The role of the gut as a primary lymphoid organ: CD8 $\alpha\alpha$ intraepithelial T lymphocytes in euthymic mice derive from very immature CD44⁺ thymocyte precursors

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Intestinal CD8 $\alpha\alpha$ intraepithelial T lymphocytes (T-IELs) have a key role in mucosal immunity and, unlike other T cells, were proposed to differentiate locally. In apparent contradiction, these cells were also shown to originate from a wave of thymus migrants colonizing the gut in the first 3 weeks after birth. We here identify previously uncharacterized very immature CD4⁻CD8⁻CD3⁻CD44⁺CD25^{int} thymocytes, which have not yet rearranged their T-cell antigen receptor (TCR), as having the capacity to leave the thymus, migrate to the blood, colonize the gut, and reconstitute CD8 $\alpha\alpha$ T-IEL, and show that this cell set is fully responsible for the generation of the CD8 $\alpha\alpha$ T-IEL pool. Thus, although the thymus may be fundamental for efficient T-cell commitment, CD8 $\alpha\alpha$ T-IEL' complete TCR rearrangements and TCR- $\alpha\beta/\gamma\delta$ lineage commitment must occur in the gut. These results demonstrate a major role of the gut environment as a primary lymphoid organ.

INTRODUCTION

Besides conventional T-cell antigen receptor (TCR)- $\alpha\beta$ ⁺ CD4⁺ or CD8 $\alpha\beta$ ⁺ cells, the gut epithelium harbors a very abundant and unique T lymphocyte population that expresses the CD8 $\alpha\alpha$ homodimeric form of the CD8 coreceptor (CD8 $\alpha\alpha$ T-IEL), and has a fundamental role in maintaining the integrity of the gut wall.¹ This cell set is enriched in TCR- $\gamma\delta$ ⁺ cells, and also harbors an agonist selected TCR- $\alpha\beta$ ⁺ set.¹ As in the absence of a thymus, these cells develop from Lineage⁻ (Lin⁻) c-kit⁺ interleukin (IL)-7R⁺ precursors found within small clusters of hematopoietic cells located in the lamina propria (LP) in-between the gut crypts, the gut cryptopatches (CP),^{2,3} it was suggested that CD8 $\alpha\alpha$ T-IEL were generated extrathymically. However, the relative role of such an extrathymic differentiation pathway in normal conditions has been questioned, as, in euthymic mice, CD8 $\alpha\alpha$ T-IEL in general and TCR- $\alpha\beta$ ⁺ cells in particular are much more abundant and have different characteristics; moreover, both TCR- $\alpha\beta$ ⁺ and TCR- $\gamma\delta$ ⁺ cells were shown to derive from a wave of thymus migrants repopulating the gut in the first 3 weeks after birth.^{4,5}

It was proposed that CD8 $\alpha\alpha$ T-IELs were the progeny of relatively mature thymocyte sets already expressing a rearranged TCR, thus excluding a T-cell differentiation process occurring in the gut in normal conditions. On the basis of the experiments conducted in mice expressing transgenic TCR specific of the HY antigen, it was proposed that TCR- $\alpha\beta$ ⁺ CD8 $\alpha\alpha$ T-IEL derive from a minority CD4⁺CD8 $\alpha\beta$ ⁺ double-positive (DP) population co-expressing the characteristic CD8 $\alpha\alpha$ homodimers of CD8 $\alpha\alpha$ T-IEL. These cells would be identified by a specific ability of CD8 $\alpha\alpha$ to bind tetramers of the thymus leukemia antigen (TL-tetramers (TL-tet)), would undergo agonist selection in the cortex, and differentiate into NK1.1⁻ TCR- $\alpha\beta$ ⁺ CD4⁻CD8⁻ (DN) cells that would reconstitute TCR- $\alpha\beta$ ⁺ CD8 $\alpha\alpha$ T-IEL.⁶ Gene mapping of ROR γ t-expressing cells and their progeny also proposed a DP origin,⁷ but these notions are somehow controversial, as other reports using either anti-HY TCR-Tg mice⁸ or ROR γ t gene mapping^{9,10} gave opposite results (SOM-1). Other data supported that the thymus contribution would rather involve immature thymocyte populations, indicating that a T-cell differentiation process could occur in the gut environment of euthymic mice. It was shown that grafts

