

# Generation of both cortical and Aire<sup>+</sup> medullary thymic epithelial compartments from CD205<sup>+</sup> progenitors

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In the adult thymus, the development of self-tolerant thymocytes requires interactions with thymic epithelial cells (TECs). Although both cortical and medullary TECs (cTECs/mTECs) are known to arise from common bipotent TEC progenitors, the phenotype of these progenitors and the timing of the emergence of these distinct lineages remain unclear. Here, we have investigated the phenotype and developmental properties of bipotent TEC progenitors during cTEC/mTEC lineage development. We show that TEC progenitors can undergo a stepwise acquisition of first cTEC and then mTEC hallmarks, resulting in the emergence of a progenitor population simultaneously expressing the cTEC marker CD205 and the mTEC regulator Receptor Activator of NF- $\kappa$ B (RANK). In vivo analysis reveals the capacity of CD205<sup>+</sup> TECs to generate functionally competent cortical and medullary microenvironments containing both cTECs and Aire<sup>+</sup> mTECs. Thus, TEC development involves a stage in which bipotent progenitors can co-express hallmarks of the cTEC and mTEC lineages through sequential acquisition, arguing against a simple binary model in which both lineages diverge simultaneously from bipotent lineage negative TEC progenitors. Rather, our data reveal an unexpected overlap in the phenotypic properties of these bipotent TECs with their lineage-restricted counterparts.

**Keywords:** Cellular immunology · Developmental immunology · Thymic epithelial cells



See accompanying Commentary by Peterson and Laan

## Introduction

The thymus supports the generation of self-tolerant CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes expressing a diverse repertoire of self-MHC restricted  $\alpha\beta$ T-cell receptors ( $\alpha\beta$ TCRs) [1, 2]. In an adult, the thymus contains anatomically distinct cortical and medullary regions, each characterized by phenotypically and functionally discrete thymic epithelial cells (TECs). Cortical TECs (cTECs), defined by ERTR4, CD205, Ly51, express IL-7 and DL4 to regulate CD4<sup>+</sup> CD8<sup>-</sup>

T-cell precursors, while expression of  $\beta$ 5t and *Prss16* underpins their specialized role in thymocyte-positive selection. In contrast, mTECs, defined by ERTR5 and MTS10, mediate tolerance induction through expression of CD80/CD86, and Aire [2]. Thus, distinct TEC compartments play nonredundant roles during T-cell development and selection.

cTECs and mTECs arise following the FoxN1-independent formation of the thymic anlagen from third pharyngeal pouch endoderm [3]. Previously, we showed that the embryonic day (E)12 thymus contains bipotent progenitors that give rise to both cTECs

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and mTECs, identifying a starting point for analysis of the emergence of these two distinct lineages [4]. Importantly, bipotent progenitors exist at postnatal stages [5], indicating their possible role in the production of cTECs/mTECs in later life. Interestingly, analysis of E12 TECs has also revealed a dominant Plet1/Mts24<sup>+</sup> population [6–8], as well as expression of molecules typically associated with either the cTEC or mTEC lineages, including  $\beta$ 5t, CD205, and Claudin3/4 [9–11]. Collectively, these findings suggest the presence of TEC progenitor populations that may represent distinct lineage-restricted progenitor pools operating downstream of bipotent progenitors during the initial emergence of the cTEC and mTEC lineages. Importantly however, the lineage transitions occurring between bipotent progenitors and their lineage-committed progeny, the phenotypic status of bipotent progenitors, as well as the timing of emergence of the cTEC and mTEC lineages, remain important unresolved issues [12].

