Brief Definitive Report

Thymic and Extrathymic Origins of Gut Intraepithelial Lymphocyte Populations in Mice

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Summary

We have investigated the origin of intraepithelial lymphocytes (IEL) populations in the murine gut, using reconstitution experiments in which the presence of thymus-derived cells of host or donor origin is rigorously controlled: $RAG^{-/-}$ mutant mice which have no T cells, were injected either with the bone marrow (BM) cells of nude mice or with selected peripheral lymph node (LN) T cells of euthymic mice. In thymectomized $RAG^{-/-}$ mice, injection of BM cells from nude mice led, after 2 mo, to the development of a peripheral B cell compartment and to the appearance, in the gut, of IEL bearing homodimeric CD8α chains and either γ/δ or α/β TCR. In $RAG^{-/-}$ mice with a thymus, a similar injection led to complete lymphoid reconstitution, with the additional appearance in the gut of $CD4^+$, CD8α/β^+ or $CD4^+$CD8α/α^+ IEL, all bearing α/β TCR. In contrast, injection of LN T cells into these mice reconstituted a gut IEL population made of $CD4^+$, CD8α/β^+, or $CD4^+$ CD8α/α^+ cells, all bearing α/β TCR; CD8α/α^+ TCR-γ/δ^+ or α/β^+ IEL were not observed. These results demonstrate that the thymus and/or thymic-derived peripheral T cells are absolutely required for the generation of $CD4^+$, CD8α/β^+, and $CD4^+$CD8α/α^+ IEL, which are thus thymus dependent. In contrast, TCR^+ CD8α/α^+ IEL appear in the absence of the thymus, and thus are thymus independent.

Intraepithelial lymphocytes (IEL)[1] present in the mouse intestinal mucosa represent, because of the length of the gut, a quantitatively major lymphocyte population of the organism (1). These cells express different surface makers and thus represent a mixture of lymphocyte populations, almost exclusively of T cell nature (i.e., bearing TCR). In adult euthymic mice bred in a conventional environment, about half of the IEL have a surface phenotype similar to most peripheral T lymphocytes, i.e., are $Thy1^+$, TCRα/β^+ and either $CD4^+$ or $CD8^+$. Their CD8 molecules are made of heterodimers of CD8α and β chains (CD8α/β^+), as on all $CD8^+$ peripheral T lymphocytes and $CD8^+$ or $CD4^+CD8^+$ (double positive [DP]) thymocytes. Another major IEL population expresses on its surface CD8 homodimeric α chains (CD8α/α) and no CD4 or CD8 heterodimeric molecules: these cells bear α/β or γ/δ TCR (or rarely have no TCR), and are most often $Thy1^-$ rather than $Thy1^+$. Small percentages of TCRα/β^+, TCRγ/δ^+, or TCR^- cells are $CD8^-CD4^-$. Finally, some $Thy1^+$, TCRα/β^+ IEL coexpress both CD4 and CD8 molecules. These cells are rare and bear high levels of TCRα/β and only CD8α/α but no CD8α/β molecules, two features which are in contrast with what is observed with DP thymocytes (2, 3).

It is generally accepted that at least part of these IEL are of extrathymic origin. It has been proposed that the gut epithelium, an endoderm derivative as the thymic epithelium, is endowed with the property of attracting circulating precursors and of inducing TCR rearrangement and expression, as well as CD8α/α expression (2). These last two events appear independent of each other (since the IEL of $RAG^{-/-}$, or of SCID mutant mice, which cannot rearrange TCR genes, are all $CD3^-$, but in part CD8α/α^+), and not necessarily coordinated (since the IEL of nude mice can be $CD3^+$CD8α/α^- or $CD3^-$CD8α/α^+) (4). However, the local pathway of differentiation of IEL, as well as the origin of some IEL populations, in particular that of the CD8α/β^+ and of the $CD4^+$ cells, remains a matter of controversy.

We have proposed a dual origin of gut IEL. In experiments performed before the description of the CD4 and CD8 molecules, and thus using the Thy1 marker to identify T cells in the gut mucosa and within the epithelium (5–7), we have shown that most $Thy1^+$ IEL derived from T blasts antigenically stimulated in Peyer's patches, migrating through the mesenteric LN to the thoracic duct lymph and then to

[1] *Abbreviations used in this paper:* BM, bone marrow; DN, double negative; DP, double positive; IEL, intraepithelial lymphocyte; TD, thymus dependent; TI, thymus independent; Tx, thymectomized.

