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Bone Marrow Ly6C<sup>high</sup> Monocytes Are Selectively Recruited to Injured Kidney and Differentiate into Functionally Distinct Populations<sup>1</sup>

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Roles for monocyte/macrophages (Mφ) in directing the development of tissue fibrosis are increasingly recognized. Macrophages form a heterogeneous group of inflammatory leukocytes, and the mechanisms by which they acquire heterogeneity and its functional significance are unclear. We used the unilateral ureteral obstruction model of progressive kidney fibrosis to explore macrophage heterogeneity and function further. Unilateral ureteral obstruction kidney Mφs form three distinct subpopulations defined by the marker Ly6C, all of which are derived from a single Ly6C<sup>high</sup> bone marrow monocyte population selectively recruited to the kidney. Conditional ablation of these Mφs in vivo in CD11b-DTR mice is potently antifibrotic. The mRNA transcription profile of these populations is consistent with differential functional roles for each subpopulation, with Ly6C<sup>low</sup> macrophages transcribing genes consistent with selective profibrotic or M2-type function. Furthermore, bone marrow chimerism studies indicate that although resident kidney macrophages proliferate markedly to comprise up to 40% of the inflammatory macrophage population, they do not contribute to fibrosis. Our data identify Ly6C as a marker of functionally discrete tissue macrophage subsets and support a model of selective recruitment of Ly6C<sup>high</sup> bone marrow monocytes to the kidney that differentiate into three populations of kidney macrophages, including a profibrotic Ly6C<sup>low</sup> population. The Journal of Immunology, 2009, 183: 6733–6743.

Understanding the mechanisms by which solid organs develop fibrosis in response to injury is pivotal to our development of new therapies to counteract fibrosis. Fibrosis is a harbinger of organ failure and reflects inadequate resolution of inflammation in response to injury or inappropriate organ remodeling (1). Although in certain circumstances a measured degree of long-term fibrosis after injury is desirable, such as in the wall of the myocardium or the wall of large blood vessels, excessive fibrosis in response to a single injurious insult or progressive fibrosis in response to repetitive injury in many studies is both associated with and is the cause of progressive organ failure. Fibrosis underlies many common diseases in many different organs.

In recent years, tremendous advances have been made in understanding some of the mechanisms that lead to fibrosis. A key role for inflammatory macrophages (Mφs)<sup>3</sup> and their circulating progenitors, monocytes, in this process has been appreciated in many different organ settings (2–11). However, the mechanisms by which Mφs cause fibrosis in diseases where there is continuous or repetitive injury have been controversial. Reports have implicated a subpopulation of bone marrow (BM)-derived cells that directly lay down a fibrous matrix known as a fibrocyte, although in the kidney and liver at least the fibrocyte appears to be a minor contributing cell type (6, 12–14). Our own in vivo studies in inflammation and fibrosis in the kidney show an unclear role for collagen-producing, myeloid-derived cells in fibrosis of the kidney (13). It is more likely that Mφs, which produce a very broad array of paracrine cell signaling molecules (cytokines), signal to neighboring scar-forming cells (pericytes and myofibroblasts) to promote their proliferation, gene transcription, and liberation of scar-forming extracellular matrix proteins such as collagen I (15).

The kidney is an organ that is frequently afflicted by fibrosis. Many different inflammatory triggers converge on a similar pattern of chronic inflammation, with fibrosis targeting the kidney interstitium (i.e., the spaces between the tubules of the kidney unit, called the nephron). This pattern of inflammatory injury has many similarities to skin wounding responses (10, 16), which are also Mφ dependent. Arguments have been made that ischemia due to peritubular capillary rarefaction is core to the problem of chronic inflammation in the kidney (17). Nevertheless, injured epithelial cells of the kidney also liberate cytokines that may contribute to fibrosis progression, and inflammatory leukocytes, many of which

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<sup>2</sup>Roles for monocyte/macrophages (Mφ) in directing the development of tissue fibrosis are increasingly recognized. Macrophages form a heterogeneous group of inflammatory leukocytes, and the mechanisms by which they acquire heterogeneity and its functional significance are unclear. We used the unilateral ureteral obstruction model of progressive kidney fibrosis to explore macrophage heterogeneity and function further. Unilateral ureteral obstruction kidney Mφs form three distinct subpopulations defined by the marker Ly6C, all of which are derived from a single Ly6C<sup>high</sup> bone marrow monocyte population selectively recruited to the kidney. Conditional ablation of these Mφs in vivo in CD11b-DTR mice is potently antifibrotic. The mRNA transcription profile of these populations is consistent with differential functional roles for each subpopulation, with Ly6C<sup>low</sup> macrophages transcribing genes consistent with selective profibrotic or M2-type function. Furthermore, bone marrow chimerism studies indicate that although resident kidney macrophages proliferate markedly to comprise up to 40% of the inflammatory macrophage population, they do not contribute to fibrosis. Our data identify Ly6C as a marker of functionally discrete tissue macrophage subsets and support a model of selective recruitment of Ly6C<sup>high</sup> bone marrow monocytes to the kidney that differentiate into three populations of kidney macrophages, including a profibrotic Ly6C<sup>low</sup> population. The Journal of Immunology, 2009, 183: 6733–6743.

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<sup>5</sup>Abbreviations used in this paper: Mφ, macrophage; BM, bone marrow; DAPI, 4’,6-diamidino-2-phenylindole; DC, dendritic cell; DT, diphtheria toxin; DTR, DT receptor; FSC, forward scatter; int, intermediate; PBM, peripheral blood monocyte; SSC, side scatter; UUO, unilateral ureteric obstruction; WT, wild type.

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are monocytes/M\(^d\)s, are major participants in many forms of chronic inflammation with fibrosis in the kidney.

In these studies we have focused on the role of the inflammatory M\(^d\)s that are such a prominent cell type in the inflammation of the kidney. Fundamental questions about the nature of inflammatory M\(^d\)s have remained unexplored in vivo. Studies dating to the 1960s from The Rockefeller Institute established that tissue M\(^d\)s derive from circulating monocytes that in turn derive from a proliferating pool of monocyte precursors in BM (18–20). However, in recent years some of the central tenets of that model (known as the mononuclear phagocyte system) have been challenged (21, 22). Many in vitro studies have indicated that M\(^d\)s show plasticity in response to extracellular cues that could potentially result in many “phenotypes” of M\(^d\)s depending on the cytokine mixture to which they are exposed (15, 23, 24). If stimulated with differentially polarizing combinations of cytokines, they can adopt a proinflammatory phenotype (M1 or classical activation) or adopt a profibrotic phenotype, known as M2 or alternative activation (25–28). However, the studies that identified these skewed phenotypes relied on cytokine combinations that may not exist in vivo and studied cultured M\(^d\)s that have been chronically exposed to selective growth factors (23, 28). More recently, it has emerged that in humans and rodents at least two kinds of circulating monocytes exist that respond differentially to injurious stimuli (29, 30). However, their respective roles in fibrosis are unclear. Thus, three schools of thought about the behavior of M\(^d\)s in vivo currently exist: 1) infinite plasticity depending on local cytokine milieu; 2) polarized phenotypes mediated by switches in function; 3) distinct lineages of precursor monocytes that exhibit predetermined functions. Furthermore, all organs have a population of resident M\(^d\)s that may have overlapping functions with myeloid dendritic cells (DCs), and their roles in inflammation and fibrosis remain untested.

