The ICOS–ICOSL pathway tunes thymic selection

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Abstract
Negative selection of developing T cells plays a significant role in T-cell tolerance to self-antigen. This process relies on thymic antigen-presenting cells which express both self-antigens and cosignaling molecules. Inducible T-cell costimulator (ICOS) belongs to the CD28 family of cosignaling molecules and binds to ICOS ligand (ICOSL). The ICOS signaling pathway plays important roles in shaping the immune response to infections, but its role in central tolerance is less well understood. Here we show that ICOSL is expressed by subsets of thymic dendritic cells and medullary thymic epithelial cells as well as thymic B cells. ICOS expression is upregulated as T cells mature in the thymus and correlates with T-cell receptor signal strength during thymic selection. We also provide evidence of a role for ICOS signaling in mediating negative selection. Our findings suggest that ICOS may fine-tune T-cell receptor signals during thymic selection contributing to the generation of a tolerant T-cell population.

INTRODUCTION
T cells express an enormous diversity of antigen receptors to recognize the myriad of foreign antigens that they may encounter. Potentially autoreactive T-cell receptors (TCR) are generated during rearrangement of antigen receptor gene segments during T-cell development in the thymus. Clonal deletion eliminates many of these autoreactive T cells in the thymus to prevent them from entering the periphery.1,2 Broadly, developing T cells scan for self-antigens presented by major histocompatibility complex (MHC) molecules on thymic antigen-presenting cells (APC). Thymocytes expressing TCRs with low to moderate affinity to self-peptide receive survival and differentiation signals (positive selection). Thymocytes bearing a TCR with high affinity for self-antigens undergo apoptosis (negative selection).3 Alternatively, some high-affinity T-cell clones can be diverted into immunosuppressive T-cell lineages that include Foxp3+CD4+ regulatory T cells (Treg) and provide an additional layer to maintain self-tolerance.3,4 Thymic negative selection occurs at both the CD4+CD8+ double-positive (DP) and the more mature CD4+ or CD8+ single-positive (SP) stages of T-cell development.3,6 A significant percentage of DP thymocytes expressing TCRs with high affinity to ubiquitous self-antigens is eliminated in the thymic
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The thymus, mTEC, thymic DCs and B cells express costimulating molecules that may modulate thymic selection. Indeed, it has been suggested that TCR signaling alone is insufficient to induce negative selection of all autoreactive thymocytes. CD28 binds to both CD80 and CD86 and emerged early as a candidate costimulatory molecule necessary for negative selection. While there is substantial evidence that negative selection requires CD28 costimulating; in some conditions, CD28 signaling alone is insufficient to induce negative selection. It is possible that other costimulatory molecules may compensate for the absence of CD28 in these instances and/or complement its function at steady state.

Inducible T-cell costimulator (ICOS) and its unique binding partner, ICOS ligand (ICOSL), belong to the CD8-B7 superfamily. CD28 and ICOS play complementary, nonredundant roles in effector T-cell and Treg functions during an immune response. ICOS is not expressed by mature naive T cells and is only upregulated after activation; ICOSL is expressed on both hematopoietic and nonhematopoietic cells. In the thymus, ICOS is expressed on some thymocytes and has been implicated in the regulation of innate T-cell subset development. In addition, ICOSL expression on mTEC may play a role in thymic Treg generation; in an in vitro system, ICOSL expression on human mTEC promoted a Treg phenotype via increased interleukin-2 production by CD4+ SP thymocytes. As interleukin-2 production by self-reactive T cells is important for inducing Foxp3 expression on Tregs, mTEC expressing ICOSL may indirectly lead to the expansion of thymic Tregs. However, reports of ICOSL expression on thymic APCs are inconsistent, and it is unclear whether ICOS is implicated in the selection of conventional T cells.

In this study, we re-examined ICOSL expression on TEC, thymic DCs and B cells, as well as ICOS expression on T-cell developmental intermediates. ICOSL is expressed by thymic APCs that coexpress other costimulating molecules and are known to support negative selection. We observed an upregulation of ICOS on DP thymocytes that have received thymic selection signals. Higher levels of ICOS expression are detected on SP thymocytes and Tregs relative to their less mature counterparts, but ICOS is down-regulated prior to thymic egress. The upregulation of ICOS correlates with the strength of TCR signaling received during T-cell development. Using ICOSL-deficient thymic slices, we identify a potential role for the ICOS pathway in fine-tuning negative selection of developing T cells bearing TCRs with high affinity for self.

