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Resident Renal Mononuclear Phagocytes Comprise Five Discrete Populations with Distinct Phenotypes and Functions

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Recent reports have highlighted greater complexity, plasticity, and functional diversity of mononuclear phagocytes (MPCs), including monocytes, macrophages, and dendritic cells (DCs), in our organs than previously understood. The functions and origins of MPCs resident within healthy organs, especially in the kidney, are less well understood, whereas studies suggest they play roles in disease states distinct from recruited monocytes. We developed an unbiased approach using flow cytometry to analyze MPCs residing in the normal mouse kidney, and identified five discrete subpopulations according to CD11b/CD11c expression as well as F4/80, CD103, CD14, CD16, and CD64 expression. In addition to distinct marker profiles, these subpopulations have different lineages and expression of genes involved in tissue homeostasis, including angiogenesis. Among them, the CD11b⁺/CD11c⁻/F4/80⁻ subpopulation notably exhibited high capacity to produce a representative anti-inflammatory cytokine, IL-10. Each subpopulation had different degrees of both macrophage (phagocytosis) and DC (Ag presentation) capacities, with a tendency to promote differentiation of regulatory T cells, whereas two of these showed expression of transcription factors reported to be highly expressed by classical DCs, and proclivity to exit the kidney following stimulation with LPS. In summary, resident kidney MPCs comprise discrete subpopulations, which cannot be simply classified into the conventional entities, and they produce anti-inflammatory and tissue-homeostatic factors to differing degrees. The Journal of Immunology, 2013, 191: 000–000.

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role of mononuclear phagocytes normally residing in the kidney in health and disease states is relatively unknown. Several studies, however, indicate that they expand in chronic disease by local proliferation, that they play different roles from inflammatory monocyte-derived cells in disease with different cytokine profiles, and that they may regulate organ growth and repair (7, 9).

MPCs are defined functionally by the name macrophage to describe phagocytic properties, or DC to describe Ag presentation properties, and thus, even this function, which is often regarded as specific for DCs, is common to both. The purpose of this study is to define resident MPC subpopulations in normal kidney to determine their potential functional contributions.

Materials and Methods

Animals

C57BL/6 (B6) and BALB/c mice were from Charles River Laboratories. FVB/N CX3CR1-GFP (CX3CR1GFP) (10), B6 CCR2-GFP (11), B6 CSF1R-GFP (12), B6 GM-CSF reporter (13), CSF1R on mixed background strain (14), and B6 IL-10–lactamase reporter (ITIB) (15) mice have been previously described. B6 LysMCre/Cre and B6 Gt(ROSA)26Sortm14(CAG-tdTomato)Hze (R26RtdTomato) mice were from The Jackson Laboratory. The whole blood was drawn into a syringe, including EDTA dissolved in PBS for anticoagulation. The blood, further diluted in PBS, was centrifuged at 300 × g for 5 min and the obtained pellet was resuspended as blood leukocytes for flow cytometry.

Processing of blood for flow cytometry

The whole blood was drawn into a syringe, including EDTA dissolved in PBS for anticoagulation. The blood, further diluted in PBS, was centrifuged at 300 × g for 5 min. The pellet was treated twice with ACK lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) for RBC lysis and washed with PBS. The obtained pellet was resuspended as blood leukocytes for flow cytometry.

Flow cytometry

Cell pellets were resuspended in ice-cold FACS buffer (PBS including 1% BSA, 2 mM EDTA, and 0.01% sodium azide), including 2% mouse serum, plated in round-bottom 96-well plate, and incubated with Ab mixture on ice for 30 min. Directly conjugated anti-CSF45 (clone 30-F11), anti-CD3e (clone 145-2C11), anti-CD19 (clone 1D3), anti-CD49b (clone DX5), anti-Ly6G (clone 1A8), anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-F4/80 (clone DM6), anti-MHC class II (clone M5/114.15.2), anti-CD103 (clone 2E7), anti-CD86 (clone GL1), anti-CD14 (clone SC12-7), anti-CD16/32/3 (clone 93), anti-CD64 a and b (clone X54-57.1), anti-Ly6C (AL-21), and anti-CD11b (AF210) Abs were from BioCentury, eBioscience, or BD Biosciences and used at 1:200 dilution. After incubation, cells were washed with FACS buffer twice, followed by analysis using BD FACSCanto II (BD Biosciences) or by sorting with BD FACSaria II (BD Biosciences). To obtain total cell numbers, in some experiments PE-conjugated counting beads (Spherotech) were added to the single-cell suspension for cell counting, according to manufacturer’s guidelines. In experiments evaluating CD135 expression, cells were incubated for 3 h after purification prior to application of Abs. Blood monocytes were defined as leukocytes that express CD45 and CD11b, but do not express Ly6G, CD19, C3e, or CD49b. Kidney MPCs were defined as cells from kidneys in which all blood had been flushed, which express CD45, but did not express Ly6G, CD19, C3e, or CD49b.

Immunofluorescence microscopy

Normal C57BL/6 kidneys were prepared and labeled, as previously described, and images were captured by confocal microscopy.

Quantitative RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen). cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR (qRT-PCR) assay was performed using an AB7900HT (Applied Biosystems) and iTaQ SYBR Green Supermix with ROX (Bio-Rad). The cycling conditions were initial denaturation at 95˚C for 2.5 min, followed by PCR cycles of 95˚C for 15 s and 58˚C for 30 s. Specificity of each primer pair was confirmed by verification of single PCR product with expected size in agarose gel electrophoresis. The sequences of primers are shown in Supplemental Table I.

T cell differentiation assay

RAG2-deficient DO11.10 (DR) mice were generated as a source of homogenous naïve Ag-specific CD4+ T cells containing no effector or regulatory T (Treg) cells (17). Purified CD4+ T cells from spleen and peripheral LNs of donor DR mice were obtained by no-touch magnetic bead isolation (Miltenyi Biotec), followed by positive cell sorting for CD4+ T cells. Bone marrow–derived dendritic cells (BM-DC) and bone marrow–derived macrophages (BM-Mφ) were prepared as described (18, 19). Briefly, single-cell suspensions of bone marrow were prepared and cultured with 20 ng/ml GM-CSF for DCs, or 20% L929-conditioned media for macrophages for 4–6 d. All APC cultures were aliquoted and then incubated with 5 μg/ml OVA (323–339) peptide for 1 h at 37˚C, and then washed three times with plain media. A total of 3 × 105 purified CFSE-labeled DR T cells were mixed with APC populations at indicated ratios in complete RPMI 1640 media supplemented with 5% FBS and cultured for 5 d. On day 5, the cultures were restimulated with plate-bound anti-CD3e (10 μg/ml, clone 145-2C11) and anti-CD28 (1 μg/ml, clone PV-1) for 5 h in the presence of Golgi inhibitor (BD Biosciences). Cells were harvested and stained for viability (Fixable Viability Dye; eBioscience), surface CD4 and DO11.10 TCR (KJ1-26 Ab), intracellular Foxp3 (clone FJK-16K), IFN-γ (clone MG1.2), IL-4 (clone BV6D-24G2), IL-10 (clone JES6-16E3), and IL-17a (clone TC11-18H10.1) (Abs from eBioscience). Negative control samples were stained with isotype control Abs. T cells were analyzed by flow cytometry, as described above.

