Regression of cardiac hypertrophy by granulocyte colony-stimulating factor-stimulated interleukin-1β synthesis

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Aims
Aortic stenosis causes cardiac hypertrophy and fibrosis, which often persists despite pressure unloading after aortic valve replacement. The persistence of myocardial fibrosis in particular leads to impaired cardiac function and increased mortality. We investigated whether granulocyte colony-stimulating factor (G-CSF) beneficially influences cardiac remodelling after pressure unloading.

Methods and results
Left ventricular hypertrophy was induced by transverse aortic constriction in C57bl6 mice followed by debanding after 8 weeks. This model closely mimics aortic stenosis and subsequent aortic valve replacement. After debanding, mice were treated with either G-CSF or saline injection. Granulocyte colony-stimulating factor treatment significantly improved systolic (ejection fraction 70.48 ± 1.17 vs. 58.41 ± 1.56%, P < 0.001) and diastolic (E/E′ 26.0 ± 1.0 vs. 32.6 ± 0.8, P < 0.05) function. Furthermore, cardiac fibrosis was significantly reduced in G-CSF-treated mice (collagen-I area fraction 7.96 ± 0.47 vs. 11.64 ± 1.22%, P < 0.05; collagen-III area fraction 10.73 ± 0.99 vs. 18.46 ± 0.71%, P < 0.001). Direct effects of G-CSF on cardiac fibroblasts or a relevant transdifferentiation of mobilized bone marrow cells could be excluded. However, a considerable infiltration of neutrophils was observed in G-CSF-treated mice. This sterile inflammation was accompanied by a selective release of interleukin-1β (IL-1β) in the absence of other proinflammatory cytokines. In vitro experiments confirmed an increased expression of IL-1β in neutrophils after G-CSF treatment. Interleukin-1β directly induced the expression of the gelatinases matrix metalloproteinase-2 (MMP-2) and MMP-9 in cardiac fibroblasts thereby providing the regression of cardiac fibrosis.

Conclusion
Granulocyte colony-stimulating factor treatment improves the cardiac function and leads to the regression of myocardial fibrosis after pressure unloading. These findings reveal a previously unknown mechanism of fibrosis regression. Granulocyte colony-stimulating factor might be a potential pharmacological treatment approach for patients suffering from congestive heart failure after aortic valve replacement, although further basic research and clinical trials are required in order to prove beneficial effects of G-CSF in the human organism.

Keywords
Aortic stenosis • Fibrosis • Hypertrophy • Inflammation • Interleukins

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Introduction
Cardiac fibrosis correlates with the post-operative clinical outcome in patients after aortic valve replacement due to severe aortic stenosis and is associated with systolic and diastolic dysfunction, myocardial ischaemia, and arrhythmias. Even though a regression of functional and structural abnormalities could be observed after pressure unloading, these reverse remodelling processes do not lead to a 'restituto ad integrum'. Wei-demann et al. demonstrated that the extent of histologically determined cardiac fibrosis closely correlated with the New York Heart Association (NYHA) functional class and the systolic function. Likewise, contemporary pharmacological approaches, e.g. application of ACE inhibitors or AT-1 receptor antagonists, fail to reduce cardiac fibrosis. Therefore, a controlled degradation of the enlarged extracellular matrix (ECM) would be an extraordinarily important therapeutic goal.

Granulocyte colony-stimulating factor (G-CSF) is a haematopoietic cytokine that regulates the proliferation, differentiation, and survival of myeloid progenitor cells and plays an eminent role in the regulation and production of neutrophil granulocytes in health and in response to infections. Apart from its immunomodulatory function, G-CSF reportedly has beneficial effects on myocardial remodelling and dysfunction in various cardiovascular pathologies, including myocardial infarction, dilated and anthracycline-induced cardiomyopathy, and the development of angiotensin-II-induced cardiac hypertrophy. Furthermore, G-CSF has shown beneficial effects in patients suffering from congestive heart failure due to ischaemic or dilated cardiomyopathy. It is well known that G-CSF induces the proliferation and differentiation of bone marrow-derived cells (BMCs). Thus, it has been proposed that G-CSF might contribute to the myocardial regeneration by mobilization and differentiation of BMCs. Furthermore, some authors have reported that G-CSF acts directly on cardiomyocytes via the Jak-Stat pathway and the PI3K-Akt pathway. In contrast to these reports, several experimental and clinical studies could not reproduce a beneficial effect of G-CSF after myocardial infarction. Therefore, the therapeutic use of G-CSF has been critically challenged because its underlying cellular and subcellular mechanisms (if any) remain widely unknown. In particular, the therapeutic potential of G-CSF after pressure unloading has not been investigated until now. We examined the molecular mechanisms of G-CSF in a mouse model that closely mimics aortic stenosis and subsequent aortic valve replacement.

