Foxp3⁺CD4⁺ T Cells Improve Healing after Myocardial Infarction by Modulating Monocyte/Macrophage Differentiation

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ABSTRACT

**Rationale:** An exaggerated or persistent inflammatory activation after myocardial infarction (MI) leads to maladaptive healing and subsequent remodeling of the left ventricle. CD4+Foxp3+ regulatory T cells (Treg cells) contribute to inflammation resolution. Therefore, Treg cells might influence cardiac healing post-MI.

**Objective:** To study the functional role of Treg cells for wound healing post-MI in a mouse model of permanent left coronary artery ligation.

**Methods and Results:** Using a model of genetic Treg cell ablation (Foxp3DTR mice), we depleted the Foxp3+ Treg cell compartment before MI induction, resulting in aggravated cardiac inflammation and deteriorated clinical outcome. Mechanistically, Treg cell depletion was associated with M1-like macrophage polarization, characterized by decreased expression of inflammation-resolving and healing-promoting factors. The phenotype of exacerbated cardiac inflammation and outcome in Treg cell-ablated mice could be confirmed in a mouse model of anti-CD25 monoclonal antibody-mediated depletion.

In contrast, therapeutic Treg cell activation by superagonistic anti-CD28 monoclonal antibody administration 2 days after MI led to improved healing and survival. Compared to control animals, CD28-SA-treated mice showed increased collagen de novo expression within the scar, correlating with decreased rates of left-ventricular ruptures. Therapeutic Treg cell activation induced an “M2-like” monocyte differentiation within the healing myocardium, associated with myofibroblast activation and increased expression of monocyte/macrophage-derived proteins fostering wound healing.

**Conclusions:** Our data indicate that Treg cells beneficially influence wound healing after MI by modulating monocyte/macrophage differentiation. Moreover, therapeutic activation of Treg cells constitutes a novel approach to improve healing post-MI.

**Keywords:** Myocardial infarction, wound healing, macrophage subsets, CD4+ T cells

**Nonstandard Abbreviations and Acronyms:**
- CD: cluster of differentiation
- DTR: diphtheria toxin receptor
- DTX: diphtheria toxin
- EDA: end-diastolic area
- FXIII: transglutaminase factor XIII
- Foxp3: forkhead box P3
- FS: fractional shortening
- IFN: interferon
- Ig: immunoglobulin
- iNOS: induced NO synthase
- IL: interleukin
- Ly-6C: lymphocyte antigen 6C
- Ly-6G: lymphocyte antigen 6G
- MI: myocardial infarction
- mLN: mediastinal lymph nodes
- MΦ: monocytes/macrophages
- OPN: osteopontin
- TGF: transforming growth factor
- TNF: tumor necrosis factor
- WT: wildtype

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INTRODUCTION

Myocardial infarction (MI) is the most common cause of cardiac injury and results in the loss of large numbers of cardiomyocytes, eventually leading to ischemic heart disease and heart failure. Cardiac injury activates innate immunity initiating an inflammatory response. Cardiomyocyte death results in replacement by scar tissue and, after large myocardial infarction, in ventricular remodeling of the remote myocardium which further compromises cardiac function. Early cardiac wound healing is characterized by infiltration of innate immune cells, especially neutrophils and monocytes/macrophages, into the myocardium.

Previously, we could show that the adaptive immunity, more precisely CD4+ T lymphocytes, crucially impact on cardiac wound healing. CD4+ T cell-deficient mouse strains show accentuated cardiac inflammation, impaired wound healing, aggravated left-ventricular remodeling and impaired survival. Activation and proliferation of both conventional and Foxp3+CD25+ regulatory CD4+ T cells (Treg cells) takes place in heart-draining mediastinal lymph nodes as early as 3 days after MI. Treg cells play a crucial role in immune homeostasis and have been described to modulate immunity in terms of malignancies, infectious diseases, and transplant rejection. Moreover, Treg cells shape innate immune responses in terms of wound healing following injury. The observed activation of Treg cells after MI and the Treg cell-immanent capacity to modulate inflammation and healing processes prompted us to hypothesize that Treg cells influence wound healing after MI.

Different approaches were employed to unravel the impact of Treg cells on cardiac healing. First, Treg cell depletion prior to MI was achieved by employing Foxp3DTR mice in which Foxp3-positive cells transgenically express the human diphtheria toxin receptor resulting in specific Treg cell ablation following diphtheria toxin (DTX) administration. In another line of experiments, an anti-CD25 monoclonal antibody was administered resulting in phagocytosis-mediated Treg cell ablation due to their high CD25 expression at the cell surface.

Gain of Treg cell function was accomplished by therapeutic administration of superagonistic CD28-specific monoclonal antibodies (CD28-SA) that preferentially activate Treg cells over conventional CD4+ T cells in vivo due to a vigorous co-stimulatory signal induced by cross-linking of CD28 molecules. Monocytes and macrophages are of paramount importance for postinfarction healing. We therefore focused especially on T cell-mediated modulation of macrophage polarization. The results of the present report indicate that Treg cells are crucial for cardiac wound healing and that therapeutic Treg cell activation could become a novel therapeutic approach to improve clinical outcome by modulating monocyte/macrophage differentiation post-MI.

METHODS

Animals and surgery.
Mice between 8 and 12 weeks of age were used for all experiments. C57BL/6J mice were purchased from Harlan Laboratories. Mice expressing the diphtheria-toxin receptor under control of the Foxp3 promotor, like DEREG mice, namely B6.129(Cg)-Foxp3tm3Ayr/J (Foxp3DTR mice) and respective wildtype controls from Jackson Laboratory, USA were used here for pharmacological ablation of Treg cells. Mice underwent left coronary artery ligation as described previously. The study conformed to the regulations for animal experimentation and was approved by the local government. Briefly, mice were anesthetized with isoflurane. After intubation, thoracotomy was performed and MI induced by ligation of the proximal part of the left coronary artery. Buprenorphine was administered for analgesia after surgery. For sham operation, thoracotomy was performed without ligating the coronary artery. In depletion experiments, 500 ng DTX...
per mouse were administered intraperitoneally on day 2 and day 1 before MI induction. To prevent a rebound of the T<sub>reg</sub> cells, 250 ng DTX per mouse were additionally injected on day 2 and day 4 after MI. For antibody-mediated T<sub>reg</sub> cell depletion, 1 mg of anti-CD25 monoclonal antibody (clone PC61) was injected. For expansion of T<sub>reg</sub> cells, mice were treated with 300 µg of a superagonistic anti-CD28 monoclonal antibody (clone D665).

**Autopsy.**
At autopsy, large amounts of blood clots around the heart and within the thoracic cavity in combination with perforation of the infarcted wall indicated rupture.

**Echocardiography.**
Echocardiography was performed on a Toshiba Apio system with a 15-MHz ultrasound probe with the mice under light anesthesia with isoflurane (1.5 vol.%).<sup>17</sup>

**Fluorescence-Activated Cell Sorting.**
Cardiac scar tissue was digested with collagenase type 2 and protease type XIV (Sigma-Aldrich, Munich) as described previously.<sup>18</sup> Staining protocols are specified in the online-only Data Supplement.

**Localization of Foxp3<sup>+</sup> cells using immunofluorescence.**
Cryosections (5 µm) were fixed with 4% formaldehyde in PBS for 10 minutes, permeabilized with 0.5% dodecyltrimethylammonium chloride in PBS (all chemicals from Sigma-Aldrich, Munich). After blocking, Foxp3 was detected using the anti-mouse Foxp3 antibody clone: FJK-16s (eBioscience, Frankfurt). The staining protocol is specified in the online-only Data Supplement.

**Real-Time Reverse Transcriptase–Polymerase Chain Reaction.**
Quantitative real-time PCR was performed (iCycler from Bio-Rad, München) with commercial TaqMan probes (Life Technologies, Darmstadt). Target gene mRNA levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase. For real-time RT-PCR analysis of sorted cells, mRNA was amplified before cDNA synthesis using the C&E Trinucleotide Pico Kit (AmpTec, Hamburg) according to the manufacturer’s protocol.

**Purification and cell culture of monocytes/macrophages, T<sub>reg</sub> and T<sub>conv</sub> cells.**
For sorting of T cell subsets and monocytes, single-cell suspensions were prepared from spleens as well as inguinal, cervical and axillary lymph nodes. Monocytic cells were defined as CD11b<sup>+</sup>Ly-6G<sup>−</sup>CD11c<sup>+</sup>. T<sub>reg</sub> cells were defined as CD4<sup>+</sup>CD25<sup>high</sup>, T<sub>conv</sub> cells as CD4<sup>+</sup>CD25<sup>+</sup>. 2x10<sup>5</sup> cells seeded per well were seeded in 96-well flat bottom plates (greiner bio-one, Frickenhausen). In co-culture experiments, 1x10<sup>5</sup> cells per cell type were used. All cell cultures were supplemented with 25 IU/ml recombinant IL-2 (Proleukin<sup>®</sup>, Novartis, Basel) and, where denoted, anti-TGFβ antibody (10 µg/ml, clone 1D11, R&D, Wiesbaden) or recombinant cytokines. CD28-SA (clone D665, AbD Serotec, Raleigh) or isotype control Ig (clone MOPC-21, Bio-X-Cell, West Lebanon) was coated on pan mouse IgG dynabeads (Invitrogen, Darmstadt) and added to cell cultures where indicated.

