CD13 is essential for inflammatory trafficking and infarct healing following permanent coronary artery occlusion in mice

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Aims
To determine the role of CD13 as an adhesion molecule in trafficking of inflammatory cells to the site of injury in vivo and its function in wound healing following myocardial infarction induced by permanent coronary artery occlusion.

Methods and results
Seven days post-permanent ligation, hearts from CD13 knockout (CD13KO) mice showed significant reductions in cardiac function, suggesting impaired healing in the absence of CD13. Mechanistically, CD13KO infarcts showed an increase in small, endothelial-lined luminal structures, but no increase in perfusion, arguing against an angiogenic defect in the absence of CD13. Cardiac myocytes of CD13KO mice showed normal basal contractile function, eliminating myocyte dysfunction as a mechanism of adverse remodelling. Conversely, immunohistochemical and flow cytometric analysis of CD13KO infarcts demonstrated a dramatic 65% reduction in infiltrating haematopoietic cells, including monocytes, macrophages, dendritic, and T cells, suggesting a critical role for CD13 adhesion in inflammatory trafficking. Accordingly, CD13KO infarcts also contained fewer myofibroblasts, consistent with attenuation of fibroblast differentiation resulting from the reduced inflammation, leading to adverse remodelling.

Conclusion
In the ischaemic heart, while compensatory mechanisms apparently relieve potential angiogenic defects, CD13 is essential for proper trafficking of the inflammatory cells necessary to prime and sustain the reparative response, thus promoting optimal post-infarction healing.

Keywords
CD13 • Myocardial infarction • Dendritic cells • Macrophages • Monocytes

1. Introduction
Tissue damage resulting from myocardial infarction (MI) induces a strong inflammatory response characterized by a dramatic increase in the infiltration of inflammatory cells. This infiltration is critically dependent on adhesion molecules expressed on both the migrating cells and the endothelium lining the blood vessels at the site of injury. After transmigration, monocytes differentiate into macrophages and dendritic cells (DCs), which play key roles in promoting beneficial inflammation and participate in the healing process by clearing the necrotic tissue, facilitating angiogenesis, promoting myofibroblast formation, collagen deposition, and healing of the infarcted myocardium. The critical role of myeloid cells in post-MI healing is illustrated by studies in which systemic depletion of various populations led to markedly impaired wound healing and increased adverse cardiac remodelling. Conversely, other studies support a negative role for myeloid cells where their...
abrogation results in improved outcomes.\textsuperscript{10–12} The current belief is that a precise balance among these cells and the regulatory factors they produce are necessary to orchestrate optimal healing.\textsuperscript{13–15}

CD13 is a cell surface, zinc-dependent metalloprotease that is expressed on all myeloid cells, activated endothelial cells and epithelium of the kidney and intestine.\textsuperscript{16} CD13 functions as a peptidase, a viral receptor,\textsuperscript{17} and a signal transduction molecule\textsuperscript{18,19} in both enzyme-dependent and -independent manners. We have shown that global CD13 knockout (CD13\textsuperscript{KO}) mice are healthy and fertile and have normal haematopoietic profiles and myeloid cell functions.\textsuperscript{20} Recently, research from our laboratory has demonstrated that CD13 is also a homotypic adhesion molecule, where cross-linking of monocyte CD13 with activating monoclonal antibodies significantly induces adhesion to CD13 expressed on activated endothelial cells\textsuperscript{18,21} forming a complex containing both monocyte and endothelial CD13. This homotypic adhesion is independent of CD13 enzymatic activity, but requires tyrosine phosphorylation and cytoskeletal rearrangement. Cross-linking also induces CD13 clustering and redistribution to the sites of monocye–endothelial cell contacts.\textsuperscript{18} However, the role of CD13 as an inflammatory adhesion molecule \textit{in vivo} is unclear.

Finally, CD13 is up-regulated in the angiogenic vessels in the infarct area and border zone following MI, where it has been proposed as a target for imaging of myocardial angiogenesis.\textsuperscript{22} We postulated that CD13 on the endothelial cells at the site of injury may mediate inflammatory cell adhesion following MI and thus, contribute to healing. In addition, CD13 may impact the response in its capacity as an angiogenic regulator.\textsuperscript{23} To address these possibilities, we subjected global CD13\textsuperscript{KO} mice to permanent ligation of the left coronary artery and assessed heart function and infarct pathology. We chose permanent left anterior descending coronary artery (LAD) ligation to investigate the role of CD13 in the acute phase and effects on remodelling after infarct.