Methods
Only a short description of methods is given here. An expanded version can be found in Supplementary material online.

Animals and animal procedures
All mice used in this study were 12–14-week-old females. All investigations conformed with the principles of GV-SOLAS and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996) and were approved by the appropriate authorities (Regierungspräsidium Darmstadt, Hessen, Germany).

Bone marrow transplantation (BMTx) was performed as described recently. C57BL/6-TgN(ActbEGFP)1Osb transgenic mice (Jackson Laboratory) served as bone marrow donors. Three months later, animals were subjected to transverse aortic constriction (TAC).

Bone marrow-transplanted C57BL/6 mice (n = 20) as well as healthy female C57BL/6 mice (n = 40) without preceding irradiation and BMTx were initially sedated in an induction chamber by exposure to isoflurane. A topical depilatory agent was applied to the neck and chest, and the area was cleaned and disinfected with Braunol (B. Braun, Germany). Mice were placed in supine position at a heating pad and temperature was maintained at 37°C. Mice were connected to a funnel and anaesthesia was kept up with the inhalation of isoflurane. A 1.0-cm skin incision was made at the level of the suprasternal notch. A 2–3-mm longitudinal cut was made in the cranial portion of the sternum, and the thymus lobes were carefully pushed aside with the forceps. This allowed the visualization of the aortic arch. The transverse aorta was isolated between the origin of the right innominate and left common carotid arteries. It was incised by a 7-0 polypropylene suture and constricted by tying firmly against a 27-gauge needle. The latter was promptly removed to yield a constriction of 0.4 mm in diameter. The sternum was adapted with a 5-0 polypropylene suture. The skin was closed with a 5-0 silk suture and mice were allowed to recover from anaesthesia on the heating pad at 37°C. In sham-operated mice, the aorta was not ligated. The debanding procedure was performed 8 weeks after aortic constriction in the same way as TAC except that the narrowing suture was cut and removed from the aortic arch.

Human recombinant G-CSF (Lenograstim, Granocyte, Chugai Pharmaceutical Co.) was administered subcutaneously in the first 4 days after the debanding procedure (300 µg/kg of bodyweight). Mice were euthanized after 4 days (G-CSF 4d), and 14 days (G-CSF 14d).

The overall mortality in the present study was 40%; 8.7% of mice died during TAC surgery. In the following 8 weeks after TAC, 20.2% of mice, who survived TAC surgery, died most probably due to heart failure. The debanding surgery was associated with a mortality rate of 19.5%; 7.3% of mice died within the 2 weeks after debanding.

High-resolution echocardiography
Anaesthesia was induced with 3% isoflurane gas and maintained with 1.0–1.5% isoflurane in 100% O2. Mice were laid supine on a heating platform with all legs taped to ECG electrodes for heart rate monitoring. Body temperature was monitored via a rectal thermometer (Indus Instruments, Houston, TX, USA) and maintained at 36.5–37.5°C using a heating pad and an infra-red lamp. The chest of the mice was shaved and treated with a chemical hair remover to reduce ultrasound attenuation. To provide a coupling medium for the transducer, a pre-warmed ultrasound gel was spread over the chest wall.

Transtracheal two-dimensional, M-mode and Doppler imaging were performed with VisualSonics Vevo770 high-resolution imaging system equipped by 30-MHz transducer (VisualSonics, Toronto, Canada).

Left ventricular internal dimensions at end-systole and end-diastole, interventricular septal wall thickness, and left ventricular posterior wall thickness as well as ejection fraction (EF) and fractional shortening were measured ECG-triggered in the parasternal long-axis as well as short-axis view. Ejection fraction was calculated by the methods of Teichholz and Simpson. Left ventricular mass was calculated as described previously. Left ventricular outflow tract (LVOT) dimension was measured from the LVOT view at the level of the aortic valve.

