Induction of Cardiac Angiogenesis Requires Killer Cell Lectin-Like Receptor 1 and α4β7 Integrin Expression by NK Cells

Manaf Bouchentouf, Kathy-Ann Forner, Jessica Cuerquis, Véronique Michaud, Jiamin Zheng, Pierre Paradis, Ernesto L. Schiffrin and Jacques Galipeau

*J Immunol* published online 22 October 2010
http://www.jimmunol.org/content/early/2010/10/22/jimmunol.1001888
Induction of Cardiac Angiogenesis Requires Killer Cell Lectin-Like Receptor 1 and α4β7 Integrin Expression by NK Cells


Recent findings indicate that NK cells are involved in cardiac repair following myocardial infarction. The aim of this study is to investigate the role NK cells in infarct angiogenesis and cardiac remodeling. In normal C57BL/6 mice, myelomonocytic inflammatory cells invaded infarcted heart within 24 h followed by a lymphoid/NK cell infiltrate by day 6, accompanied by substantial expression of IL-2, TNF-α, and CCL2. In contrast, NOD SCID mice had virtually no lymphoid cells infiltrating the heart and did not upregulate IL-2 levels. In vitro and in vivo, IL-2–activated NK cells promoted TNF-α–stimulated endothelial cell proliferation, enhanced angiogenesis and reduced fibrosis within the infarcted myocardium. Adoptive transfer of IL-2–activated NK cells to NOD SCID mice improved post-myocardial infarction angiogenesis. RNA silencing technology and neutralizing Abs demonstrated that this process involved α4β7 integrin/VCAM-1 and killer cell lectin-like receptor I/N-cadherin–specific binding. In this study, we show that IL-2–activated NK cells reduce myocardial collagen deposition along with an increase in neovascularization following acute cardiac ischemia through specific interaction with endothelial cells. These data define a potential role of activated NK cells in cardiac angiogenesis and open new perspectives for the treatment of ischemic diseases. The Journal of Immunology, 2010, 185: 000–000.

Cardiac remodeling involves several mechanisms and various components of the immune system to efficiently eliminate dead cells, induce angiogenesis, and initiate damaged cardiac tissue replacement. To date, little is known about the faculty of immune cells on cardiac tissue repair. Therefore, studying the infiltration of different types of immune cells and their relationship to wound healing in myocardial remodeling represents a key element for the development of strategies of intervention to reduce injury and promote repair.

The mechanisms responsible of triggering the inflammatory cascade are well established. The inflammatory process is initiated by early acting complement C1–C5 (1–3) and reactive oxygen species (4, 5) causing locally mast cell degranulation (6–8). The latter has been reported to be specific to the ischemic area and results in rapid release of TNF-α and IL-6 (9). Mononuclear cells infiltrate the infarcted myocardium in the first few hours following ischemia. Complement activation plays an important role in neutrophil and monocyte chemotaxis in the infarcted myocardium and has been associated with increased levels of chemokines and cytokines (IL-8 and IL-1 in particular), MCP-1, and TGF-β1 (10–12). After recruitment to the infarcted myocardium, monocytes differentiate into macrophages serving as a source of cytokines and growth factors that regulate extracellular matrix metabolism (13, 14). The inflammatory response leads to healing of injured tissue. In this context, monocyte-derived IL-10 has been widely investigated. It is an inhibitor of IL-1, IL-6, IL-8, and TNF-α production and has been reported to modulate the function and phenotype of monocytes/macrophages (15, 16). In a recent study, it was shown that c-kit–mediated mobilization of bone marrow NK cells rescues hearts post-myocardial infarction (MI), contributing to improved remodeling and cardiac function (17). The literature is, however, missing the precise mechanism of NK cell–mediated cardiac repair following infarction.

In this study, we show a previously uncharacterized function of NK cells. We demonstrate that IL-2–activated NK cells interact with TNF-α–stimulated endothelial cells to induce their proliferation and promote angiogenesis through a mechanism involving α4β7 integrin and killer cell lectin-like receptor (KLRG) 1.

Materials and Methods

Reagents

Anti–NK-1.1 (clone PK136) and -CD3 (clone 17A2) Abs were obtained from R&D Systems (Minneapolis, MN). Anti-CCR2 (clone E68), α4β7 integrin (clone DATK32), –VCAM-1 (clone M-K/2), –KLRG1 (clone 2F1), –N-cadherin (clone 32/NC), and Ki67 (clone SP6) Abs were from Abcam (Cambridge, MA). Phospho-Akt (clone 587F11), cyclin D1 (clone DC56), and the cyclin-dependent kinase inhibitor P27 (clone S10) Abs were purchased from Cell Signaling Technology (Danvers, MA). Small interfering RNA (siRNA) against KLRG1 and α4β7 integrin were from
Invitrogen (Montreal, Quebec, Canada). Anti-C9D4 (clone 20D5) and CD144 (clone 11D4.1) Abs were from BD Biosciences (Mississauga, Ontario, Canada). ELISA for cardiac troponin I (CtTnI) was obtained from Life Diagnostics (Westchester, PA). RPMI 1640 and DMEM were purchased from HyClone (Logan, UT).

