells. In contrast, the CD11b^{hi}/F4/80^{lo}/CD11c^{lo} population (population v in Figure 1) is round with a kidney shaped nucleus, indicating a monocytic origin (Figure 2J). These cells will hereafter be referred to as F4/80^{lo} cells. They are infrequent in NZB/W mice but variably increase in number in nephritic NZW/BXSB mice (Figure 1G).

Isolated cells survived for several days *in vitro* (Figure 3). The CD103^{hi} population (population i.) had numerous dendrites (Figure 3A) on which they showed spider-like movement (Movie S1). The CD11b^{hi}/CD11c^{hi} myeloid DC population (population iii) was circular to oval with many contracting and expanding filopods and dendrite-like projections (Figure 3B). When these cells exhibited movement, the filopods were at the forward edge and the long dendrites formed a tail. (Movie S2). The F4/80^{hi} population (population iv) was ellipsoidal to spindle shaped and spread over the surface of the culture dish (Figure 3C). There were densely stained nucleoli and the cytoplasm contained vesicles (data not shown). These cells were non motile, but formed large pseudopod like structures that exhibited exaggerated protrusion and retraction (Movie S3). In addition these cells had long dendrite like structures with pseudopods at their tips that attached the cells to the plate surface

(arrows in Fig 3C). The F480^{lo} population (population v) was round and firmly adherent when cultured with M-CSF for 24 hrs (Figure 3D).

Functional characterization of the sorted populations

Antigen presenting function—Since local T cells may be pathogenic in lupus nephritis, the antigen presenting function of the 4 subpopulations was studied by mixed lymphocyte reaction. Figure 3E shows representative proliferation of CFSE labeled CD4⁺ T cells in presence of the different sorted populations from a NZB/W mouse. CD103⁺ DCs had the highest relative proliferative index followed by the F4/80^{hi} macrophages and CD11b^{hi}/CD11c^{hi} DCs. Little proliferation was observed when T cells were co-incubated with the F480^{lo} cells (Figure 3F). Similar results were observed for cells from NZW/BXSB mice (not shown).

Arginase and iNOS activity—To understand whether the dominant F4/80^{hi} population displays either an M1 or M2 phenotype we tested for iNOS (M1) and arginase (M2) activity. The F4/80^{hi} population from either NZB/W or NZW/BXSB mice exhibited little iNOS (Figure 4A) or arginase activity (Figure 4B) even after stimulation with cytokines for 48 hrs, irrespective of the clinical status of the mice. In contrast, BM derived macrophages from the same mice were responsive to cytokine stimulation and could readily be differentiated either into M1 or M2 cells. To control for differences in the conditions of cell isolation, bone marrow cells from NZW/BXSB mice were passed through the AutoMACS and cell sorter before exposure to polarizing conditions. Arginase activity was readily detected after 48 hours of culture and iNOS activity was detected if the cells were matured for 5 days before exposure to cytokines (not shown).

Phagocytosis—F4/80^{hi} macrophages displayed the most phagocytic activity *in vitro*, with no differences between pre-diseased and diseased mice (Figure 4C). We obtained the same result when fluorescent latex beads were administered *in vivo* and kidneys were examined 3 days later ((22) and data not shown).

Gene expression studies—We have previously reported the results of gene expression analyses in amplified DNA from the F4/80^{hi} population from young and nephritic NZB/W mice (22). To determine whether the same profiles were present in NZW/BXSB mice, gene expression studies were performed using qRT-PCR of informative markers from unamplified cDNA. The CD11b^{hi}/F4/80^{hi} population from NZW/BXSB mice, like that of NZB/W mice (22), had a hybrid expression pattern of gene that includes the chemokine CCL2, the anti-inflammatory cytokine IL-10, the C-type lectin Mincle, and tissue remodeling genes MMP-14 and TIMP-1 (Figure 5A). qPCR analysis of single cells sorted from the F480^{hi} population of nephritic NZB/W mice indicated that the same cells expressed both CCL2 (pro-inflammatory) and IL-10 (anti-inflammatory - 29 cells), CCL2 and complement factor B (pro-inflammatory - 14 cells), CCL2 and IL1RA (anti-inflammatory - 8 cells) or CCL2 and TIMP1 (reparative - 9 cells). Finally, the same cells expressed CCL2, IL-10, CFB and TIMP1 (14 cells). Within each experiment the level of mRNA expression for each gene was similar in all the cells, confirming the mixed phenotype of this cell population.