2. Methods

Detailed methods are described in Supplementary material online.

2.1 Animals

The global C57Bl/6–CD13\textsuperscript{KO} was generated at the Gene Targeting and Transgenic Facility.\textsuperscript{20} CD11c-mcherry mice were a gift from Dr Khanna.

2.2 Induction of MI

MI was induced by permanent LAD ligation according to the established procedure.\textsuperscript{24} Surgical-grade anaesthesia was induced by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg).

2.3 Echocardiography/intact heart function

Transthoracic echocardiography and \textit{ex vivo} heart functions were determined as described previously\textsuperscript{24} and Supplementary material online. Methods.

2.4 Histology/immunohistochemistry

The infarct size was determined by Masson’s trichrome \textit{7 days} post-MI and by triphenyltetrazolium chloride (TTC)/Evans blue staining 24 h post-MI. Collagen content was determined by Masson’s trichrome, which stains collagen blush-grey. Immunostaining used paraffin-embedded or cryosections (Supplementary material online).

2.5 Flow cytometry

Infarct and border zone tissue (7 days) were minced, collagenase-digested, RBCs lysed, filtered (40 \(\mu\)m), and antibody-stained at 4°C, 30 min: anti-CD45.1-PE, anti-F480-FITC, anti-Gr-1-APCe780, anti-CD3/CD19-NK1.1/ Ly6G-AF700, anti-CD11c-PECy7, anti-CD11b-Pacific-blue, anti-CD115-APC, anti-Ly6C-PerCp-Cy5.5, and UV live/dead dye. Flow cytometry was performed on LSRII (Becton Dickinson) and analysed with FlowJo (Tree-Star).

2.6 Western blot analysis

Indicated regions of the infarcted heart were isolated 7 days post-MI, tissue lysates separated by SDS–PAGE, and probed for CD13.

2.7 Isolation and functional measurement of cardiac myocytes

As described by Shen et al.\textsuperscript{25} and Supplementary material online.

2.8 Endothelial network assay

Lung endothelial cells were isolated as described by Winnicka et al.\textsuperscript{20} and Supplementary material online.

2.9 Methylene blue staining and lectin perfusion

Methylene blue staining and lectin perfusion used to determine the perfusion of the infarcted region of the heart 7 days after MI (Supplementary material online).

2.10 Isolation of cardiac fibroblasts and RT–PCR

Isolated by collagenase digestion of minced cardiac tissue (Supplementary material online).

2.11 Quantitative RT–PCR for TGF-\(\beta\)

Different regions of the infarcted heart were isolated \textit{7 days} after MI and transforming growth factor-\(\beta\) (TGF-\(\beta\)) levels were quantitated (Supplementary material online).

2.12 Statistical analysis

Results are presented as mean \(\pm\) SEM. Statistical analysis was performed using the unpaired, two-tailed \(t\)-test or one-way analysis of variance followed by the Newman–Keuls multiple comparison test, significant at \(P < 0.05\).

3. Results

3.1 CD13 expression is induced in the infarct border zone

To analyse the role of CD13 in ischaemic heart injury, we initially assessed the expression of CD13 following MI in wild-type (WT) mice induced by permanent ligation of the left anterior descending artery. We found that while CD13 showed little expression in the sham-operated heart, it was strikingly up-regulated in the infarct area and particularly in the infarct border zone by 3 days following ligation (\textit{Figure 1A}). Expression peaked by 5–7 days and largely disappeared by 10 days after MI. Protein quantitation by western blot analysis (\textit{Figure 1B}) of WT and CD13\textsuperscript{KO} hearts or CD13 immunofluorescence in the infarcted and sham-operated heart tissue (\textit{Figure 1C}) indicated that CD13 expression increased by 15-fold at 7 days.
3.2 CD13 is expressed on multiple cell types in the infarct

