Vascular-Resident CD169-Positive Monocytes and Macrophages Control Neutrophil Accumulation in the Kidney with Ischemia-Reperfusion Injury

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ABSTRACT

Monocytes and kidney-resident macrophages are considered to be involved in the pathogenesis of renal ischemia-reperfusion injury (IRI). Several subsets of monocytes and macrophages are localized in the injured tissue, but the pathologic roles of these cells are not fully understood. Here, we show that CD169+ monocytes and macrophages have a critical role in preventing excessive inflammation in IRI by downregulating intercellular adhesion molecule-1 (ICAM-1) expression on vascular endothelial cells. Mice depleted of CD169+ cells showed enhanced endothelial ICAM-1 expression and developed irreversible renal damage associated with infiltration of a large number of neutrophils. The perivascular localization of CD169+ monocytes and macrophages indicated direct interaction with blood vessels, and coculture experiments showed that the direct interaction of CD169- cell-depleted peripheral blood leukocytes augments the expression levels of ICAM-1 on endothelial cells. Notably, the transfer of Ly6Clo monocytes into CD169- cell-depleted mice rescued the mice from lethal renal injury and normalized renal ICAM-1 expression levels, indicating that the Ly6Clo subset of CD169+ monocytes has a major role in the regulation of inflammation. Our findings highlight the previously unknown role of CD169+ monocytes and macrophages in the maintenance of vascular homeostasis and provide new approaches to the treatment of renal IRI.


Renal ischemia-reperfusion injury (IRI) is a mouse model of AKI, and it is manifested by enhanced fluid filtration and leukocyte plugging in capillaries.1 The severity of renal injury in this model is determined by not only the number of tubular epithelial cells undergoing cell death caused by hypoxia but also, the degree of endothelial activation and subsequent influx of inflammatory blood cells, including neutrophils and monocytes.2,3 The pathologic roles of monocytes and macrophages have been recently highlighted in renal IRI.4 During the early phase of IRI, Ly6Chi monocytes infiltrate injured tissue and are considered to differentiate into inflammatory macrophages. These macrophages produce a set of inflammatory cytokines, such as TNF-α and IL-1β. Depletion of macrophages by injection of clodronate liposome or deficiency of CCR2 chemokine prevented IRI, indicating the critical roles of inflammation induced by macrophages in IRI.5,6 However, kidney macrophages are also considered to promote the tissue...
repair and regeneration during the late phase of IRI.7–8 Because several subsets of monocytes and macrophages are located in the IRI tissue, it is very important to determine the pathologic roles of each subset to understand the pathogenesis of IRI.

Monocytes in peripheral blood are heterogeneous in terms of phenotype, function, and fate. It is widely accepted that they differentiate from myeloid progenitor cells in the bone marrow (BM). There are two distinct subsets of peripheral monocytes that differ in the expression levels of CX3CR1 and Ly6C.9,10 CX3CR1hiLy6Clo monocytes crawl along the surface of endothelial cells to maintain endothelial integrity; thus, they are referred to as patrolling monocytes.11,12 Although CX3CR1lo monocytes are thought to maintain vascular homeostasis, their roles in disease development in vivo remain unknown. A second population of peripheral blood monocytes, CX3CR1loLy6Chilo monocytes, is the so-called inflammatory monocytes, because they are recruited selectively to the inflamed tissue, where they differentiate into macrophages and dendritic cells.13–15

A subset of macrophages that express CD169 resides in lymphoid organs, such as spleen, lymph node, and BM.16–18 Previously, we reported that CD169+ macrophages in the marginal zone of spleen and lymph node sinus capture apoptotic cells and induce dead cell antigen-specific immune responses.19–21 It is also reported that some other tissue-resident macrophages express CD169, but their physiologic or pathologic roles remain unclear.

In this study, we identified a subset of peripheral blood monocytes and kidney-resident F4/80+ macrophages using the mice in which CD169+ cells and their descendants were visualized. We found that the transient depletion of these cells results in the exacerbation of renal IRI. We categorized CX3CR1hiLy6CloCD169+ cells as vascular-resident monocytes/macrophages that suppress the excessive activation of endothelial cells. This finding may pave the way to the establishment of a novel therapeutic approach to the suppression of tissue injury in a broad range of human ischemic diseases.

