Activation of CD4+ T-Lymphocytes Improves Wound Healing and Survival after Experimental Myocardial Infarction in Mice

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Ulrich Hofmann, MD¹*; Niklas Beyersdorf, MD²*; Johannes Weirather¹; Anna Podolskaya, MD¹; Johann Bauersachs, MD³; George Ertl, MD¹; Thomas Kerkau, MD²*; Stefan Frantz, MD¹*

¹University Clinic Wuerzburg, Comprehensive Heart Failure Center; ²University of Wuerzburg, Institute for Virology and Immunobiology, Wuerzburg; ³Medizinische Hochschule Hannover, Department of Cardiology, Hannover, Germany
* These authors contributed equally

Correspondence:
Ulrich Hofmann, MD
University of Wuerzburg, University Clinic
Comprehensive Heart Failure Center
Department of Internal Medicine I
Oberdürrbacherstraße 6
D-97080 Würzburg, Germany
Tel: ++49 931 201 1
Fax: 49 931 639110
Email: hofmann_u2@klinik.uni-wuerzburg.de

Abstract:

**Background** - The role of the adaptive immunity, especially CD4+ T-helper cells, has not yet been systematically investigated in wound healing and remodelling after myocardial infarction (MI). Therefore, we studied, whether CD4+ T-cells become activated and influence wound healing after experimental MI in mice.

**Methods and Results** - When comparing sham vs. MI in wild-type (WT) mice, T-cell receptor dependent activation of both conventional Foxp3- and regulatory Foxp3+ CD4+ T-cells could be demonstrated in heart-draining lymph nodes within the first week after MI. Concomitantly, we found infiltration of CD4+ T-cells in infarcted myocardium. To study the role of CD4+ T-cells in wound healing and remodelling, CD4+ T-cell deficient mice (CD4 KO, MHCIIΔ/Δ) and T-cell receptor-transgenic OT-II mice recognizing an irrelevant ovalbumin-derived peptide were studied. Serial echocardiography up to day 56 after MI revealed increased left ventricular dilation in CD4 KO when compared to WT mice. Within the infarcted myocardium, CD4 KO mice displayed higher total numbers of leukocytes and of pro-inflammatory monocytes (18.3 ± 3.0 WT vs. 75.7 ± 17.0 CD4 KO, p<0.05). MHCIIΔ/Δ and OT-II mice displayed significantly higher mortality (21% WT vs. 48% OT-II, p<0.05 and WT 22% vs. 52% MHCIIΔ/Δ, p<0.05) and myocardial rupture rates than WT mice. Collagen matrix formation in the infarct zone was severely disturbed in CD4 KO and MHCIIΔ/Δ as well as OT-II mice.

**Conclusions** - The study provides first evidence that CD4+ T-cells become activated after myocardial infarction, presumably driven by recognition of cardiac autoantigens, and facilitate wound healing of the myocardium.

**Key words:** T-lymphocytes, myocardial infarction, ventricular rupture, wound healing

**Non-standard abbreviations and acronyms:** CD - cluster of differentiation; CD4 KO - CD4 knockout mouse model; ECM - extracellular matrix; IFN – Interferon; IL - Interleukin MI - myocardial infarction; MMP - matrix-metalloproteinase; MHCIIΔ/Δ - major histocompatibility complex-II deficient mouse model; OT-II - mice expressing an ovalbumin-specific T-cell receptor as a transgene; Treg - regulatory T-cells; LV - left ventricle; RV - right ventricle; Th1, Th2 - T-helper type 1, -2 subsets
Introduction

Ischemic heart disease develops from a single large or from recurrent small myocardial infarctions (MI) and is the major etiology of heart failure. Today, standard therapy of acute myocardial infarction is prompt reperfusion by balloon-catheter facilitated vessel dilatation or pharmacological thrombus lysis. However, if reperfusion is established too late, a large transmural infarction may develop. Despite standardized therapy, prognosis still remains poor in these patients which regularly display severe left ventricular dysfunction after myocardial infarction \(^1\). Thus it would be highly desirable to develop new therapeutic options to improve healing after MI.

Myocardial injury induces a massive infiltration of innate immune cells into the infarct border zone starting within a few hours after the onset of ischemia in the mouse model \(^2-4\). Granulocytes and monocytes/ macrophages have long been recognized as the main innate effector cells modulating myocardial healing. However, the detailed mechanisms controlling recruitment, differentiation and effector function of these heart-infiltrating cells have still not been comprehensively defined.

The possible contribution of adaptive immunity in this context has been widely disregarded - probably for two main reasons: First, the infiltration of lymphocytes into the infarcted myocardium is scarce and is therefore often not monitored in experimental studies. Second, at first glance, activation of lymphocytes contradicts the classical notion that the adaptive immunity is not stimulated by self-antigens. However, there are several findings from both clinical and experimental animal studies implicating activation of cells of the adaptive immunity after myocardial infarction. Maisel et al. e.g. reported that ex vivo activated splenocytes collected from rats after experimental MI infiltrate the myocardium of non-infarcted
recipient animals. Splenocytes from infarcted rats contain a subset of CD8+ T-lymphocytes with cytotoxic activity against cardiomyocytes ex vivo. Another line of evidence comes from experimental autoimmune myocarditis models demonstrating that T-cells specific for myocardial proteins do exist in mice. Here, both, immunization with troponin- or myosin-derived peptides as well as transfer of myosin peptide-loaded dendritic cells induces myocarditis in susceptible mouse strains.

In patients with known coronary heart disease the prevalence of antibodies to contractile proteins is increased. As production of antibodies recognizing protein antigens by B-cells normally requires the concomitant activation of CD4+ helper T-cells, this observation indirectly implicates that also in humans CD4+ T-cells exist that can be activated by autoantigens released during ischemic heart injury. Accordingly, Cheng et al. reported activation of circulating T-lymphocytes with a Th1/Th2 functional imbalance towards Th1 in patients with acute myocardial infarction. Moreover, Abbate et al. demonstrated infiltration of lymphocytes into infarcted and remote myocardium of patients with myocardial infarction. However, the contribution of CD4+ T-lymphocyte subsets to and the relevance of CD4+ T-cell activation for myocardial wound healing and remodelling have not yet been studied systematically.