Here, we have investigated cTEC/mTEC lineage emergence during early stages of thymus development. We show that a subset of FoxN1<sup>+</sup> TECs expressing the cTEC marker CD205 is detectable as early as E11. During thymus ontogeny, this subset upregulates CD40, forming a contiguous stream with a progressively emerging, mTEC-encompassing, CD205<sup>-</sup>CD40<sup>+</sup> subset previously shown to express the medullary-associated genes RANK (receptor activator of NF- $\kappa$ B), Cathepsin-S, and Osteoprotegerin (OPG) [10]. We show that the E11–E12 transition identifies a developmental window in which functional responsiveness to the mTEC regulator RANK [13] occurs within both CD205<sup>-</sup> and CD205<sup>+</sup> subsets of FoxN1:eGFP<sup>+</sup> TECs. Finally, we show that purified CD205<sup>+</sup> TEC progenitors can generate both cortical and medullary microenvironments, including the Aire<sup>+</sup> mTEC subset in vivo. Collectively, our data provide evidence that at early stages of thymus development, progenitor cells can co-express features of both cTECs and mTECs that are acquired in a sequential manner, arguing against a simple binary model of bipotent TEC commitment to the cTEC and mTEC lineages.

## Results and discussion

### Ontogenetic analysis of cTEC and mTEC lineages

TECs arise from third pharyngeal pouch endoderm, with the subsequent development of bipotent cTEC/mTEC progenitors being controlled by the transcription factor FoxN1 [14, 15]. At E11–E12, the thymus and parathyroid have not yet physically separated and still exist as a shared epithelial rudiment [16]. Thus, flow cytometric analysis of E11/E12 embryos using the pan-epithelial marker EpCAM1 does not distinguish TECs from parathyroid epithelial cells, making it difficult to perform specific analysis of early TEC development in WT mice. To circumvent this problem, we utilized FoxN1:eGFP reporter mice, in which TECs express high levels of GFP [17].

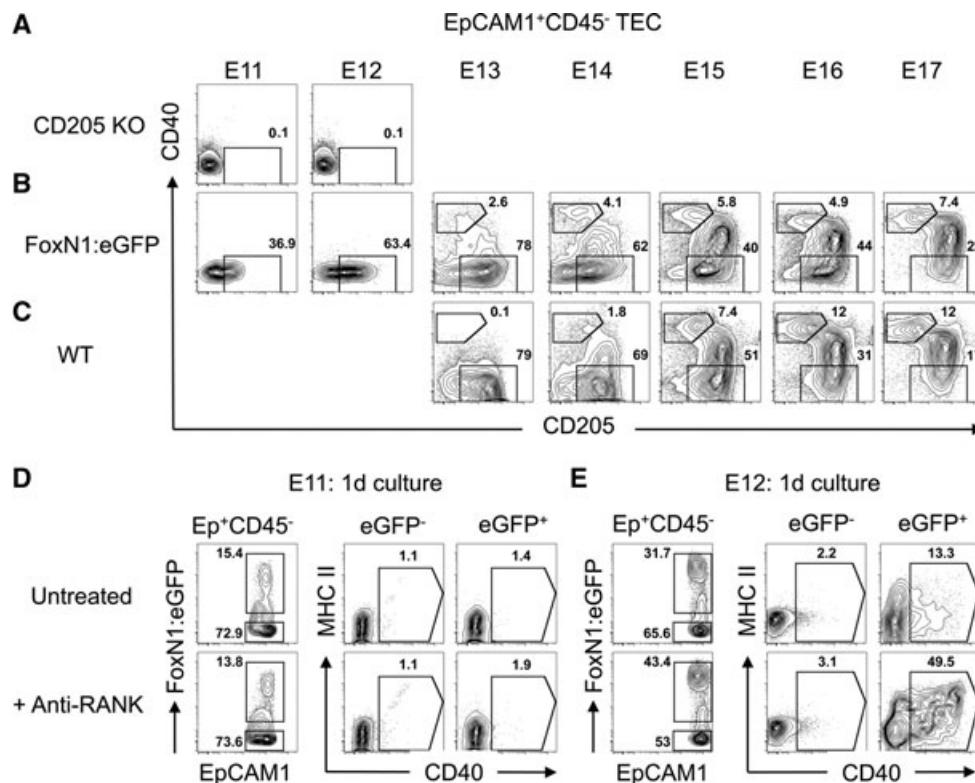
Shared parathyroid/thymus rudiments from CD205-deficient *Ly75*<sup>-/-</sup> (Fig. 1A) and FoxN1:eGFP (Fig. 1B) E11 and E12 embryos were dissected while physical exclusion of the parathy-

