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Hyaluronan Synthase 3 is Protective After Cardiac Ischemia-Reperfusion by preserving the T cell Response

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Non-standard Abbreviations and Acronyms

ECM - extracellular matrix

HA - hyaluronan

HAS - hyaluronan synthase

HF- heart failure

I/R - ischemia reperfusion

IHD - ischemic heart disease

LAD - left anterior descending artery

LVEV - left ventricular ejection fraction

LVESV - left ventricular endsystolic volume

LVEDV - left ventricular enddiastolic volume

STEMI - ST-elevation myocardial infarction

Highlights

- Mice with systemic deletion of *hyaluronan synthase 3* (*Has3*) exhibit adverse cardiac remodeling and impaired heart function in a model of acute myocardial infarction
- Detrimental post-infarct outcome in *Has3* deficient mice is associated with reduced numbers of cardiac T helper 1 and regulatory T cells
- Targeting hyaluronan-matrix to preserve T cell function may be beneficial for patients with myocardial infarction

ABSTRACT

Dysregulated extracellular matrix (ECM) is a hallmark of adverse cardiac remodeling after myocardial infarction (MI). Previous work from our laboratory suggests that synthesis of the major ECM component hyaluronan (HA) may be beneficial for post-infarct healing. Here, we aimed to investigate the mechanisms of hyaluronan synthase 3 (HAS3) in cardiac healing after MI. Mice with genetic deletion of *Has3* (*Has3* KO) and wildtype mice (WT) underwent 45 minutes of ischemia with subsequent reperfusion (I/R), followed by monitoring of heart function and analysis of tissue remodeling for up to three weeks. *Has3* KO mice exhibited impaired cardiac function as evidenced by a reduced ejection fraction. Accordingly, *Has3* deficiency also resulted in an increased scar size. Cardiac fibroblast activation and $CD68^+$ macrophage counts were similar between genotypes. However, we found a significant decrease in CD4 T cells in the hearts of *Has3* KO mice seven days post-MI, in particular reduced numbers of $CD4^+CXCR3^+$ Th1 and $CD4^+CD25^+$ Treg cells. Furthermore, *Has3* deficient cardiac T cells were less activated and more apoptotic as shown by decreased $CD69^+$ and increased annexin V^+ cells, respectively. *In vitro* assays using activated splenic CD3 T cells demonstrated that *Has3* deficiency resulted in reduced expression of the main HA receptor CD44 and diminished T cell proliferation. T cell transendothelial migration was similar between genotypes. Of note, analysis of peripheral blood from patients with ST-elevation myocardial infarction (STEMI) revealed that *HAS3* is the predominant HAS isoenzyme also in human T cells. In conclusion, our data suggest that HAS3 is required for mounting a physiological T cell response after MI to support cardiac healing. Therefore, our study may serve as a foundation for the development of novel strategies targeting HA-matrix to preserve T cell function after MI.

Keywords: Extracellular matrix, hyaluronan synthase 3, myocardial infarction, T cells

INTRODUCTION

Ischemic heart disease (IHD) is a major global health burden estimated to affect over 120 million people worldwide[1]. As its most severe clinical manifestation, myocardial infarction (MI) is a leading cause of heart failure (HF), a syndrome defined by signs of structural and/or functional cardiac abnormalities such as hypertrophy and fibrosis[2, 3]. While the success of current pharmacological and device-based measures is undisputed[4, 5], clinical outcome after MI remains highly variable, with 20-30% of patients developing HF at one year after MI hospitalization[6].

Driven by the medical need to identify novel targets for prevention of HF, our laboratory has put a focus on defining early mechanisms of cardiac remodeling. In this context, the extracellular matrix component hyaluronan (HA) is an interesting candidate molecule. HA is a highly hygroscopic glycosaminoglycan synthesized by three membrane-bound HA synthases (HAS1-3) at the inner surface of the plasma membrane, with the HA chains being extruded through pore-like structures into the extracellular space[7]. In addition to its role in maintaining tissue integrity, HA has potent immunoregulatory properties mediated by binding to a variety of HA receptors, foremost CD44 and RHAMM[7, 8]. Using genetic ablation of HAS expression in a model of MI, we have previously shown that HAS2 is critical for cardiac healing by promoting cardiac fibroblast activation and macrophage survival[9]. Of note, infarcted *Has1* deficient mice had no overt phenotype[9], while the effects of HAS3 were not investigated.

We and others have addressed the role of HAS3 in human pathology in several non-infarct models, revealing both protective and pathogenic functions of HAS3, depending on the type of disease and the cell types affected[9-14]. For example, while being protective in a model of periphery artery disease through promoting arteriogenesis[13], HAS3 expression was detrimental in models of inflammatory bowel disease[12, 15] and atherosclerosis[11, 14]. In *ApoE* knockout mice, we showed that *Has3* deficiency decreased atherosclerotic plaque burden and ameliorated the response of proinflammatory T helper 1 (Th1) cells[11]. Since T cells, in particular the CD4 subsets Th1 and regulatory T cells (Tregs), have emerged as important determinants of cardiac outcome after infarction[16] , we hypothesized that HAS3 may affect cardiac remodeling by modulating the T cell response.

RESULTS

Impaired cardiac healing and function in Has3 KO mice

To investigate the role of HAS3 in acute myocardial infarction, we subjected WT and *Has3* deficient mice[10] to 45 min of ischemia followed by reperfusion and monitoring of cardiac function and remodeling. Gomori trichrome staining of hearts 3 weeks post-I/R showed significantly increased scar sizes in *Has3* deficient mice (Figure 1 A, B), along with increased collagen content as revealed by picrosirius red (PSR) staining (Figure 1 C, D). In contrast, collagen density as analyzed by visualization of PSR staining under polarized light was similar between genotypes (Figure 1 E, F). Further, hemodynamic function was analyzed by echocardiography. A significantly reduced left ventricular ejection fraction (LVEF) was measured in *Has3* deficient mice 2 and 3 weeks after I/R (Figure 1 G). This was accompanied by a lower fractional area change (Figure 1 H) and an elevated left ventricular endsystolic volume (LVESV), while the left ventricular enddiastolic volume (LVEDV) was not significantly altered (Online Table I). Additional parameters including cardiac output, stroke volume and baseline heart function were unchanged between genotypes (Online Table I). In addition, we observed signs of increased cardiac hypertrophy in the *Has*3 KO mice: heart weight and left ventricular mass were increased 3 weeks post-I/R as compared to WT. Further analysis of hypertrophic markers ANP and BNP in the remote myocardium showed increased mRNA expression of BNP in *Has3* deficient mice while ANP expression was highly variable and not significantly changed. Cardiac hypertrophy was confirmed by WGA-stained heart sections revealing significantly larger cardiomyocytes in *Has3* KO mice (Online Figure I). Taken together, these data show that *Has3* deficiency worsens the outcome after I/R.

Similar fibroblast and macrophage response in WT and Has3 KO mice

Since we have previously shown that post-infarct fibroblast activation is impaired in *Has2* deficient mice[9], we were interested whether lack of *Has3* would also impact the fibroblast response. To profile fibroblast activation, we evaluated markers of activated cardiac fibroblasts, alpha-smooth muscle actin (α-SMA)[17], periostin (POSTN)[18] and collagen triple helix repeat containing 1 (CTHRC1)[19] with IHC. At day 3, we found a

decrease in α-SMA$^+$ cells in *Has3* KO mice (Figure 2 A, B) but no difference in POSTN and CTHRC1 (Figure 2 C-F). At day 7, expression of all three markers were similar between genotypes (Figure 2 B, D, F). Fibroblast proliferation was also unchanged as determined by flow cytometric analysis of cardiac CD45$^-$CD31$^-$ITGA7$^-$MEFSK4$^+$BrdU$^+$ cells, and so was expression of *Tgfb1*, the dominant driver of myofibroblast activation (Online Figure II). Notably, we also detected similar amounts of HA in the hearts of WT and *Has3* KO mice (Online Figure III). Together, these data indicate that *Has3* deficiency does not significantly alter fibroblast activation after I/R.

Evaluating cardiac macrophages by CD68 staining of heart sections did not reveal differences between the WT and the *Has3* KO animals (Figure 2 G, H). This result was confirmed by flow-cytometric analysis of total CD11b$^+$CD64^{high} cardiac macrophages (Figure 2 I). Further, a more detailed subtype analysis of tissue resident versus monocyte derived macrophages based on expression of CCR2 and MHCII[20] did not show differences between the genotypes either (Online Figure IV and V). In summary, *Has3* deficiency did not affect the macrophage phenotype in our model of I/R. Together with the absence of major changes in the fibroblast response, these data suggest differential roles of HAS2 and HAS3 in cardiac remodeling.

Decreased cardiac CD4 T cell numbers in infarcted Has3* KO *mice

In addition to innate immune responses early after infarction, it has become clear that activation of the adaptive immune system is also critical for cardiac healing [16, 21, 22]. Mainly two subsets of CD4 T cells were found to influence post-infarct outcome: Proinflammatory IFN-γ producing T helper 1 (Th1) cells contributed to increased infarct sizes[23], whereas CD4 regulatory T cells (Tregs) were shown to improve healing after MI[24]. We hypothesized that *Has3* may impact cardiac outcome post-IR due to modulation of the T cell phenotype. Therefore, we analyzed T cell subsets in the blood, the spleen, the mediastinal lymph nodes and the heart of WT and *Has3* KO mice at day 3 and day 7 post-I/R using flow cytometry. Specifically, we aimed for the detection of CD8 T cells, Treg cells and the CD4 T helper cell subsets Th1, Th2 and Th17. Due to the limited number of cardiac T cells, we refrained from intracellular cytokine staining and instead used chemokine receptor expression as surrogate for CD4 lineage definition: CXCR3 for Th1, CCR4 for Th2 and CCR6 for Th17 cells[25]. Treg cells were defined as CD4$^+$CD25$^+$ T cells[26] (Figure 3A).

