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Compartmentalization of neutrophils in the kidney and lung following acute ischemic kidney injury

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During renal ischemia-reperfusion, local and distant tissue injury is caused by an influx of neutrophils into the affected tissues. Here we measured the kinetics of margination and transmigration of neutrophils *in vivo* in the kidney and lungs following renal ischemia-reperfusion. After bilateral renal injury, kidney neutrophil content increased threefold at 24 h. The neutrophils were found primarily in the interstitium and to a lesser degree marginated to the vascular endothelium. These interstitial neutrophils had significantly lower levels of intracellular IFN-γ, IL-4, IL-6, and IL-10 a tendency for decreased amounts of IL-4 and TNF-α compared to the marginated neutrophils. Localization of the neutrophils to the kidney interstitium was confirmed by high resolution microscopy and these sites of transmigration were directly associated with areas of increased vascular permeability. Activation of the adenosine 2A receptor significantly decreased both kidney neutrophil transmigration by about half and vascular permeability by about a third. After unilateral renal ischemia-reperfusion, the unclipped kidney and lungs did not accumulate interstitial neutrophils or have increased vascular permeability despite a marked increase of neutrophil margination in the lungs. Our findings suggest there is a sequential recruitment and transmigration of neutrophils from the vasculature into the kidney interstitium at the site of tissue injury following renal ischemia-reperfusion.

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Following kidney ischemia-reperfusion injury (IRI), the kidney content of immune cells increases and contributes to apoptosis and/or necrosis.[1] Neutrophils, T and B lymphocytes, and macrophages are important in the pathogenesis of kidney IRI.[1,2] Neutrophils marginate to vascular endothelium, plug the microvasculature and subsequently release oxygen-free radicals and proteases. These marginated cells are unlikely to have direct effects on renal interstitial cells and/or tubular function.[2] Neutrophils secrete cytokines including interferon (IFN)-γ,[3,4] interleukin (IL)-4,[5] IL-6,[6] IL-10,[7] and tumor necrosis factor-α (TNF-α).[4] To date, studies have not addressed whether neutrophils transmigrate following kidney IRI. Furthermore, IRI is exacerbated[8] or attenuated[9–11] by experimental maneuvers that increase or reduce neutrophil infiltration, respectively. Thus, determining the localization of polymorphonuclear cell (PMN) in tissue compartments (intravascular vs marginated vs extravascular) may improve our understanding of the pathogenesis of kidney IRI.

We have shown earlier that adenosine 2A receptor ($A_{2A}R$) agonists reduce inflammation by reducing adhesion molecule expression and neutrophil adherence to endothelial cells following renal IRI.[12] Blocking adhesion of neutrophils may be the first step in preventing neutrophil transmigration, a mechanism that is most likely to be important in the mechanism of A_{2A}-agonist-mediated renal tissue protection.

Acute kidney injury (AKI) contributes to acute lung injury (ALI), factors which increase the overall morbidity and mortality associated with AKI.[13] Defining the mechanism of AKI-induced ALI could reduce the high mortality of combined AKI and ALI, which approaches 80%.[14] Compartmentation of neutrophils has been clearly described in other tissues including lungs[15] but not in the kidney due to methodological limitations. Therefore, understanding the differential kinetics of kidney neutrophil accumulation in tissue compartments following kidney IRI using refined methodologies and in response to novel compounds is necessary.

We report neutrophil transmigration from the vascular to the interstitial compartments following renal IRI. Our present method distinguishes between marginated and interstitial compartments in the kidneys and lungs as well

as the direct role of A_{2A}-agonists on neutrophil distribution in tissue compartments. We show that a non-vascular, interstitial neutrophil population has direct effects on vascular permeability mediating kidney IRI and that the kidney interstitial neutrophil population, whose cytokine content is reduced, is functionally distinct from the marginated neutrophil population. This study highlights the potential importance of leukocyte transmigration across vascular endothelium to the pathogenesis of renal IRI and shows that compounds such as A_{2A}-agonists may ameliorate injury by blocking leukocyte transmigration.

RESULTS

Renal IRI increases total kidney neutrophil content

We assessed the kinetics of neutrophil accumulation and compartmentalization to either marginated or non-vascular (interstitial) compartments following kidney IRI. Total kidney content of neutrophils (sum of marginated and interstitial PMNs) was $2.8 \pm 0.7 \times 10^5$ cells in sham, increased by 159% after 2 h following IRI ($7.3 \pm 1.1 \times 10^5$ cells; $P<0.05$), peaked at 24 h ($8.4 \pm 1.1 \times 10^5$ cells; a 200% increase above sham; $P<0.005$) and persisted at 48 and 72 h after IRI ($7.5 \pm 0.9 \times 10^5$ cells; $P<0.01$ and $5.7 \pm 1.2 \times 10^5$ cells, a 165 and 102% increase above sham), respectively, following renal IRI (Figure 1).

