PRE-CLINICAL RESEARCH

Interleukin-17A Contributes to Myocardial Ischemia/Reperfusion Injury by Regulating Cardiomyocyte Apoptosis and Neutrophil Infiltration

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Objectives	This study tested whether interleukin (IL)-17A is involved in the pathogenesis of mouse myocardial ischemia/reperfusion (I/R) injury and investigated the mechanisms.
Background	Inflammatory processes play a major role in myocardial I/R injury. We recently identified IL-17A as an important cytokine in inflammatory cardiovascular diseases such as atherosclerosis and viral myocarditis. However, its role in myocardial I/R injury remains unknown.
Methods	The involvement of IL-17A was assessed in functional assays in mouse myocardial I/R injury by neutralization/repletion or genetic deficiency of IL-17A, and its mechanism on cardiomyocyte apoptosis and neutrophil infiltration were further studied in vivo and in vitro.
Results	Interleukin-17A was elevated after murine left coronary artery ligation and reperfusion. Intracellular cytokine staining revealed that $\gamma\delta$T lymphocytes but not CD4$^+$ helper T cells were a major source of IL-17A. Anti–IL-17A monoclonal antibody treatment or IL-17A knockout markedly ameliorated I/R injury, as demonstrated by reduced infarct size, reduced cardiac troponin T levels, and improved cardiac function. This improvement was associated with a reduction in cardiomyocyte apoptosis and neutrophil infiltration. In contrast, repletion of exogenous IL-17A induced the opposite effect. In vitro study showed that IL-17A mediated cardiomyocyte apoptosis through regulating the Bax/Bcl-2 ratio, induced CXC chemokine-mediated neutrophil migration and promoted neutrophil-endothelial cell adherence through induction of endothelial cell E-selectin and inter-cellular adhesion molecule-1 expression.
Conclusions	IL-17A mainly produced by $\gamma\delta$T cells plays a pathogenic role in myocardial I/R injury by inducing cardiomyocyte apoptosis and neutrophil infiltration. (J Am Coll Cardiol 2012;59:420–9) © 2012 by the American College of Cardiology Foundation

The rapid restoration of blood flow through the occluded coronary artery by mechanical or pharmacological intervention is the most effective therapy to limit infarct size and improve the clinical outcome after acute myocardial infarction (1). However, reperfusion after ischemia itself causes additional cardiomyocyte death and increases infarct size in a process called myocardial ischemia/reperfusion (I/R) injury. Potential mediators of reperfusion injury include oxidative stress and inflammation (2).

Interleukin (IL)-17A is a member of the IL-17 family, which includes six structurally related isoforms: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (3). Multiple cell types, including CD4$^+$ $\alpha\beta$ T cells, $\gamma\delta$ T cells, natural killer cells, and neutrophils, produce IL-17A (4). Interleukin-17A acts on a variety of cells through its ubiquitous receptors, IL-17RA and IL-17RC, and is a critical mediator of neutrophil recruitment and migration through the induction of granulopoiesis and the production of neutrophil chemokines, including lipopolysaccharide-

induced CXC chemokine (LIX), cytokine-induced neutrophil chemoattractant (KC), and macrophage inflammatory protein-2 (MIP-2) (5).

IL-17A plays an important role in host defense and mediates autoimmune diseases (6). We and others have reported that IL-17A is linked to the pathogenesis of several cardiovascular diseases, including atherosclerosis (7–9), hypertension, viral myocarditis, and dilated cardiomyopathy (7–12). Recently, IL-17A has also been found to contribute to brain, kidney, and intestine I/R injury (13–15), but the mechanisms involved are still largely unknown.

In this study, we investigated the expression of IL-17A in reperfused ischemic myocardium and the subsets of IL-17A–releasing cells and characterized the functional involvement of IL-17A in myocardial I/R injury. We show that IL-17A is largely produced by γδT cells and plays a pathogenic role in myocardial I/R injury by inducing cardiomyocyte apoptosis and neutrophil infiltration.

Methods

The Methods section is available in the Online Appendix.

Results

IL-17A increases after myocardial I/R injury. To test the involvement of IL-17A in myocardial I/R injury, we first investigated IL-17A levels in the myocardium at different reperfusion time points after I/R. IL-17A messenger ribonucleic acid (mRNA) and protein expression increased significantly as early as 1 h after reperfusion, rose progressively until 24 h, and then began to decrease, although it remained at a high level compared with the sham group until 72 h after I/R (Online Figs. 1A and 1B).

In addition to Th17 cells, γδT cells, natural killer cells, and neutrophils were reported to secret IL-17A (4). To identify which leukocytes were responsible for the elevation of IL-17A in myocardial I/R, we used intracellular cytokine staining combined with staining for various surface markers. Over 95% of the IL-17A–secreting leukocytes were $CD3^+$ T cells, including approximately 70% γδ T cell receptor-positive ($γδTCR^+$) cells and 13% $CD4^+$ cells, indicating that γδT cells rather than Th17 cells were the major source of IL-17A (Online Fig. 1C). Natural killer cells and neutrophils comprised only a minor proportion of IL-17A-secreting leukocytes. After 24 h of reperfusion, there was a marked increase in the myocardial content of γδT cells. Simultaneously, the percentage of $IL-17^+$ $γδTCR^+$ cells relative to γδT cells also increased (Online Tables 1 and 2). These results suggest that myocardial I/R led to an increase not only in the number of infiltrated γδT cells but also in the IL-17A production of these cells, which accounted for a large part of the elevated myocardial IL-17A.

The IL-17 family consists of 5 members, including IL-17A. Among these, IL-17F has the highest degree of homology with IL-17A and exerts similar effects but to a lesser extent (8). We therefore also examined mRNA expression of IL-17B, C, D, E, and F in hearts after I/R. The level of IL-17A mRNA was much higher than that of the other IL-17 family members, approximately 5-fold greater than that of IL-17F (Online Fig. 2).

