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Original Article

Implications for the role of macrophages in a model of myocardial fibrosis: CCR2$^{-/-}$ mice exhibit an M2 phenotypic shift in resident cardiac macrophages

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ABSTRACT

Background: Macrophages (MΦ) are functionally diverse and dynamic. Until recently, cardiac MΦ were assumed to be monocyte derived; however, resident cardiac MΦ (rCMΦ), present at baseline, were identified in myocardia and have been implicated in cardiac healing. Previously, we demonstrated that CCR2$^{-/-}$ mice are protected from myocardial fibrosis — an observation initially attributed to changes in infiltrating monocytes. Here, we reexplored this observation in the context of our new understanding of rCMΦ.
Methods: Male CCR2$^{-/-}$ and C57BL/6 hearts were digested and purified to a single cell suspension, incubated with fluorophore-linked antibodies (CCR2, CX3CR1, CD11b, Ly6C, TNF-α, and IL-10), and assessed by flow cytometry. Differentiated MΦ were cocultured with fibroblasts in order to characterize how MΦ phenotype influences fibroblast activation. Fibroblasts were characterized for their expression of smooth muscle cell actin (SMA).
Results: A significant decrease in Ly6C expression was observed in the CCR2$^{-/-}$ cardiac MΦ population relative to WT, which corresponded with significantly lower TNF-α expression and significantly higher IL-10 expression. Using in vitro coculture system, classical MΦ promoted fibroblast activation relative to nonclassical MΦ.
Conclusion: CCR2$^{-/-}$ rCMΦ favor a more antiinflammatory phenotype relative to WT controls. Moreover, a shift toward the antiinflammatory promotes proliferation, but not activation in vitro. Together, these observations suggest that antiinflammatory cardiac MΦ populations may inhibit myocardial fibrosis in a pathological setting by preventing the activation of fibroblasts.
News and noteworthy: Here, we provide novel evidence for baseline differences in rCMΦ phenotypes (i.e. classical vs. nonclassical) and how these differences could modulate cardiac healing. Importantly, we observed differences in how classical vs. nonclassical MΦ influenced fibroblast activation, which could, in turn, affect fibrosis.

1. Introduction

MΦ are important mediators in tissue development, homeostasis, and disease progression [1]. As resident MΦ localize to different tissues during embryonic development, they acquire tissue/organ-specific functions [2–5]. These tissue/organ-specific MΦ are now known to exhibit distinct functions and phenotypes in tissues including the central nervous system, liver, spleen, lungs, peritoneum, skin, and myocardium. This is particularly important in tissues like myocardium, in which the ability to regenerate tissue after injury is assumed to be minimal [6]. Moreover, circulation can contribute to tissue MΦ populations in function-specific manors, such that monocyte-derived MΦ can promote, resolve, or regulate inflammation [7,8].

Until recently, cardiac MΦ were thought to be exclusively derived from circulating monocytes during injurious stimuli [9]. This was supported by extensive research in different cardiac models, including angiotensin II (AngII) infusion and myocardial infarction, in which MΦ inundate the heart from circulating monocytes [7,8,10]. Using a GFP-linked CX3CR1, Pinto et al. demonstrated a CX3CR1^{+} MΦ population in the adult murine myocardium that persisted at baseline and was distinct from bone marrow-derived monocytes [9]. This observation has generated a resurgence in cardiac MΦ research that has focused on their phenotype, function, turnover, and etiology [1–4,7–28]. As our understanding of MΦ evolves, researchers have been increasingly able to separate the MΦ into a broad phenotypic spectra [13]. Notably, MΦ could be differentiated by their expression of the chemokine receptors CCR2 and CX3CR1 [8,14]. The *classical* MΦ phenotype expressed high levels of CCR2 and low level of CX3CR1. Conversely, the *nonclassical* MΦ phenotype was either CCR2 low or negative and expressed high

