Nephrogenic Systemic Fibrosis Is Mediated by Myeloid C-C Chemokine Receptor 2

Catherine Do1,2,6, Viktor Drel2,6, Chunyan Tan2, Doug Lee2 and Brent Wagner3,4,5

Gadolinium-based contrast agents are implicated in several pathologic abnormalities (long-term retention in vital organs such as the skin and the brain) and are the cause of a sometimes fatal condition in patients, nephrogenic systemic fibrosis. Bone marrow–derived fibrocytes and the monocyte chemoattractant protein-1 inflammatory pathway have been implicated as mediators of the adverse effects induced by gadolinium-based contrast agents. Mechanistic studies are scant; therefore, a mouse model of nephrogenic systemic fibrosis was established. Dermal cellularity was increased in contrast-treated green fluorescent protein (GFP) chimeric mice. GFP in the skin and fibrosis were increased in the contrast-treated chimeric animals. Monocyte chemoattractant protein-1 and C-C chemokine receptor 2 were increased in the tissues from contrast-treated mice. C-C chemokine receptor 2–deficient bone marrow recipients of GFP-expressing marrow had an abrogation of gadolinium-induced pathology and displayed less GFP-positive cells in the skin. Wild-type animals that received C-C chemokine receptor 2–deficient bone marrow had a complete abrogation of dermal pathology. That GFP levels and expression increase in the skin, in tandem with a fibrocyte marker, supports the blood-borne circulating fibrocyte hypothesis of the disease. As of now, fibrocyte trafficking has yet to be demonstrated. Importantly, our data demonstrate that the monocyte chemoattractant protein-1/C-C chemokine receptor 2 axis plays a critical role in the pathogenesis of nephrogenic systemic fibrosis.

INTRODUCTION

Gadolinium-based contrast, essential for certain magnetic resonance imaging techniques (Diop et al., 2013), causes nephrogenic systemic fibrosis (NSF) in patients with renal insufficiency. This condition is characterized by a variable onset and symmetric involvement of the bilateral extremities. Skin biopsies exhibit fibrocyte markers, such as CD34, pro-collagen type I, and factor XIIIa, which implies that circulating, blood-borne fibrocytes mediate the disease (Cowper and Bucala, 2003). This has been demonstrated experimentally in rats, that is, a significant proportion of fibrotic lesions indeed involve myeloid fibrocytes (Wagner et al., 2012). However, there are no studies addressing the role of bone marrow in gadolinium-based contrast-induced disease.

In the dermis, gadolinium-induced fibrotic lesions are characterized by an increased number of CD34-positive spindle-shaped cells (Knopp and Cowper, 2008). Such CD34-positive, bone marrow–derived, and circulating cells have been named fibrocytes (Cowper et al., 2001). These fibrocytes are important in the proliferative stages of wound repair and are unique in that they are peripheral blood cells that have the potential to generate matrix (Quan et al., 2004). It has been postulated that these bone marrow–derived cells are the mediators of NSF (Jiménez et al., 2004; Quan et al., 2004).

We conducted experiments to demonstrate that a gadolinium-based contrast agent induces systemic fibrosis in mice. To gauge myeloid involvement in this process, mice with renal insufficiency underwent lethal irradiation followed by salvage bone marrow transplant from donors expressing green fluorescent protein (GFP). This permitted tracing the myeloid lineage of the skin cellularity. As the monocyte chemoattractant protein-1 (MCP-1) and its primary receptor, the C-C chemokine receptor 2 (CCR2), have been implicated in mediating the effects of gadolinium-based contrast agent–induced fibrosis (Drel et al., 2016), bone marrow transplantation experiments were performed to identify this system as a primary mechanism of the disease.

