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Developmentally Regulated Availability of RANKL and CD40 Ligand Reveals Distinct Mechanisms of Fetal and Adult Cross-Talk in the Thymus Medulla

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The thymus provides a specialized microenvironment for the generation and selection of self-tolerant T cells. During their intrathymic development, T cell progenitors migrate through distinct cortical and medullary microenvironments that contain phenotypically and functionally distinct epithelial cell types (1). Initial stages in T cell development occur in the cortex, and include the transition of immature CD4^+^ thymocytes to the CD4^+^8^+^ stage, a process that involves the generation of, and signaling through, the pre-TCR complex (2). T cell progenitor development is also dependent upon interactions between Notch and DL4 (3, 4), the latter being expressed by cortical thymic epithelial cells that can be further defined by their expression of MHC class II and CD205 (5, 6). Positive selection of CD4^+^8^+^ thymocytes also occurs within the cortex (7), with cortical thymic epithelial cell-specific expression of the thymoproteasomal subunit β5t (8) and the protease prss16 (9) enabling expression of peptide/MHC complexes to drive a process of thymocyte differentiation that includes the generation and migration of newly generated CD4^+^ and CD8^+^ cells into the thymic medulla (1).

Within thymic medullary regions, medullary thymic epithelial cells (mTEC), including those expressing the autoimmune regulator (Aire) gene, play a key role in imposing tolerance on positively selected CD4^+^ and CD8^+^ thymocytes (7, 10, 11). For example, mTEC expression of self-Ags can trigger negative selection of thymocytes bearing TCRs with potentially autoreactive specificities (12–14), a process that can also include transfer of mTEC-derived Ags to dendritic cells (15–17). In addition, mTEC are involved in the intrathymic emergence of CD4^Foxp3^ regulatory T cells (T-Reg), which play an important role in peripheral tolerance mechanisms (18, 19). Most recently, the Aire^+^ subset of mTEC has been shown to play a role in the intrathymic positioning of dendritic cells, which themselves are potent mediators of tolerance induction in the thymus (19).

Given the importance of Aire^+^ mTEC in multiple aspects of intrathymic tolerance induction, several studies have examined the cellular and molecular requirements for their maturation. Importantly, early stages in mTEC development were shown to involve the formation of clonal islets from individual mTEC progenitors within medullary areas (20). Whereas further studies have shown that progenitors for Aire^CD80^ mTEC reside within the CD80^−^ MHCI^low^ (mTEC^low^) fraction (21, 22) that are also defined by claudin-3/4 expression (23), earlier experiments demonstrated...
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a role for signals from hemopoietic cells for thymus medulla development (24, 25), a process termed thymus cross-talk. Although our own studies have revealed a role for innate immune cells, namely lymphoid tissue inducer (LTI) and invariant Vγ5+ dendritic epidermal T cell (DETC) progenitors, in the generation of the first cohorts of Aire+ mTEC in the fetal thymus (22, 26, 27), other studies demonstrated the importance of oβTCR+ CD4+ thymocytes during postnatal and adult stages (28, 29). Importantly, these studies also collectively helped to identify a role for multiple members of the TNFR superfamily in the cross-talk mechanisms that control the development of mTEC. Thus, whereas deficiency in LTβR signaling resulted in an overall reduction of murfuryllization and oβTCR+ RANKL and CD40L interactions during mTEC development (26, 29), and in vitro ligation of CD40 appears less effective than ligation of RANK in inducing Aire+ mTEC development (31). Thus, whereas CD40L- and CD40-deficient mice have a reduction in overall mTEC numbers (26, 29, 31, 32), the precise role of CD40–CD40L interactions during mTEC development is unclear.

Whereas these studies explain some aspects of the mechanisms controlling mTEC development, others remain poorly understood, including the timing of availability of the cellular sources of receptor activator for NF-κB ligand (RANKL) and CD40L during mTEC/hemopoietic cross-talk. For example, much of the data on thymic RANKL and CD40L expression comes from either PCR analysis of cell populations to measure mRNA expression, or flow cytometric analysis in which CD40L/RANKL have been analyzed individually but never simultaneously, and sometimes following pharmacological stimulation (28, 29, 31, 33, 34). As a result, potential heterogeneity in physiological levels of RANKL and CD40L expression within LTI, DETC progenitors, and mature oβTCR+ thymocytes has not been fully addressed, meaning that their ability to provide these signals either singularly or in combination is not known. Moreover, the requirement for both RANK and CD40 signaling, and the possible interplay between these molecules in mTEC development, has not been fully explored.