These observations prompted us to hypothesize that CD4+ T-cells might become activated after ischemic injury of the heart and influence myocardial wound healing. The results of our study indicate that, that both conventional and Foxp3+ regulatory CD4+ T cells are activated in the wake of myocardial infarction and that CD4+ T cell activation beneficially influences wound healing and survival.
Materials and methods

Animals

Animals were maintained at least two weeks for acclimatization on a 12:12 hour light-dark cycle with free access to standard diet and water. All mice were kept under specific pathogen-free conditions. 8-10 week old male C57BL/6J (stock Nr. 000664), CD4 KO (stock Nr. 002663), OT-II (stock Nr. 004194), MHCII\(\Delta\)\(\Delta\) (stock Nr. 003584) and CD4-EGFP stock (Nr. 005334) were purchased from The Jackson Laboratory.

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All animal procedures were approved by the local government (Ref. Nr. 01/08, 90/10).

Infarct model

MI was induced in mice as described previously\(^{13}\). In brief, mice were anaesthetized with isoflurane, intubated and put on a mechanical small animal ventilator. After a left-sided thoracotomy, MI was induced by ligating the proximal portion of the left coronary artery. Buprenorphine was given i.p. for analgesia perioperatively. In parallel subgroups, thoracotomy was performed to expose the heart but no suture was made to the coronary artery (sham operation).

Echocardiography

Echocardiography was performed on a Toshiba Aplio system with a 15-MHz ultrasound probe under light anaesthesia with isoflurane (about 1.5 volume %) and spontaneous respiration\(^{13}\).

FACS analysis

Cell suspensions from individual hearts were prepared by digestion with collagenase type 2 and protease type XIV (Sigma Aldrich, Germany) as has recently been described in detail\(^{14}\).
Staining protocols were described recently and are further specified in the supplemental methods section. For antibodies used see Supplemental Table 1.

**In vitro proliferation assay**

Single-cell suspensions from lymph nodes and spleens were prepared using a 70 μm cell strainer (BD Falcon, Heidelberg). Spleen cell suspensions were further subjected to hypo-osmotic shock for erythrocyte depletion.

Proliferation/antigen recall assays were performed in 96-well U-bottom plates. 2 x 10⁵ lymph node cells/well were diluted in a final volume of 200 μl. All cultures were done in triplicates. Proliferation was measured by determining [3H]thymidine incorporation (1.25 μCi of [3H]thymidine per well; GE Healthcare) during the final 16 h of a 3-day culturing period. Radioactive incorporation was quantified by a beta counter.

**Light microscopy**

Sections of mouse myocardium were fixed in 4% paraformaldehyde over night and embedded in paraffin. 5 μm sections were stained according to standard H.E. and Ladewig staining protocols, for determination of infarct size collagen was stained by picrosirious red (PSR) on 7 μm sections, as recently described. Immunohistochemistry for neutrophils, endothelial cells (CD31), collagen expressing cells (P4HB), and smooth muscle actin (SMA) was performed on 5 μm paraffin sections; immunohistochemistry for macrophages/monocytes was performed on 5 μm cryostat sections. Protocols and antibodies were specified in the supplemental methods section.

**Real-time reverse-transcriptase –PCR, qPCR arrays**

RNA isolation and real-time polymerase chain reaction procedures were performed with commercially available TaqMan probes (Applied biosystems, Foster City, USA) as reported
previously. Pathway-focused gene expression profiling was performed using commercial real-time quantitative PCR arrays (SAbioscience, Hilden, Germany).

**Statistical analysis**

Results are presented as means±SE per group. For single comparison of data, an unpaired t-Test was performed. If the data were not suitable for t-tests, e.g. non-parametric data, independent groups were compared by a Mann-Whitney-U-test. 2-way ANOVA was performed for multiple comparisons. Survival data were shown as Kaplan-Meier curves and data were analysed by a Log-rank test. Differences were considered significant at p<0.05. Data analysis was performed by StatView and WinStat statistics programs.

**Results**

**Myocardial infarction activates CD4⁺ T-cells**

Activation of a T-cell response requires antigen presentation to T-cells, which typically occurs not at the site of inflammation but in the respective draining lymph nodes. Therefore, we injected fluorescent microparticles into the anterior LV free wall during infarct surgery to identify heart-draining lymph nodes. These microparticles are designed to be readily incorporated by phagocytosing cells. FACS analysis 16 hours after MI surgery revealed labelled microparticles in CD11b⁺ cells isolated from infarcted myocardium and in cells from lymph nodes of the right upper mediastinum, but not in cervical or inguinal lymph nodes, spleen, and bone marrow (Supplemental Figure 1). These mediastinal lymph nodes were consistently enlarged after MI, but not after sham surgery. In wild type mice (WT) the total cell number in these lymph nodes was significantly greater than in sham-operated or naïve (not operated) mice 7 days after MI (5.4 ± 0.8 x 10⁶ WT MI, 1.1 ± 0.4 x 10⁶ WT sham, 0.4 ± 0.0 x 10⁶ naïve, p<0.05, Figure 1A). These
data provided a first indication that there was an activation process specifically induced by myocardial injury and not merely by the surgical procedure (sham surgery).

Next, we cultured freshly isolated cells from mediastinal lymph nodes of mice with MI or after sham treatment in vitro and determined their spontaneous proliferative activity by measuring 3H-thymidine incorporation. We found that the basal proliferative activity was significantly higher in animals with MI than in sham animals, both, 7 days (3.7 ± 0.4 x 10^6 cpm sham vs. 5.4 ± 1.1 x 10^6 cpm MI, p<0.05,) and 21 days after MI (0.07 ± 0.01 x 10^6 cpm sham vs. 0.13 ± 0.03 x 10^6 cpm MI, p<0.05). This suggests that the increased cellularity we observed in vivo was probably due to cell proliferation in lymph nodes in response to MI.