RESULTS

Ten percent of patients with NSF have never received hemodialysis (Rosenkranz et al., 2007); therefore, the disorder is not exclusive to those afflicted with end-stage renal disease. Wild-type mice (with normal renal function) were randomized into control and gadolinium-based contrast agent treatment groups. Skin fold thickness tended to be increased in the contrast-treated group (Figure 1a). Similar to what has been described in humans and in our own rat models (Do et al., 2014; Drel et al., 2016; Wagner et al., 2012), there was an increase in dermal cellularity (Figure 1b and c). The nuclei were dense and spindle-shaped, as we have reported in rats.
and this is similar to what has been described in patients with NSF (Sanyal et al., 2011), and there is increasing concern about metal retention in patients with normal renal function exposed to gadolinium-based contrast agents (McDonald et al., 2015). We have found evidence of gadolinium in nearly every organ tissue in contrast-treated rats (Do et al., 2014). Sections of paraffin-embedded skin were processed for transmission electron microscopy without heavy metal staining (e.g., without lead, osmium, or uranium) to assess for electron densities (Figure 1f). Several electron-dense materials, upon high magnification, demonstrated mesh-like nanowire and urchin-shaped structures. These same electron-dense crystalline nanostructures peppered the vacuoles of the renal proximal tubules (arrows). Electron-dense deposits demonstrated the presence of gadolinium as assessed by scanning transmission electron microscopy with energy-dispersive X-ray spectroscopy (Figure 1g). In total, these data demonstrate that gadolinium-based contrast agent treatment induced a hypercellular skin fibrosis with the formation of multinucleated giant cells and the presence of mesh-like nanowire electron densities concomitantly with gadolinium-enriched electron-dense deposits.

Since its discovery, the hypercellularity of NSF was theorized to be from bone marrow–derived fibrocytes (Wagner et al., 2016). This has been experimentally demonstrated in rats (Wagner et al., 2012), but not to date in mice. Lethally irradiated mice with five-sixths nephrectomy (to model renal insufficiency) were salvaged with tagged bone marrow in order to trace the lineage of infiltrating cells (Figure 2a). After several weeks for engraftment, recipients were randomized into control and gadolinium-based contrast agent treatment (2.5 mmol/kg intraperitoneally, 20 doses over 4 weeks) groups. At the endpoint, skin fold thickness was greater in the dermal contrast-treated recipients than the gadolinium-treated wild-type recipients (Figure 5e). Dermal fibronectin was similarly reduced in the contrast-treated CCR2-deficient recipients compared to the contrast-treated wild-type recipients (Figure 5d). In parallel, skin fibronectin and the myeloid marker, GFP, were reduced in the contrast-treated CCR2-deficient recipients compared to the contrast-treated wild-type recipients (Figure 5e). Dermal myeloid infiltration and α-smooth muscle actin-positive stress fibers were both suppressed in the CCR2-deficient recipients despite gadolinium-based contrast treatment (Figure 5f). Similarly, gadolinium-based contrast agent treatment led to less dermal CD34 and CD45RO when the recipients were deficient of the CCR2 (Figure 5g and h). Recipients devoid of CCR2 had a marked decrease in dermal CD163 expression in response to gadolinium-based contrast agent treatment (Figure 5i).

MCP-1 and its receptor, the CCR2, are elevated in the skin of contrast-treated rats (Drel et al., 2016). When gadolinium-based contrast-treated rats receive an antagonist of the CCR2, skin fibrosis and dermal cellularity are abrogated (Drel et al., 2016). Therefore, these effectors were examined in the skin of the chimeric mice (Figure 4). Both MCP-1 (Figure 4a) and the CCR2 (Figure 4b and c) were increased. These data imply that the MCP-1/CCR2 are involved in fibrocyte trafficking to the skin.

In order to validate the role of this receptor and the impact on bone marrow–derived fibrocyte trafficking, groups of wild-type (CCR2+/+) and CCR2-deficient (CCR2−/−) mice (with normal renal function) underwent lethal irradiation followed by salvage with bone marrow cells from GFP-expressing donors (Figure 5a). After an engraftment period, these groups were subrandomized to untreated and gadolinium-based contrast agent treatment groups (2.5 mmol/kg intraperitoneally, 20 doses over 4 weeks). The dermis from the contrast-treated CCR2-deficient recipients showed less cellularity than the gadolinium-treated wild-type recipients (Figure 5b and c). Dermal fibronectin was similarly reduced in the contrast-treated CCR2-deficient recipients with respect to the contrast-treated wild-type recipients (Figure 5d). In parallel, skin fibronectin and the myeloid marker, GFP, were reduced in the contrast-treated CCR2-deficient recipients compared to the contrast-treated wild-type recipients (Figure 5e). Dermal myeloid infiltration and α-smooth muscle actin were both suppressed in the CCR2-deficient recipients despite gadolinium-based contrast treatment (Figure 5f).

MCP-1 was increased in the dermis of the gadolinium-based contrast agent–treated wild-type recipients but not in the CCR2-deficient ones (Figure 5j). It was expected that the GFP-expressing bone marrow donor expressed the CCR2. In the wild-type recipients, gadolinium-based contrast agent treatment induced an increase in dermal CCR2; this effect was not evident in the receptor-deficient animals (Figure 5k).