In this study, we define, at a per cell level, the provision of RANKL and CD40L during fetal and adult stages of mTEC development. We find that hemopoietic cross-talk from innate LTI and invariant DETC progenitors that control Aire+ mTEC development in fetal stages involves RANKL, but not CD40L, whereas postnatal control of mTEC development by oβTCR+ high CD4+ thymocytes involves both RANKL and CD40L. Separation of thymic resident CD4+ T cells into distinct subsets based on their activation status (CD69), lineage (CD25), and maturation stage (Rag2-GFP) shows that positive selection of conventional CD4+ T cells involves the sequential acquisition of first RANKL and then CD40L, whereas CD25+ T-Reg provide RANKL, but not CD40L, regardless of their maturation stage. Finally, we show that initial RANK signaling is required to upregulate CD40 expression on developing mTECs at a stage that occurs prior to the induction of functionally relevant mTEC molecules, including Aire and CD80, and provide evidence that CD40–CD40L signaling controls proliferation, but not the rate of apoptosis, within the mTEC compartment. From our data, we propose that mTEC development in the adult is controlled by a sequence of events initially involving RANK and then CD40, with both stages being influenced by distinct CD4 thymocyte subsets.

Materials and Methods

Mice

BALB/c, C57BL/6, BoyJ, RANK-deficient (22) (Tnfrsf11a−/−), CD40L-deficient (35) (Cd40lg−/−), and FVB/N Rag2-GFP transgenic mice (36) were housed at the Biomedical Services Unit, University of Birmingham, under United Kingdom Home Office guidelines. For the generation of timed BALB/c embryos, the day of detection of the vaginal plug was designated as day 0.

Cell isolation

Thymocyte suspensions were obtained from adult thymus or fetal thymus organ cultures (FTOC) by mechanical dissociation. Stromal cells from 2-deoxyxyanosine (2-DG)-treated FTOC were obtained by disaggregation in 0.25% trypsin/0.02% EDTA (Sigma-Aldrich) solution at 37 °C for 5–10 min, with a single-cell suspension made by gentle repetitive pipetting (37). Trypsin was removed by washing prior to FACS staining. Stromal cells from postnatal thymuses were isolated, as previously described (38). In brief, thymus lobes were cut into 1-mm3 pieces, washed, and digested with 8–5 medium (HEPES and l-glutamine–supplemented RPMI 1640 [Sigma-Aldrich]), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES [all from Life Technologies-Invitrogen], 5% FCS) containing 0.32 Wunsch U/ml liberase/thermolyasin (Roche) and 50 Kunitz U/ml DNase I (Sigma-Aldrich) at 37 °C for 35 min using an orbital shaker (180 rpm; New Brunswick Scientific). Enzymatic treatment was repeated for an additional 20 min, followed by incubation with 5 mM EDTA on ice for 5–10 min. Remaining tissue fragments were mechanically dispersed by careful pipetting. Cell suspensions from each digestion were pooled and washed in ice-cold PBS containing 2% FCS and 2 mM EDTA to prevent aggregate formation. Stromal cells were enriched by MACS immunomagnetic depletion of CD45+ cells (Miltenyi Biotech), according to the manufacturer’s protocol. Cell surface FACS staining was performed in 2% FCS and 2 mM EDTA to prevent aggregate formation.

Cell suspension culture and RANKL/CD40L flow cytometry staining

Hematopoietic cells from adult thymus and FTOC were isolated by mechanical dissociation. Cells were counted, and 2 ml vol of DMEM plus 10% FCS containing 4 × 106 cells were cultured in each well of a polystyrene 6-well plate (Falcon) at 37 °C, 5% CO2, for 4.5 h. After culture, cell suspensions were harvested and washed before being stained with the Abs below. DAPI (Invitrogen) was used to exclude dead cells.