**Cell culture and isolation**

NK cells were obtained from mouse spleen by immunomagnetic selection using the EasySep mouse NK Enrichment Kit following the manufacturer’s protocol (StemCell Technologies, Vancouver, British Columbia, Canada). Purified NK cells were then resuspended in RPMI 1640-based medium supplemented with 10% FBS, 1% HEPES, 1% sodium pyruvate, and 1% penicillin/streptomycin and kept in culture at 37°C in a tissue culture incubator (5% CO₂). Medium was changed daily following cell purification. Mouse cardiac endothelial cells (CECs) were prepared as previously described (18). CECs were cultured in DMEM supplemented with 2% FBS, 2 mM glucose, 1% penicillin/streptomycin mixture, 1 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 20% FBS, 150 μg/ml endothelial growth supplement, and 12 U/ml heparin. Cells were kept in a humidified atmosphere of 5% CO₂/95% air.

**Cell labeling for FACS analysis**

Cells were resuspended in PBS containing 1% FBS buffer and incubated with either a unique Ab conjugated to APC or a biotin-conjugated Ab for 2 h at 4°C. The latter were then incubated with streptavidin PE-Cy7 Ab for 1 h at room temperature. FACS was performed using a FACS Calibur analyzer (BD Biosciences).

**Cytotoxicity assay**

NK cell cytotoxic function was tested by a standard lactate dehydrogenase release assay using YAC-1 cells (a murine T lymphoma cell line sensitive to NK cells) as target cells according to the manufacturer’s protocol (Roche, Montreal, Quebec, Canada). A total of 10⁵ YAC-1 cells were cultured with untreated or IL-2-activated NK cells obtained either from immune-competent (C57BL/6 and CB17) or immune-deficient (NOD SCID) mouse spleens. Three different E:T ratios were tested (40:1, 20:1, and 1:1). To evaluate the number of dead YAC-1 cells, culture supernatant was collected, cells were removed, and the cell-free supernatant was incubated with the substrate mixture from the kit. The mixture was transferred to a 96-well plate, and absorbance was read at 500 nm. During the assay, lactate dehydrogenase enzyme activity in the culture supernatant increases as the number of dead cells (or cells with damaged plasma membranes) increases. Dead cell number was determined by running a colorimetric cell dose curve using 10⁻³, 5 × 10⁻³, 5 × 10⁻², 10⁻¹, and 5 × 10⁻¹ YAC-1 cells.

**Chemotaxis assay**

Twenty-four-well cell invasion assay (Millipore, Billerica, MA) was used to examine the migration of NK cells according to the manufacturer’s protocol. A total of 4 × 10⁵ untreated or IL-2–stimulated NK cells (50 U/ml; Novartis, Quebec, Canada) suspended in 0.5 ml serum-free medium were loaded into the upper chamber of the transwell. Lower chambers were loaded with 1% RPMI 1640 medium in presence or absence of MCP-1 (100 ng/ml). Following a 5-h incubation period at 37°C, cells remaining on the upper surface of the insert (nonmigrated cells) were removed gently and placed into a sterile 24-well plate containing a cell detachment solution for 30 min at 37°C. Dislodged cells were treated with a lysis buffer and incubated with the CyQuant GR dye for 15 min at room temperature. The mixture was transferred to a 96-well plate, and fluorescence was read at 480 nm. Invaded cell number was determined by running a fluorescence cell dose curve using 10⁻², 5 × 10⁻³, 2 × 10⁻³, and 5 × 10⁻¹ NK cells.

**Evaluation of proliferation of CECs**

Cells were seeded in 24-well plates at a density of 2 × 10⁵ cells (98% to 99% confluent) per plate in basal medium. IL-2–activated or untreated NK cells were fixed with paraformaldehyde (PFA), then added to some wells over confluent CECs. Cells were trypsinized (1 ml/well) and harvested at the beginning of the experiments (T0) and 7 d later. CECs were counted using Z2 Beckman Coulter particle counter (Beckman Coulter, Miami, FL).

**Angiogenesis assay**

Ninety-six–well plates were first coated at 37°C for 2 h with a matrix containing laminin, collagen type IV, heparin sulfate proteoglycans, entactin, and nidogen (Millipore). A total of 10⁵ CECs were seeded in each well and incubated in presence or absence of naive or IL-2–stimulated and PFA-fixed NK cells for 18 h.

**In vitro angiogenesis score quantification**

Visual patterns were defined on photos of five random view fields per well. A numerical score was then assigned to each condition according to the degree of angiogenesis progression as previously described (18).

**Western blot**

CECs were harvested and resuspended in a lysis buffer. Protein (40 μg) was fractionated on a 12% polyacrylamide SDS gels and then transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Ontario, Canada). The membrane was then incubated with a primary Ab (anti-mouse phospho-Akt, cyclin D1, and P27) overnight at 4°C. The blots were then incubated for 1 h with their respective secondary Ab at room temperature. Blots were visualized using a chemiluminescence kit (Millipore).

**Animal models**

All experiments were carried out in agreement with local guidelines for the care and use of laboratory animals and in accordance to the guidelines of the McGill University animal care authority (Montreal, Quebec, Canada). Investigations conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. In vivo experiments were performed using 5–10 mice per group. NK cells were obtained from C57BL/6, CB17 (used as immune-competent mouse model), or NOD.CB17-Prkdc<sup>scid</sup> (also known as NOD SCID, used as immune-deficient mouse model) mice. Ten and 30–60 d after NOD SCID naïve mice or NOD.CB17-Prkdc<sup>scid</sup> mice were injected i.v. with 6.7 ng/ml and a heart rate between 490 and 535 beats/min after the MI. Only mice with cTnI concentration >10 ng/ml were excluded from data analysis, as it reflects a minor MI and may overestimate the effect of the injection of IL-2–activated NK cells.