All echocardiographic parameters were calculated off-line using the tool section of the VisualSonics Vevo770 system. All the studies were analysed by two experienced sonographers who were blinded to the study.
Cardiac magnetic resonance imaging

The mice were first sedated in an induction chamber by exposure to isoflurane. Anaesthesia was induced with 3% isoflurane gas and maintained with 1.0–1.5% isoflurane in 100% O₂. Mice were placed in a supine position and an electrocardiogram was continually registered. In vivo magnetic resonance studies were performed on a 7.05 T horizontal bore MR scanner (BRUKER, Germany), which was equipped with a 60-mm microscopy gradient system capable of 870 mT/m gradient strength and a rise time of 280 μs at complete gradient switching. For transmission and reception of the signal, a birdcage probe head with an inner diameter of 35 mm was used. Magnetic resonance imaging (MRI) measurements were conducted by applying an ECG-triggered fast gradient echo cine sequence with the following imaging parameters: echo time, 1.6 ms; repetition time, 4.3 ms; field of view, 3.0 cm²; acquisition matrix, 256 × 256; maximal in-plane resolution, 117 μm²; and slice thickness, 1.0 mm. Analysis was performed using Mass4Mice® (Medis Medical Imaging Systems, The Netherlands). All mice that underwent the debanding procedure were subjected to MRI in order to exclude the residual stenosis of the aortic arch by adhesions.

Flow cytometry

Absolute peripheral blood leukocyte subpopulations in mice were enumerated using the TruCount flow cytometry assay (BD Biosciences) 4 days after the debanding procedure. Briefly, 50 μL of freshly drawn peripheral blood were stained with 2 μL of Cy5-conjugated anti-CD45 and 2 μL of FITC-conjugated anti-11b antibodies in a TruCount tube, and incubated for 20 min at room temperature. Prior to analysis by flow cytometer, red blood cells were lysed in the tube by adding 1500 μL of ammonium chloride-based lysing buffer (BD Biosciences) and incubating for 15 min at room temperature. Prior to analysis by flow cytometer, red blood cells were lysed in the tube by adding 1500 μL of ammonium chloride-based lysing buffer (BD Biosciences) and incubating for 15 min at room temperature. Data acquisition was performed on a BD LSR2 flow cytometer using fluorescence-activated cell sorting DIVA software (BD Biosciences). At least 50 000 CD45⁺ events and 3000 TruCount beads were acquired in a single measurement (threshold set on SSC and CD45 adjusted for optimal visualization of beads). Data were analysed using FlowJo software (V 9.1 for Macintosh, Tree Star, Inc.).

Statistics

All values are reported as the mean ± SEM. All statistical analyses were performed using GraphPad Prism® version 4.00 for Windows. Normality of data was ascertained using the Kolmogorov–Smirnov test. Multiple group comparisons were performed by analysis of variance testing followed by the Bonferroni procedure for comparison of means. Comparison between two groups was performed using an unpaired Student’s t-test. A two-tailed P-value of < 0.05 was considered as statistically significant.

Results

Incomplete regression of cardiac hypertrophy after pressure unloading

Our animal model, with TAC for 8 weeks and subsequent pressure unloading through debanding, closely mimics aortic stenosis and therapeutic valve replacement. Transverse aortic constriction reproducibly induced a pressure gradient of 48 ± 4.3 mmHg across the aortic arch (see Supplementary material online, Figure S1a) leading to severe cardiac hypertrophy and depressed contractile function. Cardiac hypertrophy and fibrosis were confirmed histologically by an increased cardiomyocyte cross-section area (357.55 ± 19.74 vs. 204.77 ± 14.23 μm², P < 0.001), myocardial collagen-I area fraction (14.32 ± 1.48 vs. 7.74 ± 1.10%, P < 0.001), and myocardial collagen-III area fraction (19.30 ± 0.86 vs. 10.61 ± 1.01%, P < 0.001), as well as decreased capillary density (2313 ± 169.9 vs. 2911 ± 198.3 m²/mm², P < 0.001). After debanding, MRI and echocardiography revealed continuous undisturbed flow through the aortic arch (Figure 1A). However, pressure unloading failed to significantly improve cardiac functional (Figure 1B–D) and morphological parameters (Figure 2).

Effects of granulocyte colony-stimulating factor on cardiac function, cardiac fibrosis, and morphology after pressure unloading

After debanding, human recombinant G-CSF was administered for the first 4 days after debanding (G-CSF-14d). A control group underwent the same procedure and received saline (control-14d) instead of G-CSF. Two weeks after debanding, we measured cardiac function by high-resolution echocardiography and histologically assessed morphological alterations. Echocardiography revealed significantly improved left ventricular systolic function (EF 70.48 ± 1.17 vs. 58.41 ± 1.56%, P < 0.001; Figure 1B), with considerably decreased myocardial dimensions (see Supplementary material online, Figure S1b) in G-CSF-14d-treated mice compared with control. Accordingly, the calculated left ventricular mass was decreased in G-CSF-treated mice (84.1 ± 2.68 vs. 128.3 ± 4.41 mg, P < 0.001; see Supplementary material online, Figure S1b), and brain natriuretic peptide expression was reduced compared with control mice (1.01 ± 0.21 vs. 1.86 ± 0.35, P < 0.05; see Supplementary material online, Figure S1b).

