Interleukin-10 From Transplanted Bone Marrow Mononuclear Cells Contributes to Cardiac Protection After Myocardial Infarction

Jana S. Burchfield, Masayoshi Iwasaki, Masamichi Koyanagi, Carmen Urbich, Nadia Rosenthal, Andreas M. Zeiher, Stefanie Dimmeler

Abstract—Bone marrow mononuclear cells (BM-MNCs) have successfully been used as a therapy for the improvement of left ventricular (LV) function after myocardial infarction (MI). It has been suggested that paracrine factors from BM-MNCs may be a key mechanism mediating cardiac protection. We previously performed microarray analysis and found that the pleiotropic cytokine interleukin (IL)-10 was highly upregulated in human progenitor cells in comparison with adult endothelial cells and CD14+ cells. Moreover, BM-MNCs secrete significant amounts of IL-10, and IL-10 could be detected from progenitor cells transplanted in infarcted mouse hearts. Specifically, intramyocardial injection of wild-type BM-MNCs led to a significant increase in LV end-diastolic pressure (LVEDP) and LV end-systolic volume (LVESV) compared to hearts injected with either diluent or IL-10 knock-out BM-MNCs. Furthermore, intramyocardial injection of wild-type BM-MNCs led to a significant increase in stroke volume (SV) and rate of the development of pressure over time (+dP/dt) compared to hearts injected with either diluent or IL-10 knock-out BM-MNCs. The IL-10–dependent improvement provided by transplanted cells was not caused by reduced infarct size, neutrophil infiltration, or capillary density, but rather was associated with decreased T lymphocyte accumulation, reactive hypertrophy, and myocardial collagen deposition. These results suggest that BM-MNCs mediate cardiac protection after myocardial infarction and this is, at least in part, dependent on IL-10. (Circ Res. 2008;103:203-211.)

Key Words: acute myocardial infarction ■ remodeling ■ growth factors/cytokines ■ cell therapy

Bone marrow–derived progenitor cells have been used clinically to improve left ventricular (LV) function in patients after acute myocardial infarction (MI),1–4 however the exact mechanisms for this improvement in cardiac function have not clearly been defined. Several mechanisms have been proposed to explain the beneficial effects of this cell-based therapy including cell transdifferentiation, cell fusion, and the release of paracrine growth factors and cytokines. In support of the latter mechanism, various cytokines and growth factors from transplanted progenitor cells have been shown to be important in progenitor cell-mediated cardiac protection such as basic fibroblast growth factor, vascular endothelial growth factor, stromal-derived factor,5,6 angiopoietin-1, interleukin (IL)-1β, tumor necrosis factor-α,7,8 hepatocyte growth factor, insulin-like growth factor-1, thymosin β4,8 secreted frizzled related protein 2,9 IL-6, placental growth factor, transforming growth factor, monocye chemoattractant protein-1, platelet-derived growth factor, plasminogen activator, and metalloproteinase-9.10

We previously performed microarray analysis on peripheral blood-derived cultured human precursor cells11 and found one paracrine factor highly expressed, the pleiotropic cytokine, IL-10. IL-10 is expressed and secreted by a variety of cell types such as T cells, monocytes/macrophages, dendritic cells, B cells, and natural killer cells. IL-10 prevents production of proinflammatory cytokines and chemokines by monocytes/macrophages, which are involved in the recruitment of additional monocytes, neutrophils, dendritic cells, and T cells to sites of injury or infection (as reviewed by12). These potent antiinflammatory effects suggests that this cytokine may lead to rapid resolution of the inflammatory response or limit innate immunity in the heart after myocardial infarction. Indeed, IL-10 enhances resolution of pulmonary inflammation in vivo by promoting apoptosis of neutrophils,13 and IL-10–deficient mice revealed increased neutrophil infiltration, infarct size, and myocardial necrosis after acute MI.14 Furthermore, IL-10 exogenously administered into rats 15 minutes before reperfusion significantly attenuated myocardial injury,15 and elevated serum levels of IL-10 are associated with a more favorable prognosis in patients with acute coronary syndromes.16–18 Conversely, it has been shown that infarcted IL-10–deficient mice showed
no differences in neutrophil infiltration, infarct healing, and LV remodeling and that IL-10 serum levels are of no or little use to determine prognosis of patients with a previous MI.20,21

