Renal F4/80⁺CD11c⁺ Mononuclear Phagocytes Display Phenotypic and Functional Characteristics of Macrophages in Health and in Adriamycin Nephropathy

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ABSTRACT

Conventional markers of macrophages (Mφs) and dendritic cells (DCs) lack specificity and often overlap, leading to confusion and controversy regarding the precise function of these cells in kidney and other diseases. This study aimed to identify the phenotype and function of renal mononuclear phagocytes (rMPs) expressing key markers of both Mφs and DCs. F4/80⁺CD11c⁺ cells accounted for 45% of total rMPs in normal kidneys and in those from mice with Adriamycin nephropathy (AN). Despite expression of the DC marker CD11c, these double-positive rMPs displayed the features of Mφs, including Mφ-like morphology, high expression of CD68, CD204, and CD206, and high phagocytic ability but low antigen-presenting ability. F4/80⁺CD11c⁺ cells were found in the cortex but not in the medulla of the kidney. In AN, F4/80⁺CD11c⁺ cells displayed an M1 Mφ phenotype with high expression of inflammatory mediators and costimulatory factors. Adoptive transfer of F4/80⁺CD11c⁺ cells separated from diseased kidney aggravated renal injury in AN mice. Furthermore, adoptive transfer of common progenitors revealed that kidney F4/80⁺CD11c⁺ cells were derived predominantly from monocytes, but not from pre-DCs. In conclusion, renal F4/80⁺CD11c⁺ cells are a major subset of rMPs and display Mφ-like phenotypic and functional characteristics in health and in AN.


Macrophages (Mφs) and dendritic cells (DC) belong to the mononuclear phagocytic system and play distinct roles in homeostasis and immune responses in normal and diseased kidneys.¹⁻⁴ Mφ and DCs in mice and humans are classified according to their expression of various surface markers. F4/80 is the most specific marker for murine Mφ, whereas CD68 is the most commonly used marker for human Mφ.⁵,⁶ DCs were initially identified on the basis of MHC class II expression.⁷ CD11c is considered to be a specific marker for murine DCs and is thought to be the most reliable marker identifying DCs when combined with MHC-II.⁸ In humans, HLA-DR, DC-SIGN, BDCA-1, and BDCA-3 have all been used to define DC subsets.⁹⁻¹¹ Recent studies have shown that Mφs and DCs share similar properties, including surface markers and functional characteristics, in mice and humans.¹²⁻¹⁵ In the past, renal F4/80⁺ cells in mice and CD68⁺ cells in humans were uniformly defined.

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as Mφs without knowing whether they expressed DC markers. With the discovery of DCs, murine renal CD11c+ cells and human BDCA-1+ and DC-SIGN+ cells were considered to be renal DCs whether they expressed Mφ markers. In murine and human kidneys, a subset of mononuclear phagocytes expresses both Mφ and DC markers. Krüger et al. examined renal DCs in mice and classified kidney MHC-II+ CD11c+ cells as DCs, which also expressed the Mφ markers F4/80 and CD11b; however, this study did not further define MHC-II+CD11c+ cells according to whether they expressed F4/80 or not. Subsequently, other studies also regarded kidney F4/80+CD11c+ cells as being DCs and demonstrated that this so-called DC subset exhibited an inflammatory phenotype in renal ischemia reperfusion injury (IRI). However, the classification of F4/80+CD11c+ cells as DCs has been questioned by another study in which kidney F4/80+CD11c+ cells displayed Mφ-like morphology in chronic lupus nephritis, but the function of these cells was not characterized in vitro and in vivo. Similarly, a substantial number of cells expressing both Mφ and DC markers (CD68+BDCA-1+DC-SIGN+ cells) were found in normal and diseased human kidney, a population of renal mononuclear phagocytes (rMPs) that was also regarded as a DC subset. However, the functional characteristics of these rMPs were not assessed. By ignoring the presence of rMP that express both Mφ and DC markers, many studies designed to define the role of either Mφ or DCs are open to question and have confused our understanding of the role of Mφs and DCs in kidney disease. It is important to clarify whether these cells that express both Mφ and DC markers are predominantly Mφ or DCs, and whether these cells play a pro- or anti-inflammatory role in kidney disease.

This study aimed to characterize rMPs expressing both F4/80 and CD11c and to define their function in kidney disease. We demonstrated that the expression of MHC-II and CD11c was not sufficient to distinguish DCs from Mφs in kidney and that F4/80+CD11c+ cells displayed the features of Mφs in healthy and diseased kidney, namely Mφ-like morphology, expression of Mφ-specific surface markers and transcription profiles, Mφ function, and Mφ ontogeny. Thus, these experiments are the first to demonstrate that renal F4/80+CD11c+ mononuclear phagocytes act functionally as macrophages in health and in Adriamycin nephropathy (AN).

