

Resolution of chronic IL-33 signaling improves adult macrophage polarization and tendon healing

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INTRODUCTION: Although excessive or chronic inflammation is a feature of scar-mediated tendon healing [1, 2], the cells that regulate chronic inflammation have not been fully elucidated. Recently, we showed that enhanced tendon healing in neonatal mice is driven by acute but transient inflammation that is resolved by regulatory T cells (Tregs) that polarize macrophages from inflammatory to anti-inflammatory profiles [3]. Ablation of neonatal Tregs impaired functional healing with reversal of inflammation state to inflammatory macrophages, with adoptive transfer of neonatal Tregs into adult injury improving functional adult tendon healing [3]. IL-33 (a member of the IL1 cytokine family) is associated with fibrotic adult tendon healing [4]. IL-33 signaling *in vivo* is controlled by its bound receptor ST2 (encoded by *Il1rl1* transcript). Although IL-33 is associated with both type I and II immune responses [5], the role of IL-33 in the context of tendon healing is unclear. We previously showed IL-33 is associated with adult tendon inflammation suggesting it may induce a type I response unfavorable for functionally effective healing [6]. In this study, we use scRNA-seq to determine global T cell and Treg subpopulations enriched after neonatal and adult tendon injury and determine the role of IL-33 using loss of function (*IL33*^{-/-} mutants) and gain of function (injection of recombinant IL-33) experiments. We hypothesized that excessive IL33 signaling is the basis for chronic inflammation leading to poor tendon healing.

METHODS: scRNA-seq. At 14 days post Achilles tendon transection without repair (12 neonates at P5, 4 adults at 4 weeks), live, CD45+ CD3+ T cells were isolated from injured tendons using FACS. 10X Chromium 3' sequencing was carried out and clustering analysis performed using Seurat. IL-33 injections in neonates. Recombinant mIL-33 (R&D) was injected IP in neonates (10ng/g wt, 5-14 DPI) during the phase when IL-33 is typically resolved [6]. IL33^{-/-} adults. *IL33*^{-/-} mice were generated from *IL33*^{fl/fl} crossed to the ubiquitous *CMVCre*. Mice were aged to 4-6 weeks and Achilles injuries carried out. Structure/Function. At 56 DPI, collagen fibril orientation was assessed in tendon resin sections using FiberFit (Boise State University). At 28 DPI, gait was determined using Catwalk XT (Noldus) [7] and mechanical testing of dissected tendons carried out (ramp to failure at 0.05cm/s). All animal studies were carried out according under approved IACUC.

RESULTS: 6,741 cells from 4 adults and 1,635 cells from 12 neonates were sequenced. Expression analyses confirmed that the majority of cells were *Cd3*⁺ with only a small population of *Cd3*⁻ macrophages (Fig 1A). Analyses also revealed a surprisingly large proportion of gamma delta T cells (Tgd clusters 0 and 3, annotated as Tgd_1 and Tgd_2, respectively) (Fig 1A). Tgd populations comprised 60% and 37% of neonatal and adult CD3⁺ T cells, respectively and were confirmed by flow cytometry (not shown). We also identified a significant proportion of Foxp3⁺ Tregs within the CD4⁺ population (Fig 1A, cluster 2). Subclustering of the Foxp3⁺ Treg population revealed distinctive Treg sub-populations (Fig 1B, clusters 0-2). We found that sub-cluster 1 comprised the majority of neonatal Tregs, which were also enriched for *Il1rl1* (encoding the ST2 receptor for IL-33) and genes associated with tissue homing (Fig 1C, D). To determine the consequence of excessive IL-33 signaling in neonates, we induced chronic signaling with injections of rmIL-33 from 5-14 DPI. Consistent with our hypothesis, chronic IL-33 resulted in impaired functional and structural recovery, indicative of poor healing (Fig 2). Surprisingly, macrophage polarization at 14 DPI showed a reduction in inflammatory Ly6C^{hi} macrophages, suggesting that IL-33 did not induce a type I chronic inflammatory response in neonates (Fig 3B). To determine whether resolution of IL-33 in adults could improve tendon healing, we injured *IL33*^{-/-} mice and observed improved functional properties at 28 DPI (Fig 3A). In the adult context, we also observed reduced inflammatory Ly6C^{hi} macrophages at 14 DPI (Fig 3B), suggesting that IL-33 is normally associated with failed anti-inflammatory polarization in adult tendon healing and that IL-33 may have distinctive roles in neonates vs adults.

