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Ton C. Doan, BA, Brian M. Jeong, MSc, Mackenzie E. Coden, BA, Lucas F. Loffredo, BSc, Swati Bhattacharyya, PhD, Sergio E. Chiarella, MD, John Varga, MD, Hiam Abdala-Valencia, PhD, Sergejs Berdnikovs, PhD

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Matrix protein Tenascin C expands and reversibly blocks maturation of murine eosinophil progenitors**RUNNING TITLE:** Tenascin C maintains eosinophil progenitors

AUTHORS: Ton C. Doan, BA¹, Brian M. Jeong, MSc¹, Mackenzie E. Coden, BA¹, Lucas F. Loffredo, BSc¹, Swati Bhattacharyya, PhD², Sergio E. Chiarella, MD¹, John Varga, MD², Hiam Abdala-Valencia, PhD³ and Sergejs Berdnikovs, PhD^{1*}

¹Division of Allergy and Immunology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA.

²Division of Rheumatology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA.

³Division of Pulmonary and Critical Care, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA.

*Correspondence to: Sergejs Berdnikovs, Northwestern University Feinberg School of Medicine, Division of Allergy and Immunology, 240 East Huron Street, McGaw M-317, Chicago, IL 60611, USA; phone: 1-312-503-6924; fax number: 1-312-503-0078; e-mail address: s-berdnikovs@northwestern.edu

CAPSULE SUMMARY

We show that Tenascin-C, an asthma-associated extracellular matrix glycoprotein, promotes hematopoietic progenitors and suppresses the IL-5-driven maturation of murine lung eosinophils. The extracellular matrix's regulation of *in situ* hematopoiesis has significant implications for targeting tissue eosinophils.

KEYWORDS

Extracellular matrix, eosinophils, allergy, asthma, Tenascin-C, bone marrow, lung tissue, hematopoietic stem cells, *in situ* hematopoiesis

To the Editor:

Eosinophils are effector cells in allergy that are both recruited from circulation and develop via *in situ* hematopoiesis in the local tissue environment. While eosinophil depletion in tissues is a highly sought-after therapeutic strategy in treating allergic disease, little is known about the role of tissue factors in promoting eosinophilia. In disease, tissue eosinophils typically encounter provisional extracellular matrix (ECM) glycoproteins, such as periostin, versican, thrombospondins and tenascins¹. Particularly, eosinophil expansion in tissues strongly correlates with Tenascin-C (TNC) deposition². While highly restricted in healthy adult tissues, TNC is strongly expressed *de novo* during wound healing and pathologic tissue remodeling. TNC is a hexabrachion featuring epidermal growth factor (EGF)-like repeats, fibronectin type III (FN3)-like repeats and a fibrinogen-like globe that interface integrins and other matrix components in tissues³. Importantly, TNC is a known hematopoietic niche component in the bone marrow stroma⁴. Moreover, exposing naïve murine eosinophils to TNC-enriched provisional matrices significantly upregulates the gene expression of immaturity markers (*Ly6a* (*Sca-1*), *CD34*) and suppresses IL-5R α (*IL5ra* (*CD125*)) expression, as determined by RNA-Seq (our unpublished data). This led us to hypothesize that TNC represents a factor in the provisional allergic tissue microenvironment that could potentially support *in situ* eosinophil progenitor expansion. In support, we show that in murine bone marrow-derived cultures, Tenascin-C: 1) 3-fold expanded the available Lin⁻Sca1⁺ early precursor pool; 2) significantly downregulated IL-5R α expression on Lin⁻CD45⁺c-kit⁺ cells during the eosinophil lineage commitment phase; 3) served as a reversible block of eosinophil maturation, as TNC withdrawal from cultures rescued maturation and increased final eosinophil yield. Furthermore, we show that TNC knockout mice lack *in situ* lung expansion of Lin⁻c-kit⁺CD34⁺ common myeloid progenitors and Lin⁻Siglec-F⁺Sca-1⁺ cells in an allergic inflammation model, while exhibiting accelerated maturation of lung eosinophils *ex vivo*. Adding TNC to lung homogenates cultured in IL-5 *ex vivo* suppressed eosinophil maturation in a manner consistent with the TNC eosinophil maturation block in bone marrow-derived cultures. Experimental procedures and additional references can be found in this article's Online Repository at www.jacionline.org.