Alternatively, IL-10 may play a critical role in the adaptive immune response to cardiac injury. IL-10 was originally described as cytokine synthesis inhibitory factor produced by T-helper type 2 cells that inhibit T-helper 1 function, by preventing production of T-helper 1 cytokines, such as interferon-gamma and IL-12.22 IL-10 also exerts its effects on T lymphocyte infiltration in that IL-10 expression inversely correlated with CD8+ T lymphocyte infiltration in patients with gastric cancer,23 and IL-10 treatment prevented antigen-induced CD4+ T lymphocyte accumulation in the peritoneal cavity.24 Therefore, we hypothesized that progenitor cell expression of IL-10 is an important paracrine mechanism, contributing to progenitor cell–mediated improvement in cardiac function after myocardial infarction by modulation of innate and adaptive immune responses.

Materials and Methods

Cell Culture, Isolation, and IL-10 Measurement

Human cells were isolated, cultured, and microarray analysis was performed as described in Supplementary Methods (available online at http://circres.ahajournals.org). Bone marrow mononuclear cells were isolated by density gradient centrifugation25 from male C57BL6/J and IL-10 knock-out (KO) mice (Jackson Laboratories, Bar Harbor, Me) and cultured for 24 hours at 37°C in C57BL6/J mice (Charles Rivers, Sulzfeld, Germany) or NMRI nu/nu hearts immediately after LAD ligation.

Measurement of LV Function

At 14 days postinfarction, animals were anesthetized with 1.5% isoflurane, and a micromanometer-tipped Millar catheter (Millar Instruments) was advanced into the left ventricle via the right carotid artery to obtain pressure-volume loops for measuring left ventricular end-diastolic pressure (LVEDP), left ventricular end-systolic volume (LVESV), development of pressure over time (+dP/dt), and stroke volume (SV).

Myeloperoxidase Activity

Hearts from sham-operated mice or infarcted myocardium were divided into infarct and noninfarct regions and snap frozen in liquid nitrogen. Myeloperoxidase (MPO) activity was assessed similarly as described in Supplementary Methods available online, normalized to heart weight, and expressed as fold change over sham-operated control hearts.

Immunohistochemistry

For quantification of neutrophil and T lymphocyte number, 10 μm frozen sections of 1- or 4-day postinfarcted hearts were incubated with rat antimonuse Ly6G (1:10; HyCult Biotechnology) or with rat antimonuse CD3 (1:100; AbD Serotec).

Histology

Fourteen days after MI, hearts were perfused fixed in Z-fixative, paraffin-embedded, and picrosirius red staining was performed on 5-μm sections as previously described.27 Percent collagen was quantitated using Carl Zeiss Imaging and expressed as percent collagen/mm² of tissue. Triticum vulgaris Lectin-TRITC staining (Sigma) was performed as described,28 and cross-sectional area of myocytes in remote and border zones were analyzed. For vessel density quantification, sections were stained with biotinylated Griffonia Simplicifolia Lectin I-isolectin B4 (Vector Labs) followed by streptavidin Alexa 555 secondary antibody staining. Infarct size was measured 4 days after MI using 2,3,5-Triphenyltetrazolium chloride staining.

Additional methods and statistical analysis are provided as online supplementary methods.

Results

IL-10 Is Highly Expressed in Progenitor Cells and in Cell-Transplanted Myocardium

Using oligonucleotide array analysis, we determined that IL-10 messenger RNA expression was significantly higher in human endothelial progenitor cells (EPCs) than in human microvascular endothelial cells, human microvascular endothelial cells, or CD14+ monocytes (Figure 1A). Furthermore,
IL-10 From Transplanted BM-MNCs Improves LV Function After Myocardial Infarction

To determine whether IL-10 is necessary for BM-MNC-mediated improvement in LV function, we obtained pressure-volume loops using a Millar catheter. The induction of MI led to a significant increase in LVEDP and LVESV compared with sham-operated controls, which was decreased in hearts injected with WT BM-MNCs (Figure 3A through 3C). This decrease in LVEDP and LVESV was attenuated in hearts injected with IL-10 KO BM-MNCs (Figure 3A through 3C). Furthermore, MI led to a significant decrease in SV and rate of +dP/dt compared to sham-operated controls (Figure 3D and 3E). Injection of WT BM-MNCs led to a significant increase in SV and +dP/dt compared to hearts injected with diluents (Figure 3D and 3E). However, this decrease in SV and +dP/dt from WT BM-MNC injection was not seen on injection of IL-10 KO BM-MNCs (Figure 3D and 3E). These data demonstrate that the protective effects of BM-MNCs are at least in part dependent on the presence of IL-10.