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from CD3⁺CD4⁺CD8⁺ (triple negative (TN)) thymocytes¹¹ or Lin⁺ CP cells from euthymic mice¹² reconstitute “euthymic-like” CD8 $\alpha\alpha$ T-IEL and that Lin⁺ cells isolated from the CP or the epithelium show clear evidence of an ongoing T-cell differentiation in both mouse and man.^{9,11–15} However, limitations of data obtained from transfer experiments, in which cell sets are injected artificially into the blood, and the complexity of the TN populations, in which many T-cell differentiation events could have already taken place, do not allow identifying the relative contribution of thymic and extrathymic T-cell differentiation events in CD8 $\alpha\alpha$ T-IEL generation. Moreover, in normal mice, the majority of CD8 $\alpha\alpha$ T-IEL could derive from mature thymocyte sets and the contribution of TN populations could be minor.

We here used several complementary approaches to identify which thymocyte sets were able to leave the thymus, colonize the gut, and generate CD8 $\alpha\alpha$ T-IEL. We found that a previously uncharacterized TN sub-population that had not yet rearranged the TCR was the thymus precursor of the CD8 $\alpha\alpha$ T-IEL. Therefore, the gut environment should guarantee both TCR rearrangements and TCR- $\alpha\beta/\gamma\delta$ lineage commitment of CD8 $\alpha\alpha$ T-IEL. In this context, it must be considered as a primary lymphoid organ responsible for the generation of this major IEL subset in normal conditions in euthymic mice.

RESULTS

Identification of the TN subsets capable of thymus egress and gut reconstitution

To characterize which TN subsets are able to generate CD8 $\alpha\alpha$ T-IEL, we initially sorted TN subsets based on their expression of CD44 and CD25 and injected them intravenously into nude mice. Only CD44⁺ TN thymocytes were able to generate “euthymic-like” CD8 $\alpha\alpha$ T-IEL (SOM-2, **Supplementary Figure S1** online), but in these experimental conditions TN cells gain the blood stream artificially. To study if any immature subset is able to exit an intact thymus, CD45.1⁺ Rag-2/ $\gamma_c^{-/-}$ mice were transplanted with CD45.2⁺ neonatal thymi and studied shortly after grafting (SOM-3). This combination was selected as adapted to the characteristics of gut colonization. Thus, as CD8 $\alpha\alpha$ T-IELs are generated after birth, we used neonatal rather than fetal thymi in these experiments. Rag-2/ $\gamma_c^{-/-}$ mice were used as hosts as they are devoid of CP, allowing the colonization of the gut by putative precursors. We found that besides mature T cells the thymus transplants also exported Lin⁺ CD45.2⁺ cells into the blood (**Figure 1a**, **Supplementary Figure S2A** online). All migrants expressed CD44, but the bulk of thymus export appeared to be constituted by a previously non-identified cell type with a phenotype corresponding to the transition between the TN1 and the TN2 thymocyte sets. Indeed, early TN1 thymocyte progenitors (ETP) are CD44⁺ CD25⁺ Thy1⁺ IL-7R α ⁺ Sca-1⁺ c-kit⁺, and during their differentiation into TN2 they acquire the expression of CD25, Thy1, and IL-7R, whereas Sca-1 expression is down-regulated^{16,17} (**Supplementary Figure S3** online). Although rare migrants with the phenotype of ETP or TN2 could be visualized in the blood, the vast majority of Lin⁺ CD45.2⁺ was c-kit⁺ IL-7R α ^{int/-} and expressed intermediary levels of CD25, Sca-1 and

Thy1 as compared to TN1 and TN2 (**Figure 1a**, **Supplementary Figure S3** online). CD44⁺ thymus migrants also colonized the gut LP (**Figure 1a**), where they have the unique Sca-1^{high}, CD25⁺/low c-kit^{high} phenotype characteristic of Lin⁺ CP cells.¹² These results indicate that thymus migrants change their phenotype in the LP environment, as their compound phenotype does not correspond to any thymocyte set. Moreover, such high c-kit expression level is not found in the thymus, but is characteristic of the gut CP cells.^{12,18} Lin⁺ migrants of thymus origin expressed *rag1* and a smaller number expressed *cd3e* (**Figure 1b**), as characteristic of early TN precursors.