In order to further confirm that T-cells proliferate in vivo in response to MI in heart draining lymph nodes, we directly analyzed expression of the proliferation marker Ki67 by CD4+ T-cells isolated from mediastinal lymph nodes on day 7 after MI. As shown in Figure 1B, a significantly higher proportion of CD4+ T-cells were positive for the proliferation marker Ki67 after MI than in sham-operated animals. Thus, we could demonstrate that MI induces proliferation of CD4+ T-cells in heart draining lymph nodes in vivo.

**Distribution and differentiation of CD4+ T-cells within the myocardium after MI**

After having demonstrated that myocardial infarction induces proliferation of CD4+ T-cells in heart draining lymph nodes we investigated whether T-cells invade the infarcted myocardium. Determination of the absolute numbers of myocardial CD3+ CD4+ T-cells as identified by FACS analysis revealed recruitment to the infarct zone by day 7 after MI (Figure 1C). We further utilized a transgenic mouse model expressing EGFP under the murine CD4 promoter for regional assessment of T-cell distribution in myocardium by immunohistology. On day 3, CD4+ T-cells could be detected by anti-EGFP staining in immunohistology within the infarct/infarct border.
zone, while CD4+ T-cells were hardly detectable in the myocardium of naïve/ sham-operated mice (data not shown). By day 5, EGFP+ T-cells were evenly distributed in infarcted and non-infarcted LV myocardium (Figure 1D). The frequency of EGFP+ T-cells then decreased within the infarct/ infarct border zone until day 21 while it did not change within the septum. However, even at the peak of intracardial CD4+ T-cell accumulation the prevalence of CD4+ T-cells remained low and accounted for less than 1% of all cells, including cardiomyocytes, as determined by FACS analysis on day 7 (data not shown).

Despite their paucity, intracardial CD4+ T-cells might play an important role in wound healing. Therefore, we analyzed their differentiation state by FACS analysis of intracellular cytokines. We could detect intracellular expression of IL-10, IL-17, and IFN-γ (Figure 1E). IFN-γ was the most abundantly expressed cytokine both in CD4+ T-cells from sham and MI hearts. The expression level (MFI) of IFN-γ was significantly higher in CD4+ T-cells derived from infarcted than from sham operated myocardium. The MFI of IL-17 and IL-10 was not increased after MI. While there was no significant increase in the individual relative frequencies of cytokine expressing cells among CD4+ T-cells from sham vs. MI hearts, the recruitment of CD4+ T-cells (Figure 1C) results in a significant net increase in cytokine producing T-cells.

Collectively, MI induces an overall increase of cytokine-producing CD4+ T-cells both within infarcted and remote myocardium.

**Increased LV dilation and disturbed scar formation in CD4+ T-cell deficient mice**

To gain insight into the functional role of CD4+ T-cells for wound healing after MI, we performed permanent coronary artery ligation in CD4 KO animals which lack CD4+ T-cells. Baseline LV-function and -geometry were not different in CD4 KO and WT animals. Cumulative survival, infarct size, heart and lung weights, and papillary echocardiographic
parameters (Table 1) were also not different up to day 56 post MI. However, there was a significantly greater enlargement of the LV area determined at the apical level, representing the infarct zone, over time (day 56 apical end-diastolic area: $42.2 \pm 1.6$ CD4 KO vs. $34.7 \pm 6.6$ mm$^2$ WT, $p<0.05$, Figure 2A).

Histology of the infarct zone demonstrated qualitative and quantitative differences in the collagen composition of the scars. Ladewig collagen staining revealed disarrayed collagen fibre alignment within the infarct zone in CD4 KO mice on day 7 (Figure 3A). Quantification of extracellular collagen content based on PSR stained sections revealed significantly lower amounts in CD4 KO compared to WT (Figure 3C).

In summary, the absence of CD4$^+$ T-cells leads to increased early LV dilation without affecting mortality. This was accompanied by disturbed collagen de novo formation within the infarct zone.

**CD4$^+$ T-cells modulate recruitment of innate immune cells to the infarcted heart**

Neutrophils and monocytes infiltrating the infarcted myocardium critically modulate myocardial wound healing. Therefore, we studied whether CD4$^+$ T-cells modulate innate immune cell recruitment to the infarcted myocardium. Immunohistology showed a greater density of, both, granulocytes and monocytes/ macrophages within the infarcted myocardium of CD4 KO as compared to WT mice on day 7 (Figure 4 A,B). As CD11b$^+$ granulocytes and monocytes/ macrophages represent the main leukocyte subsets infiltrating the infarct zone within the first week after MI these cells were further studied by FACS analysis. Granulocytes were defined as CD11b$^+$ GR1/ Ly6G$^+$ and monocytes/ macrophages were defined as CD11b$^+$ GR1/ Ly6G$^-$ (Supplemental Figure 2). There was no difference in leukocyte density and in innate leukocyte subset composition between WT and CD4 KO on day 3 (data not shown). On day 7, leukocyte
density in the infarct/infarct border zone was significantly higher in CD4 KO than in WT mice (Figure 4C) reflecting a parallel increase in both, neutrophils and monocytes/macrophages on day 7 (data not shown). Analysis of the monocyte differentiation marker Ly6C on the CD11b⁺ GR1⁻ Ly6G⁻ subset revealed that in CD4 KO mice a significantly higher proportion consisted of proinflammatory Ly6C<sup>high</sup> monocytes (34.0 ± 6.0 % CD4 KO vs. 20.1 ± 1.6 % WT, p<0.05, Figure 4D) on day 7. Ly6C<sup>high</sup> monocytes are preferentially recruited during the first 3-5 days by the chemokine MCP-1. Accordingly, MCP-1 expression was significantly higher in the infarct/infarct border zone of CD4 KO mice (246.2 ± 54.1 pg/mg CD4 KO vs. 81.0 ± 17.8 pg/mg WT, p<0.05, Figure 4E).