IL-10 From Transplanted BM-MNCs Reduces LV Remodeling

To determine how IL-10 from myocardially-transplanted BM-MNCs improves cardiac function, we ascertained LV structure and remodeling. Although the infusion of BM-MNC reduced the infarct size, there was no statistically significant difference in % infarct size 4 days after infarction between WT BM-MNC- or IL-10 KO BM-MNC-injected hearts (32.6±3.5% versus 41.1±6.1%, respectively, P=0.2669, n=6 to 7) as measured using TTC staining, however, collagen quantitation at 14 days after infarction, assessed by picrosirius red staining, demonstrated a significant decrease in fibrosis described as percent collagen/mm² of tissue in the LV free wall in hearts injected with WT BM-MNCs compared to hearts injected with diluent (0.45±0.15% versus 1.57±0.64%, P<0.05; Figure 4A). Furthermore, the ratio of left ventricular free wall/septal wall thickness was significantly greater in myocardium transplanted with WT BM-MNCs compared to hearts injected with diluent, consistent with improved remodeling (Figure 4B). Assessment of the infarct border zones and remote regions revealed that hearts transplanted with WT BM-MNCs had smaller cardiac myocyte cross-sectional area than did diluent-injected controls, and this decrease in cardiac myocyte hypertrophy was not seen in hearts injected with IL-10 KO BM-MNCs (Figure 4C). These results indicate that the transplantation of BM-MNCs leads to improved ventricular remodeling after 2 weeks, which is dependent
on IL-10 and independent of differences in infarct size at day 4 after MI.

No Differences in BM-MNC Subpopulations Between WT and IL-10−/− Mice
To ensure that there were no differences between the cell populations of BM-MNCs isolated from WT and IL-10 KO mice, we performed flow cytometry using cell-specific markers on freshly isolated BM-MNCs. Figure 5A shows that there was no difference in the percent of lineage negative (lin−) cells expressing Sca1−/kit−, Sca1−/flk1−, or c-kit+/flk1− between WT and IL-10 KO BM-MNCs. Furthermore, the percent of lineage positive cells was not different (data not shown). These data suggest that the BM-MNC-mediated improvement in LV function after MI is attributable to the paracrine effects of IL-10 and is unlikely to be caused by

Figure 3. Interleukin (IL)-10 from transplanted wild-type (WT) bone marrow mononuclear cells (BM-MNCs) leads to improvement in left ventricular (LV) functional recovery after myocardial infarction (MI). A, Representative pressure-volume loops from sham-operated control hearts or MI hearts injected with either diluent, WT BM-MNCs, or BM-MNCs from IL-10 knockout mice. LV end-diastolic pressure (LVEDP) (B), LV end-systolic volume (LVESV) (C), stroke volume (SV) (D), and development of pressure over time (+dP/dt) (E). *P<0.05 compared to sham, †P<0.05 vs diluents, #P<0.05 vs WT BM-MNC, n=3 (sham) to 7 in each group.
differences in the composition of the transplanted cell subpopulations.

Effect of IL-10 on Neovascularization and Cytokine Release

Transplantation of BM-MNCs has been shown to enhance neovascularization in ischemic tissue. Therefore, we questioned whether IL-10 deficiency in BM-MNCs may interfere with cell-mediated improvement of neovascularization. Although transplantation of WT BM-MNCs led to an increase in the number of vessels compared to diluent-injected hearts at 14 days post-MI, the lack of IL-10 in transplanted IL-10 KO BM-MNCs did not lead to a decrease in neovascularization as shown in Figure 6A and, therefore, does not explain the observed differences in LV function. We also determined whether other cytokines/growth factors, which have previously been suggested to be involved in stem cell–mediated neovascularization and cardiac protection, are differentially secreted because of the presence or absence of IL-10. No significant differences exist in the amount of IL-6, MCP-1, VEGF, or IGF-1 released into the supernatants of cultured BM-MNCs from WT or IL-10 KO mice (supplemental Figure I). In summary, IL-10 deficiency does not interfere with BM-MNC–mediated neovascularization improvement and the release of other proangiogenic cytokines.

