Interleukin-23 Deficiency Leads to Impaired Wound Healing and Adverse Prognosis after Myocardial Infarction

Savvatis et al: Interleukin-23 in Myocardial Infarction

Konstantinos Savvatis, MD1,2; Kathleen Pappritz1,6; Peter Moritz Becher, MD5;
Diana Lindner, PhD5; Christin Zietsch, PhD1; Hans-Dieter Volk, MD, PhD2,3;
Dirk Westermann, MD5; Heinz-Peter Schultheiss, MD1; Carsten Tschöpe, MD1,2,4

1Department of Cardiology and Pneumology, Campus Benjamin Franklin (CBF), Charité – Universitätsmedizin Berlin, Berlin, Germany
2Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité – Universitätsmedizin Berlin, Berlin, Germany
3Institute for Medical Immunology, Charité Universitätsmedizin Berlin, Berlin, Germany
4DZHK, German Center for Cardiovascular Research, partner site Berlin – Charité
5University Heart Center Hamburg, Hamburg, Germany
6Department of Biology, Chemistry and Pharmacy, Free University Berlin, Berlin, Germany

Correspondence to
Konstantinos Savvatis, MD
Charité - Universitätsmedizin Berlin
Hindenburgdamm 30
Berlin 12203
Germany
Telephone: 00493084452349
Fax: 00493084454648
Email: ksavvatis@gmail.com

DOI: 10.1161/CIRCHEARTFAILURE.113.000604

Journal Subject Code: Basic science research:[130] Animal models of human disease
Abstract

Background—CD4+ cells are implicated in the healing process after myocardial infarction (MI). We sought to investigate the role of interleukin-23 (IL23) deficiency, a cytokine important in differentiation of CD4+ cells, in scar formation of the ischemic heart.

Methods and Results—MI was performed in wild type (WT) and interleukin-23p19-/- (IL23KO) mice. Thirty-days mortality, hemodynamic function 4 days after MI and myocardial inflammation and remodeling 4 and 30 days after MI were examined. Differentiation of fibroblasts from infarcted and non-infarcted hearts into myofibroblasts was examined under basal conditions and after stimulation with IFN-γ, IL-17α and IL-23. IL23KO mice showed higher expression of proinflammatory cytokines and immune cell infiltration in the scar early after MI compared to WT mice. A stronger IFN-γ/Th1 reaction seemed to be responsible for the increased inflammation under IL23 deficiency. Expression of α-smooth muscle actin (α-SMA), collagen I and III was significantly higher in the heart tissue and isolated cardiac fibroblasts 4 days after MI in the WT mice. IL23KO mice showed impaired healing compared to WT mice, as seen by significantly higher mortality due to ventricular rupture (40% higher after 30 days) and stronger LV dilation early after MI. Stimulation of cardiac fibroblasts with IFN-γ, the main Th1 cytokine, but not with IL-23 or IL-17α, led to a significant downregulation of α-SMA, collagen I and III and decreased migration and differentiation to myofibroblasts.

Conclusions—IL-23 deficiency leads to increased myocardial inflammation and decreased cardiac fibroblast activation, associated with impaired scar formation and adverse remodeling after MI.

Key Words: interleukin-23, myocardial infarction, fibroblast, remodeling, left ventricular rupture
In the last years, extensive research work has shown that the immune system is centrally involved in the wound healing processes after myocardial infarction. Immune cells cooperate with cardiac innate cells for clearance of cell debris, angiogenesis and coordination of the reparative mechanisms. However, immunosuppressive therapeutic interventions in the myocardial inflammatory response have largely been disappointing, showing that not a generalized immunosuppression but rather a target-specific immunomodulation might be the right strategy.1 Therefore a better understanding of the inflammatory response after myocardial infarction is necessary in order to develop proper therapeutic strategies.

Apart from the innate, the adaptive immune system is also activated early after MI and influences the myocardial remodeling and scar formation. CD4+ helper T cells infiltrate the myocardium after MI and are necessary for effective wound healing.2 Cytokines involved in the differentiation of helper T cells into their different subsets, such as interferon-γ (IFN-γ) and interleukin-17α (IL-17α) are found upregulated in the myocardium after MI.3 Deficiency in CD4+ cells leads to adverse remodeling and increased ventricular rupture.2 In our previous work we could also show that administration of an IL-2-IgG2b fusion protein after MI could reduce myocardial inflammation and improve myocardial remodeling through upregulation of CD4+ regulatory T cells, an antiinflammatory group of CD4+ T cells.4

