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Interleukin-10 Producing T Lymphocytes Attenuate Dermal Scarring

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Objective: Demonstrate the impact of IL-10 producing T lymphocytes on mediating dermal scarring.

Summary Background Data: We demonstrated that $CD4^+$ cells are essential to improving postinjury wound healing and preventing fibrosis. $CD4^+$ subsets secrete differential cytokine and growth factor profiles, though their role in fibrosis is not known. IL-10, a key anti-inflammatory cytokine shown to promote regenerative wound healing, is secreted by some $CD4^+$ subsets. We, therefore, hypothesize that IL-10 producing $CD4^+$ T lymphocyte subsets selectively attenuate dermal wound fibrosis.

Methods: IL-$10^{-/-}$ and wild-type murine splenocytes were enriched for $CD4^+$ lymphocytes and adoptively transferred into severe combined immunodeficient (SCID) mice that received full-thickness wounds which were analyzed at days 7 and 28 for inflammation and collagen content. We then sorted $CD4^+CD44^{int/low}FoxP3^-CD62L^+$ T cells (Tnaive) or $CD4^+CD44^{Hi}FoxP3^-$ type 1 regulatory (Tr1) T cell subsets from 10BiT murine splenocytes, activated them, and transferred them into wounds. *In vitro*, dermal fibroblasts were cocultured with Tnaive or Tr1 and the effect on extracellular matrix (ECM) regulation was analyzed.

Results: The anti-inflammatory and antifibrotic effects of $CD4^+$ cells on SCID wounds were lost with cells from IL-$10^{-/-}$ mice. Adoptive transfer of Tr1 into SCID mice resulted in accelerated wound closure at d7 with reduced fibrosis at d28, with Tr1 favoring hyaluronan production by fibroblasts, an ECM molecule implicated in IL-10-induced regenerative healing.

Conclusions: IL-10 producing T-lymphocytes, specifically Tr1, regulate inflammatory cell cytokine expression to promote HA-rich ECM deposition and attenuate fibrosis. Promoting IL-10 producing lymphocytes in wounds may be a therapeutic target to promote regenerative wound healing.

Keywords: Cutaneous, fibrosis, immunity, inflammation, lymphocytes, scarring, wound healing

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Postnatal healing of dermal wounds in most mammals leads to scar formation, and involves innate and adaptive inflammatory immune responses in concert with dermal fibroblasts and keratinocyte activation to optimize wound healing, prevent pathogen invasion, and improve cell survival and function. In contrast, midgestational fetal wounds heal regeneratively without scar formation, with reconstitution of dermal appendages and neurovasculature, which is dependent upon interleukin (IL)-10 induction of a hyaluronan (HA) rich wound microenvironment.[1–4] Notably, therapeutic interventions which promote mechanisms that closely mirror fetal-like wound healing without pathogenic scarring remain elusive, despite the significant understanding of the cell and molecular players engaged in the process. Studies on dermal fibrosis not only lead to advances that reduce the psychosocial impact of scarring and morbidity of burn contractures, but also implement research models to investigate mechanisms of scarring that translate into innovative treatments of fibrosis in other organ systems.[5]

The influence of various immune cells on wound healing and scarring has been heavily studied, including early mast cell activation and neutrophil and macrophage infiltration. Recently, lymphocytes have been highlighted as important cells that regulate wound healing responses.[6–8] We demonstrated a role for lymphocytes, specifically $CD4^+$ lymphocytes, in regulation of dermal fibrosis in the severe combined immune deficient (SCID) mouse model.[9] Of the $CD4^+$ subtypes, two immunoregulatory subsets stand out as producers of IL-10, $CD4^+Foxp3^+$ regulatory T cells (Treg) which also produce transforming growth factor (TGF)-β and $CD4^+Foxp3^-CD49b^+Lag3^+$ type 1 regulatory T cells (Tr1) which additionally produce TGF-β, IFN-γ, and low levels of IL-4.[10] Macrophage transition from an inflammatory (M1) to a reparative (M2) phenotype assists in propagation of the wound healing phases, with M2 macrophage subtypes playing a greater role in reducing collagen deposition and scarring.[11–13] IL-10 levels in the wound microenvironment contribute to the anti-inflammatory M2 macrophage polarization and function, which further propagates M2 mediated IL-10 expression in the local milieu.[14] IL-10 is also well established to induce a postnatal regenerative wound healing phenotype with scarless dermal architecture.[15–25] Besides its well-established role as an anti-inflammatory mediator, IL-10 has been shown to signal via STAT3 to induce HA synthesis, which plays key molecular weight dependent functions in wound healing.[23]

High molecular weight-HA (HMW-HA) is defined as being >1000 kDa, whereas low molecular weight-HA (LMW-HA) consists of degraded HA <500 kDa.[26] Post wounding tissue damage upregulates both hyaluronan synthase (HAS) and hyaluronidase (HYAL) expression, leading to a greater turnover of HA that yields

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proinflammatory LMW-HA fragments, which contrast the regenerative anti-inflammatory and anti-fibrotic properties of HMW-HA isoforms.[3, 26,27] Therefore, an imperative unmet clinical need to achieve scarless wound repair involves a conceivable strategy to reprogram immune responses to increase in situ IL-10 secretion, inducing HMW-HA synthesis, and remodeling fibroblast HA extracellular matrix (ECM) deposition.

We recently reported that adoptive transfer of total unfractionated $CD4^+$ T cells into SCID mice, that lack functional T- and B cells, reduce inflammation and collagen deposition.[9] In view of the known contribution of IL-10 to regenerative wound healing, we hypothesize that IL-10 producing $CD4^+$ lymphocyte subsets produce the beneficial role that we have shown $CD4^+$ cells to have on wound healing. Here, we first demonstrate that IL-10 capacity is essential for $CD4^+$ lymphocytes to exert their function on fibrosis, as unfractionated $CD4^+$ cells from IL-10 deficient mice were unable to reduce postwounding fibrosis in SCID mice. Using lymphocytes isolated from 10BiT mice, with reporter genes for GFP and CD90.1 to respectively confirm FoxP3 and IL-10 expression, we are able to further support that individual IL-10 competent $CD4^+$ T cell subsets promote HA production by fibroblasts *in vitro*, with their individual beneficial effects on wound healing demonstrated in adoptive transfer experiments, with Tr1 promoting the most antifibrotic phenotype.[28]

METHODS

Please refer to supplemental materials, http://links.lww.com/SLA/D189.

