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The role of Tenascin C in the lymphoid progenitor cell niche

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Abstract:

Hemopoietic stem cells (HSC) are extrinsically controlled by the bone marrow (BM) microenvironment. Mice devoid of the extracellular matrix molecule Tenascin-C (TNC) were reported to develop normally. The current study explores the relationship between TNC and hemopoiesis, from HSC within their niche to maturing progenitors in alternate niches. While the absence of TNC did not alter the size of the BM stem cell pool, we report decreased thymic T-cell progenitors with redistribution to other lymphoid organs, suggesting an anchoring role for TNC. TNC did not play an essential role in stem and progenitor cell homing to BM, but significantly altered lymphoid primed progenitor cell homing. These cells express the TNC receptor, integrin $\alpha_9\beta_1$, with the same reduced homing evident in the absence of this integrin. The absence of TNC also resulted in an increased proportion and number of mature circulating T-cells. In addition, the absence of TNC significantly impaired hemopoietic reconstitution post-transplant and increased stem and progenitor cell mobilization. In summary, our analysis revealed unidentified roles for TNC in hemopoiesis: in lineage commitment of thymic T-cell progenitors, peripheral T-cell migration and hemopoietic reconstitution.

Introduction:

In adults, hemopoiesis originates in the bone marrow (BM), where hemopoietic stem cells (HSC) reside in a three dimensional niche. However, not all hemopoietic development occurs in the BM, with some lineage specific progenitors completing their differentiation in other organs. For example, lymphoid-primed multipotent progenitors (LMPP) [1, 2] migrate to the thymus to differentiate into thymocytes.

The BM HSC niche attracts, regulates and retains HSC through intricate networks of finely tuned interactions, many of which remain unknown or uncharacterized. We recently revealed that transplanted hemopoietic stem and progenitors preferentially home to the trabecular-rich metaphysis in non-ablated recipients [3]. This region is rich in extracellular matrix (ECM) molecules such as hyaluronan (HA) and osteopontin (Opn), which serve multiple roles in HSC regulation [4-7]. By inference, preferentially expressed metaphysal ECM molecules may also contribute to the HSC niche. Furthermore, ECM molecules are critical in other hemopoietic organs, where they are involved in many regulatory functions such as cell adhesion, migration and differentiation. One such candidate ECM molecule is Tenascin-C (TNC), a tenascin glycoprotein family member. The expression of TNC is regulated by a variety of growth factors, cytokines, vasoactive peptides and ECM molecules. Tenascins exhibit both adhesive and counter-adhesive activities through binding to other ECM components and cell surface receptors [8].

Following birth, TNC is observed as thin fibers permeating amongst BM cells, but in adults TNC expression is significantly decreased and found in endosteal areas extending between hemopoietic cells and around blood vessels [9-11]. In addition, TNC has also been observed in the spleen and thymus as part of the basement membrane and surrounding blood vessels [12-14].

Mice devoid of TNC were independently generated in two laboratories and thought to develop normally [15, 16]. Hemopoiesis in $TNC^{-/-}$ adult mice is similar to wild type (WT) mice in terms of hematocrits, BM

architecture, peripheral blood (PB) nucleated cell counts and spleen weights [17]. However, an observed decrease in BM colony-forming ability and proliferative capacity and longevity of long-term BM cultures (LT-BMC) led to the proposed role for TNC as a mediator between the microenvironment and hemopoietic progenitors. In addition, Schreiber *et al.* recently revealed that TNC acts as a cytoadhesive on human $CD34^+$ cells [18].

Since Ohta *et al.* described these hemopoietic defects in $TNC^{-/-}$ established LT-BMC [17], there has been considerable advances in the identification and isolation of purified populations of HSC and progenitors, enabling putative roles for TNC in hemopoiesis to be investigated. Using the $TNC^{-/-}$ mouse model, the current study explores the role of TNC in hemopoiesis. A role for TNC in lineage commitment, hemopoietic reconstitution and progenitor retention within their niche is unveiled.

Materials and Methods:

Mice

All mice were bred at Monash Animal Services (Monash University, Victoria, Australia) and maintained on a C57BL/6J background. $TNC^{-/-}$ mice were provided by Professor Eleanor Mackie (University of Melbourne, Victoria, Australia). Red fluorescent protein (RFP) mice were provided by Professor Patrick Tam (Children's Medical Research Institute, NSW, Australia). Mice floxed for the α_9 gene ($\alpha_9^{flox/flox}$) provided by Dean Sheppard (University of California, San Francisco, USA) were mated with Vav-Cre mice provided by Warren Alexander (WEHI, Victoria, Australia) to generate $\alpha_9^{-/-}$ mice. Mice were 6-8 weeks old and sex matched for experiments approved by the Monash Animal Research Platform Ethics Committee. Irradiation was administered in a split dose (4.5Gy each) 6 hours apart and 24 hours prior to transplant. Unlabelled irradiated ($2x10^5$, 15Gy) C57BL/6J BM cells were used as carrier cells for transplants.

