Prominin-1$^+$/CD133$^+$ bone marrow-derived heart-resident cells suppress experimental autoimmune myocarditis

Gabriela Kania[1]*, Przemyslaw Blyszczuk[1], Alan Valaperti[1], Thomas Dieterle[2], Bernd Leimenstoll[3], Stephan Dirnhofer[4], Henryk Zulewski[5], and Urs Eriksson[1,6]

[1]Experimental Critical Care, Department of Biomedicine, University Hospital, Hebelstrasse 20, CH-4031 Basle, Switzerland; [2]Clinical Cardiology, Department of Internal Medicine, University Hospital, Hebelstrasse 20, CH-4031 Basle, Switzerland; [3]General Internal Medicine, Department of Internal Medicine, University Hospital, Hebelstrasse 20, CH-4031 Basle, Switzerland; [4]Institute of Pathology, University Hospital, Hebelstrasse 20, CH-4031 Basle, Switzerland; [5]Division of Endocrinology, Diabetes and Clinical Nutrition, University Hospital, Hebelstrasse 20, CH-4031 Basle, Switzerland; and [6]Department of Cardiology, University Hospital, Rämistrasse 100, CH-8091 Zurich, Switzerland

Aims Experimental autoimmune myocarditis (EAM) is a CD4$^+$ T cell-mediated mouse model of inflammatory heart disease. Tissue-resident bone marrow-derived cells adopt different cellular phenotypes depending on the local milieu. We expanded a specific population of bone marrow-derived prominin-1-expressing progenitor cells (PPC) from healthy heart tissue, analysed their plasticity, and evaluated their capacity to protect mice from EAM and heart failure.

Methods and results PPC were expanded from healthy mouse hearts. Analysis of CD45.1/CD45.2 chimera mice confirmed bone marrow origin of PPC. Depending on *in vitro* culture conditions, PPC differentiated into macrophages, dendritic cells, or cardiomyocyte-like cells. *In vivo*, PPC acquired a cardiac phenotype after direct injection into healthy hearts. Intravenous injection of PPC into myosin alpha heavy chain/complete Freund's adjuvant (MyHC-α/CFA)-immunized BALB/c mice resulted in heart-specific homing and differentiation into the macrophage phenotype. Histology revealed reduced severity scores for PPC-treated mice compared with control animals [treated with phosphate-buffered saline (PBS) or crude bone marrow at day 21 after MyHC-α/CFA immunization]. Echocardiography showed preserved fractional shortening and velocity of circumferential shortening in PPC but not PBS-treated MyHC-α/CFA-immunized mice. *In vitro* and *in vivo* data suggested that interferon-γ signalling on PPC was critical for nitric oxide-mediated suppression of heart-specific CD4$^+$ T cells. Accordingly, PPC from interferon-γ receptor-deficient mice failed to protect MyHC-α/CFA-immunized mice from EAM.

Conclusion Prominin-1-expressing, heart-resident, bone marrow-derived cells combine high plasticity, T cell-suppressing capacity, and anti-inflammatory *in vivo* effects.

1. Introduction

Dilated cardiomyopathy is a common cause of heart failure and frequently results from viral infections.[1] Ongoing inflammation can be due to viral persistence and continuous release of inflammatory cytokines, or to an autoimmune response against cardiac tissues.[2–4]

Experimental autoimmune myocarditis (EAM) is a CD4$^+$ T cell-mediated mouse model of post-infectious cardiomyopathy and can be induced in susceptible strains by immunization with self-peptides derived from the myosin alpha heavy chain (MyHC-α) together with a strong adjuvant,[5,6] or by injection of activated, MyHC-α-loaded dendritic cells.[7,8] Severity scores peak 21 days after immunization and the extent of inflammatory infiltrates resolves slowly thereafter. Nevertheless, many animals develop dilated cardiomyopathy on follow-up.[9]

The healthy mouse heart contains between 5 and 8% CD45$^+$ expressing, bone marrow-derived cells. Among this population, a CD11b$^+$ fraction is supposed to reflect immature monocyte-like cells.[9] The role of heart-resident bone marrow-derived cells in inflammatory heart disease, however, remains largely speculative. In the context of other inflammation models, recent data suggest that monocyte-like precursor cells can differentiate into diverse

* Corresponding author. Tel: +41 61 265 3524; fax: +41 61 265 2350.
E-mail address: g.kania@unibas.ch

subpopulations of inflammatory cells such as dendritic cells and macrophages, depending on the cytokine/chemokine environment.[10–12] Other studies point to an amazing regenerative potential of bone marrow-derived tissue-resident cells.[13] For example, in heart disease models, there is evidence that bone marrow-derived precursor cells can adopt tissue-specific phenotypes depending on the local milieu.[14]

We hypothesize that bone marrow-derived precursor cells with high plasticity can be expanded from healthy heart tissue. As a selection marker, we chose prominin-1 (CD133), a well-described indicator of haematopoietic, embryonic, and adult progenitor cells.[15,16] Here, we describe the expansion of high numbers of immunomodulating prominin-1-expressing bone marrow-derived precursor cells (PPC) with multilineage differentiation potential from healthy mouse hearts. In addition, we provide *in vivo* evidence that PPC efficiently suppress EAM and heart failure development in MyHC-α/complete Freund's adjuvant (CFA)-immunized mice.

2. Methods

2.1 Animals

BALB/c mice, C57Bl/6-GFP transgenic mice (GFP under the control of β-actin promoter), and *IFN-γ* receptor knockout (*IFN-γR⁻/⁻*) mice were purchased from Jackson Laboratory and housed in a specific pathogen-free environment. The local authorities approved the study and all experiments were performed in strict accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Swiss Federal Law.