RESULTS

Increased Inflammation and Collagen Deposition in SCID Mice Treated With IL-$10^{-/-}$ $CD4^+$ Cells

To confirm if IL-10 production by $CD4^+$ cells is essential for its effects on scarring, we adoptively transferred WT or IL$10^{-/-}$ $CD4^+$ cells to SCID mice at time of wounding. We studied the inflammatory infiltration of macrophages and the differentiation and migration of myofibroblasts in wounds 7 days post wounding (Fig. 1A, B). In comparison to mice receiving IL-$10^{-/-}$ $CD4^+$ (2.1 ± 0.5 versus 45.8 ± 11.5 positive F4/80 %/HPF, $P < 0.01$), WT $CD4^+$ cells-treated mice showed a significant reduction of macrophage content. There was no difference in myofibroblast content in wounds of mice treated with WT $CD4^+$ cells vs IL-$10^{-/-}$ cells (37.0 ± 16.7 versus 42.3 ± 14.3 positive αSMA %/HPF, $P = 0.62$). Quantification of Masson trichrome staining of day 28 wounds showed that the decrease in collagen content in mice treated with WT $CD4^+$ cells was lost when mice were treated with IL-$10^{-/-}$ $CD4^+$ cells (135.44 ± 29.1vs 410.98 ± 122.10 pixel/HPF, $P < 0.01$)

To validate our hypothesis *in vitro*, we harvested media from activated WT and IL-$10^{-/-}$ total lymphocytes or unfractionated $CD4^+$ T-cells and used this media to incubate WT fibroblasts, measuring mRNA 24 hours later (Fig. 1C). Fibrosis RT2 PCR profile arrays revealed that in comparison to IL-$10^{-/-}$ lymphocytes-treated fibroblasts, inflammation markers such as TNF, CCL3, TGF-β1 and TGF-β2 were reduced in WT lymphocytes-treated fibroblasts, particularly in $CD4^+$ subsets. Moreover, we found that in contrast to IL-$10^{-/-}$ lymphocyte-treated fibroblasts, the ECM remodeling enzyme, MMP9 was similarly reduced in WT T lymphocytes treated fibroblasts. Our results suggest that *in vivo* and *in vitro* IL-10 deficiency can lead to increased inflammation and pathologic remodeling.

Characterization of Culture-expanded Tnaive and Tr1 Cells

To examine the specific roles of IL-10 producing $CD4^+$ lymphocyte subsets in wound healing, effective isolation, differentiation, expansion, and lineage maintenance of the subsets are critical to this study. We isolated $CD4^+CD44^{int/low}$-$FoxP3^-CD62L^+$ T cells (Tnaive) and $CD4^+CD44^{Hi}FoxP3^-$ Tr1 precursors from 10BiT mouse splenocytes using fluorescence-activated cell sorting (FACS) (Supplemental Figure 1, http://links.lww.com/SLA/D189), and activated the cells for 6 days. We then performed flow cytometry, gating for live singlet lymphocytes, to analyze IL-10 production by CD90.1, surface markers CD49b and LAG3, and FoxP3 expression by GFP. As depicted in Figure 2, our results show that activated Tr1 precursors express higher production of IL-10 by increased CD90.1 versus Tnaive (47.9 ± 24.05 vs 1.18 ± 1.1%positive cells, $P < 0.01$). To further confirm successful enrichment of Tr1 from precursors, flow cytometry analysis of surface CD49b and LAG3 was conducted, which revealed characteristic coexpression by Tr1.[10] Specifically, a mean of 41.88% of cells gated for live singlet lymphocytes out of the Tr1 culture coexpressed CD49b and LAG3 while the proportion of $Foxp3^+$ cells, marked by GFP fluorescence in 10BiT mice, was negligible in both Tr1 and Tnaive, substantiating that these IL-10 producing cells are not polarized $Foxp3^+$ Tregs (Fig. 2A–C). These collective data support the effective Tr1 polarization of 10BiT mouse-derived Tr1 precursors with a significant proportion of them being IL-10 producers.

Coculture of Dermal Fibroblasts With Tr1 Cells Increases HAS and Downregulates Ctgf

To test if the polarized Tr1 produces functional IL-10, we quantified IL-10 and performed trans-well Tr1/fibroblasts coculture experiments and assessed the effects on HA synthesis and degradation. IL-10 production by Tr1 cells was measured by ELISA using coculture media after 24 hours of treatment (Fig. 3A). Because we have previously reported that IL-10 induces *in vivo* and *in vitro* HA production, we determined the expression of HA synthases (*Has)1, 2,* and *3* (Fig. 3B). We observed that in comparison to fibroblasts cocultured with Tnaive or untreated controls, *Has1* expression by fibroblasts exposed to Tr1 was significantly increased (2.76 ± 0.83 $P < 0.001$; 1.189 ± 0.77 $P < 0.0001$; 15.06 ± 1.99 fold expression). Has2 expression was decreased in fibroblasts cocultured with Tr1 in comparison to untreated fibroblasts (0.26 ± 0.04 vs 1.04 ± 0.34 fold expression, $P < 0.001$) or exposure to Tnaive (0.638 ± 0.26 fold expression, $P < 0.05$) while *Has3* expression was virtually unaltered (Fig. 3C). Expression of major HA degradation enzyme *Hyal1* decreased in fibroblasts cocultured with Tr1 in comparison to untreated ($0.43 \pm 0.0.17$ vs 1.02 ± 0.23 fold expression, $P < 0.01$) and when compared to Tnaive (0.81 ± 0.095 fold expression, $P < 0.05$). A similar decrease was seen in *Kiaa1199* expression (0.13 ± 0.05 vs 1.02 ± 0.23, $P < 0.0001$ vs 0.773 ± 0.39 fold expression, $P < 0.001$), the latter of which is predominantly active in dermal fibroblasts.[29,30] At the same time, Figure 3C displays a qualitative increase in pericellular HABP staining of fibroblasts cocultured with Tr1, though HA levels in supernatant of treated fibroblasts did not reflect the increase 48 hours post-treatment (Fig. 3D). Although fibroblasts exposed to Tr1 cocultures did not reveal a significant reduction in collagen I, collagen III, or αSMA gene expression, we noted a significant reduction of *Ctgf* expression, a potent fibrogenic ECM mediator of myofibroblast transition, thus underscoring the dermal regenerative potential of Tr1 exposure (Fig. 3E). Although the exposure of fibroblasts to Tr1 did result in a relative decrease in *αSMA* protein levels after 48 hours, the change did not reach statistical significance (Fig. 3F, G). These collective findings highlight a physiological balance to regulate HA synthesis and degradation to promote an anti-inflammatory/regenerative HMW-HA over a proinflammatory/fibrotic LMW-HA ECM by Tr1.