Phagocytosis assay

Sorted mononuclear phagocytes were incubated in DMEM/F12 medium, including 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, with latex beads–FITC complex in Phagocytosis Assay Kit (Cayman Chemical) for 2 h, following analysis by FACSCanto II.

LPS stimulation of renal mononuclear phagocytes

Sorted cell subpopulations were incubated in DMEM/F12 medium, including 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml LPS (Sigma-Aldrich) for 16 h. The stimulated cells were used for qRT-PCR analysis, as mentioned above.

IL-10 reporter mouse experiments

Single cells from ITIB mouse kidney were incubated in PBS, including 1.8 μM CCF4-AM (Invitrogen) and 7.2 mM probenecid (Sigma-Aldrich), at room temperature for 90 min with gentle shaking. Following one wash with PBS, cells were stained with Abs, as described above, prior to flow cytometry analysis.

Statistics

All data are given as mean ± SEM. Significant differences were analyzed using Student t test between two groups and ANOVA among groups more than three. The p values <0.05 were regarded as significant.
The integrins CD11b and CD11c in conjunction with F4/80, CD103, CD16, CD14, and CD64 discriminate five discrete mononuclear phagocyte populations

Although it has been thought that CD11b is expressed by macrophages, whereas CD11c is expressed by DCs, no clear functional correlate has been demonstrated for these receptors, and recent studies have challenged the specificity, particularly in nonlymphoid organs. We analyzed all tissue leukocytes from normal adult kidneys by flow cytometry from C57BL/6, FVB/N, and BALB/c strains of mice. After excluding all lymphocytes and granulocytes, the remaining cells were identified as MPCs (Fig. 1A). Kidney MPCs were assessed for cell surface expression of the markers CD11b and CD11c (Fig. 1B). In each strain, five populations of MPCs were discriminated according to expression levels of CD11b and CD11c, as follows: CD11bhighCD11chigh (MPC1); CD11bhighCD11clow (MPC2); CD11blowCD11cint (MPC3); CD11blowCD11clow (MPC4); and CD11blowCD11c int (MPC5). However, the ratio of each subpopulation was distinct in the different strains, and young mice (P14) also showed distinct distribution of subpopulations. To evaluate this further, total leukocyte numbers were quantified (Fig. 1C), and these showed substantially higher levels of MPC1 and MPC3 that could not be explained simply by increased kidney size (Fig. 1D). Moreover, MPC5 was the smallest population and was lower in BALB/c and FVB/N mice. The separation of these MPC populations was readily apparent with concurrent use of F4/80 expression, which has been used as a classical cell surface marker of kidney macrophages, and more recently has been found to be expressed by CD11c+ MPCs residing in the kidney (20, 21). We found that F4/80 expression, which has been used as a classical cell surface marker of kidney macrophages, and more recently has been found to be expressed by CD11c+ MPCs residing in the kidney (20, 21). We found that F4/80 expression, which has been used as a classical cell surface marker of kidney macrophages, and more recently has been found to be expressed by CD11c+ MPCs residing in the kidney (20, 21). We found that F4/80 expression, which has been used as a classical cell surface marker of kidney macrophages, and more recently has been found to be expressed by CD11c+ MPCs residing in the kidney (20, 21). We found that F4/80 expression, which has been used as a classical cell surface marker of kidney macrophages, and more recently has been found to be expressed by CD11c+ MPCs residing in the kidney (20, 21). We found that F4/80 expression, which has been used as a classical cell surface marker of kidney macrophages, and more recently has been found to be expressed by CD11c+ MPCs residing in the kidney (20, 21). We found that F4/80 expression, which has been used as a classical cell surface marker of kidney macrophages, and more recently has been found to be expressed by CD11c+ MPCs residing in the kidney (20, 21). We found that F4/80 expression, which has been used as a classical cell surface marker of kidney macrophages, and more recently has been found to be expressed by CD11c+ MPCs residing in the kidney (20, 21).

The five subpopulations of MPCs expressed distinct patterns of these receptors. Importantly, CD14 and CD64 were expressed most highly in the CD11bhighCD11chigh MPC3 population, whereas CD11bhighCD11clow MPC1 population had the highest expression of CD16 (Fig. 2A, Table I). Although DCs are often defined as CD11c+ MHCII+ cells, MPC3 cells expressed these receptors, but concurrently expressed these macrophage markers at high levels (Table II). CD11b+CD11c− MPCs were negative in all the assessed surface markers and excluded in the subsequent studies (discussed below).

As would be predicted by the flow cytometric analysis, these distinct subpopulations can be discriminated within the interstitium of the kidney cortex by fluorescence microscopy and are not distributed to any particular location by subpopulation (Fig. 2B).

Kidney mononuclear phagocyte subpopulations can be discriminated by chemokine receptor expression, ontogeny, and growth factor dependence

Chemokine receptors play an important role in monocyte, macrophage, and DC trafficking. To explore chemokine receptor expression in the MPC subpopulations, we assessed expression of CX3CR1, a receptor thought to affect steady state trafficking of monocytes, in Cx3cr1GFP mice of FVB/N strain. As noted above, kidney from FVB/N wild-type mice has reduced numbers of CD11b+CD11cint MPC5 (Fig. 1B), and this minor population did not express CX3CR1. The remaining kidney MPCs expressed different levels of CX3CR1, with CD11bhighCD11cint, F4/80high; MPC3 expressing the highest levels (Fig. 3A–C), and CD11blow CD11cint high MPC4 expressing these receptors at the lowest levels.

Similarly, we assessed expression of CCR2 (Fig. 3D–F), another receptor implicated in monocyte trafficking, using CCR2GFP mice on C57BL/6 background. In most CD11bhighCD11cint and CD11blowCD11cint+ MPCs, receptor expression was not detected. All of the remaining three populations of MPCs expressed CCR2, but CD11bhighCD11cint+, F4/80high; MPCs had lower levels. The fact that at steady state key chemokine receptor levels are distinct lends weight to the hypothesis that these cells exist as separate populations.

