Heart-Infiltrating Prominin-1<sup>+</sup>/CD133<sup>+</sup> Progenitor Cells Represent the Cellular Source of Transforming Growth Factor β–Mediated Cardiac Fibrosis in Experimental Autoimmune Myocarditis

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Rationale: Myocardial fibrosis is a hallmark of inflammation-triggered end-stage heart disease, a common cause of heart failure in young patients.

Objective: We used CD4<sup>+</sup> T-cell–mediated experimental autoimmune myocarditis model to determine the parameters regulating cardiac fibrosis in inflammatory heart disease.

Methods and Results: α-Myosin heavy chain peptide/complete Freund’s adjuvant immunization was used to induce experimental autoimmune myocarditis in BALB/c mice. Chimeric mice, reconstituted with enhanced green fluorescence protein (EGFP)<sup>+</sup> bone marrow, were used to track the fate of inflammatory cells. Prominin-1<sup>+</sup> cells were isolated from the inflamed hearts, cultured in vitro and injected intracardially at different stages of experimental autoimmune myocarditis. Transforming growth factor (TGF)-β–mediated fibrosis was addressed using anti–TGF-β antibody treatment. Myocarditis peaked 21 days after immunization and numbers of cardiac fibroblasts progressively increased on follow-up. In chimeric mice, >60% of cardiac fibroblasts were EGFP<sup>+</sup> 46 days after immunization. At day 21, cardiac infiltrates contained ~30% of prominin-1<sup>+</sup> progenitors. In vitro and in vivo experiments confirmed that prominin-1<sup>+</sup> but not prominin-1<sup>−</sup> cells isolated from acutely inflamed hearts represented the cellular source of cardiac fibroblasts at late stages of disease, characterized by increased TGF-β levels within the myocardium. Mechanistically, the in vitro differentiation of heart-infiltrating prominin-1<sup>+</sup> cells into fibroblasts depended on TGF-β–mediated phosphorylation of Smad proteins. Accordingly, anti–TGF-β antibody treatment prevented myocardial fibrosis in immunized mice.

Conclusions: Taken together, heart-infiltrating prominin-1<sup>+</sup> progenitors are the major source of subsequent TGF-β–triggered cardiac fibrosis in experimental autoimmune myocarditis. Recognizing the critical, cytokine-dependent role of bone marrow–derived progenitors in cardiac remodeling might result in novel treatment concepts against inflammatory heart failure. (Circ Res. 2009;105:462-470.)

Key Words: prominin-1<sup>+</sup> progenitor cells ■ myocarditis ■ cardiac fibrosis ■ TGF-β
bone marrow (BM)–derived dendritic cells loaded with heart-specific self peptide.9,11 In general, grading of EAM severity scores bases on the extent of inflammatory infiltrates at the peak of inflammation. In the ensuing late phase of disease, cardiac inflammation slowly resolves. However, many of the affected hearts show progressive dilation and increasing tissue fibrosis on follow-up.2

Insight from ischemic heart disease models suggests that the transition from acute inflammation to fibrosis is mediated by TGF-β.8 Along this line, resolution of inflammation and progressive remodeling are associated with high levels of TGF-β in the myocardium.12,13 TGF-β promotes the synthesis of various cytokines and growth factors, that are involved in formation/progression of cardiac fibrosis.8 In contrast, the cause of cardiac fibrosis in the context of inflammatory heart disease remains unclear. BM-derived progenitor cells recruited to the inflamed heart represent a potential cellular source for fibrosis,14,15 but this idea is largely speculative. In particular, the role of BM-derived progenitor cells in the process of regeneration/pathological remodeling in EAM is unknown.

Here, we show that prominin-1+ progenitor cells represent a subpopulation of heart-infiltrating BM-derived CD45+ cells at the peak of inflammation in EAM. These cells represent a common progenitor source for both fibroblasts and monocytes/macrophages, and play a critical role in the pathogenesis of cardiac fibrosis, a hallmark of end-stage heart failure. Fibroblast differentiation of prominin-1+ cells was promoted during the late stage of EAM because of a progressive shift of the intracardiac cytokine balance, reflected by increasing TGF-β levels.

Methods

Mice
BALB/c and C57BL/6-EGFP mice (enhanced green fluorescent protein [EGFP] under the control of β-actin promoter) were purchased from The Jackson Laboratory. C57BL/6-EGFP mice were backcrossed onto the BALB/c background for >10 generations (hereafter referred to as BALB/c-EGFP). The local authorities approved the animal protocol and all experiments were performed in accordance with Swiss Federal Law.

In Vitro Expansion/Differentiation of Heart-Infiltrating Prominin-1+ Cells
Mouse hearts were processed as described previously.16 Prominin-1+ cells were isolated by magnetic cell sorting and further expanded in vitro in culture expansion medium (Online Data Supplement, available at http://circres.ahajournals.org). To generate single-cell–derived clones, a single prominin-1+/EGFP+ cell was copurified with prominin-1−/EGFP− feeder cells derived from the healthy heart and cultured for 2 to 3 weeks. Cardiac differentiation was induced with 100 μM/mL oxytocin (Sigma, Basel, Switzerland); macrophage differentiation was induced with 10 ng/mL macrophage colony-stimulating factor (M-CSF) (PeproTech, London, UK); fibroblast differentiation was induced with 10 ng/mL TGF-β (PeproTech).