Effect on Neutrophil Infiltration in Hearts Transplanted With BM-MNCs

Because IL-10 is known to have antiinflammatory activities, we hypothesized that transplantation of BM-MNCs may lead to a decrease in inflammatory cell infiltration. Specifically,
IL-10 KO mice subjected to myocardial ischemia/reperfusion injury showed an increase in the number of infiltrating neutrophils, increased infarct size, and increased myocardial necrosis. Therefore, we sought to determine whether the cardioprotective effects of transplanted BM-MNCs were attributable to a decrease in the number of infiltrated neutrophils. Neutrophil infiltration was indirectly assessed by measuring MPO activity in the infarcted and noninfarcted regions of hearts intramyocardially injected with either BM-MNCs or diluent at 1 day and 4 days postinfarction. Figure 6B shows no difference in MPO activity between diluent or BM-MNC–injected hearts, suggesting that the improvement in LV function was not attributable to differences in neutrophil infiltration. To confirm these data, we performed immunohistochemistry to directly detect infiltrated neutrophils. In agreement with the MPO activity of diluent or BM-MNC–injected hearts, suggesting that the improvement in LV function was not attributable to differences in neutrophil infiltration. To confirm these data, we performed immunohistochemistry to directly detect infiltrated neutrophils. In agreement with the MPO activity of diluent or BM-MNC–injected hearts, there was no difference in the number of infiltrated neutrophils at 1 day and 4 days postinfarction (Figure 6C). These data suggest that the primary cardioprotective role for BM-MNC–derived IL-10 is not attributable to limiting the number of infiltrating neutrophils after MI, and therefore this cannot account for the improvement in LV function.

**Decrease in T Lymphocytes in Hearts Transplanted With BM-MNCs Is Dependent on IL-10**

T lymphocytes have been shown to mediate cardiotoxicity, LV remodeling, and depression of LV function. Because IL-10 is known to have several immunoregulatory activities, specifically on T lymphocytes, we sought to investigate the number of T lymphocytes that had accumulated into the MI heart after BM-MNC transplantation. Immunohistochemistry using CD3+ specific antibodies was performed to detect the number of T lymphocytes in the heart at 4 days postinfarction. Intramyocardial injection of WT BM-MNCs led to a decrease in average number of T lymphocytes per field as compared to hearts injected with diluent, and this decrease was not seen with injected IL-10 BM-MNCs (Figure 7). These data suggest that secretion of IL-10 by injected BM-MNCs may play an important cardioprotective role by limiting the number of infiltrating T lymphocytes following MI.
Discussion

The data of the present study demonstrate that cultured human EPCs and murine BM-MNCs release the antiinflammatory cytokine IL-10. Using BM-MNCs from WT or IL-10 KO mice, we demonstrate that myocardial transplantation of WT BM-MNCs contributes to improvement in LV functional recovery after MI and is dependent, at least in part, on the presence of the antiinflammatory cytokine IL-10. Although the reduced functional improvement seen after transplantation of IL-10–deficient BM-MNCs compared to WT BM-MNCs was clearly evident, the mechanisms by which IL-10 mediates these beneficial effects are more complex. Surprisingly, IL-10 deficiency of the transplanted cells did not affect the infarct size, neutrophil accumulation, and neovascularization but interfered with T-cell recruitment, collagen deposition, and reactive hypertrophy.