The receptor for the growth factor CSF-1, which is also called M-CSF, is thought to be critical in MPC differentiation and functions (14). In CSF1R−/− mice that report CSF-1 receptor expression by GFP fluorescence, the distribution of CSF-1R expression was also discriminatory (Fig. 3G–I). Whereas the CD11bhighCD11cint low MPC2 and CD11blowCD11cint MPC3 expressed high levels of the receptor, CD11bhighCD11cint high MPC1 and CD11blowCD11cint high, CD103+ MPC4 expressed intermediate levels, and the CD11b−CD11cint MPC5 did not express it. The absence of the receptor on this latter population suggests that it may have silenced expression or that it functions independently of CSF-1 receptor signaling. To test whether any of the MPCs had absolute dependence on the receptor expression for differentiation and survival, we evaluated MPCs in kidneys from P14 Csf1r−/− mice, because these mice rarely survive beyond weaning and because P14 is the time point when kidney development is complete. In P14 Csf1r−/− mice, CD11bhighCD11cint, F4/80high subpopulation was selectively absent, suggesting that such cells may require CSF1R for differentiation (Fig. 3J, 3K). The absence of F4/80high cells was also observed in Csf1r−/− mice at day 0 after birth (Fig. 3L). Importantly, all other subpopulations were preserved. MPC4 was increased in these juvenile mice, although the difference was not significant.

CSF-2, GM-CSF, is another growth factor that plays important roles in myeloid cell differentiation and activation. To test its role in the appearance/maintenance of MPCs, we evaluated kidney MPCs in Csf2−/− mice of C57BL/6 background compared with
FIGURE 1. Five distinct subpopulations of resident MPCs in normal kidney show both macrophage and DC markers by flow cytometry. (A) Gating strategy for identification of MPCs in normal mouse kidney concluded by a representative CD11b/CD11c flow cytometry plot of resident MPCs in normal kidney with right-hand plot showing key and nomenclature for each population. (B) Representative CD11b/CD11c MPC plots for three different strains of adult mice and 14-d-old C57BL/6 mice with representative percentages. (C) Total number of each MPC subpopulation per kidney. (D) Average kidney weight by mouse strain. (E) Representative CD11b, F4/80 plot of total MPCs (left) and CD11b/CD11c plots for F4/80\textsuperscript{high} MPCs (right, upper) and F4/80\textsuperscript{low} MPCs (right, lower). Most F4/80\textsuperscript{high} MPCs reside in CD11b\textsuperscript{int}CD11c\textsuperscript{int} subpopulation. Graphs show total numbers of F4/80\textsuperscript{high} and F4/80\textsuperscript{low} MPCs in each MPC population. (F) Plots showing CD103\textsuperscript{+} cells in young and adult kidneys and their distribution in CD11b/CD11c plots. (G) Plots (%) showing rare Flt3\textsuperscript{+} MPCs in young and adult normal kidney and their distribution (red events) in CD11b/CD11c plots. For (A) and (B), experiments were independently performed >30 times, using a total of >80 mice. For others, experiments were independently performed twice or more, using three or more mice each time.
strain-matched controls. In these adult mice, CD11blowCD11c\text{high} MPC4 were markedly reduced (Fig. 3M, 3N), as were CD11b^2CD11c\text{int} MPC5 (Fig. 3M, 3O), suggesting they are dependent on the expression of GM-CSF for differentiation and/or survival. In addition, the proportion of CD103\text{+} MPCs was reduced to 1% from 4–6% in controls. Recent studies have suggested that CD103\text{+} MPC may derive from a circulating precDC rather than monocyte (4). Consistent with that, some CD11blowCD11c\text{high}, CD103\text{+} MPCs express Flt3 (Fig. 1G), but these observations in Csf2^-/- kidney suggest that the majority of MPC4 are not Flt3 dependent, rather they are GM-CSF dependent. In addition, in Csf2^-/- mice, there was a trend toward an increase in MPC1, although this was not significant.

When monocyte-DC precursors in bone marrow differentiate into monocytes, they activate expression of the lysosomal protein, lysozyme M (2). To test the origins of the five MPC subpopulations, we assessed whether they had ever been monocytes by fate mapping monocytes by their expression of LysM during bone marrow monocyte differentiation using LysM\text{Cre}^+/+;R26\text{R}^{tdTomato/+} reporter mice (Fig. 3P). Compared with monocytes, the MPC2 showed similar levels of tdTomato reporter and MPC3 showed increased levels. These suggest that MPC2 bears a close relationship to monocytes, and, consistent with that, many express low levels of Ly6C (Fig. 2A). MPC1 population showed somewhat lower proportion with tdTomato reporter and MPC4 (CD11blowCD11c\text{high}, CD103\text{+}), and MPC5 (CD11b^2CD11c\text{int}) comprised fewer that have previously expressed the monocyte marker LysM. These findings indicate that MPC4 and MPC5 populations appear in the kidney without having trafficked as monocytes, suggesting they either arise in development from precDCs or arise from an-

FIGURE 2. Heterogeneous expression of typical DC and macrophage markers by kidney MPCs. (A) Histogram plots showing expression of cell surface markers in resident MPC subpopulations in normal C57BL/6 mouse kidney. Representative plots from three independent experiments are shown. Bars show percentage in gated area. (B) Fluorescence confocal micrographs (x400) showing CD11b, CD11c, F4/80, and CD103 expression in kidney cortex. Within the cortex, F4/80\text{high} and F4/80\text{low} cells can be seen. Rare CD103\text{+}CD3^- cells can be seen, and colabeling with CD11b and CD11c identifies cells that express CD11b alone (arrowhead), CD11b and CD11c at high levels (broad arrows), CD11c alone (upper panel, small closed arrow), and CD11c at high levels with CD11b at low levels (lower panel, small closed arrow). g, Glomerulus; v, venule.
other tissue resident progenitor. In addition, MPC1 may comprise two subsets, one of which may arise from pre-DCs or another tissue progenitor. Consistent with this, a subpopulation within MPC1 expresses Flt3 (Fig. 1G).

CD11b\textsubscript{low}CD11c\textsuperscript{high} and CD11b\textsuperscript{high}CD11c\textsuperscript{high} subpopulations have higher expression of transcription factors characteristic of classical DCs

A single study has reported that two kinds of classical DCs (cDCs) exist in nonlymphoid tissues, as follows: CD103\textsuperscript{+} DCs and CD11b\textsuperscript{+} DCs (4). Several transcription factors have been found to be involved in their development. We analyzed expression of these transcription factors in purified kidney MPC subpopulations. Zbtb46 is a transcription factor that has been identified as a marker specifically expressed by both CD103\textsuperscript{+} DCs and CD11b\textsuperscript{+} DCs, but not by other MPCs (22, 23). It was expressed in CD11b\textsubscript{low}CD11c\textsuperscript{high}, CD103\textsuperscript{+}, MPC4, and CD11b\textsuperscript{high}CD11c\textsuperscript{high} MPC1 at significantly higher levels (Fig. 4A), suggesting that the former subpopulation is equivalent to the reported CD103\textsuperscript{+} DCs and the latter is equivalent to CD11b\textsuperscript{+} DCs. Other transcription factors, Batf3 and Id2, are reported as indispensable for development of CD103\textsuperscript{+} DCs, but not CD11b\textsuperscript{+} DCs, although they are expressed in both types of DCs in peripheral tissues (4, 24). This is consistent with our findings that MPC4 had significantly higher expression of these two transcription factors and MPC1 had intermediate expression (Fig. 4A). However, only MPC4 expressed Irf8 (Fig. 4A), a transcription factor necessary for development of both CD103\textsuperscript{+} and CD11b\textsuperscript{+} cDCs, but reported to be expressed only in CD103\textsuperscript{+} DCs in peripheral tissues (4, 25). These results are consistent with CD11b\textsuperscript{high}CD11c\textsuperscript{high} MPC4 corresponding to CD103\textsuperscript{+} DCs and CD11b\textsuperscript{high}CD11c\textsuperscript{high} MPC1 corresponding to CD11b\textsuperscript{+} DCs.

