Thymic Medullary Epithelium and Thymocyte Self-Tolerance Require Cooperation between CD28–CD80/86 and CD40–CD40L Costimulatory Pathways


*J Immunol* 2014; 192:630-640; Prepublished online 13 December 2013; doi: 10.4049/jimmunol.1302550
http://www.jimmunol.org/content/192/2/630

Supplementary Material http://www.jimmunol.org/content/suppl/2013/12/13/jimmunol.1302550.DC1

References This article cites 54 articles, 27 of which you can access for free at: http://www.jimmunol.org/content/192/2/630.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Thymic Medullary Epithelium and Thymocyte Self-Tolerance Require Cooperation between CD28–CD80/86 and CD40–CD40L Costimulatory Pathways

Joy A. Williams,*1 Jingjing Zhang,*1 Hyein Jeon,* Takeshi Nitta,† Izumi Ohigashi,‡ David Klug,* Michael J. Kruhlak,* Baishakhi Choudhury,* Susan O. Sharrow,* Larry Granger,* Anthony Adams,* Michael A. Eckhaus,‡ S. Rhiannon Jenkinson,* Ellen R. Richie,§ Ronald E. Gress,§ Yousuke Takahama,† and Richard J. Hodes*

A critical process during thymic development of the T cell repertoire is the induction of self-tolerance. Tolerance in developing T cells is highly dependent on medullary thymic epithelial cells (mTEC), and mTEC development in turn requires signals from mature single-positive thymocytes, a bidirectional relationship termed thymus crosstalk. We show that CD28–CD80/86 and CD40–CD40L costimulatory interactions, which mediate negative selection and self-tolerance, upregulate expression of LTα, LTβ, and receptor activator for NF-κB in the thymus and are necessary for medullary development. Combined absence of CD28–CD80/86 and CD40–CD40L results in profound deficiency in mTEC development comparable to that observed in the absence of single-positive thymocytes. This requirement for costimulatory signaling is maintained even in a TCR transgenic model of high-affinity TCR-ligand interactions. CD4 thymocytes maturing in the altered thymic epithelial environment of CD40/CD80/86 knockout mice are highly autoreactive in vitro and are lethal in congenic adoptive transfer in vivo, demonstrating a critical role for these costimulatory pathways in self-tolerance as well as thymic epithelial development. These findings demonstrate that cooperation between CD28–CD80/86 and CD40–CD40L pathways is required for normal medullary epithelium and for maintenance of self-tolerance in thymocyte development. The Journal of Immunology, 2014, 192: 630–640.

An important aspect of thymic T cell development is the generation of a functional T cell repertoire that is capable of responding to a broad universe of foreign Ags but that is not reactive to self. Self-tolerance is accomplished by a variety of mechanisms, including deletion of thymocytes with high affinity for self and diversion of developing self-reactive thymocytes to a T regulatory (Treg) cell fate. The thymic medulla plays a central role in this tolerization process, providing an environment in which developing thymocytes are exposed to a spectrum of self Ags during maturation and selection of CD4 and CD8 single-positive (SP) lineages. Medullary thymic epithelial cells (mTEC) are essential to successful induction of self-tolerance, expressing a wide array of peripheral Ags, at least in part due to expression of the autoimmune regulator (AIRE) gene (1, 2). The critical role of AIRE-expressing mTEC in tolerance induction is evidenced by the severe autoimmune disease that occurs when expression of AIRE protein is disrupted (2, 3). The dialogue between CD4 SP thymocytes and stromal cells, termed thymic crosstalk, is in turn important in supporting development of a normal mTEC compartment (4–7). There has therefore been considerable interest in defining the molecular interactions that mediate this critical crosstalk. We undertook studies to determine whether molecular interactions known to be important in the thymic tolerization process, by both promoting negative selection and supporting generation of Treg cells, might also have critical roles in maintaining a normal thymic medulla. Thymic Treg cell development is critically dependent on CD28–CD80/86 interactions, whereas negative selection of self-reactive SP thymocytes is mediated by both CD28–CD80/86 (8, 9) and CD40–CD40L (10, 11) interactions. We therefore addressed the role of CD28–CD80/CD86 and CD40–CD40L costimulatory pathways in interactions between thymocytes and thymic stromal cells during thymic development.

We found that thymic epithelial cell (TEC) development was only modestly perturbed by inactivation of the CD40–CD40L or CD28–CD80/86 pathway alone, whereas, in striking contrast, the combined absence of CD28–CD80/86 and CD40–CD40L interactions in CD40/CD80/86 knockout (KO) mice resulted in a decrease in mTEC numbers as profound as that observed in the complete absence of SP thymocytes. Examination of thymocyte development in the altered thymic environment of CD40/CD80/86 KO mice revealed that CD40/CD80/86-deficient SP thymocytes were highly autoreactive, responding strongly in vitro to syngeneic APCs, in contrast to the minimal responses of either CD80/86- or CD40-deficient thymocytes, and causing accelerated death when transferred into congenic nude mice. These findings dem-
onstrate that a strong cooperativity between CD28–CD80/86 and CD40–CD40L pathways is required for both normal epithelial and thymocyte development; in their absence, the tolerance-inducing thymic medullary compartment fails to properly develop and SP thymocytes are autoreactive.

