# Endothelial Cx40 limits myocardial ischaemia/reperfusion injury in mice

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| **Aims** | Gap junctions are indispensable for the function of heart and blood vessels by providing electrical coupling and direct cell-to-cell transfer of small signalling molecules. Gap junction channels between neighbouring cells are composed of 12 connexins (Cx). Changes in Cx43 expression, localization, and channel properties in cardiomyocytes contribute to infarction and reperfusion injury of the heart. It is increasingly recognized that deleterious consequences of ischaemia/reperfusion (IR) are modulated by the inflammatory response and endothelial function. The role of the endothelial connexins, i.e. Cx40 and Cx37, in cardiac IR injury is, however, not known. |
| **Methods and results** | Following 30 min ischaemia and 24 h reperfusion, we found a significant increase in myocardial infarct size in mice with endothelial-specific deletion of Cx40 (Cx40del), but not in Cx37-deficient mice. The cardioprotective effect of endothelial Cx40 was associated with a decrease in neutrophil infiltration. Moreover, beneficial effects of endothelial Cx40 were not observed in isolated Langendorff-perfused hearts, suggesting direct involvement of endothelial–leucocyte interactions in the cardiac injury. Single-dose administration of methotrexate, a CD73 activator, reduced infarct size and neutrophil infiltration into the infarcted myocardium in Cx40del but not in control mice. Similar to Cx40del mice, CD73-deficient mice showed increased sensitivity to cardiac IR injury, which could not be conversed by methotrexate. |
| **Conclusion** | Endothelial Cx40, but not Cx37, is implicated in resistance of the heart to IR injury by activation of the CD73 pathway. Thus, the Cx40–CD73 axis may represent an interesting target for controlling reperfusion damage associated with revascularization in coronary disease. |
| **Keywords** | Gap junction • Cx40 • Cardiac ischaemia/reperfusion injury • Neutrophil recruitment |

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## 1. Introduction

Ischaemic heart disease is the leading cause of death and disability in industrialized countries. Following acute myocardial infarction, the rapid re-establishment of blood flow to the ischaemic zone with the use of thrombolytic therapy or primary percutaneous coronary intervention is essential to rescue the myocardium and improve clinical outcome.[1–3] However, the process of restoration of blood flow to the ischaemic myocardium (reperfusion) may lead to further complications such as diminished contractile function (stunning), arrhythmias, and ultimately heart failure.[1,3] Paradoxically, myocardial reperfusion can itself induce death of cardiomyocytes that were viable immediately before the procedure and thus increase infarct size, a phenomenon that has been called myocardial ischaemia–reperfusion (IR) injury.[1,2] Experimental studies show that IR injury may account for up to 50% of the final myocardial infarct size.[1,2] Thus, the development of therapeutic

strategies to protect the heart against the detrimental effects of reperfusion injury is of great clinical importance.[4]

Increasing evidence indicates that an inappropriate inflammatory response in the microcirculation may be at the basis of IR injury.[5,6] Shortly after the onset of reperfusion, neutrophil activation and accumulation occur in the damaged myocardium.[7] Neutrophils are important for the development of reperfusion injury by releasing free radicals, proteases, and pro-inflammatory mediators that further amplify the infiltration of leucocytes in the jeopardized myocardium. In addition, the haemorrheological properties of neutrophils contribute to leucocyte entrapment in the capillaries, leading to microvascular plugging and the no-reflow phenomenon.[8]

Endothelial cells are key players in the orchestration of neutrophil recruitment by controlling their rolling, adhesion, crawling and finally transmigration via the expression of chemokines, selectins, cell adhesion molecules, integrins, and junctional adhesion molecules.[9–11] In an earlier study, we have shown that targeting the endothelial gap junction protein Cx40 promotes leucocyte adhesion to the endothelium, thus accelerating atherosclerosis.[12] Connexins are a family of 21 transmembrane proteins that are widely expressed in the human body. They play an important role in cell–cell communication and homeostasis in various tissues by forming gap junction channels, which enable a direct passage of ions or metabolites from one cell to another. Gap junction channels are formed by the docking of two hemi-channels, which are hexamers of Cxs, in the plasma membrane of two neighbouring cells. Isolated hemi-channels can also be functional under specific conditions and may then allow for the transfer of small molecules between the cytoplasm and the extracellular space. In the heart, Cx40 is expressed in atrial myocytes, the conduction system, and the endothelium.[13] Endothelial expression of this gap junction protein is influenced by different factors such as oxidative stress, pro-thrombotic molecules, pro-inflammatory cytokines, and classical cardiovascular risk factors.[14,15] In the present study, we investigated the possibility that this endothelial gap junction protein confers cardioprotection in IR injury.

# 2. Methods

## 2.1 Mice

Animal experimentation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1966) and was approved by the cantonal veterinary authorities.

To generate mice with the endothelial-specific deletion of Cx40, we interbred *ApoE*$^{-/-}$ mice expressing the Cre under control of Tie2 transcriptional elements with *Cx40*$^{fl/fl}$*ApoE*$^{-/-}$ mice, as previously described.[12] We thus obtained two control groups in which the expression of Cx40 is normal: *Tie2Cre*$^{+}$*ApoE*$^{-/-}$ (Control group 1: C1) mice and *Cx40*$^{fl/fl}$*ApoE*$^{-/-}$ (Control group 2: C2) mice, and mice with the endothelial-specific deletion of Cx40: *Tie2Cre*$^{+}$*Cx40*$^{fl/fl}$*ApoE*$^{-/-}$ (Cx40del mice).[12] *Cx37*$^{-/-}$*ApoE*$^{-/-}$ and *CD73*$^{-/-}$*ApoE*$^{-/-}$ mice have been generated, as previously described.[16,17] Genotypes of the different mice were verified by polymerase chain reaction using previously described protocols.[12,16,17]