We also performed comparative analysis of mRNA expression for selected genes among mononuclear phagocyte subsets (Figure 5 and Table 1). The F4/80^{hi} population could be distinguished from the others based on expression of TLR3, TLR5, TLR9 (Figure 5B), ITGAM, CCL7, and CX3CR1 (Figure 5C). CD103⁺ DCs expressed TLR3, as described for this population in other organs, however unlike the CD103⁺ cell population in the gut it did not express TLRs 5, 7 or 9. This population also expressed CCR2. CD11b^{hi}/CD11c^{hi} DCs were distinguished from CD103⁺ DCs by lower expression of TLR3, and higher expression of ITGAM. The two DC populations expressed lower levels of iNOS than the macrophage populations. The F4/80^{lo} population expressed low levels of all the markers studied. Results were similar for both strains of mice.

DISCUSSION

Macrophages and DCs are key players in acute renal inflammation (36-37) where they function both to foster inflammation and to help repair the kidney once the eliciting stimulus has dissipated. Macrophage and DC infiltration also occurs in chronic renal diseases such as SLE and correlates with poor renal outcome in SLE patients (25-26). The present work describes a method for functional evaluation several different subsets of macrophages and DCs simultaneously isolated from the kidneys of pre-diseased and nephritic mice with SLE.

Normal mouse kidneys have a heterogeneous resident population of mononuclear cells that can sense injury or pathogens. The dominant population is F4/80^{hi}/CX3CR1^{hi}/MHCII^{hi}/Gr1^{lo-int}/CD86^{int}/CD11b^{int}/CD11c^{int} and forms a network that is most dense in the renal interstitium (21). We show here that this population constitutes the majority of the CD11b⁺ population in the kidneys of normal and young SLE mice, but is less commonly found in other organs, a finding recently confirmed by others (38-39). In normal mice these cells are yolk sac derived, express CX3CR1, depend on the M-CSF receptor and are independent of Flt3, Id2 and IRF8 (10, 39). Functional studies have shown that they can present antigen, they use dendrites to sample their local environment, and are poor NO producers, suggesting that they might be DCs (17, 21). However because these cells express F4/80 they are also often referred to as resident macrophages (40). Studies of human kidneys have similarly found a network of CD68 positive cells throughout the interstitium (28-29, 41).

Studies using *in vitro* stimulation protocols have classified activated macrophages into inflammatory IL12 producing M1 and alternatively activated IL-10 producing M2 macrophages which themselves are divided into M2a, M2b and M2c subtypes. Of these, M2a, induced by Th2 cytokines are thought to be anti-inflammatory, M2b, induced by TLR ligation or immune complexes also secrete the pro-inflammatory cytokines TNF, IL1 and IL6 and M2c, induced by glucocorticoids, are involved in wound healing (23, 42). Another population of “regulatory” macrophages may be associated with tumors. These macrophage phenotypes are not stable and mixed phenotypes inconsistent with any of the polarized *in vitro* phenotypes have been described *in vivo*.

It is not surprising therefore, that macrophage function in injured kidneys may be quite variable. During acute inflammation, infection or ischemia, CD11b⁺/F4/80⁺/Gr1^{hi}/CCR2⁺/CD62L⁺ macrophages that secrete pro-inflammatory cytokines are rapidly recruited to the

kidneys. In contrast, exposure of intrinsic renal macrophages to apoptotic cells may promote an anti-inflammatory M2 phenotype associated with release of IL-10 and TGF β and promotion of tubular repair; prolonged exposure to M2 macrophages may however result in renal fibrosis. Finally, macrophages may have fibrolytic functions that although beneficial in repair of acute injury, may foster excessive remodeling and damage in chronic nephritis (reviewed (23)).

Using flow cytometry, we identified distinct populations of renal macrophage-like cells in both mouse strains. The dominant resident cell population is CD11b^{hi}/F4/80^{hi}/Ly6C^{lo-int}/MHCII^{hi} and has characteristics of macrophages including adherent and phagocytic properties, but it also has dendrites, it continually forms and retracts processes when cultured *in vitro*, and has antigen presenting function, characteristic of DCs. These cells become activated at proteinuria onset with an increase in CD11b expression and upregulation of CD80 and CD86 (20, 22, 35, 43). We have shown in NZB/W mice that renal F4/80^{hi} cells derive from circulating Ly6C^{lo-int} monocytes and that their activation occurs *in situ* in nephritic kidneys (22). We further show here that these cells cannot be induced *in vitro* by cytokines to differentiate into either an M1 or M2 phenotype suggesting that they are terminally differentiated.