The presence of a dominant TN1 to TN2 transitional population in the blood of transplanted mice indicated a selected export, i.e., that neonatal thymi were not passively leaky (SOM-4, **Supplementary Figure S2B** online). However, as thymus transplantation also involves an artificial manipulation and it is generally believed that the thymus only exports mature T cells, we next studied all thymocyte populations directly including the TN1 to TN2 transitional set, to investigate their potential to exit the thymus and migrate to the gut. Thymus egress requires the expression of S1P₁, a receptor of sphingosine-1-phosphate (S1P),¹⁹ whereas migration to the gut requires the expression of the $\alpha_4\beta_7$ integrin.^{20,21} We found that besides mature thymocytes, only CD44⁺ TN cells expressed these molecules, but expression frequencies were maximal in the TN1 to TN2 transition. Indeed, although ~20% of TN1 and TN2 cells expressed *s1p1*, the *s1p1* expression frequencies of the TN1 to TN2 intermediate populations were much higher, approaching those found in mature TCR⁺CD4⁺CD8⁺ thymocytes. Neither TN3, TN4 nor DP cells expressed *s1p1* (**Figure 2a**). Similarly, we found an important upregulation of $\alpha_4\beta_7$ in the TN1 to TN2 transition, whereas $\alpha_4\beta_7$ expression was lost in the TN2 to TN3 transition: double labeling with CD44 and $\alpha_4\beta_7$ of TN2 + TN3 sets showed that the loss of CD44 directly correlated to $\alpha_4\beta_7$ decline (**Figure 2b**). Neither TN3, TN4 nor DP cells expressed $\alpha_4\beta_7$ (not shown). We conclude that besides mature T cells, only CD44⁺ TN populations harbor a significant fraction of cells that can egress the thymus and migrate to the gut. Both the direct study of thymocyte population properties and the characterization of TN migrants in the blood after thymus transplantation indicate that the bulk of TN egress should occur during the TN1 to TN2 transition. It must be noted that the TN1 to TN2 transition population is particularly abundant during the perinatal period when the gut colonization is known to occur (**Figure 2b**).

Characterization of more mature CD8 $\alpha\alpha$ T-IEL precursors in normal mice

The repertoire of TCR- $\alpha\beta$ CD8 $\alpha\alpha$ T-IEL is not deleted by the presence of self-antigens, but it is rather agonist selected.²² Studies mostly performed in TCR-Tg mice postulated that CD8 $\alpha\alpha$ T-IEL rather than being generated from immature thymocyte precursors would issue from a DP population that, alike these IEL, would also express CD8 $\alpha\alpha$.⁶ It was postulated that this minority could be recognized by TL-tet, because TL-tet would only bind CD8 $\alpha\alpha$ homodimers. These cells would be selected

Figure 1 Characterization of triple negative populations able to egress the thymus. Thymus lobes from newborn CD45.2 T-cell antigen receptor (TCR)- $\alpha^{-/-}$ mice were grafted into CD45.1 Rag-2/ $\gamma_c^{-/-}$ mice, and Lin $^{-}$ CD45.2 $^{+}$ cells of graft origin were studied in the blood, the gut lamina propria, and the spleen 10 days after transplant, as described in **Supplementary Figure S2** online. **(a)** Phenotype of Lin $^{-}$ CD45.2 cells recovered from the blood and the gut lamina propria. Similar results were obtained in two experiments. **(b)** Lin $^{-}$ CD45.2 $^{+}$ cells were purified from the spleen, sorted as individual cells, and tested simultaneously for the expression of *cd3ε* and *rag1* mRNA. The simultaneous expression of *rbpsuh* mRNA was tested to verify plating efficiency. Note: Although Lin $^{-}$ cells were clearly identified during analysis in all organs, the relative small blood volume that we could recover, the abundance of “nonspecific” contaminants in the blood and gut suspensions (platelets, bacteria, epithelial cells), and the rarity of precursors did not allow us to purify them from these organs.