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the blood, from which they rapidly seed to the whole length of the gut mucosa thanks to their peculiar homing properties. This origin of Thy1$^+$ IEL was established by a series of experiments exploring each step of this traffic, namely selective irradiation of the Peyer's patches and continuous drainage of the thoracic duct (which each led within a few days to a profound depletion in Thy1$^+$ gut lymphocytes) and transfer experiments with labeled blasts obtained from the thoracic duct (leading to the accumulation of labeled Thy1$^+$ IEL in the gut of the recipient, an observation also made by Sprent [8]). With the availability of other markers, it became apparent that the Thy1$^+$ IEL we had dealt with are CD4$^+$ and CD8α/β^+ IEL, since the majority of other IEL are Thy1$^-$. Furthermore, all the Thy1$^+$ blasts of the thoracic duct lymph are CD4 or CD8β blasts, circulating CD8α/α^+ blasts having not been observed (2). On the basis of these experiments and of observations showing that CD4$^+$ and CD8β^+ IEL have been submitted to processes of repertoire selection similar to those occurring in the thymus (1), whereas CD4$^-$CD8β^- IEL escape both "negative" and "positive" thymic selection events (1, 9), we have proposed that CD4$^+$ and CD8β^+ IEL are indeed the progeny of peripheral thymus-derived cells antigenically stimulated in the Peyer's patches, and that only CD3$^+$CD8α/α^+ or double negative (DN) IEL result from extrathymic, in situ differentiation. The small percentage of TCRα/β^{high}, CD4$^+$ CD8α/α^+ DP IEL are interpreted, in this scheme, as thymus-derived CD4$^+$ cells induced to express CD8α/α chains in the gut, as described above.

In contrast, others have proposed that most IEL are of extrathymic origin. This concept is based on the observation that injection of bone marrow (BM) (10) or fetal liver cells (11) from normal mice into thymectomized (Tx) irradiated recipients leads to the appearance of IEL of donor origin which also contain, among other phenotypes, CD4$^+$ and CD8β IEL. In addition, parabionts experiments (11) showed that chimerism in all IEL populations is established only very slowly, suggesting that traffic of T cells from the periphery to the gut epithelium is, in nonirradiated mice, slow, if present at all. These experiments were interpreted as evidence for the local origin of most if not all IEL. It was thus proposed that differentiation of IEL T lymphocytes might be somewhat comparable to that of T lymphocytes in the thymus, the first recognizable step in this pathway being the expression of CD8α/α, followed by an intermediate stage of DP CD4CD8α/α^+ IEL from which CD4$^+$CD8$^-$ and CD4$^-$ CD8α/β^+ IEL would originate (11). Differences in TCRβ repertoire of CD4CD8β^- and of CD4CD8β^+ IEL were interpreted as reflecting the immaturity of the first population, not yet negatively selected, and it was proposed that IEL undergo locally a process of negative and positive selection comparable to that occurring in the thymus, to give rise to lamina propria T lymphocytes (11, 12). As an extension of this scheme, it has been reported that the gut environment might even substitute for the thymus: gut fetal grafts, in Tx irradiated hosts receiving BM precursors, would induce the differentiation of TCRα/β^+, CD4$^+$, or CD8$^+$ lymphocytes, repopulating from the gut graft the whole peripheral T cell pool (13).

We have suggested that the generation of CD4$^+$ and CD8β^+ IEL in Tx, irradiated hosts might result from the persistence and expansion of a few thymus-derived T cell contaminants (4). A peculiar characteristic of the gut microenvironment is its continuous stimulation by exogenous antigens, which may lead to the progressive expansion and local accumulation of a small number of thymus-derived T cell contaminants. In reconstitution experiments, using Tx irradiated hosts, we have also observed that all IEL subsets are strikingly radioresistant, and that usually only a minority of these cells are of donor origin (2 and our unpublished observations). We have thus reevaluated the origin of the IEL population using transfer experiments in which any contamination of thymus-derived cells can be completely excluded, in the hosts because being RAG$^{-/-}$ mutant, they cannot rearrange TCRs and thus have no T cells, and in the donors because being nude mice they have no thymus.

Materials and Methods

Animals. C57/Bl6 euthymic or nude mice, and RAG$^{-/-}$ mutant mice (14) were obtained from the Centre National de Recherche Scientifique (Orléans, France). Thymectomies were performed under anaesthesia with forceps (Tx mice). It must be pointed out that we commonly find small thymic remnants in our Tx mice, either RAG$^{-/-}$ mutants or normal mice. These were easily detectable only in mice previously bled, and their identity was ascertained by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) of their cell content, which showed the expected phenotypes of the various thymocyte subsets. Mice with such thymic remnants were not included in these experiments.