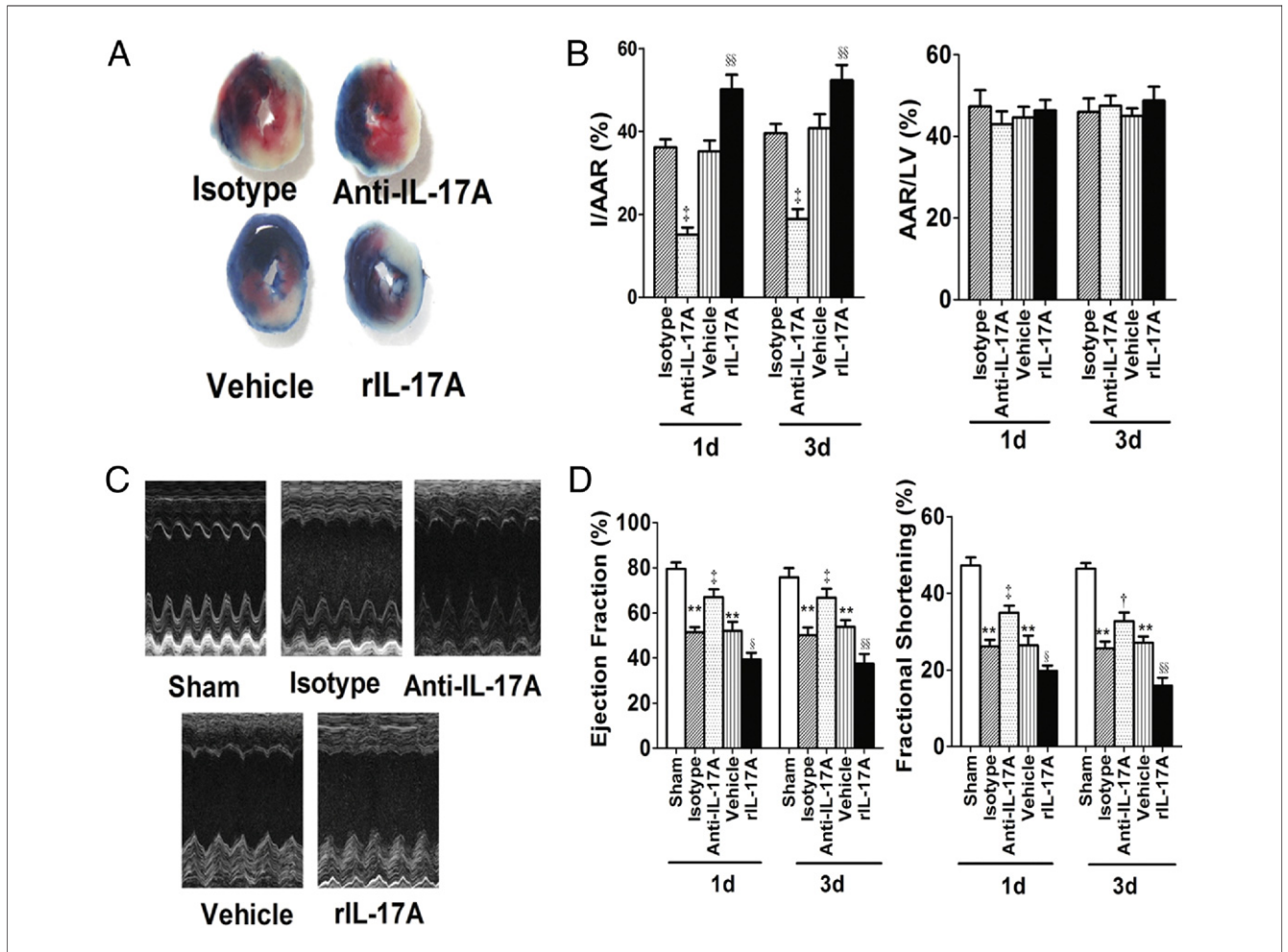**Neutralization of endogenous IL-17A protects against whereas repletion of exogenous IL-17A exacerbates myocardial I/R injury.** To determine the causative role of IL-17A in the development of myocardial I/R injury, we treated the myocardial I/R mice systemically with neutralizing anti–IL-17A monoclonal antibody (mAb) or recombinant IL-17A before reperfusion. Interleukin-17A expression reached a peak at 24 h after myocardial I/R and remained elevated for 72 h; therefore, we measured infarct size at 1 day or 3 days after reperfusion. The ratio of area at risk (AAR) to left ventricle (LV) area was the same in experimental and control groups at both time points, indicating that ligature was reproducibly performed at the same level of the left anterior coronary artery. However, the ratio of infarct area to AAR was significantly lower in mice treated with anti–IL-17A mAb relative to those treated with isotype and greater in mice treated with recombinant IL-17A relative to vehicle alone at both time points (Figs. 1A and 1B). Serum cardiac troponin T (cTnT) level, an index of myocyte injury, was also significantly lower in the anti–IL-17A mAb group and higher in the recombinant interleukin (rIL)-17A group (Online Fig. 3A).

To determine the effect of IL-17A on cardiac function after I/R and to demonstrate the clinical relevance of our findings, we measured ejection fraction (EF) and fractional shortening (FS) by echocardiography. The EF and FS were both remarkably reduced at 1 day and 3 days after myocardial I/R compared with the sham group. Furthermore, treatment with anti–IL-17A mAb increased EF and FS after 1 day and 3 days reperfusion, indicating improved cardiac function. Consistent with infarct size, a marked deterioration of cardiac function, as shown by a decrease of EF and FS, was observed in the rIL-17A group (Figs. 1C and 1D). To test whether IL-17A affects hemodynamic properties, we measured left ventricular end-diastolic pres-

Figure 1   IL-17A Neutralization and Repletion Affected Mouse Myocardial I/R Injury

(A) Representative images of left ventricular (LV) slices from different groups at 1 day (d) after reperfusion. The nonischemic area is indicated in **blue**, the area at risk (AAR) in **red**, and the infarct area in **white**. **(B)** Quantification of infarct size of myocardial tissues 1 day or 3 d after reperfusion (n = 6 to 8). **(C)** Representative M-mode echocardiography images of the LV 1 d after reperfusion. **(D)** Ejection fraction and LV fractional shortening (n = 8). **p < 0.01 versus sham; †p < 0.05; ‡p < 0.01 versus isotype; §p < 0.05; §§p < 0.01 versus vehicle. IL = interleukin; I/R = ischemia/reperfusion.

sure (LVEDP) and the derivative of LV pressure (dP/dt) with a Millar catheter (SPR-671, Millar Instruments, Houston, Texas). The LV hemodynamic statuses were augmented in the anti–IL-17A mAb-treated group compared with the isotype group. Specifically, LVEDP was lower, whereas maximum and minimum dP/dt, the indexes of contractility, were increased in the anti–IL-17A mAb-treated group. Conversely, treatment with rIL-17A led to a reduction of dP/dt and an elevation of LVEDP (Table 1). Taken together, changes in the level of IL-17A can have profound reciprocal effects on myocardial I/R injury, in terms of both infarct area and cardiac function.

IL-17A knockout ameliorates myocardial I/R injury. To elucidate the impact of IL-17A on myocardial I/R injury in the genetically IL-17A free situation, we compared myocardial I/R injury levels between *Il17a$^{-/-}$* and wild-type C57BL/6 mice. Before surgery, we excluded the possibility

Table 1   Hemodynamic Status Measurements of Myocardial I/R Mice

	Reperfusion Time (Day)	LVEDP (mm Hg)	dP/dt$_{max}$ (mm Hg/s)	−dP/dt$_{min}$ (mm Hg/s)
Sham	1	2.7 ± 0.6	5,786 ± 289	−5,471 ± 238
Isotype	1	8.8 ± 0.8*	3,760 ± 257*	−3,575 ± 218*
Anti–IL-17A	1	4.4 ± 0.6†	4,846 ± 245‡	−4,412 ± 263‡
Vehicle	1	8.4 ± 0.8*	3,945 ± 298*	−3,743 ± 304*
rIL-17A	1	14.6 ± 1.3§	2,447 ± 165§	−2,727 ± 345‖
Sham	3	3.3 ± 0.3	5,488 ± 247	−5,659 ± 336
Isotype	3	9.2 ± 0.9*	3,662 ± 254*	−3,320 ± 192*
Anti–IL-17A	3	4.8 ± 0.4†	4,763 ± 325‡	−4,617 ± 166†
Vehicle	3	9.0 ± 1.3*	3,759 ± 201*	−3,627 ± 166*
rIL-17A	3	15.7 ± 1.2§	2,450 ± 244§	−2,683 ± 275‖

Values are mean ± SEM. N = 6 to 8. *p < 0.01 versus sham; †p < 0.05 ; ‡p < 0.01 versus isotype; ‖p < 0.05; §p < 0.01 versus vehicle.