Kidney mononuclear phagocytes differentially express angiogenic, developmental, and migratory genes

Macrophages are well known for their roles in angiogenesis and organ growth, as well as roles in innate immunity and phagocytosis. To explore whether kidney MPCs play roles in these processes, we assessed gene transcript levels for functionally related genes in each purified subpopulation, together with blood monocytes as control.

The angiogenic factor, vascular endothelial growth factor-A (Vegfa), was expressed in all MPCs at higher levels than monocytes (Fig. 4B), indicating the resident MPC potential for angiogenesis. Although blood monocytes are similar to CD11b\textsuperscript{high}CD11c\textsuperscript{low} MPCs in CD11b and CD11c expression, CD11b\textsuperscript{high}CD11c\textsuperscript{low} MPC2 express significantly higher levels of Vegfa. In addition, MPC4 population had significantly higher expression of another angiogenic factor, Angiopoietin-1 (Angpt1) (Fig. 4B). These cells also had a tendency to have higher expression of platelet-derived growth factor B and C (Pdgfb and Pdgfc) (Fig. 4B).

As well as roles in angiogenesis, macrophages play important roles in organogenesis and determining organ size (9). They can also promote repair (8). Two developmental pathways implicated in these processes are the insulin-like growth factor pathway and the Wingless/Int (WNT) pathway. As with angiogenic factors, CD11b\textsuperscript{high}CD11c\textsuperscript{high} MPC4 expressed significantly more Igf1 and Wnt7b than other subpopulations (Fig. 4C).

Because CD68, a lysosome-associated phagocytic receptor, is often used as macrophage marker, but not DC marker, we assessed its expression level in each MPC subset. Strikingly, all MPC populations express similar levels of CD68 (Fig. 4D).

Next, we assessed in more detail chemokine receptors, CCR7 and CCR8, implicated in emigration of MPCs to LNs, where they can function as APCs (26, 27). CD11b\textsuperscript{low}CD11c\textsuperscript{high} MPC4 and CD11b\textsuperscript{+} CD11c\textsuperscript{int} MPC5 had higher expression of Ccr7 and Ccr8 (Fig. 4E). Ccr7 is also highly expressed in CD11b\textsuperscript{high}CD11c\textsuperscript{high} MPC1. These results suggest that these three subpopulations might present Ags to T cells in vivo.

CXCR4 plays a role in angiogenic myeloid cell recruitment (28). All MPC populations express similar levels of this receptor (Fig. 4E), implicating potential functions in angiogenesis. The IL-4R has been implicated in reactive proliferation of resident macrophages (29) and DC maturation (30), and it is also expressed in all MPC populations (Fig. 4E).

Macrophages and DCs are sentinels in mounting innate immune responses to tissue injury. In addition, macrophages can play an anti-inflammatory role, depending on the context. However, little is known about the pro- and anti-inflammatory proclivity of resident kidney MPCs. Thus, we assessed the transcriptional expression of representative cytokines in MPC subpopulations. Tnf and Il1b, proinflammatory cytokine genes, were significantly highly expressed in CD11b\textsuperscript{high}CD11c\textsuperscript{high} MPC1 and CD11b\textsuperscript{high}CD11c\textsuperscript{low} MPC3, implicating potential functions in angiogenesis.

Table I. Mean fluorescence intensity of surface markers in MPC subpopulations

<table>
<thead>
<tr>
<th>Subpopulations</th>
<th>CD103</th>
<th>MHCII</th>
<th>CD86</th>
<th>CD14</th>
<th>CD16</th>
<th>CD64</th>
<th>Ly6C</th>
<th>F4/80</th>
</tr>
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<tr>
<td>CD11b\textsuperscript{high}CD11c\textsuperscript{high}</td>
<td>2.933 ± 224</td>
<td>523 ± 52</td>
<td>90,043 ± 6,707</td>
<td>1,629 ± 484</td>
<td>32,893 ± 5,721</td>
<td>3,929 ± 1,194</td>
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<td>2,473 ± 68</td>
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<td>CD11b\textsuperscript{low}CD11c\textsuperscript{low}</td>
<td>4,115 ± 1,336</td>
<td>360 ± 91</td>
<td>26,134 ± 11,707</td>
<td>8,297 ± 5,539</td>
<td>13,460 ± 4,040</td>
<td>3,292 ± 1,223</td>
<td>2,473 ± 68</td>
<td>212 ± 35</td>
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<tr>
<td>CD11b\textsuperscript{high}CD11c\textsuperscript{low}</td>
<td>8,462 ± 2,681</td>
<td>764 ± 218</td>
<td>60,825 ± 15,701</td>
<td>21,402 ± 7,608</td>
<td>8,087 ± 3,922</td>
<td>4,381 ± 1,846</td>
<td>212 ± 35</td>
<td>118 ± 20</td>
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<tr>
<td>CD11b\textsuperscript{+}CD11c\textsuperscript{int}</td>
<td>576 ± 35</td>
<td>6,180 ± 1,013</td>
<td>18,026 ± 11,080</td>
<td>714 ± 40</td>
<td>15,727 ± 6,625</td>
<td>427 ± 303</td>
<td>118 ± 20</td>
<td>135 ± 10</td>
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<tr>
<td>CD11b\textsuperscript{high}CD11c\textsuperscript{int}</td>
<td>573 ± 38</td>
<td>487 ± 26</td>
<td>2,619 ± 427</td>
<td>222 ± 17</td>
<td>904 ± 36</td>
<td>378 ± 44</td>
<td>135 ± 10</td>
<td>1 ± 10⁻¹⁶</td>
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Table II. Summary of markers in MPC subpopulations