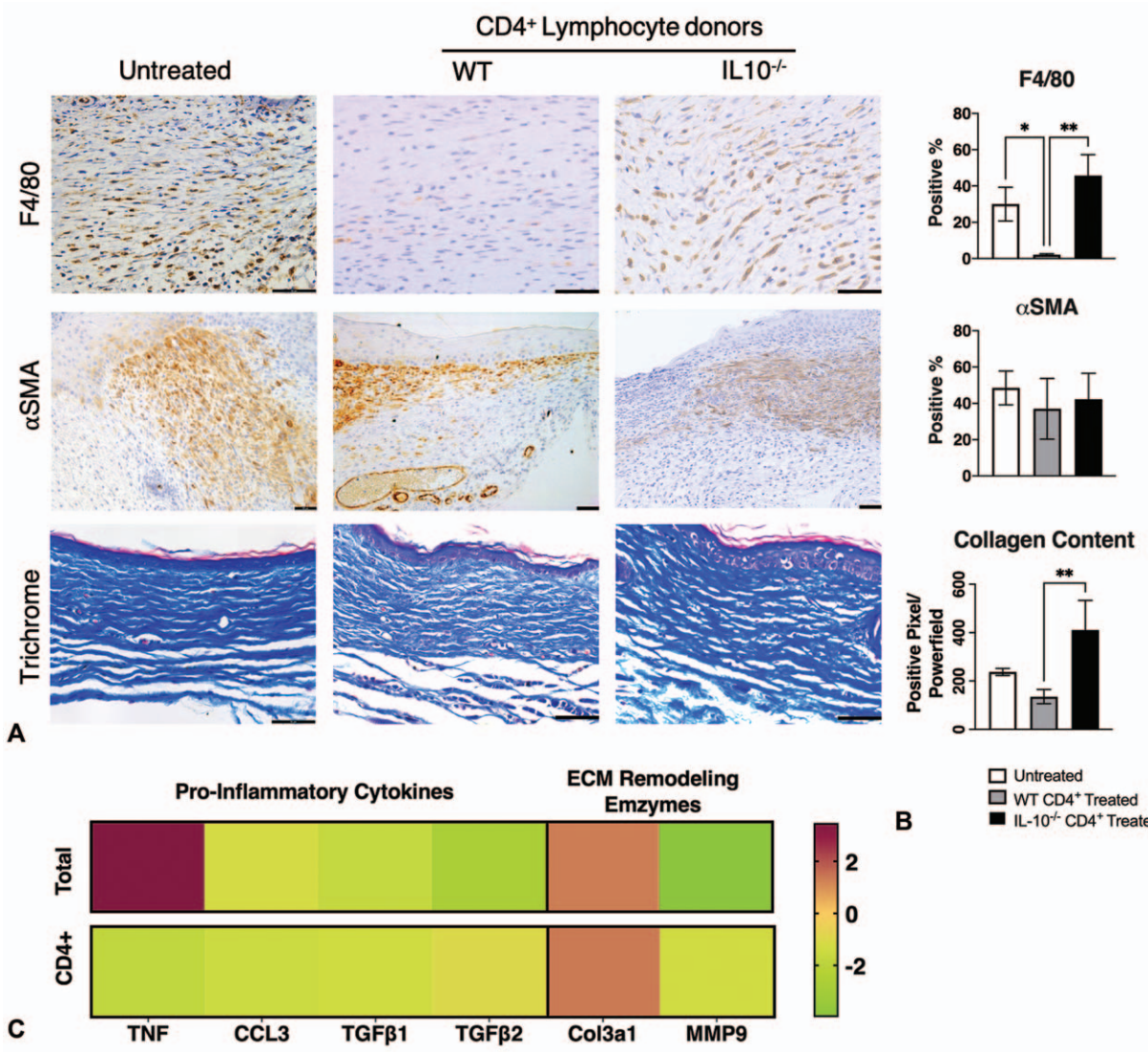

FIGURE 1. Increased inflammation, myofibroblasts, and ECM deposition in SCID mice treated with IL-10$^{-/-}$ CD4^{+} cells. A, Representative images of IHC-labeled F4/80^{+} and αSMA^{+} cells at 7 days and trichome at 30 days post wounding in untreated SCID mice and SCID mice adoptively transferred with CD4^{+} cells from WT and IL-10$^{-/-}$ donors. n=3 mice per treatment group. B, Quantification of F4/80^{+}, αSMA^{+} cells and collagen content (trichrome) levels from A. C, Heatmap comparing relative expression of genes in dermal fibroblasts cultured in conditioned medium from activated total lymphocytes and CD4^{+} cells from WT and IL-10$^{-/-}$ mice, expressed as WT cells over IL10$^{-/-}$ cells. Scale bars: 50 μm. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Adoptive Transfer of Tr1 Reduces Inflammation and Improves Wound Healing Outcome

Regulatory T lymphocytes feature multifaceted IL-10 and other cytokine-driven interactions with effector cells to orchestrate measured homeostatic responses and immune tolerance. In the present study, we sought to take a further step to examine the effects of Tr1 as a distinct IL-10 producing CD4^{+} subset with antiscarring effects and compare to our previously demonstrated effects of FoxP3^{+} Treg.[9] To that end, we adoptively transferred Tr1-enriched CD4^{+} lymphocytes into SCID mice, which subsequently received dermal wounds that were harvested at 7 days and 4 weeks post-wounding.

