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Intestinal CD8$\alpha\alpha$ and CD8$\alpha\beta$ Intraepithelial Lymphocytes Are Thymus Derived and Exhibit Subtle Differences in TCRβ Repertoires[1]

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Intraepithelial lymphocytes (IEL) of the small intestine are anatomically positioned to be in the first line of cellular defense against enteric pathogens. Therefore, determining the origin of these cells has important implications for the mechanisms of T cell maturation and repertoire selection. Recent evidence suggests that murine CD8$\alpha\alpha$ intestinal IELs (iIELs) can mature and undergo selection in the absence of a thymus. We analyzed IEL origin by cell transfer, using two congenic chicken strains. Embryonic day 14 and adult thymocytes did not contain any detectable CD8$\alpha\alpha$ T cells. However, when TCR$^+$ thymocytes were injected into congenic animals, they migrated to the gut and developed into CD8$\alpha\alpha$ iIELs, while TCR$^-$ T cell progenitors did not. The TCR Vβ1 repertoire of CD8$\alpha\alpha^+$ TCR Vβ1$^+$ iIELs contained only part of the TCR Vβ1 repertoire of total iIELs, and it exhibited no new members compared with CD8$^+$ T cells in the thymus. This indicated that these T cells emigrated from the thymus at an early stage in their developmental process. In conclusion, we show that while CD8$\alpha\alpha$ iIELs originate in the thymus, T cells acquire the expression of CD8$\alpha\alpha$ homodimers in the gut microenvironment. *The Journal of Immunology,* **2000, 165: 6716–6722.**

The intestinal epithelium harbors a large number of $\alpha\beta$ and $\gamma\delta$ TCR-positive lymphocytes functioning as the first line of defense against enteric pathogens. Although most peripheral T cells arise and expand in the thymus before they migrate to the periphery, it was proposed that the intestinal epithelium contains a thymus-independent T cell population that differentiates according to the epithelial microenvironment (1–4). These cells express the coreceptor CD8α as a homodimer, whereas thymus-derived T cells bear CD8$\alpha\beta$ heterodimers as well as CD8$\alpha\alpha$ homodimers (5–9). This unusual CD8$\alpha\alpha$ expression is also shared by an intestinal TCR$^+$, NK-like cell population (10–12). The experiments suggesting an extrathymic origin for TCR$^+$ cells were conducted in immunocompromised mice (1, 2). However, in sheep, thymectomy in utero results in severe persistent depletion of peripheral T cells and a dramatic drop in the number of intraepithelial lymphocytes (IELs)[4] in the intestine of lambs during the first year of life (13). In athymic mice the IEL population is only 20% of normal numbers, and reconstitution can be obtained by thymus grafts (3). Current experiments in chickens show that TCR$\gamma\delta^+$ thymocytes home to the intestine, whereas a TCR$\gamma\delta^-$ thymocyte population enriched for hemopoietic progenitors fails to give rise to $\gamma\delta$ IELs (14, 15). Other chicken hemopoietic tissues, such as bone marrow and spleen, were also ineffective sources for $\gamma\delta$ or $\alpha\beta$ intestinal IELs (iIELs) (14, 16, 17). Therefore, these data showed that iIELs in the chicken are primarily derived from the thymus.

The chicken $\alpha\beta$ and $\gamma\delta$ TCR proteins closely resemble those of mammals (10, 16, 18–22). The variable TCR β-chain comprises the two major gene families Vβ1 and Vβ2, and T cells expressing either of these genes differ in their ontogeny and function (19, 20). Vβ2 T cells appear later in the thymus and periphery than Vβ1 T cells and infrequently migrate to the intestine; the two subsets also differ in their graft-vs-host reaction capacity (18, 23). Furthermore, Vβ1 T cells are mainly located within the lamina propria, although a CD8$^+$ subpopulation of them subsequently enters the epithelial layer (18, 23, 24).

Mapping of the TCRβ genomic region to date has identified six Vβ1, four Vβ2, one Dβ, four Jβ, and one Cβ segments (16, 18–20). No Vβ pseudogenes have been found, and the four Jβ segments are very similar to each other (16). The TCRβ rearrangement can start either by the Vβ1Dβ or the DβJβ step and is restricted to the thymus at least during embryogenesis (25). Because of its relatively small heterogeneity among germline V, D, and Jβ elements, TCRβ diversity is largely maintained by the variable N nucleotide addition at the coding joints of VD and DJ recombinations (16, 19, 20).

T cell precursors enter the chick thymus in three waves during embryonic life, and these waves of precursors also generate waves of T cell progenies that emigrate sequentially to the periphery during development (14–17, 22, 26, 27). Thymus-derived iIELs are not characterized by preselection of TCR Vβ1 repertoires in the thymus (16). The low frequencies of nonproductive rearrangements in iIELs suggest that negative selection might occur in the intestine.

Here we studied whether $\alpha\beta$ or $\gamma\delta$ TCR$^+$ CD8$^+\alpha\alpha$ iIELs are derived from the thymus or whether they have an extrathymic origin. These studies were performed by cell transfer experiments with novel chicken strains exhibiting polymorphic differences in

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[4] Abbreviations used in this paper: IEL, intraepithelial lymphocyte; iIEL, intestinal IEL; E14, embryonic day 14.

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the CD8 α-chain and Abs that recognized them. Furthermore, we determined differences in TCR Vβ1 repertoire usage between thymus-derived CD8$\alpha\alpha$ and CD8$\alpha\beta$ iIELs.