RESULTS

Inducible T-cell costimulator ligand is expressed by subsets of thymic antigen-presenting cells

CD80 and CD86, ligands for CD28, are expressed at high levels on a subset of mTEC that also express high levels of MHC class II (MHC-II). Thymic DCs and B cells generally have high expression of CD80, while CD86 expression is lower. However, ICOSL expression on the surface of these cells has either not been examined, or the reported results are inconsistent. The inconsistencies in the literature may be a result of stromal cell isolation methods that could cleave ICOSL from the cell surface and therefore limit its assessment by flow cytometry. From microarray data deposited on the Immunological Genome Project Consortium and from published single-cell RNA-sequencing data, there is evidence that ICOSL is significantly expressed on CD80hiMHC-IIhi mTEC (mTECthi) and thymic DCs (Figure 1a). There is no transcriptomic data available for thymic B cells in this database for direct comparison. To understand which thymic APCs express ICOSL on the cell surface, we tested different thymic stromal cell isolation approaches (Figure 1b, c, Supplementary figure 1a, b). The use of Liberase and DNase I allowed us to detect ICOSL expression and compare its levels on cTEC, CD80hiMHC-IIhi mTEC (mTECthi), mTECthi and thymic CD11c+ DC subsets (Figure 1b, c, Supplementary figure 1a, b). ICOSL expression on ICOSL+ cTEC and mTECthi are indistinguishable from those of ICOSL− origin, suggesting that it is not expressed at significant levels on these populations; by contrast, ICOSL is highly expressed on ICOSL+ mTECthi (Figure 1b, c). Among thymic DC subsets, expression of ICOSL is highest on SIRPα+ DCs and, on average, lowest on plasmacytoid DCs (Figure 1b, c, Supplementary figure 1a). Similar to their peripheral B-cell counterparts, we found that thymic B cells also express high levels of ICOSL (Figure 1b, c and Supplementary figure 1c). Therefore, ICOSL is
preferentially expressed on many of the thymic APC populations that also express high levels of other cosignaling molecules such as CD80/CD86 and support negative selection.

**Figure 1.** Inducible T-cell costimulator ligand (ICOSL) is expressed on subsets of medullary thymic epithelial cells (mTEC) and thymic dendritic cells (DCs) as well as thymic B cells. (a) Relative levels of ICOSL expression extracted from the ImmGen database on the Ep.8wk.CEChi. Th.v2 (cortical thymic epithelial cells (cTECs)), Ep.8wk.MEClo. Th.v2 (mTEC low (mTEClo)) and Ep.8wk.MEChi. Th.v2 (mTEC high (mTECh)) as well as DC.8–Th and DC.8+.Th (thymic CD8– and CD8+ DC) subsets. (b) Representative histograms and (c) relative expression of ICOSL from cTEC, mTEClo, mTECh, thymic B cells, CD8+ DC, SIRPα+ DC and plasmacytoid DC (pDC) analyzed by flow cytometry. The gating strategies are depicted in Supplementary figure 1. The relative fluorescence intensity for ICOSL expression is normalized to the median fluorescence intensity of each subset from ICOSL+/+ mice. Dots indicate individual mice; n = 4 or 6 mice from a minimum of two independent experiments.