CD4+ helper T cells consist of several distinct subsets, which play various roles in the inflammatory response.5 Central role in their differentiation play cytokines of the interleukin 12-family, mainly interleukin 12 and interleukin 23. IL-12 drives mainly the Th1 response, while IL-23 stabilizes the Th17 cells. Both cytokines are heterodimeric cytokines and share a common subunit, the IL12p40 unit, showing their potential interactions in the coordination of the immune response.6 The exact microenvironment in the inflammation area decides which subunits bind together in order to form either IL12 or IL-23. Th17 and Th1 cells are implicated in the pathogenesis of atherosclerosis and are upregulated in patients with acute
coronary syndromes. Furthermore, IL-23 and Th17 cells play a pathogenic role in transplant rejection after heart transplantation, as well as in viral myocarditis, where they drive myocardial inflammation and viral replication. However, little is known about the exact role of Th1 and Th17 subsets and IL-23 in the remodeling processes of the myocardium itself after myocardial infarction. Therefore we examined the effects of IL-23 in myocardial inflammation, wound healing and survival in IL-23 knockout animals using a mouse model of myocardial infarction.

**Methods**

**Animals, surgical procedures and hemodynamic measurements.**

Male C57BL/6j mice (8-12 weeks old) were purchased from Charles River Laboratories, Sulzfeld, Germany. Interleukin-23p19-/- (IL23KO) mice on the C57BL/6j background (WT), were a kind gift from Genentech Inc., San Francisco, USA and were housed in the Research Facility for Experimental Medicine, Charité Universitätsmedizin, Berlin, Germany. MI was induced by permanent occlusion of the left anterior descending artery (LAD) as previously described. Hemodynamic measurements with a microconductance catheter are described elsewhere. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and was approved by the local ethics committee (LaGeSo).

**Immunohistology**

Frozen tissue samples were embedded in Tissue-Tek (Sakura Finetek USA, CA, USA) and cut into 5μm thick sections. Staining for following antigens was performed: CD4 (BD Biosciences, Heidelberg, Germany), CD11b (Mac-1) (BD Biosciences, Heidelberg, Germany), γδ TCR (GeneTex, CA, USA), α-SMA (Abcam, Cambridge, UK), collagen I (Merck Millipore, MA, USA), collagen III (Merck Millipore, MA, USA), neutrophils
(Abcam, Cambridge, UK). Analysis of stained sections was made in a blinded fashion by digital image analysis on a Leica DMRB microscope (Leica Microsystems, Wetzlar, Germany) at a 200x magnification, as previously described. The number of cells is expressed as cells/mm² and amount of the different proteins as the percentage of the area of the examined section which was red stained (area fraction %).

**Isolation and culture of murine cardiac fibroblasts**

For the characterization of adult murine cardiac fibroblasts tissue from the infarcted, non-infarcted and sham-operated left ventricle was obtained 4 days after operation. Obtained tissue was cut in small pieces, which were fixed in 12-well culture plates. The outgrowing primary fibroblasts were cultured in DMEM medium (PAA, Cölbe, Germany) containing 10% fetal calf serum, 100U/mL penicillin and 100μg/mL streptomycin (PAA, Cölbe, Germany) at 37°C with 95% air and 5% CO₂. Fibroblasts from passage 0 (P0) were used for basal characterization. Cells were lysed at 80-90% confluence in RLT-Buffer (Qiagen, Hilden, Germany) containing 1% mercaptoethanol. RNA isolation samples were stored at -80°C until used.

**Stimulation of cardiac fibroblasts**

Cardiac fibroblasts at passage 4-8 from healthy and infarcted WT hearts were used for stimulation with IFN-γ, IL-23 and IL-17α and assessment of their wound healing capacity.

Before starting the stimulation cells were washed once with 1xPBS (PAA, Cölbe, Germany) and starved in DMEM medium (PAA, Cölbe, Germany) containing 0.5% fetal calf serum, 100U/mL penicillin and 100μg/mL streptomycin (PAA, Cölbe, Germany) for 18 hours. Then they were treated either with 10ng/mL IFN-γ (Peprotech, Hamburg, Germany), 10ng/mL IL-23 (Miltenyi Biotech, Bergisch Gladbach, Germany) or 10ng/ml IL-17α (Peprotech, Hamburg, Germany) for up to 24 hours. The untreated control cells were incubated for the
same time without addition of IFN-γ, IL-23 or IL-17α. The experiments were repeated three times with n = 6 per group.

**Assessment of fibroblast wound healing capacity**

For assessment of the healing capacity of cardiac fibroblasts a scratch healing assay was used. Fibroblasts from sham hearts stimulated with IFN-γ and untreated fibroblasts were used as described above. A wound was made by scratching across each well with a 1000μl pipette tip. Healing capacity of the fibroblasts was assessed as the percentage of the initial scratched area at 0 h that was repopulated by fibroblasts after 6 and 24h.

