Thymic crosstalk restrains the pool of cortical thymic epithelial cells with progenitor properties

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Originally published in European Journal of Immunology 47(6): 958-969, April 13, 2017.

doi: 10.1002/eji.201746922

Cortical (cTEC) and medullary (mTEC) thymic epithelial cells establish key microenvironments for T-cell differentiation and arise from thymic epithelial cell progenitors (TEP). However, the nature of TEPs and the mechanism controlling their stemness in the postnatal thymus remain poorly defined. Using TEC clonogenic assays as a surrogate to survey TEP activity, we found that a fraction of cTECs generates specialized clonal-derived colonies, which contain cells with sustained colony-forming capacity (*Clono*TECs). These *Clono*TECs are EpCAM+MHCII-Foxn1lo cells that lack traits of mature cTECs or mTECs but co-express stem-cell markers, including CD24 and Sca-1. Supportive of their progenitor identity, *Clono*TECs reintegrate within native thymic microenvironments and generate cTECs or mTECs in vivo. Strikingly, the frequency of cTECs with the potential to generate *Clono*TECs wanes between the postnatal and young adult immunocompetent thymus, but it is sustained in alymphoid *Rag2-/-Il2rg-/-* counterparts. Conversely, transplantation of wild-type bone marrow hematopoietic progenitors into *Rag2-/-Il2rg-/-* mice and consequent restoration of thymocyte-mediated TEC differentiation diminishes the frequency of colony-forming units within cTECs. Our findings provide evidence that the cortical epithelium contains a reservoir of epithelial progenitors whose abundance is dynamically controlled by continual interactions with developing thymocytes across lifespan.

Keywords: Lympho-epithelial interactions; Progenitor; Thymic epithelial cells; Thymocytes; Thymus

Introduction

The development and selection of highly diverse T cells, which are responsive against pathogens while tolerant to one's own organs, takes place in dedicated niches of the thymus. Central to this instructive process are thymic epithelial cells (TECs) that segregate into specialized cortical (cTEC) and medullary (mTEC) microenvironments [1]. While cTECs instruct the commitment of hematopoietic precursors into the T cell lineage and positively select thymocytes expressing major histocompatibility complex (MHC)-restricted T cell receptors (TCRs), mTECs contribute to the elimination of thymocytes expressing autoreactive TCR and the development of regulatory T cells [2]. Consequently, genetic alterations that affect the differentiation of TECs lead to pathologies that range from immunodeficiency to autoimmunity [1]. Since TECs are sensitive to aging and conditioning regiments linked to bone marrow transplantation or cancer therapy [3], the functionalization of thymic epithelial niches emerges as a direct approach to improve thymopoiesis in disorders associated with ineffective T-cell responses.
The two-prototypical cTEC and mTEC subsets differentiate from common bipotent TEC progenitors (TEP) that exist in the embryonic [4–6] and postnatal [7] thymus. Deciphering how bipotent TEPs self-renew and transmogrify into cTECs and mTECs has been under intense

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investigation. The discovery of mTECrestricted precursors [8–10] led to the concept that TEP give rise to cortical and medullary lineages through unrelated differentiation pathways. More recently, evidence that embryonic cTEC-like progenitors have the potential to generate cTECs and mTECs [11–13] suggests that TEPs might progress through the cortical lineage prior to commitment to mTECs [14]. These findings equally implicate that TEPs nestle within the embryonic cortex. Recent studies identify distinct subsets of TECs in the postnatal thymus that contain, without exclusively marking, purportedly TEPs [15–17]. Yet, the singular identity and anatomical location of TEPs are still elusive. Moreover, whether TEC differentiation follows the same precursor-product relationships in the postnatal thymus is not airtight.

Thymic epithelial cell microenvironments turnover more rapidly than previously recognized, with an estimated replacement rate of one-two weeks to mTECs of the young adult thymus [18, 19]. These results suggest a requirement for regular differentiation of new mature TECs from their upstream progenitors. Two, not necessarily mutually exclusive, scenarios can coincide. On one hand, long-lasting TEPs must continually produce lineagecommitted precursors lacking self-renewal capacity. Alternatively, the abundance of functional TEPs might drop with age, being the replenishment of cortical and medullary epithelial niches assured by downstream compartment-restricted precursors. Fate-mapping studies show that the majority of adult mTEC network arise from fetal- and newborn-derived TEPs expressing beta5t (β5t), a prototypical cTEC marker [20, 21]. Furthermore, mTEC-restricted SSEA-1+ progenitors [22, 23] and specialized podoplanin+ (PDPN) mTEPs residing at the cortico-medullary junction (CMJ)

[24] have been identified, both contributing to the maintenance of mTEC compartment. Together, these findings infer that the bipotent capacity of TEPs is preserved beyond birth, but might be progressively lost with age. Consequently, the maintenance of adult medullary epithelial network seems to be secured by unipotent mTEPs.

Despite recent advances, it remains unclear how changes in the bioavailability of TEPs impact on the maintenance of TEC microenvironment across life, and ultimately on thymic output. Another unexplored area pertains to the physiological causes underlying the presumed age-dependent decrease and/or senescence of TEPs. Since the amount of embryonic TEP dictates the size of functional TEC microenvironments [25], it is conceivable that the loss in the TEC network with age might be coupled to the loss in TEP stemness. Nevertheless, we lack experimental evidence that argues in favor, or against, this possibility. Herein, we identify a subset of cTECs that generates TEC colonies of clonal origin, harbouring cells with progenitor traits, including continual colony-forming capacity, lack of mature TEC markers and bearing the potential to generate cTECs and mTECs. Detailed temporal analysis reveals that the abundance of cTECs with clonogenic activity decreases with the entry into the adulthood, in a process that is directly regulated by lympho–epithelial interactions.

Results

The postnatal cortical thymic epithelium contains cells with clonogenic capacity

The postnatal thymus represents a period of active growth of the TEC network, which plateaus during young adulthood followed by a progressive reduction with age [18, 26]. We conjectured that these dynamic changes in TEC niches might be coupled to a gradual exhaustion of TEPs. To seek for epithelial stemness within the postnatal thymus, we established clonogenic assays that were previously reported to select and support the growth of stem cells from other stratified epithelial cells and the rat thymus [27, 28]. In these midscale assays, bulk postnatal cellsorted TECs ($CD45^{-}EpCAM^{+}MHCII^{+}$) were cultivated in specialized medium onto a monolayer of feeder cells (irradiated 3T3) (Supporting Information Fig. 1A). TEC-derived colonies emerged around day 6 and grew in size up to day 12, containing tightly packed cells that express the pan-epithelial marker EpCAM (Supporting Information Fig. 1B and C) and cytokeratin 8 (K8) [29] (Fig. 1A). A limitation of experiments with “bulk” cultivated TECs is that they might hinder a possible heterogeneity at the single cell level. Thus, we determined whether the clonogenic potential was a property of all, or only a fraction of postnatal-TECs. First, we performed co-culture assays with equal amounts of postnatal cell-sorted TECs isolated from mice that constitutively express Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP) under the control of β-actin promoter. In this setting, colonyforming units could be surveyed on the basis of their live-cell fluorescence. The development of either single GFP^{+} or single RFP^{+} TEC-derived colonies suggested their clonal