Inflammation in the heart after myocardial infarction is considered to be a normal part of the healing process; however, when not tightly regulated, it can cause deleterious effects. Accordingly, analysis of gene expression profiles revealed that the genes highly expressed in BM-MNCs were involved in the inflammatory response under hypoxic conditions. Furthermore, an antiinflammatory role for mesenchymal stem cells was demonstrated. One of the first inflammatory cell types to infiltrate into the heart after myocardial infarction is neutrophils, which may disrupt tissue homeostasis and cause injury by using their myeloperoxidase activity. Therefore, we tested whether the intramyocardial injection of BM-MNCs leads to a decrease in the number of infiltrating neutrophils. Although, IL-10 has been shown to target neutrophils, specifically promoting neutrophil apoptosis, we observed no difference in MPO activity, an indirect measurement of neutrophil number, and no difference in the number of immunohistochemically-detected neutrophils at day 1 or day 4 after MI between hearts injected with BM-MNCs or diluent. This result contrasts with previous studies where exogenously administered IL-10 led to a decrease in MPO activity after 20 minutes of ischemia followed by 24 hours reperfusion, and where subjecting IL-10–deficient mice to 30 minutes of ischemia followed by 6 hours of reperfusion led to an increase in MPO activity. Alternatively, our data are consistent with a previous study showing no differences in neutrophil infiltration or resolution in IL-10–deficient mice subjected to myocardial ischemia reperfusion. Altogether, these data suggest that the ability of IL-10 to mediate cardiac protection may be dependent on the particular model of injury or the cellular source of IL-10.

There is emerging evidence that progenitor cells may have immunomodulatory properties and may specifically suppress the activation or proliferation of T lymphocytes. Bone marrow stromal cells suppressed both CD4+ and CD8+ T lymphocyte proliferation. Specifically, cocultured MSCs with purified T cells and antigen-presenting cells (APCs; either monocytes or dendritic cells) secrete large quantities of IL-10, leading to inhibition of T-cell cytokine secretion and proliferation. In addition, MSCs downregulate T cell responses, and coculture of these 2 cell types resulted in upregulation of antiinflammatory T helper (Th) 2 cytokines and downregulation of Th1 cytokines. Furthermore, MSCs have also been shown to inhibit the cytotoxic effects of antigen-primed cytotoxic T lymphocyte. Although most studies on immune cells in the heart involve autoimmune myocarditis or organ transplantation models, it is becoming increasingly clear that T lymphocytes play a role in MI. Specifically, Varda-Bloom et al have shown infiltration of T lymphocytes into the heart after MI, and in vitro, coculture of T lymphocytes from post-MI rats with isolated cardiac myocytes from a noninfarcted rat heart resulted in cytotoxicity of the cardiac myocytes. Furthermore, the injection of splenocytes from MI rats into noninfarcted rat hearts led to adverse LV remodeling and depressed LV function. Interestingly, in patients with gastric cancer, IL-10 expression inversely correlated with CD8+ T lymphocyte infiltration, and IL-10 treatment prevented antigen-induced CD4+ T lymphocyte accumulation in the peritoneal cavity. Accordingly, our data demonstrate a decrease in the number of T lymphocytes in the heart after WT BM-MNC transplantation, but not after IL-10 KO BM-MNC transplantation. These data suggest that the IL-10 from myocardially-transplanted progenitor cells may play a role in decreasing the accumulation of T lymphocytes in the heart after MI, which may directly protect the cardiac myocytes against cell death and therefore, limit adverse LV remodeling and improve LV function.

Several reports from different investigators have shown that myocardial transplantation of progenitor/stem cells leads to a decrease in myocardial fibrosis following myocardial infarction. Recently, it was shown that mesenchymal stem cells directly attenuate cardiac fibroblast proliferation and collagen synthesis via the release of paracrine factors. These investigators found that mesenchymal stem cell–conditioned medium upregulated cardiac fibroblast antiproliferation-related genes such as elastin, myocardin, and DNA damage-inducible transcript 3, and downregulated type I and III collagen expression. Moreover, T lymphocytes cocultured with cardiac fibroblasts led to an increase in cardiac fibroblast procollagen expression, suggesting that accumulation of T lymphocytes in the heart after MI may lead to increased collagen deposition and may be modulated by IL-10. Specifically, IL-10 has been shown to decrease collagen I expression in skin fibroblasts, and collagen content was significantly increased in dermal wounds of IL-10 KO mice. Our data show that transplantation of WT BM-MNCs, but not IL-10 KO BM-MNCs, led to a decrease in collagen deposition. These data indicate that IL-10 from progenitor cells may limit LV remodeling associated with a reduced reactive hypertrophy possibly attributable to decreased T lymphocyte accumulation and decreased collagen deposition.