To test the degree that myeloid CCR2 plays in mediating gadolinium-based contrast agent–induced systemic fibrosis,
bone marrow cells from CCR2-deficient mice (with normal renal function) were transplanted into lethally irradiated wild-type recipients (see Supplementary Figure S1a online). After an engraftment period, mice were randomized into untreated and contrast-treated groups (as above). Gadolinium content in flash-frozen skin, as assessed by inductively coupled plasma mass spectroscopy, was zero in the untreated group and 23 ± 1 (mean ± standard error) μg/g in the contrast-treated group.

There was no difference in dermal cellularity between the gadolinium-based contrast agent–treated and untreated recipients of the CCR2-deficient marrow (see Supplementary Figure S1b). Gadolinium-based contrast agent treatment failed to increase skin fibronectin content in the recipients of CCR2-deficient marrow (see Supplementary Figure S1c).

Comparing the untreated and contrast-treated groups, there was no difference in dermal α-smooth muscle actin (see Supplementary Figure S1d), CD34 (see Supplementary Figure S1e), CD163 (see Supplementary Figure S1f), or MCP-1 (see Supplementary Figure S1g). That the contrast agent–induced increase in dermal cellularity and development of fibrosis were completely abrogated in the recipients of CCR2-deficient marrow (when gadolinium was detectable in the tissue) demonstrates that the expression of MCP-1 receptor by myeloid cells is requisite for the disease despite the presence of gadolinium in the tissue.

In order to repeat this using a myeloid marker (analogous to our experimental method to date), lethally irradiated mice...
(with normal renal function) were salvaged with bone marrow cells from red fluorescent protein-expressing and CCR2-deficient donors (Figure 6a). After several weeks for engraftment, these animals were randomized into untreated and gadolinium-based contrast agent treatment groups (as above). Skin gadolinium (154, 156, and 157) was 36 ± 7 (mean ± standard error) μg/g in the contrast-treated group. There was no evidence of an increase in dermal cellularity in
the contrast-treated group (Figure 6b). Gadolinium-based contrast agent treatment did not induce fibronectin accumulation in the skin (Figure 6c and d). There was little increase in dermal CD163 in the contrast-treated animals (Figure 6e), and no increase of the fibrocyte markers CD34 or CD45RO (Figure 6f and g). The dermal expression of the myeloid marker, red fluorescent protein, was essentially the same in both groups (Figure 6h).

An in situ coculture assay (described previously by Drel et al. [2016]) was used to further demonstrate that the CCR2 is requisite for gadolinium-based contrast agent–induced myeloid infiltration in the skin (Figure 6c and d). There was little increase in dermal CD163 in the contrast-treated animals (Figure 6c), and no increase of the fibrocyte markers CD34 or CD45RO (Figure 6f and g). The dermal expression of the myeloid marker, red fluorescent protein, was essentially the same in both groups (Figure 6h).

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DISCUSSION

Up to 4.5 million Americans are exposed to gadolinium-based contrast agents annually (in non-federal hospitals) (FDA, 2017). The United States has a higher rate of utilization of magnetic resonance imaging than other industrialized countries (Papanicolas et al., 2018). As of this writing, the United States Food & Drug Administration Adverse Events Reporting System lists 3,094 total cases of NSF; these include 2,962 serious cases and 742 deaths. There are more sequelae than incurable systemic fibrosis that result from gadolinium-based contrast agent administration (Leyba and Wagner, 2019). The top five gadolinium-based contrast agents register 16,517 adverse event cases (57% of these being serious cases). Given the ubiquity and frequency of gadolinium-enhanced magnetic resonance imaging tests, the economic impact is large. Gadolinium-induced disease is a man-made entity. Medical science cannot be indifferent to the patients at risk or to those who are currently suffering from gadolinium-induced symptoms. Heretofore, there has yet to be a report of gadolinium-based contrast agents stimulating the recruitment of bone marrow–derived cells to an affected organ, the skin. This was demonstrable in both renal insufficiency and normal renal function. Multinucleated giant cells, similar to those observed in humans (Kim et al., 2006), were found in the dermis using this model. The morphology of the electron-dense deposits was similar to what has been described for gadolinium oxide in simulated phagolysosomal fluid in vitro (Li et al., 2014). That electron-dense deposits peppered these cells suggests a role in gadolinium-based contrast agent–induced pathology.
Hypercellular skin fibrosis and the formation of multinucleated giant cells—each found in our model—are characteristic of NSF. These data demonstrate that the dermal hypercellularity and fibrosis of NSF can be modeled in the mouse. The increase in the myeloid marker shows that gadolinium-based contrast agents promote the infiltration of bone marrow–derived cells—fibrocytes and CD163-positive macrophages—to the dermis. These experiments also suggest that gadolinium-based contrast agents can initiate pathologic responses in healthy individuals.

