nature portfolio

Peer Review File

Tregs delivered post-myocardial infarction adopt an injury-specific phenotype promoting cardiac repair via macrophages in mice

Editorial Note: This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. This document only contains reviewer comments and rebuttal letters for versions considered at *Nature Communications*.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This revised manuscript focuses on the therapeutic benefit of exogenous Treg treatment following MI. The authors have addressed many of the concerns previously raised by the reviewers. The following issues remain unresolved.

1. It would be recommended to directly compare the transcriptomes of endogenous and exogenously delivered Tregs.

2. The authors utilize CD206 expression and bulk RNAseq to support the conclusion that exogenous delivery of Tregs influences monocyte and macrophage behavior. These data are supportive and suggest substantial shifts in macrophage subsets. The authors should consider further supporting their findings with either focused validation assays (immunostaining/RNAscope) or scRNAseq.

3. A causative link between exogenous Treg delivery and monocyte/macrophage phenotyping shifts is somewhat circumstantial. The authors identify IL-10 and nidogen-1 has potential mediators by which Tregs might signal to infiltrating monocytes and macrophages. Exogenous delivery of Tregs that are unable to express IL-10 and/or nidogen-1 (knockout or siRNA knockdown) would provide rigorous data to support their conclusion.

Reviewer #2 (Remarks to the Author):

The study addresses an important question; namely the impact of regulatory T cells on post-myocardial infarction inflammation and repair, using adequate methodologies with scientific rigour. The flow cytometry gating strategy is convincing, the in vivo experiments include all adequate control groups and authors were careful to not over-interpret their data. The study not only includes some important observations that help confirm previous reports in the field, but also provides important additional mechanistic insights on how exogenous Tregs support myocardial repair via a crosstalk with monocyte / macrophages. As mentioned in my previous assessment, the experimental approach used in Figure 3c-g is particularly informative and neat, and these observations have now been expanded with new data presented in Figures 5-6. My previous comments have been satisfactorily addressed, and I am convinced that this study will foster major advancements in the field.

I would have only one minor suggestion. The data shown in Supl Fig 3 b-c is important and could be integrated into Fig 1.

Reviewer #3 (Remarks to the Author):

The authors provided more supportive data and were able to answer most of the issues that were raised by the reviewers, which resulted in an improved version of the manuscript.

Reviewer #4 (Remarks to the Author):

The authors have now removed the IL2C experiment (given that they now accept that the comparison with exogenous Tregs was irrelevant). They kept the data with exogenous Treg supplementation and with deletion of endogenous Tregs. However; I am still convinced that the data presented here is only incremental and does not provide major advance in our understanding compared to existing published literature.
They argue that the clodronate experiment reproduces previously published data. It doesn't. The Frantz paper (which they consider fit with their results) reported more than 50% mortality in mice treated with clodronate compared to less than 10% in mice with no clodronate. This is a huge difference and certainly does not support the claim that treatment with clodronate has no major impact on heart repair after MI. The clinical relevance is highly uncertain.

Point-to-point response to Referee #1:

This revised manuscript focuses on the therapeutic benefit of exogenous Treg treatment following MI. The authors have addressed many of the concerns previously raised by the reviewers. The following issues remain unresolved.

1. *It would be recommended to directly compare the transcriptomes of endogenous and exogenously delivered Tregs.*

