Differential Ly6C Expression after Renal Ischemia-Reperfusion Identifies Unique Macrophage Populations

Meghan Clements,* Michael Gershenovich,* Christopher Chaber,* Juanita Campos-Rivera,† Pan Du,‡ Mindy Zhang,‡ Steve Ledbetter,* and Anna Zuk*

*Tissue Protection and Repair Unit, Renal Science, †Flow Cytometry Core Facility, and ‡Functional Genomics, Genzyme R&D Center, Genzyme, a Sanofi Company, Framingham, Massachusetts

ABSTRACT

Macrophages are a heterogeneous cell type implicated in injury, repair, and fibrosis after AKI, but the macrophage population associated with each phase is unclear. In this study, we used a renal bilateral ischemia-reperfusion injury mouse model to identify unique monocyte/macrophage populations by differential expression of Ly6C in CD11b+ cells and to define the function of these cells in the pathophysiology of disease on the basis of microarray gene signatures and reduction strategies. Macrophage populations were isolated from kidney homogenates by fluorescence-activated cell sorting for whole genome microarray analysis. The CD11b+/Ly6C\textsuperscript{high} population associated with the onset of renal injury and increase in proinflammatory cytokines, whereas the CD11b+/Ly6C\textsuperscript{intermediate} population peaked during kidney repair. The CD11b+/Ly6C\textsuperscript{low} population emerged with developing renal fibrosis. Principal component and hierarchical cluster analyses identified gene signatures unique to each population. The CD11b+/Ly6C\textsuperscript{intermediate} population had a distinct phenotype of wound healing, confirmed by results of studies inhibiting the macrophage colony-stimulating factor 1 receptor, whereas the CD11b+/Ly6C\textsuperscript{low} population had a profibrotic phenotype. All populations, including the CD11b+/Ly6C\textsuperscript{intermediate} population, carried differential inflammatory signatures. The expression of M2-specific markers was detected in both the CD11b+/Ly6C\textsuperscript{intermediate} and CD11b+/Ly6C\textsuperscript{low} populations, suggesting these in vivo populations do not fit into the traditional classifications defined by in vitro systems. Results of this study in a renal ischemia-reperfusion injury model allow phenotype and function to be assigned to CD11b+/Ly6C\textsuperscript{+} monocyte/macrophage populations in the pathophysiology of disease after AKI.


Renal ischemia-reperfusion injury (IRI) is one of the main clinical causes of AKI,\textsuperscript{1,2} which is defined as an abrupt reduction in kidney function. Repair of the damaged tubular epithelium will result in full recovery in a subset of patients\textsuperscript{3,4}; however, numerous recent clinical studies have identified associations between AKI, CKD, and progression to ESRD.\textsuperscript{5–8} Maladaptive repair leads to permanent structural and functional changes which culminate in tubulointerstitial fibrosis and chronic inflammation, central to CKD.\textsuperscript{1,9–11} Macrophages have been implicated in the injury, repair, and fibrosis associated with various renal diseases including AKI.\textsuperscript{12,13} In fact, CKD patients with high levels of interstitial macrophage infiltration have the poorest renal outcome.\textsuperscript{14}

Macrophages are a heterogeneous cell population that can switch from one phenotype to another in response to extracellular cues.\textsuperscript{15} In vitro studies have defined the classically activated macrophages as proinflammatory, M1 macrophages, whereas the

Received November 25, 2014. Accepted March 27, 2015.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Anna Zuk, Tissue Protection and Repair Unit, Renal Science, Genzyme R&D Center, Genzyme, a Sanofi Company, 49 New York Avenue, Framingham, MA 01701. Email: drazukphd@yahoo.com

Copyright © 2016 by the American Society of Nephrology
alternatively activated, M2 macrophages typically modulate the immune response to promote repair and fibrosis.\textsuperscript{15,16} The M2 classification has been further subdivided into $M_2_{\text{reg}}$,\textsuperscript{13,17} However, it is unclear how these populations defined in the \textit{in vitro} setting with limited cytokine exposure will translate \textit{in vivo} with more diverse cytokine signaling. Therefore, we have focused our efforts on defining macrophage populations based on their function during the pathophysiology of disease.\textsuperscript{18}

In this study, we use a murine model of renal IRI characterized by three different phases postreperfusion: injury, repair, and fibrosis, to identify the macrophage population associated with each phase. The populations are distinguished by differential Ly6C expression, which has been widely used to identify functionally discrete monocyte/macrophage populations.\textsuperscript{18–25} To establish their role in the pathophysiology of AKI, a small molecule c-fms inhibitor was used to inhibit macrophage proliferation and activation at different stages of disease. Furthermore, the newly identified populations of monocytes/macrophages were isolated and sorted from the kidney for microarray analysis. Our study is the first to show in the renal IRI model that each of the three populations carries a unique gene signature that provides insight into their functional role in the pathophysiology of acute and chronic disease following AKI.

RESULTS

\textbf{In Vivo Model of AKI Shows Distinct Phases of Injury, Repair, and Developing Fibrosis}

Samples were harvested for up to 35 days postreperfusion to define the timeframe of the various phases of the AKI model (Figure 1A). Plasma creatinine levels show that the loss of renal function is greatest 1 day postreperfusion, returning to baseline by 7 days (Figure 1B). A proinflammatory burst is measured in the kidney at 3 hours postreperfusion with peaks in mRNA expression of IL-6 and CXCL1 (Figure 1C). The return of plasma creatinine to normal levels is due to the surviving tubular epithelium dedifferentiating, proliferating and repolarizing to restore tubular structure and function.\textsuperscript{4} Repair of the tubular epithelium is measured by proliferating cell nuclear antigen (PCNA) staining and vimentin mRNA expression with peaks in proliferation and dedifferentiation, respectively, quantitated 3 days postreperfusion (Figure 1D). Significant increases in extracellular matrix genes, fibronec tin, collagen $\alpha_1$, and $\alpha$-actin as well as the profibrotic gene, TGF-$\beta_1$, are measured between 3 and 10 days postreperfusion (Figure 1E and data not shown). This translates into histologic evidence of tubulointerstitial fibrosis measured by picrosirius red at 14 and 35 days (Figure 1F), demonstrating the long-term effects of renal IRI. Thus, based on the time points examined in this model, injury predominates in the acute phase for 1 day postreperfusion followed by repair that peaks at 3 days postreperfusion and declines by day 7 postreperfusion in agreement with published studies.\textsuperscript{10,26} Importantly, our study highlights that fibrosis develops from 7 to 35 days postreperfusion following bilateral renal IRI.