2.2 Generation, expansion, and *in vitro* differentiation of PPC

We generated and expanded PPC using a two-step protocol similar to that described by Tang *et al.*[17] Mouse heart tissues were prepared according to reports[17,18] with minor modifications (Supplementary methods). We purified prominin-1⁺ cells from heart cell cultures by magnetic cell sorting or FACS sorting. To generate single-cell-derived clones, a single PPC-GFP⁺ cell was co-plated with PPC-GFP⁻ feeder cells, cultured for 2–3 weeks. Cardiac differentiation was induced with 100 μM oxytocin (Sigma, Basel, Switzerland). Macrophage differentiation was induced with 10 ng/mL macrophage-colony-stimulating factor (M-CSF) (PeproTech, UK). Dendritic cell differentiation was induced with 10 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF) (PeproTech).

2.3 Immunization and treatment protocols

For immunization, all animals were transferred into conventional housing. Mice were injected subcutaneously with 100 μg/mouse of MyHC-α-(Ac-SLKLMATLFSTYASADOH) emulsified 1:1 with CFA on days 0 and 7, as described.[6] Control mice were immunized with CFA/phosphate-buffered saline (PBS) only.

Depending on the experiment, PPC-GFP⁺ cells derived from GFP transgenic mice or PPC labelled with fluorochrome-conjugated nanocrystals (Quantum dots: QD, Molecular Probes, Invitrogen, OR, USA) were injected either intravenously (2 × 10⁶ cells per mouse) at days 7 and 14 after immunization or directly into the left ventricle of anesthetized healthy animals (5 × 10⁴ cells per mouse). The number of intravenously injected PPC recruited to the heart was calculated as percentage of PPC-QD⁺ detected by FACS, multiplied by the total number of cells isolated from heart tissue. The integration of intra-cardially injected PPC-GFP⁺ was analysed using fluorescent microscopy on cryo- and paraffin-sections. Mean percentages of integrated PPC-GFP⁺, 2–3 weeks after cells injection, were calculated as mean percentage of GFP⁺ cardiomyocytes at the site of injection, multiplied by the

total number of cells in the region of injection and number of analysed slides.

To block *in vivo* nitric oxide (NO) production, mice were intraperitoneally injected daily with 10 mg/kg body weight of L-NAME (Sigma) from day 7 until they were killed at day 21, as described.[19] Control mice received D-NAME (Sigma) under the same experimental conditions. Groups of L-NAME- and D-NAME-treated mice were injected with PPC on days 7 and 14.

2.4 Generation of bone marrow chimera mice

Using a Gammatron (Co-60) system, 5–8-week-old C57/Bl6 (CD45.2⁺) mice were lethally irradiated with two doses of 6.5 Gy, and reconstituted with 2 × 10⁷ donor bone marrow cells from C57/Bl6-Ly5.1 (CD45.1⁺) mice. After reconstitution, all mice received prophylactic antibiotics in the drinking water and were housed in a specific pathogen-free environment.

2.5 Histology

Animals were sacrificed at day 21 after the first immunization. Hearts were removed and stained with haematoxylin–eosin (HE). Myocarditis severity was assessed on HE sections and graded from 0 to 4, as described before.[8]

2.6 Reverse transcription–polymerase chain reaction

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 material online, *Table S1*). 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.

2.7 Immunocytochemistry

Cells were cultured on gelatine-coated cover slips. Fixation and immunostaining procedures were performed according to Wobus *et al.*[20] (see Supplementary material online, *Methods*).

2.8 Flow cytometry and cell sorting

Cells were incubated for 30 min on ice with the appropriate combination of fluorochrome conjugated and/or primary and secondary antibodies (see Supplementary material online, *Methods*), then washed and analysed on a FACS Calibur (BD Bioscience) using FloJo 6.1.4 software (TreeStar, Ashland, OR, USA). PPC were separated on a FACSVantage SE (BD Bioscience).

2.9 T cell lines, proliferation assays, and cytokine ELISA

For proliferation assays, CD4⁺ T cell lines were either stimulated for 24 h in anti-CD3-coated 96-well plates or for 48 h on irradiated MyHC-α peptide-pulsed splenocytes in RPMI 1640 medium (Cambrex BioWhittaker, Vervier, Belgium) with additives (see Supplementary material online, *Methods*). With titrating amounts of PPC, 10⁵ CD4⁺ MyHC-α-specific T cell clones and 2 × 10⁵ antigen presenting cells (APC)/well were co-cultured. Proliferation was assessed by measuring ³H-thymidine incorporation (Amersham Biosciences, Otelfingen, Switzerland). Nitrite (NO₂⁻) levels reflecting NO production were determined using the Griess Reagent System (Promega, Madison, WI, USA). IFN-γ and TNF-α were

measured using commercially available OptEIA™ Mouse Elisa systems (both BD Biosciences).

2.10 Functional analysis

Echocardiographic assessments were carried out as described.[7] For analysis, we used healthy control mice, and animals immunized with MyHC-α/CFA, injected with either PPC or PBS.

2.11 Statistics

The Mann–Whitney *U*-test was used for the evaluation of non-parametrical data. Proliferation responses and cytokine levels were compared using ANOVA and Student's *t*-test.