Materials and Methods

Mice

BALB/c (BALB) mice were obtained from the Frederick Cancer Research Facility (Frederick, MD) and maintained at Bioqual (Rockville, MD). BALB CD40-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained at Bioqual; and BALB CD80/86-deficient mice were a gift of A. Sharpe (Boston, MA). BALB CD40/CD80/86 KO mice were generated through crosses of BALB CD40 KO and CD80/86 KO mice on a BALB background. BALB CD40L-deficient mice were generated by backcrossing C57BL/6 (B6) CD40L KO mice from The Jackson Laboratory for five generations onto the BALB background. BALB CD28/CD40L KO mice were generated by crossing the BALB CD40L KO mice with BALB CD28 KO mice obtained from The Jackson Laboratory. LTβR KO (12) mice have been previously described and were crossed to either B6 CD40 KO or B6 CD80 KO (Jackson Laboratory) mice to generate LTβR/CD40 KO or LTβR/CD80 KO mice, respectively. DO11 thymus-specific (tg) mice were crossed to BALB/CD40/CD80/86 KO or to B6 CD40/CD80/86 KO mice to generate DO11 thymocytes lacking expression of CD40, CD80, or CD40/CD80/86 on H2a or H2b backgrounds, respectively. TCRα KO mice have been previously described (14). Athymic BALB nu/nu mice were obtained from Frederick National Laboratory for Cancer Research.

Cell proliferation assays

CD8-depleted thymocytes (>80% CD4+ SP thymocytes) and T-depleted splenocytes were prepared using CD8-specific, or CD4- and CD8-specific magnetic beads (Miltenyi Biotec), respectively. T-depleted splenocytes were irradiated at 500 rad, and then 2 × 10^5 CD8-depleted thymocytes were added to titrated numbers of irradiated splenocyte APCs in 96-well round-bottom plates. Cultures were incubated for 3–4 d prior to addition of [3H]thymidine for 16 h.

Preparation of thymic stromal cells for flow cytometric analysis and sorting

Thymic stromal cell preparations were made using methods modified from those reported by Gray et al. (15). Following release of thymocytes by gentle teasing of the thymus, thymic thymisks were digested with collagenase/dispase at 0.25% w/v plus Dounce I at 0.125% w/v (Roche) in four sequential 15-min incubations at 37°C. Reactions were stopped by addition of FCS to 20%. For TEC analysis, single-cell suspensions were stained with anti-CD45, anti-MHC class II allophycocyanin (M5-114; eBioscience), and anti-Ly51 (mTEC = UEA+; cTEC = UEAnegLy51+) (Fig. 2A). The cortical thymic epithelial cell (cTEC) based on staining with UEA-1 to stain thymic medulla, and Ly-51 (cortex specific) or anti-keratin 8 (stains cortex and some medullary epithelium) to reveal the cortex. In contrast, the medullary epithelium was much reduced in the CD40/CD80/86 KO thymus relative to other genotypes and was comparable to the profound reduction seen in TCRα KO thymus lacking SP thymocytes. This analysis was extended by flow cytometric characterisation. TEC were defined as CD45+CD45R+MHCII+ and assigned further as mTEC and cortical thymic epithelial cell (cTEC) based on staining with UEA-1 and Ly51 (mTEC = UEA+; cTEC = UEA- Ly51+) (Fig. 2A). The CD40 KO had reduced numbers of mTEC relative to both WT and CD80/86 KO mice. As can be seen in Fig. 1, deletion of CD40 alone or of CD80/86 alone had little effect on cortico-medullary architecture as defined using either anti-keratin 14 or the lectin UEA-1 to stain thymic medulla, and Ly-51 (cortex specific) or anti-keratin 8 (stains cortex and some medullary epithelium) to reveal the cortex. In contrast, the medullary epithelium was much reduced in the CD40/CD80/86 KO thymus relative to other genotypes and was comparable to the profound reduction seen in TCRα KO thymus lacking SP thymocytes. This analysis was extended by flow cytometric characterisation. TEC were defined as CD45+MHCII+ and assigned further as mTEC and cortical thymic epithelial cell (cTEC) based on staining with UEA-1 and Ly51 (mTEC = UEA+; cTEC = UEA- Ly51+) (Fig. 2A). The CD40 KO had reduced numbers of mTEC relative to both WT and CD80/86 KO mice (Fig. 2B). Strikingly, however, the CD40/80/86 KO had a profound reduction in mTEC numbers relative to all other groups, including the CD40 KO. Notably, the number of CD4 or CD8 SP thymocytes in CD40/CD80/86 KO mice was not decreased, indicating that the defect in mTEC development does not result from a decrease in SP cells known to play a role in medullary development (Fig. 2C). CD4 SP cells were in fact increased in CD80/86 and CD40 KO thymuses, consistent with previous observations (19–21). A significant reduction in mTEC numbers was also observed in BALB CD40L/CD28 KO mice relative to WT, CD28 or CD40L single KOs, confirming that it is

Quantitative PCR

Total cellular RNA was DNase I treated and reverse transcribed with oligo(dt) primer and SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using SYBR Premix Ex Taq (TakaRa) and Light Cycler DX400 (Roche). Amplified products were confirmed to be single bands by gel electrophoresis. The primer sequences are described previously (6, 18).