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origin (Fig. 1B). Next, combining high-speed cell sorting and high-content imaging analysis, we microscaled the assay down to single-cell level and showed that TEC colonies were clonally derived from singlesorted TECs. Interestingly, not all TECs gave origin to colonies (Fig. 1C). Hence, serial dilution clonogenic assays offered a mean to quantify the colony-forming precursor frequency within TECs throughout normal and altered pathophysiological settings (a point addressed later in this study). Given their clonal origin, we referred to the cells that emerge from these cultures as *Clono*TECs. We also cultured postnatal-derived non-epithelial thymic stromal cells ($CD45^{-}EpCAM^{-}$) under clonogenic conditions, but these lack distinct colony-forming potential and did not generate *Clono*TECs as their $CD45^{-}EpCAM^{+}$ counterparts (Supporting Information Fig. 1D). Interestingly, *Clono*TECs contain a small fraction of cells with the capacity to regrow and re-establish clonal-derived colonies upon serial passages in vitro (Fig. 1D and Supporting Information Fig. 1E). The observations that *Clono*TECs were generated from a portion of TECs led us to investigate whether the clonogenic capacity was restricted to a subpopulation of cTECs and/or mTECs. To do so, we first purified by cell sorting cTECs ($Ly51^{+}$) and mTECs (UEA^{+}) from actinGFP reporter mice and found that the clonogenic capacity was markedly enriched within cTECs (Fig. 1E). Additionally, we used $CCRL1^{GFP}$ reporter mice, in which the combined analysis of the expression of $CCRL1^{GFP}$ and CD80 defines discrete subsets of cTECs and mTECs in the postnatal thymus [26] (Fig. 1F). While high levels of $CCRL1^{GFP}$ cells (CCRL1hi) identifies Ly51+cTECs, intermediate levels of $CCRL1^{GFP}$ define additional subsets of mTECs (UEA+CD80− and UEA+CD80+, as described in [26]) that arise in the postnatal thymus (Fig. 1F and Supporting Information Fig. 1F) Analysis of the discrete TEC subsets confirmed that the clonogenic capacity was mostly restricted to CCRL1hi cTECs (Fig. 1F). Limiting dilution clonogenic assays confirmed that colonies were of clonal origin and a property of a fraction of CCRL1hi cTECs (Supporting Information Fig. 1G). Yet, we also found residual clonogenic activity in CCRL1intCD80+/− and CCRL1−CD80+ expressing subsets (Fig. 1F). Together, these results identified that most of colony-forming cells existed within cTECs (CD45−EpCAM+Ly51+CCRL1hi), suggesting that the postnatal cortical epithelial niche harbors cells with potential progenitor activity.

*Clono*TECs display phenotypic and molecular traits of TEP-like cells

To determine the epithelial lineage identity of cTEC-derived *Clono*TECs that arise upon culture, we characterized them at phenotypic and molecular levels using a panel of pan-TEC, cTEC- and mTEC-restricted markers. As reference, we co-analyzed freshly isolated total TECs, cTECs and mTECs. Contrarily to ex vivo total TECs, *Clono*TECs lacked MHCII and expressed minute amounts of Foxn1 both at protein and mRNA levels (Fig. 2A and B). To test whether *Clono*TECs derived from Foxn1-expressing cells that downregulated Foxn1 expression, we established clonogenic assays with cell-sorted cTECs from $Foxn1^{eGFP}$ reporter mouse strain [30], in which nearly all TECs were marked by $Foxn1^{eGFP}$ expression (Supporting Information Figure 2A). Notably, total, or even the highest, $Foxn1^{eGFP}$-expressing cTECs generated detectable colonies (containing *Clono*TECs) that lost GFP expression, as measured by live-cell fluorescence imaging and flow cytometry analyses (Supporting Information Fig. 2B–D). Interestingly, *Clono*TECs expressed CD24 and Sca-1, which have been reported to identify epithelial stem cells in other anatomical sites, including breast and lung [31, 32] (Fig. 2A). Furthermore, *Clono*TECs expressed low levels of CD205 and lacked Ly51 when compared to cTECs, and displayed little UEA binding capacity and lower levels of CD80 and lacked CD40 relatively to mTECs (Fig. 2A). Additionally, we analyzed the molecular profile of purified *Clono*TECs for a restricted set of genes associated with cTECs (Kitl, Dll4, Il7, Psmb11 (β5t), Cxcl12 and Ccl25) or mTECs (Ccl19, Ccl21, Tnfrs11a (RANK) and Aire), and found that *Clono*TECs expressed lower to undetectable levels of cortical- and medullary-associated transcripts. Collectively, these findings infer that *Clono*TECs segregate from prototypical mature TECs and typify instead a subset with TEP-like properties.

*Clono*TECs generate cTECs and mTECs in vivo

To define the in vivo lineage potential of *Clono*TECs, we combined the usage of clonogenic assays with reaggregate thymic organ cultures (RTOC) and thymic transplantation under the kidney capsule. As *Clono*TECs generated from $CCRL1^{GFP}$ cTEC lose GFP expression in culture (data not shown), they were not the most appropriate for fate mapping experiments. Since Ly51+cTEC from $Actin^{GFP}$ reporter mice exhibited similar clonogenic capacity to $CCRL1^{GFP}$ cTEC from $CCRL1^{GFP}$ reporter mice (Fig. 1), we used purified *Clono*TECs (*Clono*$TEC^{ActinGFP+}$) generated from the first

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subset, in which constitutive active GFP expression provides an intrinsic label for subsequent lineage tracing in vivo. To reconstruct thymic epithelia microenvironments, we mixed *Clono*TEC$^{ActinGFP+}$ with dGUO-treated E14.5 thymic cells and the resulting hybrid RTOC was transplanted under the kidney capsule of WT mice to allow the ectopic formation of a thymus (Supporting Information Fig. 3A). As control, dGUO-treated E14.5 thymus (*Clono*TEC$^{ActinGFP+}$-free) were reaggregated and subjected to similar procedure. From 6 control and 11 hybrid engrafted RTOCs, 4 and 8 ectopic thymi were respectively recovered 4 weeks post-thymic transplantation and analysed by flow cytometry and immunofluorescence microscopy. The progeny of *Clono*TEC$^{ActinGFP+}$ was distinctly present in all RTOCs as CD45−EpCAM+ cells, with a fraction of them expressing MHCII (Supporting Information Fig. 3B). These findings indicate that *Clono*TECs contained cells competent to engage into the TEC differentiation pathway. Nevertheless, we recovered few TECs (either from embryonic or *Clono*TEC origin) from individual RTOCs for flow cytometry analysis. Thus, to gain insights about the phenotypic properties and spatial distribution of *Clono*TECs within native thymic niches, transplanted RTOCs were further analyzed by immunofluorescence microscopy. *Clono*TEC$^{ActinGFP+}$- derived cells were found in all recovered RTOCs (Supporting Information Figure 3C-D), embedded within cortical (Ly51+ or K8+) or medullary (UEA+) microenvironments or positioned at the CMJ (intersection of K8+ and UEA+ areas) (Fig. 3A). Although a fraction of *Clono*TEC progeny (GFP+) lacked typical cTEC/mTEC markers, we found that some located within cTEC areas expressed cTEC traits, such as K8+ or Ly51+, while others residing within mTEC areas displayed mTEC features, including UEA+ and MHCII^{hi} (Fig. 3A and B). We also found rare Aireexpressing *Clono*TEC-derived cells within mTEC compartment, indicating their potential to complete mTEC maturation (Fig. 3A). Additionally, to validate that *Clono*TEC-derived cells engaged in a TEC differentiation program, we performed similar reaggregation experiments using *Clono*TECs generated from Foxn1^{eGFP+} cTECs (Supporting Information Fig. 3E). As Foxn1^{eGFP+} cTECs lose Foxn1^{eGFP} expression in vitro (Supporting Information Fig. 2), GFP expression provides in this case a dual label for lineage tracing and assessment of Foxn1^{eGFP} re-induction by *Clono*TECsderived cells. We detected GFP-expressing cells only in RTOCs spiked with *Clono*TECs that derived from Foxn1^{eGFP}+ cTECs (Supporting Information Fig. 3F). *Clono*TECs-derived cells expressed EpCAM, with a fraction co-expressing MHCII and binding UEA (Supporting Information Fig. 3G). Overall, these results indicate that cTEC-derived *Clono*TECs contain cells with the potential to generate cTECs and/or mTECs upon integration in native thymic microenvironments.