*Inducible T-cell costimulator expression is developmentally regulated in the thymus*

It has been long thought that only activated effector T cells and Treg express ICOS. Although recent evidence
suggests that ICOS is expressed by some thymocytes, it is still unclear which developing thymocyte populations express this costimulating molecule.\(^39\) We examined ICOS expression on TCR\(^{\beta}\) CD4\(^{+}\) CD8\(^{-}\) double-negative (DN), CD4\(^{+}\)CD8\(^{-}\)CD69\(^{-}\) preselection and CD4\(^{+}\)CD8\(^{-}\)CD69\(^{+}\) postselection DP, mature (TCR\(^{hi}\)) CD4\(^{+}\)CD25\(^{-}\)Foxp3\(^{30}\) and CD8\(^{+}\) SP thymocytes as well as CD4\(^{+}\)CD25\(^{-}\)Foxp3\(^{+}\) Treg from wild-type (WT) mice (Supplementary figure 2a); of note, the DN population using this gating strategy will contain a small percentage of \(\gamma\delta\) T cells. The expression of ICOS is low on DN and preselection DP cells. ICOS expression is upregulated as thymocytes receive positive selection signals in the postselection DP stage, and it is further elevated on CD4\(^{+}\) and CD8\(^{+}\) SP thymocytes (Figure 2a, b). This suggests the expression of ICOS is upregulated coincident with TCR signals received during T-cell development. Interestingly, however, ICOS expression begins to decrease from the CD69\(^{-}\)MHC-1\(^{-}\) (M1) to the more mature CD69 MHC-1\(^{-}\) (M2) CD4\(^{+}\) and CD8\(^{+}\) SP stages of development as TCR sensitivity for self-peptide diminishes just prior to thymic egress (Figure 2c, Supplementary figure 2a).\(^49\)

In contrast to the homogenous expression of ICOS in postselection DP and SP thymocyte populations, there appears to be a second, small population of Treg with significantly higher expression of ICOS (Figure 2a). To investigate which Treg subpopulations contribute to the heterogeneity in ICOS expression, we first examined ICOS levels on \textit{de novo} and mature Treg that have recirculated to the thymus from the periphery. In our experiments, we used mice between 6 and 12 weeks of age, where often more than 30% of the Treg population within the thymus is composed of mature Treg that have recirculated from the periphery (Figure 2d).\(^50\) Recirculated Tregs, in general, exhibit an activated phenotype.\(^50\) Indeed, we observed that the relative fluorescence intensity of ICOS is about fivefold higher on recirculated Tregs than \textit{de novo} thymic Tregs (Figure 2e).

In addition, \textit{de novo} Foxp3\(^{+}\)CD25\(^{+}\) thymic Tregs can be derived from either CD25\(^{-}\)Foxp3\(^{30}\) or CD25\(^{+}\) Foxp3\(^{30}\) CD4\(^{+}\) precursors.\(^51\) After excluding recirculated CD4\(^{+}\) T cells (Supplementary figure 2b), we compared ICOS levels between the two thymic Treg precursors with Foxp3\(^{+}\)CD25\(^{+}\) Treg and conventional CD4\(^{+}\) SP thymocytes (Figure 2f). Interestingly, CD25\(^{+}\) Treg precursors expressed higher ICOS levels, while Foxp3\(^{+}\) Treg precursors and the Foxp3\(^{+}\)CD25\(^{+}\) Tregs expressed lower levels of ICOS (Figure 2g). CD25\(^{+}\) Treg precursors are thought to develop from CD4\(^{+}\) T cells bearing antigen receptors with a relatively higher affinity for self-antigen than Foxp3\(^{+}\) Treg precursors.\(^51\) Indeed, we observed that cell surface expression of CD5, a marker that correlates with the strength of TCR signals,\(^52\) is higher on CD25\(^{+}\) Treg precursors than those that are Foxp3\(^{30}\) Treg progenitors (Figure 2h). These findings indicate that ICOS expression is upregulated during \textit{de novo} thymic Treg development and that it is preferentially expressed on Treg progenitors with higher affinity for self-antigens.