Adaptive transfer to athymic nude recipients

Thymocytes were prepared from wild-type (WT), CD40 KO, CD80/86 KO, and CD40/CD80/86 KO mice on a BALB background and injected i.v. into BALB nu/nu recipient mice. Mice were weighed weekly and monitored daily for signs of morbidity.

Histopathology

For histological analysis, organs (liver, small intestine, colon, stomach, heart, spleen, lymph nodes, and lung) were fixed in 10% neutral buffered formalin (Sigma-Aldrich), embedded in paraffin, sectioned, and stained with H&E.

Statistical analysis

Student t test (unpaired, two tailed) was used to determine p values.

Results

Normal thymic medullary development requires CD40–CD80/86 and CD28–CD80/86 interactions

Previous work has documented the importance of thymocyte/stromal cell crosstalk in establishing normal thymic medullary structure (4, 5). Both CD28–CD80/86 and CD40–CD40L pathways are known to be important interactions for deletion of autoreactive thymocytes and mediation of self-tolerance in the thymus (8–11), which prompted us to examine the roles of these costimulatory pathways in the thymic crosstalk that results in development of the thymic medulla. To accomplish this, we analyzed thymic sections from BALB WT, CD80/86 KO, CD40 KO, and CD40/CD80/86 KO mice. As can be seen in Fig. 1, deletion of CD40 alone or of CD80/86 alone had little effect on cortico-medullary architecture as defined using either anti-keratin 14 or the lectin UEA-1 to stain thymic medulla, and Ly-51 (cortex specific) or anti-keratin 8 (stains cortex and some medullary epithelium) to reveal the cortex. In contrast, the medullary epithelium was much reduced in the CD40/CD80/86 KO thymus relative to other genotypes and was comparable to the profound reduction seen in TCRα KO thymus lacking SP thymocytes. This analysis was extended by flow cytometric characterisation. TEC were defined as CD45+MHCII+ and assigned further as mTEC and cortical thymic epithelial cell (cTEC) based on staining with UEA-1 and Ly51 (mTEC = UEA+; cTEC = UEA- Ly51+) (Fig. 2A). The CD40 KO had reduced numbers of mTEC relative to both WT and CD80/86 KO mice. As can be seen in Fig. 1, deletion of CD40 alone or of CD80/86 alone had little effect on cortico-medullary architecture as defined using either anti-keratin 14 or the lectin UEA-1 to stain thymic medulla, and Ly-51 (cortex specific) or anti-keratin 8 (stains cortex and some medullary epithelium) to reveal the cortex. In contrast, the medullary epithelium was much reduced in the CD40/CD80/86 KO thymus relative to other genotypes and was comparable to the profound reduction seen in TCRα KO thymus lacking SP thymocytes. This analysis was extended by flow cytometric characterisation. TEC were defined as CD45+MHCII+ and assigned further as mTEC and cortical thymic epithelial cell (cTEC) based on staining with UEA-1 and Ly51 (mTEC = UEA+; cTEC = UEA- Ly51+) (Fig. 2A). The CD40 KO had reduced numbers of mTEC relative to both WT and CD80/86 KO mice (Fig. 2B). Strikingly, however, the CD40/80/86 KO had a profound reduction in mTEC numbers relative to all other groups, including the CD40 KO. Notably, the number of CD4 or CD8 SP thymocytes in CD40/CD80/86 KO mice was not decreased, indicating that the defect in mTEC development does not result from a decrease in SP cells known to play a role in medullary development (Fig. 2C). CD4 SP cells were in fact increased in CD80/86 and CD40 KO thymuses, consistent with previous observations (19–21). A significant reduction in mTEC numbers was also observed in BALB CD40L/CD28 KO mice relative to WT, CD28 or CD40L single KOs, confirming that it is

Immunohistology

Sections (6 μm) of OCT-embedded frozen tissue were air dried for 15 min and then incubated 2 h with optimal dilutions of the primary rabbit polyclonal anti-keratin 14 (Covance Research Products) and rat anti-keratin 8 (Troma-I; Developmental Studies Hybridoma Bank, Iowa University) or anti-Ly51 PE (BD Pharmingen) and UEA-1 FITC (Vector). Tissues were washed and, after an amplification step (anti-rabbit Alexa 546 for K14, anti-rat Alexa 488 for K8, or biotinylated anti-PE, followed by streptavidin Alexa 546 for Ly51), were mounted on microscope slides and imaged using a Zeiss axiovert 200 inverted epifluorescence microscope equipped with appropriate fluorescence filters (Chroma Technologies), a motorized scanning stage, a 10× plan-apochromat (N.A. 0.45) objective lens, and a Photometrics CoolSnapES CCD camera (Roper Scientific).
simultaneous disruption of the CD40–CD80/86 and CD28–CD80/86 pathways that results in a deficient mTEC compartment (Supplemental Fig. 1).

