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Cutting Edge: A Thymocyte-Thymic Epithelial Cell Cross-Talk Dynamically Regulates Intrathymic IL-7 Expression In Vivo

Nuno L. Alves,*†‡ Nicholas D. Huntington,*†‡ Jean-Jacques Mention,*† Odile Richard-Le Goff,*† and James P. Di Santo*†

Thymic epithelial cells (TECs) are the predominant intrathymic source of the essential thymopoietin IL-7. Whether thymocyte-TEC interactions have a role in the regulation of IL-7 expression is not known. By exploiting IL-7 reporter mice in which yellow fluorescent protein expression identifies TECs expressing high levels of IL-7 (Il7+ TECs), we show that Il7+ TECs segregate from emerging medullary TECs during thymic organogenesis. Although Il7+ TECs normally diminish with age, we found that Il7+ TECs are markedly retained in alymphoid Rag2−/−Il2rg−/− IL-7 reporter mice that manifest a profound thymopoietic arrest. Transfer of Tcrα−/− or wild-type (but not Rag2−/−) hematopoietic progenitors to alymphoid IL-7 reporter recipients normalizes the frequency of Il7+ TECs and re-establishes cortical TEC/medullary TEC segregation. Although thymocyte-derived signals are often considered stimulatory for TEC maturation, our findings identify a negative feedback mechanism in which signals derived from TCRβ-selected thymocytes modulate TEC-dependent IL-7 expression.


Thymocyte differentiation involves a complex integration of exogenous signals provided by a three-dimensional thymic stromal cell network. Thymic epithelial cells (TECs) represent a predominant stromal cell component and have a paramount role in T cell development and selection by providing essential thymopoietic signals, including the Notch ligand D-like 4 (Dll4), IL-7, and self-peptide–MHC complexes (1). The thymic epithelium is classically divided into two specialized and spatially distinct subsets, cortical TECs (cTECs) and medullary TECs (mTECs), that manifest different functional properties (2). The generation of a functionally competent and diversified TEC compartment is a prerequisite for normal thymopoiesis. Importantly, functional maturation of TECs depends on instructive signals provided by thymocytes, thus defining the symbiotic bidirectional thymic cross-talk (1, 2). Nevertheless, the molecular events underlying the functional segregation of distinct TEC subsets are not fully elucidated.

Although compelling evidence points to IL-7 as a master regulator of thymopoiesis in man and mice, we know surprisingly little about the mechanisms that control IL-7 expression in vivo. One possibility is that IL-7 is expressed at constant and low levels in normal conditions such that the number of IL-7-consuming cells in vivo would ultimately determine IL-7 bioavailability. Alternatively, IL-7 expression might be dynamically regulated and could thereby dictate lymphoid homeostasis (3, 4). Although these different models have been considered in the context of peripheral T cell homeostasis, they may also apply to the regulation of thymocyte homeostasis. Our understanding of the mechanism that controls IL-7 expression remains speculative, in part due to the paucity of experimental models that can monitor IL-7–expressing cells.

We have recently provided a temporal-spatial analysis of IL-7–expressing TECs in vivo using bacterial artificial chromosome transgenic reporter mice in which IL-7 promoter elements control yellow fluorescent protein (YFP) expression [B6.Cg-Tg(Il7-EYFP)5Pas mice] (5). YFP+ (referred hereafter as Il7+) TECs expressed abundant Il7 transcripts, localized to the corticomedullary junction in the adult thymus and their frequency declined during postnatal life (5). Based on these findings, we hypothesized that thymocyte-TEC interactions might regulate TEC production of thymopoietic factors (including Dll4 and IL-7) that are required for early T cell development while promoting functional TEC diversification (6). In this regard, it has been recently shown that thymocyte-specific signals downregulate Dll4 expression in cTECs (7). In this study, we exploit IL-7 reporter mice to dissect the intrathymic regulation of IL-7 expression. Our results indicate that thymocyte-derived...
signals curb IL-7 expression by TECs while promoting their functional maturation.

**Materials and Methods**

**Mice**

IL-7 reporter mice (5) were backcrossed to the \( \text{Rag}^{2-/-}\text{Il2rg}^{2-/-} \) background (8). Mice were housed under specific pathogen-free conditions and experiments performed in accordance with institutional guidelines. For fetal studies, embryonic day (E) 0.5 was the day of vaginal plug detection.

**Bone marrow chimeras**

A total of \( 10^7 \) bone marrow (BM) cells from 6-wk-old wild-type (WT), \( \text{Tcra}^{2-/-} \) (9), and \( \text{Rag}^{2-/-}\text{C57BL/6} \) donors were injected i.v. in 4-wk-old sublethally irradiated (0.4 Gy) \( \text{Rag}^{2-/-}\text{Il2rg}^{2-/-} \) IL-7 reporter mice (8).