by self-antigens in the thymus cortex, differentiate into agonist selected mature TCR- $\alpha\beta^{+}$ DN cells that would then repopulate the TCR- $\alpha\beta$ CD8 $\alpha\alpha$ T-IEL.⁶ We first wished to characterize these putative DP CD8 $\alpha\alpha^{+}$ precursors, but found that TL-tet did not discriminate CD8 $\alpha\alpha^{+}$ cells in normal mice. The vast majority of CD8 $\alpha\beta^{+}$ DP thymocytes and CD8 $\alpha\beta^{+}$ T-IEL were TL-tet $^{+}$, whereas an average of 15% of CD8 $\alpha\beta^{-}$ CD8 $\alpha\alpha^{+}$ T-IEL did not interact with these tetramers (**Figure 3a**). To further investigate the capacity of TL-tet to always recognize CD8 $\alpha\alpha$ complexes, we also studied CD8 $^{+}$ T cells from CD8 β^{-} -deficient mice that can only express CD8 $\alpha\alpha$ at the cell surface. As in normal mice, an average of 15% of CD8 $^{+}$ T-IEL did not bind TL-tet (not shown) and the majority of peripheral T cells remained TL-tet $^{-/low}$ (**Figure 3a**). We conclude that TL-tet does not allow the identification of cells expressing CD8 $\alpha\alpha$ homodimers. Therefore, it remains to be determined if any TL-tet $^{+}$ DP cells co-expresses CD8 $\alpha\alpha$, as the expression of these homodimers by DP cells was never directly investigated.

We next studied if TCR- $\alpha\beta^{+}$ DN cells were agonist selected in normal conditions. We used mice expressing the endogenous mammary tumor virus (MMTV-Mls1^a) that induces the deletion of self-reactive V β 6 $^{+}$ cells in the thymus. We found that the bulk of NK1.1 $^{-}$ TCR- $\alpha\beta^{+}$ DN thymocytes had undergone V β 6 $^{+}$ deletion (**Figure 3b**). These results contrast to the agonist selected CD8 $\alpha\alpha$ T-IEL, in which V β 6-bearing cells are

not deleted,²³ and indicate that the vast majority of NK1.1 $^{+}$ TCR- $\alpha\beta^{+}$ DN thymocytes cannot originate CD8 $\alpha\alpha$ T-IEL.

The relative role of TN and mature thymocyte sets in the generation of CD8 $\alpha\alpha$ T-IEL

The above results did not exclude that part or even all CD8 $\alpha\alpha$ T-IEL would derive from a minority TCR $^{+}$ DN sub-population or from a yet other non-identified DP thymocyte set. To determine the relative contribution of the TN vs. the other more mature thymocytes in T-IEL generation, we compared T-IEL reconstitution after neonatal thymic transplantation in either Rag-2 $^{-/-}$ or Rag-2/ $\gamma_c^{-/-}$ mice. We reasoned that CD8 $\alpha\alpha$ T-IEL deriving from early TN cells should be preferentially produced in Rag-2/ $\gamma_c^{-/-}$ mice, whereas CD8 $\alpha\alpha$ T-IEL originating from more mature thymocytes (DP, conventional CD4 $^{+}$ /CD8 $\alpha\beta^{+}$, or mature DN TCR $^{+}$ thymocytes) should be generated equally in either host (**Figure 4a**). Indeed, in Rag-2/ $\gamma_c^{-/-}$ mice, the CP and immature local precursors are virtually absent.³ This empty niche should allow TN colonization. In contrast, very few TN precursors should be able to repopulate the full CP niche of the Rag-2 $^{-/-}$ mice, which should hinder any new TN entry from the thymus. These mice were studied in detail in the first 3 weeks. As expected, the gut of both strains was equally colonized by conventional T cells (CD4 $^{+}$ or CD8 $\alpha\beta^{+}$ T-IEL) 2 weeks after grafting (**Figure 4b**). Similarly, CD8 $\alpha\alpha$

Figure 2 Expression of *S1p1* and $\alpha 4\beta 7$ during thymocyte development. **(a)** Results show the frequency of *s1p1* expression determined by single-cell reverse transcription-PCR in adult (6–8 weeks) or newborn (1–2 days) mice thymocyte sub-populations. The simultaneous expression of *28s* mRNA was tested to verify plating efficiency. When frequency estimates were very low or *s1p1* was not detected, the number of positive vs. total cells studied is shown in parentheses. **(b)** Lin[−] thymocytes of adult or newborn mice were labeled with anti-CD44, anti-CD25, and anti- $\alpha 4\beta 7$ monoclonal antibodies. Left: gates used to define different triple negative (TN) types. Right: $\alpha 4\beta 7$ expression by different TN sub-populations. Data are representative of four experiments for adult mice and two experiments for newborn mice.