**Analysis of cytokine concentrations.**
Cell culture supernatants were analysed for presence of osteopontin by ELISA (R&D, Wiesbaden) as well as TNF, IFNγ, IL-6, IL-2, IL-4, IL-13 and IL-17 by Cytometric Bead Array (BD Biosciences, Heidelberg) according to the manufacturer's protocol. The latter results were analyzed with FCAP Array Version 2.0 (Soft Flow, Duesseldorf).

**Western blot.**
After blocking, nitrocellulose membranes were incubated overnight in presence of rabbit anti-rat collagen type I (Cedarlane, Burlington, CL50141AP) and rabbit anti-rat collagen type III (Cedarlane, Burlington,
The membrane was developed using an ECL detection system. After membrane stripping, total protein amount per lane was assessed by detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Statistical analysis.**

All data are presented as mean values per group and SEM. For comparison of two groups, an unpaired *t* test or, if a *t* test was not suitable, a Wilcoxon signed rank-sum test was performed. For multiple comparisons, two-way ANOVA was used. Survival is shown as Kaplan-Meier curve and data was analyzed by a log-rank test. Variance in a group was assessed using the χ² test. Differences were considered as statistically significant at *P*<0.05. Data analysis was performed using GraphPad Prism 4.03 (GraphPad Software Inc, San Diego).

**RESULTS**

**Foxp³⁺CD4⁺ regulatory T cells become activated in response to MI.**

In order to monitor Treg cell activation after MI we analysed heart-draining lymph nodes. There, the activation markers CD25 (Fig. 1A) and Helios (Online Figure I) were upregulated in Foxp³⁺CD4⁺ Treg cells 7 days post-MI compared to sham-operated animals. As previously reported by us, increased frequencies of Treg cells were found in mediastinal lymph nodes on days 3 and 7 post-MI as compared to sham-operated animals analyzed on day 7 after surgery (Fig 1B). Remarkably, compared to sham-operated mice, the percentage of Foxp³⁺ cells among CD4⁺ T cells stayed elevated for at least 56 days (Fig. 1B). Absolute cell numbers of Treg cells were also increased peaking on day 7 after MI (Fig. 1B). In addition to Treg cell expansion kinetics in heart-draining lymph nodes, we evaluated Treg cell infiltration into the healing infarct. Compared to sham-operated mice, both frequencies and absolute numbers of Treg cells were increased post-MI (1C, D). Moreover, using immunofluorescence microscopy, single Foxp3-positive cells were detected in the infarct border and within the remote myocardium (Fig. 1E, Online Figure II).

Hence, we could show that Treg cells become activated in response to MI, followed by Treg cell expansion in heart-draining lymph nodes and successive infiltration into the healing infarct.

**Specific Treg cell ablation in Foxp³DTR mice leads to increased infarct size and cardiac deterioration.**

To evaluate the influence of Treg cells on wound healing after MI, we employed Foxp³DTR mice to specifically ablate Foxp3-expressing cells that transgenically express the human diphtheria toxin receptor. Two days after diphtheria toxin administration, Foxp3⁺ cells were effectively depleted from blood, mediastinal lymph nodes and hearts as compared to DTX-treated wildtype (WT) littermates (Fig. 2A). Survival was not significantly different between the two groups, but infarct sizes in Treg cell-depleted mice were significantly increased as compared to the control group. Further, Treg cell ablation resulted in a tendency towards a more pronounced left-ventricular dilation in line with the impaired cardiac function evaluated by apical fractional shortening on both day 3 and day 7 post-MI (Fig. 2C, Online Tables I and II). Consistently, Foxp³DTR mice showed increased lung weight/ body weight ratios underlining the compromised cardiac function in these mice (Fig. 2D). With respect to procollagen synthesis, expression of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs), no differences were identifiable between the groups (Online Figure III). In accordance with this observation, there were no differences regarding the frequencies of left-ventricular ruptures (data not shown). Treg cell ablation, thus, resulted in poorer clinical outcome after MI without, however, directly affecting collagen turnover and scar formation.
**T**reg cell ablation results in increased numbers of both inflammatory myeloid cells and lymphocytes associated with M1-like macrophage polarization.

The observation that specific Treg cell depletion leads to increased infarct sizes and impaired cardiac function prompted us to focus on the leukocyte influx into the infarct zone. Both monocyte subsets and neutrophils have been demonstrated to influence cardiac wound healing after MI. Using FACS analyses, neutrophils were defined as Ly-6G\(^{+}\)CD11b\(^{+}\). Compared to control mice, neutrophil numbers were significantly increased in the infarct zone of Treg cell-depleted animals (Fig. 3A). Furthermore, we discriminated between monocyte subsets based on Ly-6C surface expression. In comparison to control mice, the proportion of inflammatory Ly-6C\(^{\text{high}}\) cells among CD11b\(^{+}\)F4-80 Ly-6G\(^{-}\) monocytes was elevated in the infarct zone of Treg cell-ablated mice (Fig. 3B).

Since Treg cells are capable of regulating inflammatory reactions, we evaluated macrophage polarization in Treg cell-sufficient and Treg cell-ablated mice. Expression of prototypic markers for inflammatory M1 macrophage polarization was assessed in CD11b\(^{+}\)Ly-6G\(^{-}\) monocytes/macrophages sorted from the healing infarct 5 days post-MI. Compared to diphtheria toxin-treated WT mice, monocytic cells sorted from Foxp3\(^{\text{DTR}}\) mice showed significantly higher mRNA expression of inducible NO synthase (iNOS), but no difference regarding tumor necrosis alpha (TNF\(\alpha\)) mRNA synthesis (Fig. 3C). Moreover, compared to wildtype littermates, mRNA expression of M2-associated anti-inflammatory interleukin-10 (IL-10) and transforming growth factor beta 1 (TGF\(\beta\)) in line with mRNA synthesis of wound-stabilizing osteopontin (OPN) and transglutaminase factor XIII (FXIII) were downregulated in monocytic cells sorted from the healing infarct of Treg cell-ablated mice (Fig. 3C).

In addition to characterizing the myeloid cell compartment, both T cell infiltration into the infarct zone and expression of T cell mediators were assessed. Compared to WT littermates, absolute numbers of CD4\(^{+}\) and CD8\(^{+}\) T cells were increased in the healing infarct of Treg cell-ablated mice 5 days post-MI (Fig. 3D). Analysis of IFN\(\gamma\) and TNF\(\alpha\) mRNA synthesis in bulk scar tissue homogenates (Fig. 3D) and increased frequencies of IFN\(\gamma\) and TNF\(\alpha\)-positive cells among CD4\(^{+}\) and CD8\(^{+}\) T cells in heart-draining lymph nodes (Fig. 3E) suggest increased production of these pro-inflammatory mediators by heart-infiltrating T cells in Treg cell-depleted mice.

Therefore, the poor clinical outcome of Treg cell-depleted mice was associated with impaired M2-like differentiation of cardiac macrophages and pronounced infiltration of the heart by pro-inflammatory T cells.

Anti-CD25 monoclonal antibody-mediated Treg cell depletion prior to MI results in impaired remodeling and survival.

To confirm the phenotype of deteriorated clinical outcome in Foxp3\(^{\text{DTR}}\) mice post-MI, we employed an additional approach of Treg cell ablation. The Treg cell compartment was depleted prior to MI induction by administration of a monoclonal rat anti-mouse CD25 antibody 8 days prior to MI. In anti-CD25 antibody-treated animals, Treg cells were nearly completely absent from peripheral blood on the day of MI induction and still significantly reduced in mediastinal lymph nodes on day 7 post-MI, whereas treatment with an isotype-matched control antibody of irrelevant specificity (isotype control Ig) did not provoke Treg cell depletion (Fig. 4A).

Antibody-mediated Treg cell depletion led to a significantly higher mortality as compared to control mice (Fig. 4B). By day 7, survival was 55.9% in control mice and 25% in Treg cell-ablated animals (Fig. 4B). Regarding frequencies of left-ventricular ruptures, no differences could be found between treatment groups. Thus, Treg-cell depleted mice died presumably due to a higher incidence of cardiac failure as
indicated by greater echocardiographic expansion of the LV area on day 7 after MI (Fig. 4C, Online Table III). Congruently with the Foxp3DTR model, the infarct zone of the anti-CD25 antibody-treated mice showed increased numbers of neutrophils 7 days after MI as compared to the control group, in line with higher frequencies of pro-inflammatory Ly-6C\textsuperscript{high} cells among monocytes (Fig. 4D).

Thus, both in Foxp3DTR mice as well as in anti-CD25 antibody-treated mice T\textsubscript{reg} cell depletion was associated with enhanced recruitment of neutrophils and Ly-6C\textsuperscript{high} monocytes into the infarcted myocardium, impaired cardiac function and anti-CD25 antibody treatment even increased mortality after MI.