mTEC are a heterogeneous population consisting of MHCII\textsuperscript{low} CD80/86\textsuperscript{low} and MHCII\textsuperscript{high} CD80/86\textsuperscript{high} cells. A subpopulation of the MHCII\textsuperscript{high} CD80/86\textsuperscript{high} cells is AIRE\textsuperscript{+} and has a critical role in the induction of thymic tolerance [reviewed in (22)]. Interestingly, whereas the total number of UEA\textsuperscript{+} mTEC was significantly decreased in CD40/CD80/86 KO mice, the frequencies of MHCII\textsuperscript{high} and MHCII\textsuperscript{low} mTEC and the frequency of AIRE\textsuperscript{+} MHCII\textsuperscript{high} cells within the mTEC population were similar to that of WT mice (Supplemental Fig. 2A, 2B). Overall, however, there was a dramatic reduction in the absolute number of AIRE\textsuperscript{+} mTEC in CD40/CD80/86 KO mice (Supplemental Fig. 2C).

To further characterize the decrease in mTEC observed in CD40/CD80/86 KO mice, we compared the number of mTEC present in WT and CD40/CD80/86 KO mice at various time points after birth. Numbers of mTEC were comparable in WT and CD40/CD80/86 KO mice shortly after birth (Fig. 2D). With increasing age, the number of mTEC increased progressively in WT mice, but not in KO mice. Thus, the absence of CD40–CD80/86 and CD28–CD80/86 interactions does not affect the numbers of mTEC present until several days after birth, a time at which the number of SP thymocytes begins to dramatically increase.

Costimulation influences expression of genes critical for mTEC development and SP thymocyte recruitment to the medulla

A number of the factors implicated in mTEC development are members of the TNF/TNFR family known to signal through the nonclassical NF-κB pathway central to lympho-organogenesis and mTEC formation [reviewed in (23)]. Therefore, to identify the mechanisms underlying collaborative effects of CD28 and CD40 pathways in mTEC development, we determined whether disruption of CD28–CD80/86 and CD40–CD40L interactions affected expression of a panel of TNF/TNFR family members in both CD4 SP cells and mTEC. Relative to WT CD4 thymocytes, levels of LTA and LTβ mRNA were significantly decreased in the CD80/86 KO and CD40/CD80/86 KO but not in CD40 KO CD4 SPs (Fig. 3A). In addition, expression of the chemokine CCL19, known to be up-regulated in mTEC in response to LTA/LTβ (24), was decreased in CD40L/CD28 KO relative to WT mTEC. Expression of mTEC CCL21 was not significantly decreased (p = 0.1). CD4 SP thymocyte expression of receptor activator for NF-κB ligand (RANKL) was not reduced in the absence of CD40–CD40L and/or CD28–CD80/86 interactions, whereas comparison of WT and CD40/CD28 KO mTEC revealed that expression of receptor activator for NF-κB (RANK) was significantly decreased in CD28/CD40L KOs (Fig. 3B). Expression of LTβR and AIRE in WT and CD28/CD40L KO mTEC was comparable. Taken together, the gene expression data from CD4 SP thymocytes and from mTEC indicate that mice lacking both CD28–CD80/86 and CD40–CD40L interactions have deficiencies in at least three TNFR family pathways that are known to promote mTEC development: CD40L–CD40, LT–LTβR, and RANKL–RANK, as well as reduction in the CCL19 chemokine important for recruiting SP thymocytes to the medulla, where they are subject to negative selection.

CD28 acts via regulation of LTA/β expression in cooperating with CD40 signals to promote mTEC development

The observations that CD28–CD80/86 interactions contribute significantly to production of LTαβ in CD4 SP thymocytes and that CD40/CD80/86 KO mice have a profound reduction in mTEC prompted us to test the hypothesis that CD28 signals cooperate with CD40 signals via regulation of the LTαβ pathway to promote mTEC development. To do this, we examined the medullary phenotype of LTβR/CD40 KO mice. As previously described (25, 26), we observed a significant reduction in the number of UEA\textsuperscript{+} mTEC in LTβR KOs relative to WT mice (Fig. 4). Interestingly, mTEC were significantly further reduced when the LTβR KO is combined with CD40 KO in LTβR/CD40 KO mice but not when combined with CD28 KO in LTβR/CD28 KO mice (Fig. 4). The failure to observe a further deficit in mTEC numbers in LTβR/CD28 KOs relative to LTβR KO mice suggests that the major contribution of CD28–CD80/86 interactions to mTEC development is via increased SP thymocyte expression of LTαβ. Conversely, the finding that the LTβR/CD40 KO does have a more profound deficit in mTEC relative to the LTβR KO is consistent with CD28–CD80/86 cooperating with CD40–CD40L interactions to facilitate mTEC development by promoting SP thymocyte production of LTαβ.

Expression of a TCR with high affinity for self does not overcome the requirement for costimulation to promote mTEC development

Requirements for costimulation in the activation of peripheral T cells are influenced by the strength of TCR–ligand interactions, such that strong TCR signaling can reduce or obviate the re-
quirement for costimulation (27, 28). It has also been reported that high-affinity TCR–ligand interactions are important in driving mTEC development (7). We therefore asked whether expression of a TCR with known high affinity for self Ag would overcome the requirement for CD40–CD40L and CD28–CD80/86 interactions in promoting mTEC development. To this end we generated DO11 TCRtg mice on either a H2d or H2b background and compared development of mTEC in the presence or absence of CD40–CD40L and CD28–CD80/86 interactions. The DO11 TCR transgene (tg) has been shown to have a high affinity for self I-Ab ligand.