**Isolation and flow cytometric analysis of TECs**

TECs were isolated as described (5). Cell suspensions were stained with anti-CD4, anti-CD80, anti–I-A/I-E, anti-Ly51 (PE), anti-CD8, anti-CD80 (allophycocyanin), anti-CD45.2 (PerCPCy5.5), anti-CD205, and anti-Ly51 (biotin) Abs, and streptavidin (PE-Cy7) (BD Biosciences, San Jose, CA); anti–I-A/I-E (allophycocyanin-Cy7) and anti-EpCAM (allophycocyanin) Abs (eBioscience, San Diego, CA).

**Immunohistological analysis**

Thymi were prepared as described (5). Briefly, samples were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), and 8-µm sections were stained with anti-GFP, Alexa 488–anti-rabbit, Alexa 555–anti-rat Abs, Alexa 647- or Alexa 555-streptavidins (Invitrogen, Carlsbad, CA), and rhodamine-UEA 1 (Vector Laboratories, Burlingame, CA). CDR-1 and MTS-10 were kindly provided by Dr. R. Boyd, Monash Immunology and Stem Cell Laboratories, Monash University, Australia (5). Counterstaining and image analysis were as described (5).

**Results and Discussion**

**Ontogeny of thymic Il7+ TECs**

We first analyzed the developmental origins of \( \text{Il7}^{+} \) TECs during thymic organogenesis. TEC ontogeny commences during embryonic life between day 9.5 and 11.5 of murine gestation (E9.5–E11.5), with endodermal outbudding of the third and the fourth pharyngeal pouches and subsequent formation of the thymus anlagen at E12.5 (10). To characterize the emergence of intrathymic \( \text{Il7}^{+} \) TECs, we performed a combinatory phenotypic-temporal analysis using cTEC and mTEC markers CD205 and CD80, respectively (10). The colonization of the thymic anlagen by hematopoietic cells (CD45+) was already visible by E12.5, and hematopoietic representation increased steadily thereafter, such that CD45+ cells comprised the majority of cells at later stages of gestation and in the neonatal thymus (Fig. 1). At E12, YFP expression was not yet detected within the anatomical region, including the third and fourth pharyngeal pouches (Supplemental Fig. 1). Analysis of TEC dynamics showed that YFP expression was first detected around E12.5, suggesting that IL-7 expression in TECs starts between E12 and E12.5 of gestation (Fig. 1B). Interestingly, the ratio between YFP+ and YFP− TECs declined concomitantly with increased gestational age and the increased number of thymocytes (Fig. 1B). This observation suggested the existence of a putative thymocyte-induced negative feedback mechanism on IL-7–expressing TECs. Moreover, YFP expression preceded that of MHC class II (MHC-II) and coincided with the initiation of expression of CD205 (Fig. 1Bii, Bii). From E14.5 onwards, YFP+ and YFP− TECs expressed MHC-II at the same level, and \( \text{Il7}^{+} \) TECs continuously segregated from CD80+ mTECs that were first detected at E17.5 (Fig. 1Bii). These results suggest that the initiation of IL-7 expression is a primitive molecular event associated with early stages of TEC specification.

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**FIGURE 1.** \( \text{Il7}^{+} \) TECs comprise a primitive thymic epithelium subset that emerges during early thymic organogenesis. Fetal and neonatal thymus from IL-7 reporter mice were analyzed. A, The frequency of CD45+ cells augments during organogenesis. Bi, The frequency of \( \text{Il7}^{+} \) TECs (CD45− gate) declines throughout organogenesis. Bii, \( \text{Il7}^{+} \) TECs segregate from CD80+ mTECs. Comparative phenotypic analysis of color-coded YFP+ (black dot plot) and YFP− (gray contour plot) TECs in relation to the expression of CD205 and CD80. A and B, Numbers indicate the percentage of gated cells. B, Italic numbers correspond to the ratio between YFP+ and YFP− TECs. Data are representative of two to three experiments/time point.
The results above indicated that thymic IL-7 expression might be regulated by TEC-extrinsic cellular signals, perhaps delivered by developing thymocytes. If true, ablation of thymocyte differentiation might result in sustained IL-7 expression by TECs. We therefore crossed IL-7 reporter mice onto the severely lymphopenic Rag2⁻/⁻ Il2rg⁻/⁻ background (8). As expected, the number of CD45⁺ hematopoietic cells was 1000-fold reduced in Rag2⁻/⁻ Il2rg⁻/⁻ IL-7 reporter mice compared with age-matched immune-competent counterparts (Supplemental Fig. 2A). Strikingly, the proportion of Il7⁺ TECs was markedly increased in lymphopenic mice compared with immune-competent mice, with most TECs expressing YFP (Fig. 2A, 2B).