Changes in Macrophage Ly6C expression and Function are Measured During the Different Phases Following Renal IRI

Because differential Ly6C expression identifies functionally discrete monocyte/macrophage populations in various tissues,\textsuperscript{18–25} flow cytometry analysis of the cell surface marker Ly6C was carried out to identify distinct monocyte/macrophage subsets in kidney homogenates following renal IRI. Populations of monocytes/macrophages are defined as CD11b$^+$ and Ly6C$^{\text{high}}$, Ly6C$^{\text{intermediate (int)}}$, or Ly6C$^{\text{low}}$ (Figure 2A). All three populations are present in the normal kidney at low levels. Correlating with the proinflammatory burst at 3 hours postreperfusion (Figure 1C), elevated numbers of the Ly6C$^{\text{high}}$ population are measured, which are F4/80$^{\text{low}}$ (Figure 2, A+inset, C). The Ly6C$^{\text{int}}$ population appears at 3 hours postreperfusion and persists as the dominant population at 3 days with varying levels of F4/80 expression (Figure 2, A+inset, C), PCNA staining and vimentin expression peak at this time (Figure 1D), suggesting this population may be involved in repair. Finally, the Ly6C$^{\text{low}}$ population emerges at 3 days postreperfusion and peaks at 10 days with F4/80$^{\text{high}}$ expression (Figure 2, A+inset, C). Elevations in extracellular matrix and profibrotic genes are measured (Figure 1E), suggesting this population may play a role in the fibrosis that develops in this \textit{in vivo} model.

Further, flow-cytometric analysis of F4/80 expression in CD11b$^+$ cells indicates that as early as 3 hours postreperfusion and extending to 3 days, the majority of cells are F4/80$^{\text{low}}$ (Figure 2, B and D). However, 10 days postreperfusion, the number of F4/80$^{\text{high}}$ cells has peaked and persists at 35 days (Figure 2, B and D), suggesting this population represents mature macrophages.\textsuperscript{27}

Moreover, inhibition of the c-fms receptor which mediates proliferation, differentiation and survival of monocytes/macrophages\textsuperscript{28} when bound to colony-stimulating factor demonstrates that inhibition of monocytes/macrophages prior to IRI (Figure 3A) dampens the proinflammatory milieu leading to renoprotection. An approximately 50% decrease is measured 2 hours postreperfusion in kidney mRNA expression of IL-6 (Figure 3F) and CXCL1 (Figure 3G), with significant reductions in plasma creatinine (Figure 3A) and neutrophil gelatinase-associated lipocalin (NGAL; data not shown), kidney injury molecule-1 (KIM-1; Figure 3E), and neutrophil gelatinase-associated lipocalin (NGAL; data not shown) are measured and a decrease in kidney CD11b$^+/$/F4/80 cells (Figure 3B) and circulating monocytes (Figure 3C) persists.

However, when the c-fms inhibitor is administered 1–3 days postreperfusion (Figure 4A) when tubular epithelial proliferation and vimentin expression are at peak levels (Figure 1D), there is a significant decline in kidney repair. Cellular proliferation measured by PCNA staining of the tubular epithelium in the cortex and outer medulla is significantly decreased (Figure 4D) concomitant with a significant increase in cell death measured by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL; Figure 4E). There are trends of increased KIM-1 and NGAL mRNA.
Figure 1. Renal IRI model shows distinct phases of injury, repair, and developing fibrosis. (A) Schematic representation of time course experiments in which C57/BL6 mice underwent bilateral ischemia followed by sacrifice at different reperfusion times. (B) Plasma creatinine indicates peak loss in renal function at 1 day postreperfusion with a return to baseline levels by 7 days. *P<0.05 indicates a significant difference from the sham. Sham plasma collected at 24 hours postreperfusion. **P<0.05 indicates a significant difference from all other groups. (C) RT-PCR of whole-kidney lysates indicates an inflammatory burst at 3 hours postreperfusion measured by peak levels of IL-6 and CXCL1. *P≤0.05 indicates a significant difference from normal and 35 days. **P≤0.05 indicates a significant difference from all other groups. (D) PCNA staining in the outer medulla and vimentin mRNA expression show peak proliferation and de-differentiation, respectively, at 3 days postreperfusion during repair of the tubular epithelium when kidney function returns. *P≤0.05 indicates a significant difference from 35 days and 1 day. **P≤0.05 indicates a significant difference from all other groups. (E) RT-PCR of whole-kidney lysates shows significant elevations from 3 to 10 days postreperfusion of fibrosis associated genes, fibronectin and TGF-β1, suggesting developing fibrosis *P≤0.05 indicates a significant difference from normal. (F) Picrosirius red staining of kidney sections at 14 and 35 days postreperfusion highlights fibrosis in the outer medulla in contrast to normal kidney which shows minimal staining. Scale bars, 100 μm. n=4 for all groups except 10 days which has n=6.

expression (P=0.19 and 0.10, respectively; data not shown) yet no difference in plasma creatinine is measured (data not shown). Total kidney CD11b+/F4/80+ cells (Figure 4B) are significantly decreased with c-fms inhibitor treatment. Importantly, further analysis of CD11b+/Ly6C+ cells demonstrates that the Ly6Cint population is significantly reduced by approximately 50% (P<0.05), whereas the Ly6Chigh and Ly6Clow populations remain equivalent (Figure 4C). Thus, the cell proliferation and death data jointly suggest a role of the Ly6Cint population in kidney repair.

