Interleukin 7 Induces Preferential Expansion of Vβ8.2$^+$CD4$^-$8$^-$ and Vβ8.2$^+$CD4$^+$8$^-$ Murine Thymocytes Positively Selected by Class I Molecules

By Alain Vicari,* Maria Do Carmo Leite de Moraes,‡ Jean-Marc Gombert,§ Michel Dy,‡ Claude Penit,* Martine Papiernik,* and André Herbelin§

*From the *INSERM U345, Institut Necker, 75730 Paris Cedex 15, France; and ‡CNRS URA 1461, §INSERM U25, Hôpital Necker, 75743 Paris Cedex 15, France*

Summary

We analyzed the phenotype and Vβ-T cell receptor (TCR) repertoire, together with interleukin 7 receptor (IL-7R) expression in unfractionated thymocytes stimulated in vitro with IL-7. This culture system results in a specific proliferation of mature thymocytes belonging to the CD3$^+$CD4$^-$, CD4$^+$8$^-$, and CD4$^-$8$^+$ subsets. IL-7 induced a preferential expansion of Vβ8.2$^+$CD4$^-$8$^-$ and Vβ8.2$^+$CD4$^-$8$^-$ thymocytes. This phenomenon is not observed in β2-microglobulin–deficient mice, showing that a fraction of CD4$^+$8$^-$ thymocytes, enriched in Vβ8.2$^+$ cells, is selected by class I molecules in normal mice, as are a large proportion of CD4$^-$8$^-$αβTCR$^+$ thymocytes. Our findings also establish that IL-7 plays a major role in the expansion of rare thymocyte subsets, which could exert important functions in inflammatory and immune responses.

T lymphocyte maturation involves coordinate rearrangement of TCR genes in committed precursor cells which will be selected to differentiate into mature T cells. Those steps are closely dependent on the thymic microenvironment and implicate cell-to-cell interactions or cytokines (1). Concerning the latter, it is now generally acknowledged that they induce a preferential expansion of particular thymocyte subsets (1).

Recent data point to the involvement of IL-7 in intrathymic development in adult mice. Its implication in thymopoiesis is suggested both by its predominant mRNA expression in the thymus and the studies on IL-7 transgenic mice (2, 3, 4). This notion is also supported by in vitro experiments showing that IL-7 exerts biological effects on distinct thymic subsets. The earliest IL-7–responsive cells are the immature CD44$^+$ CD25$^+$, CD3$^-$4$^-$8$^-$ thymocytes, whose viability is maintained, apparently without proliferation or differentiation (5, 6). Furthermore, IL-7 exerts a proliferative effect on mature CD3$^+$ thymocytes, namely CD4$^-$8$^-$TCR$^+$, CD4$^+$8$^-$, and CD4$^-$8$^+$ thymocyte subsets (6–8).

A particular feature of CD4$^-$8$^-$TCRαβ$^+$ thymocytes, relative to mature CD4$^+$8$^-$ and CD4$^-$8$^+$ thymocytes and peripheral T lymphocytes, consists of overexpression of the Vβ8.2 gene family (9, 10). It has recently been shown that the majority of these supernumerary CD4$^-$8$^-$Vβ8.2$^+$ thymocytes are positively selected by class I molecules, since they are absent from β2-microglobulin–deficient mice (11). In keeping with these data, a particular subset of CD4$^+$8$^-$ thymocytes, called Thy0, also presents overusage of the Vβ8.2 gene family, but little is known about the way they are selected (12). No such subset has been described among CD4$^-$8$^+$ thymocytes.

We have recently found that stimulation of total thymocytes by IL-7 results in preferential proliferation of CD4$^-$8$^-$ TCRαβ$^+$ and CD4$^-$8$^-$TCRγδ$^+$ cells and, to a lesser extent, in CD4$^+$8$^-$ and CD4$^-$8$^+$ cell expansion (13, 13a). The aim of the present study was to analyze the IL-7R expression in relation to the VβTCR repertoire in IL-7–cultured thymocytes. We demonstrate herein that IL-7 preferentially expands CD4$^-$8$^-$Vβ8.2$^+$ and CD4$^+$8$^-$Vβ8.2$^+$ thymocytes in normal but not in β2-microglobulin–deficient mice, indicating that IL-7 plays a major role in the expansion of rare thymocyte subsets.