Moreover, the frequency of apoptosis, as analysed by Annexin V and propidium iodide staining of CD11b⁺ leukocytes, was not different between genotypes on day 7 (data not shown). As intramyocardial monocyte proliferation is marginal<sup>16</sup>, differential recruitment is most likely the main mechanism for the observed differences in monocyte subset composition.

As MMP-9 is the main matrix-metalloproteinase secreted by infiltrating leukocytes during the first days after MI and as MMP activity is associated with adverse LV remodelling and dysfunctional wound healing<sup>17</sup>, we analysed intracellular MMP-9 expression in CD11b⁺ cells derived from the infarct/infarct border zone by FACS analysis. Single cell MMP-9 expression (MFI of the MMP-9⁺ subset) was significantly higher in CD4 KO as compared to WT mice (MFI: 277.5 ± 66.1 CD4 KO vs. 48.5 ± 0.4 WT, p<0.05, Figure 4F).

To elucidate, whether CD4<sup>+</sup> T-cells affect the cytokine/chemokine milieu within the infarct zone, we assessed their relative expression in CD4 KO and WT mice by means of qPCR arrays. In general, expression of proinflammatory as well as anti-inflammatory cytokines, chemokines and their receptors revealed a complex pattern of expression. E.g. both the relative
mRNA and protein expression of the prototypical CD4+ T-cell derived anti-inflammatory and pro-inflammatory cytokines IL-10 and IFN-γ were reduced in CD4 KO MIs (Supplemental Table 3, Figure 4G), which is also in accordance with our data showing expression of both cytokines in intramyocardial CD4+ T-cells (Figure 1E).

Collectively, absence of CD4+ T-cells results in an increased density of innate immune cells (granulocytes and monocytes/macrophages), with a higher prevalence proinflammatory monocytes on day 7.

**Higher mortality in CD4+ T-cell deficient MHCIIΔ/Δ mice**

As there is a subset of MHC class II-restricted CD8+ T-cells in the CD4 KO mouse, which can, at least in part, functionally compensate for the lack of CD4+ T-cells we additionally studied class II MHC-deficient MHCIIΔ/Δ mice. In MHCIIΔ/Δ mice there is a nearly complete absence of CD4+ T cells with a normal development of the CD8+ T-cell compartment.

MHCIIΔ/Δ mice showed significantly impaired survival compared to WT animals (Figure 2B). By day 7, mortality was 21% in WT and 48% in MHCIIΔ/Δ (n=4/19 WT vs. n=10/21 MHCIIΔ/Δ). Unlike CD4 KO mice there was no enhanced LV dilatation after MI (Table 2). Infarct size was also not different between genotypes. In analogy to the findings in the CD4 KO model there was a trend towards a higher prevalence of proinflammatory monocytes (7.3 ± 1.1 MHCIIΔ/Δ vs. 3.8 ± 1.0 WT, n=4-5, p=0.08, n=4-5/group) in MHCIIΔ/Δ mice on day 8.

**Formation of the collagenous scar is disturbed in CD4+ T-cell deficient mice**

Extracellular matrix cleavage, de novo formation, and angiogenesis are hallmarks of granulation tissue formation during early wound healing. The common feature of the infarct zone of CD4+ T-cell deficient CD4 KO and MHCIIΔ/Δ mice was less and disarrayed collagen fibres within the infarct zone compared to WT mice (Figure 3). This was not accompanied by a reduced
frequency of collagen producing cells within the infarct zone (Figure 5A). Accordingly, analysis of collagen I de novo expression by rt-PCR revealed no differences between WT and CD4 KO after MI (Figure 5B).

Besides collagen de novo synthesis the collagenolytic activity of matrix-metalloproteinases determines extracellular collagen composition. However, there was also no difference in total collagenolytic activity between WT and CD4 KO arguing against a role of matrix degradation for the disturbed collagenous scar composition (Figure 5C).

Immunohistology for the endothelial cell surface marker CD31 demonstrated that capillary density, reflecting angiogenesis, within the infarct border zone of CD4 KO mice was lower than in WT mice (Figure 5D).

Collectively, absence of CD4+ T-cells impairs collagen matrix de novo formation and angiogenesis without influencing collagen breakdown.

**Increased mortality and myocardial rupture in OT-II mice**

In order to assess whether the mere absence of CD4+ T-cells or lack of antigen recognition by the CD4+ T-cells accounted for the observed differences between WT and CD4+ T-cell deficient mice, we further studied OT-II mice. The majority of CD4+ T-cells in OT-II mice exclusively recognize an irrelevant ovalbumin-derived peptide via their T-cell receptor.

Baseline echocardiographic geometry and function was not different between genotypes. 56 days after permanent MI, infarct size, heart weight/ body weight and lung weight/ body weight ratios were not different from WT (Table 3). OT-II mice, however, showed significantly impaired survival compared to WT animals (Figure 6A). Cumulative 56-day mortality was 23% (WT, n= 10/ 44) vs. 51% (OT-II, n= 20/ 39). In both groups, most animals died within the first
week from heart failure or ventricular ruptures but the incidence of LV ruptures in mice with MI was significantly higher in OT-II (19%) than in WT mice (3%).

Hence, the increased early mortality in OT-II mice indicates that an intact T-cell receptor repertoire is vital for the protective function of CD4+ T-cells after MI.

Immunohistology revealed no difference in granulocyte and monocyte/macrophage density within the infarct zone on day 7 (data not shown). As the day 7 data might be biased by differential survival, we further analysed inflammatory cell infiltration on day 3. FACS analysis of infiltrating innate immune cells within the infarct zone on both days 3 and 7 revealed no differences between OT-II and WT (Supplemental Table 2).

Similar to CD4+ T-cell deficient mice, collagen fibres within the infarct zones in OT-II mice were more loosely distributed and disarrayed on day 7. As in CD4 KO and MHCIImice, total extracellular collagen density within infarct zone was less in OT-II than in WT (Figure 6C). PCR analysis revealed no difference in collagen I, III and MMP/TIMP expression between OT-II and WT (data not shown).