RESULTS

Identification of Novel Subsets of Peripheral Blood Monocytes and Kidney-Resident Macrophages

To explore the distribution of CD169+ cells outside secondary lymphoid organs, we generated mice that harbor the improved Cre recombinase gene22 in the CD169 loci (CD169-Cre mice) and crossed those mice with ROSA26-yellow fluorescent protein (YFP) reporter mice23 to analyze recombinase activity (Supplemental Figure 1). In those CD169-Cre×ROSA26-YFP mice (hereafter, CD169-Cre-YFP mice), YFP reporter visualizes CD169+ cells and their descendants independently of continuous or transient CD169 expression. Consistent with previous findings obtained by immunohistochemical staining with anti-CD169 antibody, YFP+ cells localized at the marginal zone of the spleen (Supplemental Figure 2). We further confirmed gene recombination in the cultured BM cells. As shown in Supplemental Figure 3, CD169-expressing BM-derived macrophages cultured with M-CSF became YFP-positive. The time course suggests that it takes a few days to complete gene recombination.

To our surprise, we detected also gene recombination in peripheral blood Gr-1int-lo, the CD11b+ fraction that contains Ly6Chi and Ly6Clo monocytes (Figure 1A). Flow cytometry analysis with Ly6C antibody revealed that both subpopulations of Ly6Chi and Ly6Clo monocytes include YFP-positive cells. These YFP-positive cells expressed CD115, another marker of blood monocytes (Supplemental Figure 4). We decided to focus on characterizing the novel subset of YFP+ (CD169+)Gr-1int-lo CD11b+ monocytic cells in additional experiments.

Macrophages are classified into cells originating from yolk sac or fetal liver and hematopoietic stem cells.24–27 The major population of tissue macrophages, which includes Kupffer cells,28 Langerhans cells of the skin,29,30 and lung alveolar macrophages,31 proliferates locally independent of the BM. In contrast, the maintenance of tissue macrophages in the kidney and the intestine is dependent on constant CX3CR1+ monocyte supply from blood stream.9,11,12 Consistent with those reports, all of the CD11b+F4/80+ macrophages and nearly 50% of CD11b+F4/80lo cells in the kidney express CX3CR1, supporting the CX3CR1+ monocyte origin of kidney myeloid cells (Figure 1B). This finding led us to ask if any of the kidney macrophage subsets are YFP-positive in CD169-Cre-YFP mice. Among three subpopulations, 60% of CD11b+F4/80+ macrophages, all of which were CX3CR1+, were YFP-positive, whereas two other subpopulations, CD11b+F4/80lo and CD11b+ fractions, did not contain YFP+ cells (Figure 1C). Both YFP-positive and YFP-negative macrophages in the DP subpopulation were CD11c-positive. Immunohistochemical observation revealed the localization of YFP+ cells exclusively in the medullary interstitium (Figure 1D, middle and bottom panels), whereas CX3CR1+ cells distributed throughout the cortex and the medulla (Figure 1D, top panel). We next tried to reveal the origin of the YFP-positive kidney macrophages. For this purpose, we parabiotically joined CD169-Cre-YFP mice and ROSA26-YFP mice. Three weeks after parabiosis, a part of CD11b+F4/80+ kidney macrophages became YFP-positive (Figure 1E) in ROSA26-YFP mice, indicating that this subpopulation of kidney macrophages, at least in part, is derived from blood monocytes.