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levels of CX3CR1. The nonclassical population was associated with a mature phenotype, typically observed in both late-infiltrating phases and resident populations [8,9].

rCMΦ have since been separated into at least four distinct populations that can be distinguished based on their expression of markers including CX3CR1, MHC class II, Ly6C, CD11b, CD64, CD11c, and CD206 [9,11,15,16]. Molawi et al. have classified the populations into (1) CX3CR1$^-$MHC II$^-$, (2) CX3CR1$^-$MHC II$^+$, (3) CX3CR1$^+$MHC II$^-$, and (4) CX3CR1$^+$MHC II$^+$, which we have also characterized by CX3CR1, CCR2, CD11b, and Ly6C. rCMΦ originate during embryonic development as CX3CR1$^+$MHC$^-$ cells. Notably, these embryonic-derived rCMΦ can confer protection against and favor resolution of injury by promoting cardiac regeneration and preventing remodeling [16,12]. While still under debate, rCMΦ appear to be replenished primarily from circulating CCR2$^+$ monocytes that express CX3CR1 and MHC class II [16]. Despite extensive work to characterize rCMΦ phenotypes, the functions of these different populations remain poorly characterized, particularly in models of disease.

It is known that animals lacking CCR2 exhibit impaired ability to turnover rCMΦ from circulating monocytes [16]. Moreover, work in our laboratory using CCR2$^{-/-}$ mice suggested that these animals were partially protected from AngII-mediated inflammation and fibrosis [17,18]. When we first described the benefits of CCR2$^{-/-}$ deficiency, we did not appreciate or explore the role of this genetic defect on resident cardiac MΦ. Notably, early MΦ infiltrate did not significantly differ from WT mice and the underlying mechanism(s) for the benefits in CCR2$^{-/-}$ mice remained unclear. Given the close relationship between MΦ activation and cardiac healing, comparing baseline resident cardiac MΦ populations between CCR2$^{-/-}$ and WT mice warrants investigation.

In order to elucidate the role of CCR2 on rCMΦ phenotype, we compared myocardial tissue and rCMΦ from CCR2$^{-/-}$ mice relative to WT controls. When we discovered that CCR2$^{-/-}$ displayed a significant shift in rCMΦ Ly6C expression relative to WT, we investigated the functional significance of such a shift in terms of MΦ phenotype and how this would influence fibroblast activation and, thus, fibrotic remodeling.

2. Methods

2.1. Mice

Animal experiments were performed in accordance with the Canadian Council on Animal Care and approved by the Dalhousie *University Committee on Laboratory Animals*. Male C57BL/6 mice (8–10 weeks old; wild type: WT) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and male CCR2$^{-/-}$ mice (8–10 weeks old) on a C57BL/6 background were provided by one of the authors (T.I.) from a routinely genotyped colony. The absence of CCR2 was confirmed using conventional PCR, as previously demonstrated [17].

Mice were housed in the Carleton Animal Care Facility at Dalhousie University and provided food and water ad libitum. At the time of harvest, animals were weighed and hearts were extracted, flushed with saline, and weighed.

2.2. Immunohistochemistry

Hearts were processed in 10% buffered formalin overnight and paraffin embedded. Serial sections (5 μm) were stained for histological analysis. Basic myocardial histology and cellular infiltrate were assessed using heart sections stained with hematoxylin and eosin (H&E). The area of the heart affected was calculated as previously described [17,19,20]. Fibrotic deposition was examined using heart sections stained with picrosirius red (SR) and the counter stain fast green (FG). Collagen content was semiquantified by photographing representative SR/FG whole-heart sections at magnification ×5. Using Adobe Photoshop CS6, red pixels were positively selected and summed for a total number of red (collagen) pixels. Subsequently, nonbackground pixels were summed for the total heart pixels. Collagen pixels were divided by the total heart pixels to provide a semiquantitative measurement of the percent collagen content in the heart. All tissues were processed simultaneously for SR/FG and the same red color palette was used to select red pixels.