Collectively, as in the CD4+ T-cell deficient mice, OT-II mice demonstrated impaired wound healing, which was associated with increased early mortality.

**Antigen recognition is vital for early CD4+ T-cell activation after MI**

After having demonstrated that the mere presence of CD4+ T-cells is not sufficient but antigen recognition via the T-cell receptor seems to be a prerequisite for proper wound healing, we next studied the activation of conventional Foxp3− and regulatory Foxp3+ CD4+ T-cells in heart-draining lymph nodes of WT and OT-II mice. As the differences in clinical outcome between WT and CD4 KO, MHCIImice, as well as OT-II mice were already evident within the first seven days we also studied CD4+ T-cell activation during the first week after MI. In particular, we
analysed the expression of the activation marker CD25 on conventional Foxp3- CD4+ T-cells on day 3 after MI or sham operation (Figure 7A). WT but not OT-II mice showed significantly increased frequencies of CD25+ cells among conventional T-cells after MI (Figure 7A, C). Moreover, absolute cell numbers of conventional CD4+ T-cells, expressed as the ratio MI over sham (Figure 7D), were in tendency also increased in WT but not in OT-II.

Early antigen-dependent activation did, however, not seem to be restricted to conventional CD4+ T-cells, but was probably even greater in Foxp3+ Treg cells as their proportion among all CD4+ T-cells in mediastinal lymph nodes of WT mice was significantly increased on day 3 (Figure 7B,C). Concomitantly, the ratio of the absolute cell numbers of Tregs from WT mice significantly increased after MI (Figure 7E). However, in contrast to day 7 (data not shown), on day 3 neither CD4+ Foxp3- nor CD4+ Foxp3+ cells showed increased frequencies of proliferating, Ki67-expressing, cells (data not shown).

Collectively, the data indicate that in WT mice MI and the ensuing release of cardiac antigens impacts on, both, conventional, putatively autoreactive, CD4+ T-cells as well as on Tregs whose autoreactivity is well documented. T-cell receptor activation in WT mice induced both early expansion of Tregs and activation of conventional CD4+ T-cells. The increase in Treg frequencies among CD4+ T-cells observed on day 3 after MI, however, did not prevent subsequent CD4+ T-cell proliferation (Figure 1B) and infiltration of CD4+ T-cells into the myocardium (Figure 1C).

Discussion

Our study provided the first evidence that CD4+ T-cells are activated and beneficially modulate myocardial wound healing after experimental myocardial infarction.
There are several reports from clinical studies indicating that cells of the adaptive immunity, especially T-lymphocytes, become activated in patients with coronary artery diseases or a history of myocardial infarction\textsuperscript{9,11,20}. However, it is important to remember that components of activated plaques, which typically trigger coronary artery thrombus formation leading to myocardial infarction, also activate T-lymphocytes\textsuperscript{21}. Therefore, analysis of lymphocyte activation, especially when lymphocytes were derived from peripheral blood of humans, does not allow distinguishing coronary artery from myocardial inflammation. The mouse model of myocardial infarction in which myocardial infarction is induced by ligature of a coronary vessel uniquely allows to study, whether adaptive immunity is activated by cardiac injury without possible interfering effects from coronary artery inflammation/atherosclerosis.

There is further recent evidence from studies in the mouse myocardial ischemia-reperfusion model that CD4\textsuperscript{+} T-cells modulate ischemia-reperfusion injury\textsuperscript{22,23}. However, it is important to emphasize that the present study was conducted for studying the role of CD4\textsuperscript{+} T-cells on wound healing and remodelling. Therefore, all experiments were performed in a model of permanent ischemia to obviate possible effects of CD4\textsuperscript{+} T-cells on primary infarct size.

Classical activation of T-cells requires the presentation of antigens by antigen presenting cells (dendritic cells, macrophages, B-cells) to the T-cell receptor, which typically takes place in the organ’s draining lymph nodes. We hypothesize that myocardial antigens which are normally not accessible to the immune system or neo-antigens created during ischemic injury e.g. by oxidative modifications, are released from the myocardium, become phagocytosed and presented to CD4\textsuperscript{+} T-cells. Monocytes infiltrate the injured myocardium\textsuperscript{16} and can differentiate into macrophages and dendritic cells which can also be found within the infarcted myocardium\textsuperscript{24}. Both cell types are able to present antigens on MHC-II complexes to CD4\textsuperscript{+} T-cells. By means of
intramyocardial injection of labelled particles we could identify heart-draining mediastinal lymph nodes. Within these lymph nodes we were able to demonstrate that MI specifically induces activation and proliferation of CD4$^+$ T-cells.

The functional role of CD4$^+$ T-cell activation was therefore studied in vivo in independent animal models that either lack CD4$^+$ T-cells (CD4 KO, MHCII$^{\Delta/\Delta}$ mice) or express a transgenic T-cell receptor specific for an ovalbumin-derived peptide (OT-II mice) leading to strongly reduced reactivity towards other antigens, including self-antigens. All mouse models demonstrated dysfunctional wound healing during the first week after myocardial infarction, which was histologically most evident as extracellular matrix disarray within the infarct zone. The OT-II and MHCII$^{\Delta/\Delta}$ models showed the more pronounced phenotype, as survival was significantly worse and the LV rupture rate was increased, whereas in the CD4 KO model aggravated LV dilation was not associated with increased mortality. The differences in the two phenotypes might most likely result from the presence of a considerable population of MHC-II restricted CD8$^+$ T-cells in the CD4 KO model, which can, at least in part, functionally compensate for the lack of CD4$^+$ T-cells$^{18}$. This consideration in turn further underlines the importance of MHC-II restricted antigen recognition for the observed effects.