## 2.2 *In vivo* IR protocol

*In vivo* IR was performed as previously described.[18,19] Briefly, 16-week-old mice were anaesthetized with 4% isoflurane, intubated through a tracheotomy, and ventilated mechanically (tidal volume 150 μL; ventilation rate 120 breaths/min; rodent respirator model 683, Harvard Apparatus). Before intubation, the adequacy of anaesthesia was confirmed with the absence of pedal reflex. Then, anaesthesia was maintained with 2% isoflurane delivered

in 100% $O_2$ through the ventilator. Before the surgical procedure, buprenorphine HCl (0.05 mg/kg in 100 μL) was subcutaneously administered. Left thoracotomy was performed to allow access to the heart, an 8-0 Prolene suture was passed around the left anterior descending (LAD) coronary artery, and a small piece of polyethylene catheter was used to form a snare. Ischaemia was induced by pulling the snare and the occluded position was maintained for 30 min. Reperfusion was generated for 24 h by releasing the snare. The chest was closed, the air was evacuated from the chest cavity, and the ventilator was removed. Some mice were treated 5 min before the reperfusion with an intraperitoneal injection of methotrexate (10 mg/kg). After 24 h of reperfusion, mice were anaesthetized with an intraperitoneal injection of ketamine−xylazine (120 and 16 mg/kg, respectively) and the heart was removed for post-mortem analyses.

## 2.3 Immunohistochemistry

Hearts were embedded in optimum cutting temperature compound and frozen. Seven micrometre cryosections from heart were stained with haematoxylin−eosin or immunolabelled with antibodies against Ly-6G (1/50, BD Pharmingen) for neutrophils, against Cx37, Cx40, and Cx43 (1/20, 1/50, and 1/50, respectively, Alpha Diagnostic International) or against CD31 (1/200, Santa Cruz) to label the endothelium. Sections immunolabelled with antibodies against Ly-6G or Cxs only were counterstained with Evans blue. Nuclei were counterstained with DAPI. Sections used for immunostainings were fixed at −20°C for 5 min with acetone (Ly6G) or with methanol (CD31 and Cxs). For neutrophil quantification, three images/heart were taken in the infarcted area and neutrophil content was quantified by dividing the number of positive cells for Ly-6G by the total number of cells (around 50 cells/image) in the infarcted area. Quantification was performed by computer image analysis using the MetaMorph6 software (Zeiss).

## 2.4 Infarct size quantification

After 24 h of reperfusion, the LAD was re-occluded and 2% Evans blue dye (Sigma-Aldrich) was injected retro-orbitally to delineate the area at risk. The heart was quickly removed, rinsed with 0.9% NaCl, frozen, and sectioned into five or six transverse slices (1 mm thick). Slices were incubated in 1% triphenyltetrazolium chloride (TTC) in sodium phosphate buffer (pH 7.4) at 37°C for 20 min to stain the viable cells in the zone at risk in red, and the slices were post-fixed in 10% formaldehyde solution for 24 h. Surfaces of areas at risk and infarct areas were calculated from computed images of the slices using the NIH Image software (NIH AutoExtractor 1.51; National Institutes of Health). The area at risk was expressed as a percentage of total ventricular surface, and the infarcted area was expressed as a percentage of the area at risk.

## 2.5 *Ex vivo* Langendorff perfusion

Sixteen-week-old mice were anaesthetized by an intraperitoneal injection of ketamine−xylazine (120 and 16 mg/kg, respectively), and the adequacy of anaesthesia was confirmed with the absence of pedal reflex. The hearts ($n = 5$/group) were isolated and perfused in a modified Langendorff system under constant pressure (70 mmHg) with gassed (94% $O_2$−6% $CO_2$) Krebs-Henseleit buffer solution at 37°C, as described previously.[20] An 8-0 Prolene suture was passed around the LAD and a small piece of polyethylene catheter was used to form a snare. The hearts were stabilized for 20 min and ischaemia was induced for 30 min by pulling the snare. Reperfusion was generated for 60 min by releasing the snare. At the end of the reperfusion, the LAD was re-occluded and 2% Evans blue dye was injected via the aorta to delineate the area at risk. The heart was quickly removed, rinsed in 0.9% NaCl, frozen, sectioned into five or six transverse slices, and incubated in TTC to stain viable myocardium as described above.

## 2.6 Western blot

Proteins were extracted from the heart ventricles of wild-type and $ApoE^{-/-}$ mice in modified RIPA buffer [Tris−HCl (pH 8.0) 20 mmol/L, NaCl 1 mmol/L, NP-40 1%, NaF 50 mmol/L, Na-orthovanadate 10 mmol/L, phenyl methyl sulfonyl fluoride 1 mmol/L, ethylene diamine tetraacetic acid 5 mmol/L, sodium dodecyl sulfate 0.25%, Na-deoxycholate 1%, and cocktail of protease inhibitors), as previously described.[20] Western blotting was performed using antibodies against Cx43 (1/1000, BD Transduction Laboratories), Cx40 (1/500, Alpha Diagnostic International), or Cx37 (1/1000, Alpha Diagnostic International).

## 2.7 Statistical analysis

All analyses were performed with the GraphPad Prism 5.01 software, and results are expressed as mean $\pm$ SEM. For two groups' comparison, unpaired $t$-test was performed, and for multiple groups' comparison, one-way or two-way ANOVA with Bonferroni's post-test was used. Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1 Myocardial localization of Cx43, Cx40, and Cx37 after *in vivo* IR

IR induces morphological changes in the myocardial tissue that can be visualized by classical histochemistry. *Figure 1A* shows representative images of haematoxylin−eosin staining performed on cryosections of a heart submitted to 30 min of LAD ligation and 24 h of reperfusion. This staining defines the three different regions that can be observed in a heart submitted to regional IR: the healthy myocardium (H), the infarcted area (I), and the border zone (B). An 1-day-old infarct is characterized by the presence of coagulative necrotic tissue with elongated and narrow fibres and by the apparition of leucocytes, typically neutrophils, in the spaces between these fibres (*Figure 1A4*). No structural modifications were observed in the non-infarcted healthy part of the heart (*Figure 1A2*). In the border zone, necrotic cells were in close contact with surviving cardiomyocytes (*Figure 1A3*).

