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Checkpoints in the Development of Thymic Cortical Epithelial Cells

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In the thymus, interactions between immature thymocytes and thymic epithelial cells (TECs) regulate the development and selection of self-tolerant MHC-restricted T cells. Despite the importance of cortical (cTEC) and medullary (mTEC) thymic epithelial cells in fostering T cell production, events in TEC development are still unclear. Although precursor-product relationships during mTEC development have been reported, and some genetic regulators of mTEC development have been identified, stages in cTEC development occurring downstream of recently identified bipotent cTEC/mTEC progenitors remain poorly defined. In this study, we combine analysis of differentiation, proliferation, and gene expression of TECs in the murine thymus, that has enabled us to identify cTEC progenitors, define multiple stages in cTEC development, and identify novel checkpoints in development of the cTEC lineage. We show an essential requirement for FoxN1 in the initial development of cTEC from bipotent progenitors, and demonstrate a stage-specific requirement for CD4<sup>+</sup> thymocytes in later stages of cTEC development. Collectively, our data establish a program of cTEC development that should provide insight into the formation and function of the thymic cortex for T cell development. The Journal of Immunology, 2009, 182: 130–137.

The thymus provides a specialized microenvironment for the development and selection of T cells bearing the αβ TCR complex. Thymic microenvironments can be anatomically compartmentalized into cortical and medullary regions, each of which are responsible for key stages in the development of thymocytes (1, 2). Thymic cortical regions foster the development of CD4<sup>+</sup> T cell progenitors through various steps that involve proliferation, rearrangement, and expression of TCR genes, and pre-TCR mediated selection (3, 4). This latter event, which is thought to occur in outer cortical regions, results in the formation of a large number of CD4<sup>+</sup> thymocytes and is followed by migration back through the thymic cortex, where cells undergo selection based on their ability to recognize self-peptides bound to MHC class I and class II molecules. Positive selection in the thymic cortex results in the cessation of TCR-α gene rearrangement, down-regulation of either CD4 or CD8 coreceptor expression, and CCR7-mediated migration of newly selected thymocytes into the thymic medulla (5–7). Migration into the thymic medulla is associated with the purging of potentially autoreactive TCR specificities by medullary thymic epithelial cells (mTECs) as well as dendritic cells that accumulate at the corticomedullary junction (8–10). Within the medulla, newly generated CD4<sup>+</sup> and CD4<sup>+</sup> thymocytes undergo a series of developmental steps over a 4–5 day period including further screening for self-tolerance to peripheral Ags, before thymus emigration (8, 11–13).

It is now well accepted that multiple stages of T cell development require constant input from surrounding thymic stromal cells in the form of cell-cell interactions and soluble factors (1, 8). Of the various types of stromal cells within the thymus, thymic epithelial cells (TEC) in particular provide an array of signals to developing thymocytes that include peptide/MHC ligands for selection (14), soluble factors such as Wnts (15), IL-7 and SCF for survival and expansion (16), and Notch ligands for commitment and differentiation (17, 18). Indeed, the efficacy of thymic T cell output is directly linked to TEC frequency (19), while the decline in T cell output as a result of age-related thymus atrophy has been linked to a reduction in TEC frequency and/or function (20). TECs are heterogeneous and cortical and medullary areas of the thymus that house distinct stages of T cell development also contain phenotypically and functionally distinct cortical and medullary TEC subsets (cTEC and mTEC). The importance of TEC for the development of self-tolerant T cells is highlighted by the autoimmunity and immunodeficiencies that can occur as a result of abnormal TEC development (21). Thus, gaining a better understanding of the stages and mechanisms of cTEC and mTEC development is essential to understanding intrathymic T cell production.

The identification of TEC progenitors capable of giving rise to both cTEC and mTEC provides a clear starting point from which to study the development of distinct cTEC and mTEC lineages (22, 23). With regard to mTEC development, most notably the subset expressing the Autoimmune Regulator Gene (AIRE), several molecular mediators have now been identified including Aire itself (24), the cell surface receptors RANK and CD40 (25–29), lymphoxygen-β receptor (30), as well as the signaling components RelB (31), NIK (32), and TRAF6 (33). Moreover, precursor-product relationships in the mTEC lineage have been identified (25, 34) and shown to require input from a...
variety of hematopoietic cells, including Lymphoid Tissue Inducer cells and mature single positive thymocytes (25–29, 35). In contrast to advances in our understanding of mTEC development, the stages and mechanisms regulating development of cTEC are still poorly understood. Indeed, while several genetic deficiencies have been identified that result in abnormal mTEC maturation, there are few reports of abnormalities in cTEC development. In this study, we have dissected the stages of cTEC development beginning with bipotent TEC progenitors and culminating in the generation of mature cTEC expressing high levels of MHC class II. This survey identifies for the first time multiple stages of cTEC development, reveals distinct checkpoints in the developmental program of cTEC, and pinpoints the requirement for thymocyte cross-talk in cTEC development.