To determine the cell types expressing CD13 in the injured heart tissue, we co-immunostained 7 days post-MI WT hearts for CD13 and cell-type-specific markers (Figure 1D). We found that CD13 is expressed on some but not all CD31+ vessels of the infarct area/border zone (Figure 1D1). F4/80-positive macrophages are highly CD13 positive, as are many CD11c+ DCs, as previously established (Figure 1D2,3). In contrast, CD1+ T cells and alpha smooth muscle actin (αSMA)-positive myofibroblasts distinctly did not express CD13 (Figure 1D4,5). Interestingly, some of the CD13+ cells appear to be intercalated among the myofibroblasts, perhaps indicating a functional interaction. To confirm that these are closely apposed but distinct cell types, we purified RNA from isolated cardiac fibroblasts differentiated into myofibroblasts with TGF-β. While αSMA transcripts are readily detectable from both control and differentiated cells by RT–PCR, they are clearly negative for CD13 (Figure 1E). Therefore, CD13 is highly expressed on a number of cell types in the infarct border zone, prompting us to further investigate its role in wound healing after MI.

3.3 Loss of CD13 results in adverse remodelling of the left ventricular wall after MI and long-term functional impairment

Our global CD13KO mice are essentially normal with respect to physiological myeloid cell functions.20 To analyse the potential role of CD13 in cardiac tissue remodelling, we subjected WT and CD13KO mice to permanent MI and measured heart function after 7 and 60 days. Importantly, sham echocardiographic measurements revealed no functional differences between genotypes (not shown). Infarct sizes were also similar at 24 h and 7 days post-MI ruling out the effects of CD13 on acute myocyte death (Figure 2A and B and Supplementary material online, Figure S1). However, at 7 days post-MI, the CD13KO mice showed a marked reduction in fractional shortening (FS) and ejection fraction, an increase in the left ventricular internal dimension, and a significant thinning of the left ventricular posterior wall (LVPW) consistent with adverse remodelling26 (Figure 2C, representative echocardiography images, see Supplementary material online, Figure S1). Comparable heart weight (HW) with body weight (BW) ratios ruled out any difference in cardiac hypertrophy as a contributing factor (Table 1). Importantly, reductions in FS and ejection fraction persisted at 2 months after MI (Figure 2C). Further analysis of intact infarcted hearts using the ex vivo working heart model at 7 days following MI corroborated our echocardiography data, where CD13KO mice showed decreases in cardiac output, left ventricular developed pressure, and the rate of left ventricular contraction pressure when compared with CD13WT mice (Figure 2D). Taken together, these functional results are consistent with a protective role for CD13 in wound healing after MI, and the lack of CD13 can result in long-term functional impairment of the heart.

3.4 Decreased infiltration of inflammatory cells in the absence of CD13

The process of healing following MI involves inflammation, angiogenesis, and tissue remodelling. During inflammation, adhesion molecules mediate the transmigration of functionally distinct subsets of monocytes through the blood vessels into the injured tissue, which subsequently differentiate into macrophages and DCs. These cells are responsible for orchestrating subsequent wound healing21 by clearing the necrotic tissue,4,5,28 facilitating angiogenesis3 facilitating angiogenesis3 and myofibroblast production.6,29,30 We have previously shown that in vitro, CD13 functions as a homotypic adhesion molecule that mediates the monocyte–endothelial cell interactions critical for inflammatory trafficking.18 To address whether CD13 might also regulate these interactions in vivo, we evaluated inflammatory cell trafficking in infarcted hearts of WT and CD13KO animals. Flow cytometric analysis of cells isolated from the...
Infarct regions showed a nearly 70% decrease in the numbers of CD45+ haematopoietic cells entering the CD13KO infarct with concomitant decreases in the numbers of CD11b+Gr-1high inflammatory monocytes, CD11b+Gr-1low reparative monocytes, F4/80+ macrophages, CD11c+ DCs, and CD3+ T cells at 7 days post-MI (Figure 3A). Quantitation of immunostained tissue sections corroborated the macrophage (Figure 3B) and T cell (Figure 3C) data. To confirm the striking degree of DC infiltration, we permanently ligated hearts from WT transgenic mice harbouring the gene encoding the mCherry fluorescent protein under the control of the CD11c promoter. While the remote heart

