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Peer Review File

Local administration of regulatory T cells promotes tissue healing

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The ms by Nayer et al addresses the phenotype and function of Tregs administered locally (in hydrogel) to injured tissue in three experimental contexts. This is a potentially exciting story but the current version has several issues that should be dealt with before publication.
1) Probably the biggest problem is their reliance on clodronate-liposome-mediated depletion of macrophages. This procedure is well known to release a burst of cytokines, eg TNFα, which can impact surrounding cells, notably Tregs. Most people in the field don't use this method to deplete macrophages anymore, at least not without independent validation. So the investigators need to re-examine key observations using a genetic-deletion model.
2) Another big issue is the superficial method of measuring tissue repair, which is not field-standard. Supporting methods – e.g. fiber analysis for the muscle – need to be added.
3) For the Treg-depletion experiments: the investigators need to look at the degree of depletion in the tissues, which can be very different from that in blood (Ext. Data 1b, c)
4) Especially considering point #1 above, the investigators are focusing too narrowly on macrophage-related mechanisms. They should look at what the Tregs are doing to T and NK cells (in particular their production of IFNγ) as well as local stem/progenitor cells.
5) One of the strong points of this study is its use of three injury models. To exploit this strength, the investigators should compare the transcriptomes taken on by the transferred Tregs in the 3 tissue contexts [not via cherry-picking (which, btw, there is too much of)].
6) Since the investigators frequently allude to the clinical potential of this method, it is important to know how late the Tregs can be delivered and still significantly improve tissue repair. This could also provide important information about what events during the repair process Tregs can impact.
7) The result with allogeneic and xenogeneic Tregs is potentially very exciting but seems a bit "magic" as it stands. The investigators need to provide a convincing explanation for why these cells are not rejected to buttress the reader's confidence. Is the hydrogel totally impervious to cell infiltration? If so, for how long?
Less crucial:
8) Can the investigators please provide flow cytometry evidence of the Ly6C and F4/80 subset overlaps?

Reviewer #2 (Remarks to the Author):

Nayer et. al. report a comprehensive program of research providing proof-of-principal that exogenous Treg cell therapy delivered at the injury site can enhance tissue regeneration in models of bone, muscle, and skin healing. This included demonstration that allogeneic and human Tregs are also effective. Evidence is provided that the overarching mechanism is via accelerated switching of the localised immune response from a pro- to anti-inflammatory state, with modulation of monocyte/macrophage biology a key component of this mechanism. Generally, the experiments are rigorously designed but I have a few concerns that would need to be addressed by the Authors. Analysis approaches are consistent with field expectations but in situ verification of ex vivo generated data with more rigorous exploration of the progression of various wound healing stages would strengthen the study's conclusions. The merged discussion of all three injury models does somewhat confound clear understanding of the variations that are apparent between each, but I acknowledge that this was not an analysis priority of the research program. While potential key molecular mediators were identified from gene lists based on existing literature, conclusive demonstration of their role was not thoroughly explored. Like the previous point, this was clearly not a priority for this research program with Authors prioritising evidence gathering for translation of a cell-based therapy and have submitted relevant patent applications as noted in the conflicts statement. Overall, the manuscript will be of interest to a broad readership and does advance knowledge within the field of regenerative medicine as well as fields relevant to each of the injury models explored. The Authors should provide more rigorous consideration of the limitations of their study.

Recommended improvements:
While the manuscript is well written, it is a challenging read, which was contributed to by the merging of reporting and discussion of each injury model and that the bulk of the experimental data has been relegated to extended data (4 main figures versus 15 extended data figures). I think it helpful for readability that the Authors relocate a portion of the extended data figures back into the main body of the paper.
Throughout the manuscript the Authors provide representative data of experimental approaches, predominantly ex vivo flow cytometry gating examples provided, without specifically indicating which injury model the 'representative data' was collected from. It is expected that there would be variation in the isolated single cell profiles not only between each of the injury models but also at different time points within an injury model. At a minimum the Authors should specifically state within the Figure legends which injury model the representative data has been sourced from. My preferred recommendation is that at least once, the Authors provide as extended data, direct comparison of the gating strategies for Tregs and monocyte/macrophages between the 3 injury models.
Can the Authors please review and verify whether data is normally distributed and therefore whether the most appropriate statistical tests have been applied to infer significant differences. As an example, the spread of individual data points in Figure 1b and d as well as Extended data Figures 1d, f and h, warrants confirmation of normal disruption.
The FSC-A versus SSC-A plots shown in Extended data Figure 3a, as well as other representative data presented throughout the manuscript, indicate a lot of large and/or dense cell clusters/other debris is present within the injury site cell preparation despite reasonable measures taken to alleviate this during the isolation protocol. It validates the expectation that isolation of single cell suspensions from these injury sites has a high risk of variability and therefore may not be fully representative of all cells within the tissue. Cells will be lost during the manual handling stages, incomplete digestion and/or restriction of analysis to flow cytometry events meeting single cell criteria. While this experimental strategy meets field norms, these limitations should be more clearly acknowledged. What 'between sample' quality control was undertaken? It is common that such ex vivo strategies are accompanied by complimentary in situ approaches. Therefore, my recommendation is that the conclusions of the study would be greatly strengthened by provision of accompanying in situ analysis to: 1) validate Treg cell in vivo depletion; 2) verify efficiency of retention of exogenous T regs within injury sites; and 3) validate monocyte/macrophage in vivo depletion. The latter is particularly critical given monocyte/macrophage depletion alone has been demonstrated to impair healing in similar injury models to those used herein. Consequently, the fact that the monocyte/macrophage depletion alone has minimal impact on healing outcomes as presented in Figure 3 is a major concern (noting that accurate comparison was impaired as PBS liposome control experimental groups, which were undertaken according to the Methods, were not presented in the paper). Additionally, accompanying detailed in situ analysis would provide richer information relating to the mechanism of action of Treg cell therapy as it would allow assessment of the associated with other cells during the dynamic and complex mechanism of tissue repair.
It is established that some of the cell surface markers utilised in the study are sensitive to enzymatic digestion. What is the impact of the tissue preparation approaches employed herein on the integrity of the cell surface markers analysed and how was this compensated for in gating strategy optimisation?
In Figure 2e-g data the Authors should clarify the X-axis unit as it is not anticipated that gene expression would fall below zero. I suspect it is likely that the unit is actually a log to the base 2 scale but this hasn't been clearly represented in the data output generated in the Degust web tool.
In extended data Figure 3, to ensure that the gating strategy is capturing all RFP+ Treg cells within the generated tissue cells suspensions, the Authors should assess for detection of RFP outside the initial lymphocyte gate and determine if any of the cell events in the excluded myeloid cell gate are RFP+. This would systematically inform if the experimental strategy is at least capturing all Tregs isolated from the injury sites. The legend indicates that panel b and c has been represented differently to the final plot shown in the example gating strategy, which is a bit perplexing given the axis in panel b (Y axis is FSC-A) and c (Y axis is CD4-APC) are not the same, and therefore unlikely represent the same gating approach. The Authors need to clarify and review their data analysis approach in this experiment (noting use of FCS-A is not a precision approach to gating lymphocytes).
The bulk sequencing data for both exogenous and endogenous Treg upregulation of Il10, Anxa1, Fgl2, Lgals1 and Lgals3 at injury sites, that are specifically noted by the Authors as genes

upregulated in support of influencing macrophage function, should be validated by qPCR in independent samples from each of the injury sites.
The Authors have carefully used the joint terminology of monocyte/macrophage throughout the manuscript based on the fact that their myeloid marker gating strategy does not achieve definitive distinction of these cells. However, it is disappointing that more definitive macrophage makers such as CD169, VCAM-1, and/or MerTK, that should have been relatively easy to include in the staining panel, were not utilised. Can the Authors confirm that the sorted cells indeed include bonafide macrophages by confirming robust detection of macrophage marker genes in the isolated RNA (e.g. Csf1r, Siglec1, Adgre1, Mertk etc)?
With respect to the data presented in Figure 3h-m, as stated in the legend, only a selection of differentially regulated genes are represented. What was the criteria used to determine which genes would be represented? What was the total number of up or down regulated genes within each population and can multi-sample comparisons be undertaken to show the number of these that overlap either within the same injury at different time points or between different injury sites at the same timepoints? Providing similar analysis for Treg sequencing data would also strengthen the Authors' conclusions and allow readers to more wholistically appreciated difference/similarities between the injury models as well as impact of cell treatment.
Gene ontogeny results presented in Figure 3l show that genes associated with bone development are upregulated in monocyte/macrophages isolated from Treg-treated muscle injury sites. Heterotopic ossification in muscles is a pathological outcome driven by macrophages in some trauma circumstances. The Authors need to specifically acknowledge that this transcriptional profile exposes a potential risk of use of this therapy for muscle repair in certain clinical settings. The Authors need to also consider and discuss why this GO profile was not apparent in the bone injury model?
I would argue that the Authors have overextended the interpretation of the somewhat conservative increase of human Treg cells compared to exogenous syngeneic or allogenic mouse Treg cell recovery from injury sites as evidence of human Treg improved stability. They would in the very least need to perform comparison with ex vivo expansion of murine Tregs to affirm this interpretation.
With respect to data presented in Extended Figure 8b. Graph shows %F4/80 low/total F4/80+ cells at two time points in the 3 injury models. Can the Authors please clarify is the total F4/80+ cell frequency also varied between the healing time points analysed.

