Early T-cell progenitors are the major granulocyte precursors in the adult mouse thymus

Maria Elena De Obaldia, J. Jeremiah Bell, Avinash Bhandoola

Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, 3620 Hamilton Walk, Philadelphia, PA 19104

To whom correspondence should be addressed: Avinash Bhandoola, 266 John Morgan Building, 3620 Hamilton Walk, Philadelphia, PA 19104, bhandooa@mail.med.upenn.edu; phone: (215) 573-0274; fax: (215) 573-2350.

Running head: Thymic granulocytes share origin with T-cells

Keywords: Hematopoiesis, T-cell development, thymic granulocytes, thymic dendritic cells
Abstract

The mouse thymus supports T-cell development, but also contains non-T-cell lineages such as dendritic cells (DC), macrophages, and granulocytes that are necessary for T-cell repertoire selection and apoptotic thymocyte clearance. Early thymic progenitors (ETP) are not committed to the T-cell lineage as demonstrated by both in vitro and in vivo assays. Whether ETP realize non-T-cell lineage potentials in vivo is not well understood and indeed controversial. Here, we examine whether ETP are the major precursors of any non-T-lineage cells in the thymus. We analyzed development of these populations under experimental circumstances in which ETP are nearly absent, either due to abrogated thymic settling, or due to inhibition of early thymic development by genetic ablation of IL-7Rα or Hes1. Results obtained using multiple in vivo approaches indicate that the majority of thymic granulocytes derive from ETP. These data indicate that myelo-lymphoid progenitors settle the thymus, and thus clarify the pathways by which stem cells give rise to downstream blood cell lineages.


**Introduction**

Since the identification of early T-lineage precursors (ETP)\(^1\), much work has characterized the developmental potentials possessed by this population.\(^2-13\) In addition to robust T-cell developmental potential, ETP have been shown to possess B-cell, dendritic cell (DC), natural killer (NK) cell potential, and a degree of myeloid potential. As ETP progress along the T-cell developmental pathway, they gradually lose non-T-cell potentials and generate CD4/CD8-double negative 2 (DN2) progenitors and finally DN3 cells that are committed to the T-cell lineage.

Single cell assays using a stromal cell culture system have shown that the majority of individual ETP can give rise to both T- and myeloid cells, including granulocytes and macrophages.\(^10,11\) However, less is known about the extent to which ETP realize this myeloid potential and other non-T-cell lineage potentials *in vivo*. We previously reported that about half of ETP and a similar fraction of thymic granulocytes were labeled in H2-VEX V(D)J recombination reporter mice.\(^10\) These data are consistent with the notion that thymic granulocytes share a common origin with T-cells. Since then, another study examining thymic myeloid cells using an IL-7R/Cre lineage tracing approach yielded discordant results.\(^12\) Thus, further examination was needed to determine whether ETP can produce granulocytes *in vivo*, and whether ETP are the major precursors of thymic granulocytes. An understanding of whether ETP generate both myeloid and T-cell progeny *in vivo* will contribute to a more complete model of hematopoietic development.

We examined thymic granulocyte development in experimental contexts where ETP are nearly absent. We reasoned that non-T-lineage cells in the thymus that are unperturbed in the absence of ETP in mixed-bone marrow (BM) chimeras must not predominantly derive from ETP. Previous studies used a similar approach to investigate whether non-T-lineage cells in the thymus originate from T-cell progenitors; yet, these studies did not examine thymic granulocytes.\(^14,15\) Studies using models that exclusively block intrathymic ETP development (for example, by ablating Notch signaling) may fail to detect a common
origin with T-cell progenitors. This is because progenitors continue to settle the thymus and may still generate non-T-lineage cells even when ETP development is abrogated. To address this concern, we chose to study development of non-T-lineage cells in the thymus by eliminating T-cell progenitors prior to thymic entry. Specifically, we have examined mixed chimeras using CCR7/CCR9 double-deficient donor BM, in which T-cell progenitors display defective thymic settling and hence generate almost no ETP. In addition, we have examined thymic granulocyte development when factors necessary for early thymic development, including IL-7Rα and the Notch target Hes1, are genetically ablated. Thus, we have undertaken multiple complementary approaches to investigate the origin of thymic granulocytes and other non-T-lineage cells in the thymus in order to account for possible confounding factors associated with a single approach.