Left ventricular diastolic function reflecting the degree of cardiac fibrosis was normalized in G-CSF-14d, while diastolic dysfunction was still present in control-14d (Figure 1C and D).

Histological analysis showed a regression of a cardiomyocyte cross-section area (206.26 ± 16.54 vs. 255.67 ± 16.45 μm², P < 0.001; Figure 2A) and an increase in capillary density in G-CSF-14d compared with controls (3420 ± 229.4 vs. 2586 ± 143.7 n/mm², P < 0.001; Figure 2B). Most importantly, a remarkable reduction in fibrosis, ascertained by decreased collagen-I area fraction (7.96 ± 0.47 vs. 11.64 ± 1.22%, P < 0.05) and collagen-III staining area fraction (10.73 ± 0.99 vs. 18.46 ± 0.71%, P < 0.001), could be observed in G-CSF-14d. Consistent with the still affected diastolic function, the relative collagen-I and -III area was not decreased in control-14d (Figure 2C).

Differentiation of bone marrow-derived cells

To examine the impact of G-CSF on the potential cardiac homing and transdifferentiation of BMCs after pressure unloading, we used a mouse model in which the original bone marrow was replaced by a transgenic enhanced green fluorescent protein (eGFP)-marked stem cell pool. The efficiency of the BMTx was evaluated by flow cytometric analysis of the peripheral blood at different time points after transplantation. Fluorescence intensity revealed eGFP
expression in 86 ± 6% of all nucleated cells 6 weeks after BMTx and 82 ± 7% 12 weeks after BMTx, indicating the successful replacement of nearly the entire original stem cell population. Nonetheless, the quantification of invaded BMCs into the myocardium excluded significant differences in all study groups (see Supplementary material online, Figure S2A). Furthermore, co-staining of eGFP with CD31 and vimentin showed only very rare events of BMC transdifferentiation in endothelial cells or fibroblasts (see Figure 1: Granulocyte colony-stimulating factor improves the left ventricular function after pressure unloading. (A) Cardiac magnetic resonance imaging. Arrow shows the aortic constriction after transverse aortic constriction and the complete resolution of the constriction after debanding. (B) Representative images of M-mode echocardiography. Transverse aortic constriction led to severe cardiac hypertrophy and impaired left ventricular function. After debanding, parameters improved in terms of reverse remodelling. Granulocyte colony-stimulating factor-treated mice showed significantly improved the left ventricular function and increased the cardiac output. n = 12–15 mice per group. Data show the mean ± SEM. **p < 0.01 vs. sham, ***p < 0.001 vs. sham, ###p < 0.001 vs. control as determined by one-way analysis of variance. (C) Posterior wall Doppler flow signal and (D) tissue Doppler of the left ventricular lateral wall obtained from the apical four-chamber view. After transverse aortic constriction, mice exhibited severely affected diastolic function, represented by a decreased E/A ratio, an increased Tei index and an increased E/E' ratio. Granulocyte colony-stimulating factor-treated mice showed an improvement of diastolic functional parameters, while diastolic function was still affected in the control group. n = 12–15 mice per group. Data show the mean ± SEM. Significance was determined by one-way analysis of variance. *p < 0.05 vs. sham, **p < 0.01 vs. sham, #p < 0.05 vs. control, and ##p < 0.01 vs. control. All data were normalized to sham and the average of all sham values was defined as 100%.
Supplementary material online, Figure S2B and C. Only few eGFP-positive cardiomyocytes have been detected in all examined hearts, excluding a functional meaning of BMC transdifferentiation into cardiomyocytes (see Supplementary material online, Figure S2D).

**Granulocyte colony-stimulating factor triggers cardiac inflammatory cell recruitment and cytokine synthesis**

It is well established that G-CSF leads to the mobilization of neutrophil granulocytes and promotes neutrophil recruitment into inflammatory tissue. Nevertheless, it is unclear whether G-CSF can induce an inflammatory response despite the absence of other inflammatory stimuli. Fluorescence-activated cell sorting analysis of the peripheral blood of G-CSF-treated mice showed a significant increase in circulating inflammatory cells, particularly neutrophils (Figure 3A). To determine whether G-CSF also induces a local inflammatory process within the myocardium, immunohistochemical staining of the specific neutrophil marker NIMP-R14 was performed. According to the results of the serum flow cytometric analysis, G-CSF treatment significantly increased the number of neutrophils in the myocardium after 4 days (G-CSF-4d) compared with control-4d (Figure 3B). The number of invaded inflammatory cells normalized to baseline values after 14 days in G-CSF-treated mice (Figure 3B).

