Thymus medulla under construction: Time and space oddities

Alves NL1,2, Ribeiro AR1,2.

1 - Instituto de Investigação e Inovação em Saúde (I3S), Universidade do Porto, Portugal.
2 - Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Portugal.

*Correspondence: nalves@ibmc.up.pt (N.L. Alves).

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The development of effective T-cell-based immunotherapies to treat infection, cancer, and autoimmunity should incorporate the ground rules that control differentiation of T cells in the thymus. Within the thymus, thymic epithelial cells (TECs) provide microenvironments supportive of the generation and selection of T cells that are responsive to pathogen-derived antigens, and yet tolerant to self-determinants. Defects in TEC differentiation cause syndromes that range from immunodeficiency to autoimmunity, which makes the study of TECs of fundamental and clinical importance to comprehend how immunity and tolerance are balanced. Critical to tolerance induction are medullary thymic epithelial cells (mTECs), which purge autoreactive T cells, or redirect them to a regulatory T-cell lineage. In this issue of the European Journal of Immunology, studies by Baik et al. and Mayer et al. [Eur. J. Immunol. 2016. 46: 857-862 and 46: 846-856) document novel spatial–temporal singularities in the lineage specification and maintenance of mTECs. While Baik et al. define a developmental checkpoint during mTEC specification in the embryo, Mayer et al. reveal that the generation and maintenance of the adult mTEC compartment is temporally controlled in vivo. The two reports described new developmentally related, but temporally distinct principles that underlie the homeostasis of the thymic medulla across life.

Within the thymus, thymic epithelial cells (TECs) span along the outer cortex and inner medulla to form specialized niches capable of generating T cells, which are simultaneously reactive to pathogens and tolerant to one's own organs. To solve the conundrum imposed by the random assortment of αβ T-cell receptors (TCR), TECs select T cells with a broad range of reactivity against foreign antigens, while generally controlling the fate of self-reactive ones. Cortical TEC (cTEC) and medullary TEC (mTEC) sublineages constitute the two main stromal components of the preinvoluted thymus (reviewed in 1). While cTECs promote T-cell lineage commitment and positive selection, mTECs regulate the elimination of autoreactive T cells and the differentiation of regulatory T cells (reviewed in 2). The particular relevance of mTECs to tolerance induction is illustrated by studies in mice and humans showing a direct link between genetic defects in mTEC differentiation and the development of autoimmunity (reviewed in 1). Intrinsic to the role of mTECs is their capacity to express tissue-restricted antigens (TRAs), a process that depends in part on autoimmune regulator (Aire) and the recently described Fezf2 3, 4. These two transcription factors control the expression of highly diverse and complementary TRAs in mTECs 3, 4, so that the coverage of virtually all self-antigens is organized in random patterns of gene expression in just a few hundred mTECs 3, 5, 6. This seemingly stochastic process secures the repeated representation of the entire genome to developing T cells within the thymic medulla. In this regard, understanding the foundation of the mTEC microenvironment is important to comprehend how the thymus establishes the limits of tolerance to peripheral tissues.

The identification of bipotent TEC progenitors (TEPs) in both the embryonic 7 and postnatal 8 thymus provided evidence that mTECs and cTECs share a common origin. The initial

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Rua Alfredo Allen, 208
4200-135 Porto
Portugal
+351 220 408 800
info@i3s.up.pt
www.i3s.up.pt

descriptions of mTEC precursors (mTEPs) 9-11 led to the notion that mTECs undergo a diversification route unrelated from cTECs. Nonetheless, the blueprint of TEC development became more complex with the observations that embryonic TEPs expressing cortical markers can generate both cTECs and mTECs 12-14. These findings support a refined model whereby progenitors transverse through the cortical lineage prior to commitment to mTEC differentiation in the embryonic thymus (reviewed in 15). Still, we lack critical information on the nature of TEPs as well as on their functional contribution to the maintenance of thymic epithelial niches across life. Another area of uncertainty deals with the molecular networks that underlie the precursor–product relationship between TEPs, lineage-restricted precursors, and mature TEC subsets. Research in TEC progenitors has been under intense scrutiny in the past years, regularly providing new advances to our understanding of thymic biology. In this issue, reports by Baik et al. 16 and Mayer et al. 17 reveal novel spatial–temporal peculiarities in the generation of mTEC lineages that sprout in the fetal and postnatal life, respectively. These discoveries extend our knowledge on the program that regulates mTEC differentiation.