The phenotype of the OT-II model demonstrates that an antigen-specific activation of CD4$^+$ T-cells rather than their mere presence is necessary for proper wound healing in WT mice. This finding differs from the recently demonstrated detrimental effects of T-cells in a mouse model of cerebral ischemia-reperfusion injury, where the impact of T-cells on the clinical phenotype was shown to be unrelated to antigen recognition via the T-cell receptor$^{25}$. Therefore, in contrast to cerebral ischemia-reperfusion, one might speculate that after MI an antigen-specific immunomodulating therapy might be successful in humans.
However, for translation of the present data derived from young male mice into the clinic the effect of gender and age deserves further investigation. Comparative data from male and female aged animals post MI are widely lacking. However, it has been demonstrated that in old mice there was reduced inflammation but enhanced LV remodelling and impaired preservation of systolic function compared to young mice. Immunosenescence has also been described for T-cell responses. Therefore, impaired activation of T-cells by myocardial antigens might contribute to the worse outcome in aged mice and man after MI.

For an exact understanding of the role of the adaptive immunity in cardiac wound healing it remains to be determined however, where and how CD4+ T-cells exert their beneficial effects. One could hypothesize that heart infiltrating CD4+ T-cells modulate the local cytokine/chemokine milieu and, thus, influence recruitment and activation of innate immune cells. Correspondingly, we found a greater leukocyte infiltrate and a higher proportion of proinflammatory Ly6C+ monocytes in CD4 KO myocardium. It has been demonstrated that a timely recruitment of the different monocyte subsets, which can be characterized by expression of the surface molecule Ly6C, is a prerequisite for proper myocardial wound healing.

However, a prolonged pro-inflammatory monocyte infiltration was only observed in CD4+ T-cell-deficient mice, which rules out monocytes as the key effectors inducing impaired collagen de novo synthesis which we observed in all three models. Our results rather suggest that heart infiltrating CD4+ T-cells might directly modulate the function of collagen secreting cells. Both CD4+ T-cell-deficient mice (CD4 KO, MHCIIΔ/Δ) and OT-II mice demonstrated disarrayed collagen fibres within the de novo formed collagenous scar. Neither parameters of collagen de novo formation (collagen producing cell density, collagen expression) nor total matrix-proteinase expression and activity were changed in CD4 KO and OT-II as compared to WT mice. Thus,
most likely, impaired post-transcriptinal processing of matrix proteins accounts for the observed phenotype. Several anti-inflammatory T-cell-derived cytokines like IL-10 \(^{28}\) and IL-13 \(^{29}\) have been demonstrated to influence extracellular matrix formation by modification of (myo)fibroblast differentiation and proliferation in various disease models. We found that both cytokines are downregulated in CD4 KO mice after MI. Although we cannot provide definite experimental evidence, those CD4\(^+\) T-cell-derived cytokines are potential mediators linking CD4\(^+\) T-cell activation and extracellular collagen synthesis in myocardial wound healing. Moreover, while monocytes/macrophages are well described effectors in wound healing and remodelling after MI, our data now pin-point to CD4\(^+\) T-cells as additional players either directly or indirectly influencing these processes.

Apart from CD4\(^+\) T-cells we also observed increased proliferation of CD8\(^+\) T-cells seven days after MI (data not shown). Preliminary data further show that this was not the case in MHCII\(^{\Delta /\Delta}\) mice suggesting that class-II MHC restricted CD4\(^+\) T-cells are needed for CD8\(^+\) T-cells to become activated after MI. As the activation of CD8\(^+\) T-cells in WT mice may influence wound healing, it cannot be totally excluded that the altered proportion of CD8\(^+\) T-cells in both CD4 deficient and OT-II mice as compared to WT mice might contribute to the phenotypes observed in these mutants.

Whereas conventional CD4\(^+\) T-cells often exert their biological effects after proinflammatory differentiation (e.g. Th1, Th17), many of the effects of regulatory T-cells can be attributed to the expression of anti-inflammatory cytokines. Especially, so-called natural regulatory T-cells are well-known for preventing autoimmunity \(^{12}\). We could demonstrate an early relative increase in the frequencies of these CD4\(^+\) Foxp3\(^+\) regulatory T-cells (Tregs) among all CD4\(^+\) T-cells in heart draining lymph nodes as early as 3 days after myocardial infarction.
This early shift towards higher regulatory T-cell frequencies was only seen in WT but not in OT-II mice indicating that antigens released during MI induce an early response of both conventional T-cells and even more so of Tregs. The observation that Treg frequencies were increased in the absence of overt changes in proliferative activity, as read out by frequencies of Ki67+ cells on day 3 after MI, suggests either, that in WT mice Tregs are selectively recruited to or retained in mediastinal lymph nodes or, alternatively, might have proliferated earlier. This CD4+ T-cell response triggered by MI in heart draining lymph nodes is strikingly similar to that observed after immunization of mice with complete Freund’s adjuvants containing foreign antigens. Tregs are also concomitantly activated with conventional CD4+ T-cells in a mouse model of mucosal herpes simplex infection, where they play a crucial role in fine tuning the immune response, thereby allowing for resolution of the infection.

In accordance with the similarity of the early CD4+ T-cell activation pattern that we observed after myocardial infarction to that triggered by foreign antigens, it was demonstrated very recently, that both in mice and humans the cardiomyocyte specific protein α-myosin heavy chain, at least in part, resembles an foreign antigen as it is not expressed within the thymus. In autoimmune myocarditis, release of α-myosin heavy chains leads to CD4+ T-cell activation, which essentially contributes to the disease pathology, as its expression in the thymus abolished the development of myocarditis in mice. It was further demonstrated that the peripheral T-cell compartment of mice not only harbours α-myosin heavy chain reactive T-cells, but that there are also CD4+ T-cells reactive to troponin. Thus, it appears that several myocardial proteins released during MI are not negatively selected against in the thymus and therefore, when released by cardiac injury, evoke a CD4+ T-cell response similar to that towards a non-self antigen. In the setting of MI this coordinate activation process of conventional and regulatory T-
cells obviously does not lead to detrimental auto-immunity, as in the case of myocarditis, but seems to tune the immune response finally promoting resolution of inflammation and wound healing.

Taken together, we could demonstrate for the first time that activation of CD4\(^+\) T-cells is a prerequisite for proper wound healing and subsequent remodelling of the myocardium after MI. These findings may, thus, pave the way for identifying novel therapeutic targets to improve clinical outcome in patients with MI.