**FIGURE 2.** Number of mTEC is decreased in CD40/CD80/86 KO mice, whereas number of CD4 SP thymocytes is increased. (A) Thymic stromal cells from BALB WT, CD80/86 KO, CD40 KO, and CD40/CD80/86 KO mice were prepared, as described in Materials and Methods, and stained with anti-CD45, UEA-1, anti-Ly51, and anti-–I-A/I-E. After first gating on CD45neg, I-A/I-E+ cells, mTEC were identified as UEA+ and cTEC as UEA–Ly51+. Flow cytometry dot plots are representative of >9 mice per strain. (B) Thymic stromal cells were prepared as in (A). After gating on CD45neg, I-A/I-E+ cells, numbers of mTEC (UEA+) and cTEC (UEA–Ly51+) were calculated for each group. Data shown are mean ± SE for >9 mice per strain. All mice were between 3 and 5 wk of age. **p ≤ 0.01, ***p ≤ 0.001. (C) Thymocytes from 4-wk-old WT, CD80/86 KO, CD40 KO, and CD40/CD80/86 KO mice were stained with anti-CD4 and anti-CD8, and numbers of CD4 and CD8 SP were calculated after gating on the respective single-positive populations. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. (D) mTEC population in CD40/CD80/86 KO mice fails to expand after birth. Thymic stromal cells from BALB WT and CD40/80/86 KO mice were prepared from mice 1–32 d of age and stained as in (B). After gating on CD45neg, I-A/I-E+ cells, numbers of mTEC (UEA-1+) were determined. Data shown are the mean ± SE for ≥3 mice per strain.
gand, as evidenced by increased CD5 levels on DO11 Tg+ in comparison with DO11 Tg−/− CD4+ SP thymocytes in I-Aβ− expressing H2b mice (29, 30), consistent with results shown in Fig. 5A (left panel). The lower affinity of the DO11 Tg for H-2d ligand is reflected in the reduced CD5 levels of DO11 Tg+ T cells in H2d mice (Fig. 5A, right panel). When we examined the thymic epithelial compartment of DO11 Tg+ H2b×d mice, we observed no significant difference in the numbers of mTEC recovered relative to those found in DO11 Tg−/− H2b×d mice, whereas numbers of mTEC are profoundly reduced in DO11 Tg+ H2d mice compared with DO11 Tg−/− H2d mice. These results are consistent with an enhanced ability of high-affinity TCR to mediate the robust interactions required to support mTEC development (Fig. 5B). Interestingly, we found that generation of the mTEC compartment even in the presence of high-affinity TCR is strongly dependent on CD40–CD40L and CD28–CD80/86 costimulatory interactions, as evidenced by the mTEC defects in CD40 KO and CD40/CD80/86 KO mTEC. (A) mRNA expression of LTA, LTβ, RANKL, and CCR7 in CD4 SP thymocytes was normalized to GAPDH mRNA, and values for the WT DP samples were arbitrarily set to 1. Data shown are mean ± SE for five independent measurements for each strain (*p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001). All mice were 4 wk of age. (B) mTEC were sorted, as described in Materials and Methods, by gating on CD45−, MHC II+, UEA+ cells. mRNA expression of RANK, CCL19, CCL21, AIRE, and LTβR was normalized to GAPDH. Data shown are mean ± SE for n = 5 (WT) or n = 4 (CD28/CD40L K0) independent measurements. For each independent measurement, RNA was prepared from mTEC sorted from a minimum of four pooled thymuses per strain. All mice were 2 wk of age (p = 0.1 for CCL21, **p < 0.01, ****p < 0.0001).
mTEC numbers in DO11tg+ CD40/CD80/86 KO H2bxd mice are dramatically reduced relative to DO11tg+ WT H2bxd mice. Notably, the number of CD4 SP thymocytes in DO11tg+ CD40/CD80/86 KO H2bxd mice was not reduced, and in fact was significantly increased, in costimulation-deficient mice, indicating that defects in mTEC development were not the result of decreased SP thymocytes (Fig. 5B). Thus, the requirement for CD40–CD40L and CD28–CD80/86 costimulatory interactions is stringently maintained even in the presence of a high-affinity TCR–ligand interaction.

Altered thymocyte subpopulations in CD40/CD80/86 KO mice: increased CD4 SP cells and severe reductions of Treg and invariant NKT cell subpopulations

As shown above in Fig. 2C, the proportion and number of CD4 SP thymocytes were increased in CD40 KO and CD80/86 KO mice, and were further increased in CD40/CD80/86 KO mice relative to WT, CD40 KO, and CD80/86 KOs, whereas numbers of CD8 SP thymocytes were not significantly different. The significant changes in the thymic medullary compartment of CD40/CD80/86 KO mice prompted us to further characterize the thymocyte subpopulations present in these animals.