There are striking similarities between the F4/80^{hi} renal mononuclear phagocyte population and the analogous population of steady state mononuclear phagocytes in the gut. In the gut this population is constitutively activated, is highly vacuolated and produces large amounts of IL-10 and IL1RA that serve to control the pro-inflammatory response to commensal bacteria (12, 44). Although the renal F4/80^{hi} population from mice with active nephritis shares this anti-inflammatory profile it also has pro-inflammatory properties (22); notably, this phenotype is acquired only during active disease (22). A similar, but not identical, profile has been reported in a murine model of SLE induced by activated lymphocyte derived DNA (45); we show here that this profile is shared by a third SLE model, the NZW/BXSB mouse. It has recently been reported that gut F4/80^{hi} mononuclear cells can be divided into two phenotypically similar subpopulations that vary in their level of expression of CX3CR1 (46). CX3CR1^{lo} cells produce pro-inflammatory cytokines and upregulate expression of the inflammatory marker Trem-1 during colonic inflammation whereas CX3CR1^{hi} cells are large and highly vacuolated and produce large amounts of IL-10, even during periods of inflammation (12). In contrast, previous flow cytometry experiments (22), as well as the single cell PCR experiments shown here indicate that the same cells within renal F4/80^{hi} population in nephritic kidneys can produce both pro- and anti-inflammatory mediators. A similarly mixed M1, M2 phenotype has been reported in the gut in the context of mucosal infection (47) and other mixed phenotypes have been reported in the context of resolution of inflammation (48). Interestingly the F4/80^{hi} population in the gut expresses genes that are associated with wound healing and tissue repair (12, 46). We also found an increase in expression of genes associated with tissue repair in the renal F4/80^{hi} population from nephritic mice (22); this may result in excessive tissue remodeling during chronic inflammation. In sum, the phenotype of the F4/80^{hi} population in nephritic kidneys is a mixed phenotype with a profile that suggests a failure to resolve chronic inflammation, most

likely as the result of continued insult by nucleic acid containing immune complexes and exposure to circulating inflammatory mediators.

In addition to the F4/80^{hi} population we found a small population of non-phagocytic, CD11b^{hi}/F4/80^{lo}/Ly6C^{int-lo} renal monocytes with poor antigen presentation capabilities. These cells are similar in their cell surface phenotype and expression profile to renal myeloid DCs but they have lower levels of MHCII and costimulatory molecules; they do not, however, express CD11c, making it unlikely that they are immature DCs. Neither are they immature neutrophils because they do not express Ly6G. Importantly, we were unable to detect an inflammatory Ly6C^{hi} population of M1 macrophages in either mouse strain at any disease stage.

At least three distinct populations of DCs are present in kidneys. There is a small population of plasmacytoid DCs that does not increase in number by more than two-fold during disease. The other two populations with clear DC morphology are morphologically, phenotypically and functionally distinct from each other. Previous work has shown that the renal CD11b^{lo}/CD103⁺ DC population in normal mice is of non-lymphoid origin and is derived from pre-cDCs (10, 15). We show here that this cell is CD8 α ^{lo}/CX3CR1⁻, has high levels of TLR3 (49), and, like CD8⁺ DC, expresses the coinhibitory molecule BTLA and the C type lectin DEC205. It is easily distinguished from the CD11b^{hi}/CD11c^{hi} population of DCs by differential expression of MHCI and II and of CD14 and CD43. The role of these cells in the kidneys is not known. In the lung they cross-present antigen to CD8 T cells and are needed for optimal antiviral responses (50) but in the gut they may have a regulatory role since they can induce expression of Foxp3 in CD4 T cells (51). Although, as we show here, CD103⁺ DCs are the most potent antigen presenting cells in the kidneys for CD4 T cells; they do not however increase in number during nephritis in either mouse strain. Thus, if they have a regulatory function, this may be overcome by the influx of other pro-inflammatory cells.