DISCUSSION: Our studies showed a unique population of Foxp3⁺ Tregs that are enriched in neonates with enhanced sensitivity for IL-33 signaling. While we initially hypothesized that IL-33 is associated with an inflammatory environment in tendon healing, rmIL-33 injection resulted in a dysregulated type II environment in neonates that was also associated with poor tendon healing. This suggests that either dysregulated type I or II immune responses can both impair tendon healing [8], or that IL-33 may also target other cell populations associated with healing, independent of macrophages and Tregs. In previous studies for example, we showed that IL-33 inhibits tenocyte migration *in vitro* [6]. Our single cell dataset also identified intriguing populations of Tgd cells that may also play critical roles in tendon healing and will be the focus of future studies. Although Tgd cells have been extensively studied in the context of arthritic diseases and are reported within the tendon-bone enthesis, the presence of such a large population after mid-substance tendon injury is previously unreported. The single cell transcriptomic data will be further analyzed to determine the distinctive gene expression between Tgd_1 and Tgd_2 populations and identify cytokines expressed by these cells (including IL-17, IL-4, and IFN γ) to determine whether these populations may contribute to a pro- or anti-inflammatory immune environment. Finally, improved healing in the adult environment in the absence of IL-33 demonstrate a potential avenue for therapeutic intervention. Temporal deletion of IL-33 will determine whether the destructive effects are due to the initial (ie. excessive inflammation) or later phases (ie. chronic inflammation) of tendon healing.

SIGNIFICANCE: Elucidating the role of IL33 during tendon healing is crucial to promote a tendon immune environment favorable for regeneration.

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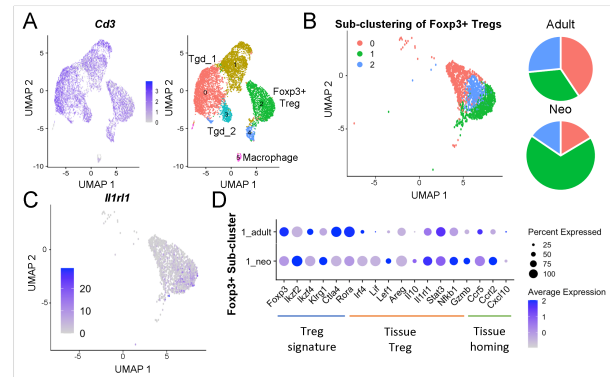


Fig 1. Adult and neonatal scRNA-seq reveal novel Treg population enriched in neonates at 14 DPI. A. Combined cells show enriched *Cd3*⁺ cells. Clustering identified Treg and gamma delta T cell (Tgd) populations. B. Sub-clustering of Tregs revealed sub-cluster 1 enriched in neonates. C. *Il1rl1* enrichment in sub-cluster 1. D. Dot plot of Treg and tissue homing genes for sub-cluster 1.

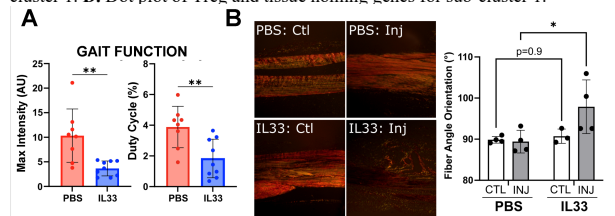


Fig 2. Chronic IL-33 impairs neonatal tendon healing. A. Impaired gait at 28 DPI with IL-33 injection (n=7-9). B. Poor structural recovery at 56 DPI with IL-33 injection (n=4). *p<0.05

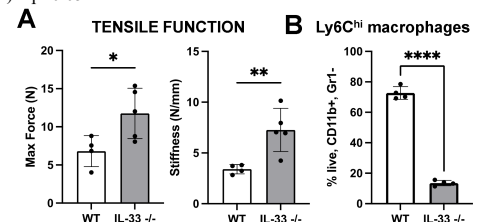


Fig 3. IL-33 deletion improves adult tendon healing. A. Improved tensile properties at 28 DPI with IL33 deletion (n=4-5). B. Reduced M1-like macrophages at 14 DPI (n=4). *p<0.05 **p<0.01 ****p<0.0001