These studies confirm a preeminent role for inflammatory M\(^d\)s in fibrosis progression during continuous injury of the kidney, uncover novel facets of the trafficking of monocytes to the injured kidney, and establish that a single lineage of monocytes differentiate into polarized phenotypes, and establish mechanisms by which M\(^d\)s achieve a profibrotic phenotype.

**Materials and Methods**

**Mouse breeding and genotyping**

Wild-type (WT) C57BL6 mice (males: weight, 25 g; age, 8–12 wk old) were from Charles River Laboratories. CD11b-DTR mice (FVB/N; where DTR is diphtheria toxin receptor gene) were generated and maintained as previously described (2). The presence of the transgene was confirmed by PCR using the following primers: 5′-TTCACGTGATCTACCGACC-3′ and 5′-TGGCGGATGATAGGCG-3′. C57BL/6 mice were from The Jackson Laboratory or Charles River Laboratories, and CD45.1 (B6.SJL-PepcPchBoy) mice and ACTB-GFP mice (C57BL6 background) were from The Jackson Laboratory. Collagen Ia-GFP (Colla1-GFP) mice have been reported previously (13).

**Adoptive transfer and bone marrow chimerism**

Citrated whole blood was collected from CD45.1 mice, and PBMCs were separated from erythrocytes and granulocytes by Ficoll-Paque gradient separation according to the manufacturer’s instructions (GE Healthcare). Whole BM was harvested from CD45.1 mice in HBSS as previously described and resuspended in sterile FACS buffer (PBS, 1% BSA, and 2 mM EDTA (pH 7.4)) on ice. After washing, BM cells and whole BM were labeled with Ly6C-GFP Abs (BD Pharmingen) on ice and immediately sorted by using a FACSAria cell sorter. For PBMCs, high forward scatter (FSC) and low side scatter (SSC) leukocytes were selected, sorted for Ly6C+ or Ly6C++ expression, and collected in PBS with 1% BSA and 2 mM EDTA (pH 7.4). For BM cells, monocytes were collected as high FSC, low SSC cells with high Ly6C expression. In preliminary experiments, this method excluded the population of Ly6G+ BM neutrophils (data not shown) and B220+ lymphocytes. Sorted cells were counted and 1 × 10^6 cells (the number monocytes in one blood volume) were given to CD45.2 C57BL6 mice with unilateral ureteric obstruction (UUO) on day 5 by tail vein injection. For adoptive transfer to CD11b-DTR mice, whole BM from FVB/N strain-matched healthy control mice was harvested, filtered, and passed through two separate MACS columns for negative selection of monocytes using beads conjugated with Abs against the cell surface markers Ly6G, CD3, NK.1.1, and B220. In preliminary studies, the resulting leukocytes were depleted by >95% of NK cells, B cells, and T cells and by >80% of neutrophils (not shown). CD11b-DTR mice with day 7 UUO received diptheria toxin (DT) (List Biological Laboratories) injection and within 1 h also received 2 million enriched BM monocytes or vehicle in FACS buffer by i.v. injection (see above). This number (twice the number of monocytes in one blood volume) was decided upon to immediately replenish the ablated monocytes and leave a reserve for those recruited out of the circulation following the 24 h. The adoptive transfer was repeated on days 8 and 9 with kidneys with days 7 and days 10. BM chimeric mice were generated as previously described (31). In brief, 10 million BM cells from ACTB-GFP, Colla1-GFP, and CD11b-DTR male donors or FVB/N male donors in 200 μl of PBS were injected into the lateral tail vein of lethally irradiated (1000 rad over 30 min) isogenic female recipients. Chimerism was confirmed after 6 wk by fluorescence in situ hybridization (FISH) of buffy coat cells according to the previously described protocol (2). The resulting monocytes were incubated with 1 M sodium thiocyanate (at 80°C for 10 min), digested with proteinase K (at 37°C for 15 min.), incubated with 0.1 M HCl (at 37°C for 10 min.), fixed with 4% PFA, dehydrated, and air-dried. A StarFISH mouse Y chromosome-specific probe (Cambio) was added to the manufacturer’s buffer. Slides were heated (at 80°C for 10 min.), incubated (for 1 h at 37°C), and then stringently washed in buffers and finally mounted with vectashield/4-diamino-2-phenylinodole (DAPI) (Vector Laboratories). In all cases, >95% of leukocytes labeled positively for the Y chromosome. For WT-GFP chimeric mice, chimerism was confirmed by 100-μl tail vein bleed and by labeling buffy coats with CD45-PE Abs. In all cases, >95% of CD45+ cells were strongly positive for GFP expression.

**Mouse UUO model of fibrosis**

Adult (12–20 wk) mice (C57BL6 and CD11b-DTR FVB/N background) were anesthetized with ketamine/xylazine (100/10 mg/kg i.p.) before surgery. UUO was conducted as previously described (13) and kidneys and blood collected on days 0, 3, 5, 7, 10, and 14. In some experiments the ureter was exposed but not tied (sham). In other experiments DT (List Biological Laboratories) or vehicle (PBS) was given i.v. at 25 ng/g on day 6, at 25 ng/g on day 4, and at 25 ng/g on days 5 and 6 and kidneys were harvested on day 7. Alternatively, DT at 25 ng/g or vehicle was given on day 7. Animals (31). BM cells were strongly positive for GFP expression.

**Tissue preparation and histology**

Mouse tissues were prepared and stained as described previously (2, 31). Primary Abs against the following proteins were used for immunolabeling: α-smooth muscle actin-Cy3 (1/200; clone 1A4; Sigma-Aldrich), CD11b (1/200; BD Pharmingen), CD34 (1/200; CalTag Laboratories), collagen III (Southern Biotech), and Ki-67 (1/200; clone SP6; Fisher Scientific). Fluorescent conjugated, affinity-purified secondary Ab labeling (1/400 to 1/800; Jackson ImmunoResearch Laboratories), mounting with Vectashield/DAPI or Prolong Gold, and image capture and processing were conducted as described previously (2, 31, 32). To study the proliferation of resident and monocyte-derived M\(^d\)s, kidneys of WT controls and mice labeled with anti-Ki-67 Abs (1/200; clone SP6; Fisher Scientific), Alexa Fluor 350-conjugated anti-rabbit Ab (1/500; Molecular Probes), and anti-CD11b Abs (e Bioscience) followed by affinity-purified, Cy3-conjugated anti-rat Abs (Jackson ImmunoResearch Laboratories) and then postfixed and mounted with Prolong Gold. For morphometric analysis of collagen fibril staining, 3-μm paraffin sagittal sections were stained with 0.1% Picrosirius red using established methods (2, 13).

**Single cell preparation from blood, spleen, and kidney**

Blood (500 μl) was collected from the inferior vena cava in sodium citrate (0.38%). The remaining blood was flushed out as described above with ice-cold PBS and then kidney and BM were harvested. PBMCs were isolated from citrated whole blood using Ficoll-Paque PLUS (GE Healthcare). Kidney was decapsulated, diced, incubated (at 37°C for 30 min) with Liberase (0.1% (1/200; Roche)) and DNAse (1000 U/ml; Roche) in HBSS, resuspended in 10 μl of FACS buffer or PBS and 1% BSA, and cells were filtered (40 μm). In some cases, leukocyte enrichment was conducted by resuspending the single cell suspension in PBS and then overlaying it on a
discontinuous Percoll gradient (33 and 66% in PBS) followed by centrifuga-
tion (for 20 min at 620 × g). All sorting of cells was performed using a FACSAria apparatus. Sorted Ly6Chigh or Ly6Clow kidney Møs were viewed microscopically following centrifugation at 300 × g for 5 min (Cytospin), air drying, methanol fixation, and mounting with Vectashield/ DAPI onto glass slides. Sorting of resident kidney Møs from normal kidney was performed using MACS columns (Miltenyi Biotec) and CD11b positive selection.