The clonogenic activity of cTECs decreases with the entry into the adulthood

The observation that a fraction cTEC-derived *Clono*TECs maintains their clonogenic potential in vitro and generates cTECs and/or mTECs indicate that the cortical niche harbors TEC progenitors. Thus, we inferred that the measurement of the clonogenic potential of TEC subsets at neonatal, pre-puberty and young adult stages could be used as a proxy to survey alterations in TEP dynamics throughout time. In CCRL1 reporter mice, analysis of CCRL1^{GFP} and CD80 expression allowed us to discriminate heterogeneous TEC subsets in the postnatal thymus [26]. While CCRL1^{GFPhi} cTECs (gate D) were dominant in the neonatal period, distinct mTEC subsets (gates A, B, C), which either lacked or expressed intermediate levels of CCRL1) [26], expanded during the first weeks of age and predominated in the young adult thymus (Fig. 4A). To determine the colony-precursor frequency within these detailed TEC subsets, we combined cell sorting, limiting dilutions in microscale clonogenic assays and high-content imaging microscopy. Given the clonal origin of TEC-derived colonies, we estimated the frequency of colony-forming cells by dividing the number of colonies by the number of sorted cells per well for each detailed subset (Fig. 4B). We observed that clonogenic activity was highly restricted to the CCRL1^{GFPhi} cTEC subset (gate D) at all time points analyzed (Fig. 4B). Furthermore, we noticed that the clonogenic potential of CCRL1^{GFP} cTECs gradually decreased from postnatal day 5 to young adult thymus (Fig. 4C). To further evaluate the reduction in colony-forming potential of cTECs with time, we established competitive clonogenic assays with age-matched or age-mismatched cTECs isolated from Actin^{GFP} and Actin^{RFP} report mice. While coculture experiments with cTEC^{ActinGFP} and cTEC^{ActinRFP} from postnatal day 5 thymus yielded a similar respective proportion of GFP+ or RFP+ *Clono*TECs, cTECs purified from postnatal day 5 thymus showed a marked clonogenic advantage over cTECs isolated from 2-week-old

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counterparts (Supporting Information Figure 4). Together, our findings suggest that TEP-like cells within cTECs autonomously lose the clonogenic capacity with the entry into the adulthood.

The clonogenic potential of TECs is sustained in alymphoid thymus

Just as TECs have a central function in T-cell development, thymocytes are in turn vital to TEC maturation [2]. Albeit lymphoepithelial interactions are often considered stimulatory to TEC differentiation, and in particular to the expansion of the mTEC network, we previously conjectured that signals provided by developing thymocytes might restrain functional properties coupled to immature TECs [2]. To determine whether the loss in clonogenic potential of cTECs was directly linked to thymocyte-driven TEC differentiation, we used *Rag2−/−Il2rg−/−* CCRL1^{GFP}-reporter mice, in which T-cell development is profoundly blocked at early stage of development. Consequently, TEC maturation is severely arrested in these mice due the lack of maturation signals delivered by lymphoid cells. Specifically, apart of virtually lacking mature CD80+ mTECs, Rag2−/−Il2rg−/− thymus display a partial blockade in full CCRL1 and MHCII expression [26, 33] (Fig. 5A). We performed limiting dilution clonogenic assays (as described in Fig. 4) with TECs that either lack or express intermediate and higher levels of CCRL1 isolated from 2- and 6-week-old *Rag2$^{-/-}$Il2rg$^{-/-}$* thymus. We found that the capacity to generate colonies was scattered among the three TEC subtypes in the 2-week-old thymus, progressively increasing within TECs expressing higher levels of CCRL1 (CCRL1^{GFPhi}) (Fig. 5B and C). Notably, the frequency of colony-precursor cells was sustained within the three TEC subsets in the 6-week-old *Rag2$^{-/-}$Il2rg$^{-/-}$* thymus (Fig. 5C). The maintenance of the pool of cells with clonogenic capacity was also notorious when their absolute cellularity was extrapolated within the total number of TECs of the alymphoid thymi and longitudinally compared to their abundance in immunocompetent counterparts (Fig. 5D). These results indicate that thymocyte-derived signals might negatively affect the clonogenic potential of TECs during life.

Lympho-epithelial interactions control the clonogenic potential of TECs

To directly assess the influence of thymocyte-TEC crosstalk on TEC clonogenic capacity we reconstituted 6-week-old *Rag2−/−Il2rg−/−* CCRL1-reporter mice with bone marrow (BM) precursors from WT mice and analyzed the frequency of colony-forming cells of recipient mice 6 weeks post-transplantation. As expected, the number of CD45+ cells increased and T-cell development was corrected in the thymus of WT BM-reconstituted *Rag2−/−Il2rg−/−* CCRL1^{GFP}-reporter mice (data not shown), restoring the differentiation of CD80+ mTECs and the normal cTEC/mTEC segregation (Fig. 6A). The conditioning sub-lethal irradiation protocol prior to BM transplantation and age did not alter the clonogenic activity of TECs from 12-week-old non-reconstituted *Rag2−/−Il2rg−/−* CCRL1^{GFP} -reporter mice (Fig. 6B and C), as the frequency of colony-forming cells in TECs that either lack or express intermediate and higher levels of CCRL1 was similar to the ones in unconditioned 6-week-old mice (Fig. 5C). Strikingly, the clonogenic potential of purified cortical/medullary subsets from WT BM-reconstituted *Rag2−/−Il2rg−/−* CCRL1-reportermice exhibited a notorious reduction and restriction to the CCRL1^{GFPhi} TEC subset (Fig. 6B and C), normalizing to the profile observed in young immunocompetent mice. Collectively, our findings demonstrate that continual thymic crosstalk negatively regulates TEC clonogenic activity.

Discussion

Comprehending the principles that underlie the maintenance of cortical and medullary thymic epithelial compartments is chief to harness thymopoiesis in the elderly and in patients with immunodeficiency disorders or autoimmunity. Therefore, the prospective isolation of TEPs within the adult thymus has emerged as a central objective in thymic biology, as it would provide means for reconstructing functionalized thymic epithelial microenvironments in therapies targeting thymus disorders. An aspect of equal importance that has been overlooked pertains to the principles that control the bioavailability and functionality of those progenitors in the adulthood. Given that TEC network expands vigorously during the period between birth and early adulthood, we centred our attention in this temporal window with the premise that it might reveal new

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insights on how TEP homeostasis is balanced in vivo. Our temporal analysis of TEC clonogenic potential suggests that the postnatal life defines a period of intense stem cell-like activity within the thymic cortex, which gradually diminished thereafter with the entry into the adulthood as a result of lympho-epithelial interactions. These findings support the notion that the pool of TEPs and/or their replenish rate deteriorates across life, providing a possible explanation to their incapacity to sustain functional epithelial niches in the aged thymus.