RESULTS

Characteristics of F4/80+CD11c+ Cells in Normal and Diseased Kidney

To characterize the phenotype of kidney Mφ and DC populations, we used a combination of CD45, MHC-II, lineage makers (lin; CD3, CD19, T cell receptor (TCR)-β, TCR-γδ, CD49b), CD11c, and F4/80. After pregating on CD45+ cells to exclude contaminating kidney epithelial cells, total rMPs, gated as MHC-II−lin− cells, could be subgrouped into three populations based on CD11c and F4/80 expression (Figure 1A). Most F4/80+ cells coexpressed MHC-II in kidney (Figure 1B). Two classic F4/80+CD11c+ Mφs and F4/80+CD11c+ DC populations represented 24.2%±3.3% and 9.7%±2.2% of total kidney CD45+ leukocytes, respectively. Interestingly, the kidney also contained a large population of cells (28.2%±2.8%) coexpressing F4/80 and intermediate CD11c (Figure 1C). For clarity, the three described kidney Mφ/DC populations will be referred to hereafter as subsets R1 (F4/80+CD11c+ DCs), R2 (F4/80+CD11c+ cells), and R3 (F4/80+CD11c− Mφs), as illustrated in Figure 1A. Wright–Giemsa staining revealed that cells of both R2 and R3 subsets exhibited classic morphologic characteristics of Mφs with numerous phagocytic vacuoles, eccentric nuclei, and lysosomal granules. By contrast, cells of the R1 subset were of smaller size and had more typical DC morphology, with less cytoplasm, fewer cell surface dendritic projections, and an absence of large intracytoplasmic vesicles (Figure 1D).

We next looked for F4/80+CD11c+ cells in other organs including lymphoid tissues and nonlymphoid tissues using the same gating strategy. In lymphoid tissues, classic F4/80+CD11c+MHC-II+ DCs and F4/80+CD11c−MHC-II− Mφ populations were identified in spleen, and F4/80+CD11c+MHC-II+ DCs and a small population of F4/80+CD11c−MHC-II− Mφs were identified in lymph nodes. In nonlymphoid tissues, F4/80+CD11c+MHC-II+ DCs, F4/80+CD11c−MHC-II− Mφs, and F4/80+CD11c+MHC-II+ Mφ populations were identified in liver and pancreas, and F4/80+CD11c+MHC-II+ DCs, F4/80+CD11c+MHC-II− cells, and F4/80+CD11c−MHC-II− Mφ populations were identified in the lung. Interestingly, the intestine contained the three populations (F4/80+CD11c+ Mφs, F4/80+CD11c+ DCs, and F4/80+CD11c+ cells), similar to those in kidney (Supplemental Figure 1). These data indicate that MHC-II and CD11c are specific DC markers in lymphoid organs and are not expressed on F4/80+ cells of lymphoid organs, whereas they are not specific DC markers in nonlymphoid organs and are expressed on F4/80+ cells of nonlymphoid organs. We also examined the population of F4/80+CD11c+ cells (R2) in diseased kidney using the AN model. The number of CD45+ leukocytes as a percentage of total kidney cells was significantly decreased at day 3 and gradually increased from week 1 to week 4 in AN mice (Figure 1E). The number of R2 cells as a percentage of total kidney cells changed in parallel with subsets R1 and R3, decreasing at day 3 and gradually increasing from week 1 to week 4 (Figure 1F). The number of R2 cells among kidney CD45+ leukocytes was significantly increased at week 2 and week 4, but this was not the case for subsets R2 and R3 (Figure 1G).

Furthermore, we sought to determine the physical location of rMP subsets within kidney. Immunofluorescence staining of frozen kidney sections revealed that F4/80+CD11c+ cells (R1, red) and F4/80+CD11c+ cells (R2, orange) were only distributed in the cortex of normal kidney, whereas F4/80+CD11c− cells (R3, green) were scattered throughout the whole kidney, including cortex and medulla (Figure 2A). The rMP subsets...
R1–R3 displayed the same distributions in AN kidney as in normal kidney, and the numbers of cells in each rMP subset were significantly increased in AN kidney compared with those in normal kidney (Figures 1F and 2B). Together, these results identified a unique population of cells expressing both Mφ and DC markers (F4/80⁺CD11c⁺ cells, R2) in normal and diseased kidney.

**Kidney F4/80⁺CD11c⁺ Cells Display Mφ-Like Phenotype and Function**

To further characterize the three populations of kidney rMP (R1–R3), an extensive flow cytometry phenotypic analysis of the DC and Mφ markers was performed. Subset R1 expressed the classic DC markers CD103 and CD205, but not PDCA-1 (pDC marker) or 33D1 (spleenic DC marker) in normal kidney (Figure 3A). The expression of CD103 and CD205 on subset R1 was significantly increased in AN kidney compared with that in normal kidney (Figure 3C). Both subsets R2 and R3 highly expressed Mφ markers CD11b, CD68, CD204 (scavenger receptor), and CD206 (mannose receptor) in normal kidney (Figure 3B). The expression of CD204 and CD206 on subsets R2 and R3 was significantly increased in AN kidney compared with that in normal kidney (Figure 3D).