At day 3, T cell counts in blood, mediastinal lymph nodes and cardiac tissue were similar between WT and *Has3* KO mice. Splenic CD4 and CD8 T cell numbers were significantly decreased in mice lacking *Has3*, however, this decrease was rather transient as it was not observed at day 7 (Online Figure VI and VII). At day 7, differences between the genotypes became apparent in changes of cardiac T cell counts. Although leukocyte and total CD3 T cell numbers were similar (Figure 3 B, C), CD4 T cells were significantly decreased in *Has3* KO mice (Figure 3 D). CD8 and $CD4^-$ $CD8^-$ double negative (DN) T cells were not significantly changed (Figure 3 E, F). Notably, within the CD4 T cells, the decrease was significant for Th1 and Treg cells but not for Th17 cells (Figure 3 G-I). Th2 cells were hardly detected (data not shown). In addition to flow cytometry, we used immunofluorescent staining of heart sections to identify Treg cells by expression of transcription factor FoxP3. As shown in Online Figure VIII, we counted fewer cardiac $FoxP3^+$ cells in the *Has3* deficient mice, although statistical significance was not reached ($p = 0.15$). Potential reasons for this discrepancy between our flow cytometric and immunofluorescence analysis are addressed in the Discussion section.

Surprisingly, reduced counts of cardiac T cells in the *Has3* KO mice were accompanied by increased numbers of Th17 and Treg cells in the blood (Online Figure VII). Other immune cell populations including monocytes, neutrophils and B cells were unchanged between the genotypes at any timepoint (Online Figures II, IX and X). In summary, these data show that *Has3* deficiency modulates the T cell response post I/R.

Reduced numbers of activated T cells and increased T cell apoptosis in infarcted hearts of Has3 KO

To gain insight into the underlying mechanisms of the altered T cell response in *Has3* deficient mice, we asked whether T cell activation might be different between the genotypes. Interleukin-2 (IL-2) is a cytokine released by CD4 and CD8 T cells in response to T cell receptor (TCR)- activation through their cognate antigens. It is critically involved in many aspects of T cell biology including T cell homeostasis, proliferation and differentiation[27]. A first indication that T cell activation might be dysregulated in *Has3* KO mice was obtained from cytokine plasma analyses showing significantly reduced IL-2 concentrations at day 3 post-I/R as compared to the WT (Figure 4 A). This led us to assess the activation status of cardiac T cells at day 7, the

timepoint where T cell counts were reduced compared to the controls. Choosing CD69 as a marker for early T cell activation[28], we found a significant decrease in activated total CD3 as well as CD4 and CD8 T cells (Figure 4 B-D). Unfortunately, calculation of percent activated $CD69^+$ cells proofed challenging due to the low number of recorded T cells in some samples. We hypothesized that impaired activation of *Has3* deficient T cells might result in increased T cell apoptosis, similar to the known effects of IL-2 withdrawal[29]. In fact, we detected a highly significant increase in the percentage of annexin V^+ total CD3 as well as CD4 and CD8 T cells in the heart of *Has3* KO mice as compared to WT mice (Figure 4 E-G). Interestingly, there were differences within the CD4 subsets, reflected by a profound increase in apoptotic Th1 but not Th17 or Treg cells (Figure 4 H-J). While this result seems surprising, it is in line with the differential IL-2 requirements for survival of T cell helper subsets which will be discussed later. Further, we investigated whether the observed changes in T cells would, alter cytokine concentrations in the left ventricle. There were no significant differences in single cytokines between WT and *Has3* KO mice, however there were subtle shifts towards reduced IL-2 and regulatory cytokines (IL-4, IL-10, IL-13) in *Has3* deficient mice, while the pleiotropic cytokine IL-6 was increased (Online figure XI). In conclusion, these results demonstrate that at day 7 post-I/R, cardiac T cells of *Has3* deficient mice are less activated and more apoptotic than WT T cells.

Diminished HA synthesis and CD44 protein abundance in T cells from Has3 KO mice associates with decreased T cell proliferation

To better understand the impact of T cell- expressed *Has3* for T cell function, we performed *in vitro* proliferation and transendothelial migration assays using purified splenic CD3 T cells from infarcted WT and *Has3* KO mice. First, we measured *Has* expression using qPCR. *Has3* was the only detected HAS isoform in T cells from WT mice at day 7 post-I/R (Figure 5 A). As expected, we did not detect *Has3* transcript in *Has3* deficient T cells and of note, other *Has* isoforms were not upregulated (data not shown). Interestingly, HAS3 was also the predominant isoenzyme in T cells from patients with acute myocardial infarction (Figure 5 B). Immunofluorescent staining of anti-CD3/CD28 activated murine T cells plated on collagen-coated slides, revealed that T cells lacking *Has3* not only displayed significantly less HA but also less CD44, the principal HA receptor required for CD44-HA mediated T cell adhesion and diapedesis[30]

(Fig. 5 C-E). Interestingly, we also observed profoundly reduced CD44 staining after activated T cells were treated with hyaluronidase. This suggests that CD44 is held near the T cell surface by a pericellular HA network and not only by integration into the cell membrane (Online figure XII). Strikingly, induction of *Cd44, Il2* and *Ifnγ* mRNA expression following TCR activation was similar between genotypes (Online Figure XIII). Given the role of CD44 in T cell extravasation[30], we next set up a transmigration assay using splenic T cells from infarcted WT and *Has3* KO mice allowing them to migrate through a layer of murine endothelial cells in response to CXCL12, a potent T cell chemoattractant[31]. However, despite strong induction of T cell transmigration by CXCL12, no difference in T cell migration between the genotypes was detected (Online Figure XIV). Since TCR signal strength is linked to expansion of both CD4 and CD8 T cells[32], we next asked whether the suspected impairment of *Has3* deficient T cells to become activated would translate into compromised cell proliferation. Indeed, using activated CD3 T cells in a CFSE-based assay, we found a significant decrease in proliferation of T cells from *Has3* KO mice (Figure 5 F). T cells from uninfarcted *Has3* deficient mice also exhibited reduced proliferation compared to the WT. No differences in splenic T cell abundance or CD69 expression were detected in uninfarcted mice prior to *in vitro* activation (Online Figures XV and XVI).

Altogether, our data suggest that expression of HAS3 is required for adaptive cardiac healing by ensuring adequate T cell activation after myocardial infarction. We provide evidence that reduced counts of cardiac CD4 T helper and regulatory T cells in the infarcted heart of *Has3* KO mice are due to compromised activation and increased apoptosis, leading to reduced T cell proliferation and survival and ultimately, adverse cardiac remodeling. The main findings of our study are summarized in Figure 6.

DISCUSSION

Targeting early events in adverse cardiac remodeling is an unmet medical need to reduce the incidence of heart failure post MI. Changes in the ECM occur within minutes after infarction and continue throughout all phases of cardiac healing[33]. Increasing evidence suggests a critical role for hyaluronan (HA) in supporting adaptive remodeling.

Here, we show that mice deficient for the HA synthase 3, *Has3,* have a significantly worse outcome after I/R compared to WT animals, as evident by impaired left ventricular function and increased scar size. We further demonstrate that this phenotype is associated with reduced cardiac CD4 T cells at day 7 post-infarction and that these T cells are characterized by reduced activation and increased apoptosis. Moreover, we found decreased CD44 protein abundance in T cells from *Has3* KO mice, which were less able to proliferate *in vitro* than WT cells. Finally, we show that *HAS3* is the predominant isoform in human T cells from patients with STEMI. To the best of our knowledge, this study is the first to demonstrate a critical role of HA in regulating the T cell response after myocardial infarction.

Interestingly, we observed that the total myocardial HA content was unchanged and that there were no major differences in the fibroblast and macrophage response between WT and *Has3* KO mice, despite significantly impaired left ventricular function and increased scar size in the infarcted *Has3* KO animals on day 21. This is in contrast to our observations using *Has2* KO mice, which exhibited less HA, fewer cardiac macrophages and reduced fibroblast activation[9]. We believe the difference in these phenotypes is due to the fact *Has* isoform expression is cell type specific. We have previously shown that *Has2* is the most highly expressed *Has* isoform in murine cardiac fibroblasts, with *Has3* levels being orders of magnitude lower in comparison[9]. Moreover, in contrast to *Has1* and *Has2*, we found *Has3* insensitive to upregulation by TGF-β1[9], the most critical factor for conversion of fibroblasts into myofibroblasts[34]. Since the large majority of HA in the post-infarct environment is produced by fibroblasts, it is likely that the proportion of HAS3-produced HA in the infarcted heart is very small. Therefore, *Has3* deficiency will not result in detectable changes in gross HA content, but rather alters the HA abundance in the T cell niche. However, we cannot exclude the possibility that also *Has* compensation plays a role. Although we did not see such a mechanism in isolated *Has3* deficient T cells, *Has*1/3 double deficient skin

fibroblasts, for example, expressed higher levels of *Has2* and produced more HA than WT fibroblasts[35]. Besides increased *Has2* mRNA, the authors discuss a potential role for the formation of HAS2 homo- and heterodimers: HAS2 enzymatic activity was found to be inhibited by the formation of HAS2/HAS3 heterodimers[36], so while in WT cells such dimers will be generated, *Has*1/3 null cells will only form HAS2 homodimers with enhanced activity. Hence, it is conceivable that also in *Has3* deficient cells, the proportion of HAS2 homodimers is increased compared to the WT. Future studies may address this question.