Depletion and Recovery Kinetics of Peripheral Blood and Kidney-Resident CD169+ Cells in CD169-DTR Mice

To understand the turnover kinetics of CD169+ cells in peripheral blood and the kidney, we injected diphtheria toxin (DT) into CD169-DTR mice and analyzed the frequency of CD169+ cells up to 7 days after the injection. The percentages of Gr-1int-loCD11b+ cells dropped from 14.5% to 4.3% 1 day after the DT injection (Figure 2, A and B) followed by a decrease of CD11b+F4/80+ kidney macrophages at day 2 (Figure 2, C and D). The frequency of CD11b+F4/80lo cells was unchanged in the kidney during this period, indicating selective depletion of resident macrophages. To further confirm the depletion of CD169+ cells among the CX3CR1+ monocytes and
Kidney macrophages, we injected DT into CD169-DTR mice that were crossed with CX3CR1-green fluorescent protein (GFP) mice. In peripheral blood, the DT injection induced approximately 57% (from 1.94 to 0.82) and 36% (from 6.6 to 4.2) reduction of CX3CR1hi and CX3CR1lo monocytes, respectively (Figure 2E) and 50% (from 68 to 34) reduction of kidney CD11b+CX3CR1+ macrophages (Figure 2F, upper panel). Notably, the depletion was restricted to the CD11b+F4/80+ macrophage fraction in the kidney (Figure 2F, lower panel). The degree of peripheral blood monocyte reduction was more profound than the frequency of YFP-positive cells among peripheral blood CD11b+ cells. This difference could be explained by a delayed gene recombination (Supplemental Material). Consistent with the results from CD169-Cre-YFP mice, these data show that CX3CR1+ monocytes and CX3CR1+CD11b+F4/80+ kidney macrophages are composed of CD169+ and CD169− fractions.

Role of CD169+ Cells in the Development of Renal IRI
To further investigate the roles of CD169+ monocytes in peripheral blood and kidney macrophages under an inflammatory condition, we performed renal IRI. In this model, clamping of unilateral renal pedicles combined with contralateral nephrectomy induces tubular necrosis and massive infiltration of polymorphonuclear cells in the interstitium that manifest as a clinical symptom of AKI in wild-type (WT) mice. The improved surgical approach to kidney pedicles from retroperitoneum enabled us to perform 1-hour clamping for AKI followed by complete recovery in WT mice. Surprisingly, the depletion of CD169+ cells resulted in progressive renal injury by IR (Figure 3, A–D). WT mice showed transient increases of serum creatinine and BUN levels, both of which returned to their basal levels within 2 days from IR (Figure 3B). Tissue injury in WT mice was characterized by transient loss of brush border and casts in the lumen that will recover within 7 days. Moderate interstitial infiltration was also observed (Figure 3C, center panel). However, kidneys of CD169-DTR mice presented with progressive renal dysfunction (Figure 3B) and larger necrotic areas.
lesion than WT mice (Figure 3D, left panel) caused by sustained tissue injury that is characterized by tubular necrosis and inflammatory cell infiltration (Figure 3D, center panel), and they could not survive the IR-induced AKI (Figure 3A). CD169-DTR mice survived unilateral IRI of the kidney, but the kidneys from those mice were totally destructed at day 7 (Figure 3D, right panel). Damage was most prominent in the corticomedullary junction. In parallel with the development of AKI, we observed substantial increases of proinflammatory cytokine in the kidneys of CD169-DTR mice compared with WT mice after IR (Figure 3E). Collectively, these results suggest that CD169+ cells in peripheral blood and the kidney contribute to the suppression and/or resolution of IRI. To deny the possibility that the depletion of CD169+ cells results in inflammation, even in the pre-injury state of DT-treated CD169-DTR mice, we assessed vascular hyperpermeability of the kidneys. As shown in Supplemental Figure 5A, there were no significant differences in the leakage of Evans blue in the kidneys between DT-treated WT and DT-treated CD169-DTR mice. In addition, there was no increase in the number of neutrophils between these mice in the preinjury state (Supplemental Figure 5B).

Aggravation of Renal Injury in the Absence of CD169+ Cells Is Induced by Accumulated Neutrophils

Immunohistochemistry revealed larger number of neutrophils in the kidney of CD169-DTR mice than WT mice after IR (Figure 4A). Flow cytometry also confirmed increase in the absolute number of neutrophils (Figure 4B). These findings suggest that the infiltrated neutrophils are responsible for the development of lethal AKI in CD169-DTR mice. To confirm this hypothesis, we depleted neutrophils in peripheral blood and the kidney by using anti–Gr-1 antibody before IR. CD169-DTR mice were rescued by the neutrophil depletion (Figure 4, C and D, Supplemental Figure 6). These results indicate that neutrophil accumulation promoted by the absence of CD169+ cells is the primary cause of AKI.