We next investigated the functional properties of rMP. First, their capacity to internalize fluorescent microbeads was assessed, as a measure of their phagocytic activity. The subsets R2 and R3 phagocytosed more fluorescent microbeads than subset R1 under resting and LPS-activated conditions. In
addition, the phagocytic ability of subset R2 was stronger than that of subset R3 (Figure 4A). Furthermore, the unstimulated subsets R2 and R3 separated from AN kidney displayed stronger phagocytic activity than those from normal kidney (Figure 4B). Second, nitric oxide in supernatant of subsets R1 – R3 was examined. The subsets R2 and R3 stimulated with LPS or separated from AN kidney produced more nitric oxide than subset R1 (Figure 4, C and D). The subsets R2 and R3 separated from normal and AN kidney exhibited low T cell priming capacity, similar to splenic Mϕs, and lower than that of subsets R1 or splenic DCs in the antigen-specific T cell proliferation assay (Figure 4, E and F). The subset R1 separated from normal kidney exhibited higher T cell priming capacity than subsets R2 and R3 in the allogeneic T cell proliferation assay (Figure 4G). Furthermore, the subset R1 was uniquely able to upregulate CCR7 expression after 24-hour stimulation with LPS, consistent with their potential selective ability to migrate to kidney draining lymph nodes to prime T cells (Supplemental Figure 2). Together, these data indicate that the rMP subset R2 exhibited Mϕ-like phenotypic and functional characteristics in vitro. Furthermore, these findings underline the fact that CD11c integrin expression, even when combined with high levels of expression of MHC-II, is not a sufficient criterion to define DCs in normal and diseased kidney and identify MHC-II^+CD4^+CD11b^+CD68^+CD103^− subset R2 as a major Mϕ (rather than DC) subset in kidney.

Kidney F4/80^+CD11c^+ Cells Display an M1 Mϕ Phenotype in AN

To understand the role of F4/80^+CD11c^+ cells in diseased kidney, we performed phenotypic analysis by real-time PCR and flow cytometry in AN mice. The mRNA expression of inducible nitric-oxide synthase, STAT1, and IRF5 in subsets R2 and R3 separated from AN kidney was increased compared with those separated from normal kidney (Figure 5A). The subsets R2 and R3 separated from AN kidney produced much more IL-1β, IL-6, TNF-α, chemokine ligand 2 (CCL2), and CCL5 than those from normal kidney. The subset R1 only produced higher levels of IL-6 in AN mice. In addition, R2 cells separated from AN mice produced higher levels of IL-10 than from normal mice. There was no increased production of IL-12 and IFN-γ in these three populations in AN mice (Figure 5B). In addition, CD86 was highly expressed on subsets R2 and R3 in normal kidney, and the expression of CD80, CD86, and B7-H1 on subsets R1 – R3 was significantly increased in AN mice compared with that in normal mice (Supplemental Figure 3). These data indicate that F4/80^+CD11c^+ cells (subset R2) displayed an M1 Mϕ phenotype in AN kidney.

Kidney F4/80^+CD11c^+ Cells Separated from AN Mice Aggravate Renal Injury in AN Mice

We next investigated the in vivo function of F4/80^+CD11c^+ cells by an adoptive transfer study. Adoptive transfer of subsets R2 and R3 each significantly exacerbated renal dysfunction in AN mice, as shown by an increase in proteinuria and serum creatinine and a decrease in creatinine clearance at day 28, whereas the transfer of subset R1 had no effect on renal function in AN mice (Figure 6, A–C). Renal injury was characterized by glomerulosclerosis, tubular atrophy, and interstitial expansion in AN. Adoptive transfer of subsets R2 and R3 significantly aggravated renal injury, including glomerulosclerosis, tubular damage, and interstitial volume expansion in AN.
mice, whereas transfusion of subset R1 did not affect renal injury (Figure 6, D–G).

**Kidney F4/80⁺CD11c⁺ Cells Were Generated from Monocytes In Vivo**

To understand how the F4/80⁺CD11c⁺ cells develop in kidney, we examined transcripts involved in DC and Mφ development. First, we examined expression of growth factor receptors: Flt3 (the receptor for the DC growth factor, Flt3-L) and MCSF-R (the receptor for Mφ growth factor, M-CSF) in rMP subsets (R1–R3). Flt3 was highly expressed in subset R1, whereas the MCSF-R transcript was abundant in both subsets R2 and R3 (Figure 7A). Second, we compared the expression of transcription factors that are selectively expressed by rMP subsets (Figure 7, B and C). Basic leucine zipper transcription factor ATF-like 3, interferon regulatory factor 8, PU1, and inhibitor of DNA binding 2, which are required for CD8α⁺ DC and CD8α⁻ DC development, were selectively expressed in subset R1. V-Maf musculoaponeurotic fibrosarcoma oncogene family, protein B, avian musculoaponeurotic fibrosarcoma AS42 oncogene homolog, forkhead box P1, interferon regulatory factor 4, runt-related transcription factor 3,
CCAAT/enhancer binding protein-α, and CCAAT/enhancer binding protein-β, which are reported to be required for development and differentiation of monocyte/macrophages, were selectively expressed in both subsets R2 and R3 (Supplemental Figure 4). These data indicate that the R2 and R3 subsets expressed similar high levels of growth factor receptor and transcription factors, and may be derived from the same bone marrow progenitors.