Response: In Figure 2, we compare the differentially expressed genes (DEGs) from both endogenous and exogenous Tregs. The exogenous Tregs were sequenced using minibulk RNA-seq, a technique different from the bulk RNA-seq used for the endogenous Tregs. Therefore, a direct comparison is not feasible. Each sequencing technique has inherent biases and limitations due to differences in sample preparation, sequencing depth, analysis pipelines, and normalisation methods. These variables can introduce variability, complicating direct comparisons between datasets derived from different techniques. Bulk RNA-seq was performed on at least 1000 endogenous Tregs per sample providing an average gene expression profile for the entire population. In contrast, minibulk RNA-seq involves sequencing RNA from 100-200 cells which was not possible with standard bulk RNA-seq. Because of these differences, attempting to directly compare the results of minibulk RNA-seq for exogenous Tregs with bulk RNA-seq for endogenous Tregs would likely yield misleading or unreliable conclusions. Instead, we performed the analysis separately and compared the DEGs from each dataset in Figure 2. This is because our aim was not to enable a direct comparison or quantitation of similarity for each gene, rather to reveal how the DEGs detected in the exogenous Tregs from the minibulk RNA-seq dataset overlapped with DEGs detected in the endogenous Tregs from the bulk RNA-seq dataset.

2. *The authors utilize CD206 expression and bulk RNAseq to support the conclusion that exogenous delivery of Tregs influences monocyte and macrophage behaviour. These data are supportive and suggest substantial shifts in macrophage subsets. The authors should consider further supporting their findings with either focused validation assays (immunostaining/RNAscope) or scRNAseq.*

Response: As suggested by the referee, we now provide further validation with a focused validation assay at the protein level. We performed immunostaining to validate protein

expression levels of the surface markers CD206, LYVE1, CD163 and the ECM protein SPARC in F4/80^{+} monocyte/macrophages (Mo/MΦ) on day 7 post-MI (**Figure 4d, e and lines 222-226**). We show that the levels of these proteins, which are typical markers of pro-repair macrophages, are significantly higher in Treg-treated animals. Thus, these new data validate the RNA sequencing gene expression results and demonstrate that macrophages polarise to a pro-repair phenotype in response to Treg treatment.

3. *A causative link between exogenous Treg delivery and monocyte/macrophage phenotyping shifts is somewhat circumstantial. The authors identify IL-10 and nidogen-1 has potential mediators by which Tregs might signal to infiltrating monocytes and macrophages. Exogenous delivery of Tregs that are unable to express IL-10 and/or nidogen-1 (knockout or siRNA knockdown) would provide rigorous data to support their conclusion.*

Response: We agree that further mechanistic validations would strengthen the study. Therefore, we decided to validate IL-10, as we observed that the cytokine had the strongest effect on Ly6C expression in comparison to nidogen-1. Additionally, the IL-10 knockout mouse model (B6.129P2-*Il10*^{tm1Cgn}/J) was readily available; however, the nidogen-1 knockout mouse was not, necessitating over a year to breed a new mouse line. While siRNA knockdown was considered, it was ultimately not chosen due to the potential for significant viability loss when transfecting Tregs *in vitro* prior to administration. Moreover, transient and partial gene knockdown via siRNA may not be suitable for a validation assay in this model, as sustained inhibition of gene expression over several days is likely required.

Using the IL-10 knockout mouse, we sorted CD4^{+} CD25^{+} Tregs (Tregs$^{IL10-/-}$) and delivered them intravenously one day post-MI. Compared to saline control, Tregs knocked out for IL-10 did not significantly improve healing outcomes post-MI, suggesting that exogenous Tregs primarily exert their therapeutic effect through IL-10 (**Figure 7 and lines 289-295**).

Point-to-point response to Referee #4:

The authors have now removed the IL2C experiment (given that they now accept that the comparison with exogenous Tregs was irrelevant). They kept the data with exogenous Treg supplementation and with deletion of endogenous Tregs. However; I am still convinced that:

1. *The data presented here is only incremental and does not provide major advance in our understanding compared to existing published literature.*