**RNA isolation and gene expression analysis**

Total RNA from tissue was isolated using the Trizol method as described previously.14 Gene expression analyses were carried out with the 7900 TaqMan System (Applied Biosystems). Following gene expression assays were used: Col1A1 (Mm01302043_m1), Col3A1 (Mm00802331_m1), Acta2 (Mm00725412_s1), TNF-α (Mm00443258_m1), IL-1β (Mm00434228_m1), IL-10 (Mm00439616_m1), IL-6 (Mm00446190_m1), TGF-β (Mm00441724_m1), monocyte chemoattractant protein-1 (MCP-1) (Mm99999056_m1), MCP-3 (Mm00443113_m1), T-bet (Mm00450960_m1), RORγt (Mm03682796_m1), IFN-γ (Mm00801778_m1), IL-23r (Mm00519942_m1). The expression of IL-17α was examined with a semiquantitative method with following custom made primers: forward 5'-CCTCAGACTACCTCAACCGTTC-3' and reverse 5'-TTCATGTGGTGGTGTCAGCTTTT-3'.

The quantification of the relative mRNA expression was performed by using CDKN1b (Mm00438167_g1) for each sample as an internal control and expressed in the 2-ΔCt formula. For the comparison of the effect of different stimulation experiments the mRNA expression was normalised to the WT sham using the 2-ΔΔCt method.15
Zymography of matrix metalloproteinase activity

Gelatin zymography from frozen heart tissue was performed to determine the gelatinolytic activities of matrix metalloproteinase-2 (MMP-2) and MMP-9 in the scar and the non-infarcted area as previously described.\textsuperscript{13}

Western blot

LV samples of the infarcted, non-infarcted and sham-operated area of the heart were homogenized in lysis buffer containing proteinase and phosphatase inhibitors. Collagen I, GAPDH, as well as the total and phosphorylated forms of the signal-regulated kinase (ERK) 1/2 and Stat3, involved in the IL-23 signaling, were detected with specific antibodies (Cell Signaling Technology) as previously described.\textsuperscript{12}

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software, La Jolla, USA). Data are expressed as means ± SEM. Data were tested for normality with the Kolmogorov–Smirnov and the Shapiro-Wilk tests. Statistical differences between groups were assessed with one way ANOVA and the Bonferroni post-hoc test for normally distributed data or the Kruskal-Wallis test with the Dunn’s post-hoc test for non-parametric data. Due to the small number of 8-10 animals per group a non-parametric approach was used. Survival analysis was performed with the Kaplan-Meier method and survival curves were compared with the log-rank test. Differences were considered to be statistically significant at a two-sided value of $p<0.05$.

Results

Left ventricular function after MI

IL23KO mice showed significant dilation of the left ventricle 4 days after MI. LV enddiastolic volume was 35% and endsystolic volume 33% higher in the IL23KO mice.
compared to their WT controls (p=0.02 and 0.05 respectively). Accordingly, LV enddiastolic pressure was higher in the IL23KO mice (p=0.0016 vs. WT) (Table).

**Effects of interleukin-23 on mortality after MI**

Deficiency of IL-23 led to a 40% higher mortality in the knockout mice compared to the WT mice (p=0.04). All deaths in both strains were due to ventricular rupture in the subacute phase after MI (Figure 1). IL23KO mice showed a higher rate of ventricular rupture. No further deaths were observed in any of the two groups between day 8 and 30 after MI.

**Role of interleukin-23 in early myocardial inflammation after MI**

The mRNA amount of IL-23R was significantly upregulated by 4.2 times in the infarcted area (p=0.0003) compared to the sham operated animals and by 1.5 times compared to the non-infarcted area (p=0.017) in WT animals 4 days after MI. The IL23KO mice showed a 40% lower mRNA expression in the infarcted area (p=0.003) and a 50% lower mRNA expression in the non-infarcted area (p=0.0076) compared to the WT mice (Figure 2a).

Gene expression of IL-1β was 60% higher in the infarcted area in the IL23KO mice compared to WT (p=0.006) and that of IL-10 in the infarction zone was reduced by 40% due to IL-23 deficiency (p=0.0379 vs. WT). Gene expression of IL-1β and IL-10 in the non-infarcted area was the same between the two strains (Figure 2c-d). Analysis of TNF-α, TGF-β, and IL-6 showed no significant difference in the mRNA expression between the 2 strains (Figure 2b, e-f). Gene expression of the chemokines MCP-1 and MCP-3 did not differ in the scar, however, the IL23KO mice showed a 1.8fold increase of MCP-1 and a 1.7fold increase of MCP-3 mRNA in the non-infarcted area (p=0.049 and p=0.049 respectively, Figure 2g-h).