3. Results

3.1 Expansion of PPC from healthy hearts

To specifically enrich heart-resident monocyte precursor cells, we adopted a modified two-step protocol, originally described for the expansion of cardiac progenitor cells from heart cell suspensions.[17] Healthy adult heart contains up to 2% prominin-1$^+$ cells (see Supplementary material online, Figure S1). On the basis of the hypothesis that the expansion of bone marrow-derived precursor cells critically depends on various tissue factors creating a specific milieu, we seeded the cell suspensions at high density ($>10^6$ cells per 60 mm culture dish) and screened proliferating cells for the expression of both bone marrow-specific and progenitor cell markers. After a few days of culture, we observed small, round, semi-attached, and highly proliferating cells which contained 80–90% CD45$^+$CD11b$^+$ cells co-expressing the stem/progenitor cell marker prominin-1 (*Figure 1A*). Prominin-1$^+$ cells were then purified by cell sorting, re-plated onto gelatine-coated plates, and expanded for another 5–6 passages. The resulting PPC were clonogenic; co-plating of single PPC-GFP$^+$ cell together with 5×10^4 PPC-GFP$^-$ feeder cells resulted in highly proliferating clones (see Supplementary material online, *Figure S1A*). All PPC were CD11b$^+$CD45$^+$ and chemokine receptor (CXCR) 4 expressing cells (*Figure 1A, C,* and *D*). In addition, PPC co-expressed stem/progenitor cell markers, such as stem cell antigen (Sca) 1 and c-kit (CD117) (*Figure 1A* and *E*), suggesting a monocyte precursor phenotype. PPC, however, did not express the endothelial/haematopoietic progenitor marker CD34 or the granulocyte specific marker Gr-1 (not shown). On the genetic level, PPC showed enhanced expression of genes characteristic for stem/progenitor cells such as nanog, Sca-1, and c-kit, and markers of early lineage precursors like Islet-1 and nestin (see *Figure 3A*).

Isolation of PPC from hearts of CD45.1/CD45.2 chimera mice confirmed the bone marrow origin of PPC (*Figure 1F*). Importantly, the efficient generation of clonogenic PPC critically required the presence of a heart-derived feeder cell layer.

Thus, we have established an efficient *in vitro* system to specifically expand high numbers of clonogenic PPC with stem/progenitor and haematopoietic cell characteristic from mouse hearts.

3.2 Differentiation capacity of PPC

PPC co-expressed CD45 and CD11b, but no markers indicating a mature monocyte or macrophage phenotype such

as F4/80 (*Figure 2A*). In the presence of M-CSF, PPC lost their stem/progenitor characteristic gene expression pattern; they became F4/80$-$ and major histocompatibility complex (MHC) class II-positive, acquired a large, flat morphology, and showed granular cytoplasm and phagocytic activity (*Figure 2D* and *E*). On the other hand, in the presence of GM-CSF, PPC expressed CD11c and MHCII and acquired a dendritic cell phenotype (*Figure 2F* and *G*).

The observation that PPC expressed transcription factors characteristic for the developing myocardium (*Figure 3B*) prompted us to evaluate the capacity of PPC to differentiate into cardiomyocyte-like cells. To induce cardiac differentiation, we cultured PPC in the presence of oxytocin.[21,22] As illustrated in *Figure 3*, oxytocin-treated PPC formed clusters (*Figure 3C*), expressed genes characteristic for cardiomyocytes such as Nkx 2.5, cardiac actin, cardiac troponin (cTn) I, αMyHC, βMyHC, atrial natriuretic peptide, and myosin light chain-2V[23] (*Figure 3B*), and produced the corresponding proteins (*Figure 3E–H*). Co-cultures of differentiated, GFP-expressing PPC together with adult, cell-tracker-labelled rat cardiomyocytes resulted in spontaneous contraction of the clusters (see Supplementary material online, *Movie*), excluding cell fusion between rat and GFP$^+$ mouse cells (*Figure 3D*; see Supplementary material online, *Figure S3B*).

In order to confirm the capacity of the PPC to differentiate into cardiomyocytes *in vivo*, 5×10^4 PPC-GFP$^+$ were injected directly into the left ventricular wall of healthy heart. One day after injection, PPC were detectable as small, round, and prominin-1-expressing cells (*Figure 3I*). Three weeks later, GFP-expressing cells were clearly integrated into the myocardium and co-expressed cardiomyocyte-specific markers, such as αMyHC, sarcomeric actin, and cTnI (*Figure 3K–M*). We determined that the region of cell insertion into the myocardium contained 500–2500 of injected GFP$^+$, which corresponds to 1–5% of the total number of injected PPC-GFP$^+$. Notably, none of GFP$^+$ cells expressed vimentin or αSMA, specific for fibroblast differentiation (*Figure 3N* and *O*). Importantly, PPC did not accumulate specifically in the healthy heart after intravenous injection (not shown).

Taken together, these results demonstrate the capacity of PPC to differentiate into cardiomyocyte-like cells *in vitro* and *in vivo* and to integrate themselves into the myocardium of healthy mice after direct intra-cardial administration.

3.3 PPC suppress experimental autoimmune myocarditis

Autoimmune myocarditis development depends on the presence of heart-specific autoreactive CD4$^+$ T cells.[24,25] The first inflammatory infiltrates develop 10 and 14 days after immunization and maximal disease scores are usually evident at days 20–23.[9] Given the high plasticity of the PPC, we queried how these cells affect the disease course of EAM.