Across several different in vivo experimental systems, we have consistently implicated ETP as the major precursors of thymic granulocytes. In all models we have analyzed, we found that thymic granulocytes have distinct developmental history and developmental requirements from their extrathymic counterparts. Thymic granulocytes, like ETP, but unlike other granulocytes, show a history of RAG-1 expression and depend on CCR7/CCR9, IL-7Rα, and Hes1 for their development. These data are compatible with the notion that ETP give rise to the bulk of thymic granulocytes in vivo. ETP may also contribute to other non-T-lineage cells in the thymus for which they have demonstrated potential, such as macrophages, DCs, and B-cells; however, these lineages derive predominantly from progenitors other than ETP. Hence, although ETP possess many non-T-cell lineage potentials, they are the major precursors for only a select subset of thymic non-T-cell lineages.
Methods

Mice
Female C57BL/6 (CD45.2⁺) and B6.Ly5.2 (CD45.1⁺) mice were purchased from the National Cancer Institute and were used at 5-8 weeks of age. RAG-1/Cre mice were obtained from Terry Rabbitts and bred to Rosa26-YFP reporter mice. CCR9⁻/⁻ mice were a gift of Dr Paul Love (NIH, Bethesda, MD). CCR7⁻/⁻CCR9⁻/⁻ mice were generated by inter-crossing single knockout mice. Mice with the Hes1-deficient allele were provided by Lori Raetzman (University of Illinois at Urbana-Champaign) with the permission of Ryoichiro Kageyama (Kyoto University Institute for Virus Research). Hes1⁻/⁻ mice were crossed with C57BL/6 mice. CCR7⁻/⁻, IL-7Rα⁻/⁻, and C/EBPαF/F mice were obtained from Jackson Laboratories. All animal experiments were done according to protocols approved by the Office of Regulatory Affairs of the Perelman School of Medicine at the University of Pennsylvania (Philadelphia, Pennsylvania) in accordance with guidelines set forth by the National Institutes of Health.

Cell preparations, flow cytometry, and cell sorting
Bone marrow cells were obtained from mouse femurs and red blood cells were lysed. Fetal liver cells were obtained from day 16.5 embryos and red blood cells were lysed. Prior to sorting of ETP, thymocyte cell suspensions were depleted using anti-CD4 (GK1.5), anti CD8α (53.6-7), and magnetic beads conjugated to goat anti-rat IgG. In most experiments, the lineage antibody cocktail (Lin) used included anti-B220 (RA3-6B3) and anti-CD19 for exclusion of B-cells; anti-CD11b (M1/70), anti-Gr-1 (8C5) for exclusion of myeloid cells; anti-CD11c for exclusion of dendritic cells; anti-Ter119 for exclusion of erythroid cells; anti-NK1.1 (PK136) for exclusion of natural killer cells; and anti-CD3ε (2C11), anti-CD8α (53-6.7), anti-CD8β (53-5.8), anti-TCRβ (H57) and anti-TCRγ (GL-3) for exclusion of T-lineage cells. Additional antibodies used include: anti-CD45.1(A20) and anti-CD45.2 (104). Cells were sorted on a FACSAria. Cells were analyzed on a two-laser FACS Canto or a four-laser LSR II cytometer (Becton D."
Dickinson). 4,6-diamidino-2-phenylindole was used for exclusion of dead cells. Data were analyzed using FlowJo version 8.8.6 (TreeStar).

**Intravenous transfers and intrathymic transfers**

Female CD45.1\(^+\) host mice were irradiated with 9 Gray (Gy) and transplanted with a total of 10\(^6\) bone marrow cells. Prior to transplantation, BM was depleted using anti-CD4 (GK1.5), anti CD8\(\alpha\) (53.6-7), and magnetic beads conjugated to goat anti-rat IgG. Donor reconstitution was analyzed 8-10 weeks post-transplant. For intrathymic transfers, female CD45.1\(^+\) host mice were irradiated with 6 Gy. Intrathymic transfers were done as described.\(^{21}\) Briefly, mice were anesthetized and a thoracic incision was made to expose the thymus. Freshly sorted thymocyte progenitors were injected directly into the thymus in a 10\(\mu\)L total volume.

**Wright-Giemsa staining and microscopy**

Sorted populations were spun onto glass slides using a Shandon cytocentrifuge. Cells were fixed in fresh methanol and stained in Wright-Giemsa reagent (Fisher Scientific) for 3 min, followed by Wright-Giemsa with Original Azure Blend (Harleco) for 10 min, then Wright-Giemsa with phosphate buffer pH 6.8 (Fisher Scientific) for 2 min. Stained slides were washed with ddH\(_2\)O, allowed to dry, coverslipped, and examined under the microscope. Microscopy pictures shown are 100X. Equipment and software used include: Leica DMRBE widefield microscope, Qimaging MicroPublisher 5.0 MP camera, iVision for Mac acquisition software.