Therapeutic T\textsubscript{reg} cell activation after MI induction results in enhanced recruitment of T\textsubscript{reg} cells into the infarct zone and improves scar tissue formation and survival.

Since the lack of T\textsubscript{reg} cells aggravated clinical outcome, we focused on T\textsubscript{reg} cell expansion in order to improve wound healing post-MI. T\textsubscript{reg} cell activation was accomplished in a therapeutic fashion, i.e. after MI. The peak of the physiological T\textsubscript{reg} cell response was found to be between day 3 and day 7 after MI induction (Fig. 1B). For therapeutic T\textsubscript{reg} expansion we used a superagonistic anti-CD28 antibody (CD28-SA). The CD28-SA-mediated T\textsubscript{reg} cell expansion reaches its full effect at the earliest 2 days after a single administration.\textsuperscript{15} Thus, the CD28-SA was administered on day 2 after MI induction in order to support the physiological expansion of T\textsubscript{reg} cells in response to MI. By day 3 after CD28 administration, T\textsubscript{reg} cell frequencies among CD4\textsuperscript{+} T cells in blood and heart-draining lymph nodes were more than 2-fold elevated in CD28-SA-treated animals (Fig. 5A).

In comparison to control animals, CD28-SA-treated animals had significantly improved survival owing to fewer left-ventricular ruptures during the first 7 days after MI (Fig. 5B, C). After CD28-SA treatment, 5\% of the infarcted animals suffered from left-ventricular ruptures. In control animals, 23\% of infarcted mice died from cardiac rupture which is in accordance with former studies.\textsuperscript{19, 20}

By day 56, survival was 47.1\% in isotype control Ig-treated mice and 76.6\% in CD28-SA-treated animals. In surviving mice, echocardiography on day 7 and day 56 revealed no statistically significant differences between the treatment groups (Online Table IV). Better survival of mice with large MI after CD28-SA treatment presumably explains the observation that no attenuation of adverse remodeling was found in the CD28-SA group compared to isotype control Ig-treated mice.

Having observed that therapeutic T\textsubscript{reg} cell activation resulted in decreased rates of heart ruptures, we focused on scar tissue formation and the extracellular collagen matrix (ECM). On day 5 post-MI, CD28-SA-treated mice showed, compared to control antibody treated animals, increased mRNA expression of both pro-collagen alpha-1 (I) and pro-collagen alpha-1 (III) that constitute integral components of the reparative scar (Fig. 5D). Elevated expression of both collagen I and collagen III in CD28-SA-treated mice was confirmed at the protein level (Fig. 5E). Consistently, mesenchymal vimentin and alpha smooth muscle actin (\(\alpha\)-SMA) mRNA levels were upregulated in the healing myocardium 5 days post-MI, but not mRNA synthesis of von Willebrand factor (vWF), reflecting increased myofibroblast numbers rather than accelerated angiogenesis after CD28-SA treatment (Online Figure IVA, B). Increased myofibroblast numbers were confirmed by \(\alpha\)-SMA immunohistochemistry staining (Online Figure IVC). Moreover, compared to control mice, CD28-treated mice showed increased mRNA expression of both TIMP-1 and TIMP-2, endogenous tissue inhibitors of matrix metalloproteinases preventing ECM degradation (Online Figure IVD).

To better understand how CD28-SA-activated T\textsubscript{reg} cells improve scar tissue formation, we evaluated T\textsubscript{reg} cell infiltration into the healing myocardium. In CD28-SA-treated animals, the frequency of bulk CD4\textsuperscript{+} T cells among CD45\textsuperscript{+} leukocytes was slightly reduced on day 5 post-MI as compared to control
animals (Fig. 6A). However, the proportion of Foxp3⁺ Treg cells was about 50% in CD28-SA-treated mice as compared to approximately 25% in isotype control Ig-treated animals (Fig. 6B, C). Absolute Treg cell numbers were increased in CD28-SA-treated mice on day 5 post-MI (Fig. 6D). The increased prevalence of Foxp3⁺ cells in the scar tissue of CD28-SA-treated mice could be confirmed by immunohistochemistry stainings (Fig. 6E), while CD28-SA treatment in the absence of tissue injury did not lead to Treg cell accumulation in the heart (Fig. 6F).

Collectively, therapeutic Treg cell activation leads to increased recruitment of Foxp3⁺ regulatory CD4⁺ T cells to the infarcted myocardium, enhances de novo extracellular matrix formation in the infarct zone, and consequently reduces ventricular rupture and mortality after MI.

**Therapeutic Treg cell activation induces macrophage M2 differentiation in the scar tissue.**

As monocytic cells are key players in wound healing post-MI, we focused on monocyte recruitment to the infarct zone. Regarding absolute cell numbers and monocyte subset composition, defined on the basis of Ly-6C expression levels, we could not detect significant differences between groups (data not shown). However, having observed that Treg cell ablation leads to M1-like macrophage polarization, we further assessed the monocyte/macrophage activation state in the healing myocardium of CD28-SA-treated mice. In scar tissue homogenates of CD28-SA-treated mice, M2-inducing IL-13, IL-10 and TGFβ1 mRNA expression were upregulated on day 5 post-MI as compared to isotype control Ig-treated mice (Fig. 7A). Increased expression of prototypical M2-associated arginase I and the ECM-bracing M2 effectors OPN and FXIII in bulk scar tissue homogenates further indicated that M2 cells are generated in these hearts (Fig. 7B). 21, 22

In order to directly follow expression of M2 markers in monocytes/macrophages after CD28-SA treatment, we sorted CD11b⁺Ly-6G⁻ monocytic cells from the scar tissue and evaluated mRNA expression of both M1 and M2 marker genes. Compared to isotype control Ig-treated mice, M2-associated expression of arginase I and CD206 were significantly upregulated in monocytic cells sorted from the scar of CD28-SA-treated animals (Fig. 7C). Consistently, the M2-mediators TGFβ1 and IL-10 were also upregulated in these cells, while M1-associated TNFα (Fig. 7D), but not IL-1β or IL-6 (Online Figure V), was downregulated.

Conclusively, therapeutic CD28-SA treatment leads to M2-like macrophage differentiation in the healing myocardium and expression of mediators such as OPN and FXIII, factors well-known to contribute to myocardial healing.

**Treg cell-derived soluble mediators induce M2-like macrophage polarization in vitro.**

Given that Treg cell numbers were elevated in the infarct zone of CD28-SA-treated mice, we speculated that Treg cells might modulate expression of M2-associated genes in monocytic cells. In order to assess how Treg cells modulate monocyte/macrophage differentiation in CD28-SA-treated mice, we set up an in vitro cell culture system with primary CD4⁺ T cells and monocytic cells sorted from secondary lymphoid organs of naïve animals (Online Figure VI, VII). Mono-cultures of sorted Treg cells, conventional T cells or monocytes showed comparable minor secretion of OPN in the presence of CD28-SA and IL-2 after 4 days of incubation (Fig. 8A). However, co-culture of monocytes and Treg cells in the presence of CD28-SA and IL-2 resulted in a dramatic increase in OPN secretion whereas an unspecific isotype control Ig and IL-2 induced only mild OPN release (Fig. 8A). Since Treg cell numbers are, compared to monocyte/macrophage numbers, relatively small, we titrated Treg cells in the co-culture system. Even at a Treg cell:macrophage ratio of 1 : 25, OPN release was still higher as compared to monocytes cultivated in complete absence of Treg cells (Fig. 8B).
In order to test, if T_{reg} cell-derived soluble mediators modulate OPN secretion by monocytes and to show that OPN is, indeed, secreted by the monocytes in our co-cultures, we activated T_{reg} cells for 4 days by CD28-SA and IL-2 stimulation in vitro and subsequently cultured monocytes with a volume ratio of T_{reg} cell supernatant to fresh culture medium containing purified monocytes of 1:1. Monocytes incubated with supernatant from T_{reg} cells that had been cultivated in the presence of isotype control Ig and IL-2 were used as reference. After 3 days of incubation, monocytic cells had released high amounts of OPN only in response to mediators in the supernatant of CD28-SA-activated T_{reg} cells (Fig. 8C). Moreover, OPN release from monocytic cells was accompanied by CD206 and arginase I mRNA upregulation indicating M2-like macrophage polarization (Fig. 8C).

To identify the T_{reg} cell-derived mediators that might modulate the monocyte phenotype and function in our test system, we analyzed cytokine secretion into the supernatants of T_{reg} cells after CD28-SA stimulation for 4 days in vitro. Compared to T_{reg} cells cultivated in the presence of isotype control Ig and IL-2, stimulation with CD28-SA and IL-2 resulted in a modest release of TGF{\beta}1 as well as strong secretion of both IL-10 and IL-13, but no release of IL-2 or IL-4 (Fig. 8D).