Abs and flow cytometry

The following Abs were used for flow cytometric analysis (all obtained from eBioscience, unless stated otherwise): anti-B220 FITC (RA3-6B2), anti-CD3e Alexa700 (17A2), anti-CD4 Alexa700 or PECy7 (GK1.5), anti-CD4 V500 (RM4-5; BD Biosciences), anti-CD8a FITC, allophycocyanin, or V500 (53-6.7; BD Biosciences), anti-CD11c FITC (N418), anti-CD25 allophycocyanin (PC61; Biologend), anti-CD40 PE (3/23; BD Biosciences), anti-CD40L PE (MR1; BD Biosciences), anti-CD45 allophycocyanin 780 (30-F11), anti-CD69 PerCpCy5.5 (H1.2F3), anti-CD80 V421 (16-10A1; Biologend), anti-CD127 Alexa647 (ATR34), anti-Aire Alexa488 (clone H512; gift of H. Scott, Adelaide University), anti-EpCAM1 Alexa647 (G8.8; gift of A. Farr, University of Washington), anti-FOXP3 PE (FJK-16s), anti-IAA FITC, or biotin (AF6-120.1; BD Biosciences), anti-Ki67 PECy7 (B56; BD Biosciences), and anti-Ly51 biotin or PE (6C3), anti-RANKL biotin (IK22.5), anti-TCR δ allophycocyanin 780 (H57-597), anti-TCR δ FITC (GL3), anti-Vγ5 FITC (536; BD Biosciences), isotype mouse IgG1 PECy7 (BD Biosciences), isotype rat IgG1 eB(eB)G1, isotype rat IgG2a biotin (eB2a), and annexin V-FITC (BD Biosciences). Biotinylated Abs were revealed with streptavidin PECy7 or PerCpCy5.5. Surface staining of cell suspensions was performed in PBS/3% FCS solution at 4°C. Annexin V staining was performed in binding buffer (BD Biosciences) for 20 min at room temperature, and staining was stopped by adding an excess of binding buffer containing DAPI (Invitrogen). Intracellular staining for Foxp3 was performed using fixation buffer (eBioscience) and permeabilization buffer (eBioscience), according to the manufacturer’s protocol. Intracellular staining...
for Aire was performed, as described (27). Intracellular staining for Ki67 was performed following the same protocol. Flow cytometric acquisition was performed on a BD-LSR Fortessa machine using FACSDiva 6.2 software (BD Biosciences), and data were analyzed with FlowJo 8.7 software (Tree Star).

**RANKL stimulation of 2dGuo-treated FTOC**

The 2dGuo-treated FTOC were established from freshly isolated E15 thymus lobes, as previously described (39). After 7-d organ culture in 1.35 mM 2dGuo, thymic lobes were transferred in center-well organ culture dishes (Falcon) containing fresh DMEM plus 10% FCS. A total of 5 mg/ml agonist anti-RANK Ab (R&D Systems) was added where indicated to induce mTEC maturation (22), and lobes were harvested over an additional 4 d at the indicated time points.

**Real-time quantitative PCR**

Expression of the tissue-restricted Ags casein-α (Coun) and salivary protein 1 (Sp1) was analyzed before and after RANK stimulation of 2dGuo-treated FTOC. The generation of cDNA and quantitative PCR was performed exactly as described (27). Expression values for each sample were normalized to β-actin, and fold levels of the indicated genes represent the mean (±SEM) of replicate reactions. Primer sequences are as follows: b-actin (Actb), QuantiTect Min Actb 1 SG Primer Assay (Qiagen QT00095242); casein α (Coun), forward, 5’-CATCATCCAAGACTGAA-GCCAG-3’, and reverse, 5’-CCTGTGGAAAGTACGCACAGGCAGAAG-3’; salivary protein 1 (Sp1), forward, 5’-GGCTCTGAAACTCAGGAGA-3’, and reverse, 5’-TGCAAACTCATCCACGTTT-3’.

**Results**

**Differential expression of RANKL and CD40L in cells regulating fetal and adult hemopoietic cross-talk during Aire+ mTEC development**

To compare the way in which fetal innate and adult adaptive immune cells influence mTEC development, we first investigated expression of RANKL and CD40L protein simultaneously by flow cytometry on defined cell types, to provide a detailed analysis of the provision of these molecules at the single cell level. Note that isotype control Abs were used to set flow cytometric gates for levels of RANKL expression, whereas for CD40L staining, both isotype controls and cells from CD40L- mice were used.