IL = interleukin; I/R = ischemia-reperfusion; LVEDP = left ventricular end-diastolic pressure.
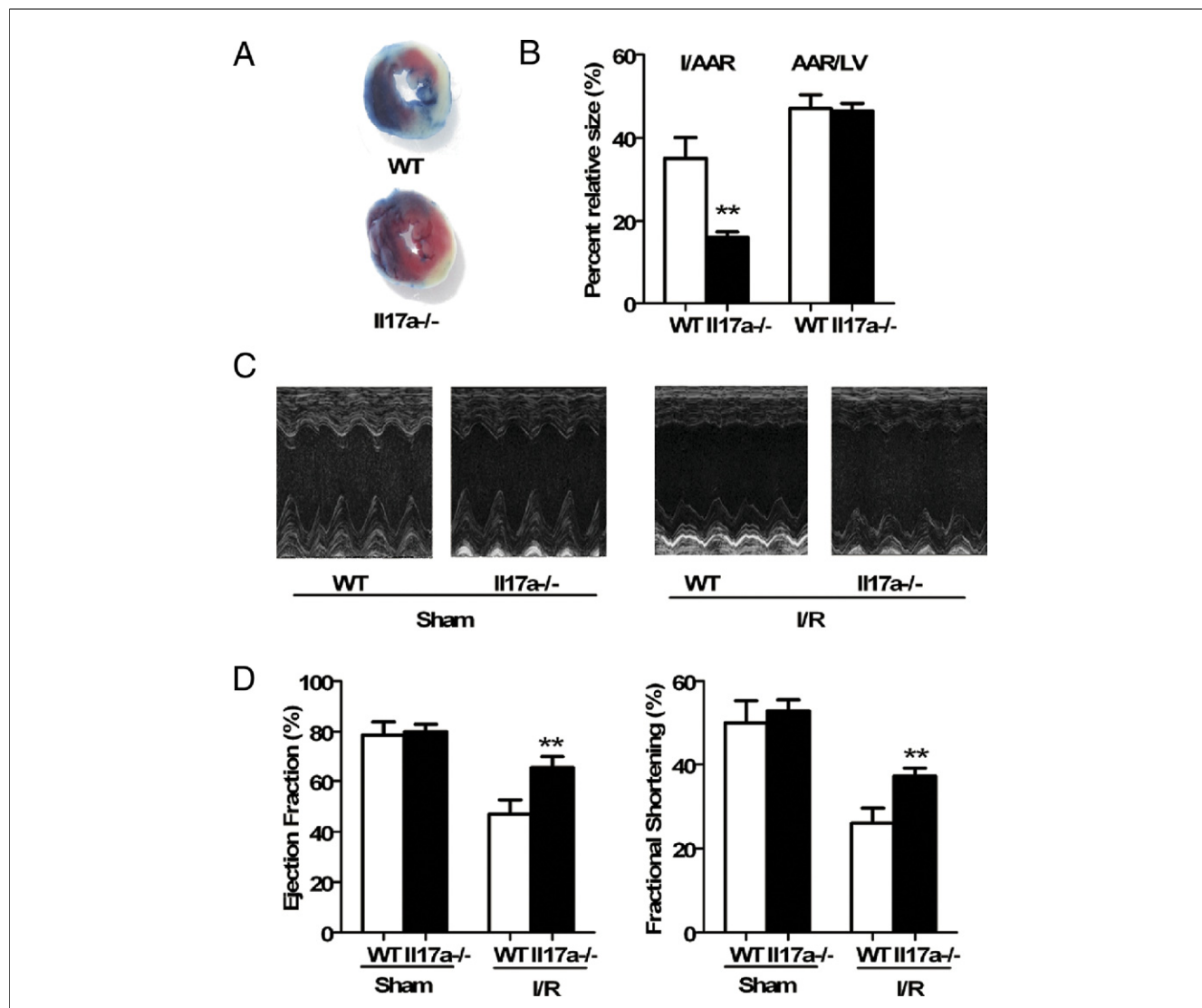
Figure 2   IL-17A Knockout Ameliorated Myocardial I/R Injury

(A) Representative images of LV slices from wild-type (WT) and *Il17a*$^{-/-}$ mice at 1 day after I/R. **(B)** Quantification of infarct size of myocardium 1 day after reperfusion (n = 8). **(C)** Representative M-mode images of the LV after sham and myocardial I/R from WT and *Il17a*$^{-/-}$ mice. **(D)** Ejection fraction and LV fractional shortening (n = 8). **p < 0.01 versus WT. Abbreviations as in Figure 1.

of cardiac abnormality in *Il17a*$^{-/-}$ mice by echocardiography. Echocardiographic parameters of *Il17a*$^{-/-}$ mice were comparable to those of wild-type mice (Online Table 3). The *Il17a*$^{-/-}$ and wild-type mice were then subjected to myocardial I/R. The extent of injury and heart functions were assayed 1 day after initiation of reperfusion. With similar AAR, the infarct size/AAR was significantly smaller in *Il17a*$^{-/-}$ than in wild-type mice (Figs. 2A and 2B). Less serious injury was also manifested by lower levels of serum cTnT in *Il17a*$^{-/-}$ mice (Online Fig. 3B). In parallel, we observed improved cardiac function, as indicated by elevated EF and FS, in *Il17a*$^{-/-}$ mice after myocardial I/R compared with wild-type (Figs. 2C and 2D). This protection was also demonstrated by hemodynamic measurements. The LVEDP was lower, whereas LV dP/dt was higher in

Il17a$^{-/-}$ mice at 1 day after reperfusion (Table 2). These results confirm that IL-17A plays a pathogenic role in myocardial I/R injury.

Table 2	Hemodynamic Status Measurements of Il17a$^{-/-}$ Mice After I/R			
	Reperfusion Time (Day)	LVEDP (mm Hg)	dP/dt$_{max}$ (mm Hg/s)	−dP/dt$_{min}$ (mm Hg/s)
WT sham	1	3.0 ± 0.5	5,898 ± 282	−5,564 ± 172
WT I/R	1	8.36 ± 1.1	3,832 ± 155	−3,534 ± 157
Il17a$^{-/-}$ sham	1	2.8 ± 0.4	5,784 ± 291	−5,369 ± 148
Il17a$^{-/-}$ I/R	1	4.3 ± 0.6*	4,903 ± 265†	−4,717 ± 172*