Isolation and analysis of BM stem and progenitor cells

Populations of murine stem and progenitors (lineage $(Lin)^-Sca\text{-}1^+c\text{-}kit^+CD150^+CD48^-$, LSKSLAM cells and $Lin^-Sca\text{-}1^+c\text{-}kit^+$, LSK cells) were isolated from the endosteal (e) and central (c) BM regions as previously described [6]. Alternatively, enriched populations of LSK cells were isolated by grinding and enzymatically digesting whole bones as previously described [19].

Analysis of lineage composition in $TNC^{-/-}$ and WT mice

Peripheral blood (PB) was lysed with 0.83% ammonium chloride. Single cell suspensions of spleen, thymus and lymph nodes (LN) (superficial cervical, axillary, brachial and inguinal) were obtained and analyzed. In a subset of experiments, cell cycling was analyzed by staining with 10μM Hoechst33342.

Flow cytometry

Fluorescence activated cell sorting (FACS) was performed using a Cytopeia Influx 516SH cell sorter (Cytopeia, WA, USA) and analysis post-transplant performed using a LSR II analyser (BD, Franklin Lakes, NJ, USA) as previously described [3]. For analysis of stem and progenitor cell numbers, lineage composition and T-cell differentiation in BM, spleen, thymus, LN and PB, 0.3-5x10^6 events were analyzed.

Homing assays

For homing experiments, sorted cells were labeled with the fluorescent tracking dye 5- (and 6-) Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE, CFSE when incorporated, Molecular Probes, Eugene, Oregon, USA) or Seminaphtorhodafluor-1-Carboxylic Acid Acetate Succinimidyl Ester (SNARF) (Invitrogen, USA) as previously described [6]. Briefly, either eLSK or cLSK (~3-8x10^4 cells), LMPP or LSK-LMPP cells from WT, $TNC^{-/-}$ and $\alpha_9^{-/-}$ mice (~1-8x10^4 cells) were transplanted into the lateral tail vein of non-myeloablated recipients and homing assessed after 15 hours. To ensure 50-400 positive events were analyzed, 1-2.5x10^7 nucleated cells were processed. The homing efficiency was expressed as the percentage of injected cells assuming one femur, tibia and iliac crest represents 15% of total BM mass [20].

Transplantation assay

Two hundred RFP LSK cells were transplanted into lethally irradiated WT or $TNC^{-/-}$ recipients. Peripheral blood reconstitution was assessed after 6, 12 and 20 weeks and BM after 20 weeks. Mice with greater than 1% donor lymphoid and myeloid reconstitution were designated as multi-lineage.

Mobilization

$TNC^{-/-}$ and WT mice were mobilized using subcutaneous injections of G-CSF (Filgrastrim, Amgen) at 250μg/kg in saline twice daily, 6-8 hours apart, for 2, 4 or 6 consecutive days. Control animals received an equivalent volume of saline. PB was analyzed for nucleated as well as stem and progenitor cell incidence. Colony-forming potential of 4000 nucleated PB cells were assayed in a double-layer nutrient agar culture system as previously described [21, 22].

$\alpha_9\beta_1$ expression on murine hemopoietic progenitors

The expression of α_9 on LMPP and ETP was assessed using anti-mouse α_9 antibody (R&D, 20 μg/ml) followed by secondary conjugated to AF594 (Molecular Probes, 2.5 μg/ml). The expression of β_1 on LMPP and ETP was assessed using anti-mouse β_1 antibody (R&D, 10 μg/ml) followed by secondary conjugated to AF594 (Molecular Probes, 2.5 μg/ml).

Immunohistochemistry

Immunolabelling of thymus sections was performed as previously described [3]. Briefly, sections were incubated with rat anti-mouse TNC antibody (5 μg/ml) and the signal amplified using tyramide signal amplification (PerkinElmer, MA, USA). Biotinyl-tyramide was visualized using streptavidin-AF488 (Invitrogen, 1 μg/ml). Sections were counterstained with 0.25 μg/ml DAPI. Immunofluorescent images were recorded using a SPOT RT-SE6 Slider cooled CCD camera (Diagnostic Instruments, Miami, USA) fitted on an Olympus BX51 microscope (Australia) equipped with conventional epifluorescence.