Wounds were first examined at day 7 to assess wound closure, angiogenesis, and macrophage quantity (Fig. 4). We observed that in comparison to untreated SCID mouse controls, adoptively transplanted cohorts with enriched activated Tr1 CD4^{+} lymphocytes displayed rapid wound closure defined by the percentage of re-epithelialization at day 7 (41.0 ± 9.9% vs 83.0 ± 18.6% closure, $P < 0.01$). The accelerated wound closure in the Tr1 group was accompanied by an increased number of myofibroblasts in the wound, detected by positive αSMA staining, which contrasted with the levels detected in untreated SCID mice (47.6 ± 2.9% vs 32.8 ± 12.0% positive cells/HPF, $P < 0.05$). Wounds of mice treated with Tr1 also displayed greater microvascular density than untreated mice (23.1 ± 2.5 vs 10.5 ± 4.1 CD31 positive vessels/HPF, $P < 0.0001$). In SCID

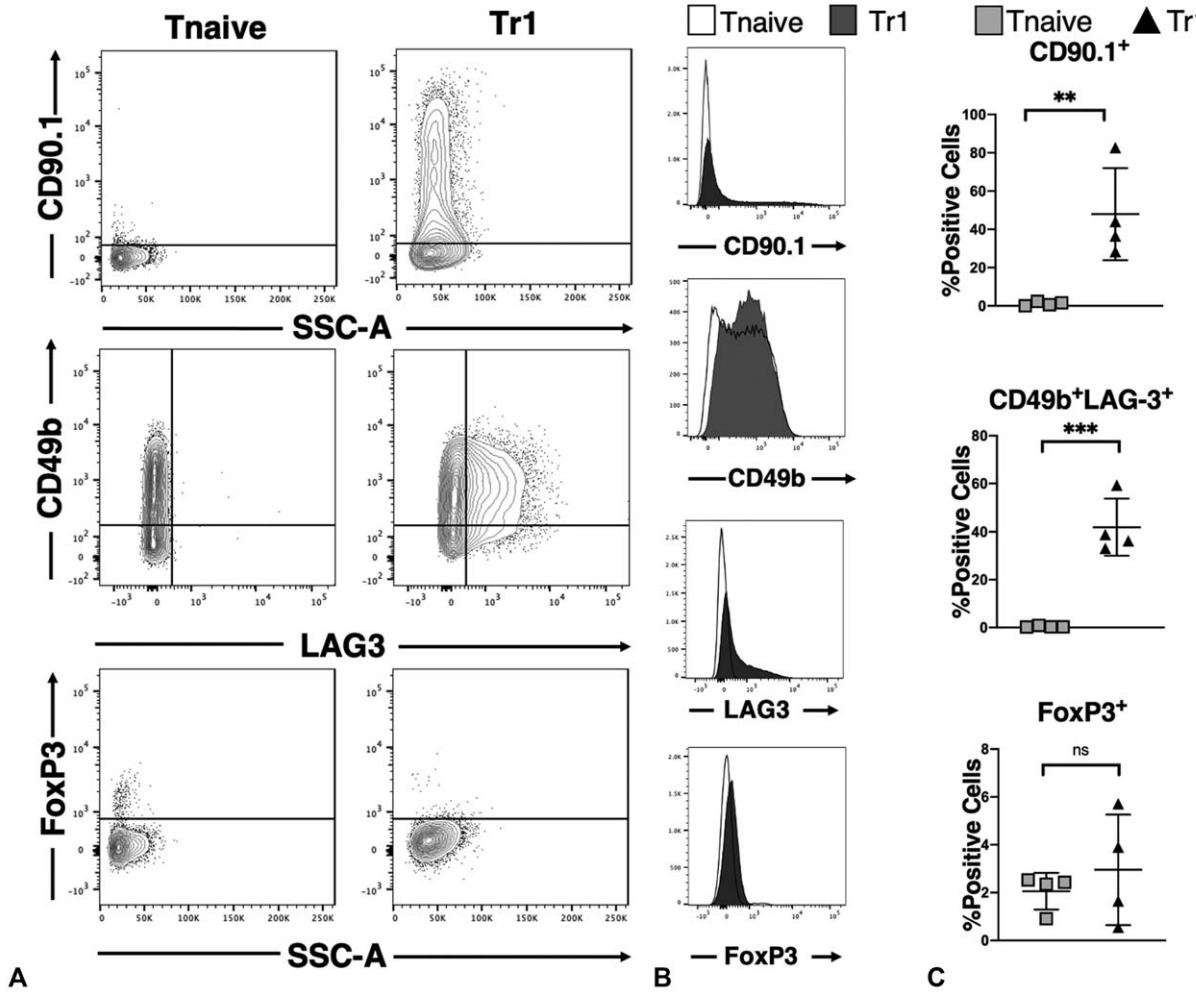

FIGURE 2. Characterization of culture-expanded Tnaive and Tr1 cells. A, *In vitro* differentiation of Tnaive and Tr1 cells from splenic $CD4^+$ cells of 10BiT mice was analyzed by flow cytometry using surface CD90.1, CD49b, and Lag3 staining as well as GFP marking $FoxP3^+$ Expression. B, Representative histograms of A. C, Quantification of A. Expression of CD90.1, coexpression of CD49b/LAG-3, and expression of FoxP3. Data are presented as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

mice adoptively transplanted with Tr1 lymphocytes, there was a significant decrease in macrophages in the wound compared to the untreated cohorts (10.2 ± 6.5% vs 28.6 ± 11.7% F4/80 positive cells/HPF, $P < 0.05$) (Fig. 4B).

To illustrate the spatial relationship of our cellular subsets of interest, such as $CD4^+$ T cells, Tr1, and macrophages, in the wound healing process, we utilized high-dimensional imaging mass cytometry (IMC), which enables the simultaneous visualization of up to 40 different epitopes using antibodies conjugated to lanthanide metals, avoiding the pitfalls of spectral overlap in traditional immunofluorescence.[31] Using this technique, we are able to uncover the spatial relationship of various cells in the Tr1 treated SCID wounds (Fig. 4C, D). $CD4^+$ cells can be seen concentrated in the base of the wound in SCID wounds treated with Tr1, and are surrounded by macrophages, with a greater concentration of CD206 staining, implying M2 polarization of these macrophages. This cellular distribution suggests that Tr1 may be secreting factors that act locoregionally to influence the polarization of macrophages to M2.[32]

Tr1-Enriched $CD4^+$ Cells Attenuate Murine Cutaneous Fibrosis

Wounds at 4 weeks were examined for dermal architecture and collagen content (Fig. 5A–C). Adoptive transfer of Tr1-enriched $CD4^+$ lymphocytes resulted in significantly reduced collagen content in the healed scar compared to untreated mice (128.49±8.55 vs 155.67 ± 8.39 pixels/HPF, $P < 0.05$), as measured by trichrome staining. The decrease in collagen content implicates Tr1 as anti-fibrotic cells, and the histologic structure in the scar seen in H&E staining demonstrates a basket-weave pattern of dermal collagen similar to that of unwounded skin. Quantified data in Figure 5C.