Materials and Methods

Animals

MHC-homozygous embryos of White Leghorn chicken strains H.B15.H7 (H7) and H.B15.H12 (H12) were derived from animals kept at the Department of Medical Microbiology, Turku University (Turku, Finland).

The two strains are different with respect to their reactivity with the allotypic CD8-specific mouse mAb 11-13, which only recognizes H.B15.H12 cells (28–30). The H.B19 chickens were from colonies at the Basel Institute for Immunology (Gipf-Oberfrick, Switzerland). The H.B19 strain was subdivided into the congenic lines H.B19ov$^+$ and H.B19ov$^-$. These are distinguished by the ov Ag, which is present only on thymocytes and T cells of H.B19ov$^+$ animals and is recognized by mAb 11A9 (31). These experimental animals were treated according to Swiss governmental veterinary guidelines. Fertilized eggs were incubated at 38°C and 80% humidity in a ventilated incubator.

Abs and FACS

For three-color analysis we used mouse-anti-TCR Vβ1 mAb coupled to biotin (TCR2, Southern Biotechnology, Birmingham, AL), mouse anti-CD8β mAb (Ep42, an IgG2a, gift from M. Ratcliffe, Montreal, Canada), and mouse anti-CD8α mAbs (11-39, an IgG1 recognizing all polymorhic forms; 11-7 and 11-13, both IgG1 recognizing only CD8 of the strain H.B15.H12; (32)). These Abs were detected by streptavidin-Tricolor (Caltag, South San Francisco, CA), polyclonal anti-mouse-IgG2a-FITC, or anti-mouse-IgG1-PE, respectively (Southern Biotechnology). Reanalysis of sorted CD8$\alpha\alpha$ and CD8$\alpha\beta$ cells showed no contamination (0%) by one or the other population.

COS cell labeling

COS-7 cell transfections and their staining with anti-CD8α mAb was performed as previously described (33). Transfections were performed with pCDM8 plasmids carrying CD8α from the inbred chicken lines H.B15.H12 and H.B15.H7, respectively. Transfected lines were grown for 2 days to express the protein. The cells were then fixed and stained with the CD8α allotypic mAbs 11-7 or 11-13, respectively. Staining of the cells with 11-39 served as a positive control. Abs were detected with HRP-conjugated rabbit anti mouse-Ig (Dako, Copenhagen, Denmark).

Injection of lymphoid cells into congenic chickens

Embryonic day 14 (E14) thymocytes of H12 animals were injected into a large vein at the end of the air sac of E16 H7 embryos (34). Alternatively, thymocytes of 2-day-old H12 chicks were injected into the jugular vein of 2-day-old H7 chicks. Before injection, thymocytes were suspended in PBS containing 10% chicken serum, filtered through a nylon sieve (mesh width, 25 μm; Nytal P-25 my, SST, Thal, Switzerland), and centrifuged at 255 $\times$ *g* for 7 min. The cells were then resuspended in PBS and adjusted to 2 $\times$ 10^8 cells/ml, and 100 μl was injected into E16 embryos or into chicks 2 days after hatching.

cDNA synthesis

Total cellular RNA from sorted CD8$\alpha\alpha$ or CD8$\alpha\beta$ IELs (3 $\times$ 10^4 cells for each population) of a 21-day-old chick was isolated by the guanidium isothiocyanate method and purified on a CsCl gradient (35). About 5 μg of RNA was used as a template for the synthesis of randomly primed single-stranded cDNA using murine Moloney leukemia virus reverse transcriptase (BRL, Gaithersburg, MD) in a reaction volume of 20 μl (according to the supplier's instructions). This cDNA was subsequently diluted in 100 μl of water and heated to 94°C for 2 min to inactivate the reverse transcriptase enzyme.

PCR, semiquantitative PCR, and cloning of Vβ transcripts

A PCR technique employing nucleotide primers was used to amplify the expressed TCR Vβ regions. One nucleotide primer, 5872, was specific for the sequence contained in the chicken TCR Cβ region located just upstream of the stop codon (16, 19). The expressed TCR Vβ1 regions were specifically amplified using the second oligonucleotide primer, 5349, which starts at the position corresponding to amino acid residue 15 of the Vβ1 segment. The procedure used for semiquantitative PCR was described previously (36). The amount of cDNA synthesized was calibrated using the relative expression level of β-actin as a standard. The two actin oligonucleotide primers, 4611 and 4612, generated a band of 283 nucleotides (37).

The oligonucleotides used are as follows: 5872 (3′ of Cβ, antisense), ACAGGTCGACGTACCAAAGCATCATCCCCATCACAAAT; 5349 (5′ of Vβ1), ACAGGTCGACCTGGGAGACTCTCTGACTCTGAACTGT; 4611 (5′ of actin), TACCACAATGTACCCTGGC; 4612 (3′ of actin), CTCGTCTTGTTTTATGCGC; Vβ18.b, 5′-ACACAAAGAGAGTGGAAA-3′; and Jβ1280 (antisense), 5′-GCCATCACCGAAAATCATG-3′.

PCR were performed in 30 μl using 1 U of Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT). The PCR buffer was prepared (as suggested by Perkin-Elmer) with the addition of 10 mM 2-ME. Reaction mixtures were denatured at 96°C for 5 min and then subjected to 30 rounds of amplification using a Trio Thermoblock 48 thermocycler (Biometra, Tampa, FL). The following conditions were used: 96°C for 5 s, 50°C for 15 s, and 72°C for 1 min. For cloning of rearranged TCR V, final extension was at 72°C for 10 min. For cloning of rearranged TCR Vβ1 cDNA, the PCR were performed with 5 times more cDNA template than that used for the semiquantitative analysis. Amplified DNA fragments were purified and cloned into the PCRII plasmid (Invitrogen, San Diego, CA).

