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Extrathymic T Cell Lymphopoiesis: Ontogeny and Contribution to Gut Intraepithelial Lymphocytes in Athymic and Euthymic Mice

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Abstract

In the absence of thymopoiesis, T lymphocytes are nevertheless present, mainly in the gut epithelium. Ontogeny of the extrathymic pathway and the extent of its involvement in euthymic mice are controversial. These questions have been addressed by assessing the expression of recombinase activating gene (RAG) through the use of green fluorescent protein RAG2 transgenic mouse models. In athymic mice, T lymphopoiesis occurs mainly in the mesenteric lymph node and less in the Peyer's patches. Ontogenic steps of this lymphopoiesis resemble those of thymopoiesis, but with an apparent bias toward $\gamma\delta$ T cell production and with a paucity of oligoclonal $\alpha\beta$ T cells possibly resulting from a deficit in positive selection. Whether in athymic or euthymic mice, neither T intraepithelial lymphocytes (IEL) nor cryptopatch cells (reported to contain precursors of IEL) displayed fluorescence indicating recent RAG protein synthesis. Newly made T cells migrate from the mesenteric node into the thoracic duct lymph to reach the gut mucosa. In euthymic mice, this extrathymic pathway is totally repressed, except in conditions of severe lymphocytic depletion. Thus, in normal animals, all gut T IEL, including CD8$\alpha\alpha^+$ cells, are of thymic origin, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ IEL being the likely progeny of double negative NK1-1$^-$ thymocytes, which show polyclonal Vα and Vβ repertoires.

Key words: T lymphocytes • extrathymic differentiation • gut intraepithelial lymphocytes • recombinase activating gene • mucosal immunity

Introduction

T cell lymphopoiesis can take place in the absence of a thymus because nude mice have T cells. This extrathymic lymphopoiesis, however, has special features. It populates mainly the gut mucosa leading to the accumulation of T intraepithelial lymphocytes (IEL).* It generates mostly $\gamma\delta^+$ T cells with $\alpha\beta^+$ T cells being scarce. In euthymic mice, on the other hand, T IEL are much more abundant and consist of $\gamma\delta^+$ T cells (30–40%) and $\alpha\beta^+$ T cells, not only CD4$^+$ or CD8$\alpha\beta^+$ but also CD8$\alpha\alpha^+$, which are a variety of $\alpha\beta^+$ T cells clearly identified only in the gut epithelium. The TCRs of this last population have a special recognition pattern directed mainly toward MHC I–like antigens (1–3) and also contain superantigen-reactive TCRβ chains (4), suggesting that these cells have not been submitted to negative selection. Because they do not appear to belong to the double positive (DP) main thymic pathway, we (4) and others (5) have called them "thymus-independent." However, experiments with grafts of fetal or neonatal thymus have suggested that at least part of these cells might be of thymus origin (6, 7). All of these observations have accredited the notion that in normal mice T IEL may have a dual, thymic and extrathymic, origin. It has been proposed that gut cryptopatches, small aggregates of mucosal lymphocytes

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**Abbreviations used in this paper:* DN, double negative; DP, double positive; GFP, green fluorescent protein; IEL, intraepithelial lymphocytes; LP, lamina propria; MLN, mesenteric lymph node; PP, Peyer's patches; SP, single positive; Tg, transgenic; TN, triple negative; Tx, thymectomized.

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at close contact of the epithelium (8), are the extrathymic source of lymphopoiesis contributing to $\gamma\delta^+$ and $\alpha\beta^+$ T IEL in euthymic or athymic mice (9, 10).

To identify lymphopoietic sites and analyze ontogenic pathways, occurrence of the cardinal event of B and T cell lymphopoiesis, namely the initiation of gene rearrangements allowed by synthesis of the RAG proteins, can be used as a landmark (11–13). To this end, animals used in this study were transgenic (Tg) and bore a green fluorescent protein (GFP) gene placed under the control of the RAG2 promoter (14). In such mice, high GFP-expressing cells (GFP^H) are in the process of rearranging Ig or TCR genes or will rearrange them shortly later. Cells with low fluorescence (GFP^L) represent previously GFP^H cells in which GFP synthesis has stopped such that the GFP protein is decaying. This feature allows to identify, among mature cells, those in which gene rearrangement has occurred recently (14). Nonfluorescent cells (GFP^-) of the T or B lineages are either very immature at a stage before RAG synthesis, or mature but distant from RAG synthesis. Observations made with this system allowed us to identify the sites of extrathymic T lymphopoiesis, determine the ontogenic pathways involved, and completely reassess thymic or extrathymic contributions to gut IEL in various conditions.

Materials and Methods

Animals. Euthymic Tg mice (FVB) were used (14) or bred with nude mice (Swiss nu/nu or Swiss nu/+), $TCR\delta^{-/-}$ (15), or $TCR\beta^{-/-}$ (16) mutant mice. Mice were maintained in sterile isolators at the Centre Des Techniques Avancées pour l'Expérimentation Animale, Orléans, France. Presence of the transgene was detected by blood cell analysis.

For reconstitution experiments, euthymic mice or thymectomized (Tx) mice 2 wk before transfer were 12 (FVB mice) or 6 Gy ($RAG\gamma c^{-/-}$; reference 17) irradiated and intravenously injected on the same day with 10^7 bone marrow cells of Tg nude mice.

Cell Isolation, Flow Cytometry, Sorting, PCR Analyses, and Studies of TCRβ Repertoire. Cells from the gut and the thoracic duct lymph were isolated as previously described (18). Lungs were treated as lamina propria (LP). Peripheral lymph nodes were pooled inguinal, brachial, and axillary lymph nodes. Isolated cells were three-color stained (FL-1 channel being used for detection of GFP) and analyzed with a FACSCalibur® (Becton Dickinson). The mAbs used were allophycocyanin or Phycoerythrin labeled, or were biotinylated and revealed with streptavidin PerCP (BD Biosciences or Bioscience). Minor populations were electronically gated during the acquisition process after staining by a combination of allophycocyanin-labeled mAbs (anti-CD3, anti-CD19, with or without the addition of anti-CD4, anti-CD8α).