CD169+ Cells Suppress Intercellular Adhesion Molecule-1 Expression on Endothelial Cell Surface to Prevent Accumulation of Neutrophils

We next sought signals that drive neutrophil accumulation in the kidney. Such chemokines as CXCL1 and CXCL2 are responsible for neutrophil recruitment to an inflammatory site.
In parallel with previous results (Figure 4, A and B), the production of CXCL2 was higher in the kidneys of CD169-DTR mice than in WT mice after IR (Figure 5A). However, we could not answer whether the upregulation of those chemokines was the direct outcome of CD169+ cell depletion or whether the upregulation represented one of the secondary results of inflammation, because the chemokine expression was similar in preinjury kidneys of WT and CD169-DTR mice. To further explore the primary cause of neutrophil accumulation, we quantitated the expression levels of adhesion molecules. We found that the depletion of CD169+ cells resulted in the substantial increase of intercellular adhesion molecule-1 (ICAM-1) mRNA expression even before IRI (Figure 5B). As shown in Figure 5C, the injection of blocking anti–ICAM-1 antibody before IR into CD169-DTR mice rescued them from lethal AKI. These results suggest that the suppression of ICAM-1 expression on endothelial cell surface by vascular CD169+ cells under physiologic conditions is critical for the maintenance of tissue homeostasis.

We next tried to reveal the mechanisms of regulation of ICAM-1 expression by CD169+ cells. It was recently reported that the direct interaction between leukocytes and endothelial cells regulates ICAM-1 expression on endothelial cell surface.34 Therefore, we first analyzed the spatial relationship between CD169+ cells and blood vessels in the kidney. As shown in Figure 5D, the majority of YFP+ cells localized in close contact with blood vessel, suggesting their direct interaction with blood vessels. We also examined localization of CD169+ monocytes in blood vessels. When we injected anti-CD11b antibody into CD169-Cre-YFP mice, the frequency of YFP+ cells in Gr-1int-loCD11b+CD115+ fraction of peripheral blood leukocytes

Inflammatory cytokine mRNA levels in kidneys. Bilateral IR was performed in WT or CD169-DTR mice as described in A and B. Quantity of tnf and il1b mRNA levels in the kidneys relative to WT preinjury mice at the indicated time points after IR were determined by quantitative PCR. Mean±SD, n=5 mice/group. *P<0.05.
increased (Supplemental Figure 7). These results also suggest that CD169+ monocytes on vascular surface interact with the vascular endothelial cells in a CD11b-dependent manner.

To examine the possibility that the direct interaction of peripheral blood leukocytes regulates the expression of ICAM-1 on endothelial cells, we cocultured endothelial cells and peripheral blood leukocytes from the mice. We found that upregulation of ICAM-1 expression was higher in the endothelial cells cocultured with leukocytes obtained from DT-treated CD169-DTR mice than in those cocultured with leukocytes from WT mice (Figure 5E, left panel); 10 μM butylated hydroxyanisole suppressed ICAM-1 mRNA expression in endothelial cells cocultured with CD169-DTR leukocytes (Figure 5E, center panel), indicating the oxidant-dependent upregulation of ICAM-1. Intriguingly, the transwell experiments clearly showed that the direct interaction between these cells was definitely required for the upregulation of ICAM-1 expression on the endothelial cells (Figure 5E, right panel).