Response: The therapeutic potential of Tregs to induce cardiac repair is known, and perhaps that is why the Referee feels that our study does not add major advance in the field. However, we disagree as we strongly believe that our findings answer many unknown questions in the field, shedding light upon the specific mechanisms by which Tregs and exogenous Tregs promote cardiac repair. Previous research showed that Treg delivery or *in vivo* expansion of Tregs is beneficial for cardiac repair following MI[1, 2, 3, 4, 5, 6, 7] (discussed in **lines 325-327**). For example, studies have shown that Treg attenuated ventricular remodelling upon intravenous injection immediately after MI[3], or one day post-MI[1]. Addionally, the *in vivo* expansion of Tregs with superagonistic anti-CD28 antibody has been shown to improve survival[4] and cardiac function[3], while multiple injections of IL-2 complex over one-two weeks have been shown to promote cardiac repair[7, 8]. Lastly, intramyocardial delivery of Tregs (which might not be ideal from a therapeutic point of view) was found to promote cardiomyocyte proliferation[6]. Altogether, these studies demonstrated the therapeutic potential of Tregs in cardiac repair, however the molecular mechanisms by which they elicit their effect directly on cardiomyoctes and/or other immune cells remained very elusive.

In our study, we investigated this in a model where Tregs were delivered in a therapeutically relevant manner, that is, systemically, one day post-MI. Firstly, we demonstrated that exogenous Tregs home to the injury site and stay there for a few days: a mechanism that was previously unknown. Most importantly, we demonstrated that they adopt a phenotype specific to the injured cardiac microenvironment, akin to the endogenous Tregs that accumulate one-week post-MI. To our knowledge, this has never been demonstrated before. Secondly, we demonstrated that the therapeutic effect of Tregs is ultimately dependent on monocytes/macrophages (Mo/MΦ) via modulation of the pro-inflammatory Ly6C^{Hi} CCR2^{+} Mo/MΦ subset. Moreover, we showed the dynamic changes in cardiac Mo/MΦ from Treg treated mice and contrasted these to the Mo/MΦ from Treg depleted mice, thereby revealing how Treg administration leads to the rapid shift of Mo/MΦ towards a pro-repair phenotype. In

addition, we delved deeper into the mechanisms and found that the reduction of Ly6C^{Hi} CCR2^{+} Mo/MΦ is likely due to a direct effect via the Treg-derived factors nidogen-1 and IL-10, as well as an indirect consequence of the lower CD8^{+} T cell number. Subsequently, we validated Treg-derived IL-10 as one of the main factors responsible for the Treg mediated cardiac repair. All together, these mechanisms have not been previously demonstrated.

Overall, our study highlights the important mechanisms of action, paving the way for novel Treg-centric therapies following MI. In addition, we believe that our findings have broad implications not only in the development of therapeutic strategies for cardiac repair but also in enhancing our understanding the immune milieu in MI, particularly regarding Tregs and macrophages. The discussion section, in particular sections from **lines 361-483,** highlights the significance of these new findings.

2. *They argue that the clodronate experiment reproduces previously published data. It doesn't. The Frantz paper (which they consider fit with their results) reported more than 50% mortality in mice treated with clodronate compared to less than 10% in mice with no clodronate. This is a huge difference and certainly does not support the claim that treatment with clodronate has no major impact on heart repair after MI.*

Response: As we previously mentioned in our last response, a detrimental effect of CL-mediated Mo/MΦ depletion on cardiac function has not been reported by many investigators in the context of permanent left coronary artery ligation in mice. On the contrary, multiple studies have reported no significant effects on cardiac function regardless of the effect on mortality (**lines 371-372**). We would like to once again emphasise that Frantz *et al.,* reported reduction in survival without any deterioration in cardiac function parameters[9]. Increased death was likely due to ventricular thrombi. In our study, we did not assess thrombi formation and survival post-MI since it was beyond the scope of our studies objectives. Below is a summary of the research articles that depleted Mo/MΦ with CL however saw no change in cardiac function parameters, and only some of which have assessed survival.

- Frantz *et al.,*[9] showed that CL-depletion led to no change in infarct size. The study reported 33% survival in the CL-depleted group with increase mortality due to ventricular thrombi.

- Zhao *et al.,*[10] showed no effect on infarct size, ejection fraction and fractional shortening. Survival rates are not mentioned.