Despite their phenotypic and functional differences, the major subsets of mononuclear phagocytes in lymphoid tissue and nonlymphoid tissue arise from common progenitors, Mφs and DC precursors (MDPs), preclassical dendritic cells (pre-cDCs), and monocytes. To determine the relative contribution of DC progenitors and monocytes to the development of rMP subsets, we adoptively transferred MDPs, pre-cDCs, and monocytes from CD45.1+ C57BL/6 mice into untreated congenic CD45.2+ C57BL/6 recipient mice. We then measured the presence of CD45.1+ donor-derived rMP subsets 1 week and 2 weeks after adoptive transfer. As expected, MDPs, which produce monocytes and DCs in bone marrow, gave rise to rMP subsets R1–R3 in normal kidney. The ratio of subsets R1–R3 derived from MDPs was comparable with that in normal kidney (Figures 1 and 7D). Pre-cDCs, which are restricted to producing classic dendritic cells (cDCs), only gave rise to subset R1 in normal kidney. Monocytes, which produce Mφs and some cDCs, mainly gave rise to subsets R2 and R3, and a small population of subset R1 in normal kidney (Figure 7D). These data indicate that kidney F4/80+CD11c+ cells (R2) and F4/80+CD11c2Mφs (R3) were mainly derived from monocytes, whereas kidney F4/80−CD11c+ DCs are heterogenous (derived from pre-cDCs and monocytes).

DISCUSSION

Mφs and DCs have been regarded as relatively discrete cell types with different functions. However, recent studies have found an increasing overlap in function and surface marker expression of Mφs and DCs. Indeed, distinguishing Mφs from DCs has largely relied on the use of specific cell surface markers. Traditionally, murine Mφs have been defined as F4/80+ cells and DCs as CD11c+MHC-II+ cells. However, a large proportion of rMPs in kidney coexpress both Mφ and DC markers and have been regarded variously as Mφ or DC subsets in different kidney diseases. For example, Mφs were demonstrated to mediate tissue injury and fibrosis or shown to have protective and reparative roles in renal IRI, unilateral ureteric obstruction, and lupus nephritis. These contradictory effects of Mφs could be due to functional changes of Mφs during the disease process. M1 Mφs play a predominant
pathogenic role during early stages of some kidney diseases. Subsequently, M2 M\(\text{ф}\)s contribute to wound healing and resolution of inflammation at later stages of kidney disease. Because of the existence of these F4/80\(^+\)CD11c\(^+\) cells, depletion of either M\(\phi\)s or DCs based on these markers is in fact nonspecific and likely contributes to the contradictory conclusions about M\(\phi\) function in previous reports. For example, in those studies, M\(\phi\)s were depleted using either liposomal clodronate, antibodies, or transgenic mice (CD11b-DTR mice), each of which would have depleted both F4/80\(^+\)CD11c\(^+\) cells and DCs.

Figure 5. Kidney F4/80\(^+\)CD11c\(^+\) cells display an M1 M\(\phi\) phenotype in AN mice. (A) Real-time PCR analysis of mRNA expression of transcription factors in FACS-sorted rMP subsets from normal and AN BALB/c mice (week 2). (B) Quantification of the cytokines and chemokines secreted by rMP subsets from normal and AN BALB/c mice after 24 hours of culture, using ELISA and cytometric bead array. Data represent the mean \(\pm\) SEM of at least three experiments with four mice each. \(*P<0.05; **P<0.01; ***P<0.001\) versus normal. iNOS, inducible nitric-oxide synthase; STAT1, signal transducer and activator of transcription 1; IRF5, interferon regulatory factor 5.
cells and F4/80⁺CD11c⁻ MΦs in kidney. Similarly, the use of CD11c⁻DTR mice, the most common DC depletion strategy in murine models of kidney disease, would deplete both kidney F4/80⁺CD11c⁺ cells and F4/80⁺CD11c⁺ DCs, and could explain contradictory conclusions about DC function. For example, DCs were shown to play a protective role in nephrotoxic nephritis and cisplatin nephrotoxicity but a pathogenic role in antiglomerular basement membrane nephritis, IRI, and unilateral ureteric obstruction in CD11c⁻DTR mice. Therefore, it is necessary to define the function of

Figure 6. Kidney F4/80⁺CD11c⁺ cells separated from AN mice aggravate renal injury in AN mice. (A) The rMP subsets (R1–R3) are separated from kidney at day 14 after Adriamycin injection, as described in the Concise Methods, and are adoptively transferred into BALB/c mice at day 5 after Adriamycin injection. Mice are euthanized on day 28. (B–D) Proteinuria, serum creatinine, and creatinine clearance are assessed in normal, AN+vehicle, AN+R1, AN+R2, and AN+R3 at day 28 after Adriamycin injection. (E) PAS-stained sections of renal cortices at day 28. (F–H) Kidney injury (glomerulosclerosis, damaged tubules, and interstitial volume) is assessed quantitatively. The values represent the mean±SEM of evaluations from each group (n=7 per group). *P<0.05; **P<0.01 versus AN+vehicle. Original magnification, ×200.
Figure 7. Kidney F4/80^CD11c^ cells are derived from monocytes in vivo. (A–C) Real-time PCR analysis of mRNA expression of growth factor receptors and transcription factors in freshly isolated rMP subsets from BALB/c mice. Data represent the mean±SEM of four experiments with 4–8 pooled kidneys each. *P<0.05 compared with R2 and R3; #P<0.05 versus R1. (D) MDPs, pre-DCs, and monocytes are purified from the BM of CD45.1^ mice, as described in the Concise Methods, and adoptively transferred into CD45.2^ congenic
rMPs coexpressing both Mφ and DC markers in order to clarify the confusion about the role of macrophages and DCs in previous studies. MHC-II and CD11c have been considered as reliable markers to identify DCs in most organs and tissues. However, this study shows that both Mφs and DCs in kidney are MHC-II positive and that a large proportion of Mφ are CD11c positive, indicating that neither MHC-II nor CD11c is a specific marker for kidney DCs, unless F4/80- cells have been excluded. This finding should be applied to separate DCs from Mφs in future studies of kidney diseases.