**Inducible T-cell costimulator expression correlates with the strength of TCR signals**

Among thymic Treg populations, ICOS is preferentially expressed among cells that receive stronger TCR signals during differentiation. In addition, there is some evidence that the level of expression of ICOS on peripheral T cells may be dependent on signal strength.\(^53,54\) To better understand the correlation of ICOS with TCR signals received during thymic development, we compared the levels of ICOS on CD4\(^{+}\) and CD8\(^{+}\) SP thymocytes gated on the top and bottom 15% of CD5 expression as well as a CD5 intermediate population (Figure 3a). We observed that CD4\(^{+}\) and CD8\(^{+}\) SP thymocytes expressing higher levels of CD5 also express higher levels of ICOS (Figure 3a, b and Supplementary figure 3a). We also sought to examine to what extent the positive correlation between CD5 and ICOS holds true in TCR transgenic mouse models where all T cells express a single TCR. We compared CD5 and ICOS levels on mature thymocytes from WT mice with MHC-II-restricted SMARTA (Figure 3c, d) and MHC-I-restricted OT-I (Figure 3e, f) TCR transgenic mice. Relative to WT CD4\(^{+}\) SP thymocytes, those from SMARTA TCR transgenic mice express lower levels of CD5 (Figure 3c, d, left panels). SMARTA CD4\(^{+}\) SP thymocytes also expressed significantly lower ICOS levels than their WT counterparts (Figure 3c, d, right panels). By contrast, CD8\(^{+}\) SP thymocytes from OT-I mice express higher levels of CD5 and ICOS than their counterparts in WT mice (Figure 3e, f). It is also possible that ICOS–ICOSL interactions may, in turn, influence TCR signals in response to self-antigen at later stages of development. As such, we assessed CD5 levels on thymocytes from WT (ICOS\(^{+/+}\)ICOSL\(^{+/+}\)), ICOS\(^{-/-}\) and ICOSL\(^{-/-}\) mice. Ultimately, we do not detect any statistically significant differences in CD5 levels among conventional CD4\(^{+}\) and CD8\(^{+}\) SP thymocytes nor Treg from WT versus ICOS- or ICOSL-deficient mice (Figure 3g, Supplementary figure 3b). Overall, our results suggest that ICOS expression on thymocytes correlates with the strength of TCR signaling they received during thymic selection but that ICOS signaling during T-cell differentiation does not globally change the strength of TCR signals perceived during development.
Figure 2. Differential expression of inducible T-cell costimulator (ICOS) on thymocyte subsets. (a) Representative histograms and (b) relative expression of ICOS on thymic developmental subsets in wild-type (WT) adult mice analyzed by flow cytometry: CD4\(^+\)CD8\(^-\) TCR\(\beta\)\(^{hi}\) double-negative (DN), CD4\(^+\)CD8\(^-\)CD69\(^{hi}\) preselection double-positive (Presel DP), CD4\(^+\)CD8\(^+\)CD69\(^{hi}\) postselection DP (Postsel DP), TCR\(\beta\)\(^{hi}\)CD25\(^+\)Foxp3\(^+\)CD4\(^+\) single-positive (SP), TCR\(\beta\)\(^{hi}\)CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T (Treg), TCR\(\beta\)\(^{hi}\)CD8\(^+\) SP. Dots in (b) indicate individual mice (\(n = 22\)) from a minimum of three independent experiments. Relative fluorescence intensity (RFI) is calculated after normalization to ICOS median fluorescence intensity (MFI) of the DN subset in each experiment. (c) Relative expression of ICOS on distinct maturation stages of CD4\(^+\) SP and CD8\(^+\) SP thymocytes (CD69\(^{hi}\)MHC-I\(^{M1}\) and the more mature CD69\(^{lo}\)MHC-I\(^{M2}\)). Dots in (c) indicate individual mice (\(n = 6\)) from two independent experiments. The RFI for ICOS is normalized to the MFI of the M1 CD4\(^+\) SP subset in each experiment. (d) Representative flow plots of recirculated (CD73\(^+\)CD44\(^+\)) and de novo (CD73\(^{lo}\)) thymic Tregs. (e) Representative histograms and quantification of ICOS expression on recirculated and de novo thymic Tregs. The RFI is normalized to the MFI of de novo thymic Tregs in each experiment. (f) Representative flow plots for two thymic Treg precursors (CD25\(^+\) and Foxp3\(^{lo}\)) gated on de novo, nonrecirculated thymocytes. (g) Representative histograms and quantification of ICOS expression on thymic Treg precursors and Foxp3\(^{lo}\)CD25\(^+\) Tregs. (h) Quantification of CD5 expression on thymic Treg precursors and mature Foxp3\(^{lo}\)CD25\(^+\) Tregs. The RFI for ICOS or CD5 is normalized to the MFI of the CD4\(^+\) SP subset in each experiment. Dots in (f, g, and h) indicate individual mice (\(n = 8\)) from three independent experiments. *\(P < 0.05\), ***\(P < 0.001\), Welch’s t-test (e) or Brown–Forsythe and Welch ANOVA followed by Dunnett’s T3 multiple comparisons test (b, c, g, h). FMO, fluorescence minus one control; MHC, major histocompatibility complex; TCR, T-cell receptor.
Inducible T-cell costimulator may fine-tune negative selection