roid was possible in all strains from E13. Tissues were digested, and EpCAM1<sup>+</sup>CD45<sup>-</sup> TECs analyzed for CD205, CD40, and FoxN1:eGFP expression. Figure 1B shows that the emergence of FoxN1:eGFP<sup>+</sup> TECs can be detected at E11, and at all stages GFP was only detectable within EpCAM1<sup>+</sup> cells (data not shown). When we analyzed markers used previously in defining the emergence of cTECs/mTECs during thymus development [10], we found that while FoxN1:eGFP<sup>+</sup> TECs at early stages were uniformly CD40<sup>-</sup>, a distinct subset of CD205<sup>+</sup> FoxN1:eGFP<sup>+</sup> TECs was detectable at both E11 and E12 (Fig. 1B). E11 and E12 embryos from CD205-deficient (*Ly75*<sup>-/-</sup> mice) [18] confirmed the specificity of CD205 antibody staining at these stages (Fig. 1A). Thus, at an early stage in thymus development, a proportion of TECs expresses the cTEC-associated marker CD205.

Later stages of TEC development in FoxN1:eGFP embryos showed a progressive increase in the size of the CD205<sup>+</sup> subset, which gradually acquired CD40 expression from E13 onward (Fig. 1B), a pattern of TEC maturation also observed in WT embryos (Fig. 1C). By E14–E15 of gestation, both FoxN1:eGFP and WT CD205<sup>+</sup> TECs appear linked to an emerging CD205<sup>-</sup> TEC subset that expresses high levels of CD40 (Fig. 1B and C). Taken together with earlier findings that CD205<sup>+</sup>CD40<sup>-</sup> and CD205<sup>-</sup>CD40<sup>+</sup> TEC subsets express a genetic profile typically associated with the cTEC and mTEC lineages, respectively [10], these data suggested a developmental relationship between mTECs and cells expressing the cTEC marker CD205.

### Responsiveness to the mTEC regulator RANK in both CD205<sup>+</sup> and CD205<sup>-</sup> TEC subsets

The TNF-receptor superfamily member RANK controls mTEC development in the embryo [13]. However, the current lack of RANK-reporter mice and antibodies to monitor RANK expression by flow cytometry precludes analysis of RANK expression in relation to bipotent TEC progenitors and their RANK<sup>+</sup> progeny. To investigate the timing of emergence of RANK<sup>+</sup> TECs, we established short-term organ cultures of FoxN1:eGFP E11 and E12 thymus rudiments, in the presence or absence of agonistic RANK antibodies, shown previously to induce mTEC progenitor development [13]. Thus, RANK responsiveness as indicated by upregulation of the mTEC maturation markers CD40 and MHC class II [10], is used here as a surrogate marker of RANK expression. RANK stimulation of E11 thymus rudiments failed to induce maturation of FoxN1:eGFP<sup>+</sup> TECs (Fig. 1D), as indicated by the absence of MHC class II and CD40 upregulation. In contrast, while E12 EpCAM1<sup>+</sup>FoxN1:eGFP<sup>-</sup> cells failed to respond to RANK stimulation, a subset of E12 FoxN1:eGFP<sup>+</sup> TEC responded to RANK stimulation as indicated by induction of MHC class II and CD40 expression (Fig. 1E). Importantly, the lack of response in E11 cultures was not due to suboptimal conditions, as E11 rudiments cultured for 2 days showed induction of CD40 and MHC class II expression within FoxN1:eGFP<sup>+</sup> cells (data not shown). Rather, these data suggest the ability of developing TECs to respond to RANK stimulation, a property normally associated with development of the mTEC lineage, first occurs within the E11–E12