Statistical analysis

Data are mean±SEM, with differences evaluated using SigmaPlot 11.0 (Germany) or Statcalc (StatPac Inc. MN, USA). Students t-test, Mann Whitney Rank Sum test, one-way ANOVA or two-way ANOVA were used where appropriate and considered statistically significant at a P value of <0.05. For

comparisons of the proportion of transplant recipients with multi-lineage reconstitution, a two-sample t-test between proportions was performed.

Results:

The absence of TNC results in an increased proportion and number of BM T-cells.

Consistent with previous data [17], $TNC^{-/-}$ mice were equivalent to WT mice in terms of BM cellularity and body weights (data not shown). Compared to WT, analysis of both central and endosteal BM revealed significant increases in the incidence and total number of $CD3^{+}$ T-cells in $TNC^{-/-}$ mice (Fig. 1) but no differences in the incidence or total number of other cell lineages ($B220^{+}$, MAC-1^{+} or GR-1^{+} cells) (data not shown).

The absence of TNC does not alter BM stem and progenitor incidence, numbers or homing ability.

To determine if the absence of TNC in the BM microenvironment affected hemopoietic stem and progenitor cells, endosteal and central BM was analyzed for LSK and LSKSLAM (Fig. 2A, B). No differences in the incidence or number of these cells were detected in either region (Fig. 2C-F). Our results are consistent with and expand upon previously published data [11], suggesting TNC does not play a critical role in regulating the size of the HSC pool.

The preferential expression of TNC in the metaphysis [11] suggests a role in the HSC niche. To determine if TNC is involved in the attraction of stem and progenitors to the niche, short-term homing assays were performed. As previously described [23], endosteal WT LSK cells have a significantly enhanced ability to home to BM 15 hours post-transplant compared to their central counterparts (Fig. 2G, H). However, when transplanted into $TNC^{-/-}$ recipients, homing of endosteal and central WT LSK cells was equivalent (Fig. 2H), suggesting that TNC plays a role in the normally observed enhanced homing ability of endosteally located stem and progenitor cells. Since the absence of TNC did not overtly decrease homing, next we wanted to examine the reconstitution ability of WT stem and progenitors following transplant into lethally ablated $TNC^{-/-}$ recipients.

The absence of TNC significantly impairs hemopoietic reconstitution and recapitulates the lineage distribution skewing towards T-cells.

Enriched populations of WT LSK cells were transplanted into lethally irradiated WT and $TNC^{-/-}$ recipients (Fig. 3A). Early post-transplant (6 weeks), donor cell recovery was reduced in the PB of $TNC^{-/-}$ recipients (Fig. 3B), but this delay in hemopoietic reconstitution was no longer detectable after 12 weeks (data not shown). In addition, 6 weeks post-transplant, a significantly higher proportion of circulating T-cells ($CD3^{+}$) was detected in the absence of TNC (Fig. 3C), recapitulating the lineage skewing evident in unmanipulated $TNC^{-/-}$ mice (Fig. 1). This change in PB lineage distribution of transplanted $TNC^{-/-}$ recipients was maintained long-term post-transplant, with a significant increase in the proportion of T-cells accompanied by a significant decrease in both the proportion and absolute number of myeloid cells (GR-1/MAC-1^{+}) evident at both 12 (Fig. 3D-F) and 20 weeks (Fig. 3G-I) post-transplant.

In addition, $TNC^{-/-}$ recipients also revealed a significant increase in the proportion of BM T-cells (Fig. 3J) accompanied by a significant decrease in the proportion and total number of BM myeloid cells (Fig. 3K-L). Furthermore, long-term transplant analysis revealed a significantly reduced proportion of multi-lineage reconstitution in the BM of $TNC^{-/-}$ recipients compared to their WT counterparts (10/15, 67% and 12/12, 100% respectively, $p = 0.03$): with all recipients lacking multi-lineage reconstitution revealing an absence of myeloid cells. No changes in overall cellularity of PB or BM were detected at any of the time-points (data not shown). Together, this further suggests a role for TNC in hemopoietic lineage commitment.

The absence of TNC reduces the proportion and number of T-cell progenitors.

Recently, a population of cells primed for lymphoid commitment, LMPP, was identified in BM [1, 2]. These cells migrate to the thymus to give rise to early thymic progenitors (ETP) [24] and ultimately thymocytes. As an increase in mature $CD3^{+}$ T-cells was observed in the absence of TNC, we analyzed the

incidence and number of LMPP in WT and $TNC^{-/-}$ BM (Fig. 4A). No significant differences in the incidence or total number of LMPP were detected (data not shown).

