*F*oxp3*+ CD4*+ T Cells Improve Healing After Myocardial Infarction by Modulating Monocyte/Macrophage Differentiation

Johannes Weirather,* Ulrich D.W. Hofmann,* Niklas Beyersdorf,* Gustavo C. Ramos, Benjamin Vogel, Anna Frey, Georg Ertl, Thomas Kerkau,* Stefan Frantz*

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

**Objective:** Our aim was to study the functional role of Treg cells in 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 Treg-cell compartment before MI induction, resulting in aggravated cardiac inflammation and deteriorated clinical outcome. Mechanically, Treg-cell depletion was associated with MI-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 with 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 macrophage 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. *(Circ Res. 2014;115:55-67.)*

**Key Words:** myocardial infarction ▪ wound healing

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.1 Cardiomyocyte death results in replacement by scar tissue and, after large MI, in ventricular remodeling of the remote myocardium, which further compromises cardiac function.2,3 Early cardiac wound healing is characterized by infiltration of innate immune cells, especially neutrophils and monocytes/macrophages, into the myocardium.2,4

**Editorial, see p 7**

**In This Issue, see p 1**

Previously, we could show that the adaptive immunity, more precisely CD4+ T lymphocytes, crucially affects cardiac wound healing. CD4+ T-cell–deficient mouse strains show accentuated cardiac inflammation, impaired wound healing, aggravated left ventricular remodeling, and impaired survival.5 Activation and proliferation of both conventional and Foxp3+ CD4+ regulatory T cells (Treg cells) take place in heart-draining mediastinal lymph nodes (mLNs) 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.6–9 Moreover, Treg cells shape innate immune responses in terms of wound healing after injury.10 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 used to unravel the impact of Treg cells on cardiac healing. First, Treg-cell depletion before MI was achieved by using Foxp3<sup>ΔT</sup> mice in which Foxp3<sup>+</sup> cells transgenically express the human diphtheria toxin (DTX) receptor (DTR) resulting in specific Treg-cell ablation after DTX administration. In another line of experiments, an anti-CD25 monoclonal antibody was administered resulting in phagoctosis-mediated Treg-cell ablation because of 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 compared with conventional CD4<sup>+</sup> T cells in vivo because of a vigorous costimulatory 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/6 mice were purchased from Harlan Laboratories. Mice expressing the DTX under control of the Foxp3 promoter, such as deletion of regulatory T cell mice, were purchased from Jackson Laboratory. In a separate set of experiments, wild-type control mice were used here for pharmacological ablation of Treg<sup>+</sup> 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 Treg<sup>+</sup> cells, 250 ng DTX per mouse were additionally injected on day 2 and day 4 after MI. For antibody-mediated Treg-depletion, 1 mg of anti-CD25 monoclonal antibody (clone PC61) was injected. For expansion of Treg cells, mice were treated with 300 µg of a superagonistic anti-CD28 monoclonal antibody (clone D665).

#### Fluorescence-Activated Cell Sorting

Cardiac scar tissue was digested with collagenase type 2 and protease type XIV (Sigma-Aldrich, Munich, Germany) as described previously. Staining protocols are specified in the Online Data Supplement.

#### Purification and Cell Culture of Monocytes/ Macrophages, Treg, and Conventional T 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>. Treg cells were defined as CD4<sup>+</sup>CD25<sup>high</sup>/<sup>+</sup>CD69<sup>-</sup> cells, and conventional T cells as CD4<sup>+</sup>CD25<sup>−</sup>/<sup>+</sup>CD69<sup>+</sup> cells (2×10<sup>6</sup> per well) were seeded in 96-well flat-bottom plates (Greiner bio-one, Frickenhausen, Germany). In coculture experiments, 1×10<sup>5</sup> cells per cell type were used. All cell cultures were supplemented with 25 IU/mL recombinant interleukin (IL)-2 (Prolifekt; Novartis, Basel, Germany) and, where denoted, anti-TGFβ antibody (10 µg/mL; clone 1D11; R&D, Wiesbaden, Germany) or recombinant cytokines. CD28-SA (clone D665; AbD Serotec, Raleigh, NC) or isotype control immunoglobulin G (clone MOPC-21; Bio-X-Cell, West Lebanon, NH) was coated on pan mouse immunoglobulin G dynabeads (Invitrogen, Darmstadt, Germany) or recombinant cytokines. CD28-SA (clone D665; AbD Serotec, Raleigh, NC) or isotype control immunoglobulin G (clone MOPC-21; Bio-X-Cell, West Lebanon, NH) was coated on pan mouse immunoglobulin G dynabeads (Invitrogen, Darmstadt, Germany) and added to cell cultures where indicated.