In healthy ventricular tissue, three connexins are expressed. The major connexin Cx43 localized mainly in the intercalated disk between cardiomyocytes (*Figure 1B1−2*), but was absent from the endothelium and endocardium (see Supplementary material online, *Figure S1A3, 1B3,* and *1C3*). Cx40 and Cx37 were typically found in endothelial cells of arteries and in the microvasculature (*Figure 1C1−2* and *D1−2*, respectively), where it co-localized with CD31 (see Supplementary material online, *Figure S1B1−2* and *C1−2*). Moreover, Cx37 was also expressed in the endocardium (see Supplementary material online, *Figure S1A2*). Twenty-four hours after LAD ligation, Cx43, Cx40, and Cx37 were no longer expressed in injured myocardium and in CD31-positive cells in the infarcted area with a sharp demarcation at the border zone (*Figure 1B3−4, C3−4,* and *D3−4* as well as Supplementary material online, *Figure S1D1−3*). Thus, IR of the heart leads to a reduction in endothelial Cx40 and Cx37 in the infarct zone.

### 3.2 Endothelial-specific deletion of Cx40 increases neutrophil infiltration and *in vivo* cell death associated with IR

In the cardiovascular system, Cx40 is expressed in atrium, conduction system, endothelial cells throughout the vascular tree, and smooth muscle cells of resistance arteries.[13] Cx40 plays an important role in electrical propagation between atrial cells and in arteriolar vasomotion.[21,22]
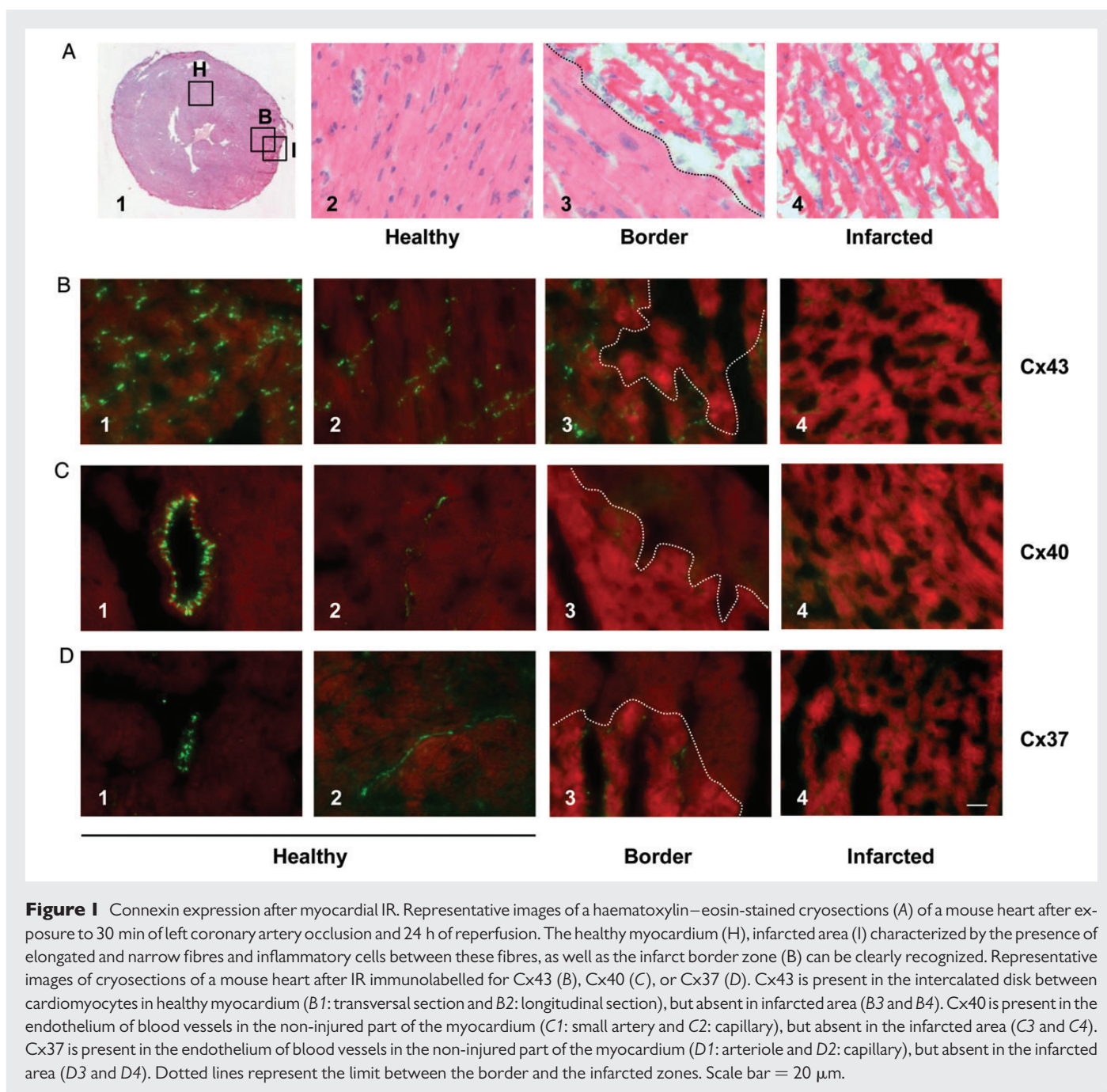
To specifically study the role of endothelial Cx40 in a clinically relevant setting of IR injury, we used a previously characterized $ApoE^{-/-}$ mouse line in which Cx40 is deleted from the endothelium only, i.e. $Tie2Cre^{+}$-$Cx40^{fl/fl}ApoE^{-/-}$ (Cx40del) mice, as well as $Tie2Cre^{+}ApoE^{-/-}$ (C1) and $Cx40^{fl/fl}ApoE^{-/-}$ (C2) control mice.[12] $ApoE^{-/-}$ mice show modest elevation of serum cholesterol levels.[23] We verified the absence of Cx40 in the endothelium of Cx40del mice (see Supplementary material online, *Figure S2A*). Of note, endothelial expression of Cx37 was reduced and expression of Cx43 in the myocardium was not affected by endothelial-specific deletion of Cx40 (see Supplementary material online, *Figure S2B* and *C*) as previously reported;[12] however, the expression of the three connexins was not altered between wild-type and $ApoE^{-/-}$ mice (see Supplementary material online, *Figure S2D*). The three groups of mice were submitted to an *in vivo* protocol of acute myocardial infarction to investigate the role of endothelial Cx40 in leucocyte recruitment and cell death associated with IR. The infiltration of inflammatory cells starts quickly after the onset of reperfusion and the peak of neutrophil recruitment is usually observed at 1 day of reperfusion.[24] In our study, neutrophil infiltration into the infarcted area 24 h after the onset of reperfusion was higher ($P < 0.05$) in Cx40del mice in comparison with control mice (C1 and C2) (*Figure 2A* and *B*). Of note, we observed minimal neutrophil infiltration ($4.8 \pm 1.3\%$, $n = 6$) in a similar perfusion territory of sham-operated mice. The higher proportion of inflammatory cells in Cx40del mice was associated with a larger infarcted area ($P < 0.05$) in Cx40del mice in comparison with both groups of control mice (*Figure 3A* and *B*—right panel). Importantly, the area at risk was not different between the three groups of mice, illustrating the reproducibility of the LAD ligation procedure (*Figure 3A* and *B*—left panel). To further investigate the role of leucocytes in the increased infarct size in Cx40del mice, *ex vivo* Langendorff perfusion with crystalloid buffer were performed. As expected, neither the area at risk nor the infarct size was different between the three groups of mice in the absence of leucocytes (*Figure 4*). These results demonstrate the importance of endothelial Cx40 in limiting leucocyte recruitment for the protection of the heart against IR injury.