Using a CD1 tetramer to identify thymic invariant NKT (iNKT) cells, we observed a significant reduction in these cells in CD80/86 KO mice, as previously reported (31, 32), and, interestingly, also found that the absence of CD40 was accompanied by a significant decrease in thymic iNKTs, a finding, to our knowledge, not previously reported (Fig. 6). A significantly more profound decrease of iNKT cells occurred in CD40/CD80/86 KO mice.
CD4\(^{+}\)Foxp3\(^{+}\) (Treg) thymocytes were clearly decreased in the absence of CD80/86, as previously reported (33, 34) (Fig. 6). Also consistent with previous reports, we found a decrease in the frequency of CD4\(^{+}\) Treg thymocytes in CD40 KO mice (35, 36), but we did not see a decrease in absolute Treg numbers. Notably, however, both Treg cell frequency and number were decreased in CD40/CD80/86 KO relative to WT, CD40 KO, and even CD80/86 KOs (Fig. 6). Thus, iNKT and Treg thymic populations are dependent on both CD28–CD80/86 and CD40 signaling.

**CD40/CD80/86 KO thymocytes are autoreactive**

The increase in CD4 SP thymocytes observed in CD40/CD80/86 KO mice suggested that tolerance induction via clonal deletion may not be occurring normally in these mice. The deficiency in regulatory iNKT cells and Treg further suggested that defects in tolerance might occur in this setting. To determine whether these defects in CD40/CD80/86 KO mice result in the appearance of an autoreactive T cell repertoire, we first examined the TCR Vβ repertoire (Fig. 6). Also an autoreactive T cell repertoire, we first examined the TCR Vβ repertoire. Thymocytes from 4-wk-old BALB WT, CD80/86 KO, and CD40/CD80/86 KO mice were stained with anti-CD3 and CD1-PBS-57 tetramers to identify iNKT cells (top panels), or anti-CD4, anti-CD8, and anti-Foxp3 to identify Treg cells (bottom panels), and analyzed by flow cytometry. Bar graphs show the mean ± SE for four to nine mice per strain (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

Upon expression of CD40 and CD80/86 costimulatory ligands on APC, it was possible that the absence of in vivo manifestations of autoreactivity in CD40/CD80/86 KO mice was due to the absence of CD40–CD40L and CD28–CD80/86 costimulatory interactions required to drive an autoimmune response. To overcome the absence of costimulatory interactions and to test whether the autoreactive response displayed by CD40/CD80/86 KO thymocytes in vitro would be manifest in vivo, thymocytes from BALB WT, CD40 KO, CD80/86 KO, and CD40/CD80/86 KO mice were adoptively transferred to congenic athymic BALC nude CD40\(^{-}\)/CD80/86\(^{-}\) recipients. Transfer of CD40/CD80/86 KO thymocytes resulted in death of the majority of recipient mice by 2 mo posttransfer, a time point at which no deaths had occurred in recipients of BALB WT, CD40 KO, or CD80/86 KO thymocytes (Fig. 8A). Detailed histological examination revealed no consistent cause of death, but striking lymphadenopathy and splenomegaly were significantly more pronounced in recipients of CD40/CD80/86 KO thymocytes, and splenic CD8 T cells from these mice were significantly increased in numbers relative to BALB nudes receiving WT, CD80/86 KO, or CD40 KO thymocytes (Fig. 8B). In addition, the proportion of CD69\(^{+}\) CD4 and CD8 T cells was significantly higher in spleens of recipients of CD40/CD80/86 KO thymocytes relative to recipients of individual KO or WT thymocytes. Taken together, the autoreactive phenotype observed in the BALB CD40/CD80/86 KO thymocytes in vitro and the high mortality rates accompanied by significant T cell activation observed in vivo transfer indicate that there has been a breakdown of functional self-tolerance in the CD40/CD80/86 KO thymus.

**Discussion**

In the studies reported in this work, we found that simultaneous disruption of CD28–CD80/86 and CD40–CD40L costimulatory pathways results in a profound defect in mTEC development that is as severe as that which results from absence of all SP thymocytes. Moreover, CD4 SP thymocytes that matured in the altered thymic epithelial environment of CD40/CD80/86 KO mice were highly autoreactive in vivo and proliferative responses and were lethal when adoptively transferred to congenic athymic recipients in vivo. Collectively, these findings demonstrate a critical and cooperative role for these costimulatory pathways in the bidirectional interactions between medullary epithelium and developing
thymocytes that are critical for self-tolerance as well as thymic epithelial development.

Consistent with previous reports, we observed that disruption of the CD40–CD40L pathway alone resulted in a significant defect in mTEC development, but one that was far less severe than that in mice deficient in both CD40–CD40L and CD28–CD80/86. We found no effect of disruption of CD28–CD80/86, although a recent report employing an elegant technique of three-dimensional reconstruction did identify an effect of CD80/86 deletion on mTEC (37).