Immunization and Treatment Protocols
Six- to 8-week-old mice were injected subcutaneously with 150 μg/mouse of αMyHC (Ac-HN-SKLMLATFLSTYASAD-OH, Coslo) emulsified 1:1 with complete Freund’s adjuvant (CFA)/PBS on days 0 and 7, as described.16 Control mice were immunized with CFA/PBS only. Prominin-1+/EGFP+ cells were injected either directly into the left ventricle of anesthetized animals (5×106 cells per mouse) 0, 14, or 32 days after immunization using a Hamilton microsyringe; or intravenously (1×106 cells per mouse) at days 7 and 14 after immunization.

A neutralizing monoclonal antibody α-TGF-β (clone1D11; R&D Systems) was used at 15 μg/mL for in vitro experiments. For in vivo neutralization, mice received intraperitoneal injections of α-TGF-β (20 μg in 100 μL of PBS) or mouse IgG1 isotype control antibody every third day between days 17 to 46 after αMyHC/CFA immunization.

Generation of Chimera
Six- to 8-week-old BALB/c mice were lethally irradiated with 2×6.5 Gy using a Gammatron (Co-60) system and reconstituted with 2×106 donor BM cells from C57BL/6-EGFP mice (hereafter referred to as BALB/c-EGFP) (Online Data Supplement).

Western Blotting
Prominin-1+ cells were cultivated in vitro with TGF-β (PeproTech) for 1 hour and 3 days. Control cells were cultivated in the absence of TGF-β. Cell lysates were blotted and incubated with appropriate antibodies (Online Data Supplement).

Flow Cytometry
Cell suspensions were stained using fluorochrome-conjugated mouse-specific antibodies (Online Data Supplement) and analyzed with the FACSVerse (Becton Coulter) using FlowJo software (Tree Star). Heart infiltrates were identified as CD45+ cells from heart tissue suspensions gated on CD45/side scatter plots as described.3

Cytokine ELISA
Heart-infiltrating mononuclear cells were isolated as described3 with slight modifications (Online Data Supplement). Cytokine levels of...
interferon-γ, tumor necrosis factor-α, IL-17 and TGF-β were measured by ELISA in supernatants of heart-infiltrating mononuclear cells (Online Data Supplement).

Statistics
The Mann–Whitney U test was used for the evaluation of nonparametric data. Normally distributed data were compared using Student t test. Differences were considered as statistically significant for P<0.05.

Results
BM Cells Represent the Cellular Source of Cardiac Fibrosis in Late EAM
Accumulation of collagen-producing fibroblasts is a hallmark of pathological remodeling in end-stage heart failure. For addressing the contribution of the BM compartment to tissue fibrosis during the late stage of EAM, we created chimeric mice using lethally irradiated BALB/c mice reconstituted with EGFP+ syngeneic BM. Six weeks after BM reconstitution, only a few EGFP+ cells were found in healthy hearts (data not shown). Immunization of chimeric mice with αMyHC/CFA, however, resulted in severe myocarditis at day 21 (Figure 1A). Flow cytometry revealed that nearly all EGFP+ cells isolated from the heart of chimeric mice at this time point coexpressed CD45, suggesting an inflammatory phenotype (Figure 1B). We also identified a significant fraction of prominin-1+ cells among the EGFP+/CD45+ heart-infiltrating cells (Figure 1B). Nonetheless, in CFA-only immunized control chimeric mice, just few EGFP+ cells were observed (Figure 1B).

After day 21, inflammatory infiltrates largely resolved and progressive fibrosis developed by day 46 (Figure 1C through 1F). Analysis of these fibrotic hearts demonstrated that >60% of collagen-producing, α smooth muscle actin (αSMA)-producing, and fibronectin-producing fibroblasts expressed EGFP (Figure 1C through 1E). Importantly, we observed no EGFP signal in αMyHC+ cardiomyocytes (Figure 1F). CFA-only immunized control chimeric mice did not develop any fibrosis as expected (data not shown).

These findings indicate that the BM compartment represents the cellular source for both heart-infiltrating progenitor cells at the peak, as well as fibroblasts during the late stage of disease.

Inflammatory Prominin-1+ Progenitor Cells Showed Bilineage Differentiation Potential
Next, we aimed to identify the potential fibroblast progenitors within the inflammatory infiltrates at the peak of disease. Analysis of inflamed hearts of immunized chimeric mice revealed a fraction of ~30% of EGFP+ cells coexpressing the progenitor cell marker prominin-1 (Figure 1B). Nearly all prominin-1+ cells coexpressed CD45 and stem/progenitor cell markers c-kit, Sca-1, and Cxcr4 (Figure 2A and 2B).