Genomic Analysis Identifies a Unique Gene Signature for Each Macrophage Population

As there are conflicting reports on the role of macrophages in fibrosis based on studies with liposome clodronate29 and the c-fms inhibitor,30 a genomic approach was taken. Single-cell suspensions of kidney homogenates were flow-sorted based on CD11b/Ly6C expression to isolate each population followed by whole genome array. Based on hierarchical cluster analysis, a unique gene signature is identified for each population (Figure 5A). Principal component analysis confirms the differential gene expression in each population (Figure 5B).

A number of differentially expressed genes highlight the distinct phenotypes of the populations. The CD11b+/Ly6Chigh population carries a gene signature associated with the monocyte/macrophage immune response and monocyte/macrophage differentiation (Figure 6D). In contrast, the CD11b+/Ly6Cint population expresses numerous genes associated with wound healing (Figure 6A) and the CD11b+/Ly6Clow population expresses genes associated with fibrosis (Figure 6A). Interestingly, both macrophage populations express a variety of chemokines, membrane receptors, and inflammation-associated genes, suggesting both carry an inflammatory signature (Figure 6B); however, complement-associated genes are exclusively expressed by the Ly6Clow population (Figure 6A). The expression of M2-specific macrophage markers such as Arg1, CD74, Chi3l3, and Mrc1 suggest that both populations may be considered M2 macrophages; however, only Chi3l3 and Mrc1 would be useful to distinguish between them (Figure 6A). A subset of genes was confirmed by quantitative RT-PCR (qRT-PCR) and the results verify the accuracy of the microarray analysis (Figure 6C).

The top 15 cell processes associated with the genes uniquely upregulated in the Ly6Cint population were identified using Pathway Studio software. The majority of the cell processes...
Figure 2. Changes in Ly6C expression are measured in the kidney following IRI. (A) Flow-cytometric analysis of CD11b^+ cells identifies three distinct populations based on Ly6C^high, int or low, which vary in prevalence postreperfusion. Gating of the CD11b^+ /Ly6C^high population at 3 hours postreperfusion shows that these cells are also F4/80^low (inset), whereas the CD11b^+ /Ly6C^int population at 24 hours is primarily F4/80^low with some cells F4/80^high (inset). Ten days post reperfusion the CD11b^+ /Ly6C^low population is only F4/80^high (inset). (B) Flow-cytometric analysis of CD11b^+ /F4/80^high or low cells indicates changes in F4/80 over time. The CD11b^+ /F4/80^low population is the major population from 3 hours to 1 day postreperfusion, whereas the CD11b^+ /F4/80^high population predominates by 10 days. (C) Quantitation of total cell number indicates the Ly6C^high population is present at 3 hours postreperfusion during the inflammatory burst, the Ly6C^int population is dominant during the repair phase, while the Ly6C^low population emerges during the fibrotic phase. *P<0.05 indicates a significant difference from all other time points of respective Ly6C population. #P<0.05 indicates a significant difference from all other time points of respective Ly6C population except 10 days. ^P<0.05 indicates a significant difference from all other time points of respective Ly6C population except 3 hours and 35 days. ¶P<0.05 indicates a significant difference of respective Ly6C population compared with 10 days, 35 days, and normal. (D) Quantitation of total cell number indicates that CD11b^+ /F4/80^high cells increase over time, peaking at 10 days, whereas CD11b^+ /F4/80^low cells peak at
identifiers associated with inflammation and wound healing, including: immune response, innate immune response, inflammatory response, cell differentiation, tissue remodeling, phagocytosis, and vascularization (Table 1). Importantly, these data further support our hypothesis that the Ly6C<sup>int</sup> population plays a role in repair following AKI. Querying published microarray data sets using NextBio identified statistically significant strong correlations between the genes upregulated in the Ly6C<sup>int</sup> population and several studies of macrophages activated in culture systems with various stimuli, indicating that the Ly6C<sup>int</sup> population represents activated macrophages (Table 2).

Statistically significant, strong correlations also were identified between the genes upregulated in the Ly6C<sup>low</sup> population and several studies analyzing fibrotic tissues, including mouse lung tissue treated with bleomycin to induce fibrosis, and cirrhotic liver and kidney biopsies from human patients with interstitial fibrosis at 1 year post-transplant (Table 3). Importantly, these data support our hypothesis that the Ly6C<sup>low</sup> population is involved with the fibrosis that develops following renal IRI.

**DISCUSSION**

Macrophage infiltration into the kidney correlates with decline in kidney function of patients with CKD. These data suggest that macrophages play a critical role in the progression of kidney disease; however, the mechanism remains unclear. Our study is the first to phenotype and define a functional role of monocytes/macrophages based on differential Ly6C expression over time in the pathophysiology of renal IRI. Three unique subsets of monocytes/macrophages that correlate with injury, repair, or fibrosis were identified. Whole-genome analysis of the three different subsets identified a distinct gene signature for each that provided insight into the role of the
Ly6<sup>C</sup><sub>high</sub> in injury, the Ly6<sup>C</sup><sub>int</sub> population in repair, and the Ly6<sup>C</sup><sub>low</sub> population in fibrosis. It also highlighted that these populations do not fit into the canonical M1/M2 classifications defined by <em>in vitro</em> assays. Finally, these data provide insight into mechanisms by which monocytes/macrophages contribute to acute and chronic disease following renal IRI.

Differential Ly6C expression has been widely used to identify functionally discrete monocyte/macrophage populations. In a murine model of kidney fibrosis by unilateral ureteral obstruction (UUO), three different populations of macrophages were identified by differential Ly6C expression. Our data confirm and extend these findings. Similarly, we show that one of the first populations to appear in the renal IRI model is the CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>high</sub> population, which correlates with the proinflammatory phase characteristic of this model and which carries an inflammatory signature unique from the CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>int</sub> and CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>low</sub> populations. However, as the renal IRI model is a reversible model that undergoes repair, our c-fms inhibition study, which was confirmed by the microarray data, demonstrates the CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>int</sub> population mediates kidney recovery. Duffield also identified a CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>int</sub> population, but based on mRNA expression of a subset of monocyte/macrophage genes in the UUO model, characterized this population in transition from the proinflammatory CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>high</sub> to profibrotic CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>low</sub>. In contrast, our microarray data identify a unique gene signature of wound healing for the CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>int</sub> population, which is distinct from that of the Ly6<sup>C</sup><sub>high</sub> and Ly6<sup>C</sup><sub>low</sub> populations. Thus, in aggregate, the data suggest that the functional definition and phenotype of the macrophage populations reflects the pathophysiology of the animal model from which they are isolated. Thus, it is not surprising that the CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>int</sub> population in the UUO model which does not have a repair phase carries a gene signature reflective of a transition population, in contrast to the CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>int</sub> population in the renal IRI model which undergoes recovery.