In this study, the phenotype and function of kidney F4/80+CD11c+ cells were examined in detail in normal and AN mice. Kidney F4/80+CD11c+ cells exhibited classic morphologic characteristics of Mφs, which is in line with a previous study in chronic lupus nephritis in which F4/80highCD11c+ cells contained multiple double-membrane phagocytic vacuoles and fewer dendrites. However, Kaissling et al. reported that MHC-II+ cells have DC-like morphology in rat kidney. The discrepancy between the studies by Kaissling et al. and our study is likely related to the use antibodies against different surface markers and the use of different animal species. Kidney F4/80+CD11c+ cells have been described in several previous studies. For example, approximately 70% of CD11c+ DCs expressed F4/80 in healthy kidney and around 50% of CX3CR1+ DCs expressed both F4/80 and CD11c in CX3CR1GFP/+ mice. A study by Kawakami et al. recently reported a group of kidney CD11bintCD11cintF4/80high cells that have features similar to the kidney F4/80+CD11c+ cells of our study, including high ability to phagocytose, production of high levels of IL-10, and expression of IL-10 in mice with AN is increased, kidney F4/80+CD11c+ cells predominantly expressed high levels of inflammatory mediators, including IL-1β, IL-6, TNF-α, CCL2, and CCL5, and promoted renal inflammation and injury.

In this study, kidney F4/80+CD11c+ cells were different from kidney F4/80-CD11c+ DCs in various aspects, including morphology, cell surface marker expression, cell function, and transcription factor expression, whereas they share the same distribution in kidney, being present mainly in cortex of kidney. In AN mice, kidney F4/80+CD11c+ cells produced large amounts of inflammatory mediators including IL-1β, IL-6, TNF-α, CCL2 and CCL5, whereas kidney F4/80-CD11c+ DCs did not. In interpreting this difference it must be acknowledged that a prolonged modulatory effect of Adriamycin on mononuclear phagocyte phenotype cannot be fully excluded. Although kidney F4/80+CD11c+ cells and F4/80+CD11c+ Mφs shared features characteristic of Mφs in normal and AN mice, they exhibited different distributions within kidney, suggesting that they may have position-specific functions in kidney diseases. Moreover, F4/80+CD11c+ cells had stronger phagocytic ability and produced more nitric oxide than did F4/80+CD11c− Mφs, suggesting a more potent defense or destructive role in kidney diseases. In addition, F4/80+CD11c+ cells produced high levels of IL-10 in AN mice, whereas F4/80+CD11c− Mφs did not, indicating that F4/80+CD11c+ cells may play an immune regulatory role in the AN model of CKD. Nevertheless, adoptive transfer of F4/80+CD11c+ cells or F4/80+CD11c− Mφs separated from AN kidney exacerbated renal injury in AN to the same degree, indicating a similar in vivo function in this model of CKD. The in vitro functional differences between these two subsets deserve further investigation.

Mononuclear phagocytes coexpressing F4/80 and CD11c have also been found in other organs. In intestine, mononuclear phagocytes that expressed F4/80 and CD11c were initially described as DCs. However, this classification has been challenged by other studies in which F4/80+CD11c+ cells in intestinal lamina propria (LP) displayed a Mφ-like vacuolar system, poor ability to prime T cell stimulation, and an inability to migrate to draining lymph nodes. On the basis of intensive phenotypic and functional analysis, the LP F4/80+CD11c+ cells were recently redefined as a Mφ subset rather than a DC subset. LP F4/80+CD11c+ Mφs produce a large amount of IL-10 and maintain immune tolerance through expansion of Foxp3+ regulatory T cells in noninflammatory conditions. Kidney F4/80+CD11c+ cells were also initially described as DCs. However, this classiﬁcation has been called into question by other studies in which F4/80+CD11c+ cells in kidney displayed intermediate capacity to stimulate T cells, which differs from the kidney F4/80+CD11c+ cells of our study that have a low T cell priming capacity. Lung alveolar macrophages were shown to express F4/80 and CD11c and displayed Mφ features in vivo. Adoptive transfer of alveolar macrophages did not restore Th2 cytokine secretion in CD11c+ cell depleted mice, indicating low T cell priming capability of alveolar macrophages in vivo. Therefore, lung alveolar F4/80+CD11c+ cells were identiﬁed as a Mφ subset. They mediated allergic lung inﬂammation through releasing inflammatory mediators including vascular endothelial growth factor, IL-1β, IL-10, IL-17, IL-22, IL-23, and TNF-α. Kidney F4/80+CD11c+ cells and lung alveolar macrophages exhibit Mφ features in vitro and a similar M1 phenotype under disease conditions. Interestingly, these cells were localized selectively to certain compartments within organs: Lung F4/80+CD11c+ alveolar macrophages were present only in alveolar space and kidney F4/80+CD11c+ Mφ in cortex, but not medulla of kidney.
The transcript analysis of rMP subsets in our study showed that F4/80+CD11c+ cells and F4/80+CD11c+ Mφs expressed similar high levels of Mφ-associated growth factor receptors and transcription factors, suggesting that kidney F4/80+CD11c+ cells belong to the Mφ lineage. A recent transcriptomic study showed that kidney CD11b+ cDCs clustered near cDCs and away from Mφs in principal-component analysis, suggesting that the kidney CD11b+ cDCs belonged to the DC lineage.60 The discrepancies between that study and ours could be explained by the fact that kidney CD11b+ cDCs would include both F4/80+CD11c+ cells and F4/80+CD11c+CD11b+ DCs. Moreover, the kidney F4/80+CD11c+ cells in our study selectively expressed CD64, Maf-b, c-Maf, CEBP-α, and CEBP-β, which were recently identified as core Mφ gene signatures in mouse tissue Mφs,61 suggesting that kidney F4/80+CD11c+ cells belong to the Mφ lineage.