Immunofluorescence was performed on fixed cocultures for KI-67 (eBioscience, San Diego, CA, USA), collagen type I (Rockland, Gilbertsville, PA, USA), and α-smooth muscle cell actin (α-SMA; Sigma Aldrich) [15]. In brief, cells were permeabilized with 0.1% Triton-X-100/PBS, blocked with 5% BSA/PBS, and then incubated with primary antibodies overnight at 4°C. Sections were then incubated with AlexaFluor488, AlexaFluor555, or AlexaFluor647. Nuclei were stained with Hoechst. Slides were visualized using a Zeiss Axiovert 200M and photographed with a Hamamatsu Orca R2 Camera.

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2.3. Coculture

The MΦ fibroblast coculture was conducted as previously described with modification [21,22,15]. Bone marrow-derived MΦ were generated as previously described [23]. In brief, animals were anesthetized then sacrificed and the femurs and tibias were isolated. Cells were flushed from the marrow using a 30G needle attached to a syringe containing DMEM (Dulbecco's modified Eagle media) complete (DMEM-C: DMEM, L-glutamine, Pen Strep, 10% FBS). Following washes and cell straining, bone marrow-derived MΦ were resuspended in DMEM complete with 15% L929 conditioned medium and plated in T75 flasks. Media was replaced on day 3. On day 7, media was changed to (1) DMEM-C for control, (2) DMEM-C + 100 ng/ml LPS + 10 ng/ml IFN-γ for M1 differentiation, or (2) DMEM-C + 10 ng/ml IL-4 for M2 differentiation. On day 9, MΦ populations were lifted using 0.25% trypsin, washed with DMEM-C, counted, and replated in 12-well plates at a density of 2×10^5/well. Samples of MΦ populations were screened for F4/80, CD11b, Ly6C, CD206, TNF-α, and IL-10 expression using flow cytometry as described below. NIH/3T3 (ATCC, Manassas, VA, USA) served as fibroblasts in monocultures and cocultures. NIH/3T3 were maintained in DMEM-C until lifted using 0.25% trypsin, washed with DMEM-C, counted, and replated at 2×10^5/well. Monocultures and cocultures were incubated for 72 h, at which point the supernatant was removed and the cells were washed with PBS then fixed in 4% PFA. The cells were immunofluorescently labeled as described above and wells were read for fluorescence using a Tecan infinite M200 Pro (Tecan, Männedorf, Germany) plate reader. α-SMA expression was standardized to DAPI fluorescence intensity for cocultures and fibroblast monocultures. The coculture expression was then calculated relative to fibroblast monoculture expression. Immunofluorescence and flow cytometry were used to quantify cell purity.

2.4. Isolation of cells from myocardium

Hearts from WT and KO mice were harvested under sterile conditions and used for cell isolation as previously described with modification [17,24]. In brief, hearts were mechanically and enzymatically digested in a collagenase solution (1 mg/ml collagenase II; Cedarlane Laboratories, Ltd., Burlington, ON, Canada) in DMEM-C at 37°C, with agitation for 45 min. Cell isolates were twice washed in DMEM-C and purified over a Percoll gradient (30% and 70%). Cells were then counted and used for flow cytometric analysis. Cells were washed with FACS buffer (DPBS, 1% BSA, 0.1% NaN_3) and incubated the antibodies αCD11b-APC (eBioscience, San Diego, CA, USA), αF4/80-PerCP-Cy5.5 (Biolegend, San Diego, CA, USA), αCX3CR1-PerCP-Cy5.5 (Biolegend), αCCR2-FITC (Biolegend), αTNF-PE (Biolegend), αIL-10-PE (Biolegend), and αLy6C-PE (Biolegend). Following incubation, cells were twice washed with FACS buffer and fixed with 1% formalin/FACS buffer. Isotype control (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was