Moreover, similar to Duffield’s findings in the UUO model, our microarray studies in the renal IRI model implicate the CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>low</sub> population as profibrotic. We identified similar (Igf1) yet novel genes (Parp1, TRPM7, SPARC, ROCK1), confirming a role in kidney fibrosis of the CD11b<sup>+</sup>/Ly6<sup>C</sup><sub>low</sub> population. However, in other organs, such as the liver in a model of reversible fibrosis, the Ly6<sup>C</sup><sub>low</sub> population is involved in the resolution of fibrosis while the Ly6<sup>C</sup><sub>high</sub> population drives fibrosis. In a model of myocardial infarction, the Ly6<sup>C</sup><sub>high</sub> population scavenged necrotic debris while the Ly6<sup>C</sup><sub>low</sub> population was implicated in reparative processes. Thus, the phenotype of a particular Ly6C population may differ based on organ and pathophysiology of disease. Therefore, as suggested by Murray et al., studies should focus on the function of the macrophage rather than definition by cell surface marker expression.

Additional rationale to focus on macrophage function comes from our data in which we show that the Ly6<sup>C</sup><sub>int</sub> and Ly6<sup>C</sup><sub>low</sub> populations do not fit into the canonical M1/M2 classifications defined by <em>in vitro</em> assays. The M2 markers, Arg1 and CD74, are expressed in both populations; however, the M2 markers, Chi3l3 and Mrc1, are expressed only in the Ly6<sup>C</sup><sub>int</sub> and Ly6<sup>C</sub><sub>low</sub> populations, respectively. No M1 markers are detected in any population. The nomenclature of M1 and M2 macrophages was based on <em>in vitro</em> studies in which cells were exposed to one cytokine; however, in the disease setting there will be a milieu of various cytokines so the classification of...
macrophages based on cell surface marker expression becomes complex. Thus, in light of current efforts to harmonize nomenclature for macrophage studies, we have focused our studies on defining macrophage populations based on function.

The origin of the macrophage subsets may also vary based on organ and disease. Recruitment and differentiation of Ly6Chigh monocytes were identified as the primary source of profibrotic macrophages in a UUO model. In a study focused on reparative macrophages following renal IRI, recruitment of monocytes as well as in situ proliferation of resident macrophages were shown to contribute to increases in the reparative population. In a liver fibrosis model, Ly6Chigh monocytes differentiated into two different macrophage subsets: profibrotic and proresolution. In a myocardial infarction model, two different subsets of monocytes were recruited to differentiate into two different subsets of macrophages. Our data show that Ly6C expression decreases while F4/80 expression increases over time, suggesting that the Ly6Chigh subset may be more monocytic while the Ly6Clow subset is more mature. This is supported by the expression of genes in the low population associated with macrophage differentiation from monocytes including: Mcm6 and Nrp1 (neuropilin-1). Also, chemokines and membrane receptors expressed in both the Ly6Cint and low populations, including Cxcl14, Cxcl3, Csf1r, and Cx3cr1, have been linked to the recruitment of monocytes. Collectively, these data suggest that monocyte recruitment and resident cell proliferation are likely to be essential for the expansion of the macrophage populations in our studies, but further experiments would be required to confirm.

Several groups have examined the deleterious role of macrophages in renal IRI by demonstrating renoprotection after reducing macrophage number by genetic deletion of Ccr2 and Cx3cr1 or treatment with liposome

![Genomic analysis identifies a unique gene signature for each of the populations of macrophages.](image)

**Figure 5.** Genomic analysis identifies a unique gene signature for each of the populations of macrophages. Cells were sorted based on CD11b/Ly6C expression at time points enriched for a particular population: CD11b/Ly6Chigh at 4 hours post-reperfusion, CD11b/Ly6Cint at 1 day postreperfusion, and CD11b/Ly6Clow at 9 days postreperfusion. Next, the sorted populations underwent whole-genome mRNA profiling with Affymetrix mouse gene microarray. (A) Hierarchical clustering is carried out to identify differentially expressed genes between macrophage populations. The heat map indicates that each population has a unique gene signature. There are 72 genes upregulated in the Ly6Chigh population, represented as cluster C, 102 genes upregulated in the Ly6Cint population represented as cluster B, and 740 genes upregulated in the Ly6Clow population, represented as cluster A1. Cluster A2 represents 237 genes that are primarily upregulated in the Ly6Clow population with some overlapping upregulation in the Ly6Chigh population. (B) Principal component analysis confirms that the populations are different. n=3.
clodronate, a widely accepted means of inducing macrophage apoptosis. Macrophages have also been linked to kidney repair following AKI by using liposome clodronate during the recovery phase to deplete macrophages, leading to reduced proliferation of the tubular epithelium. In our studies, we use a small molecule inhibitor of c-fms to prevent the differentiation and activation of macrophages and confirm published reports showing that macrophages are involved in both the injury and repair phases of renal IRI. Moreover, we extend this observation by gene expression analysis. Further studies are needed to determine whether the Ly6C<sub>int</sub> population arises from the Ly6C<sub>high</sub> population or whether the gene expression profile of the Ly6C<sub>int</sub> population changes to one of repair from 3 hours to 1 day post-reperfusion.

To understand potential mechanisms by which macrophages regulate injury and repair, our strategy was to focus only on genes uniquely expressed in each population without consideration of genes that are up- or downregulated in each respective phenotype over time. A limitation of the analysis is that we are also comparing cell populations harvested at different time points when sufficient cells were present to harvest RNA. Moreover, we cannot rule out that gene expression profiles could be altered through the tissue-processing and cell-sorting procedure. Because CD11b and Ly6C can be expressed on dendritic cells and neutrophils, we must also consider that these cells could be present in our sorted populations.