**PCR**

For real-time PCR, messenger RNA was isolated using the RNeasy Kit (Qiagen) and reverse transcribed with Superscript II (Invitrogen). Resultant complementary DNAs were then amplified and detected using pre-made Taqman primer/probes against *Hes1* and *Dtx1* (Applied Biosystems). Amplification and analysis were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative
transcript abundance was determined by using the ΔΔCT method after normalization with GAPDH. All samples were run in triplicate. Error bars represent standard error of the mean.

Statistical analysis

Each data set was analyzed using the Student’s t-test on Microsoft Excel, with a two-tailed distribution assuming equal sample variance.
Results

Thymic granulocytes have a developmental history of RAG-1 expression

We first characterized the Mac1^+Gr1^+ thymic granulocyte population (Figure 1A). Thymic Mac1^+Gr1^+ cells had polynuclear morphology characteristic of granulocytes, and distinct from lymphocytes (Figure 1B). To verify that Mac1^+Gr1^+ cells in the thymus were myeloid and not T-lineage cells, we made competitive bone marrow (BM) chimeras by transplanting CD45.2^+ C/EBP{α}F/F;VavCre^+ fetal liver and CD45.1^+ competitor BM into lethally irradiated CD45.1^+ recipients. We verified that Mac1^+Gr1^+ cells in the thymus are dependent on the myeloid transcription factor C/EBP{α}, like granulocyte populations outside the thymus (Figure 1C-D). Therefore, this Mac1^+Gr1^+ population is not contaminated with T-lymphocytes, since T-cell development is unaffected by C/EBP{α}-deficiency (Figure 1C).

Lymphocyte progenitors form functional antigen receptors via rearrangement of antigen receptor loci during the process of V(D)J recombination. This process requires Recombinase Activating Gene (RAG) enzymes RAG-1 and RAG-2, the expression of which is restricted to lymphocyte precursors. Some thymic granulocytes were previously shown to be labeled in H2-VEX V(D)J recombination reporter mice. Based on this finding, we wished to directly test whether ETP can give rise to granulocytes labeled by a recombination reporter following direct intrathymic injection. For these experiments, we made use of RAG-1/Cre mice in which RAG-1 promoter elements control Cre recombinase expression. We bred these mice to mice with a floxed stop cassette upstream of the gene for YFP, which has been inserted into the ubiquitously expressed Rosa26 locus. Thus, in RAG-1/Cre x Rosa26-YFP mice, RAG-1-expressing cells and their progeny are permanently marked by YFP expression. The RAG-1/Cre mouse was superior to the H2-VEX V(D)J reporter for this experiment, which entailed analysis of rare thymic granulocyte populations, because the YFP reporter provides greater fluorescence intensity and sensitivity.
Similar to the labeling of ETP in H2-VEX V(D)J recombination reporter mice, we found that over half of ETP were labeled in RAG-1/Cre x Rosa26-YFP mice (Figure 2A). We also examined whether granulocytes and other non-T-lineage cells in the thymus were labeled in these mice. Since RAG expression is restricted to lymphocyte precursors, labeling of a non-lymphoid population would suggest that this population originated from a progenitor that had expressed RAG-1 at some point during its development. We examined granulocyte and DC populations in the adult mouse thymus for a history of RAG-1 expression in RAG-1/Cre x Rosa26-YFP mice. The gating strategy used to identify these and other populations examined in this study is shown in Supplemental Figure 1. We found that more than half of thymic granulocytes were labeled; however, only about 2% of splenic granulocytes were labeled (Figure 2A-B). Thymic CD11c^{hi} conventional DC (cDC), including CD8α^{+} cDC and Mac1^{+} cDC, were less than 5% labeled, which is similar to the level of labeling in splenic cDC populations (Figure 2A-B). These results are consistent with the idea that RAG-1-expressing ETP give rise to thymic granulocytes, but not thymic cDC. Moreover, thymic granulocytes have a distinct developmental history compared to peripheral granulocytes.