Although our data do not exclude the possibility that other cytokines may also be involved in mediating cardiac protection, IL-10 deficiency did not directly interfere with the release of several proangiogenic and cardioprotective cytokines in vitro. In accordance, the increase in capillary density observed in WT BM-MNCs transplanted hearts was not dependent on the presence of IL-10. Furthermore, we observed no differences in the number of stem and inflammatory cell subpopulations from freshly isolated BM-MNCs from either WT or IL-10 KO mice, suggesting that the observed improvement in LV function after MI was mediated
by the presence of IL-10 and not attributable to the abundance or absence of a particular cell type in the mononuclear cell fraction. In addition, we myocardially injected lineage negative cells from the bone marrow and found no differences in neutrophil infiltration (data not shown) suggesting that the presence of already differentiated cells in our mononuclear cell fraction did not influence the results. However, one limitation of our study is that we cannot exclude the possibility that the cell subpopulations between BM-MNCs from WT and IL-10 KO mice may have differences in cell functionality. The dependence of IL-10 on mediating cardiac protection is not entirely clear, but it is possible that a deficiency in IL-10 leads to a decrease in activation of tissue resident cardiac stem cells or a deficiency in IL-10 diminished the capacity of these transplanted cells to differentiate or fuse with preexisting myocardial cells, or alternatively, the lack of IL-10 may prevent protection against infarct expansion and continual cardiac myocyte apoptosis.

Altogether, our studies suggest that IL-10 from transplanted BM-MNCs contributes to cardiac protection after MI, decreasing T lymphocyte accumulation and the amount of collagen deposition, thereby preventing adverse LV remodeling and improving LV functional recovery after MI. Although our data exclude that IL-10 acts via changes in infarct size, neutrophil accumulation, neovascularization, or paracrine activities of infused cells, the causal relevance of IL-10-mediated reduced T-cell infiltration for LV remodeling remains to be formally proven and warrants further studies.

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Disclosures
None.

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Supplementary Methods

Cell culture, Isolation and IL-10 measurement

Human endothelial progenitor cells (EPCs), human umbilical vein endothelial cells (HUVECs), human dermal microvascular endothelial cells (HMVECs), and CD14⁺ monocytes were isolated, cultured, and microarray analysis was performed as previously described [1].

In vivo Detection of Transplanted Cells and IL-10 from Transplanted Cells

Total RNA was isolated using Trizol Reagent ™ (Invitrogen, Carlsbad, California), and subjected to reverse transcription followed by PCR using human IL-10 specific primers or primers specific for X and Y chromosome-specific genes.

human IL-10 specific primers (forward: 5' CCGAGATGCCTTCAGCAGAG 3' and reverse: 5' GGTTCTTGTTCTCAGCTTGG 3')

mouse-specific GADPH primers (forward: 5' AACTTTGGCATTGTGGAAGG 3' and reverse: 5' ACACATTGGGGGTAGGAACA 3').

For the detection of injected BM-MNCs, DNA of the hearts of female recipient mice injected with male BM-MNCs was extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer’s instructions. Screening for Y chromosomal DNA was performed with primers specific for a 404-bp fragment of the SRY gene located on the sex-determining region of the Y chromosome (SRY2: 5’ TCTTAAACTCTGAAGAAGAC 3’ and SRY4: 5’ GTCTTGCCTGTATGTGATGG 3’) and a 244-bp fragment of the DXNds3 locus, which is a polymorphic microsatellite locus located on mouse X chromosome, was chosen as a control [2] (NDS3: 5’ GAGTGCCTCATCTATGCTTACAG 3’ and NDS4: 5’ TCTAGTTCATTGATTTGATTAGTTGC 3’).
Flow Cytometry

Freshly isolated BM-MNCs from WT and IL-10 KO mice were stained with either APC conjugated antibodies recognizing Sca1, ckit, CD105 (eBioscience, San Diego, CA), and Gr1 (BD Franklin Lakes, NJ) or PE-conjugated antibodies recognizing ckit, Flk1, and CD45 (BD Franklin Lakes, NJ). 30,000 cells were analyzed using BD Canto II FACs system and Diva software (BD Franklin Lakes, NJ).