Instead, after transition from the acute to the chronic phase of EAM, the number of inflammatory infiltrates dramatically decreased, and we found only few prominin-1+ cells in the fibrotic myocardium (data not shown).

Given the progenitor phenotype of prominin-1+ cells, we addressed their specific differentiation capacity. To this aim, we isolated inflammatory prominin-1+ cells from acutely inflamed hearts at day 21 and expanded them in vitro for 2 to 3 weeks. The cultured cells expressed prominin-1, CD45, c-kit, Sca-1, and Cxcr4 antigens and nanog, c-kit, islet-1, and nestin mRNA transcripts (Figure 3A, 3B, and 3G). In the presence of TGF-β, prominin-1+ cells acquired a fibroblast-like phenotype, expressed αSMA and fibronectin (Figure 3C) and downregulated the expression of genes and proteins characteristic for stem/progenitor cells (Figure 3F and 3H). These findings demonstrate the capacity of heart-infiltrating prominin-1+ cells to differentiate into fibroblasts in the presence of TGF-β.

Alternatively, prominin-1+ cells gained a large, flat morphology with granular cytoplasm and showed high phagocytic activity in the presence of M-CSF (Figure 3D), suggesting a monocyte/macrophage phenotype. However, the prominin-1+ cells failed to upregulate cardiomyocyte-
Heart-infiltrating cells, gated on prominin-1

derived feeder layer gave rise to single-cell–derived EGFP

intojected before the onset of acute inflammation, at days 7 and

hearts prevented EAM development when intravenously in-

macrophages, we isolated prominin-1

sents a common progenitor for fibroblasts and monocytes/

clones were divided into 3 dishes containing either TGF-

Next, cocultures containing single-cell–derived EGFP

cell-colony (Figure 4A), positive for prominin-1 (Figure 4B).

prominin-1

Heart-Infiltrating Prominin-1

Microenvironment Defines the Fate of
Heart-Infiltrating Prominin-1+ Progenitors
in EAM

To address whether heart-infiltrating prominin-1+ cell repre-
sents a common progenitor for fibroblasts and monocytes/

specific genes and proteins (Figure 3E and 3H). Unlike prominin-1+/CD45+ cells, the prominin-1−/CD45+ cell fraction isolated from inflamed hearts showed only macrophage phenotype in the presence of M-CSF, but failed to differentiate into fibroblasts and cardiomyocytes (Online Figure I).

Notably, prominin-1+ cells isolated from the inflamed hearts prevented EAM development when intravenously injected before the onset of acute inflammation, at days 7 and 14, into αMyHC/CFA immunized mice (Online Figure II).

Prominin-1 Identifies a Common
Fibroblast/Macrophage Progenitor Cell in EAM

To define whether TGF-β mediates fibroblast differentiation of heart-infiltrating prominin-1+ cells. In fact, TGF-β-treated prominin-1+ cells significantly upregulated the expression of genes characteristic for fibroblasts such as collagen I, asma, and fibronectin (Figure 5A). Activation of the TGF-β receptor complex involves phosphorylation of Smad proteins, which subsequently regulate transcription.17 TGF-β-induced in vitro fibroblast differentiation of heart-infiltrating prominin-1+ cells was associated with phosphorylation of Smad proteins (Figure 5B and 5C).

Taken together, prominin-1 expression characterizes a common fibroblast/macrophage progenitor among heart-infiltrating BM-derived cells at the peak of inflammation in the EAM model. Consequently these prominin-1+ cells represent the potential cellular source of fibroblasts mediating cardiac fibrosis during the late stage of disease.

TGF-β Signaling Mediates Fibroblast Activation
via Smad Proteins

So far, we have shown that TGF-β mediates fibroblast differentiation of heart-infiltrating prominin-1+ cells. To characterize the stage-specific microenvironment of the inflamed heart that dictates the fate of prominin-1+ cells in vivo, we measured cytokine production by heart-infiltrating cells at various time points after immunization. Release of the proinflammatory cytokines: IL-17, interferon-γ, and tumor necrosis factor-α by heart-infiltrating mononuclear infiltrates was significantly reduced at day 32 (beginning of fibrotic phase) compared with day 21 (inflammatory phase; Figure 6A). In contrast, levels of TGF-β were markedly increased in heart tissue during the late stage of disease (Figure 6A). Subsequently, we isolated the inflammatory heart-infiltrating prominin-1+ cells expressed fibronectin (Figure 4C), whereas exposure to M-CSF gave rise to a macrophage phenotype with phagocytic activity (Figure 4D). In the presence of oxytocin, however, cells failed to assume cardiomyocyte-like phenotype, ie, stained negative for αMyHC (Figure 4E). Thus, the prominin-1+ cells represent a common progenitor for fibroblasts and monocytes/macrophages but not for cardiomyocytes in the acutely inflamed heart.

To test whether heart-infiltrating prominin-1+ cells undoubtedly originate from BM, we isolated and expanded prominin-1+/EGFP+ cells from the inflamed hearts of chimeric mice reconstituted with EGFP+ BM. EGFP+ cells retained an undifferentiated phenotype expressing prominin-1 and CD45 (Online Figure III, A and B), and on induction of differentiation, differentiated into fibroblasts and monocytes/macrophages but not into cardiomyocyte-like cells (Online Figure III, C through F).