**ETP can generate granulocytes in vivo**

Labeling of ETP as well as thymic granulocytes in RAG-1/Cre x Rosa26-YFP mice suggested that thymic granulocytes may derive from ETP. In order to directly test whether ETP can give rise to granulocytes with a history of RAG-1 expression, we sorted thymocyte progenitor populations from RAG-1/Cre x Rosa26-YFP mice and injected them directly into the thymus of sublethally irradiated congenic CD45.1^{+} recipients. We injected either pooled CD45.2^{+} ETP and DN2 progenitors or DN3 progenitors. We then examined donor contribution to thymic granulocyte populations 6 days after injection (Figure 2C). We found that pooled ETP and DN2 thymocytes gave rise to CD45.2^{+} thymic granulocytes which were YFP^{+}, indicating a history of RAG-1 expression. As expected, control DN3 thymocytes, which are T-lineage committed, did not generate thymic granulocytes. These data show that ETP can make granulocytes in vivo within the thymus, and that these granulocytes show a history of RAG-1 expression.
We next wished to establish the kinetics with which ETP-derived thymic granulocytes can be detected following intrathymic injection. A previous study found no ETP-derived thymic granulocytes 11 or 16 days post-injection; however, we considered that an earlier time point may be appropriate, given the short lifespan of granulocytes. Thus, we intrathymically injected CD45.2+ ETP into sublethally irradiated CD45.1+ congenic recipient mice and examined the donor contribution to thymic granulocytes 6 or 11 days post-injection. Consistent with the results in Figure 2C, we found that ETP give rise to Mac1+Gr-1+ thymic granulocytes at 6 days post-injection, but these can no longer be detected 11 days post-injection (Figure 2D). Again, DN3 thymocytes, which are T-lineage committed, did not generate thymic granulocytes. As expected, both ETP and DN3 cells produced CD25+ T-lineage progeny following intrathymic injection at both time points examined (Supplemental Figure 2). Thus, ETP have the capacity to generate granulocytes in vivo at early time points.

**Impaired thymic granulocyte development in the absence of thymic settling by T-cell progenitors**

Our results with RAG-1/Cre x Rosa26-YFP mice suggested that at least a fraction of thymic granulocytes derived from ETP. We therefore asked whether development of thymic granulocyte populations was dependent upon the ability of T-cell progenitors to settle the thymus. Thymic settling by T-cell progenitors is impaired in mice that are doubly-deficient for chemokine receptors CCR7 and CCR9. Indeed, ETP are almost completely absent from CCR7/CCR9 double-deficient BM in mixed marrow chimeras (Figure 3A-B), despite normal engraftment of BM Lin’Sca1+Kit+ (LSK) progenitors (Figure 3C-D). We therefore examined the ability of CCR7/CCR9 double-deficient BM to reconstitute non-T-lineage cells in the thymus of mixed marrow chimeras. We reasoned that if a particular thymic lineage predominantly originates from ETP, then the donor chimerism of this population would necessarily be reduced in the absence of CCR7 and CCR9. Alternatively, if a particular thymic lineage develops independently of both CCR7 and CCR9, then it is unlikely to develop from ETP. Indeed, we found that the development of thymic granulocytes was diminished from CCR7/CCR9 double-deficient donor BM
(Figure 3A-B), whereas splenic granulocyte chimerism was unaffected (Figure 3C-D). These results are consistent with the idea that thymic granulocytes develop from ETP.

We additionally examined the development of conventional DC (cDC) subsets in the thymus of CCR7/CCR9 double-deficient mixed marrow chimeras. Thymic Mac1+ cDC were unperturbed in the absence of CCR7 and CCR9; however, we consistently saw a 50% decrease in thymic CD8α+ cDC donor chimerism from CCR7/CCR9-deficient BM (Figure 3A-B). Both CD8α+ and Mac1+ cDC subsets in the spleen were unaffected by CCR7/CCR9-deficiency (Figure 3C-D). We additionally examined whether CCR7/CCR9 deficiency affected the development of macrophages and B-cells within the thymus. We saw a modest reduction in the development of thymic macrophages from CCR7/CCR9 double-deficient progenitors (Supplemental Figure 3A-B); however, this difference did not reach statistical significance. There was a decrease in contribution of CCR7/CCR9-deficient BM to CD19hiB220hi thymic B-cells (Supplemental Fig. 3A-B). As expected, CCR7/CCR9-deficiency did not alter macrophage or B-cell development in the spleen (Supplemental Figure 3C-D). Our study cannot exclude the possibility that the absence of CCR7/CCR9 leads to reduce settling of the thymus by CD8α+ cDC, macrophages, B-cells, or their precursors. In summary, these results indicate that at least 50% of thymic CD8α+ cDC, all thymic Mac1+ cDC, and the major fraction of macrophages and B-cells in the thymus develop independently of ETP.

**Thymic granulocyte development is IL-7Rα-dependent**

We next wished to determine whether cytokine signals critical for T-cell development are also required for the development of granulocytes in the thymus. IL-7 signaling is critical for the development of T- and B-lymphocytes, but is dispensable for blood granulocyte production.19,27 IL-7 signals through the IL-7 receptor (IL-7R), which is composed of the IL-7Rα (CD127) and the common γ-chain (CD132).28 We examined whether development of granulocytes in the thymus is dependent on IL-7R signaling; if so, this would support a model in which thymic granulocytes share a common origin with T-cells. We constructed
mixed BM chimeras using IL-7Rα-deficient BM and examined the development of ETP and thymic granulocytes after 8 weeks. The IL-7Rα−/− donor chimerism of both ETP and thymic granulocytes was significantly reduced compared to BM LSK chimerism (Figure 4A-C), indicating a role for IL-7R signaling in the development of both populations. Granulocytes in the spleen were unaffected by IL-7Rα deficiency (Figure 4B). These data are consistent with the notion that most thymic granulocytes derive from T-cell precursors.