Materials and Methods

Mice. 4- to 6-wk-old male C3H/HeJ low endotoxin-responder mice were purchased from CSEAL (Orléans, France). β2-microglobulin–deficient mutant mice of the 129 strain (−/−) (14), hereafter referred to as β2m$^-$, and wild-type littermates, referred to as β2m$^+$, were bred in our own facilities by backcross generation from heterozygous β2m$^{+/-}$ mice. First, β2m$^{+/-}$ breeders and

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/08/0653/09 $2.00
Volume 180 August 1994 653–661

mice lacking MHC class II molecules ($A_\beta^{\circ/\circ}$) (15) (referred to as Class II°) were obtained from CSEAL (Orléans, France).

In Vitro Culture of Thymic Cells. Single-cell thymocyte suspensions were prepared in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% heat-inactivated FCS (Flow Laboratories, Irvine, Scotland), 10 mM Hepes buffer, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from GIBCO BRL), and 0.05 mM 2-ME (culture medium). Cultures were carried out at 37°C in 24-well plates (Costar, Cambridge, MA) using 15×10^6 unseparated thymocytes/well in a total volume of 0.5 ml. In some experiments, 10×10^6 of both $\beta 2m^+$ and $\beta 2m^-$ thymocytes were mixed in a total volume of 0.5 ml, and then incubated for 4 or 9 d with 100 U/ml human recombinant IL-7 (hrIL-7[1], specific activity of 8.8×10^6 U/mg) kindly provided by Sterling Winthrop, Inc. (Collegeville, PA). At the end of culture, thymocytes were harvested and dead cells were removed by density gradient centrifugation (Lympholyte M; Cedarlane, Ontario, Canada).

Depletion of CD8⁺ Thymocytes. To analyze the Vβ-TCR repertoire of freshly isolated $CD4^-8^-$ and $CD4^+8^-$ thymocytes, together with IL-7R expression, we depleted thymocyte suspensions of $CD8^+$ cells. Briefly, total thymocytes were incubated with purified anti-CD8 (clone 3155) followed by low-tox-M rabbit complement (Cedarlane), washed extensively, and then incubated with another purified anti-CD8 (clone 53-6.7), followed by depletion with anti-rat Ig coated magnetic beads (Dynal, Oslo, Norway). Purification was assessed by staining the remaining cells with a third anti-CD8 (clone YTS-169.4). $CD8^+$ contaminants were routinely <2%.

Immunofluorescence Labeling and Flow Cytometry. For surface antigen detection, cells were resuspended in PBS containing 5% FCS and 0.02% sodium azide and incubated for 30 min with an appropriate dilution of antibody. The following antibodies were used for double or triple labeling: anti-αβTCR (clone H57-97), anti-Vβ6 (clone 44.22.1), anti-Vβ8.2 (clone F23.2), anti-Vβ14 (clone 14-2), anti-γδTCR (clone GL3), anti-HSA (clone J11d), anti-CD44 (clone 1M781), anti-CD25 (clone PC61), anti-Ia (clone M5-114), anti-H-$2K^b$ (clone 34-1-2S), anti-CD4 (clone GK1.5), and anti-CD8 (clone 53-6.7), coupled to biotin, FITC or PE. For triple labeling, biotinylated antibodies were revealed using Tricolor–streptavidin (Caltag Labs, San Francisco, CA).

Flow cytometry was performed using a FACScan apparatus (a registered trademark of Becton Dickinson, Mountain View, CA) equipped with a 488-nm argon laser. In double-labeling experiments, cell suspensions were analyzed after propidium iodide exclusion of dead cells. At least 10^4 lymphoid cells were acquired in each run and the results were analyzed using Lysys II software.

IL7R Detection. We used biotinylated murine IL-7 to detect IL-7R by means of flow cytometry (16). Briefly, 10^6 cells were incubated for 60 min on ice with 10 μl of a 10 μg/ml biotinylated murine IL-7 solution (British Biotechnology, Oxon, UK). Unbound cytokine was removed by washing the cells twice followed by another incubation for 30 min with 10 μl of PE–streptavidin second-step reagent (British Bio-technology) or Tricolor–streptavidin. The specificity of biotinylated murine IL-7 labeling was assessed by its reversal in the presence of a 80-fold molar excess of unlabeled human IL-7 (data not shown).