We immunized groups of BALB/c wild-type mice with the MyHC-α peptide together with CFA, and addressed the *in vivo* fate of PPC, labelled with fluorochrome-conjugated nanocrystals (QD), after intravenous administration at days 7 and 14 following immunization (2×10^6 of PPC-QD$^+$/animal). PPC-QD$^+$ homed to the hearts of immunized mice and were still detectable 2–3 weeks after injection (*Figure 4A* and *B*). We detected 5×10^4–2×10^5 of PPC-QD$^+$
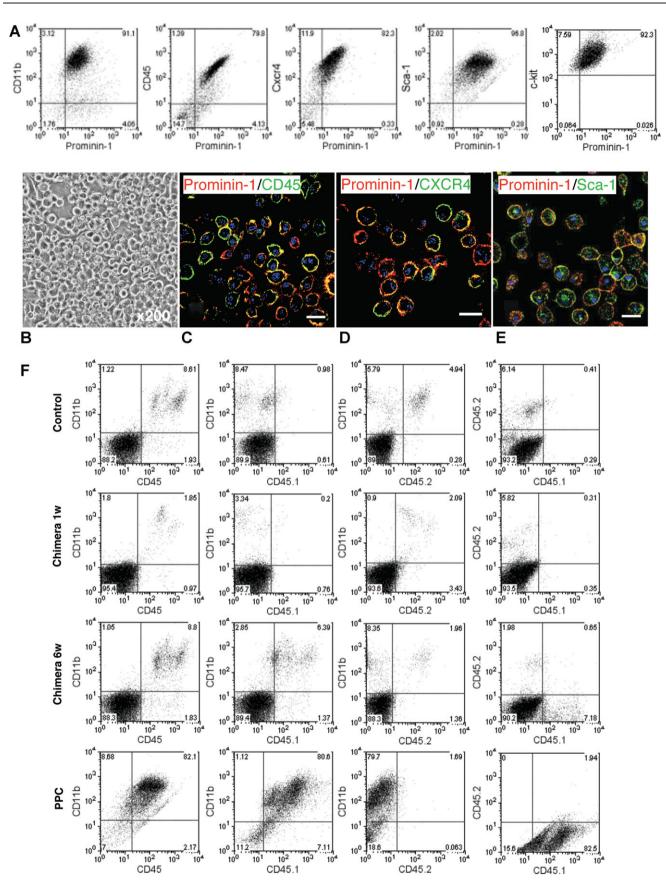
Figure 1 Characteristic of prominin-1-expressing progenitor cells (PPC). (*A*) FACS analysis of heart-derived cells before separation of prominin-1$^+$ cells. (*B*) Morphology of PPC. (*C–E*) Immunofluorescence showed that PPC co-expressed CD45 (*C*), CXCR4 (*D*), and Sca-1 (*E*). Hoechst 33342 (blue) was used to visualize cell nuclei. Bars = 20 μm. (*F*) In CD45.1/CD45.2, chimera mice population of donor CD45.1 cells replaced CD45.2 of host origin within the heart. PPC from hearts of CD45.1/CD45.2 chimera confirmed the bone marrow origin of PPC.
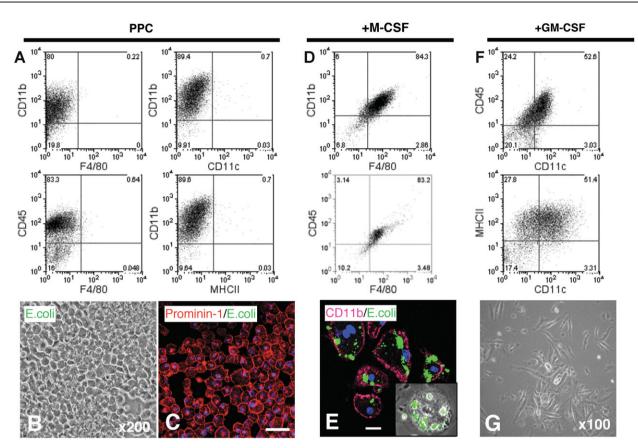
Figure 2 Sorted prominin-1-expressing progenitor cells (PPC) differentiated into macrophage- and dendritic-like cells *in vitro*. (*A–C*) Undifferentiated prominin-1^{+}/CD45^{+}/CD11b^{+} cells stained negative for F4/80, CD11c, and major histocompatibility complex (MHC) II (*A*), and did not show phagocyte activity (*B* and *C*). (*D* and *E*) PPC exposed to macrophage-colony-stimulating factor (M-CSF) co-expressed F4/80, CD11b, and CD45, and became large, oval cells with the capacity to phagocyte bacteria (*E*). (*F* and *G*) PPC exposed to granulocyte/macrophage colony-stimulating factor (GM-CSF) started to express CD11c and MHCII (*F*), and changed the morphology towards dendritic cell phenotypes (*G*). Hoechst 33342 (blue) was used to visualize cell nuclei. Bars = 20 μm.

among the total cell number in heart tissue suspensions in FACS analysis. This number corresponds to ∼5% of injected PPC-QD[+]. Importantly, QD-labelled cells accumulating within the inflamed heart gained the expression of macrophage markers F4/80 (*Figure 4A*) and CD68 (*Figure 4B*), but no fibroblast (collagen I; in *Figure 4C*), granulocyte (Gr-1; in *Figure 4D*) or cardiac markers (not shown). This suggests that in the inflammatory environment *in vivo*, PPC differentiated to a macrophage-like phenotype. No relevant homing to other organs was observed (not shown).

We next evaluated the effect of intravenously injected PPC on myocarditis severity in immunized mice. Control animals received either PBS or crude suspensions of bone marrow cells. Injection of PPC (*Figure 4E* and *G*), but not PBS (*Figure 4F* and *G*) or crude bone marrow cells (*Figure 4G*), suppressed the histological severity of myocarditis. Echocardiography showed preserved fractional shortening (*Figure 4I*) and velocity of circumferential shortening (*Figure 4K*) in PPC-treated mice compared with control PBS-treated MyHC-α/CFA-immunized mice. These data demonstrate that PPC injected during the acute phase of disease efficiently suppress EAM and prevent the progression to heart failure.
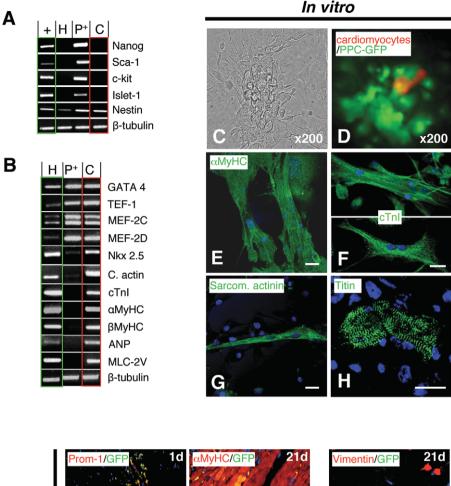
3.4 PPC suppress the expansion of heart-specific CD4^{+} T cells

As shown in *Figure 4H*, injection of PPC impaired the expansion of heart-specific, auto-reactive CD4^{+} T cells *in vivo*.