For single cell RT-PCR, lymphocytes were sorted using an automatic cell deposition unit. Single cell RT-PCR was performed as follows: after sorting, each cell sample was deposited in 5 μl PBS and kept frozen at −80°C. For RT-PCR, cells were first disrupted by heating 2 for min at 70°C and 10 μl RT mixture containing a final concentration of 0.01 M DTT, 1 mM DNTP, 0.5 μg oligodT, 40 U RNase block, 200 U M-MLV (Invitrogen), and 3 pmoles of RAG1 antisense primer (GTGGATGGAGTCAACATCTGCCTT). 1× first strand buffer was added. After 1 h of incubation at 37°C, enzyme was inactivated during 10 min at 70°C. Subsequently, CD3ε, RAG1, and pre-Tα cDNAs were coamplified by two steps of semi-nested PCR. In the first round, 65 μl PCR mixture containing 3 pmoles of the primers RAG1-S CAAGCTGCAGACATTCTAGCACTC, CD3ε-S GCCTCAGAAGCATGATAAGC, CD3ε-AS1 CTTGGCCTTCCTATTCTTG, pre–Tα-S ATGGCTAGGACATGGCTGCTG, and pre–Tα-AS1 TCAGGAGCACATCGAGCAGAA, as well as 1× buffer, 0.2 mM DNTP, and 1.5 U AmpliTaq polymerase (Applied Biosystems) was added to the 15 μl RT reaction product. Touchdown PCR was performed in a thermal cycle (Applied Biosystems) under these conditions: 5 min denaturation at 94°C, 5 cycles consisting of 30 s denaturation at 94°C, 20 s at 68–60°C, 1 min at 72°C, and 25 cycles consisting of 10 s at 94°C, 20 s at 58°C, and 1 min at 72°C. PCR was completed by elongation at 72°C for 5 min. In a second round of PCR, each gene was amplified separately. 2 μl of the first PCR were added to 18 μl PCR mixture containing the same reagents used for the first PCR and nested antisense primers (RAG1-R2 GTCGATCCGGAAAATCCTGGCAAT, CD3ε-AS2 TGACCATCAGCAAGCCCAGA, and pre–Tα-AS2 GCAGAAGCAGTTTGAAGAGGAGC). PCR products were obtained by 5 and 35 cycles and performed with the same conditions as the first PCR. Reaction products were visualized by electrophoresis on a 1.5% agarose gel. Specificities of each primer amplification were verified by sequencing (Abi Prism, 3700 Genetic Analyser; Applied Biosystems).

TCRβ and TCRα repertoires were analyzed using the immunoscope technique as previously described (18, 19).

Analysis of Lymphocytes on Tissue Sections. For analyses of GFP^H cells, tissues were fixed for 24 h with 4% paraformaldehyde, dehydrated for 24 h in 10 and 20% sucrose solutions, and then embedded in Tissue Teck OCT (Sakuta Finetek) and frozen in liquid nitrogen. Cryostat sections were examined under a confocal microscope (Zeiss LSM 510 and Microscope Axiovert 200 M). For determination of the relative percentage in various animals of IEL, pieces of duodenum were embedded in paraffin and tissue sections were stained with periodic acid-Schiff. The number of IEL was expressed in percentage of villus epithelial cells.

Online Supplemental Material. A table including data from LP and thoracic duct lymph, from very young mice and from $TCR\beta^{-/-}$ mice, is available at http://www.jem.org/cgi/content/full/jem.20021639/DC1.

Results

Localization of T Lineage GFP^H Cells in Nude Mice: Mesenteric Lymph Nodes (MLNs) and Peyer's Patches (PP). The presence of GFP^H cells of the T lineage was first studied on cells obtained from nude mice between 18 d and 7 mo of age and isolated from a variety of sites: MLNs and peripheral lymph nodes, gut mucosa LP, which includes cells from PP and cryptopatches (8), gut epithelium, spleen, liver, lung, bone marrow, and peritoneal cavity. Thy-1^H GFP^H cells were consistently observed in MLN cells (in all 36 mice tested) and in gut LP cells (in all 10 mice tested), very rarely in peripheral lymph node cells (very few cells in 3 out of 16 mice tested), and never elsewhere (Table I and see Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1). No GFP^H cells of the B lineage were found, except for the bone marrow. Histologic sections of tissues showed clusters of GFP^H cells in the medulla of MLNs (Fig. 1 a) and rare single cells in interfollicular areas of PP (Fig. 1 b). Cryptopatches and gut epithelium did

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Table I. *Percentage of T Lymphocytes and GFP Expression*