Sequencing

Sequences were determined from denatured double-stranded recombinant plasmid DNA with Sequenase (Amersham, Arlington Heights, IL) using the chain termination reaction. Oligonucleotide 6106 starting 60 bp downstream of the 5′ end of the Cβ segment in the antisense orientation was used as a primer for the chain elongation reaction (6106 (5′ of Cβ, antisense), AATCTCTTGCTTTGATGGTGA). In cases where ambiguities remained, several additional nucleotide primers were used. Sequences were assembled and analyzed with the CITI2 software package (Université de Jussieu, Paris, France).

Results

The thymus does not produce CD8$\alpha\alpha^+$ T cells

IELs in the gut are formed by an unusual T cell population that expresses the CD8 α-chain as a homodimer instead of an $\alpha\beta$ heterodimer as found on cells in most other organs (Fig. 1). To date, the expression of CD8$\alpha\alpha$ has often been taken as a nonexclusive indicator of the extrathymic origin of these intestinal IELs. We found that neither E14 chicken embryonic nor adult thymus contained CD8$\alpha\alpha$ T lymphocytes (Fig. 1; no CD8$\alpha\alpha$ cells detected from 2 $\times$ 10^8 T cells analyzed). However, in the intestine, >50% of all TCR-positive IELs expressed CD8α as a homodimer (Fig. 1). Using two congenic chicken strains, H.B19ov$^+$ and H.B19ov$^-$, we recently demonstrated that embryonic TCR$\gamma\delta^+$ thymocytes can colonize the gut where they are able to survive for months (14, 15). However, thymus-derived TCR-negative progenitors were not able to migrate into the intestinal epithelium and differentiate into T cells (14).

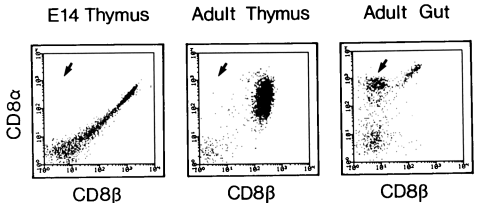

FIGURE 1. CD8$\alpha\alpha$ cells are absent from the thymus. Cytofluorometry of E14 and adult thymocytes and adult gut using anti-CD8α and CD8β Abs 11-39 and EP-42, respectively, and anti-mouse IgG-specific Abs coupled to FITC or PE. Arrows point to the locations of CD8$\alpha\alpha$ cells

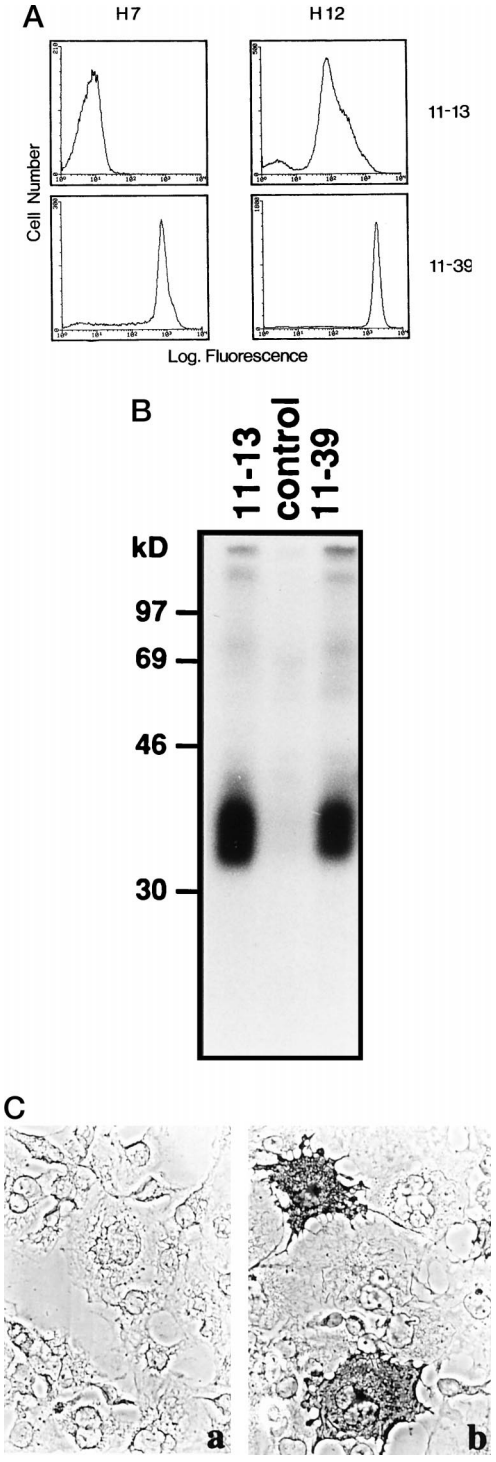

FIGURE 2. Identification of two chicken strains congenic for the CD8 α-chain. H.B15.H7 and H.B15.H12 strains express different CD8α alleles. *A*, Cytofluorometry of adult H7 and H12 chicken thymocytes using anti-CD8α Abs 11-13 and 11-39. Note that Ab 11-13 does not recognize CD8α on thymocytes of H7 animals. *B*, mAbs 11-13 and 11-39 immunoprecipitate the CD8 α-chain. The thymocytes of a 3-wk-old H12 chicken were ^{125}I labeled. The lysate was precipitated with mAbs 11-13 and 11-39. Immunoprecipitates were analyzed by SDS-PAGE on a 10% gel under reducing conditions. The molecular mass standards are indicated on the *left*. *C*, mAb 11-13 recognizes the allotypic CD8 α-chain. COS-7 cells were transfected with pCDM8 plasmids carrying CD8α from the inbred chicken lines H.B15.H7 (*a*) or H.B15.H12 (*b*), respectively. The cells were then stained with the CD8α allotypic mAb 11-13. Only H12 CD8 was recognized. Staining of the cells with mAb 11-39 served as a positive control. Abs were detected with HRP-conjugated rabbit anti-mouse-Ig.