The establishment of the murine mTEC compartment starts during early embryogenesis 15. Following the initial discovery of embryonic claudin-3^{+} and claudin-4^{+} (Cld3,4) mTEPs, which give rise to Aire^{+} mTECs 11, subsequent studies show that these mTEPs are able to restore life-long tolerance induction in defective mTEC microenvironments caused by a dysfunctional mutation in NF-κB-inducing kinase 18. Additionally, a further degree of heterogeneity has been resolved within Cld3,4^{+} TECs with the description of long-lived mTEPs typified by SSEA-1 expression 18. These findings support the notion that mTEPs sustain the breadth and function of the medullary epithelium for the duration of life 18. Importantly, mTEC differentiation depends on crosstalk with developing thymocytes 2. Past studies elucidated the chief role of members of TNF receptor superfamily receptor activator of NF-κB (RANK), lymphotoxin β receptor and CD40 in the establishment of mature mTECs 1. Nonetheless, the determinants that control the responsiveness of mTECs and their precursors to these key inductors of the mTEC lineage program remain poorly understood.

Given the role of RANK in mTEC differentiation, it is important to understand the relationship between RANK expression and mTEC lineage specification. Using reporter mice in which the RANK promoter controls Venus fluorescent protein expression 19, Baik et al. describe the first temporal functional analysis of RANK^{Venus}-expressing (RANK^{+}) mTEPs in the embryonic thymus 16. The authors start by surveying the ontogeny of RANK^{+} TECs relative to Cld3,4^{hi}SSEA1^{+} mTEPs in embryonic day (E) 13–15 thymus. While Cld3,4^{hi}SSEA1^{+} TECs exist in the E13 thymus, RANK^{+} TECs emerge one day later within the Cld3,4^{hi}SSEA1^{-} subset and become prominently detected in the E15 thymus. Interestingly, RANK^{+} TECs express higher levels of MHC class II (MHCII) and lower levels of CD205 than Cld3,4^{hi}SSEA1^{+} mTEPs 16. To address the lineage potential of RANK^{+}TECs, Baik et al. established reaggregate thymic organ cultures (RTOCs) in which MHCII-mismatched E15 Cld3,4^{hi}RANK^{+} TECs were mixed with E15 WT thymus and their progeny was traced on the basis of distinct MHCII. The authors show that in chimeric RTOCs, E15 Cld3,4^{hi}RANK^{+} cells preferentially generate MHCII^{hi}Ly51^{-}CD80$^{-/+}$TECs, indicating that RANK^{+} TECs contain mTEC unipotent progenitors 16. Although future experiments are required to map the expression of additional cTEC and mTEC markers in RANK^{+} TECs, these results uncover a novel degree of heterogeneity in early steps of the mTEC differentiation program. Additionally, the study by Baik et al. provides genetic evidence for the role of chief regulators of TEC specification, such as Foxn1 and Relb 1, in the differentiation of RANK^{+} mTEPs. The authors show that Foxn1 and Relb are differentially required for the development of Cld3,4^{hi}SSEA1^{+} and Cld3,4^{hi}RANK^{+} TEC subtypes 16. While unaltered in Relb-deficient mice, the abundance of Cld3,4^{hi}SSEA1^{+} cells was reduced in Nude mice. These observations indicate that the pool, but not the ontogeny, of primitive mTEPs is dependent on Foxn1. In this regard, Cld3,4^{+} TECs have been previously reported in wild-type and Nude E11-12 thymic anlagen 11, 20. Although these findings might indicate that mTEC commitment and maturation are independently regulated, it is challenging to demonstrate how Cld3,4^{hi}SSEA1^{+} cells derived from Nude mice relate to their functionally identified Foxn1^{+} counterparts 18. On the other hand, the ontogeny of RANK^{+} mTECs is dependent on Relb. These results implicate the non-canonical nuclear factor kappa B (NF-κB) pathway 1 as an important checkpoint in the regulation of RANK expression in mTEPs. Along these lines, previous studies demonstrate that RANK expression in embryonic TECs is controlled by activation of lymphotoxin beta receptor, a known inducer of NF-kB 21. Further experiments are required to map the spatial location of RANK^{+} and