Analysis of DNA-synthesizing cells. Total thymocytes were incubated with IL-7 for 4 d and with 100 μg/ml bromodeoxyuridine (BrdUrd) for the last 15 h of culture. BrdUrd incorporation by DNA synthesizing cells was analyzed by flow cytometry with a three color labeling technique (17). Briefly, cultured cells were surface stained with appropriate combinations of anti-CD4, anti-CD8, anti-αβTCR, anti-Vβ6, anti-Vβ8.2, anti-Vβ14, or anti-γδTCR, coupled to either biotin or PE. Biotinylated antibodies were revealed with Tricolor–streptavidin. Cells were then fixed for 48 h at 4°C in 1% paraformaldehyde containing 0.01% Tween 20 and incubated with pancreatic deoxyribonuclease I (Pharmacia, Uppsala, Sweden) for 1 h at 37°C. BrdUrd incorporated into DNA was detected with the 76/7 antibody and FITC-conjugated anti–mouse IgG1. The negative control consisted of cultured cells labeled by the same procedure but without BrdUrd incubation.

Results

The $CD4^-CD8^-$ Thymocyte Subset Is the Predominant Cell Population Recovered after IL-7 Stimulation. We have previously described that IL-7 induces a strong proliferative response of unseparated thymocytes, that peaks after 3–4 d of culture (13). As illustrated in Table 1, a 4-d incubation with IL-7 results in a preferential expansion of $CD4^-CD8^-$ thymocytes, the second major subset being $CD4^+CD8^-$. Some $CD4^-CD8^+$ thymocytes were also present, while nearly all $CD4^+CD8^+$ cells had disappeared.

Phenotypic Analysis of $CD4^-CD8^-$ and $CD4^+CD8^-$ IL-7-stimulated Thymocytes. We analyzed the expression of CD44, CD25, HSA, αβTCR, γδTCR, and IL-7R in $CD4^-8^-$ and $CD4^+8^-$ thymocyte after 4-d stimulation of total thymocytes with IL-7 relative to freshly isolated thymocytes. Table 2 shows that all $CD4^-8^-$ thymocytes recovered after 4 d of IL-7 stimulation expressed either αβTCR or γδTCR. The vast majority were mature HSA^- and activated $CD44^+$ T cells bearing IL-7R. They also homogeneously expressed intermediate CD25 levels, i.e., at a lower intensity than fresh $CD4^-8^-$ thymocytes. All $CD4^+8^-$ thymocytes were mature αβTCR T cells, as defined by low HSA expression. About one third were $IL\text{-}7R^+$, while only 10–20% were $CD25^+$ or $CD44^+$. In both $CD4^-8^-$ and $CD4^+8^-$ thymocyte populations, IL-7R expression corresponded to a larger cell size (data not shown).

IL-7 Promotes the Preferential Expansion of $V\beta 8.2^+$ Thymocytes from Normal Mice. We analyzed the actual cell recoveries of $CD4^-8^-$ and $CD4^+8^-$ thymocytes expressing different Vβs after 4 d of IL-7 stimulation and found a preferential recovery of $V\beta 8.2^+$ cells (Table 3). More precisely, $CD4^-8^-V\beta 8.2^+$ thymocytes expanded approximately 10-fold when $CD4^-8^-$ thymocytes expressing other Vβs expanded approximately three fold. Concerning $CD4^+8^-$ thymocytes, we did not observe enhanced recovery of any particular subset, but the recovery of $CD4^+CD8^-V\beta 8.2^+$ thymocytes was only slightly diminished whereas those of $CD4^+8^-$ thymocytes expressing other Vβs decreased by twofold. This result may reflect the heterogeneity of the sensitivity of $CD4^+8^-$ thymocytes to IL-7 rather than a selective survival, as only a fraction of them expressed IL-7R (Table 2) and as a notable proportion of $CD4^+8^-$ thymocytes died during the first days of culture (data not shown). We inves-

[1] *Abbreviations used in this paper:* BrdUrd, bromodeoxyuridine; hrIL-7, human recombinant IL-7.