Our data suggest that unlike *Has2,* which promotes fibroblast activation and macrophage survival[9], the beneficial role of *Has3* in myocardial infarction is promoting the activation and survival of cardiac T cells. CD4 and CD8 T cells as well as unconventional T cells like γδ T cells and Natural Killer T (NKT) cells gradually infiltrate the infarcted heart, following neutrophils and macrophages and peaking at day 3 or day 7 post-MI, depending on the model used[37]. The relevance of CD4 T cells was highlighted in several studies employing genetic or antibody-mediated T cell depletion[16]. It became clear that cardiac outcome was largely determined by two CD4 T cell subsets with opposing roles: IFN-γ producing T helper 1 (Th1) cells promoting inflammation and contributing to increased infarct sizes[23], and regulatory T cells (Tregs) supporting adaptive cardiac healing. Natural Treg cells are best defined as $CD4^+FoxP3^+CD25^+$ T cells suppressing inflammation by cell-cell contact and the release of regulatory cytokines including IL-10 and IL-35[38]. In our study, we provide evidence that both cardiac Th1 and Treg cells are decreased in *Has3* KO mice at day 7 post-I/R. With respect to FoxP3, which was not significantly reduced in heart sections of the *Has3* KO, we think that methodological differences need to be considered. In contrast to flow cytometric analysis of $CD4^+CD25^+$ cells in whole heart homogenates, $Foxp3^+$ cells were counted manually in selected heart tissue section, potentially not capturing areas of uneven Treg cell distribution. Moreover, considering that Treg cells can lose FoxP3 expression under inflammatory conditions resulting in so-called 'exTregs[39], even subtle differences in cytokine concentrations in the infarcted hearts of WT and *Has3* KO mice may have impacted $FoxP3^+$ cell counts. Although lineage tracing of Treg cells would be needed to study the fate of Treg cells in more detail in our model, we believe our data strongly suggests reduced Treg cell abundance in the infarcted hearts of *Has3* deficient mice.

Irrespective of the Th1/Treg ratio, which was unchanged compared to the WT (data not shown), this decrease in absolute T cell numbers was associated with a detrimental outcome, indicating the outstanding role of Tregs for cardiac healing[24, 40, 41]. In fact, using mice with genetic or CD25-antibody mediated ablation of Treg cells, Weirather et al. showed that Treg cell deficiency led to exacerbated cardiac inflammation with increased infiltrates of neutrophils and M1-polarized macrophages. Conversely, *in vivo* expansion of Treg cells by administration of superagonistic CD28 (CD28-SA) antibody two days post MI induced regulatory M2 macrophages and resulted in improved survival[24]. Although the mechanisms of how Tregs exert their cardioprotective function are still incompletely understood, Tregs were reported to alleviate cardiac hypertrophy and fibrosis under conditions of pressure overload, possibly by suppressing TGF-β mediated myofibroblast activation[42]. This idea of Treg cell function is in line with our findings of increased hypertrophy and reduced cardiac Tregs in the *Has3* KO mice.

Our results indicated that *Has3* deficient T cells may have a compromised capacity to activate upon antigen stimulation. Classical T cell activation occurs through T cell receptor (TCR) recognition of peptide antigens presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APC), typically dendritic cells (DCs)[43]. T cell activation can be measured by different means including cytokine release, upregulation of activation markers and cell proliferation. A critical cytokine for T cell survival and proliferation is IL-2, which is produced by activated CD8 and CD4 T cells, with the exception of Tregs. Tregs are unique in that they do not secrete IL-2 but constitutively express high levels of CD25, the IL-2Rα chain, enabling them to respond to very low IL-2 concentrations[44]. Reduced IL-2 plasma concentrations in the *Has3* KO mice at day 3 after infarction led us to evaluate T cell activation in the heart. At day 7 post-I/R, we found significantly decreased numbers of $CD69^+$ CD4 and CD8 T cells in cardiac tissue of *Has3* deficient mice compared to WT mice, indicating impaired activation of the two major T cell populations. Interestingly, with regard to Treg cell function, a subset of $CD4^+Foxp3^+CD69^+$ Tregs was shown to have enhanced immunosuppressive capacity by increased release of IL-10[45]. Hence, whether *Has3* deficient Tregs are functionally compromised, is an intriguing question that may be addressed in future studies.

Suboptimal T cell activation, for example by cytokine deprivation or insufficient engagement of costimulatory molecules like CD28 can result in apoptosis[46]. Therefore,

we stained cardiac T cells with annexin-V and indeed detected an increased percentage of annexin-V$^+$ CD4 and CD8 T cells in the *Has3* KO mice. Of note, the most affected CD4 subset were the Th1 cells while apoptosis in Tregs and Th17 cells was not significantly altered compared to the WT. This may be explained, at least in part, by distinct cytokine requirements of the T helper subsets for survival. As mentioned above, Tregs are viable in the presence of very low IL-2 concentrations due to high CD25 expression. In contrast, Th17 cells are dependent on IL-23, with IL-2 inhibiting Th17 cell development[47].

We used immunocytochemical analysis of purified splenic T cells to assess HA synthesis and CD44 expression. Remarkably, activated WT T cells showed an intense HA staining which was almost entirely due to *Has3* expression since no *Has1* and only minute amounts of *Has2* in a few samples were detected. *Has3* deficient T cells did not compensate for the lack of *Has3* by upregulation of other Has isoforms and accordingly, showed profoundly reduced HA staining. Interestingly, this reduction in HA was matched by decreased CD44 protein, despite unchanged *Cd44* transcript levels between WT and *Has3* deficient T cells. Moreover, treatment of WT cells with hyaluronidase removed CD44, suggesting that HA stabilizes CD44 at the T cell surface. The underlying mechanisms are unclear; however, it is possible that CD44 is caught and held inside the HA matrix by HA itself or a binding partner, free from the cell membrane, but close enough that it increases its chances of being re-attached to the cell and activating it's signaling cascade. It is also conceivable that HA stabilizes CD44 by protecting the ectodomain from proteolytic shedding. Like many cell surface proteins, the ectodomain of CD44 can be cleaved by the action of proteinases to release a soluble form of CD44. Specifically, membrane type 1 matrix metalloproteinase (MT1-MMP) and a disintegrin and metalloproteinase-10 (ADAM10), both of which are expressed on T cells, have been shown to mediate CD44 shedding[48, 49]. Future studies are needed to address these questions in more detail.

Because CD44 binding to HA on endothelial cells mediates T cell adhesion and extravasation[30], we performed *in vitro* transendothelial migration assays. Although WT and *Has3* deficient T cells behaved similarly, we cannot exclude the possibility that in mice with global *Has3* deficiency T cell extravasation was impaired, considering that lack of endothelial *Has3* can cause endothelial dysfunction[13]. Future experiments using

Has3 deficient endothelial cells or a mouse model of CD4 T cell specific *Has3* KO may help clarify this question.

Impaired antibody-induced *in vitro* proliferation of *Has3* deficient T cells from both uninfarcted and infarcted mice indicated a T cell intrinsic lack of activation capacity. The question of how HA and CD44 contribute to T cell activation has been addressed in several studies. In seminal work, Bollyky et al showed that Th1 cytokines induce HA synthesis in DCs, which promotes T cell-DC binding at the immune synapse[50]. Further, the beneficial effects of 4-Methylumbelliferone (4-MU)-mediated blockade of HA synthesis in a mouse model of multiple sclerosis could be attributed to reduced Th1 cell polarization and increased regulatory T cells[51]. Notably, T cell intrinsic HA synthesis was also shown to affect T cell activation. For instance, Mummert et al. found that T cells pre-treated with a peptide inhibitor of HA showed reduced mitogen-triggered IL-2 production and proliferation, indicating an autocrine mechanism by which T cells assist their own activation through synthesis of HA[52]. Reduced T cell proliferation was also seen by 4-MU inhibition of HA synthesis[53] and recently, Gebe et al. showed in a model of type 1 diabetes that 4-MU treated naïve T cells, specific for an antigen expressed in the insulin-producing beta cells of the pancreas, have a priming defect resulting in reduced CD69 expression and diminished T cell infiltration into the pancreas[54].

The impact of CD44 on cell motility and proliferation has mostly been studied in the context of cancer[55, 56]. In agreement with published data[53], blockade of CD44's HA binding site using a neutralizing antibody did not alter T cell proliferation (data not shown). One possible explanation may be that CD44 has been shown to exert its effects on proliferation through interaction with epidermal growth factor receptor (EGFR)[57]. While Treg cells express EGFR under inflammatory conditions, the receptor is generally absent in conventional CD3 T cells[58]. However, CD44 has an important role in T helper and Treg cell function. For instance, Baaten et al. showed that CD44 was required for generation of memory Th1 cells by promoting effector cell survival. In the same study, the authors also demonstrated that CD44 protected Th1 cells against apoptosis[59]. These results are in line with our finding of increased apoptotic T cells in the infarcted heart of *Has3* KO mice. In addition, CD44 has been shown to impact Treg cell function: Bollyky et. al found that CD44 acts as a co-stimulatory molecule in Treg

cells to promote FoxP3 expression and that Tregs from CD44$^{-/-}$ mice had impaired suppressive function[60]. Similar results were obtained by Liu et al[61]. Interestingly, high-molecular weight HA was also found to increase FoxP3 expression via CD44 crosslinking[60]. Taken together, it is likely that the observed phenotype of reduced Th1 and Treg cells in the infarcted hearts of *Has3* deficient mice is associated with changes in CD44 surface expression.