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SSEA1$^+$ TECs in situ and define the identity of upstream receptors controlling the expression of RANK in vivo. Although RANK$^+$mTEPs emerge temporally downstream of SSEA-1$^+$ mTEPs, the direct lineage relationship between these two subsets remains undetermined (Fig. 1). An alternative, and perhaps more speculative, scenario is that the segregation of Cld3,4^{hi}SSEA1$^+$ and Cld3,4^{hi}RANK$^+$ cells marks the initiation of alternative routes of embryonic mTEC differentiation.

The establishment of the medullary epithelial niche is a dynamic process that extends beyond embryonic life, so that the prototypical corticalmedullary compartmentalization is only achieved in the adult thymus. The observation that mTECs, in particular the Aire$^+$ subset, turn over at a rate of 7–10 days 22, 23implicates a requirement for regular replacement by their (single or multiple) upstream progenitors. How mTEC niches are maintained across life has remained enigmatic until recently. One possibility is that the adult mTEC niche results from the expansion of embryonic-derived mTEPs. Cld3,4$^+$SSEA1$^+$ cells are rare in the adult thymus, indicating that the pool of mTEPs is exhausted throughout life. In this respect, the recent identification of podoplanin$^+$ (PDPN) TECs, which reside at the corticomedullary junction (PDPN$^+$jTEC) and harbor the potential to generate nearly half of adult mTECs 24, has shed further light in the mTEC enigma. PDPN$^+$jTECs might represent one of the downstream, transiently amplifying subsets that contribute to maintain the adult medullary network (commented on 25). Future studies on the ontogeny of PDPN$^+$jTECs as well as their spatial-lineage relationship to Cld3,4$^+$SSEA1$^+$ and Cld3,4$^+$RANK$^+$mTEPs are warranted (Fig. 1). A second possibility is that alternative temporal-restricted pathways might partake in the homeostasis of the adult medullary epithelial niche. This scenario implicates the new generation of mTEPs from bipotent progenitors. Recent reports have shown that distinct types of bipotent TEPs can be isolated from the adult murine thymus 26, 27, which in line with earlier reports 8, indicate that they persist in the postnatal life. Previous studies from Ohigashi et al. have demonstrated that the majority of adult mTECs descend from TEPs expressing beta5t (β5t$^+$), a cTEC-restricted marker 12. As β5t is expressed in fetal TEPs 12, it is unclear whether the bipotent capacity is confined to embryonic progenitors, or a similar process is maintained in postnatal life. Nonetheless, the location and physiological contribution of TEPs to the maintenance and regeneration of the medulla remain unknown.