Flow cytometric analysis of Vγ5+TCR DETC progenitors and CD4+3 IL7Rα+ LTi isolated from E15 thymus, explanted for 7 d in organ culture, showed that both populations expressed readily detectable cell surface levels of RANKL (Fig. 1A), with higher levels detectable on LTi cells, a finding consistent with quantitative PCR (qPCR) analysis (27). In contrast to RANKL, both LTi and DETC progenitors were found to lack detectable expression of CD40L (Fig. 1A), whereas both RANKL and CD40L were expressed at the cell surface of adult thymocytes (Fig. 1B), most notably CD4+TCRβhigh mature thymocytes. In addition, clear heterogeneity with regard to RANKL/CD40L expression was observed within this subset (Fig. 1B), with some cells expressing either RANKL or CD40L, or both (Fig. 1C). Collectively, these data suggest that whereas RANKL expression is common to both fetal and adult hemopoietic cells that are involved in Aire+ mTEC development, the capacity to regulate mTEC development through CD40–CD40L interactions is limited to a subset of mature CD4+TCRβhigh thymocytes.

**RANKL and CD40L are expressed at defined stages of αβTCR thymocyte maturation**

CD4+8 TCRβhigh cells within the adult thymus are known to consist of a heterogeneous mixture of cell types, including mature medullary-resident thymocytes, giving rise to both Foxp3+ conventional T cells and Foxp3+ T-Reg as result of intrathymic positive selection, as well as recirculating CD4+ T cells that have re-entered the thymus from the periphery (40). To explore further the heterogeneity in RANKL/CD40L expression seen in total CD4+8 TCRβhigh cells, we took advantage of Rag2GFP mice in which decreasing levels of GFP expression are directly linked to increasing thymocyte maturity (41, 42). We also analyzed RANKL/CD40L expression in Rag2GFP mice in conjunction with the activation marker CD69, and CD25 as a marker of T-Reg. As in our hands, nuclear staining of Foxp3 using Abs impacts on detection of GFP (data not shown), we used CD25 expression as a surrogate T-Reg marker (43), as it largely overlaps with Foxp3

**FIGURE 1.** RANKL and CD40L are differentially expressed in innate cells and αβTCR thymocytes. LTi cells (CD4+IL-7Rα+CD8α- B220- CD11c+ TcRγδ+ CD3ε+ TCRβ+) and Vγ5+TCR (Vγ5+CD3ε+CD4+ CD8α-) thymocytes from 7-d FTOC (A), together with adult thymocyte subsets defined by expression of CD4, CD8, CD69, and TCRβ (B) were analyzed for expression of RANKL and CD40L by flow cytometry. Note that gates are set using isotype control Abs for RANKL, and cells obtained from CD40L- mice for CD40L expression. (C) Shows frequencies of CD40L+ and RANKL+ cells in adult thymocytes. Error bars represent SEM. Open bars, RANKL+CD40L- cells; hatched bars, RANKL+CD40L+ cells; black bars, RANKL-CD40L+ cells. Analysis of Vγ5+TCR and LTi cells was performed on pooled batches of ~60 and 100 FTOC, respectively. The dot plots shown are representative of two independent experiments. Dot plots and histograms representing RANKL and CD40L expression on adult Rag2-GFP thymocytes are representative of two independent experiments with four mice analyzed in each of these experiments.
in thymic CD4+ TCRβ\textsuperscript{high} cells (Fig. 2A), thereby enabling identification of CD25\textsuperscript{−} conventional, and CD25\textsuperscript{+} regulatory, CD4\textsuperscript{+} thymocyte subsets (Fig. 2A).

Fig. 2B shows that within the CD25\textsuperscript{−} subset of CD4\textsuperscript{+} TCRβ\textsuperscript{high} cells, a dominant Rag2GFP\textsuperscript{+}CD69\textsuperscript{−} population is present, representing newly positively selected thymocytes, and a smaller subset of Rag2GFP\textsuperscript{−}CD69\textsuperscript{−} cells, representing later stage mature CD4\textsuperscript{+} thymocytes (41, 42, 44). Analysis of RANKL/CD40L expression within the Rag2-GFP\textsuperscript{+} CD25\textsuperscript{−} CD4\textsuperscript{+} TCRβ\textsuperscript{high} subset of CD69\textsuperscript{−} thymocytes shows that newly selected Rag2GFP\textsuperscript{+}CD69\textsuperscript{−} cells are enriched for RANKL\textsuperscript{+}, but not CD40L\textsuperscript{+} cells (Fig. 2C). In contrast, Rag2GFP\textsuperscript{−} CD69\textsuperscript{+} cells contain few RANKL\textsuperscript{+} cells, whereas CD40L\textsuperscript{+} cells are readily detectable (Fig. 2C), suggesting that RANKL and CD40L expression differentially map to early and late stages of CD4 thymocyte selection. Interestingly, whereas analysis of the CD25\textsuperscript{+} subset of Rag2GFP\textsuperscript{+} CD4\textsuperscript{+} TCRβ\textsuperscript{high} thymocytes also revealed RANKL expression within the CD69\textsuperscript{−} subset (Fig. 2E), Rag2GFP\textsuperscript{−}CD69\textsuperscript{+} cells within the CD25\textsuperscript{+} subset were CD40L\textsuperscript{−} (Fig. 2E). Thus, these data suggest that although RANKL expression is a shared feature of both recently produced CD25\textsuperscript{+} T-Reg and CD25\textsuperscript{−} conventional T cells, CD40L expression is limited to the latter.