	Recovery	Percent pro/pre-T GFP^H	DP GFP^H	$TCR\alpha\beta^+$ $CD4^+$		$TCR\alpha\beta^+$ $CD8\alpha\beta^+$		$TCR\alpha\beta^+$ DN or $CD8\alpha\alpha^+$		$TCR\gamma\delta^+$ DN or $CD8\alpha\alpha^+$	
Nude Mice (8–39 mice)											
MLN[a]											
<10 wk	9×10^6	0.04	0.4	0.9	*5.6*[b]	0.14	*7.9*	0		0.3	*19.6*
>10 wk	33×10^6	0.03	1.47	7.9	*2*	2.2	*1.8*	0		0.7	*4.4*
IEL[c]	5/100EC	0	0	2.8	*1.6*	1.3	*1.7*	2		52	*0.4*
Euthymic Mice Tx at Birth (6 mice)											
MLN											
<10 wk	6×10^6	not done	1	6.7	*1.2*	0		0		0.9	7
>10 wk	31×10^6	0.007	0.14	29.6	*0.7*	8.1	*2.2*	0		1.8	*2.8*
IEL	12/100EC	0	0	49	*0.1*	15.4	*0.1*	2.1	*0.2*	21	*0.1*
Euthymic Mice (12–15 mice)											
MLN[a]											
<10 wk	22×10^6	0	0	57.6	*36*	31.8	*32*	0.02	*25*[d]	0.8	*27*
>10 wk	23.5×10^6	0	0	55.7	*11*	17.9	*4.2*	0.06	7[d]	1	*12*
IEL[c]	22/100EC	0	0	11	*2.4*	32	*1.8*	11.7	*0.1*	36	*0.5*
$TCR\delta^{-/-}$ Euthymic Mice Tx at Birth (11 mice)											
MLN											
<10 wk	9×10^6	0.006	0.57	11	*2.4*	2	*4*	0		0	
IEL	9/100EC	0	0	21	*0.3*	12	*1.7*	2.9	*2*	0	
$TCR\delta^{-/-}$ Euthymic Mice (10 mice)											
MLN											
<10 wk	28×10^6	0	0	31	*36*	14	*24.5*	#0		0	
>10 wk	14×10^6	0	0	29	*10.7*	15.5	*4.8*	#0		0	
IEL	12/100EC	0	0	15.2	*4.7*	30.7	*2.4*	30.4	*0.1*	0	

EC, epithelial cells.

[a]MLN in nude mice contained >90% $CD19^+$ B cells (~10% being GFP^L) and in euthymic mice, for a comparable cell recovery, only ~20% (with a comparable fraction of GFP^L cells).

[b]Italics indicate the percentage of GFP^L cells in the cell category tabulated.

[c]Results of IEL from mice before and after 10 wk of age are pooled because their GFP expression did not vary significantly with age. In nude mice, up to 50% of IEL were $CD3^-$ and in euthymic mice ~5–10% (absolute numbers of $CD3^-$ IEL being comparable). Some of these cells were GFP^L and in this case were either CD19 B cells or belonged to a small population that could be made of pro-B cells ($CD19^-$, Thy-1^-, $B220^+$, ± $CD4^L$, without intracytoplasmic CD3ε chains; reference 43). These cells were also detectable in the LP.

[d]These DN $\alpha\beta^+$ lymphocytes were also studied in B6 mice in order to distinguish them from $NK1.1^+$ NKT cells. Note that the bone marrow (six mice studied) did not contain Thy-1^+ DP or $CD25^H$ lymphocytes and that GFP^H lymphocytes were $CD3^-$ $CD19^-$ and $B220^+$. <2% $TCR\gamma\delta^+$ lymphocytes were detectable, all GFP^-.

not contain any GFP^H cells (Fig. 1 c). Thus, in nude mice, lymphopoiesis takes place in MLNs and to a much smaller extent in PP.

Ontogeny of T Lineage GFP^H Cells in Nude Mice: $CD25^H$ Pro-Pre T Cells and DP $CD4^+$ $CD8^+$ Cells Resembling Their Thymus Counterparts. The ontogenic steps of this lymphopoiesis were then explored by surface phenotypic analysis of GFP^H cells on one hand and by GFP content assessment of all cells of T lineage on the other, comparing each of these steps with those of thymic lymphopoiesis (Fig. 2). Quantitative analysis of these cells and other MLN cells is shown in Table I as well as Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1. All GFP^H cells were Thy-1^H and fell into two categories: ~97% were $CD4^+$ $CD8\alpha\beta^+$ (DP) and 2–3% were $CD3^-$ $CD4^-$ $CD8^-$ (triple negative [TN]) and $CD19^-$ $CD25^H$ $CD44^+$ or $CD44^-$, i.e., comparable to thymocytes at the double negative (DN) DN2 ($CD25^H$ $CD44^+$) and DN3 ($CD25^H$ $CD44^-$) stages, which can be collectively referred to as pro-pre T cells. Analysis of MLN DP cells (Fig. 2 a) showed that they were similar to their thymic counterparts (Fig. 2 c). All were GFP^H with low levels of surface TCRβ chains and were $CD25^-$, HSA^+, c-Kit^-, IL7-R^- (not depicted). Analysis of MLN TN cells (Fig. 2 b) showed $CD25^H$ cells that were DN2 ($CD44^+$) or DN3 ($CD44^-$). Virtually all DN3 cells were GFP^H as in the thymus. All other TN cells that were not $CD25^H$ were GFP^-. There was a population of $CD25^L$ cells (3–15% of all TN cells)

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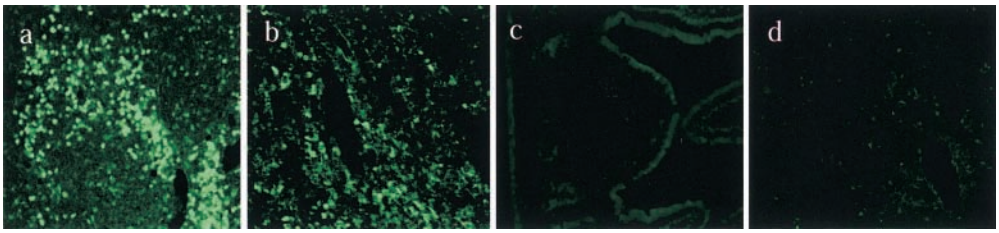

Figure 1. GFP^H lymphocytes in tissue sections from nude or euthymic mice. (a) MLN, nude mouse: clusters of fluorescent GFP^H cells located in some medullary cords (empty spaces are lymphatic vessels of the medulla). (b) PP, nude mouse: rare GFP^H cells located in an interfollicular T area near lymphatic vessels. (c) Cryptopatch, nude mouse: lack of GFP^H cells in the patch and adjacent epithelium (~10 cryptopatches were explored in various mice). (d) MLN, euthymic mouse: absence of GFP^H cells in the medulla. ×70.

not found in TN thymocytes (Fig. 2, compare b with d). These cells belonged to a distinct lineage because they were Thy-1$^{+/-}$, c-Kit$^+$, IL7-R$^+$, and by RT-PCR on 22 individual cells, found not to express RAG1, pre-Tα, and CD3ε chain mRNAs (not depicted). This is the phenotype of cryptopatch cells (8, 20) to which these cells could be related. Finally, ~60% of CD25$^-$ CD44$^+$ cells (DN1) bore markers of NK cells (not depicted).