Interestingly, the deficit that we observed in CD40/CD80/86 KO mTEC numbers was not evident in newborn mice and did not become apparent until ∼day 6 after birth, following the appearance of SP thymocytes. In the embryonic thymus, mTEC are critically dependent on RANKL-expressing LTi cells (38) and Vγ5 γ6 T cells (39). Our data suggest that this early LTi-dependent mTEC development is occurring normally in the CD40/CD80/86 KO mice, with defects in the mTEC compartment only being manifest in 5- to 6-d-old thymus. Expression of CD40L in the thymus is not detectable until the appearance of CD4 SP thymocytes (40) and is not detectable on LTi cells (41). Although CD28 is expressed on late double-negative and double-positive (DP) thymocytes, it is unlikely that these immature thymocytes would have access to or interact with CD80/86-expressing mTEC or thymic dendritic cells that are primarily localized to the medulla (42). The migration of developing thymocytes to the medulla occurs only once developing DP thymocytes receive a positive selection signal and the chemokine receptor, CCR7, is upregulated, allowing positively selected thymocytes to migrate toward CCL19/21-producing mTEC (43). Thus, CD80/86–CD28 and CD40–CD40L interactions are expected to become critical components of the circuitry that promotes mTEC development only after CD4 SP thymocytes begin to appear, precisely as we observed when tracking mTEC numbers at birth and shortly thereafter.

mTEC express a number of TNFR family members, including CD40, LTβR, and RANK (6). A feature shared by the TNFR family members expressed on mTEC is the capacity to activate...
RelB (44–46), a critical component of the nonclassical NF-κB pathway. The importance of this pathway in mediating development of a normal mTEC compartment is evidenced by the absence of UEA+ medullary epithelial cells in mice deficient for crucial components involved in the alternative NF-κB pathway, including RelB, IKKα, and NF-κB–inducing kinase (47–51). The mechanism(s) by which SP thymocytes promote mTEC development has been shown to include important roles for members of the TNFR family ligands. In particular, CD40L, RANKL, and LTβ have all been shown to be upregulated on SP thymocytes relative to double-negative and DP thymocytes (6); and mice lacking RANK–RANKL, LTβ–LTβR, or CD40–CD40L interactions have mTEC compartment defects of varying severities (6, 25, 38, 40, 41). Consistent with recent reports (37, 52), we found that expression of LTβR on CD4 SP thymocytes is decreased in the absence of CD28–CD80/86 interactions. Our finding that LTβR/CD40 combined KO mice have a more profound mTEC defect than either the LTβR or CD40 single KOs suggests that CD28–CD80/86 cooperates with CD40–CD40L interactions to promote mTEC development, in part via increased LTβR production by CD4 SP thymocytes. These findings are consistent with the recent report that combined stimulation of 2-dGUA–treated thymic lobes with agonist anti-LTβR Ab and CD40L synergized in the in vitro induction of mTEC (52), importantly extending these findings by demonstrating that

FIGURE 8. Transfer of CD40/80/86 KO thymocytes into syngeneic BALB nu/nu mice results in rapid death. Total thymocytes were purified from BALB WT, CD80/86 KO, CD40 KO, and CD40/80/86 KO mice, and 1 × 10^7 (1 ×) or 0.5 × 10^7 (0.5 ×) cells were transferred i.v. to BALB nu/nu recipient mice. (A) Mice were monitored daily for 13.5 wk, and the proportion of surviving mice is shown plotted against days posttransfer. Fifteen to 20 mice were included in each group. (B) Splenomegaly and increased numbers of activated CD8 T cells in BALB nu/nu mice receiving CD40/CD80/86 KO thymocytes. Total thymocytes were purified from BALB WT, CD80/86 KO, CD40 KO, and CD40/80/86 KO mice and transferred i.v. to BALB nu/nu recipient mice as in (A). Single-cell suspensions were prepared from spleens harvested from BALB nu/nu recipient mice 4–6.5 wk after receiving thymocytes and stained with anti-CD4, anti-CD8, and anti-CD69. Data shown are means ± SE for WT (n = 9), CD80/86 KO (n = 6), CD40 KO (n = 5), and CD40/CD80/86 KO (n = 17). A total of 1 × 10^7 thymocytes was transferred for WT, CD80/86 KO, and CD40 KO, whereas the data for the CD40/CD80/86 KO transfers represent both 1 × (1 × 10^7) and 0.5 × (0.5 × 10^7) cells transferred (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).
LTβR and CD40L pathways in fact interact cooperatively during in vivo thymic development. Furthermore, we found that RANK expression is decreased on mTEC in mice deficient for both CD40–CD40L and CD28–CD80/86 interactions, possibly a reflection of the decrease in LTβR observed in the absence of CD28 signals (37, 52). Thus, in the CD40/CD80/86 KO thymus, RANK–RANKL, LTβR–LTβR, and CD40–CD40L interactions, all shown to cooperate in promoting development of mTEC, are either significantly reduced (RANK–RANKL and LTβR–LTβR) or eliminated (CD40–CD40L), providing a basis for explaining the dramatic reduction in mTEC numbers we observe in these mice.