Detailed phenotypic and molecular analyses show that in vitrogenerated *Clono*TECs lacked traits that are typically associated with cortical or medullary lineages, but instead expressed markers linked to stem cells. These observations correlate with their reduced levels of Foxn1, which is central for the initiation and maintenance of the TEC program but appears dispensable for the maintenance of the thymic epithelial stem cell pool [7, 15, 34]. Using Foxn1^{eGFP} reporter thymus, we showed that *ClonoTECs* derived from Foxn1-expressing cTEC lose Foxn1 expression in vitro, indicating that Foxn1 levels are tightly controlled by thymic microenvironmental factors. Nonetheless, our understanding on the molecular signals that initiate and sustain the expression of this master regulator of TEC identity in vivo requires further analysis. Importantly, the findings that a fraction of *Clono*TECs retains continual clonogenic potential in vitro and contain cells that can diversify into cTECs and mTECs in vivo reinforce their TEP-like signature. Still, the progeny of *Clono*TECs that reintegrated within thymic microenvironment was limited. Whether the low engraftment of *Clono*TECs is due to experimental impediments related to the establishment of organotypic cultures or competitive disadvantage relatively to embryonic TEP within RTOCs is unclear. For technical reasons related with the establishment of RTOCs, the proportion of *Clono*TECs within the hybrid RTOCs before thymic transplanting was reduced relatively to TECs derived from E14.5 "carrier" thymus (1:5-16:1). The abundance and/or competitive fitness of ex vivo embryonic TEC progenitors might under these conditions limit the integration, maintenance and the generation of a more prominent *Clono*TEC-derived progeny 4 weeks after transplantation. Moreover, and similarly to the capacity to continual establish colony-forming units, it is possible that only a fraction of *Clono*TECs developed within the ectopic thymus and contributed to the TEC network. Future studies should address the long-term maintenance of *Clono*TECs within native thymic niches. This technical limitation seems to be common to several studies using hybrid RTOCs, which are composed of predominant embryonic thymic stromal cells mixed with adult TEC subsets purportedly enriched with TEPs [15–17]. Furthermore, due to high cell density requirements to perform RTOC experiments, we used a pool of cTEC-derived *Clono*TECs from multiple colonies. This technical impediment has precluded testing the lineage potential of cTEC-derived *Clono*TECs from individual colonies or residual mTEC-derived *Clono*TECs. Despite phenotypic similarities at a population level, possible intra- and inter-clonal heterogeneity within *Clono*TECs might also influence their engraftment and lineage potential. Hence, it is possible that *Clono*TECs contain cTEC- and mTEC-producing cells. Nonetheless, *Clono*TECs were originally generated from cells with a prototypical cTEC features, suggesting that the cortical thymic epithelium compartment harbors cTEC and/or mTEC precursors. Future refined experimental setups are required to address these possibilities at the single cell level with the purported postnatal-derived TEC progenitors, as reported earlier with embryonic cells [6]. Furthermore, apart of lineage tracing assays, it would be equally important to unravel whether postnatal-derived TEC progenitors can functionally contribute to thymopoiesis.

Using distinct experimental approaches, three recent reports have revealed the existence of TEC stem cell activity within the adult thymus. First, Ucar et al. reported the presence of EpCAM−Foxn1− cells within the thymic stroma that form under specialized in vitro culture system spheroids, so-called thymosphere, with the capacity to generate cTECs and mTECs 15. We reason that cTECs with colony-forming capacity described in our report are distinct from thymosphere-generating cells. First, although the location of thymosphere-forming cell remains undetermined, *Clono*TECs arise from a fraction of cells belonging to the prototypical cTEC lineage (EpCAM^{+}Ly51^{+}CCRL1^{+}). Secondly, CD45^{-}EpCAM^{-} thymic stromal cells failed to form *Clono*TEC-containing colonies in our experimental condition. Despite being hitherto phenotypically undistinguishable from cTECs lacking clonogenic activity, *Clono*TEC-generating cTECs might encompass a subset of progenitors cells nestling in the cortical compartment that have not completed the cTEC maturation program. In this regard, Wong et al. documented that bipotent TEPs exist within a fraction of UEA-1−MHII^{lo} cTEC-like cells of the adult thymus [16] and Ulyanchenko et al. further mapped them to a fraction of Ly51^{+}MHCII^{hi}Plet1^{+} cTECs [17], inferring

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in agreement with our results that TEPs share to some extent a cortical-associated signature. Along this line, genetic inducible cell-fate mapping studies by Ohigashi et al. and Mayer et al. found that a large fraction of adult cTECs and mTECs develop from fetal- and newborn-derived TECs expressing β5t [20, 21]. These findings suggest that TEC differentiation in the postnatal period follows a similar process to the one defined in the embryonic life [14], in which the cortex represents a reservoir of TEPs wherefrom they can potentially differentiate into cortical and medullary epithelial lineages. However, it remains to be elucidated whether cTEC-derived *Clono*TECs and other recently identified subsets enriched in TEP-like cells, contain truly bipotent progenitors or unipotent progenitors for each lineage. It is important to underline that even with the most refined subsets and distinct assays, TEC precursors are still being described at the population level, but are not yet recognized at the single-cell level. Albeit it is conceptually possible that several pools of TEP-like cells exist within the thymus, further studies are required to determine the lineage and temporal relationship between the distinct types of TEPs that are being disclosed within the postnatal thymus.

It remains unclear how TEC stem cell activity is controlled in the adult thymus. The incapacity of TEPs to undergo compensatory proliferation to maintain the mature TEC compartment [25] indicates a deficit in their stemness. The progenitor features of *Clono*TECs led us to use the colony-forming potential as a surrogate to survey the dynamics of TEP during early postnatal life and adulthood. Our findings indicate that clonogenic activity is predominantly enriched in cTECs during the first week of life. Previous observations showed that cTECs regenerate after the specific ablation of CCRL1^{hi} cTECs [35]. Nevertheless, the complete cTEC depletion was not achieved in this study and therefore resistant TEP within CCRL1-expressing subset might explain the observed regenerative capacity of cortical epithelium. The drop in the clonogenic activity of CCRL1^{hi} cTECs in the ensuing weeks might suggest that the bioavailable pool of TEPs is reduced with the entry into the adulthood. In this regard, the contribution of β5t^{+} progenitors to cTEC and mTEC lineages declines postnatally [20, 21] and TEPs isolated from the adult thymus are extremely rare cells [15, 16, 20]. The maintenance of TEC clonogenic activity in 6- and 12-week-old alymphoid thymus suggests that aging is, not per se, a determinant in this process. In particular, we observed that clonogenic activity was enriched in CCRL1^{hi} cTEC-like subset of *Rag2−/−Il2rg−/−* mice, indicating that TEPs might progress through, and settle within, the cortical lineage in a thymocyte-independent manner. In fact, bone marrow reconstitution experiments revealed that clonogenic potential is dynamically modulated by interactions with developing thymocytes. Similar observations were recently suggested to Cld3,4^{+}SSEA1^{+} mTEC-restricted cells, which are rare in the adult thymus and enriched in *Rag2−/−* mice [23]. These findings provide evidence for a negative feedback mechanism in which continual thymic crosstalk fine-tunes the homeostasis of distinct TEPs. Thymocyte-derived signals are often considered stimulatory for TEC differentiation [1]. Nonetheless, previous studies, including from our group, have shown that thymocyte-TEC crosstalk negatively regulates functional attributes coupled to cTECs, including the expression of DLL4 and IL-7 [13, 36]. Now, our findings implicate that thymocyte-derived signals might act at the root of the TEC differentiation branch, deteriorating the pool of TEPs and possibly limiting their replenishment rate. It remains however opened whether these effects are mediated by direct or indirect lympho–epithelial interactions. These notions might provide a possible explanation to the failure in sustaining TEC compartments in the aged thymus [18] and the success of inducing cTEC and mTEC niches in WT BM-reconstituted adult mice that lacked previous functional lympho-epithelial crosstalk [33, 37]. Last, our data question whether the mere prospective isolation of bipotent progenitors from the adult and aged thymus represents the more desirable strategy for cellular replacement therapies in thymic disorders. Alternative approaches might focus in unraveling active mediators of stem cell activity, which will permit a more effective functionalization of TEPs isolated the adult thymus.