Figure 2. Inflamed heart recruits prominin-1+ progenitor cells. Heart-infiltrating cells, gated on prominin-1+ cell population (A), analyzed by flow cytometry at the peak of inflammation, showed the coexpression of CD45, c-kit, Cxcr4, and Sca-1 (B).

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from EGFP \(^+\) mice at day 21, expanded in vitro and injected intracardially into healthy (d0), inflamed (d14), and fibrotic (d32) hearts. Seven to 14 days after intracardiac injection the mice were euthanized and the hearts analyzed. Prominin-1\(^+\)/EGFP\(^+\) cells maintained an undifferentiated phenotype after injection into the healthy hearts (Figure 6B). Injection of prominin-1\(^+\)/EGFP\(^+\) cells at the onset of inflammation (day 14) resulted in EGFP\(^+\) monocyte/macrophage
differentiation at day 21 (Figure 6C). Of note, some EGFP+/H11001 cells already expressed also fibronectin at this time point (Figure 6C). Injection of prominin-1/H11001/EGFP cells at day 32, characterized by enhanced tissue fibrosis, resulted in the differentiation of EGFP/H11001 cells into collagen I-, fibronectin-, and αSMA-expressing fibroblasts, analyzed at day 46 (Figure 6D).

Taken together, these findings suggest that the stage-specific cytokine milieu dictates the fate of BM-derived progenitor cells in the diseased heart during EAM.

TGF-β Is a Critical Mediator for Cardiac Fibrosis in EAM

So far, our data suggest that TGF-β promotes the fibroblast differentiation of heart-infiltrating prominin-1+ cells in vitro. Heart-infiltrating prominin-1+ cells cultivated in vitro in the presence of TGF-β for 1 hour and 3 and 7 days. A, Gene expression levels assessed by real-time RT-PCR. Bars represent means±SD from at least 5 individual cell cultures. B, Western blot analysis of Smad2 phosphorylation (P-Smad2). C, The quantification of P-Smad2 to Smad2/3 immunoreactivity. Control cells were cultivated in the absence of TGF-β. Bars represent means±SD from 3 individual experiments.

Taken together, TGF-β critically modulates fibroblast differentiation of heart-infiltrating prominin-1+ progenitor cells and tissue fibrosis in the EAM model.
Discussion

Myocarditis predisposes to dilated cardiomyopathy, the most common cause of heart failure in young patients. Cardiac fibrosis is a hallmark of the failing heart, but little is known about the cellular origins and the underlying mechanisms of accumulating fibroblasts. Using a mouse model of EAM, we demonstrate that prominin-1 expression defines a specific population of heart-infiltrating BM-derived progenitor cells. Regulated by TGF-β, they differentiate into fibroblasts, thereby promoting tissue fibrosis during the late stages of disease. Importantly, however, these cells showed no ability to differentiate into cardiomyocytes.

Fibrogenic cells can expand from at least 3 sources: (1) from local activation and proliferation of heart-resident mesenchymal cells, (2) by epithelial-to-mesenchymal transition, and (3) from BM-derived cells.18 Using immunized chimera reconstituted with EGFP+ donor BM, we illustrated that the majority (>60%) of cardiac fibroblasts promoting fibrosis in the EAM model originate from BM. Given the facts that EGFP expression was detected in 80% to 90% of EGFP transgenic cells (K Gabriela, E Urs, unpublished observations, 2009) and that BM-derived fibroblasts were not observed before the onset of cardiac inflammation, we conclude that inflammatory infiltrates represent the major source of cardiac fibroblasts in EAM. Nevertheless, we cannot exclude that endogenous cardiac fibroblasts also participate in the fibrotic process at late stages of EAM. Some authors have postulated heart resident fibroblasts as the major cellular source of tissue fibrosis associated with hypertrophy and ischemic heart failure.19,20 Moreover, El-Helou et al21 identified heart-resident nestin-expressing fibroblast progenitors, migrating to the infarcted region in the response to ischemia, suggesting involvement of these cells in the scarring processes of the injured myocardium.

Injured hearts are believed to dispose of a pool of endogenous/recruited progenitors playing a relevant role in myocardial regeneration and pathological remodeling,22 but nothing is known about a potential role of any progenitors in EAM. We demonstrated here that at the peak of EAM prominin-1+ progenitors denote a significant fraction among CD45+ heart-infiltrating inflammatory cells. Prominin-1 is a well-established marker of hematopoietic, embryonic and adult progenitors.16,23,24 We illustrated that heart-infiltrating prominin-1+ cells represent a common progenitor for fibroblasts and macrophages in EAM. However, heart-infiltrating prominin-1+ cells showed impaired regenerative capacity (new cardiomyocytes formation). This is in strong contrast to prominin-1+ cells derived from healthy heart,16 despite the high similarity regarding the expression of stem/progenitor cell markers and immunomodulatory properties. Thus, our findings point to careful evaluation of stem/progenitor cells isolated from injured organs for use in regenerative medicine. In the EAM model, inflammation in the myocardium disables the expansion of progenitors with regenerative capacity from cardiac tissue. It remains still to be elucidated whether genetic or epigenetic manipulations can revert/induce regenerative potential in prominin-1+ cells derived from inflamed heart or even BM.