Strong in vitro and lethal in vivo autoreactivity was observed in CD4 SP thymocytes that develop in CD40/CD80/86 KO thymus. A number of factors may contribute to the observed failure to induce self-tolerance in the absence of these costimulatory pathways. Autoreactivity may result, at least in part, from the defective thymic medullary epithelial compartment found in these costimulatory-deficient animals, as defective tolerance induction has been shown to accompany disruption of the mTEC compartment. RelB−, Traf6−, Bcl3/NFκB2−, and CD40−RANK-deficient mice all provide examples of this; in each of these cases, severe defects in development of the mTEC compartment are coupled with an autoreactive T cell repertoire (40, 47, 48, 53, 54). In the RelB−, Traf6−, and Bcl3/NFκB2−deficient animals, clear signs of autoreactivity were present in the knockouts themselves, whereas, in the CD40−RANK-deficient mice, similar to our CD40/CD80/86 KOs, autoreactivity was revealed only when the T cells were placed in an environment in which the ligands for T cell activation (i.e., CD40L and CD80/86) were supplied (40). However, although the severe disruption of the thymic medullary compartment may have a role in allowing self-reactive thymocytes to develop in the CD40/CD80/86 KOs, interactions between the costimulatory receptor–ligand pairs CD28–CD80/CD86 and CD40–CD40L have additional roles in shaping the repertoire of developing thymocytes that could, in their absence, lead to the emergence of autoreactive thymocytes. CD40−CD40L interactions play an essential role in negative selection of autoreactive thymocytes (10, 11), whereas CD28−CD80/86 and CD40−CD40L have additional roles in shaping the repertoire of developing thymocytes that could, in their absence, lead to the emergence of autoreactive thymocytes.

References

18. Traf6−, Traf4−, and Bcl3/NFκB2−deficient mice all provide examples of this; in each of these cases, severe defects in development of the mTEC compartment are coupled with an autoreactive T cell repertoire (40, 47, 48, 53, 54). In the RelB−, Traf6−, and Bcl3/NFκB2−deficient animals, clear signs of autoreactivity were present in the knockouts themselves, whereas, in the CD40−RANK-deficient mice, similar to our CD40/CD80/86 KOs, autoreactivity was revealed only when the T cells were placed in an environment in which the ligands for T cell activation (i.e., CD40L and CD80/86) were supplied (40). However, although the severe disruption of the thymic medullary compartment may have a role in allowing self-reactive thymocytes to develop in the CD40/CD80/86 KOs, interactions between the costimulatory receptor–ligand pairs CD28–CD80/CD86 and CD40–CD40L have additional roles in shaping the repertoire of developing thymocytes that could, in their absence, lead to the emergence of autoreactive thymocytes. CD40–CD40L interactions play an essential role in negative selection of autoreactive thymocytes (10, 11), whereas CD28–CD80/86 and CD40–CD40L have additional roles in shaping the repertoire of developing thymocytes that could, in their absence, lead to the emergence of autoreactive thymocytes.

The results reported in this work demonstrate that the effects of CD40−CD40L and CD28−CD80/86 interactions in the thymus are bidirectional and influence both CD28− and CD40L-expressing T cells and the CD80/86− and CD40-expressing cells with which these T cells interact; in the absence of these costimulatory pathways, a normal mTEC compartment fails to be formed and autoreactive thymocytes are allowed to persist. Thus, these costimulatory molecules that are vital components in the dialogue that occurs between T cells and the APCs with which they interact in the periphery are also critical elements of the crosstalk that shapes the development of thymocytes and the epithelial cells of the thymus.

Acknowledgments

We dedicate this paper to the memory of Dr. David Klug. David’s intense intellectual curiosity, keen insight, and infectious enthusiasm were an inspiration to all colleagues. We thank Dr. Alfred Singer and Karen Hathcock for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.


Figure S1. Reduction in mTEC number in CD28/CD40L KO mice. Thymic stromal cells from BALB WT, CD28 KO, CD40L KO and CD28/CD40L KO mice were prepared as described in the Materials and Methods section and stained with anti-CD45, UEA-1, anti-Ly51 and anti-IA/IE. Thymic stromal cells were prepared as in Figure 2. After gating on CD45neg, IA/IE+ cells, numbers of mTEC (UEA+) and cTEC (UEAneg, Ly51+) were calculated for each group. Data shown are averages ± SE for ≥7 mice per group. All mice were between 3-5 weeks of age. (**, p≤ 0.01; ***, p≤ 0.001 ****, p≤ 0.0001).
Thymic stromal cells from BALB WT and CD40/80/86 KO mice were prepared from 4-5 week old mice and stained with anti-CD45, UEA-1, anti-IA/IE, and anti-AIRE. (A) Representative flow cytometry dot plots are shown after first gating on CD45- IA/IE+ UEA+ mTECs. (B) Frequencies (shown as mean ± SE for n=9 mice) of MHC^hi and MHC^lo mTEC, and frequencies of AIRE^+ cells within the MHC^hi mTECs are shown in the accompanying bar graphs. (C) Total number of AIRE^+ mTECs in WT and CD40/80/86 KO mice. Numbers of AIRE^+ mTECs are the mean ± SE for 9-10 mice per strain. (**** p≤ 0.0001).
Figure S3. Negative selection is defective in BALB CD40 KO and BALB CD40/80/86 mice.

Thymocytes from 8-12 week old C57BL/6 (n=5), BALB CD80/86 KO (n=5), BALB CD40 WT (n=5) and BALB CD40/80/86 KO (n=3) mice were stained with FITC anti-CD4, PE anti-CD8 and biotin-conjugated anti-TCR Vβ Abs. The percent Vβ positive CD4 SP in each strain is shown.