**Flow cytometric analysis**

Single cells (1 × 10⁵) from kidney, PBMCs, or spleen were resuspended in FACS buffer (31) and incubated with Abs against CD14, CD11b, CD11c, CD45, and MHC class II (I-A/J-E) (conjugated to PE, allophycocyanin, or Alexa Fluor 594, 1/200; eBioscience); CD86, CD115, and CD34 (conjugated to PE, 1/200; BD Pharmingen), CD16/32, F4/80 (allophycocyanin; eBioscience); CD64 (conjugated to Alexa Fluor 647, 1/200; BD Biosciences); Ly6-C (conjugated to FITC, 1/200; BD Biosciences); Ly6C (conjugated to PE; AbD Serotec); 7/4 (conjugated to Alexa Fluor 647, 1/200; AbD Serotec); NK1.1 (conjugated to PE-Cy5.5; eBioscience); Ly6G (conjugated to FITC or PE; 30 min at 4°C; BD Pharmingen); MARCO (conjugated to FITC; BD Pharmingen); and Mac-2-FITC (galactin 3; conjugated to FITC; Cedarlane Laboratories) in the presence of 1% mouse serum. For adoptive transfer experiments, PBMCs and kidney leukocytes were labeled with Abs against CD45.1 (conjugated to PE-Cy5.5) and CD45.2 (conjugated to allophycocyanin), NK1.1, CD11b, and Ly6C. For GFP BM chimera experiments, leukocytes were labeled with CD11b, NK1.1 Ly6C, and Ly6G. After washing with FACS wash buffer (31) and resuspension in 200 μl FACS buffer, cells were analyzed using a BD FACSCalibur flow cytometer.

** Branched chain DNA amplification quantification of mRNA**

Kidney Mø subpopulations were separated at 4°C by FACS sorting using a FACSaria apparatus and collected in PBS buffer with 1% BSA and 2 mM EDTA (pH 7.4). Cells (1 × 10⁵) were centrifuged and then resus-
pended and lysed with a proprietary cell lysis solution containing a 50% lysis mixture (Panomics) and 1 mg/ml proteinase K at a ratio of 400 cells/μl (Panomics) to a final volume of up to 160 μl. The mixture was incubated at 50°C for 30 min and the cells were mechanically lysed. The lysate was stored at −80°C. mRNA transcripts were analyzed in duplicate in 96-well plates. Target gene probe sets specific to the designated genes (II-1, II-6, II-10, II-13, Tnfα, Ccl3, Ccl4, Cxc11o, Cxc12, Fizz-1, Ym-1, Tgfβ, Pdgfb, Fgf2, arginase, Ccl17, Ccl22, Fn-1, and the house keeping gene transcripts Hprt1 and Gappdh) were designed and tested for sensitivity and specificity before use by the manufacturer (Panomics). Gene probe sets containing capture extender probes, label extender probes, and probe–speci-
cific fluorescently conjugated magnetic beads were added (up to 20 differ-
ent gene targets per well), and hybridization was performed at 54°C for 18 h in a shaker incubator (600 rpm; Labnet Vortemp 56). Following hy-
bridization, samples were transferred to a filter plate, washed three times in wash buffer, then hybridized with a 2.0 preamplifier working reagent in 100 μl for 1 h at 50°C and 600 rpm. After washing twice, further amplifying hybridization was performed with a 2.0 amplifier working reagent for 1 h at 50°C and 600 rpm. Following additional washes, a final hybridization with a label extender probe, which adds biotinylated sites to the amplified hybridization sandwich, was performed (100 μl for 1 h at 50°C and 600rpm). Next, after washing, hybridized beads were incubated with 100 μl of streptavidin conjugated to R-PE at 25°C and 600 rpm for 30 min. Two further wash steps were performed before the beads were resuspended in 20 μl of buffer. Beads in each well were analyzed using a Luminex instru-
ment and the Bio-Plex 5.0/Bio-Rad platform. At least 100 beads in each well for each gene of interest were assessed. In general, the co-
efficient of variation for each gene in each well was <5%. The intensity of the PE fluorescence signal associated with individual capture beads was detected by a Luminex flow cytometer (Bio-Rad). The signal is reported as mean fluorescence intensity and is proportional to the number of target RNA molecules present in the sample. The linear range for fluorescence intensity of these multiplex beads is 10−4000. Samples were presented normalized to the housekeeping gene HPRPT-1. All sam-
ples were prepared in duplicate and each sample was tested in duplicate by branched chain DNA amplification.

**Results**

**Discrete populations of monocytes and kidney macrophages can be identified by the marker Ly6C during kidney fibrosis**

Monocyte and Mø heterogeneity in kidney fibrosis in mice was explored using the cell surface marker Ly6C. In BM a large pop-
ulation of mature monocytes was identified by high FSC, low SSC, the presence of CD11b, and the absence of the NK and granulocyte markers NK1.1 and Ly6G, respectively. Almost all of these BM monocytes had high levels of surface Ly6C (Ly6Chigh) (Fig. 1A). In peripheral blood, three discrete populations of monocytes were identified in Ficoll-enriched PBMC leukocytes expressing high,
Intermediate, and low levels of the marker Ly6C (Fig. 1B). To specifically identify these monocytes, leukocytes expressing Ly6G (granulocyte marker) and NK1.1 (NK cell marker) were excluded (supplemental Fig. 1A) because they also express CD11b. However NK cells expressed low levels of CD11b, and in purified PBMCs very few granulocytes were detected. All three populations of monocytes (CD11bhigh and Ly6Chigh, Ly6Cint, and Ly6Clow, where “int” is “intermediate”) exhibited high FSC and low SSC, a characteristic of monocytes (Fig. 1D). We therefore can be confident that CD11bhigh PBMCs are almost exclusively monocytes.

We induced UUO, a progressive model of kidney fibrosis in mice, and monitored the subpopulations of blood monocytes (Fig. 1E). There was a marked monocytes in response to UUO that was induced specifically by kidney disease, as it did not occur in response to sham surgery. Furthermore, this monocytes was exclusively to the appearance of Ly6Chigh monocytes in the blood (Fig. 1E).

