Nitric oxide synthase 2 is required for conversion of pro-fibrogenic inflammatory CD133\(^+\) progenitors into F4/80\(^+\) macrophages in experimental autoimmune myocarditis

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Aims
Experimental autoimmune myocarditis (EAM) model mirrors important mechanisms of inflammatory dilated cardiomyopathy (iDCM). In EAM, inflammatory CD133\(^+\) progenitors are a major cellular source of cardiac myofibroblasts in the post-inflammatory myocardium. We hypothesized that exogenous delivery of macrophage-colony-stimulating factor (M-CSF) can stimulate macrophage lineage differentiation of inflammatory progenitors and, therefore, prevent their naturally occurring myofibroblast fate in EAM.

Methods and results
EAM was induced in wild-type (BALB/c) and nitric oxide synthase 2-deficient (Nos2\(^-/-\)) mice and CD133\(^+\) progenitors were isolated from inflamed hearts. In vitro, M-CSF converted inflammatory CD133\(^+\) progenitors into nitric oxide-producing F4/80\(^+\) macrophages and prevented transforming growth factor-β-mediated myofibroblast differentiation. Importantly, only a subset of heart-infiltrating CD133\(^+\) progenitors expresses macrophage-specific antigen F4/80 in EAM. These CD133\(^+\)/F4/80\(^+\) cells show impaired myofibrogenic potential compared with CD133\(^+\)/F4/80\(^-\) cells. M-CSF treatment of wild-type mice with EAM at the peak of disease markedly increased CD133\(^+\)/F4/80\(^+\) cells in the myocardium, and CD133\(^+\) progenitors isolated from M-CSF-treated mice failed to differentiate into myofibroblasts. In contrast, M-CSF was not effective in converting CD133\(^+\) progenitors from inflamed hearts of Nos2\(^-/-\) mice into macrophages, and M-CSF treatment did not result in increased CD133\(^+\)/F4/80\(^+\) cell population in hearts of Nos2\(^-/-\) mice. Accordingly, M-CSF prevented post-inflammatory fibrosis and left ventricular dysfunction in wild-type but not in Nos2\(^-/-\) mice.

Conclusion
Active and NOS2-dependent induction of macrophage lineage differentiation abrogates the myofibrogenic potential of heart-infiltrating CD133\(^+\) progenitors. Modulating the in vivo differentiation fate of specific progenitors might become a novel approach for the treatment of inflammatory heart diseases.

Keywords
Experimental autoimmune myocarditis • CD133 progenitor • M-CSF • Myofibroblast • Macrophage • Nitric oxide synthase 2

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1. Introduction

Inflammatory dilated cardiomyopathy (iDCM) is an important cause of heart failure and sudden death in children and young patients.\textsuperscript{1} Progressive cardiac dilation and fibrosis are hallmarks of iDCM in humans. iDCM refers to an end-stage heart failure phenotype that often results from virus-triggered myocarditis.\textsuperscript{2} Both clinical observations and animal experiments suggest that infection-triggered autoimmune plays an important role in iDCM.\textsuperscript{3} Heart-specific autoimmune responses are a consequence of the lack of T cell tolerance to heart-specific alpha-myosin heavy chain (αMyHC) in mice and in humans.\textsuperscript{4}

Experimental autoimmune myocarditis (EAM) is a CD4\textsuperscript{+} T cell-mediated mouse model of iDCM. In susceptible mouse strains, EAM is commonly induced after immunization with αMyHC peptide together with a strong adjuvant.\textsuperscript{5} In BALB/c mice, the extent of heart failure phenotype is commonly induced after immunization with αMyHC peptide emulsified 1:1 with Freund’s Adjuvant (CFA, Difco) on Days 0 and 7. M-CSF (200 μg/kg (Peprotech)) was iv injected every second day between Days 21–29 or Days 40–48 of EAM. Control mice received solvent only.

2. Methods

2.1 Mice

BALB/c (n = 250) and Nos2\textsuperscript{−/−} (n = 50) mice on BALB/c background were used in this study. Animal experiments were performed in accordance with the Swiss federal law and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). All animal experiments were approved by the Cantonal Veterinary Office in Zurich.