Transfer of Ly6Clo Monocytes Rescues CD169-DTR Mice from IR-Induced AKI

To prove anti-inflammatory activity of CD169-positive cells in tissue injury, we transferred total peripheral blood mononuclear cells from neutrophil-depleted WT mice into CD169-DTR mice before IR. As shown in Figure 6A, the injection of WT mononuclear cells improved the survival rate of CD169-DTR mice from lethal AKI by 60%, whereas the injection of WT Ly6Ghi neutrophils did not improve the survival rate. It is reported that Ly6Clo monocytes contribute to vascular homeostasis.12 To specify the monocyte subpopulation responsible for the amelioration of the IR-induced AKI, we injected fractionated Ly6Ghi or Ly6Clo monocytes into CD169-DTR mice (Figure 6B). As shown in Figure 6C, the survival rate of CD169-DTR mice injected with Ly6Clo monocytes improved by 62.5% 7 days after IR. Contrary to our speculation, CD169-DTR mice injected with Ly6Ghi monocytes were also partially rescued (33% at day 7) from the IR-induced AKI (Figure 6C). The amelioration of AKI in mice injected with Ly6Clo monocytes may be explained by the conversion of Ly6Ghi monocytes into Ly6Clo monocytes in the host mouse.32 We further examined the ICAM-1 expression levels in the kidneys of mononuclear cell-transferred mice and found that the levels were normalized in these mice (Figure 6D). The result further supports the notion that Ly6Clo monocytes suppress ICAM-1 expression on endothelial cell surface.

DISCUSSION

In this study, we identified and characterized a novel population of peripheral blood monocytes and kidney-resident macrophages that express CD169. We showed that they reside along the length of blood vessel and suppress the activation state of endothelial cells under physiologic conditions. The analysis using CX3CR1-GFP mice crossed with CD169-DTR mice reveals that the CD169+ cell lineage constitutes a major compartment of CX3CR1+ monocytes and kidney-resident macrophages.

In sterile inflammation, activated vascular endothelial cells release an array of inflammatory mediators that upregulate the expression of adhesion molecules, such as ICAM-1, which promotes leukocyte recruitment to the site of inflammation.35 In addition, damaged cells release endogenous adjuvants that activate pattern recognition receptors on innate immune cells,
Figure 5. Upregulated ICAM-1 expression in the absence of CD169+ cells exacerbates AKI. (A) Chemokine expression in IR kidneys. DT was injected intraperitoneally into WT or CD169-DTR mice. Thirty-six hours later, bilateral IR was performed. Quantities of ccl2, cxcl1, and cxcl2 mRNA levels in the kidneys relative to WT preinjury mice were determined by quantitative PCR. n=5 mice/group. Mean±SD. *P<0.05. (B) Enhanced expression of ICAM-1 in kidneys of CD169-DTR mice. Quantity of icam1 and icam2 mRNA levels in the kidneys of DT-treated WT or CD169-DTR mice relative to WT mice without DT were determined by quantitative RT-PCR. n=8 mice/group each. The average values are plotted with SD. *P<0.05. (C) Blockade of ICAM-1 suppresses the development of AKI in CD169-DTR mice. DT was injected intraperitoneally into CD169-DTR mice. Twenty-four hours later, 100 μg anti-ICAM-1 (clone KAT1) antibody or PBS was injected intravenously into these mice. Thirty-six hours after the DT injection, bilateral IR was performed. Survival rate is shown. n=3 mice/group. *P<0.05. (D) CD169+ cells localize in the perivascular area of the renal interstitium. DyLight 488-labeled tomato lectin was injected intravenously into CD169-Cre-YFP mice 5 minutes before removing the kidney. Cryosections of the kidneys were stained for YFP (green) and observed under a confocal microscope. White arrows indicate the CD169-positive macrophages, which are located along the capillary vessels. z-Stack images were processed by ImageJ software. Red signal indicates tomato lectin on endothelium. Scale bar, 100 μm. (E) Peripheral blood leukocytes from CD169-DTR mice augment ICAM-1 expression levels in cultured endothelial cells. (Left panel) Peripheral blood leukocytes from DT-treated WT or CD169-DTR mice were cocultured with UV92 cells for 18 hours. icam1 mRNA levels of these cells were determined by quantitative RT-PCR. (Center panel) Peripheral blood leukocytes from DT-treated CD169-DTR mice were cocultured with UV92 cells for 18 hours in the presence or absence of butylated hydroxyanisole (BHA). (Right panel) Peripheral blood leukocytes from DT-treated WT or DT-treated CD169-DTR mice were cocultured with UV92 cells for 18 hours using the transwell plate. The average values are plotted with SD. Data are representative of two or three independent experiments. *P<0.05.
in the IR-induced AKI. Intriguingly, YFP+ macrophages preferentially localize in the renal medulla. Proximal tubular cells in the corticomedullary border are considered susceptible to IRI.2,41 The correlation between the damaged site and the localization of CD169+ macrophages prompted us to speculate the protective role of those macrophages in the renal IRI. It was recently shown that macrophages are also involved in tissue repair after IRI.8,42,43 It would be very intriguing to examine the roles of those cells in tissue repair subsequent to IRI.