Normal and day 7 post-UUO surgery kidneys were dissociated into single cells and analyzed by flow cytometry. Whereas there were few CD11bhigh or Csf1R-GFP macrophages in the normal kidney and the vast majority of these were Ly6Clow (Fig. 1F), in the diseased kidney there were many CD11bhigh cells and, similar to blood monocytes, these kidney Mφs also divided into three distinct populations based upon expression of the marker Ly6C (Fig. 1G). Subanalysis of CD11b− kidney cells indicated that there were very few Ly6G-contaminating leukocytes or NK1.1 or B220 high leukocytes (supplemental Fig. 2A), and the UUO kidney from Csf1R-GFP transgenic mice confirmed that there were three populations of Csf1R-GFPhigh leukocytes differentiated by Ly6C expression (Fig. 1H) and that they were not neutrophils (supplemental Fig. 2A). We can therefore be confident that the CD11bhigh cells or Csf1R-GFPhigh are kidney Mφ subpopulations. Further subpopulation analysis of these CD11b+ kidney inflammatory leukocytes was performed (Fig. 1I). CD11b+ leukocytes in the UUO kidney comprised the majority of CD45+ leukocytes. However only ~5% of leukocytes expressed markers associated with DC functions: CD11c−, I-Ahigh, and B7.2high. These data suggested that the majority of UUO kidney CD11b+ cells were Mφs rather than DCs. The majority of CD11b+ cells expressed F4/80, a minority (1%) were NK cells, and there were no detectable B220− CD11b− B cells. However, there was a small, distinct population of NKT cells that lacked NK1.1 but expressed CD11b, CD4, and Csf1R-GFP. In contrast to diseased kidney, ~30% of resident normal kidney CD11b+ leukocytes express CD11c and high levels of MHC class II and B7.2 (not shown). Importantly, some but not all of the kidney Mφs expressed the Mφ subset markers Ly6C or 7/4. We analyzed the CD11b+NK1.1−Ly6G− population of kidney leukocytes, which we have carefully defined as Mφs, during progression of UUO fibrosis (Fig. 1J). There is an early influx that persists. When kidney Mφs were subdivided by the Ly6C marker into three populations, there was a stable ratio of Ly6Chigh, Ly6Cint, and Ly6Clow, subpopulations. Ly6Chigh and Ly6Clow kidney Mφs were purified by flow cytometric sorting. The Ly6Chigh cells were discrete by flow cytometric scatter characteristics (not shown), and many had nuclear morphology similar to that of monocytes when viewed by Cytospin (Fig. 1K). By contrast, Ly6Clow Mφs had more diverse scatter characteristics and a characteristic “fried egg appearance” when viewed by Cytospin (Fig. 1K), indicating a more mature phenotype.

Conditional monocyte/macrophage ablation prevents fibrosis following ureteral obstruction

To explore further the functions of kidney Mφs, we used conditional Mφ ablation in vivo in the CD11b-dTR mouse, which is an accepted model for the specific ablation of monocytes and Mφs (2–4, 34, 35). Ureteral obstruction, a model of progressive kidney fibrosis, was induced in the kidneys of CD11b-dTR mice to study the development of fibrosis. Monocytes/Mφs accumulate progressively during the first 7 days of UUO fibrosis (Fig. 1). Administration of DT to mice with UUO kidney was highly effective at ablating all three subpopulations of circulating monocytes (Fig. 2, A and B), and this ablation persisted for >24 h (supplemental Fig. 1C). DT administration also depleted a minor population of B220+ and CD11b+ circulating lymphocytes and a population of CD4+ and CD11b+ circulating NKT cells. NK1.1+ cells were not affected by DT treatment (supplemental Fig. 1, A and B), and granulocytes in blood were also not ablated by DT administration (Ref. 3 and data not shown). DT administration was also highly effective at ablating CD11b+ cells in the kidney (Fig. 2, B and C) and effective at ablating all three subpopulations of kidney Mφs defined by the marker Ly6C (Fig. 2, C and D). Very few B220+ cells or NK1.1+ cells were detected in the UUO kidney, and those that were present were not affected by DT administration (Fig. 1F and supplemental Fig. 2). Likewise, there were few Ly6G+ neutrophils in the UUO kidney, and these were not affected by DT administration (supplemental Fig. 2). However a notable, but small population of CD4+ and CD11b+ NKT cells was recruited to the UUO kidney and also ablated by DT injection (supplemental Fig. 2). Ablation of Mφs continuously for 72 h from day 4 through day 7 or from day 7 through day 10 of fibrosis by three daily injections of diphtheria toxin DT resulted in a ~70% reduction in kidney Mφs in the diseased kidney and a 95% reduction in control kidney when assessed by morphometric quantification of F4/80 staining in kidney sections (Figs. 2, E and F). The effect of Mφ ablation on pathological collagen matrix deposition was assessed by morphometry of Picosirisirius red-stained tissues (labeling collagen I and collagen III) and collagen III immunostaining (Figs. 2, E and F). Both collagen III and Sirius red immunostaining were significantly reduced in response to Mφ ablation (Fig. 2, E and F). Our recent studies indicate that, in the kidney, Mφs do not generate pathological collagens (3, 13) but rather matrix deposition is restricted to fibroblasts. Furthermore, there are very few fibrocytes in the injured kidney (13) (see below). Fibrosis progression in the kidney must therefore proceed via paracrine signaling from Mφs to fibroblasts. To be certain of the specificity of our observations, we performed adoptive transfer experiments in CD11b-dTR mice treated with DT from day 7 to day 10 of UUO kidney disease. Mice either received vehicle or 2 × 106 purified BM monocytes from strain-matched control mice daily (days 7–9) during ablation induced by daily injection of DT (Fig. 2G). The transfer of this number of monocytes was based on calculations that there are 1 × 106 monocytes in the circulation of mice (3, 29). Kidneys and blood were assessed on day 10 UUO. Adoptive transfer resulted in partial restoration of the normal complement of circulating monocytes (not shown) and complete restoration of kidney Mφs (Fig. 2G). Kidney fibrosis progression was restored by adoptive transfer of BM monocytes (vehicle).
Bone marrow Ly6C high monocytes are selectively released and recruited to the kidney and differentiate into distinct subpopulations of macrophages

To understand the mechanisms of recruitment of monocytes to the injured kidney we performed adoptive transfer experiments, transferring CD45.1+/Ly6C high monocytes to CD45.2 mice on day 7 of UUO fibrosis. Ly6C high (Ly6C high combined with Ly6C int) or Ly6C high (Ly6C low) monocytes from peripheral blood (1 x 10^6) were transferred. Transfer of this number of monocytes was decided based on calculations that ~1 x 10^6 monocytes are retrievable from the mouse circulation at any one time (3, 29). Two hours later, transferred monocytes were confirmed to be in the circulation and retained the pretransfer level of Ly6C expression (Fig. 3A). However, 48 h later none were detected in the circulation but many in the spleen (not shown) and also the fibrotic kidney (Fig. 3A). In the fibrotic kidney, >3% of Mψs were derived from transferred BM monocytes. The BM monocytes had differentiated into three distinct populations of kidney Mψs: Ly6C high, Ly6C low, and Ly6C low (Fig. 3A). The proportion of these three populations reflected the populations of endogenous kidney Mψs (See Fig. 2). Thus, a single population of monocytes differentiated into three distinct populations of Mψs. We also transferred equal numbers of Ly6C high blood monocytes (Fig. 3B) or Ly6C low blood monocytes (Fig. 3C). After 2 h, Ly6C high peripheral blood monocytes (PBMs) and Ly6C low PBMs were detected in the circulation at similar numbers compared with Ly6C high BM monocytes (Fig. 3, B and C). The majority of the Ly6C high monocytes continued to express high levels of Ly6C in the circulation, but some of the Ly6C low cells had induced Ly6C high expression as a result of the transfer (Fig. 3C). Forty-eight hours following transfer, no transferred monocytes were detected in the circulation and some were detected in the spleen, but many fewer transferred Ly6C high or Ly6C low blood monocytes had been recruited to the UUO kidney (Fig. 3, B and C). In fact, ~8-fold fewer Ly6C low and 4-fold fewer Ly6C low blood monocytes were transferred to the fibrotic kidney compared with the transferred BM monocytes (Fig. 3D). Nevertheless Ly6C high blood monocytes had differentiated into three populations of kidney Mψs, but the Ly6C low blood monocytes that had been transferred differentiated primarily into Ly6C low kidney Mψs. The morphology of Ly6C high kidney Mψs suggested that they were more immature Mψs and that Ly6C low Mψs were mature Mψs (Fig. 1). These findings, combined with our observations that the monocytosis observed in response to UUO kidney fibrosis is due to the release of BM monocytes retained in the BM, therefore suggested that the mechanism by which Ly6C high kidney Mψs evolve is from the recruitment of Ly6C high BM monocytes to the kidney which then progressively differentiate into Ly6C low Mψs. To test this hypothesis further, we transferred BM monocytes to mice with day 5 UUO kidney fibrosis as before but collected leukocytes from the kidney after a further 96 h (Fig. 3E). After 96 h, ~1.8% of CD11b + kidney Mψs were derived from the CD45.1 donor BM monocytes. We expected that all Ly6C high-transferred monocytes would have become Ly6C low kidney Mψs. However the experiment showed that although there were now fewer CD45.1 Mψs remaining in the kidney compared with 48 h following transfer, the 96 h transferred Ly6C high BM monocytes had differentiated into three distinct Mψ populations, Ly6C high, Ly6C low, and Ly6C low (Fig. 3E). This observation indicates that rather than BM monocytes entering the kidney, becoming activated, and differentiating progressively into Ly6C low Mψs, there are kidney factors that govern the differentiation into three types of
Ly6C DEFINES MACROPHAGE HETEROGENEITY IN FIBROSIS