2.2 EAM induction and cytokine treatments

Mice were injected subcutaneously with 150 μg of αMyHC (Ac-RSLKLMATLFLTYASADROH; Caslo) peptide emulsified 1:1 with Complete Freund’s Adjuvant (CFA, Difco) on Days 0 and 7. M-CSF (200 μg/kg (Peprotech)) was iv injected every second day between Days 21–29 or Days 40–48 of EAM. Control mice received solvent only.

2.3 Histopathology and immunocytochemistry

Hearts were formalin-fixed and paraffin embedded. Heart sections were stained with rat anti-mouse CD45 (BD Bioscience), rat anti-mouse CD3 (Neomarkers), rat anti-mouse F4/80 (BMA biomedicals) and rabbit anti-rat IgG (Abcam) antibodies, and with the Bond Polymer Refine Detection kit using the BOND-MAX system (both Leica). Masson’s Trichrom staining was used to detect fibrosis. Immunopositive cells and fibrotic areas were quantified using analysis software (VIVE software (Olympus)).

2.4 Echocardiography

Mice were lightly anaesthetized with 1–1.5% isoflurane, maintaining the heart rate at 400 – 500 b.p.m.

Transthoracic echocardiography was performed using a 30 MHz probe and the Vevo 770 Ultrasound system (VisualSonics) as described\textsuperscript{17} and in Supplementary Material online.

2.5 Cell cultures

Myocarditis-positive hearts were perfused, dissected, and digested with Liberase Blendzyme (Roche) for 45 min at 37°C and tissue suspensions were passed sequentially through 70 and 40 μm cell strainers. Cardiomyocytes were separated by low speed centrifugation (50 g, 2 min). CD133\textsuperscript{+} cells were isolated by positive selection using anti-CD133-PE antibody (eBioscience) and magnetic anti-PE-microbeads (Miltenyi) or using FACS. Enriched CD133\textsuperscript{+} cells were plated onto gelatine-coated cell culture plates and cultured in the Iscove’s Modified Dulbecco’s Medium supplemented with 20% foetal bovine serum, 1:100 penicillin/streptomycin, 100 mM non-essential amino acids, 100 mM sodium pyruvate, and 50 mM β-mercaptoethanol (all Invitrogen). Macrophage differentiation was induced with 10 ng/mL M-CSF, and fibroblast differentiation with 10 ng/mL TGF-β (both PeproTech).

2.6 Flow cytometry and cell sorting

Single cell suspensions were prepared from digested hearts and cultured cells. Cells were incubated 30 min on ice with the appropriate combination of fluorochrome- or biotin-conjugated antibodies. The following antibodies were used: anti-CD45-FITC, anti-CD45-PE, anti-NOS2-FITC (all BD Bioscience), anti-CD133-PE, anti-F4/80-PE (both eBioscience), biotin-conjugated anti-F4/80 (Cedarlane), anti-CD16/32 (Milteny), anti-CD206 (Biologend), anti-CD301 (SeroTec), and anti-CD133 (eBioscience). Streptavidin-APC (BD Bioscience) was used to detect biotin-conjugated antibodies. Cells were analysed with the FACS Canto

exogenous cytokine treatments may change the default differentiation fate of inflammatory progenitors and can prevent pathological tissue remodelling in iDCM.
2.7 Quantitative RT–PCR
Total RNA was isolated using the RNeasy micro kit (Qiagen). cDNAs were amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and oligonucleotide primers to transcribe the detected genes using the 7500 Fast Real-Time PCR System (Applied Biosystems). The following oligonucleotide primers were used in this study: α-SMA (Acta2): 5′-cgtgctgcaaacctgaga-3′, 5′-cgaacgctgccttacaag-3′; collagen I (Col1a1): 5′-gtatgacgccgtcaatgaa-3′, 5′-cctctgcagccactcatcttcgta-3′; fibronectin (Fn1): 5′-taccaaggtcaatccacacccc-3′, 5′-cagatggcaaaagaaagcagagg-3′; gapdh (Gpdh): 5′-ctgaccacacacgccttacaag-3′, 5′-ggctgctctggctgctagag-3′. Transcript levels of gapdh were used as an endogenous reference, and relative gene expression was analysed using the 2−ΔΔCt method.

