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Editor's Choice

Cosmc is required for T cell persistence in the periphery

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Abstract

T lymphocytes, a key arm of adaptive immunity, are known to dynamically regulate O-glycosylation during T cell maturation and when responding to stimuli; however, the direct role of O-glycans in T cell maturation remains largely unknown. Using a conditional knockout of the gene (*C1GalT1C1* or *Cosmc*) encoding the specific chaperone Cosmc, we generated mice whose T cells lack extended O-glycans (T cell conditional *Cosmc* knock out or TCKO mice) and homogeneously express the truncated Tn antigen. Loss of *Cosmc* is highly deleterious to T cell persistence, with near-complete elimination of *Cosmc*-null T cells from spleen and lymph nodes. Total T cell counts are 20% of wild type (WT), among which only 5% express the truncated glycans, with the remaining 95% consisting of escapers from *Cre*-mediated recombination. TCKO thymocytes were able to complete thymic maturation but failed to populate the secondary lymphoid organs both natively and upon adoptive transfer to WT recipients. Our results demonstrate that extended O-glycosylation is required for the establishment and maintenance of the peripheral T cell population.

Key words: Cosmc, O-glycans, O-Glycosylation, T cells

Introduction

T lymphocytes are a crucial element of the adaptive immune system that recognize potentially harmful antigens through the T cell receptor (TCR) and coordinate the immune response (Koch and Radtke 2011). T cell dysfunction can lead to life threatening infections or auto-immune disorders. T cells must home to secondary lymph organs (SLO), including the spleen and lymph nodes, to survey antigens and protect the body from pathogen invasion (Freitas and Rocha 1999; Nolz et al. 2011; Kumar et al. 2018). In order for T cells to mature, home to their target locations, and appropriately bind foreign targets, the TCR and associated glycoproteins must be correctly expressed, folded and modified (Dustin and Chan 2000; Kawashima and Fukuda 2012; Nolz and Harty 2014).

As T cells develop from bone marrow-derived precursors in the thymus (Trnková et al. 2012), one essential step in their maturation is generation of a functional TCR by genomic rearrangement. This unique TCR is subsequently tested for functionality and self-reactivity (Huesmann 1991), where T cells that fail these tests are induced to undergo apoptosis and never reach circulation or the SLO (Dustin and Chan 2000). The signals for regulating and testing TCR generation are mediated by glycoproteins, but the role that glycosylation plays directly in these events is largely unknown. Similarly, glycoproteins mediate signaling for other major events in T cell

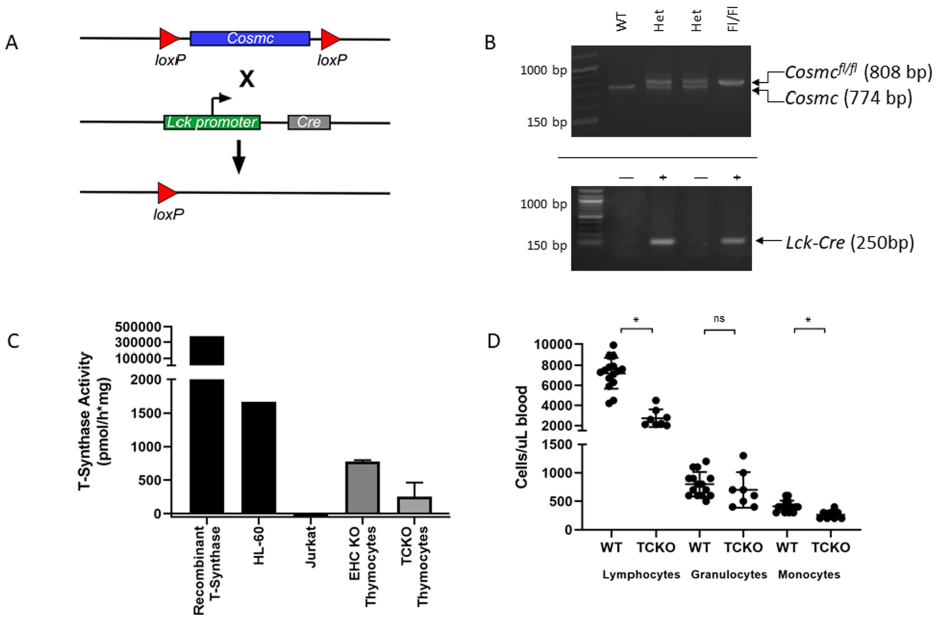

Fig. 1. Generation of a T cell-specific Cosmc knockout mouse. (**A**) Genetic approach to generate a TCKO by crossing a mouse carrying *Cosmc* flanked by *loxP* sites to a mouse driving Cre expression from the proximal *Lck* promoter. (**B**) Representative PCR genotyping results showing the presence of the WT and/or floxed *Cosmc* allele (top) and the presence or absence of the transgenic *Lck Cre* (bottom). (**C**) T-synthase enzymatic activity of purified T-synthase and lysates from immortalized human neutrophil cell line (HL-60), immortalized human T cell cell line with deficient T-synthase activity (Jurkat), T cells isolated from low penetrance EHCKO mice and T cells isolated from the TCKO mice. Analysis by one-way ANOVA, n = 2. (**D**) Populations of lymphocytes, granulocytes and monocytes in blood of WT and TCKO mice measured by CBC analysis. Analysis by t-test with Bonferroni correction for multiple comparisons. WT n = 15, TCKO n = 8, ∗ indicates $p < 0.05$, ns = not significant.

maturation, activation and homing (Daniels et al. 2002; Bi and Baum 2009; Love and Bhandoola 2011; Jones 2018; Pereira et al. 2018). The glycosylation state of these glycoproteins is temporally regulated during maturation and activation indicating a substantial regulatory role for glycosylation in these processes (Paessens et al. 2007; Balcan et al. 2008a; Balcan et al. 2008b).

More than 80% of proteins with a secretory signal sequence are predicted to be modified with O-glycosylation (Apweiler et al. 1999; Steentoft et al. 2011), and O-glycans influence a protein's structure, stability and binding interactions (Seguchi et al. 1991; Daniels et al. 2001; Wang et al. 2012; Cummings and Pierce 2014). O-glycosylation of proteins begins post-translationally in the Golgi apparatus with the addition of an α-linked *N*-acetylgalactosamine (GalNAc) to serine or threonine, catalyzed by any of more than 20 polypeptides: UDP-GalNAc transferase enzymes (Tenno et al. 2007). This initiating structure, GalNAc-linked to Ser/Thr, the Tn antigen, is almost invariably modified by the addition of β3-linked galactose to form the Core-1 disaccharide Galβ3GalNAcα1-Ser/Thr, which forms the precursor for extended O-glycan structures. This crucial reaction is catalyzed by a single enzyme, C1GalT1 or T-synthase. Production of functional T-synthase enzyme requires the chaperone Cosmc encoded on the X chromosome (*C1GalT1C1* or *Cosmc*), without which nascent T-synthase aggregates and is degraded (Ju and Cummings 2002). Loss of Cosmc or T-synthase results in the truncation of all Core-1 type O-glycans to the Tn antigen. The requirement for Cosmc chaperone is complete and quantitative, such that in cells lacking Cosmc there is no residual T-synthase activity (Ju et al. 2011). Reinforcing the importance of the enzymatic reaction from this single gene, global Cosmc KO animals result in embryonic lethality (Wang et al. 2010).