Materials and Methods

**Mice**

BALB/c (H-2b), C57BL/6 nude (H-2b), Rag1-/- (H-2d), CD3eTg26 (H-2d) mice were bred and maintained under specific pathogen-free conditions in the Biomedical Services Unit at the University of Birmingham. Day of vaginal plug detection was designated as day 0.

**Embryonic thymic stromal cell isolation and thymus organ culture**

Thymic lobes were isolated at the indication embryonic stages by microdissection and, where necessary, were disaggregated by incubation in 0.25% trypsin/0.02% EDTA for 5 to 7 min at 37°C to obtain single-cell suspensions (10). In some experiments, freshly isolated lobes were expelled in fetal thymic organ culture (FTOC), prepared as described (10).

For BrdU experiments, BrdU is added to the FTOC culture medium at a final concentration of 10 μM for an overnight incubation.

**Abs and flow cytometry**

The following Abs were used for flow cytometry: BrdU-FITC (BD Pharmingen), I-A/I-FITC (clone AF6-120.1; BD Pharmingen), I-A/I-FITC (clone AMS-32.1; BD Pharmingen), CD45.2-FITC (clone 104; BD Pharmingen), CD45.1-FITC (clone M1/9, American Type Culture Collection) supernatant. For MHC class II and BrdU staining, all samples were CD45 depleted and the purity of the CD45 negative fraction checked.

Collection supernatant. For MHC class II and BrdU staining, all samples were CD45 depleted and the purity of the CD45 negative fraction checked. CD205 purified anti-EpCAM (clone: G8.8; gift from A. Farr, 30-F11; eBioscience), CD205-biotin (NLDC-145; AbCam), streptavidin-FITC (clone AMS-32.1; BD Pharmingen), CD45.2-FITC (clone 104; BD Pharmingen), 3,3’-diaminobenzidine (DAB; Corbett Research) using standard curves generated from FTOC cDNA. Calculation of the relative expression values for each sample normalized to β-actin, was performed as described (25). Fold levels represent the mean (±SEM) of triplicate reactions and data shown is representative of two separate Dynabead cell sorting experiments.

**RT-PCR, real-time PCR, and isolation of embryonic TEC subsets**

High-purity cDNA was obtained from purified mRNA using μMACs One-step cDNA synthesis kit, according to the manufacturer’s instructions (Milenyi Biotec). Real-time PCR was performed using SYBR Green with primers specific for β-actin, Aire, Claudin3, Claudin4, RANK, OPG, Ly-6c, and reverse, 5’-TCTGGCTACCTGGAATGTTG-3’ (112 bp); OPG (PrimerBank ID 31543882a2) forward, 5’-GCCGCCAACAGGAAA-3’ (149 bp); RANK forward, 5’-GC TGGCTACCTGGAATGCT-3’ and reverse, 5’-GCCAGGAAAGCAAACGC-3’ (125 bp). Isolation of total TECs from wild-type (WT) and Nude embryos of the indicated ages was performed by MoFlo cell sorting of EpCAM CD45 cells from disaggregated thymus suspensions. For enrichment and selection of defined E15 CD40/CD205 TEC populations, an immunomagnetic separation technique was performed using Dynabeads, which were coated with Abs according to the manufacturer’s instructions. Cells and beads were mixed by centrifugation, and multiple rounds of anti-CD45 coated beads were used to deplete hematopoietic cells, with clusters of CD45 cells removed using an Eppendorf (1.5 ml) Dynal Magnetic Particle Concentrator.

The resulting supernatant, highly enriched for thymic stromal cells, was centrifuged, and multiple rounds of anti-CD45 coated beads were used to deplete hematopoietic cells, with clusters of CD45 cells removed using an Eppendorf (1.5 ml) Dynal Magnetic Particle Concentrator.