Having observed a CD28-SA-induced release of IL-10, IL-13 and TGF{\beta}1 from T_{reg} cells in vitro, we tried to induce OPN secretion from monocytes as indicator for M2-polarization by cultivation in the presence of the aforementioned cytokines. Supplementation with TGF{\beta}1 alone did not influence OPN release from monocytes, but simultaneous presence of TGF{\beta}1 and IL-10 induced OPN secretion which could be further increased by addition of IL-13. Neutralization of TGF{\beta}1 dramatically restrained IL-10- and IL-13-driven OPN release from the monocytes (Fig. 8E).

Conclusively, TGF{\beta}1, IL-13 and IL-10 produced by CD28-SA-activated T_{reg} cells synergized in inducing M2-like differentiation and subsequent OPN release from monocytes/ macrophages in vitro.

**DISCUSSION**

Wound healing post-MI requires an orchestrated inflammatory response. Temporal and spatial containment of inflammation is a prerequisite for arrayed wound healing and prevention of adverse ventricular remodeling. Based on our previous finding that T_{reg} cells become activated after MI and due to their potent immuno-suppressive capacity we hypothesized that T_{reg} cells might influence cardiac healing.

T_{reg} cell depletion deteriorates healing after MI.

To address this hypothesis we ablated T_{reg} cells prior to MI induction. T_{reg} cell ablation in Foxp3\textsuperscript{DTR} mice resulted in an increased infarct size and, thus, left-ventricular dilation that was consistently associated with impaired cardiac function. The phenotype of impaired outcome in the absence of T_{reg} cells could be confirmed in a model of anti-CD25 antibody-mediated T_{reg} cell depletion. Anti-CD25 antibody-treated mice showed a significantly increased left-ventricular dilation and, most strikingly, an impaired survival, suggesting that these mice succumbed predominantly to heart failure. This observation is in accordance with the phenotype of infarcted CCR5 knockout mice in which the attenuated recruitment of T_{reg} cells, in line with a decreased influx of other cell types, correlates with adverse remodeling and cardiac deterioration.\textsuperscript{23}

Neither of the two T_{reg} cell depletion models showed a predisposition to develop left-ventricular ruptures. This observation is seemingly at odds with the finding that CD28-SA-treated mice exhibited a reduced incidence of LV ruptures. However, CD28-SA treatment increased the expression pro-collagens as well as TIMP-1 and TIMP-2 indicating that activated T_{reg} cells and M2 cells augmented or even
accelerated scar tissue construction that likely prevented LV ruptures in these hearts. In contrast, T_{reg} cell depletion did not restrain mRNA synthesis of scar-forming collagens or collagenolytic enzymes compared to control mice implying that T_{reg} cell deficiency and M1 differentiation do not impair myofibroblast function during scar tissue formation. However, as a limitation of this study, we cannot exclude that DTX- and anti-CD25 antibody-mediated ablation of T_{reg} cells might have differentially influenced the prevalence of fatal cardiac arrhythmias. We assume that the differences in survival observed between the two models of induced T_{reg} cell deficiency are due to off-target or indirect effects that both the DTX injections\textsuperscript{24} and anti-CD25 antibody treatments\textsuperscript{25} are known to be associated with to some degree.

In both models, the healing myocardium of T_{reg} cell-depleted mice harbored increased numbers of inflammatory myeloid cells, i.e. neutrophils, Ly-6Chigh monocytes and M1-polarized macrophages. The observation of enhanced recruitment of myeloid cells to inflammatory sites is in accordance with other models of wound healing and inflammation. In a mouse model of healing after burn injury, the lack of T_{reg} cells correlated with an increased influx of innate immune cells into the lesion.\textsuperscript{10} Moreover, T_{reg} cells modulate chemokine expression during inflammation influencing myeloid cell infiltration.\textsuperscript{26} In addition to an increased proportion of inflammatory myeloid cells, both conventional CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells showed a pronounced accumulation in the infarct zone of T_{reg} cell-depleted Foxp3\textsuperscript{DTR} mice, contributing to the increased synthesis of TNF\textsubscript{α} and IFN\textsubscript{γ} that both are individually capable of inducing M1 macrophage polarization.\textsuperscript{27}

T_{reg} cell-ablated Foxp3\textsuperscript{DTR} mice exhibited a significantly increased infarct size and deteriorated cardiac function. From the mechanistic point of view, escalated TNF\textsubscript{α} synthesis in combination with a disturbed M2 differentiation likely provokes the maladaptive phenotype. TNF\textsubscript{α} is capable of depressing cardiac contractile function and elicits cardiomyocyte apoptosis as well as left-ventricular dilation.\textsuperscript{28,29} The detrimental impact of this cytokine on postinfarction remodeling is further underscored in TNF\textsubscript{α} knock-out mice that are characterized by a preservation of cardiac function.\textsuperscript{30} Macrophages in the infarct zone of T_{reg} cell-depleted mice further showed an increased synthesis of iNOS that critically compromises cardiac function after MI. Accordingly, previous reports showed that pharmacological inhibition or genetic iNOS deficiency in mice lead to infarct size reduction and ameliorated remodeling.\textsuperscript{31-34} NO-dependant cytotoxicity is mediated by NO-derived radicals and an inactivation of sulphur-iron-centered enzymes that are crucially involved in metabolic pathways.\textsuperscript{35,36} Enlarged infarct size in Foxp3\textsuperscript{DTR} mice, thus, likely arises partially from a secondary loss of cardiomyocytes.\textsuperscript{37} However, although we could not detect differential expression of collagensases that are critically involved in ECM turnover, we cannot definitely rule out an escalated overall enzyme activity in the hearts of Foxp3\textsuperscript{DTR} mice, for instance due to MMP activation by reactive nitrogen and oxygen species that may have further aggravated infarct expansion.\textsuperscript{38} Consistent with iNOS upregulation, monocytic cells showed an attenuated M2 polarization which has been shown by unrelated experimental studies to be associated with aggravated left-ventricular dilation and postinfarction cardiac dysfunction.\textsuperscript{20,39} Reduced synthesis of wound-stabilizing OPN and FXIII as well as inflammation-resolving TGFβ1 likely further deteriorated the outcome in T_{reg} cell-ablated mice.\textsuperscript{40-42}

Activation of T_{reg} improves healing after MI.

As T_{reg} depletion showed pronounced effects during the phase of myocardial healing we next studied whether the obvious beneficial effect that T_{reg} cells physiologically exert on post-infarct wound healing could be further increased by therapeutic T_{reg} cell activation with a CD28-SA after MI. This approach is similar to a recently published report stating that administration of a CD28-SA in rats beneficially influenced cardiac remodeling.\textsuperscript{43} In our mouse model, CD28-SA-treated animals had, compared to isotype control Ig-treated mice, significantly higher T_{reg} cell numbers in the healing myocardium 5 days after MI. This strengthens our interpretation that the therapeutic effect we observed after CD28-SA treatment was, indeed, due to T_{reg} cell activation and not a result of reduced T cell egress from secondary lymphoid organs as has also been observed after CD28-SA treatment in vivo.\textsuperscript{44}
Therapeutic T<sub>reg</sub> cell activation triggers M2-like monocyte differentiation.

Herein, we demonstrate for the first time that therapeutic T<sub>reg</sub> cell activation/expansion by CD28-SA treatment enhances M2-like monocyte differentiation. We believe that regulation of monocyte differentiation by T<sub>reg</sub> cells certainly is not the only mechanism but constitutes a crucial contribution to the beneficial effects of therapeutic and most likely also physiological T<sub>reg</sub> cell activation after MI. Alternatively activated “M2-like” macrophages have well known anti-inflammatory characteristics and are an integral component of wound healing processes. Consistently, experimental modulation of macrophage polarization towards an M2 state has been previously demonstrated to improve wound healing post-MI. Alternative macrophage activation is initiated in response to IL-4 or IL-13, and other stimuli like IL-10 or TGF<sub>β</sub> inducing an “M2-like” phenotype. We show here that T<sub>reg</sub> cells produced little TGF<sub>β</sub>1 but high amounts of IL-10 and IL-13 in vitro upon CD28-SA activation capacitating the cells to induce M2-like macrophage differentiation. In vivo, the accumulation of T<sub>reg</sub> cells in the hearts of CD28-SA-treated mice was associated with elevated amounts of TGF<sub>β</sub>1, IL-10, and IL-13 within the cardiac scar tissue suggesting that the activated T<sub>reg</sub> cells also induced an M2-polarizing milieu locally within the heart (see model in Online Figure VIII). Production of IL-10 and TGF<sub>β</sub>1 by M2-like cells themselves might have further contributed to the M2-creating milieu in situ. However, we cannot exclude T<sub>reg</sub> cell-mediated contact-dependent mechanisms that may also contribute to monocyte/macrophage polarization.

Molecular mediators of improved healing after therapeutic T<sub>reg</sub> cell activation.

TGF<sub>β</sub> is considered to drive collagen deposition by myofibroblasts. In CD28-SA-treated mice, both activated T<sub>reg</sub> cells and M2 macrophages likely contribute to increased TGF<sub>β</sub>1 levels in the healing myocardium as compared to control animals. Moreover, IL-13, which was strongly induced by CD28-SA treatment, synergizes with TGF<sub>β</sub>1 in promoting collagen synthesis in myofibroblasts. Consistently, before the completion of healing 5 days post-MI, the infarct zone of CD28-SA-treated mice showed an increased amount of collagen, indicating accelerated scar tissue formation.