We demonstrated that ICOSL is expressed on subsets of APCs predominantly localized to the thymic medulla and corticomedullary region, and that ICOS is upregulated at the postselection DP and SP thymocyte stages. Therefore, we considered the possibility that ICOS–ICOSL interactions may play a previously unappreciated role in the thymic selection outcome of ICOS-expressing thymocytes. Except for innate T-cell subsets, it has been suggested that thymic T-cell development is largely normal in ICOS signaling-deficient mice, in line with this, we do not observe any consistent abnormalities in the percentages or number of conventional T-cell and Treg populations in the thymus of ICOS−/− and ICOSL−/− mice as compared with WT (ICOS+/+ICOSL+) controls (Supplementary figure 4a). In addition, there is no difference in the accumulation of thymocyte populations with select markers of strong signals that accompany negative selection (Supplementary figure 4b and data not shown). The one exception is a reduction in a population of CD8+ SP thymocytes that express PD-1 in ICOS- and ICOSL-deficient mice as compared with controls (Supplementary figure 4c).

Given that PD-1 is a marker that has been associated with strong, persistent signals in the thymus and autoreactive TCRs, we sought to determine whether ICOS–ICOSL interactions influenced negative selection of MHC-I-restricted thymocytes. For this, we used TCR transgenic thymocytes that will undergo negative selection in the presence of their cognate antigen in an in situ thymic slice system. We generated thymic slices from ICOSL−/+ and ICOSL−/− mice and overlaid the slices with CellTrace Violet-labeled total OT-I thymocytes mixed 1:1 with control congenic (CD45.1) WT thymocytes. OVA (SIINFEKL) peptide, the cognate antigen for the OT-I TCR, was added in the media for the thymic slices (Figure 4a). The thymic APCs can take up the peptides from the media and present them to the overlaid thymocytes. Maximal negative selection is achieved in this model with 1 nM of peptide, and we reasoned that a role for costimulation in negative selection may be more evident in conditions where peptide availability or avidity is lower; as such, we also incubated thymic slices in the presence of 0.1 nM peptide. After 24 h of incubation, we examined the relative ratio of OT-I thymocytes as compared with overlaid control CD45.1+ cells by flow cytometry (Figure 4a, b). In the presence of OVA peptide, we observed a significant decrease in total OT-I thymocytes on ICOSL−/+ slices as compared with no peptide control slices, indicating that the OT-I thymocytes are efficiently deleted in the presence of OVA peptide (Figure 4b, c). However, the extent of negative selection of OT-I thymocytes is significantly reduced in the absence of ICOSL (Figure 4b, c). In this model, OVA peptide acts like ubiquitous self-antigen, and TCRs reactive to the peptide can be deleted in both the cortex and the medulla, ICOS expression in OT-I thymocytes is also elevated during the postselection DP stage and expressed at higher levels on CD8+ SP thymocytes (Supplementary figure 4d, e), similar to the expression pattern observed on the polyclonal populations from WT mice (Figure 2a, b). We analyzed whether DP or CD8+ SP thymocytes are more resistant to deletion in the absence of ICOSL. We observed that the difference in negative selection efficiency in the presence or absence of ICOSL is slightly less striking in the OT-I TCR transgenic DP thymocytes (Figure 4d) compared with CD8+ SP thymocytes (Figure 4e), and that ICOS–ICOSL interactions may be more important for negative selection when cognate antigen is limited. Therefore, as ICOSL is expressed at a higher level in the thymic medulla, its expression may be more critical for the negative selection of SP thymocytes expressing higher levels of ICOS.