**Figure 2** Granulocyte colony-stimulating factor leads to the regression of cardiac hypertrophy and myocardial fibrosis. (A) Time-course of changes in the cardiomyocyte cross-section area. Staining of laminin (red) and nuclei (blue). The cardiomyocyte cross-section area was significantly decreased in granulocyte colony-stimulating factor-treated animals compared with control. (B) Time-dependent changes of myocardial capillary density. Staining of CD31 (red) and nuclei (blue). Capillary density was significantly increased in granulocyte colony-stimulating factor-treated mice compared with the NaCl-treated group. (C) Time-course of changes of the fibrotic area. Staining of collagen III (red), actin (green), and nuclei (blue). Granulocyte colony-stimulating factor treatment reduced myocardial fibrosis, represented by a significant reduction in collagen-III staining. (D) Staining of collagen-I (red), actin (green), and nuclei (blue). After granulocyte colony-stimulating factor treatment, the myocardial collagen-I content was reduced significantly. n = 5–7 mice per group. Data show the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001. Significance was determined by one-way analysis of variance.
Figure 3  Granulocyte colony-stimulating factor treatment induces local inflammatory processes. (A) Fluorescence-activated cell sorting analysis of serum samples. Granulocyte colony-stimulating factor induced an increase in circulating CD11b<sup>pos</sup> cells. n = 6–8 mice per group. Data show the representative fluorescence-activated cell sorting profiles. *<i>P</i> < 0.05 vs. control and **<i>P</i> < 0.001 vs. control. Significance was determined by an unpaired Student’s t-test. (B) Neutrophil density in the myocardium of sham-operated mice, after transverse aortic constriction, in control and in granulocyte colony-stimulating factor-treated mice. Red fluorescence shows the specific neutrophil marker NIMP-R14 (red). Nuclei were counterstained with DAPI (blue). Neutrophil density was similar in sham, transverse aortic constriction, and control experiments, while granulocyte colony-stimulating factor led to a significant myocardial infiltration of neutrophil granulocytes. n = 6–8 mice per group. Data show the mean ± SEM. ***<i>P</i> < 0.001 vs. sham and ###<i>P</i> < 0.001 vs. control. Significance was determined by one-way analysis of variance. (C) Immunohistochemical co-staining for NIMP-R14 (red), interleukin-1β (green), and nuclei (blue). Accumulation of interleukin-1β is shown surrounding neutrophil clusters after granulocyte colony-stimulating factor treatment. Neutrophil granulocytes of untreated mice did not reveal positive co-staining for interleukin-1β. (D) Quantitative real-time polymerase chain reaction (qRT–PCR) of interleukin-1β. Expression levels were increased after granulocyte colony-stimulating factor treatment. n = 6–8 mice per group. Data show the mean ± SEM. *<i>P</i> < 0.05 vs. sham and #<i>P</i> < 0.05 vs. control. Significance was determined by one-way analysis of variance. (E) qRT–PCR of interleukin-1β in granulocyte colony-stimulating factor-stimulated neutrophils. Granulocyte colony-stimulating factor induced the increased expression of interleukin-1β in both short- and long-term stimulation. Data show the mean ± SEM of three experiments. n = 8 per group. *<i>P</i> < 0.05 vs. control. Significance was determined by an unpaired Student’s t-test. All data were normalized to sham and the average of all sham values was defined as 100%.
Inflammatory processes are a highly complex system involving several cell types and cytokines. We therefore investigated the influence of G-CSF on the myocardial expression of important inflammatory cytokines IL-1β, IL-6, and tumour necrosis factor-α (TNF-α). No significant differences in the expression levels of TNF-α and IL-6 were observed between G-CSF-4d and control-4d (see Supplementary material online, Figure S2A). In contrast, quantitative real-time polymerase chain reaction revealed significantly increased levels of IL-1β mRNA in G-CSF-4d, which returned to baseline values after 14 days (Figure 3D). Correspondingly, immunohistochemical co-staining of NIMP-R14 and IL-1β revealed that neutrophils expressed IL-1β in G-CSF-4d, while neutrophils in control-4d did not show positive staining (Figure 3C).

Given this selective up-regulation of IL-1β in G-CSF-4d, we hypothesized that G-CSF provokes a direct effect on neutrophil granulocytes. Therefore, cultured neutrophils were stimulated with G-CSF. Consistent with the in vivo data, G-CSF significantly increased the expression of IL-1β in neutrophils (Figure 3E), while expression levels of the proinflammatory cytokines TNF-α and IL-6 remained unaltered (see Supplementary material online, Figure S3B).