More precise comparison of MLN TN CD25^H cells with thymocytes at the DN2 and DN3 stages revealed differences between thymus and MLNs. The following data suggested that the maturation process was different, probably slower in extrathymic T lymphopoiesis, and somewhat biased toward γδ chain synthesis (21–22). The ratio of DN2 to DN3 cells was much higher in MLNs than in thymus (1:1 vs. 1:5) and GFP^H cells were fewer in the DN2 MLN cells than in DN2 thymocytes (Fig. 2, b and d, middle panels; comparable results in six comparative studies). Thus, there was an increased proportion of less differentiated cells, less commonly showing GFP accumulation in extrathymic lymphopoiesis. CD25^H cells contained four times more c-Kit$^+$ or IL7-R$^+$ cells ("transitional pro-T cells") in MLNs (c-Kit$^+$/42%, IL7-R$^+$/25%) than in thymus (c-Kit$^+$/9%, IL7-R$^+$/6%; Fig. 2, b and d, right panels; five comparative studies), consistent with an increased accumulation of less differentiated cells in extrathymic T lymphopoiesis. The possibility that this form of T lymphopoiesis was somewhat biased toward γδ chain synthesis was suggested by single cell RT-PCR analysis of DN2-DN3 cells isolated from MLNs and thymus for expression of RAG1 and pre-Tα transcripts: of RAG1-expressing cells, fewer coexpressed pre-Tα (i.e., were likely to become αβ$^+$ cells) in MLN cells (25 cells out of 62 studied, 40%) than in thymocytes (50 cells out of 72, 70%; $P < 0.001$ test $\times^2$).

Similar analysis of gut LP cells gave closely comparable results, except that the numbers of DP and CD25^H pro-pre T cells, almost all GFP^H, were very small (Fig. 2, f and g, and Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1). A point of special interest was that cryptopatch cells, identified among LP cells by their Thy1$^{+/-}$ CD44$^+$ c-Kit$^+$ IL7R$^+$ CD25$^{L/-}$ phenotype (8, 20), were all GFP$^-$ (Fig. 2 h). This indicates that cryptopatches are not lymphopoietic sites. They may, however, contain very early progenitors before synthesis of RAG

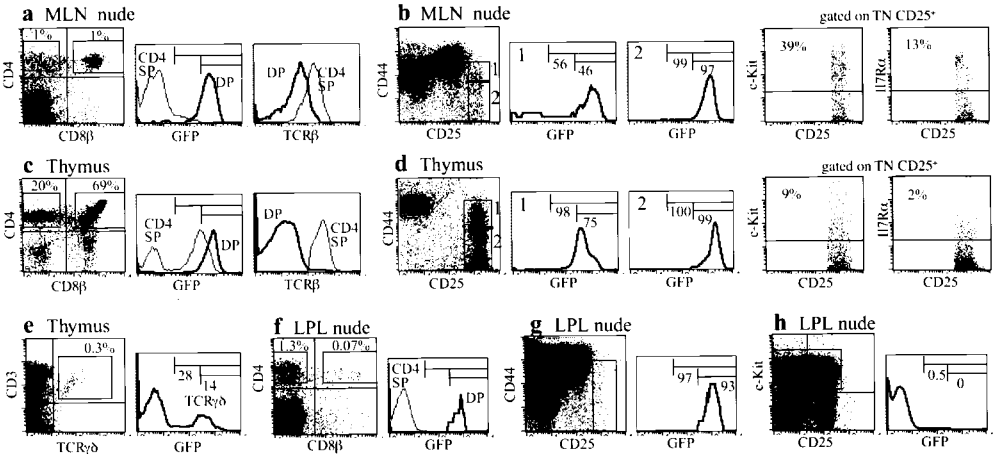

Figure 2. Analysis of GFP expression during T lymphopoiesis in athymic mice and thymopoiesis. (a–d) MLN (a and b) and thymus (c and d). (a and c) Comparative features of DP and SP cells analyzed for GFP (middle histograms) and TCRβ (right histograms) expressions. Note that DP cells express less TCRβ chains than SP and that there is a small difference between thymus and MLN DP cells in this respect. (b and d) TN CD25^H cells (after gating out CD3$^+$, CD4$^+$, CD8$^+$, and CD19$^+$ cells) analyzed for GFP content of CD44$^+$ DN2 (middle histograms) and CD44$^-$ DN3 cells (right histograms). All other TN cells were GFP$^-$ and most of the MLN CD25^L cells had the phenotype of cryptopatch cells (reference 8). Lack or rarity of DN4 cells (CD44$^-$ CD25$^-$) results from gating out CD3 and coreceptors bearing cells with potent antibodies binding even cells with trace amounts of these molecules, as is the case of most DN4 cells. The percentages of c-Kit$^+$ and IL7-Rα$^+$ in CD25$^+$ TN MLN cells and thymocytes are shown in the right panels. (e) Thymus: γδ$^+$ cells analyzed for GFP content. (f–h) LP lymphocytes analyzed for GFP content. (f) DP cells are GFP^H and (g) TN CD25^H cells (after gating out of cells as described above) are GFP^H. (h) TN c-Kit$^+$ CD25$^{+/-}$ cells, belonging to a population of CD44$^+$ Thy-1$^{+/-}$ IL7-R$^+$ CD4$^{+/-}$ cells (not depicted), i.e., with the phenotype of cryptopatch cells (references 8 and 20), are GFP$^-$.