#### Analysis of Cytokine Concentrations

Cell culture supernatants were analyzed by ELISA (R&D) as well as tumor necrosis factor (TNF), interferon-γ, IL-6, IL-2, IL-4, IL-13, and IL-17 by Cytometric Bead Array (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s protocol. The latter results were analyzed with FCAP Array Version 2.0 (Soft Flow, Duesseldorf, Germany).

#### Western Blot

After blocking, nitrocellulose membranes were incubated overnight in the presence of rabbit antirat collagen type I (CL50141AP; Cedarlane, Burlington) and rabbit antirat collagen type III (CL50341AP; Cedarlane). The membrane was developed using an enhanced chemiluminescent detection system. After membrane stripping, total protein amount per lane was assessed by detection of glyceraldehyde 3-phosphate dehydrogenase.
Statistical Analysis
All data are presented as mean values per group and SEM. For comparison of 2 groups, an unpaired t test or, if a t test was not suitable, a Wilcoxon signed rank-sum test was performed. For multiple comparisons, 2-way ANOVA was used. Survival was shown as Kaplan–Meier curve, and data were 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, CA).

Results

T<sub>reg</sub> Cells Become Activated in Response to MI
To monitor T<sub>reg</sub>-cell activation after MI, we analyzed heart-draining lymph nodes. Here, the activation markers CD25 (Figure 1A) and Helios (Online Figure I) were upregulated in T<sub>reg</sub> cells 7 days post-MI compared with sham-operated animals. As previously reported by us, increased frequencies of T<sub>reg</sub> cells were found in mLNs on days 3 and 7 post-MI as compared with sham-operated animals analyzed on day 7 after surgery (Figure 1B). Remarkably, compared with sham-operated mice, the percentage of Foxp3<sup>+</sup> cells among CD4<sup>+</sup> T cells stayed elevated for ≤56 days (Figure 1B). Absolute cell numbers of T<sub>reg</sub> cells were also increased peaking on day 7 after MI (Figure 1B). In addition to T<sub>reg</sub>-cell expansion kinetics in heart-draining lymph nodes, we evaluated T<sub>reg</sub>-cell infiltration into the healing infarct. Compared with sham-operated mice, both frequencies and absolute numbers of T<sub>reg</sub> cells were increased post-MI (Figure 1C and 1D). Moreover, using immunofluorescence microscopy, single Foxp3<sup>+</sup> cells were detected in the infarct border and within the remote myocardium (Figure 1E; Online Figure II).

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

Specific T<sub>reg</sub>-Cell Ablation in Foxp3<sup>DTR</sup> Mice Leads to Increased Infarct Size and Cardiac Deterioration
To evaluate the influence of T<sub>reg</sub> cells on wound healing after MI, we used Foxp3<sup>DTR</sup> mice to ablate Foxp3-expressing cells specifically that transgenically express the human DTR. Two days after DTX administration, Foxp3<sup>+</sup> cells were effectively depleted from blood, mLNs, and hearts as compared with DTX-treated wild-type littermates (Figure 2A). Survival was not significantly different between the 2 groups, but infarct sizes in T<sub>reg</sub>-cell–depleted mice were significantly increased as compared with the control group. Furthermore, T<sub>reg</sub>-cell ablation resulted in a tendency toward 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 (Figure 2C; Online Tables I and II). Consistently, Foxp3<sup>DTR</sup> mice showed increased lung weight/body weight ratios underlining the compromised cardiac function in these mice (Figure 2D). With respect to procollagen synthesis, expression of matrix metalloproteinases, and...
tissue inhibitors of matrix metalloproteinases, 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.

Treg-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 fluorescence-activated cell sorting analyses, neutrophils were defined as Ly-6G+CD11b+. Compared with control mice, neutrophil numbers were significantly increased in the infarct zone of Treg-cell–depleted animals (Figure 3A). Furthermore, we discriminated between monocyte subsets based on lymphocyte antigen 6C (Ly-6C) surface expression. In comparison with control mice, the proportion of inflammatory Ly-6C<sup>high</sup> cells among CD11b<sup>-</sup>F4-80<sup>-</sup>Ly-6G<sup>-</sup> monocytes was elevated in the infarct zone of Treg-cell–ablated mice (Figure 3B).