Embryonic TCR$\gamma\delta^+$ thymocytes differentiate into CD8$\alpha\alpha^+$ T cells in the intestinal epithelium

To determine whether T cells emigrating from the thymus could differentiate into CD8$\alpha\alpha$ cells after immigration into the gut epithelium, we developed a new cell transfer system with two congenic chicken strains expressing polymorphic CD8α molecules. To this end, we raised the mAb 11-13, which recognized CD8α of T cells from H.B15.H12 (H12) chickens, but not from congenic H.B15.H7 (H7) chickens (Fig. 2*A*). Immunoprecipitation using mAb 11-13 on H12 allotypic thymocytes showed CD8α bands on SDS-PAGE apparently identical with those found using mAb 11-39. The mAb 11-39 recognizes CD8α from all allotypic chicken strains (Fig. 2*B*). When COS cells were transfected with CD8α cDNA from either animal strain, the allotypic mAb 11-13 specifically recognized cells transfected with CD8α from H12 animals (Fig. 2*C*), whereas mAb 11-39 recognized both (not shown). These two mAbs were used to distinguish transferred donor H12 CD8α T cells in the intestine of H7 recipients from host H7 CD8α cells.

Colonization of the chicken gut by T lymphocytes starts during embryogenesis at around E14–E17. On E14 about 25% of all thymocytes express TCR$\gamma\delta$, and $<$3% show TCR$\alpha\beta$. Injection of sorted TCR$\gamma\delta^+$ E14 thymocytes into E16 recipients resulted in the colonization of the intestinal epithelium by these TCR$\gamma\delta$ cells (14). In contrast, injected TCR$\gamma\delta^-$ thymocytes did not migrate to the gut epithelium (14). To follow the origin of CD8$\alpha\alpha$ IELs, we then injected 2×10^7 E14 thymocytes of H12 embryos into E16 embryos of the H7 haplotype. After 18 days, iIELs were harvested from the H7 host animals and analyzed by cytofluorometry to test whether the injected thymocytes had seeded the gut epithelium. Double staining of these cells with the H12 donor-specific anti-CD8α mAb 11-13 and the anti-CD8β mAb EP42 showed that 16.5% of all CD8$^+$ cells were from the donor. Of these cells, 72% expressed CD8 as $\alpha\alpha$ homodimers and 28% as $\alpha\beta$ heterodimers (Fig. 3 and Table I). Thus, E14 TCR$\gamma\delta^+$ thymocytes are able to migrate to the intestine and differentiate into CD8$^+\alpha\alpha$ T cells.

Juvenile TCR$\alpha\beta^+$ thymocytes differentiate into CD8$^+\alpha\alpha$ T cells in the intestinal epithelium

The thymus of a 2-day-old chick contains about 60% TCR$\alpha\beta^+$ cells (38, 39). To test whether TCR$\alpha\beta^+$ thymocytes from 2-day-old animals could migrate to the intestinal epithelium and differentiate into CD8$\alpha\alpha$ T cells, we performed thymocyte transfer by injecting 2×10^7 cells from H12 chicks into age-matched H7

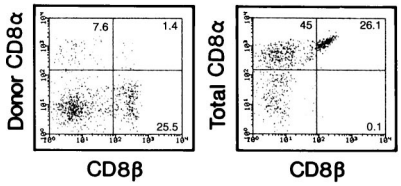

FIGURE 3. Embryonic TCR$\gamma\delta^+$ CD8$\alpha\alpha^-$ thymocytes differentiate into TCR$\gamma\delta^+$ CD8$\alpha\alpha^+$ iIELs. E14 H12 thymocytes (2×10^7) were injected i.v. into E16 H7 recipient embryos. The iIELs were analyzed 18 days after injection by cytofluorometry. CD8$\alpha\alpha^+$ donor cells were detected by Ab 11-13, which specifically recognizes the H12 CD8 α-chain (donor). Total CD8α^+ iIELs, including both host and donor cells from the same animal, were detected with Ab 11-39 recognizing donor and host CD8α^+ cells (Total). Quantification of CD8$\alpha\alpha$ and CD8$\alpha\beta$ cells was performed by double staining using anti-CD8β-chain Ab EP42. The figure shows representative data from one animal of six. TCR$\gamma\delta^+$ iIELs derive from TCR$\gamma\delta^+$ thymocytes (14), and most donor CD8$\alpha\alpha^+$ iIELs were TCR$\gamma\delta$ positive (not shown). The numbers in the graph indicate percentages.