Collagen production and array is also crucially affected by OPN which was significantly upregulated in the scar tissue of CD28-SA-treated mice. The healing myocardium of OPN KO mice has been shown to exhibit disarrayed and decreased collagen deposition, implying an essential role of OPN in matrix assembly and organization. Therefore, elevated OPN levels in the healing myocardium likely contribute to wound-stabilizing scar tissue formation in CD28-SA-treated mice.

In other situations of tissue trauma such as non-ischemic skeletal muscle damage formation of a collagenous scar can be avoided and full tissue recovery is possible. Under such circumstances T<sub>reg</sub> cells have also been shown to beneficially influence the healing process, in this case by suppressing OPN production in favour of myotrophic factors like amphiregulin with the latter being produced by muscle-infiltrating T<sub>reg</sub> cells themselves.

As after MI OPN favours the formation of a robust collagenous scar we set up an in vitro system to assess the contribution of monocytic cells and T<sub>reg</sub> cells to the amount of cardiac OPN in vivo. T<sub>reg</sub> cells stimulated with a CD28-SA in vitro secreted TGF<sub>β</sub>1, IL-10, and IL-13 which, in combination, provoked strong OPN release from monocytic cells along with M2 differentiation. TGF<sub>β</sub> neutralization dramatically restrained OPN release from monocytic cells, which is in line with the observation that a lack of TGF<sub>β</sub> receptor engagement inhibits M2 polarization. However, TGF<sub>β</sub> receptor signaling alone was not sufficient to elicit full OPN secretion, showing that TGF<sub>β</sub> is required to render the monocytic cells responsive to T<sub>reg</sub> cell-derived IL-10 and IL-13 driving OPN expression.
As monocytic cells are the predominant leukocyte fraction in the scar tissue 5 days after MI, a large proportion of OPN within the healing myocardium of CD28-SA-treated mice might be derived from monocytes/macrophages. Nevertheless, we cannot exclude the contribution of other cell types to the observed increase in OPN expression.

Apart from OPN, M2-associated transglutaminase FXIII also crucially improves scar tissue integrity by promoting cross-linking of ECM components. FXIII deficiency in mice and low expression in humans correlates with a high incidence of cardiac rupture after MI. Gain of FXIII function by intravenous FXIII administration in mice results in both increased collagen fiber density as well as in decreased rates of heart ruptures. Thus, increased FXIII expression in the myocardium of CD28-SA-treated mice very likely also contributes to improved scar tissue formation (Online Figure VIII).

Clinical implications.

The concept of manipulating the Treg cell compartment in order to alleviate inflammatory disorders in human patients has been proposed years ago. Recently, a human CD28-SA was successfully tested in a phase I clinical trial provoking a significant increase of the Treg cell signature mediator IL-10 in the plasma following treatment. Therefore, administration of a CD28-SA may become an eligible treatment modality to improve postinfarction healing in the clinical arena. Moreover, other unrelated strategies have been developed to selectively expand Treg cells in vivo. E.g. recombinant human IL-2 is used at low dosages to selectively expand Treg cells over conventional T cells in human patients. Complexing IL-2 with an anti-IL-2 monoclonal antibody, currently used only experimentally, probably holds even more clinical promise than low dose IL-2 treatment as it also increases the suppressive activity of the Treg cells on a per-cell basis. In conclusion, we have shown that on the one hand Treg cells beneficially regulate wound healing and thus improve clinical outcome mostly by attenuating inflammation within the healing myocardium. On the other hand, therapeutic activation of Treg cells especially improves the replacement of necrotic tissue by a stable collagenous scar and thus prevents left-ventricular dilation and rupture. This potentially implicates high clinical relevance as modulation of both the Treg cell compartment and monocyte differentiation was induced days after MI induction, resulting in improved survival (also see scheme in Online Figure VIII). Thus, there might be a therapeutic window of days to weeks in humans to prevent expansion of the myocardial infarct zone, left-ventricular rupture post-MI, and detrimental progressive remodeling of viable myocardium by such an approach.

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DISCLOSURES
None.
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FIGURE LEGENDS

Figure 1: T_{reg} cell activation in heart-draining lymph nodes and infiltration into the infarct zone.
A: Representative FACS plots showing CD25 expression on CD4^+ T cells and CD25 mean fluorescence intensity on Foxp3^+CD4^+ T cells from sham-operated and infarcted mice 7 days post-MI (n=3-4 per group, *P<0.05). B: Kinetics of T_{reg} cell expansion in heart draining lymph nodes of infarcted and sham-operated mice analyzed on day 7 post-surgery (n=3-5 per group, *P<0.05 vs. sham d7). C: Representative FACS plots showing Foxp3 frequencies among CD4^+ T cells in a non-infarcted heart and among CD4^+ T cells in the infarct zone 7 days post-MI. D: Kinetics of absolute T_{reg} cell numbers in the healing infarct and T_{reg} cell numbers in the hearts of sham-operated mice analyzed on day 7 after surgery (n=3-8 per group, *P<0.05 vs. sham d7). E: Immunofluorescence staining of Foxp3^+ cells (purple, arrow head) in both the infarct border zone and the remote myocardium. Cardiomyocytes were stained using Alexa 488 phalloidin (green). Nuclei are depicted in blue (DAPI). Images were acquired at 400-fold magnification.

Figure 2: Clinical parameter of T_{reg} cell-sufficient wildtype and T_{reg} cell-ablated Foxp3^{DTR} mice.
A: Representative FACS plots and quantitative analysis showing the frequency of Foxp3^+ cells among CD4^+ T cells in both blood, mediastinal lymph nodes (mLN) and the scar (n=3 per group, *P<0.05). B: Survival (n=14-17 per group) and infarct size (n=8-11 per group, *P<0.05) of diphtheria toxin-treated mice. C: Apical end-diastolic area 7 days post-MI and apical fractional shortening 3 and 7 days after MI in diphtheria toxin-treated mice (n=8-11 per group). D: Lung weight to body weight ratio 7 days after MI (n=8-11 per group, *P<0.05).

Figure 3: Infiltration and polarization of myeloid cells and T cells in wildtype and Foxp3^{DTR} mice.
A: Representative FACS plots showing frequencies of Ly-6G^+ neutrophils among all leukocytes and absolute neutrophil numbers per mg scar tissue 7 days post-MI (n=3-6 per group, *P<0.05). B: Representative FACS plot depicting the Ly-6C expression on monocytes/macrophages (MΦ) and frequency of Ly-6C^high cells among monocytes in the scar tissue 7 days after MI (n=3-5 per group, *P<0.05). C: mRNA expression of M1-associated iNOS and TNFα as well as M2-associated effectors IL-10, TGFβ1, OPN and FXIII in CD11b^+Ly-6G^+ monocytes/macrophages sorted from the scar tissue 5 days post-MI (iNOS: induced NO synthase, TNFα: tumor necrosis factor alpha, IL-10: interleukin-10, TGFβ1: transforming growth factor beta 1, OPN: osteopontin, FXIII: transglutaminase factor XIII, n=5 per group, *P<0.05, n.s. not significant). D: Absolute numbers of CD4^+ and CD8^+ T cells in the scar tissue as well as mRNA expression of T cell mediators interferon gamma (IFNγ) and TNFα in bulk scar tissue homogenates 5 days post-MI (n=3 per group, *P<0.05). E: IFNγ (left) and TNFα (right) expression in both CD4^+ and CD8^+ T cells in heart-draining lymph nodes 5 days post-MI.

Figure 4: Innate immune cell infiltrate and clinical outcome in T_{reg} cell-sufficient mice and T_{reg} cell-depleted animals (anti-CD25 antibody-mediated ablation).
A: Representative histograms and quantitative analysis showing the frequency of Foxp3^+ cells among blood and mediastinal lymph node (mLN) CD4^+ T cells after administration of an anti-CD25 monoclonal antibody or an isotype control antibody (Ig) of irrelevant specificity (n=3 per group, *P<0.05). B: Survival of anti-CD25 and isotype control Ig-treated mice after myocardial infarction (MI). C: Apical end-diastolic area (EDA) 7 days after MI in T_{reg} cell-sufficient and T_{reg} cell-deficient mice (n=4-7 per group, *P<0.05). D: Evaluation of neutrophil numbers and frequency of Ly-6C^high cells among monocytes within the scar tissue (n=3-7 per group; *P<0.05).

Figure 5: Expansion of T_{reg} cells after therapeutic CD28-SA treatment improves survival and reduces frequencies of left-ventricular ruptures.
A: Frequency of Foxp3^+ cells among blood CD4^+ T cells 3 days after administration of CD28-SA or isotype control antibody (Ig). Antibody injection was performed 2 days after MI induction. B: Survival of CD28-SA and isotype control Ig-treated mice (isotype control Ig n=47 at baseline, CD28-SA n=42 at baseline; *P<0.05). C: Frequency of left-ventricular ruptures in isotype
control Ig- or CD28-SA-treated mice during the first 7 days post-MI; n=45-62 per group, *P<0.05). D: Real-time RT-PCR analysis of pro-collagen alpha-1 (I) (Col1a1) and pro-collagen alpha-1 (III) (Col3a1) mRNA expression in the healing infarct 5 days post-MI (n=4-7 per group, *P<0.05). E: collagen I, collagen III and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels in the scar of treated mice 5 days post-MI.