Reviewer #3 (Remarks to the Author):

In this manuscript, Nayer et al provided robust experimental evidence in 3 distinct mouse models of acute tissue injuries that locally administered Tregs enhance tissue healing (Fig 1). Even more impressive was the finding that the tissue healing activities were intact across allogeneic and xenogeneic barriers (Fig 4). While the tissue repair function of Tregs has been well-established in the literature, local administration of Tregs and the use of allogeneic and xenogeneic Tregs in this context are novel contributions to the field.

The authors attempted to determine the mechanistic basis of the tissue healing function of Tregs by gene expression profiling of the administered Tregs recovered from the sites of injury and by gene expression profiling of tissue macrophages with and without Treg treatment. While results from these experiments provide some clues, they fall short of establishing the roles of Treg-expressed genes in the tissue repair function of Tregs. For example, the authors stated in the abstract that "Tregs exert their regenerative effect .... injured tissues ... via factors such as interleukin-10". This statement is not supported by experimental evidence. While IL-10 gene expression was upregulated in Tregs in all 3 models, the evidence for IL-10 involvement in tissue repair is correlative. Would IL-10KO mice have more severe tissue injury as seen in Treg-depleted mice? Would Tregs from IL-10KO mice fail to promote tissue repair? Would IL-10-producing Tr1 cells have a similar function in tissue repair?

Another conclusion that the authors proposed in the abstract is that the administered Tregs "rapidly adopt an injury-specific phenotype in response to the damaged tissue microenvironment".

This conclusion is primarily based on a comparison of Treg RNAseq data before and after applying it to the wounded tissue. Without performing a similar analysis of Tregs applied to non-injured tissue or lymphoid tissue using a fibrin scaffold, it is hard to know if the changes were injury-specific or represented an adaptation to a non-lymphoid tissue environment. For example, IL-10 is made by activated Tregs in lymphoid and nonlymphoid tissues, and Nr4a1 expression is induced by TCR engagements in Tregs and conventional T cells in lymphoid and non-lymphoid tissues. Thus, expression of these genes doesn't necessarily mean the cells are adapting to a nonlymphoid tissue environment. In addition to these non-injury controls, it would be nice to also compare the rate of wound healing in mice treated with fibrin-embedded CD4 conventional cells.

The authors state that "it is worth noting that our observations of a positive regenerative outcome following allogeneic and xenogeneic Treg delivery suggest a significant contribution of TCR stimulation-independent mechanisms" (lines 428-430). TCRs expressed by Tregs should be able to respond to allogeneic and xenogeneic MHCs. Thus, it may be argued that they are more likely to be activated via their TCR than in the syngeneic setting. The authors isolated Tregs using FACS purification after staining the cells with antibodies to CD4 and CD3 along with a fluorescent Foxp3 reporter. It should be noted that the CD3 antibody used for isolating the Tregs could remain on the Treg surface and activate the Tregs, especially in the presence of FcR-expressing myeloid cells in the tissue. The use of anti-CD3 is not needed to isolate highly pure Tregs. For the human Tregs, a more detailed method of Treg purification (FACS or MACS? What markers?) and expansion (how were the cells stimulated? How long was the expansion?) The reference provided (#76) did not provide this important information.

Response to Reviewer #1:

The ms by Nayer et al addresses the phenotype and function of Tregs administered locally (in hydrogel) to injured tissue in three experimental contexts. This is a potentially exciting story but the current version has several issues that should be dealt with before publication.

1. *Probably the biggest problem is their reliance on clodronate-liposome-mediated depletion of macrophages. This procedure is well known to release a burst of cytokines, eg TNFα, which can impact surrounding cells, notably Tregs. Most people in the field don't use this method to deplete macrophages anymore, at least not without independent validation. So the investigators need to re-examine key observations using a genetic-deletion model.*

Response: We agree that re-examining key observations using a genetic macrophage deletion model would reinforce our claims. However, to our knowledge, clodronate-based macrophage depletion is in fact quite effective for acute injury-based studies[1], and is still widely employed in the field to confirm the role of macrophages in multiple biological processes, based on recent publications (e.g. in *Nature* and *Nature Communications*)[2, 3, 4, 5, 6, 7]. We have also used this method several times[8, 9]. Additionally, previous studies on skeletal muscle injury suggest that unlike some other methods of macrophage depletion, liposomal clodronate induces selective apoptotic cell death in monocytes and macrophages without the secretion of pro-inflammatory cytokines[10]. Thus, to address the Reviewer's concerns, we decided to confirm this using our muscle injury model as an example. We quantified the levels of key pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, IFN-γ and CCL-2, by performing ELISAs on muscle tissues obtained from mice with quadriceps volumetric muscle loss injury, treated with clodronate or PBS liposomes. Indeed, we also found no significant increase in the levels of these cytokines/chemokines, on D2 and D4 post-injury (**Supplementary Fig. 11**). In fact, the levels of TNF-α, IL-1β and CCL-2 were significantly lower in clodronate-treated mice, on D2 post-injury, while their levels recovered back to normal by D4. These results suggested that at least in our model, clodronate liposome-mediated macrophage depletion does not lead to a burst of cytokines, thus supporting our choice of this technique for our experiments. We have now discussed this point on **page 10, paragraph 2.**

2. Another big issue is the superficial method of measuring tissue repair, which is not field-standard. Supporting methods – e.g. fiber analysis for the muscle – need to be added.

Response: For muscle regeneration, we have now added additional analysis for the cross-sectional area of muscle fibres (**Supplementary Fig. 3**) to further support our conclusions from the analysis of muscle fibrotic area and muscle area. Compared to models using toxin-induced muscle injuries that destroy fibres, in a volumetric muscle loss (VML) model the overall muscle area is likely more important than the area of individual muscle fibres. Indeed, our method of analysis for the VML model has recently been published, demonstrating its acceptance as a standard in the field of tissue

regeneration (e.g. in *Nature*[11, 12]). Additionally, we would like to clarify that in the full-thickness skin biopsy model, wound closure is considered a gold-standard technique for evaluating tissue healing[13]. Similarly, bone coverage and volume are gold-standard measures in the cranial bone defect model[14]. In fact, we have published with these field-standard methods of analysis since many years[8, 9, 15, 16, 17, 18, 19, 20, 21].

3. For the Treg-depletion experiments: the investigators need to look at the degree of depletion in the tissues, which can be very different from that in blood (Ext. Data 1b, c).

Response: We now provide additional analysis of Treg depletion within the injured tissues, in addition to blood, using flow cytometry. We show that Tregs are also effectively ablated in the bone, muscle, skin and spleen tissues of injured *Foxp3*$^{DTR/GFP}$ + DT mice compared to wildtype + DT mice (control) on D7 post-injury, which represents the peak of Treg accumulation in tissues (**Supplementary Fig. 1b-e**). In addition, we show *in situ* validation of Treg depletion in injured muscle and skin tissues as examples by immunostaining of tissue cryosections for Foxp3 on D7 post-injury (**Supplementary Fig. 1f**). Consistent with our flow cytometry data, wildtype + DT mice showed clear staining for Foxp3$^+$ Tregs, while the sections from *Foxp3*$^{DTR/GFP}$ + DT mice showed an absence of Foxp3$^+$ cells in both skin and muscle, thus validating the efficacy of Treg depletion in these tissues.

4. Especially considering point #1 above, the investigators are focusing too narrowly on macrophage-related mechanisms. They should look at what the Tregs are doing to T and NK cells (in particular their production of IFNγ) as well as local stem/progenitor cells.