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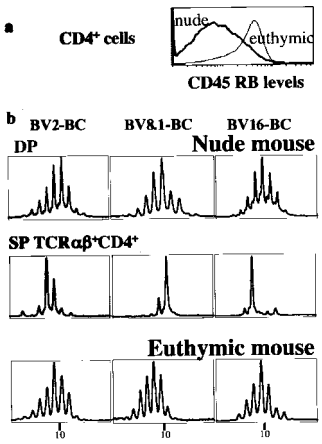

Figure 3. Exploration of the memory phenotype and of the TCRβ repertoire of MLN $\alpha\beta^+$ $CD4^+$ cells of nude and euthymic mice. (a) MLN $CD4^+$ cells from a nude mouse express the phenotype of memory cells, in contrast with comparable cells from an euthymic mouse. Cells were triply labeled with anti-TCRβ, anti-CD4, and anti-CD45RB mAbs. (b) The TCRβ repertoire of MLN DP cells from a nude mouse shows the gaussian-like profile characteristic of polyclonal populations (top), whereas that of SP $CD4^+$ cells is oligoclonal (middle), in contrast with that of comparable cells from an euthymic mouse (bottom).

proteins, with TCR rearrangements occurring after the entrance of these cells in the adjacent epithelium (10). This hypothesis is not consistent with the lack of GFP expression in IEL described below.

Mature T Cells in MLNs of Nude Mice. In nude mice, MLNs and LP also contained small numbers of mature $CD3^+$ T cells (Table I and Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1).

Their GFP content was compared with that of thymocytes with the same phenotype. Among MLN $\gamma\delta^+$ cells, ~30% were GFP^L in young mice (see Fig. 5 a) whereas in the thymus, about half of the GFP^+ $\gamma\delta^+$ cells were GFP^H (compare Fig. 5 a with Fig. 2 e), which suggests that $\gamma\delta^+$ cells produced leave the thymus more rapidly (explaining the predominance of very young cells in this location). The proportion of GFP^L $\gamma\delta^+$ in MLNs declined with age (Table I), which is consistent with observations described below that GFP^L $\gamma\delta$ cells leave MLNs to accumulate in the gut as GFP^- IEL. Regarding $TCR\alpha\beta^+$ single positive (SP) cells, either $CD4^+$ (by far the most numerous) or $CD8\alpha\beta^+$, a much lower number were GFP^+ than their thymic counterparts (Fig. 2, compare a and f to c). With age, the populations of SP cells increased but their content in GFP^L cells still decreased (Table I). This increasing paucity of GFP^L SP cells did not correspond to a weakening lymphopoiesis with decrease of RAG synthesis. On the contrary, it was accompanied by an increased percentage of GFP^H DP cells (Table I). This suggested a severe impairment in the maturation of DP precursors into GFP^L SP cells. The progressive accumulation of SP GFP^- cells might represent, in this light, the expansion of restricted populations of GFP^- memory cells. Large expansion of a few memory cells is indeed known to occur in lymphopenic mice (for review see reference 23). Two observations strongly support this interpretation. First, most of these $CD4^+$ T cells had the $CD45RB^L$ phenotype of memory cell (24), which is in contrast with comparable MLN cells of euthymic mice (Fig. 3 a). Second, the exploration of the TCRβ chain repertoire of MLN DP and $CD4^+$ cells showed that DP cells expressed a polyclonal repertoire, but $CD4^+$ cells only showed a very limited oligoclonal repertoire. Furthermore, comparable T cells in euthymic mice had a polyclonal repertoire (Fig. 3 b and reference 18). These contrasting repertoires can best be explained by a markedly decreased positive selection in MLN lymphopoiesis of nude mice. MLNs indeed lack the thymus cortical epithelial cells that appear to play a major role in positive selection of DP thymocytes (25). Along this line, the percentage of DP $CD4CD8\alpha\beta^L$ and $CD4^+$ MLN cells expressing CD69, a marker of ongoing MHC selection (26), was half that of the equivalent population of thymocytes (not depicted).

Lack of Extrathymic T Lymphopoiesis in Euthymic Mice, Except in Case of Severe Lymphocyte Depletion. In euthymic mice Tx at birth (including $TCR\gamma\delta^{-/-}$ mutant mice, described later), GFP^H DP (Fig. 4 a) and DN2-3 pro-pre T cells were identified in MLNs (Table I). However, MLNs also contained a markedly higher proportion of $\alpha\beta^+$ $CD4^+$ cells than found in nude mice (compare Fig. 4 a to Fig. 2 a), progressively increasing with age (Table I). Because these last cells were all GFP^- (Fig. 4 a), they probably expanded from of population of cells released from the thymus before thymectomy.