**Figure 6**: CD4+ T cell infiltrate in the scar tissue of CD28-SA and isotype control antibody (Ig)-treated mice 5 days after MI (A-E) or sham surgery (F). A: Representative FACS plots showing CD4+ T cell frequencies among CD45+ leukocytes in the scar tissue. Numbers indicate percentage among all CD45+ leukocytes. B: Representative histograms depicting Foxp3+ cells among CD4+ T cells. C: Proportion of Foxp3+ cells among CD4+ T cells in the scar tissue. D: Absolute numbers of Foxp3+CD4+ Treg cells in the scar tissue of treated animals (n=4-5 per group; *P<0.05). E: Immunofluorescence staining of Foxp3+ cells in the scar tissue 5 days after MI. Foxp3 is depicted in purple (arrow heads). Nuclei were stained using DAPI. The scale bar indicates 100 µm. F: Representative FACS plots showing frequencies of Foxp3-positive cells among cardiac CD4+ T cells 5 days post-surgery after administration of an isotype control antibody or CD28-SA to sham-operated mice.

**Figure 7**: Expression of M2-inducing cytokines as well as mRNA levels of macrophage polarization markers in scar tissue homogenates and monocytes sorted from the scar.
A: Real-time RT-PCR analysis of M2-inducing interleukin (IL)-13, IL-10 and transforming growth factor beta 1 (TGFβ1) in scar tissue homogenates (n=4-7 per group, *P<0.05). B: Real-time RT-PCR analysis of the M2 marker/mediators arginase I, osteopontin (OPN) and transglutaminase factor XIII (FXIII) (n=4-7 per group, *P<0.05). C: mRNA expression of M2-associated arginase I and CD206 in monocytes/macrophages sorted from the scar on day 7 post-MI (n=3-4 per group, *P<0.05). D: mRNA expression of M2-associated TGFβ1 and IL-10 as well as M1-associated TNFα in monocytes/macrophages sorted from the scar on day 7 post-MI (n=3-4 per group, *P<0.05).

**Figure 8**: Treg cell-mediated induction of M2-associated osteopontin (OPN) in monocytic cells (MΦs) in vitro.
A: OPN concentrations in the supernatant of cultivated Treg cells, Tconv cells and MΦs, cultured for 4 days either alone or in the indicated combinations in the presence of a CD28-SA or isotype control antibody (Ig) and recombinant human IL-2 (n=3-4). B: OPN concentrations in the supernatants of mononuclear cells cultured for 4 days in presence of titrated CD28-SA-activated Treg cells. C: OPN concentration in the monocyte supernatant and mRNA expression of M2-associated CD206 and arginaseI expression in monocytes cultivated for 3 days in presence or absence of supernatant from CD28-SA-activated Treg cells (n=3-4, *P<0.05). D: Cytokine concentrations in the supernatant of Treg cells cultivated for 4 days in the presence of a CD28-SA or isotype control Ig (n=2 individual experiments). TGFβ1 background in FCS-containing culture medium was subtracted from the measured concentrations in cell supernatants. E: OPN concentration in the supernatant of MΦs cultivated for 3 days in the presence of the indicated cytokines and/or a neutralizing anti-TGFβ antibody (n=4 per group, *P<0.05 vs. MΦ + TGFβ1 + IL-10).
Novelty and Significance

What Is Known?

- CD4+ T cell activation after myocardial infarction (MI) facilitates healing and improves clinical outcome.

- The CD4+ T cell subpopulation of Foxp3-expressing CD4+ regulatory T cells (T_{reg} cells) feature potent anti-inflammatory characteristics.

- Accumulation of monocytes/macrophages in the infarcted myocardium is biphasic characterized by an early Ly-6C^{high} monocyte-dominant inflammatory phase followed by an M2-like macrophage-prevalent reparative phase.

What New Information Does This Article Contribute?

- T_{reg} cells accumulate at low numbers in the infarcted myocardium.

- T_{reg} cells influence the transition from the inflammatory to the reparative phase by modulating macrophage function that critically affects clinical outcome.

- Therapeutic T_{reg} cell activation represents an eligible strategy to accelerate and improve healing after MI.

The reparative phase after MI requires inflammation resolution and is characterized by the emergence of healing-promoting M2-like macrophages. CD4+ T cells become activated after ischemic cardiac injury improving healing as well as clinical outcome. T cell activation after MI involves the expansion of T_{reg} cells that constitute a T cell subpopulation with anti-inflammatory properties. We hypothesized that T_{reg} cells influence healing and outcome post-MI. T_{reg} cells expanded in heart-draining lymph nodes and accumulated at low numbers in the infarcted myocardium. T_{reg} cell depletion prior to MI provoked an adverse activation of non-regulatory CD4+ and CD8+ T cells that numerously infiltrated the infarct zone of T_{reg} cell-deficient mice. These non-T_{reg} cells restrained M2-like macrophage polarization by secreting inflammatory factors, resulting in impaired healing and cardiac function. In contrast, therapeutic T_{reg} cell activation reinforced T_{reg} cell influx into the infarct zone and stimulated M2-like macrophage polarization by soluble mediators. Release of inflammation-resolving and healing-fostering factors from T_{reg} and M2 cells led to accelerated and improved scar tissue formation and correlated with improved survival. The study delineates the interplay between adaptive (T cells) and innate (macrophages) immunity post-MI and presents a novel treatment modality bearing therapeutic potential to treat patients with MI.
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**Figure 1**

**A**

Gated CD3+CD4+ T cells (mediastinal lymph nodes)

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**B**

% Foxp3+ of CD4+

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**C**

Gated CD3+CD4+

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**D**

Number of Foxp3+CD4+ Treg cells per mg tissue

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**E**

Infarct border

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Remote myocardium

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Figure 2

A. Gated CD4+ T cells

- Blood (before MI)
  - Wildtype: 14.0
  - Foxp3DTR: 1.8
- MLN (before MI)
  - Wildtype: 18.7
  - Foxp3DTR: 1.3
- Scar d5 post-MI
  - Wildtype: 26.2
  - Foxp3DTR: 2.5

B. Survival (%)

- WT
- Foxp3DTR

C. Apical EDA [mm²]

- Foxp3DTR
- WT

D. Lung weight / body weight ratio

- Foxp3DTR
- WT
Figure 3

Panel A: Flow cytometry analysis of Ly-6G+ neutrophils and Ly-6C+ monocytes in Foxp3DTR and wildtype mice. Statistics are presented as mean ± SEM. *p < 0.05 compared to wildtype.

Panel B: Percentage of Ly-6C+ high monocytes in Foxp3DTR and wildtype mice. Statistics are presented as mean ± SEM. *p < 0.05 compared to wildtype.

Panel C: mRNA levels of iNOS, TGFβ1, OPN, and FXIII in sorted monocytic cells. Statistics are presented as mean ± SEM. *p < 0.05 compared to wildtype.

Panel D: CD4+ and CD8+ T cell counts in Foxp3DTR and wildtype mice. Statistics are presented as mean ± SEM. *p < 0.05 compared to wildtype.

Panel E: Flow cytometry analysis of CD4+Foxp3+ T cells, gated on CD3+ cells. Statistics are presented as mean ± SEM. *p < 0.05 compared to wildtype.
Figure 4

A. gated CD4+ blood (before MI) vs mLN (d7 post-MI) blood (before MI)

B. Survival (%) vs days post MI

C. Apical EDA [mm^2]

D. Number of neutrophils per mg scar tissue x 10^3

% Ly-6Chigh of monocytic cells

* indicates statistical significance.
**Figure 5**

A. Flow cytometry analysis of Foxp3 expression in blood and mLN gated CD4+ T cells. 

B. Survival rate of infarcted mice treated with CD28-SA and isotype control Ig. 

C. Frequency of left-ventricular ruptures in infarcted mice treated with CD28-SA and isotype control Ig. 

D. mRNA expression levels of Col1a1 and Col3a1 in mice treated with CD28-SA and isotype control Ig. 

E. Western blot analysis of collagen I and III expression in mLN.
Figure 6

A. 

Gated CD45+ lymphocytes

1.1% 0.7%

isotype control Ig

CD28-SA

B. 

gated CD45+CD4+CD11b- lymphocytes

isotype control Ig

CD28-SA

C.

Frequency of Foxp3+ cells among CD4+ T cells

* p < 0.05

isotype control Ig

CD28-SA

D. 

Number of Foxp3+CD4+ Treg cells per mg tissue

* p < 0.05

isotype control Ig

CD28-SA

E. 

DAPI Foxp3

isotype control Ig

CD28-SA

F. 