Table 1. *Recovery of CD4⁻ and CD8-defined Thymocyte Subsets after 4 d of Culture with IL-7*

	*Viable cell recovery × 10^{-6}			
	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$
Day 0	‡2.2 ± 1.1	83.1 ± 1.2	9.7 ± 1.0	4.6 ± 1.0
Day 4 + IL-7	8.5 ± 3.5	0.7 ± 0.8	4.6 ± 0.9	2.6 ± 0.8

* Day 0 or day 4 IL-7–stimulated thymic cells from C3H/HeJ mice were stained with fluorescently labeled antibodies specific for CD4 and CD8 and analyzed by two-color flow cytometry. Dead cells and debris were eliminated by propidium iodide exclusion. Cultures were set up with 15 × 10^6 unseparated thymic cells in 0.5 ml of culture medium containing 100 U/ml hrIL-7.
‡ Results are expressed as mean ± SD of five separate experiments. They represent viable cell recovery calculated from 10^8 cells at the beginning of culture.

Table 2. *Phenotype of $CD4^-8^-$ and $CD4^+8^-$ Thymocytes before and after 4 d of IL-7 Stimulation*

	Percentage of positive cells in gated populations			
	$CD4^-8^-$	$CD4^+8^-$	$CD4^-8^-$	$CD4^+8^-$
	* *day 0*		*day 4 + IL-7*	
αβ-TCR^+	‡32	99	76	99
γδ-TCR^+	20	0	23	0
$CD25^+$	34	6	62	10
$CD44^+$	25	8	85	13
HSA^+	80	56	10	18
$IL\text{-}7R^+$	44	25	95	32

* Freshly isolated or cultured thymocytes exposed for 4 d to IL-7 were stained with anti-CD4-PE, anti-CD8-FITC, and the appropriate biotinylated antibody or biotinylated mrIL-7, plus Tricolor–streptavidin, and then analyzed by three-color flow cytometry. Cultures were set up with 15 × 10^6 unfractionated thymic cells in 0.5 ml of culture medium containing 100 U/ml hrIL-7.
‡ Results are expressed as the percentage of positive cells compared to staining with Tricolor–streptavidin alone, in one of three independent experiments.

Table 3. *Recovery of $CD4^-8^-$ and $CD4^+8^-$ Thymocytes Expressing Different Vβs after 4 d of Culture with IL-7*

	*Viable cell recovery × 10^{-5}			
	$CD4^-8^-$	$CD4^+8^-$	$CD4^-8^-$	$CD4^+8^-$
	day 0		*day 4 + IL-7*	
$V\beta6^+$	‡0.7 ± 0.3	10.9 ± 0.8	2.3 ± 0.5	4.5 ± 0.5
$V\beta8.2^+$	2.7 ± 0.8	12.7 ± 1	28.4 ± 1.1	10.4 ± 1.2
$V\beta14^+$	0.04 ± 0.2	5.9 ± 0.8	0.13 ± 0.2	2.4 ± 0.3

* Freshly isolated or cultured thymocytes exposed for 4 d to IL-7 were stained with anti-CD4-PE, anti-CD8-FITC, and the appropriate biotinylated anti-Vβ antibody plus Tricolor–streptavidin. Cultures were set up with 15 × 10^6 unfractionated thymic cells in 0.5 ml of culture medium containing 100 U/ml hrIL-7.
‡ Results are expressed as mean ± SD of three separate experiments. They represent viable cell recovery in each subpopulation calculated from 10^8 cells at the beginning of culture.