Mφs and DCs are generated from different cell lineages within bone marrow. It was previously demonstrated that Mφs are only generated from monocytes, but DCs are generated predominantly from pre-cDCs and from monocytes.62–64 F4/80+CD11c+ Mφs in LP of intestine are generated from Ly-6c+ monocytes or Gr-1+ monocytes in noninflammatory conditions.47,51,65 However, the origin of CD103+ DCs and CD11b+ DCs has been assessed in kidney in which pre-DCs gave rise to CD103+ DCs and CD11b+ DCs, whereas monocytes produced only CD11b+ DCs.66 In this study, we demonstrated that F4/80+CD11c+ cells are generated from monocytes, but not from pre-DCs. Therefore, our lineage studies showed that both kidney F4/80+CD11c+ cells and F4/80+CD11c- Mφs are derived from the same source (monocytes), whereas kidney F4/80-CD11c+ DCs are heterogeneous and are derived from both pre-cDCs and monocytes.

Identification of rMP subsets in humans is more complicated than in mice. However, like F4/80+CD11c+ cells in mice, a substantial number of rMPs coexpress Mφ and DC markers—CD68+BDCA-1+ cells have been found in normal and diseased human kidney.18,19 Studies before the discovery of DCs regarded all CD68+ cells in kidney as being Mφs. The human rMP subset coexpressing CD68 and DCs markers are considered as kidney DCs. However, the phenotype and functions of these cells have not been clarified. From this study, we speculate that the counterpart of murine F4/80+CD11c+ cells in humans, including CD68+BDCA-1+ cells, are likely to act like Mφs instead of DCs in healthy and diseased kidney. Further studies are necessary to verify the nature of human rMP subsets coexpressing both Mφ and DC markers.

In conclusion, this study has characterized a unique subset of murine rMPs coexpressing F4/80 and CD11c as Mφ-like cells. F4/80+CD11c+ cells demonstrated an effect (M1) Mφ phenotype in AN mice, and aggravated renal injury when adoptive transferred into AN mice. Neither MHC-II nor CD11c is a specific marker for identifying kidney DCs. Definition of Mφs and DCs should be based not only on cell surface markers, but also on their functions, transcription profiles, and lineage. This study may help explain some uncertainties about the role of Mφs and DCs in kidney diseases, and contributes to the definition of pathogenetic and protective functions of subsets of rMPs.

CONCISE METHODS

Mice
BALB/c, C57BL/6 (CD45.2+), and congenic CD45.1+ C57BL/6 mice were purchased from the Animal Resources Centre (Perth, Australia). Transgenic D011.10 BALB/c mice were bred at the Department of Animal Care at Westmead Hospital. Experiments were conducted in accordance with the protocols approved by the Animal Ethics Committee of Sydney West Area Health Service. Dose-finding studies defined an optimal dose of 10.4 mg/kg body wt of Adriamycin (doxorubicin; Pharmacia & Upjohn Pty Ltd, Australia) for BALB/c mice. Adriamycin was injected once via the tail vein of each mouse.

Cell Suspension Preparation
Kidney, liver, lung, pancreas, and intestine were perfused with saline before removal and digested with collagenase and DNase as previously described.66 Kidney, liver, lung, and pancreas were cut into 1- to 2-mm2 pieces and placed in DMEM containing 1 mg/ml collagenase IV (Sigma-Aldrich) and 100 μg/ml DNase I (Roche) for 40 minutes at 37°C with intermittent agitation. Small and large intestines were removed and carefully cleaned of their mesentery, Peyers patches were excised (small intestine), and intestines were opened longitudinally and fecal contents were washed off. Intestines then were cut into 0.5-cm pieces, and subjected to two sequential 20-minute incubations in HBSS with 5% FBS and 2 mM EDTA at 37°C with agitation to remove epithelial cells. The remaining intestinal tissue was washed and cut into smaller 2-mm2 pieces and digested in HBSS with 5% FCS, 1.5 mg/ml collagenase VIII (Sigma-Aldrich), 100 μg/ml DNase I (Roche), and 10 mM EDTA for 30 minutes at 37°C with agitation. The digested cell suspension was then passed through a 40-μm cell strainer. Mononuclear cells from organs were separated using 1.077 g/ml NycoPrep gradient (Axis-Shield, Oslo, Norway). Spleen and LNs were isolated, minced, and digested for 30 minutes at 37°C in RPMI 1640 containing 1 mg/ml collagenase D (Roche) and 100 μg/ml DNase I (Roche). The digested cell suspension was then passed through a 40-μm cell strainer.