T cell O-glycans are dynamically regulated both during maturation and immune responses (Starr et al. 2003; Hernandez et al. 2007). There is some indication that specific O-glycans participate in particular stages of T cell development and activity (Moore et al. 2008; Pereira et al. 2018), but the overall importance of O-glycans in T cell maturation remains largely unknown. Thus, we investigated the general role of O-glycans in T cell biology by taking advantage of *Cosmc* deletion to abrogate T-synthase activity. *Cosmc*$^{-/+}$ have truncated O-glycans presenting as Tn antigen, providing an ideal model to evaluate the importance of complex O-glycan structures.

To evaluate the role that O-glycans play after T lineage commitment, we generated a T cell-specific *Cosmc* knock out (TCKO) model and examined the effect on T cells in the thymus, circulation and SLO. We determined that loss of Cosmc and consequential truncation of O-glycans is highly deleterious to T cell persistence, with near-complete elimination of peripheral Tn antigen expressing T cells. TCKO thymocytes complete thymic maturation but fail to populate the SLO. Co-transfer of wild-type (WT) and TCKO thymocytes shows that TCKO cells have reduced ability to home to SLO and are not maintained in circulation. We demonstrate that Cosmc, and therefore extended O-glycosylation, is a critical element in the establishment and maintenance of the peripheral T cell population.

Results

Lck-Cre drives T cell-specific KO of Cosmc.

In order to specifically examine the role of O-glycosylation in T cells, we created a TCKO mouse. The mouse was produced by crossing the *Cosmc*$^{fl/fl}$ mouse (Wang et al. 2012) with a mouse expressing Cre recombinase from the T cell-specific *Lck* proximal promoter (Hennet et al. 1995) (Fig 1A). The proximal promoter was selected because it is most active during thymocyte maturation, beginning at the early CD8$^-$CD4$^-$ double negative (DN) stage, while *Lck* expression from the distal promoter is more active during

activation of mature T cells (Shi and Petrie 2012). Mouse genotypes were determined by PCR (Fig 1B). All TCKO mice used in subsequent experiments constitutively express Cre in a T cell-specific manner and are homozygous $Cosmc^{fl/fl}$ females or hemizygous $Cosmc^{fl/y}$ males, as *Cosmc* is located on the X chromosome. To determine whether T-synthase activity is reduced in TCKO cells, we performed a fluorescence-based T-synthase enzyme activity assay on lysate from bulk thymocytes of TCKO and endothelial and hematopoietic conditional *Cosmc* knock out (EHCKO) mice (Fig 1C). EHCKO mice express Cre recombinase from the hematopoietic specific promoter *Tie2* (Wang et al. 2012). EHCKO thymocyte derived lysate has appreciable specific activity of T-synthase, though reduced relative to an immortalized human neutrophil cell line (HL-60). This indicates very low level of *Cosmc* deletion in the T cell compartment, consistent with prior characterization of the EHCKO model (Wang et al. 2012). In contrast, TCKO thymocyte lysate exhibits a reduced specific activity relative to both HL-60 and EHCKO lysates. However, T-synthase activity in TCKO thymocyte lysate is still substantially higher than the activity of the negative control, an immortalized human T cell line with a known T-synthase deficiency (Jurkat) (Ju et al. 2008). This indicates that the T cell population in TCKO mice is somewhat heterogeneous, containing some cells that have not undergone Cre-mediated *Cosmc* deletion. Notwithstanding the incomplete deletion of *Cosmc*, T-synthase activity is significantly reduced in TCKO thymocytes, making it an effective model to examine the role of T-synthase in T cell maturation and function. We next characterized the hematopoietic compartment of TCKO mice by measuring the abundance of major blood cell types in TCKO and WT littermates. Blood counts of TCKO mice revealed significant lymphopenia relative to littermate controls (Fig 1D). The levels of granulocytes were unchanged and there was a small but significant decrease in the number of monocytes. Together these data indicate that *Cosmc* can be specifically deleted in T cells using this TCKO model, resulting in decreased T-synthase activity and reduced levels of circulating, peripheral T cells.

Cosmc KO results in dramatically reduced T cell numbers

To determine if all circulating T cell subtypes were affected equally, we examined the peripheral T cell populations in the TCKO mouse. $CD4^+$ and $CD8^+$ T cells were identified and analyzed by flow cytometry (Fig 2A). While circulating levels of B cells were unchanged in TCKO mice compared to WT, as expected, the median cell numbers of both the $CD4^+$ and the $CD8^+$ T cells were only 20% that of WT littermates (Fig 2B, Table I). This decrease in T cell numbers highlights the effect of *Cosmc* deletion. A significantly higher percentage of both $CD4^+$ and $CD8^+$ cells express Tn antigen relative to cells from WT mice, where Tn antigen is undetectable. However, surprisingly $>90\%$ of circulating T cells in TCKO mice do not express Tn antigen. The median percentage of Tn positive cells was 5% for $CD4^+$ T cells and 1% for $CD8^+$ T cells (Fig 2B, Table II). This very small percentage of cells expressing the Tn antigen led us to hypothesize that Tn antigen positive cells are depleted from circulation in one of possibly three ways: (1) Tn antigen positive T cells may be sequestered in SLO; (2) Tn antigen positive T cells may be prevented from entering circulation; or (3) Tn antigen positive T cells may be actively removed from circulation.

We then interrogated SLOs for T cell population amounts, as compared to circulation. Splenic T cells mirrored the circulating T cells in both overall T cell levels and percentage of Tn antigen expression (Fig 2C). The populations of both $CD4^+$ and $CD8^+$ T cells were significantly reduced by 3- to 5-fold, with a median count at 22% and 36% of WT levels, respectively. Splenic TCKO T cells expressed the Tn antigen at the same low percentages seen in blood (Fig 2C). In the case of the peripheral lymph nodes (PLN, pooled lymphocytes from inguinal, axillary and brachial nodes) and the mesenteric lymph node (MLN), $CD4^+$ T cell counts were significantly reduced in TCKOs and $CD8^+$ T cell counts also trended lower. The median percentage of Tn positive T cells in both PLN and MLN was even lower than observed in blood and spleen (Fig 2D and E, Table I). As the reduced T cell counts and Tn + status in blood and spleen are reflected in the lymph nodes, we conclude that Tn + T cells are not being sequestered in SLO.

Although the percentage of Tn expressing cells is consistently lower in $CD8^+$ T cells than in $CD4^+$ T cells of TCKO mice, within the total T cell population (including Tn- cells) the relative ratio of $CD8^+$ to $CD4^+$ cells in TCKO do not differ significantly from WT (Fig 2F). This indicates that the persistence of both $CD4^+$ and $CD8^+$ T cells is negatively impacted by *Cosmc* deletion, with $CD8^+$ T cells having enhanced sensitivity to *Cosmc* deficiency, but that proportional numbers of both populations escaped *Cosmc* deletion. While loss of *Cosmc* may have a more severe impact on persistence of $CD8^+$ T cells than $CD4^+$ T cells, the qualitative effect on both populations appears to be similar.

Having observed that total numbers of circulating T cells are 5-fold lower in TCKO mice than in WT and the median percentage of T cells expressing Tn antigen does not exceed 5% in any peripheral lymphoid compartment, we analyzed whether *Cosmc*-deleted cells are escaping detection. We measured the percentage of T cells expressing the Tn antigen by co-staining with the Tn-specific antibody Bags6 and the GalNAc-binding lectins *Vicia villosa* lectin and *Helix pomatia* agglutinin, both of which were consistent with the antibody-based detection (data not shown). These data suggest that the majority of peripheral T cells represent those that escaped Cre recombination and loss of *Cosmc*. Thus, these data indicate that T cells lacking *Cosmc* are either unable to reach or are deficient in their ability to populate the periphery, and that cells escaping *Cosmc* deletion have a survival advantage in establishing the peripheral T cell pool.

