Neonatal and adult mice have intrinsic tenocyte and T cell differences

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DISCLOSURES: Giulia Crosio (N), Alice H. Huang (N)

INTRODUCTION: Although the immune response is a critical driver of wound healing, the specific immune cells that mediate functionally effective vs ineffective tendon healing are only beginning to be established. While most studies focus on macrophages, we recently showed that T cells also play a critical role in effective tendon healing as Rag2$^{-/-}$ mice lacking T and B cells heal poorly after neonatal tendon injury compared to wild type (WT) mice, which was defined by failed macrophage polarization and impaired function [1]. Adoptive transfer of specific subpopulations of T cells (regulatory T cells, Tregs) into neonatal Rag2$^{-/-}$ mice improved mechanical properties, but only to a partial extent, despite restoration of macrophage polarization. To test whether other T cell subpopulations may also mediate healing via paracrine signaling independent of macrophages, we applied T cell-derived supernatants from neonatal and adult mice and assessed their effects on neonatal and adult-derived tenocytes.

METHODS: Supernatants. CD4+ T cells from P7 or adult (4-6 months) mice were isolated from spleens and thymuses using the CD4+ Negative Selection Kit (Fisher) and cultured for 3 days in Immunocult T cell media containing 1% P/S, 0.2 ng/mL IL-2, CD3/CD28 beads (stimulated) or Immunocult T cell media with 1% P/S only (unstimulated). Migration**.** Tenocytes were derived from neonatal and adult mice (collagenase I, collagenase IV and DMEM, 2.5h) and cultured in DMEM, 10% FBS, 1% P/S (tenocyte media) until passage 2. Scratch assays were performed after 24 hr serum starvation and analyzed at 0, 4, and 8h using ImageJ. Scratch areas were normalized to 0h. Proliferation. Since tenocytes were not viable in T cell medias, proliferation was assessed after 3 days of culture in 50% tenocyte media and 50% T cell medias (with or without supernatants) using the CCK8 kit. (Dojindo). Gene expression. Tenocyte gene expression was assessed after 3 days of culture with 50% tenocyte media and 50% T cell medias (with or without supernatants) using qPCR. Proteomics. The composition of unstimulated and stimulated supernatants was evaluated using the Proteome Profiler Mouse Cytokine Array Kit (R&D Systems) to quantify 40 proteins. All animal work was carried out under approved IACUC.

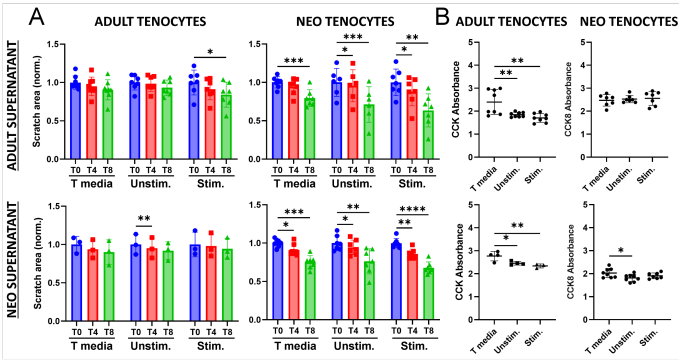

Fig 1: **T cell factors' effect on tenocyte migration. A**. Scratch assay and **B.** proliferation of adult or neonatal tenocytes treated with either adult or neonatal T cell supernatants. Scratch: 2-way ANOVA (n=4-8). CCK: 1-way ANOVA (n=4-8). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

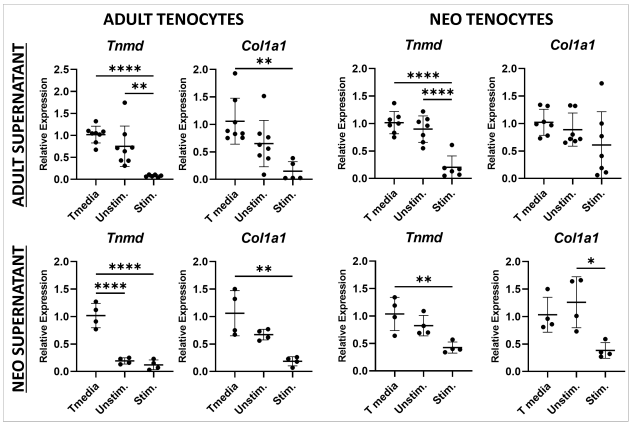

Fig 2: **Adult and neonatal T cell supernatants inhibited tendon gene expression.** Stimulated supernatants reduced tendon gene expression regardless of source. 1-way ANOVA (n=4-8) *p<0.05, **p<0.01, ****p<0.0001.