To understand further the contribution of resident kidney M\(\phi\)s (M\(\phi\)s or possibly resident DCs) to the development of kidney fibrosis, we generated BM chimeras transplating GFP mouse BM into lethally irradiated recipient mice (WT\(^{GFP}\)) (Fig. 4A). The ensuing chimeric mice were characterized and complete chimerism was confirmed (Fig. 4C) (31). UUO kidney obstruction was performed and kidneys were assessed on day 5 of the disease for the contribution of CD11b\(^+\) cells from the circulation compared with that of CD11b\(^-\) cells originating from the kidney parenchyma. All resident kidney M\(\phi\)s have the leukocyte marker CD45.1, indicating that they originate from hematopoietic stem cells and not from developing kidney mesenchyme (32) (not shown). In control kidneys (after 6 wk of chimerism), \(\sim\)15–20% of resident M\(\phi\)s expressed GFP and had therefore been replaced by cells of BM origin (Fig. 4B).

Nevertheless, \(\sim\)80% of “resident” M\(\phi\)s lacked the GFP marker and thus the absence of the GFP marker could be used to trace the fate of this pool of resident leukocytes. Five days after the induction of UUO kidney disease there was a 20-fold increase in GFP\(^-\)CD11b\(^+\) leukocytes in the kidney (Fig. 4B). Very few (<3%) had the nuclear morphology of neutrophils, consistent with a minor role for PMNs in this disease (see Fig. 2). In addition to the large increase in CD11b\(^+\) leukocytes of BM origin, there was also a substantial increase (\(\sim\)5-fold) in CD11b\(^-\) cells that lacked the BM GFP marker, indicating that they had originated from the resident pool of kidney M\(\phi\)s. This expansion must be due to proliferation of the resident kidney M\(\phi\)s. In keeping with this observation, UUO kidney sections immunolabeled for the proliferation markers Ki67 and CD11b showed CD11b cells that were proliferating. However, these cells were of both resident kidney M\(\phi\) origin and recruited BM cell origin (Fig. 4D). The kidneys from the chimeras were assessed in more detail by flow cytometry (Fig. 4C). Whereas in the blood 97.1% of CD11b\(^+\) PBMCs expressed high levels of GFP, the CD11b\(^+\) leukocytes in the kidney divided into those expressing GFP and those lacking GFP, similar to the data we acquired in tissue sections (Fig. 4B). The proportion of kidney M\(\phi\)s (CD11b\(^+\)) that derived from resident kidney M\(\phi\)s was rather underrepresented in these FACS studies. This was due to leukocyte enrichment using a Percoll gradient in which some of the resident-derived leukocytes were selectively lost from the gradient (data not shown). Nevertheless, the kidney M\(\phi\)s recruited from the BM comprised Ly6C\(^{high}\), Ly6C\(^{int}\), and Ly6C\(^{low}\) subpopulations (Fig. 4C). In contrast, the resident M\(\phi\)s-derived inflammatory M\(\phi\)s formed a discrete group of Ly6C\(^{int}\) M\(\phi\)s. In normal kidney the resident M\(\phi\)s are Ly6C\(^{low}\) (Fig. 4C and see Fig. 2), indicating that resident M\(\phi\)s induce Ly6C\(^{expression}\) in the UUO kidney and are distinct from recruited kidney M\(\phi\)s.

We were interested in understanding whether recruited and resident M\(\phi\)s played distinct or similar roles in promoting fibrosis. Initially, resident kidney M\(\phi\)s were purified from healthy kidneys and activated in vitro for 16 h with IFN-\(\gamma\) (Fig. 4E). The activation was compared with that of autologous monocytes cultured identically. Kidney resident M\(\phi\)s were less activated in vitro compared with monocytes, suggesting that these resident leukocytes were functionally distinct from monocytes and that they are less susceptible to activation in vivo. Similar resistance to activation was found when resident M\(\phi\)s were activated with LPS (not shown). To explore further the significance of the resistance of resident