The resulting supernatant was then centrifuged with biotin-conjugated CD205 streptavidin beads to positively select CD205+ cells, and CD40+ rosettes separated on a magnet and snap frozen for RT-PCR. The resulting supernatant was then treated with biotin-conjugated CD40 streptavidin beads to positively select for CD40+ cells, with CD40+ rosettes separated on a magnet and then snap frozen for RT-PCR.

**Results**

Ontogenesis of cTEC Development

Thymic epithelial cell development begins around E11 of gestation, when endodermally derived epithelial cells (36) bud off from the third pharyngeal pouch endoderm to form the thymus anlage. We recently showed that at E12 of gestation, the thymus contains a dominant population of EpCAM+cTECs that are capable of giving rise to both cTEC and mTEC (22). To investigate the timing of cTEC development in relation to these early thymus stages, we performed flow cytometric analysis of disaggregated thymus suspensions and analyzed TECs, identified on the basis of EpCAM expression, for expression of the cTEC marker CD205, also known as DEC205, a known marker of cTEC in the adult thymus (37). By gating on EpCAM+cTECs, and in contrast to the presence of a large population of CD205+cTEC present in FTOCs, Fig. 1a shows that a small but detectable EpCAM+cCD205− subset of TEC is present at E12 of gestation, with the frequency of these cells increasing during thymus ontogeny. Interestingly, using quantitative real-time PCR of isolated embryonic TECs, we also found that expression of CD205 during cTEC development at E12 is accompanied by expression of the recently described cTEC-specific proteosome subunit β5t (38), with the proportion of CD205+ TECs and β5t expression levels increasing at later developmental stages (Fig. 1, a and c). Importantly, we also analyzed early stages of TEC development in the embryonic nude thymus, which, as a result of lacking expression of FoxN1, fail to undergo a normal TEC development program (39–41). In contrast to WT E12 thymus, analysis of E12 TECs deficient in FoxN1 expression showed that both CD205 and β5t expression were absent (Fig. 1, b and d), with nude TECs remaining negative for CD205 at later
developmental stages (Fig. 1b), suggesting that acquisition of these cTEC markers during TEC development is a FoxN1-dependent process.

In addition to CD205 and β5t expression, we also analyzed expression of the costimulatory molecule CD40, which is expressed by mature TEC (42). We compared the ontogeny of expression of these molecules with CD40−/H11001 CD205−/H11001 cTEC present within fetal thymus organ cultures (Fig. 1a), that represent mature cTEC as indicated by their ability to support thymocyte positive selection (10). In contrast to CD205−/H11001 cTEC in FTOC, we found that CD205−/H11001 TECs initially emerged as CD40− cells, with CD40 expression gradually being acquired around E14–15 of gestation (Fig. 1a). As with CD205 expression, we found CD40 expression to be largely absent from FoxN1-deficient TECs at comparable developmental stages (Fig. 1b). Interestingly, however, CD205+ TEC at E15 of gestation expressed lower levels of β5t and Cathepsin-L as compared with mature CD205+ cTEC from 7 d FTOC (Fig. 1, j and k), suggesting that CD205+ cTEC at E15 of gestation are not fully mature. Collectively, our phenotypic and genotypic analysis of cTEC development during thymus ontogeny shows that the emergence of cTEC lineage cells, presumably from bipotent cTEC/mTEC progenitors, occurs as early as E12 of gestation via a FoxN1 dependent mechanism, and identifies EpCAM1+/H11001 CD205+/H11001 CD40−/H11002 cells present at E15 of gestation as putative cTEC progenitors.

Induction of CD40 and MHC class II expression and proliferative status define distinct stages during cTEC development

Acquisition of MHC molecules during TEC development is essential for their ability to mediate selection of the developing TCR repertoire. Levels of MHC class II expression have been
used to identify heterogeneity and precursor-product relationships within the mTEC lineage (42). However, changes in levels of MHC class II expression during cTEC development are not clear. To investigate this, we analyzed MHC class II expression on developing cTEC in combination with CD40 and CD205 expression. In contrast to the uniform high levels of MHC class II expression by CD205+/H11001 cTEC in FTOC (Fig. 3a), E15 CD205−CD40+ TEC largely lack MHC class II expression.