Thymic epithelial architecture is altered in lymphopenic conditions as result of lack of instructive maturation signals derived from developing thymocytes (11, 12). Under these conditions, TECs retain an immature cortical-like phenotype and do not segregate into mature cTECs and mTECs (11–13). Our analyses of thymi from Rag2⁻/⁻ mice revealed a predominance of cortical-like TECs (CD205⁺, Ly51⁺, and CDR-1⁺) with a virtual absence of CD80⁺, UEA⁺, and MTS10⁻ mTECs. Il7⁺ TECs displayed a scattered distribution throughout the thymus, with the majority expressing cortical (CD205⁺, Ly51⁺, and CDR-1⁺) traits (Fig. 2A, 2C; Supplemental Fig. 2B, 2C). In contrast, Il7⁺ TECs localized predominantly to the corticomedullary region in the immunocompetent thymus (Fig. 2C; Supplemental Fig. 2) (5). These results indicate the existence of an intrathymic negative feedback mechanism involving thymocyte-derived signals that diminish IL-7 expression by TECs.

**Thymocyte-derived signals negatively impact on TEC-driven IL-7 expression**

We next assessed the influence of thymocyte subsets on TEC IL-7 expression by reconstituting Rag2⁻/⁻ Il2rg⁻/⁻ IL-7 reporter mice with BM precursors from Rag2⁻/⁻, Tcrα⁻/⁻, or from WT mice. The thymi of recipient mice were analyzed 4 wk later. As expected, the number of CD45⁺ cells was progressively increased in the thymus of Rag2⁻/⁻ Il2rg⁻/⁻ IL-7 reporter mice reconstituted with Rag2⁻/⁻ or Tcrα⁻/⁻ and WT BM precursors, the latter restoring normal T cell development (Supplemental Fig. 3A). The conditioning irradiation protocol did not appear to alter the percentage of Il7⁺ TECs because their frequency was unchanged in nonirradiated and nonreconstituted mice (Supplemental Fig. 3B). Strikingly, although the ratio of Il7⁺ TECs compared with YFP⁺ TECs remained largely unaffected in thymi reconstituted with Rag2⁻/⁻ BM progenitors, the ratio of YFP⁺/YFP⁻ TEC subsets was markedly diminished upon reconstitution with either Tcrα⁻/⁻ or WT BM precursors and coincided with the emergence of CD80⁺ mTECs (Fig. 3A, 3B).

Although cTEC differentiation is suggested to be dependent on signals derived from early T cell progenitors, proper mTEC maturation depends on signals provided by positively selected and mature thymocytes (10, 14, 15). Accordingly, mTEC differentiation process was stalled in nonreconstituted mice (Fig. 3A, middle panels). However, the seeding of the thymus of Rag2⁻/⁻ Il2rg⁻/⁻ IL-7 reporter mice by Tcrα⁻/⁻ or WT (but not Rag2⁻/⁻) T cell precursors and the ensuing

**FIGURE 2.** The frequency of Il7⁺ TECs is augmented under severe lymphopenia. A, The expression of YFP is compared in TECs (CD45⁺ MHC-II⁺) of Rag2⁻/⁻ Il2rg⁻/⁻ control (WT), Rag2⁻/⁻ Il2rg⁻/⁻ (Tg), and immunocompetent (Tg) IL-7 reporter mice (top panels). The expression of CD205 and CD80 is shown in total TEC gate (middle panel) and colored gated for Il7⁺ (green) and total YFP⁻ TECs (gray) (bottom panel). Numbers represent the same as in Fig. 1. B, Frequency (i) and ratio (ii) of Il7⁺ and YFP⁺ TECs in immunocompetent and Rag2⁻/⁻ Il2rg⁻/⁻ IL-7 reporter mice at 4–6 wk of age. Data represent average of more than six experiments (n = 10). C, Immunohistochemical analysis (5) of Rag2⁻/⁻ Il2rg⁻/⁻ and immunocompetent IL-7 reporter thymi (upper panels, original magnification ×100; lower panels, original magnification ×200).
thymocyte differentiation up to or beyond DP stage, respectively (Supplemental Fig. 3A), allowed CD80+ mTECs to emerge (Fig. 3A, middle panels). Interestingly, Il7+ TECs retained a cortical-like phenotype (CD205+) under all conditions and clearly segregated from the emerging CD80+ mTEC subset (Fig. 3A, bottom panels), recapitulating the phenotype observed in immunocompetent thymus. These results suggest that signals provided by DP thymocytes are sufficient to decrease IL-7 expression by TECs. cTEC and mTEC progenitors can be discriminated by the expression of CD205 and claudin-3 and -4, respectively, at early phases of thymic organogenesis (16, 17). Although Il7+ TECs are predominantly CD205 low/+ (Figs. 1–3), it is not known whether mTECs (CD80+YFP+2) may derive from a putative precursor included in Il7+ TECs (CD205+YFP+) or from an alternatively (CD205+YFP+2) TEC progenitor.