Several genes upregulated in the Ly6C<sup>high</sup> population are linked with the monocyte/macrophage immune response. ApoC1 and Met associate with monocyte-macrophage differentiation, whereas Egf mediates monocyte/macrophage chemotaxis. Sel (L-selectin) facilitates cell adhesion and rolling. Importantly, analysis of the cell processes linked to the Ly6C<sub>int</sub> subset indicate a role in cell differentiation, tissue remodeling, phagocytosis and vascularization, which are all key processes in repair of the tubular epithelium. Mmp8 contributes to the resolution of inflammation, while Mmp9 regulates re-epithelialization and collagen fibril formation during

**Figure 6.** The gene signature indicates a unique phenotype and function for each CD11b/Ly6C population. (A) There is a switch from a reparative Ly6C<sub>int</sub> population to a profibrotic Ly6C<sub>low</sub> population. Genes that are associated with wound healing are uniquely up-regulated in the Ly6C<sub>int</sub> population, while genes associated with fibrosis are uniquely expressed in the Ly6C<sub>low</sub>. The Ly6C<sub>low</sub> population is enriched for complement genes. However, markers of M2 macrophages are present in both populations, with some markers differentially expressed. (B) Both the CD11b<sup>+</sup>/Ly6C<sub>int</sub> and CD11b<sup>+</sup>/Ly6C<sub>low</sub> populations carry an inflammatory signature with various membrane receptors, chemokines and other genes associated with inflammation expressed in both populations. (C) qRT-PCR on sorted macrophage populations confirms the microarray data by showing that the representative genes in (A) and (B) are uniquely upregulated in one population. (D) Genes associated with monocyte/macrophage immune responses are highly expressed in the CD11b<sup>+</sup>/Ly6C<sup>high</sup> subset. n=3.
BASIC RESEARCH

Table 1. Top 15 cell processes regulated by genes upregulated in the Ly6Cint population

<table>
<thead>
<tr>
<th>Cell Process</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response</td>
<td>6.64E-12</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>1.42E-08</td>
</tr>
<tr>
<td>Innate immune response</td>
<td>8.25E-08</td>
</tr>
<tr>
<td>Neutrophil chemotaxis</td>
<td>7.91E-07</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>1.29E-06</td>
</tr>
<tr>
<td>Epithelial to mesenchymal transition</td>
<td>2.50E-06</td>
</tr>
<tr>
<td>Cell differentiation</td>
<td>4.00E-06</td>
</tr>
<tr>
<td>Neutrophil activation</td>
<td>6.44E-06</td>
</tr>
<tr>
<td>Apoptosis of neutrophils</td>
<td>6.87E-06</td>
</tr>
<tr>
<td>Tissue remodeling</td>
<td>7.57E-06</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>1.14E-05</td>
</tr>
<tr>
<td>Cell-cell adhesion</td>
<td>1.24E-05</td>
</tr>
<tr>
<td>Leukocyte migration</td>
<td>1.43E-05</td>
</tr>
<tr>
<td>Immunity</td>
<td>2.01E-05</td>
</tr>
<tr>
<td>Vascularization</td>
<td>3.55E-05</td>
</tr>
</tbody>
</table>

Table 2. Studies of stimulated macrophages that have significant overlap with genes uniquely upregulated in the Ly6Cint population

<table>
<thead>
<tr>
<th>Study Name (Reference)</th>
<th>P Value</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow-derived macrophages infected 48 hr with <em>M. bovis</em> BCG wild-type versus MyD88 KO (J.E. Qualis, GSE22935)</td>
<td>7.9E-78</td>
<td>Mouse</td>
</tr>
<tr>
<td>J774.A1 macrophages infected 48 hr with <em>Brucella suis</em> VTRS1 versus uninfected controls (Y. He, GSE21117)</td>
<td>4.3E-56</td>
<td>Mouse</td>
</tr>
<tr>
<td>Macrophages from STAT6 KO mice cultured 10d with 10 uM rosiglitazone + 20 ng/mL IL-4 versus RSG only (A. Szanto, GSE25088)</td>
<td>6.5E-52</td>
<td>Mouse</td>
</tr>
<tr>
<td>Bone marrow-derived macrophages stimulated with Th2 cytokine IL-4 (F.O. Martinez, GSE35435)</td>
<td>1.9E-41</td>
<td>Mouse</td>
</tr>
<tr>
<td>RAW 264.7 mouse macrophages + 3.5 mM HOCl 6 hr versus untreated (C.G. Woods, GSE15457)</td>
<td>2.4E-40</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

wound repair.45 *Chi3l1* encodes a protein secreted by macrophages that mediates kidney repair by limiting tubular cell death,46 which may explain the increased cell death with c-fms inhibitor treatment. Collectively, these data clearly indicate the Ly6Cint subset plays a paracrine role in repair of the tubular epithelium.

Due to the inconsistent reports about depleting macrophages to establish a role in kidney fibrosis,29,30 we investigated the gene signature of the Ly6Clow population. One key piece of evidence supporting a role in fibrosis is the common gene signature identified between the Ly6Clow population and numerous genomic studies linked to fibrotic diseases including kidney transplant patients that have interstitial fibrosis.47,48 Additionally, several genes are directly linked to fibrosis. SPARC regulates the expression of secreted extracellular matrix proteins to mediate fibrosis while *Timp2* inhibits extracellular matrix turnover by matrix metalloproteinases (MMPs) leading to matrix accumulation.51 *Igf1* released by macrophages may attenuate myofibroblast apoptosis52 or stimulate type I collagen release.53 Activation of complement is involved in progressive tubulointerstitial disease.54 Clearly, the genetic signature of the Ly6Clow population indicates that it contributes to kidney fibrosis.