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**Table 1** Organ weights

| Organ weight | WT ligated (7 days) | KO ligated (7 days) |
|--------------|---------------------|---------------------|
| HW/BW, mg/g  | 6.73 ± 0.35         | 7.17 ± 0.40         |
| LW/BW, mg/g  | 7.500 ± 0.25        | 7.784 ± 0.29        |
| Left ventricular weight, mg | 0.1556 ± 0.004 | 0.1440 ± 0.01 |

Values are means ± SE. WT ligated (7 days) n = 5; KO ligated (7 days) n = 5.
Figure 3 Inflammatory cell infiltration. (A) Infiltrating macrophages-F4/80hi, inflammatory monocytes-Gr-1^hi^, reparative monocytes-Gr-1^low^, DCs-CD11chi, T cells-CD3^hi^ in the live CD19-NK1.1-CD45^+^ cell population were analysed 7 days post-MI in the infarct and infarct border zone by flow cytometry. (B) Macrophage infiltration (brown—F4/80^+^, indicated by arrows) and (C) T-cell infiltration (green—CD3^+^ immunofluorescence) at the border zone were quantified 7 days post-MI in paraffin sections; objective = 40 ×. (D) Infiltration of DCs in CD11c-mCherry transgenic hearts at the infarct border zone compared with the non-infarcted remote heart region was analysed 7 days post-MI in frozen tissue. (E) Apoptotic cells with DNA strand breaks detected by Apoptag-tunel (brown) at the border zone were quantified 7 days post-MI in paraffin sections; objective = 40 ×. Error bars represent mean ± SEM for CD13WT (n = 5) and CD13KO (n = 5) mice, *P < 0.05. Blue = DAPI nuclear stain.
is largely negative; we observed a remarkable increase in cells displaying cytoplasmic mCherry expression in the infarct border zone in agreement with our quantitative flow cytometric data (Figure 3D). Staining of infarct sections for apoptotic cell markers ruled out the possibility that the lower cell numbers were due to increased apoptosis in the absence of CD13 (Figure 3E). Finally, our previous analysis of CD13KO animals had shown essentially normal haematopoietic development, including macrophage and DC differentiation, arguing against defective development/differentiation in the absence of CD13.19,20 Therefore, the lack of CD13 impairs inflammatory cell trafficking.

3.5 Lack of CD13 has no effect on cardiomyocyte function, but results in fewer αSMA-positive myofibroblasts
Impaired cardiomyocyte contractile function could contribute to reduced intact heart function in CD13KO mice. The measurement of the size and contraction capabilities of cardiac myocytes isolated from non-infarcted regions of WT and CD13KO mice at 7 days post-MI showed that there was no difference in the myocyte cell length isolated from either genotype (Figure 4A). Similarly, analysis of stimulated myocyte contraction showed that cells from both genotypes behave similarly, suggesting that the lack of CD13 does not impair individual cardiomyocyte function (Figure 4B, representative tracings Figure 4C). Following MI, macrophages produce TGF-β that induces cardiac fibroblasts to differentiate to αSMA-expressing myofibroblasts, which in turn synthesize and deposit collagen to provide tensile strength to the heart tissue and form a stable scar.2 The adverse remodelling in CD13KO mice would also be consistent with an impaired myofibroblast response. Immunohistochemical staining of 7 days post-MI heart sections indicated that there were significantly fewer αSMA-expressing myofibroblasts in the infarct region of CD13KO mice (Figure 4D). Masson’s trichrome staining showed decreased in large collagen fibres in the CD13KO infarct (Figure 4E, bluish-grey area). Finally, qRT–PCR analysis of mRNA isolated from WT and CD13KO hearts showed reduced levels of TGF-β transcripts (Figure 4F), consistent with fewer macrophages in the wound. Since cardiac myofibroblasts do not express CD13 (Figure 1), the decrease in myofibroblast numbers and any subsequent effects on extracellular matrix degradation or deposition is likely the result of other CD13-dependent effects in the healing heart.