In conclusion, our study provides evidence for the requirement of HAS3-mediated HA synthesis to induce a physiological T cell response after myocardial infarction. Our results are in line with the mentioned reports on the role of HA in T cell activation in non-infarct models and support the notion that Tregs are critical for adaptive cardiac healing. Having identified HAS3 as the major isoform also in human T cells, it is conceivable that HAS3 also shapes human T cell responses.

EXPERIMENTAL PROCEDURES

The data that support the findings of this study are available from the corresponding author upon reasonable request. A detailed description of methods is provided in the online supplement.

Mice

Mice lacking the murine hyaluronan synthase 3 (Has3) were generated by genOway (Lyon Cedex, France) as described before by Kiene et al.[10]. Wildtype mice served as control. 10-13-week-old male control or *Has3* deficient mice were used for experiments. Food and water were accessible ad libitum and mice were kept in a 12-hour dark/light cycle. All animal experiments were performed in accordance with “Deutsches Tierschutzgesetz” and were permitted by Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) Nordrhein-Westfalen, Bezirksregierung Düsseldorf, Aktenzeichen 81-02.04.2018.A079 and 84-02.04.2019.A012.

Closed chest cardiac ischemia/reperfusion

Cardiac ischemia and reperfusion were induced as described before[9, 17, 18]. Mice were anesthetized by intraperitoneal injection of 90 mg ketamine and 15 mg xylazine per kg

bodyweight. Body temperature was constantly measured and kept at 38 °C with a heated plate. After lateral thoracotomy, 7-0 suture was placed 1 mm from the tip of left auricle around left anterior ascending artery (LAD). Suture was led through a 1 mm piece of a PE-tube and to the outside of the thorax, building a loose snare around the LAD. The ends of the suture were placed subcutaneously and the skin was closed. Three days later, mice were anesthetized with air containing 40% oxygen and 2% isoflurane. Temperature was kept at 38 °C. Subsequently, the skin was opened and the suture was led to both sides of the mouse. By hanging 5 g weights per side, coronary artery was occluded and ischemia was induced for 45 min. Occurrence of myocardial ischemia was controlled by ST-elevation via echocardiography.

Echocardiography

Echocardiography (ECG) was performed using high-resolution ultrasound with a 18-38 MHz transducer. During measurements, mice were anaesthetized with 2% isoflurane (Forene®, Abbott GmbH, Germany) and heart rate as well as ECG were monitored. Long and short axis were measured parasternal and hemodynamic parameters were calculated using VevoLab Software (FUJIFILM Visual Sonics, Toronto, Canada) as described before[17]. Ejection fraction, fractional area change, cardiac output, stroke volume, fractional shortening, endsystolic volume and enddiastolic volume were calculated in VevoLab according to Simpson's protocol based on 3 measurement locations in short-axis. Peak velocity and velocity time integral from PW Doppler mode in long axis, heart rate from long-axis measurement.

Histology

Hearts were fixed in solution of 4% paraformaldehyde (Histofix 4%, Roth, Karslruhe, Germany) for 24 hours. Starting from the apex, hearts were cut into 10 levels of 5 µm sections (100 µm per level), discarding 250 µm between each level. For gomori trichrome and picrosirius red stainings, levels 1-10 were used. For wheat germ agglutinin staining, levels 3-10 and for all other immunohistochemical stainings, level 5 was used.

Gomori trichrome staining was performed as described in a protocol from Sigma Aldrich (St. Louis, MO, USA; Procedure Number HT10). Collagen maturation was evaluated by picrosirius red staining and cardiomyocyte staining was performed using Alexa fluor 488 conjugated wheat germ agglutinin (Invitrogen, Carlsbad, CA, USA; Cat. -No.: W11261). Macrophages were detected with anti-CD68 antibody (ab125212, Abcam) and myofibroblasts with anti-αSMA (ab5694, Abcam), anti-POSTN (ab14041, Abcam) and anti-CTHRC1 (MABT889, Sigma Aldrich) antibodies, all followed by Alexa fluor 647 goat anti-rabbit IgG (H+L) secondary antibody (A-21245 Life technologies). For regulatory T-cells, cells were permeabilized using 0,1% Triton X100 and stained with anti-FOXP3 (Clone FJK-16s, 14-5773-80, eBioscience) antibody, followed by Alexa fluor 647 goat anti-rat IgG (H+L) secondary antibody (A-21247 Life technologies). For HA staining, slides were blocked with Avidin and Biotin. Subsequently, biotinylated HA binding protein (HAbp, 385911, Calbiochem) was applied, followed by Streptavidin-Cy3 (SA1010, Invitrogen). Isotype controls were run in all experiments. All immunohistochemically stained sections were mounted using Roti-Mount FluorCare DAPI (Roth, Karlsruhe, Germany).

Images were taken with a Zeiss AxioObserver.Z1 microscope with subsequent stitching in Zen2 software (Carl Zeiss Microscopy GmbH). Planimetric scar size analysis in gomori stained hearts was carried out using Diskus view Software (Hilgers, Königswinter, Germany) as described previously[19]. For determination of cardiomyocyte size in WGA stained hearts, 50 round shaped cardiomyocytes with centrally localized nuclei in the remote area of the left ventricular septum were analyzed in each level. Cell area was measured using ImageJ software (NIH) and the mean of all analyzed levels was calculated. Area fraction quantification for all antibody-stainings was done using ImageJ.

Flow cytometric analyses

Quantification of immune cells was performed by flow cytometric analysis of cardiac blood, cardiac tissue, spleen and mediastinal lymph nodes. Blood, spleen and cardiac

tissue were processed as described before[9, 20] to achieve single cell suspensions. Lymph nodes were homogenized with gentle MACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Absolute cell concentrations were calculated using Flow-Count Fluorospheres (Beckmann Coulter, Krefeld, Germany). After Fc-receptor blockage with anti-mouse CD16/32 antibody, single cell suspensions were stained with LIVE/DEAD Fixable Aqua dead cell stain kit (Life technologies) and different panels of antibodies. Subsets of cardiac monocytes and macrophages were defined as described before[9, 21, 22]: monocytes: $CD45^+$ $CD11b^+$ $CD64^{int}$ $MHCII^{low}$ $CCR2^+$, monocyte-derived macrophages: $CD45^+$ $CD11b^+$ $CD64^+$ $MHCII^{high}$ $CCR2^+$ and resident macrophages: $CD45^+$ $CD11b^+$ $CD64^+$ $MHCII^{high}$ $CCR2^-$ and $CD45^+$ $CD11b^+$ $CD64^+$ $MHCII^{low}$ $CCR2^-$. Lymphocyte subsets were defined as: B-lymphocytes: $CD45^+$ CD19+, cytotoxic T-lymphocytes: $CD45^+$ $CD3^+$ $CD8^+$, Th1 cells: $CD45^+$ $CD3^+$ $CD4^+$ $CXCR3^+$, Th17 cells: $CD45^+$ $CD3^+$ $CD4^+$ $CCR6^+$, Treg cells: $CD45^+$ $CD3^+$ $CD4^+$ $CD25^+$. Apoptotic cells were defined by binding of annexin V, proliferating cells by BrdU incorporation. For BrdU identification, cells were stained for surface markers, followed by permeabilization (Fix & Perm Medium, Life Technologies), incubation with DNase I (1200U/mL, Sigma Aldrich) for 1 hour and staining with BrdU antibody. A list of all antibodies is provided in Supplemental Table 2. LSRII Fortessa flow cytometer (BD Bioscience, San Jose, CA, USA) was used for all measurements. Data analysis was done using FlowJo software (Treestar, San Carlos, CA, USA). Gating schemes for flow cytometric analysis are provided in Figure 3 and Online Figure IV.

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DISCLOSURES

The authors have no disclosures.