Normal mice have a small population of CD103⁻CD11c^{hi} DCs within the renal interstitium (38). In NZB/W and NZW/BXSB, but not in NZM2410 mice, proteinuria onset is associated with the exuberant renal influx of CD11c^{hi} DCs that localize within lymphoid aggregates in NZB/W mice, separate from the F4/80^{hi} population, and in the glomeruli of NZW/BXSB mice (20, 35). These cells are CD43^{hi}/CD14^{hi}/BTLA^{lo} and they express low levels of CX3CR1 and TLRs 3, 5, 7 and 9. We further show that they have antigen presentation capabilities similar to that of the F4/80^{hi} population but most have low expression of MHCII. Whether these MHCII^{lo} cells reflect an influx of immature DCs into the kidneys (52) or whether they have downregulated MHCII as a consequence of exposure to inflammatory cytokines remains to be determined. Analysis of kidney biopsies from humans with SLE nephritis has similarly revealed that at least three types of DCs, including plasmacytoid DCs, can be identified in the interstitium in inflammatory renal diseases whereas macrophages are found in and around glomeruli (41). While the phenotype of all these different cells is being established using immunohistochemistry, their function is still unknown. Our own genomic data from human lupus renal biopsies suggests that macrophages located in and around glomeruli are functionally different from those in the interstitium (53).

While it is clear that the profile of DCs and macrophages changes in the kidneys during active SLE nephritis, it remains to be determined which of these cell types is friend and which is foe. Deletion studies in other models of renal inflammatory disease have yielded mixed results depending both on the type of disease and the disease stage. In acute nephrotoxic nephritis for example, deletion of macrophages is protective during inflammation but deleterious during recovery and repair (8). In contrast, CD11b⁺ cells are pathogenic in a mouse model of cryoglobulinemia with chronic membranoproliferative nephritis (24). Similarly, we have shown that deletion of macrophages and DCs during early nephritis using clodronate is protective in the NZB/W SLE model (54) but not in mice with established proteinuria despite adequate depletion (data not shown). The renal mononuclear cell profile in chronic SLE nephritis is clearly different from that observed in acute renal injury with tissue necrosis. The phenotypic and functional profiles we have described here will allow further characterization of renal mononuclear phagocytes and will enable specific targeting of each cell type in chronic inflammatory renal disease so as to identify protective and pathogenic subsets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A-F: Gating strategy for sorting of macrophages and DCs from kidney cell suspensions of NZB/W mice after MACS enrichment. **A, B:** After exclusion of doublets, cells were gated on the lymphocyte/monocyte gate and dead cells were excluded by DAPI staining. **C-F:** The CD11c^{hi}/CD11b^{lo} (i) population was sorted separately and the remaining CD11b⁺ population (ii) was further gated on CD11c and F4/80 to distinguish CD11c^{hi}/F4/80^{lo} (iii), CD11c^{lo-int}/F4/80^{hi} (iv), and CD11c^{lo}/F4/80^{lo} (v) populations. Note the increase in intensity of CD11b staining and the increase in the frequency of the CD11c^{hi}/F4/80^{lo} population (iii) in nephritic (D, F) vs. young mice (C, E). **G:** The frequency of the four mononuclear phagocyte cell types in the kidneys of young and nephritic mice expressed as a percentage of the total live cell suspension; p values young vs. nephritic: * p < 0.05, † p < 0.01. Stippled grey bars represent three 24w old C57BL/6 mice. **H:** Fold enrichment of populations after MACS. Note that the low abundance macrophage/DC populations are highly enriched by the bead selection in the young mice. **I:** The relative proportion of the CD11b⁺ sub-populations was maintained in the MACS enriched fractions compared with the corresponding fraction of the total kidney cell population.

Figure 2.

Post sort analysis to confirm purity and morphology of the sorted populations from kidneys of an NZB/W mouse. **A:** CD11b^{lo}/CD11c^{hi}/CD103⁺; **B:** CD11c^{hi}/F4/80^{lo}; **C:** CD11c^{lo-int}/F4/80^{hi}; **D:** CD11c^{lo}/F4/80^{lo}. Populations defined in Figure 1 are indicated in the top right hand corner of each panel. **E:** Overlay of all four populations. Panels F-J show morphologic features of isolated cells after Wright Giemsa stain. **F:** CD11b^{lo}/CD11c^{hi}/CD103⁺; **G:** CD11c^{hi}/F4/80^{lo}; **H:** CD11c^{lo-int}/F4/80^{hi} (young mouse); **I:** CD11c^{lo-int}/F4/80^{hi} (nephritic mouse) **J:** CD11c^{lo}/F4/80^{lo}. Note the increased number and size of vacuoles in panel J compared with panel I. Representative of 5 mice. **K-P:** Phenotype of the CD11b^{lo}/CD11c^{hi} populations: Rectangle in panel K shows CD11b^{lo}/CD11c^{hi} cells that are CD103⁺ and CD8a⁻ (L). Panels M-P show that the CD103⁺ CD11b^{lo}/CD11c^{hi} population (black lines) is higher for BTLA and DEC205 and lower for CD43 and CD14 expression compared with the CD11b⁺/F4/80^{lo}/CD11c^{hi} DC population (grey lines). Results are representative of 5 nephritic NZB/W mice.