To explore the potential role of TNC in thymocyte development, thymic T-cell lineage commitment in WT and $TNC^{-/-}$ mice was assessed. Expression of TNC in the thymus was confined to the medulla, capsule (insert) and around vessels (Fig. 4B-D). ETP (Fig. 4E) give rise to DN1-4 T-cell progenitors (Fig. 4F) and triple negative (TN, $CD3^-CD4^-CD8^-$) $TNCD25^+$ (Fig. 4G), followed by DP, SP4 and SP8 T-cells (Fig. 4H) [25]. In the absence of TNC, there was a significant decrease in the incidence and total number of both thymic $Flt3^+$ and $Flt3^-$ ETP (Fig. 4E, Table 1, 2), accompanied by a redistribution of ETP to extra-thymic sites where they are not usually detected, with a significant increase in the proportion and number of ETP detected in both the spleen and LN (Table 1, 2). Furthermore, in the absence of TNC a significant reduction in the proportion and total number of thymic DN1-4 cell populations was observed (Fig. 4F, Table 1, 2). In addition, a redistribution of the proportion of cells expressing CD44 and CD25 within the DN thymic fraction of $TNC^{-/-}$ mice was detected, with a significant decrease in the proportion of $CD44^+CD25^+$ cells accompanied by a significant increase of $CD44^-CD25^+$ cells (Table 1, indented). Similarly to the DN2 and 3 subpopulations, within the DN there was a redistribution of cells expressing CD25 and CD3, with a significant increase in the proportion of $CD3^-CD25^+$ and $CD3^+$ cells in $TNC^{-/-}$ mice (Table 1). To ensure the observed differences were not due to changes in cell cycle we assessed the cycling rate of ETP in the thymus, LN and spleen as well as the DN population in the thymus. We detected no significant differences in the cell cycle of these T-cell sub-populations between $TNC^{-/-}$ and WT mice (data not shown).

In the absence of TNC there was also a significant reduction in the proportion and total number of thymic $TNCD25^+$ cells (Fig. 4G, Table 1, 2) and analysis of thymocytes revealed a significant decrease in the proportion and total number of thymic naïve cytotoxic SP8 T-cells (Fig. 4H, Table 1, 2), but the

proportion and number of naïve SP4 T-cells detected in the thymus, spleen or LN was not altered. A significant decrease in the proportion and total number of $DNCD3^+$ cells was observed in the spleen, but no changes in the incidence or number of these cells in the thymus or LN was detected (Fig. 4I, Table 1, 2). The data suggest a role for TNC in lineage commitment, although this did not appear to be through the rate of progenitor cell cycling. Next we wanted to investigate the role of TNC on T-cell progenitors in the BM.

TNC plays a role in anchoring T-cell progenitors through the integrin $\alpha_9\beta_1$.

$TNC^{-/-}$ mice contained equivalent BM LSK numbers to WT mice but the observed decrease in T-cell progenitors in $TNC^{-/-}$ thymus suggests a possible change in T-cell migration and/or adhesion. To begin to understand the role of TNC in T-cell progenitor cell migration we performed short-term homing assays. LMPP isolated from $TNC^{-/-}$ donors had a significantly reduced ability to home back to WT BM post-transplant compared to their WT controls (Fig. 5A). This could be due to LMPP being primed to migrate out of the BM, an idea supported by the significant increase in the proportion of LMPP detected in the spleen in unmanipulated $TNC^{-/-}$ mice (Table 1). Although, the ability of LMPP to home to the thymus post-transplant was assessed both at 15 hours and 48 hours post transplant, the frequency of cells homing to the thymus were too small to make any definitive conclusions (data not shown; more than $40x10^6$ events processed) When WT LMPP were transplanted into $TNC^{-/-}$, no significant differences in homing to BM were detected compared to transplants into their WT controls (data not shown), suggesting that the altered microenvironment in which the $TNC^{-/-}$ LMPP developed was critical for their homing ability post-transplant.

Next we examined the effect of TNC on cell binding. TNC binds with high affinity to the integrin $\alpha_9\beta_1$ [26], which we have previously shown to be expressed on hemopoietic stem and progenitors, interacting with Opn as a negative regulator for the HSC pool [22]. To begin to understand the mechanism through

which the absence of TNC influences LMPP migration and potentially thymocyte differentiation, we analyzed $\alpha_9\beta_1$ expression on LMPP and ETP. Similarly to LSKCD34$^-$ cells, both LMPP and ETP expressed $\alpha_9\beta_1$ (Fig. 5B, C), suggesting a role for the interaction of $\alpha_9\beta_1$ and TNC in anchoring LMPP and ETP in the BM and thymus. When $\alpha_9^{-/-}$ LMPP were isolated from BM and transplanted into WT recipients, they showed reduced homing back to BM (Fig. 5D), suggesting similar priming to TNC$^{-/-}$ LMPP or an inability to bind and be retained in the microenvironment. Together, these data suggest that in BM, TNC is involved in anchoring progenitors through $\alpha_9\beta_1$. In the absence of the TNC-$\alpha_9\beta_1$ interaction, LMPP are released and "primed" for migration to other sites.