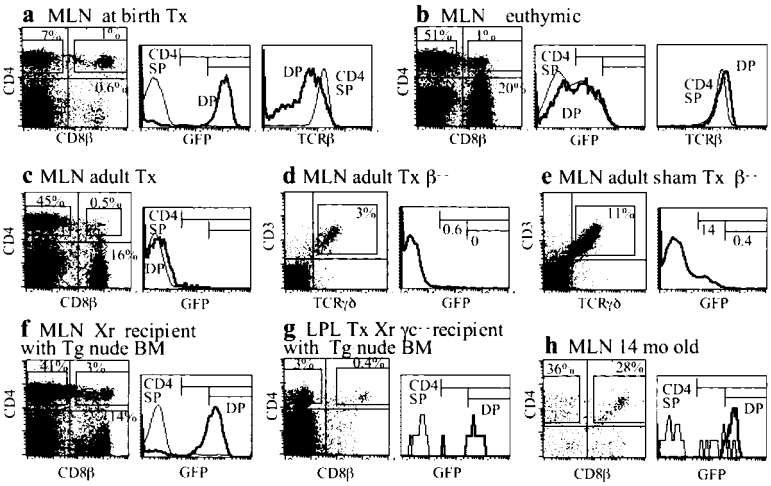

Figure 4. Analysis of lymphopoiesis in various Tx or euthymic mice. (a) MLN cells, mouse Tx at birth (compare with Fig. 2 a). (b) MLN cells, 7-wk-old euthymic mouse, sham Tx (match of mouse with adult Tx shown in c). Presence of some DP with GFP and TCRβ expression similar to that of SP $CD4^+$ cells (right panels). Note that many $CD4^+$ cells are GFP^L. (c) MLN, 7-wk-old mouse 2 wk after thymectomy. All DP and SP cells are GFP^- (compare with b). (d and e) MLN cells from 8-wk-old Tg $TCR\beta^{-/-}$ mice, Tx (d) or sham Tx (e) 2 wk earlier, used for easy study of $\gamma\delta^+$ cells. GFP^L cells are absent in the Tx mouse. (f) MLN cells of lethally irradiated non-Tg mouse reconstituted 18 d earlier with bone marrow cells from a nude Tg mouse. DP cells are GFP^H. Note that MLN cell recovery was 10-fold less than that from normal mice. (g) Lymphocytes isolated from the LP of a Tx $RAG\gamma c^{-/-}$ mutant mouse reconstituted 36 d earlier with nude Tg mouse bone marrow cells (note that some $CD4^+$ GFP^- cells, originating from the graft, are detectable). (h) MLN cells from a 14-mo-old mouse (cell recovery was ~20-fold less than in younger mice). DP cells with GFP expression comparable to that of DP thymocytes.

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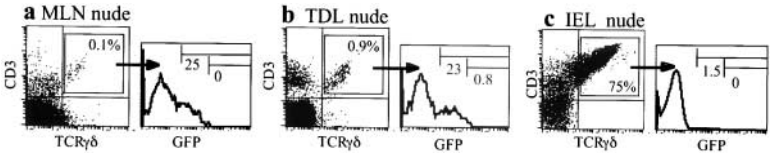

Figure 5. GFP^L $\gamma\delta^+$ T cells in nude mice; comparison between MLN, circulating cells, and IEL. $\gamma\delta^+$ T from MLN (a), thoracic duct lymphocytes (TDL; b), and IEL (c) analyzed for GFP expression.

In sharp contrast, in non-Tx mice (15 mice studied between 23 d and 5 mo), GFP^H cells, either DP or pro-pre T, were never observed in MLNs (Table I) nor PP (Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1), nor were they detected in tissue sections of MLNs (Fig. 1 d) and gut. Some DP cells were found in MLNs, but they had the features of mature T cells, in TCRβ expression and in low GFP content (Fig. 4 b). On the other hand, GFP^L cells were observed at all times among $\gamma\delta^+$ and $\alpha\beta^+$, $CD4^+$ or $CD8\alpha\beta^+$ mature cells, in higher percentages in the first 10 wk of life (for some mice, up to 50% of each of the three populations) than later (Table I and Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1). Because local GFP^H precursors were absent, these GFP^L T cells appeared to result from recent thymic emigration. To assess this point, adult mice were Tx or sham-operated between 7 and 11 wk of age (seven pairs), and their MLN cells studied between 8 d and 3 mo later. GFP^L T cells, both αβ and γδ (studied in mutant $TCR\beta^{-/-}$ mice, see legend of Fig. 4) disappeared in Tx mice (Fig. 4, c–d) and not in sham-operated controls (Fig. 4, b–e), demonstrating a continued contribution of recent GFP^L thymus-derived cells to MLN T cells. Pro-pre T and DP GFP^H cells remained undetectable under both conditions. This indicates that the lack of extrathymic T lymphopoiesis in euthymic mice does not result from the presence of a thymus per se. It appears to result from the presence of $\alpha\beta^+$ T cells because extrathymic lymphopoiesis was not found in mutant $TCR\delta^{-/-}$ mice, which have only $\alpha\beta^+$ T cells whereas euthymic mutant $TCR\beta^{-/-}$ mice, which have only $\gamma\delta^+$ T cells, had GFP^H pro-pre T cells in MLNs and PP (see Table I and Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1). This suggests that the accumulation, starting very early in life, of thymus-derived αβ T cells in MLNs and PP may inhibit local lymphopoiesis. The possibility of an inhibitory effect was first explored by reconstituting non-Tg recipient mice lacking lymphocytes, either as the result of lethal irradiation or because of a $RAG\gamma c^{-/-}$ mutation (17), with bone marrow cells of GFP Tg nude mice. Some of these recipients had been Tx 2 wk before transfer to prevent thymopoiesis. In all four mice of each group killed 3–4 wk after transfer, GFP^H pro-pre T and DP cells were found in the MLNs (Fig. 4 f) and/or LP ($RAG\gamma c^{-/-}$; Fig. 4 g), providing conclusive evidence that extrathymic T lymphopoiesis may take place even in euthymic adult mice, but in pathological conditions. Second, it was explored whether in aged euthymic mice with marked thymic atrophy, some extrathymic T lymphopoiesis may be detected. In three out of six 14-mo-old mice (thymus and MLN cell recovery ~10 and 2% of young mice, respectively), GFP^H DP cells were found, in two cases in MLNs (Fig. 4 h and see pattern resemblance with young thymus in Fig. 2 c), and in one case in PP (very atrophic; not depicted).

Contribution of Thymic and Extrathymic Lymphopoiesis to Gut T IEL. In nude mice, some mature T cells are found in small amounts in all lymphoid organs and in liver, but the only site where they accumulate is the gut epithelium, as IEL. Euthymic mice, on the other hand, have four to five times more IEL than nude mice (27), all of thymic origin because extrathymic lymphopoiesis is lacking or its contribution is insignificant. These different origins of IEL under these two conditions raise interesting questions. Do T cells maturing in MLNs and PP reach the gut epithelium by different routes than thymus-derived cells? Why do T IEL of nude and euthymic mice widely differ in subpopulation composition?