Response: We agree that it is necessary to gain a more comprehensive understanding of the immune microenvironment after Treg administration. In the manuscript, we did report changes in the dynamics of neutrophil and CD8$^+$ T cell accumulation in Treg-treated tissues compared to controls using flow cytometry (**Supplementary Fig. 19**). However, we concur that it is worth examining the expression of IFN-γ within Treg-treated tissues, especially since prior studies have shown that T cells, along with natural killer (NK) cells, produce increased levels of the pro-inflammatory cytokine IFN-γ upon Treg depletion[22, 23]. Thus, as suggested, we have quantified IFN-γ expression by CD4$^+$ T cells, CD8$^+$ T cells and NK cells in Treg-treated bone, muscle and skin tissues compared to controls. Interestingly, we found that Treg delivery led to a reduction in the percentage and number of IFN-γ-producing T cells and NK cells in the injured tissues **(Supplementary Fig. 21**). This indicated that exogenous Tregs limited IFN-γ production, which in turn is known to affect the composition and phenotype of monocytes/macrophages (Mo/MΦ) during tissue healing[22, 23]. Furthermore, this was consistent with the RNA-seq analysis of endogenous Mo/MΦ from Treg-treated mice that also showed a diminished cellular response to IFN-γ (Fig. 3). Nevertheless, while these significant changes in other immune cells likely play an important role in modulating tissue healing post Treg-delivery, the profound impairment in Treg-mediated tissue healing upon Mo/MΦ depletion suggests that exogenous Treg treatment primarily promotes tissue healing by influencing

Mo/MΦ. In the revised manuscript, we have included these results on **page 17, last paragraph**, and discussed this point on **page 25, paragraph 2**. Additionally, we do not exclude the possibility of Tregs acting on stem/progenitor cells; however, if these cells had a major impact on Treg-mediated tissue healing, we would have seen some preservation of its therapeutic effect in the absence of Mo/MΦ. Thus, our data strongly suggests Mo/MΦ modulation to be the key mechanism of the observed therapeutic effect of Tregs.

5. One of the strong points of this study is its use of three injury models. To exploit this strength, the investigators should compare the transcriptomes taken on by the transferred Tregs in the 3 tissue contexts [not via cherry-picking (which, btw, there is too much of)].

Response: We agree that the comparison of transcriptomes across injured bone, muscle and skin is important to understand mechanisms that are commonly shared between the 3 tissues. Indeed, the heatmap shown in **Fig. 2d** was included with the objective to provide this comparison, by visualising the expression of selected genes across all samples. These genes were selected based on their known function in immunomodulation and tissue regeneration. However, we now also show global gene expression changes in all three tissues through volcano plots depicting differentially expressed genes (DEGs) (using an FDR adjusted p-value < 0.05 with fold change > |1.5|) between exogenous spleen Tregs before delivery and exogenous Tregs recovered from injured tissues at D3 post-delivery (**Supplementary Fig. 6a-c**). These plots show all the significantly upregulated (red) and downregulated (blue) genes in D3 recovered Tregs. The genes that are labelled in these volcano plots correspond to the ones presented in the heatmap in **Fig. 2d**. To further address the point raised by the Reviewer, Venn diagrams have now been provided to compare the overlap of upregulated and downregulated DEGs between all three tissues, from which it can be observed that 32 genes were commonly upregulated in exogenous Tregs recovered from all 3 tissues (**Supplementary Fig. 6d**). These 32 genes have also been depicted on a heatmap (**Supplementary Fig. 6e**). Additionally, we would like to point out that amongst these commonly upregulated genes, *Il10* stands out as a key anti-inflammatory factor, known to be involved in macrophage modulation[24]. We now mention this more clearly in the Results section on **page 7, paragraph 1**.

6. Since the investigators frequently allude to the clinical potential of this method, it is important to know how late the Tregs can be delivered and still significantly improve tissue repair. This could also provide important information about what events during the repair process Tregs can impact.

Response: This study focused on delivering exogenous Tregs immediately post-injury, as the presence of endogenous Tregs is reportedly the most crucial during the early stage post skin[22] and muscle[23] injury, and delivery of exogenous Tregs either immediately or one-day post-MI has been shown to promote cardiac repair[25, 26]. Thus, we expect that augmenting Treg numbers locally as early as possible after tissue damage would likely provide the maximum benefit as a therapeutic strategy

for tissue healing. However, we agree that future research is required to determine the maximum possible timeframe within which we can administer Tregs to damaged tissue to effectively facilitate healing. We now discuss this point **in page 27, paragraph 2**. Nevertheless, regardless of the timing of delivery, we think that our study provides important mechanistic insights into the pro-regenerative effects of exogenous Tregs when administered to injured tissues.

7. The result with allogeneic and xenogeneic Tregs is potentially very exciting but seems a bit "magic" as it stands. The investigators need to provide a convincing explanation for why these cells are not rejected to buttress the reader's confidence. Is the hydrogel totally impervious to cell infiltration? If so, for how long?

Response: To clarify the point raised by the Reviewer, we are not claiming that the hydrogel is impervious to cell infiltration. In fact, it is known that fibrin hydrogels act as a scaffold allowing cells to infiltrate and interact with each other[27]. However, our results show that the Tregs are detectable for up to 5 days post-delivery, during which their effect is likely exerted within the first 3 days upon administration, based on the RNA seq analysis of exogenous Tregs recovered on D3 post-delivery (**Fig. 2**). Indeed, other cell-based therapies that have also shown some degree of efficacy for regeneration upon allogeneic or xenogeneic transfer typically exert their effects soon after delivery due to excessive cell death within 24 hours post-administration[28]. Additionally, fibrin hydrogels are known to be an effective cell carrier, protecting the administered cells from forces applied during delivery, thereby preserving their viability[27]. Hence using them as a local delivery system likely improves cell survival, compared to other modes of direct local delivery such as intradermal or intramuscular injections. In the revised manuscript, we have provided *in situ* validation of Treg retention as well (**Supplementary Fig. 5**), showing that the Tregs administered through fibrin gels mostly stay within the hydrogel, with some infiltration into the injured tissue. However, factors released by the Tregs are more likely to permeate through the hydrogel and into the tissue to interact with other cell types. Nevertheless, we do not claim that the cells are not rejected. In fact, if we compare the retention of murine and human Tregs that were cultured *in vitro* prior to *in vivo* delivery, we find that murine Tregs showed superior recovery (~80%) compared to human Tregs (45%) by D5 post-delivery, likely suggesting that human Tregs may have been subject to some rejection *in vivo* (**Supplementary Fig. 24 and 25**).

8. Less crucial: Can the investigators please provide flow cytometry evidence of the Ly6C and F4/80 subset overlaps?

Response: As suggested by the Reviewer, we have now included representative flow cytometry plots to demonstrate the inverse correlation between $F4/80^{high/low}$ and $Ly6C^{high/low}$ populations (**Supplementary Fig. 12**).

Response to Reviewer #2:

Nayer et. al. report a comprehensive program of research providing proof-of-principal that exogenous Treg cell therapy delivered at the injury site can enhance tissue regeneration in models of bone, muscle, and skin healing. This included demonstration that allogeneic and human Tregs are also effective. Evidence is provided that the overarching mechanism is via accelerated switching of the localised immune response from a pro- to anti-inflammatory state, with modulation of monocyte/macrophage biology a key component of this mechanism. Generally, the experiments are rigorously designed but I have a few concerns that would need to be addressed by the Authors. Analysis approaches are consistent with field expectations but in situ verification of ex vivo generated data with more rigorous exploration of the progression of various wound healing stages would strengthen the study's conclusions. The merged discussion of all three injury models does somewhat confound clear understanding of the variations that are apparent between each, but I acknowledge that this was not an analysis priority of the research program. While potential key molecular mediators were identified from gene lists based on existing literature, conclusive demonstration of their role was not thoroughly explored. Like the previous point, this was clearly not a priority for this research program with Authors prioritising evidence gathering for translation of a cell-based therapy and have submitted relevant patent applications as noted in the conflicts statement. Overall, the manuscript will be of interest to a broad readership and does advance knowledge within the field of regenerative medicine as well as fields relevant to each of the injury models explored. The Authors should provide more rigorous consideration of the limitations of their study.
Recommended improvements:
While the manuscript is well written, it is a challenging read, which was contributed to by the merging of reporting and discussion of each injury model and that the bulk of the experimental data has been relegated to extended data (4 main figures versus 15 extended data figures). I think it helpful for readability that the Authors relocate a portion of the extended data figures back into the main body of the paper.

Response: We apologise for the inconvenience caused by the formatting of the paper. Our manuscript was first formatted for submission to *Nature*, after which it was transferred to *Nature Communications* without the need of reformatting. Thus, it retained the initial formatting that restricted us to 4 main figures only. We have now reformatted the manuscript appropriately for publication in *Nature Communications* and have relocated some figures from the extended data into the main body of text.