Heart injury results in leukocyte recruitment and a specific inflammatory environment, promoting healing processes and scar formation. Accordingly, proinflammatory cytokines/chemokines become rapidly induced in the injured myocardium. In parallel, counter regulatory mechanisms result in release and activation of inhibitory mediators, such as profibrotic growth factor TGF-β regulating the transition from inflammation to fibrosis.25 Our experiments demonstrate that the stage-specific microenvironment dictates the fate of transplanted progenitor cells at various time points during the

Figure 6. The stage-specific cytokine milieu determines differentiation of heart-infiltrating prominin-1+ cells in EAM. A, Cytokine production of stimulated cardiac mononuclear cells isolated from inflamed hearts at the peak of inflammation (d21) (white bars) and from fibrotic hearts (d32) (black bars). Healthy heart tissue (d0) (gray bars) was used as a control. Each bar represents a mean±SD of 5 different culture wells. One of several representative experiments is shown. B through D, Prominin-1+/EGFP+ cells, isolated from the inflamed heart of BALB/c-EGFP mouse at day 21, were expanded in vitro and intracardially injected into healthy (day 0), inflamed (day 14), and fibrotic heart (day 32) of BALB/c mice and were analyzed 7 to 14 days after injection. Prominin-1+/EGFP+ cells injected into healthy hearts retained an undifferentiated phenotype coexpressing prominin-1 and CD45 (B). In the inflammatory microenvironment, prominin-1+/EGFP+ cells gained expression of macrophage-specific F4/80, and few expressed fibronectin (C). In the fibrotic heart, EGFP+ cells expressed fibroblast-specific collagen I, fibronectin, and αSMA (D). Differentiation of EGFP+ cells into αMyHC-positive cardiomyocytes was not observed at any stage. Hoechst 33342 (blue) was used to visualize cell nuclei. Bars=20 μm.
entiation only14,29 or questioned the regenerative potential of heart disease model, the present evidence supports the view that the specific microenvironment of an injured heart does not allow cardiac regeneration but promotes either the accumulation of monocytes/macrophages or tissue fibrosis/scarring. Thus, it is not surprising that most studies evaluating BM-derived progenitor cells with cardiomyogenic growth factors potentiates their cardiac differentiation and their functional regenerative capacity in vivo.33

To date, several studies on models of noninflammatory heart disease suggested a critical role of TGF-β in cardiac fibrosis.3 A broad range of different cells recruited because of cardiac issue injury, ie, macrophages, T cells, and mast cells produce TGF-β.2,5 In EAM, intracardiac TGF-β levels progressively increase in parallel with steadily resolving cardiac infiltrates. TGF-β modulates the behavior of fibroblast progenitors/mature fibroblasts by stimulating the synthesis of various extracellular matrix proteins including collagens, fibronectin, tenasin, and proteoglycans,25 factors that affect the fate of progenitor cells. In our model, the fibroblast-specific differentiation of prominin-1+ cells depended on TGF-β, suggesting that this cytokine is the key mediator of cardiac fibrosis late during the EAM course. In fact, TGF-β signaling mediated the phosphorylation of Smad2 proteins in prominin-1+ progenitors, pointing to the involvement of canonical Smad-dependent signaling pathways19,34 in the transition of prominin-1+ progenitors to fibroblasts. Given the fact that TGF-β strongly promotes fibroblast differentiation of heart-infiltrating prominin-1+ cells in vitro, it was not surprising that prominin-1+ cells differentiated into fibroblasts after intracardiac injection at late stage of EAM characterized by high levels of TGF-β release within the heart. Accordingly, TGF-β depletion efficiently blocked cardiac fibrosis in our model. Similarly, anti–TGF-β treatment markedly decreased collagen deposition in infarcted hearts.35 Given the high frequency of heart-infiltrating progenitors in EAM, further studies are needed to develop strategies that specifically block the fibroblast-specific differentiation of progenitor cells. Based on our data, we would expect that TGF-β–dependent signaling pathways might become a potential target against progressive cardiac fibrosis and end-stage heart failure in the future.