DISCUSSION

We report that Tr1-enriched $CD4^+$ lymphocytes comprise a key IL-10 producing subset with the capacity to heal murine wounds more rapidly and with less fibrosis. Here, we provide *in vitro* and *in vivo* evidence that IL-10 production is necessary to promote $CD4^+$

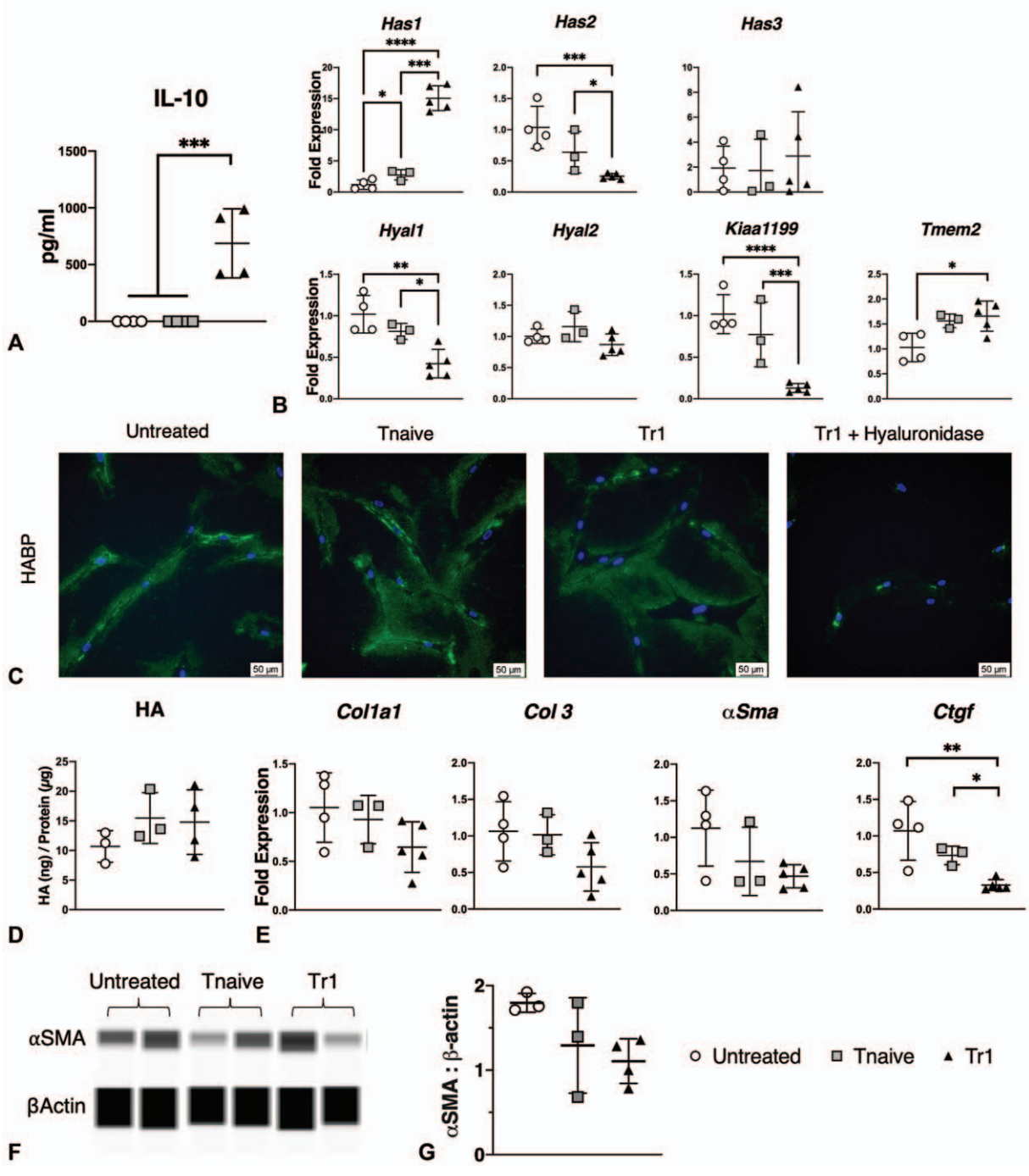

FIGURE 3. Coculture of dermal fibroblasts with Tr1 cells increases hyaluronan synthesis and downregulates Ctgf. A, IL-10 content of medium in fibroblast-lymphocyte co-culture after 24 hours of no treatment, treatment with Tnaive, or treatment with Tr1. B, Gene expression of hyaluronan synthase and degradation enzymes expressed as fold expression normalized to untreated fibroblasts compared to treatment groups of activated Tnaive and Tr1. C, HABP staining of dermal fibroblasts cultured in medium alone, with Tnaive, or Tr1. Scale bars: 50 μm. D, HA content normalized to total protein of medium in fibroblast-lymphocyte coculture after 24 hours of no treatment, treatment with Tnaive, or treatment with Tr1. E, Gene expression of *Col1a1 1, 3*, *αSma*, and *Ctgf* similarly depicted. F, Western blot image for protein isolated from dermal fibroblasts cultured in medium alone, with Tnaive, or Tr1. G, Quantification of F. Data presented as mean±SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. $^{****}P < 0.0001$.