Generation of Chimeric Mice. 2 wk after surgery, Tx or non Tx RAG$^{-/-}$ mice were irradiated (400 rad) and injected intravenously with 30 × 10^6 BM cells from nude mice. In other experiments, 20 × 10^6 peripheral LN T lymphocytes from B6 mice were injected into nonirradiated, non Tx recipients.

Cell Suspensions and Immunofluorescence Analyses. Cell suspensions from the thymus, LN, or IEL were prepared as described (6). The following antibodies, coupled to FITC or biotin (revealed with streptavidin-Tricolor) or directly coupled to PE (Caltag Laboratories, South San Francisco, CA) were used: GK1.5 (anti-CD4); H022 (anti-CD8 α chain); H35-17-1 (anti-CD8 β chain); H57-597 (anti-TCR β chain); GL-3 (anti-TCR δ chain) (1, 2); anti-B220 (PharMingen, San Diego, CA); and rabbit anti-mouse IgD antibody (kind gift of P. Truffa-Bachi, Institut Pasteur, Paris, France). Fluorescence analyses were performed using a FACScan® (Becton Dickinson & Co.), and the data processed in a Hewlett Packard computer.

Histological Studies. A 1.6-cm-long piece of small bowel taken 1 cm below the pylorus was fixed in Carnoy fluid, and tissue sections were stained with Periodic Acid Schiff (PAS) (in order to appreciate the ratio of IEL per 100 epithelial cells) or with methyl-green-pyronin and/or with rhodamin-rabbit anti-mouse IgA α chains (in order to count gut mucosal plasma cells).

Results and Discussion

Injection of BM cells from nude mice into RAG$^{-/-}$ mutant mice with a thymus led, 2 mo after the injection,