Myeloperoxidase Activity

The frozen tissues were weighed and homogenized in 500 µl of 10 mM potassium phosphate buffer (pH 7.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO) using a Tissue Tearor™ (Biospec Products, Bartlesville, OK) and MPO activity was assessed similarly as described [3]. The samples were centrifuged for 30 min at 20,000 X g and 10 µl supernatant was added to 290 µl 10 mM potassium phosphate buffer (pH 7.0) containing 0.167 mg/mL o-dianisidine hydrochloride (Sigma, St. Louis, MO) and 0.0015% hydrogen peroxide. Absorbance was measured at 450 nm every 1 min for 4 min. Human MPO was used to generate a standard curve, and 1 unit of MPO activity represented the degradation of 1 µM of hydrogen peroxide at 25°C. MPO activity was calculated as Units/g of heart tissue and was expressed as fold change over sham-operated control hearts.

Immunohistochemistry

For quantification of neutrophil infiltration, 1 and 4 day- post-infarcted hearts were submersed in O.C.T. compound, and frozen. 10 µm frozen sections were fixed in acetone for 10 min, incubated with 3% hydrogen peroxide (Dako), rabbit serum (Dako), and overnight incubation with rat anti-mouse Ly6G (1:10)(HyCult Biotechnology) at 4°C. Sections were incubated with biotinylated anti-rat IgG mouse adsorbed (1:100 dilution), Vectastain ABC reagent, 3,3'-diaminobenzidine (DAB) containing NiCl (Vector Labs), and dehydrated and counterstained with
eosin Y. Greater than 20 photos per heart at 200 x magnification were taken using a Zeiss Microscope (Carl Zeiss, Germany). Neutrophil number was quantified using Carl Zeiss Imaging (Carl Zeiss, Germany) and expressed as number of neutrophils/mm^2 of tissue. For quantification of T lymphocyte infiltration, frozen sections from 4 day-post-infarcted hearts were incubated with rat anti-mouse CD3 (1:100) (AbD Serotec) for 2 hours, detected using DAB, and counterstained with hematoxylin. The number of cells in each image was quantified by a blinded-observer.

**Histology**

For quantification of collagen deposition, cardiac myocyte hypertrophy, and vessel density, 14 day- post-infarcted hearts were perfused-fixed with 10% zinc-buffered formalin, and embedded in paraffin. 5 µm sections were deparaffinized, and stained with Sirius Red F3BA (Sigma) for detection of collagen which was quantified from fifty photos at 200 X magnification taken in the ischemic anterior wall and in the nonischemic posterior septum using a microscope equipped with a digital camera (AxioCam, Carl Zeiss) and expressed as percentage of total myocardial area. *Triticum vulgaris* Lectin-TRITC (Sigma) at a 1:500 dilution was used for cardiac myocyte membrane staining. Eighteen photos at 400 X magnification were taken in the remote and border zones of *Triticum vulgaris* Lectin-TRITC, and only cardiac myocytes identified with DAPI-stained nuclei in the cell center were counted, and average myocyte cross-sectional area was obtained using AxioVision Software (Carl Zeiss). Biotinylated Griffonia Simplicifolia Lectin I-isolectin B_4 (Vector Labs) at a 1:50 dilution was used for vascular cell membrane staining. Photos from whole heart sections were quantified for vessel density and calculated as number of vessels/field. For infarct size, five 1 mm sections of 4 day-post MI hearts were incubated for 20 min at 37°C with 1% 2,3,5-Trippheyltetrazolium chloride (TTC), and percent infarct was calculated by measuring area of infarct and weight of each section. The weight of the infarction
\[ = (A_1 \times W_1) + (A_2 \times W_2) + (A_3 \times W_3) + (A_4 \times W_4) + (A_5 \times W_5) \] and percent infarct = (weight of the infarction/weight of LV) \times 100.

**Statistical Analysis**

All data are presented as mean ± SE. Statistical analysis was performed with Student’s \( t \)-test or ANOVA followed by Bonferroni’s post-hoc test for comparisons between two groups or between more than two groups. A \( p \) value of less than 0.05 was considered to be statistically significant.
References


Cytokine/growth factors were detected in supernatant of WT and IL-10 KO BM-MNCs. Supernatants were concentrated using Amicon Ultra protein concentrators (MWCO: 5,000; Millipore, Billerica, MA), and cytokines were measured by ELISAs (R&D Systems, Minneapolis, MN). No difference in the amount of secreted IL-6, MCP-1, VEGF, or IGF-1 was detected (n=3).