Materials and methods

Mice

Transgenic Actin reporter C57BL/6J mice in which the chicken β-actin promoter respectively drives enhanced Green Fluorescent Protein (eGFP) (Actin^{GFP}) or Red Fluorescent Protein (RFP) (Actin^{RFP}) expression were purchased from Jackson Laboratory. Ccrl1:eGFP (CCRL1^{GFP}) [35] and Foxn1^{eGFP} reporter mice 30 were kindly provided by Dr. Thomas Boehm (Germany). Ccrl1:eGFP

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(CCRL1^{GFP}) reporter mice were used as such or were backcrossed onto *Rag2−/− Il2rg−/−* [33] C57BL/6J background. For thymic transplantation, 6-8-week-old C57BL/6J mice were used as recipients. Mice were housed under specific pathogen–free conditions and experiments were performed in accordance with institutional guidelines. For fetal studies, the day of vaginal plug detection was designated embryonic day (E) 0.5.

TEC Clonogenic assay

FACS sorted TECs were cultured onto a feeder layer of irradiated mouse embryonic NIH/3T3 (3T3) fibroblast cell line as described [27, 28]. 3T3 cells were regularly maintained in culture using Dulbecco-Vogt modification of Eagle's Medium (DMEM, Gibco–Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. For the preparation of feeder layer, 3T3 cells were irradiated (60 Gy) one day before the experiment, seeded onto 6-well (midscale) or 96-well culture plates (microscale) coated with 0,05mg/mL of fibronectin (Sigma-Aldrich), at a density of 12.5×10^4 cells cm^{-2} or 5×10^4 cells per cm^{-2},respectively. TEC purified by cell sorting were directly cultured onto feeder layer in a specialized medium consisting of a 3:1 mixture of DMEM and Ham's F-12 medium (Gibco–Invitrogen), supplemented with 10% FBS, hydrocortisone 0.4 µg mL^{-1}, 10^{-6} M cholera toxin, 5 µg mL^{-1} insulin, 2×10^{-9} M 3,3′,5-triiodo-L-thyronin (T3), 10 ng ml^{-1} recombinant human epidermal growth factor rhEGF, and penicillin/streptomycin (Peprotech). All cultures were performed at 37°C in a 7% CO2 atmosphere for 12 days. Colonies were firstly revealed by hemacolor staining (Merck) according to the manufacturer's instructions or processed for flow cytometry or immunofluorescence microscopy analyses as described below.

TEC and *Clono*TEC isolation and flow cytometry analysis

TECs were isolated as described [13]. *Clono*TEC were recovered from clonogenic assays using 0.05% trypsin-EDTA (Gibco-Invitrogen), followed by cold PBS supplemented with 10% FBS to stop the reaction. Single-cell suspensions were stained with anti-Ly51 (PE) (BD Biosciences); anti-Sca-1 (BV785); anti-CD24 (BV510) and anti-EpCAM (BV421) (BioLegend); anti–I-A/I-E (Alexa 780); anti-CD45.2 (PerCP-Cy5.5); anti-CD40 (PE); anti-CD205 (biotin); UEA-1 (biotin); anti-CD80 (APC) and streptavidin (PE-Cy7) (eBioscience); anti-FOXN1 (Alexa 647) [38] was kindly provided by Dr. Hans-Reimer Rodewald (Germany). For intracellular staining, cells were fixed and permeabilised with the Transcription Factor Staining Buffer Set (eBioscience) according to the manufacture's instructions. Flow cytometry analysis was performed with the LSRFortessa instrument (BD Biosciences) and FlowJo software. Cell sorting was performed using the FACSAria II (BD Biosciences), with sort purities >95%.

Immunofluorescence analysis

Immunofluorescence staining was performed directly either on midscale and microscale clonogenic assays or on 8-µm sections of reaggregate thymic organ cultures (RTOCs) samples. Cultures or Thymus were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) and stained with rabbit anti-GFP (Thermo Fisher Scientific), rat Troma-I (kindly provided by Drs. Brulet and Kemler), rat anti–I-A/I-E, rat anti-Aire, UEA1- or Ly51-biotinilated (eBioscience); and revealed with secondary Alexa Fluor 488 anti-rabbit, Alexa Fluor 647 anti-rat, or streptavidin Alexa 555 (Invitrogen). Nuclei were stained with DAPI (Invitrogen). Vectashield mounting medium (Vector Laboratories) was used to prepare the slides. Analysis was performed with IN Cell Analyzer 2000 (GE lifesciences) and collected images were processed with Fiji Software.

Gene expression

For quantitative PCR, mRNA from sorted cells was purified using the RNeasy Micro Kit (QIAGEN). RNA was reverse transcribed to cDNA, using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and Random Hexamers (Fermentas), and then subjected to real-time PCR using TaqMan Universal PCR Master Mix (Applied Biosystems) and primers for *18s, Foxn1, Il7, Psmb11, Tnfrsf11a, Aire, Kitl, Dll4, Cxcl12, Ccl25, Ccl19, Ccl21* (Applied Biosystems). All samples were analyzed as triplicates, and the ΔΔCt method was used to calculate relative levels of target mRNA compared with *18s*. Procedures were done according to the manufacturer's protocols. Real-

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time PCR was performed in an iCycler iQ5 Real-Time PCR thermocycler (Bio-Rad). Data were analyzed using iQ5 Optical System software (Bio-Rad).

RTOC

Freshly isolated E14.5 thymic lobes were used to establish RTOCs, as described 13. Previous to aggregation, embryonic lobes were cultured for 3 days in DMEM supplemented with 10% FBS and 360mg/L 2-deoxyguanosine (dGuo) (Sigma-Aldrich). *Clono*TEC^{GFP+} were sorted to high purity (>95%). RTOCs were established from mixtures of 50,000–150,000 Actin^{GFP+} or Foxn1^{eGFP} – derived *Clono*TECs with E14.5 thymic cells at 1:16 to 1:5 ratios, and transplanted under the kidney capsule of WT mice. Ectopic thymus were recovered after 4 weeks of transplantation and analyzed by flow cytometry or immunohistochemistry.

Bone marrow chimeras

A total of 10^7 CD3-depleted bone marrow (BM) cells (MACS MicroBead, Miltenyi Biotec) from 6-week-old wild-type (WT) C57BL/6J donors were injected i.v. in 6-week-old sublethally irradiated (0.4Gy) *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ CCRL1-reporter mice.

Statistical analysis

Statistical analysis of the results was made using GraphPad Prism Software. The two-tailed Mann–Whitney U test was used for analysis between groups. A 95% confidence interval was applied in the calculations, and samples with p values < 0.05 were considered significant (*).