Because Treg cells are capable of regulating inflammatory reactions, we evaluated macrophage polarization in Treg–sufficient and Treg–cell–ablated mice. Expression of prototypic markers for inflammatory M1 macrophage polarization was assessed in CD11b<sup>-</sup>Ly-6G<sup>-</sup> monocytes/macrophages sorted from the healing infarct 5 days post-MI. Compared with DTX-treated wild-type mice, monocytic cells sorted from Foxp3<sup>ΔT</sup> mice showed significantly higher mRNA expression of inducible nitric oxide (NO) synthase, but no difference regarding TNFα mRNA synthesis (Figure 3C). Moreover, compared with wild-type littermates, mRNA expression of M2-associated anti-inflammatory IL-10 and transforming growth factor β1 (TGFβ1) in line with mRNA synthesis of wound-stabilizing osteopontin and transglutaminase factor XIII (FXIII) was downregulated in monocytic cells sorted from the healing infarct of Treg-cell–ablated mice (Figure 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 with wild-type littermates, absolute numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were increased in the healing infarct of Treg-cell–ablated mice 5 days post-MI (Figure 3D). Analysis of interferon-γ and TNFα mRNA synthesis in bulk scar tissue homogenates (Figure 3D) and increased frequencies of interferon-γ and TNFα-positive cells among CD4<sup>+</sup> and CD8<sup>+</sup> T cells in heart-draining lymph nodes (Figure 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 \( T_{\text{reg}} \)-cell–depleted mice was associated with impaired M2-like differentiation of cardiac macrophages and pronounced infiltration of the heart by proinflammatory T cells.

**Anti-CD25 Monoclonal Antibody–Mediated \( T_{\text{reg}} \)-Cell Depletion Before MI Results in Impaired Remodeling and Survival**

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

Antibody-mediated \( T_{\text{reg}} \)-cell depletion led to a significantly higher mortality as compared with control mice (Figure 4B). By day 7, survival was 55.9% in control mice and 25% in \( T_{\text{reg}} \)-cell–ablated animals (Figure 4B). Regarding frequencies of left ventricular ruptures, no differences could be found between treatment groups. Thus, \( T_{\text{reg}} \)-cell–depleted mice died presumably because of a higher incidence of cardiac failure as indicated by greater echocardiographic expansion of the left ventricular area on day 7 after MI (Figure 4C; Online Table III). Congruently with the Foxp3\(^{DTR} \) model, the infarct zone of the anti-CD25 antibody–treated mice showed increased numbers of neutrophils 7 days after MI as compared with the control group, in line with higher frequencies of proinflammatory Ly-6C\(^{\text{high}} \) cells among monocytes (Figure 4D).

Therapeutic \( T_{\text{reg}} \)-cell activation after MI induction results in enhanced recruitment of \( T_{\text{reg}} \) cells into the infarct zone and improves scar tissue formation and survival.

Because the lack of \( T_{\text{reg}} \) cells aggravated clinical outcome, we focused on \( T_{\text{reg}} \)-cell expansion to improve wound healing post-MI. Therapeutic \( T_{\text{reg}} \)-cell activation was accomplished in a therapeutic fashion, that is, after MI. The peak of physiological \( T_{\text{reg}} \)-cell response was found to be between day 3 and day 7 after MI induction (Figure 1B). For therapeutic \( T_{\text{reg}} \)-cell expansion, we used a superagonistic anti-CD28 antibody (CD28-SA). The CD28-SA–mediated \( T_{\text{reg}} \)-cell expansion reaches its full effect at the earliest 2 days after a single administration.\(^{15} \) Thus, CD28-SA was administered on day 2 after MI induction to support the...
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 with isotype control immunoglobulin–treated mice.

Having observed that therapeutic $T_{reg}$-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 with control antibody–treated animals, increased mRNA expression of both procollagen α1 (I) and procollagen α1 (III) that constitute integral components of the reparative scar (Figure 5D). Elevated expression of both collagen I and collagen III in CD28-SA–treated mice was confirmed at the protein level (Figure 5E).

Consistently, mesenchymal vimentin and α-smooth muscle actin mRNA levels were upregulated in the healing myocardium 5 days post-MI, but not mRNA synthesis of von Willebrand factor, reflecting increased myofibroblast numbers rather than accelerated angiogenesis after CD28-SA treatment (Online Figure IVA and IVB). Increased myofibroblast numbers were confirmed by α-smooth muscle actin immunohistochemistry staining (Online Figure IVC). Moreover, compared with control mice, CD28-treated mice showed increased mRNA expression of both tissue inhibitors of matrix metalloproteinases 1 and 2, endogenous tissue inhibitors of matrix metalloproteinases preventing ECM degradation (Online Figure IVD).