FIGURE 3. BM Ly6C\(^{high}\) monocytes are selectively recruited to the UUO kidney and differentiate into three distinct populations of kidney macrophages, segregated by the marker Ly6C. A. Representative FACS plots of adoptive transfer (i.v.) of \(1 \times 10^6\) CD45.1\(^+\) BM monocytes (all Ly6C\(^{high}\)) into CD45.2\(^-\) mice with day 5 UUO kidney disease. CD45.1\(^+\) monocytes were identified in the circulation 2 h later, and >90% retained the Ly6C\(^{high}\) marker (left panel); 48 h later CD45.1 cells were identified in the UUO kidney (middle panel) and the CD45.1\(^+\) kidney leukocytes all expressed CD11b but had now differentiated into three populations, Ly6C\(^{high}\), Ly6C\(^{int}\), and Ly6C\(^{low}\) (right panel) (NK1.1\(^-\)). B. Adoptive transfer (i.v.) of \(10 \times 10^6\) CD45.1\(^+\) Ly6C\(^{high}\) (Ly6C\(^{high}\) combined with Ly6C\(^{int}\)) PBMs from healthy mice into CD45.2\(^-\) mice with day 5 UUO kidney disease. CD45.1\(^+\) monocytes were identified in the circulation 2 h later, and \(\sim\)70% retained the Ly6C\(^{high}\) marker (left panel); 48 h later small numbers of CD45.1\(^+\) cells were identified in the UUO kidney (middle panel), and the CD45.1\(^+\) kidney leukocytes all expressed CD11b but had now formed three populations, Ly6C\(^{high}\), Ly6C\(^{int}\), and Ly6C\(^{low}\) (right panel). C. Adoptive transfer (i.v.) of \(1 \times 10^6\) CD45.1\(^+\) Ly6C\(^{int}\) (Ly6C\(^{int}\) combined with Ly6C\(^{low}\)) PBMs from healthy mice into CD45.2\(^-\) mice with day 5 UUO kidney disease. CD45.1\(^+\) monocytes were identified in the circulation 2 h later, and \(\sim\)70% had newly acquired the Ly6C\(^{int}\) marker (left panel); 48 h later small numbers of CD45.1\(^+\) cells were identified in the UUO kidney (middle panel), and the CD45.1\(^+\) kidney leukocytes all expressed CD11b but now formed three populations, Ly6C\(^{high}\), Ly6C\(^{int}\), and Ly6C\(^{low}\) (right panel), but a large number were Ly6C\(^{int}\). D. Graph of the proportion of CD45.1\(^+\) kidney M\(\phi\)s recruited compared with the total number of M\(\phi\)s following adoptive transfer of equal numbers of leukocytes derived from BM or peripheral blood (PB) of healthy mice. Mo, Monocytes. E, Representative FACS plots of adoptive transfer (i.v.) of \(1 \times 10^6\) CD45.1\(^+\) BM monocytes (all Ly6C\(^{high}\)) into CD45.2\(^-\) mice with day 5 UUO kidney disease. Ninety-six hours later CD45.1 cells were identified in the UUO kidney (left panel, histogram), and the CD45.1\(^+\) kidney leukocytes all expressed CD11b but formed three populations, Ly6C\(^{high}\), Ly6C\(^{int}\), and Ly6C\(^{low}\) (right panel). All experiments were n = 4/group; *p < 0.05.
M\&s to activation, we generated BM chimeras to selectively ablate either resident M\&s or monocytes and monocyte-derived M\&s. BM chimeras were generated using C11b-DTR mouse BM transplanted into WT mice (WT\textsuperscript{DTR}) or WT BM transplanted into C11b-DTR mice (DTR\textsuperscript{WT}). All mice underwent UUO kidney injury and were given either DT or vehicle to ablate monocytes/M\&s on days 7 and 9. Kidneys were assessed for fibrosis on day 10. In previous studies, after 6 wk of chimerism the proportion of resident kidney M\&s that were replaced by new M\&s from BM was 15–20%. Therefore, 80–85% of kidney M\&s were of resident M\& origin (data not shown but see Fig. 4, B and C). The number of circulating monocytes that were of transplant origin was >99% (data not shown but see Fig. 4C and supplemental Fig. 3B). Ablation of monocytes or resident M\&s in WT\textsuperscript{DTR} or DTR\textsuperscript{WT} chimeras, respectively, from day 7 to day 10 of disease by daily injections of DT resulted in partial ablation of the total number of day 10 UUO kidney F4/80 cells (Fig. 4F). This partial ablation was expected, because the resident M\&s comprise ~30–40% of the total inflammatory M\& population in the UUO kidney (Fig. 4, B and C). In those chimeras that had DT-mediated ablation selectively of circulating monocytes (WT\textsuperscript{DTR}) but not resident M\&s, there was reduced fibrosis compared with littermates that had vehicle injections only (Fig. 4G). However, in those mice that had selective ablation of resident kidney M\&s (DTR\textsuperscript{WT}) but not of circulating monocytes, there was no reduction in fibrosis (Fig. 4G), indicating distinct functions for resident-derived M\&s in the UUO fibrotic kidney and also indicating that only recruited M\&s were profibrotic.

**Fibrocytes make no significant contribution to fibrosis in this model**

Although our findings indicate a key role for monocyte-derived M\&s in fibrogenesis, recent studies have implicated a circulating myeloid leukocyte capable of directly producing a fibrotic matrix known as the fibrocyte (14, 36). One explanation for our findings was that monocyte ablation in the C11b-DTR mouse resulted in fibrocyte ablation. To explore this further, we generated BM chimeric mice using BM from male Collagen\textsubscript{1a1}-GFP (Coll-GFP) reporter mice, which we recently reported (13), and lethally irradiated strain-matched female WT mice (supplemental Fig. 3A). Complete chimerism (WT\textsuperscript{coll-GFP}) was confirmed by detecting the Y chromosome in buffy coat cells (supplemental Fig. 3B) at 6 wk. In normal kidneys of these mice, no cells producing GFP indicative of Collagen\textsubscript{1a1} were detected. However 7 days after induction of UUO we detected GFP\textsuperscript{+} cells, confirming the recruitment of small numbers of BM-derived cells that generate Collagen\textsubscript{1a1} (supplemental Fig. 3, C and D). These fibrocytes were, however, detected exclusively around large- and medium-sized vessels and no fibrocytes were detected in the interstitium, where myofibroblasts are always detected and where fibrosis occurs (supplemental Fig. 3C). Furthermore, the proportion of leukocytes that generated Collagen\textsubscript{1a1} was extremely low (supplemental Fig. 3D), representing...
<1:1000 leukocytes in the kidney. Fibrocytes also represented <1:1000 myofibroblasts in the injured kidney (data not shown) (13). In addition, among purified PBMCs from these mice, GFP+ leukocytes were never detected (data not shown). Coimmunolabeling of kidney fibrocytes indicated that they expressed CD45 and CD14 and that the majority expressed CD34 (supplemental Fig. 3E). They also expressed high levels of MHC class II and CD86, markers suggestive of DC function. By contrast, however, they lacked CD11b, CD11c, F4/80, or markers of myofibroblasts. These studies confirmed the existence of fibrocytes but provided no evidence that they made a significant contribution to fibrogenesis. Furthermore, because they lacked expression of CD11b we did not expect them to be ablated in CD11b-DTR mice. To confirm that in CD11b-DTR mice there were very few fibrocytes and that ablation did not affect their number (supplemental Fig. 3F).

Ly6C defines functionally discrete macrophage populations in the fibrotic kidney

To understand whether there was any functional significance of the three kidney Mφ populations, kidney Mφs were labeled and sorted by Ly6C expression and assessed for transcript copy numbers of M1 and M2 inflammatory cytokines by using branched chain DNA amplification of hybridized mRNA methods. Ly6Chigh Mφs exhibited markers of classical or M1-biased activation with high levels of Il-1β, Mip-2α, Mip-1α, and Mip-1β (not shown). Ip-10 and Tgfβ transcripts did not increase in Ly6Chigh Mφ populations compared with monocyte controls, and Tnfα, although increased, was expressed at moderate transcript levels (Fig. 5A). The transcription factor Ym-1 was expressed at similar levels...
Table 1. Fold increase in transcript, normalized to Hprt1, 24 h following activation with IFN-γ or LPS compared with unactivated leukocytes

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<tr>
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<th>IFN-γ</th>
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<tr>
<td>Monocytes</td>
<td></td>
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<tr>
<td>Tnfα</td>
<td>4.9 ± 1.1</td>
<td>6.0 ± 2.1</td>
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<tr>
<td>IL-1β</td>
<td>14.7 ± 2.4</td>
<td>9.1 ± 1.8</td>
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<tr>
<td>Day 7 BM Mφs</td>
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<tr>
<td>Tnfα</td>
<td>43.6 ± 5.9</td>
<td>15.4 ± 3.1</td>
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<tr>
<td>IL-1β</td>
<td>67.1 ± 7.4</td>
<td>100.7 ± 8.9</td>
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*Unactivated monocyte and Mφ controls had Tnfα transcript levels of 0.15 ± 0.03 and 0.19 ± 0.04, respectively, and IL-1β transcript levels of 0.13 ± 0.04 and 0.14 ± 0.04, respectively.