### 3.3 Cx37 expression does not modify *in vivo* IR-induced cell death

A reduction in Cx37 expression is observed in the endothelium of mice with ubiquitous deletion of the Cx40 gene ($Cx40^{-/-}$)[25] and in Cx40del mice used in this study.[12] Cx37 hemi-channels in monocytes inhibit in an autocrine manner ATP-dependent adhesion of these cells.[16] Moreover, endothelial Cx37−endothelial nitric oxide synthase (eNOS) interactions lead to an altered NO production that might affect leucocyte adhesion.[26] To investigate whether the increased infarct size in Cx40del mice might be the consequence of reduced Cx37 expression, we submitted $Cx37^{-/-}ApoE^{-/-}$ mice to the *in vivo* IR protocol. As shown in *Figure 5*, the deletion of Cx37 did not affect the area at risk (*Figure 5A*) or the infarct size (*Figure 5B*) in mice.

### 3.4 CD73 activation limits infarct size development in mice with endothelial-specific deletion of Cx40

CD73 is an ectoenzyme at the surface of cells that participate in the hydrolysis of extracellular ATP into adenosine. Adenosine produced in the extracellular space limits the recruitment of leucocytes into the injured area.[27] Activation of CD73 by methotrexate enhances the formation of adenosine,[28] and endothelial Cx40 is known to regulate CD73 expression and activity.[12] To investigate the possible involvement of CD73 in

**Figure 1** Connexin expression after myocardial IR. Representative images of a haematoxylin—eosin-stained cryosections (*A*) of a mouse heart after exposure to 30 min of left coronary artery occlusion and 24 h of reperfusion. The healthy myocardium (H), infarcted area (I) characterized by the presence of elongated and narrow fibres and inflammatory cells between these fibres, as well as the infarct border zone (B) can be clearly recognized. Representative images of cryosections of a mouse heart after IR immunolabelled for Cx43 (*B*), Cx40 (*C*), or Cx37 (*D*). Cx43 is present in the intercalated disk between cardiomyocytes in healthy myocardium (*B1*: transversal section and *B2*: longitudinal section), but absent in infarcted area (*B3* and *B4*). Cx40 is present in the endothelium of blood vessels in the non-injured part of the myocardium (*C1*: small artery and *C2*: capillary), but absent in the infarcted area (*C3* and *C4*). Cx37 is present in the endothelium of blood vessels in the non-injured part of the myocardium (*D1*: arteriole and *D2*: capillary), but absent in the infarcted area (*D3* and *D4*). Dotted lines represent the limit between the border and the infarcted zones. Scale bar = 20 μm.

Cx40-mediated cardioprotection, $CD73^{-/-}ApoE^{-/-}$ mice have been submitted to 30 min of LAD ligation and 24 h of reperfusion. Similar to Cx40del mice, the infarcted area in $CD73^{-/-}ApoE^{-/-}$ mice is larger (18.1 ± 3.5%, $n = 9$, $P < 0.05$) in comparison with control (*Figure 6B*). Moreover, neutrophil infiltration into the infarcted area 24 h after the onset of reperfusion was increased in $CD73^{-/-}ApoE^{-/-}$ mice (23.2 ± 3.5%, $n = 4$, $P < 0.05$) in comparison with control mice (9.8 ± 1.8 and 8.5 ± 1.7%, see *Figure 2*). Finally, a single intraperitoneal injection of methotrexate 5 min before reperfusion significantly reduced infarct size in Cx40del mice (*Figure 6B*), whereas it had no effect on the infarcted area in C1 or C2 control mice as well as in $CD73^{-/-}ApoE^{-/-}$ mice (*Figure 6B*). In agreement, neutrophil infiltration into the infarcted area 24 h after the onset of reperfusion was decreased in methotrexate-treated Cx40del mice (13.7 ± 2.6%, $n = 5$, $P < 0.05$)

in comparison with Cx40del mice (24.3 ± 1.6%, $n = 4$, see *Figure 2*). Of note, the area at risk was unaffected by methotrexate treatment in all groups of mice (*Figure 6A*). Collectively, these results show that endothelial Cx40-induced cardioprotection may be mediated by CD73-dependent signalling.