Table 4. *Relation between Cycling Cells and Vβ-TCR Repertoire in IL-7–stimulated Thymocytes*

		*Percentage of BrdUrd-positive cells in gated populations	
		$CD4^-8^-$	$CD4^+8^-$
Gated in	Total	69 ± 3	12 ± 2
	$\alpha\beta TCR^+$	70 ± 2	12 ± 2
	$\gamma\delta TCR^+$	68	ND
	$V\beta6^+$	65 ± 1	12 ± 2
	$V\beta8.2^+$	73 ± 2	22 ± 3
	$V\beta14^+$	65 ± 6	10 ± 1

* Cultures were performed with 15 × 10^6 unseparated thymic cells from C3H/HeJ mice, incubated for 4 d in 0.5 ml of culture medium containing 100 U/ml rhIL-7. BrdUrd (100 μM) was incorporated during the last 15 h of culture. Cells were stained with anti-CD4-PE plus anti-CD8-PE, the appropriate biotinylated antibody, and Tricolor-streptavidin. BrdUrd-incorporating cells were revealed using a standard technique described in Materials and Methods. The percentage of BrdUrd-incorporating cells was determined by comparison with the staining of cells not incubated with BrdUrd. Results are expressed as mean percentages ± SD from three separate experiments, except for $\gamma\delta TCR^+$ (two experiments).

tigated DNA-synthesizing cells together with TCR repertoire in our system by incubating total thymocytes with BrdUrd during the last 15 h of a 4-d IL-7 stimulation (Table 4). According to the expansion of $CD4^-8^-$ cells, the percentage of BrdUrd-incorporating $V\beta8.2^+$ cells was higher in $CD4^-8^-$ than in $CD4^+8^-$ cells. The numbers of cycling cells of the different phenotypes calculated on the basis of the data of Tables 3 and 4 indicate that $V\beta8.2^+$ cells represent the great majority of cycling cells in both subsets. These results clearly indicate a preferential proliferative response, rather than a better survival, of $V\beta8.2^+$ cells of both $CD4^-8^-$ or $CD4^+8^-$ phenotypes, in the presence of IL-7.

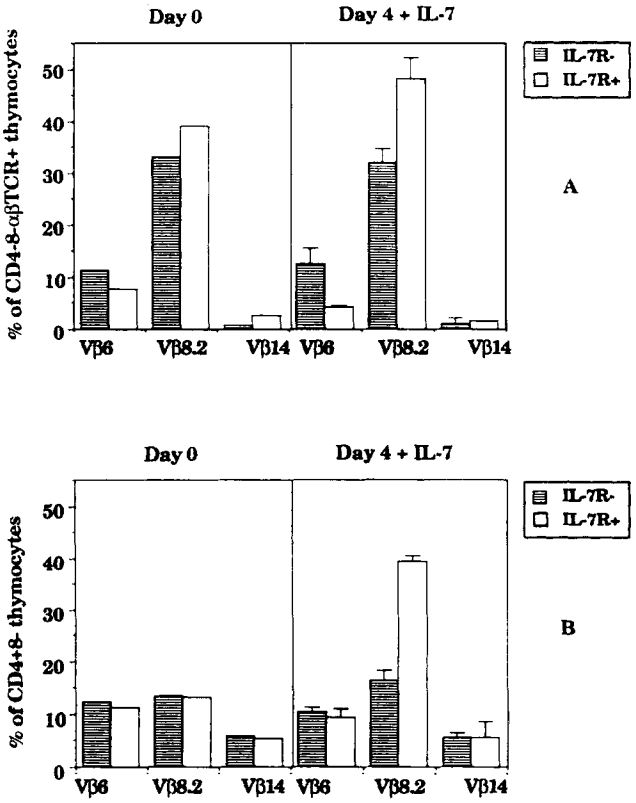

Figure 1. Vβ-TCR repertoire of $CD4^-8^-\alpha\beta TCR^+$ and $CD4^+8^-$ thymocytes according to IL-7R expression before and after 4-d IL-7 stimulation. The Vβ-TCR repertoire was analyzed by three-color flow cytometry in $CD8^+$-depleted thymocytes before (*Day 0*) and after exposure to IL-7 (*Day 4 + IL-7*). In $CD4^-8^-\alpha\beta TCR^+$ (*A*) and $CD4^+8^-$ (*B*) thymocytes. The Vβ-TCR repertoire is represented as the percentage of positive cells among IL-7R- (*hatched*) and $IL\text{-}7R^+$ (*solid*) populations. Results are mean percentages of two separate experiments for Day 0 and mean percentages ± SD of three separate experiments for Day 4.