In conclusion, we identified for the first time prominin-1+ multilineage fibroblast and monocyte/macrophage progenitors among heart-infiltrating CD45+ cells as the major cellular source of cardiac fibrosis in postinflammatory heart disease. Furthermore, we proved that the developmental fate of heart-infiltrating prominin-1+ progenitors was dictated by a specific, disease stage–dependent myocardial microenvironment. Finally, we identified TGF-β as the key cytokine promoting fibroblast differentiation of prominin-1+ progenitors during the transition from acute myocarditis to inflammatory cardiomyopathy in the EAM model. The critical role of prominin-1+ progenitors in the progression of cardiac inflammation to myocardial fibrosis identifies these cells as a promising target for improved cardiac function.32 Similarly, we showed that prominin-1+ progenitor cells expanded from both, healthy16 and inflamed hearts efficiently prevent EAM development but do not contribute to the regeneration of damaged cardiac tissue. Therefore, the development of clinically relevant cell-based therapies requires either ex vivo manipulation of progenitor cells, rendering them resistant to the inflammatory environment, or the combined in vivo targeting of specific cytokines/chemokines, impairing the regenerative capacity of progenitor cells. In fact, ex vivo pretreatment of autologous BM-derived progenitor cells with cardiomyogenic growth factors potentiates their cardiac differentiation and their functional regenerative capacity in vivo.33

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novel treatment approaches for postinflammatory cardiac fibrosis during nonischemic heart failure in the future.

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Disclosures
None.

References
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**Methods**

**Generation of chimera**

6-8 weeks old BALB/c mice were lethally irradiated with 2x6.5 Gy using a Gammatron (Co-60) system, and reconstituted with 2x10^7 donor BM cells from BALB/c-EGFP mice (hereafter referred to as EGFP->BALB/c). After reconstitution all mice received prophylactic antibiotics in the drinking water and were housed in a specific pathogen-free environment for 6 weeks.

**Isolation, in vitro expansion and differentiation of inflammatory heart-infiltrating prominin-1^+ cells**

Mouse heart tissues were prepared according to previous report^1^. Hearts were perfused, dissected and digested with 20 µg/mL Liberase Blendzyme (Roche, Basel, Switzerland) for 45 min at 37°C and tissue suspensions were passed sequentially through 70 µm and 40 µm cell strainers (BD Falcon, Belgium).

Prominin-1^+ cells were isolated from the inflamed heart at the peak of inflammation (at day 21 after αMyHC/CFA immunization) by positive selection with anti-prominin-1-PE antibody (eBioscience, California, USA) and magnetic anti-PE-microbeads (Miltenyi Biotec, Germany). Prominin-1^+ cells were plated onto gelatine-coated cell culture plates and expanded in vitro in Culture Expansion Medium (CEM) composed of Iscove’s modified Dulbecco’s medium (IMDM; Sigma, Switzerland), supplemented with 100 µM β-mercaptoethanol, 1:100 penicillin-streptomycin, and 10% heat-inactivated foetal calf serum (all from Gibco-Invitrogen, UK). To generate single cell derived clones, 1-5 prominin-1^+/EGFP^+ cells were co-plated with 10^5 of non-
transgenic prominin-1\(^+\) progenitor cells isolated from the healthy hearts (PPC)\(^1\) feeder cells, and expanded for 2-3 weeks.

Prominin-1 expressing progenitor cells (PPC) were isolated from healthy heart tissue and expanded as previously described\(^1\).

Cardiac differentiation was induced with 100 \(\mu\)M oxytocin (Sigma, Basel, Switzerland). Macrophage differentiation was induced with 10 ng/mL M-CSF (PeproTech, UK). Fibroblast differentiation was induced with 10 ng/mL TGF-\(\beta\) (PeproTech). Co-cultures containing single cell-derived EGFP\(^+\) clones were spitted into 3 dishes for in vitro differentiation.

**Immunization and treatment protocols**

For immunization, all animals were transferred into conventional housing. Mice were injected subcutaneously with 150 \(\mu\)g/mouse of \(\alpha\)MyHC- (Ac-SLKLMATLFSTYASADOH) emulsified 1:1 with CFA on days 0 and 7, as described\(^2\). Control mice were immunized with CFA/PBS only.

Heart-infiltrating prominin-1\(^+\)/EGFP\(^+\) cells after in vitro expansion were injected directly into the left ventricle of anesthetized animals (5x10\(^4\) cells per mouse) 0, 14 or 32 days after immunization. The integration and differentiation of intra-cardially injected EGFP\(^+\) was analyzed using fluorescent microscopy on cryo- and paraffin-sections.

To investigate the immunomodulatory effect of progenitor cells on the EAM severity heart-infiltrating prominin-1\(^+\) cells were injected intravenously (2x10\(^6\) cells per mouse) at day 7 and 14 after \(\alpha\)MyHC/CFA immunization.

The percentage of EGFP-positive fibroblasts was analyzed/calculated using fluorescent microscopy on paraffin-sections of fibrotic heart sections (46 days after \(\alpha\)MyHC/CFA immunization). Fibronectin-positive cells were considered as fibroblasts.

**Histology**
Animals were sacrificed at days 21 and 46 after the first immunization. Hearts were removed and stained with Hematoxylin/eosin to assess myocarditis severity graded from 0 to 4, as described before\(^3\).

Masson’s trichrome staining was used to detect fibrosis. The degree of fibrosis, analyzed as collagen I depositions, was calculated as percentage of the fibrotic area in relation to the total heart area. The fibrosis was graded from 1 to 4 (<1%, 1-5%, 5-10%, >10% collagen I-positive fibroblast areas of a cross-section). Calculations represent the average of 3 independent sections for each studied heart.