Gadolinium-based contrast agent–treated human peripheral blood mononuclear cells increase the expression of CD163, a marker of alternatively activated macrophages (Swaminathan et al., 2013). We found that myeloid CD163-positive cells also increase in the dermis of contrast-treated rats (Drel et al., 2016). Partial abrogation of dermal hypercellularity in contrast-treated chimeric GFP-expressing marrow to CCR2-deficient recipients and the complete abrogation of dermal cellularity and skin fibronectin in chimeric CCR2-deficient marrow to wild-type recipients demonstrate the importance of this C-C chemokine mechanism in gadolinium-induced fibrosis. Myeloid expression of the CCR2 is requisite for gadolinium-based contrast induction of systemic fibrosis and dermal hypercellularity even when gadolinium is present in the tissue. These studies now provide a new therapeutic target for gadolinium-induced diseases. Furthermore, that profound systemic effects are induced by gadolinium whether renal insufficiency is present or not should serve to alert patients and clinicians to the biologic activity of these compounds.

The CCR2 is important for the recruitment and activation of lung fibrocytes in a mouse model of pulmonary fibrosis (Moore et al., 2005). In a murine ureteral ligation model of renal fibrosis, there is an infiltration of CD45<sup>+</sup> fibrocytes, and a large subset of these express the CCR2 (Sakai et al., 2006). Antagonism of the MCP-1/CCR2 system did reduce kidney fibrosis in a unilateral ureteral obstruction model (Kitagawa et al., 2004). Similar to NSF, scleroderma is characterized by dermal fibrosis (Boin and Hummers, 2008). MCP-1 and the CCR2 are involved in bleomycin-induced scleroderma (Yamamoto and Nishioka, 2003) and cutaneous sclerosis (Yamamoto, 2003).

Gadolinium has been found in the brains of patients (on autopsy) with normal renal function several months after exposure to magnetic resonance imaging contrast (Kanda et al., 2015). This appears to be irrespective of the chemical formulation of the gadolinium-binding ligand (McDonald et al., 2017). Sea urchin-shaped mesh-like nanowire structures, similar to what we report in the skin, have now been found in brain tissue of humans exposed to gadolinium-based contrast agents (Rasschaert et al., 2018).

It has yet to be proven that gadolinium (dechelated or bound with the chaperone) residing in the target organ is what triggers the systemic fibrosis. We know that priming bone marrow with prior gadolinium-based contrast exposure is not sufficient to replicate the disease in contrast-naive recipients (yet such priming does increase the severity once recipients are exposed to contrast) (Drel et al., 2016). Gadolinium concentrations are higher in affected skin than in non-affected skin in patients with NSF (71.4 ± 89.4 mg/g).
Figure 5. The role of host CCR2 in systemic fibrosis induced by gadolinium-based contrast agent.

(a) Recipients that expressed CCR2 (CCR2⁺) or were devoid of this receptor (CCR2⁻) were lethally irradiated and salvaged with bone marrow from GFP-expressing donors. After an engraftment period, these recipients were randomized to non-treatment or contrast treatment.

(b) H&E. (c) Dermal nuclear quantification was performed in three randomly chosen areas from each group (n = 3 each). (d) Fibronectin expression by immunofluorescence analysis. (e) Immunoblot of skin fibronectin. (f–k) Immunofluorescence analysis of GFP (f, g, h, j), α-SMA (f), CD34 (g), CD45RO (h), CD163 (i), MCP-1 (i), and CCR2 (k). **P < 0.01, ***P < 0.001, analysis of variance and Tukey's Honest Significant Difference post hoc test. Bars = 0.05 mm. CCR2, C-C chemokine receptor 2; GFP, green fluorescent protein; H&E, hematoxylin and eosin; α-SMA, α-smooth muscle actin.
Figure 6. The role of myeloid CCR2 in systemic fibrosis induced by gadolinium-based contrast agent.