Table I. *Chimerism of iIEL obtained 18 days after injection of E14 thymocytes into E16 congenic animals*

Animal No.	Donor		Host	
	CD8αα% of host CD8αα	CD8αβ % of host CD8αβ	CD8αα% of total iIEL	CD8αβ % of total iIEL
1	13.4	8.1	35	21
2	16.85.4	45	26	
3	7.0	2.0	36	10
4	9.0	1.8	39	28
5	12.6	4.8	34	41
H7 control	0	0	37	22

[a] Thymocytes from E14 H12 animals were carefully prepared, adjusted to 2×10^7 cells and injected into E16 H7 animals. After 18 days iIELs were extracted from the gut of injected animals. Cytofluorometry was performed with the H12 donor-specific anti-CD8α or the anti-CD8α recognizing all allotypes. For double staining, the anti-CD8β Ab was applied. Numbers represent the percentage of stained cells of the total number of iIEL (host) or the percentage of chimerism between donor and host cells (donor).

congenic animals. Injected TCR^+ thymocytes differentiated into CD8αα T cells in the gut, while injected TCR^- thymocytes did not (Fig. 4*A*).The analysis of intestinal TCRαβ$^+$ IELs 18 days after transfer showed that 8.0% of all CD8αα cells and 6.2% of all CD8αβ$^+$ were from the donor (average of two animals; Fig. 4*B*). This experiment demonstrated that in young chicks TCRαβ$^+$ T cells can colonize the intestinal epithelium and are able to differentiate into CD8αα IELs. We then wondered whether CD8αα and CD8αβ T cells of these 20-day-old chicks presented different TCRαβ repertoires.

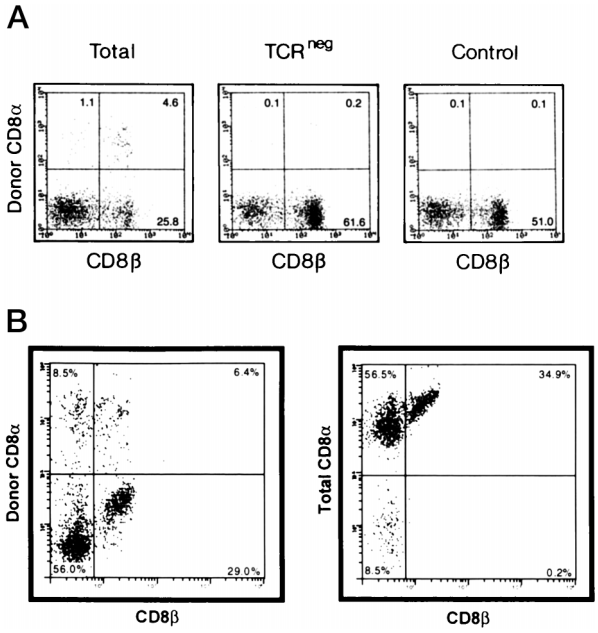

FIGURE 4. Differentiation of thymocytes into CD8αα iIELs. Total or TCR-negative thymocytes of 2-day-old H12 chicks were injected into age-matched H7 recipients. TCR^- thymocytes represented 24% of the total thymocytes. Consequently, to compare total and TCR^- thymocyte progenies, 20×10^6 total thymocytes or 5×10^6 TCR^- thymocytes were injected into each recipient. Donor CD8α iIELs were analyzed by flow cytometry using the 11-13 mAb specific for the H12 CD8 α-chain and the EP42 mAb specific for the CD8 β-chain. *A*, TCR-negative thymocytes do not differentiate into CD8αα iIELs. Total, H7 recipients injected with total thymocytes; TCRneg, H7 recipients injected with TCR^- thymocytes; Control, control recipients. The figure shows representative data from one animal of six for total thymocyte injections and one animal of three for TCR^- thymocyte injections. The numbers in the graph indicate percentages. *B*, Donor thymocytes give rise to TCRαβ$^+$, CDαα$^+$ iIELs. H7 recipients injected with total thymocytes were analyzed 18 days later for TCRαβ$^+$ IELs. *Left*, Donor CD8α vs CD8β; *right*, total (donor plus host) CD8α vs CD8β. This experiment was performed with two animals, and similar data were observed following transfer of E14 into E16 embryos.

Comparison of the TCRβ repertoire of CD8αα and CD8αβ intestinal T cells

To examine the TCRβ repertoire, we used semiquantitative PCR, employing a 3′ primer specific for Cβ and a 5′ primer specific for Vβ1, to amplify the Vβ1 regions expressed in intestinal CD8αα and CD8αβ IELs from a 20-day-old chick. Vβ2 regions were not analyzed, because Vβ2-positive iIELs are rarely found (23), as confirmed at the RNA level (16). The number of TCR Vβ1-positive cells is higher in the CD8αβ than in the CD8αα iIEL population (Fig. 5). This could reflect the fact that TCRγδ intestinal cells are better represented in the CD8αα than in the CD8αβ population. More than 30 Vβ1 cDNA clones from CD8 αα and αβ intestinal intraembryonic T cells were sequenced (Fig. 6).

To compare the TCRβ repertoire of CD8αα and CD8αβ cells with previous data, we performed a series of experiments with the chicken strain H.B19 (16, 22, 25). A single animal expressed eight members of the Vβ1 family, confirming our former hypothesis that the H.B19 strain is not homogenous at the TCRβ locus, as 17 Vβ1 members were previously identified in 13 animals (16). The different Vβ1 genes in the CD8αα or -αβ cell populations were expressed with comparable distribution (Table II). However, in this animal we cannot ignore the apparent absence of Vβ1.8b in the CD8αα population or the lower usage of Vβ1.15a in the CD8αβ population. Similarly, the usage of the Jβ segments showed subtle differences among the two IEL populations (Table III). The total $CD8^+$, TCR Vβ1 IELs preferentially rearranged Jβ-1340 compared with $CD4^+$, TCR Vβ1 IELs. Furthermore, the CD8αβ IELs

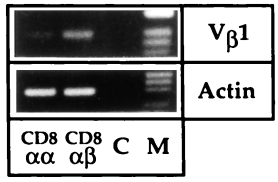

FIGURE 5. Expression of TCR Vβ1 transcripts. Identification of Vβ1-Dβ-Jβ-Cβ transcripts by PCR amplification. Actin expression was used as the parameter to standardize cDNA levels and for semiquantitative analysis. The templates were prepared from sorted CD8αα or CD8αβ iIELs of a single 20-day-old H.B19 chick. After 32 PCR cycles of cDNA amplification, the products were separated by electrophoresis, stained with ethidium bromide, and photographed. M, M_r marker; C, negative control PCR using water as template.