**Inflammatory cell infiltration**

Leukocyte content was quantified by immunohistochemistry on C57BL/6 or NOD SCID mouse heart sections at the time of LAD ligation and 4, 1, 3, 6, and 2, wk, and 4 wk after. The nonspecific binding sites were blocked with PBS (10%) in PBS for 1 h. The sections were then incubated for 2 h with anti-GR-1 and anti–Mac-1 Abs for neutrophils, with anti-CD14 and anti-F4/80 Abs for monocytes/macrophages, and with anti–NK-1.1 (C57BL/6 cells) or anti-CD94 (NOD SCID cells) and anti-CD3 Abs for NK cells/lymphocytes. Slides were then incubated, respectively, with Abs conjugated with Alexa Fluor 488 and 647 (1 h; Molecular Probes, Montreal, Quebec, Canada). The sections were washed two times with PBS and one time with DAPI (0.1%) in PBS and observed using a Leica DM-LB2 fluorescent microscope (Leica Microsystems, Deerfield, IL).

**Quantification of cytokine expression in vivo by quantitative PCR**

Quantitative PCR (qPCR) for MCP-1, TNF-α, and IL-2 was performed to assess cytokine or chemokine levels in the myocardium of NOD SCID and C57Bl6 mice. Basically, mRNA was harvested from frozen heart cross sections at the indicated time points. A reverse transcription reaction using 40 ng mRNA was performed, and the resulting cDNA was amplified by qPCR according to the manufacturer’s protocol (Model 7500, Applied Biosystems, Foster City, CA). Relative quantification of the target mRNA concentrations reached in 12–24 h postinfarction (22, 23). In our experiments, the infarct size was estimated by determination by ELISA of cardiac cTnI in mouse plasma collected 24 h post-MI. All mice had a cTnI concentration of 28.8 ± 6.7 ng/ml and a heart rate between 490 and 535 beats/min after the MI. Only mice with cTnI concentration >10 ng/ml were excluded from data analysis, as it reflects a minor MI and may overestimate the effect of the injection of IL-2–activated NK cells.

**Evaluation of proliferation of CECs**

Cells were seeded in 24-well plates at a density of 2 × 10⁵ cells (98% to 99% confluent) per plate in basal medium. IL-2–activated or untreated NK cells were fixed with parafomaldehyde (PFA), then added to some wells over confluent CECs. Cells were trypsinized (1 ml/well) and harvested at the beginning of the experiments (T0) and 7 d later. CECs were counted using Z2 Beckman Coulter particle counter (Beckman Coulter, Miami, FL).
incubated with Abs conjugated, respectively, with Alexa Fluor 488 and 546 (1 h; Molecular Probes). The sections were washed two times with PBS and one time with DAPI (0.1%) in PBS. CD144, Ki67, and DAPI stainings were observed using a fluorescent microscope (Leica DM-ILB2, Leica Microsystems). The endothelial cell proliferation index was obtained as the ratio ((Ki67+CD144+×Ki67+CD144-)×100).

Echocardiography

Echocardiography was performed as described previously (18) 1 mo postinduction of MI. Left ventricle (LV) end-diastolic internal diameter (LVDD), end-systolic internal diameter (LVIDs), end-diastolic interventricular, LV posterior wall (LVPW) thickness, and LV fractional shortening (FS) were determined as described previously (24, 25).

Dissection of the heart and histological analysis of infarct area

Hearts were obtained by performing 1 mo post-LAD ligation (18). For the long-term experiments, hearts were fixed in 10% formalin and mounted in paraffin. Paraffin sections were stained for H&E or Sirius red and processed for CD31 immunohistochemistry at the histology facility of Immunology and Cancer Research Institute of the University of Montreal (Montreal, Quebec, Canada). To evaluate the Sirius red-stained area, microphotographs of heart sections were acquired using nanozoomer. Images were then transferred to Scion Image software (version 4.0.2). Objects of interest (Sirius red) were discriminated from surrounding background using the thresholding mode. In this mode, pixels equal to or greater than threshold level are displayed in black, and all other pixels are displayed in white (background). Evaluation of stained areas was performed by surface measurement.

Statistical analysis

Data are presented as means ± SEM. Differences among groups were evaluated using ANOVA followed by a post hoc Bonferroni correction. Statistical analyses were performed using GraphPad software (GraphPad, San Diego, CA). p < 0.05 was considered significant.

Results

NOD SCID mice have worse cardiac outcome following MI compared with normal immune-competent C57BL/6

To test the hypothesis that lymphoid competence affects outcome of MI, we compared LV FS following anterior coronary ligation–induced MI in immune-deficient (NOD SCID) and immune-competent (C57BL/6) mice. The results in Fig. 1 indicate that LV FS was 24% lower in NOD SCID mice in comparison with C57BL/6 mice (p = 0.01).