Acknowledgements

We thank Thomas Boehm (Max Planck Institute of Immunology and Epigenetics, Freiburg, Germany) for Foxn1^{eGFP} reporter mice. We thank Dr. Hans-Reimer Rodewald (German Cancer Research Center, Heidelberg, Germany) for the anti-Foxn1 antibody. We thank Dr. Leonor Araújo for technical support and Drs. Chiara Perrod and Gema Romera-Cardenas for critical reading the manuscript. We also thank Dr. Sofia Lamas and the caretakers from the animal facility for the assistance with animal experimentation.
C.M., A.R.R., and N.L.A. conceived and designed experiments, performed experiments, analyzed the data, and wrote the manuscript. P.M.R., R.D.P., and C.L. performed experiments and analyzed data. N.L.A. conceptualized the original idea.
This work has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No 637843 - TEC_Pro), from FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior in the framework of the project "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274) and FEDER funds through the Operational Competitiveness Programme – COMPETE and by National Funds through FCT – Fundação para a Ciência e a Tecnologia under the project FCOMP-01-0124-FEDER-021075 (PTDC/SAU-IMU/117057/2010). N.L.A., P.M.R., A.R.R., C.M., and R.D.P. are supported by the Investigator program, Post-doctoral and PhD fellowships from FCT (Portugal).

Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Abbreviations: **Aire**: Auto-immune regulator · CCRL1: Chemokine (C-C motif) receptor-like 1 · CMJ: cortico-medullary junction · cTEC: cortical TEC · dGUO: deoxyguanosine · EpCAM: Epithelial cell adhesionmolecule · LTβR: Lymphotoxin eta receptor ·MHC:Major Histocompatibility Complex ·mTEC: medullary TECs ·RANK: Receptor activator of NF-κB · RTOC: reaggregate thymic organ culture · TCR: T cell receptor · TEC: Thymic epithelial cell · TEP: Thymic Epithelial Progenitor · WT: wild-type

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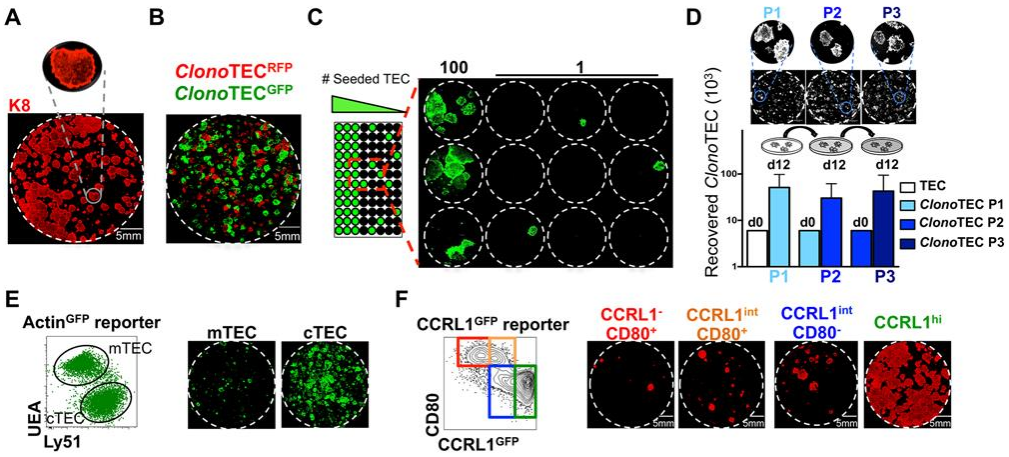

Figure 1. Colony-precursor cells are markedly restricted to the cortical thymic epithelium of the postnatal thymus. **(A)**Midscale clonogenic assays (6-well microplates) were established with cell-sorted total TECs (defined as CD45$^-$EpCAM$^+$) from postnatal day 5 (P5) thymus. After 12 days, cultures were fixed, stained with anti-cytokeratin 8 (K8) antibody and analyzed by immunofluorescence microscopy. **(B)** Cell-sorted total TECs purified from P5 Actin^{GFP} and Actin^{RFP} reporter mice were co-cultured at 1:1 ratio in midscale clonogenic assays and analyzed for the expression of GFP and RFP by fluorescence live cell imaging. **(C)** P5 Actin^{GFP} TECs were directly sorted at indicated densities into microscale clonogenic assay (96-well microplates) and the colony formation was analyzed by fluorescence live cell imaging. Green circles mark positive wells for clonogenic activity, while black circles mark wells with no activity (left). Representative live-cell fluorescence images of indicated wells are shown (right). **(D)** *Clono*TECs contain cells with continual regrowth and colony-forming potential in vitro. Clonogenic assays were established with cell-sorted TECs from P5 Actin^{GFP} or Actin^{RFP} at the indicated density (6000 cells). 12 days after culture, *Clono*TECs (P1) were analyzed by flow cytometry (EpCAM$^+$ and GFP$^+$or RFP$^+$), purified by cell sorting and re-cultured into clonogenic assays at the initial density (6000 cells) for two consecutive passages (P2 and P3). Representative scheme of the passages and live cell immunofluorescence analysis of indicated cultures at day 12 of each passage is displayed (top). The number of cells at day 0 (d0) and at day 12 (d12) from the different passages (P1-P3) is shown as mean ± SEM of a pool of 10 independent experiments. **(E)** cTECs and mTECs from P5 Actin^{GFP} were sorted and cultured under clonogenic assays. Cultures were analyzed for the expression of GFP by fluorescence live cell imaging. **(F)** TEC subsets from P5 CCRL1^{GFP} reporter mice were purified based on CCRL1^{GFP} and CD80 expression (as indicated in color gates) and cultured under clonogenic assays. Cultures were fixed, stained with anti-cytokeratin 8 (K8) antibody and analyzed by immunofluorescence microscopy. (A, B, E & F) Images represent complete individual wells from midscale clonogenic assays and are illustrative of at least three experiments. Scale bars, 5 mm.

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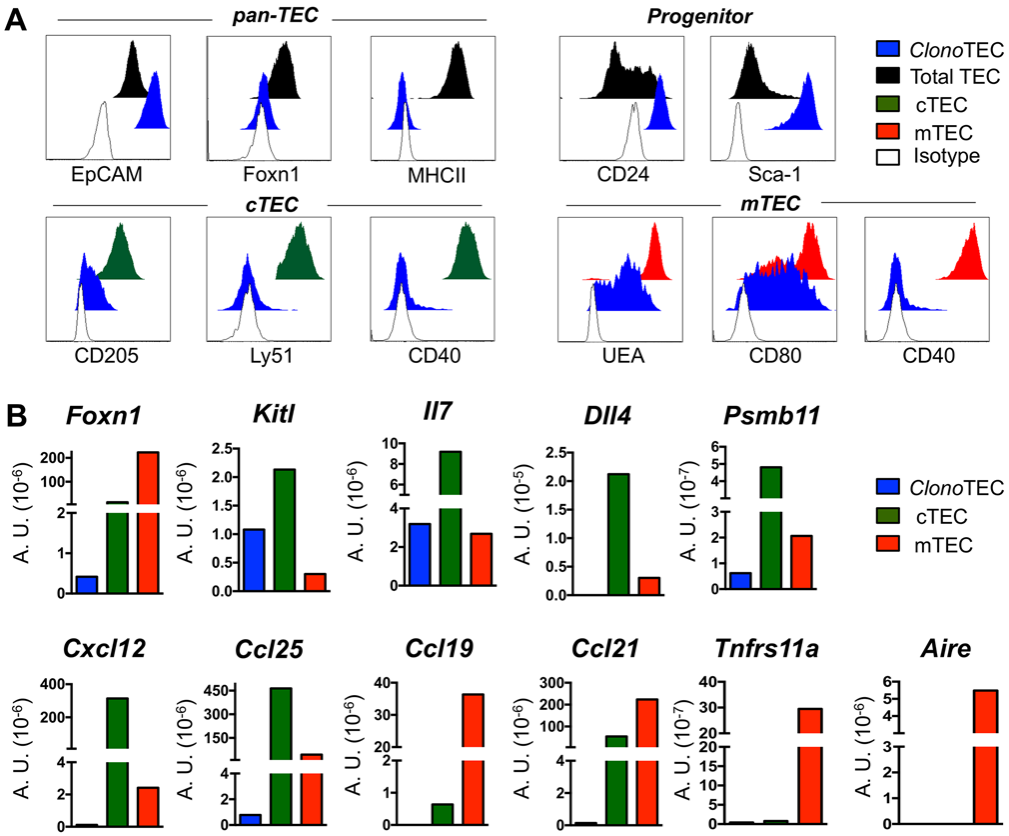