**Interleukin-1β promotes regression of fibrosis by enhancing matrix metalloproteinase activity**

Matrix metalloproteinases (MMPs) represent the major proteolytic system for the ECM. Together with their tissue inhibitors (TIMPs), MMPs are of significant importance in cardiac remodelling processes. We hypothesized that alterations in the MMP/TIMP ratio cause the observed degradation of the ECM in G-CSF-treated mice. Expression levels of both gelatinases MMP-2 and MMP-9 were significantly increased in G-CSF-4d (Figure 4A). The major inhibitors of the gelatinases in the myocardium are TIMP-2 and TIMP-4. We detected significantly decreased mRNA levels for both TIMP-2 and TIMP-4 in G-CSF-4d compared with control mice (Figure 4B). In concordance with these results, gelatine zymography revealed significantly increased MMP-2 and MMP-9 activity in G-CSF-4d (Figure 4A). Aside from the synthesis of MMPs and TIMPs, fibroblasts are known as a major source of collagens and profibrotic mediators, such as transforming growth factor-β (TGF-β) and osteopontin (OPN). We were able to demonstrate a significant decrease in the expression of both TGF-β and OPN in G-CSF-treated mice (see Supplementary material online, Figure S4).

We then further evaluated whether G-CSF exerts direct effects on cardiac fibroblasts. For this purpose, isolated cardiac fibroblasts were cultured in the absence or the presence of G-CSF at different concentrations. Granulocyte colony-stimulating factor did not directly affect the expression of MMP-2 or MMP-9 in fibroblasts (see Supplementary material online, Figure S3C). Furthermore, manual cell count and MTT assay did not detect any effect of G-CSF on cardiac fibroblast proliferation.

We next examined the interaction between G-CSF-stimulated neutrophil granulocytes and cardiac fibroblasts. Neutrophil granulocytes were treated with G-CSF for 48 h and subsequently co-cultured with cardiac fibroblasts. Fibroblasts stimulated in this way exhibited significantly increased MMP-2 and MMP-9 expression (Figure 4C), while neutrophil granulocytes did not show any changes in MMP expression. In a further experiment, the supernatant of G-CSF-stimulated neutrophils was given to cardiac fibroblasts. This stimulation of cardiac fibroblasts with supernatant led to a significantly increased expression of MMP-2 and MMP-9 in cardiac fibroblasts (see Supplementary material online, Figure S6B). Furthermore, secretion experiments using cell culture inserts could show that the increased expression of MMP-2 and MMP-9 is caused by the secretion of IL-1β of neutrophils. Possible effects of direct cell–cell interactions between neutrophils and cardiac fibroblasts could be excluded (see Supplementary material online, Figure S6C).

To further elucidate the involvement of IL-1β in the observed effects of G-CSF, we stimulated cultured cardiac fibroblasts with IL-1β in the absence of neutrophil granulocytes. This stimulation led to increased MMP-2 and MMP-9 expression, as well as increased gelatinolytic activity comparable with that seen in co-cultured fibroblasts and stimulated neutrophils (Figure 4D).

Interleukin-1β stimulation also led to a significant increase in the proliferation of cardiac fibroblasts, which was detected by an MTT assay and confirmed by a manual cell count (Figure 5A). The differentiation of fibroblasts into myofibroblasts was elucidated by immunocytochemical co-staining with vimentin and α-smooth muscle actin (α-SMA). Interleukin-1β treatment caused a significant reduction in α-SMA+ fibroblasts, indicating reduced myofibroblast differentiation (Figure 5B).

**Discussion**

Long-lasting pressure overload leads to a diffuse perivascular and interstitial distribution of collagens, which is defined as ‘reactive fibrosis’. Such an excessive extent of fibrosis in terms of adverse cardiac remodelling decreases cardiac function, subsequently leading to high morbidity and mortality. It has been shown in experimental as well as clinical studies that cardiac fibrosis and left ventricular dysfunction, once present, do not show a complete restoration despite long-lasting pressure unloading. In the present study, G-CSF treatment not only led to an impressive improvement of left ventricular functional parameters, but also significantly reduced myocardial fibrosis in pressure unloaded hearts. The beneficial degradation of the ECM is caused by an increased activity of both MMP-2 and MMP-9. These gelatinases are the only physiological enzymes with collagen-degrading properties. On the other hand, a persistent increased the activity of MMPs has detrimental effects on myocardial remodelling processes. However, in the present study, we could clearly demonstrate that the application of G-CSF provides well-controlled temporary fibrolysis without destabilizing effects on myocardial integrity.