Gated CD4+ T cells

isotype control Ig

CD28-SA

(heart, sham d5)
Figure 7
Figure 8

A

B

C

D

E

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Foxp3+CD4+ T Cells Improve Healing after Myocardial Infarction by Modulating Monocyte/Macrophage Differentiation
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Supplemental Material

Supplemental Methods

Animals and surgery
Male C57BL/6J mice (Harlan Laboratories, Netherlands) between 8 and 12 weeks of age were used for all experiments. Mice underwent left coronary artery ligation as described previously. The study conformed to the regulations for animal experimentation and was approved by the local government. Briefly, mice were anesthetized with isoflurane. After intubation, thoracotomy was performed and MI induced by ligation of the proximal part of the left coronary artery. Buprenorphine was administered for analgesia after surgery. For sham operation, thoracotomy was performed without subsequent ligation of the coronary artery.

Echocardiography
Cardiac ultrasound analysis was performed on Toshiba Aplio Systems. Mice were kept under light anesthesia with isoflurane (1.5 vol%). Short-axis 2-dimensional echocardiographic images were acquired at the mid-papillary and apical levels of the left ventricle (LV). Endocardial borders were traced at endsystole and end-diastole using the Nice software package (Toshiba Medical Systems). End-diastolic and end-systolic diameters were measured from transversal M-mode tracings. Only animals with a heart rate >450/min and histologically determined infarct size >30% were included for analysis.

Fluorescence-Activated Cell Sorting
Cardiac scar tissue was digested with collagenase type 2 and protease type XIV (Sigma-Aldrich, Munich). From heart-draining mediastinal lymph nodes, single cell suspensions were prepared. Up to $10^6$ cells were resuspended in 50 µl of PBS/0.1%BSA/0.02%NaN₃ with saturating amounts of cell culture supernatant of clone 2.4G2 to block unspecific Fc-receptor binding (15 min, 4°C). After blocking, fluorophore-conjugated antibodies were added. The cells were analyzed on an LSR II flow cytometer (BD Biosciences, Heidelberg). For further data analyses, FlowJo (TreeStar Inc, Ashland) was used. In order to exclude false-positive events resulting from cross-reactive antibody binding on different populations, gates for populations of interest, e.g. lymphocytes, were set according to the population’s FSC/SSC profile. Plots are shown as log10 fluorescence intensities. Used antibodies are shown in the Online Table 5.

Immunohistochemistry
Hearts were fixed over night with 4% paraformaldehyde and embedded in paraffin. Infarct sizes were determined by picrosirius red stainings of 7 µm sections. Alpha smooth muscle actin (α-SMA) stainings on 5 µm sections were conducted using the Dako ARK™ Peroxidase Kit in combination with anti-α-SMA monoclonal antibody clone 1A4 (Dako, Carpinteria) and, subsequently, stained with hematoxylin. Slides were dehydrated and mounted for light microscopy. Pictures were acquired on a Zeiss Axioskiop 2 plus using Spot Software 5.0 (Diagnostics Instruments, Inc, Sterling Heights).

Localization of Foxp3⁺ cells using immunofluorescence
Cryosections (5 µm) were fixed with 4% formaldehyde in PBS for 10 minutes, permeabilized with 0.5% dodecyltrimethylammonium chloride in PBS and dehydrated using methanol for 3 minutes (all chemicals from Sigma-Aldrich, Munich). Sections were blocked with 5% fetal calf serum for 30 minutes. Rat anti-mouse Foxp3 antibody (clone: FJK-16s, eBioscience, Frankfurt) was diluted 1:100 in blocking solution. After 2 washing steps, secondary antibody goat anti-rat
Alexa555 (Life Technologies, Darmstadt) was diluted 1:200 in blocking solution and incubated for 30 minutes. Nuclei were stained with DAPI, washed with PBS and mounted with vectashield (Vector Laboratories, Peterborough, UK). Images were acquired using an Axio Imager.Z1m epifluorescence microscope (Zeiss, Oberkochen).

**Real-Time Reverse Transcriptase–Polymerase Chain Reaction**

RNA was extracted from frozen scar tissue samples using a tissue RNA isolation kit (Qiagen, Hilden). cDNA was synthesized from 1µg RNA with iScript (Bio-Rad, München). Quantitative real-time PCR was performed (iCycler from Bio-Rad, München) with commercial TaqMan probes (Life Technologies, Darmstadt). Target gene mRNA levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase. For real-time RT-PCR analysis of sorted cells, mRNA was amplified before cDNA synthesis using the C&E Trinucleotide Pico Kit (AmpTec, Hamburg) according to the manufacturer’s protocol.

**Purification and cell culture of monocytes/macrophages, T\(_{\text{reg}}\) and T\(_{\text{conv}}\) cells**

For purification of monocytic cells, single-cell suspensions were prepared from spleens as well as inguinal, cervical and axillary lymph nodes. T and B cells were depleted using MACS (Miltenyi Biotec, Bergisch Gladbach) separation columns according to the manufacturer's instructions. To remove T cells and B cells, suspensions were incubated with biotinylated anti-CD3\(_{\varepsilon}\) or anti-CD19 antibodies, followed by streptavidin micobeads. CD3\(^-\)CD19\(^+\) cells were stained with anti-CD11b, anti-Ly-6G and anti-CD11c for fluorescence activated cell sorting. Monocytic cells were defined as CD11b\(^+\)Ly-6G\(^-\)CD11c\(^-\). T\(_{\text{reg}}\) and T\(_{\text{conv}}\) cells were sorted from lymph node cell suspensions stained with anti-CD4 and anti-CD25. T\(_{\text{reg}}\) cells were defined as CD4\(^+\)CD25\(_{\text{high}}\), T\(_{\text{conv}}\) cells as CD4\(^+\)CD25\(^-\). The antibodies are shown in the Online Table 5. Cell cultures were set up in 96-well flat bottom plates (greiner bio-one, Frickenhausen) and 2x10\(^5\) cells seeded per well. Cells were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, non-essential amino acids, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 U/ml penicillin, 2 mM L-glutamine and 30 µM mercaptoethanol (all from Life Technologies, Darmstadt). In co-culture experiments, 1x10\(^5\) cells per cell type were used. All cell cultures were supplemented with 25 IU/ml recombinant IL-2 (Proleukin®, Novartis, Basel) and, where denoted, anti-TGFβ antibody (10 µg/ml, clone 1D11, R&D, Wiesbaden) or recombinant human TGFβ1 (R&D, Wiesbaden), recombinant mouse IL-10 (BioLegend, San Diego) or recombinant mouse IL-13 (BioLegend, San Diego) was added to cultivated monocytes/macrophages. CD28-SA (clone D665, AbD Serotec, Raleigh) or isotype control Ig (clone MOPC-21, Bio-X-Cell, West Lebanon) was coated on paramagnetic pan mouse IgG dynabeads (Invitrogen, Darmstadt) according to the manufacturer’s instructions and added to cell cultures where indicated.

**Western blot analysis**

After tissue homogenization in sample buffer, 15 µg of total protein was separated by SDS-PAGE and subsequently blotted onto a nitrocellulose membrane. Blocking was performed using 5% non-fat dry milk solution. Membranes were incubated for 2 hours at room temperature in the presence of primary or secondary antibodies. Protein expression levels were quantified using the rabbit anti-rat collagen type I (Cedarlane, Burlington, CL50141AP) and rabbit anti-rat collagen type III (Cedarlane, Burlington, CL50341AP). The membrane was developed using an ECL detection system. After membrane stripping, total protein amount per lane was assessed by detection of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
References

Online Figure I: Representative FACS plot showing expression of the activation marker Helios in CD4+ T cells 7 days after MI (left). Right: MFI of Helios in CD4+Foxp3+ T cells in infarcted (MI) and sham-operated animals 7 days (d7) after surgery (n=4 per group; *P<0.05)
Online Figure II: Representative FACS plot showing that Foxp3 is exclusively expressed in CD4+ T cells making Foxp3 staining alone sufficient to detect T\(_{reg}\) cells.
Online Figure III: Real-time RT-PCR analyses of extracellular collagen matrix (ECM) components and factors influencing ECM integrity in bulk scar tissue of diphtheria toxin-treated Foxp3^{DTR} and wildtype (WT) mice 5 days after MI.