Figure 2. *Clono*TECs typify TEP-like cells. **(A)** cTEC-derived *Clono*TECs (blue) were analyzed by flow cytometry for the expression of the indicated markers. As a comparison, ex vivo bulk TECs (black), cTECs (dark green) or mTECs (red) isolated from postnatal day 7 thymus were co-analyzed. Isotype antibody controls for each antibody is represented (white) **(B)** *Clono*TECs, cTECs and mTECs were purified by cell sorting and analyzed by RT-qPCR for the expression of the indicated genes. Relative mRNA expression for represented target genes was normalized to 18s and values are represented in arbitrary units (A.U.). Data are from single samples representative of two to three experiments using independent samples.

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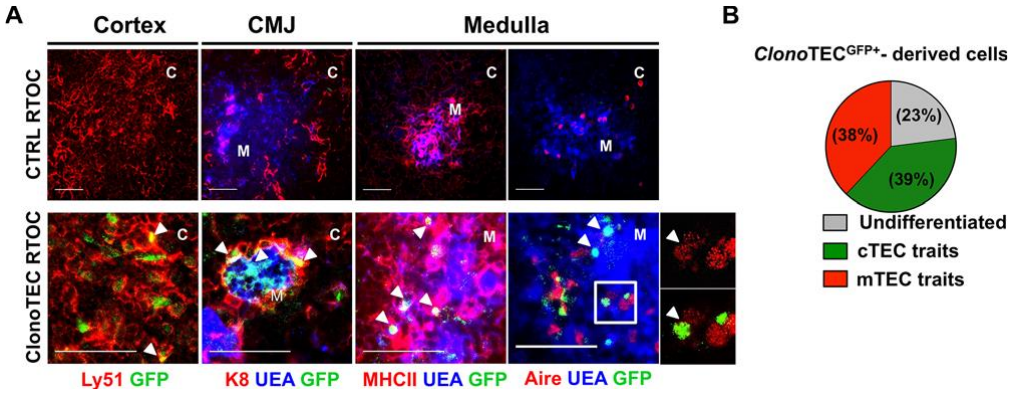

Figure 3. *Clono*TECs contain cells that are able to generate cTECs and mTECs in vivo. Generated cTEC^{ActinGFP}-derived *Clono*TECs^{GFP} were cell-sorted and aggregated with dGuo-treated E14.5 thymic lobes. RTOCs were transplanted into the kidney capsule ofWTmice, ectopic thymi were recovered 4 weeks post-transplantation (Details in Supporting Information Figure 3A). **(A)** Immunofluorescence analysis of ectopic thymus. Control and *Clono*TECs post-transplanted RTOCs were co-analyzed for the expression of GFP, K8, Ly51, MHCII and Aire with specific antibodies and UEA binding capacity, with the indicated combinations in serial sections. Cortical (C) and medullary (M) regions were defined as either Ly51$^+$ or K8$^+$ areas and UEA+ or MHCII^{bright} areas, respectively. Triangles indicate examples of *Clono*TEC^{GFP+}-derived cells that display features of either cTEC (Ly51 or K8) or mTEC (UEA, MHCII, Aire) lineage traits. 50 μm scale is shown. Images are representative of 5 ectopic thymus containing *Clono*TEC^{GFP}. **(B)** Quantification of lineage fate distribution of *Clono*TECs. Pie graph represents the proportion of *Clono*TEC^{GFP+}-derived cells within the thymic grafts that express the above-indicated cTEC (green) or mTEC (red) markers. *Clono*TEC-derived cells (GFP$^+$) found within cTEC area (K8$^+$ or Ly51$^+$) and expressing these markers were scored as cTECs. *Clono*TEC-derived cells (GFP$^+$) found within mTEC area (UEA$^+$ and MHCII^{bright}) and binding UEA or expressing high levels of MHCII were scored as mTECs. *Clono*TEC-derived cells (GFP$^+$) found within cTEC and mTEC areas that lacked respective cTEC/mTEC-markers were considered as undifferentiated (gray).

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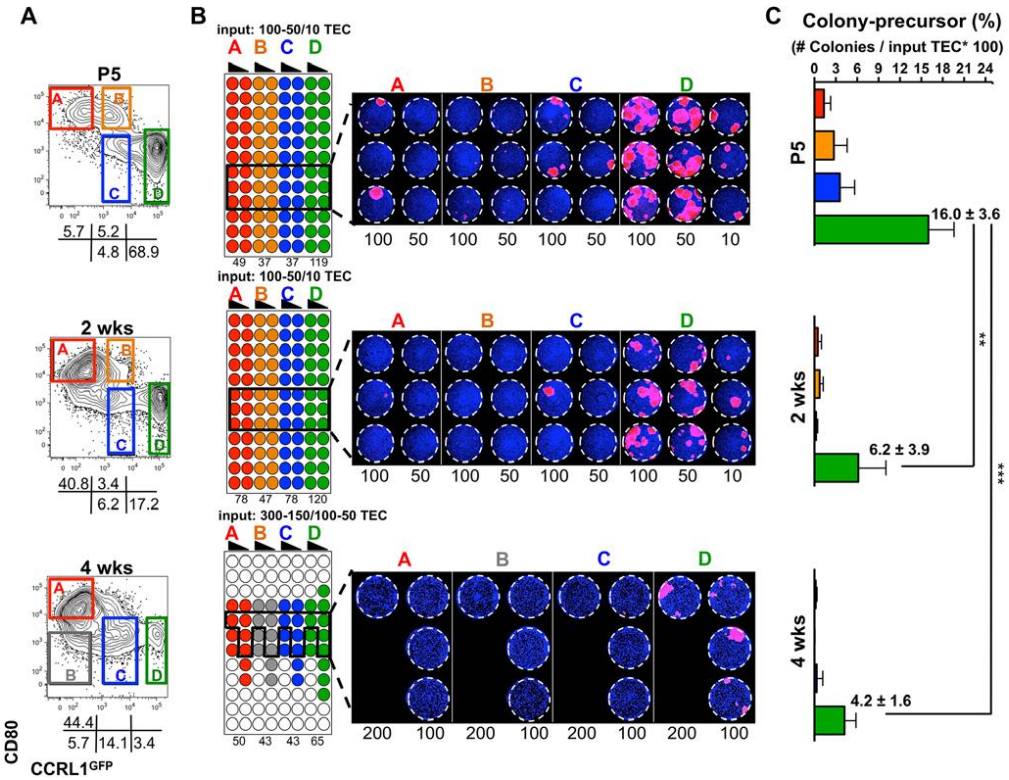