Values are mean ± SEM. N = 8. *p < 0.01; †p < 0.05 versus wild-type (WT). Abbreviations as in Table 1.
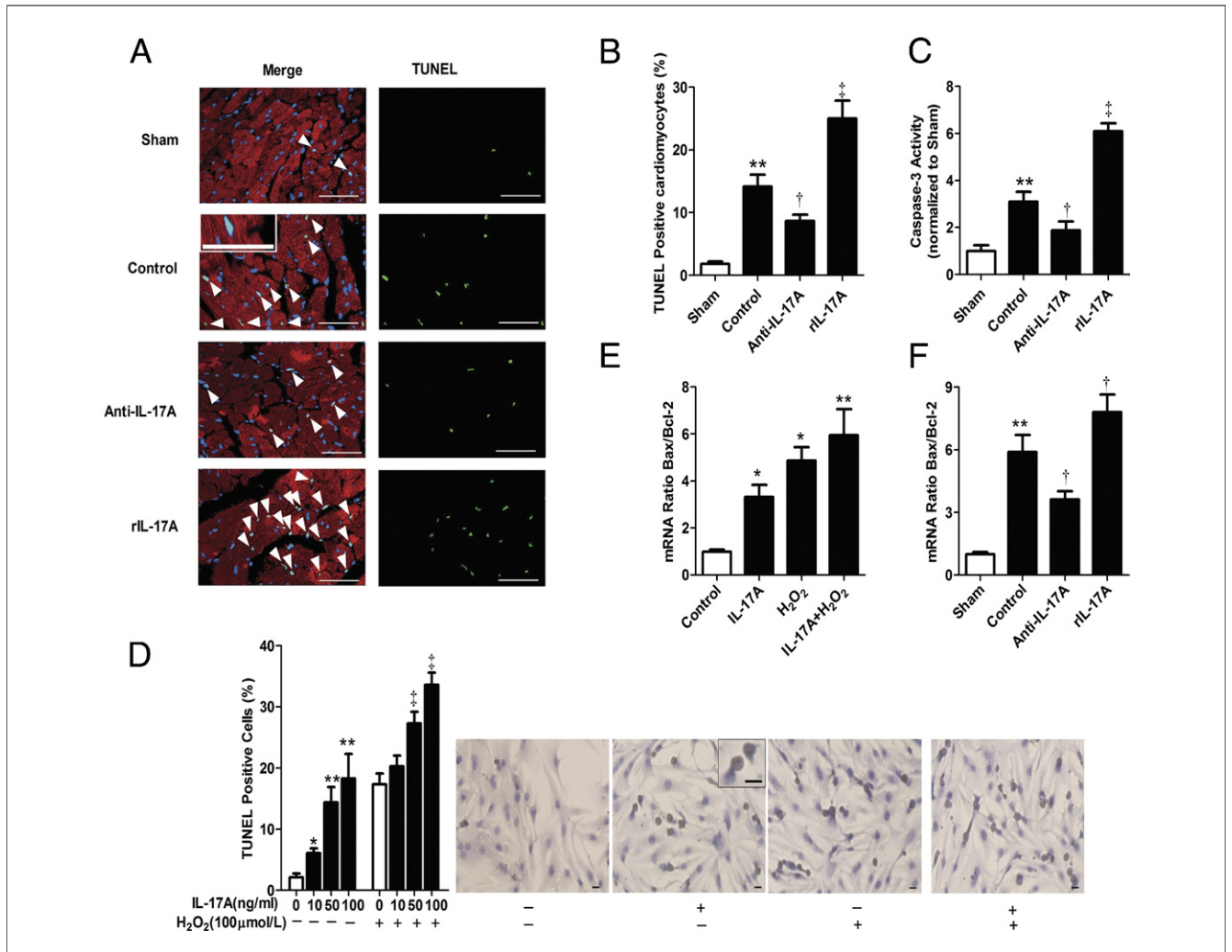
Figure 3   IL-17A–Mediated Cardiomyocyte Apoptosis In Vivo and In Vitro

(A) Representative photographs of terminal deoxynucleotidyl-transferase mediated dUTP nick-end labeling (TUNEL)-stained heart sections from different groups after 3-h reperfusion. Apoptotic nuclei were identified by TUNEL staining **(green)**, cardiomyocyte by anti-sarcomeric actin antibody **(red)**, and total nuclei by 4',6-diamidino-2-phenylindole staining **(blue)**. **Arrowheads** indicate apoptotic cardiomyocytes. Scale bar: 50 mm. **(B)** Percentages of TUNEL-positive nuclei over total number of nuclei (n = 4 to 5). **(C)** Caspase 3 activity in myocardium was assessed after 3 h of reperfusion, and the values were normalized to sham (n = 5); **$p < 0.01$ versus sham; †$p < 0.05$; ‡$p < 0.01$ versus control. **(D)** Mouse neonatal cardiomyocyte apoptosis under hydrogen peroxide (H_2O_2) and different doses of IL-17A. **Brown staining** indicates TUNEL positive cells. Results are representative of 3 independent assays. Scale bar: 100 mm. *$p < 0.05$; **$p < 0.01$ versus $H_2O_2(-)$IL-17A(-); ‡$p < 0.01$ versus $H_2O_2(+)$IL-17A(-). **(E)** Real-time polymerase chain reaction (PCR) determined mouse neonatal cardiomyocyte Bcl-2 and Bax messenger ribonucleic acid (mRNA) levels. The results were expressed as Bax/Bcl-2 ratio. *$p < 0.05$; **$p < 0.01$ versus control. **(F)** Real-time PCR determined mRNA level of Bcl-2 and Bax in ischemia myocardium. The results were expressed as ratio of Bax/Bcl-2. **$p < 0.01$ versus sham; †$p < 0.05$ versus control. Abbreviations as in Figure 1.

IL-17a facilitates cardiomyocyte apoptosis after I/R in vivo or oxidative stress in vitro. Apoptosis contributes significantly to myocardial I/R injury (16). We hypothesized that the role of IL-17A on myocardial I/R is associated with effects on cardiomyocyte apoptosis. To test this hypothesis, we carried out terminal deoxynucleotidyl-transferase mediated dUTP nick-end labeling (TUNEL) of LV sections from different experiment groups at 3 h after myocardial I/R. As shown in Figures 3A and 3B, anti–IL-17A mAb treatment remarkably decreased the number of TUNEL-positive cardiomyocytes compared with control, suggesting that IL17A neutralization inhibited the extent of cardiomyocyte apoptosis. In contrast, repletion of IL-17A conferred

the opposite effect. Caspase 3 activity determined by a caspase colorimetric assay from ischemic myocardium was concomitantly down-regulated by anti–IL-17A mAb and up-regulated by rIL-17A (Fig. 3C). These data indicate that IL-17A has a pro-apoptotic effect on cardiomyocytes after myocardial I/R in vivo.

To test whether IL-17A directly induces cardiomyocyte apoptosis, we used primary cultures of neonatal mouse ventricular myocytes that co-expressed IL-17RA and IL-17RC, as detected by real-time polymerase chain reaction and Western blot (Online Figs. 4A and 4B). Cardiomyocytes were cultured with or without hydrogen peroxide (H_2O_2), a potent inducer of oxidative stress

(17), in the presence or absence of rIL-17A. As expected, H_2O_2 treatment increased the frequency of apoptotic cardiomyocytes, whereas IL-17A incubation further augmented apoptosis. Interestingly, incubation with IL-17A alone also induced apoptosis in a dose-dependent manner (Fig. 3D). Taken together, these data indicate that IL-17A has a direct pro-apoptotic effect on cardiomyocytes.