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<tr>
<th>F4/80</th>
<th>Ly6C</th>
<th>CD14</th>
<th>CD16</th>
<th>Cx3CR1</th>
<th>CCR2</th>
<th>CSF1R</th>
<th>MHCII</th>
<th>CD103</th>
<th>Zbtb46</th>
<th>Batf3</th>
<th>Irf8</th>
<th>CCR7</th>
<th>IL-10</th>
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<td>CD11b\textsuperscript{high}CD11c\textsuperscript{high}</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
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<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>CD11b\textsuperscript{large}CD11c\textsuperscript{low}</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<tr>
<td>CD11b\textsuperscript{+}CD11c\textsuperscript{int}</td>
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<td>-</td>
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Mean ± SEM is shown (n = 3).
FIGURE 3. MPC subpopulations show differential expression of chemokine receptors, CSF dependency, and distinct ontogeny. Histograms (A, D, G) and graphs of mean fluorescence intensity (MFI) (C, F, I) of GFP in MPC subpopulations from CX3CR1<sup>GFP/+</sup> kidneys (n = 3), CCR2<sup>GFPTr</sup> mice (n = 4), and CSF1R<sup>GFPTr</sup> mice (n = 3), respectively. (J) Representative plots of P14 juvenile kidney MPCs in control Csf1r<sup>+/+</sup> and Csf1r<sup>−/−</sup> mice at day 14. In right plots, gated cells (%) are shown as red events, whereas total events (%) are shown in gray. Note that in juvenile kidneys MPC4 expresses CD11b at lower levels compared with adults. (K and L) Graphs showing the proportion of MPCs that are F4/80<sup>high</sup> CD11b<sup>int</sup> MPCs (corresponding to CD11b<sup>int</sup>CD11c<sup>int</sup> MPC3) in control Csf1r<sup>+/+</sup> and Csf1r<sup>−/−</sup> mice at day 14 (K) (n = 3) or at day 0 (L) after birth. (M–O) Representative plots (%) of kidney MPCs (M) and a graph showing the proportion (N, O) of MPCs that are the CD11b<sup>int</sup>CD11c<sup>int</sup> subpopulation (MPC4) and CD11b<sup>−</sup>CD11c<sup>int</sup> (MPC5) in adult control and adult Csf2<sup>−/−</sup> knockout mice (n = 3). (P) A graph showing the ratio of tdTomato-positive MPCs. (Figure legend continues)
MPC2 (Fig. 4F). In contrast, CD11b<sup>low</sup>CD11c<sup>high</sup> MPC4 and CD11b<sup>+</sup>CD11c<sup>int</sup> MPC5 subsets had higher expression of other proinflammatory cytokines, Ifn<sub>γ</sub> and Ifng. Anti-inflammatory cytokines were also produced at differing levels. CD11b<sup>high</sup>CD11c<sup>low</sup> MPC2 and CD11b<sup>high</sup>CD11c<sup>int</sup> MPC3 had significantly more transcripts of Il10, whereas Tgf<sub>β</sub>1 was highly expressed in CD11b<sup>high</sup>CD11c<sup>high</sup> cells (Fig. 4G). Interestingly, RALDH2, an enzyme that regulates retinoic acid synthesis and has been implicated in stimulating T<sub>reg</sub> cells, and macrophage polarization were expressed at high levels by all MPC subsets (Fig. 4G). Overall, these results suggest that MPC subpopulations have distinct functions in innate immunity.

It is well known that macrophages have much heterogeneity, including highly polarized examples named M1 and M2 macrophages, which promote and suppress inflammation, respectively. Surprisingly, CD11b<sup>low</sup>CD11c<sup>high</sup> subpopulation had significantly higher expression in both a representative M1 marker Nos2 and M2 marker Arg1 (Fig. 4H, 4I).

Kidney mononuclear phagocyte populations show differential capacity for phagocytosis

The detailed expression profiles of these kidney MPC populations suggest they all may have both phagocytic and APC functions. We therefore assessed function in ex vivo assays. Phagocytosis of latex beads has been used as a gold standard to experimentally uncover phagocytic function typical of macrophages, because only professional phagocytes have the capacity to phagocytose inert beads (31). MPCs were collected together and incubated with fluorescent latex beads for 2 h at 37°C. Internalization of beads in respective subpopulations was assessed by flow cytometry. All the MPC subpopulations showed phagocytic function, but to differing degrees (Fig. 5A). CD11b<sup>high</sup>CD11c<sup>low</sup> MPC2 exhibited the highest capacity to phagocytose whether assessed as proportion of phagocytosing cells or the mean fluorescence intensity (Fig. 5B, 5C). The rare population of CD11b<sup>low</sup>CD11c<sup>high</sup>, CD103<sup>+</sup> MPC4 also showed significant phagocytic capacity, whereas CD11b<sup>+</sup>CD11c<sup>int</sup> MPC5 showed a much lower capacity. Collectively, all MPC subpopulations can be considered to be phagocytic cells.

Kidney mononuclear phagocyte populations show differential capacity for stimulation of naive T cells and driving T cell polarization

The capacity to successfully present Ag to naive T cells is the gold standard of APC or DC function. Because all MPC populations expressed some level of MHC class II and costimulatory molecule CD86, we assessed the capacity of kidney MPC subpopulations to present Ag, in steady state, successfully to naive T cells from the DO11.10 mice with the transgenic TCR and lack other TCRs due to deficiency of its recombination. Cultured BM-DC and BM-M<sub>φ</sub> were used as controls. Purified kidney MPC populations or aliquots of bone marrow–derived cells, previously pulsed with OVA peptide, were incubated with CFSE-labeled naive T cells for 5 d in nonpolarizing conditions, and restimulated with anti-CD3e and anti-CD28 Abs, followed by assessment of T cell proliferation (Fig. 6). As expected, BM-DC showed a much higher capacity to stimulate Ag-specific T cell proliferation compared with BM-M<sub>φ</sub>. Intriguingly, however, there were marked differences in the capacity of kidney MPC populations to stimulate T cell proliferation. CD11b<sup>high</sup>CD11c<sup>low</sup> MPCs (MPC2) had a low capacity to stimulate T cells, similar to BM-M<sub>φ</sub>, but CD11b<sup>high</sup>CD11c<sup>int</sup> MPCs (MPC1) had a much higher capacity, similar to BM-DC, whereas CD11b<sup>±</sup>CD11c<sup>int</sup>, F4/80<sup>+</sup> MPCs (MPC3) had an intermediate capacity to stimulate T cells. Even more surprising was the finding that CD11b<sup>low</sup>CD11c<sup>high</sup>, CD103<sup>+</sup> cells (MPC4) showed pronounced APC capacity, much greater than CD11b<sup>high</sup>CD11c<sup>int</sup>, implicating these monocyte-derived MPCs as the most potent APCs in the kidney. CD11b<sup>1</sup>CD11<sup>int</sup> MPCs were too few in number to collect from BALB/c mice.