**DISCUSSION**

In this study, we suggest that the ICOS–ICOSL pathway may play a role in tuning thymic negative selection. We confirmed that ICOSL is mainly expressed by mTECα, SIRPα+ DCs and thymic B cells, while ICOS is expressed on postselection DP, SP thymocytes and thymic Tregs. Throughout thymic development, ICOS expression generally correlates with the strength of TCR interactions with self-antigens. In the absence of ICOSL, in situ negative selection of MHC-I-restricted T cells using a thymic slice model is less efficient.

We present data that suggest that ICOS–ICOSL interactions may play a role in modulating negative selection of CD8-lineage T cells; whether the same is true for CD4+ T cells is not yet clear. We observed a decrease in the percentage of potentially autoreactive PD-1-expressing MHC-I-restricted CD8+ SP thymocytes in the absence of ICOS signaling. In addition, we detected a decrease in deletion of MHC-I-restricted antigen-specific thymocytes in an in situ model of negative selection, suggesting that ICOS–ICOSL interactions may play a role in modulating negative selection of CD8-lineage T cells. By contrast, no consistent differences in mature CD4+ SP and thymic Treg populations were noted in the absence of ICOS signaling. Yet, it is possible that ICOS–ICOSL interactions also have an impact on the negative selection of the MHC-II-
restricted TCR repertoire. MHC-II-restricted TCR transgenic models have lower self-reactivity than that of the polyclonal T-cell population. Based on our data, this would also suggest that they may express lower levels of ICOS. Therefore, these TCR transgenic models might be less sensitive to ICOS–ICOSL interactions during thymic selection as compared with their MHC-I-restricted TCR transgenic counterparts and additional means are necessary to assess the impact of ICOS signaling on negative selection in the CD4+ T-cell lineage.

In the in situ thymic slice system, not only did we observe that negative selection of total and CD8+ OT-I was less efficient in the absence of ICOSL, but we also made similar observations with negative selection of MHC-I-restricted DP thymocytes. This is not entirely surprising considering that our results show that postselection DP

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**Figure 3.** Inducible T-cell costimulator (ICOS) expression correlates with the strength of T-cell receptor (TCR) signaling. (a) Representative histograms and (b) relative ICOS expression on CD4+ or CD8+ single-positive (SP) thymocytes gated on the top (CD5hi), intermediate (CD5int) and bottom (CD5lo) 15% of CD5 expression. The relative fluorescence intensity (RFI) of ICOS is normalized to the median fluorescence intensity (MFI) of ICOS on CD5lo CD4+ SP thymocytes in each experiment. (c) Representative histograms and (d) relative expression of CD5 (left) and ICOS (right) on CD4+ SP thymocytes from nontransgenic wild-type (WT) mice as compared with those from MHC-II-restricted TCR transgenic SMARTA mice. (e) Representative histograms and (f) relative expression of CD5 (left) and ICOS (right) on CD8+ SP thymocytes from WT mice as compared with those from MHC-I-restricted TCR transgenic OT-I mice. (g) Quantification of CD5 expression on CD4+ SP and CD8+ SP thymocytes from WT, ICOS ligand (ICOSL)- and ICOS-deficient mice. The RFI for CD5 is normalized to the MFI of CD4+ SP in each experiment. Dots indicate individual mice (n = 6) from a minimum of two independent experiments. **P < 0.01, ***P < 0.001, two-tailed unpaired Student’s t-test.
Figure 4. Negative selection of OT-I T-cell receptor (TCR) transgenic thymocytes is less efficient in the absence of inducible T-cell costimulator ligand (ICOSL). (a) Schematic representation of experimental setup. CellTrace Violet (CTV)-labeled total OT-I thymocytes were mixed at a 1:1 ratio with congenic CD45.1+ wild-type (WT) thymocytes and were overlaid atop thymic slices generated from either ICOSL+/+ or ICOSL−/− mice in the presence or absence of OVA peptide (SIINFEKL) at the indicated concentrations. The slices were analyzed by flow cytometry 24 h later. (b) Representative flow plots and (c) compilation of the relative proportion of total live OT-I thymocytes normalized to the proportion of congenic CD45.1+ thymocytes from each slice; a decrease in the proportion of OT-I thymocytes indicates negative selection. The relative ratio of (d) CD4+CD8+ double-positive (DP) OT-I thymocytes or (e) CD8+ single-positive (SP) OT-I thymocytes. n = 9 thymic slices for each condition from three independent experiments, *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed unpaired Student’s t-test within each peptide concentration condition.
thymocytes already express ICOS. A small number of thymic DCs in the cortex is sufficient to mediate negative selection, though SIRPα DCs with the highest levels of ICOSL are preferentially found at the corticomedullary junction. It is possible that some of the postselection DP thymocytes in our system have already migrated to the medulla as it was previously suggested that DP thymocytes bearing MHC-I-restricted TCRs, as in the case of our study, migrate to the medulla prior to becoming CD8 SP thymocytes. Nevertheless, the differences in negative selection efficiency between WT and ICOSL−/− thymic slices were more evident in the mature CD8 SP thymocyte compartment. Higher expression of ICOS on the SP thymocytes than on the DP thymocytes and more thymic APCs expressing ICOSL could indicate that the expression of ICOSL plays a larger role in the negative selection of thymocytes in the medulla.