Bone marrow-derived eosinophil cultures supplemented with TNC significantly expanded the Lin⁻Sca-1⁺ early precursor pool compared to controls (**Fig 1B, C and D, Supplemental Fig 1**). Maturation suppression was evidenced by constitutive Sca-1 expression (hematopoietic progenitor/stem cell marker) at all time points in TNC culture (**Fig 1D**). Terminal IL-5-driven eosinophil differentiation and maturation was significantly suppressed by TNC addition, as determined by flow cytometry (**Fig 1B and 1C**). In comparison with control samples expressing only Siglec-F, TNC-treated cultures on day 15 had a substantial proportion of eosinophils expressing both Siglec-F

(eosinophil marker) and Sca-1 (**Fig 1C**). Compared to controls, differential staining at TNC culture end points revealed morphology characteristic for class III eosinophil progenitor cells, featuring a lack of distinct eosin staining and non-segmented nuclei (**Fig 1B**). TNC may have a direct effect on maturing eosinophils via the binding of integrin-binding and fibronectin (and other ECM)-binding repeats within its fibronectin type III (FN3) domain³, which was specifically presented to eosinophils in our cultures. Among integrins, $\alpha\beta 1$ integrin specifically binds the AEIDGIEL sequence in the FN3 TNC domain⁵, and this integrin expression on human hematopoietic stem and progenitor cells was found to regulate cell proliferation and differentiation⁶. These findings are consistent with reports of TNC and other provisional ECM proteins regulating hematopoiesis. TNC sustains hematopoietic regeneration after bone marrow ablation, which is dependent on FN3 domain binding⁴. Mice lacking TNC show reduced bone marrow colony-forming capacity and hematopoietic cell production. Within the bone marrow environment, fibronectin affects progenitor fate decisions via the FN3 domain binding⁷. Hyaluronic acid scaffolds are sufficient to maintain long-term cultures of CD34⁺ hematopoietic cells obtained from human cord blood⁸. Identifying specific integrin-matrix interactions regulatory for *in situ* hematopoiesis of eosinophil progenitors is a subject of future studies by our group.

Next, we showed that TNC suppresses eosinophil progenitor differentiation by suppressing IL-5R α expression on Lin⁻CD45⁺c-kit⁺ precursor cells. By flow cytometry, IL-5R α expression on Lin⁻CD45⁺c-kit⁺ precursors significantly peaks on days 5-7 in control bone marrow-derived eosinophil cultures supplemented only with IL-5, corresponding to IL-5-driven progenitor commitment to maturation in eosinophil lineage. After day 7, IL-5 receptor expression gradually decreases until eosinophils mature (**Fig 1E**). In TNC-supplemented cultures, we detected significantly downregulated IL-5R α expression during this time (**Fig 1E**). Flow cytometry analysis showed that more than 70% of CD45⁺Lin⁻ cells expressed IL-5R α during the commitment phase in IL-5-supplemented culture. TNC addition resulted in nearly complete IL-5R α expression loss during culture days 5-7 (**Supplemental Fig 2**), suggesting this as the mechanism suppressing eosinophil progenitor maturation. TNC served as a reversible eosinophil maturation block, as its removal from cultures on day 6 (after the IL-5R α suppression phase) completely rescued normal eosinophil maturation as evidenced by morphology (**Fig 2A**). Flow cytometry confirmed the reversal to a mature phenotype, as all TNC withdrawal group eosinophils lost Sca-1 expression (**Fig 2A**). Also, TNC withdrawal from culture doubled the final mature eosinophil yield (**Fig 2B**). This is likely due to the initial eosinophil precursor expansion during the first week in TNC-enriched culture, the differentiation potential of which was fulfilled by IL-5 after TNC withdrawal. As such, temporary TNC deposition corresponding to a fluctuating provisional ECM environment may represent a mechanism by which tissues locally expand eosinophil progenitors and rapidly supply effector eosinophils during progressing repair or remodeling stages. Current eosinophil depletion strategies that target IL-5 (mepolizumab) or IL-5R α (benralizumab) achieve only partial eosinophil depletion and 50% success in clinical trials. Moreover, mepolizumab was found to reduce airway eosinophil numbers in asthma but not their functional phenotype⁹. Provisional matrix components such as TNC may explain the only partial success of these therapies, which target cytokine drivers of eosinophil maturation but fail to address tissue factors driving eosinophil progenitors *in situ*.