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

Collectively, therapeutic $T_{reg}$-cell activation leads to increased recruitment of $T_{reg}$ 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** $T_{reg}$-**Cell Activation Induces Macrophage M2 Differentiation in the Scar Tissue**

Because monocytes 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 $T_{reg}$-cell ablation leads to M1-like macrophage polarization, we also 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-induced IL-13, IL-10, and TGFB1 mRNA expressions were upregulated on day 5 post-MI as
compared with isotype control immunoglobulin–treated mice (Figure 7A). Increased expression of prototypical M2-associated arginase I and the ECM-bracing M2 effectors, osteopontin and FXIII, in bulk scar tissue homogenates further indicated that M2 cells are generated in these hearts (Figure 7B).

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 with isotype control immunoglobulin–treated mice, M2-associated expression of arginase I and CD206 was significantly upregulated in monocytes/macrophages sorted from the scar of CD28-SA–treated animals (Figure 7C).

Consistently, the M2 mediators TGFβ1 and IL-10 were also upregulated in these cells, whereas M1-associated TNFα (Figure 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 osteopontin and FXIII, factors well known to contribute to myocardial healing.

**T<sub>reg</sub>**-Cell–Derived Soluble Mediators Induce M2-Like Macrophage Polarization In Vitro

Given that T<sub>reg</sub>-cell numbers were elevated in the infarct zone of CD28-SA–treated mice, we speculated that T<sub>reg</sub> cells might modulate expression of M2-associated genes in monocyte cells. To assess how T<sub>reg</sub> 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 lympoid organs of naïve animals (Online Figures VI and VII). Monocultures of sorted T<sub>reg</sub> cells, conventional T cells, or monocytes showed comparable mRNA secretion of osteopontin in the presence of CD28-SA and IL-2 after 4 days of incubation (Figure 8A). However, coculture of monocytes and T<sub>reg</sub> cells in the presence of CD28-SA and IL-2 resulted in a dramatic increase in osteopontin secretion, whereas an unspecific isotype control immunoglobulin and IL-2 induced only mild osteopontin release (Figure 8A).

Because T<sub>reg</sub>-cell numbers are, compared with monocyte/macrophage numbers, relatively small, we titrated T<sub>reg</sub> cells in the coculture system. Even at a T<sub>reg</sub>:macrophage ratio of 1:25, osteopontin release was still higher as compared with monocytes cultivated in complete absence of T<sub>reg</sub> cells (Figure 8B).

**T<sub>reg</sub>**-Cell–Derived Soluble Mediators modulate osteopontin secretion by monocytes and to show that osteopontin is, indeed, secreted by the monocytes in our cocultures, we activated T<sub>reg</sub> cells for 4 days by CD28-SA and IL-2 stimulation in vitro and subsequently cultured monocytes with a volume ratio of T<sub>reg</sub>-cell supernatant to fresh culture medium containing purified monocytes of 1:1. Monocytes incubated...
with supernatant from T<sub>reg</sub> cells that had been cultivated in the presence of isotype control immunoglobulin and IL-2 were used as reference. After 3 days of incubation, monocytic cells had released high amounts of osteopontin only in response to mediators in the supernatant of CD28-SA–activated T<sub>reg</sub> cells (Figure 8C). Moreover, osteopontin release from monocytic cells was accompanied by CD206 and arginase I mRNA upregulation indicating M2-like macrophage polarization (Figure 8C).

To identify the T<sub>reg</sub>-cell–derived mediators that might modulate the monocyte phenotype and function in our test system, we analyzed cytokine secretion into the supernatants of T<sub>reg</sub> cells after CD28-SA stimulation for 4 days in vitro. Compared with T<sub>reg</sub> cells cultivated in the presence of isotype control immunoglobulin and IL-2, stimulation with CD28-SA and IL-2 resulted in a modest release of TGF<sub>β1</sub> as well as strong secretion of both IL-10 and IL-13, but no release of IL-2 or IL-4 (Figure 8D).

Having observed a CD28-SA–induced release of IL-10, IL-13, and TGF<sub>β1</sub> from T<sub>reg</sub> cells in vitro, we tried to induce osteopontin secretion from monocytes as indicator for M2 polarization by cultivation in the presence of the aforementioned cytokines. Supplementation with TGF<sub>β1</sub> alone did not influence osteopontin secretion from monocytes, but simultaneous presence of TGF<sub>β1</sub> and IL-10 induced osteopontin secretion, which could be further increased by the addition of IL-13. Neutralization of TGF<sub>β1</sub> dramatically restrained IL-10- and IL-13–driven osteopontin release from the monocytes (Figure 8E).

Conclusively, TGF<sub>β1</sub>, IL-13, and IL-10 produced by CD28-SA–activated T<sub>reg</sub> cells synergized in inducing M2-like differentiation and subsequent osteopontin 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<sub>reg</sub> cells become activated after MI and because of their potent immunosuppressive capacity we hypothesized that T<sub>reg</sub> cells might influence cardiac healing.