List of supplemental materials:

Expanded materials and methods

Online figures I- XVI

Online tables I-V

REFERENCES

1. Khan MA, Hashim MJ, Mustafa H, Baniyas MY, Al Suwaidi S, AlKatheeri R, Alblooshi FMK, Almatrooshi M, Alzaabi MEH, Al Darmaki RS and Lootah S. Global Epidemiology of Ischemic Heart Disease: Results from the Global Burden of Disease Study. *Cureus*. 2020;12:e9349.
2. Bozkurt B, Coats AJ, Tsutsui H, Abdelhamid M, Adamopoulos S, Albert N, Anker SD, Atherton J, Bohm M, Butler J, Drazner MH, Felker GM, Filippatos G, Fonarow GC, Fiuzat M, Gomez-Mesa JE, Heidenreich P, Imamura T, Januzzi J, Jankowska EA, Khazanie P, Kinugawa K, Lam CSP, Matsue Y, Metra M, Ohtani T, Francesco Piepoli M, Ponikowski P, Rosano GMC, Sakata Y, Seferovi CP, Starling RC, Teerlink JR, Vardeny O, Yamamoto K, Yancy C, Zhang J and Zieroth S. Universal Definition and Classification of Heart Failure: A Report of the Heart Failure Society of America, Heart Failure Association of the European Society of Cardiology, Japanese Heart Failure Society and Writing Committee of the Universal Definition of Heart Failure. *J Card Fail*. 2021.
3. Schirone L, Forte M, Palmerio S, Yee D, Nocella C, Angelini F, Pagano F, Schiavon S, Bordin A, Carrizzo A, Vecchione C, Valenti V, Chimenti I, De Falco E, Sciarretta S and Frati G. A Review of the Molecular Mechanisms Underlying the Development and Progression of Cardiac Remodeling. *Oxid Med Cell Longev*. 2017;2017:3920195.
4. Zhao W, Zhao J and Rong J. Pharmacological Modulation of Cardiac Remodeling after Myocardial Infarction. *Oxid Med Cell Longev*. 2020;2020:8815349.
5. Gustafsson F and Rogers JG. Left ventricular assist device therapy in advanced heart failure: patient selection and outcomes. *Eur J Heart Fail*. 2017;19:595-602.
6. Jenca D, Melenovsky V, Stehlik J, Stanek V, Kettner J, Kautzner J, Adamkova V and Wohlfahrt P. Heart failure after myocardial infarction: incidence and predictors. *ESC Heart Fail*. 2021;8:222-237.
7. Jiang D, Liang J and Noble PW. Hyaluronan as an immune regulator in human diseases. *Physiol Rev*. 2011;91:221-64.
8. Nagy N, Kuipers HF, Marshall PL, Wang E, Kaber G and Bollyky PL. Hyaluronan in immune dysregulation and autoimmune diseases. *Matrix Biol*. 2019;78-79:292-313.
9. Petz A, Grandoch M, Gorski DJ, Abrams M, Piroth M, Schneckmann R, Homann S, Muller J, Hartwig S, Lehr S, Yamaguchi Y, Wight TN, Gorressen S, Ding Z, Kotter S, Kruger M, Heinen A, Kelm M, Godecke A, Flogel U and Fischer JW. Cardiac Hyaluronan Synthesis Is Critically Involved in the Cardiac Macrophage Response and Promotes Healing After Ischemia Reperfusion Injury. *Circulation research*. 2019;124:1433-1447.
10. Kiene LS, Homann S, Suvorava T, Rabausch B, Muller J, Kojda G, Kretschmer I, Twarock S, Dai G, Deenen R, Hartwig S, Lehr S, Kohrer K, Savani RC, Grandoch M and Fischer JW. Deletion of Hyaluronan Synthase 3 Inhibits Neointimal Hyperplasia in Mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2016;36:e9-16.
11. Homann S, Grandoch M, Kiene LS, Podsvyadek Y, Feldmann K, Rabausch B, Nagy N, Lehr S, Kretschmer I, Oberhuber A, Bollyky P and Fischer JW. Hyaluronan synthase 3 promotes plaque inflammation and atheroprogression. *Matrix Biol*. 2018;66:67-80.
12. Hundhausen C, Schneckmann R, Ostendorf Y, Rimpler J, von Glinski A, Kohlmorgen C, Pasch N, Rolauer L, von Ameln F, Eckermann O, Altschmied J, Ale-Agha N, Haendeler J, Flogel U, Fischer JW and Grandoch M. Endothelial hyaluronan synthase 3 aggravates acute colitis in an experimental model of inflammatory bowel disease. *Matrix Biol*. 2021.
13. Schneckmann R, Suvorava T, Hundhausen C, Schuler D, Lorenz C, Freudenberger T, Kelm M, Fischer J, Flogel U and Grandoch M. Endothelial Hyaluronan Synthase 3 Augments Postischemic Arteriogenesis Through CD44/eNOS Signaling. *Arteriosclerosis, thrombosis, and vascular biology*. 2021:ATVBAHA121315478.
14. Hartmann F, Gorski DJ, Newman AAC, Homann S, Petz A, Owsiany KM, Serbulea V, Zhou YQ, Deaton RA, Bendeck M, Owens GK and Fischer JW. SMC-Derived Hyaluronan Modulates Vascular SMC Phenotype in Murine Atherosclerosis. *Circ Res*. 2021;129:992-1005.

15. Kessler SP, Obery DR and de la Motte C. Hyaluronan Synthase 3 Null Mice Exhibit Decreased Intestinal Inflammation and Tissue Damage in the DSS-Induced Colitis Model. *Int J Cell Biol*. 2015;2015:745237.
16. Hofmann U and Frantz S. Role of T-cells in myocardial infarction. *Eur Heart J*. 2016;37:873-9.
17. Frangogiannis NG, Michael LH and Entman ML. Myofibroblasts in reperfused myocardial infarcts express the embryonic form of smooth muscle myosin heavy chain (SMemb). *Cardiovasc Res*. 2000;48:89-100.
18. Shimazaki M, Nakamura K, Kii I, Kashima T, Amizuka N, Li M, Saito M, Fukuda K, Nishiyama T, Kitajima S, Saga Y, Fukayama M, Sata M and Kudo A. Periostin is essential for cardiac healing after acute myocardial infarction. *J Exp Med*. 2008;205:295-303.
19. Ruiz-Villalba A, Romero JP, Hernandez SC, Vilas-Zornoza A, Fortelny N, Castro-Labrador L, San Martin-Uriz P, Lorenzo-Vivas E, Garcia-Olloqui P, Palacio M, Gavira JJ, Bastarrika G, Janssens S, Wu M, Iglesias E, Abizanda G, de Morentin XM, Lasaga M, Planell N, Bock C, Alignani D, Medal G, Prudovsky I, Jin YR, Ryzhov S, Yin H, Pelacho B, Gomez-Cabrero D, Lindner V, Lara-Astiaso D and Prosper F. Single-Cell RNA Sequencing Analysis Reveals a Crucial Role for CTHRC1 (Collagen Triple Helix Repeat Containing 1) Cardiac Fibroblasts After Myocardial Infarction. *Circulation*. 2020;142:1831-1847.
20. Epelman S, Lavine KJ and Randolph GJ. Origin and functions of tissue macrophages. *Immunity*. 2014;41:21-35.
21. van der Laan AM, Nahrendorf M and Piek JJ. Healing and adverse remodelling after acute myocardial infarction: role of the cellular immune response. *Heart*. 2012;98:1384-90.
22. Epelman S, Liu PP and Mann DL. Role of innate and adaptive immune mechanisms in cardiac injury and repair. *Nat Rev Immunol*. 2015;15:117-29.
23. Yang Z, Day YJ, Toufektsian MC, Xu Y, Ramos SI, Marshall MA, French BA and Linden J. Myocardial infarct-sparing effect of adenosine A2A receptor activation is due to its action on CD4+ T lymphocytes. *Circulation*. 2006;114:2056-64.
24. Weirather J, Hofmann UD, Beyersdorf N, Ramos GC, Vogel B, Frey A, Ertl G, Kerkau T and Frantz S. Foxp3+ CD4+ T cells improve healing after myocardial infarction by modulating monocyte/macrophage differentiation. *Circulation research*. 2014;115:55-67.
25. Sallusto F and Lanzavecchia A. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. *Eur J Immunol*. 2009;39:2076-82.
26. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J and Sakaguchi S. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol*. 1998;10:1969-80.
27. Ross SH and Cantrell DA. Signaling and Function of Interleukin-2 in T Lymphocytes. *Annu Rev Immunol*. 2018;36:411-433.
28. Sancho D, Gomez M and Sanchez-Madrid F. CD69 is an immunoregulatory molecule induced following activation. *Trends Immunol*. 2005;26:136-40.
29. Duke RC and Cohen JJ. IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent T cells. *Lymphokine Res*. 1986;5:289-99.
30. Baaten BJ, Li CR and Bradley LM. Multifaceted regulation of T cells by CD44. *Commun Integr Biol*. 2010;3:508-12.
31. Okabe S, Fukuda S, Kim YJ, Niki M, Pelus LM, Ohyashiki K, Pandolfi PP and Broxmeyer HE. Stromal cell-derived factor-1alpha/CXCL12-induced chemotaxis of T cells involves activation of the RasGAP-associated docking protein p62Dok-1. *Blood*. 2005;105:474-80.
32. Bhattacharyya ND and Feng CG. Regulation of T Helper Cell Fate by TCR Signal Strength. *Frontiers in immunology*. 2020;11:624.
33. Dobaczewski M, Gonzalez-Quesada C and Frangogiannis NG. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. *J Mol Cell Cardiol*. 2010;48:504-11.