A

Vβ1-Jβ junctional sequence of CD8αα intestinal lymphocytes

	Vβ1	N/P	Dβ	N/P	Jβ	Vβ1	Jβ	NUMBER	IN FRAME
	ATTTCTGCGCTAAGCAAGATA		GGGACAGGGGGATC		AACACACCAC		1340		
cd8aa6	ATTTCTG	TAAGCGC	CAGG	CGTCCGATTTTGGG	AACACACCAC	3	1340	2	+
cd8aa1	ATTTCTGCGCTAA	TCATA	GACAGGG	ATATCTAC	ACACACCAC	4	1340	1	+
cd8aa9	ATTTCTGCGCTAAGCAAGA		CAGGGGGAT		CACACCAC	12	1340	5	+
cd8aa12	ATTTCTGCGCTAAGCAAG	CTTCCT	ACAGGGGG	GTCCCCCACGAGGTCT	CAC	12	1340	1	+
cd8aa19	ATTTCTGCGCTAAG	TCGGACCGGT	CAGGG	ACTGGA	AACACACCAC	12	1340	3	+
cd8aa21	ATTTCTGCGCTAAGCAAGAT	GAGCGAGGTGTTCC	GGGG	T	ACACACCAC	16	1340	1	+
cd8aa14	ATTTCTGCGCTAAGCAAG	CGCAC	GACAG	CGTG	ACACACCAC	16	1340	1	+
cd8aa7	ATTTCTGTGCTAAG	GC	GACAGGG	CCCT	ACACACCAC	17	1340	1	+
cd8aa30	ATTTCTGTGCTAAGCAAG		GGACAGGGGG	G	AACACACCAC	17	1340	1	+
					AACAGTAACA		1280		
cd8aa18	ATTTCTGCGCTAAGC	TCGTA	GAC	CCCAG	CAGTAACA	12	1280	2	+
cd8aa22	ATTTCTGCGCTAAGCAAGA	CAGTGCCC	GGG	TCT	ACAGTAACA	12	1280	1	+
cd8aa4	ATTTCTGCGCTAAGC		GGGACAGG	TATT	ACAGTAACA	15a	1280	1	+
cd8aa8	ATTTCTGCGCTAAG	GAGAG	AGGGG	CCCGA	AGTAACA	16	1280	1	+
					GTTAATATCC		1336		
cd8aa15	ATTTCTGCGCTAAG	AGGACCG	GGGGGA	GAAACTA	TAATATCC	7	1336	1	+
cd8aa17	ATTTCTGCGCTAAGCAA	CCCGA	ATC	CCCTC	TTAATATCC	7	1336	1	+
cd8aa5	ATTTCTGCGCTA	TA	GGACAGG	CCTTG	AATATCC	12	1336	1	+
cd8aa25	ATTTCTGCGCTAAG		GGACAGGGG	TCG	AATATCC	12	1336	1	+
cd8aa23	ATTTCTGCGCTAAGCAAG	TCCCTAGT	CAGG	TCGAC	ATCC	16	1336	1	+
					AACGTAAGAC		4		
cd8aa29	ATTTCTGCGCTAAGC	GA	GAC	CGCAAT	AACGTAAGAC	4	4	1	+
cd8aa16	ATTTCTGCGCTAAGCAAGATA	T	GACAGG	T	GTAAGAC	15a	4	2	+
cd8aa3	ATTTCTGCGCTAAGCAAG	T	GACAGGGGGAT		ACGTAAGAC	16	4	1	+
cd8aa28	ATTTCTGTGCTAA	ACG	GGGACAGGG	CTTCG	ACGTAAGAC	17	4	1	+