Cosmc KO does not block thymocyte maturation

We next examined whether T cells from TCKOs survive thymic maturation and selection. Although the *Lck* Cre transgene is activated very early at the $CD4^-CD8^-$ DN stage, thymocyte populations (Fig 3A) at all maturation stages were not significantly different in numbers between TCKO mice and their WT littermates. These results demonstrate that in contrast to the results regarding peripheral T cells, loss of *Cosmc*, as driven by *Lck* Cre, does not result in dramatic loss of TCKO thymocyte populations (Fig 3B). At all maturation stages the percentage of T cells expressing Tn antigen was significantly higher in TCKO than WT. The percentage of T cells expressing the Tn antigen is significantly and substantially higher in maturing T cells in the thymus than in T cells isolated from the blood or peripheral lymph organs (57% at the highest in the thymus compared to 5% at the highest in the periphery) (Fig 3C, Table II), which indicates that the strong deleterious effect of *Cosmc* loss on T cells does not occur during thymocyte maturation.

Due to the large differences in percent of Tn positive T cells at each stage of T cell maturation, we examined the maturation profile of TCKO and WT thymocytes. As T cells mature and prepare to egress from the thymus, they upregulate levels of CD62L (L-selectin). We

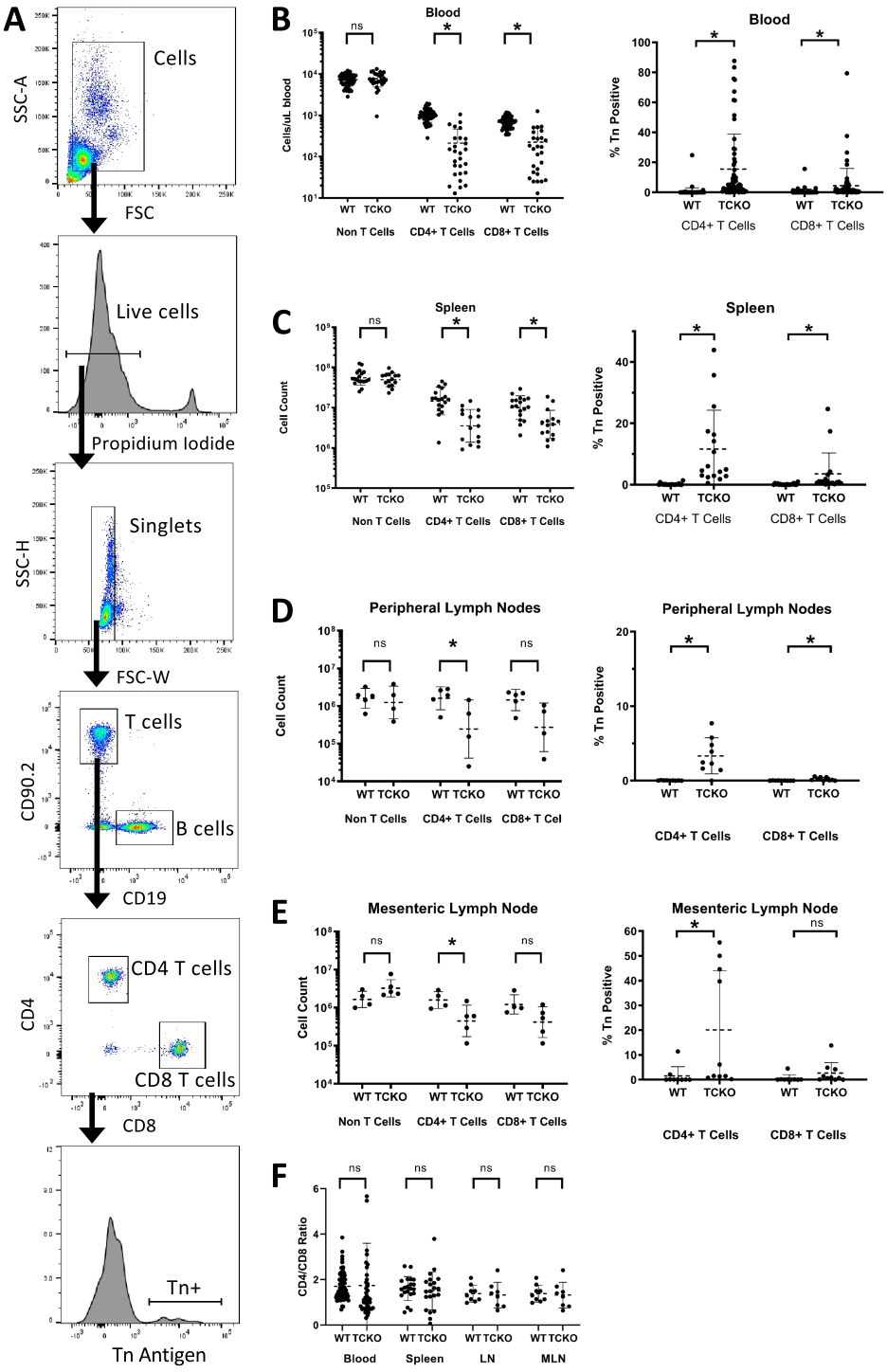

Fig. 2. Analysis of T cells from TCKO blood, spleen, and lymph nodes. (**A**) The gating strategy and representative populations for flow cytometric analysis of lymphocyte populations. (**B–E**) (left panels) The cell counts of non-T cells, $CD4^+$ T cells and $CD8^+$ T cells were measured for WT and TCKO mice in blood (**B**), spleen (**C**), PLN (inguinal, axial and brachial lymph nodes) (**D**) and MLN (**E**). (**B-E**) (right panels) The percent of $CD4^+$ and $CD8^+$ T cells that express detectable Tn antigen in each organ as noted, for WT and TCKO mice. (**F**) The ratio of $CD4^+$ T cells to $CD8^+$ T cells in secondary lymphoid organs as noted for WT and TCKO mice was calculated from the flow cytometry data. Blood: WT n = 53, TCKO n = 27, Spleen: WT n = 18, TCKO n = 15, peripheral LN: WT = 5, KO = 4, MLN: WT n = 4, TCKO n = 5. All comparisons by Mann–Whitney test with Bonferroni correction for multiple comparisons. ns = not significant.

observed that 69% of WT single positive (SP) thymocytes express high levels of CD62L, indicating that they are preparing to exit the thymus (Fig 3D). However, in TCKO SP thymocytes there was a striking difference in CD62L expression levels between Tn+ and Tn– cells (Fig 3E). Overall, 62% of TCKO SP cells were $CD62L^{hi}$, but the $CD62L^{lo}$ SP thymocytes were only 13% Tn+, while the