## 4. Discussion

Myocardial Cx43 has been suggested to play an important role in the pathophysiology of IR injury. Initial studies performed with pharmacological blockade of connexin channels and later studies on transgenic mice with reduced Cx43 expression demonstrated reduced infarct size after IR *in vivo* or in isolated perfused hearts *ex vivo*,[29–33] thus supporting a role for direct cell-to-cell communication or signalling via Cx43
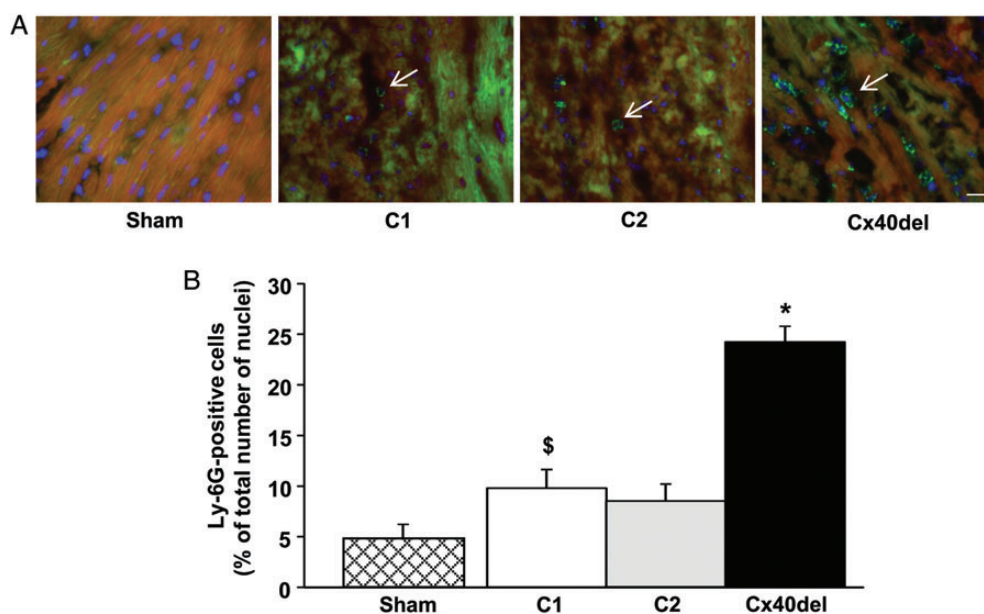
**Figure 2** Cx40del mice show increased neutrophil infiltration upon IR. (A) Representative images of sham-operated, C1, C2, and Cx40del heart immunolabelled for Ly6G (neutrophils in green) after 30 min of left coronary artery occlusion and 24 h of reperfusion. Scale bar = 20 μm. Arrows indicate examples of LyG-positive cells. (B) Quantification of neutrophil immunostainings after *in vivo* IR. Endothelial-specific deletion of Cx40 (Cx40del: black bar) induces a significant increase of neutrophil infiltration after *in vivo* IR in comparison with the two control groups (C1: white bar and C2: grey bar). Only minimal neutrophil infiltration is observed in sham-operated mice (hatched bar). Values are expressed as mean ± SEM, n = 4–6/group. *$P < 0.05$ vs. control groups, and $^{\$}P < 0.05$ vs. sham-operated group.
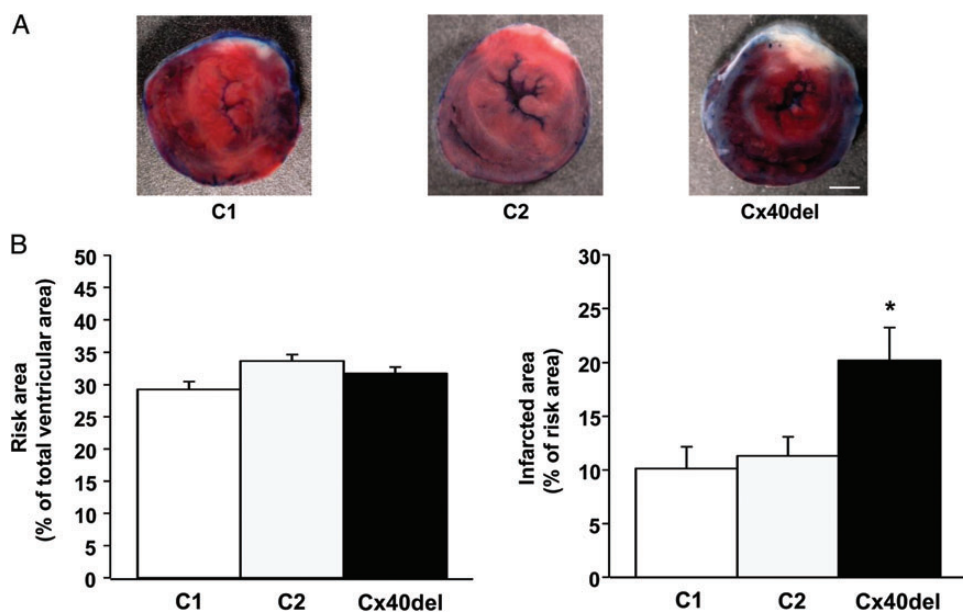