**Thymic granulocyte development depends on the Notch target gene Hes1**

The IL-7 receptor requirement shared by T-cells and granulocytes in the thymus suggested that these cells develop from a common precursor. We next examined whether thymic granulocytes require the Notch target gene Hes1, which is required for T-cell development.29 Hes1 expression is minimal in fetal liver and increases in the thymus downstream of Notch signaling, like expression of another Notch target Deltex1 (Figure 5A).30,31 Because germline Hes1-deficiency results in midgestation lethality, we constructed competitive irradiation chimeras using Hes1-deficient fetal liver progenitors. After 8-10 weeks, we analyzed the chimeric mice for donor contribution to thymic and splenic populations. We found that the contribution of Hes1-deficient cells to ETP was decreased (Figure 5B-C), consistent with published work.32 Further, contribution of Hes1-deficient cells to thymic granulocytes was also decreased (Figure 5B-C); whereas, splenic granulocytes developed independently of Hes1 (Figures 5D-E). We additionally examined whether Hes1-deficiency affected the development of cDC, macrophages, and B-cells within the thymus, but found no significant defects in these lineages in the absence of Hes1 (Figure 5B-C, Supplemental Figure 4A-D). These data demonstrate that thymic granulocytes, like ETP, depend on the transcription factor Hes1.
Discussion

The earliest progenitors of T-cells in the mouse thymus retain a variety of alternative or non-T-lineage potentials; yet, it has remained controversial whether these potentials are realized within the thymus in vivo. Using multiple in vivo models, we found that thymic granulocytes have a distinct origin from peripheral granulocytes. Furthermore, our study implicates ETP as the major precursors of thymic granulocytes. Thymic granulocytes differ from their peripheral counterparts in several ways that suggest a close developmental relationship with T-cell progenitors: 1) they are labeled by a history of RAG-1 expression; 2) they are diminished in the absence of chemokine receptors necessary for thymic settling by T-cell progenitors; 3) they rely on IL-7Rα and the Notch target gene Hes1 for their development. These properties together provide strong evidence that thymic granulocytes and T-cells derive from the same precursors.

Recent work using an IL-7R/Cre lineage tracing approach found that less than 20% of Mac1⁺Gr1⁺ thymic granulocytes were labeled with a developmental history of IL-7R expression, and concluded that T-cells and myeloid cells in the thymus have separate origins. However, we find that thymic granulocytes depend on IL-7Rα for their development. The simplest explanation for this apparent discrepancy is that lineage-tracing approaches require a threshold level of Cre recombinase expression, whereas cytokine receptors can be active at levels below this threshold. Thus, cellular responsiveness to cytokine is a more sensitive readout than reporter labeling. Hence, although thymic granulocyte precursors do not reach the threshold level of Cre expression needed to confer labeling in IL-7R/Cre reporter mice, they are clearly revealed to require IL-7Rα in our studies. Our results argue against the conclusions reached using the IL-7R/Cre lineage tracing approach, and reveal that most thymic granulocytes share a common origin with T-cells.
Because Notch signaling inhibits non-T-cell fates,\textsuperscript{10,35-38} it has been proposed that ETP may give rise to non-T-lineage cells instead of becoming T-cells when they “escape” strong Notch signals. Indeed, the Notch target gene Hes1 is specifically required for T-cell development and dispensable for peripheral myeloid development.\textsuperscript{29,32} Moreover, Notch and Hes1 can inhibit myeloid development.\textsuperscript{10,35,36} However, we found that thymic granulocytes were diminished in the absence of Hes1. These data show that thymic granulocytes develop from a progenitor that requires Hes1, like T-cell precursors, but unlike extrathymic granulocytes. Interestingly, elements of the T-cell developmental program, such as Gfi1, are both expressed by ETP\textsuperscript{39} and are involved in neutrophil fate specification.\textsuperscript{40} It is possible that components of the early T-cell developmental program allow and perhaps even potentiate the granulocyte fate from some uncommitted progenitors.

This study indicates that cells of the same lineage can arise from distinct progenitors in different sites. Similarly, DC can derive from either myeloid or lymphoid progenitors.\textsuperscript{41} We verified that thymic Mac1\textsuperscript{+}Gr1\textsuperscript{+} cells have polynuclear morphology and depend on the transcription factor C/EBP\textit{a} for their development, thus confirming their identity as myeloid cells. Although the function of thymic granulocytes is not precisely known, it is possible that these cells act as scavengers of apoptotic thymocytes. Because the vast majority of developing thymocytes will fail positive or negative selection, scavenger cells, such as macrophages, play an important role in thymopoiesis.\textsuperscript{42,43} A recent study showed that mice deficient for neutrophil migration were impaired in their ability to clear apoptotic cells following irradiation.\textsuperscript{44} Thus, it is possible that granulocytes function as scavengers of apoptotic thymocytes in the steady state.