34. Cucoranu I, Clempus R, Dikalova A, Phelan PJ, Ariyan S, Dikalov S and Sorescu D. NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circ Res*. 2005;97:900-7.
35. Wang Y, Lauer ME, Anand S, Mack JA and Maytin EV. Hyaluronan synthase 2 protects skin fibroblasts against apoptosis induced by environmental stress. *J Biol Chem*. 2014;289:32253-32265.
36. Karousou E, Kamiryo M, Skandalis SS, Ruusala A, Asteriou T, Passi A, Yamashita H, Hellman U, Heldin CH and Heldin P. The activity of hyaluronan synthase 2 is regulated by dimerization and ubiquitination. *J Biol Chem*. 2010;285:23647-54.
37. Yan X, Anzai A, Katsumata Y, Matsuhashi T, Ito K, Endo J, Yamamoto T, Takeshima A, Shinmura K, Shen W, Fukuda K and Sano M. Temporal dynamics of cardiac immune cell accumulation following acute myocardial infarction. *Journal of molecular and cellular cardiology*. 2013;62:24-35.
38. Arce-Sillas A, Alvarez-Luquin DD, Tamaya-Dominguez B, Gomez-Fuentes S, Trejo-Garcia A, Melo-Salas M, Cardenas G, Rodriguez-Ramirez J and Adalid-Peralta L. Regulatory T Cells: Molecular Actions on Effector Cells in Immune Regulation. *J Immunol Res*. 2016;2016:1720827.
39. Saxena V, Lakhan R, Iyyathurai J and Bromberg JS. Mechanisms of exTreg induction. *Eur J Immunol*. 2021;51:1956-1967.
40. Tang TT, Yuan J, Zhu ZF, Zhang WC, Xiao H, Xia N, Yan XX, Nie SF, Liu J, Zhou SF, Li JJ, Yao R, Liao MY, Tu X, Liao YH and Cheng X. Regulatory T cells ameliorate cardiac remodeling after myocardial infarction. *Basic research in cardiology*. 2012;107:232.
41. Zacchigna S, Martinelli V, Moimas S, Colliva A, Anzini M, Nordio A, Costa A, Rehman M, Vodret S, Pierro C, Colussi G, Zentilin L, Gutierrez MI, Dirkx E, Long C, Sinagra G, Klatzmann D and Giacca M. Paracrine effect of regulatory T cells promotes cardiomyocyte proliferation during pregnancy and after myocardial infarction. *Nature communications*. 2018;9:2432.
42. Kanellakis P, Dinh TN, Agrotis A and Bobik A. CD4(+)CD25(+)Foxp3(+) regulatory T cells suppress cardiac fibrosis in the hypertensive heart. *J Hypertens*. 2011;29:1820-8.
43. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI and McCluskey J. T cell antigen receptor recognition of antigen-presenting molecules. *Annu Rev Immunol*. 2015;33:169-200.
44. Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, Gasteiger G, Feng Y, Fontenot JD and Rudensky AY. An essential role for the IL-2 receptor in Treg cell function. *Nat Immunol*. 2016;17:1322-1333.
45. Yu L, Yang F, Zhang F, Guo D, Li L, Wang X, Liang T, Wang J, Cai Z and Jin H. CD69 enhances immunosuppressive function of regulatory T-cells and attenuates colitis by prompting IL-10 production. *Cell Death Dis*. 2018;9:905.
46. Watts TH. Staying alive: T cell costimulation, CD28, and Bcl-xL. *J Immunol*. 2010;185:3785-7.
47. Stockinger B and Veldhoen M. Differentiation and function of Th17 T cells. *Curr Opin Immunol*. 2007;19:281-6.
48. Anderegg U, Eichenberg T, Parthaune T, Haiduk C, Saalbach A, Milkova L, Ludwig A, Grosche J, Averbeck M, Gebhardt C, Voelcker V, Sleeman JP and Simon JC. ADAM10 is the constitutive functional sheddase of CD44 in human melanoma cells. *J Invest Dermatol*. 2009;129:1471-82.
49. Nakamura H, Suenaga N, Taniwaki K, Matsuki H, Yonezawa K, Fujii M, Okada Y and Seiki M. Constitutive and induced CD44 shedding by ADAM-like proteases and membrane-type 1 matrix metalloproteinase. *Cancer research*. 2004;64:876-82.
50. Bollyky PL, Evanko SP, Wu RP, Potter-Perigo S, Long SA, Kinsella B, Reijonen H, Guebtner K, Teng B, Chan CK, Braun KR, Gebe JA, Nepom GT and Wight TN. Th1 cytokines promote T-cell binding to antigen-presenting cells via enhanced hyaluronan production and accumulation at the immune synapse. *Cell Mol Immunol*. 2010;7:211-20.
51. Kuipers HF, Rieck M, Gurevich I, Nagy N, Butte MJ, Negrin RS, Wight TN, Steinman L and Bollyky PL. Hyaluronan synthesis is necessary for autoreactive T-cell trafficking, activation, and Th1 polarization. *Proceedings of the National Academy of Sciences of the United States of America*. 2016;113:1339-44.

52. Mummert ME, Mummert D, Edelbaum D, Hui F, Matsue H and Takashima A. Synthesis and surface expression of hyaluronan by dendritic cells and its potential role in antigen presentation. *Journal of immunology*. 2002;169:4322-31.
53. Mahaffey CL and Mummert ME. Hyaluronan synthesis is required for IL-2-mediated T cell proliferation. *Journal of immunology*. 2007;179:8191-9.
54. Gebe JA, Gooden MD, Workman G, Nagy N, Bollyky PL, Wight TN and Vernon RB. Modulation of hyaluronan synthases and involvement of T cell-derived hyaluronan in autoimmune responses to transplanted islets. *Matrix Biol Plus*. 2021;9:100052.
55. Nam K, Oh S, Lee KM, Yoo SA and Shin I. CD44 regulates cell proliferation, migration, and invasion via modulation of c-Src transcription in human breast cancer cells. *Cellular signalling*. 2015;27:1882-94.
56. Hu B, Ma Y, Yang Y, Zhang L, Han H and Chen J. CD44 promotes cell proliferation in non-small cell lung cancer. *Oncol Lett*. 2018;15:5627-5633.
57. Wang SJ and Bourguignon LY. Hyaluronan and the interaction between CD44 and epidermal growth factor receptor in oncogenic signaling and chemotherapy resistance in head and neck cancer. *Arch Otolaryngol Head Neck Surg*. 2006;132:771-8.
58. Zaiss DM, van Loosdregt J, Gorlani A, Bekker CP, Grone A, Sibilia M, van Bergen en Henegouwen PM, Roovers RC, Coffer PJ and Sijts AJ. Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. *Immunity*. 2013;38:275-84.
59. Baaten BJ, Li CR, Deiro MF, Lin MM, Linton PJ and Bradley LM. CD44 regulates survival and memory development in Th1 cells. *Immunity*. 2010;32:104-15.
60. Bollyky PL, Falk BA, Long SA, Preisinger A, Braun KR, Wu RP, Evanko SP, Buckner JH, Wight TN and Nepom GT. CD44 costimulation promotes FoxP3+ regulatory T cell persistence and function via production of IL-2, IL-10, and TGF-beta. *Journal of immunology*. 2009;183:2232-41.
61. Liu T, Soong L, Liu G, Konig R and Chopra AK. CD44 expression positively correlates with Foxp3 expression and suppressive function of CD4+ Treg cells. *Biol Direct*. 2009;4:40.

FIGURES AND FIGURE LEGENDS

Figure 1. *Has3* deficiency results in adverse remodeling and impaired cardiac function

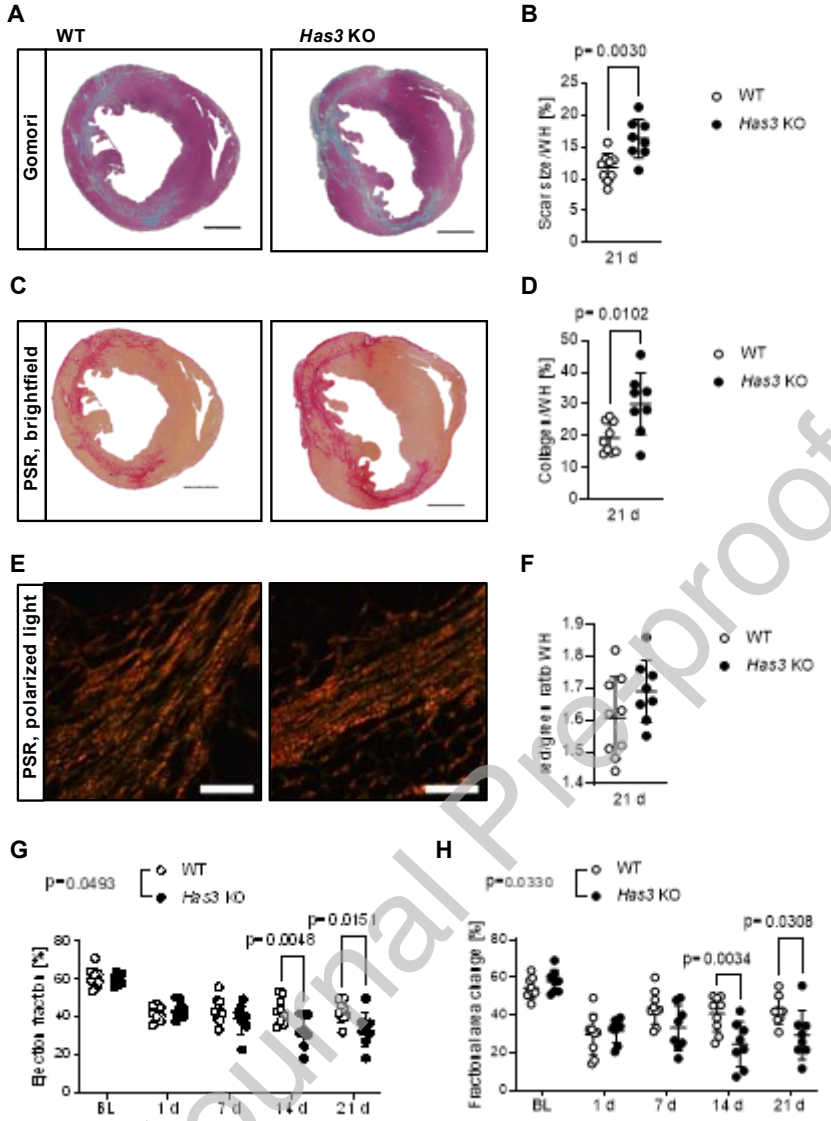

Figure 1. *Has3* deficiency results in adverse remodeling and impaired cardiac function. WT and *Has3* deficient mice underwent I/R injury. Scar size and hemodynamic function were determined by histology and echocardiography. **A.** Representative pictures of gomori trichrome staining 21 days post I/R. **B.** Scar size quantification in whole heart (WH), n=9,8. Statistical test: Unpaired t-test. **C.** Representative brightfield pictures of picrosirius red staining 21 days post I/R. **D.** Total collagen quantification in whole heart (WH), n=9,8. Statistical test: Unpaired t-test. **E.** Representative pictures of picrosirius red staining in polarized light 21 days post I/R. Red indicates dense collagen fibres, green loosely packed collagen. **F.** Collagen density analysis in whole heart (WH), n=9,8. Statistical test: Unpaired t-test. **G, H.** Left

ventricular ejection fraction and fractional area change determined by short-axis echocardiography at baseline (BL), 1 day, 7 days, 14 days and 21 days post I/R, n=9,8. Statistical test: Two-way ANOVA. P-value above the graphs indicates genotype effect over all timepoints as determined by Two-way ANOVA. Scale bar is 1000µm. Data represent mean ± SD.