CD4^{+} T cells were isolated at day 21 from immunized mice treated with either PPC or PBS. CD4^{+} T cells were then re-stimulated on MyHC-α pulsed antigen presenting cells. As illustrated in *Figure 4H*, CD4^{+} T cells from PPC-treated, MyHC-α/CFA-immunized mice show a markedly reduced recall response upon MyHC-α stimulation. This, together with the fact that PPC were injected 7 and 14 days after immunization (i.e. after the priming phase of T cells), illustrates the markedly impaired *in vivo* expansion of MyHC-α-specific T cells in PPC-treated mice.

Because the frequency of the pathogenic Th17 CD4^{+} T cell subset within the whole peripheral CD4^{+} T cell population largely exceeds 1–2% in the EAM model,[24] we specifically addressed the effect of the PPC on *in vitro* expanded, highly MyHC-α-specific CD4^{+} T cell lines containing between 20 and 60% IL-17 producing T cells, together with 10–20% of IFN-γ producing T cells (*Figure 5A* and not shown). As illustrated in *Figure 5B*, PPC strongly suppressed the proliferation of MyHC-α-specific, activated CD4^{+} T cells *in vitro* and reduced the release of TNF-α, one of the key cytokines in myocarditis development[26] (*Figure 5D*).

Given the fact that PPC differentiate to mature macrophages in the inflammatory microenvironment *in vivo* (*Figure 4*), we hypothesized that PPC mediate T cell suppression by NOS2 up-regulation and release of NO *in vivo*. Indeed, we observed markedly enhanced release of NO (*Figure 5E*) in supernatants of T cell/APC cultures in the presence of PPC. In addition, T cell responses were largely restored in the
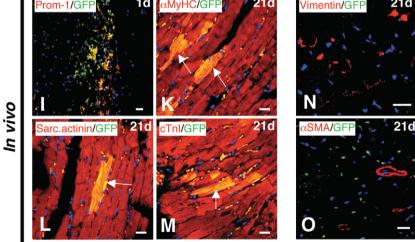
Figure 3 Sorted prominin-1-expressing progenitor cells (PPC) differentiated to cardiomyocyte-like cells. (*A*) mRNA levels of stem and progenitor cell specific markers: nanog, sca-1, c-kit, Islet-1, nestin. (*B*) mRNA levels of genes specific for developing and mature cardiomyocytes like GATA4, Nkx2.5, MEF-2C, MEF-2D, TEF-1, cardiac actin, cardiac troponin (cTn) I, α myosin heavy chain (αMyHC), βMyHC, atrial natriuretic peptide, myosin light chain-2V, in sorted PPC (P$^+$), and after differentiation into cardiac (C) lineages. (+), positive control; H, adult heart. (*C–H*) Oxytocin induced cardiac *in vitro* differentiation of PPC. PPC formed cardiomyocyte-like clusters (*C*). Co-culture of PPC-GFP$^+$ cells with cell-tracker red CMTPX-labelled adult rat cardiomyocytes showed no cell fusion between mouse and rat cells (*D*). Cardiomyocyte-like cells differentiated from PPC expressed αMyHC (*E*), cTnI (*F*), and showed a typical pattern of sarcomeric actin (*G*) and titin (*H*). (*I–O*) PPC injected into healthy hearts were integrated in the myocardium. Prominin-1$^+$/GFP$^+$ cells were found in clusters 1 day post-injection (*I*), and acquired the cardiac phenotype 3 weeks after injection expressing αMyHC (*K*), sarcomeric actinin (*L*), cTnI (*M*), but not vimentin (*N*) or αSMA (*O*). Hoechst 33342 (blue) was used to visualize cell nuclei. Bars = 20 μm.

presence of the NOS inhibitor L-NAME (*Figure 5C*), suggesting that PPC-mediated NO release indeed accounts for the observed suppression of heart-reactive T cells *in vitro*. The critical role of NO in the PPC-mediated suppression of auto-reactive T cell responses and myocarditis was confirmed *in vivo*. As shown in *Figure 6A* and *B*, treatment of immunized

and PPC injected mice with the non-specific NOS inhibitor L-NAME, but not with the inactive D-NAME, abolished the pro-tective effects of PPC treatment.

Several lines of evidence suggest that IFN-γ is critically involved in the up-regulation of NOS2 on activated mono-cytes/macrophages. We therefore hypothesized that IFN-γ
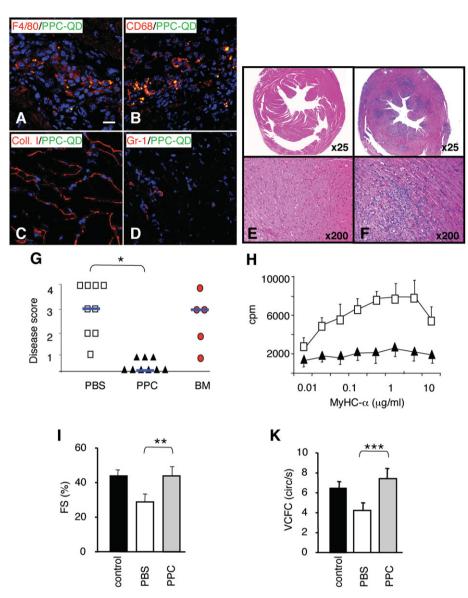
Figure 4 Prominin-1-expressing progenitor cells (PPC) suppressed experimental autoimmune myocarditis. (A–D) Intravenously injected PPC-QD$^+$ (2×10^6 cells per mouse at days 7 and 14) homed to the hearts of myosin alpha heavy chain/complete Freund's adjuvant (MyHC-α/CFA)-immunized mice, and 21 days post-immunization showed characteristic of macrophages expressing F4/80 (A) and CD68 (B), but not collagen I$^+$ fibroblasts (C) or Gr-1$^+$ granulocytes (D). (E and F) Heart sections from MyHC-α/CFA-immunized mice treated either with PPC (E) or PBS (F). (G) Myocarditis scores of individual immunized mice treated with PPC (black triangles), PBS (white squares), or bone marrow suspensions (red circles), analysed 21 days after immunization. Median values for each group are shown. (H) Proliferation assay of CD4$^+$ T cells isolated from spleens of MyHC-α/CFA-immunized mice treated with PPC (black triangles) or PBS (white squares). Means $\pm$ SD of 23 individual mice (nine per experimental and control group, five per bone marrow treated mice) are shown. (I–K) Echocardiography of mice treated with PBS or PPC after MyHC-α/CFA immunization. Fractional shortening (FS, %) (I) and velocity of circumferential shortening (VCFC; circ/s) (K) were determined 34 days after immunization on PBS- or PPC-treated mice and on healthy mice used as controls. Average values $\pm$ SD of six individual mice are shown.