FIGURE 2. CD40−CD205+ cells have a genotypic profile consistent with cTEC progenitors. Real-time PCR was used to compare the relative mRNA expression level of a panel of TEC expressed genes (a–i) associated with cTEC and mTEC lineages. The relative mRNA expression levels were assessed from E15 CD45− TECs subdivided into CD40− CD205+ (□) and CD40+CD205− (■) subsets. The β5t (j) and Cathepsin-L (k) mRNA levels in immature E15 CD45+CD205+ cTECs (□) and mature CD45−CD205+ cTECs (■) was also compared. Data are the mean ± SEM from technical triplicate and are representative of at least two distinct sorting experiments.

FIGURE 3. Expression of MHC class II, CD40, and proliferative status define distinct stages of cTEC differentiation. FACS analysis of E15 (left panels) and 7-day FTOC (right) TECs, determining expression of MHC class II (a) and BrdU incorporation (b) in the EpCAM+ thymic epithelial cells at E15 and in 7 days cultured FTOC. Histograms shown are generated by electronic gating on CD40/CD205 subsets of EpCAM+ TECs, depleted of CD45+ cells using magnetic beads before staining. Numbers in the histograms are the percentage of cells within the gates. Data are representative of at least three separate experiments.
Interestingly, E15 CD205+CD40− cTECs were found to be heterogeneous with regard to expression of MHC class II, with a MHC class II+ subset still evident (Fig. 3a), while CD205+CD40+ cTECs at E15 of gestation were largely MHC class II (Fig. 3a). Collectively, these data suggest that induction of expression of MHC class II within the CD205+ cTEC lineage occurs before expression of CD40.

Finally, while TEC are known to undergo phases of proliferation during their development (42, 44), how this relates to distinct developing cTEC and mTEC lineages is not clear. To investigate the proliferative status of cTEC subsets identified here, freshly isolated E15 thymus lobes and E15 lobes placed in culture for 7 d were pulsed with BrdU for an overnight period. Analysis of the proliferative status of TEC subsets at E15 of gestation showed that the majority of CD205+CD40+ cTEC incorporated BrdU (Fig. 3b). Consistent with analysis of cTEC in neonatal thymus after in vivo BrdU labeling (42), the large majority of CD205+CD40+ cTEC in 7 d FTOCs, representing the in vitro age-matched equivalent to the in vivo neonatal thymus, are nondividing cells. In marked contrast, approximately one-third of E15 CD205+CD40− and CD205+CD40+ cTEC incorporated BrdU (Fig. 3b). Thus, the sequential acquisition of the markers CD205 and then CD40 during cTEC development correlates with a decline in proliferation.

Stage-specific requirement for thymocyte cross-talk during later stages of cTEC development

Several studies have now reported the importance of interactions between hemopoietic cells and thymic epithelial cells for the formation of cortical and medullary microenvironments (45). For example, thymic epithelial cell development in the medulla can be regulated by CD4+3+ Lymphoid Tissue Inducer cells and mature...
single positive thymocytes (25–29, 35). Although a normal program of T cell development is thought to play a role in the development of cortical thymic epithelial cells, the precise thymocyte subsets and the stages of cTEC development they regulate are not clear (45). To investigate this, we analyzed cTEC development in mutant mice in which thymocyte development is blocked at different stages. Specifically, we analyzed cTEC in Rag$^{-/-}$ mice where thymocyte development is blocked at the CD4$^{-}$ 8$^{-}$ 25$^{-}$ 44$^{-}$ (double negative (DN)3) stage due to an absence of TCR gene rearrangement (46). We also analyzed cTECs in CD3etag26 mice that display an even earlier block in T cell development (47). Although the reasons for this block are unclear, experiments demonstrating normal development of WT T cell precursors in CD3etag26 thymus lobes argues in favor of the defect mapping to CD3$^{-}$/H9255$^{-}$tg26 (Fig. 4$^{g}$), suggesting that the presence of a normal program of thymocyte cross-talk. Fig. 4,$^{c}$ shows that CD205$^{+}$ EpCAM1$^{+}$ cTEC are present in the E15 thymus of both WT and Rag-deficient thymus, in agreement with previous reports that cTEC development can occur in the absence of a normal T cell development program (42). However, we also found a similar proportion of EpCAM1$^{+}$ CD205$^{+}$ cTEC in the thymus E15 CD3etag26 embryonic thymus (Fig. 4$^{c}$), which is in contrast to other reports suggesting a link between cTEC development and normal thymocyte development (47). Importantly, however, while CD205$^{+}$ cTEC in both E15 WT and Rag$^{-/-}$ thymuses show evidence of further maturation as indicated by similar levels of CD40 (Fig. 4, $d$ and $e$) and MHC class II expression (Fig. 4, $g$ and $h$), there was a marked absence of CD40 (Fig. 4$^{f}$) and MHC class II (Fig. 4$^{i}$) expression by CD205$^{+}$ cTEC in the CD3etag26 thymus. Thus, these data suggest that while the initial stages of cTEC development (i.e., expression of CD205) can occur independently of thymocyte-derived signals, the presence of DN1–3 thymocytes is necessary and sufficient for the further maturation of CD205$^{+}$ cTEC to the stage where they are expressing MHC class II and CD40. Interestingly however, analysis of BrdU incorporation showed that CD205$^{+}$ TECs present in Rag$^{-/-}$ (Fig. 4$k$) and CD3etag26 (Fig. 4$f$) are still able to undergo proliferation, albeit at a lesser extent as compared with WT TECs (Fig. 4$j$), suggesting that the presence of a normal program of developing thymocytes is not an essential requirement for the proliferation of developing cTECs.