Myocardial lymphocyte and NK cell infiltration is impaired following MI in NOD SCID mice

The main defining immunological impairment of NOD SCID mice relative to normals is the deficiency affecting T and B lymphocyte development and a deficit in NK cell number and function (19–21). We therefore assessed inflammatory cell infiltration over time following MI by immunohistochemical analysis of C57BL/6 and NOD SCID mouse heart cross sections (Fig. 2). Neutrophils were the first cells to invade the infarcted myocardium, followed in chronological order by monocytes, macrophages, lymphocytes, and NK cells. There were 12% less neutrophils (p = 0.03), 44% less monocytes (p = 0.01), and 77% less macrophages (p = 0.0001) in NOD SCID mouse hearts following MI in comparison with C57BL/6 mouse. Neither lymphocytes nor NK cells were detected in NOD SCID mouse hearts at any time point postinfarct.

Postinfarct myocardial MCP-1, TNF-α, and IL-2 expression pattern is altered in NOD SCID mice

In light of the substantial decrease in lymphocyte and NK cell infiltration in NOD SCID hearts post-MI, we examined the contemporaneous myocardial inflammatory cytokine expression pattern. Myocardial levels of MCP-1, TNF-α, and IL-2 were estimated by immunohistochemistry in C57BL/6 and NOD SCID mouse heart cross sections post-MI. Fig. 3A–E show quantification of cytokine density at different time points. MCP-1 has been described to be produced by cardiac endothelial cells, monocytes/macrophages, and myofibroblasts. In the myocardium, TNF-α is produced mainly by macrophages but has been described to be generated also by lymphoid cells, mast cells, endothelial cells, cardiac myocytes, and fibroblasts. IL-2 is produced by activated T lymphocytes (mainly Th0 and Th1 CD4+ cells) and by NK cells. MCP-1 and TNF-α were expressed from days 1–6 in both C57BL/6 and NOD SCID mouse hearts. IL-2 expression was detected typically at day 3 with a peak at day 6 in C57BL/6 mouse hearts and was virtually absent at any time point in NOD SCID mice.

NOD SCID NK cells display impaired cytotoxicity and chemotaxis relative to C57BL/6 mouse NK cells

There were virtually no infiltrating lymphocytes and NK cells in NOD SCID hearts post-MI, suggesting that these cells, among other lymphoid cells, may play a cardioprotective role. In an effort to distinguish the cell biological features of NOD SCID NK cells from normals, we compared cytotoxicity and chemotaxis of NK cells obtained from immune-defective NOD SCID and normal immune-competent mice (C57BL/6 and CB17). Fig. 4A shows NK cell purity following isolation. In all mouse strains, >90% of isolated cells expressed NK-1.1, and <7% expressed CD3. The cytotoxicity assay consisted in culturing YAC-1 cells either with IL-2–activated or untreated NK cells using three E:T ratios. Results in Fig. 4B demonstrate that untreated or IL-2–stimulated NK cells obtained from C57BL/6 and CB17 mice mediated YAC-1 cell death at an E:T ratio of 40:1. IL-2–activated C57BL/6- and CB17-derived NK cells induced, respectively, 48 ± 5% and 39 ± 3% YAC-1 cell death. Untreated C57BL/6- and CB17-derived NK cells were able to induce, respectively, 18 ± 2% and 24 ± 4% YAC-1 cell death. In contrast, NOD SCID-derived NK cells induced 5 ± 1% YAC-1 cell death. NOD SCID-derived NK cells also has a limited chemotactic capability in comparison with NK cells obtained from C57BL/6 and CB17 mice. Indeed, as shown in Fig. 4C, MCP-1 induced chemoinvasion of 1456 ± 375 IL-2–activated C57BL/6-derived NK cells and 1317 ± 239 NK cells obtained from CB17 mice. MCP-1 induced the chemotaxis of 127 ± 31 NOD SCID-derived NK cells. Interestingly, flow cytometry showed that CCR2 (receptor for MCP-1) expression was induced in 98% and 99% of IL-2–stimulated NK cells obtained, respectively, from C57BL/6 and CB17 mice, whereas only 6% of NOD SCID-derived NK cells expressed CCR2 post-treatment with IL-2.

NK cells enhance cardiac endothelial cell proliferation and angiogenesis through IL-2–dependent expression of αβ7 integrin

It has been suggested that NK cells are proangiogenic. Considering the defect in NK cell function and cardiac homing observed in postinfarct NOD SCID hearts, we determined whether NK cells may stimulate primary mouse cardiac endothelial cell proliferation in vitro via a contact mechanism. IL-2 induced αβ7 integrin expression in 92% of C57BL/6 and 97% of CB17 NK cells, whereas only 13% of NOD SCID-derived NK cells expressed αβ7 integrin post–IL-2 treatment (Fig. 5A, right panel). PFA-fixed NK cells had no effect on TNF-α–stimulated CEC proliferation or angiogenesis. However, NK cells obtained from C57BL/6 mice and treated with IL-2 prefixation improved CEC proliferation and angiogenesis, respectively, by 20% and 25% (p = 0.02), which suggests a role for a NK cell surface contact effector molecule independent of metabolic activity. Because PFA cross-links proteins, we also tested whether NK cell fixation could affect the conformation of αβ7 integrin and reduce their
promitotic effect. Our data indicate that IL-2–activated NK cells obtained from C57BL/6 mice but without fixation improved CEC proliferation and angiogenesis, respectively, in a similar manner to IL-2–activated and PFA-fixed NK cells (Fig. 5 B, 5D). In an effort to identify a candidate molecule, we demonstrated that VCAM-1 (the ligand of α4β7 integrin) expression was inducible in C57BL/6 and NOD SCID mouse CEC upon stimulation with TNF-α (Fig. 5A, left panel). Neutralization of α4β7 integrin inhibited promitotic and proangiogenic effects of IL-2–activated NK cells. NOD SCID-derived NK cells treated with IL-2 prefixation had no effect on TNF-α–stimulated CEC proliferation and angiogenesis (Fig. 5C, 5E). IL-2–activated NK cells had no effect on naive CECs not treated with TNF-α (data not shown). Altogether, these data suggest that IL-2 activation of NK cells upregulates α4β7 integrin, which then allows binding to activated VCAM-1–expressing CECs, with angiogenic effects, features that are absent in NOD SCID NK cells.