In athymic or euthymic mice, T IEL were GFP^-, whatever their phenotype, even in young adult mice, when the corresponding MLN populations, $\gamma\delta^+$ or $\alpha\beta^+$, were ~30% GFP^L (Table I and Fig. 5, a and c). This suggests that recent GFP^L T cells do not rapidly enter the gut epithelium but become IEL after some delay during which their residual GFP disappears. In euthymic mice, it is well documented that $\alpha\beta^+$ IEL can result from seeding of the gut epithelium by blood-borne cells of MLN and PP origin, which reach the blood through their release from these lymphoid structures into the thoracic duct lymph. In addition to gut epithelium, these cells also return to MLNs and PP and can circulate for several rounds before becoming IEL (18). To explore if recent GFP^+ T cells may follow such a traffic, the thoracic duct of 7-wk-old nude and euthymic mice were canulated overnight and lymph cells were analyzed for the presence of $\gamma\delta^+$ or $\alpha\beta^+$ GFP^+ cells. In nude mice, $TCR\gamma\delta^+$ lymph cells (1% of collected cells, Fig. 5 b compared with MLN in a and IEL in c) and in euthymic mice $TCR\alpha\beta^+$ lymph cells as well, $CD4^+$, $CD8^+$, or DN (discussed below) contained 15–30% of GFP^+ cells, these percentages decreasing with age (Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1). This is consistent with the hypothesis that recent T cells of the gut lymphoid system, whether locally produced by extrathymic lymphopoiesis or of thymic origin, follow a lymph-blood circuit before becoming IEL. This also argues strongly against the idea that some IEL may result from rapid crossing of the gut mucosal basement membrane by early GFP^+ T cells or precursors emerging from mucosal lymphoid structures, such as cryptopatches (10) or PP.

Judged from quantitative and qualitative analysis of T IEL in euthymic and athymic mice (Table I), extrathymic T lymphopoiesis is approximately ~4 times less efficient

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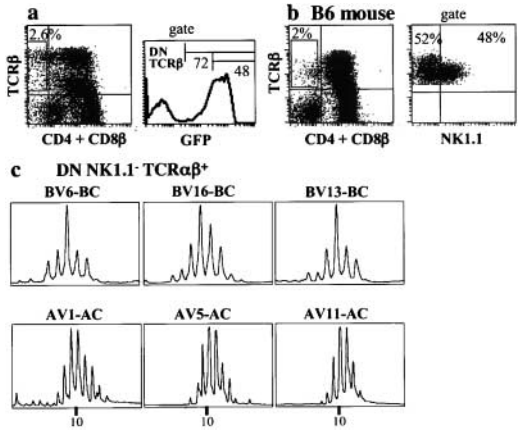

Figure 6. Analyses of DN $\alpha\beta^+$ thymocytes. Studies of (a) GFP content and (b) NK1-1 expression (in this last case, cells were from a B6 mouse because the Tg mice do not express NK1.1). (c) TCRα and β repertoires of DN NK1.1$^-$ thymocytes: polyclonal repertoires expressing chains different from that peculiar to NKT cells.

than thymopoiesis at populating the gut epithelium with $\gamma\delta^+$ IEL, but 30–40 times less efficient for $\alpha\beta^+$ IEL. In euthymic mice, however, $\alpha\beta^+$ IEL not only consist of CD8$\alpha\beta^+$ and CD4$^+$ cells but also of DN and CD8$\alpha\alpha^+$ populations (here collectively referred to as CD8$\alpha\alpha^+$). CD8$\alpha\alpha^+$ IEL are at least as severely depleted as $\alpha\beta^+$ CD8$\alpha\beta^+$ and CD4 IEL in athymic mice. This was most clearly seen here with IEL from TCR$\delta^{-/-}$ mutant mice, in which, in the absence $\gamma\delta^+$ IEL, $\alpha\beta^+$ CD8$\alpha\alpha^+$ IEL may represent as much as 30% of all IEL. After neonatal thymectomy, this population was by far the most strongly decreased (more than 10 times in absolute numbers) of the $\alpha\beta^+$ IEL populations (Table I). This is compelling evidence that most of the $\alpha\beta^+$ CD8$\alpha\alpha^+$ IEL observed in euthymic mice derive from the thymus (for review see reference 28), as was also recently suggested by studies performed with mice bearing Tg αβ TCRs (29, 30).

What could be the thymic precursors of $\alpha\beta^+$ CD8$\alpha\alpha^+$ IEL, and why is extrathymic T lymphopoiesis so inefficient at generating these IEL? There is a small population of DN $\alpha\beta^+$ thymocytes, mostly GFP^H (Fig. 6 a), which consists of classical NKT cells (NK1.1$^+$ expressing canonical Vα14 chains together with some polyclonal but restricted Vβ chains; for review see reference 31) and of NK1.1$^-$ cells (Fig. 6 b, right) with a polyclonal α and β chain repertoire (Fig. 6 c). These last cells are likely precursors of the CD8$\alpha\alpha^+$ IEL, which display diverse, though oligoclonal, TCR α and β chain repertoires (32 and unpublished data). If ligands to their peculiar αβ receptors were expressed mainly in the thymus and on the gut epithelium and not elsewhere, as appears to be the case for some MHC I–like antigens, these cells could not be effectively selected during extrathymic T lymphopoiesis, explaining their rarity in nude mice. In euthymic mice, DN $\alpha\beta^+$ cells of thymic origin would not accumulate significantly in MLNs, explaining their paucity in this location (Table I), but rapidly leave in the thoracic duct lymph (which contained 0.2–0.6% of cells with this phenotype; Table S1, available at http://www.jem.org/cgi/content/full/jem.20021639/DC1) to reach the gut epithelium and acquire CD8αα dimers.

Discussion

The use of a RAG-GFP transgene introduced into mice of different genetic backgrounds allowed us to identify cells of the T lineage at three different steps of their ontogeny and life history: close to the time of RAG proteins synthesis (GFP^H cells), recently matured virgin T cells (GFP^L cells), and older virgin or memory T cells (GFP$^-$ cells). This interpretation was validated by the study of thymocytes and MLN T cells in euthymic mice. Among these last cells, GFP^L T cells interpreted as resulting from recent ongoing thymus emigration indeed disappeared rapidly and selectively after adult thymectomy.