**Figure 3** Infarct size is increased in Cx40del mice after IR. (A) Representative photographs of heart slices of C1, C2, and Cx40del mice stained with Evans blue and TTC after 30 min of left coronary artery occlusion and 24 h of reperfusion. Scale bar = 1 mm. (B) Quantification of areas at risk and infarct areas after *in vivo* IR. Areas at risk (left panel) are not different between C1 (white bar), C2 (grey bar), and Cx40del mice (black bar). Infarct area (right panel) is significantly increased in Cx40del mice in comparison with the two control groups. Values are expressed as mean ± SEM, n = 9–11/group. *$P < 0.05$ vs. control groups.

hemi-channels in the propagation of cell death. It is increasingly recognized that myocardial post-ischaemic damage during reperfusion is also influenced by the inflammatory response. As the endothelial Cx40 is known to affect leucocyte recruitment in atherosclerosis,[12] lung inflammation,[12] and hindlimb ischaemia,[34] we sought to determine in this study whether this endothelial connexin plays a role in cardiac IR injury.
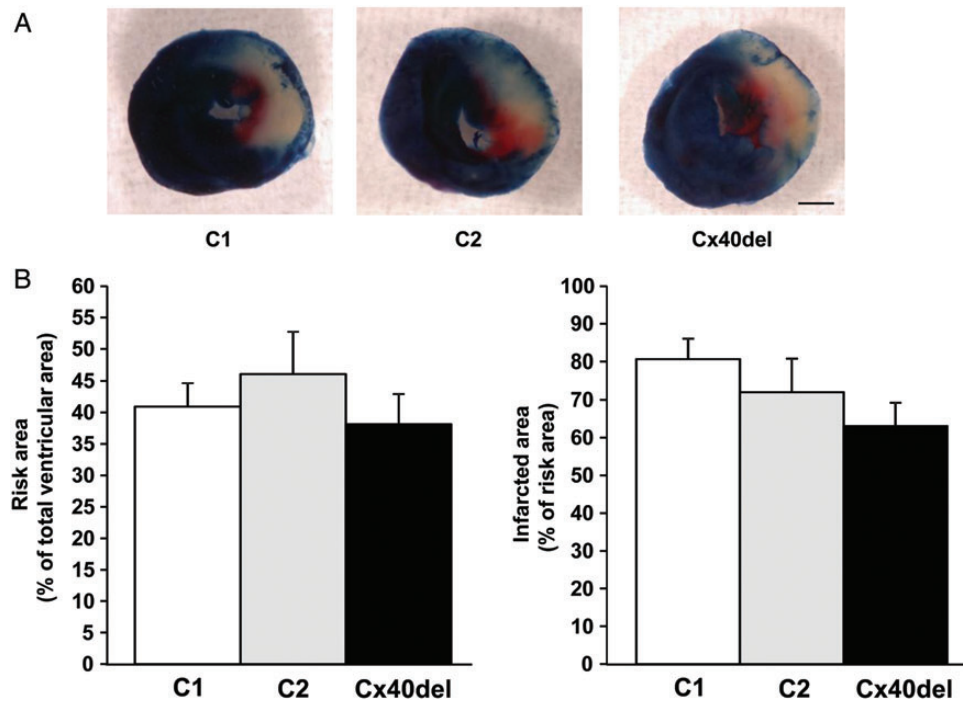
**Figure 4** No beneficial effects in isolated perfused hearts of Cx40del mice. (*A*) Representative photographs of heart slices of C1, C2, and Cx40del mice stained with Evans blue and TTC after 30 min of left coronary artery occlusion and 1 h of reperfusion (*ex vivo* Langendorff perfusion). Scale bar = 1 mm. (*B*) Quantification of areas at risk and infarct areas after *ex vivo* IR. Areas at risk (left panel) and infarct area (right panel) are not different between control (C1: white bar and C2: grey bar) and Cx40del mice (black bar). Values are expressed as mean ± SEM, $n = 5$/group.
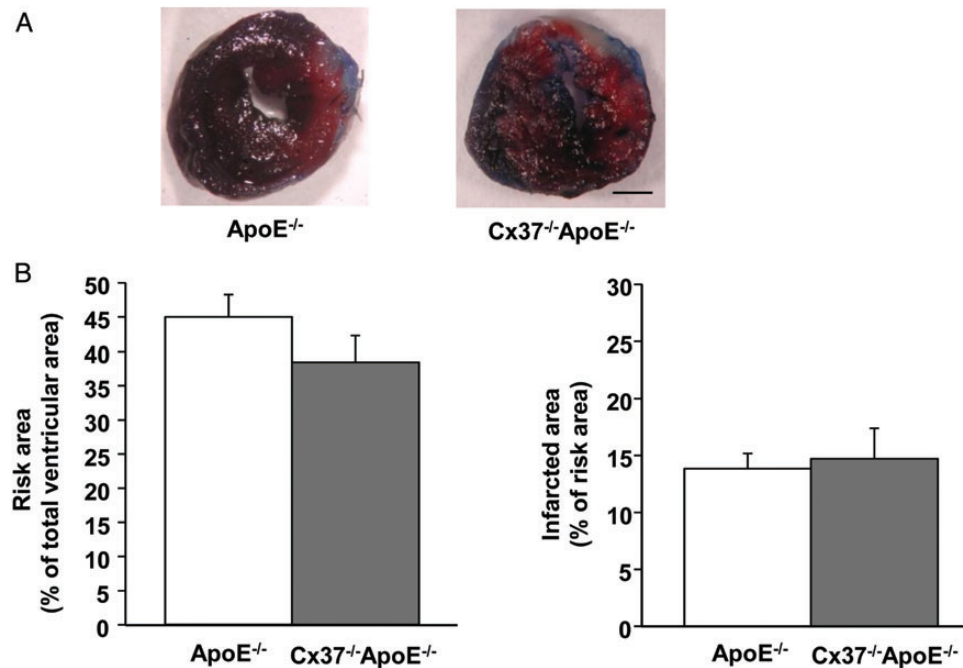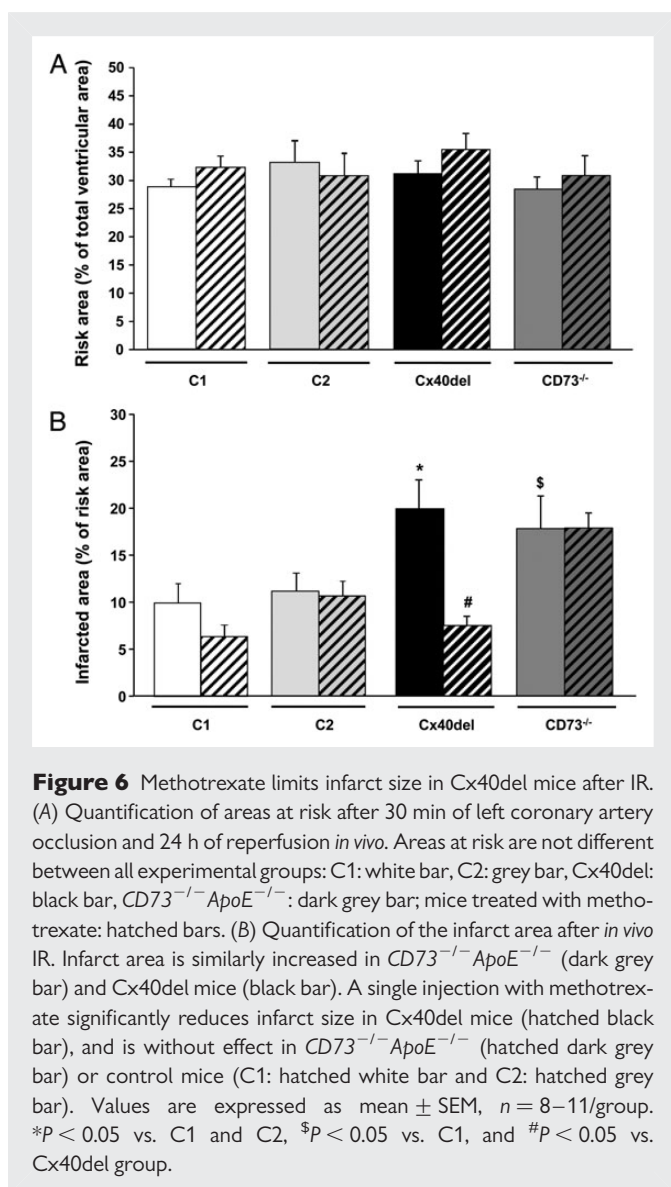