To understand mechanisms through which IL-17A induces cardiomyocyte apoptosis, we examined expression of the Bcl-2 family by real-time polymerase chain reaction. The Bax/Bcl-2 ratio was significantly increased in cardiomyocytes exposed to H_2O_2. Interleukin-17A alone also increased the Bax/Bcl-2 ratio, which was further elevated under an oxidative stress situation. Administration of rIL-17 in vivo similarly increased the Bax/Bcl-2 ratio after myocardial I/R, whereas that of anti–IL-17A mAb decreased the ratio (Figs. 3E and 3F, Online Fig. 5). These results show that IL-17A induces cardiomyocyte apoptosis, at least in part, through the regulation of pro- to anti-apoptotic protein ratio of Bcl-2 family.

IL-17A mediates neutrophil recruitment through regulation of CXC glutamic acid–leucine-arginine chemokine expression after I/R in vivo and oxidative stress in vitro. Neutrophil infiltration is a hallmark of inflammatory injury after myocardial I/R (17), and 1 of the main functions of IL-17A is neutrophil recruitment (18). As determined by myeloperoxidase activity and fluorescence activated cell sorting analysis of $CD11b^+Gr-1^+$ neutrophils, myocardial I/R induced a surge in neutrophil recruitment to myocardium. Anti–IL-17A mAb treatment reduced neutrophil recruitment, whereas treatment with rIL-17A promoted this effect (Figs. 4A to 4C).

The CXC glutamic acid–leucine-arginine (ELR^+) chemokines KC, MIP-2, and LIX are not only potent neutrophil chemoattractants but also IL-17A target genes (19). Myocardial I/R caused a significant induction of mRNA and protein levels of all 3 chemokines, consistent with increased neutrophil infiltration. Moreover, neutralization or repletion of IL-17A had the opposite effect on expression of these chemokines after myocardial I/R (Fig. 4D, Online Fig. 6A). Interleukin-17A also increased expression of cardiomyocyte of these 3 chemokines, in vitro as determined by enzyme-linked immunoadsorbent assay. Stimulation with IL-17A in addition to H_2O_2 led to a dose-dependent potentiation of the effect of H_2O_2, markedly up-regulating expression of these chemokines. Preincubation with anti–IL-17A Ab could completely abolish IL-17A activity (Fig. 4E, Online Fig. 6B). Furthermore, in vitro neutrophil migration assay showed a marked enhancement of neutrophil migration to media from cells treated with IL-17A or H_2O_2 alone and was synergistically enhanced by conditioned media from cells treated with both agents (Fig. 4F). The activity of IL-17A could be completely abolished by anti–IL-17A mAb but not isotype mAb, indicating an IL-17A–specific effect.

IL-17A regulates inter-cellular adhesion molecule-1 and E-selectin expression and stimulates endothelial activation and neutrophil-endothelial cell adhesion. Adhesion molecule expression, an indicator of endothelium activation, is a prerequisite for neutrophil-endothelium adherence, which in turn contributes to myocardial I/R injury (20). To further explore the mechanisms by which IL-17A affects myocardial I/R injury, we focused on inter-cellular adhesion molecule (ICAM)-1 and E-selectin expression. After 30 min of ischemia and 3 h of reperfusion, we detected a substantial increase of ICAM-1 mRNA level and a moderate but significant increase of E-selectin mRNA levels within I/R myocardium. Treatment with anti–IL-17A mAb attenuated this induction, whereas rIL-17A treatment had the opposite effect (Fig. 5A). This effect of IL-17A on the expression of ICAM-1 and E-selectin was further confirmed by Western blotting (Online Fig. 6C). As to the cellular level: stimulation with IL-17A led to a higher surface expression of ICAM-1 and E-selectin on endothelial cells (ECs), as detected by cell-based enzyme-linked immunoadsorbent assay and an increased adherence of neutrophils to ECs as detected by neutrophil-EC adhesion assays. Moreover, addition of IL-17A to H_2O_2 led to a potentiation of the increased ICAM-1 and E-selectin surface expression and neutrophil adherence caused by H_2O_2 alone. When anti–IL-17A mAb was added, this augmentation could be absolutely blocked (Figs. 5B to 5D, Online Fig. 6D).

Discussion

The present study reveals a critical role for IL-17A in mediating mouse myocardial I/R injury. Myocardial infiltrated γδ T lymphocytes but not Th17 cells were a major source of IL-17A. Anti–IL-17A mAb treatment or IL-17A knockout markedly ameliorated I/R injury, which was associated with a reduction in cardiomyocyte apoptosis and neutrophil infiltration. Repletion of exogenous IL-17A induced the opposite effect. In vitro experiments demonstrated that IL-17A could induce cardiomyocyte apoptosis through regulation of the Bax/Bcl-2 ratio. Moreover, IL-17A enhanced neutrophil infiltration by promoting EC E-selectin and ICAM-1 expression and inducing CXC chemokine-mediated neutrophil migration.

Ischemia-reperfusion triggers a vigorous inflammatory response, augmented by the generation and release of various cytokines, chemokines, and adhesion molecules, which ultimately exacerbates tissue injury (21). Increasing evidence indicates that the elements of both adaptive immunity and innate immunity participate in I/R injury (22). Notably, IL-17A acts as a bridge between adaptive and innate immunity through the potent induction of a gene expression program typical of the inflammatory response, presenting a unique position in the immune response process (3). This finding has prompted new insight into the role of IL-17A in I/R injury. A growing body of evidence demonstrates that IL-17A is involved in the
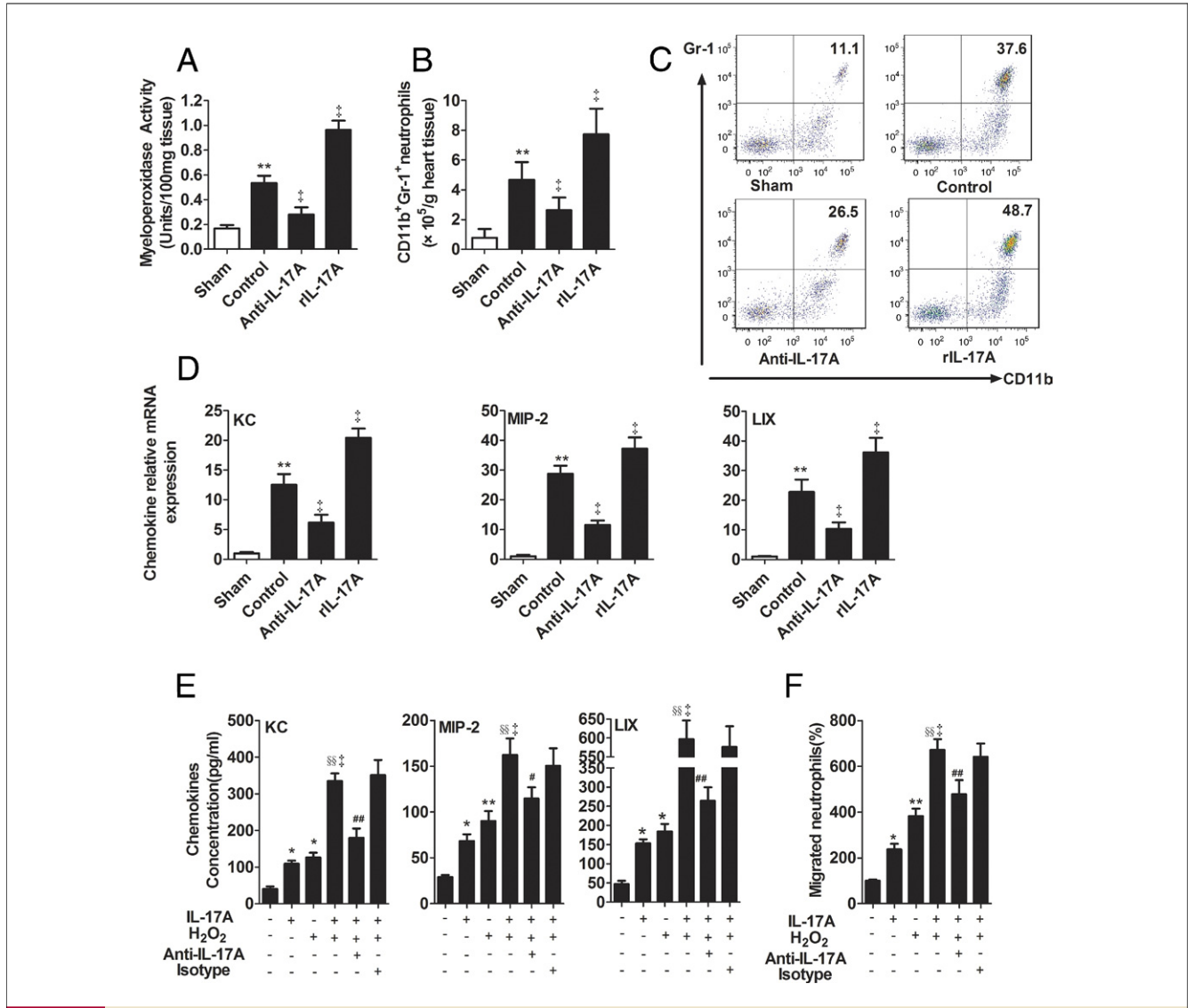
Figure 4   IL-17A Increased Cardiac Neutrophil Recruitment and Migration