2.8 Immunohistochemistry and phagocytosis assays
Cells were cultured on gelatin-coated cover slips and fixed with 4% paraformaldehyde or methanol/acetic acid (7:3). One per cent bovine serum albumin was used as blocking solution. The following antibodies were used: mouse IgG anti-α-SMA (Sigma), rabbit IgG anti-fibronectin (Milenpor), AlexaFluor488 anti-mouse IgG, and AlexaFluor546 anti-rabbit IgG 1:600 (both Invitrogen). 4′,6-diamidino-2-phenylindole was used to label nuclei. Alexa Fluor 488-conjugated Escherichia coli BioParticles (Invitrogen) were used according to manufacturer’s recommendations. Immunofluorescence was analysed using the Olympus BX51 fluorescence microscope.

2.9 Statistics
Normally distributed data were analysed by the unpaired, two-tailed Student’s t-test. Severity scores were analysed by the one-way Kruskal−Wallis analysis. For correlation analysis, Pearson’s correlation coefficient was calculated. All analyses were computed using the GraphPad Prism 5 software. Differences were considered as statistically significant for $P < 0.05$.

3. Results
3.1 M-CSF converts heart-infiltrating CD133+ progenitors into functional F4/80+ macrophages, and prevents TGF-β-induced myofibroblast differentiation in vitro
BALB/c mice receiving two subcutaneous injections of αMyHC/CFA at Days 0 and 7 develop severe myocarditis at Day 21. At this stage, CD133+ progenitors represent ~30% of all CD45+ inflammatory cells in the heart. To assess the differentiation capacity of these cells at the peak of disease, CD133+ cells were isolated from diseased hearts at Day 21 of EAM, plated, and expanded for 2 weeks in vitro. Expanded cells were cultured in the presence or absence of M-CSF or TGF-β for additional 14 days. In the presence of TGF-β, CD133+ cells differentiated into myofibroblasts, positive for α-SMA and fibronectin (Figure 1A and B). In contrast, the addition of M-CSF failed to trigger myofibroblast differentiation (Figure 1A and B) and pre-treatment of CD133+ cells with M-CSF for 3 days prevented TGF-β-triggered myofibroblast formation (Figure 1C and D).

In cytokine-free cultures, most expanded cells remained positive for CD133 and were mainly negative for macrophage marker F4/80 (Supplementary material online, Figure S1A and B). In the presence of M-CSF, however, CD133 expression was reduced (Supplementary material online, Figure S1A) and cells up-regulated F4/80, CD11b, and CD16/32, suggesting macrophage lineage differentiation (Supplementary material online, Figure S1B–D). Cells cultured with M-CSF developed into fully active macrophages producing NO2, secreting nitric oxide, and phagocyting Escherichia coli bacteria (Supplementary material online, Figure S1E, F, and J). Of note, M-CSF treatment did not affect expression of markers characteristic for alternatively activated M2 macrophages (CD206, CD301, Supplementary material online, Figure S1G–H) or markers characteristic for granulocytes or dendritic cells, i.e. Gr-1 (Ly6C, Supplementary material online, Figure S1J), MHC class II and CD11c (not shown).