Finally, using an allergic airway inflammation model, we showed that the allergen-challenged lungs of TNC-deficient (TNC^{-/-}) mice lacked both CD45⁺c-kit⁺CD34⁺ common myeloid progenitors and CD45⁺Lin⁻Siglec-F⁺Sca-1⁺ eosinophil precursors compared to wild type (WT) controls (**Fig 2C**, **Supplemental Fig 3**). To control for the potentially confounding recruitment of eosinophils and their

progenitors from bone marrow during allergic inflammation, we cultured complete lung homogenates from WT and TNC-/- mice in the presence of IL-5 *ex vivo*. Remarkably, the untreated lung homogenates culture with IL-5 was sufficient to conservatively yield up to 20% mature eosinophils (out of CD45⁺ hematopoietic cells, measured by flow cytometry), implicating the significant recruitment-independent *in situ* hematopoietic potential of resting lung tissue. Eosinophil maturation was significantly accelerated in the *ex vivo* culture of TNC-/- lungs (**Fig 2D**). Adding TNC to lung homogenates promoted CD45⁺Lin⁻Siglec-F⁺Sca-1⁺ cell expansion and significantly inhibited eosinophil maturation in WT and TNC-/- cultures (**Fig 2D**), consistent with TNC's function as an eosinophil maturation block. Interestingly, eosinophil morphology was dramatically different in TNC-/- lung cultures with hyper-segmented nuclei and vacuoles typical of airway eosinophils during allergic inflammation. Adding TNC "rescued" the wild type eosinophil phenotype, with a ring-shaped nucleus and no vacuoles. This phenotype is reported for stromal, sub-epithelial eosinophils (where TNC is found *in vivo*) which may implicate TNC as a factor dictating the compartment-specific eosinophil phenotype *in vivo*.

Collectively, our results illustrate the significant potential of the provisional ECM to support a hematopoietic niche environment and control eosinophil progenitor *in situ* expansion and maturation, which has significant implications for future strategies looking to limit tissue eosinophils in allergic diseases.

Ton Doan, BA^a

Brian M. Jeong, MSc^a

Mackenzie E. Coden, BA^a

Lucas F. Loffredo, BSc^a

Swati Bhattacharyya, PhD^b

Sergio E. Chiarella, MD^a

John Varga, MD^b

Hiam Abdala-Valencia, PhD^c

Sergejs Berdnikovs, PhD^a

From the ^aDivision of Allergy and Immunology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA; ^bDivision of Rheumatology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA and ^cDivision of Pulmonary and Critical Care, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA. E-mail: s-berdnikovs@northwestern.edu.

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AUTHOR CONTRIBUTIONS

S.B., T.D., H.A.V. and L.F.L. conceived and designed the study. T.D. and L.F.L. cultured bone marrow eosinophils. Sw.B. and J.V. established TNC-/- models and assisted in the design of experiments. B.M.J., M.E.C., and S.E.C. performed lung hematopoiesis experiments *in vivo* and *ex vivo*. T.D., B.M.J. and M.E.C. performed flow cytometry. T.D. and S.B. analyzed data. S.B., M.E.C. and T.D. prepared the figures. S.B., J.V., L.F.L., H.A.V. and T.D. interpreted the results. The manuscript was written by T.D. and S.B. and edited by H.A.V. Final version of the manuscript was approved by S.B.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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FIGURE LEGENDS