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**Conflict of Interest Disclosures:** None

**References:**


Table 1. Infarct size, organ weights and echocardiographic data of CD4 KO and WT animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase</th>
<th>WT</th>
<th>CD4 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infarct size, %</td>
<td>Day 56</td>
<td>51.9 ± 4.8</td>
<td>53.9 ± 5.0</td>
</tr>
<tr>
<td>LV weight/ BW weight, mg/g</td>
<td>Day 56</td>
<td>4.5 ± 0.0</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Lung weight/ BW, mg/g</td>
<td>Day 56</td>
<td>7.0 ± 1.0</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>Day 56</td>
<td>31.4 ± 0.5</td>
<td>30.9 ± 0.7</td>
</tr>
<tr>
<td>Echocardiographic measurements:</td>
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<td></td>
</tr>
<tr>
<td>papillary EDA, mm²</td>
<td>baseline</td>
<td>7.4 ± 1.0</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>15.1 ± 0.3</td>
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</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>24.6 ± 2.5</td>
<td>23.4 ± 1.4</td>
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<tr>
<td></td>
<td>Day 21</td>
<td>31.9 ± 1.6</td>
<td>31.6 ± 2.3</td>
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<tr>
<td></td>
<td>Day 56</td>
<td>44.6 ± 1.1</td>
<td>36.7 ± 3.1</td>
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<tr>
<td></td>
<td>Day 56 sham</td>
<td>6.7 ± 0.2</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>papillary M-mode FS, %</td>
<td>baseline</td>
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<td>30.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>11.8 ± 1.8</td>
<td>13.6 ± 1.9</td>
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<tr>
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<td>Day 7</td>
<td>10.4 ± 0.1</td>
<td>9.2 ± 0.9</td>
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<tr>
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<td>Day 21</td>
<td>7.0 ± 0.5</td>
<td>6.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Day 56</td>
<td>7.2 ± 0.9</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Day 56 sham</td>
<td>35.2 ± 1.1</td>
<td>29.0 ± 4.9</td>
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Table 2. Infarct size and echocardiographic data of MHCII<sup>Δ/Δ</sup> and WT animals.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>WT</th>
<th>MHCII&lt;sup&gt;Δ/Δ&lt;/sup&gt;</th>
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<tr>
<td>Infarct size, %</td>
<td>Day 8</td>
<td>52.8 ± 7.3</td>
<td>54.8 ± 6.6</td>
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<tr>
<td>papillary EDA, mm²</td>
<td>baseline</td>
<td>4.8 ± 0.5</td>
<td>4.5 ± 0.3</td>
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<td></td>
<td>Day 8</td>
<td>20.3 ± 2.5</td>
<td>20.2 ± 2.3</td>
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<tr>
<td></td>
<td>Day 8 sham</td>
<td>8.9 ± 0.6</td>
<td>6.5 ± 0.5</td>
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<tr>
<td>papillary M-mode FS, %</td>
<td>baseline</td>
<td>41.3 ± 2.1</td>
<td>44.7 ± 2.4</td>
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<tr>
<td></td>
<td>Day 8</td>
<td>14.29 ± 4.9</td>
<td>14.6 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>Day 8 sham</td>
<td>34.4 ± 4.4</td>
<td>25.2 ± 1.5</td>
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<tr>
<td>apical EDA, mm²</td>
<td>baseline</td>
<td>4.8 ± 0.6</td>
<td>4.5 ± 0.3</td>
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<tr>
<td></td>
<td>Day 8</td>
<td>21.6 ± 2.9</td>
<td>21.6 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>Day 8 sham</td>
<td>7.9 ± 1.3</td>
<td>8.2 ± 0.8</td>
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<tr>
<td>apical M-mode FS, %</td>
<td>baseline</td>
<td>46 ± 3.1</td>
<td>47.6 ± 2.8</td>
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<td>Day 8</td>
<td>8.7 ± 1.7</td>
<td>13.5 ± 5.0</td>
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<tr>
<td></td>
<td>Day 8 sham</td>
<td>30.9 ± 4.7</td>
<td>23.6 ± 0.0</td>
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Table 3. Infarct size, organ weights and echocardiographic data of OT-II and WT animals.

<table>
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<th>Parameter</th>
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<th>OT-II</th>
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<td>48.8 ± 3.6</td>
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<td>LV weight/ BW weight, mg/g</td>
<td>Day 56</td>
<td>4.3 ± 0.5</td>
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<tr>
<td>Lung weight/ BW, mg/g</td>
<td>Day 56</td>
<td>6.4 ± 0.8</td>
<td>7.7 ± 1.1</td>
</tr>
<tr>
<td>body weight, g</td>
<td>Day 56</td>
<td>30.2 ± 0.8</td>
<td>27.6 ± 0.5</td>
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<tr>
<td>Echocardiographic measurements:</td>
<td>baseline</td>
<td>8.9 ± 0.6</td>
<td>8.1 ± 0.5</td>
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<td></td>
<td>Day 3</td>
<td>13.1 ± 0.8</td>
<td>12.6 ± 0.7</td>
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<tr>
<td></td>
<td>Day 7</td>
<td>24.6 ± 2.1</td>
<td>27.9 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Day 21</td>
<td>28.3 ± 7.3</td>
<td>34.8 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>Day 56</td>
<td>32.8 ± 9.0</td>
<td>43.1 ± 10.6</td>
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<tr>
<td></td>
<td>Day 56 sham</td>
<td>7.6 ± 0.5</td>
<td>6.5 ± 0.4</td>
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<tr>
<td>papillary EDA, mm²</td>
<td>baseline</td>
<td>33.0 ± 0.9</td>
<td>28.7 ± 2.1</td>
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<td>11.4 ± 2.1</td>
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<td>Day 7</td>
<td>8.6 ± 1.0</td>
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<td>Day 21</td>
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<tr>
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<td>Day 56 sham</td>
<td>30.1 ± 1.7</td>
<td>32.5 ± 2.7</td>
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</table>
**Figure Legends:**