Table I. Total cell counts

		WT			TCKO		
Organ	*Cell type*	*Median cell count*	*n*	*SD*	*Median cell count*	*n*	*SD*
Thymus	DN	4.60E+06	19	6.23E+06	5.11E+06	20	1.63E+07
Thymus	DP	7.67E+07	19	3.11E+07	5.18E+07	20	2.32E+07
Thymus	SP4	7.03E+06	19	3.77E+06	4.22E+06	20	1.26E+07
Thymus	SP8	1.90E+06	19	2.89E+06	1.07E+06	20	1.40E+07
Blood	B	7.29E+03	53	2.28E+03	7.21E+03	27	2.83E+03
Blood	CD4	1.03E+03	53	3.61E+02	1.21E+02	27	2.54E+02
Blood	CD8	6.83E+02	53	2.20E+02	1.45E+02	27	2.65E+02
Spleen	B	5.08E+07	18	2.71E+07	5.10E+07	15	1.98E+07
Spleen	CD4	1.62E+07	18	6.97E+06	3.50E+06	15	5.07E+06
Spleen	CD8	1.08E+07	18	1.16E+07	3.88E+06	15	4.24E+06
PLN	B	1.86E+06	5	9.51E+05	1.41E+06	4	1.54E+06
PLN	CD4	1.93E+06	5	9.41E+05	3.93E+05	4	6.47E+05
PLN	CD8	1.98E+06	5	7.57E+05	4.53E+05	4	4.56E+05
MLN	B	1.62E+06	4	9.08E+05	2.62E+06	5	2.30E+06
MLN	CD4	1.59E+06	4	9.00E+05	5.94E+05	5	5.29E+05
MLN	CD8	1.01E+06	4	9.65E+05	5.33E+05	5	4.33E+05

Notes: DN = double negative, DP = double positive, SP = single positive, SD = standard deviation

Table II. Percent Tn positive cells

		WT			TCKO		
Organ	*Cell type*	*Median percent Tn positive*	*n*	*SD*	*Median percent Tn positive*	*n*	*SD*
Thymus	DN	0.335	20	0.5734	2.810	23	5.105
Thymus	DP	0.055	20	0.8024	14.500	23	16.76
Thymus	SP4	0.335	20	0.5415	52.600	23	24.56
Thymus	SP8	0.105	20	0.7404	23.400	23	22.29
Blood	CD4	0.000	88	2.669	4.700	63	23.52
Blood	CD8	0.000	98	2.253	1.000	64	11.53
Spleen	CD4	0.044	19	0.3522	4.980	17	12.72
Spleen	CD8	0.024	19	0.2593	1.000	17	6.81
PLN	CD4	0.035	9	0.02917	2.450	9	2.427
PLN	CD8	0.008	9	0.01833	0.110	9	0.2215
MLN	CD4	0.062	9	3.761	3.825	10	23.88
MLN	CD8	0.088	9	1.48	0.770	10	4.283

Notes: DN = double negative, DP = double positive, SP = single positive, SD = standard deviation

$CD62L^{hi}$ SP thymocytes were 50% Tn+ (Fig 3F). This result indicates that T cells with *Cosmc* deletion are successfully maturing, and Tn + cells are enriched in the most mature T cell populations. We hypothesize that this may be due to inappropriate retention of Tn+ cells or accelerated passage through checkpoints intended to ensure selection of immune competent, non-autoreactive cells. Accelerated passage is better supported by the data than retention because CD4 SP thymocytes from TCKO contain a reduced percentage of $CD62L^{hi}$ cells than WT, indicating that mature pre-egress thymocytes are not being enriched, though no significant difference was detected in the proportion of cells expressing high levels of other maturation markers CD24 and Qa-2 (Fig 3G). These data imply that the Tn+ cells are not depleted, even at the latest maturation stages of thymic development.

The high percentage of Tn + T cells in the thymus is in sharp contrast to the trends seen for peripheral T cells. Whereas the mice with the highest penetrance of Tn antigen expression and therefore *Cosmc* deletion, had the most pronounced T cell loss and those with the highest T cell numbers had the least penetrance of Tn antigen expression, no such trend exists within the thymus (Fig 4A). Plotting the loss in cell numbers by compartment and population (Fig 4B) clearly demonstrates that the powerful effect of *Cosmc* deletion occurs after the events of thymic maturation. Comparing $CD4^+$ and $CD8^+$ T cell populations for Tn antigen expression shows that $CD8^+$ T cells are more strongly affected by the selective pressure against *Cosmc* deletion, and that this difference begins to appear even at the SP stage of thymic maturation (Fig 4C). Together, these data demonstrate that the selective pressure decreasing TCKO T cell persistence exists in the thymus, but predominantly acts after thymic maturation. TCKO T cells are able to mature through thymic checkpoints and are not strongly selected against within the thymus. Thus, we hypothesize that the T cell numbers in general and Tn antigen positive T cells specifically are so drastically reduced in the periphery due to one or both of two likely explanations: (1) T cells are unable to leave the thymus; (2) T cells are actively selected against in the periphery.