Throughout the manuscript the Authors provide representative data of experimental approaches, predominantly ex vivo flow cytometry gating examples provided, without specifically indicating which injury model the 'representative data' was collected from. It is expected that there would be variation in the isolated single cell profiles not only between each of the injury models but also at different time

points within an injury model. At a minimum the Authors should specifically state within the Figure legends which injury model the representative data has been sourced from. My preferred recommendation is that at least once, the Authors provide as extended data, direct comparison of the gating strategies for Tregs and monocyte/macrophages between the 3 injury models.

Response: As suggested by the Reviewer, we have ensured that the name of the tissue used for generating the representative gating strategies or FACS plots has been added in every figure that contains flow cytometry plots. Additionally, we would like to clarify that once the tissue is digested and single cell suspensions are prepared for running on flow cytometers, they generate similar dot plots for injured bone, muscle and skin tissues (example shown below for macrophage sorting). However, for some critical data, such as those presented in **Supplementary Fig. 5b**, we have shown the representative flow cytometry plots of RFP^+ Tregs recovered from all three Treg-treated tissues (bone, muscle, and skin), at different time points (D1, D3 and D5) post-injury, compared to untreated controls. Nonetheless, we would like to mention that the slight differences observed between different tissues or different time points of the same tissue do not impact the gating strategy itself. Therefore, we did not deem it necessary to include direct comparisons between the 3 injury models for all gating strategies as supplementary data in the manuscript.

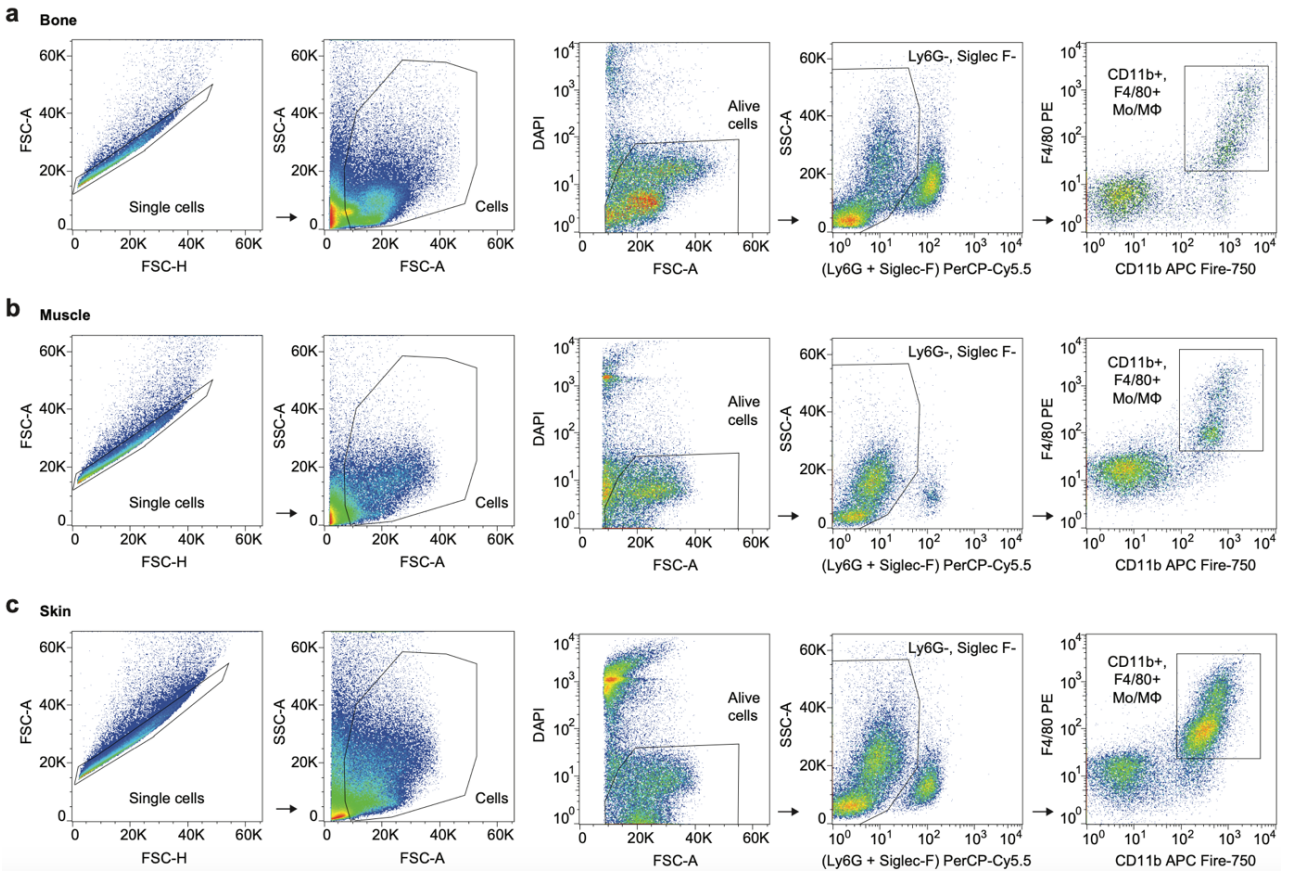

Fig 1: Flow cytometry gating strategies for isolating tissue Mo/MΦ from injured tissues. a-c, Representative flow cytometry gating strategies for sorting endogenous Mo/MΦ from injured bone, muscle, and skin tissues, by excluding $Ly6G^+$ neutrophils, and Siglec-F^+ eosinophils, before gating for $CD11b^+$, $F4/80^+$ cells in bone (**a**), muscle (**b**), and skin (**c**) tissues.

Can the Authors please review and verify whether data is normally distributed and therefore whether the most appropriate statistical tests have been applied to infer significant differences. As an example, the spread of individual data points in Figure 1b and d as well as Extended data Figures 1d, f and h, warrants confirmation of normal disruption.

Response: We thank the Reviewer for pointing out the possibility of data not following a normal distribution, hence requiring a non-parametric statistical test. We have now tested all our data for normality using the Shapiro-Wilk test and found that the following datasets were not normally distributed: Fig. 1d, Fig. 7d, f, Supplementary Fig. 1e, i, k, and Supplementary Fig. 3b, e. For these non-normally distributed data, a Mann Whitney U Test was performed for pairwise comparisons, while a Kruskal-Wallis test was performed for 3 or more groups followed by the Dunn's *post hoc* test for multiple comparisons. $P < 0.05$ was considered statistically significant. The statistical methods, relevant figures and figure legends have been updated to reflect this change.

The FSC-A versus SSC-A plots shown in Extended data Figure 3a, as well as other representative data presented throughout the manuscript, indicate a lot of large and/or dense cell clusters/other debris is present within the injury site cell preparation despite reasonable measures taken to alleviate this during the isolation protocol. It validates the expectation that isolation of single cell suspensions from these injury sites has a high risk of variability and therefore may not be fully representative of all cells within the tissue. Cells will be lost during the manual handling stages, incomplete digestion and/or restriction of analysis to flow cytometry events meeting single cell criteria. While this experimental strategy meets field norms, these limitations should be more clearly acknowledged. What 'between sample' quality control was undertaken?

Response: The cellular debris observed in the FACS plots does not affect the conclusions drawn from our experiments, as they are always excluded in the gating strategy. Furthermore, we ensured that samples with very poor viability (<30%) were not included in any analyses.

It is common that such ex vivo strategies are accompanied by complimentary in situ approaches. Therefore, my recommendation is that the conclusions of the study would be greatly strengthened by provision of accompanying in situ analysis to:

a) validate Treg cell in vivo depletion
b) verify efficiency of retention of exogenous T regs within injury sites
c) validate monocyte/macrophage in vivo depletion. The latter is particularly critical given monocyte/macrophage depletion alone has been demonstrated to impair healing in similar injury models to those used herein. Consequently, the fact that the monocyte/macrophage depletion alone has minimal impact on healing outcomes as presented in Figure 3 is a major concern (noting that accurate comparison was impaired as PBS liposome control experimental groups, which were undertaken according to the Methods, were not presented in the paper). Additionally,

accompanying detailed in situ analysis would provide richer information relating to the mechanism of action of Treg cell therapy as it would allow assessment of the associated with other cells during the dynamic and complex mechanism of tissue repair.