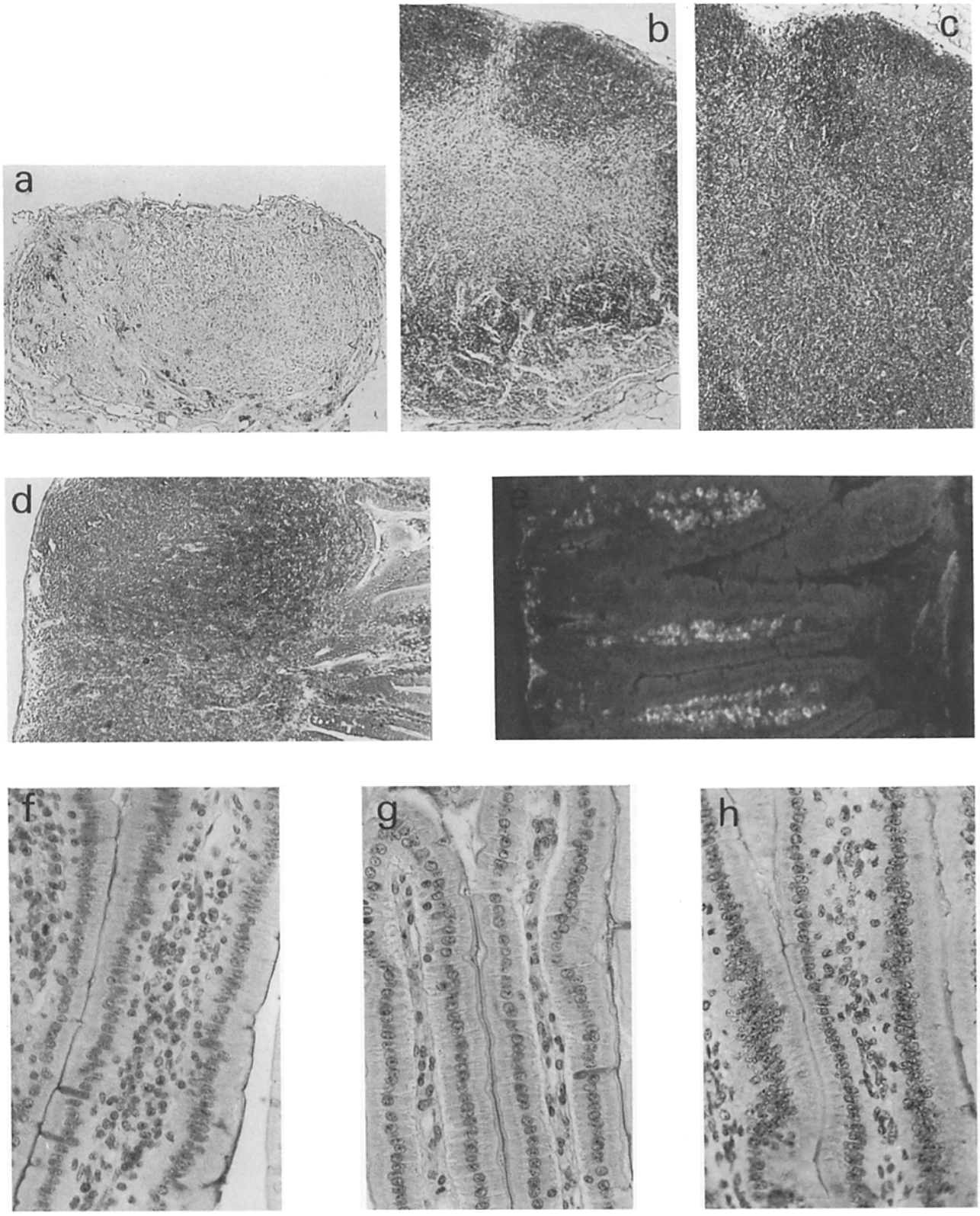

Figure 1. Histologic finding in various reconstituted $RAG^{-/-}$ mutant mice. (*Top*) (*a–c*) Brachial LNs. (*a*) Untreated mouse; the LN contains no lymphocytes. (*b*) Tx mouse injected with nude mice BM cells; lymphoid follicles are present. (*c*) Non Tx mouse, similarly treated; the LN is fully reconstituted as are the Peyer's patches. (*d*) Note the LN size increase from *a* to *c*; both LN and Peyer's patches show dense lymphocyte accumulation in the T and B cell areas and the presence of germinal centers in the follicles. (*e*) In the same mouse as shown in *c* and *d*, numerous IgA plasma cells are found in the lamina propria. (*Bottom*) Gut from (*f*); a non Tx mouse reconstituted with BM cells. (*g*) A Tx mouse similarly treated. (*h*) A non Tx mouse reconstituted with peripheral T cells; lymphocytes are rare only in the Tx mouse, both in the epithelium and in the lamina propria.

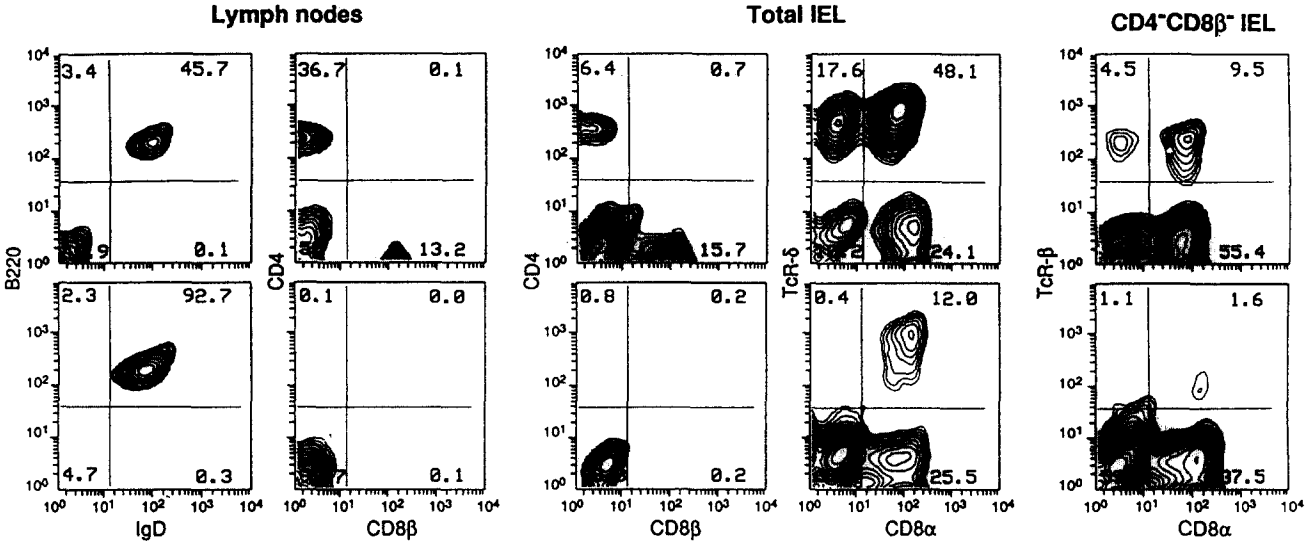

Figure 2. Flow cytometry analyses of cell populations from LN and gut IEL from a RAG$^{-/-}$ mouse reconstituted with nude mice BM cells. Non Tx (*top*) or Tx mice are shown.