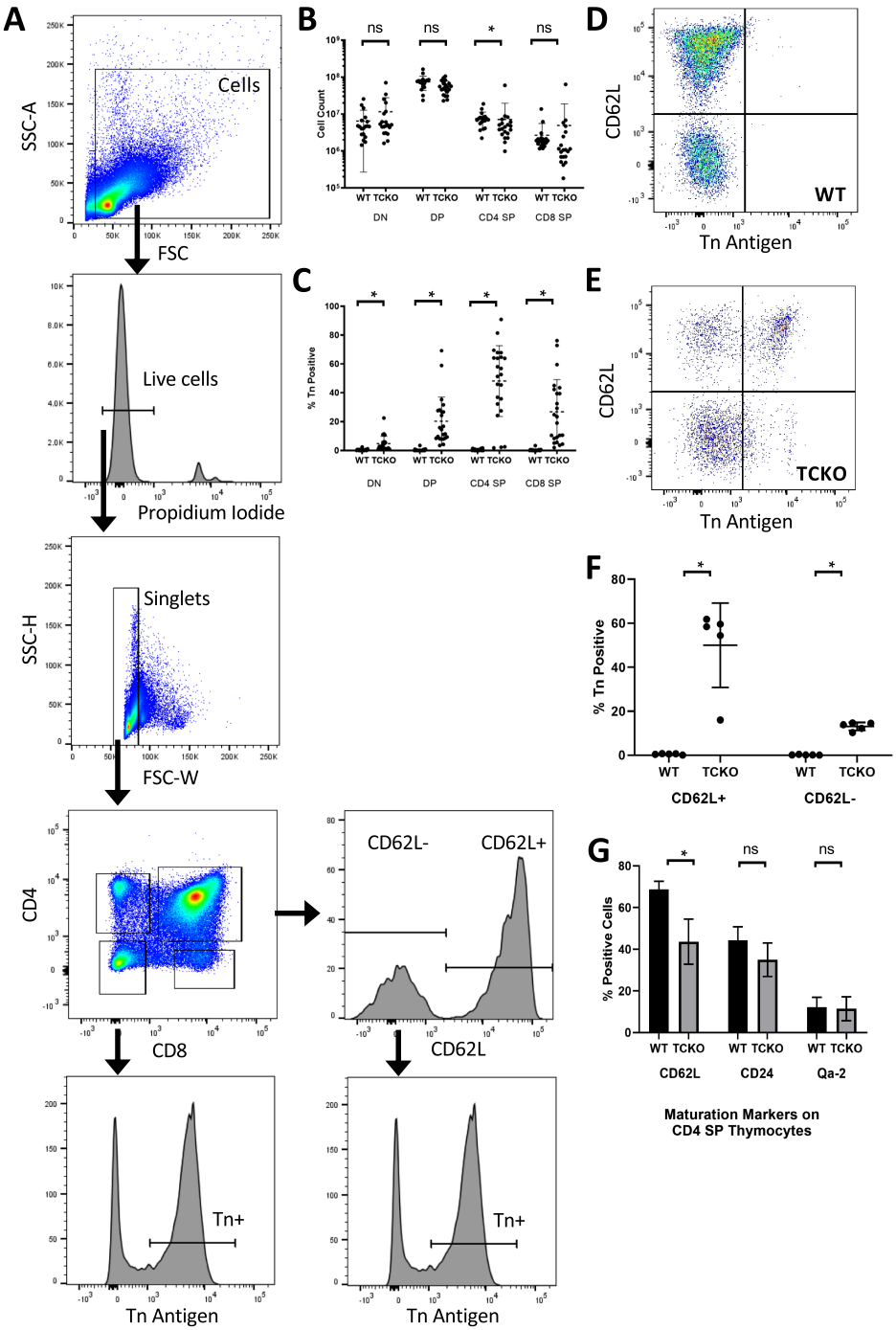

Fig. 3. Effect of TCKO on thymocyte populations. (**A**) Gating strategy for thymocyte analysis. (**B**) Absolute counts of thymocytes from each stage of T cell maturation (DN, DP, SP) recovered from the thymus of WT and TCKO quantified by flow cytometry. Median and quartiles are indicated by dashed lines. (**C**) Percent of thymocytes that were positive for Tn antigen are presented for each stage of T cell maturation. Median and quartiles are indicated by dashed lines. (**D–E**) Representative flow plots of CD4 SP thymocytes from WT (**D**) and TCKO (**E**) for Tn antigen and CD62L expression. (**F**) Quantification of CD4 SP thymocytes analyzed for simultaneous expression of L-selectin (CD62L) and Tn antigen (n = 5). (**G**) Percent of CD4 SP cells expressing high levels of maturation markers (CD62L, CD24, Qa-2) in WT or TCKO mice were analyzed. Error bars represent standard deviation. For **B** and **C**, WT n = 19 TCKO n = 20. For **F** and **G**, n = 5 for both WT and TCKO. Comparisons for **B, C** and **F** were made by Mann–Whitney test with Bonferroni correction for multiple comparisons, and for **G** a 2-way ANOVA was used; ∗ indicates $P < 0.05$, ns = not significant.

TCKO T cells are deficient in homing to SLO

To determine whether Tn + T cells encountered negative survival selection after leaving the thymus, we adoptively transferred thymocytes into WT animals (Fig 5A). Thymocytes isolated from the WT and TCKO mice were differentially fluorescently labeled, mixed in a 1:1 ratio, and injected into a WT host. Animals were allowed to rest for 16 h post injection (the time required for T cells to home to SLO and recirculate; Oostendorp et al. 2000; Srour et al. 2001;

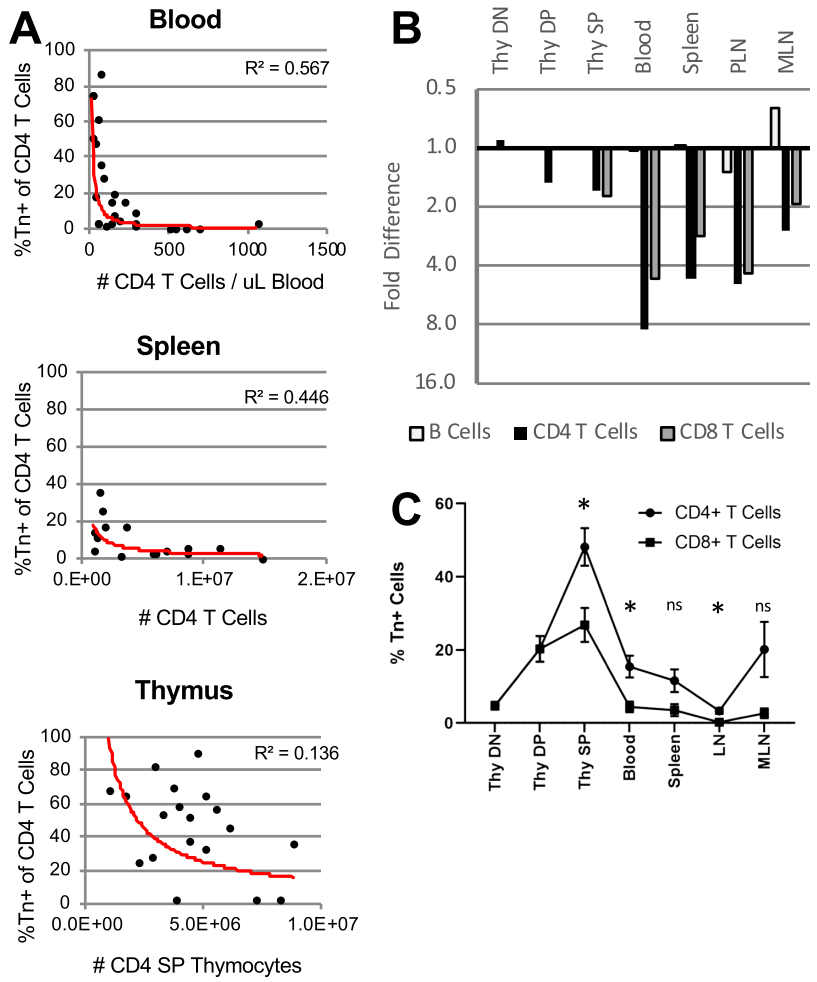

Fig. 4. Difference in Tn expressing cells across T cell maturation. (**A**) Total number of CD4 T cells in the blood, spleen and thymus of individual TCKO mice, plotted against the percent of those cells expressing the Tn antigen. Trendline is a power curve fit, with R^2 value displayed. (**B**) Visual representation of the difference in total cell count between TCKO and WT mice, calculated by dividing the median WT count by the median TCKO count for B cells, $CD4^+$ T cells and $CD8^+$ T cells. Bars below the axis indicate a reduced number of cells in TCKO. (**C**) Percentage of T cells expressing Tn antigen at specified development stages and compartments. Mean values and standard error are plotted for $CD4^+$ and $CD8^+$ populations. Statistical differences between CD4 and CD8 cells are indicated, as calculated by Student's t-test with Bonferroni correction for multiple comparisons.

Ganusov and Auerbach 2014), at which point the recipient tissues were collected and analyzed. Donor splenocytes correctly homed to all peripheral lymphoid organs indicating that cells from both the TCKO and WT mice survived isolation and the adoptive transfer procedure. As expected, WT SP4 thymocytes homed at lower efficiency than WT $CD4^+$ splenocytes but were still able to home to all SLO (Fig 5B); however, TCKO thymocytes homed with significantly lower efficiency than WT for each SLO measured. Strikingly, TCKO SP4 thymocytes engrafted into the PLN at less than 20% the efficiency as WT SP4 thymocytes. The results from this adoptive transfer experiment dramatically illustrate that *Cosmc* KO is detrimental to the ability of T cells to home to SLO, and that negative selective pressure from the loss of elaborated O-glycans acts on post-thymic T cells.

Discussion

We discovered that loss of *Cosmc* causes a profound loss of T cells from the peripheral lymph system while T cell maturation was undisturbed. The dramatic global reduction of peripheral T cell numbers in TCKO animals and the further disproportionate loss of T cells expressing Tn antigen on their surface, demonstrates that appropriate elaboration of extended O-glycans is critical to persistence of T cells in the blood or SLO. Nevertheless, the presence of some Tn + T cells in the periphery, however few, indicates that extended O-glycans are not an absolute requirement to maturation and survival. These findings are consistent with previous studies (Vainchenker et al. 1985; Berger 1999; Kao and Sandau 2006) and further indicate that T-synthase activity is especially important in T cells.