In agreement with an earlier study (42), within CD4\textsuperscript{+} TCRβ\textsuperscript{high} cells, a Rag2GFP\textsuperscript{+} population is detectable within both CD25\textsuperscript{−} and CD25\textsuperscript{+} subsets, and most predominately in the latter (Fig. 2B). As previously discussed (42), we cannot currently determine what proportions of these Rag2GFP\textsuperscript{+} cells represent either peripheral T cells that have re-entered the thymus, or thymocytes that have lost GFP expression intrathymically. However, it is interesting to note that CD25\textsuperscript{−} Rag2GFP\textsuperscript{−} cells within the CD4\textsuperscript{+} TCRβ\textsuperscript{high} subset are enriched for CD40L\textsuperscript{+} cells (Fig. 2D). In

**FIGURE 2.** RANKL and CD40L define early and late stages of αβ T cell maturation. (A) Shows CD25 expression and intracellular Foxp3 expression in total CD4\textsuperscript{+} TCRβ\textsuperscript{high} adult thymocytes. (B) CD4\textsuperscript{+} TCRβ\textsuperscript{high} adult thymocytes from adult Rag2GFP mice were divided into CD25\textsuperscript{−} and CD25\textsuperscript{+} subsets shown in the right dot plot of (A), and subsequently analyzed for Rag2GFP in conjunction with CD69 expression. (C-F) Show frequencies of RANKL- and CD40L-expressing cells in the indicated CD25\textsuperscript{−}Rag2GFP/CD69\textsuperscript{−} subsets of CD4\textsuperscript{+} TCRβ\textsuperscript{high} thymocytes. Error bars represent SEM. Open bars, RANKL\textsuperscript{+}CD40L\textsuperscript{−} cells; hatched bars, RANKL\textsuperscript{+}CD40L\textsuperscript{+} cells; black bars, RANKL\textsuperscript{−}CD40L\textsuperscript{+} cells. The dot plot showing Foxp3 expression is representative of four independent experiments, with one mouse analyzed in each of these experiments. Dot plots and histograms showing RANKL and CD40L expression alongside CD25, GFP, and CD69 on adult RAG2-GFP thymocytes are representative of two independent experiments, with four mice analyzed in each of these experiments.

**FIGURE 3.** CD40 expression by mTEC is reduced in the absence of the TNFR superfamily member RANK. (A) Shows expression of MHC class II and CD80, used to define mTEC\textsuperscript{low} and mTEC\textsuperscript{high} compartments, in total CD45\textsuperscript{+}EpCAM\textsuperscript{−}Ly51\textsuperscript{−} mTEC within freshly digested thymuses from 3- to 4-wk-old WT and Tnfrsf11a\textsuperscript{−/−} mice. mTEC\textsuperscript{low} and mTEC\textsuperscript{high} fractions in WT and Tnfrsf11a\textsuperscript{−/−} mice were compared for expression of CD40 (B), and (C) shows mean fluorescence intensity analysis of CD40 expression on the indicated mTEC subsets. Dot plots are representative of two independent experiments on 3- to 6-wk-old mice. In total, five mice of each group were studied. Statistical analysis performed used a Mann–Whitney U test (unpaired, two tailed, 95% of confidence); *p < 0.05 and N.S., nonsignificant.
marked contrast, CD25*Rag2GFP* cells within the CD4+ TCRβhigh subset (Fig. 2F) are predominantly CD40L− RANKL−, irrespective of their CD69 expression. Thus, regardless of whether Rag2GFP CD4+ TCRβhigh cells represent recirculating or longer-term thymus resident cells, expression of RANKL but not CD40L within the CD25− subset suggests that these cells are able to stimulate RANK-mediated but not CD40-mediated mTEC cross-talk.