T-IEL were rapidly generated in Rag-2/ $\gamma_c^{-/-}$ mice (**Figure 4b**), but the presence of immature precursors in the gut Rag-2^{−/−} mice had a major impact on CD8 $\alpha\alpha$ T-IEL generation. Rather than reduced production we found a virtually complete block. Two weeks after transplantation, CD8 $\alpha\alpha$ T-IELs were barely detectable. By 3 weeks they were still very rare (**Figure 4b**). Although a very minor contribution of other thymocyte sets cannot be formally excluded, these experiments show that reconstitution of this compartment that takes place in the first 3 weeks after birth depends on colonization of the gut by TN precursors and cannot be ensured by more mature thymocyte sets.

DISCUSSION

Although it is generally accepted that CD8 $\alpha\alpha$ T-IEL derive from precursor cells located in the gut CP in athymic mice, the role of a local T-differentiation process in normal conditions has been controversial. The reduced number of CD8 $\alpha\alpha$ T-IEL and the virtual absence of TCR- $\alpha\beta^+$ cells in athymic mice,¹ as well as the demonstration that these cells are thymus derived in normal mice,⁵ suggested that when the thymus was present, T-cell differentiation events in the gut might have little or no impact in CD8 $\alpha\alpha$ T-IEL generation. In contrast, the characterization of the thymus contribution to the CD8 $\alpha\alpha$ T-IEL pool that we describe here confers a fundamental role to the gut in local T cells' differentiation.

The thymus was believed to export only mature T cells, but our results identify another phase of thymus differentiation when immature thymocytes acquire the capacity to exit the thymus, circulate in the blood, and migrate to the gut. Indeed, we found that during the transition from the TN1 to the TN2 differentiation steps, thymocytes showed a major upregulation of the expression of the $\alpha 4\beta 7$ integrin, in particular, during the post-natal period, and that their *S1p1* expression frequencies were similar to those found in mature thymocytes. The location of this cell set in the thymus could favor thymus egress, described to take place through capillaries located at cortex/medullary boundary. Indeed, TN1 cells are around this boundary and must cross this zone to reach the inner cortex, where TN2 cells reside.²⁴ As TN1 to TN2 transition is accompanied by progressive CD25 upregulation, CD25^{int} TN1 to TN2 transition cells might yet be near the cortex/medullary boundary and have a better access to the blood than their more mature counterparts. The study of Lin[−] precursors of thymus origin circulating in the blood confirmed that the bulk of the thymus egress occurs during the TN1 to TN2 transition, as the vast majority Lin[−] migrants recovered from the blood have the compound phenotype of CD44⁺ CD25^{int} transitional cells, sharing their Thy1, Sca-1, IL-7R, and c-kit phenotypes. We also demonstrate directly that Lin[−] cells of thymus origin repopulate the gut LP. These cells are not just blood contaminants of gut suspensions, as the phenotype of recent thymus

Figure 3 Thymus leukemia antigen-tetramers (TL-tet) binding and agonist selection in T cells. **(a)** TL-tet binding in CD8⁺ T cells. Upper graphs: left: CD8αβ⁺ double-positive (DP) thymocytes; middle: CD8αβ⁺ splenocytes; right: CD8α⁺ splenocytes from CD8β^{-/-} mice. Lower graphs: intestinal CD8α intraepithelial T lymphocytes (T-IELs). Left: T-cell antigen receptor (TCR)-αβ⁺; right: TCR-γδ⁺. Cells from all organs were studied simultaneously. Data are representative of six experiments for normal mice and two experiments for CD8β^{-/-} mice with the same results. **(b)** Frequency of Vβ6⁺ cells within TCR-αβ⁺ SP and NK1.1⁻ TCR-αβ⁺ DN thymocytes from C57Bl/6 (Mls1^b) and DBA/2 (Mls1^a) mice.

migrants in the blood and in the gut did not overlap. Both populations were CD44⁺ but virtually all cells recovered from the gut have acquired the characteristic phenotype of the Lin⁻ CP cells.