We discovered that, following *Cosmc* deletion in TCKO mice, there was an overall reduction in T cell levels relative to WT, and in that reduced population a surprisingly low percentage (<5%) expressed the Tn antigen. While the number of peripheral T cells expressing the Tn antigen was vanishingly small, an appreciable number of circulating T cells that escaped *Cosmc* deletion remained. Reduced total T cell numbers indicates that "escaper" T cells, i.e. those in which *Cosmc* was not deleted efficiently by Cre activity, fail to expand and fill the available niche. This is an important

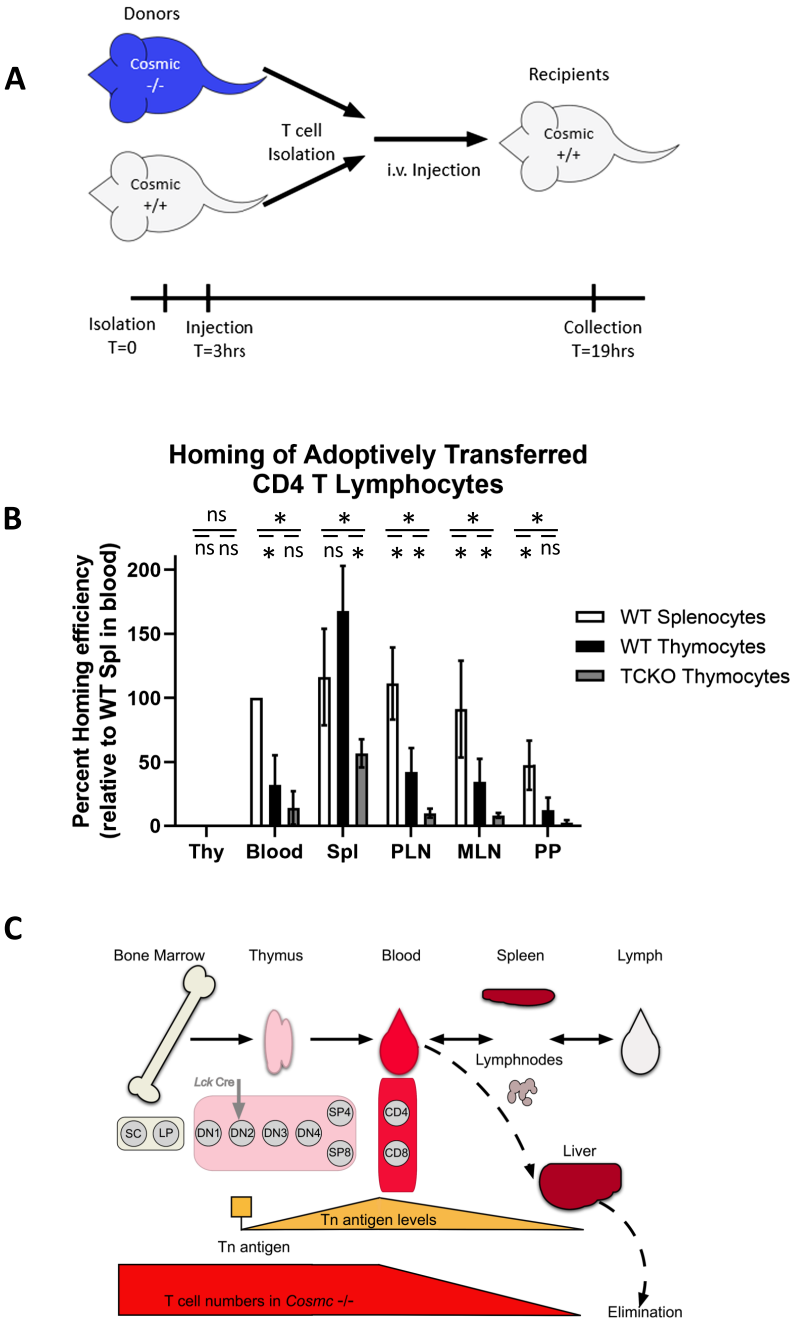

Fig. 5. Adoptive transfer of WT and TCKO Thymocytes. (**A**) Thymocytes from WT and TCKO mice were isolated, fluorescently labeled, then mixed at a 1:1 ratio before transfer into WT hosts by tail vein injection. Hosts were sacrificed 16 h post injection, and their tissues analyzed by flow cytometry for the presence of transferred cells. (**B**) Quantification of distribution of adoptively transferred WT and TCKO CD4 SP thymocytes and WT CD4 splenocytes, calculated as a percent of injected ratio relative to host CD4 T cells, normalized to transferred splenocytes in blood. Two outliers were excluded by Grubbs test, and statistical significance calculated by Student's t-test with Bonferroni correction for multiple comparisons. $*$ indicates $P < 0.05$, ns = not significant. (**C**) Proposed model of Tn antigen positive T cell clearance from circulation.

result, as compensatory expansion of a minor subset of escaper T cells dividing to achieve comparable cell counts to WT animals is common in cases where a majority of T cells have a survival defect and the lack of such expansion in the TCKO mice was surprising (Martin et al. 2013). The reduction in total T cell numbers observed in TCKO mice indicates that another mechanism is preventing the usual compensatory expansion. One possibility is that T cells in TCKO mice are activated in the periphery, inducing *Lck* expression and inappropriate production of Cre. *Lck* expression upon activation is predominantly driven from the distal promoter (Chiang and Hodes 2016); however, if expression of the proximal promoter were also induced, Cre would be expressed resulting in post-thymic *Cosmc* deletion. These T-synthase-deficient cells would experience the same selective pressure that eliminated the earlier Tn expressing thymic emigrants, maintaining the lymphopenia. However, because extrathymic expression of *Lck* is controlled by the distal rather than

the proximal promoter, it is unlikely for recombination to occur in peripheral T cells that had already escaped recombination. Therefore, the Tn positive T cells in the periphery are most likely to be persistent Tn positive thymic emigrants, rather than mature Tn negative cells converted to Tn positive by post-egress deletion of *Cosmc*.

The dramatically low number of Tn antigen-expressing T cells we detected in the TCKO model parallels findings in human patients with Tn syndrome. Tn syndrome is a relatively rare disorder arising from spontaneous somatic mutation of *Cosmc* in hematopoietic stem cells, and is characterized by Tn antigen expression on all hematopoietic subpopulations (Vainchenker et al. 1985; Berger 1999; Ju and Richard 2005). Examination of peripheral blood cells from these patients revealed that while Tn antigen occurs on a moderate to high proportion of most blood cell types (70–95% of erythrocytes, 50–80% of megakaryocytes, 25–90% of granulocytes and 15–65% of B cells) the occurrence on T cells was far lower, ranging only between 2–5% of total T cells (Brouet JC 1983; Judson PA 1983; Berger EG 1994). This finding matches closely to our TCKO results, in which fewer than 5% of surviving T cells expressed the Tn antigen. Thus, T cells are particularly susceptible to perturbation of O-glycosylation and the TCKO model is an ideal method for further study on the unique requirements of T cells for extended O-glycosylation and modeling Tn syndrome.

We observed that <60% of CD4 SP thymocytes expressed the Tn antigen, the marker of Cre mediated *Cosmc* deletion, and that the percentage in other thymic subpopulations was even lower. Previous studies that also used the *Lck* Cre construct for gene deletion documented greater than 80% recombination efficiency in T cells (Shi and Petrie 2012). This divergence in recombination efficiency suggests that the loss of *Cosmc* is impacting not only the survival and homing of peripheral T cells, but possibly also the development of thymocytes and/or their ability to successfully pass positive and negative selection checkpoints. We observed a steady increase in the proportion of each subpopulation that expressed the Tn antigen, with more mature stages having a higher percentage of Tn positive cells.