to a complete reconstitution of all lymphoid organs in T and B cells, indistinguishable from the pattern observed in normal mice. The thymus, spleen, LN, and Peyer's patches (Fig. 1) had a normal histology, with lymphoid follicles containing germinal centers (Fig. 1). Peripheral CD4$^+$, CD8α/β^+ T lymphocytes, as well as B cells, were present in normal amounts (Fig. 2). Gut IEL had the same phenotypes and distribution as found in normal mice (Fig. 2). The IEL/epithelial cell ratio was comparable to that found in normal mice (~15%). The lamina propria was thick, containing lymphocytes and a normal number of IgA plasmocytes (about 40 per villus) (Fig. 1).

Injection of BM cells from nude mouse into RAG$^{-/-}$ mutant mice previously thymectomized led, 2 mo later, to a completely different pattern. Virtually all cells present in the LN (Fig. 2) or spleen expressed B cell markers, with no CD4$^+$ or CD8β^+ cells. On tissue sections, the T cell–dependent areas of the spleen and of the LN (Fig. 1) appeared empty, whereas lymphoid follicles were well formed but without germinal centers. Peyer's patches were not detectable. Very few IgA plasma cells were found in the gut wall (about 1 per villus). Gut IEL were present in reduced number, the ratio of IEL/100 epithelial cells being about 25% of that of reconstituted RAG$^{-/-}$ mice bearing a thymus (Fig. 1). FACS® analysis showed the lack of CD4$^+$ or CD8α/β^+ TCRα/β^+ IEL and of CD4$^+$CD8α/α^+ IEL, whereas on the contrary, CD4CD8β^-, TCRα/β^+ or γ/δ^+ IEL were present (Fig. 2), confirming that these IEL T cells can be generated in the absence of a thymus. These cells, however, were in reduced percentages compared to what was found in reconstituted mice bearing a thymus, the majority of IEL being TCR$^-$, CD8α/α^+ or $^-$. Thus, the phenotype of IEL in these mice was, qualitatively and quantitatively, similar to that observed in nude mice (2, 15, 16).

We then explored whether reconstituting RAG$^{-/-}$ mice with peripheral LN T cells of euthymic mice leads to the appearance of CD4$^+$CD8β^+ IEL. After the injection of 20 × 10^6 LN cells into these mice, the thymic structure was unchanged compared to that of uninjected RAG$^{-/-}$ control mice, with recovery of no more than 1–2 × 10^6 cells and

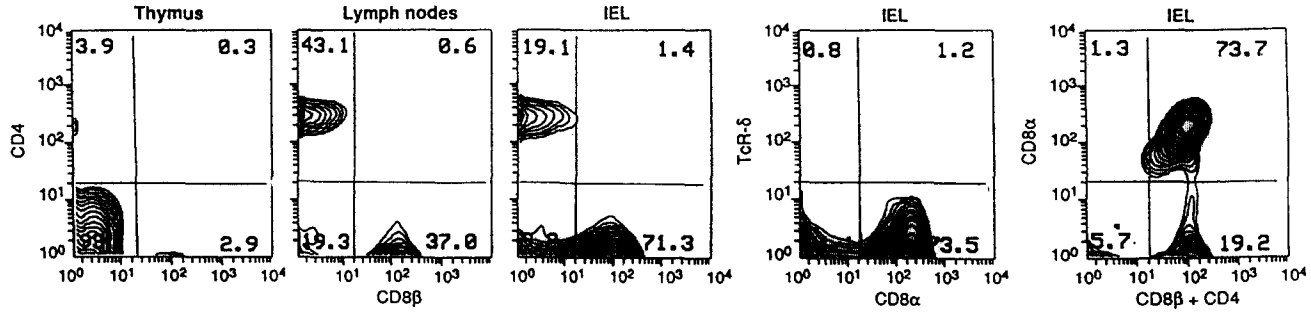

Figure 3. Flow cytometry of cell populations from the thymus, LN, and gut IEL from RAG$^-$ $^-$ mice injected with LN T cells from euthymic mice.