**NK cell-induced endothelial cell proliferation and angiogenesis require KLRG-1 and N-cadherin**

This experiment was performed to verify whether KLRG1 and N-cadherin were involved in modulating CEC proliferation and angiogenesis in response to NK cells in vitro. Fig. 6A shows that N-cadherin as well as KLRG1 expression were constitutive in CEC and NK cells. In addition, 69% and 76% of NK cells obtained, respectively, from C57BL/6 and CB17 mice, but only 22% of NOD SCID-derived NK cells, expressed KLRG1. Fig. 6B

---

**FIGURE 1.** Lymphoid cells are required to preserve cardiac function following MI. A, Left ventricular FS (% FS). B, Left ventricular FS (% FS) normalized to sham. C, Size in millimeters of LVIDs, LVIDd, interventricular septum in systoles (IVSs), and LVPW in systole (LVPWs). D, LV mass corrected. Data are reported as means ± SEM (n = 10). *p < 0.05; #p < 0.05.
and 6D demonstrate that PFA-fixed NK cells had no effect on CEC proliferation or angiogenesis. However, NK cells obtained from C57BL/6 mice treated with IL-2 prefixation improved CEC proliferation and angiogenesis. Inhibition of KLRG1 using siRNA reduced by 31% promitotic and proangiogenic effects of IL-2–stimulated NK cells obtained from C57BL/6 mice (p = 0.03). NOD SCID-derived NK cells treated with IL-2 had no effect on TNF-α–stimulated CEC proliferation and angiogenesis (Fig. 6C, 6E).

Enhancement of endothelial cell proliferation is associated with upregulation of p-Akt and cyclin D1 expression and sequestration of p27

To evaluate expression levels of proteins involved in endothelial cell proliferation and survival following contact with IL-2–activated NK cells, Western blots were performed with proteins harvested from CECs to demonstrate the phosphorylated form of protein kinase B (p-Akt) and cyclin-dependent kinase inhibitor
p27. We also included in our analysis G1/S-specific cyclin D1. Results clearly indicated that PFA-fixed NK cells obtained from C57BL/6 mice had no effect on expression of these proteins. However, IL-2–activated and PFA-fixed NK cells derived from C57BL/6 mice enhanced p-Akt expression 2-fold and cyclin D1 levels 1.8-fold and reduced p27 expression 1.7-fold. Neutralizing α4β7 integrin and inhibition of KLRG1 suppressed p-Akt and cyclin D1 expression and restored p27 expression to basal levels (Fig. 7).

Adoptive transfer of IL-2–activated NK cells into NOD SCID mice promotes post-MI endothelial cell proliferation and reduces cardiac fibrosis

To test the hypothesis that IL-2–activated NK cells affect cardiac angiogenesis post-MI, we infused i.v., 3 d following MI, 10⁶ untreated or IL-2–activated NK cells obtained from C57BL/6 mice into NOD SCID mice. Immunohistochemistry allowed identification of proliferating endothelial cells (Ki67⁺ and CD144⁺) and the in vivo endothelial cell proliferation index. Fig. 8A and 8B demonstrate that in basal conditions, 12% of endothelial cells in NOD SCID mice were undergoing proliferation and that injection of IL-2–activated NK cells enhanced 2.2-fold the proportion of proliferating endothelial cell (p = 0.003). The increase in the endothelial cell proliferation index was consistent with the number of NK cells infiltrating the heart that occurred in the border and the scar zones (Fig. 8C). We noticed that homing in the infarct was variable from the border to infarct zone as it is dependent on neoangiogenesis. In contrast, injection of untreated C57BL/6-derived NK cells had no effect on the endothelial cell proliferation index. Inhibition of α4β7 integrin and inhibition of...
KLRG1 expression by siRNA significantly reduced IL-2–activated NK cell mitotic effects relative to controls. In a separate set of in vivo experiments, we verified whether improvement of angiogenesis mediated by IL-2–activated NK cells would improve cardiac function. An identical infarct/NK adoptive transfer assay and evaluation of cardiac function demonstrated that injection of IL-2–activated NK cells following MI induced a trend, albeit without achieving significance, to improve LV FS and LVPW (Fig. 8D, 8E), suggesting that cardiac repair is controlled by multiple mediators. Moreover, injection of IL-2–activated NK cells reduced by 37% ($p = 0.04$) the Sirius red staining area, indicating a diminution in cardiac fibrosis, and improved by 42% ($p = 0.005$) capillary density in the border and the scar zones of the infarct (Fig. 8F–H).
The importance of the inflammatory cascade in MI has been recognized and investigated in numerous experimental studies (26–30). Understanding the mechanisms regulating the reaction to injury is crucial for development of efficient and innovative cell-based therapies for MI.