**RT-PCR and Real-Time RT-PCR**

Samples were collected in Tri Reagent (Luzerna Chem, Luzern, Switzerland) and total RNA was isolated according to manufacturer’s recommendations. mRNA was reverse transcribed using Oligo d(T) primers (Invitrogen) and RevertAid M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). cDNA samples were amplified using the TaqPCR Master Mix Kit (Qiagen, Basel, Switzerland) and the appropriate oligonucleotides (see Supplementary Table 1). Positive controls included R1 embryonic stem cells (kindly provided by Prof. A. M. Wobus, IPK, Gatersleben, Germany) for nanog, c-kit and sca-1, and brain tissue of adult mouse for islet-1 and nestin. For real-time RT-PCR cDNA was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and oligonucleotides complementary to transcripts of the analyzed genes. The following oligonucleotides were used in this study: procollagen I: 5’-gatgacgtgcaatgcaatgaa-3’, 5’-ccctcgactctacatcttcta-3’; gapdh: 5’-ctgcaccaccaactgtgctg-3’, 5’-ggcatggaagctggtagctacag-3’; αsma: 5’-cgtgtgcaacgctagtata-3’, 5’-cgaagccgggcttacaga-3’, fibronectin: 5’-tccaaggtcactcatacctc-3’, 5’-cagatggcaaaagaacgag-3’. Transcript levels of gapdh were used as endogenous reference.
Flow cytometry

Hearts were perfused with phosphate buffered saline (PBS), excised, dissected into small pieces and digested as described above. Single cell suspensions were obtained by straining through 70 µm and 40 µm nylon mesh (BD Falcon). Cells were washed twice with 50 mL FACS buffer (1% FCS, 1 mM EDTA in PBS, all from Gibco) and incubated 30 min on ice with the appropriate combination of fluorochrome conjugated and/or primary and secondary antibodies. Labelled cells were washed and re-suspended in FACS buffer. The following antibodies and dilutions were used: 1) Biotin-conjugated antibodies: anti-CXCR4 1:200, anti-Sca-1 1:200, anti-CD45 1:600, anti-c-kit 1:200 (all from Cedarlane, Ontario, Canada), anti-prominin-1 1:200 (eBioscience). 2) Fluorochrome-conjugated antibodies: anti-CD45-PE 1:400 (BD Bioscience, Switzerland), anti-prominin-1-PE (eBioscience), 3) For biotin labelled cells: Streptavidin-APC 1:400 (BD Bioscience) was used. Cells were analyzed with a CyAn ADP analyzer (Beckman Coulter) and FlowJo software (Tree Star). Heart infiltrates were identified as CD45+ cells from heart tissue suspensions gated on CD45/side scatter plots.

Immunohistochemistry

Cells were cultured on gelatine-coated cover slips. For immunohistochemistry, cells were fixed either in PBS containing 4% paraformaldehyde for 20 minutes at room temperature or with methanol:acetone (7:3) for 10 minutes at -20°C, as described. PBS supplemented with 1% BSA was used as blocking solution and Hoechst 33342 (5 µg/ml) was used to label nuclei. Hearts were harvested and cell engraftment was analyzed in cryo- and paraffin-sections. The following fluorochrome-conjugated antibodies were used: anti-CD45-PE 1:600, anti-F4/80-PE 1:400, anti-CD11b-PE 1:400 (all from BD Bioscience), anti-prominin-1-PE 1:200 (eBioscience).
The following primary antibodies and dilutions were used: mouse IgG anti-smooth muscle actin (Sigma) 1:1000, rat IgG anti-prominin-1 (Chemicon, Hofheim, Germany) 1:100, mouse IgG anti-myosin heavy chain alpha (Abcam) 1:200, rabbit IgG anti-collagen I (Sigma), rabbit IgG anti-desmin (Chemicon), rabbit IgG anti-fibronectin (Milipore, California, USA). The following secondary antibodies and dilutions were used: AlexaFluor488 chicken anti-mouse IgG 1:100, AlexaFluor546 goat anti-mouse IgG 1:600, AlexaFluor488 donkey anti-rat IgG 1:100, AlexaFluor546 donkey anti-rat IgG 1:600, AlexaFluor488 chicken anti-rabbit IgG 1:100, AlexaFluor546 goat anti-rabbit IgG 1:600 (all from Molecular Probes, Invitrogen).

For phagocytosis assays, *Escherichia coli* BioParticles Alexa Fluor 488- or Texas Red-conjugated (Molecular Probes, Invitrogen) were used according to the instructions of the manufacturer. Samples were analyzed using an Axiophot fluorescence microscope and a LSM 510 META confocal laser-scanning microscope (both Carl Zeiss, Jena, Germany).