the presence of a few TCRα/β^{high} cells with a mature $CD4^+$ or $CD8\alpha/\beta^+$ phenotype (Fig. 3). In contrast, the spleen and LN contained numerous $CD4^+$ and $CD8\alpha/\beta^+$ cells (Fig. 3). In the gut, Peyer's patches without germinal centers were seen, and IEL (Fig. 1), present in normal amounts (~15% of the epithelial cells), were TCRα/β^{high} (data not shown) and $CD4^+$, $CD8\alpha/\beta^+$ or $CD4^+CD8\alpha/\alpha^+$. In contrast, no $CD4CD8\beta^-$ TCRα/β^+ or γ/δ^+ IEL were detected (Fig. 3). The TCR^- $CD8\alpha/\alpha^+$ IEL which are present in $RAG^{-/-}$ mutant mice were not observed, probably because their dilution by the IEL of donor origin made them undetectable. These results are in agreement with our previous experiments of reconstitution of the TCRα/β^+ $CD4^+$ or $CD8\alpha/\beta^+$ IEL populations in nude mice, injected with syngeneic LN T cells of euthymic mice (4).

Altogether, these experiments demonstrate that: (*a*) The thymus is absolutely required for the generation of $CD4^+$, $CD8\alpha/\beta^+$ or $CD4^+CD8\alpha/\alpha^+$ IEL bearing α/β TCR; (*b*) only these three subpopulations of IEL can be restored by the transfer of peripheral thymus–dependent T cells; and (*c*) other IEL populations may be generated in the absence of a thymus. Thus, the TCRα/β^+ $CD4^+$, $CD8\beta^+$ and $CD4^+CD8\alpha/\alpha^+$ IEL can be called thymus dependent (TD) and the others thymus-independent (TI). This dual origin of IEL is in keeping with their differences in TCR repertoire (1) and in signal-transducing modules (17).

Why can reconstitution experiments lead to misleading interpretations? We have previously shown that mature T lymphocytes have a large capacity to expand in mice lacking a thymus. For instance, a single CD4 mature T lymphocyte can generate in nude mice a progeny of as many as 8×10^5 cells, and injection of 100 $CD4^+$ or $CD8\alpha/\beta^+$ T cells is sufficient to reconstitute the peripheral T cell pool of a nude mouse (18). Peripheral T cell expansion requires antigen recognition (19, 20) and thus it may be easy to induce proliferation of TD IEL precursors in the Peyer's patches which are constantly stimulated by exogenous antigens. What could be, in reconstitution experiments, the origin of a small number of residual T cells which could be progressively amplified in Peyer's patches and lead to the accumulation of TD IEL? These cells could have remained in T cell–depleted BM, if as few as 100 thymus-derived cells are sufficient to eventually regenerate the TD IEL. Another unexpected source of T cell contaminants is represented by the small thymic remnants that we found in the majority of our Tx mice (not easily recognizable: see Materials and Methods). These remnants are colonized by BM precursors, and appear capable of producing the few thymic-derived cells that may be required for TD IEL repopulation. In mice with these remnants (whether $RAG^{-/-}$ mutants or lethally irradiated mice) we have indeed observed some mature T cells in the LN and numerous TD IEL of donor origin in the gut (our unpublished observations), in addition, in lethally irradiated mice, to the host IEL which resist irradiation, as mentioned above.

Finally, the role of the thymus in the expansion of TI IEL remains to be clarified, since, as mentioned, in nude mice and in neonatal Tx mice (21), as well as in the present experiments using Tx $RAG^{-/-}$ mice, TI IEL are markedly decreased in numbers compared to what is found in euthymic animals. Furthermore, in all these conditions the generation of TI IEL bearing α/β TCR remains very limited. It may be that the expansion of TI IEL is facilitated by cytokines released by TD-IEL. However, reconstitution of nude mice with peripheral T cells does not lead to significant modifications in TI IEL, arguing against a simple influence of that sort. The thymus could also act through the production of soluble factors, such as IL-7. It has been shown that implantation of a thymic stroma within a cell-impermeable chamber can increase some T cell populations in nude mice (22). Alternatively, immature precursors may reach not only the gut epithelium but also the thymus, in which they might expand, then migrate and complete their differentiation in the gut wall. Part of the TI IEL might be the progeny of TCR^{high} α/β or γ/δ DN thymocytes, a cell population that has some common characteristics with TI IEL. However, at least in adult mice, this population uses only ζ chain homodimeric modules, in contrast with TI IEL (17).

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