Immunohistologic analysis of immune cell infiltration in the myocardium 4 days after MI revealed a 2.9fold increase of CD4+ cells (p=0.013 vs. WT), an 1.4fold increase in neutrophils (p=0.036 vs. WT) and an 1.4fold increase in CD11b cells (p=0.021 vs. WT) in the infarction zone in the IL23KO mice 4 days after MI, with no significant differences in the
non-infarcted area of the LV (Figure 3a-c). Finally, we found no significant difference in the number of $\gamma$δ-T cells between the WT and IL23KO mice (Figure 3d).

**Effect of IL-23 on balance between Th1/Th17 reaction**

We examined the myocardial gene expression of signature cytokines, IFN-γ for the Th1 and IL-17α for Th17 cells, and the gene expression of their transcription factors, Tbet and RORγt respectively 4 days after MI.

IL23KO mice showed a significant 3.3fold higher gene expression of IFN-γ in the infarcted area ($p=0.046$) and a 2.4fold higher gene expression in the non-infarcted LV ($p=0.025$) vs. the WT mice. On the other hand, WT mice showed a significant upregulation of IL-17α in both the infarcted and the non-infarcted area, while the IL23KO failed to upregulate IL-17α after MI. Accordingly, mRNA amount of Tbet in the scar was significantly higher in the IL23KO mice compared to the WT mice ($p=0.0078$ vs. WT), whereas gene expression of RORγt was significantly lower ($p=0.0012$ vs. WT), indicating a stronger activation of the Th1 over Th17 immune reaction under IL-23 deficiency (Figure 4a-d).

**Myocardial Stat3 and ERK1/2 activation**

In order to investigate the effects of IL-23 on the myocardium we examined by western blot analysis the phosphorylation of Stat3, the main transcription factor over which IL-23r signals 4 days after induction of myocardial infarction. A significantly enhanced phosphorylation of Stat3 was observed in both the infarcted and the non-infarcted area compared to sham operated hearts. However, we found a 2.8fold increase in the phosphorylation of Stat3 in the infarcted area in the WT compared to the IL23KO mice ($p=0.0056$). Likewise a significantly higher activation of Stat3 by 2.6 times was observed in the non-infarcted area of the WT compared to the IL23KO mice ($p=0.0009$, Figure 5a).

The phosphorylation status of ERK1/2 was significantly upregulated in the infarction area compared to healthy hearts in WT mice. We observed no significant activation of ERK1/2 in
the IL23KO mice neither in the infarcted or non-infarcted area. In comparison, activation of 
ERK1/2 in the infarcted area was 1.5 times higher in the WT than in the IL23KO mice 
(p=0.028, Figure 5b).

**Myocardial wound healing and remodeling**

We analysed myocardial wound healing parameters and myofibroblast differentiation early 
after MI in order to understand the mechanisms leading to increased rupture. Gene expression 
of both Col1a1 and Col3a1 in the infarcted area 4 days after MI was 20% higher in the WT 
mice compared to the IL-23KO (p=0.021 and 0.029 respectively, Figure 6a). We performed a 
zymography in order to assess the gelatinolytic activity of MMP-2 and MMP-9 4 days after 
MI. We observed no difference in their activity between the two strains (Figure 5c-d). Gene 
expression of Acta2 as a marker of myofibroblast differentiation in the scar was 25% higher 
in the WT than in the IL23KO mice (p=0.026, Figure 6a).

Increased mRNA expression of Col1a1 in the infarcted area in WT mice persisted until 30 
days after MI and was significantly higher by 1.6 times (p=0.04) and that of Col3a1 by 1.7 
times (p=0.042) compared to the IL23KO mice. Accordingly protein abundance of collagen I 
in the infarcted area was higher in the WT mice by 3.6 times (p=0.002) compared to the 
IL23KO mice, while collagen III protein levels were the same in both groups (Figure 6a and 
c). Western blot analysis of collagen I confirmed the data from immunohistochemistry and 
showed a 1.9 times higher amount of protein in the scar 4 days and 1.7 times 30 days after MI 
(p=0.0312 and p=0.0048 respectively Figure 6d). Higher gene expression of Acta2 persisted 
also until 30 days after MI (1.6 fold higher in the scar in the WT mice vs. IL23KO, p=0.0028, 
Figure 6a). Similarly, protein amount of α-SMA in the infarcted area was 2 times higher in 
the WT than in the knockout mice 30 days after MI (p=0.028, Figure 6b-c). No significant 
difference could be observed in the non-infarcted area between the two strains.
**Myofibroblast differentiation under basal conditions**

Cardiac fibroblasts from heart 4 days after MI were examined. Basal mRNA expression of Acta2 in WT scar fibroblasts was 1.9 times higher than in IL23KO fibroblasts (p<0.0001). Furthermore, WT fibroblasts isolated from the scar produced 1.4 times more Col1a1 mRNA (p=0.0147) and 1.5 times more Col3a1 RNA (p=0.0039) than IL23KO fibroblasts (Figure 7a-d).