A: mRNA expression level of pro-collagen alpha-1 (III) (Col3a1), pro-collagen alpha-1 (I) (Col1a1, n=4 per group). B: mRNA expression of matrix metalloproteinase (MMP)-2 and MMP-9 (n=4 per group). C: mRNA expression of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 (n=4 per group).
Online Figure IV: Real-time RT-PCR analyses and immunohistochemical detection of myofibroblast markers as well as mRNA expression level of tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2.
A: mRNA level of alpha smooth muscle actin (α-SMA) and vimentin 5 days after MI (n=4-7 per group, *P<0.05) B: mRNA expression level of von Willebrand factor (vWF) 5 days post-MI (n=4-7 per group; *P<0.05). C: representative immunohistochemistry staining of alpha smooth muscle actin in the scar tissue 7 days after MI (brown: α-SMA-positive cells indicated by arrow head, blue: cell nuclei, bar represents 50 μm). D: mRNA level of tissue inhibitor of metalloproteinases 1 (TIMP-1) and tissue inhibitor of metalloproteinases 2 (TIMP-2) on day 5 after MI in scar tissue homogenates (n=4-7 per group; *P<0.05).
Online Figure V: Real-time RT-PCR analysis of M1-associated IL-1β and IL-6 in monocyteic cells sorted from the scar of treated mice 7 days after (n=3-4 per group, n.s. not significant).
Online Figure VI: Purification strategy for isolation of primary monocytes/macrophages and T cells from naïve mice.
A: Purification of monocytic cells. Depletion of CD3+ and CD19+ cells from spleen and lymph node cell suspensions by MACS separation columns was followed by fluorescence-activated cell sorting. Monocytes/macrophages were defined as CD45+CD11b+Ly-6G−CD11c−. B: Purification of T cells. CD4+ T cells were sorted from bulk lymph node cell suspensions. Treg cells were defined as CD4+CD25high and T_conv cells as CD4+CD25−.
Online Figure VII: Evaluation of T cell purification (S1) on the basis of Foxp3 expression and CD28-SA-mediated activation.

Expression of Foxp3 and the proliferation marker Ki67 in sorted CD4+CD25− (A) and CD4+CD25<sup>high</sup> (B) T cells after 4 days incubation in the presence of CD28-SA and IL-2.
Online Figure VIII: Model for T_{reg}-cell-mediated improvement of scar tissue formation after myocardial infarction in CD28-SA-treated mice.
Therapeutic T_{reg} cell activation leads to enhanced T_{reg} cell recruitment into the healing myocardium, along with increased cytokine secretion. T_{reg} cell-derived TGF-1, IL-10 and IL-13 synergistically induce alternative macrophage activation, resulting in upregulation of both FXIII and osteopontin in M2-like cells. FXIII directly improves scar tissue integrity by cross-linking of ECM components. Osteopontin has been shown to restrain IL-1β-mediated upregulation of matrix metalloproteinases (MMPs) and, moreover, to induce tissue inhibitors of MMPs in myofibroblasts within the scar tissue after MI. M2-derived TGF-1 and T_{reg}-cell-derived TGF-1 and IL-13 function cooperatively to drive collagen synthesis in myofibroblasts.
Online Table 1: Echocardiographic characterization of Foxp3\textsuperscript{DTR} and wildtype mice at baseline

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Foxp3\textsuperscript{DTR}</th>
<th>wildtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>body weight, g</td>
<td>20 ± 3.5</td>
<td>23 ± 1.8</td>
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<tr>
<td>Echocardiographic measurements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary EDA, mm\textsuperscript{2}</td>
<td>8.4 ± 1.4</td>
<td>7.4 ± 0.8</td>
</tr>
<tr>
<td>Papillary M-mode FS, %</td>
<td>42.6 ± 7.7</td>
<td>43.3 ± 5.8</td>
</tr>
<tr>
<td>Apical EDA, mm\textsuperscript{2}</td>
<td>6.6 ± 0.6</td>
<td>6.4 ± 0.8</td>
</tr>
<tr>
<td>Apical M-mode FS, %</td>
<td>47 ± 9.7</td>
<td>43.8 ± 6.8</td>
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<tr>
<td>Heart rate</td>
<td>617 ± 4.7</td>
<td>610 ± 16</td>
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<tr>
<td>Apical end-diastolic anterior wall, cm</td>
<td>0.08 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Apical end-diastolic posterior wall, cm</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.01</td>
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</table>
Online Table 2: Infarct sizes, organ weights and echocardiographic data of diphtheria toxin-treated Foxp3<sup>DTR</sup> and wildtype mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase</th>
<th>Foxp3&lt;sup&gt;DTR&lt;/sup&gt;</th>
<th>wildtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>body weight, g</td>
<td>d7</td>
<td>19 ± 2.5</td>
<td>21.9 ± 1.8</td>
</tr>
<tr>
<td>LV weight/ body weight</td>
<td>d7</td>
<td>5.4 ± 0.6</td>
<td>4.8 ± 0.4</td>
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<tr>
<td>Echocardiographic measurements</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Papillary end-diastolic area, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>d3</td>
<td>13.8 ± 3</td>
<td>14 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>d7</td>
<td>20.1 ± 4.9</td>
<td>18.2 ± 5.2</td>
</tr>
<tr>
<td>Papillary M-mode FS, %</td>
<td>d3</td>
<td>10.9 ± 5.2</td>
<td>13.8 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>d7</td>
<td>8.8 ± 2.3</td>
<td>11.3 ± 6.5</td>
</tr>
<tr>
<td>Apical end-diastolic area, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>d3</td>
<td>13.8 ± 2.8</td>
<td>13.2 ± 3</td>
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<tr>
<td></td>
<td>d7</td>
<td>19.9 ± 4.8</td>
<td>17.9 ± 4.5</td>
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<tr>
<td>Heart rate</td>
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<td>500 ± 30</td>
<td>510 ± 36</td>
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<td>Apical end-diastolic anterior wall, cm</td>
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<td>0.050 ± 0.003</td>
<td>0.055 ± 0.0100</td>
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<tr>
<td>Apical end-diastolic posterior wall, cm</td>
<td>d7</td>
<td>0.050 ± 0.005</td>
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Online Table 3: Echocardiographic characterization of anti-CD25 antibody and isotype control antibody (Ig) treated mice

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<th>Parameter</th>
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<th>Isotype control Ig</th>
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</thead>
<tbody>
<tr>
<td>Infarct size, %</td>
<td>d7</td>
<td>63.1 ± 7.6</td>
<td>58.2 ± 12.3</td>
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<tr>
<td>Echocardiographic measurements</td>
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<td></td>
</tr>
<tr>
<td>Papillary end-diastolic area, mm²</td>
<td>d7</td>
<td>26.4 ± 4.9</td>
<td>22.9 ± 1.6</td>
</tr>
<tr>
<td>Papillary M-mode FS, %</td>
<td>d7</td>
<td>8.1 ± 2.9</td>
<td>8.4 ± 2.3</td>
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<tr>
<td>Apical M-mode FS, %</td>
<td>d7</td>
<td>7.8 ± 1.8</td>
<td>7.7 ± 0.3</td>
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<tr>
<td>Heart rate</td>
<td></td>
<td>590 ± 43</td>
<td>570 ± 40</td>
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<tr>
<td>Apical end-diastolic anterior wall, cm</td>
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<td>0.04 ± 0.02</td>
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<tr>
<td>Apical end-diastolic posterior wall, cm</td>
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<td>0.04 ± 0.01</td>
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### Online Table 4: Infarct sizes, organ weights and echocardiographic parameters of isotype control Ig and CD28-SA-treated mice

<table>
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<th>Parameter</th>
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<th>Isotype control Ig</th>
<th>CD28-SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infarct size, %</td>
<td>d7</td>
<td>59.5 ± 4.5</td>
<td>65.8 ± 5.6</td>
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<tr>
<td>Infarct size, %</td>
<td>d56</td>
<td>56.8 ± 10.8</td>
<td>55.6 ± 9.4</td>
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<tr>
<td>body weight, g</td>
<td>d7</td>
<td>23.6 ± 2</td>
<td>23.5 ± 1.3</td>
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<tr>
<td>LV weight/body weight</td>
<td>d7</td>
<td>5 ± 0.3*</td>
<td>5.7 ± 0.5</td>
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<tr>
<td>Lung weight/body weight</td>
<td>d7</td>
<td>7.7 ± 1*</td>
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</table>

Echocardiographic measurements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase</th>
<th>Isotype control Ig</th>
<th>CD28-SA</th>
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</thead>
<tbody>
<tr>
<td>Papillary EDA, mm²</td>
<td>d7</td>
<td>25.9 ± 4.1</td>
<td>26.6 ± 5.6</td>
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<td></td>
<td>d56</td>
<td>28.5 ± 3.6</td>
<td>33.2 ± 8.8</td>
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<td>d7</td>
<td>8.2 ± 3.9</td>
<td>7.5 ± 3.7</td>
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<td></td>
<td>d56</td>
<td>10.3 ± 3.4</td>
<td>8.8 ± 5</td>
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<tr>
<td>Apical EDA, mm²</td>
<td>d7</td>
<td>26.6 ± 6.6</td>
<td>28.4 ± 6.3</td>
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<td>d56</td>
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<td></td>
<td>d56</td>
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<tr>
<td>Heart rate</td>
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<td>591.7 ± 56.7</td>
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<td>Apical end-diastolic anterior wall, cm</td>
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<td>Apical end-diastolic posterior wall, cm</td>
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<td>0.03 ± 0.01</td>
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*P<0.05 vs. CD28-SA
Online Table 5: Antibodies used for FACS analyses

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<td>CD11b-PE</td>
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<td>eBioscience</td>
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<tr>
<td>CD45-efluor 450</td>
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<td>BM8</td>
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<td>Foxp3-PE-Cy5.5</td>
<td>FJK-16s</td>
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