To further test the role of TNC plays a role in anchoring progenitor cells in their niche, we examined G-CSF induced mobilization in TNC$^{-/-}$ mice. The administration of G-CSF to TNC$^{-/-}$ mice resulted in significantly more nucleated cells in the PB on days 2, 4, and 6 post-G-CSF compared to WT (Fig. 5E) and this was accompanied by the detection of significantly more LSK cells in the PB on days 4 and 6 post-G-CSF (Fig. 5F, G). *In vitro* colony-forming assays confirmed increased mobilization in the PB of TNC$^{-/-}$ mice (Fig. 5H). No statistical differences were observed between spleen weights of TNC$^{-/-}$ and WT mice (data not shown). Collectively, this data supports a role for TNC in retaining HSC within the BM niche, via the integrin $\alpha_9\beta_1$.

Discussion

In this study we have identified a novel role for the ECM molecule, TNC, in thymic T-cell development; with decreased ETP, committed thymic progenitors and naïve cytotoxic T-cells evident in $TNC^{-/-}$ thymus. This decrease in thymic T-cell progenitors was not a result of changes in cell cycling and was accompanied by a redistribution of ETP to LN and spleen, organs where these cells are not usually detected. Together, these data suggest that TNC is playing a cytoadhesive role that is important to thymic T-cell development. In support of this premise, a role for TNC in cytoadhesion, acting either directly [10, 18, 27] or indirectly [10, 28-30], has been demonstrated for other cell types and in addition, we showed that G-CSF induced mobilization is more efficient in microenvironments devoid of TNC.

The mechanism by which TNC acts as a cytoadhesive for HSC and sub-populations of T-cell progenitors in both the BM niche and thymus is unclear. We have previously demonstrated that $\alpha_9\beta_1$, a high-affinity receptor for TNC [26], is expressed on stem and progenitors and plays a critical role in the negative regulation of the HSC pool as well homing of transplanted HSC to the BM via binding to thrombin cleaved Opn [22]. In the current study we extend these observations, demonstrating the hemopoietic progenitor sub-populations LMPP as well as ETP express $\alpha_9\beta_1$. Consequently, we hypothesize that in the absence of TNC, LMPP BM thymocyte progenitors are not 'held' in the BM and are thus primed to leave. Subsequently, when transplanted into WT mice, $TNC^{-/-}$ LMPP showed a significantly reduced ability to home back to the BM. The decreased number of ETP, progenitors and T cells in the thymus of $TNC^{-/-}$ mice in concert with the appearance of ETP in the LN and spleen suggests TNC binding to $\alpha_9\beta_1$ on LMPP and ETP is important for their anchoring within the thymus.

Support for TNC activating integrin mediated binding stems from a recent study where a TNC derived small peptide was shown to activate β_1 integrins, stimulating β_1 integrin-mediated cell adhesion to

fibronectin [31]. The region corresponding to the peptide is exposed in TNC cleavage by matrix-metalloproteinase 2 or via the interaction of TNC with other ECM molecules. The interaction of $\alpha_9\beta_1$ with tenascins has been shown to stimulate cell proliferation [32]. Recently, Nakamura-Ishizu *et al.* revealed TNC increased the *in vitro* proliferation of stem and progenitor cells via an α_9-dependent mechanism [11]. As a consequence, the absence of TNC in the thymus may also result in the loss of a normal proliferative stimulus for early thymic progenitors and may be a contributing factor to the observed decrease in ETP, thymic progenitors and naïve cytotoxic T-cells. However, as no differences were detected following cell cycle analysis of $TNC^{-/-}$ and WT thymic progenitors, this would not appear to be the dominant mechanism.

Progenitors migrating to the thymus interact with thymic epithelial reticular cells, with the resulting activation of Notch signaling marking the initiation of T-cell lineage commitment [24, 33]. The absence of TNC potentially compromises progenitor cell anchoring post thymic migration, leading to fewer progenitors undergoing T-cell lineage commitment and consequently the redistribution of LMPP and ETP to other lymphoid organs. In contrast to TNC, Opn, the other ECM molecule known to bind to $\alpha_9\beta_1$, is predominantly intracellular in the thymus and plays a role in effector T-cell development [34], rendering it unlikely to play a compensatory role in the anchoring of thymic progenitors in the absence of TNC.