Figure 1. (A) Protocol for bone marrow and *ex vivo* lung eosinophil differentiation culture, separated into three critical phases: precursor expansion, eosinophil commitment, and eosinophil maturation. Cells were cultured with SCF and FLT3L for 4 days before induction to eosinophil commitment via IL-5. (B) Expression of Siglec-F (eosinophil marker) and Sca-1 (stem cell marker) on CD45⁺ cells at terminal bone marrow culture endpoint on day 15. Control culture consistently resulted in >90% Siglec-F⁺Sca-1⁻ eosin-positive eosinophils. Morphology of these cells is shown in cytopsin preparations stained with differential staining solution. (C) Kinetics of bone marrow derived eosinophil differentiation and Sca-1 expression on CD45⁺Siglec-F⁺ by flow cytometry. Asterisks denote differences between each time point and day 3 culture start time point for each of the treatment curves, asterisk color matches treatment curve color. (D) Quantification of CD45⁺Sca-1⁺ cells by flow cytometry in bone marrow derived eosinophil cultures. Blue, control culture; green, TNC supplemented culture, asterisks denote differences between two cultures. (E) Kinetics of expression of IL-5R α on cells throughout bone marrow culture quantified by flow cytometry. TNC significantly downregulated IL-5 receptor during days 5 and 7 of culture (IL-5 induced eosinophil lineage commitment phase). 3 wells/treatment/culture time point, N=3 independent donor cultures. *, $p < 0.05$, ****, $p < 0.0001$.

Figure 2. (A) Withdrawal of TNC from day 6 bone marrow culture rescued IL-5-induced eosinophil commitment. MFI levels of Sca-1 on CD45⁺Siglec-F⁺ eosinophils on day 15 of the culture are shown. Cytopsin preparations confirmed restoration of mature eosinophil phenotype after TNC withdrawal. (B) Quantification of CD45⁺Siglec-F⁺ eosinophils on day 15 of bone marrow culture. Withdrawal of TNC on day 6 doubled the yield of mature eosinophils. (C) In a model of allergic inflammation, TNC^{-/-} mice had a significant reduction in both common myeloid progenitors (CD45⁺c-kit⁺CD34⁺ population, top scatterplots) and Lin⁻ CD45⁺Siglec-F⁺Sca-1⁺ eosinophil progenitors (Lineage expression shown out of CD45⁺Siglec-F⁺Sca-1⁺ parent population, bottom histograms). N=8 for WT, N=4 for TNC^{-/-} mice. The experiment was repeated twice. (D) IL-5 culture of *ex vivo* lung homogenates. Lineage histograms shown are out of CD45⁺Siglec-F⁺Sca-1⁺ parent gates. Eosinophils from TNC^{-/-} lungs showed accelerated maturation and reduced numbers of Lin⁻Siglec-F⁺Sca-1⁺ cells, while TNC peptide addition reversed this effect. Bar graphs on the left show normalized mature eosinophil counts (out of total structural and hematopoietic cells) in cytopsin preparations of the whole lung *ex vivo* cultures (40x, 10 different fields scored/treatment). Bar graphs on the right quantify the percentage of Sca-1⁺Lin⁻ cells out of total population of Siglec-F⁺ cells in IL-5 culture of *ex vivo* lung homogenates. 3 wells/treatment/culture time point, N=2-3 independent donor cultures. *, $p < 0.05$; **, $p < 0.01$.