**FIGURE 5.** In vitro evaluation of the role of α4β7 integrin expressed by NK cells on VCAM-1 expressing CEC proliferation and angiogenesis. A, Flow cytometry analysis of VCAM-1 in untreated or TNF-α–stimulated CECs and α4β7 integrin in naïve or IL-2–activated NK cells derived from NOD SCID, CB17, and C57BL/6 mice. CECs were counted after 7 d in culture without NK cells or with untreated or IL-2–activated NK cells obtained from C57BL/6 (B) and NOD SCID (C) mice. Estimation of the angiogenesis score of CECs cultured for 18 h either without NK cells or with untreated or IL-2–activated NK cells obtained from C57BL/6 (D) or NOD SCID (E) mice. Three different conditions were performed, and they are represented in each panel using three different colors. Black bars: no treatment (NT), without neutralizing Ab; gray bars (anti-α4β7 integrin): with α4β7 integrin neutralizing Ab; white bars: with isotypic Ab control (ISOT). Data are reported as means ± SEM (n = 5). * p < 0.05.

**FIGURE 6.** In vitro evaluation of the role of KLRG1 expressed by NK cells on N-cadherin expressing CEC proliferation and angiogenesis. A, Flow cytometry analysis of N-cadherin and KLRG1 expression, respectively, in CECs and in untreated or IL-2–stimulated NK cells derived from NOD SCID, CB17, and C57BL/6 mice. Number of CECs cultured for 7 d either without NK cells or with untreated or IL-2–activated NK cells obtained from C57BL/6 (B) and NOD SCID (C) mice. Estimation of the angiogenesis score of CECs cultured for 18 h either without NK cells or with untreated or IL-2–activated NK cells obtained from C57BL/6 (D) or NOD SCID (E) mice. Three different conditions were compared, and they are represented in each panel using three different colors. Black bars: mock transfection (MOCK) without siRNA; gray bars: NK cells transfected with siRNA against KLRG1 (siKLRG1); white bars: NK cells transfected with scrambled siRNA (SCR). Data are reported as means ± SEM (n = 5). * p < 0.05.
We evaluated cardiac function of immune-deficient and immune-competent mice following MI. Our data demonstrate that the absence of cardiac-infiltrating lymphoid cells is associated with a poorer outcome. Previous publications underlined the importance of NK cell dependence in heart recovery following MI (17). In their study, Ayach and coworkers (17) showed that specific NK cell depletion in immune-competent mice reduced their survival by 30% as a result of heart failure. We hypothesized that injection of IL-2–activated NK cells promote angiogenesis and preserve cardiac function following infarction. This process may explain the better outcome observed in immune-competent mice. To validate our hypothesis, we first established the dynamic of inflammatory cell infiltration and quantified levels of MCP-1, TNF-α, and IL-2 in NOD SCID and C57BL/6 mouse hearts following sham treatment and MI. A typical inflammatory cascade consisting of neutrophil, monocyte, macrophage, lymphocyte, and NK cell infiltration was associated with high levels of MCP-1, TNF-α, and IL-2. Neither NK cells nor lymphocytes or IL-2 could be detected in the ischemic area and the border zone of the heart of NOD SCID mice. We focused on the evaluation of the amount of inflammatory cells infiltrating the myocardium between day 0 and day 7 post-MI as it is well established that healing process of the myocardium takes place mainly during this time frame. Indeed, leukocytes invade the myocardial wound within the first day post-MI and regulate extracellular matrix metabolism through the presence of cardiac-infiltrating lymphoid cells and myofibroblasts (35, 36).

In vitro, we compared chemotaxis and cytotoxicity of immune-deficient and immune-competent mouse-derived NK cells. Our data show failure of NOD SCID NK cells to respond to IL-2. Indeed, IL-2–treated NOD SCID NK cells were unable to induce significant YAC-1 cell death, and only a few cells expressed CCR2 and migrated toward a MCP-1 gradient. In contrast, IL-2–stimulated immune-competent NK cell cytotoxic activity and induced CCR2 expression, allowing them to migrate toward an MCP-1 bait.

In another series of experiments, we showed in vitro and in vivo that NK cells derived from C57BL/6 mice induced CEC proliferation and angiogenesis through a previously uncharacterized mechanism involving α4β7 integrin and KLRG1. In vivo, this process required migration of injected NK cells toward the inflammation site and binding to CECs. Previous reports indicated adhesive properties of IL-2–activated NK cells and the role of some integrins in regulating their transmigration through the vascular endothelium (37, 38). In particular, α4β7 integrin has been shown to function as a receptor for fibronectin and VCAM-1 on B, T, and NK cells (39–41). In this study, VCAM-1 expression was inducible in endothelial cells following TNF-α treatment, consistent with previous publications (42, 43). Our data also indicate that association of VCAM-1 found on TNF-α–activated CECs and α4β7 integrin expressed by IL-2–stimulated NK cells is required, but their binding is incapable of inducing CEC proliferation due to inability of VCAM-1 to induce intracellular signaling.