Figure 4. The pool of TEC colony-precursor cells residing within the postnatal cortex decreases during the transition from postnatal to adult life. **(A)** TECs from CCRL1^{GFP} reporter mice were analyzed by flow cytometry at indicated time-points for the expression of CCRL1^{GFP} and CD80. Proportions of indicated subsets are shown below the plots. **(B)** Colored TEC subsets (A-B-C-D) illustrated in (A) were purified by cell sorting and cultured inmicroscale clonogenic assay (96-well microplates) at designated cell densities (input: as represented on the top of the schematic plate). Numbers below each schematic plate represent the total amount of wells analyzed per subset (left). Representative images of full individual wells for each time point and TEC subset are shown (right). Number of plated cells (input) is shown below. 96-well plate cultures were fixed, stained with anti-cytokeratin 8 (K8) Ab and analyzed by high-content immunofluorescence microscopy. Data shown are representative of three to four experiments performed per time point. **(C)** Colony-precursor cell frequency (%) was estimated by dividing the number of colonies obtained by the number of seeded TEC (input) x 100. Data are shown as mean ± SEM of a pool of 5 (p5), 6 (2 wks) and 9 (4 wks) independent experiments. **p< 0.005 ***p< 0.001, the two-tailed Mann–Whitney U test.

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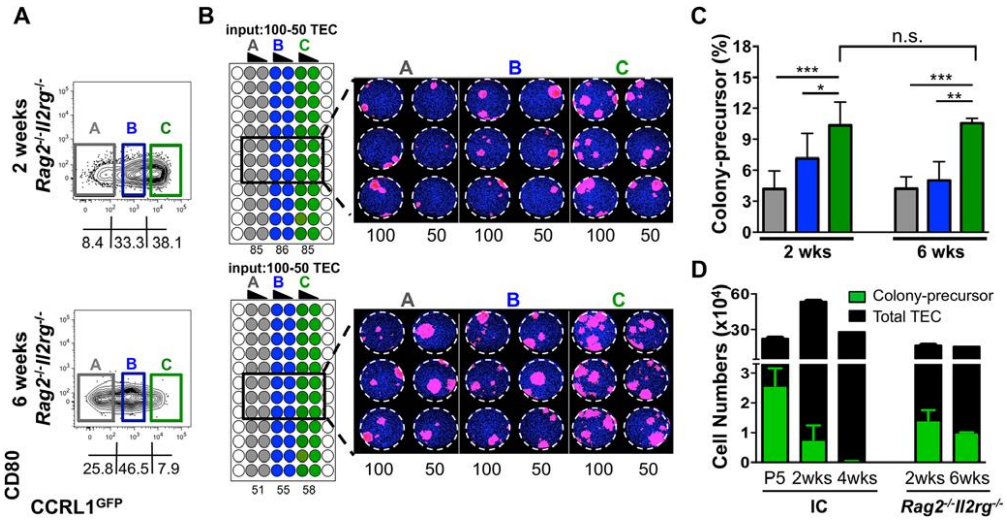

Figure 5. The pool of TEC colony-precursor cells is sustained in the adult *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ thymus. **(A)** TECs from *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ CCRL1^{GFP} mice were analyzed by flow cytometry at indicated time-points for the expression of CCRL1^{GFP} and CD80. Proportion of indicated subsets are shown below the plots. **(B)** Colored TEC subsets (A-B-C) illustrated in (A) were purified by cell sorting and cultured in microscale clonogenic assay (96-well microplates) at designated cell densities (input: as represented on the top of the schematic plate). Numbers below each schematic plate represent the total amount of wells analyzed per subset (left). Representative images of full individual wells for each time point and TEC subset are shown (right). Number of plated cells (input) is shown below. 96-well plate cultures were fixed, stained with anti-cytokeratin 8 (K8) Ab and analyzed using a high-content immunofluorescencemicroscopy. Data shown are representative of three to four experiments per time point. **(C)** Colony-precursor cell frequency (%) was estimated based on the number of colonies relatively to the number of seeded TECs (as in Fig. 4C). Data are shown as mean ± SEM of a pool of 6 (2 wks) and 4 (6 wks) independent experiments. *p<0.05 **p<0.005 ***p<0.001, the two-tailed Mann–Whitney U test. (D) Number of colony-precursor cells (green) within total TEC (black) for immunocompetent (IC) (Fig. 4) and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (this figure) mice. The number of colony-precursor cell was estimated based on the frequency of these cells within the total TEC cellularity for a given time. IC: Data are shown as mean ± SEM of a pool of 3 (p5), 4 (2 weeks) and 3 (4 weeks) independent experiments; *Rag2*$^{-/-}$*Il2rg*$^{-/-}$: Data are shown as mean ± SEM of a pool of 3 (2 weeks) and 3 (6 weeks) independent experiments.

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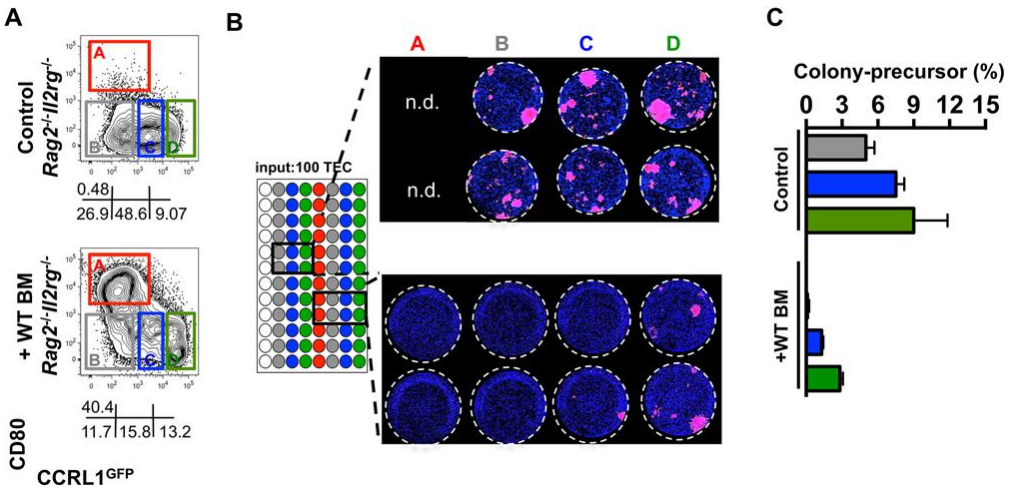

Figure 6. Thymocyte-derived signals negatively impact the pool of TEC colony-precursor cells. *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ CCRL1^{GFP} mice were reconstituted with WT BM precursors (+WT BM) or left non-reconstituted (Control). **(A)** TECs from control and WT BM-reconstituted *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ CCRL1^{GFP} mice were analyzed by flow cytometry for the expression of CCRL1^{GFP} and CD80. The proportion of indicated subsets is shown below the plots. **(B)** TEC subsets (A-B-C-D) illustrated in (A) were purified by cell sorting and cultured inmicroscale clonogenic assay (96-well microplates) at designated cell densities (input: as represented on the top of the schematic plate). Representative images of full individual wells for each time point and TEC subset (right). 96-well plate cultures were fixed, stained with anti-cytokeratin 8 (K8) Ab and analyzed using a high-content immunofluorescence microscopy. n.d. (not determined). Data are representative of two experiments. **(C)** Colony-precursor cell frequency (%) was estimated by dividing the number of colonies obtained by the number of seeded TEC (input) x 100. Data are shown as mean ± SEM of 3 control and 4WT BM-reconstituted samples from a pool of two independent experiments.

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