Mice were assessed for chemokine expression and neutrophil infiltration at 3 h after myocardial I/R. **(A)** Cardiac myeloperoxidase activity in tissue samples. **(B)** The number of CD11b⁺Gr-1⁺ neutrophils infiltrated in myocardium were analyzed by flow cytometry. **(C)** The representative flow cytometry dot plots of CD11b⁺Gr-1⁺ neutrophils infiltrated in myocardium. **(D)** Lipopolysaccharide-induced CXC chemokine (LIX), cytokine-induced neutrophil chemoattractant (KC), and macrophage inflammatory protein-2 (MIP-2) mRNA levels were analyzed by real-time PCR. **p < 0.01 versus sham; ‡p < 0.01 versus control. **(E)** Enzyme-linked immunoadsorbent assay determined mouse cardiomyocytes KC, MIP-2, and LIX levels in the supernatants. **(F)** Neutrophil migration, means ± SEM. The value was normalized relative to medium alone. *p < 0.05; **p < 0.01 versus medium; ‡p < 0.01 versus H₂O₂; §§p < 0.01 versus IL-17A; #p < 0.05; ##p < 0.01 versus IL-17A/H₂O₂. All experiments were repeated 3 times. N = 4 to 5. Abbreviations as in Figures 1 and 3.

immune response during tissue I/R injury in brain, kidney, and intestine (13–15). However, little was previously known about myocardial I/R, although IL-17A is involved in the pathogenesis of diverse cardiovascular diseases (7–12). Most importantly, we have found elevated circulating levels of IL-17A in patients with acute myocardial infarction (23). Therefore, we focused on IL-17A in myocardial I/R with a mouse model. Here, we observed elevated IL-17A in the myocardium beginning at 1 h after I/R, peaking at 24 h, and decreasing thereafter, which suggests that IL17A acts in the early stages of myocardial I/R.

Although prior studies of IL-17A have focused largely on CD4⁺ Th17 cells, several reports indicate that γδT cells are also a major source of this cytokine (4). In our study, most of the IL-17A⁺ cells were CD3⁺ T lymphocytes. To our surprise, approximately 70% of the IL-17A–producing T lymphocytes were CD4⁻ but TCRγδ⁺. The differentiation of Th17 cells from naïve-CD4 cells usually takes several days in vivo and is currently thought to depend on TCR engagement. However, IL-17–producing γδT cells can immediately respond to stimuli—such as pathogens or IL-1/IL-23—with no need for prior stimulation via the
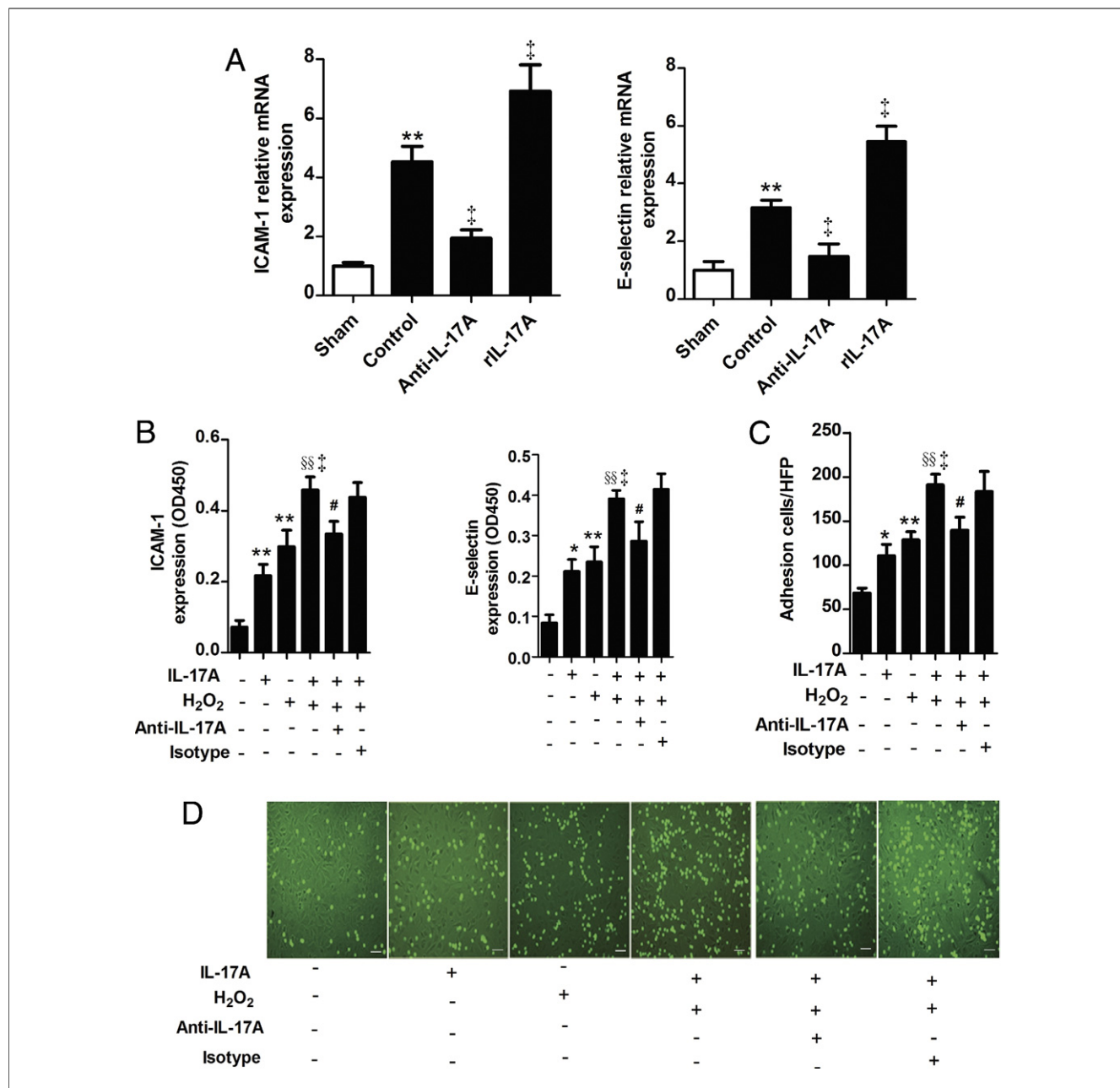
Figure 5   IL-17A–Mediated Neutrophil-EC Adhesion