NK cells express KLRG1, an inhibitory receptor that binds to a highly conserved site on E-, N-, and R-cadherins on target cells (44). Cadherins are a class of type 1 transmembrane proteins that play a critical role in establishing homophilic adherens-type junctions by mediating calcium-dependent cell–cell adhesion. Cadherins were first shown to maintain tissue adhesion but lately have been demonstrated to act as cell signaling receptors by regulating the location of β-catenin, a class of protein involved in the control of cell survival, proliferation, and migration (45). Loss of cadherins and high frequency mutations in them have been detected in tumors, suggesting that a decrease in cadherin–β-catenin association contributes to cell proliferation and neoplastic transformation (46). Moreover, Levenberg et al. (47) demonstrated that adhesion-mediated signals, triggered by N-cadherin homophilic association, suppress cell growth by arresting the cell cycle at the G1 phase. Growth inhibition by cadherin stimulation induced an increase in the cell cycle inhibitory protein p27 levels in CHO cells (47). In addition, proteolytic shedding of N-cadherin–β-catenin system promotes vascular smooth muscle cell proliferation in a manner similar to that observed in carcinogenesis. In particular, disruption of N-cadherin cell–cell contacts, mediated in part by MMPs, releases β-catenin into the cytoplasm.
and triggers intracellular signaling leading to cell growth (48). It has been recently demonstrated that N-cadherin–mediated cell–cell contacts also initiate antiapoptotic signaling by increasing Akt phosphorylation (49). In this paper, we showed in vitro and in vivo that specific inhibition of KLRG1 or \(\alpha_4\beta_7\) integrin blocked the promitotic effect of IL-2–activated NK cells obtained from immune-competent mice, indicating a key role of these receptors in controlling CEC proliferation and angiogenesis. In support of this observation, Western blot analysis of proteins extracted from CECs cultured with IL-2–activated and PFA-fixed NK cells, either with anti-\(\alpha_4\beta_7\) integrin neutralizing Ab or following transfection with an siRNA against KLRG1, demonstrated that IL-2–activated

**FIGURE 8.** In vivo evaluation of angiogenesis in immune-deficient mice following adoptive transfer of IL-2–activated NK cells obtained from immune-competent mice. Post-MI, mice with similar cTnI levels were classed into six groups: MI only (NT); mice injected with untreated NK cells (NK); mice injected with IL-2–activated NK cells (NK, IL-2); mice infused with IL-2–activated NK cells and transfected with siRNA against KLRG1 (NK, IL-2, siKLRG1); and mice infused with IL-2–activated NK cells and transfected with siRNA against \(\alpha_4\beta_7\) integrin (NK, IL-2, si\(\alpha_4\beta_7\)). A, In vivo endothelial cell proliferation index corresponding to the percentage of proliferating endothelial cells in the infarct area. B, Representative images of mouse hearts stained for Ki67 (yellow), CD144 (green), and DAPI (blue). C, Number of PKH26 labeled NK cells infiltrating the heart (red). D, Left ventricular FS (% FS). E, Size in millimeters of LVIDd, LVIDd, IVSs, and LVPW in systole (LVPWs). F, Sirius red-stained surface as percentage of heart section area. G, Capillary density (number/mm²) within the scar and the border zones. H, Representative cross-sections of hearts stained for H&E, Sirius red (SR), and CD31. Original magnification \(\times 3\) for heart cross-sections and \(\times 40\) for immunohistochemistry images. Data are reported as means ± SEM (n = 4 for the short-term experiments and n = 5 for the long-term experiments). *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\), ****\(p<0.0001\), & indicate a significant difference versus bar with identical symbol with \(p<0.05\).
NK cells enhanced the expression of the antiapoptotic protein p-Akt, promoted the expression of the cell cycle regulator protein cyclin D1, and reduced the expression of p27. This underlines the proangiogenic effects of NK cells. The injection of NK cells reduced cardiac fibrosis and enhanced the number of capillaries in the scar and the border zones (the latter defined as the viable myocardial tissue immediately adjacent to the infarct scar). Smaller scars in NOD SCID mice infused with IL-2-activated NK cells may reflect altered scar contraction or attenuated cardiomyocyte loss. Effects on the composition of the scar are more likely, as late activation of angiogenesis would be unlikely to protect cardiomyocytes from ischemic death. It is well established that among various mediators involved in cardiac extracellular matrix (ECM) homeostasis, TGF-β1, connective tissue growth factor, and angiotensin II play a key role in promoting infarct fibrogenesis (50, 51). In particular, TGF-β1 stimulates fibroblast-like cell growth, enhances collagen synthesis, and suppresses collagen degradation (52). Furthermore, an increased enzymatic activity of MMP-9 and -2 was detectable in the cardiac infarct and border zones where inflammatory cells were abundant. MMPs degrade injured structural ECM components in the infarcted myocardium, a process that is essential for subsequent reconstruction of the ECM for infarct healing and affects remodeling events in the infarcted myocardium. It is thus very likely that IL-2–activated NK cells influence composition of the scar by secreting factors that may interfere with some fibrogenesis mediator (e.g., TGF-β1). Lastly, we showed that adoptive transfer of IL-2–activated NK cells palliated in part the MI outcome of NOD SCID mice. Adoptive transfer experiments on NOD SCID animals were performed to validate our hypothesis, and these were not designed to examine the role of endogenous NK cells in cardiac repair. Actually, the fact that adoptive transfer of NK cells in NOD SCID mice did not improve cardiac function and remodeling following MI indicates that other cells (e.g., B cells, T cells, and monocytes/macrophages) may be involved in the healing process. Therefore, we suggest that progenitor or other lymphoid- and myeloid-derived cells may be involved in cardiac angiogenesis.