Despite finding significantly decreased T-cell progenitors in the thymus of $TNC^{-/-}$ mice, we found significantly increased $CD3^+$ mature T-cells in the BM. This may be due to the previously identified role of TNC in down regulating $CD3^+$ lymphocyte proliferation through blocking early signaling events following T-cell receptor stimulation [35]. In addition, previous reports have shown that during steady-state conditions, the thymus is not responsible for the maintenance of the peripheral T-cell pool, although it does regulate the repertoire of cells within the pool [36] and that T-cell recovery after T-cell depletion is predominantly due to peripheral expansion of resistant mature T-cells [37].

Interestingly, the previously reported enhanced ability of endosteal stem and progenitor cells to home to BM compared to their central counterparts was not observed in the absence of TNC [23]. This suggests a role for TNC in the functional differences evident for these cells isolated from different BM regions. Furthermore, although Opn forms part of the BM ECM, our data suggests it is not playing a significant compensatory role in anchoring BM LMPP.

Analysis of hemopoietic reconstitution in a $TNC^{-/-}$ microenvironment revealed an impaired ability to support WT stem and progenitor cells post-transplant. In addition, there was a significant decrease in the number of recipients with long-term multi-lineage reconstitution. This was evidenced by a recapitulation of the lymphoid skewing evident in steady-state $TNC^{-/-}$ mice, with an increase in the proportion of $CD3^{+}$ T-cells in the peripheral blood and BM, accompanied by a significant decrease in the proportion and total number of myeloid committed cells. Our data is supported by Nakamura-Ishizu *et al.* [11] who showed increased TNC expression within BM following myeloablation with 5-fluoruracil, as well as a defect in BM reconstitution in $TNC^{-/-}$ mice following BM ablation. Although the authors reported a slight elevation in BM $CD3^{+}$ cell frequency following ablation, they did not observe an increase in $CD3^{+}$ cells during steady-state hemopoiesis. Discrepancies between data sets could be due to the age and sex of mice assessed.

Together, our data demonstrate previously unidentified roles for this ECM glycoprotein in hemopoiesis: T-cell progenitor lineage commitment, hemopoietic reconstitution and T-cell migration. Analysis suggests that the integrin $\alpha_9\beta_1$ is integral in these processes, although further studies are required to delineate the specific mechanisms of its role.

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Authorship Contributions

S.E., S.H. and S.N. designed and performed experiments, analyzed data and wrote the manuscript. B.W., A.R, J.G. and J.B. performed experiments. C.H performed experiments and critically assessed the manuscript. A.C. contributed intellectually and helped prepare the manuscript.

Disclosure of Conflict of Interest

There are no conflicts of interest

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Table 1: Incidence of T-cell progenitors and maturing T-cells within the spleen, thymus and lymph nodes of WT and $TNC^{-/-}$ mice

	Spleen	**Thymus**	**Lymph Nodes**
LMPP ($Lin^-Sca^+c\text{-}it^+Flt3^{high}CD34^+$)	p=0.02 0.0008±0.0002, 0.001±0.0002	NS	NS
%LSK $Flt3^{high}CD34^+$	NS	NS	NS
ETP ($Lin^-CD4^-CD8^-CD44^+c\text{-}kit^+CD25^-Flt3^+$)	p=0.002 0.004±0.0008, 0.01±0.002	p=0.002 0.008±0.0007, 0.004±0.0006	p=0.04 0.003±0.0008, 0.005±0.0007
ETP ($Lin^-CD4^-CD8^-CD44^+c\text{-}kit^+CD25\text{-}Flt3^-$)	NS	p=0.009 0.05±0.005, 0.03±0.005	NS
DN ($CD4^-CD8^-$)	NS	p=0.002 3.4±0.3, 2.3±0.1	p=0.03 17.5±2.2, 23.8±0.9
$B220^-CD3^-CD44^+CD25^-$	ND	NS	ND
$CD44^+CD25^+$	ND	p=0.03 5.4±0.4, 4.4±0.2	ND
$CD44^-CD25^+$	ND	p<0.001 41.9±0.6, 47.2±0.9	ND
$CD25^-CD44^-$	ND	NS	ND
$CD3^-CD25^+$	ND	p<0.001 52.8±0.6, 57.5±0.3	ND
$CD3^+$	p=0.009 2.5±0.2, 1.5±0.2	p=0.001 11.7±0.3, 13.9±0.4	NS
DN1 ($B220^-CD3^-CD44^+CD25^-$)	NS	p<0.001 0.4±0.02, 0.3±0.02	p=0.03 16.4±2.4, 22.6±0.9
DN2 ($CD4^-CD8^-CD44^+CD25^+$)	ND	p<0.001 0.2±0.01, 0.1±0.008	ND
DN3 ($CD4^-CD8^-CD44^-CD25^+$)	ND	p=0.03 1.4±0.1, 1.1±0.04	ND
DN4 ($CD4^-CD8^-CD25^-CD44^-$)	ND	p=0.03 1.2±0.1, 0.8±0.07	ND
TN $CD25^+$ ($CD4^-CD8^-CD3^-CD25^+$)	ND	p=0.03 1.8±0.2, 1.3±0.06	ND