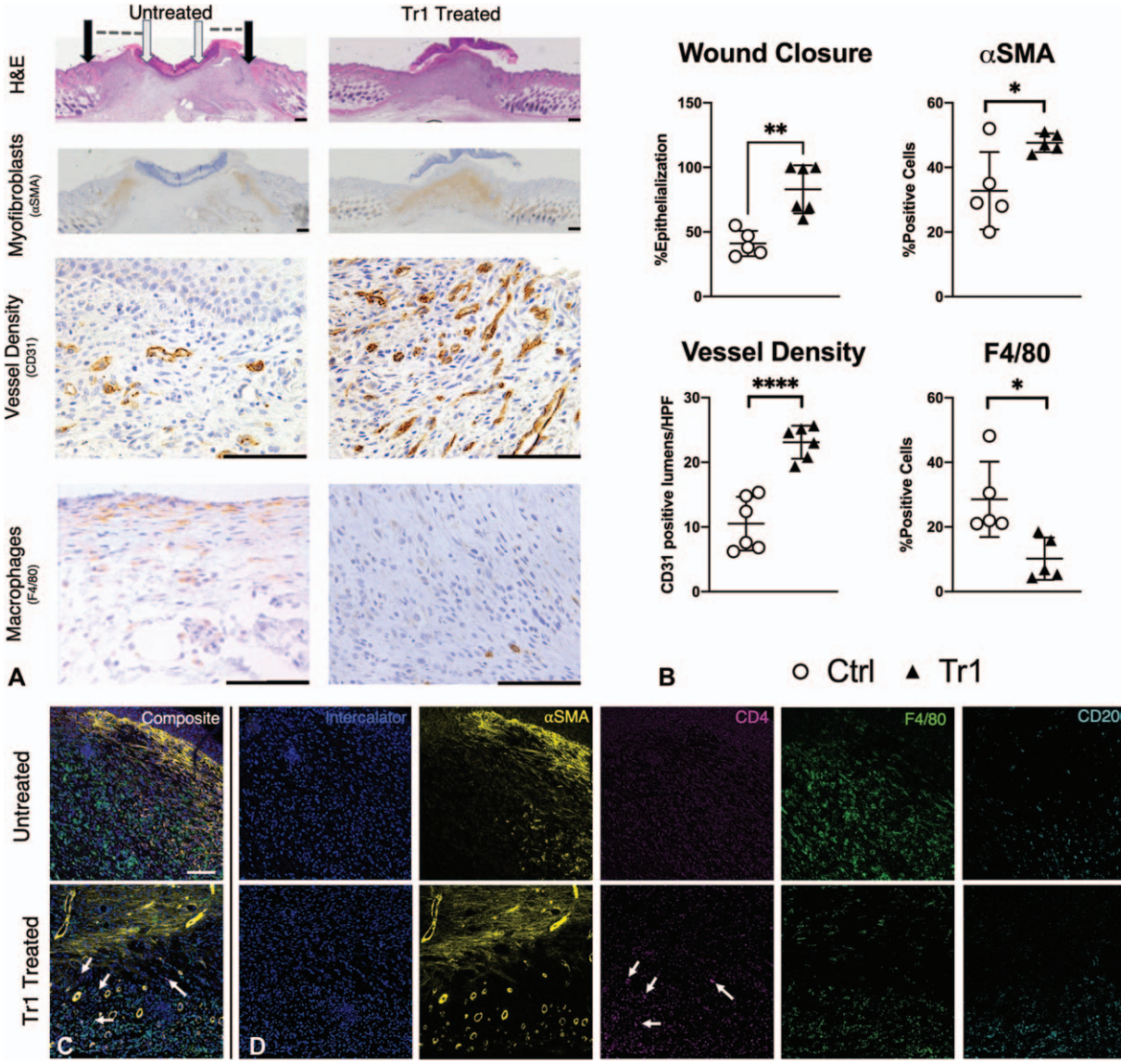

FIGURE 4. Adoptive transfer of Tr1 reduces inflammation and improves wound healing outcome. A, Representative images of H&E, αSMA, CD31, and F/480 staining at day 7 postwounding in untreated control SCID mice and mice adoptively transferred with Tr1 cells. Black arrows indicate the original wound edges, and gray arrows indicate the extent of neoepithelialization. B, Quantification of A. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. $^{****}P < 0.0001$. C-D. $CD4^{+}$ cells are spatially associated with M2 macrophages and blood vessels in wounds of mice treated with Tr1 visualized using imaging mass cytometry. C, Composite image from the edge of a day 7 wound comparing cellular architecture of untreated SCID mice and mice adoptively transferred with Tr1 cells. D, individual channels of C. White arrows mark $CD4^{+}$ cells. Scale bars: 100 μm.

lymphocyte-driven anti-fibrotic wound healing.[9] We found that Tr1, in particular, show a potential to regulate the fibrogenic responses in the healing wound with one of the key mechanisms comprising proteoglycan-rich ECM production, specifically HA, by fibroblasts while concurrently inhibiting hyaluronidase activity.

Our data suggest that IL-10 secretion by lymphocytes is essential for their effect on tissue repair. Furthermore, as we have shown that lymphocytes are present early in the wound repair process, it is conceivable that IL-10 production by these lymphocytes in the wound microenvironment may promote macrophage M1 to M2 polarization, thus activating anti-inflammatory and anti-fibrotic processes.[33] Tr1 were first identified in sera of patients who exhibited immune tolerance after HLA-mismatched stem cell transplants, highlighting their regulatory role to dampen inflammatory responses.[34] Although Tr1 have not been studied in the context of cutaneous wound repair, lymphocytes are known to regulate macrophage differentiation via cytokine secretion, thus we posit that the lymphocytes may be governors of the inflammatory wound repair process upstream of macrophages.[35]

Although IL-10 was previously believed to exert its antifibrotic effects via an immunomodulatory mechanism, we reported an essential mechanism of IL-10 to stimulate fibroblast induction of