Our data indicate that infiltration of monocytes/macrophages is profoundly reduced in NOD-SCID mice as compared with C57BL/6 mice. As monocytes/macrophages have been shown to play a crucial role in myocardial angiogenesis and remodeling post-infarction (53), their reduced infiltration might also explain the phenotype of NOD SCID mice. Several studies reported EPCs to be subpopulation of these cells with a critical role in angiogenesis (54–56).

Mechanisms responsible for worse cardiac function in NOD SCID mice are not fully identified. Although our results show that injection of IL-2–activated NK cells improved cardiac angiogenesis through a cell-contact mechanism, adoptive transfer of NK cells did not significantly improve cardiac function in infarcted NOD SCID mice. These data suggest that NK cells are not the only inflammatory cell population implicated in cardiac healing following infarction but that NK cell-independent mechanisms may also be involved (e.g., T or B cell-mediated pathways). Indeed, NOD SCID (NOD.CB17-Prkdcscid) are characterized by an absence of functional T cells, B cells, and circulating complement and by NK cell deficit in number and function (19, 57). It has also been suggested that T lymphocytes may play a healing role after acute ischemia depending on the stage of injury. Indeed, depletion of CD4+ impaired collateral vessel formation, perfusion recovery, and rate of perfusion recovery in a murine model of hind limb ischemia (58). Regulatory T cells or suppressor T cells have been found to have a variety of functions including modulating the immune system. This occurs through many different mechanisms, including inhibiting the activation and proliferation of other T cells, a process likely mediated by IL-10, TGF-β, and CTLA-4. In particular, it has been shown that regulatory T cell recruitment to the site of ischemia restrains postinfarction inflammation, preventing excessive matrix degradation and attenuating adverse remodeling (59). All of these data together suggest that cardiac repair is controlled by multiple mediators including but not exclusive to NK cells. In another series of experiments, we investigated whether NK cells could promote angiogenesis in a paracrine manner. Evaluation of NK cell secretome revealed that they do not secrete anti-inflammatory cytokines (IL-10, soluble TNFR-1, and MCP-1) or growth factors (insulin-like growth factor-1, hepatocyte growth factor, vascular endothelial growth factor, endothelial growth factor, and stromal cell-derived factor) that may influence angiogenesis (data not shown).

In the current study, we reveal a new mechanism, to our knowledge, whereby injection of IL-2–activated NK cells contribute to vascular remodeling via α4β7 integrin and KLRG1 without taking part in vascular formation (Supplemental Fig. 1). Basically, activated NK cells first bind to CEC through α4β7 integrin and VCAM-1 and disrupt N-cadherin associations via KLRG1. This process dislocates β-catenins from the cytoplasm to the nucleus and removes the cell contact inhibition of proliferation. In this study, we provide new insights, to our knowledge, into the regenerative capability of inflammatory cells and NK cells in particular. These data open new perspectives for the treatment of ischemic diseases.

Disclosures
The authors have no financial conflicts of interest.

References
NK CELL-MEDIATED CARDIAC ANGIOGENESIS

20. NK CELL-MEDIATED CARDIAC ANGIOGENESIS

28. Frangogiannis, N. G. 2004. Chemokines in the ischemic myocardium: from
31. Lindsey, M. L., J. Gannon, M. Aikawa, F. J. Schoen, E. Rabkin, L. Lopresti-
32. Lipocalin-2 regulates the inflammatory response during ischemia and reperfu-
33. Blankesteijn, W. M., E. Creemers, E. Lutgens, J. P. Cleutjens, M. J. Daemen, and
39. de Landa´ zuri. 1993. Alpha 4 beta 7 integrin mediates B cell binding to
42. H. P. Schultheiss, and C. Tscho¨pe. 2010. Immunosuppression
43. Botta, R., E. Gao, G. Stassi, D. Bonci, E. Pelosi, D. Zwas, M. Patti, L. Colonna,
45. Lampugnani, M. G., and E. Dejana. 2007. The control of endothelial cell
46. Koutsouki, E., C. A. Beeching, S. C. Slater, O. W. Blaschuk, G. B. Sala-Newby,
49. Koutsouki, E., C. A. Beeching, S. C. Slater, O. W. Blaschuk, G. B. Sala-Newby,
52. Botta, R., E. Gao, G. Stassi, D. Bonci, E. Pelosi, D. Zwas, M. Patti, L. Colonna,
54. Botta, R., E. Gao, G. Stassi, D. Bonci, E. Pelosi, D. Zwas, M. Patti, L. Colonna,
55. Fo¨rster. 2006. Cardioprotective c-kit+ cells are
56. R. D. Weisel, A. Keating, and R. K. Li. 2006. Cardioprotective c-kit+ cells are
57. M. Eldar, and D. Hasin. 2000. Cytotoxic T lymphocytes are activated following
58. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
59. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
60. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
61. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
63. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
64. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
65. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
66. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
68. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
69. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
70. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
71. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
72. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
73. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
74. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
75. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
76. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
77. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
78. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
79. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
80. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,
81. Koch, M., K. Savvatis, M. Scheeler, S. Dhayat, K. Bonaventura, T. Pohl, A. Riad,