Next, we studied the polarization of naive T cells induced by MPC subpopulations, by assessing Th1 (IFN-γ), Th2 (IL-4), Th17 (IL-17), and T<sub>reg</sub> (Foxp3) differentiation (Fig. 6B, 6C). No Th17 differentiation was observed (data not shown), but CD11b<sup>high</sup>CD11c<sup>high</sup> and CD11b<sup>±</sup>CD11c<sup>int</sup> MPCs induced a high proportion of T<sub>reg</sub> and did not stimulate Th2 differentiation. By contrast, CD11b<sup>low</sup>CD11c<sup>int</sup>, F4/80<sup>+</sup> MPCs, the most abundant subpopulation in steady-state kidney, induced a high proportion of T<sub>reg</sub> differentiation. By contrast, BM-DC stimulated higher proportions of Th1 and Th2 and lower proportions of T<sub>reg</sub>. Collectively, these studies implicate MPC1, 3, and 4 in maintenance of immunological tolerance.

In response to the pathogen-associated molecule, LPS, kidney mononuclear phagocyte populations show distinct cytokine repertoires

We were interested to understand how resident kidney MPCs respond to a proinflammatory agent. To test this, MPC subpopulations and autologous blood monocytes were purified, and gene expression was assessed after 16-h exposure, ex vivo, to the innate activator LPS, which activates cells via TLR4 and CD14 (32) (Fig. 7). CD11b<sup>high</sup>CD11c<sup>high</sup> and CD11b<sup>±</sup>CD11c<sup>int</sup> subpopulations had higher expression of proinflammatory Ifnγ and Il1b, especially when compared with monocytes, whereas another proinflammatory cytokine Il6 was more highly expressed in CD11b<sup>high</sup>CD11c<sup>low</sup> and CD11b<sup>±</sup>CD11c<sup>int</sup> as well as CD11b<sup>high</sup>CD11c<sup>int</sup> (Fig. 7A). The expression of an anti-inflammatory cytokine, Il10, showed a similar pattern to Ilb (Fig. 7B). The expression of another anti-inflammatory cytokine Tgfβ1 was extremely low or could not be detected (data not shown).

LPS is a typical inducer of M1 activation in macrophages. M1 markers, Cxcl10 and Il23a, were highly expressed in CD11b<sup>low</sup>CD11c<sup>high</sup> and CD11b<sup>±</sup>CD11c<sup>low</sup> MPCs (Fig. 7C). These two MPC subsets also had higher expression of another M1 inducer, Ifng. Intriguingly, a typical M2 activation marker, Arg1, was also expressed in these two subpopulations with significantly higher levels (Fig. 7D). We also assessed other M1 markers, Nos2, Il12a, and Il12b, but their expression levels were extremely low (data not shown).

Next, we evaluated expression of inflammatory chemokines, including Cxcl2, Ccl2, and Ccl5, which showed differential expression in MPC subpopulations (Fig. 7E). Ccr7, a chemokine receptor related to MPC migration to LNs, was highest in CD11b<sup>low</sup>CD11c<sup>high</sup>, CD103<sup>+</sup> cells (Fig. 7F). This population as well as CD11b<sup>high</sup>CD11c<sup>low</sup> cells had higher expression of genes related to tissue repair, Vegfa, Pdgfb, and Il7a, which showed differential expression in MPC subpopulations (Fig. 7E).

Because the resident MPCs predominantly stimulated T<sub>reg</sub> cell proliferation in APC assays, we reasoned that some of the MPCs

kidney MPCs normalized to monocytes in Lysm<sup>Cre+/−</sup>R26R<sup>tdTomato+/−</sup> monocyte fate reporter mice. 1, CD11b<sup>high</sup>CD11c<sup>high</sup>; 2, CD11b<sup>high</sup>CD11c<sup>low</sup>; 3, CD11b<sup>±</sup>CD11c<sup>int</sup>; 4, CD11b<sup>±</sup>CD11c<sup>high</sup>; 5, CD11b<sup>+</sup>CD11c<sup>int</sup>. Data are represented as mean ± SEM.
may play an anti-inflammatory role. To study this, we analyzed IL-10 production in vivo using a novel, highly sensitive IL-10 reporter mouse. These mice have the knock-in transgene \( \beta \)-lactamase under the IL-10 promoter, endogenous IL-10 gene, and an internal ribosomal entry site, and \( \beta \)-lactamase expression in respective cells can be detected by flow cytometry due to its enzymatic ability to convert green fluorescence to blue (15). In steady state, most MPCs do not synthesize IL-10, but a proportion of CD11b\(^{hi}\)CD11c\(^{hi}\), F4/80\(^{hi}\) MPCs generate IL-10 in healthy kidneys (Fig. 8A), suggesting they may have a unique role in

FIGURE 4. qRT-PCR analysis of gene expression in normal kidney MPC subpopulations. (A–I) The expression levels of the gene relative to GAPDH are shown \( (n = 3) \). 1, CD11b\(^{hi}\)CD11c\(^{hi}\); 2, CD11b\(^{hi}\)CD11c\(^{lo}\); 3, CD11b\(^{lo}\)CD11c\(^{hi}\); 4, CD11b\(^{lo}\)CD11c\(^{lo}\); 5, CD11b\(^{lo}\)CD11c\(^{hi}\). Mo, blood monocytes. Data are represented as mean ± SEM. \( *p < 0.05, \#p < 0.01, \{p < 1 \times 10^{-2} \).
preventing inflammatory responses. To investigate IL-10 upregulation in kidney MPCs in the context of systemic inflammation, we used a model of systemic administration of LPS to mice. It is known that IL-10 is elicited as a counterbalance against systemic inflammatory response. Following systemic LPS administration, ~40% of MPCs activated IL-10 production (Fig. 8B). Analysis of the MPC subpopulations that expressed IL-10 indicated that the CD11b<sup>hi</sup>CD11c<sup>int</sup>, F4/80<sup>hi</sup> subpopulation showed the highest ratio of IL-10-producing cells (Fig. 8C). These findings show that this subpopulation has a greater capacity to IL-10 production in vivo.

Mononuclear phagocyte populations show differential evanescence from the kidney following systemic LPS administration in vivo

To study the in vivo response of MPCs to systemic administration of LPS, we compared resident kidney MPC populations with those 48 h after LPS treatment. Over a 48-h time period, two MPC populations showed almost complete disappearance from the kidney in response to LPS (Fig. 5D, 5E). These populations are the CD11b<sup>hi</sup>CD11c<sup>hi</sup> MPC1 and CD11b<sup>lo</sup>CD11c<sup>hi</sup>, CD103<sup>+</sup> MPC4. CD11b<sup>-</sup>CD11c<sup>int</sup> MPC5 also declined in number, although this was not complete (Fig. 5E). In addition, there was a significant reduction in the number of CD11b<sup>hi</sup>CD11c<sup>int</sup> F4/80<sup>hi</sup> MPC3 population (Fig. 5E). Although this population did not disappear, numerically, the greatest number of MPCs disappeared from the kidney from this population of cells. To evaluate the meaning of this evanescence, we assessed kidney draining LNs for the migration of MPCs. Forty-eight hours after LPS administration, there was a substantial increase in CD11b<sup>hi</sup>, CD11c<sup>hi</sup>, F4/80<sup>hi</sup> MPCs in the LNs, but no clear population of CD103<sup>+</sup> MPCs (Fig. 8F, 8G). Although this is not a fate map, these studies are consistent with a substantial number of MPC1 and CD103<sup>+</sup> MPCs (Fig. 8F, 8G). Although this is not a fate map, these findings show that the MPC subpopulations that expressed IL-10 indicated that the CD11b<sup>int</sup>CD11c<sup>int</sup>, F4/80<sup>hi</sup>v population showed the highest ratio of IL-10-producing cells (Fig. 8C). These findings show that this subpopulation has a greater capacity to IL-10 production in vivo.