Response: To provide additional evidence for endogenous Treg depletion, exogenous Treg retention and endogenous monocyte/macrophage (Mo/MΦ) depletion, we performed *in situ* validation through immunostaining of injured muscle and skin tissue cryosections. We were unable to perform the same for bone due to technical difficulties encountered in cryosectioning of injured calvarial bone. As such, we used only skin and muscle injured tissues as examples to validate the following:

a) Treg depletion: For *Foxp3*$^{DTR/GFP}$ and wildtype mice administered with diphtheria toxin (DT) as per our standard protocol, we harvested injured muscle and skin on D7 post-injury, which represents the peak of Treg accumulation in tissues, to stain for Foxp3. Consistent with our flow cytometry data, immunostaining of tissues from wildtype + DT mice showed clear Foxp3^{+} Tregs, while the sections from *Foxp3*$^{DTR/GFP}$ + DT mice showed an absence of Foxp3^{+} cells, in both skin and muscle, thus validating the efficacy of Treg depletion (**Supplementary Fig. 1f**).

b) Treg retention: For wild-type mice administered with exogenous RFP^{+} Tregs via fibrin gels, we harvested injured muscle and skin on D3 post-Treg delivery, which represents the time point at which we performed RNA-seq on the recovered Tregs, and directly examined the expression of RFP within the tissue cryosections. We now show that Tregs administered through fibrin gels mostly stay within the hydrogel, with some infiltration into the injured tissue (**Supplementary Fig. 5c**).

c) Mo/MΦ depletion using clodronate liposomes: For wildtype mice administered with clodronate or control (PBS) liposomes as per our standard protocol, we harvested injured muscle on D4 post-injury, which represents the peak of Mo/MΦ accumulation, to stain for the macrophage marker F4/80. Consistent with flow cytometry analysis of Mo/MΦ depletion, immunostaining of muscle and skin tissues from PBS liposome-treated mice showed an abundance of F4/80^{+} cells, which were absent in clodronate liposome-treated mice, thus validating the efficacy of Mo/MΦ depletion by this method (**Supplementary Fig. 10c**).

Additionally, we have now included the PBS liposome control group in **Fig. 3**, to enable a direct comparison with clodronate liposome-treated groups. Indeed, Mo/MΦ depletion in clodronate liposome-treated mice does show a trend of slight impairment in wound healing (lower average wound closure), muscle regeneration (higher average fibrotic area and lower muscle area) and bone regeneration (lower average bone volume), albeit not significantly, for the sample numbers tested.

It is established that some of the cell surface markers utilised in the study are sensitive to enzymatic digestion. What is the impact of the tissue preparation approaches employed herein on the integrity

of the cell surface markers analysed and how was this compensated for in gating strategy optimisation?

Response: Indeed, we observed that CD11b, and to a lesser extent, F4/80, were slightly sensitive to enzymatic digestion by collagenase, but we had optimised the type and concentration of collagenase as well as the duration of digestion, prior to performing any experiments. We found that at a concentration of 2 mg/ml, the use of collagenase II for bone and muscle, and collagenase XI for skin, were optimal to preserve CD11b and F4/80 expression, while enabling sufficient tissue digestion to release the majority of cells. Furthermore, we found that performing two sequential digestions of 20 minutes each produced the highest cell viability. We performed all tissue dissociation experiments only after these conditions were optimised. Lastly, all the population gates were based on fluorescence-minus-one (FMO) controls, which were also derived from injured tissues and were all digested and treated in the same way as the samples, thereby improving the accuracy of gating.

In Figure 2e-g data the Authors should clarify the X-axis unit as it is not anticipated that gene expression would fall below zero. I suspect it is likely that the unit is actually a log to the base 2 scale but this hasn't been clearly represented in the data output generated in the Degust web tool.??

Response: The x-axis of Fig. 2e-g depicts the "Average expression", obtained from differential gene expression analysis performed with limma/voom. It denotes the average expression across all samples, in $\log_2$ CPM (counts per million). The CPM values for some of the genes in this dataset are extremely low (<1), especially for healthy spleen Tregs. This yields negative values when performing a $\log_2$ transformation of the average expression.

In extended data Figure 3, to ensure that the gating strategy is capturing all RFP+ Treg cells within the generated tissue cells suspensions, the Authors should assess for detection of RFP outside the initial lymphocyte gate and determine if any of the cell events in the excluded myeloid cell gate are RFP+. This would systematically inform if the experimental strategy is at least capturing all Tregs isolated from the injury sites.

Response: Yes, a small percentage of RFP^+ events (around 1-5%) are indeed detected outside the initial lymphocyte gate, often present within the excluded myeloid cell gate. However, we do not include them in our analysis of recovered Tregs, as these cells have likely been phagocytosed by macrophages, or have adhered to fragmented remnants of macrophages while preparing the tissue for flow cytometric analysis[29]. Thus, it was difficult to determine whether these cells were healthy and represented bona fide Tregs at the time of tissue harvest. As such, we decided to draw conclusions only from cells that showed typical T cell characteristics in flow cytometry.

The legend indicates that panel b and c has been represented differently to the final plot shown in the example gating strategy, which is a bit perplexing given the axis in panel b (Y axis is FSC-A) and c (Y axis is CD4-APC) are not the same, and therefore unlikely represent the same gating approach.

The Authors need to clarify and review their data analysis approach in this experiment (noting use of FCS-A is not a precision approach to gating lymphocytes).

Response: We had previously indicated in the figure legend of extended data Fig. 3 (now **Supplementary Fig. 5**), that the "RFP$^+$ Tregs in (b) were gated as described, on the total CD3+, CD4+ population, and represented as the RFP+ population within all live lymphocytes of each respective tissue." However, we acknowledge that this representation of the data may seem less intuitive and apologise for the confusion. To address this concern, we replaced the original plots in panel (b) with those that match the final plot of the gating strategy in panel (a), showing the exogenous Tregs gated as CD4$^+$, RFP$^+$ cells in the different tissues, across all time points (**Supplementary Fig. 5**).

The bulk sequencing data for both exogenous and endogenous Treg upregulation of Il10, Anxa1, Fgl2, Lgals1 and Lgals3 at injury sites, that are specifically noted by the Authors as genes upregulated in support of influencing macrophage function, should be validated by qPCR in independent samples from each of the injury sites.

Response: In the revised manuscript, we have included RT-qPCR validation of selected genes that were significantly upregulated in endogenous and exogenous Tregs, compared to healthy spleen Tregs (*Areg*, *Il10*, *Anxa1*, *Lgals3* and *Fgl2*) (**Supplementary Fig. 9**) and we now mention it in Results on **page 9, last paragraph**. It should be noted that while RNA-seq data of endogenous Tregs from all tissues (bone, muscle, and skin) showed a significant upregulation of all these genes, RNA-seq data of exogenous Tregs showed a significant upregulation for all these genes only from injured muscle. Thus, we decided to perform this validation for muscle injury only, as an example. Consistent with our RNA-seq results, qPCR analysis showed that the $\log_2$ fold change in expression of these genes in injured muscle was significantly higher compared to the control (spleen Tregs) (**Supplementary Fig. 9**). However, among these genes, we would like to draw the Reviewer's attention to *Il10*, which was one of the few genes commonly upregulated in exogenous Tregs recovered from all three tissues. As such, we validated the expression of this cytokine in exogenous Tregs recovered on D3 post-delivery at the protein level using flow cytometry. Interestingly, exogenous Tregs recovered from all three injured tissues showed a significantly higher expression of IL-10 compared to Tregs before delivery (**Supplementary Fig. 23**). Additionally, we now show that IL-10 was the only Treg-derived factor that led to a significant reduction in the proportion of Ly6C$^+$ (pro-inflammatory) Mo/MΦ using *in vitro* and *ex vivo* Mo/MΦ culture systems, while none of the other factors demonstrated this effect (**Supplementary Fig. 22**). Finally, validating the key role of IL-10 in exogenous Treg-mediated repair and regeneration, the pro-healing capacity of these immune cells is lost when *Il10* is knocked out (**Fig. 6**). Altogether, these findings suggested that while other Treg-derived factors likely play some role in modulating Mo/MΦ, the profound impairment in the pro-healing capacity of *Il10*-deficient Tregs, suggests that exogenous Tregs mainly exerted their effects on Mo/MΦ via IL-10.

The Authors have carefully used the joint terminology of monocyte/macrophage throughout the manuscript based on the fact that their myeloid marker gating strategy does not achieve definitive distinction of these cells. However, it is disappointing that more definitive macrophage makers such as CD169, VCAM-1, and/or MerTK, that should have been relatively easy to include in the staining panel, were not utilised. Can the Authors confirm that the sorted cells indeed include bonafide macrophages by confirming robust detection of macrophage marker genes in the isolated RNA (e.g. Csf1r, Siglec1, Adgre1, Mertk etc)?