RESULTS: Surprisingly, adult and neonatal tenocyte migration were insensitive to T cell supernatant source, with enhanced migration observed for neonatal tenocytes compared to adult. The presence of stimulated T cell supernatant also accelerated neonatal tenocyte migration compared to T cell media alone (**Fig 1A**). Similarly, adult tenocyte proliferation was suppressed with both adult and neonatal supernatants with no difference observed between unstimulated and stimulated supernatants (**Fig 1B**). In contrast, neonatal tenocyte proliferation appeared relatively insensitive to supernatant culture (**Fig 1B**). In terms of tendon phenotype, *Tnmd* and *Col1a1* were generally responsive to both adult and neonatal supernatants with suppression observed in nearly all stimulated supernatant conditions for both adult and neonatal tenocytes (**Fig 2**). Of the 13 cytokines/chemokines detected in the proteomic array (including multiple inflammatory molecules such as IFNg, IL-16, IL-17, CCL3, CCL4, and TNFa), we found 4 proteins that were significantly elevated in the adult stimulated supernatant compared to neonate (**Fig 3**). Only IL-13 was detected in unstimulated supernatant samples, with higher levels observed for adult samples compared to neonate (not shown).

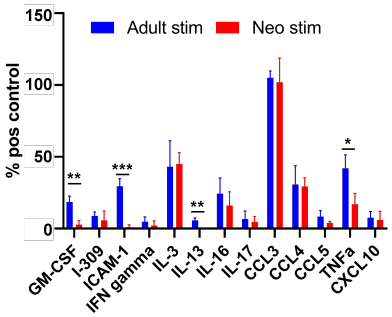

Fig 3: **Elevated cytokines in adult stimulated T cell supernatants.** Proteome array detected 13 proteins with 4 increased in adult supernatant. 2-sided t-tests (n=4), *p<0.05, **p<0.1, ***p<0.01.

DISCUSSION: In these studies, we observed strong tenocyte-intrinsic effects in terms of migration and proliferation, with neonatal tenocytes demonstrating enhanced migratory potential. Although stimulated supernatant enhanced neonatal migration, the effects were independent of supernatant source (ie. adult vs neonate-derived). Interestingly, although supernatants enhanced migration, tendon gene expression was generally suppressed for both adult and neonatal tenocytes, suggesting that cells may be adopting a de-differentiated in the presence of T cell supernatants. In previous studies, we found that the majority of CD4+ T cells in culture were Th1 cells with minimal Th2 and Treg cells, suggesting that the supernatant collected in these studies may model a type I inflammatory environment [2]. This is consistent with other studies showing that T cell activation results in expression of inflammatory cytokines in tenocytes leading to continued T cell activation and a chronic inflammatory loop [3]. Type I inflammation is also supported by our proteomic analyses which revealed the presence of several inflammatory cytokines in both adult and neonatal supernatants. Although a few proteins were detected at higher levels in adult supernatant, it is likely that threshold levels were already achieved in the neonatal supernatant which is why outcomes were largely similar. These exciting results indicate that neonatal tenocytes are intrinsically more migratory under inflammatory conditions, which may explain the successful *in vivo* recruitment of neonatal tenocytes after injury but not adults [4]. Although recruitment is a key factor in neonatal tendon healing, the resolution of inflammation is also important for subsequent re-differentiation, which is observed by 14 days post-injury *in vivo* [4]. Ongoing studies will test the effects of identified proteins on tenocyte behavior and the effects of type II T cell supernatants (derived from Th2 or Treg cells).

SIGNIFICANCE: Establishing the mechanisms underlying tenocyte and T cell interactions under inflammatory conditions are required to prevent maladaptive tendon healing.

ACKNOWLEDGEMENTS: This work was funded by NIH R56 AR076984 and NIH R01 AR081674.

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