**T<sub>reg</sub>-Cell Depletion Deteriorates Healing After MI**

To address this hypothesis, we ablated T<sub>reg</sub> cells before MI induction. T<sub>reg</sub>-cell ablation in Foxp3<sup> ΔT</sup> 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<sub>reg</sub> cells could be confirmed in a model of anti-CD25 antibody–mediated T<sub>reg</sub>-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<sub>reg</sub> cells, in line with a decreased influx of other cell types, correlates with adverse remodeling and cardiac deterioration.23

Neither of the 2 T<sub>reg</sub>-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 left ventricular ruptures. However, CD28-SA treatment increased the expression of procollagens as well as tissue inhibitors of matrix metalloproteinases 1 and 2, indicating that activated Treg cells and M2 cells augmented or even accelerated scar tissue construction that likely prevented left ventricular ruptures in these hearts.

In contrast, T<sub>reg</sub>-cell depletion did not restrain mRNA synthesis of scar-forming collagens or collagenolytic enzymes compared with control mice implying that T<sub>reg</sub>-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 Treg cells might have differentially influenced the prevalence of fatal cardiac arrhythmias. We assume that the differences in survival observed between the 2 models of induced T<sub>reg</sub>-cell deficiency are because of off-target or indirect effects that both DTX injections and anti-CD25 antibody treatments are known to be associated with to some degree.

In both models, the healing myocardium of T<sub>reg</sub>-cell–depleted mice harbored increased numbers of inflammatory myeloid cells, that is, 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<sub>reg</sub> cells correlated with an increased influx of innate immune cells into the lesion.10 Moreover, Treg cells modulate chemokine expression during inflammation influencing myeloid cell infiltration.26 In addition to an increased proportion of inflammatory myeloid cells, both conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells showed a pronounced accumulation in the infarct zone of T<sub>reg</sub>-cell–depleted Foxp3<sup>DTR</sup> mice, contributing to the increased synthesis of TNFα and interferon-γ that both are individually capable of inducing M1 macrophage polarization.27

T<sub>reg</sub>-cell–ablated Foxp3<sup>DTR</sup> mice exhibited a significantly increased infarct size and deteriorated cardiac function. From the mechanistic point of view, escalated TNFα synthesis in combination with a disturbed M2 differentiation likely provokes the maladaptive phenotype. TNFα is capable of depressing cardiac contractile function and elicits cardiomyocyte apoptosis as well as left ventricular dilation.28,29 The detrimental impact of this cytokine on postinfarction remodeling is further underscored in TNFα- knockout mice that are characterized by a preservation of cardiac function.30 Macrophages in the infarct zone of T<sub>reg</sub>-cell–depleted mice further showed an increased synthesis of inducible NO synthase that critically compromises cardiac function after MI. Accordingly, previous reports showed that pharmacological inhibition or genetic inducible NO synthase deficiency in...
mice leads to infarct size reduction and ameliorated remodeling.\textsuperscript{31–34} NO-dependant cytotoxicity is mediated by NO-derived radicals and an inactivation of sulfur/iron-centered enzymes that are crucially involved in metabolic pathways.\textsuperscript{35,36} Enlarged infarct size in Foxp3 DTR mice, thus, likely arises partially from a secondary loss of cardiomyocytes.\textsuperscript{37} However, although we could not detect differential expression of collagenases that are critically involved in ECM turnover, we cannot definitely rule out an escalated overall enzyme activity in the hearts of Foxp3DTR mice, for instance, because of matrix metalloproteinase activation by reactive nitrogen and oxygen species that may have further aggravated infarct expansion.\textsuperscript{38} Consistent with inducible NO synthase 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 osteopontin and FXIII as well as inflammation-resolving TGF\textsubscript{β}\textsubscript{1} likely further deteriorated the outcome in Treg-cell–ablated mice.\textsuperscript{40–42}

Activation of T\textsubscript{reg} Improves Healing After MI

Because T\textsubscript{reg} depletion showed pronounced effects during the phase of myocardial healing, we next studied whether the obvious beneficial effect that T\textsubscript{reg} cells physiologically exert on postinfarct wound healing could be further increased by therapeutic T\textsubscript{reg} cell activation with a CD28-SA after MI. This approach is similar to a recently published report stating that administration of CD28-SA in rats beneficially influenced cardiac remodeling.\textsuperscript{43} In our mouse model, CD28-SA–treated animals had, compared with isotype control immunoglobulin–treated mice, significantly higher T\textsubscript{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, because of Treg-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\textsubscript{reg}-Cell Activation Triggers M2-Like Macrophage Differentiation