Flow Cytometry and Cell Sorting
For FACS analysis or sorting of different organ samples, single-cell suspensions were stained with Fc block/anti-CD16/32 (2.4G2) and antibodies to CD45.2 (104), MHC-II (M5/114), CD11c (N418), and F4/80 (BM8), as well as antibodies to natural killer (NK) cell, B cell, and T cell lineages (referred hereafter as lin): CD49b (DX5), CD19 (1D3), CD3 (145-2C11), TCR-β (H57-597), and TCR-γδ (eBioGL3), all from eBioscience. When FACS sorting was performed on the digested kidney cell suspension, cells were pregated on hematopoietic cells using anti-CD45.2 antibody, and lineages (CD49b/CD19/CD3/TCR-β/TCR-γδ) were then used to exclude NK cells and lymphocytes and 4,6-diamidino-2-phenylindole was used to exclude dead cells. F4/80+CD11c+ (R1), F4/80-CD11c+ (R2) and F4/80-CD11c- were included.
(R3) cells were sorted using a FACSArria machine (BD). For the FACS sorting of splenic DCs and MΦs, lin– (CD49b/Cd19/Cd11c/Cd123/Tcr-β/Tcr-γδ) cells were first excluded. Splenic DCs were then sorted as MHC-II+CD11c+ cells, whereas splenic MΦs were sorted as F4/80+ CD11c− cells (Supplemental Figure 1). After sorting, cells were used for phenotypic and functional assays.

Other antibodies used in this study include CD45.1 (A20), CD1103 (2E7), CD205 (205yekta), PDCA-1 (2F4), CD11b (M1/70), CD68 (EA-11), CD204 (2F8), CD206 (MR3D3), CD40 (1C10), CD80 (16-10A1), CD86 (GL1), B7-H1 (MIH5), CCR5 (7A4), CCR7 (4B12), NK1.1 (PK136), Ter119 (Ter119), B220 (RA3-682), Gr-1 (RB6-8C5), CD115 (AF598), c-kit (B8), Flt3 (A2F10), and SIRPα (P84) as well as corresponding isotype controls, all purchased from eBioscience or Biolegend. Cells were analyzed on an LSR II flow cytometer (BD).

Imaging
To analyze cell morphology, sorted rMP subsets were spun onto glass slides using a CytoSpin centrifuge, allowed to air dry for 1 hour, fixed in 100% methanol, and then stained using Wright–Giemsa (Sigma-Aldrich) stain.

Immunofluorescence Staining of Kidney Tissue Sections
Kidney sections from normal and AN mice were double stained with hamster anti-mouse CD11c (N418; 1:100) and rat anti-mouse F4/80 (BM4; 1:200) antibodies, and were then incubated with AF546 goat anti-hamster IgG (1:500) and AF488 goat anti-rat IgG (1:500). Isotype control IgGs to these rat, hamster, and goat antibodies were included in immunofluorescence staining. Tissue sections were analyzed by an inverted fluorescence microscopy (BX50; Olympus). Consecutive photos from kidney cortex to kidney medulla were taken in each section.

Phagocytic Activity
FACS-sorted rMP subsets (R1–R3) of normal mice were culture with or without LPS (100 ng/ml; Sigma-Aldrich) in complete RPMI 1640 medium (10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml M-CSF, 10 ng/ml GM-CSF) for 24 hours. Cytokine production was analyzed by sandwich ELISA (IL-1β and CCL2, R&D Systems) or cytometric bead array (Mouse inflammation CBA kits; BD Biosciences). ELISA was performed according to the manufacturer’s protocol. For the CBA assay, 50 μl of supernatant was incubated with a mixture of beads coated with capture antibodies for IL−6, IL−10, and TNF−α, or IL−12p70. The addition of the phycoerythrin (PE)-conjugated detection antibodies forms a sandwich complex. After 2 hours of incubation and one wash, samples were analyzed by flow cytometry. Standard curves were generated from analysis of titrated cytokine standards using BD CBA analysis software.

Cytokine Assays
FACS-sorted rMP subsets (R1–R3) of normal and AN mice were plated at 2×10^5 cells/well in 24-well plates cultured in complete RPMI 1640 medium for 24 hours. Cytokine production was analyzed by sandwich ELISA (IL-1β and CCL2 kits; R&D Systems) or cytometric bead array (Mouse inflammation CBA kits; BD Biosciences). ELISA was performed according to the manufacturer’s protocol. For the CBA assay, 50 μl of supernatant was incubated with a mixture of beads coated with capture antibodies for IL−6, IL−10, CCL2, IFN−γ, TNF−α, or IL−12p70. The addition of the phycoerythrin (PE)-conjugated detection antibodies forms a sandwich complex. After 2 hours of incubation and one wash, samples were analyzed by flow cytometry. Standard curves were generated from analysis of titrated cytokine standards using BD CBA analysis software.