We observed that negative selection is less efficient in the absence of ICOSL in situ, but it did not completely abrogate deletion of the autoreactive cells. In addition, there are no gross defects in T-cell development in ICOS- and ICOSL-deficient mice. Although rare, a study reported autoimmune symptoms such as autoimmune arthritis in an ICOS-deficient patient. This raises the question as to whether the thymic selection defect we observed in mice may contribute to some autoimmune diseases. However, multiple mouse studies suggest autoimmune symptoms observed in ICOS-mutant mouse models are largely attributable to weakened Treg functions in the periphery as opposed to increased autoreactive effector T cells. In NOD mice, germline ICOS or ICOSL deficiency dramatically reduces the incidence of type 1 diabetes, emphasizing a positive costimulatory role of ICOS in pathogenic effector cells. By contrast, in non-obese diabetic (NOD) mice in which most T cells recognize an autoantigen (BDC2.5 TCR transgenic model), the dominant role of ICOS seems to be the maintenance of Treg cells in the inflamed pancreas to prevent the onset of diabetes. Therefore, germline deficiency of ICOS or ICOSL in BDC2.5-NOD mice accelerates type 1 diabetes. Collectively, these data support the notion that the role of ICOS in thymic selection is to fine-tune the TCR repertoire but the absence of ICOS or ICOSL can be largely compensated by other mechanisms in this context. Indeed, it is possible that multiple cosignaling molecules work together to mediate negative selection; cosignaling through molecules such as CD28, CD40, CD43 and others have been implicated in the deletion of autoreactive thymocytes. Our observations add an additional layer of complexity to the network of cosignaling molecules that may be involved in sculpting the TCR repertoire.

METHODS

Mice

C56BL/6, B6.SJL-PtprcPepcB/Bom (B6SJJ), B6.129P- Icos tm1Mak /J (ICOSL−/−)69 and B6.129P2-Icos tm1Mak/J (ICOSL−/−)70 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6-Tg(OT-I)-Rag1−/−mice were obtained through the National Institute of Allergy and Infectious Diseases Exchange Program, National Institutes of Health (Bethesda, MD, USA). SMARTA TCR transgenic mice were kindly provided by Dr Judith Mandl (McGill University, Montreal, QC, Canada). All mice were bred and maintained in specific pathogen-free animal facilities at the Maisonneuve-Rosemont Hospital Research Centre and Institut de Recherches Cliniques de Montréal. Both male and female mice 6–12 weeks of age were used. All animal protocols have been approved by Animal Care Committees at the Maisonneuve-Rosemont Hospital Research Centre and Institut de Recherches Cliniques de Montréal.

Cell Isolation

Single-cell suspensions of thymus were prepared with a tissue grinder. Red blood cells were lysed with Ack lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA). The digestion was stopped with Roswell Park Memorial Institute 1640 (RPMI 1640) media (Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum. The sample was centrifuged at 200 × g for 7 min and the pellet resuspended. All samples were filtered and counted with a hemocytometer using trypan blue; an equal number of cells from each sample was stained for flow cytometry.