Herein, we demonstrate for the first time that therapeutic T\textsubscript{reg} cell activation/expansion by CD28-SA treatment enhances M2-like monocyte differentiation. We think that regulation of monocyte differentiation by T\textsubscript{reg} cells certainly is not the only mechanism but constitutes a crucial contribution to the beneficial effects of therapeutic and most likely also physiological T\textsubscript{reg}-cell activation after MI. Alternatively, activated M2-like macrophages have well known anti-inflammatory characteristics and are an integral component of wound-healing processes.\textsuperscript{45} Consistently, experimental modulation of macrophage polarization toward an M2 state has been previously demonstrated to improve wound healing post-MI.\textsuperscript{46}
Alternative macrophage activation is initiated in response to IL-4 or IL-13 and other stimuli such as IL-10 or TGFβ inducing an M2-like phenotype. We show here that T cells produced little TGFβ1 but high amounts of IL-10 and IL-13 in vitro on CD28-SA activation, capacitating the cells to induce M2-like macrophage differentiation. In vivo, the accumulation of T cells in the hearts of CD28-SA–treated mice was associated with elevated amounts of TGFβ1, IL-10, and IL-13 within the cardiac scar tissue, suggesting that the activated T cells also induced an M2-polarizing milieu locally within the heart (see model in Online Figure VIII). Production of IL-10 and TGFβ1 by M2-like cells themselves might have further contributed to the M2-creating milieu in situ. However, we cannot exclude T–cell–mediated contact-dependent mechanisms that may also contribute to monocyte/macrophage polarization.

**Molecular Mediators of Improved Healing After Therapeutic T Cell Activation**

TGFβ is considered to drive collagen deposition by myofibroblasts. In CD28-SA–treated mice, both activated T cells and M2 macrophages likely contribute to increased TGFβ1 levels in the healing myocardium as compared with control animals. Moreover, IL-13, which was strongly induced by CD28-SA treatment, synergizes with TGFβ1 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 osteopontin, which was significantly upregulated in the scar tissue of CD28-SA–treated mice. The healing myocardium of osteopontin-knockout mice has been shown to exhibit disarrayed and decreased collagen deposition, implying an essential role of osteopontin in matrix assembly and organization. Therefore, elevated osteopontin 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 nonischemic skeletal muscle damage, formation of a collagenous scar can be avoided and full tissue recovery is possible. Under such circumstances, T cells have also been shown to influence the healing process beneficially, in this case by suppressing osteopontin production in favor of myotrophic factors such as amphiregulin with the latter being produced by muscle-infiltrating T cells themselves. 

Because, after MI, osteopontin favors the formation of a robust collagenous scar, we set up an in vitro system to assess the contribution of monocytic cells and T cells to the amount of cardiac osteopontin in vivo. T cells stimulated with a CD28-SA in vitro secreted TGFβ1, IL-10, and IL-13, which, in combination, provoked strong osteopontin release from monocytic cells along with M2 differentiation. TGFβ neutralization dramatically restrained osteopontin release from monocytic cells, which is in line with the observation that a lack of TGFβ receptor engagement inhibits M2 polarization. However, TGFβ receptor signaling alone was not sufficient to elicit full osteopontin secretion, showing that TGFβ is required to render the monocytic cells responsive to T–cell–derived IL-10 and IL-13 driving osteopontin expression.

Because monocytic cells are the predominant leukocyte fraction in the scar tissue 5 days after MI, a large proportion of osteopontin 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 osteopontin expression.

Apart from osteopontin, 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 correlate 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 decreased rates of heart ruptures. Thus, increased FXIII expression in the myocardium of CD28-SA–treated mice likely also contributes to improved scar tissue formation (Online Figure VIII).

**Clinical Implications**

The concept of manipulating the T cell compartment 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 T signature mediator IL-10 in the plasma after treatment. Therefore, administration of 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 T cells in vivo. For example, recombinant human IL-2 is used at low dosages to selectively expand T cells compared with 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 because it also increases the suppressive activity of T cells on a per-cell basis.

In conclusion, we have shown that, on the one hand, T 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 T 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 because modulation of both T cells 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.

**Acknowledgments**

We greatly appreciate the excellent technical assistance of S. Knorr, B. Bayer, H. Wagner, C. Diensch, C. Linden, S. Umbenhauer, and M. Göbel.
Sources of Funding
The study was supported by grants of the Bundesministerium für Bildung und Forschung (BMBF01 EO1004, to G. Ertl and S. Frantz). G.C. Ramos was supported by the Brazilian National Council for Scientific and Technological Development.