Although these data demonstrated that the thymus could export precursor cells to the gut, it was possible that these cells had a minor contribution to CD8α T-IEL generation. Several independent experiments demonstrate that reconstitution of the CD8α T-IEL compartment is a post-natal event, and that the thymus contribution to CD8α T-IEL generation is restricted to the first 3 weeks after birth. At birth, IEL are virtually absent and only very rare TCR-γδ cells are detected.²⁵ Lin⁻ CP cells are first dispersed in the LP and only start to aggregate around day 10 after birth.⁹ In neonatal thymectomized mice, CD8α T-IEL are as found in nude mice, but when thymectomy is performed in 3-week-old mice, CD8α T-IEL is not affected.⁵ Other studies indicate that after this period, the gut CD8α T-IEL compartment is not renewed from outside. In thymus transplanted mice,

we found that CPs of thymus origin maintained constant size and persisted until 5 months after transplant (the later point we studied), suggesting permanent colonization.¹¹ Importantly, parabiosis experiments showed absence of CD8α T-IEL chimerism between partners, demonstrating that the CD8α T-IEL compartment is self-maintained in adult mice.⁴ In this context, our experiments to investigate the relative contribution of mature and immature thymocyte sets in transplanted mice were designed to focus this crucial 3 weeks reconstitution period, either mimicking the conditions found in the post-natal period (no precursor, no T cell as found in Rag-2/γ_c^{-/-} mice) or the conditions allowing the migration of TCR⁺ cells, but hindering precursor colonization (Rag-2^{-/-} mice). We were surprised to observe that when precursors were prevented to colonize the gut, CD8α T-IEL generation was virtually abrogated. Thus, when thymus lobes that can export both mature and immature thymocytes were transplanted into Rag-2/γ_c^{-/-} mice both the conventional CD4⁺/CD8αβ⁺ and CD8α T-IEL compartments

Figure 4 The relative role of triple negative (TN) cells and other thymocyte populations in CD8 $\alpha\alpha$ intraepithelial T lymphocytes (T-IEL) generation. Thymectomized CD45.1 $Rag^{-/-}$ mice or CD45.1 $Rag^{-\gamma_c -/-}$ mice were grafted with newborn CD45.2 thymi. **(a)** Schematic representation of the experimental strategy. **(b)** Upper graphs: numbers of CD45.2 $^{+}$ T-IEL studied at different time points after transplant; lower graphs: phenotype of CD45.2 $^{+}$ T-IEL 3 weeks after grafting. Note: CD8 $\alpha\alpha$ T-IEL were virtually absent in $Rag^{-/-}$ mice by 2 weeks after thymus transplant. Data are from one full kinetics experiment out of two full kinetic studies with identical results.

were rapidly reconstituted, demonstrating that the thymus is able to generate both cell types. In contrast, when thymus lobes were transplanted into Rag-2^{-/-} mice, in which the gut immature precursor pool is present, only CD4⁺/CD8 α β ⁺ were rapidly generated. During the 3 weeks period required for T-cell reconstitution, CD8 α α T-IEL were virtually absent by 2 weeks and yet very rare by 3 weeks. It is possible that the rare CD8 α α T-IEL generated in Rag-2^{-/-} transplanted mice issue from more mature thymocytes. However, parabiosis experiments using Rag-2^{-/-} mice demonstrated that their precursor pools are not fully impermeable, allowing the entry of rare precursors.²⁶ Therefore, our results are fully compatible with the notion that the rare CD8 α α T-IEL cells accumulating at late time points in Rag-2^{-/-} transplanted mice issue from rare TN precursors yet able to enter their CP. Overall, these experiments demonstrate that CD8 α α T-IEL in euthymic mice derive from very immature thymocyte precursors, the contribution of more mature thymocytes to their generation being very minor or null.