signalling might also be critical for NOS2 induction in PPC within the inflammatory microenvironment. To address this question directly *in vivo*, we treated MyHC-α/CFA-immunized mice with PPC generated from hearts of IFN-γR$^{-/-}$ mice. As illustrated in *Figure 6C*, IFN-γR$^{-/-}$ PPC did not protect against myocarditis development.

Taken together, these data suggest that PPC exert a direct inhibitory effect on heart-specific autoreactive CD4$^+$ T cells. This effect requires IFN-γ signalling on PPC and is mediated by NO.

4. Discussion

We have developed an efficient *in vitro* system to expand high numbers of a specific population of bone marrow-derived prominin-1-expressing monocyte precursor cells from healthy heart tissue with immunomodulating and multilineage differentiation capacity. PPC homed to the inflamed heart, protected against EAM, and inhibited pathogenic CD4$^+$ T cell responses. Importantly, the PPC are self-renewing, clonogenic, and exhibit a capacity to differentiate not only to macrophage/monocyte or dendritic cell phenotypes but also to cardiomyocyte-like cells depending on the cell culture conditions and applied signalling. From this point of view, our cells fulfil the required criteria for multipotent progenitor cells and share many features of mesenchymal stem cells (MSC).[27]

EAM is a CD4$^+$ T cell-mediated disease. Th17 cells are critical for the induction of heart-specific autoimmunity, whereas Th1 and Th2 subsets are supposed to modulate
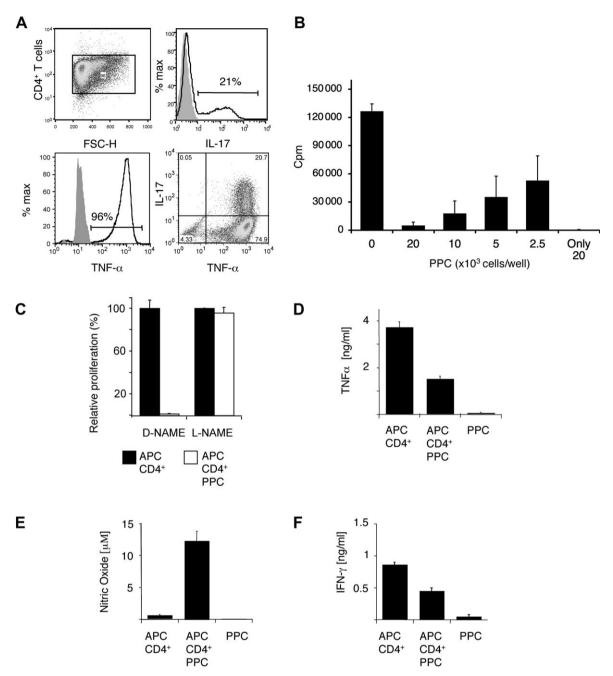
Figure 5 Prominin-1-expressing progenitor cells (PPC) inhibited the proliferation of myosin alpha heavy chain (MyHC-α)-specific Th17 T cell *in vitro*. (*A*) FACS analysis confirmed that T cell lines contained between 20 and 60% of interleukin-17 producing CD4+ T cells. (*B*) Titrating amounts of PPC suppressed the proliferation of MyHC-α-specific CD4+ T17 cells. CD4+ T17 cells were re-stimulated with the MyHC-α antigen on irradiated APC cells. (*C*) The nitric oxide synthase (NOS) inhibitor L-NAME prevented PPC-mediated suppression of antigen re-stimulated MyHC-α-specific CD4+ T17 cells. TNF-α (*D*) and IFN-γ (*F*) production of *in vitro* MyHC-α antigen re-stimulated CD4+ T cells are reduced in the presence of PPC. (*E*) Enhanced NO production in supernatants of T cell/APC cultures in the presence of PPC. Each bar represents the mean ± SD from five different culture wells.

the disease phenotype.[24,28] Our data show that PPC suppress the expansion of autoreactive CD4+ T cells *in vivo* and *in vitro*. Mechanistically, the protective capacity of the PPC depends on their differentiation to a monocyte/macrophage-like phenotype suppressing heart-specific autoreactive CD4+ T cells IFN-γ-dependently by release of NO. This observation is in line with earlier studies suggesting immunomodulatory effects of NO, including the induction of reversible growth arrest in proliferating human Th1 and Th2 T cell lines.[29] T cell suppression could also be observed in the absence of APC, if T cells were stimulated by plate-bound anti-CD3 antibodies (not shown), suggesting direct