**Figure 5** Cx37 deletion does not affect infarct size in mice. (*A*) Representative photographs of heart slices of $ApoE^{-/-}$ and $Cx37^{-/-}ApoE^{-/-}$ mice stained with Evans blue and TTC after 30 min of left coronary artery occlusion and 24 h of reperfusion. Scale bar = 1 mm. (*B*) Quantification of areas at risk (left panel) and infarct area (right panel) after *in vivo* IR. Areas at risk and infarct areas are not different between $ApoE^{-/-}$ (white bars) and $Cx37^{-/-}ApoE^{-/-}$ mice (grey bars). Values are expressed as mean ± SEM, $n = 10–12$/group.

**Figure 6** Methotrexate limits infarct size in Cx40del mice after IR. (A) Quantification of areas at risk after 30 min of left coronary artery occlusion and 24 h of reperfusion *in vivo*. Areas at risk are not different between all experimental groups: C1: white bar, C2: grey bar, Cx40del: black bar, $CD73^{-/-}ApoE^{-/-}$: dark grey bar; mice treated with methotrexate: hatched bars. (B) Quantification of the infarct area after *in vivo* IR. Infarct area is similarly increased in $CD73^{-/-}ApoE^{-/-}$ (dark grey bar) and Cx40del mice (black bar). A single injection with methotrexate significantly reduces infarct size in Cx40del mice (hatched black bar), and is without effect in $CD73^{-/-}ApoE^{-/-}$ (hatched dark grey bar) or control mice (C1: hatched white bar and C2: hatched grey bar). Values are expressed as mean ± SEM, $n = 8–11$/group. $*P < 0.05$ vs. C1 and C2, $^{\$}P < 0.05$ vs. C1, and $^{\#}P < 0.05$ vs. Cx40del group.

Reperfusion injury reflects both the initial degree of tissue damage and the subsequent consequences of leucocyte infiltration. In order to separate both events, we have compared two different experimental protocols of cardiac IR in our study. First, we have performed 30 min ischaemia by LAD occlusion followed by 24 h reperfusion *in vivo*. This protocol that is most frequently used[35] induced relatively small infarcts with virtually no loss of animals and allowed us to analyse the peak of neutrophil infiltration.[24] A possible drawback of this experimental protocol is that it is more difficult to measure putative cardioprotective effects with a small infarct size. Secondly, to investigate the contribution of Cx40 to the initial tissue damage, we have performed an *ex vivo* protocol (without leucocytes) with 30 min of ischaemia induced by LAD occlusion followed by 1 h of reperfusion. This commonly used *ex vivo* experimental protocol[35] induces larger infarcts on top of which putative cardioprotective and cardiodamaging effects may be easier to observe when compared with the *in vivo* protocol.