Figure 3.

Analysis of cell morphology and antigen presentation function: **A-D**: Phase contrast images of the different sorted cell subpopulations after 24hrs of culture in 10% DMEM with M-CSF (A and B) or GM-CSF (C and D). **A**: CD103⁺; **B**: CD11c^{lo-int}/F4/80^{hi} - arrows indicate structures that probably attach the cells to the plate; **C**: CD11c^{hi}/F4/80^{lo}; **D**: CD11c^{lo}/F4/80^{lo}. Pictures taken at 20X magnification. Data are representation of 3-5 independent experiments. **E-F**: Alloresponses of CD4 T cells to each of the four sorted subpopulations. Relative proliferation index (RPI) of each population calculated by FlowJo software was compared with the index of the CD11c^{lo}/F4/80^{lo} population for each experiment. **E**: Representative data from an NZB/W mouse shows proliferation of CFSE labeled CD4⁺ cells after 5 days in culture. **F**: Pooled data from young and nephritic mice (mean + 1SD). † p < 0.01 vs. age matched F4/80^{lo} population.

Figure 4.

Functional profile of the F4/80^{hi} sub-populations from NZB/W and NZW/BXSB mice. **A:** Arginase activity was determined by monitoring production of urea from cell lysates. **B:** iNOS expression was determined by monitoring production of nitrite in supernatant of cultured cells. Data shown are mean $\pm$ 1SD. For each experiment, bone marrow macrophages from the same mice were differentiated with M-CSF and used as a reference. **C:** Phagocytic activity of the different sorted populations. * $p < 0.05$; ** $p < 0.01$ vs. the F4/80^{hi} population.

Figure 5. Real-time PCR analysis of selected genes. **A:** Analysis of the transcriptional levels of selected inflammatory genes in the F480^{hi} population from young and nephritic mice of both strains. **B, C:** Comparison of transcriptional levels of selected genes including chemokines receptors and TLRs in F480^{hi} populations from young and nephritic NZB/W mice (B) compared with sorted F4/80^{lo} and CD11c^{hi} and CD103⁺ DC populations from the nephritic NZW/BXSB mice (C). Similar results as those shown in Panel C were obtained with samples from the same populations from NZB/W mice (not shown). Pellets from 3-5

individual mice were used per group, and experiments were repeated once. Data was normalized to the mean of young NZB/W F4/80^{hi} controls, given an arbitrary value of 1.

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Table 1

Phenotype of renal mononuclear phagocyte sub-populations

	CD103 ⁺ (DC)	CD11b ^{hi} CD11c ^{hi} (DC)	CD11b ^{hi} F4/80 ^{hi} (MΦ)	CD11b ^{hi} F4/80 ^{lo}
CD11b	+	++	+ to +++ [†]	++
CD11c	+++	++	- to +	-
F4/80	-	- to + [‡]	+ to ++ [†]	-
VLA4	+	+	-	-
MHC II	++	+++ to + [†]	+++	+
MHC I	+++	++	++	++
CD8α	-	-	-	-
CD103	++	-	-	-
BTLA	++	-	-	-
CD14	+/-	++	+++	++
CD43	-	+++	-	mixed
CD80/86	+	+	+ to ++ [†]	+/-
OX40L	-	-	- to + [†]	-
CD62L	-	-	-	-
Gr1	+/-	+/-	+/-	+/-
DEC205	+++	-	-	-
DCIR2 (33D1)	-	-	-	-
Ly6G	-	-	-	-
Ly6C	-	-	lo/int	-
RALDH2 [*]	+	+	+	-
TLR3 [*]	+++	-	+	-
TLR5 [*]	-	-	+	-
TLR7 [*]	-	+/-	+	+/-
TLR9 [*]	-	-	-	-
IL12 [*]	+	+	+	+
iNOS [*]	+/-	+/-	+/-	-
ITGAM [*]	-	+	++ to +++ [†]	+
CCL7 [*]	-	-	++ to +++ [†]	-
CCR2 [*]	+	+	+ to ++ [†]	+
CX3CR1 [*]	-	-	+	-
Mean fold increase in cell number during nephritis (NZB/W)	2.0	11.9	6.1	3.7

* by PCR

[†] In nephritic mice

[‡]In NZW/BXSB mice

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