**Figure 1.** Emergence of the CD205<sup>+</sup> TEC compartment during thymus ontogeny. (A) Thymus/parathyroid rudiments from Ly75<sup>-/-</sup> embryos were analyzed for CD205 and CD40 after gating on EpCAM1<sup>+</sup>CD45<sup>-</sup> TECs. (B) FoxN1:eGFP embryos were analyzed for CD205 and CD40 expression, and data are shown after gating on EpCAM1<sup>+</sup>CD45<sup>-</sup>FoxN1:eGFP<sup>+</sup> cells. (C) WT E13–E17 thymus rudiments were isolated and analyzed as above. (D) E11 and (E) E12 thymus/parathyroid rudiments from FoxN1:eGFP embryos were cultured for 1 day in the presence (bottom) or absence (top) of anti-RANK, and FoxN1:eGFP<sup>+</sup> and FoxN1:eGFP<sup>-</sup> EpCAM1<sup>+</sup> subsets were analyzed for CD40 and MHC class II expression. Gates to discriminate FoxN1:eGFP<sup>+</sup> and FoxN1:eGFP<sup>-</sup> TEC subsets were set using WT thymus preparations. Data shown are representative of at least three separate experiments.

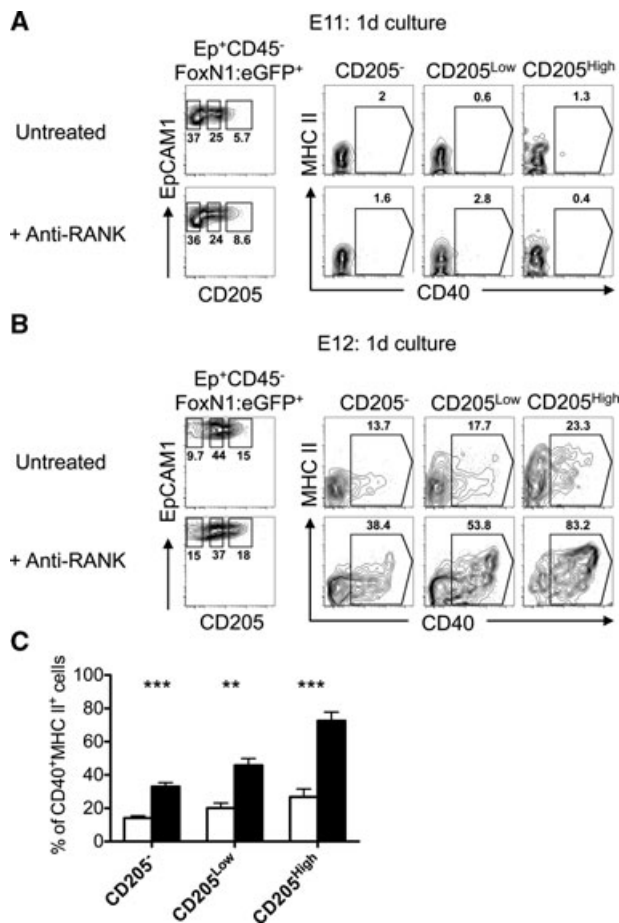
transition, indicating that this window in thymus development marks the initial emergence of RANK<sup>+</sup> TEC progenitors.

Given our ontogenetic analysis demonstrated CD205 expression by TECs at E11 and E12, we next investigated the emergence of the mTEC lineage, as revealed by RANK stimulation, in the context of CD205 expression. When we separated TECs into CD205<sup>-</sup>, CD205<sup>Low</sup>, and CD205<sup>High</sup> subsets, in line with data shown in Figure 1, all E11 TEC subsets failed to respond to RANK ligation (Fig. 2A). In contrast however, upregulation of CD40 and MHC class II by RANK stimulation at day 1 was observed at E12 in both CD205<sup>-</sup> and CD205<sup>+</sup> FoxN1:eGFP<sup>+</sup> subsets, including those expressing the highest levels of CD205 (Fig. 2B and C). It is currently unclear whether the RANK-responsive TECs contained within the CD205<sup>-</sup> fraction represent cells that have already transitioned through a CD205<sup>+</sup> stage and subsequently lost CD205 expression by E12. Alternatively, RANK<sup>+</sup>CD205<sup>-</sup> cells may represent mTEC progenitors that acquire RANK responsiveness without passing through a CD205<sup>+</sup> stage, a scenario that would suggest two pathways for the emergence of mTEC progenitors. Nevertheless, while dual expression of cTEC/mTEC markers initially suggested the possible existence of bipotent TEC progenitors in both mouse [19] and human [20], our data demonstrating that CD205<sup>+</sup> TEC respond to the mTEC regulator RANK provide functional evi-