Evidence for a sequential involvement of RANK and CD40 in mTEC differentiation and proliferation

As the data above suggest that, in conventional CD4 thymocyte positive selection, RANKL expression appears to map to earlier CD69+ stages, whereas CD40L appears at later CD69− stages, we investigated the possibility that RANK, and then CD40 signals, are sequentially involved in mTEC maturation. Thus, thymi from 3- to 6-wk-old wild-type (WT) and Tnfrsf11a−/− (RANK−/−) littermate controls were enzymatically digested, and the CD45− EpCAM+Ly51− mTEC compartment was analyzed. Consistent with previous studies (22, 28, 31), a reduced proportion of mature CD80+MHChigh mTEC was detected in adult Tnfrsf11a−/− mice (Fig. 3A). Interestingly, comparison of the levels of CD40 expression in both CD80−MHClow mTEClow and CD80+ MHChigh mTEChigh cells revealed a slightly lower level of expression of CD40 in mTEChigh cells from Tnfrsf11a−/− mice compared with WT controls (Fig. 3B, 3C), suggesting that in vivo levels of CD40 expression within mTEC may be RANK dependent.

To explore this possibility further, we stimulated 2dGuo-treated FTOC, deprived of hemopoietic cross-talk and devoid of mature mTEC (22), with agonistic RANK Abs, and analyzed expression of a panel of mTEC markers, namely CD40, Aire, and the tissue-restricted Ags casein-α (Csna) and salivary protein 1 (Spt1), over a time course of 1–4 d. At the indicated time points, lobes were disaggregated and analyzed by flow cytometry in the case of Aire and CD40, and qPCR for Csna and Spt1. Interestingly, analysis of mTEC expression of CD40 prior to RANK stimulation shows that these cells are CD40− (Fig. 4A), suggesting that CD40 expression is cross-talk dependent. Moreover, in line with the in vivo analysis shown in Fig. 3, upregulation of CD40 by mTEC occurred as a consequence of RANK signaling (Fig. 4B), with RANK-mediated CD40 upregulation occurring prior to the expression of Aire and CD80 (Fig. 4B) and the tissue-restricted Ags Csna and Spt1 (Fig. 4C). Thus, anti-RANK stimulation of mTEC progenitors rapidly promotes the upregulation of CD40, consistent with the idea that RANK and CD40 are sequentially involved in mTEC development.

Although several studies have examined the mTEC compartment in CD40- and/or CD40L-deficient mice, the role played by CD40–CD40L-mediated cross-talk on mTEC development and/or homeostasis, and the timing of its involvement at particular stages of mTEC development, remain unclear (29, 31, 32). To investi-
gate further the role of CD40–CD40L interactions in mTEC development, we investigated whether this signaling axis may play a role in regulating cellular proliferation within the mTEC compartment. To this end, thymuses from adult WT and Cd40lg$^{-/-}$ mice were disaggregated, and the proliferative status of mTEC subsets was analyzed using anti-Ki67 Abs. Fig. 5A shows that the proportion of cells expressing Ki67 within the total mTEC population is similar in WT and Cd40lg$^{-/-}$ mice. Interestingly, however, by subdividing total mTEC into mTEC$^{\text{low}}$ and mTEC$^{\text{high}}$ compartments, we saw a significant disruption of the proliferative status of mTEC from Cd40lg$^{-/-}$ mice, with a reduction in the frequency of Ki67$^+$ cells in the mTEC$^{\text{low}}$ subset (Fig. 5B, 5C), and an increase in the frequency of Ki67$^+$ cells within the mTEC$^{\text{high}}$ population (Fig. 5B, 5C), suggesting that CD40–CD40L-mediated cross-talk may play a key role in controlling mTEC proliferation. Finally, given the observed perturbations in mTEC proliferation in Cd40lg$^{-/-}$ mice reported in this study, together with the relatively rapid turnover time of mature mTEC (21, 45) and the possible link between Aire expression by mTEC and the induction of apoptosis (45, 46), we next investigated the frequency of apoptotic cells within distinct subsets of mTEC. Freshly digested thymic stromal preparations were analyzed by flow cytometry for the presence of apoptotic cells within subsets of mTEC distinguished on the basis of their levels of MHC class II expression, using a combination of annexin V and DAPI. Interestingly, however, despite an increase in the frequency of Ki67$^+$ mTEC$^{\text{high}}$ cells in Cd40lg$^{-/-}$ mice, we did not see a sig-