In vivo neutrophil compartmentation following kidney IRI

As the total kidney content of neutrophils peaked at 24 h, we sought to distinguish between the marginated ($7/4^+$GR-1^+) and interstitial ($7/4^+$GR-1^-) compartments of neutrophils in the kidneys and lungs, following renal IRI as shown in schematic Figure 2. We assessed compartmentation of neutrophils by harvesting kidneys 5 min after injection of anti-GR-1 antibody to selectively labeled neutrophils in the vascular and marginated compartments. It was shown earlier that 99.2% of blood neutrophils were stained without detectable vascular leakage 5 min after anti-GR-1 antibody injection.[15] In our study, it was important to systemically perfuse mice at the end of the study to eliminate all non-adherent vascular cells, including neutrophils, and any free antibody. Thus, we could not draw an accurate conclusion about vascular neutrophil concentration. Kidney IRI followed by 24 h reperfusion increased kidney neutrophil interstitial content ($7/4^+$GR-1^-) by 375% above sham, whereas there was a minimal increase (123% above sham) in marginated cells ($7/4^+$GR-1^+) (Figure 3). In contrast, lung neutrophil interstitial content did not change despite a marked increase in marginated neutrophils (261% above sham).

Our results also indicate that there were no differences between normal (non-operated) and sham-operated mice for both the kidney and lung marginated and interstitial neutrophil content (data not shown).

To exclude the possibility that poorly perfused areas of the vasculature that might be inaccessible for an intravenously GR-1 injected antibody, we used the monoclonal antibody to TER-119, an antigen associated with cells of the erythroid lineage.[16] Five minutes after injection, blood, kidneys, and lungs were harvested from both sham and 24 h renal IRI. Erythrocytes were defined by their typical appearance in the forward-side scatter, and the TER-119^+ erythrocytes in each organ were expressed as percentage of total erythrocytes by flow cytometry. We found 98 ± 0.2% of all blood erythrocytes to be TER-119^+. At the same time, 83 ± 3 and 96 ± 0.6% of all erythrocytes were TER-119^+ in the kidney and lung homogenates, respectively ($n = 6$–11 each group). This could result in an overestimation of the interstitial neutrophil concentration in the kidney by 17%. Because of the heterogeneity of kidney injury following IRI, the overestimation of neutrophil concentration resides primarily in the outer medulla, as this is the region with the greatest extent of injury. Therefore, we took this into consideration and corrected for this value in all subsequent experiments.

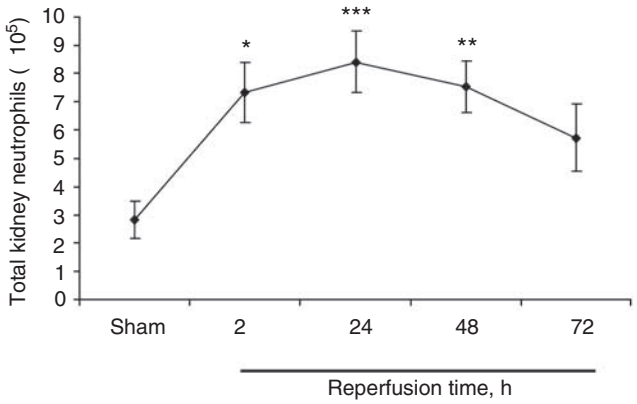

Figure 1 | Total kidney neutrophil content. Mouse kidneys were subjected to sham or 32 min ischemia and reperfusion for 2, 24, 48, or 72 h. Kidney suspensions were prepared for flow cytometry. Neutrophils were identified by (1) their typical appearance in the forward-side scatter, (2) their CD45^+ expression, and (3) two independent neutrophil markers, GR-1 APC and 7/4 FITC. $N = 3$–8 for all groups. *$P<0.05$, **$P<0.01$, ***$P<0.005$ compared with sham.

Histological and immunofluorescence localization of neutrophils in the kidney and lung

Interstitial neutrophils in the kidney identified by flow cytometry were confirmed by Periodic acid-Schiff stain and immunofluorescence labeling (Figure 4) using monoclonal antibody to 7/4 (neutrophils) and CD31 (to demarcate the vascular endothelium). Histological staining shows neutrophils in the peritubular capillary, vascular (Figure 4a), and interstitial (Figure 4b and c) compartments as well as within the tubular lumen (Figure 4b), indicating transmigration of neutrophils across the interstitial compartment. Immunofluorescence labeling confirmed these results and revealed neutrophils in both marginated and non-vascular (interstitial) compartments as well as a neutrophil transmigrating through the vascular wall in the kidney (Figure 4d). We also combined Z-stack images of 12 optical slices to identify several groups of kidney interstitial neutrophils (square; Figure 4e). Combined Z-stack images identify the borders of