Response: We performed Mo/MΦ sorting using standard established markers of CD11b and F4/80 (also known as Adgre1 or Emr1), similar to previously published studies[30, 31, 32]. However, to address the Reviewer's concerns, we performed flow cytometric analysis to determine the expression level of CD115/ Csf-1r, Siglec-1/ CD169, VCAM-1 and Mertk, within our CD11b$^+$, F4/80$^+$ Mo/MΦ population (Fig. 2 below) in injured bone, muscle and skin tissues. We found that all these markers were expressed within the CD11b$^+$, F4/80$^+$ Mo/MΦ, albeit at different levels, suggesting that these markers likely captured only a subset of Mo/MΦ within the CD11b$^+$, F4/80$^+$ population.

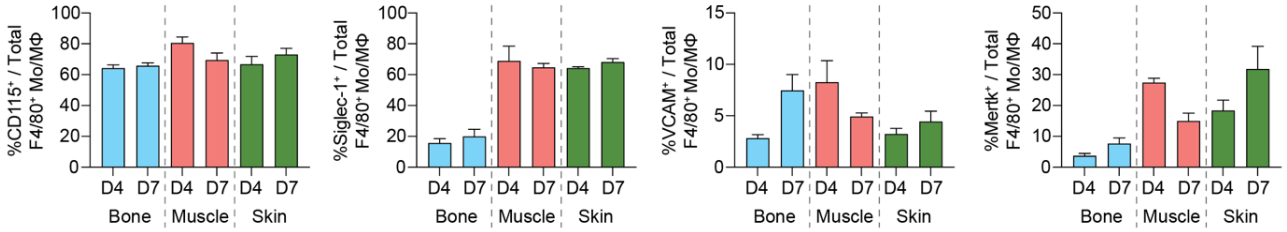

Fig. 2: Expression of CD115, Siglec-1, VCAM-1 and Mertk within the CD11b$^+$, F4/80$^+$ population of Mo/MΦ from injured bone, muscle and skin tissues at D4 and D7 post-injury.

Similar results were observed from our RNA-seq analysis on Mo/MΦ from Treg-treated and Treg-depleted mice, which confirmed that these genes could be clearly detected within the isolated RNA from the sorted populations, but the expression levels varied greatly across different tissues, with *Adgre1* (F4/80) expression being the most consistent between samples (shown in Fig. 3 below for Treg-treated samples as an example).

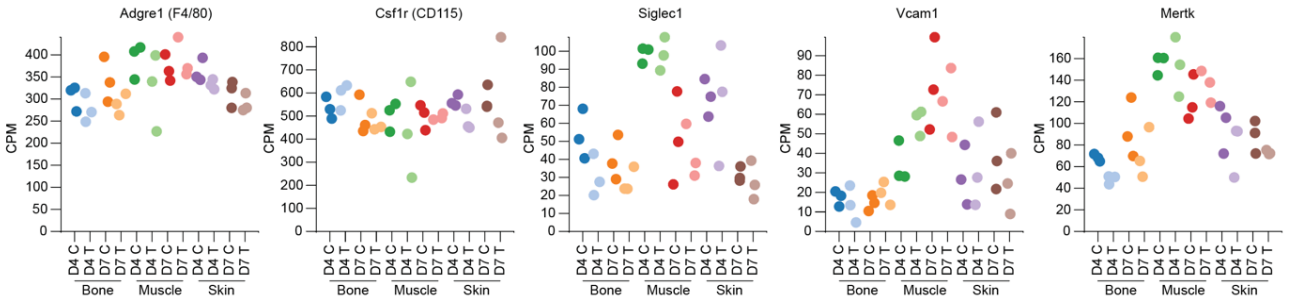

Fig. 3: CPM values of *Adgre1* (F4/80), *Csf1r* (CD115), *Siglec-1*, *Vcam1* and *Mertk* within the RNA-seq data of $CD11b^+$, $F4/80^+$ Mo/MΦ from Treg-treated injured bone, muscle and skin tissues sorted at D4 and D7 post-injury.

Therefore, we believe that sorting of Mo/MΦ as $CD11b^+$, $F4/80^+$ cells provides a robust method to obtain bona fide macrophages for analysis.

With respect to the data presented in Figure 3h-m, as stated in the legend, only a selection of differentially regulated genes are represented. What was the criteria used to determine which genes would be represented? What was the total number of up or down regulated genes within each population and can multi-sample comparisons be undertaken to show the number of these that overlap either within the same injury at different time points or between different injury sites at the same timepoints?

Response: In the revised manuscript, we added this information in **Supplementary Fig. 14**, in the form of Venn diagrams depicting the total number of DEGs, along with the overlap between all significantly upregulated and downregulated DEGs in Mo/MΦ from all three tissues, at both time points (D4 and D7 post-injury). As seen in the figure, there was minimal overlap in the DEGs between two time points of the same tissue, as well as between different tissues for both time points combined. We have now mentioned this more clearly in the Results on **page 12, paragraph 2**. Therefore, the selection of DEGs presented in the heatmaps of **Fig. 4b-d** (previously Fig. 3h-m) was simply based on their known function and role in tissue healing post-injury, according to published literature.

Providing similar analysis for Treg sequencing data would also strengthen the Authors' conclusions and allow readers to more wholistically appreciated difference/similarities between the injury models as well as impact of cell treatment.

Response: In the revised manuscript, we added volcano plots for the exogenous Treg sequencing data, showing global gene expression changes in all three tissues (**Supplementary Fig. 6a-c**). These plots depict differentially expressed genes (DEGs) (using an FDR adjusted p-value < 0.0.5 with fold change > |1.5|) between exogenous spleen Tregs before delivery and exogenous Tregs recovered from injured tissues at D3 post-delivery, with all the significantly upregulated (red) and downregulated (blue) genes in D3 recovered Tregs. The genes that are labelled in these volcano plots correspond to the ones presented in the heatmap in **Fig. 2d**. Furthermore, Venn diagrams are now provided to compare the overlap of upregulated and downregulated DEGs between all three tissues, from which it can be observed that only 32 genes were commonly upregulated in exogenous Tregs recovered from all 3 tissues (**Supplementary Fig. 6d**). These 32 genes are also depicted on a heatmap (**Supplementary Fig. 6e**).

Gene ontogeny results presented in Figure 3l show that genes associated with bone development are upregulated in monocyte/macrophages isolated from Treg-treated muscle injury sites. Heterotopic ossification in muscles is a pathological outcome driven by macrophages in some trauma circumstances. The Authors need to specifically acknowledge that this transcriptional profile exposes a potential risk of use of this therapy for muscle repair in certain clinical settings. The Authors need to also consider and discuss why this GO profile was not apparent in the bone injury model?

Response: The Reviewer has raised an interesting point, however, GO terms are known to provide a structured and controlled set of terminology, based on existing databases and published literature. Thus, they may not necessarily implicate the exact biological process that they map the genes to. In this case, the genes upregulated in Mo/MΦ from Treg-treated muscle, which are mapped to the term "bone development", include *Cadm1*, *Serpinh1*, *Acp5*, *Gpr68* and *Src* (**Supplementary Fig. 15**). Although these genes may have been generally associated with bone development, thus explaining the GO term annotation, there are some publications that have linked their expression to anti-inflammatory effects in tissues. For instance, *Cadm1* expression has previously been reported on Trem2^{+} macrophages[33], which have been shown to contribute to cardiac repair in infarcted hearts[34]. Similarly, *Gpr68* (*Ogr1*) expression has been associated with anti-fibrotic effects in the lung[35], while upregulation of *Serpinh1*, which encodes Hsp47, has been reported in regenerating muscle fibres during cellular response to injury[36]. Thus, we speculate that the expression of these genes in Mo/MΦ from Treg-treated injured muscle is associated with the anti-inflammatory status of these cells and does not necessarily suggest heterotopic ossification.

I would argue that the Authors have overextended the interpretation of the somewhat conservative increase of human Treg cells compared to exogenous syngeneic or allogenic mouse Treg cell recovery from injury sites as evidence of human Treg improved stability. They would in the very least need to perform comparison with ex vivo expansion of murine Tregs to affirm this interpretation.

Response: As suggested, to determine whether the improved recovery of human Tregs was due to their prior *in vitro* culture, we performed a parallel Treg delivery experiment with sorted mouse Tregs that were cultured for 1 week *in vitro*, prior to *in vivo* delivery. Indeed, we observed that *in vitro* expanded murine Tregs showed a remarkably higher recovery, with an average retention of 80% on D5 post-delivery (**Supplementary Fig. 25**). This suggested that *in vitro* expansion of Tregs may have conferred upon them improved stability and survivability, following *in vivo* administration[37]. We now discuss these points on **page 21, paragraph 1**, while its implications for future Treg-based therapies have been discussed on **page 26, last paragraph**.