**Cytokine production of heart-infiltrating cells**

Heart-infiltrating mononuclear cells were isolated 21 and 32 days after αMyHC/CFA immunization, as described previously with the following modifications: Collagenase D (Worthington Biochemical Corporation) was used at a concentration of 0.895 mg/ml and tissue suspensions were passed sequentially through 70µm, 40µm cell strainers (BD Biosciences) and finally through 15µm self-assembled strainers (Sefar AG). Healthy heart tissue (d0, grey bars) was used as a control. IFN-γ, TNF-α, IL-17 and TGF-β levels in heart-infiltrating monocyte cells were measured using commercially available OptEIA™ Mouse Elisa systems (both BD Biosciences).

Cytokine levels of TGF-β and TNF-α were measured in supernatants after culturing 1 x 10^5 heart-infiltrating mononuclear cells/well for 48 hours in RPMI 1640 medium (Cambrex) supplemented with 10% FBS, penicillin/streptomycin, 100 mM non essential amino acids, 100
mM sodium pyruvate, 50mM β-mercaptoethanol (all from Gibco) at 37°C in a humidified atmosphere with 5% CO₂ using commercially available Quantikine ELISA kits (R&D Biosystems). For INF-γ and Il-17 measurements heart-infiltrating mononuclear cells were stimulated for 24 hours with plate bound anti-CD3/anti-CD28 (BD Biosciences).

**Western blotting**

Prominin-1⁺ cells were cultivated *in vitro* with TGF-β (PeproTech) for 1 hour, 3 and 7 days. Control cells were cultivated in the absence of TGF-β. Cell lysates were blotted and incubated with rabbit anti-Phospho-Smad2 (1:500) and rabbit anti-Smad2/3 (1:1000; both from Cell Signaling Technology).

**References**

Online table 1

Primer sequences, size of the amplified products and annealing temperatures (Tm).

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<th>Analyzed gene</th>
<th>Forward primer</th>
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Online Figure 1. Heart-infiltrating prominin-1+/CD45+ cells differentiate into macrophages/monocytes but not fibroblasts or cardiomyocytes.

Morphology and immunofluorescence images of heart-infiltrating prominin-1- cells after culture in CEM medium (A), with TGF-β (B), M-CSF (C), or oxytocin (D).

Online Figure 2. Heart-infiltrating prominin-1+ cells suppress EAM development.

(A) Myocarditis scores of individual αMyHC/CFA immunized mice intravenously injected with 1x10^6 heart-infiltrating prominin-1+ cells (grey squares), or PBS (white circles) at day 7 and 14. Analysis was performed at day 21 after immunization. Heart-infiltrating prominin-1+ cells were isolated from myocarditis hearts of αMyHC/CFA immunized mice at day 21. Median values for each group are shown. * p<0.05.

Online Figure 3. Heart-infiltrating prominin-1+ cells, representing common progenitors for fibroblast and monocyte/macrophage differentiation, undoubtedly originated from bone marrow.

A-B, Prominin-1+ cells isolated from inflamed hearts of EGFP->BALB/c chimeric mice at day 21 were EGFP-positive and retained an undifferentiated phenotype expressing prominin-1 and CD45 after expansion in CEM medium. C-F, Prominin-1+/EGFP+ cells differentiate into fibroblasts and monocytes/macrophages, but not cardiac phenotypes. Hoechst 33342 (blue) was used to visualize cell nuclei. Bars = 20µm.

Online Figure 4. Anti-TGF-β blocking antibody treatment abrogates in vivo fibroblast differentiation of heart-infiltrating prominin-1+ cells.
Heart-infiltrating prominin-1\(^+\)/EGFP\(^+\) cells, isolated from inflamed hearts of EGFP mice, were expanded in CEM, and injected into hearts at the early stage of the fibrotic phase (day 32). αTGF-β blocking antibody was repetitively injected in mice between days 32-46 days post αMyHC/CFA immunization. Fibroblast-specific \textit{in vivo} differentiation of heart-infiltrating prominin-1\(^+\)/EGFP\(^+\) cells was abrogated (A). EGFP\(^+\) cells did not acquire cardiac phenotype (B). Hoechst 33342 (blue) was used to visualize cell nuclei. Bars = 20μm.

**Online Figure 5.** Prominin-1\(^+\) progenitor cells (PPC), isolated from the healthy heart, differentiated into fibroblasts \textit{in vitro} upon stimulation with TGF-β or \textit{in vivo}- in the specific microenvironment of the fibrotic heart.

\textbf{A}, Undifferentiated PPC expressed prominin-1 but not fibronectin and αSMA before exposure to TGF-β. \textbf{B}, PPC exposed \textit{in vitro} to TGF-β developed a fibroblast-specific morphology expressing fibronectin, desmin and αSMA, but not prominin-1. \textbf{C}, Quantum dots (QD) FITC-labelled PPC, injected intracardially into the fibrotic heart 32 days after αMyHC/CFA immunization, acquired fibroblast but not cardiac phenotype. Hoechst 33342 (blue) was used to visualize cell nuclei. Bars = 20μm.
Online figure 1; Kania et al.
A disease score

Heart-infiltrating prominin-1+ cells

PBS

0 1 2 3 4

disease score

*
Online figure 3; Kania et al.
Online figure 4; Kania et al.
Online figure 5; Kania et al.