Discussion

We used an unbiased approach to identify five distinct subpopulations of resident MPCs in the normal kidney, based on the different CD11b/CD11c integrin expression patterns in conjunction with F4/80, CD103, and CD14. By using this strategy, we were able to discriminate five distinct subpopulations. To our knowledge, this is the first study that comprehensively analyzed and classified kidney MPCs, although other studies have previously discriminated MPCs in other organs (16, 33). These kidney subpopulations also show different patterns in MPC-related surface markers, monokine receptor expression, and gene expression profiles at steady state, and respond to LPS stimulation with distinct cytokine expressions. However, these resident subpopulations all express a range of growth factors that may regulate vasculature and tissue homeostasis. A potent anti-inflammatory cytokine, IL-10, is expressed by these resident MPCs, and it is remarkably elevated with LPS stimulation. They also share the capacity of phagocytosis and Ag presentation to naive T cells, but to different degrees. The presentation to naive T cells predominantly promotes differentiation of T<sub>reg</sub> cells. These results therefore identify discrete resident MPC subpopulations that share both macrophage and DC functions as well as macrophage and DC markers even within each subpopulation. A major conclusion of these findings is that conventional dichotomous macrophage and DC functions, phagocytosis and Ag presentation, respectively, are context-specific functions of resident MPCs rather than functions restricted to distinct cells such as macrophages or DCs.

Among the five MPC subpopulations, CD11b<sup>hi</sup>CD11c<sup>hi</sup>, F4/80<sup>hi</sup> MPC3 are the most abundant in normal kidney. Their most striking feature is high proclivity for IL-10 production both at steady state and in response to pathogenic stimulus. This subpopulation also has a tendency to stimulate differentiation of T<sub>reg</sub> cells. Furthermore, these MPCs appear to be the same MPCs described by Schulz et al. (6, 34) as arriving from yolk sac progenitors, adding further weight to their distinct function in kidney. Lower CCR2 expression in this population than in CD11b<sup>hi</sup>CD11c<sup>hi</sup> F4/80<sup>low</sup> populations (CD11b<sup>hi</sup>CD11c<sup>hi</sup> and CD11b<sup>hi</sup>CD11c<sup>lo</sup>) might reflect the difference of their ontogeny. In addition, only this CD11c<sup>int</sup>CD11c<sup>int</sup> MPC3 subpopulation requires CSF1R for their existence in the kidney, although other subpopulations also express this receptor at a lower level than CD11b<sup>int</sup>CD11c<sup>int</sup> population. A recent study reported that CSF1R signaling promotes repair from kidney injury through skewing MPC functions (35). Collectively, these findings have several important implications. First, CD11b<sup>int</sup> CD11c<sup>int</sup>, F4/80<sup>hi</sup> MPC3 may serve as endogenous defenders against inflammation. Second, because resident MPCs may expand in chronic diseases (7), it is quite possible that they represent a significant population of reparative macrophages (also known as regulatory or M2 macrophages). In addition, because the number of MPC3 cells differs significantly between mouse strains, it is
possible that some of the immunological differences noted between these strains relate to the differences in this cell population. Finally, although MPC3 have many characteristics of macrophages, nevertheless, numerically, they leave the kidney in greatest numbers after systemic LPS injection and have significant APC capacity.

A second major finding from this study is that there exists a relatively rare resident population of CD103+ (CD11blowCD11chigh) MPCs that have extremely higher capacity for Ag presentation with a potentially greater tendency to stimulate Th1 cells. These CD103+ MPCs also express the migratory chemokine receptor CCR7 and have an inclination to disappear from the kidney in response to LPS exposure, although at this time their fate is unclear. These findings suggest that they may have distinct APC roles in the kidney, potentially in local Ag presentation. In addition, relatively few of these

FIGURE 6. Kidney MPC populations exhibit unique differential capacities for stimulating Ag-specific CD4+ T cell proliferation and polarization. (A) Graph showing the proportion of viable DO11.10/RAG2−/− T cells that have proliferated, as measured by CFSE dilution after incubation with aliquots of OVA peptide-pulsed MPCs from each purified kidney MPC subpopulation (MPC1, CD11bhighCD11chigh; MPC2, CD11bhighCD11clow; MPC3, CD11bintCD11cint; MPC4, CD11blowCD11chigh), or from BM-MΦ or BM-DC. (B) Representative flow cytometric plots showing T cell survival and proliferative responses (CSFE dilution) and expression of markers of differentiation (Foxp3, IFN-γ, IL-4) in response to incubation of 30,000 naive T cells with 2,000 OVA peptide-pulsed APCs. Gates were set using isotype control and no APC experimental control. (C) Graphs showing the proportion of viable proliferated T cells that expressed intracellular Foxp3 (left panel) or produced intracellular IFN-γ (middle) or IL-4 (right) after restimulation following initial incubation with 2,000 APCs. BM-MΦ and BM-DC were used as controls. MPC2 and BM-MΦ were excluded due to failure to stimulate >75% of T cells to proliferate. Data are represented as mean ± SEM. The experimental conditions were reproduced in duplicates or triplicates, and the experiment was performed twice.
MPCs appear to derive from monocytes. Consistent with this, some of the MPC4 population also express Flt3, a receptor central for DC development, and had the highest expression of all the cDC development-related transcription factors that we assessed, including Zbtb46, Batf3, Id2, and Irf8 (36). Nevertheless, this population was selectively dependent on GM-CSF for its existence. These results suggest that CD11b^low^CD11c^high^ MPC4 correspond to CD103^+^ cDCs, although the latter cells have been conventionally limited to populations of CD11c^+^MHCII^+^ cells. Furthermore, CD11b^low^CD11c^high^ MPCs had higher expression of angiogenic factors, especially the ones related to pericyte recruitment, including Angpt1, Pdgfb, and Pdgfc, and, in response to LPS, activate genes consistent with M2 macrophage phenotype. Therefore, they may also have an important role in angiogenesis and/or maintaining the homeostasis of vasculature as well as regulating inflammation. In addition to this relatively rare, but functionally important population, there is a small population of CD11b^2^CD11c^int^ MPC5 that expresses CD11c, but most do not express CD103 or Flt3. These cells also do not derive from a monocyte precursor. The function of this subpopulation is currently unclear because it is rare, and this has rendered functional studies incomplete. They are also dependent on CSF2 (GM-CSF), which has a role in development of DCs in vivo (37). One possibility is that they are a quiescent precursor of CD11b^low^CD11c^high^, CD103^+^ MPCs, which is also supported by the finding that they have intermediate expression of Irf8, a transcription factor known to be expressed in earlier stages of DC development (25). However, further studies will be required to validate this hypothesis. The alternative is that they represent a novel population of MPCs that have higher Ccr8 expression and capacity to emigrate from the kidney.