Figure 2. Cardiac fibroblast and macrophage responses are similar between WT and *Has3* KO mice

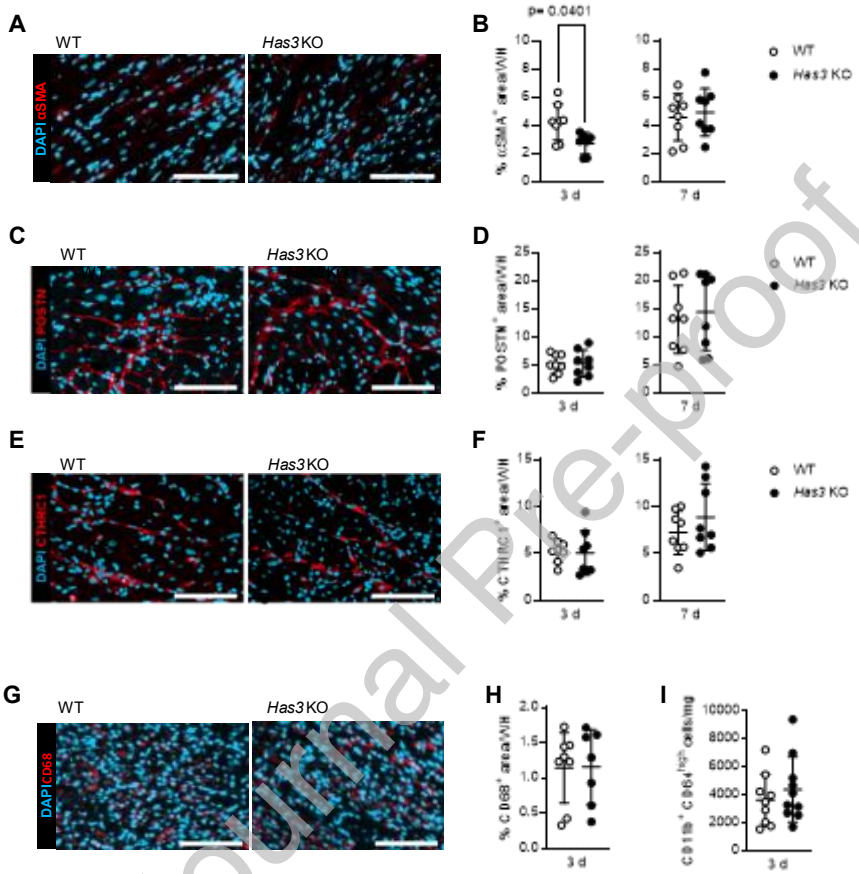

Figure 2. Cardiac fibroblast and macrophage responses are similar between WT and *Has3* KO mice. Heart sections were immunohistochemically stained for αSMA, periostin and CTHRC1 at day 3 and day 7 post I/R. In addition, heart sections were stained for $CD68^+$ macrophages 3 days post I/R, and macrophages were analyzed in digested heart tissue using flow cytometry. **A.** Representative pictures of αSMA staining. **B.** Quantification of $\alpha\text{-SMA}^+$ area in whole heart 3 days post I/R, n=8,7 and 7

days post I/R, n=8,8. Statistical test: Mann-Whitney-U (3 d), Unpaired t-test (7 d). **C.** Representative pictures of periostin staining. **D.** Quantification of POSTN$^+$ area in whole heart 3 days post I/R, n=8,8 and 7 days post I/R, n=8,8. Statistical test: Unpaired t-test. **E.** Representative pictures of CTHRC1$^+$ staining. **F.** Quantification of CTHRC1$^+$ area in whole heart 3 days post I/R, n=8,8 and 7 days post I/R, n=8,8. Statistical test: Unpaired t-test. **G.** Representative pictures of heart sections stained for CD68. **H.** Histological quantification of CD68$^+$ monocytes/macrophages 3 days post I/R, n=8,8. Statistical test: Unpaired t-test. **I.** Flow-cytometric quantification of CD11b$^+$ CD64$^+$ macrophages per mg cardiac tissue 3 days post I/R, n=9,10. Statistical test: Unpaired t-test. Scale bar is 100µm. Data represent mean ± SD.

Figure 3. Reduced CD4 T cells in cardiac tissue of *Has3* KO mice on day 7 after MI

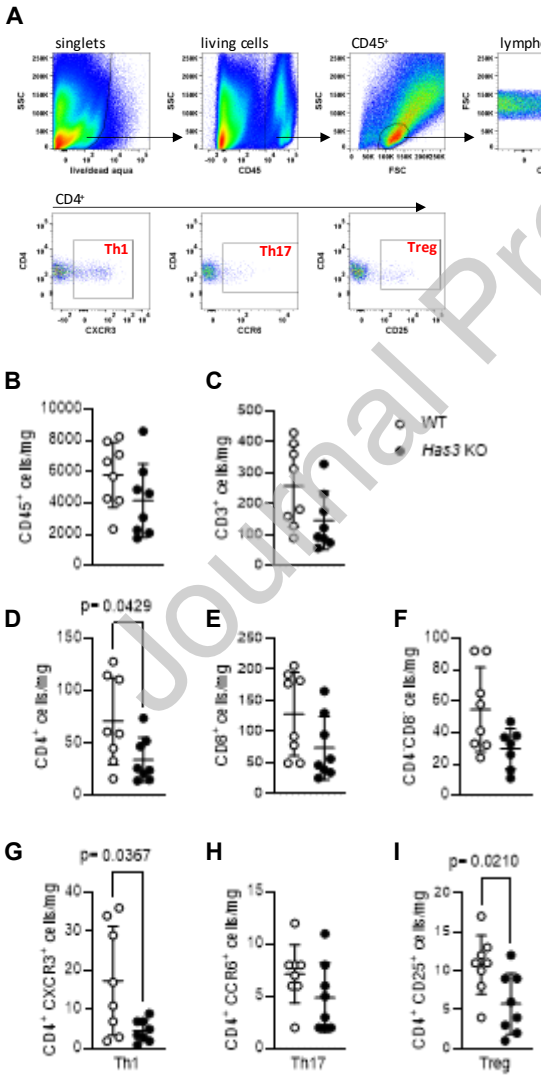

Figure 3. Reduced CD4 T cells in cardiac tissue of *Has3* KO mice on day 7 after MI. WT and *Has3* deficient mice underwent I/R injury. 7 days post I/R, cardiac tissue was analyzed by flow cytometry and cells per mg cardiac tissue were quantified. **A.** Gating scheme for T-helper cell subsets. **B.** Leukocytes, **C.** T-lymphocytes, **D.** CD4 T cells, **E.** CD8 T cells, statistical tests: Unpaired t-tests, **F**. $CD4^{-}CD8^{-}$ T cells, statistical test: Mann-Whitney-U, **G.** Th1 cells, statistical test: Welch's t-test, **H.** Th17 cells and **I.** regulatory T cells, statistical tests: Unpaired t-tests. n=8,8 (B-H). Data represent mean ± SD.

Figure 4. Decrease in $CD69^{+}$ T cells and increased T cell apoptosis in hearts of *Has3* KO mice