**Effects of IFN-γ, IL-23 and IL-17α on cardiac fibroblasts**

WT fibroblasts were isolated from heart and stimulated with 10 ng/ml of IL-23, IFN-γ or IL-17α. Both IL-23 and IL-17α showed no effects on the mRNA expression of Acta2, Col1a1 and Col3a1 after 24 hours of stimulation on healthy fibroblasts (data not shown). However, we observed significant effects of IFN-γ on fibroblast differentiation. Therefore we stimulated fibroblasts isolated from sham and infarcted hearts with IFN-γ. Stimulation with IFN-γ led to a significant downregulation of the gene expression of Col1a1, Col3a1 and Acta2 after 24 hours stimulation in fibroblasts isolated from sham-operated hearts or from the scar. The effects of IFN-γ were not so strong on fibroblasts isolated from the non-infarcted LV, where only a reduction in Col3a1 mRNA expression was significant after 24h of stimulation (Figure 7e).

**IFN-γ effects on wound healing capacity of cardiac fibroblasts**

We analysed the effects of IFN-γ on the migratory capacity of cardiac fibroblasts using an in vitro wound healing model. Stimulation of cardiac fibroblasts with 10 ng/ml IFN-γ decreased their migratory capacity by 5% after 24 hours compared to control untreated fibroblasts as assessed by the area they covered 24 hours after induction of a scratch in the culture well (p=0.0054, Figure 8).
Discussion

The salient finding of our study is that IL-23 deficiency leads to an impaired scar formation in the ischemic heart. IL-23 controls the direction of the myocardial immune response early after MI and inhibits the Th1 reaction in the scar with its deleterious IFN-γ production. Through indirect effects on myofibroblast differentiation and migration in the infarcted area, IL-23 deficiency led to adverse myocardial remodeling, ventricular rupture and increased mortality.

**IL-23 controls the differentiation of CD4+ T cells after MI**

Activation of the adaptive immune system after MI is important for the orchestration of the wound healing process in the heart. IL-23 is centrally involved in the survival and stabilization of Th17 cells but also indirectly by inhibiting the development of Th1 cells. In the early stages after MI activation of the innate immune cells is followed by migration of cells of the adaptive immune system into the myocardium, mainly CD4+ T cells. Our data show that 4 days after MI the expression of IL-23 receptor and the transcription factor RORγt, mainly expressed on Th17 cells, is upregulated both in the infarcted as well as in the non-infarcted area. Ablation of IL-23 led to a change of balance between the Th1 and Th17 reaction in the IL-23KO mice, which was associated with an aggravated myocardial inflammation. Analysis of the number of the heart infiltrating γδ-T cells, which also express the IL-23 receptor, revealed no difference, which implies that in our model IL-23 mainly acted through activation of the Th17 cells.

Immunohistological analysis of the heart 4 days after MI demonstrated a significantly higher infiltration with CD4+ T cells compared to non-ischemic hearts. However, CD4+ cells can be divided in several subgroups with both proinflammatory as well as antiinflammatory actions. Our data demonstrate that IL-23 acts mainly by suppressing the deleterious Th1/IFN-γ reaction in the early stages after MI, reducing thus excessive myocardial inflammation.
The Th1 reaction and IFN-γ production are traditionally thought to lead to deleterious effects in several immune-mediated diseases. Similar adverse effects of Th1 cells have been demonstrated in the progression of atherosclerosis, development of vascular inflammation and adverse vascular remodeling.18 IFN-γ is mainly driving the development of atherosclerotic plaques and vascular inflammation and is associated with adverse prognosis.19, 20 IFN-γ and IL-17α producing cells are both upregulated in patients with acute coronary syndromes, implying an activation of Th1 and Th17 cells respectively.7, 21 Furthermore, these cells infiltrate the coronary arteries and affect the function of smooth muscle cells.22 In a recent clinical study in patients with coronary artery disease, low levels of IL-17α after an acute coronary syndrome were associated with a worse prognosis and increased rate of death or reinfarction.7 Accordingly, our data demonstrate a regulatory and antiinflammatory role for Th17 after MI, which seems to be associated with inhibition of the deleterious Th1 reaction and IFN-γ production. However, a recent study by Yan et al. demonstrated a protective effect of IL-23 deficiency after myocardial infarction, associated with a decreased myocardial inflammation in the IL-23 knockout mice. These contradicting results may in part be due to different genetic background of the wild type mice, since the authors exhibited a mortality rate twice as high as in our own study. Furthermore, selection of different time points in our study makes direct comparisons difficult, since the inflammatory reaction after myocardial infarction is a continuously changing process.23