To address the immunomodulating potential of CD133+ progenitors and CD133+-derived M-CSF-differentiated macrophages, we co-cultured them with αMyHC-reactive Th17 cell lines in the presence of αMyHC-pulsed irradiated splenocytes as antigen presenting cells. On irradiated splenocytes, αMyHC-reactive CD4+ T cells proliferated rapidly. In the presence of CD133+ progenitors or M-CSF-differentiated macrophages, however, proliferation was completely abolished (Supplementary material online, Figure S2A). Further, we induced EAM in BALB/c mice and additionally administered iv 2 × 106 of either in vitro expanded CD133+ progenitors or CD133+-derived mature macrophages at Day 7 after EAM induction and analysed myocarditis severity at Day 21. In animals treated with both CD133+ progenitors and M-CSF-differentiated macrophages, practically no myocarditis was observed compared with sham-treated mice (Supplementary material online, Figure S2B). In contrast, the administration of M-CSF-differentiated macrophages expanded from Nos2+− mice failed to protect from myocarditis (Supplementary material online, Figure S2C). These latter findings confirm the protective and NO2-dependent role of CD133+-derived M-CSF-differentiated macrophages in EAM.

3.2 M-CSF treatment promotes accumulation of F4/80+ macrophages in the post-inflammatory heart
At Day 21, inflamed hearts contained a substantial pool of CD133+ progenitors, but fibrosis was not evident at this time point. As illustrated above, M-CSF effectively directs CD133+ progenitors into functional macrophages and prevents their myofibroblast differentiation. We, therefore, analysed how systemic M-CSF treatment of αMyHC/CFA-immunized mice affects the pattern of heart-infiltrating cell subsets. Accordingly, αMyHC/CFA-immunized mice received M-CSF injections between Days 21 and 29 of EAM (Supplementary material online, Figure S3A). Three days after the first M-CSF injection, we observed significantly increased number of F4/80+ and CD133+ cells in the myocardium of M-CSF-treated mice (Day 24, Figure 2A and B). Immunopositive F4/80+ macrophages in the myocardium decreased over time and remained at low numbers at Day 40 (Figure 2A; Supplementary material online, Figure S4A and B). M-CSF treatments, however, did not affect the expression of CD16/32, CD206, and CD301 in inflammatory macrophages (Figure 2E–G). Instead, we observed significantly increased Nos2 on F4/80+ cells in the heart after M-CSF treatment of wild-type mice (Figure 2D).

3.3 M-CSF inhibits formation of myofibroblasts from inflammatory CD133+ progenitors in EAM
Heart-infiltrating CD133+ progenitors represent the major cellular source of myofibroblasts in post-inflammatory EAM. At Day 24 of

analysed (BD Bioscience) and FlowJo software (Tree Star). In some experiments, cells were sorted with FACSaria III (BD Bioscience).
EAM, we sorted inflammatory (CD45-positive) CD133+/F4/80− and CD133+/F4/80hi cells from the inflamed myocardium of BALB/c mice and found elevated levels of myofibroblast-specific genes in CD133+ cells negative for F4/80 (Figure 3A and B). These findings suggest that CD133+/F4/80− rather than CD133+/F4/80hi cells contribute to fibrogenesis in post-inflammatory EAM.

Figure 1. M-CSF prevents differentiation of heart-infiltrating CD133+ progenitors into myofibroblasts. (A and B) Heart-infiltrating CD133+ cells were expanded from myocarditis-positive hearts at Day 21 of EAM and stimulated without (control, white) or with 10 ng/mL M-CSF (grey), or 10 ng/mL TGF-β (black) for 14 days. Relative mRNA expression of myofibroblast-specific fibronectin (Fn1), collagen I (Col1a1), and αSMA (Acta2) (A, n = 5). Representative immunofluorescence and quantification analysis of αSMA (top) and fibronectin (bottom) in cultured cells (B, n = 5). (C and D) Expanded heart-infiltrating CD133+ cells (EAM d21) pre-treated for 3 days without (control, black) or with 10 ng/mL M-CSF (+M-CSF d-3, hatch), washed and stimulated with 10 ng/mL TGF-β for 14 days. Relative mRNA levels of myofibroblast-specific genes (C, n = 5) and representative immunofluorescence and quantification analysis of αSMA (D, top) and fibronectin (D, bottom) are shown (n = 5). ***P < 0.001 (two-tailed Student’s t-test vs. control), bar = 20 μm.