Incomplete repair of the tubular epithelium following AKI leads to progressive kidney disease driven by chronic inflammation, tubulointerstitial fibrosis, and vascular dropout.9,10,55 Our data indicate that one subset of macrophages has a beneficial role in repair of the tubular epithelium, while another has a detrimental role in the progression to chronic disease following AKI. Interestingly, both populations have an inflammatory signature and we propose, based on our data and clinical studies showing that CKD is a proinflammatory state,56 that it is the chronic inflammation which develops following acute injury that drives the progression to CKD. Because it is unknown whether there is a dynamic relationship between the different subsets of macrophages, the best therapeutic approach may be to target macrophage paracrine signaling. Our microarray analyses identified several genes which may be key contributors to repair of the kidney or progressive fibrosis. Future studies should aim to determine whether enhancing repair or minimizing fibrosis and inflammation would improve patient outcome.

**CONCISE METHODS**

Please refer to the Detailed Methods in the online Supplemental Material for additional information.

**Mouse Model of Bilateral Renal IRI**

*In vivo* studies were approved by the Genzyme Institutional Animal Care and Use Committee. C57BL/6 male mice (8–10 weeks, Taconic, Germantown, NY) underwent bilateral renal IRI for 28 min or sham surgery followed by reperfusion for various times as previously described.11 Surgery was carried out through bilateral flank incisions with animals on homeothermic operating tables to maintain body temperature at 37°C through rectal probe during the ischemia and reperfusion episodes. After surgery, animals were hydrated with intraperitoneal injection of 1 ml sterile saline. Pain medication
Table 3. Studies associated with fibrosis that have significant overlap with genes uniquely upregulated in the Ly6C\textsubscript{low} population

<table>
<thead>
<tr>
<th>Study Name (Reference)</th>
<th>P Value</th>
<th>Species</th>
<th>Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse lung wild-type 7d after intratracheal bleomycin 1 mg/kg versus no bleomycin (T. Oga\textsuperscript{49})</td>
<td>7.10E-27</td>
<td>Mouse</td>
<td>Lung</td>
</tr>
<tr>
<td>Lungs from wild-type mice 21d after endotracheal injection of bleomycin versus vehicle (T. Liu\textsuperscript{61})</td>
<td>1.10E-26</td>
<td>Mouse</td>
<td>Lung</td>
</tr>
<tr>
<td>Renal allografts: subclinical interstitial fibrosis 12mo post-transplant versus time zero (W.D. Park\textsuperscript{48})</td>
<td>4.20E-24</td>
<td>Human</td>
<td>Kidney</td>
</tr>
<tr>
<td>Cirrhotic hepatic inflammatory cells - adipose stem cells treatment 2\times every 2wk versus PBS control (Y. Sakai, GSE40395)</td>
<td>2.80E-23</td>
<td>Mouse</td>
<td>Liver</td>
</tr>
<tr>
<td>Kidney biopsy 1yr post-Tx with histology interstitial fibrosis + cellular infiltration versus normal (W.D. Park\textsuperscript{47})</td>
<td>1.00E-19</td>
<td>Human</td>
<td>Kidney</td>
</tr>
</tbody>
</table>

Morphologic Analysis
Picrosirius red staining and immunostaining of proliferating cell nuclear antigen (PCNA; Abcam, Inc.) and TUNEL (ApopTag Red Apoptosis Determination Kit; EMD Millipore) were carried out as previously detailed.\textsuperscript{1,58,59} To quantify reactivity in kidney tubules, four to six random fields of the outer medulla or cortex per section per animal were photographed at 20\times followed by Metamorph analysis (Molecular Devices, Sunnyvale, CA). Data are expressed as mean±SEM.

Quantitative RT-PCR
For qRT-PCR, total RNA was extracted from snap-frozen tissues with the RNeasy Kit (Qiagen, Valencia, CA) and reverse-transcribed to cDNA (Clontech, Mountain View, CA), as previously described.\textsuperscript{11} Probes and primers specific for mouse (Applied Biosystems, Foster City, CA) included IL-6 (Mm00446190\_m1), CXCL1 (Mm04207460\_m1), fibropectin (Mm01256744\_m1), TGFB1 (Mm01178820\_m1), KIM-1 (Mm00506686\_m1), NGAL (Mm01324470\_m1), CXCL3 (Mm01701838\_m1), IGF1 (Mm00439560\_m1), C3AR1 (Mm02620006\_s1), PARP1 (Mm01321084\_m1), CD74 (Mm00658576\_m1), MMP8 (Mm00439509\_m1), and MMP9 (Mm00442991\_m1). Data were normalized to 18S and expressed as fold change compared with normal.

Flow Cytometry
For flow cytometry, single-cell suspensions of the kidney were made by digesting the tissue in 1 mg/ml collagenase B, 1.2 U/ml dispase, 5 U/ml DNase II followed by a series of filtering and washing as described.\textsuperscript{58} Fc receptors on single cells (1\times10\textsuperscript{6}) were blocked with RPMI containing 10% normal mouse serum/0.05% NaAzide followed by incubation of cells with an antibody cocktail containing Alexa Fluor 488 rat anti-mouse CD11b (BD Biosciences, Franklin Lakes, NJ), APC rat anti-mouse F4/80 (eBioscience, San Diego, CA) and APC Cy7 rat anti-mouse Ly6C (BD Biosciences), each at a 1:20 dilution. After washing, cells were incubated with UV-fixable live-dead dye (Invitrogen, Carlsbad, CA) followed by analysis on a BD LSRII flow cytometer (BD Biosciences) with FlowJo 7.6 software (TreeStar, Ashland, OR). For gating, only live cells were included in the analysis followed by dot plots to identify cell populations of Ly6C versus CD11b and F4.80 versus CD11b. Once the CD11b\textsuperscript{+}Ly6C\textsuperscript{high}, int and low populations were gated, each population was then plotted for CD11b versus F4/80 to determine the expression levels of F4/80 of each individual population. To accurately measure cell number, counting beads (Invitrogen) were used following the manufacturer’s recommendation.