Granulocytes are unique among non-T-lineage cells in the thymus, in that their major precursor appears to be ETP. Our data suggest that there are not other efficient sources of thymic granulocytes apart from ETP. The frequencies of donor-derived thymic macrophages and B-cells from CCR7/CCR9 double-deficient
progenitors were reduced compared to wild-type controls (25% and 37%, respectively), and for B-cells this achieved statistical significance (Supplemental Figure 3). Our study cannot exclude the possibility that the absence of CCR7 and/or CCR9 leads to reduced settling of the thymus by macrophages, B-cells, or their precursors. However, our results are in line with other work that examined the development of thymic macrophages and B-cells and concluded that despite the demonstrated macrophage and B-cell potential of ETP\textsuperscript{2,4,7,10,11,13}, the major fraction of these thymic lineages develop independently of ETP\textsuperscript{11,15}. Hence, ETP may contribute to lineages in the thymus besides granulocytes, but there are likely to be more efficient or abundant precursors for thymic macrophages and B-cells than ETP. It is important to note that our conclusions apply only to adult mice. The frequency of ETP with B-lineage potential is increased in neonatal mice\textsuperscript{13,45}, and ETP may be a more significant source of thymic B cells in early life.

ETP have been shown to have dendritic cell potential in vitro and in vivo upon intrathymic injection.\textsuperscript{3,46} Specifically, CD8α\textsuperscript{+} thymic cDC have been proposed to originate from ETP.\textsuperscript{47,48} We found that thymic cDC subsets are unlabeled in RAG-1/Cre reporter mice, consistent with previous reports that this population lacks T-cell receptor rearrangements.\textsuperscript{15} Although thymic cDC development can be uncoupled from T-cell development in the context of Notch1 deficiency,\textsuperscript{14,15} these studies cannot exclude the possibility that thymic cDC develop from thymus settling progenitors upstream of the ETP. In the absence of Notch, progenitors continue to arrive at the thymus and fail to become ETP, but may develop into other lineages. Thus, we asked whether thymic cDC development would be perturbed by deficiency for CCR7 and CCR9, which results in near complete ablation of ETP in competitive chimeras. We found that about half of CD8α\textsuperscript{+} thymic cDC derive completely independently of ETP in CCR7/CCR9-deficient mixed marrow chimeras. We cannot exclude the possibility that the absence of CCR7 and/or CCR9 leads to reduced settling of the thymus by DC or DC progenitors. Additionally, CD8α\textsuperscript{+} thymic cDC are not perturbed in the context of Hes1-deficiency, despite substantial reductions in ETP. Consistently, a recent study\textsuperscript{49} proposed that thymic CD8α\textsuperscript{+} cDC derive from DN1c thymocytes, which are not considered
canonical T-cell progenitors. These data indicate that most or all thymic cDC, including the CD8α+ subset, derive independently of ETP.

In summary, this study establishes that T-cells and granulocytes in the adult mouse thymus derive from a common progenitor. ETP may generate other non-T-lineage cells in the thymus; however, ETP are not the major precursors of these cells (Supplemental Figure 5). These data together with other recent work support the notion that some thymus settling progenitors are myelo-lymphoid progenitors. Further, the myeloid potential of uncommitted T-cell progenitors is realized in vivo in the adult mouse thymus. Future studies are needed to clarify the mechanisms that control the lineage fate decisions of ETP and their descendants.

Acknowledgments

We thank R. Kageyama for permission to use Hes1−/− mice. We thank P. Love for CCR9−/− mice, and T. Rabbitts for RAG-1/Cre mice. We thank David Allman, Dan Zlotoff, and Will Bailis for critical comments on the manuscript. This work was supported by NIH grants R01-AI059621, R01-HL110741 (A.B), T32 GM-07229, T32 CA-09140 (M.E.D.), 1-F32-AI-080091-01A1 (J.J.B).

Authorship

Contribution: M.E.D., J.J.B, and A.B. designed experiments, performed experiments, and analyzed data. M.E.D. and A.B. wrote the paper.

Conflict-of-interest disclosure: The authors declare no conflicts of interest.