This expected increase in percentage of Tn positive cells with maturation does not indicate a survival advantage for Tn positive cells during the selection process, because in cultured cell lines there is a seven-day delay between loss of Cosmc and the appearance of Tn antigen on the cell surface (Hofmann et al. 2015). This delay between *Cosmc* deletion and Tn antigen expression corresponds to the expected time to turn over functional Cosmc and T-synthase. Thymic maturation from $CD4^{-}CD8^{-}$ DN T cell progenitor to mature $CD4^{+}$ or $CD8^{+}$ SP cell takes approximately 2 weeks, a gradual increase of Tn positive cells in maturing populations is expected. While we detected increases in the percentage of Tn positive T cells, there was high variability between biological replicates in expression levels (SD = 25%). This high degree of variability between individual mice in the eventual proportion of Tn + cells is surprising and suggests that the effect of *Cosmc* deletion is subject to stochastic processes. One such stochastic process is TCR recombination, which is the target of the thymic selection events. Therefore, signaling through the TCR and associated coreceptors is a promising potential mechanism by which O-glycan truncation impacts thymocyte fitness. However, it is clear from the proportion of Tn positive cells that pass selection and reach the mature SP stage that loss of extended O-glycans does not preclude productive TCR engagement and associated signaling.

Of additional interest is our finding that the most mature cells, i.e. those expressing high levels of L-selectin (CD62L), are highly enriched for Tn antigen expression (58% Tn positive among $CD62L^{hi}$ cells, compared to 14% Tn positive among $CD62L^{lo}$ cells). This enrichment of Tn positive mature thymocytes might indicate an enhancement of maturation signals in Tn positive cells, such that they are driven toward a mature phenotype earlier than their Tn negative counterparts, or it might indicate that Tn positive cells are slower to egress from the thymus. Because normal thymocyte development results in upwards of 90% of cells undergoing apoptosis due to failure to pass positive or negative selection checkpoints, and such apoptotic cells are quickly cleared by resident phagocytes (Surh and Sprent 1994), it is not possible to easily determine the relative survival rate of Tn positive and Tn negative thymocytes in TCKO animals. Determining the effect of Cosmc loss on thymocyte survival will require new experimental models and further study, but could further illuminate the role of O-glycosylation in thymocyte development, selection and survival.

Because T cells are required for B cell development, complete loss of peripheral T cells results in an accompanying absence of B cells, as observed in severe combined immunodeficiency syndrome consequent to deficiency of the common gamma chain receptor (Buckley 2004). However, very small numbers of T cells are apparently sufficient to provide the necessary factors for B cell development, as patients with DiGeorge syndrome have severe T lymphopenia but do not lack T cells entirely, yet have no reduction in B cell numbers (Derfalvi et al. 2016). Therefore, it is not unexpected that the B cell numbers in our TCKO model would not differ from WT, despite the extensive crosstalk between the two cell types. Once the T cell compartment of the TCKO model is more fully characterized, it would be interesting to evaluate the ways in which B cells are affected by the altered and reduced T cell population.

The TCKO model we describe here exhibits reduced overall T cell levels, appreciable Tn expression and a low percentage of Tn antigen positive T cells in circulation and the peripheral lymphoid organs. In contrast, the EHCKO mouse, in which *Cosmc* deletion is driven by the earlier *Tie2* promoter, had no detectable Tn expression in the thymus (Wang et al. 2010). This indicates that in addition to a survival and homing defect in thymic and peripheral T lymphocytes, *Cosmc* deletion also affects the differentiation or homing of early thymic progenitors. This deficiency could present as impaired development in the bone marrow, egress from the bone marrow, homing to the thymus, a combination of those three effects or others. This *Cosmc* requirement in early T cell maturation combined with our present findings indicate that proper elaboration of O-glycans is essential to all stages of T cell development, from early differentiation in the bone marrow to maturation in the thymus and to appropriate homing to immune organs. Investigating the mechanism by which extended O-glycosylation modulates each of these stages of T cell development will illuminate the role of carbohydrates in T cell survival signaling events, the quality control systems ensuring only correctly mature T cells are released into the body and in the signaling pathways influencing T cell homing.

We established that murine L-selectin (CD62L) is upregulated disproportionately on mature Tn + thymocytes (Fig 3). In addition to serving as a marker of thymocyte maturation, CD62L is an adhesion molecule necessary for the homing of T cells to SLO. CD62L binds to O-glycosylated ligands on endothelial cells, but it is unknown whether O-glycans themselves are present on CD62L, and whether they play any role in protein function. Prediction algorithms indicate that murine, but not human, L-selectin might bear an O-glycosylation site on the protein's stem region (Steentoft 2013). Thus, it is possible that the defect in T cell homing in the TCKO mouse model is partially mediated by dysfunction of L-selectin due to improper glycosylation.

Investigating the impact of post translational modification on selectin function could lead to better understanding of leukocyte tethering and homing and provide avenues to manipulate the trafficking of immune cells.

We employed the well-established adoptive transfer method to analyze homing and migration characteristics of T cells (Matheu 2011). We found that TCKO T cells engrafted at less than 30% the efficiency of WT T cells. While adoptive transfer is widely accepted in the field, it is primarily used for the transfer of cells isolated from spleen or other SLO. A few others have undertaken adoptive transfer of mature thymocytes to determine the homing and survival of recent thymic emigrants (Houston et al. 2011; Kim et al. 2016). In agreement with their findings, we found that mature $CD4^+$ SP thymocytes are less competent at entering SLO than splenic $CD4^+$ T cells on a short time scale (16 h) corresponding to tissue homing (Fig 5). We also discovered that TCKO cells are further deficient relative to WT counterparts, only entering the SLO at approximately one-fourth the efficiency of WT cells. An important caveat to interpreting the findings that TCKO cells engrafted to SLO at lower rates than WT T cells, is that the TCKO cells are also less numerous in the blood, meaning that the effect is not simply due to a defect in homing. If the only effect of *Cosmc* deletion in TCKO cells was reduced ability to enter SLO, there would be an enrichment of TCKO cells in the blood. Thus, the data support our conclusion that T cells expressing the Tn antigen are actively removed from circulation. Indeed, a series of early transplant studies support targeted removal of partly de-glycosylated T cells from circulation. In these studies, splenocytes and thymocytes were desialylated prior to transfusion into recipient rats. Desialylated splenocytes successfully arrived to recipient SLO at approximately half the frequency of mock treated cells, and a corresponding increase was seen in the number of cells sequestered in the liver (Gesner and Ginsburg 1964; Woodruff and Gesner 1969). In the case of thymocytes, the effect of desialylation was even greater with near complete abrogation of splenic localization of the transfused cells, with the bulk of transfused cells located in the liver (Berney and Gesner 1970). While these classic experiments did not distinguish between lymphocyte subsets, the results are consistent with specific capture of T cells in the liver. This effect would be unique to T cells with perturbed glycosylation, as no loss or hepatic capture of circulating B cells, granulocytes, erythrocytes or platelets has been observed in studies with genetic or enzymatic alteration of glycosylation in those cells (Wang et al. 2010; Wang et al. 2012). Thus, if there is a specific and novel glycan-receptor in the liver for partly-de-glycosylated T cells or TCKO cells, it cannot simply recognize the Tn antigen. The targeted removal of Tn antigen expressing T cells that we observe indicates that the effect is T cell-specific and not a general response to cells expressing Tn antigen (Fig 5C).