M-CSF-stimulated CD133+ progenitors acquired a functional macrophage phenotype within 3 days in vitro (Figure 2H). We, therefore, analysed F4/80 expression on heart-infiltrating CD133+ progenitors in M-CSF-treated mice. We found that heart-infiltrating CD133+/CD45+ cells of M-CSF-treated mice had higher expression of F4/80 at Day 24 (i.e. 3 days after the cytokine treatment, Figure 3C and D).
Next, we sorted heart-infiltrating CD133+/CD45+ cells from PBS- and M-CSF-treated mice and found reduced mRNA levels of myofibroblast-specific genes in the M-CSF-treated group (Figure 3E). Furthermore, sorted heart-infiltrating CD133+/CD45+ cells from both groups were plated and cultured in the presence of TGF-β for 10 days. CD133+/CD45+ cells isolated from the myocardium of M-CSF-treated mice failed to differentiate into αSMA- and fibronectin-expressing myofibroblasts (Figure 3F).

3.4 NOS2 controls M-CSF-dependent macrophage differentiation of heart-infiltrating CD133+ progenitors

F4/80+ macrophages derived from CD133+ progenitors up-regulate NOS2 and produce nitric oxide. We, therefore, addressed the role of NOS2 in the macrophage differentiation processes. Accordingly, CD133+ progenitors were isolated at Day 21 after immunization...
from inflamed hearts of wild-type or Nos2<sup>−/−</sup> mice and cultivated in the presence of M-CSF. Nos2<sup>−/−</sup> CD133<sup>+</sup> progenitors showed reduced F4/80 and CD14 expression and were functionally impaired as indicated by reduced E. coli phagocytosis (Figure 4A and E). To determine whether macrophage differentiation was mediated by NOS activity, CD133<sup>+</sup> progenitors isolated from inflamed hearts of BALB/c mice were cultured for 3 days in the presence of M-CSF with or without l-NAME, a non-specific NOS inhibitor. Cells cultured

**Figure 3** M-CSF inhibits myofibroblast lineage differentiation of inflammatory CD133<sup>+</sup> in EAM. (A) Representative flow cytometry analysis of CD133<sup>+</sup> and F4/80 (right) gated on heart-infiltrating CD45<sup>+</sup> cells (left) at Day 24 of EAM. Numbers indicate the percentage of positive cells in the adjacent gates. (B) Heart-infiltrating CD45<sup>+</sup>/CD133<sup>+</sup>/F4/80<sup>−</sup> (white) and CD45<sup>+</sup>/CD133<sup>+</sup>/F4/80<sup>+</sup> (black) cells were FACS sorted from myocarditis-positive hearts at Day 24 of EAM. Relative mRNA levels of myofibroblast-specific genes are shown for one out of two independent experiments (n = 5). (C) Representative flow cytometry analysis of CD133<sup>+</sup> and F4/80 gated on heart-infiltrating CD45<sup>+</sup>/CD133<sup>+</sup> progenitor cells (left) of PBS- (+PBS) and M-CSF-treated (+M-CSF) mice at Day 24 of EAM. Staining with anti-CD45 and anit-CD133 and IgG control to anti-F4/80 antibodies (IgG control) was used to set the gates. Numbers indicate the percentage of positive cells in the adjacent gates. (D) The quantification of F4/80<sup>+</sup> (left) and F4/80<sup>−</sup> (right) cells gated of CD45<sup>+</sup>/CD133<sup>+</sup> progenitor cells (gated as shown in C) of PBS- (black) and M-CSF-treated (white) mice at Day 24 of EAM. (E) Relative mRNA level of myofibroblast-specific genes of sorted cells is shown (n = 5). In addition, sorted cells were plated and stimulated with 10 ng/mL TGF-β for 10 days. Representative immunofluorescence and quantification analysis of αSMA (F, top) and fibronectin (F, bottom) are shown (n = 5). Bar = 20 μm. ns P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed Student’s t-test).
with L-NAME showed significantly reduced E. coli phagocytosis (Figure 4F) and reduced nitric oxide levels (Figure 4G).