Thymic slices

Thymic selection on thymic slices was performed as previously described. Briefly, the thymus of ICOSL−/− and ICOSL+/+ controls were harvested, cleaned, embedded in 4% NuSieve GTG agarose (Lonza, Basel, Switzerland) and cut into 500-µm slices using a vibratome (VT1000S, Leica Biosystems, Wetzlar, Germany). Thymic slices were placed...
atop 0.4-µm cell culture inserts (BD Falcon, Corning Inc., Corning, NY, USA) over 1.5 mL of complete Roswell Park Memorial Institute 1640 (RPMI 1640) media containing 2 mM l-glutamine, 100 IU penicillin and 100 µg mL⁻¹ streptomycin (Wisent) and 10% fetal bovine serum (GE Life Sciences) in a 6-well plate. Total thymocytes from OT-I mice were stained with CellTrace Violet (Thermo Fisher) according to the manufacturer’s protocol. CellTrace Violet-stained OT-I thymocytes were mixed 1:1 with B6SJL thymocytes; 1–3 million thymocytes were overlaid on top of each thymic slice and incubated for 2–3 h at 37°C prior to washing off excess cells that had not migrated into the tissue. For experiments using OVA (SIINFEKL) peptide (AnaSpec, Fremont, CA, USA), OVA peptide was added to the media at the indicated concentrations after the initial 2–3-h incubation. The slices were further incubated at 37°C for 24 h, at which point the thymic slices were harvested, dissociated with a tissue grinder and analyzed by flow cytometry.

Antibodies and flow cytometry
Fluorescently labeled anti-mouse CD4 (GK1.5 and RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD11c (N418), CD25 (PC61), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), CD80 (16-10A1), EpCAM (CD326, G8.8), I-A/I-E (M5/114.15.2), ICOS (CD278, C398.4A), ICOSL (CD275, HK5.3), Ly-51 (6C3), TCRβ (H57-597), TCR Vα2 (B20.1), SIRPα (CD172a, P84), B220 (RA3-6B2), H-2Kb (AF6-88.5), CD44 (IM7), PD-1 (CD279, 29F.1A12), CD62L (MEL-14), CD19 (6D5), CD73 (RTY/11.8), CD11b (M1/70) and Zombie fixable viability dye were purchased from BioLegend (San Diego, CA, USA). Biotinylated anti-UEA-1 was purchased from Vector Laboratories (Burlingame, CA, USA), and anti-Foxp3 (150D/E4) was purchased from eBioscience (Thermo Fisher). Cells were incubated with viability dye according to the manufacturer’s protocol followed by incubation with cell surface antibodies for 20 min at 4°C (10 min in the case of thymic stromal cells). Intranuclear staining of Foxp3 was performed using a Foxp3 staining kit for fixation and permeabilization (eBioscience/Thermo Fisher) according to the manufacturer’s protocol. All data were acquired on an LSR Fortessa X-20 or LSR II flow cytometer using FACSDiva software and analyzed with FlowJo version 10 (BD Biosciences, San Jose, CA, USA).

Statistical analysis
Statistical analyses were performed using Prism version 8 (GraphPad, San Diego, CA, USA). Statistical significance is indicated by P-values: *P < 0.05, **P < 0.01, ***P < 0.001.

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CONFLICT OF INTEREST
All authors declare not conflicts of interest.

AUTHOR CONTRIBUTIONS
Mengqi Dong: Conceptualization; Data curation; Formal analysis; Methodology; Writing – original draft; Writing – review & editing. Jinsam Chang: Conceptualization; Data curation; Formal analysis; Investigation; Writing – review & editing. Marie-Eve Lebel: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Supervision; Writing – review & editing. Noémie Gervais: Data curation; Methodology. Marilaine Fournier: Data curation; Visualization; Writing – review & editing. Ève Mallet Gauthier: Methodology; Writing – review & editing. Woong-Kyung Suh: Conceptualization; Funding acquisition; Supervision; Writing – original draft; Writing – review & editing. Heather J Melichar: Conceptualization; Funding acquisition; Writing – original draft; Writing – review & editing.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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