**Figure 1.** CD4⁺ T-cell activation after MI. **A.** Heart draining mediastinal lymph nodes demonstrated significantly higher cellularity 7 days after MI (n=3/group, * p<0.05 t-test). **B.** CD4⁺ T-cells proliferate in response to MI as demonstrated by a higher expression of the proliferation marker Ki67 (n= 4/group, * p<0.05 U-test). **C.** The prevalence of CD4⁺ T-cells in myocardial tissue was significantly increased 7 days post MI (n= 3/group, * p<0.05 U-test). **D.** In a mouse model expressing EGFP under the CD4 promoter T-cells localisation after MI was identified by immunohistology for EGFP. Representative picture demonstrated the distribution of T-cells within the infarct zone (blue: DAPI staining; red: EGFP signal marked by arrows). **E.** CD4⁺ T-cells within infarcted myocardium express IFN-γ, IL-17 and IL-10 (representative plots gated on CD45⁺ CD4⁺ cells from MI) after ex vivo PMA/ Ionomycin restimulation. There was a significant upregulation of IFN-γ expression/cell (MFI) after MI (n=6, p<0.05, t-test).

**Figure 2.** LV dilation after MI in WT and CD4 KO mice. **A.** Apical end-diastolic short-axis area (EDA) reflects the ventricular geometry within the infarct zone. CD4 KO (KO n=6) mice demonstrated significantly greater EDA than WT (n=5) mice over the course of 56 days after MI (* p<0.05 ANOVA for repeated measures). **B.** Cumulative survival of MHCIIΔΔ and WT animals after MI (MHCIIΔΔ n= 21 at baseline, WT n=19 at baseline, * p<0.05 log rank test) and sham operation (MHCIIΔΔ n=2, WT n=2).

**Figure 3.** Extracellular collagen in CD4⁺ T-cell deficient mice. **A.** Infarct/ infarct border zone (*) in WT, CD4 KO, and MHC-IIΔΔ mice. Collagen fibres are stained blue by Ladewig staining
(100-fold, bar represents 100μm). **B.** 400-fold magnification displays fibre disarray in MHCII^{A/A} and CD4 KO. **C:** Collagen fibre density quantification in infarct zone based on PSR stained sections (n=5-8/ group, * p< 0.05, † p=0.06, U-test).

**Figure 4.** Innate immune cells in CD4 KO infarcts. Immunohistology for monocytes/macrophages (Mono/MΦ) (**A**) and neutrophil granulocytes (**B**) for semiquantitative grading of cell density within the infarct zone (n=7/ group, *p<0.05 U-test). **C.** Total leukocyte numbers within single cell suspensions prepared from the infarct zone (n=6/ group, *p<0.05 t-test). **D.** Ly6C^{+} Mono/MΦ within the leukocyte gate analysed by FACS (n=8/ group, *p<0.05 t-test). **E.** MCP-1 expression within infarct zone (n=6 WT, n=8 CD4 KO, *p<0.05 t-test). **F.** Intracellular MMP-9 expression (absolute MFI) in CD11b^{+} cells within the infarct zone (n=6/ group, *p<0.05 U-test). **G.** IFN-γ, IL-10 protein expression within infarct zone. (n=6 WT/ group, n=8/ group CD4 KO, *p<0.05 t-test).

**Figure 5.** Collagen de novo formation and angiogenesis in CD4 KO mice. **A.** Collagen secreting cells within the infarct zone were identified by staining for prolyl-4-hydroxylase (P4HB^{+}), an enzyme, which is vital for collagen formation and highly expressed in collagen-secreting cells (n=6/ group, n.s.). **B.** Procollagen-Ia2 expression was analysed by rt-PCR and normalized to 18S (n=6/ group, n.s.). **C.** Matrix protease activity in infarct tissue (n=7/ group, n.s.) **D.** Endothelial cells in WT (white bars) and CD4 KO (grey bars) mice were identified as CD31^{+} (endothelial cell marker stained in blue) without adjacent SMA^{+} cells (smooth muscle actin stained in brown) within the LV infarct border zone and the basal adjacent intact myocardium. Bar indicates 100 μm. (n=4/ group, * p<0.05 U-test).
Figure 6. Survival and LV dilation after MI in WT and OT-II mice. A. Cumulative survival of OT-II and WT animals after MI (OT-II n= 41 at baseline, WT n=45 at baseline, * p<0.05 log rank test) and sham operation (OT-II n=5, WT n=4). B. Collagen fibres are stained blue by Ladewig staining. Bar represents 100 μm. Grading of collagen fibre density in infarct zone on day 7 (n=5/ group, * p< 0.05 U-test).

Figure 7. CD4⁺ T-cell activation in WT and OT-II. Mediastinal lymph node cells were harvested from WT and OT-II mice on day 3 and Trypan blue negative cells were manually counted. A. Percent CD25⁺ / conventional CD4⁺ Foxp3⁻. B. Percent regulatory Foxp3⁺ CD4⁺ T-cells. (* p<0.05 vs WT MI, ANOVA) C. Representative FACS plots of mediastinal lymph nodes gated on CD45⁺CD4⁺ T-cells. D. Ratio of absolute conventional Foxp3⁻ CD4⁺ T-cell numbers in heart draining lymph nodes from MI vs sham animals (p= 0.1 t-test). E. Ratio of absolute regulatory Foxp3⁺ CD4⁺ T-cell numbers in heart draining lymph nodes from MI vs sham animals (* p<0.05, t-test).
A

Cumulative survival

B

Cumulative survival
**A**

WT and CD4 KO immunofluorescence images showing procollagen Ia2 expression.

**B**

Comparison of procollagen Ia2 expression levels between WT and CD4 KO groups.

**C**

Comparison of MMP activity levels between WT and CD4 KO groups.

**D**

Comparison of CD3+ capillaries/field between WT and CD4 KO groups in basal myocardium and mid-ventricular border zone.