as those of circulating monocytes (not shown), and the M2-biased cytokines Ccl17 (Tarc) and Ccl22 (Mdc) were expressed at low levels. The profibrotic growth factors Pdgfb and Igf-1 were difficult to detect. Overall, the Ly6Chigh Mφs exhibited an M1-biased pattern, although notably lacking high Tnfα (and also iNos' data not shown) expression. The paucity of Tnfα message induction in Ly6Chigh Mφs may reflect the preference that mouse monocytes exhibit for induction of IL-1β transcripts over Tnfα transcripts in vitro (Table I). In contrast Ly6Clow kidney Mφs exhibited a very different pattern of gene expression. IL-1β and Mip-2α were expressed at much lower levels and, strikingly, the M2-biased cytokines Ccl17, Ccl22, Igf-1, and Pdgfb were substantially induced relative to the Ly6Chhigh kidney Mφs, indicating that Ly6Clow kidney Mφs generate profibrotic transcripts that have the capacity to promote fibrosis by paracrine signaling. However the transcripts for Ym1 and Fizz1, markers of IL-4-activated induced Mφs, were down-regulated and not detected respectively, indicating that kidney Ly6Clow Mφs were different from M2 Mφs that are studied in vitro. Tnfα and Tgfβ were expressed in Ly6Clow Mφs at levels similar to those of Ly6Chhigh Mφs. The Ly6Chhigh population of Mφs showed intermediate expression of transcripts, suggesting that they are a group of Mφs in transition between Ly6Chhigh and Ly6Clow Mφs. IL-10, IL-6, IL-13, fibronectin, and Fgf-2 transcripts were also not detected by any population of kidney Mφs, and arginase transcripts were down-regulated in Ly6Clow macrophages (data not shown). Using a FACS-based approach to identify cell surface markers for denoting functional differences, Galectin-3, reported to be a profibrotic factor in the kidney and elsewhere (4) and potentially a selective Mφ M2 marker, was up-regulated in Ly6Clow Mφs. MARCO, a scavenger receptor highly expressed by M2 Mφs in vitro, was detected at low levels in UUO kidney Mφs but was not selectively induced by Ly6Clow Mφs, although other scavenger receptor transcripts, including SRA, are selectively induced in Ly6Clow Mφs (data not shown). Finally, FcγRI was highly expressed on the surface of Ly6Chhigh Mφs but down-regulated in the Ly6Clow population.

Discussion

In these studies we identified inflammatory kidney macrophages as a major contributor to the progression of interstitial fibrosis in a sterile mechanical model of progressive inflammation that is independent of glomerular inflammation, immune complex deposition, or autoimmunity. These observations are similar to our findings in the nephrotic nephritis model of anti-GBM glomerulonephritis and the thymic stromal lymphopoietin model of cryoglobulinemic glomerulonephritis, as well as the findings of others, suggesting that in progressive inflammation of the kidney, macrophages are profibrotic regardless of the initiating injury (3, 9, 37–40) (Z. Guo, A. S. Muhlfeld, K. L. Hudkins, J. S. Duffield, and C. E. Alpers, submitted for publication). The detection of a clear profibrotic function of Mφs in vivo also mirrors reports of Mφ function during chronic injury in other organs, including skin, kidney, heart, lung, and liver, and where repeated injury or chronic injury leading to fibrosis is dependent on Mφs (2–10). Although fibrocytes have been identified in other organs (13, 36), our comprehensive study of fibrocytes using robust methods did not find this myeloid cell type to be a significant contributor to fibrosis in the kidney (13). Therefore, in the kidney Mφ profibrotic function must be primarily via cellular crosstalk.

Our studies identify three populations of peripheral blood monocytes defined by expression of the marker Ly6C. Identification of discrete monocyte subpopulations supports those reported elsewhere (29, 34, 41), but whereas other studies relying on GFP expression driven by the fractalkine receptor (CX3CR1) promoter identified only two populations of monocytes, our methods identify a third population that expresses Ly6C at an intermediate level. Adoptive transfer studies in our system of progressive kidney injury confirm that Ly6Chigh monocytes are preferentially recruited to sites of injury compared with Ly6Clow monocytes and, in addition, our findings suggest that a pool of mature Ly6Chhigh inflammatory monocytes is released from BM in response to kidney injury, implying that injured kidney signals to the BM to release inflammatory monocytes, which then track preferentially to the kidney. Ly6Chhigh monocytes from normal peripheral blood were, by comparison, poorly recruited to the injured kidney.

In our model of interstitial kidney disease, we have defined three populations of inflammatory kidney Mφs that are also distinguished by the marker Ly6C. Administration of DT to the CD11b-DTR mouse selectively ablates all three populations of kidney Mφs, and repletion with BM monocytes is sufficient to restore fibrosis, underscoring the role of Mφs in kidney disease progression and the utility of the CD11b-DTR mouse for studying them (2, 3, 35). Although other leukocytes express CD11b, our comprehensive studies underscore both the utility of the CD11b-DTR mouse as a model of selective monocyte/Mφ deletion, because NK cells and neutrophils are not affected by ablation.

We used BM chimerism to dissect the relative contributions of recruited vs resident monocytes/Mφs to kidney fibrosis and used adoptive transfer to test whether Ly6C expression by monocytes was a monocyte fate/function marker or a marker of the state of activation/differentiation. Importantly, although several studies have identified resident Mφs in other tissue beds as important cells in the recruitment of monocytes following injury, we did not find the resident Mφ pool of cells significantly affected monocyte recruitment (42). However, our studies ablated resident Mφs several days following injury onset, so it is possible that once injury is established the resident Mφs no longer function in that capacity. It is interesting, in that context, that IFN-γ-activated resident Mφs were resistant to the induction of chemokine transcripts in vitro but that other cell types in the kidney, including epithelial cells, are a potent source of chemotactants (43), suggesting that resident Mφs may not be a potent source of proinflammatory chemokines in vivo. In BM chimeras, resident kidney Mφs represented a substantial discrete population contributing to the total kidney Mφ population. By microscopic counting, as many as 40% of all UUO kidney Mφs derived from the initial population of resident Mφs, and the presence of Ki-67+ resident-derived kidney Mφs strongly suggests that the expanded resident-derived Mφ population is due to local proliferation. Whereas in healthy kidneys resident Mφs expressed Ly6C at low levels, in UUO kidneys the resident-derived inflammatory Mφs exclusively expressed Ly6C at an intermediate level. This pattern of Ly6C expression was quite discrete from that of the kidney Mφs recruited from circulating monocytes.
which formed three separate Mφ populations, Ly6C\textsuperscript{high}, Ly6C\textsuperscript{int}, and Ly6C\textsuperscript{low}, indicating that resident kidney Mφs were discrete from monocyte-derived macrophages. We generated BM chimeras in CD11b-DTR mice to selectively test the profibrotic role of monocyte-recruited or resident-derived kidney M\textsuperscript{\textdagger}. These studies confirmed that only monocyte-recruited M\textsuperscript{\textdagger} had the capacity to promote fibrosis. It is likely that the reason resident kidney macrophages do not contribute to fibrosis is because they do not become activated in the same way as recruited monocytes. Supporting this hypothesis, the transcriptional profiling studies of M\textsuperscript{\textdagger} subpopulations in vivo suggest that resident-derived M\textsuperscript{\textdagger}, which contribute a major proportion of the Ly6C\textsuperscript{int} subpopulation, generate low levels of cytokines because Ly6C\textsuperscript{int} M\textsuperscript{\textdagger} have lower Tnf\alpha, Cxcl10, and Tgf\beta (data not shown) levels. In addition the ex vivo IFN-\gamma-stimulated resident M\textsuperscript{\textdagger} generated low levels of inflammatory cytokine transcripts. However, a second possibility is that resident M\textsuperscript{\textdagger} fail to differentiate into profibrotic M2-type M\textsuperscript{\textdagger}. Whereas monocyte-derived M\textsuperscript{\textdagger} lose Ly6C as they differentiate into M2-type M\textsuperscript{\textdagger}, all resident M\textsuperscript{\textdagger} acquire intermediate expression of Ly6C. One explanation is that they are partially activated but not differentiated. Further studies beyond the scope of the present ones will be required to understand the function of resident M\textsuperscript{\textdagger} in healthy kidney and during the injury process.