(a) Wild-type mice (n = 20) underwent lethal irradiation followed by salvage bone marrow transplantation from CCR2-deficient mice expressing red fluorescent protein (Jackson Laboratory, stock 017586). Weeks after engraftment, mice were randomized to non-treated and contrast-treated (n = 10, each).

(b) Representative dermal histology with H&E staining. Bar chart depicts quantification of dermal cellularity (triplicate randomly chosen fields, n = 3).

(c) Immunoblot of skin fibronectin. (d–g) Immunofluorescence expression analysis of dermal fibronectin (d), dermal CD163 (e), dermal CD34 (f), and CD45RO (g). (h) Red fluorescent protein—the myeloid marker—expression by immunofluorescence. Bars = 0.05 mm. CCR2, C-C chemokine receptor 2; H&E, hematoxylin and eosin.
Table 1. Immunofluorescence stain synopsis

<table>
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<th>Marker (Code)</th>
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<th>Company</th>
<th>City, State</th>
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<td>1:100</td>
<td>Abcam</td>
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<tr>
<td>CD34 (ab81289)</td>
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<tr>
<td>CD163 (bs-2527R)</td>
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<td>Bioss</td>
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<tr>
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Abbreviations: CCR2, C-C chemokine receptor 2; GFP, green fluorescent protein; MCP-1, monocyte chemoattractant protein-1; α-SMA, α-smooth muscle actin.

compared to 23.1 ± 26.6 [mean ± SD] μg/g, respectively (Christensen et al., 2011). These concentrations match what we found in our samples.

Our mouse model provides a method of studying the mechanism of gadolinium-based contrast-induced disease and demonstrates the centrality of the CCR2 to this disease process. Gadolinium-based contrast treatment induces the myeloid cellular infiltration of a target organ, the skin, in a systemic manner. Furthermore, our experiments pinpoint the CCR2 as a critical mediator of fibrocyte trafficking. This work is limited by the concentrations of gadolinium-based contrast agent and the repetitive dosing. Nonetheless, elucidation of this pathway may permit sharply focusing on the multitude of undiscovered risk factors that permeate in patients with gadolinium-induced affictions. There is a thread that consists of gadolinium-based contrast agents, the CCR2, and myeloid cell-induced fibrosis. This study provides a rational target for therapy in gadolinium-based contrast agent—induced diseases.

MATERIALS AND METHODS

Animals

All experimental procedures and protocols were in accordance with the Guide for the Care and Use of Laboratory Animals published by the NIH and approved by the Institutional Animal Care and Use Committee. Endpoints were the following: (i) weight loss of 10%, (ii) dermatologic findings previously described (Wagner et al., 2012), or (iii) completion of 4 weeks of contrast treatment (unless otherwise noted). Animals were examined daily for any signs of systemic fibrosis.

NSF model

Analogous to the gadolinium-based contrast agent—induced systemic fibrosis model established in rats (Do et al., 2014; Drel et al., 2016; Wagner et al., 2012; Wagner et al., 2016), weight-matched female C57 black mice 33–34 weeks of age were randomized to control and gadolinium-based contrast agent (Omniscan—gadodiamide/caldiamide—2.5 mmol/kg intraperitoneally, 5 doses per week aiming for 40 doses over 8 weeks or gross evidence of systemic effect).

GFP chimeric mice

For all bone marrow transplant experiments, fully acclimated (either C57 black or on a C57 black background) mice underwent lethal irradiation (950 rads divided into two sessions spaced 4 hours apart [GammaCell 40, Atomic Energy of Canada Limited, Mississauga, Canada]) followed by bone marrow cells (1 × 10⁷) harvested from donors. Recipients were the following: (i) wild-type mice with 5/6 nephrectomies at 10–12 weeks salvaged with GFP-expressing marrow (C57BL/6-Tg(CAG-EGFP)1130s8/LeySopJ [The Jackson Laboratory, Bar Harbor, ME]), (ii) CCR2-deficient mice salvaged with GFP-expressing marrow, and (iii) wild-type mice salvaged with CCR-deficient marrow (C57BL/6-129S4-Ccr2tmIfc/J and C57BL/6-6.129(C57-BL/6)-129(S4)-Ifc). For the first group, partial nephrectomies were performed at 10–12 weeks following by 2 weeks acclimation. For all groups, 4 weeks were used for engraftment, and then animals were randomized into control or gadolinium contrast-treated groups (2.5 mmol/kg intraperitoneally, aiming for 20 doses over 4 weeks [Omniscan, General Electric HealthCare, Little Chalfont, UK]).