The identification of the CD44⁺CD25^{int} TN population, as responsible for the generation of CD8 α α T-IEL, allows to predict the properties of the thymus precursors colonizing the gut, and thus to discriminate thymus differentiation events from further T-cell differentiation steps occurring in the gut. TN1 cells have not initiated TCR rearrangements, and single cell cultures revealed that they have the capacity to generate both TCR- α β ⁺ and TCR- γ δ ⁺ T cells. TCR rearrangements are yet rare in the TN2 set, and about 50% of TN2 thymocytes are yet bipotential.²⁷ It is therefore likely that at least the majority of TN1 to TN2 cells have not yet rearranged their TCR and are bipotential, and that TCR rearrangements and TCR- α β and TCR- γ δ lineage commitment occurs in the gut. This notion is fully supported by other data. It was shown that transplants of Rag-deficient thymic reconstitute the gut CP,¹¹ indicating that CP colonization may precede TCR rearrangements. Alike TN1 and TN2 sets,²⁸ precursors located in the CP cells only express rare incomplete TCR- β DJ rearrangements. They originate more mature precursor cells with TCR- α β and TCR- γ δ rearrangements located in the epithelium,¹² where both β -selection^{9,12} and TCR- γ δ rearrangements²⁹ were shown to occur. Finally, TN1 and TN2 thymocytes have yet the potential to generate NK (natural killer) cells. It was recently described that CP could be involved in the generation of a unique population of Nkp46⁺ cells in the gut.¹⁰ It remains to be established if these cells also derive from TN precursors.

The present data contrast with previous reports proposing that CD8 α α T-IEL would originate from a TL-tet⁺ DP minority sharing with these IEL, the expression of CD8 α α . This cell set would be agonist selected in the thymus cortex, originating agonist selected TCR- α β ⁺ DN cells that would repopulate the gut.⁶ Our present data exclude this differentiation pathway. Thus, the expression of CD8 α α homodimers in DP cells was never directly demonstrated, and we now show that TL-tet binding is not CD8 α α specific, as these tetramers fail to bind many CD8 α α ⁺ cells while adhering to several populations expressing CD8 α β . It was reported that binding of CD8 α β ⁺ cells to tetramers of classic major histocompatibility complex class

I molecules is maximal in DP thymocytes and declines during differentiation into mature CD8 α β ⁺ T cells, and that such differential binding is determined by complex changes in the glycosylation status of the CD8 chains during ontogeny.³⁰ We found that TL-tet binding to CD8 complexes had very similar characteristics suggesting that CD8 glycosylation also has a major influence in TL binding. It must be noted that the notion that TL-tet recognizes CD8 α α specifically results from binding studies performed with non-glycosylated CD8 α and CD8 β molecules.³¹ Our data show that it does not apply to the glycosylated CD8 molecules present *in vivo*. We also demonstrate that the vast majority of TCR- α β ⁺ DN cells is deleted of self-reactive cells, and thus cannot originate the self-reactive agonist selected CD8 α α T-IEL pool.

To conclude, as the thymus and the gut epithelia share the same embryonic origin it was suggested that both would be able to sustain a T-cell differentiation process. The present results indicate that in the normal conditions the thymus is fundamental to ensure efficient T-cell commitment, as CD8 α α T-IEL are rare in athymic mice. However, the gut must ensure all further T-cell differentiation steps, and thus has all characteristics of a primary lymphoid organ.

METHODS

Mice. DBA/2 mice were obtained from Charles River Laboratories (L'Abresle, France), CD45.2 mice from the Centre d'Elevage R Janvier (Le Genest St Isles, France), and CD45.1 mice from the Centre de Distribution, Typage et Archivage Animal (CDTA, Orléans, France). Nude (nu/nu) mice were from CDTA or Taconic (Denmark). CD45.1 Rag-2^{-/-} and CD45.1 Rag-2^{-/-} γ_c ^{-/-} mice were a gift from JP Di Santo (Institut Pasteur, Paris, France). CD45.2 TCR- α ^{-/-} mice were from A Lehuen (Hôpital Saint Vincent de Paul, Paris, France) and CD8 β ^{-/-} mice were a gift from A Singer (National Cancer Institute, Bethesda, MD).

Thymectomy, thymus transplants, and cell transfers. To completely remove the very small thymus rudiment of CD45.1 Rag-2^{-/-} and CD45.1 Rag-2/ γ_c ^{-/-} mice, we use a special surgical procedure described elsewhere.³² Neonatal thymi were recovered 1–2 days after birth using the same procedure, and one thymus lobe was transplanted under the kidney capsule of each recipient.