### Discussion

The development of thymic cortical epithelial cells represents an essential step in the formation of functional thymic microenvironments. cTEC provide essential signals during early stages of T cell development, and perhaps most notably are efficient mediators of thymic positive selection that restricts the developing TCR repertoire to self-MHC recognition. In contrast to recent advances in our understanding of the stages of mTEC development (25–29), relatively little is known about the stages and processes regulating the development of cTEC.

Based on the data reported in this study, we propose a model of distinct stages of cTEC development (Fig. 5). Analysis of the cTEC marker CD205 during thymus ontogeny reveals the presence of a population of TEC with a previously unreported CD205$^{+}$ CD40$^{-}$ population that differ from mature cTEC in FTOCs in relation to their surface levels of expression of CD40 and MHC class II. Moreover, these E15 CD205$^{+}$ CD40$^{-}$ TEC express lower levels of mRNA for cTEC expressed genes, namely β5t and cathepsin-L. Interestingly, CD205$^{+}$ 40$^{-}$ TEC are enriched for proliferating cells as compared with mature CD205$^{+}$ CD40$^{+}$ cTEC, leading us to propose that CD205$^{+}$ CD40$^{-}$ cells represent a cTEC progenitor population that lies between bipotent cTEC/mTEC progenitors and mature cTEC.

In addition to defining sequential stages in cTEC development, we have also analyzed the requirement for thymocyte cross-talk in progression through these stages. Although other studies have analyzed cytokeratin 5 and 8 expression in relation to early TEC development (50, 51), our study has analyzed the ontogenetic sequence leading to the generation of mature CD205$^{+}$ CD40$^{+}$ MHC class II$^{+}$ cTEC in addition to analyzing expression of the recently described cTEC specific proteosome subunit β5t (38). We show that the initial appearance of cells expressing the cTEC markers CD205 and β5t occurs as early as E12 of gestation, and occurs via a FoxN1 dependent mechanism. This latter finding agrees well with the notion that FoxN1-deficient TECs may represent bipotent progenitors that have not yet undergone commitment to the cTEC and mTEC lineages (23). Moreover, our analysis of cTEC development in relation to thymocyte cross-talk has revealed a series of checkpoints in cTEC development that differ in relation to their requirement for thymocyte derived signals. Although initial studies
showed the emergence of Keratin (K)5+8+ TEC occurs in the absence of thymocyte development (51), our study extends this analysis to show that while the initial steps in cTEC development and advances our understanding of the role of thymocyte-derived signals for differentiation as shown here, they also are important in remodelling developing cTEC and the formation of the typical 3-dimensional reticular cortical network (53). Interestingly, analysis of TEC proliferation in the absence of a normal program of T cell development shows that developing cTECs are still able to undergo some degree of proliferation. Although the signals driving this thymocyte-independent TEC proliferation are not known, as thymic mesenchyme is known to be capable of driving proliferation of bipotent TEC progenitors (44), these cells may also play a role in TEC proliferation at later developmental stages.

In summary, our studies helps to define the developmental program that leads to the production of mature MHC class II+ cTEC and advances our understanding of the role of thymocyte-derived signals in this process. As has been recently shown for the mTEC lineage, current studies are focusing on studying the mechanisms regulating the turnover and lifespan of cells at different stages of cTEC development.

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Disclosures

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