(A) Mice were assessed for myocardium inter-cellular adhesion molecule (ICAM-1)-1 and E-selectin expression by real-time PCR at 3 h after I/R (n = 4 to 5). **$p <$ 0.01 versus sham; †$p < 0.05$; ‡$p < 0.01$ versus control. (B) Mouse myocardial endothelial cell (EC) expression of ICAM-1 and E-selectin was measured by a cell-based enzyme-linked immunoadsorbent assay, as indicated by the optical density 450 nm. (C) Neutrophil adhesion on ECs was determined by fluorescence microscopy. (D) Representative photomicrograph of neutrophils adhering to ECs. Neutrophils are identified by **green fluorescence**. Scale bar: 50 mm. Values are means ± SEM; *$p <$ 0.05; **$p < 0.01$ versus medium; †$p < 0.01$ versus H_2O_2; §§$p < 0.01$ versus IL-17A; #$p < 0.05$ versus IL-17A/H_2O_2. All experiments were repeated 3 times. Abbreviations as in Figures 1 and 3.

TCR and produce high amounts of IL-17A within hours (24). It is no wonder, considering the nearly instantaneous elevation of IL-17A in myocardium after I/R, that γδT lymphocytes but not Th17 cells were a major source of IL-17A, which is consistent with cerebral I/R (13). However, IL-17A–producing cells detected in kidneys after 3 h of reperfusion were $CD11b^+GR-1^+$ neutrophils (14),

which differs from our results that little of IL-17A–producing cells are neutrophils. In intestinal I/R, although Edgerton et al. (15) focused on $CD4^+$ T cells, they only examined the co-localization of CD3 and IL-17A but performed no further examination of T cell subsets. This discrepancy might be attributed to differences between tissues of I/R model.

The $Il17a^{-/-}$ mice showed a significant reduction in infarct volume after cerebral I/R (13). The $Il17a^{-/-}$ and $Il17r^{-/-}$ mice also showed protective effects on kidney function and morphology after renal I/R. Such protection was also observed by treatment with neutralizing anti–IL-17A mAb (14). In the present study, both neutralization of IL-17A just before induction of reperfusion and IL-17A genetic deficiency markedly ameliorated I/R injury, as demonstrated by reduced infarct size, cTnT levels, and improved cardiac function, whereas exogenous IL-17A significantly exacerbated I/R injury, demonstrating a critical role for IL-17A in myocardial I/R injury.

Apoptosis has been proposed to be an important mechanism for a significant amount of cell death in reperfused ischemic myocardium (16). It could be regulated by oxygen free radicals, cytokines such as TNF-α and IL-6, and neutrophil accumulation (25). The Bcl-2 family consists of pro- and anti-apoptotic members. The balance between pro-apoptotic and anti-apoptotic proteins determines the possibility of cells to either survive or undergo apoptosis after a certain stimulus or injury. IL-17A has been shown to induce vascular smooth muscle cell and airway epithelial cell apoptosis, but it has no effect on keratinocyte and osteoblast cell apoptosis (9,26–28). Our in vivo study showed that neutralization or repletion of IL-17A could regulate cardiomyocyte apoptosis, as confirmed by the change of TUNEL positive cardiomyocytes, caspase-3 activity, and the ratio of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins. Interleukin-17A might trigger apoptosis directly in cardiomyocytes or indirectly as a mediator that orchestrates other factors. Our in vitro study further confirmed that IL-17A had direct pro-apoptosis effect on cardiomyocyte. When the cardiomyocytes are exposed to oxidative stress, extrinsic and intrinsic signal pathways are activated, Fas mRNA and Bcl-2 family proteins are up-regulated, and the redox state is changed, which can ultimately drive cardiomyocyte into apoptosis (25,29,30). The regulation of caspase-3 activity and ratio of Bax/Bcl-2 demonstrated that IL-17A activated intrinsic signal pathways. Remarkably, myocardial IL-17R expression was not affected by I/R in vivo or oxidative stress in vitro (data not shown), therefore the mechanisms through which IL-17A alone or interaction with oxidative stress modifies the apoptosis signal pathway in myocardial I/R remain to be elucidated.

Neutrophil recruitment plays a major role in myocardial damage after I/R (18). Neutrophil chemotaxis and activation might be strongly regulated by CXC chemokines, particularly ELR$^+$ chemokines with the tripeptide ELR motif (31). Interestingly, IL-17A specifically induced the expression of all ELR$^+$CXCL chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8) in cultured human synoviocytes but had no effect on the expression of non-ELR CXC chemokines (CXCL9, CXCL10, and CXCL11) (32). Interleukin-17A has also been shown to induce KC, MIP-2, and LIX, which are rodent homologs of CXCL1, CXCL2, and CXCL5, in mouse osteoblastic cells (33) and lung fibroblasts (34). Moreover, the importance of ELR$^+$CXC chemokines in myocardial I/R has been implicated in vivo (35). Here, we found that inhibition of IL-17A markedly decreased cardiac LIX, KC, and MIP-2 expression and neutrophil infiltration in mouse I/R myocardium, whereas exogenous IL-17A increased LIX, KC, and MIP-2 expression and neutrophil infiltration in mouse I/R myocardium. In vitro experiments demonstrated that IL-17A could enhance cardiomyocyte LIX, KC, and MIP-2 production and neutrophil migration to the supernatant from conditioned cardiomyocytes. Taken together, these results indicate that IL-17A contributes to myocardial I/R injury at least partially through the regulation of neutrophil infiltration to the myocardium via CXC chemokine expression.

The neutrophil-endothelium interaction is critical for neutrophil-mediated inflammation. The molecular interactions required for neutrophils to cross the endothelium are regulated via the expression of adhesion molecules on the endothelium (18). E-selectin is a key molecule for rolling, whereas ICAM-1 is important in adhesion. Deficiency in either ICAM-1 or E-selectin expression results in a marked reduction in neutrophil accumulation and myocardial injury after I/R (36). IL-17A was reported to be a potential upstream inducer of ICAM-1 and E-selectin in lung microvascular ECs (37) and human umbilical vein ECs (19). We found that anti–IL-17A mAb and rIL-17A treatments regulate the expression of ICAM-1 and E-selectin in I/R myocardium at both mRNA and protein levels. In vitro studies showed that stimulation with IL-17A resulted in endothelial activation as revealed by elevated surface expression of E-selectin and ICAM-1 as well as neutrophil-endothelial adhesion. These results, along with the neutrophil migration data, support the notion that IL-17A has a potent effect on neutrophil recruitment and adherence, which are critical processes in myocardial I/R injury.