Tissue fixation, sectioning, and histology

Sections of dorsal skin were dissected off the underlying fascia and sliced into equal pieces. These were processed and the dermal nuclei were quantified as previously described (Do et al., 2014; Wagner et al., 2012). A portion was fixed in 10% neutral-buffered formalin, and the remnant was flash-frozen (for immunoblot or immunofluorescence). Unless otherwise noted, all photomicrographs were obtained with a Nikon Eclipse 55i with a ×40 objective.

Immunofluorescence

Paraffin-embedded tissue was sectioned on glass slides, deparaffinized in xylene, and rehydrated. Tissues were incubated with citric acid-based unmasking solution (Vector Laboratories, Burlingame, CA) and blocked with 1% BSA and 10% goat serum in phosphate buffered saline (pH 7.4) for 2 hours. Antibodies are listed in Table 1. For mouse antibodies, blocking was with a mouse on mouse immunodetection kit (Vector Laboratories) per the manufacturer’s instructions. Goat secondary antibodies to mouse and rabbit immunoglobulins were AlexaFluor594 and AlexaFluor488 (Thermo Fisher Scientific Life Sciences, Waltham, MA).

Immunoblot

Tissue was homogenized in radioimmunoprecipitation assay buffer as previously described (Do et al., 2014; Wagner et al., 2012). Antibodies for fibronectin (F3648) were obtained from Sigma-Aldrich (St. Louis, MO), CCR2 (3415R) from Biovision Inc (Milpitas, CA), GFP (ab290) and collagen type I (ab34710) from Abcam (Cambridge, MA), and glyceraldehyde 3-phosphate dehydrogenase from Santa Cruz Biotechnology (Dallas, TX).

Statistics

Unless otherwise indicated, multigroup comparisons were analyzed using analysis of variance with Tukey’s Honest Significant Difference post hoc test using the statistical software R, version 3.4.3.

Data availability statement

Datasets related to this article can be found at https://digitalrepository.unm.edu/hsc_data/KINM/1/ hosted at the University of New Mexico (Wagner, 2019).

ORCIDs

Brent Wagner: https://orcid.org/0000-0002-7063-0142
Catherine Do: https://orcid.org/0000-0002-9688-1820
Viktor Drel: https://orcid.org/0000-0003-4542-0132
Chunyan Tan: https://orcid.org/0000-0003-4789-8527
Doug Lee: https://orcid.org/0000-0002-8018-4043
CONFLICT OF INTEREST

BW serves as an expert witness for a firm representing patients with adverse events from gadolinium-based contrast agents. The remaining authors do not have any relationships that pose conflicts of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: BW, YG; Data Curation: BW, CD; Formal Analysis: BW; Funding Acquisition: BW; Investigation: BW, YG, CD, CT, DL; Methodology: BW, Resources: BW; Supervision: BW; Visualization: BW; Writing - Original Draft Preparation: BW; Writing - Review and Editing: YG

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at 10.1016/j.jid.2019.03.1145.

REFERENCES

Supplementary Figure S1. The role of myeloid CCR2 in systemic fibrosis induced by gadolinium-based contrast agent. (a) Wild-type mice (n = 4) were lethally irradiated and salvaged with bone marrow from CCR2-deficient donors. After several weeks for engraftment, they were randomized to non-treated and contrast-treated. (b) Dermal histology with H&E staining. Bar chart depicts quantification of dermal nuclei per HPF of triplicate randomly chosen fields. (c) Immunoblot of fibronectin expression in the skin. (d–g) Immunofluorescence expression of α-SMA (d), dermal CD34 (e), dermal CD163 and CD45RO (f), and dermal MCP-1 (g). Bars = 0.05 mm. CCR2, C-C chemokine receptor 2; H&E, hematoxylin and eosin; HPF, high-power field; MCP-1, monocyte chemoattractant protein-1; α-SMA, α-smooth muscle actin.
Supplementary Figure S2. CCR2 is required for gadolinium-based contrast agent–induced myeloid cell infiltration in the skin. Immunofluorescence analysis of bone marrow cells that were isolated from wild-type and CCR2-deficient mice, stained with CFSE, and cocultured (50,000 cells per well) with 3 mm skin punches overnight. Bar = 0.05 mm. CFSE, 5(6)-carboxyfluorescein diacetate N-hydroxysuccinimidyl ester.