Figure 1

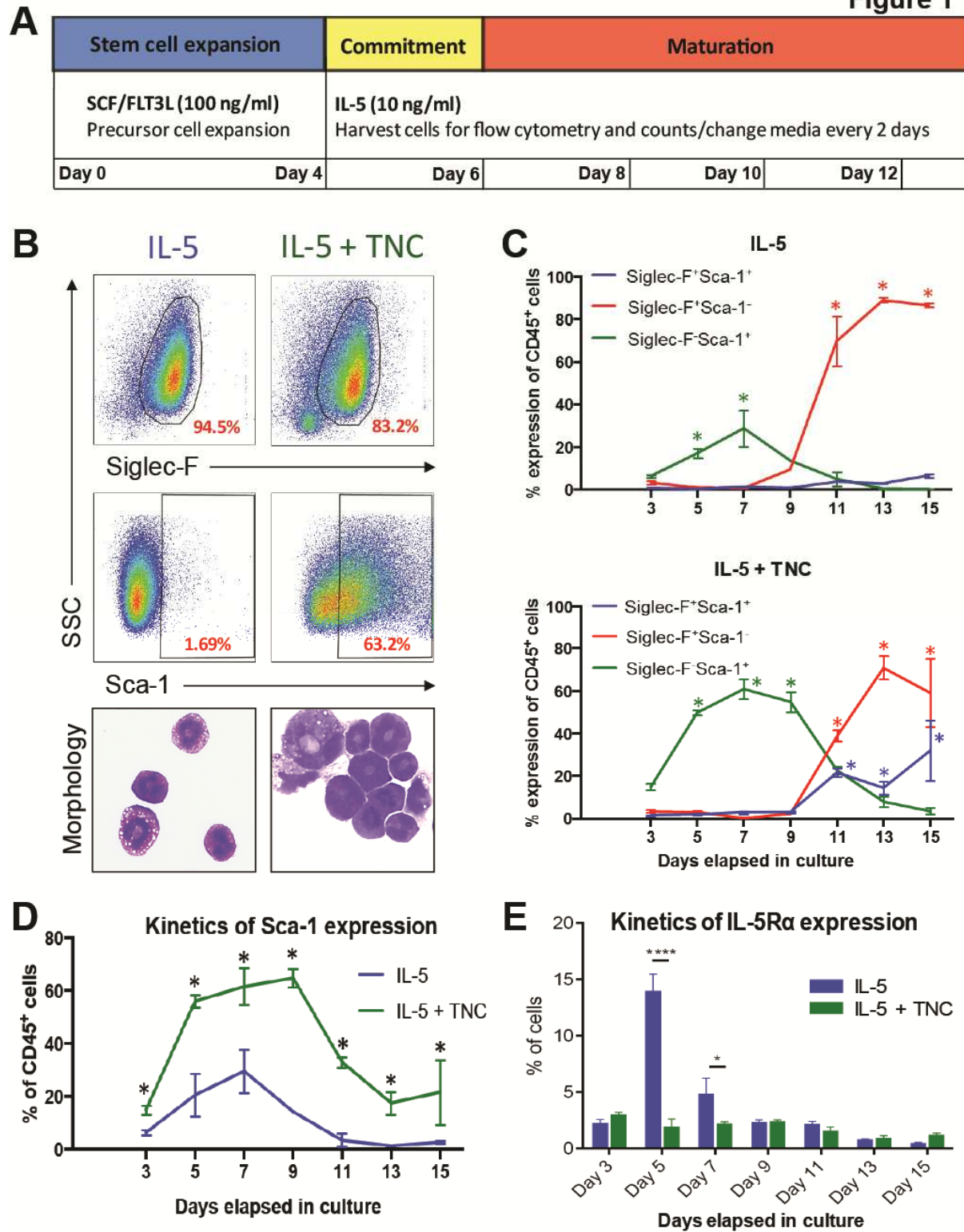