To test whether Ly6C\textsuperscript{low} profibrotic kidney M\textsuperscript{\textdagger} derive selectively from Ly6C\textsuperscript{low} blood monocytes and whether Ly6C\textsuperscript{high} kidney M\textsuperscript{\textdagger} derive selectively from Ly6C\textsuperscript{high} blood monocytes, as has been suggested elsewhere (29, 41), adoptive transfer experiments were performed. These experiments indicated that firstly Ly6C\textsuperscript{high} monocytes residing in BM are selectively recruited to the kidney and indicated that Ly6C\textsuperscript{high} monocytes differentiate into Ly6C\textsuperscript{high}, Ly6C\textsuperscript{int}, and Ly6C\textsuperscript{low} populations of kidney M\textsuperscript{\textdagger}. Thus, one monocyte population can differentiate into three different M\textsuperscript{\textdagger} populations. Whereas Ly6C\textsuperscript{low} monocytes are recruited to the kidney and differentiate predominantly into Ly6C\textsuperscript{low} M\textsuperscript{\textdagger}, the recruitment of these monocytes was poor and did not appear to be the major mechanism by which Ly6C\textsuperscript{low} kidney M\textsuperscript{\textdagger} appeared. These studies highlight the fact that monocyte Ly6C expression in our experiments is not a lineage marker for M1- or M2-fated monocytes but does mark monocytes with differential capacity for recruitment to the injured kidney.

To study further the significance of Ly6C expression by kidney M\textsuperscript{\textdagger}, transcript expression was tested in Ly6C\textsuperscript{high}, Ly6C\textsuperscript{int}, and Ly6C\textsuperscript{low} M\textsuperscript{\textdagger} by using branched DNA amplification technology. Ly6C\textsuperscript{low} M\textsuperscript{\textdagger} are the most abundant population in the UUO kidney, derive exclusively from recruited monocytes, have low expression of markers of classical activation such as Il-1\beta and Cxcl2, but high expression of the profibrotic, M2-biased (or wound healing) M\textsuperscript{\textdagger} markers Ccl17, Ccl22, Igf-1, and Pdgfb, and Galectin-3. In addition, Galectin-3, Pdgf-\beta, and Igf-1 have all been shown to be paracrine factors that promote fibrosis (4, 44–48). Therefore our studies indicate that Ly6C\textsuperscript{low} kidney M\textsuperscript{\textdagger} are selectively profibrotic. However, Ly6C\textsuperscript{low} kidney M\textsuperscript{\textdagger} lack expression of the M2 (or wound healing) markers Fizz-1, Ym-1, or Arginase and do not up-regulate MARCO, although they do up-regulate transcripts for other scavenger receptors. In addition, they have intermediate levels of Tnf\alpha and have high levels of Ccl3 transcripts. Therefore Ly6C\textsuperscript{low} kidney M\textsuperscript{\textdagger} have some differences from IL-4/IL-13- or steroid-induced M2 polarization of M\textsuperscript{\textdagger} in vitro (27) or M2-biased M\textsuperscript{\textdagger} reported in the lung. These findings indicate that Ly6C\textsuperscript{low} M\textsuperscript{\textdagger} are generative of cytokines that render them profibrotic and, therefore, M2-like, but suggest that the kidney environment is different from the lung or in vitro environments (49–51) because Fizz-1 and Ym-1 have been widely described in lung M\textsuperscript{\textdagger} and DCs. It is quite likely therefore that there will be a kidney M\textsuperscript{\textdagger}-specific transcriptional signature. Further studies will be required to define that signature. It is interesting to note that other mechanisms have been described by which M\textsuperscript{\textdagger} acquire an M2 or (wound healing) signature, including adenosine receptor-mediated differentiation, G protein-coupled receptor-mediated differentiation, and chemokine receptors (26, 52); these receptor classes could play a functional role in kidney injury. Furthermore, although M2 has been used to describe both angiogenic functions and profibrotic functions of M\textsuperscript{\textdagger} and, at least in lung and skin, this M2 phenotype is readily apparent, it is clear that multiple phenotypes of activated M\textsuperscript{\textdagger} can occur that may be contextual, i.e., specified by organ environment as well as activation and differentiation signals (28).

Ly6C\textsuperscript{high} kidney M\textsuperscript{\textdagger} have high levels of Il-1\beta and Cxcl2 and low levels of Ccl17, Ccl22, Igf-1, and Pdgfb, indicating that they are similar to M1-polarized M\textsuperscript{\textdagger}. However, they do not have high levels of Tnf\alpha transcripts, suggesting that they are activated by factors other than TLR-2 and TLR-4 ligands. Although it has been reported that activated M\textsuperscript{\textdagger} may generate high levels of TNF-\alpha protein in the absence of high levels of transcript (53), it is increasingly recognized that danger-associated molecular pattern recognition receptors other than TLRs can activate monocytes in an injured tissue and that this may result in a much lower level induction of TNF-\alpha than that achieved through signaling via TLR4 (54). In addition, whereas in these studies mature cultured M\textsuperscript{\textdagger} respond to activation by IFN-\gamma or LPS with high Tnf\alpha and high Il-1\beta transcripts, BM monocytes are less responsive and, in particular, generate much lower Tnf\alpha responses than Il-1\beta responses, underscoring the importance of studying monocytes in preference to cultured M\textsuperscript{\textdagger}. Further studies will be required to elucidate the mechanism by which kidney M\textsuperscript{\textdagger} become activated.

The Ly6C\textsuperscript{int} kidney M\textsuperscript{\textdagger} overall have intermediate level expression of the transcripts quantified, suggesting that they represent a functionally intermediate leukocyte population. Morphologically, Ly6C\textsuperscript{high} kidney M\textsuperscript{\textdagger} appear more immature and monocyte like, whereas Ly6C\textsuperscript{low} kidney M\textsuperscript{\textdagger} appear larger by SSC and more like mature M\textsuperscript{\textdagger}. Because Ly6C\textsuperscript{high} monocytes give rise to both Ly6C\textsuperscript{high} and Ly6C\textsuperscript{low} kidney M\textsuperscript{\textdagger}, there does not appear to be distinct monocyte lineage that is fated to be profibrotic. Furthermore, Ly6C\textsuperscript{low} more correctly represents a M\textsuperscript{\textdagger} differentiation marker rather than as a specific M1/M2 marker. Our findings indicate that the kidney environment is responsible for triggering the differentiation of inflammatory Ly6C\textsuperscript{high} monocytes into profibrotic M\textsuperscript{\textdagger} rather than being responsible for recruitment of monocytes with lineage-prescribed functions from the circulation. However, the parenchymal factors that govern those differentiation steps remain obscure. The fact that there is a stable population of M1-type inflammatory Ly6C\textsuperscript{high} kidney M\textsuperscript{\textdagger} and also a stable population of intermediate Ly6C-expressing M\textsuperscript{\textdagger} suggests that these subpopulations play distinct roles in kidney pathology rather than simply representing M\textsuperscript{\textdagger} in transition to Ly6C\textsuperscript{low} profibrotic M\textsuperscript{\textdagger}.

We conclude that kidney M\textsuperscript{\textdagger} play important roles in fibrosis progression in progressive kidney injury and this is dependent on the differentiation of kidney M\textsuperscript{\textdagger} into Ly6C\textsuperscript{low} profibrotic M\textsuperscript{\textdagger}.

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Disclosures

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