**IL-23 deficiency reduces Stat3 and ERK1/2 activation**

Stat3 is the main transcription factor over which signaling of the IL-23 receptor occurs. Apart from its role in the differentitation of Th17 cells, Stat3 is known to play a protective role in the heart.24 IL-23 deficiency led to a significantly lower activation of Stat3 in the myocardium in our study. Decreased Stat3 activation is associated with an adverse prognosis after myocardial infarction by reducing the myocardial capillary growth and interstitial
Furthermore, Stat3 activation is protective in viral myocarditis. Importantly however, Stat3 activation leads to increased differentiation of fibroblasts into myofibroblasts, associated with increased production of α-SMA and collagen gel contraction, which might in part explain the impaired wound healing induced by IL-23 deficiency in our model.

Furthermore, we observed a significant phosphorylation of ERK1/2. ERK1/2 activation is associated with protective effects in myocardial ischemia, mainly due to its antiapoptotic function and effects in hypertrophy and growth. Moreover, ERK1/2 seems to act downstream of Stat3 signaling and be partially responsible for the actions of Stat3 in the myocardium. Another important aspect of ERK1/2 activation is that it seems to be taking part in the IL-23 functions on cell differentiation and moreover to induce IL-23 production in a positive feedback manner, potentiating possibly the effects of IL-23 in our model. Finally, ERK1/2 activation takes also part in the activation of fibroblasts to myofibroblasts, an effect we could also observe in our study.

**Deficiency of IL-23 leads to impaired wound healing and adverse remodeling**

We observed a decreased collagen deposition in the scar already in the early stages after MI which persisted throughout the whole observation time. Impaired scar formation in the early stages led to an increased rate of LV rupture in the IL23KO mice. Isolation of cardiac fibroblasts from the scar 4 days after MI showed a higher gene expression of collagen I, III and α-SMA in the WT mice, implying a stronger differentiation into myofibroblasts in the WT mice. Furthermore, protein amount and gene expression of α-SMA 30 days after MI was stronger in the WT mice as a marker for myofibroblast persistence in the scar for a longer time than in the IL23KO mice. Formation of a collagenous scar is important for preventing infarct expansion and LV dilation and is mainly mediated by myofibroblasts. Myofibroblasts migrate into the scar after their activation, express α-SMA and produce large

...
amounts of collagen I and III already few days after MI. Presence of myofibroblasts and persistence in the scar is of utmost importance and early removal of myofibroblasts leads to impaired wound healing with adverse remodeling, LV dilation and LV rupture.31,33

**IFN-γ reduces the activation of myofibroblasts**

The interaction between cardiac fibroblasts and immune cells and the transition from the inflammatory phase to wound healing and formation of a stable scar is gaining continuously attention.34 We identified IFN-γ as the main coordinating cytokine in our model. Stimulation of cardiac fibroblasts with IFN-γ led to a significant downregulation of α-SMA, collagen I and III. These effects were retained in healthy cardiac fibroblasts as well as in fibroblasts isolated from the scar. Interestingly, IFN-γ affected less fibroblasts isolated from the non-infarcted LV. Furthermore, IFN-γ-stimulated fibroblasts showed a decreased migratory ability in the wound healing scratch experiment. However, the effects on the migratory capacity were less pronounced than the effects on the activation status of fibroblasts, which implies that IFN-γ acts mainly through induction of differentiation in the fibroblasts and that their migratory capacity might be controlled mainly by other factors in our study. On the other hand, stimulation with IL-23 or IL-17α had no significant effects on the expression of α-SMA or collagen by cardiac fibroblasts. However, a positive effect of IL-17α on the migratory capability and proliferation of fibroblasts has been previously described and cannot be excluded in our study.35

**Study limitations**

Our study was performed in a knockout mouse model in which deletion of the IL23p19 gene led to a loss of IL23 throughout the experiment. A time- or dose-specific action of IL23 can therefore not be excluded in our study; however, our data indicate that IL23 acts mainly in the early stages after MI. Furthermore, we examined cardiac inflammation, myocardial wound healing and myofibroblasts differentiation 4 days after MI, which we found to be the
important time point for impaired wound healing, therefore time-dependent changes in inflammation and wound healing parameters at other time points cannot be excluded.

**Conclusion**

In conclusion, IL-23 seems to play a key role in regulation of the myocardial inflammatory response after MI and its interaction with cardiac fibroblasts. IL-23 deficiency leads to an aggravated myocardial inflammation and impaired wound healing in the ischemic heart associated with high LV rupture rate and adverse remodeling.

**Acknowledgements**

The authors would like to thank K. Puhl and G. Zingler for their excellent technical assistance.