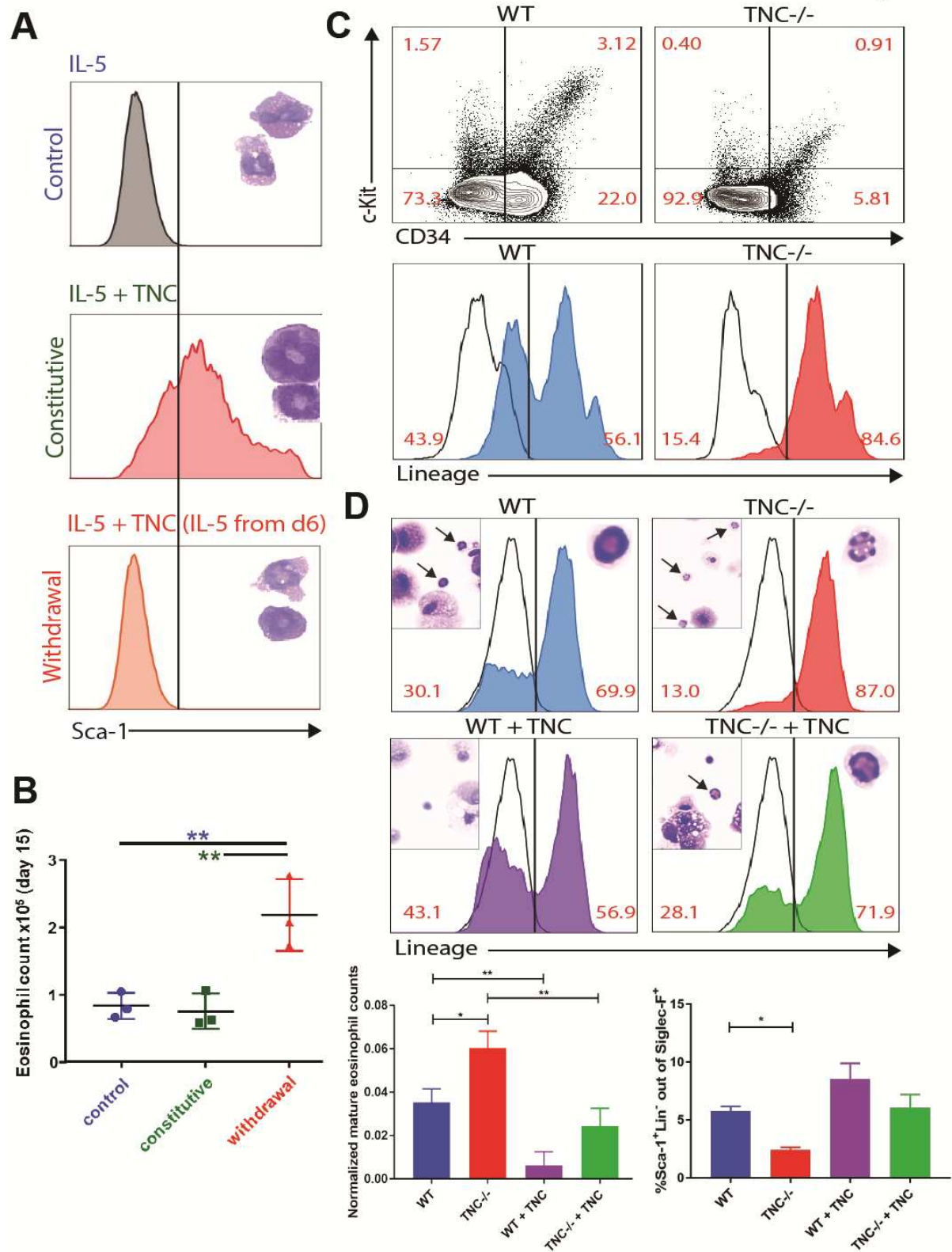