DNCD3^{+} ($CD4^{-}CD8^{-}CD3^{+}$)	p=0.001 1.2±0.08, 0.8±0.06	NS	NS
DP ($CD4^{+}CD8^{+}$)	NS	p=0.006 81.3±0.6, 83.8±0.4	NS
SP_4 ($CD4^{+}CD8^{-}CD25^{-}CD44^{-}$)	NS	NS	NS
SP_8 ($CD8^{+}CD4^{-}CD25^{-}CD44^{-}$)	NS	NS	NS
Naïve helper T-cells ($CD4^{+}CD8^{-}CD62L^{+}CD69^{-}$ $CD44^{-}$)	NS	NS	NS
Naïve cytotoxic T-cells ($CD8^{+}CD4^{-}CD62L^{+}CD69^{-}$ $CD44^{-}$)	NS	p=0.009 2±0.1, 1.5±0.06	NS

Data is mean%±SEM of WT and $TNC^{-/-}$ respectively. Depending on cell frequency, at least $3x10^{5}$-$5x10^{6}$ events were analyzed per sample. Repeats≥2, number of mice≥5. ND=Not done, NS=Not significant. Indentation=proportion of sub-population within DN population. Green represents a significant decrease in $TNC^{-/-}$ mice. Red represents a significant increase in $TNC^{-/-}$ mice.

Table 2: Absolute numbers of T-cell progenitors and maturing T-cells within the spleen, thymus and lymph nodes of WT and $TNC^{-/-}$ mice

	Spleen x10^3	**Thymus x10^6**	**Lymph Nodes x10^3**
LMPP ($Lin^-Sca^+c\text{-}kit^+Flt3^{high}CD34^+$)	NS	NS	NS
%LSK $Flt3^{high}CD34^+$	NS	NS	NS
ETP ($Lin^-CD4^-CD8^-CD44^+c\text{-}kit^+CD25^-Flt3^+$)	p=0.03 3.3±0.6, 6.4±1	p=0.008 0.01±0.002, 0.006±0.001	p=0.006 0.3±0.1, 0.8±0.1
ETP ($Lin^-CD4^-CD8^-CD44^+c\text{-}kit^+CD25\text{-}Flt3^-$)	ND	p=0.02 0.09±0.01, 0.05±0.008	ND
DN ($CD4^-CD8^-$	NS	p=0.004 5.1±0.5, 3.1±0.3	NS
DN1 ($B220^-$ $CD3^-CD44^+CD25^-$)	ND	p=0.001 0.7±0.05, 0.4±0.03	NS
DN2 ($CD4^-CD8^-CD44^+CD25^+$)	ND	p<0.001 0.3±0.02, 0.1±0.02	ND
DN3 ($CD4^-CD8^-CD44^-CD25^+$)	ND	p=0.01 2.1±0.2, 1.4±0.1	ND
DN4 ($CD4^-CD8^-CD25^-CD44^-$)	ND	p=0.01 1.9±0.2, 1.1±0.1	ND
$TNCD25^+$ ($CD4^-CD8^-CD3^-CD25^+$)	ND	p=0.01 2.6±0.3, 1.8±0.1	ND
DN $CD3^+$ ($CD4^-CD8^-CD3^+$)	p=0.004 943.1±126.8, 470.5±58.1	ND	ND
DP ($CD4^+CD8^+$)	ND	NS	ND
SP_4 ($CD4^+CD8^-CD25^-CD44^-$)	ND	ND	ND
SP_8 ($CD8^+CD4^-CD25^-CD44^-$)	ND	ND	ND
Naïve helper T-cells ($CD4^+CD8^-CD62L^+CD69^-CD44^-$)	ND	ND	ND
Naïve cytotoxic T-cells ($CD8^+CD4^-CD62L^+CD69^-CD44^-$)	ND	p=0.01 3±0.3, 2±0.2	ND

Data is mean cell number±SEM of WT and $TNC^{-/-}$ respectively. Depending on cell frequency, at least $3x10^5$-$5x10^6$ events were analyzed per sample. Repeats≥2, number of mice≥5. ND=Not done, NS=Not significant. Green represents a significant decrease in $TNC^{-/-}$ mice. Red represents a significant increase in $TNC^{-/-}$ mice.