Conclusions

We have presented evidence that IL-17A produced primarily by $\gamma\delta$T cells plays a pathogenic role in myocardial I/R injury by inducing cardiomyocyte apoptosis and neutrophil infiltration. These data suggest a novel IL-17A-dependent pathway by which the immune system might influence the myocardial I/R injury. Control of IL-17A production might be of benefit for minimizing I/R-instigated myocardial damage.

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REFERENCES

1. Hillis LD, Lange RA. Myocardial infarction and the open-artery hypothesis. N Engl J Med 2006;355:2475–7.
2. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med 2007;357:1121–35.
3. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity 2004;21:467–76.
4. Roark CL, Simonian PL, Fontenot AP, Born WK, O'Brien RL. gammadelta T cells: an important source of IL-17. Curr Opin Immunol 2008;20:353–7.
5. Ley K, Smith E, Stark MA. IL-17A-producing neutrophil-regulatory Tn lymphocytes. Immunol Res 2006;34:229–42.
6. Miossec P. IL-17 and Th17 cells in human inflammatory diseases. Microbes Infect 2009;11:625–30.
7. Cheng X, Taleb S, Wang J, et al. Inhibition of IL-17A in atherosclerosis. Atherosclerosis 2011;215:471–4.
8. Xie JJ, Wang J, Tang TT, et al. The Th17/Treg functional imbalance during atherogenesis in ApoE(-/-) mice. Cytokine 2010;49:185–93.
9. Erbel C, Chen L, Bea F, et al. Inhibition of IL-17A attenuates atherosclerotic lesion development in apoE-deficient mice. J Immunol 2009;183:8167–75.
10. Madhur MS, Lob HE, McCann LA, et al. Interleukin 17 promotes angiotensin II-induced hypertension and vascular dysfunction. Hypertension 2010;55:500–7.
11. Yuan J, Yu M, Lin QW, et al. Th17 cells contribute to viral replication in coxsackievirus B3-induced acute viral myocarditis. J Immunol 2010;185:4004–10.
12. Baldeviano GC, Barin JG, Talor MV, et al. Interleukin-17A is dispensable for myocarditis but essential for the progression to dilated cardiomyopathy. Circ Res 2010;106:1646–55.
13. Shichita T, Sugiyama Y, Ooboshi H, et al. Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. Nat Med 2009;15:946–50.
14. Li L, Huang L, Vergis AL, et al. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. J Clin Invest 2010;120:331–42.
15. Edgerton C, Crispin JC, Moratz CM, et al. IL-17 producing CD4+ T cells mediate accelerated ischemia/reperfusion-induced injury in autoimmunity-prone mice. Clin Immunol 2009;130:313–21.
16. Hamacher-Brady A, Brady NR, Gottlieb RA. The interplay between pro-death and pro-survival signaling pathways in myocardial ischemia/reperfusion injury: apoptosis meets autophagy. Cardiovasc Drugs Ther 2006;20:445–62.
17. Chandrasekar B, Colston JT, de la Rosa SD, Rao PP, Freeman GL. TNF-alpha and H2O2 induce IL-18 and IL-18R beta expression in cardiomyocytes via NF-kappa B activation. Biochem Biophys Res Commun 2003;303:1152–8.
18. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. Nature 2008;453:1051–7.
19. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology 2010;129: 311–21.
20. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc Res 2004;61:481–97.
21. Steffens S, Montecucco F, Mach F. The inflammatory response as a target to reduce myocardial ischaemia and reperfusion injury. Thromb Haemost 2009;102:240–7.
22. Linfert D, Chowdhry T, Rabb H. Lymphocytes and ischemia-reperfusion injury. Transplant Rev (Orlando) 2009;23:1–10.
23. Cheng X, Yu X, Ding YJ, et al. The Th17/Treg imbalance in patients with acute coronary syndrome. Clin Immunol 2008;127:89–97.
24. Kapsenberg ML. Gammadelta T cell receptors without a job. Immunity 2009;31:181–3.
25. Nakamura M, Wang NP, Zhao ZQ, et al. Preconditioning decreases Bax expression, PMN accumulation and apoptosis in reperfused rat heart. Cardiovasc Res 2000;45:661–70.
26. Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. J Exp Med 2010;207:1293–305.
27. Pennino D, Eyerich K, Scarponi C, et al. IL-17 amplifies human contact hypersensitivity by licensing hapten nonspecific Th1 cells to kill autologous keratinocytes. J Immunol 2010;184:4880–8.
28. Shen F, Ruddy MJ, Plamondon P, Gaffen SL. Cytokines link osteoblasts and inflammation: microarray analysis of interleukin-17- and TNF-alpha-induced genes in bone cells. J Leukoc Biol 2005;77: 388–99.
29. Tanaka M, Ito H, Adachi S, et al. Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Circ Res 1994;75:426–33.
30. Kubasiak LA, Hernandez OM, Bishopric NH, Webster KA. Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. Proc Natl Acad Sci U S A 2002;99:12825–30.
31. Frangogiannis NG. Chemokines in ischemia and reperfusion. Thromb Haemost 2007;97:738–47.
32. Zrioual S, Toh ML, Tournadre A, et al. IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR+ CXC chemokine expression in synoviocytes and are overexpressed in rheumatoid blood. J Immunol 2008;180:655–63.
33. Ruddy MJ, Shen F, Smith JB, Sharma A, Gaffen SL. Interleukin-17 regulates expression of the CXC chemokine LIX/CXCL5 in osteoblasts: implications for inflammation and neutrophil recruitment. J Leukoc Biol 2004;76:135–44.
34. Zhou X, Chen Q, Moore J, Kolls JK, Halperin S, Wang J. Critical role of the interleukin-17/interleukin-17 receptor axis in regulating host susceptibility to respiratory infection with Chlamydia species. Infect Immun 2009;77:5059–70.
35. Chandrasekar B, Smith JB, Freeman GL. Ischemia-reperfusion of rat myocardium activates nuclear factor-KappaB and induces neutrophil infiltration via lipopolysaccharide-induced CXC chemokine. Circulation 2001;103:2296–302.
36. Jones SP, Trocha SD, Strange MB, et al. Leukocyte and endothelial cell adhesion molecules in a chronic murine model of myocardial reperfusion injury. Am J Physiol Heart Circ Physiol 2000;279: H2196–201.
37. Roussel L, Houle F, Chan C, et al. IL-17 promotes p38 MAPK-dependent endothelial activation enhancing neutrophil recruitment to sites of inflammation. J Immunol 2010;184:4531–7.

Key Words: inflammation ■ interleukin-17 ■ ischemia/reperfusion ■ $\gamma\delta$T cell.

▶ **APPENDIX**

For supplementary figures, tables, and Methods, please see the online version of this article.