Cell isolation, antibodies, and TL-tet labeling. Intraepithelial and LP lymphocytes from the small intestine were obtained as described.¹² For flow cytometry, cells were incubated with an anti-CD16/CD32 Fc-receptor monoclonal antibody (mAb) (2.4G2) to block Fc-mediated antibody binding, previous to cell surface staining. The following mAbs obtained from BD Pharmingen (San Diego, CA) were used: anti-CD3 (145-2C11), anti-CD8 α (53-6.7), anti-CD11b/Mac1 (M1/70), anti-CD19 (1D3), anti-CD25 (PC-61), anti-CD45.1/Ly5.1 (A20-1.7), anti-CD45.2/Ly5.2 (104-2.1), anti-CD90.2/Thy1.2 (53-2.1), anti-CD117/c-kit (2B8), anti-TCR β (H57-597), anti-TCR δ (GL3), anti-V β 6 (44.22.1), anti-LPAM1/ α 4 β 7 integrin (DATK/32), anti-NK1.1 (PK136), anti-GR1 (8C5), anti-erythroid cells (TER-119), and anti-Sca-1 (E13-161.7). Anti-CD4 (GK1.5), anti-CD8 β (H35-172), and anti-CD127/IL-7 α (A7R34, a gift from Dr S-I Nishikawa, Kyoto University and RIKEN Center, Kyoto, Japan) were purified and conjugated in our laboratory (Necker Institute, Paris, France). Anti-CD44 (1M781) was obtained from eBiosciences (San Diego, CA). All the above-mentioned mAbs were directly coupled to fluorescein isothiocyanate, phycoerythrin,

PerCP-Cy5.5, PEcy7, allophycocyanin, allophycocyanin-Alexa Fluor 750, Pacific Blue, or conjugated with biotin. Biotinylated mAbs were revealed with PEcy7-streptavidin (BD Pharmingen), phycoerythrin-Alexa Fluor 750-streptavidin (Invitrogen, Cammarillo, CA), Pacific Blue-streptavidin (Invitrogen) or Pacific Orange-streptavidin (Molecular Probes). Cells were analyzed on a FACSCalibur or a FACSCanto (Becton Dickinson, San Diego, CA). Phycoerythrin-conjugated TL-tet were obtained as described previously.³³ We found that adequate TL-tet binding required particular approaches. Each lot had to be tested for maximal efficiency and TL-tet/cell number ratios should be strictly maintained. Moreover, any previous labeling with mAbs reduced considerably TL-tet staining. Therefore cells were first incubated with TL-tet for 1 h at 4°C. Cells were then washed and incubated with the indicated antibodies.

For analysis and sorting of CD4⁺CD8⁺CD3⁺ (TN) subpopulations, thymocytes were initially enriched for TN cells by depletion with beads coated with anti-CD8 α (Dynabeads M-450; Dynal AS, Oslo, Norway). Antibodies recognizing mature lineage-positive hematopoietic cells in all experiments were TER-119, NK1.1, GR1, Mac1, DX5, CD3, TCR- β , TCR- δ , CD8 β , and CD19. Cells were sorted on a FACS ARIA (Becton Dickinson). The efficiency of purification was verified after each sorting and was higher than 99%.

PCR analysis. Single cells were sorted by a FACS ARIA equipped with an automatic cell deposition unit (Becton Dickinson), and multiplex single-cell reverse transcription-PCR analysis was carried out as previously described.³⁴ To allow evaluation of the expression of several mRNAs in the same cell, primers were selected to have the same efficiency, to amplify fragments of similar relatively small size and to provide amplifications where neither the primers nor the amplicons competed between themselves, and were the following: *cd3e*: 5'-TGGAACTCTTCTGGGGCAT-3', 5'-AAGTCTCCATCTCAGGAACC-3' (nested), and 3' primer 5'-CTCCACACAGTACTCACA CA-3'; *rag1*: 5'-5'-CAACCAAGCTGCAGACATTC-3'; 5'-GCAGACATTTCTAGCACTCTG-3' (nested), and 3': 5'-CTACTGGAGACTGTTCTAGG-3'; *rbpsuh* 5' primers: 5'-TGTGCATTGCTTCAGGAACG-3', 5'-GAAGGTGGCACTGTTCAA TC-3' (nested), and 3' primer 5'-GGTCGTCTGCATCCAGTAAT-3'; 28S 5' primer: 5'-TACCGGACCCTGAACAGAAT-3', and 3' primer 5'-GTAGATCCTCCGGCATGTTT-3'; *s1p1* primers were as described.³⁵

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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