We suspect that Tn + T cells are being captured and removed from circulation in the liver. The liver is a presumed site of homeostatic T cell death (Crispe and Huang 1994; Mehal et al. 2001) and the majority of thymocytes transplanted by Berney and Gesner (1970) localized to the liver. However, despite these suggestive findings, the swift disappearance of dying cells in our adoptive transfer experiments makes it difficult for us to conclusively determine and how the Tn + T cells would be specifically targeted. The canonical mediator of glycan-based removal of circulating factors is the Ashwell–Morell receptor (AMR), which is expressed primarily in the liver and is responsible for extracting prothrombotic factors from the blood, including von-Willebrand factor (Grewal 2010). However, the pathway for AMR-mediated removal has never been shown to function on cell-sized targets, and the known ligands for AMR are moieties of N-linked glycans, so it is unlikely that AMR is mediating the sequestration of TCKO T cells in the liver.

It is possible that altered glycosylation of cell surface proteins makes TCKO mutant T cells targets for receptor-mediated recognition and destruction. Alternatively, the truncated glycans could influence the innate hardiness and durability of the circulating cells, resulting in increased fragility to stressors of circulation. Whether the liver mediates removal of Tn positive T cells, and how Tn expressing T cells are selectively targeted are exciting mechanistic questions to pursue.

Our discovery that truncated O-glycosylation adversely affects both $CD4^+$ and $CD8^+$ T cells contrasts prior work on two other enzymes in the pathway. Both ST3Gal-I and C2GnT act immediately after T-synthase, catalyzing the addition of a sialic acid (Sia) or an *N*-acetylgalactosamine (GalNAc) residue, respectively. When ST3Gal-I was deleted, preventing the sialylation of Core-1 O-glycans, $CD8^+$ T cells were almost completely absent from blood and SLO, while peripheral $CD4^+$ T cells were unaffected; neither CD4 nor CD8 SP thymocytes were reduced in number (Priatel et al. 2000). The authors ruled out a direct effect on CD8 itself or the associated signaling through the TCR, so the reason for the dichotomy between $CD4^+$ and $CD8^+$ cells remains unclear (Kao and Sandau 2006). The ST3Gal-I KO partially phenocopies our TCKO model, and the similar mechanisms may be responsible for the loss of $CD8^+$ T cells in both models, especially since we observed enhanced clearance of Tn expressing $CD8^+$ T cells relative to CD4 T cells in TCKO animals. In contrast, ectopic expression of C2GnT, forcing the branching of Core 1 glycans into Core 2 based structures, resulted in no defect of T cell development but did cause reduced immune function in affected T cells (Tsuboi and Fukuda 1997). That result demonstrates that the composition and structure of O-glycans on T cells impacts and modulates the cell–cell interactions necessary for effector function. However, our work shows that beyond modulation of signals the presence of those glycans is also a requirement for survival.

Taken together our results demonstrate that T cells require extended O-glycosylation for proper physiology in the thymus and SLO. One of the most commonly studied cultured T cell lines, the Jurkat cells, express the Tn antigen (Piller et al. 1990), due to dramatically reduced expression of T-synthase resulting from a mutation in the *Cosmc* gene (Ju and Cummings 2002), resulting in near-complete loss of enzyme activity and global expression of the Tn antigen. Much of the foundational work describing TCR signal ligand effector function has been carried out in this T-synthase deficient cell line and likely differs substantially from T-synthase-sufficient cells and from physiological T cell activity. This highlights a potentially major caveat of the many studies regarding T cell signaling and response carried out in Jurkat cell lines and the need to examine T cell function and functional glycoproteins utilizing primary T cells ex vivo or in vivo under physiological conditions.

Materials and methods

Animals

Mice were kept in a specific pathogen-free barrier facility. Animal studies were performed according to the Institutional Animal Care and Use Committee protocol approved by Emory University and Beth Israel Deaconess Medical Center and in compliance with guidelines from the National Institutes of Health. T cell-specific Cosmc KO mice were generated by crossing $Cosmc^{flox/flox}$ animals (Wang et al. 2012) with *Lck-Cre* transgenic mice (JAX Lab #003802;

Hennet et al. 1995). Mouse genotypes were determined by PCR of DNA from tail snips, using the primer sequences: CosmcFor: GCAACACAAAGAAACCCTGGG, CosmcRev: TCGTCTTTGT TAGGGGCTTGC, LckCreFor: TGTGAACTTGGTGCTTGAGG, LckCreRev: CAGGTTCTTGCGAACCTCAT.

T cell isolation

Blood was collected by cheek bleed. For organ collection, mice were euthanized by CO_2 asphyxiation and organs of interest were collected, including the thymus, spleen, mesenteric, axial, inguinal and brachial lymph nodes. Organs were pressed through a 70 μm cell strainer in cold phosphate buffered saline, supplemented with 2% FBS. Splenocytes and whole blood were treated with red blood cell lysis buffer. Once isolated, cells were immunolabeled with a panel of antibodies against cell surface markers CD4 (RM4-5, Biolegend 100553), CD8 (5H10, Invitrogen MCD0804), CD90.2 (30-H12, Invitrogen 12-0903-82), CD62L (MEL-14, Biolegend 104405), CD24 (30-F1, Invitrogen 12-0241-82), Qa2 (695H1-9-9, Biolegend 121703), Tn antigen (Bags6; Cao et al. 1995), VVA lectin (Vector Labs B-1235). Secondary staining with anti-IgM (Invitrogen A 21042), anti-IgG (Invitrogen A-11029) and streptavidin (Biolegend 405206) was used when necessary. Live/Dead staining was performed with propidium iodide (Invitrogen BMS500PI). Whole blood was analyzed on a Drew Scientific Hemavet to obtain complete blood counts (CBSs).

Flow cytometry

Cells were suspended in PBS containing 2% FBS at 10^7/ml and immunolabeled on ice with appropriate panels of antibodies at 1–5 ug/ml, washed, and analyzed on BD LSR II or Beckman Coulter CytoFLEX LX flow cytometer. Data were collected with FACSDiVa (BD Biosciences) and analyzed using Flowjo (v 10) (BD Biosciences) software.

Adoptive transfer

Splenic T cells were isolated from WT mice by magnetic depletion (CD4 T cell isolation kit, Miltenyi 130-104-454). Thymocytes isolated from WT and TCKO mice and were enriched for CD4 SP cells by magnetic depletion against CD8 (CD8 microbeads, Miltenyi 130-117-044). WT and TCKO cells were then fluorescently labeled with 1:1000 and 1:10,000 dilution of CellTrace Violet (Biolegend 100553) for TCKO and WT cells, respectively. TCKO and WT cells were mixed 1:1. A total of 10^8 cells were injected into WT hosts through the tail vein. Hosts were sacrificed after 16 h and blood and lymphoid organs collected and analyzed by flow cytometry as described above.

Glycosyltransferase assays

T-synthase activity of purified T-synthase and cell extracts were measured using the acceptor GalNAcα-phenyl as previously described (Ju 2013). Briefly, cell extract or purified enzyme was incubated with 100 mM MES, pH 6.8, 0.2% Triton X-100, 20 mM MnCl2, 1 mM GalNAcα1-O-phenyl, 0.4 4-methylumbelliferyl-UDP-Gal, 2 mM ATP and excess O-glycosidase, at 37°C for 60 min and stopped by raising the pH to 9.6. Fluorescence of released 4MU was measured on a PerkinElmer Victor3 plate reader (Ex: 355 nm; Em: 460 nm.)

Statistical analysis

Data were analyzed for statistical significance by Mann–Whitney, t-test and ANOVA as indicated in the individual figure legends. Tests were performed using GraphPad Prism version 8 (Graphpad Software). A P-value of less than 0.05 was considered statistically significant. Regression was performed in Microsoft Excel, using the power fit and R-squared values are reported for each regression.

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Conflict of interest statement.

None declared.

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