With respect to data presented in Extended Figure 8b. Graph shows %F4/80 low/total F4/80+ cells at two time points in the 3 injury models. Can the Authors please clarify is the total F4/80+ cell frequency also varied between the healing time points analysed.

Response: The data that was in Extended Fig. 8b is now in **Supplementary Fig. 16b**. Yes indeed, the total Mo/MΦ are indeed significantly higher in Treg-depleted tissues compared to controls, and we now include this data in **Supplementary Fig. 16a** and mention it in Results on **page 15, paragraph 1**.

Response to Reviewer #3:

In this manuscript, Nayer et al provided robust experimental evidence in 3 distinct mouse models of acute tissue injuries that locally administered Tregs enhance tissue healing (Fig 1). Even more impressive was the finding that the tissue healing activities were intact across allogeneic and xenogeneic barriers (Fig 4). While the tissue repair function of Tregs has been well-established in the literature, local administration of Tregs and the use of allogeneic and xenogeneic Tregs in this context are novel contributions to the field. The authors attempted to determine the mechanistic basis of the tissue healing function of Tregs by gene expression profiling of the administered Tregs recovered from the sites of injury and by gene expression profiling of tissue macrophages with and without Treg treatment.

While results from these experiments provide some clues, they fall short of establishing the roles of Treg-expressed genes in the tissue repair function of Tregs. For example, the authors stated in the abstract that "Tregs exert their regenerative effect .... injured tissues ... via factors such as interleukin-10". This statement is not supported by experimental evidence. While IL-10 gene expression was upregulated in Tregs in all 3 models, the evidence for IL-10 involvement in tissue repair is correlative. Would IL-10KO mice have more severe tissue injury as seen in Treg-depleted mice? Would Tregs from IL-10KO mice fail to promote tissue repair? Would IL-10-producing Tr1 cells have a similar function in tissue repair?

Response: We agree that further validation of the role of Treg-derived factors such as IL-10 would strengthen our conclusions and provide deeper mechanistic insights. Thus, as suggested, we decided to deliver Tregs from IL-10 knockout mice and test their ability to promote tissue healing in all our injury models. For this, we sorted $CD4^+$, $CD25^+$ Tregs from IL-10 knockout mice ($Tregs^{Il10-/-}$) and delivered them into bone, muscle and skin injuries via fibrin hydrogel (**Fig. 6**), as previously done for normal Treg delivery (Fig. 1). Interestingly, we observed that *Il10*-deficient Tregs were unable to promote bone, muscle and skin healing post-injury, thereby highlighting the key role of this cytokine in mediating the pro-healing effects of exogenous Tregs. We now include this data in **Fig. 6**, along with a new sub-section in Results **(page 18**), and discussed this point on **page 26, paragraph 2**.

Another conclusion that the authors proposed in the abstract is that the administered Tregs "rapidly adopt an injury-specific phenotype in response to the damaged tissue microenvironment". This conclusion is primarily based on a comparison of Treg RNAseq data before and after applying it to the wounded tissue. Without performing a similar analysis of Tregs applied to non-injured tissue or lymphoid tissue using a fibrin scaffold, it is hard to know if the changes were injury-specific or represented an adaptation to a non-lymphoid tissue environment. For example, IL-10 is made by activated Tregs in lymphoid and nonlymphoid tissues, and Nr4a1 expression is induced by TCR engagements in Tregs and conventional T cells in lymphoid and non-lymphoid tissues. Thus,

expression of these genes doesn't necessarily mean the cells are adapting to a nonlymphoid tissue environment.

Response: To address the point raised by the Reviewer, we performed a new experiment in which we delivered Tregs to uninjured tissue by implanting a fibrin hydrogel subcutaneously. The implant was placed through a small pocket formed in the subcutaneous space by blunt dissection to accommodate the fibrin hydrogel. This technique has been described in a new sub-section of Methods, on **page 32, paragraph 2**. We found that exogenous Tregs recovered from uninjured tissue on D3 post-delivery did not show a significant increase in IL-10 expression, compared to Tregs before delivery, measured by flow cytometric analysis of the percentage of IL-10^{+} Tregs and IL-10 mean fluorescence intensity (MFI). This contrasted with exogenous Tregs recovered from all three injured tissues (bone, muscle, and skin), which showed a significantly higher expression of IL-10 compared to Tregs before delivery. These results are now included in **Supplementary Fig. 23** and discussed on **page 18, last paragraph**. This finding demonstrated that the increased IL-10 expression was truly reflective of exogenous Treg adaptation to the damaged microenvironment, rather than a mere response to a non-lymphoid tissue environment.

In addition to these non-injury controls, it would be nice to also compare the rate of wound healing in mice treated with fibrin-embedded CD4 conventional cells.

Response: As suggested by the Reviewer, we assessed the ability of CD4^{+} conventional T cells (Tconvs) to promote tissue healing in all our injury models. For this, we sorted Tconvs as CD4^{+}GFP^{-} T cells from *Foxp3*$^{DTR/GFP}$ mice and delivered them into bone, muscle and skin injuries via a fibrin hydrogel (**Supplementary Fig. 4),** as previously done for normal Treg delivery (Fig. 1). Our results showed that Tconvs were unable to promote tissue healing post-injury, thus emphasising the unique potential of Tregs as an effective immune cell therapeutic for regenerative medicine applications. We have included this data in Supplementary Fig. 4, mentioned this in the Results on **page 5, paragraph 1**, and discussed this point on **page 23, paragraph 1**.

The authors state that "it is worth noting that our observations of a positive regenerative outcome following allogeneic and xenogeneic Treg delivery suggest a significant contribution of TCR stimulation-independent mechanisms" (lines 428-430). TCRs expressed by Tregs should be able to respond to allogeneic and xenogeneic MHCs. Thus, it may be argued that they are more likely to be activated via their TCR than in the syngeneic setting.

Response: Indeed, TCRs expressed by Tregs can respond to allogeneic and xenogeneic MHCs, which suggests a potential for activation via their TCR in these settings. As such, we acknowledge that additional experiments specifically designed to dissect the relative contributions of TCR-dependent and independent pathways in exogenous Treg-mediated regeneration are required to test this. However, this may be beyond the scope of our study. Thus, we have now removed this claim from our discussion.

The authors isolated Tregs using FACS purification after staining the cells with antibodies to CD4 and CD3 along with a fluorescent Foxp3 reporter. It should be noted that the CD3 antibody used for isolating the Tregs could remain on the Treg surface and activate the Tregs, especially in the presence of FcR-expressing myeloid cells in the tissue. The use of anti-CD3 is not needed to isolate highly pure Tregs.

Response: We apologise as this was a mistake from our end. We agree with the Reviewer that anti-CD3 is not needed for Treg isolation by FACS. Indeed, when we sorted Tregs from spleens on the BD Influx Cell Sorter or the BD FACSAria Fusion flow cytometer for fibrin-mediated *in vivo* cell delivery, we only used mouse anti-CD4 antibody, along with GFP or RFP expression, to obtain highly pure Tregs. This is now reflected in the updated gating strategies shown in **Supplementary Fig. 2**, which have been corrected to show that Tregs were gated only on $CD4^+$ T cells. The anti-CD3 antibody was only used when we analysed Treg populations within injured tissues (bone, muscle, or skin), on the BD LSR Fortessa X-20 flow cytometer, to reliably gate pure Tregs in immune cell kinetic experiments. We had mistakenly added that into the sorting strategy figure as well. Nevertheless, we would like to clarify that we used the 17A2 clone of the anti-mouse CD3 antibody, which is considered a non-activating clone, while the 145-2C11 clone of anti-mouse CD3 antibody is known to cause T cell activation[38, 39]. Thus, to our understanding, staining the Tregs with anti-CD3 (clone 17A2) would not have impacted their activation status in any case.

For the human Tregs, a more detailed method of Treg purification (FACS or MACS? What markers?) and expansion (how were the cells stimulated? How long was the expansion?) The reference provided (#76) did not provide this important information.

Response: For human Treg purification and expansion, $CD4^+CD25^{hi}FOXP3^+$ were isolated by negative enrichment of $CD4^+$ T cells (RosetteSep, STEMCELL Technologies) followed by $CD25^{hi}$ positive selection (RoboSep; STEMCELL Technologies). The isolated Tregs were activated and expanded *in vitro* for 1-2 weeks using Dynabeads Human CD3/CD28 T-cell expander beads (Thermo Fisher Scientific), cultured in RPMI 1640 with 10% heat-inactivated FBS. The expansion was supported by addition of high-dose IL-2 (500 IU/ mL, Roche) and 100 nM rapamycin (Sigma-Aldrich) in the culture medium. We now include these details in the revised manuscript under the Methods section (**page 32, paragraph 3**).