### 3.5 M-CSF treatment prevents cardiac fibrosis and left ventricular dysfunction in EAM

M-CSF prevents TGF-β-mediated myofibroblast differentiation and promotes the formation of macrophages from inflammatory CD133+ progenitors. Given that CD133+ progenitors represent the major source for myofibroblasts in EAM, we addressed whether M-CSF treatment reduces fibrogenesis in the post-inflammatory heart. Accordingly, we treated αMyHC/CFA-immunized mice with M-CSF between Days 21–29 or 40–48 of EAM, and analysed the hearts at Days 40 and 60, respectively (Supplementary material online, Figure S3A). As illustrated in the Figure 5, M-CSF treatment completely prevented accumulation of fibroblasts in the post-inflammatory heart (Figure 5A–C; Supplementary material online, Figure S4B). Of note, M-CSF treatment failed to attenuate fibrosis when delivered between Days 40 and 48 (Figure 5A). These findings suggest that M-CSF treatment prevented the fibrotic process, but did not revert already established cardiac fibrosis.

Relapses of inflammatory cells are quite common in autoimmune diseases. In BALB/c mice, we observed spontaneous inflammatory relapses 40 days after the first immunization (Figure 5D and E). During relapses, CD45+ inflammatory cells were mainly detected in the pericardium and largely represented CD3+ T lymphocytes (Figure 5D and E, Supplementary material online, Figure S3C). Interestingly, M-CSF treatment between Days 21 and 29 inhibited relapses in αMyHC/CFA-immunized mice as reflected by reduced numbers of CD45+ and CD3+ T cells in the myocardium at Day 40 (Figure 5D and E; Supplementary material online, Figure S3C).
Cardiac fibrosis parallels both, diastolic dysfunction, and reduced cardiac contractility. Not all immunized mice develop severe myocarditis. Consequently, the extent of post-inflammatory fibrosis varies in the EAM model. In our series, 11 out of 20 αMyHC/CFA-immunized BALB/c mice showed substantial fibrosis (2% of total heart area) on Day 40. At this time point, echocardiography revealed reduced ejection fraction and fractional shortening in mice with significant fibrosis (Figure 5F). In addition, the extent of fibrosis correlated with increased mass, systolic and diastolic volume, and isovolumetric contraction time of the left ventricle (Supplementary material online, Figure S5A). Echocardiography of αMyHC/CFA-immunized and M-CSF-treated mice showed unaffected cardiac function at Day 40 (Figure 5F, Supplementary material online, Figure S5E).

Next, we immunized Nos2−/− mice with αMyHC/CFA and treated with M-CSF or PBS between Days 21 and 29. αMyHC/CFA immunization resulted in myocarditis in Nos2−/− mice (not shown), but M-CSF treatment failed to affect fibrosis and heart functions during the post-inflammatory phase of EAM in Nos2−/− mice (Figure 6A, Supplementary material online, Figure S6). Similar results were observed if mice were M-CSF treated between Days 14 and 22 (not shown). M-CSF
treatment of Nos2−/− mice failed to increase the numbers of F4/80+ macrophages and CD133+ cells in the myocardium at Days 24 and 40 of EAM (Figure 6A–C). We also observed no differences in F4/80 expression on inflammatory CD133+/CD45+ progenitors (not shown) and in the expression of M1/M2 markers on heart-infiltrating F4/80+/CD45+ macrophages between M-CSF- and PBS-treated Nos2−/− mice (Figure 6D).

4. Discussion

We previously reported that in the EAM model post-inflammatory pathogenic myofibroblasts mainly originate from heart-infiltrating CD133+ progenitor cells.6 In line with our previous findings, herein we demonstrate that M-CSF controls the formation of anti-inflammatory macrophages from inflammatory CD133+ progenitors and prevents TGF-β-mediated differentiation into pathogenic myofibroblasts. Thus, our data suggest that in myocarditis, a significant pool of cells infiltrating the myocardium at the peak of disease represent non-committed progenitors.