Disclosures
None.

References
27. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A. 1990;87:1620–1624.
Therapeutic Treg-cell activation represents an eligible strategy to achieve healing-fostering factors from Treg and M2 cells leading 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.

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+ CD4+ regulatory T cells (Treg cells) feature potent anti-inflammatory characteristics.
- Accumulation of monocytes/macrophages in the infarcted myocardium is biphasic characterized by an early lymphocyte antigen 60α monocyte–dominant inflammatory phase followed by an M2-like macrophage–prevalent reparative phase.

What New Information Does This Article Contribute?

- Treg cells accumulate at low numbers in the infarcted myocardium.
- Treg cells influence the transition from the inflammatory to the reparative phase by modulating macrophage function that critically affects clinical outcome.
- Therapeutic Treg-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 Treg cells that constitute a T-cell subpopulation with anti-inflammatory properties. We hypothesized that Treg cells influence healing and outcome post-MI. Treg cells expanded in heart-draining lymph nodes and accumulated at low numbers in the infarcted myocardium. Treg-cell depletion before MI provoked an adverse activation of nonregulatory CD4+ and CD8+ T cells that numerously infiltrated the infarct zone of Treg-cell–deficient mice. These non-Treg cells restrained M2-like macrophage polarization by secreting inflammatory factors, resulting in impaired healing and cardiac function. In contrast, therapeutic Treg-cell activation reinforced Treg-cell influx into the infarct zone and stimulated M2-like macrophage polarization by soluble mediators. Release of inflammation-resolving and healing-fostering factors from Treg 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.
Foxp3\(^+\) CD4\(^+\) T Cells Improve Healing After Myocardial Infarction by Modulating Monocyte/Macrophage Differentiation

Johannes Weirather, Ulrich D.W. Hofmann, Niklas Beyersdorf, Gustavo C. Ramos, Benjamin Vogel, Anna Frey, Georg Ertl, Thomas Kerkau and Stefan Frantz

Circ Res. 2014;115:55-67; originally published online April 30, 2014; doi: 10.1161/CIRCRESAHA.115.303895

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/115/1/55

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/04/30/CIRCRESAHA.115.303895.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
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%NaN$_3$ 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. For intracellular cytokine stainings, cells were restimulated for 5 hours in vitro in presence of ionomycin (Sigma), phorbol-12-myristate-13-acetate (Sigma) and monensin (BD Biosciences).

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_{reg} and T_{conv} cells
For purification of monocytes, 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-CD3ε 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_{reg} and T_{conv} cells were sorted from lymph node cell suspensions stained with anti-CD4 and anti-CD25. T_{reg} cells were defined as CD4^+CD25^{high}, T_{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).
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\textsuperscript{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 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 α-SMA 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 monocytic 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. T_{reg} cells were defined as CD4+CD25^{high} 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^{high} (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-$eta$ 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-1$eta$-mediated upregulation of matrix metalloproteinases (MMPs) and, moreover, to induce tissue inhibitors of MMPs in myofibroblasts within the scar tissue after MI.$^1$ M2-derived TGF-$eta$1 and T_{reg}-cell-derived TGF-$eta$1 and IL-13 function cooperatively to drive collagen synthesis in myofibroblasts.
Online Table 1: Echocardiographic characterization of Foxp3^{DTR} and wildtype mice at baseline

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Foxp3^{DTR}</th>
<th>wildtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>body weight, g</td>
<td>20 ± 3.5</td>
<td>23 ± 1.8</td>
</tr>
<tr>
<td>Echocardiographic measurements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary EDA, mm²</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, mm²</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>
</tr>
<tr>
<td>Heart rate</td>
<td>617 ± 4.7</td>
<td>610 ± 16</td>
</tr>
<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>
</tr>
</tbody>
</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>
</tr>
<tr>
<td><strong>Echocardiographic measurements</strong></td>
<td></td>
<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>
</tr>
<tr>
<td></td>
<td>d7</td>
<td>19.9 ± 4.8</td>
<td>17.9 ± 4.5</td>
</tr>
<tr>
<td>Heart rate</td>
<td></td>
<td>500 ± 30</td>
<td>510 ± 36</td>
</tr>
<tr>
<td>Apical end-diastolic anterior wall, cm</td>
<td>d7</td>
<td>0.050 ±0.003</td>
<td>0.055 ±0.0100</td>
</tr>
<tr>
<td>Apical end-diastolic posterior wall, cm</td>
<td>d7</td>
<td>0.050 ±0.005</td>
<td>0.055 ±0.009</td>
</tr>
<tr>
<td>Parameter</td>
<td>Phase</td>
<td>Anti-CD25</td>
<td>Isotype control Ig</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-------</td>
<td>-----------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Infarct size, %</td>
<td>d7</td>
<td>63.1 ± 7.6</td>
<td>58.2 ± 12.3</td>
</tr>
</tbody>
</table>