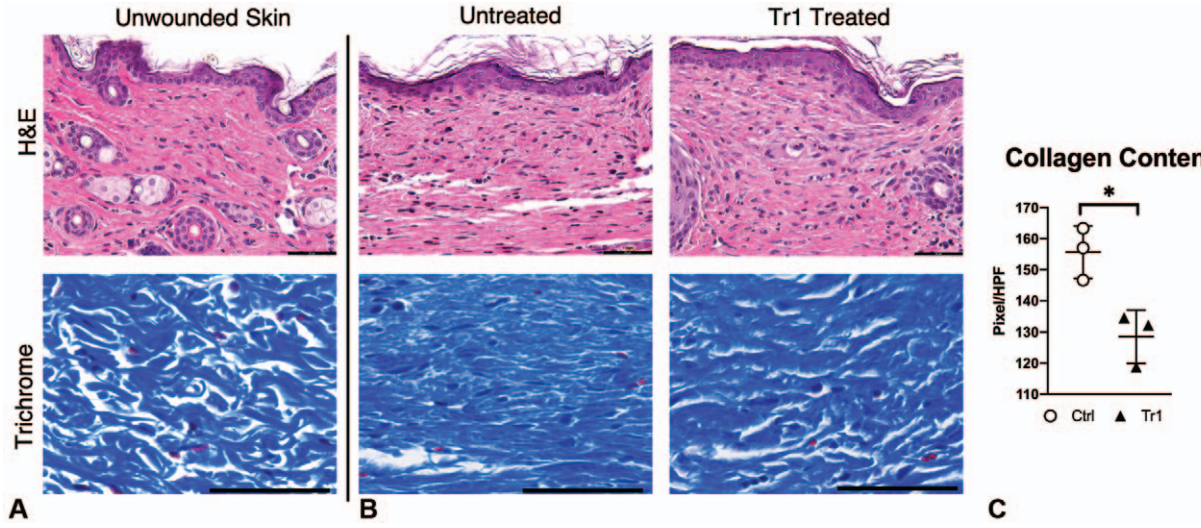

FIGURE 5. Adoptive transfer of Tr1 reduces dermal fibrosis at day 28 postwounding. A, Unwounded normal skin stained with H&E and Trichrome for qualitative comparison to treatment groups. B, Representative images of H&E and trichrome staining at day 28 postwounding in untreated control SCID mice and mice adoptively transferred with Tr1 cells. C, Quantification of B. Scale Bar: 50 μm. Data are presented as mean ± SD. $^{*}P < 0.05$.

HMW-HA that mimics the fetal wound microenvironment to promote regenerative wound healing in dermal wounds.[3,4,23] We chose to study SCID mice in our wound healing models as their cell and molecular microenvironment remain responsive to key mediators like IL-10, which can be readily tested in the wound bed to unveil the mechanism of action on key regulators of wound healing such as fibroblasts, macrophages, keratinocytes, and endothelial cells. SCID mice only lack functional T and B lymphocytes, and display accelerated healing with more fibrosis when compared to WT mice.[9] Similarly, wound healing in IL-$10^{-/-}$ mice has been previously shown to have accelerated wound closure and increased inflammation that culminates with worsened fibrosis than WT mice.[36] Furthermore, IL-$10^{-/-}$ mice demonstrate systemic effects due to the loss of this cytokine, including spontaneous inflammation at barrier sites such as alopecia, colitis, and rectal prolapse.[37] Lymphocyte reconstitution studies in IL-$10^{-/-}$ mice would preclude the important sequence of effects on IL-10 production by other immune cells in the wound environment which are regulated by these T lymphocytes.

Our observation of Tr1 as potent producers of IL-10 renders these cells as capable of not only modulating the immune response but also as regulators of antifibrotic ECM milieu. In support of this notion, we observed in Tr1 treated wounds the pattern of αSMA expression closely follows the re-epithelializing wound margins and regressed once the wound was healed with no difference in αSMA in day 28 wounds between Tr1-treated and untreated SCID mice (data not shown), with overall reduced fibrosis in the Tr1 treated wounds. Further, our *in vitro* Tr1/fibroblast coculture data demonstrated a potent suppression of *Ctgf* which is a key growth factor that regulates fibrosis, suggesting that Tr1 reduces the fibrotic phenotype of fibroblasts.[38] Tr1 secrete IL-22 in addition to IL-10 and TGF-ß1, which aids in supporting intestinal epithelial barrier function, which could explain our findings of increased epithelial migration resulting in accelerated wound closure.[39] A regenerative role of Tr1 is seen in their ability to promote differentiation of goblet cells from intestinal organoid cultures, which is independent of IL-10 or IL-22.[39] This function of Tr1 indicates that their beneficial effects in tissue repair are far more complex than IL-10 production alone, and will require further investigation. Notably, our previously published work demonstrated that Treg were not able to achieve an antifibrotic effect when adoptively transferred in this wound model, despite being a known producer of IL-10.[9] One explanation may be that although equal numbers of cells were adoptively transferred, more Tr1 seem to be recruited to the wound as compared to Treg (Supplemental Figure 3, http://links.lww.com/SLA/D189), suggesting there are inherent differences in reparative capacities of these cells which is not only limited to IL-10. Notably, knockdown of Treg in mice leads to spontaneous dermal fibrosis, thus underscoring their regulatory impact to influence scarless tissue repair.[40] This work suggests that Treg require effector cells to regulate in order for their antifibrotic effects to become apparent, thus in SCID mice we may not see this effect. Knockdown of Tr1 in normal wound healing is a challenge, as there is no single transcription factor or surface marker that can be attributed specifically to Tr1. For instance, co-expression of CD49b and LAG3 is currently the most accepted method to identify $Foxp3^{-}$ Tr1; however, coexpression of these surface markers has also been demonstrated in $Foxp3^{+}$ Treg, highlighting the importance of our verification studies confirming the specific polarization of $Foxp3^{-}$ Tr1 lymphocytes.[41] Although the immunoregulatory and cytokine profiles of Tr1 and Treg are similar, major differences in the proportional secretion of cytokines and growth factors between these cells may help to explain the varying success these cells have on final wound healing outsomes. Treg produce greater quantities of TGF-β1 than Tr1 while the latter yields higher IL-10 levels, which can be a decisive factor controlling the magnitude of fibrosis.[42] Tr1 have also been demonstrated to suppress NLRP3 inflammasome activation, an effect not seen by Treg.[10] Others have demonstrated varying roles for the NRLP3 inflammasome in dermal wound healing, including antagonizing macrophage polarization towards the M2 phenotype.[43–45] NLRP3 inflammasome activity is responsible for cellular activation of IL-1β, which has an established role in promoting tissue fibrosis across many organ systems, including systemic sclerosis.[46,47] The inhibitory action by Tr1, but not Treg, on the NLRP3 inflammasome may help to explain why antifibrotic effects are not seen with adoptive transfer of Treg into SCID mice, even though this effect is dependent on IL-10 signaling.[10] Additionally, our IMC data reveals that $CD4^{+}$ cells in wound bed of adoptively transferred Tr1

lymphocytes appear to be spatially associated closely with an increased proportion of M2 macrophages, suggesting a role in M2 polarization and thus improved healing outcomes, though Tr1 interactions with macrophages remain to be fully explored.