From a clinical perspective, it is important that the differentiation fate of multipotent, non-committed precursor cells infiltrating the myocardium, can be modulated via specific cytokine signalling in a controlled fashion. M-CSF is a key cytokine guiding macrophage differentiation in vitro⁸ and in vivo.¹⁸ In EAM, M-CSF treatment up-regulated F4/80 expression on CD133+ inflammatory progenitors promoting macrophage differentiation. In untreated mice, however, heart-infiltrating CD133+ progenitors spontaneously differentiate into pathogenic myofibroblasts. To our knowledge, this is the first report describing the change of in vivo fate of inflammatory progenitors naturally present in the inflamed organ. So far, it has been
described that M-CSF treatment improved cardiac function in a viral model of myocarditis, after myocardial infarction and in the ischaemia-reperfusion model. Similar to our findings, M-CSF treatment promoted monocyte/macrophage accumulation in the heart after myocardial infarction or virus inoculation.

Myocardial fibrosis plays a dual role in remodelling after cardiac injury. On one hand, it is a pre-requisite for wound healing, as in the case of ischaemic injuries, for example. On the other hand, it contributes to ventricular stiffening and typical pathological remodelling in heart failure. Our previous and current data clearly show that excessive cardiac fibrosis parallels impaired cardiac function in EAM. Thus, heart failure.

It has been proposed that differential macrophage activation defines protective and pathogenic functions. Accordingly, adoptive transfer of M1 macrophages promoted histological disease scores, whereas injection of M2 macrophages was largely protective in Coxackievirus-induced myocarditis. Also most heart-infiltrating macrophages were classified as M2 macrophages expressing mannose receptor and Gr-1 in αMyHC/CFA- and Coxackievirus-induced myocarditis.

However, macrophages expressing M1 marker Nos2 have been identified in myocarditis as well. We critically analysed macrophages in the myocardium after the peak of disease, during the post-inflammatory phase of EAM, and detected both, M1- and M2-specific markers. We were, however, unable to clearly delineate two distinct activation states. We assume that during EAM, heart-infiltrating macrophages were activated classically (M1) and alternatively (M2) at the same time. In fact, it is well known that T cells and other heart-infiltrating cells massively produce both M1-activating IFN-γ as well as M2-activating IL-4 and IL-13. Importantly, M-CSF treatment did not affect the expression of M1 and M2 markers except of Nos2. M-CSF-induced macrophage accumulation in the post-inflammatory heart was not pathogenic in our model, because resolution of inflammation, defined by the extent of inflammatory cells was clearly M-CSF independent. Moreover, M-CSF-induced macrophages showed strong immunosuppressive activity in vitro and in vivo. We believe that the accumulation of the immunosuppressive macrophages in M-CSF-treated mice protected from spontaneous T cell relapse. Nevertheless, we cannot, entirely rule out that M-CSF also mediates protective effects by acting on other target cells.

We previously found that M-CSF-induced CD11b+ monocytes prevented EAM development and suppressed CD4+ T cell proliferation in a nitric oxide-dependent manner. M-CSF treatment promoted inflammatory macrophages expressing Nos2 in EAM, We, therefore, hypothesized that macrophage-produced nitric oxide might control both; T cell-mediated early inflammation as well as post-inflammatory fibrosis in EAM. Indeed, mice lacking Nos2 or treated with nitric oxide inhibitors showed enhanced cardiac inflammation and fibrotic lesions in a model of Coxackievirus-induced myocarditis. Similarly, Nos2−/− mice also develop more severe myocarditis in the EAM model (Kania et al., unpublished observations).

In summary, our data demonstrate that a single haematopoietic cytokine, such as M-CSF, can modulate the in vivo fate of inflammatory CD133+ progenitors in a way to promote their differentiation into macrophages instead of myofibroblasts in the post-inflammatory phase of the EAM. Furthermore, our data suggest that mature inflammatory macrophages, in contrast to progenitor-derived fibroblasts, do not promote heart failure after acute myocarditis. These findings are in line with the clinical observation that fulminant myocarditis implies an excellent long-term prognosis, whereas subacute myocarditis usually progresses to end-stage heart failure. Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References

Altering in vivo fate of inflammatory progenitors