Repertoire of IL-7R- Versus $IL\text{-}7R^+$ Cells among $CD4^-8^-\alpha\beta TCR^+$ and $CD4^+8^-$ Thymocytes. As $V\beta8.2^+$ cells proliferated preferentially in our IL-7–dependent culture system, we analyzed the VβTCR repertoire of $CD4^-8^-\alpha\beta TCR^+$

Table 5. *IL-7R Expression According to the Vβ-TCR Repertoire in $CD4^-8^-$ and $CD4^+8^-$ Thymocytes before and after 4 d of IL-7 Stimulation*

	*Percentage of $IL\text{-}7R^+$ in $CD4^-8^-$		Percentage of $IL\text{-}7R^+$ in $CD4^+8^-$	
	Day 0	Day 4 + IL-7	Day 0	Day 4 + IL-7
$\alpha\beta TCR^+$	‡93	93	29	32
$V\beta6^+$	92	90	32	28
$V\beta8.2^+$	98	96	31	49
$V\beta14^+$	98	95	32	32

* Day 0 $CD8^+$ depleted or 4-d IL-7–stimulated thymic cells from C3H/HeJ mice were stained with anti-CD4PE, the appropriate antibody coupled to FITC and biotinylated mrIL-7, revealed by Tricolor-streptavidin, then analyzed by three-color flow cytometry. Cultures were performed with 15 × 10^6 unseparated thymic cells incubated for 4 d in 0.5 ml of culture medium containing 100 U/ml rhIL-7.
‡ Results are expressed as the percentage of positive cells for IL-7R compared to control, in one representative experiment out of three.

Table 6. *Recovery of CD4⁻, CD8⁻, and αβ TCR⁺-defined Thymocyte Subsets after a 4-d Exposure to IL-7 in β2m⁺ and β2m⁻ Mice*

	*Viable cell recovery × 10^{-6}				
	CD4⁻CD8⁻	CD4⁺8⁺	CD4⁺8⁻	CD4⁻8⁺	CD4−8− αβTCR⁺
Day 0 β2m⁺	‡3.3	84.6	10.3	1.8	1.1
Day 0 β2m⁻	5.3	87.6	6.4	0.7	1.0
Day 4 + IL-7 β2m⁺	13.6	1.6	7.6	2.2	8.1
Day 4 + IL-7 β2m⁻	9.4	1.3	11.5	0.7	5.2

* Day 0 or 4-d IL-7-stimulated thymic cells from β2m⁺ or β2m⁻ mice were stained with fluorescence-labeled antibodies specific for CD4, CD8, αβTCR, and analyzed by three-color flow cytometry. Cultures were performed with 15 × 10^6 unseparated thymic cells incubated for 4 d in 0.5 ml of culture medium containing 100 U/ml hrIL-7.

‡ Results are expressed as viable cell recovery in each subset calculated from 10^8 cells at the beginning of culture, in one representative experiment out of three.

and CD4⁺8⁻ thymocytes before and after 4 d of IL-7 stimulation of total thymocytes, according to their IL-7R expression (Fig. 1). Before stimulation, Vβ expression was not significantly different between CD4⁻8⁻αβTCR⁺ and CD4⁺8⁻ subpopulations whether they expressed IL-7R or not (Fig. 1). After a 4-d stimulation with IL-7, there appeared a significant bias in the repertoire towards overusage of the Vβ8.2 gene family among IL-7R⁺ thymocytes (Fig. 1). This was the case for both CD4⁻8⁻αβTCR⁺ and CD4⁺8⁻ thymocytes, whereas no such bias existed in the Vβ repertoire of cultured CD4⁻8⁺ thymocytes (data not shown).