B

Vβ1-Jβ junctional sequence of CD8αβ intestinal lymphocytes

	Vβ1	N/P	Dβ	N/P	Jβ	Vβ1	Jβ	NUMBER	IN FRAME
	ATTTCTGCGCTAAGCAAGATA		GGGACAGGGGGATC		AACACACCAC		1340		
cd8ab20	ATTTCTGCGCTAA		CAG	ATCTTT	AC	3	1340	1	+
cd8ab11	ATTTCTGCGCTAAG	AAAGCCGGC	ACAGGG	T	CACCAC	7	1340	1	+
cd8ab7	ATTTCTGCGCTAAGCAAG	T	GACAGGG	AAA	AACACACCAC	12	1340	1	+
cd8ab13	ATTTCTGCGCTAAGC	GAGTTAGACGGA	ACAGGGGGATC	GCCGGG	CCAC	12	1340	1	+
cd8ab16	ATTTCTGCGCTAAGCAAG	CAGCT	ACAG	AA	AACACACCAC	12	1340	1	+
cd8ab17	ATTTCTGCGCTAAGCAAG	GCGGCC	CAGGGGG	GCCTATCGGTC	ACACACCAC	12	1340	1	+
cd8ab18	ATTTCTGCGCTAAGC	GAGTTAGAC	GGGACAGGGGGATC	GCCGGG	CCAC	12	1340	2	+
cd8ab23	ATTTCTGCGCTAAGCAAG	GA	AGGGGGATC	GTC	AACACACCAC	12	1340	1	+
cd8ab4	ATTTCTGCGCTA	CCGGTT	CAGGGGGA	CTCCCT	AACACACCAC	16	1340	2	+
cd8ab10	ATTTCTGCGCTAAG		GACAG	TGAA	AACACACCAC	16	1340	1	+
cd8ab15	ATTTCTGCGCTAAGCAAGA	CAC	GGACA	AAGGA	AACACACCAC	16	1340	1	+
cd8ab2	ATTTCTGTGCTAAGCA		CAGGGGGATC	GATAAA	AACACACCAC	17	1340	1	+
cd8ab29	ATTTCTGTGCTAAGCAAG	TAGAATT	GGGACAGGG	TCG	AC	17	1340	1	+
					AACAGTAACA		1280		
cd8ab28	ATTTCTGCGCTAAG		GGGGGATC	GATCGAGT	ACAGTAACA	7	1280	1	+
cd8ab31	ATTTCTGTGCTAAG	T	ACAGGGGGATC		AGTAACA	17	1280	1	+
					GTTAATATCC		1336		
cd8ab22	ATTTCTGCGCTAAGCAAGATA	TTCCGTCAGACT	GGAC	G	AATATCC	3	1336	1	+
cd8ab26	ATTTCTGCGCTAAGC	GT	GGGACAGGGGGAT	TACAC	ATCC	3	1336	1	+
cd8ab1	ATTTCTGCGCTAAG	GTACCTCGA	ACAGG	A	AATATCC	4	1336	1	+
cd8ab12	ATTTCTGCGCTAAGCAAGATA	T	AGGGGG	CGGGCAGGGC	AATATCC	7	1336	1	+
cd8ab14	AT	ACGA	GAT	GC	GTTAATATCC	12	1336	1	+
cd8ab30	ATTTCTGCGCTAAGC	TGCTC	CAGGG	ACGTTCG	AATATCC	12	1336	1	+
cd8ab9	ATTTCTGCGCTAAGCAAGAT	TTGG	GGGACAGGG	ATTCCCTTAATTAT	GTTAATATCC	15a	1336	1	+
cd8ab6	ATTTCTGCGCTAAGCAAGA	CGCAATGTCGCACGAC	GGGACAGGGGG	GTATGG	TATCC	16	1336	1	+
cd8ab24	ATTTCTGTGCTAAGCAAG	TGGTTTCATT	GGGA		ATCC	17	1336	1	+
					AACGTAAGAC		4		
cd8ab3	ATTTCTGCGCTA		GGACAGGG	TAT	AACGAAAGAC	8b	4	2	+
cd8ab8	ATTTCTGCGCTAAGCAAGAT	CC	GGACAGGG	TGTATATTAAT	AACGTAAGAC	12	4	1	+
cd8ab21	ATTTGTGCGCTA		GGGACAGGGGG	GTGATTAAT	AACGTAAGAC	12	4	1	+
cd8ab25	ATTTCTGCGCTATGCAAGATA	TTTCCGCCCTGGT	ACAGGGG	ATCTGTCGGG	GAC	12	4	1	+

FIGURE 6. Comparison of the TCR Vβ1 repertoire between CD8αα and CD8αβ iIELs. The cDNA was prepared from iIELs of a single 20-day-old H.B19 chick and sorted for CD8αα or CD8αβ expression. Nucleotide sequences are shown for the Vβ1-Dβ-Jβ junctions. The sequences of Vβ1, Dβ, and Jβ segments are given for individual clones. The sequence of N and P nucleotides are indicated as N/P. All sequences that were found in-frame are indicated (+). The identification number for each Vβ1 and Jβ clone is indicated to the *right* of the figure (16). The clones that were encountered several times in the repertoire are also indicated in a column (NUMBER). *A*, TCRβ1 repertoire of CD8αα iIELs. *B*, TCR Vβ1 repertoire of CD8αβ iIELs

presented lower usage of Jβ-1280 segments and more Jβ-1336 than the CD8αα IELs. This was later confirmed by PCR using the Jβ-1280-specific and Cβ-specific primers (not shown).

Analysis of additional animals also revealed subtle TCRβ1 repertoire differences. However, these differences varied from one animal to the other (data not shown). Notably, Jβ-1280 was frequently used in the CD8αα population of animal 3, and Vb1.8b was used in CD8αα population of two animals. Analysis of junctional diversity did not reveal oligoclonality in either CD8 cell population. However, diversity appeared to be slightly larger in CD8αβ than in CD8αα cells, as suggested by the number of independent clones sequenced, 28 clones for CD8αβ cells and 22

Table II. *Vβ1 usage in rearranged Vβ1 genes*[a]

	Whole iIELs	CD8αα	CD8αβ
Vβ1.3	6	6	10
Vβ1.4	3	6	3
Vβ1.7	13	6	10
Vβ1.8b	1	0	6
Vβ1.12	39	45	39
Vβ1.15a	6	10	3
Vβ1.16	10	16	16
Vβ1.17	22	9	13

[a] Results are expressed in percentage of cDNAs of rearranged genes that contains the corresponding Vβ1 member. The intestine of the analyzed chick had a total of 8 Vβ1 members. TCRβ clones analyzed in whole sorted IEL, CD8αα, and CD8αβ cell population were standardized for display of distribuiton of Vβ1 members. The listed Vβ1 members were previously idenitifed in iIELs (16)

Table III. *Jβ usage in rearranged Vβ1 genes*[a]

	Whole iIELs	CD8αα	CD8αβ
Jβ-1340	20	52	48
Jβ-1280	22	16	6
Jβ-1336	30	16	29
Jβ-4	28	16	16

[a] Results are expressed in the percentage of cDNAs of rearranged genes that contains the corresponding Jβ segment. The intestine of the analyzed chick used the four Jβ members. TCRβ clones analyzed in whole sorted IEL, CD8αα, and CD8αβ cell population were standardized for display of distribution of Jβ segments. The listed Jβ segments were previously identified in iIELs (16).

clones for CD8αα cells of 31 clones each. Taken together, these results suggest that CD8αα and CD8αβ IELs express TCR Vβ1 repertoires with subtle differences. More precisely they indicate that CD8αβ cells exhibit a slightly larger repertoire than CD8αα cells, in accordance with the total number of TCR Vβ1 transcripts in both populations (Fig. 6).