The physiological roles of G-CSF, namely mobilization, proliferation, and differentiation of progenitor cells, do not overtly imply this increased MMP activity. We therefore aimed to elucidate the observed beneficial fibrolytic properties of G-CSF on hypertrophied myocardium.

It has been proposed that cardiac remodelling may be beneficially influenced by the mobilization, homing, and (trans-) differentiation of BMCs. To track and distinguish the cells of
BM origin from sedentary cells in the myocardium, we successfully transplanted stem cell-depleted mice with BM from their eGFP-transgenic littermates. However, although a differentiation of BMCs in selected cell types was registered, the number of these cells was negligible, indicating that G-CSF-induced effects cannot be sufficiently explained by BMC differentiation.

Figure 4: Mechanisms of the beneficial effects of granulocyte colony-stimulating factor. (A) qRT–PCR and gelatine zymography of matrix metalloproteinase-2 and -9. Expression levels and gelatinolytic activity of matrix metalloproteinase-2 and -9 were significantly increased in granulocyte colony-stimulating factor-treated mice. $n = 6–8$ mice per group. Data show the mean $\pm$ SEM. * $p < 0.05$ vs. sham and $p < 0.05$ vs. control, $***p < 0.001$ vs. sham and $****p < 0.001$ vs. control as determined by one-way analysis of variance. (B) qRT–PCR of tissue inhibitor of metalloproteinase-2 and -4. Bar graphs represent the decreased expression in the granulocyte colony-stimulating factor group. $n = 6–8$ mice per group. Data show the mean $\pm$ SEM. * $p < 0.05$ vs. sham and $p < 0.05$ vs. control. Significance was determined by one-way analysis of variance. (C) Co-culture of cardiac fibroblasts with human neutrophils. Preceding stimulation of neutrophils with granulocyte colony-stimulating factor caused the up-regulation of matrix metalloproteinase-2 and -9 in cardiac fibroblasts. In the absence of granulocyte colony-stimulating factor, co-culture had no influence on matrix metalloproteinase expression. $n = 6$ per group. * $p < 0.05$ vs. sham, $#p < 0.05$ vs. control, $***p < 0.001$ vs. sham and $****p < 0.001$ vs. control. Significance was determined by an unpaired Student’s t-test. (D) qRT–PCR and gelatine zymography of matrix metalloproteinase-2 and -9. Cardiac fibroblasts were stimulated with 10 ng/mL of interleukin-1β. Interleukin-1β stimulation caused a significant up-regulation of matrix metalloproteinase-2 and -9. Data show the mean $\pm$ SEM of two experiments. $n = 4$ per group. * $p < 0.05$ vs. control. Significance was determined by an unpaired Student’s t-test. All data were normalized to sham and the average of all sham values was defined as 100%.
In addition to the effects of BMCs, it has been clearly shown that G-CSF directly affects the expression profile of cardiomyocytes via the Jak-Stat and PI3K-Akt pathways.\textsuperscript{18,19} Given the fact that cardiac fibroblasts also express the G-CSF receptor, we assumed that G-CSF-induced fibrolysis might stimulate MMP synthesis in cardiac fibroblasts. However, G-CSF did not alter the expression of MMP-2 or MMP-9 in cultured cardiac fibroblasts, excluding the possibility that G-CSF directly mediates MMP expression in these cells.

Further investigations revealed that G-CSF initiated a significant increase in circulating inflammatory cells. It is well established that inflammatory cells play an important role in myocardial remodelling processes, as previously shown after myocardial infarction.\textsuperscript{33–36} Although beneficial at early stages after myocardial injury, a persistence of inflammatory cells initiates a vicious cycle of cytokine stress.\textsuperscript{37,38} Cardiac remodelling after pressure unloading is normally not accompanied by an infiltration of inflammatory cells. However, we were able to demonstrate in the present study that G-CSF induces the significant infiltration of inflammatory cells, mainly neutrophils, into the myocardium. Furthermore, we have shown that these cells mainly synthesize IL-1β.