dence that bipotent TEC progenitors can simultaneously express hallmarks of the cTEC and mTEC lineages, including known functional regulators of the latter, and that they are acquired sequentially. Moreover, the data also suggest that mTECs may be generated from TEC progenitors defined by a CD205<sup>+</sup>CD40<sup>-</sup> phenotype typically associated with the cTEC lineage.

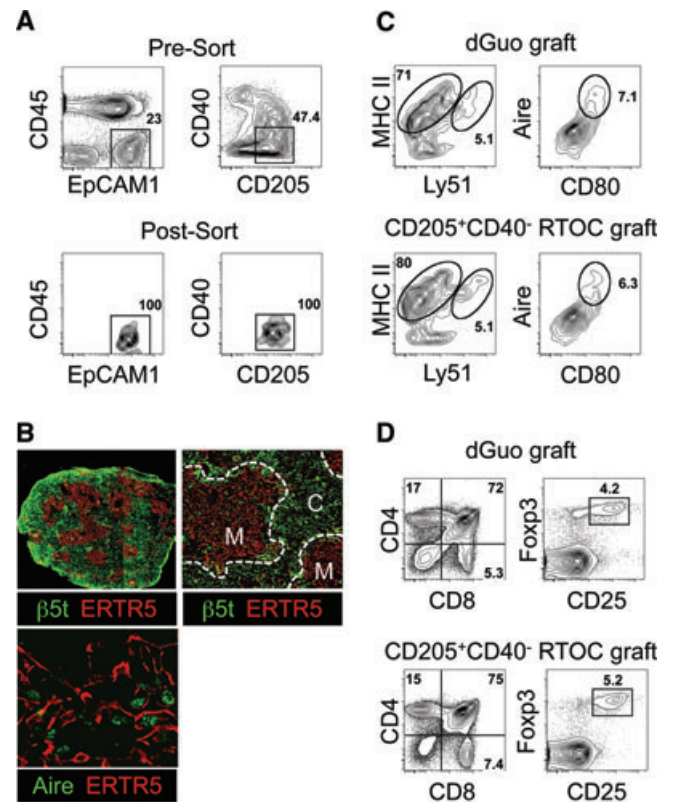
### CD205<sup>+</sup> progenitors give rise to mTECs, including the Aire<sup>+</sup> subset, in vivo

To investigate the developmental potential of CD205<sup>+</sup> TEC progenitors, we purified CD205<sup>+</sup>CD40<sup>-</sup> TECs from E15 thymus lobes (Fig. 3A), and used them to form reaggregate thymus organ cultures (RTOCs), which were transplanted under the kidney capsule of WT mice, as described [13]. After 6–8 weeks, a total of 8/8 CD205<sup>+</sup>CD40<sup>-</sup> RTOC grafts were recovered, a frequency comparable with the recovery of unmanipulated 2dGuO Fetal Thymus Organ Cultures (FTOCs) grafts (not shown). Recovered grafts were processed for either immunohistochemical or flow cytometric analysis of thymocyte and TEC subsets. CD205<sup>+</sup>CD40<sup>-</sup> RTOC grafts consisted of clearly demarcated cortical and medullary thymic regions (Fig. 3B), with the cTEC markers  $\beta$ 5t (Fig. 3B)