**Sources of Funding**

This study was supported by the “Deutsche Forschungsgemeinschaft” (SFB/TR 19, Project Z3 and B5 to C.T.), the Berlin-Brandenburg Center for Regenerative Therapies (Platform F to C.T.) and the Hellenic Society of Cardiology (Research grant to K.S.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Disclosures**

None.

**References**


34. van Nieuwenhoven FA, Turner NA. The role of cardiac fibroblasts in the transition from inflammation to fibrosis following myocardial infarction. Vascular pharmacology. 2013;58:182-188.

Table. Left ventricular function 4 days after myocardial infarction

<table>
<thead>
<tr>
<th></th>
<th>WT-sham</th>
<th>IL23KO-sham</th>
<th>WT-MI4d</th>
<th>IL23KO-MI4d</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beats/min)</td>
<td>546±19</td>
<td>542±21</td>
<td>450±17</td>
<td>§ 437±24</td>
</tr>
<tr>
<td>LVPmax (mmHg)</td>
<td>98.5±6</td>
<td>93.4±2.7</td>
<td>72.2±3.3</td>
<td>§ 70.7±4.1</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4.6±0.4</td>
<td>4.3±0.5</td>
<td>6.8±0.48</td>
<td>§ 12.2±0.6</td>
</tr>
<tr>
<td>dP/dt max (mmHg/ms)</td>
<td>7241±384</td>
<td>7861±521</td>
<td>3676±398</td>
<td>§ 3309±384</td>
</tr>
<tr>
<td>dP/dt min (mmHg/ms)</td>
<td>-6298±650</td>
<td>-5886±337</td>
<td>-3085±194</td>
<td>§ 3184±356</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>10.1±0.4</td>
<td>10.3±0.5</td>
<td>13.7±0.4</td>
<td>§ 13.8±0.6</td>
</tr>
<tr>
<td>PHT (ms)</td>
<td>21.3±3</td>
<td>27.8±4</td>
<td>61.5±5.8</td>
<td>§ 62.5±8.4</td>
</tr>
<tr>
<td>LVESV (µl)</td>
<td>21.3±3</td>
<td>27.8±3.7</td>
<td>57.2±4.5</td>
<td>§ 76.2±6</td>
</tr>
<tr>
<td>LVEDV (µl)</td>
<td>61.2±5.6</td>
<td>59±4.4</td>
<td>73.1±6.3</td>
<td>§ 98.5±4.5</td>
</tr>
<tr>
<td>SV (µl)</td>
<td>40.3±3.6</td>
<td>35.6±1.7</td>
<td>26.3±6.9</td>
<td>§ 20.7±2.8</td>
</tr>
<tr>
<td>EF (%)</td>
<td>65.5±3</td>
<td>60.7±2.4</td>
<td>28.2±4.5</td>
<td>§ 22.1±3.7</td>
</tr>
<tr>
<td>CO (µl/min)</td>
<td>22111±1756</td>
<td>18658±665</td>
<td>9994±2532</td>
<td>§ 10568±1470</td>
</tr>
</tbody>
</table>

IL23KO mice showed already 4 days after MI a LV dilation with increase of the LV enddiastolic and endsystolic volumes and increased LV enddiastolic pressure. CO, cardiac output; dP/dt max, maximal rate of LV pressure increase; dP/dt min, maximal rate of LV pressure fall; EF, ejection fraction; HR, heart rate; LVPmax, maximal LV pressure; LVEDV, LV enddiastolic volume; LVEDP, LV enddiastolic pressure; LVESV, LV endsystolic volume; PHT, pressure half-time; SV, stroke volume. Parameters are presented as mean ± SEM. § p<0.05 vs. respective sham group, ¶ p<0.05 vs. respective WT group. n=8-10 mice per group
Figure Legends

Figure 1. Effect of IL-23 deficiency on survival after MI. Kaplan-Meier analysis of survival and subjects at risk at various time points after MI.

Figure 2. Gene expression of cytokines and chemokines 4 days after MI. a. IL-23 receptor was significantly upregulated in the WT mice compared to IL23KO mice. b-c. IL23KO mice showed a stronger inflammation, indicated by higher gene expression of IL-1β and lower IL-10 mRNA in the scar and higher MCP-1 and MCP-3 mRNA in the non-infarcted area. * p<0.05 vs. respective WT group; # p<0.05 vs. LV from the same group; § p<0.05 vs. respective sham group. n=8-10 per group.

Figure 3. Immunohistochemical analysis of immune cell infiltration in the myocardium 4 days after MI. a-d. IL23KO mice showed a significant higher infiltration with CD4+ T cells, CD11b+ cells and neutrophils in the scar. However, no difference was observed in the number of the γδ T cells, which are also stimulated by IL-23 to produce IL-17α. e. Representative images of CD4+ cells and neutrophils in the border zone in infarction-operated WT and IL23KO mice 4 days after MI (100x magnification, scale bar represents 100 μm). * p<0.05 vs. respective WT group; # p<0.05 vs. LV from the same group; § p<0.05 vs. respective sham group. n=8-10 per group.