3.6 Loss of CD13 results in increased but non-functional CD31-positive vessels in vivo
Angiogenesis is an important step in myocardial infarct repair and healing. We have previously shown that CD13 is an angiogenic regulator,21,22 and thus, the lack of CD13 may also affect angiogenesis and subsequent heart repair. Surprisingly, analysis of heart sections for CD31-positive endothelial-lined lumens showed that, at 7 days post-MI, CD13KO animals have significantly higher numbers of these structures when compared with WT hearts (Figure 5A), which were no longer evident at the longer 2-month post-MI time-point (not shown), consistent with normal vessel regression during healing.33 However, many of the lumens in the CD13KO hearts were quite small and accounted for differences in vessel numbers (Figure 5A). These small vessels were apparently not perfused since CD13KO infarcted hearts excluded methylene blue dye to the same extent as WT infarcts (Figure 5B). To confirm this notion, we perfused WT and CD13KO infarcted hearts with FITC-labeled IB4-lectin to detect patent vessels and counterstained sectioned tissue with Dylight-594-conjugated IB4-lectin to identify total blood vessels. Fluorescence quantitation indicated that there was no significant difference in the perfused green area between genotypes (Figure 5C), suggesting that in vivo vessel function is not affected by the lack of CD13. However, in agreement with our previous findings, in vitro assessment of endothelial morphogenesis using primary lung endothelial cells confirmed that cells from CD13KO mice are severely impaired in their ability to form capillary networks (Figure 5D), suggesting that compensatory mechanisms support angiogenesis in vivo.

4. Discussion
This study highlights the role of CD13 as an adhesion molecule modulating inflammatory cell trafficking and its functional role in wound healing using MI as an in vivo model. While induction of CD13 expression has previously been reported,23,24 the specific cell-type-expressing CD13 or its functional role in the injured/healing myocardium has not been investigated. In the current study, we find that the pattern of CD13 immunostaining is consistent with its expression on both infiltrating myeloid cells and blood vessels in the infarct area and border zone. In addition, a large proportion of the CD13 expression in the injured tissue appears to belong to a remarkably abundant population of infiltrating DCs. Functional evaluation of hearts following infarction shows that CD13 plays a protective role in cardiac repair as the hearts of mice lacking CD13 show both acute and sustained functional impairment accompanied by significant decreases in inflammatory and reparative monocyte, macrophage, DC, and αSMA-positive myofibroblast populations and left ventricular dilatation, but apparently no overall effect on angiogenesis or individual cardiac myocyte function. Thus, abnormal trafficking is the likely mechanism responsible for adverse remodelling after MI in CD13KO hearts. The multifunctional nature of this molecule and its expression on a number of cell types that are critical to the healing process suggest that CD13 may regulate multiple aspects of immune and cardiac cell function following acute MI.

CD13KO mice have normal myeloid profiles, function, and differentiation.20 Following MI, we observe fewer total CD45+ haematopoietic cells in the infarcts of global CD13KO mice suggesting a generalized defect in inflammatory trafficking, consistent with our characterization of CD13 as an adhesion molecule regulating the monocyte–endothelial interactions critical for inflammatory trafficking.18 Logically, fewer infiltrating monocytes would result in fewer macrophages and DCs and concomitant reductions in the cytokines they elicit. Indeed, we see lower levels of TGF-β transcripts, which very likely is responsible for the diminished myofibroblast numbers as well. Elegant studies have demonstrated that infarct healing occurs in a biphasic manner that depends on the sequential trafficking of pro-inflammatory monocytes (Ly6Chigh phase I, peaking at day 3) followed by a reparative subset (Ly6Clow phase II, peaking at day 7). Importantly, these phases must occur in sequence, as eliminating phase I alone resulted in a suboptimal phase II and consequent impaired healing. CD13 expression is significantly higher in pro-inflammatory monocytes than in the reparative subset,25 raising the intriguing possibility that CD13 regulates trafficking of Gr-1high, but not Gr-1low monocytes. In our study, the reduced numbers of pro-inflammatory monocytes in phase I would in turn affect the recruitment of the reparative monocytes in phase II, leading to impaired healing of the infarcted tissue and impaired function in CD13KO hearts. In agreement with our data, studies where monocyte or macrophage populations were either depleted or functionally impaired, the response to
infarction phenocopies the decrease in cell numbers that we see in the CD13KO animals and results in significant reductions in heart function. These findings would suggest that the decrease in infiltrating inflammatory cells in CD13KO animals could be largely responsible for the adverse remodelling in CD13KO hearts. Paradoxically, other studies suggest the reverse; that decreased monocyte/macrophage infiltration promotes tissue repair and less adverse remodelling. Taken together, these contradictory findings are believed to reflect a critical balance between pro- and anti-inflammatory activities in the healing heart. Evidence suggests that optimal healing following permanent ligation depends on the precise co-ordination of the timing, phenotype, and quantity of infiltrating inflammatory cells in that either excessive inflammation or repression of inflammatory activity may derail repair. The question whether CD13 exclusively regulates pro-inflammatory monocyte trafficking or may designate an additional subset is currently under investigation in our laboratory.