Figure Legends

Figure 1. The absence of TNC results in an increased proportion and number of BM T-cells.

A significant increase in the incidence and total number of $CD3^+$ T-cells in the central **(A-B)** and endosteal **(C-D)** BM regions of $TNC^{-/-}$ mice compared to WT controls (n = 9, biological repeats = 3). Error bars are mean±SEM.

Figure 2. The absence of TNC does not alter BM stem and progenitor incidence, numbers or general homing ability.

Central and endosteal BM progenitors (LSK R1, **A**) and stem cells (LSKSLAM R2, **B**) were assessed in WT and $TNC^{-/-}$ mice. No significant differences in the incidence or total numbers of LSK cells **(C-D)** or LSKSLAM cells **(E-F)** were detected. Each symbol represents an individual animal and each color a biological repeat. $p > 0.05$. i.c,f,t = iliac crest, femur and tibia. WT endosteal and central BM LSK cells were transplanted into WT or $TNC^{-/-}$ recipients and the percentage of donor cells homed to the BM analyzed using flow cytometry **(G-H)**. ($n \geq 9$, biological repeats = 3). The white region in the bottom left hand corner of **(G)** is a result of gating out the double negatives to allow the maximum number of positive events to be saved and analyzed in each file. Error bars are the mean±SEM.

Figure 3. The absence of TNC significantly impairs hemopoietic reconstitution and recapitulates the lineage distribution skewing towards T-cells.

A limited number (200) of enriched RFP LSK were transplanted into lethally irradiated WT and $TNC^{-/-}$ recipients **(A)**. Six weeks post-transplant analysis of peripheral blood revealed significantly reduced numbers of donor RFP cells in $TNC^{-/-}$ recipients compared to WT controls **(B)**, associated with a significant increase in the incidence of circulating $CD3^+$ T-cells **(C)**. At 12 and 20 weeks post-transplant the significant increase in the incidence of circulating T-cells was still evident **(D** and **G** respectively**)** and

was associated with a significant decrease in incidence and total numbers of circulating myeloid (GR-1^+ / MAC-1^+) cells **(E-F**, and **H-I** respectively**)**. Furthermore, analysis of the BM after 20 weeks also revealed an increase in the incidence of T-cells **(J)** accompanied by a significant decrease in incidence and total numbers of myeloid cells **(K-L** respectively**)**. Each symbol represents an individual animal and each color a biological repeat. Error bars are mean±SEM.

Figure 4. The absence of TNC reduces the proportion and number of hemopoietic T-cell progenitors.

Lymphoid-primed multipotent progenitors (LMPP) were assessed in WT and $TNC^{-/-}$ mice following sequential gating through LSK gate **(A).** TNC expression in the medulla and capsule (insert) of the thymus **(B)**, **(C) =** isotype control, **(D)** = $TNC^{-/-}$ control. Micrographs captured using x20 objective. Analysis of WT and $TNC^{-/-}$ thymic early thymic progenitors (ETP) **(E)**; T-cell progenitors (DN1, DN2, DN3 and DN4) **(F)**; $TNCD25^+$ **(G)**; T-cells (DN, DP, SP_4 and SP_8) **(H)**; and $DNCD3^+$ **(I)**.

Figure 5. TNC plays a role in anchoring T-cell progenitor through integrin $\alpha_9\beta_1$ binding and $TNC^{-/-}$ mice show increased mobilization following G-CSF administration.

The homing ability of WT and $TNC^{-/-}$ LSK cells, subdivided into LMPP and LSK-LMPP, into WT recipients was assessed (n = 3-5, **biological repeats** = 2) **(A)**. Flow cytometry and fluorescence microscopy analysis revealed BM LMPP and thymic ETP express α_9 and β_1 **(B-C)**. Images are representative of at least two biological repeats. The homing ability of WT and $\alpha_9^{-/-}$ LSK cells, subdivided into LSK-LMPP and LMPP, into WT recipients was assessed (n = 2-5, biological repeats = 1-2) **(D)**. Following G-CSF administration, $TNC^{-/-}$ mice (purple line) exhibited significantly higher numbers of nucleated cells in the PB on days 2, 4 and 6 **(E**) and significantly higher numbers of LSK on days 4 and 6 **(F-G)**. Per mouse, 4000 PB nucleated cells were plated in agar assays and colonies were scored after 14

days **(H).** * = $p < 0.05$. ** = $p < 0.001$. $n \geq 4$. Error bars represent the mean±SEM and results normalized to saline injected controls.

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