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed my comments except for #6. It is not true that Tregs are required only at the beginning of the skeletal muscle response to acute injury. Their ref #23 refers only to Treg control of the IFNγ response. It is necessary to shut down the inflammatory response to have effective muscle regeneration, which is accomplished by one class of Tregs that peaks early. But effective muscle regeneration also requires growth factors such as Areg, which are provided by a different class of Tregs with a reparative transcriptional program that don't peak until later – see Kuswanto et al Immunity 2016; Hanna et al Immunity 2023.
I guess I can let it go if the authors state this caveat.

Reviewer #2 (Remarks to the Author):

The revised version of Nayer et. al. reporting proof-of-principal that exogenous Treg cell therapy delivered at injury sites can enhance tissue regeneration in models of bone, muscle, and skin healing has been much improved. The Authors have provided new data or clarifying argument/information to sufficiently address most of the Reviewers' concerns. Some of this new data has raised new considerations that, if implemented, would further improve the manuscript.
Additional points for consideration:
The new experiments showing that IL-10 deficient Tregs almost completely abrogates the beneficial effects of this therapeutic strategy. This raised the question of with IL-10 delivery in fibrin hydrogel would be sufficient to promote healing. Delivery of a recombinant cytokine product would presumably present a therapeutic with fewer barriers to clinical translation and be more cost effective. The Authors should consider undertaking this additional experiment or at least discuss this as an alternative strategy worth exploring.
I note in Fig. 1f, particularly at D7 post injury, there is a subgroup of mice in the Treg treated group for which healing seems to progress at a similar rate to the control group, whereas another subgroup has a robust response to therapy. Is there any local healing response characteristics that differentiates between these extremes of therapy outcome in the skin wound repair model?
Supplemental Fig 2 seems to be missing reference to panel (c) in the legend.
In Line 86, I suggest the appropriate figure panels to be referred to are Supplementary Fig. 1g-l as panel f relates to validation of cell depletion versus healing outcomes.

Reviewer #3 (Remarks to the Author):

The authors have addressed the critiques I raised previously. I have only two minor suggestions:

1. The new IL-10KO data is nice. However, the section title (Tregs primarily exert their therapeutic effect via IL-10 expression)and Figure 6 caption (IL-10 is the main Treg-derived factor that promotes repair and regeneration)are not exactly accurate. The data demonstrate IL10 dependency but not its sufficiency implied by the title and caption. It would be more accurate to state that Treg acceleration of tissue repair depended on their IL-10 production.

2. Thanks for adding the human Treg isolation and expansion protocol. The purity of Tregs using MACS is typically subpar, although the use of rapamycin in the expansion may help to ensure the use of high-purity Tregs. Please add a panel to show the purity of human Tregs, by FOXP3 and HELIOS staining and/or TSDR analyses, prior to injection.

Response to Reviewer #1:

The authors have adequately addressed my comments except for #6. It is not true that Tregs are required only at the beginning of the skeletal muscle response to acute injury. Their ref #23 refers only to Treg control of the IFNγ response. It is necessary to shut down the inflammatory response to have effective muscle regeneration, which is accomplished by one class of Tregs that peaks early. But effective muscle regeneration also requires growth factors such as Areg, which are provided by a different class of Tregs with a reparative transcriptional program that don't peak until later – see Kuswanto et al Immunity 2016; Hanna et al Immunity 2023. I guess I can let it go if the authors state this caveat.

Response: We agree that Treg presence is crucial during both early and late stages of tissue regeneration. Similar to previous reports[1, 2], our data suggest that endogenous Tregs naturally peak about a week post-injury, and we expect that they likely accumulate in sufficient numbers by then to accomplish the reparative activities. However, our exogenous Treg administration strategy soon after injury augments their numbers in the injured site beyond what would naturally accumulate during the first few days. This rationale, along with studies that have shown improved cardiac repair upon delivery of exogenous Tregs either immediately or one-day post-myocardial infarction[3, 4], underlies our expectation that early exogenous Treg administration may likely provide the maximum benefit as a therapeutic strategy for tissue healing. Nevertheless, we did mention on page 19 of the manuscript that future research is required to determine the maximum possible timeframe within which we can administer Tregs to damaged tissue to effectively facilitate healing. Additionally, we now also include a statement on page 19 to acknowledge the importance of Tregs at a later stage of tissue healing due to their ability to express growth factors such as AREG which can directly stimulate progenitor cell proliferation[2, 5]. Specifically, we mention that it would be interesting to test whether exogenous Treg administration at a more advanced stage of healing can provide additional regenerative benefit, perhaps by supplementing the activities of endogenous Tregs that naturally accumulate at later time points post-injury.

Response to Reviewer #2:

The revised version of Nayer et. al. reporting proof-of-principal that exogenous Treg cell therapy delivered at injury sites can enhance tissue regeneration in models of bone, muscle, and skin healing has been much improved. The Authors have provided new data or clarifying argument/information to sufficiently address most of the Reviewers' concerns. Some of this new data has raised new considerations that, if implemented, would further improve the manuscript.

Additional points for consideration:
The new experiments showing that IL-10 deficient Tregs almost completely abrogates the beneficial effects of this therapeutic strategy. This raised the question of with IL-10 delivery in fibrin hydrogel would be sufficient to promote healing. Delivery of a recombinant cytokine product would presumably present a therapeutic with fewer barriers to clinical translation and be more cost effective. The Authors should consider undertaking this additional experiment or at least discuss this as an alternative strategy worth exploring.

Response: We agree with the Reviewer's suggestion and indeed previous research has demonstrated that IL-10 overexpression or sustained administration can improve cutaneous wound healing[6] and muscle regeneration[7], respectively. In fact, we are currently exploring similar approaches with our mouse injury models and if successful, they may contribute to potential future publications. However, to address the Reviewer's comment, we now discuss this point about exploring the direct use of the recombinant cytokine IL-10 as an alternative strategy to promote tissue healing on page 19 of the manuscript.

I note in Fig. 1f, particularly at D7 post injury, there is a subgroup of mice in the Treg treated group for which healing seems to progress at a similar rate to the control group, whereas another subgroup has a robust response to therapy. Is there any local healing response characteristics that differentiates between these extremes of therapy outcome in the skin wound repair model?

Response: Indeed, wound closure in the excisional full-thickness skin-punch biopsy model can be quite variable, which likely led to the relatively wide distribution observed in our data. Despite it being a gold standard method for evaluating skin wound healing[8], the nature of the model is such that even slight differences can cause substantial intra-mouse and inter-group variability[9]. To account for this, we have included a sufficient number of biological replicates (n=8 to 12) for our analysis.

Supplemental Fig 2 seems to be missing reference to panel (c) in the legend. In Line 86, I suggest the appropriate figure panels to be referred to are Supplementary Fig. 1g-l as panel f relates to validation of cell depletion versus healing outcomes.

Response: We have now added panel (c) in the legend for Supplementary Figure 2 and have made the necessary correction in the figure panels referred to in line 86 (by changing it to Supplementary Fig. 1g-l).

Response to Reviewer #3:

The authors have addressed the critiques I raised previously. I have only two minor suggestions:
1. The new IL-10KO data is nice. However, the section title (Tregs primarily exert their therapeutic effect via IL-10 expression)and Figure 6 caption (IL-10 is the main Treg-derived factor that promotes repair and regeneration)are not exactly accurate. The data demonstrate IL10 dependency but not its sufficiency implied by the title and caption. It would be more accurate to state that Treg acceleration of tissue repair depended on their IL-10 production.

Response: We understand the Reviewer's concern regarding the accuracy of wording used in the title and figure caption of the section incorporating the IL-10 knockout Treg data. To address this, we have now changed the title of that section to "The therapeutic effect of exogenous Tregs depends on IL-10 expression" and changed the caption of Figure 6 to "Treg-mediated acceleration of tissue healing depends on their production of IL-10".

2. Thanks for adding the human Treg isolation and expansion protocol. The purity of Tregs using MACS is typically subpar, although the use of rapamycin in the expansion may help to ensure the use of high-purity Tregs. Please add a panel to show the purity of human Tregs, by FOXP3 and HELIOS staining and/or TSDR analyses, prior to injection.

Response: The human $CD4^{+}CD25^{hi}FOXP3^{+}$ Tregs were isolated and cryopreserved during experiments performed for previous publications, wherein 95% of expanded Tregs were reported to express high levels of FOXP3, which was also maintained after cryopreservation and thawing[10, 11]. However, as suggested by the Reviewer, we now include data showing expression of FOXP3 (>98%) and HELIOS (>95%) in these human Tregs by flow cytometry (Supplementary Figure 24a), thus confirming their purity.

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