**Figure 2.** A TEC subset expressing the cTEC marker CD205 responds to RANK stimulation. (A, B) FoxN1:eGFP thymus/parathyroid rudiments from (A) E11 and (B) E12 embryos were cultured for 1 day in the absence (top) or presence (bottom) of 10  $\mu$ g/mL anti-RANK. CD205 expression is shown following gating on EpCAM1<sup>+</sup>CD45<sup>-</sup>FoxN1:eGFP<sup>+</sup> cells, with CD205<sup>-</sup> CD205<sup>Low</sup> and CD205<sup>High</sup> subsets analyzed for MHC class II and CD40 expression. (C) Quantitative analysis of CD40 and MHC class II induction in the presence (black bars) or absence (open bars) of RANK stimulation in E12 FoxN1:eGFP<sup>+</sup> TECs is shown. Data are shown as mean  $\pm$  SEM of  $n = 4$  and are representative of three experiments performed. An unpaired student t-test was performed, \*\* $p < 0.005$  and \*\*\* $p < 0.0005$ .

and CD205 (not shown) being restricted to cortical regions, while medullary regions contained ERTR5<sup>+</sup> mTEC, including the Aire<sup>+</sup> subset (Fig. 3B). Flow cytometric analysis of digested grafts initiated from CD205<sup>+</sup>CD40<sup>-</sup> TEC revealed, as in 2dGuo-treated FTOC grafts, both Ly51<sup>+</sup> cortical and Ly51<sup>-</sup> medullary subsets, the latter containing CD80<sup>+</sup>Aire<sup>+</sup> cells (Fig. 3C). Moreover, analysis of thymocytes recovered from both CD205<sup>+</sup>CD40<sup>-</sup> RTOC and 2dGuo-treated FTOC grafts revealed a normal program of thymocyte development, including all CD4/CD8 subsets, including FoxP3<sup>+</sup>CD25<sup>+</sup> regulatory CD4 single positive cells (Fig. 3D). Taken together, these findings provide evidence that embryonic CD205<sup>+</sup> TEC progenitors can give rise to functionally organized cortical and medullary thymic microenvironments capable of supporting thymocyte maturation.



**Figure 3.** CD205<sup>+</sup> TECs generate both cTECs and Aire<sup>+</sup> mTECs in vivo. (A) E15 thymus lobes stained for CD45, EpCAM1, CD40, and CD205 before (top) and after (bottom) cell sorting are shown. (B) Confocal microscopy of grafts initiated from CD205<sup>+</sup>CD40<sup>-</sup> TECs, after transplantation into WT mice is shown. “C” denotes cortex, “M” denotes medulla. Top left panel is a composite tile scan at 250 $\times$  final magnification, top right and lower panels taken at 250 $\times$  and 400 $\times$  final magnification, respectively. (C) Flow cytometric analysis of the EpCAM1<sup>+</sup>CD45<sup>-</sup> TEC compartment of CD205<sup>+</sup>CD40<sup>-</sup> TEC grafts (bottom), with unmanipulated dGuo FTOC grafts as comparison (top) is shown. (D) Analysis of thymocytes from 6-week dGuo FTOC grafts (top) and CD205<sup>+</sup>CD40<sup>-</sup> E15 TEC grafts (bottom) for the expression of CD4, CD8, FoxP3, and CD25 is shown. Dot plots (right) show FoxP3/CD25 expression in CD4<sup>+</sup>8<sup>-</sup> $\alpha$  $\beta$ TCR<sup>hi</sup> cells. Data shown are representative of at least three experiments.

## Concluding remarks

We have investigated the emergence of the cTEC and mTEC lineages from bipotent progenitors in the early thymus rudiment. The identification of a subset of FoxN1:eGFP<sup>+</sup> TECs co-expressing the cTEC marker CD205 and the mTEC regulator RANK indicates that maturation of bipotent TEC progenitors includes a potential developmental route defined by the simultaneous expression of markers typically associated with either the cTEC or the mTEC lineage. Interestingly, the co-expression of cTEC/mTEC markers by TEC progenitors in mouse thymus, and the ability of CD205<sup>+</sup> progenitors to generate mTECs, fits well with recent studies analyzing TEC development following transplantation of human thymus for the treatment of diGeorge syndrome [20], in which progenitors expressing the cTEC marker CDR2 were suggested to give rise to cytokeratin-14 expressing mTECs. Interestingly, during development of the central nervous system, both glial and neuronal cell

lineages were shown to emerge from a common progenitor that expresses proteolipid protein [21], a major myelin component, suggesting that blurring of progenitor and lineage phenotypes may occur within multiple tissues. Collectively, these findings argue against current models of TEC development based solely upon the simultaneous divergence of distinct cTEC and mTEC progenitor populations, each separately expressing lineage-restricted markers. Given that both cTECs and mTECs shape the  $\alpha\beta$  T-cell repertoire, further analysis of the stepwise generation of the cTEC and mTEC lineages, and the regulation of bipotent TEC progenitor development, will be important in informing strategies aimed at regenerating both cortical and medullary compartments of the thymus.