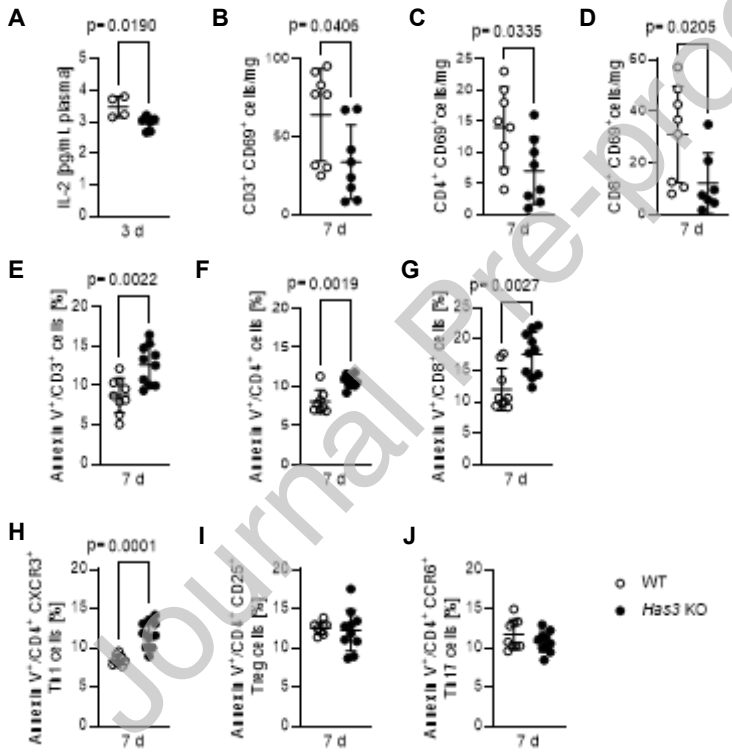

Figure 4. Decrease in $CD69^{+}$ T cells and increased T cell apoptosis in hearts of *Has3* KO mice. WT and *Has3* deficient mice underwent I/R injury. 7 days post I/R, cardiac tissue was analyzed by flow cytometry. **A.** Quantification of IL-2 in plasma of

mice 3 days post I/R. n=4,6. Statistical test: Unpaired t-test. **B-D.** Quantification of activated CD3, CD4, and CD8 cells as cells per mg cardiac tissue. n=8,8. Statistical tests: Unpaired t-tests **E.** Proportion of apoptotic CD3 T cells. n=9,10. Statistical test: Unpaired t-test **F, G.** Proportion of apoptotic cells in CD4 and CD8 T cells, statistical tests: Mann-Whitney-U (F) and Unpaired t-test (G). n=8,10. **H-J.** Proportion of apoptotic Th1, regulatory and Th17 cells, n=9,10. Statistical tests: Welch's t-tests. Data represent mean ± SD.

Figure 5. Reduced HA and CD44 protein expression in *Has3* deficient T cells associates with reduced proliferation

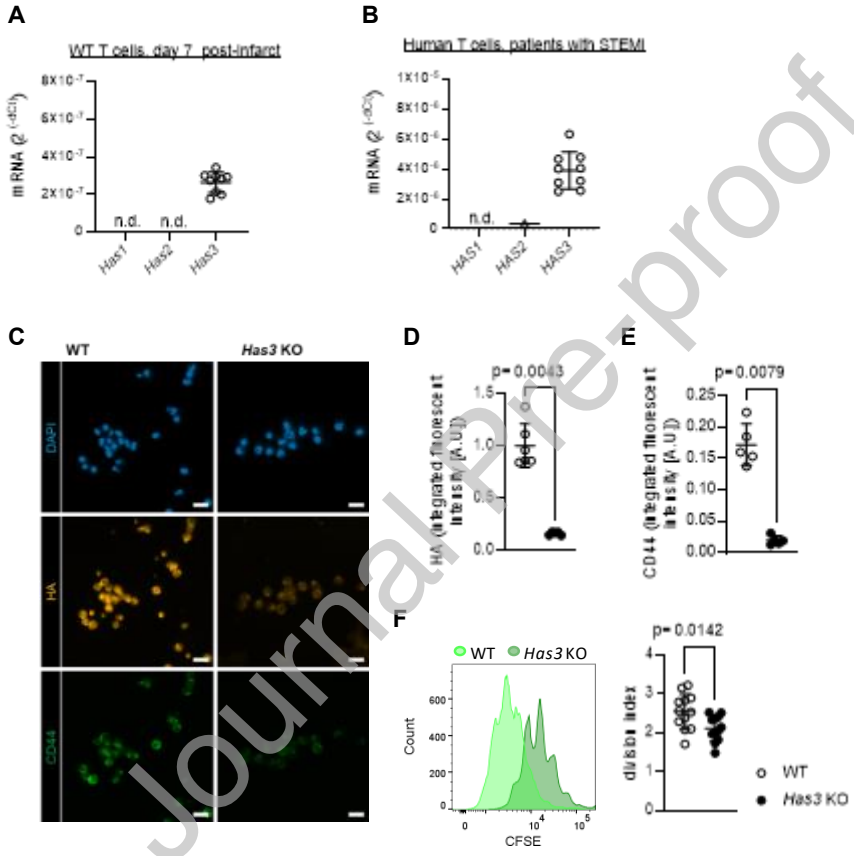

Figure 5. Reduced HA and CD44 protein in T cells from *Has3* KO mice associates with reduced proliferation. WT and *Has3* deficient mice underwent I/R injury. 7 days post I/R, splenic CD3 T cells were isolated and further investigated. For mRNA analysis, cells were processed directly after isolation. Human CD3 T cells were isolated

from peripheral blood of patients 5 days after STEMI. For ICC, cells were cultured in the presence of CD3/CD28 (1/5 µg/mL) antibodies for 24 hours. To monitor proliferation, cells were labeled with CFSE and incubated in the presence of CD3/CD28 (10/1 µg/mL) antibodies for 5 days. For transmigration, T cells were seeded on a monolayer of endothelial cells and were allowed to migrate for 24 hours. **A.** mRNA expression of HAS isoenzymes in splenic T cells of mice 7 days post I/R. n=9. n.d. indicates not detected. **B.** mRNA expression of HAS isoenzymes in T cells from patients with STEMI. n =9. **C.** Representative pictures of HA and CD44 co-staining in mouse splenic T cells. **D, E.** Quantification of fluorescence intensity of HA and CD44 stainings. n=5,5. Statistical test: Mann-Whitney-U. **F.** Representative histogram of CFSE labeled T cells illustrating proliferation after 5 days of culture. Division index of cells calculated using proliferation modeling in FlowJo. n=12,12. Statistical test: Unpaired t-test. Data represent mean ± SD.

Figure 6. Schematic of compromised T cell activation in the infarcted heart of *Has3* KO mice

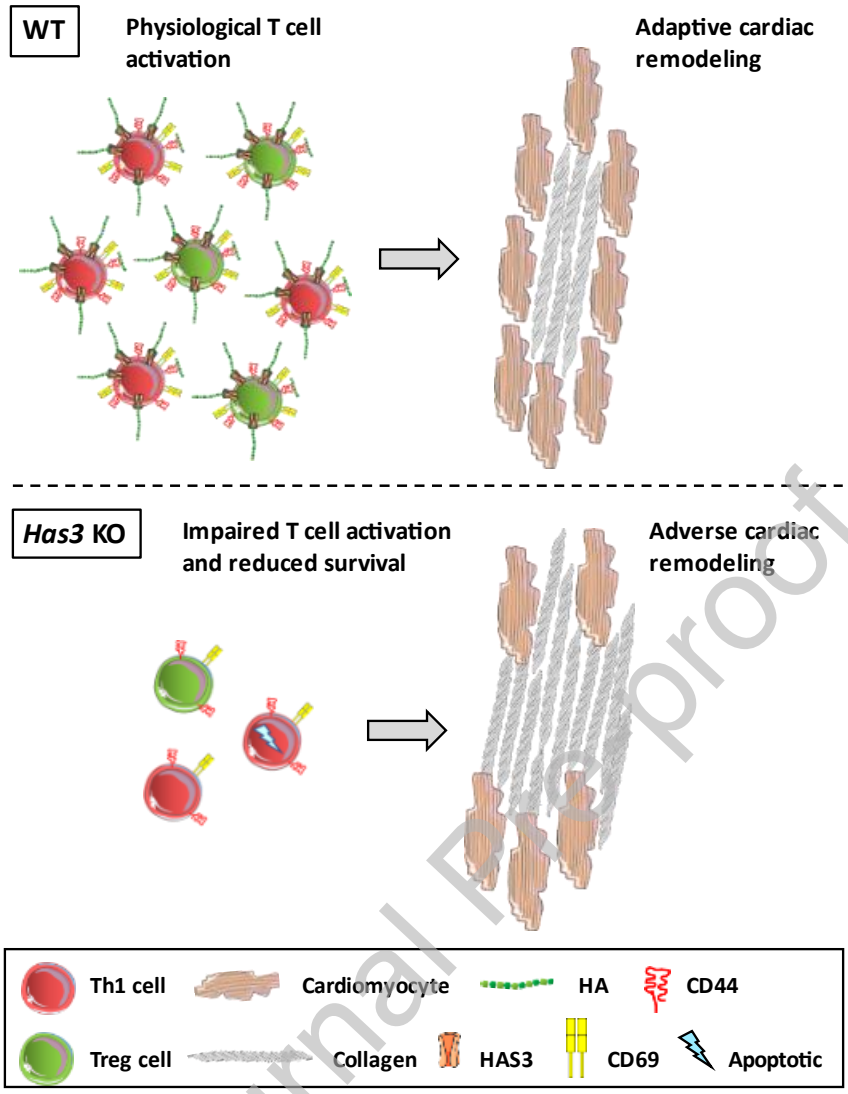

Figure 6. Schematic of compromised T cell activation in the infarcted heart of *Has3* KO mice. T cells play an important role in cardiac healing after myocardial infarction. Studies of the kinetics of T cell accumulation in the infarcted heart have shown that T cell numbers peak between day 3 and day 7 post-infarct[37]. In our model of acute myocardial infarction, adverse cardiac remodeling and impaired heart function in *Has3* deficient mice was associated with significantly reduced numbers of cardiac CD4 T cells at day 7 post-infarct, in particular fewer Th1 and Treg cells. Lack of *Has3* in T cells was not compensated for by expression of other Has isoenzymes, and *Has3* deficient T cells were less activated and more apoptotic as indicated by reduced expression of CD69 and increased numbers of annexin V positive cells, respectively. In line with these results, *in vitro* proliferation of splenic T cells lacking *Has3* was decreased. Altogether, the current study provides evidence for a critical role of *Has3*

in mounting a physiological T cell response that ensures adaptive cardiac healing after MI.