Discussion

This study describes the thymic origin of the majority of CD8αα iIELs in birds. It is discordant with some of the results obtained with adult mice, in which CD8αα TCRαβ$^+$ and TCRγδ$^+$ lymphocytes are considered to be primarily of extrathymic origin (1, 2). However, several studies suggest that a subset of both TCRαβ and TCRγδ murine CD8αα iIELs is derived from the fetal/neonatal thymus (5–8). In cell transfer experiments using congenic chickens, neither embryonic nor adult thymus contained CD8αα cells, while injection of TCRαβ$^+$ or γδ$^+$ thymocytes into congenic animals gave rise to CD8αα iIELs. The intestinal intraembryonic CD8αα TCR$^+$ cells seem to emerge by a secondary T cell differentiation event and present a TCR repertoire different from that of CD8αβ T cells.

Do TCR$^+$ cells from the thymus acquire CD8αα in the gut? We found that only thymocytes that expressed the TCR would migrate to the gut. Embryonic TCR-negative thymocytes, embryonic bone marrow and spleen cells do not migrate to the gut (14, 16). Most of our former experiments were performed with embryonic thymocytes injected into embryonic or juvenile recipients. Here we show that donor cells from juvenile thymus also need to express the TCR to home to the gut. An open question was whether these cells could acquire CD8 in the thymus or whether this would happen in the gut. Of 2×10^8 thymocytes analyzed, we did not detect a single CD8αα-positive cell, indicating that the expression of this molecule is dependent on the gut microenvironment. However, due to technical limits we cannot exclude that some CD8αα IELs derive from CD8αα thymocytes.

Do CD8αα thymus-derived T cells show normal characteristics? Most TCRγδ cells in the thymus and peripheral lymphoid organs are CD4$^-$ CD8$^-$ (40, 41). In gut epithelium the majority of the TCRγδ cells express CD8 as αα homodimeric chains. Our former and present experiments show that in young animals these cells derive from the thymus. Because most of them did not express CD8 before they left the thymus, they may have undergone a secondary differentiation and selection process in the gut. However, this gives rise to the problem of expansion of potential self-reacting CD8$^+$ T cells. Does an MHC class I-restricted negative selection occur in the gut, or do MHC class I recognizing inhibitory receptors exist on iIELs, leading to inactivation of self-reacting CD8$^+$ T cells? In fact, both these mechanisms seem to exist simultaneously in the gut as represented either by classical T cells (CD8αα or αβ) or by CD8αα cells bearing molecules that are characteristic of NK cells. Either cell type could receive inhibitory signals for proliferation upon interaction with MHC (11, 42). Therefore, while CD8αα T cells in the gut are thymus derived, those that have circumvented negative selection in the thymus have an alternative selection mechanism in the gut.

A previous report indicated that colonization of the intestine by thymocytes was not related to TCR Vβ1 repertoire selection (16). Here we show that the CD8$^+$ TCR Vβ1$^+$ iIELs do not represent the entire TCR Vβ1 repertoire of iIELs as exhibited by the different usage of Jβ-1340 and Vβ1.8b in this subpopulation. Further analysis suggested that the repertoires expressed by the CD8αα and CD8αβ iIEL populations are different. Thus, each TCRαβ subpopulation exhibits a part of the TCRαβ repertoire of all iIELs. These results are somewhat in agreement with murine studies indicating that CD8αα and CD8αβ TCRαβ iIEL populations are oligoclonal with no overlap between the two subsets (16). The structure of the TCRβ locus is different in birds and mice. Only two Vβ families are found in the chicken (18), whereas 20 Vβ families are described in the mouse (43, 44). At first sight, repertoire analysis would seem to be easier in mice, because Abs against the different families are readily available. However, the study of the diversity within each family would require a large number of clones. Due to the relative simplicity of the Vβ repertoires in chicken, this endeavor can be performed with fewer clones. In addition, chicken Vβ2 cells are extremely rare in the intestine, making analysis of this family unnecessary.

By sequencing the isolated Vβ1 clones, we found all TCR Vβ1 members in the two CD8$^+$ iIEL subpopulations. This suggests that all resulting TCRs may recognize gut Ags. Although the CDR3 size is constant in each CD8 iIEL subpopulation in chicken and mouse, our data show that the diversity of the TCRβ repertoire of CD8 iIELs is larger in chicken than in mouse. Our previous studies in chick embryos indicated that thymocytes migrating to the gut contained the entire TCR Vβ1 repertoire (16). Here, we also show that no Vβ1 member, Jβ segment, or Vβ1-Dβ-Jβ rearrangement is expressed solely in CD8αα iIELs. The presence of a low frequency of nonproductive TCR Vβ1 rearrangements in CD8 iIEL subpopulations indicates an active negative or positive TCR selection process in the gut, similar to the establishment of locally restricted repertoires in the mouse (45).

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