Echocardiographic measurements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase</th>
<th>Anti-CD25</th>
<th>Isotype control Ig</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>Apical M-mode FS, %</td>
<td>d7</td>
<td>7.8 ± 1.8</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>Heart rate</td>
<td></td>
<td>590 ± 43</td>
<td>570 ± 40</td>
</tr>
<tr>
<td>Apical end-diastolic anterior wall, cm</td>
<td>d7</td>
<td>0.04 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Apical end-diastolic posterior wall, cm</td>
<td>d7</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

Online Table 3: Echocardiographic characterization of anti-CD25 antibody and isotype control antibody (Ig) treated mice
Online Table 4: Infarct sizes, organ weights and echocardiographic parameters of isotype control Ig and CD28-SA-treated mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase</th>
<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>
</tr>
<tr>
<td>Infarct size, %</td>
<td>d56</td>
<td>56.8 ± 10.8</td>
<td>55.6 ± 9.4</td>
</tr>
<tr>
<td>body weight, g</td>
<td>d7</td>
<td>23.6 ± 2</td>
<td>23.5 ± 1.3</td>
</tr>
<tr>
<td>LV weight/body weight</td>
<td>d7</td>
<td>5 ± 0.3*</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>Lung weight/body weight</td>
<td>d7</td>
<td>7.7 ± 1*</td>
<td>11 ± 1.5</td>
</tr>
</tbody>
</table>

Echocardiographic measurements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase</th>
<th>Isotype control Ig</th>
<th>CD28-SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary EDA, mm²</td>
<td>d7</td>
<td>25.9 ± 4.1</td>
<td>26.6 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>d56</td>
<td>28.5 ± 3.6</td>
<td>33.2 ± 8.8</td>
</tr>
<tr>
<td>Papillary M-mode FS, %</td>
<td>d7</td>
<td>8.2 ± 3.9</td>
<td>7.5 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>d56</td>
<td>10.3 ± 3.4</td>
<td>8.8 ± 5</td>
</tr>
<tr>
<td>Apical EDA, mm²</td>
<td>d7</td>
<td>26.6 ± 6.6</td>
<td>28.4 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>d56</td>
<td>29.8 ± 4.2</td>
<td>36.4 ± 10.3</td>
</tr>
<tr>
<td>Apical M-mode FS, %</td>
<td>d7</td>
<td>6 ± 3.3</td>
<td>6.3 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>d56</td>
<td>5 ± 3.7</td>
<td>6 ± 3.8</td>
</tr>
<tr>
<td>Heart rate</td>
<td>d7/d56</td>
<td>622.5 ± 22.8</td>
<td>591.7 ± 56.7</td>
</tr>
<tr>
<td>Apical end-diastolic anterior wall, cm</td>
<td>d56</td>
<td>0.020 ± 0.001*</td>
<td>0.040 ± 0.001</td>
</tr>
<tr>
<td>Apical end-diastolic posterior wall, cm</td>
<td>d56</td>
<td>0.03 ± 0.01</td>
<td>0.06 ± 0.07</td>
</tr>
</tbody>
</table>

*P<0.05 vs. CD28-SA
## Online Table 5: Antibodies used for FACS analyses

<table>
<thead>
<tr>
<th>specificity</th>
<th>clone</th>
<th>supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b-PE</td>
<td>M1/70</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD45-efluor 450</td>
<td>30-F11</td>
<td>ebioscience</td>
</tr>
<tr>
<td>F4/80-FITC</td>
<td>BM8</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Foxp3-PE-Cy5.5</td>
<td>FJK-16s</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Ly-6C-PerCP-Cy5.5</td>
<td>HK1.4</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD4-Alexa647</td>
<td>RM4-5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD8a-Pe-Cy7</td>
<td>53-6.7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Helios-Pacific Blue</td>
<td>22F6</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD19-Biotin</td>
<td>6D5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Ly-6G-Alexa647</td>
<td>1A8</td>
<td>BioLegend</td>
</tr>
<tr>
<td>IFNγ-PE</td>
<td>XMG1.2</td>
<td>BioLegend</td>
</tr>
<tr>
<td>TNFα-Brilliant Violet 421</td>
<td>MP6-XT22</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD3ε</td>
<td>145-2C11</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>