**FIGURE 5.** CD40–CD40L interactions control the balance of proliferation within the mTEC compartment. (A) Shows quantitative analysis of Ki67$^+$ cells within the total mTEC population, obtained by flow cytometry analysis, in WT and Cd40lg$^{-/-}$ mice. (B) Shows flow cytometric analysis of digested thymuses from 7- to 8-wk-old WT (upper panels) and Cd40lg$^{-/-}$ (lower panels) mice, gated on CD80$^{+}$ MHCII$^{\text{low}}$ and CD80$^{+}$ MHCII$^{\text{high}}$ subsets of CD45$^{+}$ EpCAM$^{+}$Ly5.1$^{\text{low}}$ mTEC. Staining is shown for levels for MHC class II and the proliferation marker Ki67. (C) Shows quantitative analysis of Ki67$^+$ cells within the mTEC$^{\text{low}}$ and mTEC$^{\text{high}}$ subsets in WT and Cd40lg$^{-/-}$ mice. The histogram in (A) is a summary of two independent experiments. In total, a minimum of four mice of each group were studied. The dot plots are representative of three independent experiments on 6- to 9-wk-old male mice. In total, seven WT and six Cd40lg$^{-/-}$ mice were studied in each experiment. Statistical analysis performed used a Mann–Whitney U test (unpaired, two-tailed, 95% of confidence); **p < 0.005 and N.S., nonsignificant.

**FIGURE 6.** CD40–CD40L interactions do not control the rate of apoptosis within the mTEC compartment. (A) Shows flow cytometric analysis of DAPI/annexin V staining in digested thymuses from 11- to 15-wk-old WT (upper panels) and Cd40lg$^{-/-}$ (lower panels) mice, gated on MHC-II$^{\text{low}}$ and MHC-II$^{\text{high}}$ subsets of CD45$^{+}$ EpCAM$^{+}$Ly5.1$^{\text{low}}$ mTEC. (B) Shows quantitative analysis of annexin V$^+$ DAPI$^{\text{low}}$ cells within mTEC$^{\text{low}}$ and mTEC$^{\text{high}}$ subsets in WT and Cd40lg$^{-/-}$ mice. (C) Shows quantitative analysis of annexin V$^+$ DAPI$^{\text{high}}$ cells within mTEC$^{\text{low}}$ and mTEC$^{\text{high}}$ subsets in WT and Cd40lg$^{-/-}$ mice. The dot plots are representative of two independent experiments. The histograms are the summary of these two independent experiments. Three WT and three Cd40lg$^{-/-}$ mice were studied in each experiment. Statistical analysis performed used a Mann–Whitney U test (unpaired, two-tailed, 95% of confidence); ns, nonsignificant.
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significant difference in the levels of apoptosis in WT and CD40lg−/− mTEClow and mTEChigh compartments (Fig. 6), suggesting that different mechanisms regulate proliferation and apoptosis within the mTEC compartment (Fig. 7).

Discussion

The thymic medulla represents a key site for T cell tolerance induction, a process that is mediated by several cell types, including Aire+ medullary epithelial cells (7). Exploring the mechanisms by which mTEC microenvironments are induced to develop represents an important aspect in determining how central tolerance mechanisms are controlled. In this study, we have investigated a panel of cell types linked to Aire+ mTEC development, with regard to their ability to provide RANKL and CD40L during cross-talk for thymus medulla development at distinct developmental stages. Importantly, our experiments involved simultaneous analysis of cell surface RANKL and CD40L expression in cells without the need for exogenous stimulation.