The search for Thy-1$^+$ GFP^H cells in athymic mice (nude or Tx at birth) led to identify MLNs as the major site of extrathymic T lymphopoiesis and PP as a minor site, with ontogenic steps of pro-pre T cell and DP cells corresponding, perhaps not so surprisingly, to those found in the thymus but with two distinctive features: some apparent bias of pro-pre T cells toward γδ chain synthesis, perhaps resulting from a different local exposure to some critical cytokines (e.g., IL-7), and a marked oligoclonality of mature αβ T cells as judged by their TCRβ repertoire, contrasting with the polyclonality of the repertoire of comparable cells in euthymic mice and best explained by a decreased process of positive selection of DP cells. This may reflect the lack of local cells especially effective for this process, such as the thymus cortical epithelial cells. On the other hand, cryptopatches in situ and cells isolated from LP cells with the phenotype of cryptopatch cells were all GFP$^-$, as were gut T IEL, providing no evidence for a lymphopoietic process involving cryptopatch cells migrating into the gut epithelium to undergo gene rearrangement and maturation into T IEL (9).

Because extrathymic lymphopoiesis in nude mice contributes mainly to gut IEL, and because in euthymic mice some $\alpha\beta^+$ subpopulations of T IEL do not appear to originate from the main DP thymic pathway, one is generally inclined to believe that the peculiar complexity of gut T IEL reflects a dual origin from thymus-derived cells and from an extrathymic, locally adapted, form of lymphopoiesis. Thus, it came as a surprise to observe that extrathymic T lymphopoiesis is shut off in normal euthymic mice and can resume or be unmasked only in conditions of severe lymphocytic depletion (as the result of irradiation or genetic defect), total lack of $\alpha\beta^+$ T cells (in TCR$\beta^{-/-}$ mice), or marked thymic atrophy (in old mice). This raised interesting questions.

Why is extrathymic lymphopoiesis inhibited whereas MLNs contain large amounts of thymus-derived cells, many, especially in young mice, recently released from the thymus as indicated by their GFP^L content? Inhibition appears to result from the presence of $\alpha\beta^+$ T cells because it was complete in mutant TCR$\delta^{-/-}$ mice, which contain only $\alpha\beta^+$ T cells (where it was relieved, as in normal mice, by neonatal thymectomy). Inhibition of extrathymic lymphopoiesis may result from competition for some local cy-

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tokines (23) between $\alpha\beta^+$ T cells of thymic origin and extrathymic T cell progenitors. It is striking in this respect to note that when euthymic mice are exposed to increased levels of oncostatin M, a massive extrathymic T lymphopoiesis develops in MLNs accompanied by thymic atrophy (33, 34).

What is the ontogeny of the CD8$\alpha\alpha^+$ $\alpha\beta^+$ T IEL? Because, in normal euthymic mice, all T IEL including $\gamma\delta^+$ and CD8$\alpha\alpha^+$ $\alpha\beta^+$ IEL are of thymic origin, it follows that the complexity of gut T IEL populations in normal conditions reflects a complexity of thymic lymphopoiesis that had been described so far only for Vα14$^+$ NKT cells, which are not significantly found among IEL (31). There also exists a small population of DN $\alpha\beta^+$ NK1.1$^-$ thymocytes with a polyclonal TCR β and α repertoire (Fig. 6), and these cells contain likely precursors of the gut CD8$\alpha\alpha^+$ $\alpha\beta^+$ IEL. It is of interest that the two subpopulations of CD8$\alpha\alpha^+$ IEL, $\gamma\delta^+$ and $\alpha\beta^+$, share special features absent in CD4$^+$ and CD8$\alpha\beta^+$ IEL. They display NK cytotoxic abilities and $\alpha\beta^+$ IEL bear Ly49 NK receptors (we have proposed that they be considered as "gut NK-T cells"; reference 35). In addition, they use both ζ and γFcεRI chains as CD3-associated signal transmitting module (36, 37). By intracellular staining, we have recently observed that DN NK1.1$^-$ TCR$\alpha\beta^+$ and $\gamma\delta^+$ thymocytes all contain ζ chains, with a small minority of cells also containing γFcε-RI chains. In IEL, in contrast, virtually all cells of the two CD8$\alpha\alpha^+$ populations contain both chains whereas CD4$^+$ and CD8$\alpha\beta^+$ cells contain only ζ chains (unpublished data). Thus, it appears that it is in the gut epithelium that cells of DN thymus origin acquire full expression of γFcεRI chains. Stimulation by cytokines released from the gut epithelium (e.g., IL-15 and IL-7; reference 38) might be necessary for the survival of these CD8$\alpha\alpha^+$ populations because they are severely and selectively depleted in IL15$^{-/-}$ or IL2-R$\beta^{-/-}$ mutant mice (39, 40). All of these features would now appear as hallmarks of an origin from a DN pathway of differentiation, perhaps with recognition of peculiar ligands, rather than from an extrathymic site of lymphopoiesis as we previously postulated (41).

Finally, why are all T IEL GFP$^-$, whereas GFP^L T cells are present in MLNs? This indicates that colonization of the gut epithelium is not occurring early after T cell maturation. Presence of GPL^L T cells of various phenotypes circulating in the thoracic duct lymph, which drains MLNs, suggests that it is during lymph-blood circuits (known to be a step in the colonization of the gut epithelium by T cells; reference 18) that GFP disappears from recently generated T cells released from MLNs, including in nude mice.

In conclusion, extrathymic lymphopoiesis is a minor pathway of T cell differentiation, taking place mostly in the MLNs and operative only in conditions of defective thymopoiesis but with comparable ontogenic steps. This explains why in nude mice antigenic stimulation may lead to the production of small numbers of specific T cells displaying MHC restriction (42).

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