Figure 2



Matrix protein Tenascin C expands and reversibly blocks maturation of murine eosinophil progenitors

Supplemental Document 1. SUPPLEMENTAL METHODS

EXPERIMENTAL PROCEDURES

Mouse model of allergic lung inflammation

All experiments were performed with 6-12 week-old female C57BL/6J mice (Jackson Labs) and age matched female TNC^{-/-} mice (C57BL/6 N-TgH, from RIKEN, Japan). The mice were sensitized by an intraperitoneal injection (200 μ l) of OVA grade V 10 μ g/alum or saline/alum on days 0 and 7 and then challenged intranasally on days 15 (Ch1), 17 (Ch2) and 19 (Ch3) with OVA grade VI (50 μ g/50 μ l saline) or saline alone. Tissues were harvested and processed for analysis at 24 hours after the last OVA challenge.

Ex vivo bone marrow and lung homogenate cultures and Tenascin-C treatments

All experiments were performed with 6-12 week-old female C57BL/6J mice (Jackson Labs) and age matched female TNC^{-/-} mice (C57BL/6 N-TgH, from RIKEN, Japan). Eosinophils were cultured following the established murine bone marrow culture protocol (see reference 17 in Supplemental Document 3). The cytokine treatment timeline and stages of eosinophil development in this culture are illustrated in **Figure 1A**. Samples underwent three different TNC treatments: non-treated (cytokines only, no TNC), TNC treatment (cytokines plus constitutively administered TNC), and TNC withdrawal (cytokines, TNC withdrawal from culture at day 7, further supplemented only with 10 ng/mL mIL-5). For Tenascin-C treatments, we used 25 μ g/mL of MAPTrix Tenascin-C peptide corresponding to fibronectin type III (FN3) binding domain (MAPTrix motif: M-VAEIDGIEL) (Sigma 168314K; Kollodis Biosciences). For *ex vivo* lung cultures, lungs were harvested in sterile conditions and cultured in SCF, FLT3 and IL-5 according to the protocol described in **Figure 1A**. To preserve all lung tissue cellular interactions, structural cells were allowed to adhere and form a monolayer under the proliferating hematopoietic cells, which was maintained at all time points in the culture. *Ex vivo* culture of the lung tissue in IL-5 resulted in a significant *in situ* mature eosinophil yield and allowed us to control for confounding eosinophil progenitor recruitment from the bone marrow.

Flow cytometry

Cells were harvested from plates and washed in 1x PBS in preparation for flow cytometry. Prior to antibody staining, cells were incubated in 500 μ L 1x PBS with 0.25 μ L Zombie Live/Dead dye (Biolegend) in the dark at room temperature for 20 minutes followed by wash steps and incubation at 4°C for 10 minutes with 0.75 μ L/50 μ L murine FC Block (BD Biosciences). Antibody cocktail was added directly to blocked samples and incubated for 30 min at 4°C. All centrifugation steps were carried out at 300 g for 5 minutes in a swing bucket centrifuge. We used the following panel of antibodies and fluorescent dyes to characterize eosinophil cultures: (i) Zombie Live/Dead Fixable Viability Dye (Biolegend/Aqua/0.25 μ L); (ii) Lineage antibody mix [CD11b (clone M1/70), Ter119 (clone TER119), CD3 (clone 17A2), CD4 (clone RM4-5), CD8a (clone 53-6.7), CD19 (clone 6D5), Ly-6G/Ly-6C (Gr1) (clone RB6-8C5), B220 (clone RA3-6B2)] (Biolegend/PerCP-Cy5.5/0.3 μ L each); (iii) Ly-6A/E (Sca-1) (clone D7/Biolegend/PE-Cy7/0.5 μ L); (iv) CD127 (IL-7R α) (clone A7R34/Biolegend/PE/0.4 μ L); (v) CD117 (c-kit) (clone 2B8/Biolegend/PE Dazzle 594/0.4 μ L); (vi) CD125 (IL-5R α) (clone T21/BD Biosciences/Alexa Fluor 488/0.5 μ L); (vii) CD45 (clone 30-

F11/Biolegend/Alexa Fluor 700/0.5 μ L); (viii) Siglec-F (clone E50-2440/BD Biosciences/APC-Cy7/1 μ L); (ix) CD34 (clone MEC14.7/Biolegend/Alexa Fluor 647/1.25 μ L); (x) IL-33Ra (IL1RL1, ST2) (clone DIH9/Biolegend/Brilliant Violet 421/1.75 μ L). Samples were fixed in 2% paraformaldehyde and kept in the dark at 4°C prior to acquisition. Samples were acquired on a BD LSRII flow cytometer (BD Biosciences). Bead compensation (OneComp; eBioscience, San Diego, CA, USA, and ArC; Molecular Probes beads), gating and data analysis were performed using FlowJo v.10 (TreeStar, Inc., Ashland, OR, USA). Only live, single, hematopoietic (CD45+) cells were used in analyses. Fluorescence Minus One (FMO) controls were used to determine gate boundaries during analysis steps.

Cytospin differential staining and counts

Cells cytospun from bronchoalveolar lavages or *ex vivo* lung homogenates cultured in IL-5 were stained with initial fixation/staining in Wright Giemsa solution (EMD Millipore) for 2 minutes, incubation in Eosin Xanthene dye for 3 minutes, and dipping twice in Hematoxylin/Blue/Azure (Electron Microscopy Science). Nuclear morphology and eosin granule staining were visualized at 40x on Olympus DSU microscope (Olympus, USA). Mature eosinophil counts were performed in a blinded fashion on 10 different 40x fields per slide. Eosinophil counts were normalized to total cell counts.