- Haider *et al.,*[11] reported no difference in ejection fraction. Survival rates are not mentioned.

- Wang F *et al.,*[12] reported no effect on ejection fraction and systolic left ventricular internal diameter. Survival rates are not mentioned.

- Hess A *et al.,*[13] showed no difference in ejection fraction in the CL-depleted group for the permanent left coronary artery ligation model, but some differences with an ischemia/reperfusion model which is less severe. The study reported 60% survival in the CL-depleted group.

Therefore, in contrast to the concerns expressed by the Referee, an increase in mortality is not necessarily an outcome of reduced cardiac function measured by ejection fraction and infarct size. In our study, we claim no differences in cardiac functions, as reported by Frantz *et al.* and others.

3. The clinical relevance is highly uncertain.

Response: A Treg-centric approach to promote cardiac is currently being explored clinically by expanding endogenous Tregs with low dose of IL-2[14]. Such a protein-based therapeutic approach may offer many advantages from a clinical perspective, but potential drawbacks and limitations could emerge, similar to most therapeutic approaches currently being explored. Again, we are not comparing IL-2-based approaches with exogenous Tregs, nor are we claiming that one is more clinically relevant than the other.

Cellular therapies based on immune cells are novel and very important approaches in regenerative medicine that deserve exploration and may ultimately prove to be very useful. Indeed, many pharmaceutical companies have heavily invested in cell-based therapeutic programs for multiple applications, particularly those involving T cells and Tregs[15, 16]. Our study highlights the important mechanisms by which exogenous Tregs promote cardiac repair post-MI, serving as a stepping stone. The novel mechanisms we revealed (homing of exogenous Tregs to the MI site, Tregs phenotypic changes, effects on CD8 T cells and Ly6C^{Hi} CCR2^{+} Mo/MΦ, critical role of Treg-derived IL-10, etc.) are indeed directly relevant for the further development of cell-based therapeutics and could also be relevant for therapeutics that aim at increasing Treg number (e.g., IL-2, IL-2 complex or other methods). All the new mechanisms of action we revealed, and their relevance to the development of therapeutic approaches, are discussed from **lines 361-483.**

Lastly, we want to mention again that GMP-grade Tregs banks do exist and Tregs are being explored and used in clinical trials for adoptive cell therapy to facilitate organ transplantation or for autoimmune diseases[17, 18, 19]. Furthermore, methods to obtain large numbers of Tregs are being developed[20]. It is also important to note that in those cases, a high number of Tregs and multiple deliveries are required to maintain exogenous Tregs in the body for a long period. However, in the case of MI, our data suggest that Tregs persistence is likely crucial only during the first few days following injury when they modulate the infarcted heart immune microenvironment. In addition, the efficacy of the Treg adoptive cell therapy described in our study could very likely be improved, enabling the utilisation of fewer Tregs with methods described in a recent review[17]. Those aspects are discussed in lines **342-360.**

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed the questions and concerns raised by the reviewers. As a minor comment, the authors should be careful to appropriately interpret the phenotypic shift observed in cardiac macrophages following Treg delivery. Changes seen could be reflective of shifts in monocyte phenotype or preservation of cardiac resident macrophages. This should be mentioned in the discussion.

Response to Referee #1:

The authors have adequately addressed the questions and concerns raised by the reviewers. As a minor comment, the authors should be careful to appropriately interpret the phenotypic shift observed in cardiac macrophages following Treg delivery. Changes seen could be reflective of shifts in monocyte phenotype or preservation of cardiac resident macrophages. This should be mentioned in the discussion.

Response: We agree that the phenotypic shift observed in macrophages could be related to changes in monocytic phenotypes, such as the Ly6C^{Hi} subset profiled in Figure 6, and/or the preservation of cardiac resident macrophages, as indicated by the upregulation of key markers *Lyve1* and *Cd163*. We discuss this point further in the discussion section (lines 405-406).