## Materials and methods

### Mice

WT BALB/c, C57BL/6, *Ly75<sup>-/-</sup>* [18], and *FoxN1:eGFP* [17] mice were maintained in the Biomedical Services Unit, University of Birmingham. The morning of vaginal plug detection was designated day 0 of gestation. Animal experiments were performed with local and national (UK Home Office) permission.

### Antibodies and flow cytometry

The following antibodies were used (eBioscience unless otherwise indicated): PE-Cy7/PerCP-Cy5.5 anti-CD4 (GK1.5/RM4-5), eFluor 450/FITC anti-CD8 (53–6.7), allophycocyanin anti-CD25 (PC61, Biolegend), allophycocyanin eFluor780 anti-TCR- $\beta$  (H57–597), allophycocyanin eFluor780 anti-CD45 (30F-11), allophycocyanin anti-EpCAM (G8.8), PE anti-CD40 (3/23, BD Bioscience), Pacific Blue anti-CD80 (16–10A1, Biolegend), PE anti-Ly51 (D7), PE-Cy7/biotinylated anti-CD205 (205yekt/NLDC-145, eBioscience/Abcam), biotinylated anti-IA<sup>d</sup> (AMS-32.1, BD Bioscience), Pacific Blue anti-IA<sup>b</sup> (AF6–120.1, Biolegend), PE anti-FoxP3 (FJK-16s), eFluor488 anti-AIRE (5H12). Streptavidin PE-Cy7 was used to pick up biotinylated antibodies. The FoxP3/Transcription Factor Staining Buffer Set was used for intracellular FoxP3 staining, and IC Fixation Buffer (both eBioscience) was used for intracellular Aire staining. Flow cytometry was performed using a BD Fortessa, with scatter gates set to exclude nonviable cells.

### Thymus organ culture

Shared thymus/parathyroid rudiments were dissected from E11/E12 embryos, while from E13 onward, isolated thymus lobes were dissected following physical removal of the parathyroid. A total of 10  $\mu$ g/mL agonistic anti-RANK antibody (AF692, R&D Systems) was added to cultures where indicated [13].

### Immunohistochemistry

Thymus grafts were processed as described [13]. The following antibodies were used for section staining: eFluor488 anti-Aire (5H12, eBioscience), rabbit IgG anti- $\beta$ 5t (MBL International), anti-mTEC (ERTR5, kind gift of W. van Ewijk), which were detected with the secondary antibodies eFluor594 anti-rat IgM (Invitrogen), and eFluor488 anti-rabbit IgG (Invitrogen).

### RTOC

Purified CD205<sup>+</sup>CD40<sup>-</sup> E15 TECs (typically,  $1 \times 10^5$  cells) were used to prepare RTOC as described [13]. Intact RTOC, and unmanipulated dGuo FTOC as controls, were transplanted under the kidney capsule of WT mice, and harvested after 6–8 weeks. Analysis of thymic stromal cells in transplanted RTOC was performed as described [22]. For the analysis of thymocyte development, grafts were mechanically disrupted, and liberated thymocytes stained with antibodies described above.

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**Abbreviations:**  $\alpha\beta$ TCR:  $\alpha\beta$ T-cell receptor · 2dGuo: 2-deoxyguanosine · cTEC: cortical TEC · mTEC: medullary TEC · RANK: receptor activator of nuclear factor kappa B (NF- $\kappa$ B) · RTOC: reaggregate thymus organ culture · TEC: thymic epithelial cell

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