Detailed flow cytometric analysis revealed that LTi cells and Vγ5 Tdendritic epidermal T cell progenitors, both of which are involved in development of the first cohorts of Aire+ mTEC at stages prior to αβ T cell selection (27), express RANKL, but not CD40L. Thus, despite expression of CD40 and RANK by fetal mTEC (6, 22), the cells of the innate immune system that drive initial medulla formation do so through provision of RANKL, but not CD40L, a finding that fits well with the absence of Aire+ mTEC in the fetal period of RANK-deficient mice (22), as well as PCR (31) and histological analysis (33), demonstrating the absence of CD40L expression in total embryonic thymus. Interestingly, a more complex RANKL and CD40L expression pattern was observed when subsets of cells within the αβ T cell lineage of the adult thymus were analyzed (Fig. 7). In agreement with earlier findings, CD4+8 TCRRBhigh, but not CD4+8 TCRRBlow thymocytes, were found to be the major source of RANKL and CD40L (28, 29). Importantly, however, in the experiments described in this study, use of Rag2GFP mice together with expression of the activation marker CD69 as well as CD25 expression allowed us to reveal marked heterogeneity within CD4+8 TCRRBhigh thymocytes with regard to cell surface expression of RANKL and CD40L. Thus, RANKL+ cells were abundant within recently positively selected CD69+Rag2GFP+ cells, whereas CD40L+ cells mapped to later CD69− stages. Interestingly, analysis of the CD25+ subset of CD4+ thymocytes, the majority of which are Foxp3+ T-Reg, showed this population to express RANKL, but not CD40L, regardless of their Rag2GFP and CD69 status. Thus, conventional and regulatory subsets of CD4+ thymocytes appear differently equipped with regard to RANKL/CD40L expression to stimulate mTEC development.

That positive selection and maturation of CD4+ thymocytes appear to first enable RANKL- and then CD40L-mediated mTEC cross-talk led us to explore the idea that mTEC development involves RANK and CD40 acting in sequence during mTEC development. In support of this idea, mTEC from Tnfrsf11a−/−-deficient mice expressed lower levels of CD40 in vivo, and in vitro RANK stimulation of mTEC induced the rapid upregulation of CD40, prior to expression of Aire, tissue-restricted Ags Csmn and Sp1, and CD80. Whereas our data show that RANK signaling in mTEC precursors involves the rapid upregulation of CD40 expression, a recent study showed that RANK expression by mTEC is controlled by LTβR signaling (47). Collectively, these observations further support the idea that signaling through individual TNFRs controls the availability of other family members during mTEC development. Moreover, whereas proliferation within the total mTEC population was not perturbed in CD40lg−/− mice, analysis of mTEClow and mTEChigh compartments revealed significant alterations. Indeed, reduced proliferation within the mTEClow compartment was observed, together with enhanced proliferation within mTEChigh cells. Given that proliferation within total mTEC was unaltered, these findings suggest that, rather than triggering proliferation directly, CD40 may play a role in controlling its timing during mTEC development, with a delay in proliferation occurring in the absence of CD40 signaling. Interestingly, however, no significant difference in the frequency of apoptotic mTEC was found in CD40lg−/− mice, suggesting that whereas CD40−CD40L interactions impact upon proliferative control of the mTEC compartment, a different mechanism regulates the rate of mTEC apoptosis. Of note, our findings that CD40 influences proliferation within mTEC are of interest in relation to studies linking it to the regulation of epithelial cells of other tissues such as the skin, where it plays a role in the homeostatic control of keratinocyte proliferation (48, 49). Given other studies have shown that epithelial cells within skin and thymus can share certain molecular characteristics (50, 51), including FoxN1 (52) and RANK expression (53), these findings suggest that CD40 represents an additional example of molecules that are involved in the regulation of epithelial compartments within both these tissues.

Taken together, our data reveal complexity in the intrathymic availability of key TNFR ligands that control mTEC development, and show that whereas embryonic and adult stages of mTEC development share a common requirement for RANK signaling, provision of CD40L signaling occurs only in the postnatal thymus from interactions with discrete subsets of CD4 thymocytes (Fig. 7). Finally, the control of CD40 expression, shown in this work to be a regulator of mTEC proliferation, by RANK stimulation supports a stepwise involvement for these molecules in mTEC development, in which upregulation of CD40 is an early event, occurring prior to the induction of CD80, Aire, and genes encoding tissue-restricted Ags.

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Disclosures

The authors have no financial conflicts of interest.

FIGURE 7. The involvement of RANK and CD40 during fetal and adult mTEC development. A model of mTEC development involving the TNFR superfamily members RANK and CD40 is presented. During the fetal program of thymus development, the generation of the first cohorts of Aire+ mTEC involves RANK, but not CD40 signaling, via interactions with RANKL−CD40L− innate-like cells. In contrast, in an adult program of mTEC development, RANK signaling is followed by CD40 upregulation and signaling, a two-step process involving interactions with distinct CD4 thymocyte subsets.
References