Statistical analysis

Analysis of Variance (ANOVA) followed by Tukey's post-hoc pairwise comparisons was used to assess differences between groups. Kinetics data was analyzed by repeated measures ANOVA (SYSTAT 11, Systat Software, Inc.). 0.05 alpha level was used for significance cut-offs. All data are presented as the means $\pm$ SEM.

Matrix protein Tenascin C expands and reversibly blocks maturation of murine eosinophil progenitors

Supplemental Document 2. SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Total cell counts in bone marrow derived eosinophil cultures. Blue, control culture; green, TNC supplemented culture. 3 wells/treatment/culture time point, N=3 independent donor cultures. Asterisks denote differences between two cultures for each corresponding time point. *, $p < 0.05$.

Supplemental Figure 2. Expression of IL-5R α on CD45⁺Siglec-F⁺ eosinophil progenitors at day 6 in bone marrow culture.

Supplemental Figure 3. Quantification of flow cytometry data shown in Fig 2C. *, $p < 0.05$; ****, $p < 0.0001$.

Matrix protein Tenascin C expands and reversibly blocks maturation of murine eosinophil progenitors

Supplemental Document 3. ADDITIONAL REFERENCES

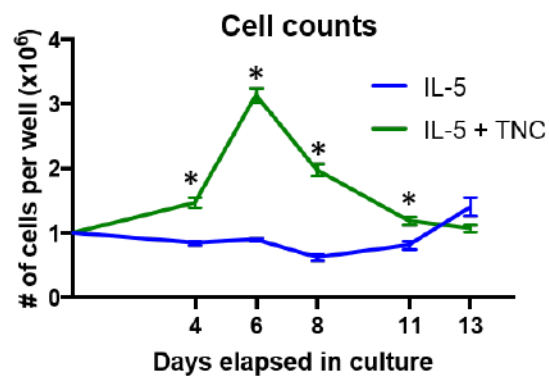
9 references used in the main manuscript are marked in bold

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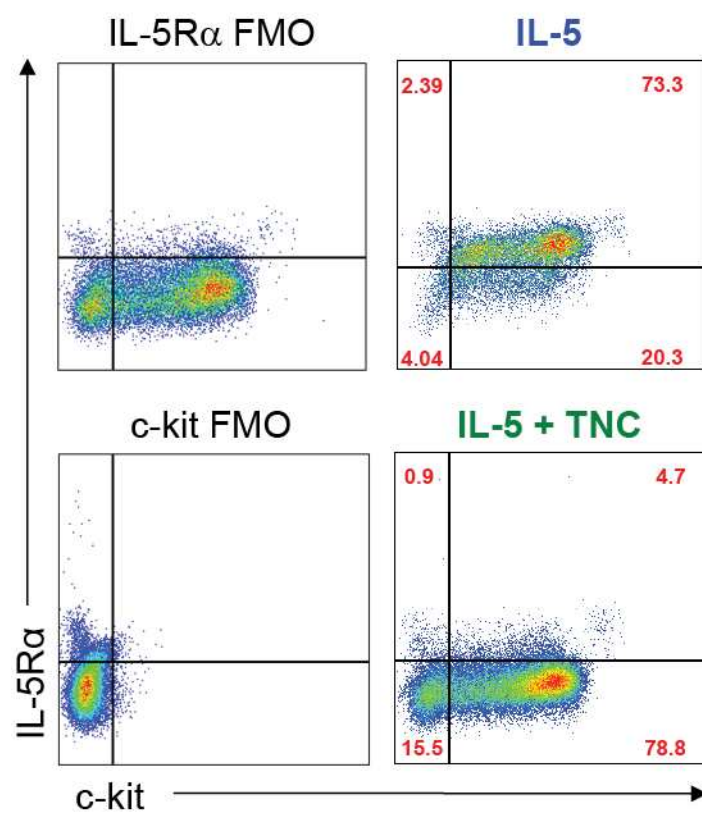
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Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