NO release from PPC. PPC, injected into MyHC-α/CFA-immunized mice differentiated into monocyte-like cells up-regulating NOS2 synthetase. Importantly, this effect was dependent upon IFN-γ, suggesting that the immunomodulating effects of PPC require the presence of IFN-γ, released by Th1 cells as part of a heterogeneous autoreactive CD4+ T cell response, CD8+ T cells,[24] or natural killer (NK) cells. In autoimmune myocarditis, autoreactive heart infiltrating CD4+ T cells belong to the Th17 phenotype. Accordingly, only minimal IFN-γ is released from CD4+ T cells within the acutely inflamed heart.[24] However, it has been shown very recently that heart-infiltrating CD8+ T
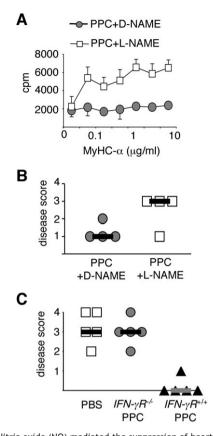
Figure 6 Nitric oxide (NO) mediated the suppression of heart-specific CD4^{+} T cells *in vivo*. (*A*) Proliferation assay of CD4^{+} T cells isolated from spleens of myosin alpha heavy chain/complete Freund's adjuvant (MyHC-α/CFA)-immunized mice treated with prominin-1-expressing progenitor cells (PPC) (at days 7 and 14) and D-NAME (daily between days 7–21; grey circles), or with PPC (at days 7 and 14) and L-NAME (daily between days 7–21; black squares). Means $\pm$ SD of 8 individual mice are shown. (*B*) Myocarditis scores of individual immunized mice analysed at day 21, after treatments with PPC (at days 7 and 14) and D-NAME (daily between days 7–21; grey circles), or with PPC (at days 7 and 14) and L-NAME (daily between days 7–21; black squares). (*C*) Myocarditis scores of individual wt (*INF-γR$^{+/+}$*) mice 21 days after MyHC-α/CFA immunization, after treatments, at days 7 and 14, with PPC derived from the hearts of *IFN-γR$^{-/-}$* mice (grey circles), with PPC derived from the wild-type hearts of *IFN-γR$^{+/+}$* mice (black triangles), or with PBS as control (white squares). Median values for each group are shown.

cells release IFN-γ and mediate non-specific bystander suppression of autoreactive Th17 cells by induction of NOS2 and NO release from macrophages.[24] Nevertheless, we cannot exclude other PPC-mediated anti-inflammatory mechanisms and further studies are certainly needed to clarify these issues.

Given the progenitor cell characteristics of the PPC, it should be mentioned that human MSC are also supposed to exert anti-inflammatory effects by changing the cytokine secretion profile of dendritic cells, naive and effector T cells, and NK cells.[30] In the animal system, MSC reduced EAM severity in rats.[31,32] Interestingly, Sato *et al.*[32] recently showed that MSC induce an NO-dependent reversible T cell growth arrest *in vitro*. In humans, cell-based immunomodulatory treatments are mostly applied to patients with autoimmune disease such as severe systemic lupus erythematosus[33] or as adjuvant for severe graft vs. host disease after bone marrow transplantation in patients with leukaemia.[34] In both cases, however, the precise mechanisms responsible for the beneficial effect of cell-based immune-modulating

treatment remain unknown. The PPC provide some explanation on the cellular level as to how such modulation of the immune system could work. To our knowledge and in contrast to the heterogeneous population of MSC, these PPC are the first haematopoietic progenitor cells with such well-defined properties.

Appropriate timing of PPC injections was essential for the prevention of EAM. These findings are in line with observations on the effects of embryonic stem cells in a model of viral myocarditis[35] or MSC in a rat model of acute myocarditis.[36] Our data suggest that the protective effects of PPC depend rather on their immunomodulatory capacity than on their regenerative potential. Injection of PPC resulted in the differentiation of macrophages/mature monocytes in the inflammatory environment *in vivo*, and we found no evidence for heart-specific differentiation. On the other hand, in the microenvironment of a healthy heart PPC integrated themselves into the myocardium and differentiated into cardiomyocytes. Nevertheless, the multilineage differentiation capacity of the PPC offers the potential to develop strategies that specifically promote tissue regeneration from PPC in the inflamed heart. Potential strategies might include combined PPC treatment/cytokine targeting, and epigenetic or genetic manipulation of *in vitro* expanded PPC.

PPC represent a population of prominin-1-expressing, bone marrow-derived heart-resident cells. Prominin-1-expressing cells are also present in bone marrow of healthy animals but show no comparable regenerative and immunomodulatory capacity (unpublished observation). We believe that either heart-specific tissue factors, the specific microenvironment due to the disintegration of whole heart tissue, or inflammatory processes are critical for the activation and expansion of immunomodulatory and regenerative PPC. If it is possible to precisely specify these conditions and to adopt them on crude bone marrow, it might become feasible to isolate huge numbers of immunomodulatory/regenerative cells from bone marrow. This would be a further step towards an innovative treatment strategy against inflammatory heart diseases.

In conclusion, the healthy mouse heart contains a specific population of prominin-1[+] bone marrow-derived heart-resident precursor cells that can be expanded from disintegrated cardiac tissue. These cells combine regenerative and immunomodulatory capacities and suppress EAM in mice. If it will become feasible to specifically control the differentiation potential of the PPC within the specific cytokine/chemokine milieu of the chronically inflamed heart, PPC-based treatment strategies could become a promising therapeutic option for devastating inflammatory heart diseases in the future.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Funding