Microarray Analysis
Time points for microarray analysis were selected when the Ly6C population of interest dominated to optimize mRNA isolation and amplification. Cell populations were sorted on a BD FACSCARIA II cell sorter (BD Biosciences) based on CD11b\textsuperscript{+}Ly6C\textsuperscript{high} intermediate or low for RNA isolation (Qiagen) and amplification (NuGen Ovation Pico WTA v2, NuGen Technologies, San Carlos, CA) for cDNA production. The amplified cDNA library was further fragmented and labeled with NuGen Encore Biotin Module (NuGen Technologies) followed by hybridization to Affymetrix GeneChip Mouse Genome 430 2.0 array. Raw CEL files were robust multivariate average preprocessed in Affymetrix Expression Console and imported into Array Studio v6.1 (OmicSoft, Cary, NC) for further downstream statistical analysis. The expression data set was filtered to reduce background noise and median normalized across filtered probes. Principal Component Analysis separated ischemia and sham kidneys. Inference reports (t tests) were generated for IRI and sham group separated by population and time course as well as overall IRI versus sham. Similarly, hierarchical clustering was performed to show differentially expressed genes between IRI versus Sham. Statistical significance was set at P<0.01 with no multiple testing correction. To understand potential mechanisms by which macrophages regulate injury, repair and fibrosis, our strategy was to focus only on genes uniquely expressed in each population without consideration of genes that are up- or downregulated in each respective phenotype over time. Differentially expressed gene lists (FC±1.5, P<0.01) from comparative analysis were correlated to public gene expression repository with commercial software NextBio (NextBio, Cupertino, CA). Links
between genes and cell processes/phenotype were identified with Pathway Studio (Ariadne Genomics, Rockville, MD).

One-way ANOVA followed by the Newman–Keuls multiple comparison test and two-way ANOVA followed by Sidak’s multiple comparison test or t tests were used when appropriate to determine statistical differences using GraphPad Prism v6.0 software (GraphPad Software, Inc., La Jolla, CA). Data are expressed as mean±SD, unless otherwise indicated.

ACKNOWLEDGMENTS

M. Gershenovich, J. Campos-Rivera, M. Zhang, S. Ledbetter and A. Zuk are employees of Genzyme, a Sanofi Company. M. Clements and P. Du are employees of Biogen Idec and C. Chaber is an employee of Roche Diagnostics.

DISCLOSURE

None.

REFERENCES

43. Lamb DJ, Modjtahedi H, Plant NJ, Fems GAA: EGF mediates monocyte chemotaxis and macrophage proliferation and EGF receptor is expressed in atherosclerotic plaques. Atherosclerosis 176: 21–26, 2004
52. Wynes MW, Frankel SK, Riches DW: IL-4-induced macrophage-derived IG-F1 protects myofibroblasts from apoptosis following growth factor withdrawal. J Leukoc Biol 76: 1019–1027, 2004

This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014111138/-/DCSupplemental.
Detailed Methods

*In vivo model of renal IRI*

All studies were approved by the Genzyme Institutional Animal Care and Use Committee. C57BL/6 male mice (8-10 weeks) purchased from Taconic (Germantown, NY) were housed in a virus- and parasite-free barrier facility with a standard 12:12 hr light-dark cycle and had ad libitum access to water and standard chow. For surgery, the procedure of Clements et al was followed. Briefly, animals were anesthetized with sodium pentobarbital (50-70 mg/kg, IP), aseptically prepped, and positioned on homeothermic operating tables to rigorously maintain body temperature at 37°C through rectal probe. The kidneys were exposed by bilateral flank incisions and the renal pedicles were cleaned of adherent connective tissue. The renal artery and vein of each kidney were clamped with non-traumatic microaneurysm clamps (Roboz Surgical, Holliston MA) for 28 minutes. Clamps were released and reflow of each kidney was visually confirmed. After suturing, 1 ml of sterile saline was injected IP and the animals were recovered on surgical heating pads at 37°C for 24-48 hrs. Buprenorphine (0.05 mg/kg, SC) was administered 2.5 hrs prior to surgery and again at 24, 36 and 48 hrs after the initial dose. Sham surgeries were identical without the bilateral clamping.

At various times post-reperfusion, blood was collected from the retro-orbital sinus followed immediately by euthanasia and whole body perfusion with Hank’s Balanced Salt Solution (HBSS). Kidneys were harvested and the kidney capsules were removed. One kidney was snap frozen in liquid nitrogen for molecular analysis while the second kidney was divided in half, one half for flow cytometric analysis and one half fixed in 10 mM sodium periodate-75 mM L-lysine-2% paraformaldehyde. Plasma creatinine and blood urea nitrogen (BUN) values were measured on a Roche Integra 400 Bioanalyzer (Roche Diagnostics, Indianapolis IN). Whole blood was analyzed for circulating monocytes (Sysmex XT-V Automated Hematology Analyzer, Sysmex, Japan).

To determine monocyte/macrophage function in the different phases following renal IRI, a small molecule inhibitor of c-fms kinase activity was administered at 30 mg/kg, PO, following the dosing regimens described in Figures 3 and 4 to reduce monocyte/macrophage proliferation, differentiation and survival, based on published reports.

**Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)**

To extract total RNA, snap frozen tissues were lysed for 2 min at RT in RLT Lysis Buffer containing β-mercaptoethenol (Qiagen, Valencia, CA) using zirconium beads placed in a bead beater (Biospec, Bartlesville, OK) at 1:5 ratio. Lysate was then purified using RNAeasy column and dissolved in RNAse free water. RNA quality and concentration was determined. Total RNA (2 ug) was reverse-transcribed to cDNA by Clontech Sprint RT Reagent (Clontech, Mountain View, CA) using random hexamer primers with the following cycling conditions: 42°C for 60 min, 70°C for 15 min, 4°C end. qRT-PCR was done using 4 ug of newly synthesized cDNA, TaqMan RT-PCR Mastermix (Applied Biosystems, Foster City, CA), and FAM/TAMRA labeled primers/probes (Applied BioSystems) following standard RT-PCR reaction. Probes and primers specific for mouse were as follows: IL-6 (Mm00446190_m1), CXCL1 (Mm04207460_m1), fibronectin (Mm01256744_m1), TGFβ1 (Mm01178820_m1), KIM-1 (Mm00506686_m1), NGAL (Mm01324470_m1), CXCL3 (Mm01701838_m1),
IGF1 (Mm00439560_m1), C3AR1 (Mm02620006_s1), PARP1 (Mm01321084_m1), CD74 (Mm00658576_m1), MMP8 (Mm00439509_m1), MMP9 (Mm00442991_m1) and 18S (Hs99999901_s1). Data were normalized to 18S and expressed as fold change compared to normal.