Now, Mayer et al. 17 and Ohigashi et al. 28 provide novel, and complementary, evidence that a large fraction of adult mTECs develop from fetal- and newborn-derived β5t$^+$ TEPs under physiological conditions. The groups of Holländer and Takahama engineered a new mouse line that expresses the reverse tetracycline transactivator (rtTA) under the control of Psmb11 (β5t) locus. β5t-rtTA knock-in mice were then crossed to transgenic mice which express the Cre recombinase under the control of doxycycline (Dox) and activate ZsGreen 17 or eGFP 28reporter expression only after Cre-mediated recombination. Using this genetic inducible cell-fate mapping strategy, both studies follow the progeny of β5t$^+$ TEPs during adult life. The sensitivity of the new model was confirmed by performing prolonged Dox treatments, which span from early embryogenesis until birth or adulthood. In line with past observations 12, extended Dox treatment showed that β5t$^+$ progenitors generate the majorly of cTECs and mTECs in the young adult thymus. Interestingly, using a series of temporally restricted Dox regimens, both groups documented a differential involvement of β5t$^+$ TEPs of the embryonic, postnatal and adult thymus for the establishment of young adult mTECs. While Dox administration in the embryo labelled circa 70–80% of mTEC, the labelling efficiency declined to circa 20% when Dox was provided between birth and one week of age, and became marginal once Dox-treatment was administrated from 1 week onwards 17, 28. These results indicate that the contribution of β5t$^+$ TEPs to the adult mTEC niche decreases with age. Worth noting, the labelling efficiency of cTECs also decreased, although to a lesser extent than mTECs, following Dox-treatment in young adult mice 17, 28. A reduction in β5t$^+$ transcription in cTECs with age 28 might explain the discrepancy between the broad expression of β5t protein 12 and the limited labelling penetrance in adult cTECs. One cannot formally exclude the possibility that other (non-labelled) cell lineage(s) residing within the cortex contribute to the mTEC network. Nonetheless, both studies provide evidence that the majority of young adult mTECs arise from progenitors that express β5t during embryonic development, up to the first week of life (Fig. 1). Moreover, long-term analysis of embryonically and neonatally β5t-derived mTECs indicates that the maintenance of the adult medullary epithelium is likely assured by mTEPs which develop downstream of β5t$^+$ TEPs. In line with this view, Ohigashi and colleagues show that embryonic and postnatal β5t$^+$ progenitors give rise to Cld3,4^{hi}SSEA-1$^+$mTEPs 28. On the other hand, not all mTECs of the aged thymus have a β5t-positive past. Despite the particularities

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of different reporter lines, several studies have described phenotypic heterogeneity within mTECs 4, 5, 19, 29. Future studies are needed to address whether these examples point to the existence of alternative lineages of medullary differentiation. Further experiments by Ohigashi et al. 28 indicate that embryonically/neonatally β5t-derived mTEPs are major participants in models of adult thymic medullary regeneration trigged by sub-lethal radiation or polyinosinic-polycytidylic acid (poly I:C) treatments. It is important to consider that complete mTEC depletion was, in this case, not achieved and thus intrathymic competition with resistant mTEPs might hinder the contribution of adult β5t progenitors in thymic regenerative responses 28. Whatever the case may be, both studies imply the existence of distinct mechanisms controlling embryonic mTEC specification and postnatal mTEC maintenance. A second important finding is that β5t$^+$ progenitors preserved their bipotent capacity in the early postnatal period, indicating that TEPs might nestle in the cortical areas of the adult thymus. Using RTOC and a creative model which permits in vivo analysis of β5t$^+$ progeny at the clonal level, Mayer et al. present additional insights on the clonal progeny of β5t$^+$-derived mTEPs 17. The authors show that β5t$^+$-derived TECs integrate in both the cortical and medullary region and form distinct clusters of clonal origin at the corticomedullary area, which presumably mark transit-amplifying mTEPs. Supportive of this view, postnatal β5t$^+$ progenitors are able to generate PDPN$^+$jTECs, inferring that the de novo formation of mTECs after birth contributes to the expansion of the medulla 24. Given their particular spatial location, one can speculate that postnatal β5t$^+$-derived mTECs bridge discrete clonally derived medullary islets generated during embryonic period, providing the basis for the development of larger medullary areas of the adult thymus 9. Although embryonically and neonatally β5t-derived mTECs express similar levels of genes coupled to the TNF receptor superfamily pathway, they differ in the expression of TNF receptor-associated factor 3 (TRAF3) and in some TRAs 28. Future experiments must address whether postnatal-derived mTECs developed by the reiteration of the same differentiation pathways described to the fetal thymus 1. Collectively, both reports highlight a critical, and yet restricted spatiotemporal contribution of β5t$^+$ progenitors to the mTEC differentiation (Fig. 1), supporting the notion that the adult mTEC compartment is (in part) maintained by unipotent mTEP(s).