Although proposing the use of Tr1 as a therapeutic means to ameliorate dermal scarring may seem farfetched, this unique lymphocyte subset is currently being tested in clinical trials of graft versus host disease and refractory Crohn's disease. Although the strategies used in these treatments involve expansion of Tr1 *ex vivo*, it is possible to enrich the wound environment with Tr1 without the need for expensive *ex vivo* expansion strategies that require further regulatory approval. For example, HA has the capacity to induce Tr1 proliferation and cytokine production, making biomaterial wound dressings a plausible player to consolidate Tr1 and IL-10 in the wound.[48] Naturally occurring Tr1 are also activated in an antigen-specific manner, functioning to promote immune tolerance to recognized environmental antigens. The significance of immune tolerance to wound healing has been demonstrated in studies where oral administration of proteins such as ovalbumin and zein (a protein found in corn) to mice leads to reduced scarring after intraperitoneal immunization with the respective desensitizing protein.[49,50] Though no explicit link to Tr1 as drivers of the reduced scarring is made, the authors emphasize the attenuated inflammatory responses and increased CD3 content in the wounds.[50] Physiologic Tr1 activation *in vivo* requires cognate antigens, which upon activation regulate non-specific immune responses.[51,52] As ovalbumin administration is a validated *in vitro* and *in vivo* method of priming Tr1 towards antigen-specific activation, it reveals potential mechanisms that could apply to wound healing.[53] Although cell therapy with *ex vivo* expanded or engineered Tr1 has proven to be safe and feasible, these cells can also be induced *in vivo* from effector memory T cell precursors through cognate HMW-HA/CD44 interactions.[48,52] We therefore posit that Tr1 can be attracted to the wound environment by means of specific HA formulations to increase endogenous IL-10 production in the wound and support dermal wound repair.

In that context, we envision to harness the learned knowledge in the present study to further investigate the contributions of lymphocyte subsets to wound repair. While results from this study support our original premise that adoptively transferred Tr1 improve SCID mice wound healing, the activity or even presence of these regulatory immune cells in the wound under physiologic healing conditions have yet to be demonstrated. To that end, we aim to characterize these cells in wound physiology and develop new methods of recruiting Tr1 to cutaneous wounds to promote endogenous IL-10 production.

In summary, we unveiled beneficial effects of Tr1 in wound healing, which follows upon our findings on total unfractionated $CD4^+$ lymphocytes that showed positive roles of IL-10's regenerative effect on wound healing. Future work will investigate how Tr1 mechanistically influence physiologic wound healing by examining their spatiotemporal interaction with other $CD4^+$ lymphocytes, non-lymphoid cell lineages, and molecular mediators in the healing wound, which will place our research a step closer to translating our discoveries on the bench into bedside applications to achieve regenerative wound healing.

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DISCUSSANT

Dr. George Yang

I thank the American Surgical Association for giving me the privilege of commenting on this paper, and I thank the authors for allowing me to see an early copy of the manuscript. Dr. Short presents work examining the role of various T lymphocytes in scar formation during wound repair. He builds upon work from the Keswani lab over the last decade showing IL-10 decreases scarring in part through its effect on hyaluronic acid metabolism. In this particular study, they examine the role of regulatory T-lymphocytes (Treg) and type-1 regulatory T-lymphocytes (Tr1) in this process. They conclude that Tr1 cells are most capable of promoting scarless wound healing. I have the following questions for the authors.

The authors mention initially that IL-10 secretion is a hallmark of M2 macrophages. How did the authors decide upon studying the various T-lymphocyte populations instead?

When examining the role of IL-10 in wound healing, why did they decide to place $CD4^+$ cells from KO mice into SCID mice rather than just wounding the IL-10 KO mice that they had?

If both Treg and Tr1 cells secrete IL-10, suppress SMA and CTGF expression, and increase hyaluronic acid content, why do only Tr1 cells lead to decreased scarring? Does this suggest that the biology is more complex than just increased IL-10 secretion?

I thank the authors for the opportunity to comment on this manuscript.

Response from Dr. Walker D. Short

Thank you for the tremendous opportunity to present my work and thank you Dr. Yang for these insightful comments and taking the time to review our manuscript, we greatly appreciate it, and I think you really highlight the complexities of the cellular interactions we are studying.

You're absolutely right, IL-10 secretion is the hallmark of many cells including macrophages. As many know, macrophages are recruited to the wound and activated/polarized by the wound environment into either a proinflammatory or anti-inflammatory phenotype. In other words, macrophages react to the wound. Interestingly, lymphocytes, which are very understudied within dermal wound repair have recently been shown to have an active role in the tissue repair process, in fact, we recently published a very significant role for lymphocytes in the dermal wound repair process. There is also a good body of literature that suggests lymphocytes actually regulate macrophage differentiation, making them upstream of the macrophage response. That's why we have such an interest in lymphocytes as governors or regulators of the wound repair process and the specific subpopulations of lymphocytes that secrete IL-10 to see what role *they* have in modulating the scarring process.

This is an excellent question, because there are some limitations to this model. What we were trying to accomplish with this experiment is to determine if the positive effects on wound repair that we've previously shown with CD4 lymphocytes is dependent on their IL-10 production. So to do that we had to eliminate any other lymphocyte population, because that's the model we have previously used when showing the beneficial effects of $CD4^+$ cells on wound healing. it is certainly a complex system and what We really wanted to determine is if the effects we were seeing with adoptive transfer of CD4 cells were dependent on IL-10. By taking CD4 cells from an IL-10 KO mouse, we introduce those cells into a mouse that has no lymphocytes, but still contains cells that can produce IL-10. This we thought was an experiment that more directly answered the question ''are the positive effects we saw on wound repair by $CD4^+$ lymphocytes dependent on IL-10.'' And in this model, every other cell in that mouse represented the normal physiologic phenotype.

This again a very insightful question, and one that we have been investigating. The biology is absolutely more complex than just IL-10 secretion. What we've shown in this presentation is that while Treg and Tr1 both produce IL-10 and have similar effects on gene expression in fibroblasts, there are many other avenues to explore for why there's a difference. For example, we've subsequently done imaging mass cytometry that allows us to spatially see where these Tr1 cells are functioning in the wound, and what we find is in the Tr1 wounds specifically compared to Treg, there are Tr1 cells at the base of the wounds and an increased proportion of M2 antifibrotic macrophages associated with these Tr1. So while you're completely correct that Treg and Tr1 have similar effects on fibroblasts, we see a striking difference in the effect they have on macrophages. We also see in our 28 days time point, the wounds of mice treated with Tr1 still have lymphocytes present whereas Treg don't, suggesting that perhaps Tr1 are exerting their effects in the wound healing process for a longer duration. So these reasons may explain why Tr1 have a greater effect on fibrosis while the earlier timepoints are similar to Treg.