The expression of Cx40 disappeared from the endothelium in the infarct zone 24 h after reperfusion. This observation is consistent with previous reports that endothelial Cx40 expression is down-regulated during acute inflammatory conditions.[12,36,37] However, the infarct size at this early time was increased in mice with specific deletion of Cx40

from the endothelium, thus suggesting that endothelial Cx40 plays a protective role at the onset of the inflammatory response or that the absence of this protein in the adjacent non-injured part of the heart modifies the inflammatory response. It is known that genetic deletion of one connexin can modulate the expression of other connexins expressed in the same cells. For instance, a reduction of endothelial Cx37 expression was observed in Cx40del mice as well as in mice with ubiquitous Cx40 deletion ($Cx40^{-/-}$).[12,25] Cx37–eNOS interactions are known to alter NO production by endothelial cells, which might affect leucocyte adhesion and reperfusion injury.[26] To determine whether the enhanced neutrophil recruitment after reperfusion in Cx40del mice could be explained, in part, by reduced endothelial Cx37, we submitted $Cx37^{-/-}ApoE^{-/-}$ mice to the same *in vivo* IR protocol as Cx40del mice. In our Cx37-deficient mice, infarct size was not different in comparison with control mice, suggesting that Cx37 is not implicated in acute injury associated with IR.

Neutrophils are the first inflammatory cells to arrive within 24 h in the infarct zone, shortly thereafter followed by the enrolment of monocytes that differentiate into macrophages.[24] By releasing reactive oxygen species, proteases, and pro-inflammatory molecules, neutrophils significantly contribute to reperfusion injury.[38] Taken together with the macrophages, they degrade extracellular matrix constituents and macromolecules released by injured cells, and aid to clearance of dead cardiomyocytes.[2] In our Cx40del mice, the enhanced neutrophil infiltration after IR was indeed associated with increased infarct size in comparison with control mice. Infarct size was, however, not different between controls and Cx40del mice in the absence of inflammatory cells as revealed by *ex vivo* Langendorff perfusion, thus confirming the importance of neutrophils in IR-induced cardiomyocyte death in Cx40del mice. Furthermore, these results downgrade the probability that an altered recovery of tissue perfusion was responsible for the larger *in vivo* myocardial infarcts in Cx40del mice, as was reported for a severe model of hindlimb ischaemia in $Cx40^{-/-}$ mice.[34] Of note, we investigated cardiomyocyte death only after 24 h of reperfusion, a time point corresponding to the peak of neutrophil recruitment but not yet monocyte recruitment.[24] As Cx37 is expressed in monocytes and regulates their adhesion to the endothelium in an ATP-dependent manner,[16] we cannot exclude that deletion of Cx37 may reduce monocyte infiltration at a later time point and influences heart remodelling at a later stage. Thus, our results point to the potential of endothelial Cx40 as a specific target in preventing early IR injury.

The most widely recognized molecule with endogenous protective properties in the context of IR injury is adenosine.[39,40] The production of adenosine in the extracellular space results from the hydrolysis of ATP by the sequential action of ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5′-nucleotidase (CD73). Adenosine accumulates in the extracellular space in response to prolonged ischaemic stress and cell damage. In turn, adenosine activates cell surface receptors to induce ATP synthesis, to favour survival of cardiac cells,[41] and to mediate a variety of actions on the vascular endothelium ranging from suppression of vascular leakage and leucocyte extravasation to promotion of vasodilation.[27] In agreement, we observed an increased infarct size after IR in CD73-deficient mice. In this context, we have previously shown that endothelial Cx40 may intersect with CD73-dependent signalling to regulate leucocyte recruitment. Indeed, the *in vivo* deletion of Cx40 in endothelial cells was associated with reduced expression of CD73, increased expression of adhesion molecules, and consequently enhanced neutrophil infiltration in acute and chronic models of inflammation.[12] In addition, *in vitro* silencing of

Cx40 by siRNA or antisense led to reduced CD73 expression and activity, and increased leucocyte adhesion to the surface of endothelial cells. These effects were reversed by the adenosine receptor agonist 5′-(N-ethylcarboxamido) adenosine, indicating for the importance of the CD73–adenosine signalling axis in the function of endothelial Cx40.[12] Finally, co-culture experiments with Cx40-expressing and wild-type communication-incompetent HeLa cells revealed that Cx40-mediated gap junctional communication was both necessary and sufficient for the propagation of adenosine-evoked anti-inflammatory signals between cells. Altogether, these data suggest that the enhanced neutrophil infiltration in the infarcted area in Cx40del mice may be the consequence of reduced CD73 activity in the microcirculation of the heart. Whether Cx40-mediated gap junctional communication plays an additional role in the expression and activity of CD39 is presently not known. We can also not completely rule out the possibility that endothelial Cx40 hemi-channels might mediate ATP release from cells (this way contributing to enhanced production of extracellular adenosine).

It has been postulated that the use of methotrexate, an activator of CD73, in patients with rheumatoid arthritis has protective properties in cardiovascular disease, including acute myocardial infarction.[42–44] Methotrexate, or its derivative MX68, was shown to limit infarct size via adenosine-dependent mechanisms in canine heart.[45] Importantly, we also found that infarct size in Cx40del mice decreased upon a single-dose administration of methotrexate shortly before reperfusion. Moreover, this cardioprotective effect in Cx40del mice was mechanistically linked to reduced infiltration of neutrophils to the infarcted area. As expected, cardioprotection was not observed in the methotrexate-insensitive CD73$^{-/-}$ mice. Although a single dose of methotrexate had no effects on our control mice, the use of this drug allowed us to unveil the key role for endothelial Cx40 in resistance of the heart to IR injury. In the absence of effective therapy, investigations have recently turned towards peptidic strategies to modulate myocardial Cx43 channel function during IR injury.[33,46] In this context, the development of new peptides to maintain the functional integrity of endothelial Cx40 might be beneficial to prevent myocardial reperfusion injury. As for other compounds, the ideal peptidic therapy to fight reperfusion injury would be a single dose, locally applied (catheter-based) at the time of reperfusion.

# Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

**Conflict of interest:** none declared.

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