Figure 4. Gene expression of the transcription factors T-bet and RORγt and the cytokines IFN-γ and IL-17α 4 days after MI. Deficiency in IL-23 diverses the immune reaction into the Th1 direction with an upregulation of T-bet and IFN-γ production in both the non-infarcted area and the scar. Absence of IL-23 abrogated the Th17 reaction in the IL-23KO mice. *
p<0.05 vs. respective WT group; # p<0.05 vs. LV from the same group; § p<0.05 vs. respective sham group. n=8-10 per group.

Figure 5. Western blot analysis of total and phosphorylated Stat3 and ERK1/2 MAPK and zymography 4 days after MI. a. Stat3, as a downstream mediator of IL23 signaling, was significantly stronger activated in both the infarcted and non-infarcted area of the WT compared to the IL23KO mice. b This was also associated with a stronger activation of ERK1/2 in the infarcted area, which exerted protective effects. c. No difference was observed in the activity of MMP-2 or MMP-9 between the IL23KO and WT mice. d. Representative pictures of zymography. * p<0.05 vs. respective WT group; # p<0.05 vs. LV from the same group; § p<0.05 vs. respective sham group. n=8-10 per group.

Figure 6. Gene and protein expression of collagen I, III and α-SMA after MI. a-b. WT mice showed a higher deposition of collagen I and III already 4 days after MI, which persisted till day 30 after MI. This was associated with increased presence of myofibroblasts in the scar early after MI, which persisted in the late stage, as measured by α-SMA. c. Representative immunohistochemical images of collagen I, III and α-SMA from the border zone in WT and IL23KO mice 30 days after MI (100x magnification, scale bar represents 100 μm). d. Western blot analysis of collagen I 4 and 30 days after MI confirmed the increased collagen I abundance in the scar in the WT mice compared to the IL23KO mice. * p<0.05 vs. respective WT group; # p<0.05 vs. LV from the same group; § p<0.05 vs. respective sham group. n=8-10 per group.
Figure 7. Analysis of cardiac fibroblasts isolated from the heart 4 days after MI and after stimulation with IFN-γ. a-c. Fibroblasts isolated from the non-infarcted area and scar of WT mice showed a significantly higher gene expression of collagen I, III and α-SMA as markers for differentiation into myofibroblasts compared to IL23KO fibroblasts. d. Representative immunofluorescence images of cardiac fibroblasts stained for α-SMA. Blue: nuclei; red: collagen I. 200x magnification, scale bars represent 100μm. e. IFN-γ stimulation led to significant suppression of the gene expression of collagen I, III and α-SMA in cardiac fibroblasts from sham-operated hearts and the scar, implying that their differentiation into myofibroblasts and their collagen producing capacity is reduced. * p<0.05 vs. respective WT group; # p<0.05 vs. LV from the same group; § p<0.05 vs. respective sham group. n=18 wells per group.

Figure 8. Wound healing scratch assay and effects of IFN-γ (10ng/ml) stimulation on migration of cardiac fibroblasts. a. Stimulation with IFN-γ reduced the migratory capacity of cardiac fibroblasts after 24h and led to impaired reoccupation of the scratched area. b. Representative images of stimulated and unstimulated cardiac fibroblasts at 0, 6 and 24h after scratching. Untreated fibroblasts reoccupy faster the scratched area. Contrast phase microscopy, 100x magnification. § p<0.05 vs. unstimulated. n=18 wells per group. Scale bar represents 200μm.
**CD4+ cells**

- WT
- IL23KO

**CD11b+ cells**

- WT
- IL23KO

**Neutrophils**

- WT
- IL23KO

**γδ T cells**

- WT
- IL23KO

**CD4+ cells**

- WT
- IL23KO

**Neutrophils**

- WT
- IL23KO
Scratch wound healing assay

untreated

IFN-γ (10ng/ml)

0h

6h

24h

% of occupied wound area

untreated

IFNg 10ng/ml

6h

24h

§
Interleukin-23 Deficiency Leads to Impaired Wound Healing and Adverse Prognosis after Myocardial Infarction

Kostantinos Savvatis, Kathleen Pappritz, Peter Moritz Becher, Diana Lindner, Christin Zietsch, Hans-Dieter Volk, Dirk Westermann, Heinz-Peter Schultheiss and Carsten Tschöpe

Circ Heart Fail. published online December 3, 2013;
Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/early/2013/12/03/CIRCHEARTFAILURE.113.000604.1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Heart Failure can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Heart Failure is online at:
http://circheartfailure.ahajournals.org//subscriptions/