Correspondence: Avinash Bhandoola, 266 John Morgan Building, 3620 Hamilton Walk, Philadelphia, PA 19104; email: bhandooa@mail.med.upenn.edu; phone: 215-573-0274; fax: 215-573-2350.
References


Figure Legends

Figure 1. Mac1⁺Gr1⁺ cells in the thymus are C/EBPα-dependent and show polynuclear morphology. 
(A) Gating of Mac1⁺Gr1⁺ granulocytes in the adult mouse thymus and spleen. (B) Sorted Mac1⁺Gr1⁺ cells in the thymus and CD4/CD8 double-negative (DN) T-lymphocytes were cytopsin and examined by Wright-Giemsa stain at 100X. Scale bars represent 10 microns. Equipment and software used include: Leica DMRBE widefield microscope, Qiumaging MicroPublisher 5.0 MP camera, iVision for Mac acquisition software. (C) The development of total CD4+ thymocytes and thymic granulocytes was examined in mixed chimeras of C/EBPαF/F;VavCre⁺ fetal liver or C/EBPαF/FVavCre⁻ control fetal liver and congenic CD45.1⁺ bone marrow (BM). Two mice were examined per group 10 weeks after reconstitution of lethally irradiated CD45.1⁺ hosts. (D) B-cells and granulocyte development in the spleen was examined in mixed chimeras of C/EBPαF/F;VavCre⁺ fetal liver and congenic CD45.1⁺ BM. Two mice were examined per group 8-10 weeks post-reconstitution.

Figure 2: ETP generate granulocytes with a developmental history of RAG-1 expression. (A) Granulocytes and conventional dendritic cell (cDC) subsets in the thymus and spleen were examined in RAG-1/Cre x Rosa26YFP mice for the presence of YFP labeling. Shaded histograms represent the same population in YFP⁻ control mice. (B) The mean percentage of YFP⁺ cells in each population was quantified. Thymus populations are in black bars. Splenic populations are in gray bars. There is no corresponding splenic population for ETP, which are found only in the thymus. Three mice per group were examined. Error bars represent standard error of the mean (SEM). **P<0.01 for the percentage of YFP⁺ cells of the indicated population in the thymus compared with the analogous population in the spleen. (C) ETP plus DN2 thymocytes or DN3 thymocytes were sorted from CD45.2⁺ RAG-1/Cre-Rosa26YFP mice and intrathymically injected into congenic CD45.1⁺ sublethally irradiated recipients (7,000 ETP+DN2/recipient and 70,000 DN3/recipient). Donor contribution to YFP⁺ thymic Mac1⁺Gr1⁺ cells was examined 6 days post-injection. (D) ETP (10,000/recipient) and DN3 thymocytes
(50,000/recipient) were sorted from CD45.2+ donor mice and intrathymically injected into sublethally irradiated CD45.1+ recipients. Mice were sacrificed 6 or 11 days post-injection and examined for donor contribution to Mac1+Gr1+ thymic granulocytes. Four mice were examined per group.

**Figure 3. Thymic granulocytes are reduced in the absence of thymic settling by T-cell progenitors.** (A) The development of CD45.2+ thymic ETP, granulocytes, and cDC in the absence of both CCR7 and CCR9 was examined in competitive BM chimeras, 8-10 weeks after reconstitution of lethally irradiated CD45.1+ hosts. (B) Shown is the mean CD45.2+ donor contribution of CCR7/CCR9+/+ (black bars) or CCR7/CCR9−/− (gray bars) BM to thymic ETP, granulocytes, and cDC. Eight animals per group were examined, over three independent experiments. Error bars represent SEM. ***P<0.001 for the CD45.2+ donor chimerism of the indicated population compared with BM LSK CD45.2+ donor chimerism. (C) The development of CD45.2+ BM LSK and splenic granulocytes and cDC in the absence of both CCR7 and CCR9 was examined in competitive BM chimeras 8-10 weeks after reconstitution of lethally irradiated CD45.1+ hosts. (D) Shown is the mean CD45.2+ donor contribution by CCR7/CCR9+/+ (black bars) or CCR7/CCR9−/− (gray bars) donor BM to BM LSK, splenic granulocytes, and cDC populations. Eight animals per group were examined, over three independent experiments. Error bars represent SEM.