**Figure 4** Cardiomyocyte function, myofibroblasts, and collagen deposition. (A) Myocyte length, (B) myocyte shortening, and (C) representative traces of myocyte shortening at the frequency of 1 Hz. Error bars represent mean ± SEM for 26 cells from CD13WT and 25 from CD13KO mice. (D) Myofibroblasts at the infarct border zone were analysed 7 days post-MI by αSMA immunostaining and quantifying paraffin sections; objective = 40 ×. (E) Collagen deposition was quantitated with Masson’s trichrome staining (blue or bluish-grey stain) for CD13WT (n = 5) and CD13KO (n = 5) mice. (F) qRT–PCR of RNA isolated from the indicated areas of CD13WT (n = 6) and CD13KO (n = 6) hearts 7 days post-MI for relative transcript levels of TGF-β and GAPDH. Error bars represent mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant difference between CD13WT and CD13KO mice.
Figure 5  Angiogenesis. (A) Angiogenesis was analysed by enumerating total CD31+ lumens, lumens ≤ 36 μm² indicated by arrows, or lumens > 36 μm² but ≤ 143 μm² by immunostaining of paraffin sections 7 days post-MI; CD13WT (n = 5) and CD13KO (n = 5) mice, bar = 100 μm. (B) Perfusion of the infarct region at 7 days was analysed by injecting methylene blue dye through the aorta of the excised intact heart; CD13WT (n = 3) and CD13KO (n = 3) mice or (C) by injecting FITC-conjugated IB4-lectin to detect functional blood vessels and by counterstaining fixed frozen tissues with Dylight 594-conjugated IB4-lectin to identify total blood vessels; CD13WT (n = 3) and CD13KO (n = 3) mice; objective = 5 ×. (D) Primary lung endothelial cells isolated from CD13KO mice are unable to form capillary networks and form fewer branches; n = 3 experiments, objective = 2 ×. Error bars represent mean ± SEM, *p < 0.05.
In addition to monocytes and macrophages, CD13 is highly expressed in classical DC populations and is one of a panel of signature genes that are specifically up-regulated in DCs. Similar to our observations, the few studies addressing cardiac DCs have described them as aligned with and interdigitated among myocytes and were found to accumulate in rat and human hearts following acute MI. We have recently shown a novel function for CD13 in DCs where it regulates antigen uptake and presentation, suggesting that in addition to trafficking, the lack of CD13 may also impact the function of DCs in the wound. Endogenous antigens released from damaged tissue are recognized by DCs via pattern recognition receptors and often trigger extreme inflammatory responses resulting in further damage. Alternatively, a clear role for DCs has been demonstrated in repair where global depletion of DCs exacerbated adverse remodelling post-MI in agreement with our results. In contrast, however, the DC-ablated infarcts showed substantially increased pro-inflammatory monocyte/macrophage infiltration in striking opposition to the decreased numbers found in the CD13KO infarcts. Because DCs are present in the CD13KO infarcted hearts, this discrepancy strongly suggests that the lack of CD13 alters the normally protective function of DCs in the wound healing process. The role of DCs in remodelling and the novel CD13-dependent mechanisms regulating their function in vivo are an exciting and unexplored field that is currently under study in our laboratory.

We have previously shown that CD13 is also expressed on activated endothelial cells where it positively regulates cdc42 activation and filopodia formation, thus controlling endothelial invasion and angiogenesis. Based on these findings, we would predict a decrease in podia formation, thus controlling endothelial invasion and angiogenesis. It is well established that the vigorous or persistent inflammation induced following myocardial ischaemia intensifies injury, impedes repair, and promotes left ventricular dilatation. Therefore, increasing our understanding of the adhesion molecules and mechanisms regulating inflammatory infiltration into the infarct will form the basis for rational, targeted therapies for the millions of patients suffering from heart failure worldwide.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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