Collectively, our findings indicate that Il7+ TECs comprise a primitive TEC population and that signals delivered by thymocytes beyond the stage of TCRβ selection can negatively influence IL-7 expression by TECs. Although thymocyte-derived signals are often considered stimulatory for proper TEC differentiation (1, 2, 14, 15), recent evidence suggests that developing thymocytes may also negatively impact functional properties of TECs, includingDll4 (7) and IL-7 expression (described in this study), required to foster early stages of thymopoiesis. Whether Dll4 and IL-7 expression are coordinately regulated in TECs is not known. Nevertheless, this notion may be taken in consideration for future clinical applications aimed at boosting T lymphopoiesis. Inadequate T cell reconstitution has been shown in adult patients suffering from conditioned lymphocyte depletion (18). One may envisage that in this scenario, despite the transitory lymphopenia, TECs had previously become silenced for thymopoietic factors required at early stages of thymopoiesis (e.g., IL-7). This functional insufficiency could be due to preceding and continuous thymocyte-derived signals. As such, further studies are warranted to elucidate the molecular events involved in the initiation of IL-7 expression as well as to identify the molecular players responsible for the downregulation of IL-7 expression by TEC, which can include the concerted action of multiple thymocyte-derived cell surface or soluble factors. Understanding the mechanisms that allow the functional rejuvenation of TECs may open the door to new strategies that can enhance thymus reconstitution and functioning in clinical settings in which more robust T cell responses are required, including the induced (e.g., post-BM

**FIGURE 3.** Thymocyte-derived signals negatively impinge on IL-7 expression by TECs. Both Rag2−/− Il2rg−/− IL-7 reporter mice (Tg) and control (WT) mice were reconstituted with Rag2−/−, Tcra−/−, and WT BM precursors or were left nonreconstituted. A, The expression of YFP in TECs is compared in nonreconstituted and reconstituted thymi (top panels). WT Rag2−/− Il2rg−/− (± BM) mice were used to set the YFP+ gate (data not shown). Plots in middle and bottom panels represent the same as in Fig. 2A: YFP+ (black dot plot) and YFP− (gray contour plot) TECs. Numbers represent the same as in Fig. 1. B, Frequency of CD80+ TECs (squared), Il7+ (gray), and total YFP− (black) TECs isolated/thymus from unreconstituted and reconstituted thymi (i). Ratios between Il7+ and YFP− (left panel) and between total CD80+ and Il7+ TECs (right panel) are represented (ii). Data represent average of four to five independent experiments (n = 4–6 mice/group).
transplantation settings) or acquired (e.g., HIV infection) immunodeficiencies.

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Disclosures
The authors have no financial conflicts of interest.

References

Supplemental figure legends:

Supplemental figure 1 - Il7+TECs emerge beyond E12. Flow cytometric analysis of E12 and E13.5 stages of fetal thymi from IL-7 reporter mice following stromal isolation. (i) Augment in the frequency of CD45+ cells. (ii) Analysis was performed on thymic stromal cells (CD45- EpCAM+ gate). Il7+TECs emerge possibly upon E12. Comparative phenotypic analysis of Il7+(color-coded blue) and YFP+ (color-coded red) TECs in relation to the expression of CD205 and Ly51.

Supplemental figure 2 – A) Numbers of CD45+ cells isolated from immuno-competent and Rag2-/-Il2rg-/ IL-7 reporter mice at 4-6 wks of age. B) Immunohistochemical analysis of Rag2-/-Il2rg-/ non-transgenic littermate (as in Fig. 2 C). (C) Immunohistochemical analysis of Rag2-/-Il2rg-/ (Tg) and immuno-competent (Tg) IL-7 reporter mice thymi stained with the indicated Ab combinations.

Supplemental figure 3 – A) i) Numbers of CD45+ cells isolated from Rag2-/-Il2rg-/ IL-7 reporter transgenic mice at 4 wks post-reconstitution with Rag2-/-, Tcra-/-, and WT precursors. ii) Representative flow cytometric analysis of T cell development in lymphopenic (Rag2-/-Il2rg-/ ) IL-7 reporter mice 4 wks post-reconstitution with Tcra-/- and WT BM precursors. B) i) Flow cytometric analysis of Rag2-/-Il2rg-/ IL-7 reporter transgenic mice non-irradiated and analyzed at 8 wks of age (control). ii) Comparison of the frequency of Il7+ and YFP- TECs in 8 wks old control (n=3) and Rag2-/-Il2rg-/ IL-7 reporter mice irradiated at 4 wks and left unreconstituted for 4 wks (conditioned) (n=12, same as unreconstituted mice figure 3).