Adoptive Transfer of rMP Subsets into AN Mice
To examine in vivo functions of rMP subsets in AN mice, 1×10^6 rMP subsets (R1–R3) isolated from AN mice (week 2) were transferred into treated BALB/c mice by a single tail-vein injection at day 5 after Adriamycin. Mice were divided into five groups: normal, AN with saline, and AN with R1, R2, or R3 treatment. Mice were euthanized at weeks 4 after Adriamycin injection. Blood, urine, kidney draining lymph nodes, and kidneys were harvested for analysis. All urine and blood specimens were analyzed by the Institute of Clinical Pathology and Medical Research (Westmead Hospital), using a BM/Hitachi 747 analyzer (Tokyo, Japan).

Adaptive Transfer of MDPs, Pre-DCs, and Monocytes
Bone marrow (BM) cells were harvested from the femora and tibiae of CD45.1 C57BL/6 mice. For adaptive transfer experiment, MDPs and pre-DCs were pre-enriched by biotin anti-Flt3 antibody, followed by antibiotin microbeads (Miltenyi Biotec), and the positive fraction from columns was eluted. MDPs were sorted as lin− (CD3, CD19, NK1.1, Ter119, and B220), CD11b−/CD11c−/CD115+/Flt3+/c-kit+ cells. Pre-DCs were sorted as lin−CD11b+/HMC-II−/CD11c+/Flt3+/c-kit−/SIRPa+ cells. BM monocytes were pre-enriched by anti-CD11b microbeads (Miltenyi

3H Thymidine Incorporation Assay
In the antigen-specific assay, FACS-sorted rMP subsets (R1–R3) of normal and AN BALB/c mice and splenic DCs/MΦ subsets of normal BALB/c mice were incubated with 10 μg/ml OVA_{323-339} peptide (Mimotopes, Australia) for 2 hours at 37°C, then cocultured with OVA specific CD4+ T cells (2×10^5 per well) isolated from DO11.10 mouse spleen at different ratios (1:80, 1:40, 1:20, 1:10) in U-bottom 96-well plates for 4 days. In mixed lymphocyte reactions, rMP subsets (R1–R3) and splenic DCs/MΦ subsets were sorted from BALB/c mice and cocultured with magnetic cell sorting-enriched CD4+ T cells (2×10^5 per well) from C57BL6 mouse spleens at the above ratios for 4 days. In all assays, 3H-thymidine (1 μCi/well) was added for the last 16 hours of culture, and cells were harvested using a Packard Filtermate Harvester 96 and counted by Microbeta counter (PerkinElmer, Beaconsfield, UK).

Nitric Oxide Production
Nitric oxide production by rMP subsets was determined by the measurement of the nitrite concentration with the Griess assay.8 Briefly, sorted rMP subsets (R1–R3) of normal mouse were cultured with or without LPS (100 ng/ml; Sigma-Aldrich) in complete RPMI 1640 medium for 24 hours. In controls for nonspecific dextran attachment, cells were added to 0.02% azide to stop energy-dependent cellular functions. Cell viability was >95%. To determine phagocytic activity, the uptake of FITC-labeled dextran was detected by flow cytometry.

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Quantitative RT-PCR
Total RNA were isolated from rMP subsets (R1–R3) of normal and AN mice by an RNeasy Mini Kit (Qiagen, Australia), and were then reverse-transcribed with the First Strand cDNA Synthesis Kit (Fermantas, Australia). Real-time PCR was performed on the Rotorgene-6000 Real-Time Thermo cycler (Corbett Research, Australia) using the SYBR Master Mix (Invitrogen). The analysis method was as previously described7 and the PCR primer sequences are presented in Supplemental Table I.

Histology and Immunofluorescence Staining
Coronal sections of kidney tissue were stained with periodic acid–Schiff (PAS). Glomerulosclerosis, tubular damage and interstitial volume were evaluated using previously described methods.8 The degree of glomerulosclerosis was measured using a quantitative method. The outline of the glomerular capillary tuft was traced, and the computed area covered by PAS-positive staining in the same glomerulus was then determined. The percentage of glomerulosclerosis for each glomerulus was calculated by dividing the total PAS-positive area by the total glomerular area. The mean value of 20 randomly selected glomeruli was determined for each section. Damaged tubules were identified by the presence of diffuse tubular dilation, intraluminal casts, and/or tubular cell vacuolization and detachment in cortex and medulla in 10–15 high-power fields (×200 magnification) per PAS-stained section, in a blinded fashion. The number of damaged tubules was divided by the number of the total tubules in the same field to obtain the percentage of damaged tubules. The degree of interstitial expansion was determined by quantification of the relative interstitial volume in 10–15 high-power fields (×200 magnification) per PAS-stained section. The percentage of relative interstitial volume was calculated by dividing the total interstitial area by the total area. To avoid selection bias, the areas to be viewed for quantitative analysis were anatomically identical for each section and positioned before microscopic visualization.

Statistical Analyses
Renal functional data (serum creatinine, creatinine clearance, and proteinuria) were log-transformed before analysis to stabilize the variance. Statistical tests included unpaired, two-tailed t tests using Welch’s correction for unequal variances and one-way ANOVA with Tukey’s multiple comparison tests. Statistical analyses were done using Prism software (version 5; GraphPad). Results are expressed as the mean±SEM. P<0.05 was considered statistically significant.

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REFERENCES

DISCLOSURES
None.


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