Taken together with other recent discoveries 24, 26-29, the three new studies spark further avenues of investigation that need to be translated into experimental approaches to underpin, or argue against, current models of TEC differentiation 15, 30. The plot thickens. One must consider that even with the most refined subsets, distinct TEC precursors are defined at the population level, but cannot be yet recognized as single cell. A major challenge is to define the nature and abundance of bipotent TEPs in the adult thymus. Furthermore, the physiologic contribution of all recently discovered mTEPs to the medullary compartment remains to be addressed. As a corollary, it will be of clinical relevance that researchers start applying the knowledge acquired in the last decade to potentially modulate TEC function to treat a broad range of thymic disorders, such as immunodeficiency and autoimmune disease.

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

ABBREVIATIONS

Aire: autoimmune regulator · Cld: claudin · cTECs: cortical TECs · mTECs: medullary TECs · PDPN+j: podoplanin+ TECs at the corticomedullary junction · RANK: receptor activator of NF-κB · TECs: thymic epithelial cells · TEPs: thymic epithelial progenitors · TRAs: tissue-restricted antigens

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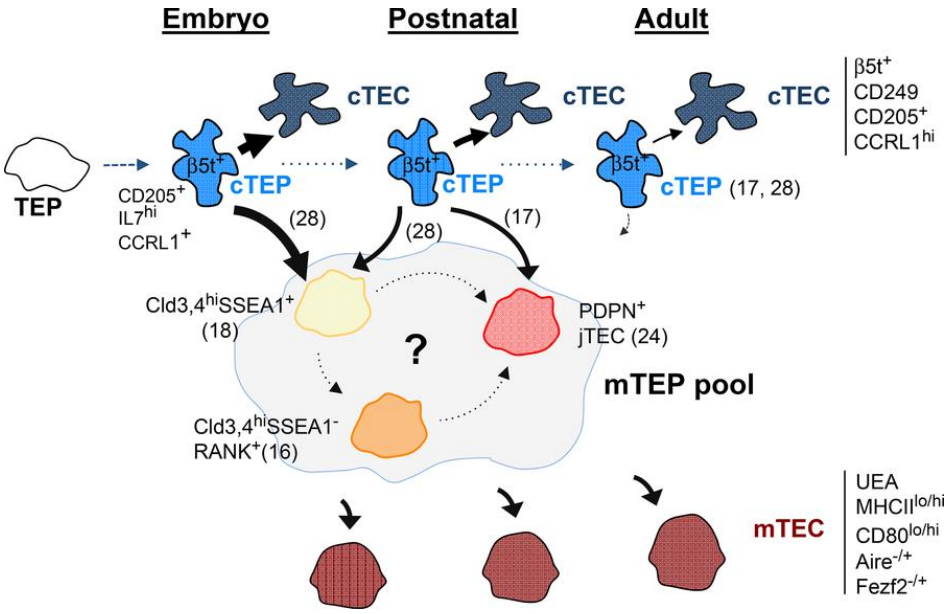

Figure 1 - Spatial–temporal principles that underlie the development and maintenance of mTEC niches. This figure is based on the reports of Baik et al. 16, Mayer et al. 17, Ohigashi et al. 28, and other recent reports 18, 24. Embryonic cTEC-like progenitors (cTEP) progress through cortical lineages and contribute to the development of cTECs and mTECs. The contribution of β5t+ progenitors to the generation and maintenance of cTECs and mTECs is a developmental process that fades as life progresses (top). Three novel subsets of mTEC-restricted progenitors (mTEP) have been recently defined and represent the mTEP pool. Although β5t+ progenitors are able to generate Cld3,4^{hi}SSEA1+ 28 and PDPN+jTECmTEPs 17, the direct precursors of Cld3,4^{hi}SSEA1−RANK+ cells remain unknown. Also, it is an open question whether the new mTEP subsets represent distinct mTEC lineages or various stages of a single differentiation program. Note: For simplicity other references used in the manuscript were excluded from the figure.

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