In conclusion, our study identified CD169+ monocytes/macrophages as a novel anti-inflammatory cell type in the initiation of IRI, which may be relevant to clinical treatment of human ischemic diseases.

CONCISE METHODS

Mice

C57BL/6J (8–12 weeks old) male mice were obtained from CLEA Japan, Inc. CD169-DTR knockin mice (C57BL/6J background) were established in our laboratory. CX3CR1-GFP mice and ROSA26-YFP mice were kindly provided from D. Littman (New York University School of Medicine) and F. Costantini (Columbia University), respectively. All mice were housed under specific pathogen-free conditions in the RIKEN RCAI and Tokyo University of Pharmacy and Life Sciences animal facility. All experiments using the mice described herein were approved by the RCAI and Tokyo University of Pharmacy and Life Sciences animal use committee and performed in accordance with the applicable guideline and regulations.

For systemic CD169+ cell depletion, CD169-DTR mice were injected intraperitoneally with 20–40 μg DT/kg body wt (CALBIOCHEM) 24–36 hours before IR and experiments. The generation of CD169-Cre knockin mice was performed essentially as described previously.13 Briefly, the improved Cre recombinase gene with a polyA tail and the neomycin resistance gene were introduced by replacing the first 28-bp fragment including the endogenous ATG start site in exon 1 of the CD169 gene. HSV-thymidine kinase gene was inserted downstream of the 3′ arm to select against random integrants. Then, B6N/1 ES cells were transfected with the linearized targeting vector by electroporation. G-418 and ganciclovir-resistant clones were screened for homologous recombination by Southern blot analysis. Germ-line chimeric mice were generated by aggregation methods. Chimeric mice with a high ES cell contribution were crossed with C57BL/6J mice to produce +/Cre mice. +/Cre mice were crossed to ROSA26-YFP mice.

endothelial cells in coordination with CD169+ monocytes. There are several works on the characterization of the mononuclear phagocyte system in the kidney. Mononuclear phagocytes in the kidney is composed of mesangial and interstitial cells. Mesangial cells in the glomeruli are considered macrophages, because they show some phagocytic function, although they do not express panmacrophage marker F4/80. However, medulary interstitial macrophages that localize in direct contact with tubules express both macrophage marker F4/80 and DC marker CD11c.38,39 In addition, there are solid lines of evidence that infiltrating monocyte-derived macrophages initiate and propagate the renal IRI and that the inhibition of macrophage infiltration ameliorates renal damage.5,40 In contrast, we propose that kidney-resident CD169+ macrophages play protective roles.
Flow Cytometry Analyses

We evaluated kidney cells with a flow cytometer. To obtain single-cell suspensions, whole kidneys were minced and digested with a digestion mixture (500 μg/ml Collagenase IIs [Sigma-Aldrich] and 10 μg/ml DNase I [Roche] in 1× HBSS buffer containing Ca²⁺ and Mg²⁺) for 20 minutes at 37°C under vigorous agitation (200 rpm/min). The cells were suspended in 5 ml 2 mM EDTA in 1× HBSS buffer not containing Ca²⁺ and Mg²⁺ and further incubated for 20 minutes at 37°C under gentle agitation (50 rpm/min). Single-cell suspensions thus prepared were incubated with Fc-blocker (anti-CD16/32; 2.4G2). Then, CD11b- and/or CD11c-positive cells were enriched by magnetic sorting (Miltenyi).