**Figure 4. Thymic granulocyte development is IL-7Rα-dependent.** (A) The development of CD45.2+ ETP and thymic granulocytes in the absence of IL-7Rα was examined in competitive BM chimeras, 8 weeks after reconstitution of lethally irradiated CD45.1+ hosts. (B) The development of CD45.2+ BM LSK and splenic granulocytes in the absence of IL-7Rα was examined in mixed marrow chimeras, 8 weeks after reconstitution of lethally irradiated CD45.1+ hosts. (C) Shown is the mean percent CD45.2+ donor contribution to BM LSK, splenic granulocytes, ETP, or thymic granulocytes by IL-7Rα+/+ (black bars) or IL-7Rα−/− (gray bars) BM. Three mice per group were examined. Error bars represent SEM. ***P<0.001 for the CD45.2+ donor chimerism of the indicated population compared with BM LSK CD45.2+ donor chimerism.
Figure 5. Thymic granulocyte development requires the Notch target gene Hes1.  (A) Hes1 is expressed in the thymus, like another Notch target, Deltex1. Hes1 and Deltex1 expression in fetal liver are minimal. (B) The development of CD45.2+ thymic ETP, granulocytes, and cDC was examined in the absence of Hes1 in mixed fetal liver chimeras, 8-10 weeks after reconstitution of lethally irradiated CD45.1+ hosts. (C) Shown is the mean percent CD45.2+ donor contribution to each intrathymic lineage by Hes1+/+ (black bars) or Hes1-/- (gray bars) fetal liver. Four mice per group were examined. ***P<0.001 for the CD45.2+ donor chimerism of the indicated population compared with BM LSK CD45.2+ donor chimerism. (D) The development of CD45.2+ BM LSK, splenic granulocytes, and cDC was examined in the absence of Hes1 in mixed fetal liver chimeras, 8-10 weeks after reconstitution of lethally irradiated CD45.1+ hosts. (E) Shown is the mean percentage CD45.2+ donor contribution to each lineage by Hes1+/+ (black bars) or Hes1-/- (gray bars) fetal liver. Four mice per group were examined. Error bars represent SEM.
Figure 1

A

Thymus

Spleen

Mac1

Gr-1

Mac1

Gr-1

0.01

B

Thymus

Mac1+Gr-1+

DN Thymocytes

C

C/EBPα

F/F

VavCre+

C/EBPα

F/F

VavCre-

CD45.1 (host)

CD45.2 (donor)

Mac1+Gr-1+B220+

1

0

0

1

D

Spleen

B220+

Mac1+Gr-1+

Mac1+Gr-1+

CD45.2 (donor)

CD45.2 (donor)

43

92

43

92

77

69

34

0

0

34

0

0
**Figure 2**

A. Thymus

- ETP
- Mac1⁺Gr-1⁺
- CD8α⁺ cDC
- Mac1⁺ cDC

RAG-1/Cre-YFP

Spleen

- ETP
- Mac1⁺Gr-1⁺
- CD8α⁺ cDC
- Mac1⁺ cDC

RAG-1/Cre-YFP

B. 

<table>
<thead>
<tr>
<th>ETP</th>
<th>Mac1⁺Gr-1⁺</th>
<th>CD8α⁺ cDC</th>
<th>Mac1⁺ cDC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>78</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Thymus | Spleen

C. Injected population:

- ETP + DN2
- DN3

Gated on CD45.2⁺CD69⁺CD4⁻SSChi⁻:

- Day 6 post-injection
- Day 11 post-injection

D. Injected population:

- ETP
- DN3

Mac1⁺Gr-1⁺

CD45.2 (donor)

Day 6 post-injection

Day 11 post-injection
Figure 3

A

Thymus

<table>
<thead>
<tr>
<th>ETP</th>
<th>Mac1(^+)Gr-1(^+)</th>
<th>CD8α(^+)cDC</th>
<th>Mac1(^+)cDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR7/9(^++)</td>
<td>85</td>
<td>81</td>
<td>92</td>
</tr>
<tr>
<td>CCR7/9(^-)</td>
<td>0</td>
<td>17</td>
<td>55</td>
</tr>
</tbody>
</table>

B

Intrathymic populations

<table>
<thead>
<tr>
<th>ETP</th>
<th>Mac1(^+)CD8α(^-)Gr-1(^+)</th>
<th>Mac1(^+)cDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45.2 (host)</td>
<td>*** ***</td>
<td>***</td>
</tr>
</tbody>
</table>

C

Spleen

<table>
<thead>
<tr>
<th>BM LSK</th>
<th>Mac1(^+)Gr-1(^+)</th>
<th>CD8α(^+)cDC</th>
<th>Mac1(^+)cDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR7/9(^++)</td>
<td>89</td>
<td>85</td>
<td>92</td>
</tr>
<tr>
<td>CCR7/9(^-)</td>
<td>81</td>
<td>80</td>
<td>92</td>
</tr>
</tbody>
</table>

D

Extrathymic populations

<table>
<thead>
<tr>
<th>LSK</th>
<th>Mac1(^+)CD8α(^-)Gr-1(^+)</th>
<th>Mac1(^+)cDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45.2 (donor)</td>
<td>*** ***</td>
<td>***</td>
</tr>
</tbody>
</table>
Early T-cell progenitors are the major granulocyte precursors in the adult mouse thymus

Maria Elena De Obaldia, J. Jeremiah Bell and Avinash Bhandoola