FIGURE 7. qRT-PCR analysis of gene expression in MPC subpopulations stimulated ex vivo with LPS. (A–G) The expression levels of the gene relative to GAPDH are shown (n = 3). 1, CD11b^high^CD11c^high^; 2, CD11b^high^CD11c^low^; 3, CD11b^low^CD11c^int^; 4, CD11b^low^CD11e^high^; 5, CD11b^-CD11c^low^; Mo, blood monocytes. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 1 × 10^{-3}, ****p < 1 × 10^{-4}.
The CD11b^{high}CD11c^{high} MPC1 subpopulation exhibits complicated characteristics. A minority of these cells express Flt3 and MPC1 and have high expression of \( Zbtb46 \), suggesting that they are equivalent to CD11b^{+} DCs. Indeed, they have high capacity for Ag presentation, stimulating differentiation of Treg cells; they express the migratory chemokine receptor \( Ccr7 \) highly; and they appear to migrate avidly to kidney LNs in response to LPS (Table II). In contrast, this subpopulation has monokine receptor \( CX3CR1 \) and \( CCR2 \) expression at high levels, whereas the CD11b^{low}CD11c^{high}, CD103^{+} subpopulation does not. These two subsets also differ in inflammatory cytokine expression, suggesting the possibility that they have distinct roles in inflammatory states, although further studies are needed to clarify their lineage and features in detail.

One possibility is that the Flt3^{+} subpopulation within MPC1 are CD11b^{+} DCs derived from a pre-DC, as described elsewhere (4), but that the remaining Flt3^{−} cells within MPC1 can also function as CD11b^{+} DCs. Other studies support the notion that circulating monocytes can give rise to nonconventional DCs in nonlymphoid tissues (16, 33, 38), and MPC1 bears similarities to CD64^{+} monocyte-derived tissue DCs described by others (39, 40). Our studies indicate that MPCs that are not derived from pre-DCs have the capacity to function as DCs in the normal kidney. Further studies are required to dissect the origins of these MPC populations in the normal kidney and their differential responses to homeostatic and inflammatory stimuli.

The CD11b^{high}CD11c^{low} subpopulation is similar to blood monocytes in terms of CD11b and CD11c expression. However, we found clear and significant differences between these two types of cells. The former resident cells have significantly higher expression of \( Vegfa \), \( Tnf \), \( Il1b \), and \( Il10 \) at steady state than the latter circulating cells, suggesting that CD11b^{high}CD11c^{low} subpopulation plays a role as sentinels in tissues relevant to maintaining homeostasis.

We did not analyze CD11b^{low}CD11c^{low} MPC subpopulation because they lack all the MPC-related markers that we assessed. According to the gating strategy that we used, they also lack lymphocyte (T, B, and NK cells) and granulocyte markers. One possibility is that they are atypical lymphocytes, such as innate lymphoid cells, although further studies are needed for their characterization.

**FIGURE 8.** IL-10 expression and quantification of kidney MPC subpopulations in vivo in steady state or following systemic LPS exposure. (A) Representative flow cytometry plots of kidney MPCs in highly sensitive IL-10 reporter \( Il10^{+/-} \) and control \( Il10^{+/-} \) mice. The right panel shows the distribution of IL-10-positive cells by F4/80 and CD11b (\( n = 4 \)). (B) Representative plots and a graph of IL-10–positive cells showing a remarkable increase in IL-10 expression of kidney MPCs with systemic LPS treatment, with the right panel showing the distribution of IL-10^{+} cells by F4/80 and CD11b. (C) IL-10 histograms in CD11b^{high}CD11c^{low} (2), CD11b^{int}CD11c^{int} (3), and CD11b^{low}CD11c^{low} (5) cells, and a graph showing the proportion of IL-10^{+} cells within each subpopulation (\( n = 3 \)), indicating that the majority of IL-10^{+} cells are MPC3. (D) Representative flow cytometry panels showing the distribution of MPCs with or without systemic LPS stimulation. (E) The graph showing the changes in the absolute number of MPCs within each subpopulation normalized to kidney weight. The filled bars correspond to those treated with vehicle, whereas the blank bars correspond to those treated with LPS (\( n = 3 \)). (F and G) Representative plots showing nonlymphocyte cells in LNs in steady state or 48 h after LPS injection. Note marked increase in F4/80^{+} CD11b^{+} cells as well as CD11c^{+} cells. Data are represented as mean ± SEM. *\( p < 0.05 \), #\( p < 0.01 \), \{\( p < 1 \times 10^{-3} \).
Another major observation is that all of the MPCs most likely play concurrent roles in vascular homeostasis. All of the subpopulations express typical angio-regulatory factors Vegf, Angpt1, and Pdgf genes in steady state. Recent studies have implicated macrophages and CSF-1 in tissue growth, including kidney during development (9). A major factor in organ size is angiogenesis, but it will be interesting to understand whether the MPC subpopulations play similar or distinct roles in vascular regulation.

Inflammatory cytokines are expressed with different levels among MPC subpopulations in normal kidney. For example, Tnf and Il1b are more highly expressed in CD11blowCD11clow, CD11bhiCD11clow, and CD11bintCD11cint populations, whereas Il6 and Il1b are more highly expressed in CD11bPinhCD11clihi and CD11bhiCD11cint populations. However, when they are stimulated with LPS ex vivo, proinflammatory cytokines Tnf and Il1b are generally downregulated compared with steady state. Furthermore, an M1 marker Nos2 is remarkably decreased (undetectable by qRT-PCR), whereas an M2 marker Arg1 is increased by LPS stimulation. Taken together, these findings may further support the idea that resident MPCs have anti-inflammatory and reparative roles in tissue injury.

We conclude that the normal kidney has five discrete MPC subpopulations that exhibit distinct characteristics, functions, and ontogeny. These functions are likely to be important in homeostasis and immunological tolerance. The subpopulations share both macrophage and DC characteristics, suggesting that these functions are more related to context than separate lineage. Finally, both macrophage and DC characteristics, suggesting that these subpopulations share functions are more related to context than separate lineage. Finally, both macrophage and DC characteristics, suggesting that these subpopulations share functions are more related to context than separate lineage.

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Disclosures
J.S.D. holds patents related to leukocytes in kidney repair after injury and is cofounder of Muregen LLC. The other authors have no financial conflicts of interest.

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