For the analysis of peripheral blood mononuclear cells, peripheral blood was suspended in 2 mM EDTA in PBS. Then, red blood cells were lysed with deionized water. For flow cytometry analysis, cells were stained with the following antibodies: anti-CD11b (M1/70), anti-F4/80 (CJ:A3–8C5), anti-Gr-1 (RB6–8C5), and anti-Ly6C (AL-21). In some experiments, 7-AAD (BD Biosciences) was used to exclude dead cells. Then, the cells were analyzed by a flow cytometer (FACS Verse; BD Biosciences). The data were analyzed with FlowJo software (Tree Star, Inc.).

Immunohistochemistry

To detect neutrophils, kidneys were snap-frozen in OCT compound. Six-micrometer-thick cryosections were fixed in ice-cold acetone. Endogenous peroxidase activity and nonspecific binding in the sections were blocked by 3% H₂O₂. The sections were then incubated with a Biotin Blocking System (DAKO). The sections were stained with biotinylated anti-Ly6G antibody (clone: 1A8) using a TSA BIOTIN SYSTEM Kit (PerkinElmer Life and Analytical Sciences, Inc.).

For the detection of GFP or YFP, tissues were fixed in 4% paraformaldehyde/4% sucrose in 0.1 M phosphate buffer (pH 7.2), snap-frozen in OCT compound, and subsequently submerged in 10% and 20% sucrose for 1–16 hours each. Fourteen-micrometer-thick cryosections were stained with biotinylated anti-GFP (Abcam, Inc.) using the TSA BIOTIN SYSTEM Kit. Sections were observed under a fluorescence microscope (Keyence) or a confocal microscope (Olympus). In some experiments, DyLight 488-labeled Lycopersicon Esculentum (Tomato) lectin (Vector Laboratories) was injected intravenously into mice 5 minutes before euthanasia to visualize blood vessel. The z stack of confocal images was processed using ImageJ software (National Institutes of Health).

In Vitro Endothelium Activation Assay

The UV-Φ2 cells, a mouse vascular endothelial cell line, were obtained from RIKEN Cell Bank. The cells were seeded (5×10⁶ cells/well) on six-well plates in DMEM/10% FCS and cultured overnight. Then, the cells were cocultured with total leukocytes obtained from either WT or DT-injected CD169-DTR mice for 18 hours.

In some experiments, to confirm the role of oxidants derived from leukocyte in the mechanism of ICAM-1 expression on the endothelial cells, butylated hydroxyanisole (100 μM; WAKO) was added into the coculture system. To exclude the influence of some humoral factors, endothelial cells and total leukocytes were cocultured using transwell plates (Corning). Endothelial cells were seeded (2.5×10⁶ cells/well) on the bottom chamber in DMEM/10% FCS and cultured overnight. Then, the total leukocytes obtained from either WT or DT-injected CD169-DTR mice were added to the upper chamber and cocultured for 18 hours. Total RNA was isolated with an RNAspin Mini RNA Isolation Kit (GE Healthcare). ICAM-1 mRNA levels were determined by quantitative PCR.

Monocyte Isolation Using Cell Sorter

For monocyte transfer experiments, peripheral blood was obtained from WT mice. Red blood cells were then lysed with 1× Pharmlyse (Pharmingen) or distilled water. For transfer of monocyte subsets, white blood cells were stained with FITC–anti-mouse Ly6C (AL-21), PE–anti-mouse Gr-1 (RB6–8C5), and APC–anti-mouse CD11b (M1/70). Then, Ly6Clo or Ly6Chi monocytes were sorted by a cell sorter (SH-800; SONY). Sorted monocytes (3.5–6.0×10⁶) were injected intravenously into a CD169-DTR mouse.

Statistical Analyses

For analyses, t test or ANOVA was used. Some datasets had a statistical difference in the variation between groups. The survival rate was estimated by the Kaplan–Meier method, and rates were compared using the log-rank test. Differences in values were considered significant at P<0.05.
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DISCLOSURES

None.

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