Acknowledgements

References

1. Feldman AM, McNamara D. Myocarditis. *N Engl J Med* 2000;**343**:1388–1398.
2. Rose NR. Infection, mimics, and autoimmune disease. *J Clin Invest* 2001;**107**:943–944.
3. Fairweather D, Kaya Z, Shellam GR, Lawson CM, Rose NR. From infection to autoimmunity. *J Autoimmun* 2001;**16**:175–186.
4. Eriksson U, Penninger JM. Autoimmune heart failure: new understandings of pathogenesis. *Int J Biochem Cell Biol* 2005;**37**:27–32.
5. Neu N, Rose NR, Beisel KW, Herskowitz A, Gurri-Glass G, Craig SW. Cardiac myosin induces myocarditis in genetically predisposed mice. *J Immunol* 1987;**139**:3630–3636.
6. Eriksson U, Kurrer MO, Schmitz N, Marsch SC, Fontana A, Eugster HP *et al.* Interleukin-6-deficient mice resist development of autoimmune myocarditis associated with impaired upregulation of complement C3. *Circulation* 2003;**107**:320–325.
7. Eriksson U, Ricci R, Hunziker L, Kurrer MO, Oudit GY, Watts TH *et al.* Dendritic cell-induced autoimmune heart failure requires cooperation between adaptive and innate immunity. *Nat Med* 2003;**9**:1484–1490.
8. Marty RR, Dirnhofer S, Mauermann N, Schweikert S, Akira S, Hunziker L *et al.* MyD88 signaling controls autoimmune myocarditis induction. *Circulation* 2006;**113**:258–265.
9. Afanasyeva M, Georgakopoulos D, Belardi DF, Ramsundar AC, Barin JG, Kass DA *et al.* Quantitative analysis of myocardial inflammation by flow cytometry in murine autoimmune myocarditis: correlation with cardiac function. *Am J Pathol* 2004;**164**:807–815.
10. Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G *et al.* Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 2005;**436**:266–271.
11. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL *et al.* The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med* 2007;**204**:3037–3047.
12. Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A *et al.* Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med* 2007;**204**:1057–1069.
13. Rota M, Kajstura J, Hosoda T, Bearzi C, Vitale S, Esposito G *et al.* Bone marrow cells adopt the cardiomyogenic fate in vivo. *Proc Natl Acad Sci USA* 2007;**104**:17783–17788.
14. Xu Q. Stem cells and transplant arteriosclerosis. *Circ Res* 2008;**102**:1011–1024.
15. Kania G, Corbeil D, Fuchs J, Tarasov KV, Blyszczuk P, Huttner WB *et al.* Somatic stem cell marker prominin-1/CD133 is expressed in embryonic stem cell-derived progenitors. *Stem Cells* 2005;**23**:791–804.
16. Mizrak D, Brittan M, Alison MR. CD133: molecule of the moment. *J Pathol* 2008;**214**:3–9.
17. Tang YL, Shen L, Qian K, Phillips MI. A novel two-step procedure to expand cardiac Sca-1+ cells clonally. *Biochem Biophys Res Commun* 2007;**359**:877–883.
18. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* 2004;**95**:911–921.
19. Eriksson U, Kurrer MO, Bingisser R, Eugster HP, Saremaslani P, Follath F *et al.* Lethal autoimmune myocarditis in interferon-gamma receptor-deficient mice: enhanced disease severity by impaired inducible nitric oxide synthase induction. *Circulation* 2001;**103**:18–21.
20. Wobus AM, Guan K, Yang HT, Boheler KR. Embryonic stem cells as a model to study cardiac, skeletal muscle, and vascular smooth muscle cell differentiation. *Methods Mol Biol* 2002;**185**:127–156.
21. Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H *et al.* Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. *J Biol Chem* 2004;**279**:11384–11391.
22. Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K *et al.* Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. *J Cell Biol* 2007;**176**:329–341.
23. Lints TJ, Parsons LM, Hartley L, Lyons I, Harvey RP. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* 1993;**119**:969.
24. Rangachari M, Mauermann N, Marty RR, Dirnhofer S, Kurrer MO, Komnenovic V *et al.* T-bet negatively regulates autoimmune myocarditis by suppressing local production of interleukin 17. *J Exp Med* 2006;**203**:2009–2019.
25. Donermeyer DL, Beisel KW, Allen PM, Smith SC. Myocarditis-inducing epitope of myosin binds constitutively and stably to I-Ak on antigen-presenting cells in the heart. *J Exp Med* 1995;**182**:1291–1300.
26. Bachmaier K, Pummerer C, Kozieradzki I, Pfeffer K, Mak TW, Neu N *et al.* Low-molecular-weight tumor necrosis factor receptor p55 controls induction of autoimmune heart disease. *Circulation* 1997;**95**:655–661.
27. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;**418**:41–49.
28. Sonderegger I, Rohn TA, Kurrer MO, Iezzi G, Zou Y, Kastelein RA *et al.* Neutralization of IL-17 by active vaccination inhibits IL-23-dependent autoimmune myocarditis. *Eur J Immunol* 2006;**36**:2849–2856.
29. Eriksson U, Egermann U, Bihl MP, Gambazzi F, Tamm M, Holt PG *et al.* Human bronchial epithelium controls TH2 responses by TH1-induced, nitric oxide-mediated STAT5 dephosphorylation: implications for the pathogenesis of asthma. *J Immunol* 2005;**175**:2715–2720.
30. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**:1815–1822.
31. Okada H, Suzuki J, Futamatsu H, Maejima Y, Hirao K, Isobe M. Attenuation of autoimmune myocarditis in rats by mesenchymal stem cell transplantation through enhanced expression of hepatocyte growth factor. *Int Heart J* 2007;**48**:649–661.
32. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T *et al.* Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* 2007;**109**:228–234.
33. Traynor AE, Schroeder J, Rosa RM, Cheng D, Stefka J, Mujais S *et al.* Treatment of severe systemic lupus erythematosus with high-dose chemotherapy and haemopoietic stem-cell transplantation: a phase I study. *Lancet* 2000;**356**:701–707.
34. Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lonnies H *et al.* Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* 2006;**81**:1390–1397.
35. Malek S, Kaplan E, Wang JF, Ke Q, Rana JS, Chen Y *et al.* Successful implantation of intravenously administered stem cells correlates with severity of inflammation in murine myocarditis. *Pflugers Arch* 2006;**452**:268–275.
36. Ohnishi S, Yanagawa B, Tanaka K, Miyahara Y, Obata H, Kataoka M *et al.* Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. *J Mol Cell Cardiol* 2007;**42**:88–97.