**Morphology**

Fixed kidneys were used for all histological and immunohistochemical analysis following paraffin embedding, sectioning (5 μm) and deparaffinization, as previously described.\(^{11, 58, 59}\) To detect fibrosis, sections were stained with picrosirius red.\(^{11}\)

To identify proliferating cells, sections were washed with 0.05% Tween-20 in PBS, quenched in 10 mM citric acid buffer containing 0.05% Tween-20, pH 6 by boiling for 30 min, washed again and blocked in 10% normal donkey serum. Samples were then incubated with 1 μg/mL rabbit anti-PCNA (proliferating cell nuclear antigen) antibody (Abcam, Cambridge, MA) followed by 25 μg/mL Alexafluor 488 donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hr each at RT. Samples were mounted with hard set mounting medium containing DAPI after washing (Vector, Burlingame, CA) and coverslipped.

To detect cell death, TUNEL positive nuclei were detected by an ApopTag Red Apoptosis Determination kit (Millipore, Billerica, MA) according to the manufacturer’s recommendations.

To quantify cell proliferation and cell death, four to six random fields within the cortex and outer medulla per animal were photographed with a Nikon Eclipse 80i microscope (Nikon, Melville, NY) at 20X magnification. Metamorph analysis (Molecular Devices, Sunnyvale, CA) was used to quantify PCNA, TUNEL and DAPI positive nuclei in kidney tubules. Data are expressed as mean ± standard error (SEM) of percent PCNA or TUNEL positive cells per total cell number.

**Flow cytometric analysis**

Single cell preparations of the kidney were made by digesting the tissue in dissociation media (HBSS containing 1 mg/mL collagenase B, 1.2 U/mL dispase, 5 U/mL DNase II) for 45 min at 37°C with agitation. The digested homogenate was passed through an 18 gauge needle and filtered through a 100 μm cell strainer. After washing with ice cold PBS, cells were centrifuged at 2000 rpm for 5 min. The cell pellet was lysed in BD Pharm Lyse buffer (BD Bioscience, Franklin Lakes, NJ) for 3 min at RT to remove red blood cells. Lysed cells were washed twice with PBS containing 2% fetal bovine serum (FBS), resuspended in PBS and filtered through a 40 μm cell strainer. The number of cells in each kidney homogenate was counted (Vicell, Beckman Coulter, Brea CA) followed by incubation of single cells (1 x 10⁶) with RPMI containing 10% normal mouse serum/0.05% NaAzide, for 10 min at RT to block Fc receptors. Cells were then incubated for 20 min at RT in the dark with an antibody cocktail which included, Alexa Fluor 488 rat anti-mouse CD11b (BD Bioscience) APC rat anti-mouse F4/80 (eBioscience, San Diego, CA) and APC Cy7 rat anti-mouse Ly6C (BD Bioscience), each at a 1:20 dilution. Cells were washed with PBS, 2% FBS followed by incubation with UV-fixable live-dead dye (Invitrogen, Carlsbad, CA) for 30 min at 4°C in the dark to allow gating only of viable cell populations. After washing, cells were fixed in 1%
methanol-free formaldehyde and analyzed on a BD LSRII flow cytometer (BD Bioscience) using FlowJo 7.6 software (TreeStar, Ashland, OR).

For gating, only live cells were included in the analysis followed by dot plots to identify cell populations of Ly6C vs CD11b and F4.80 vs CD11b. Once the CD11b/Ly6C<sup>high</sup>, int and low populations were gated, each population was then plotted for CD11b vs F4/80 to determine the expression levels of F4/80 of each individual population. To accurately measure cell number, counting beads (Invitrogen) were used following the manufacturer’s recommendation. Data are expressed as total cell number +/- standard deviation.

Microarray analysis

Timepoints for microarray analysis were selected based on when the Ly6C population of interest dominated to optimize mRNA isolation and amplification. Cell populations were sorted on a BD FACSARIA II cell sorter (BD Bioscience) based on CD11b/Ly6C high, intermediate or low into RLT Lysis Buffer containing β-mercaptoethanol for RNA isolation (Qiagen). Total RNA was extracted with RNeasy Micro kit (Qiagen) with on-column DNase I digestion. RNA quality control was tested with Bioanalyzer running RNA Pico 6000. Samples were linearly amplified with NuGen Ovation Pico WTA v2 (NuGen Technologies, San Carlos, CA) to obtain a sufficient quantity of cDNA for downstream microarray application. The amplified cDNA library was further fragmented and labeled with NuGen Encore Biotin Module (NuGen Technologies) then hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 array. Subsequent washing and scanning were performed according to a validated Affymetrix protocol in a CLIA certified service lab.

Raw CEL files were RMA (robust multi-array average) preprocessed in Affymetrix Expression Console and imported into Array Studio v6.1 (OmicSoft, Cary, NC) for further downstream statistical analysis. In short, the expression dataset was filtered to reduce background noise. Probe intensities <5 in log2 scale in at least 67% of any individual group were removed. 30512 (67.7%) probes were kept from 45101. The data was median normalized across filtered probes. Primary separation for Principal Component Analysis was between ischemia and sham kidneys. Inference reports (t-tests) were generated for I/R and sham group separated by population and time course as well as overall I/R vs. sham. Similarly, hierarchical clustering was performed to show differentially expressed genes (DEG) between I/R vs. sham. Statistical significance was set at p < 0.01 with no multiple testing correction. DEG lists (FC +/- 1.5, p<0.01) from comparative analysis were correlated to public gene expression repository with commercial software NextBio (NextBio, Cupertino, CA). Furthermore, links between genes and cell processes/phenotype were identified with Pathway Studio (Ariadne Genomics).

Statistical analysis

One way ANOVA followed by the Newman-Keuls multiple comparison test and two way ANOVA followed by Sidak’s multiple comparison test or t-tests were used when appropriate to determine statistical differences using GraphPad Prism v6.0 software (GraphPad Software, Inc, Lo Jolla, CA). Data are expressed as mean ± standard deviation (SD), unless otherwise indicated.