Figure 5 Interleukin-1β promotes the proliferation of cardiac fibroblasts. (A) MTT assay and manual cell count. After stimulation with 10 ng/mL of interleukin-1β, cardiac fibroblasts revealed a significantly higher proliferation rate compared with control. Data show the mean ± SEM in one representative experiment out of two. n = 7 per group. *P < 0.05 vs. control. Significance was determined by an unpaired Student’s t-test. (B) Immunocytochemical staining of myofibroblasts. Cardiac fibroblasts were incubated with antibodies to α-smooth muscle actin (green), vimentin (red), and nuclei (blue). After pre-incubation with interleukin-1β for 24 h, a significantly lesser number of differentiated myofibroblasts (α-SMA$^{\text{pos}}$/Vim$^{\text{pos}}$) could be observed. Data show the mean ± SEM in one representative experiment out of two. n = 7 per group. #P < 0.05 vs. control. Significance was determined by an unpaired Student’s t-test. (C) Schematic depiction of granulocyte colony-stimulating factor-mediated effects.
Interleukin-1β is critically involved in the pathogenesis of cardiac remodelling by modulating fibroblast function and regulating ECM metabolism. However, recent studies regarding the pathophysiological impact of IL-1β exhibited mainly detrimental effects after myocardial infarction. These effects are due to the fact that the release of IL-1β is accompanied by a number of other cytokines, such as IL-6 and TNF-α. This ‘cytokine storm’ ultimately leads to an increased expression of the profibrotic mediators TGF-β and OPN, thereby promoting interstitial fibrosis and collagen deposition. It has been shown that TNF-α as well as IL-6 play a crucial role in the pathogenesis of cardiovascular diseases and especially in the development of myocardial fibrosis and the progression to heart failure. Sakata et al. revealed a direct link between overexpression of TNF-α and increased expression of TGF-β in inflammatory heart disease. Furthermore, they could demonstrate that the inhibition of TGF-β attenuated the increase in cardiac fibrosis observed in TNF-α-overexpressing mice. However, TGF-β can also exert protective effects via the up-regulation of laminin receptors in cardiomyocytes.

Because G-CSF increases the proliferation and migration of neutrophil granulocytes, one might assume that G-CSF treatment would aggravate inflammatory processes, and subsequently tissue destruction. In the present study, we have demonstrated that G-CSF induces the selective up-regulation of IL-1β, while the destructive potential of neutrophils is absent given the lack of TNF-α and IL-6 up-regulation. These findings are supported by haematologic studies, in which G-CSF treatment attenuated the increase in IL-6 and TNF-α levels in serum after exposure to endotoxin. Stimulation of cultured neutrophils with G-CSF revealed that the expression of IL-1β is directly mediated by G-CSF. We have shown in three ways that this selective up-regulation of IL-1β is the deciding mechanism, leading to the increased expression of MMPs in fibroblasts. First, IL-1β inhibited the transformation of cardiac fibroblasts into collagen-synthesizing myofibroblasts. Second, the co-culture of G-CSF-pre-treated neutrophils with cardiac fibroblasts caused significant increases in MMP-2 and MMP-9 expression in fibroblasts. These effects were completely abolished in the absence of either G-CSF or neutrophils. Third, direct stimulation of cultured fibroblasts with IL-1β led to a likewise increased MMP expression. This increased MMP expression, together with decreased TIMP-2 and TIMP-4 levels, ultimately results in increased proteolytic activity and explains the reduction in cardiac fibrosis.

Persisting cardiac fibrosis still exhibits a problem after successful aortic valve replacement and the current standard therapy for patients with congestive heart failure (antagonization of the RAS-system, β-blocker, diuretics) failed to improve functional parameters and the medical condition of such patients. Given this lack of current treatment options for patients with increased cardiac fibrosis, further experimental studies and clinical trials should be carried out to evaluate the effects of G-CSF administration in humans. In summary, the G-CSF-induced sterile inflammation might be a promising therapeutic approach for the treatment of patients with congestive heart failure after aortic valve replacement.

Limitations
The present study is based on experiments, which were performed in vivo and in vitro. Due to the artificial character of cell culture experiments and the complex interplay of different cell types in the myocardium, our in vitro experiments cannot exactly reflect the underlying mechanisms in vivo. Therefore, it cannot be definitely ruled out that further mechanisms apart from the G-CSF/IL-1β axis take part in the observed remodelling processes.

Our data do not indicate a relevant synthesis of MMP-2 and MMP-9 in neutrophils. However, given the fact that neutrophils are a known source of MMPs in inflammatory processes, we cannot rule out that neutrophils take part in the observed increase in MMP expression and activity in the present study.

Although there was no numerical difference in eGFPpos cells between the study groups, we cannot fully exclude that the mobilization of bone marrow cells contribute to the beneficial effects of G-CSF observed in the present study.

The results of the present are based on experiments, which were performed in female, young-aged mice. Given the fact that gender as well as age lead to significant differences in the gene expression profile and response to pathophysiological stimuli, it cannot definitely ruled out that G-CSF might have less pronounced or completely attenuated effects in male and/or older mice.

The observation period after debanding only comprises 2 weeks. Actually, it has not been sufficiently investigated, if the regression of cardiac fibrosis can be also observed at a longer period after pressure unloading. Therefore, it cannot be excluded that differences between the study groups vanish over longer time periods.

Supplementary material
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