IL-7R Expression Among CD4⁻8⁻αβTCR⁺ and CD4⁺8⁻ Thymocytes According to Vβ Usage. The overexpression of the Vβ8.2 gene gamily by IL-7R⁺ thymocytes upon exposure to IL-7 is apparently not due to a more important expression of IL-7R on Vβ8.2⁺ cells at the onset of culture since we could not detect significant differences in this respect between Vβ6⁺, Vβ8.2⁺, and Vβ14⁺, CD4⁻8⁻, or CD4⁺8⁻ thymocytes (Table 5). After 4 d of IL-7 stimulation, the vast majority of CD4⁻8⁻ cells were IL-7R⁺, whatever the Vβ subset considered (Tables 2 and 5). Among CD4⁺8⁻ thymocytes the proportion of IL-7R⁺ cells was significantly higher in the Vβ8.2⁺ than in the Vβ6⁺, Vβ14⁺, or the total CD4⁺8⁻ subset (Table 5).

IL-7 Does Not Promote Preferential Expansion of Vβ8.2⁺ Thymocytes from β2 Microglobulin-deficient Mice. It has recently been demonstrated that overexpression of the Vβ8.2 gene family among CD4⁻8⁻αβTCR⁺ thymocytes is largely due to positive selection of Vβ8.2⁺ cells by β2-microglobulin-associated class I molecules (11). This finding together with the preferential growth of Vβ8.2⁺ thymocytes in response to IL-7 prompted us to analyze the Vβ-TCR repertoire of CD4⁻8⁻ and CD4⁺8⁻ cells from β2-microglobulin-deficient mice (β2m⁻), as compared to control β2m⁺ littermates, before and after IL-7 stimulation for 4 or 9 d. IL-7 promoted the expansion of CD4⁻8⁻αβTCR⁺ and CD4⁺8⁻ thymocytes from both β2m⁺ and β2m⁻ thymocytes and with similar efficacy (Table 6). In addition, CD4⁻8⁻αβTCR⁺ and CD4⁺8⁻ thymocytes from β2m⁺ and β2m⁻ mice expressed similar levels of IL-7R in vivo (data not shown).

On analyzing the repertoire of freshly isolated CD4⁻8⁻αβTCR⁺ thymocytes from β2m⁻ mice, we found, as described by others (11), no bias towards Vβ8.2 expression (Fig. 2). Similarly to C3H/HeJ mice, Vβ8.2 expression by both CD4⁻8⁻ and CD4⁺8⁻αβTCR⁺ IL-7-stimulated thymocytes from β2m⁺ mice, is enhanced. Yet, the progressive overexpression of Vβ8.2 was more pronounced in CD4⁺8⁻ thymocytes. In β2m⁻ mice, the repertoire of CD4⁻8⁻αβTCR⁺ and CD4⁺8⁻ thymocytes was unchanged, even after 9 d of IL-7 stimulation (Fig. 2). These results show that in our model IL-7 does not induce a specific bias towards Vβ8.2 expression in CD4⁻8⁻αβTCR⁺ or CD4⁺8⁻ thymocytes from β2m⁻ mice. They thus provide evidence that either CD4⁻8⁻αβTCR⁺ or CD4⁺8⁻ thymocytes selected by class I molecules preferentially expand in response to IL-7.

CD4⁺8⁻ Thymocytes and Peripheral Lymphocytes from Class II-deficient Mice Are Enriched in Vβ8.2⁺ Cells. Mice lacking MHC class II molecules are not completely devoid of CD4⁺8⁻ thymocytes or peripheral T lymphocytes (15). Therefore, the rare CD4⁺8⁻ thymocytes could have been selected in the absence of class II molecules, and may represent a subpopulation of class I-selected thymocytes. We found that not only peripheral CD4⁺8⁻ lymphocytes (15) but also CD4⁺8⁻ thymocytes from class II-deficient mice were enriched in Vβ8.2⁺ cells (Fig. 3). These in vivo findings strengthen our in vitro demonstration that a fraction of normal CD4⁺8⁻ thymocytes characterized by overexpression of the Vβ8.2 gene family are selected by class I molecules. Moreover, class I-selected cells could also represent a fraction of peripheral CD4⁺8⁻ lymphocytes.

Co-culture of β2m⁺ and β2m⁻ Thymocytes Does Not Change Vβ8.2 Expression in CD4⁻8⁻αβ TCR⁺ or CD4⁺8⁻ Thymocytes from β2m⁻ Mice. It has previously been demonstrated that Vβ8.2⁺CD4⁻8⁻αβTCR⁺ thymocytes are selected by class I molecules expressed by bone marrow-de-

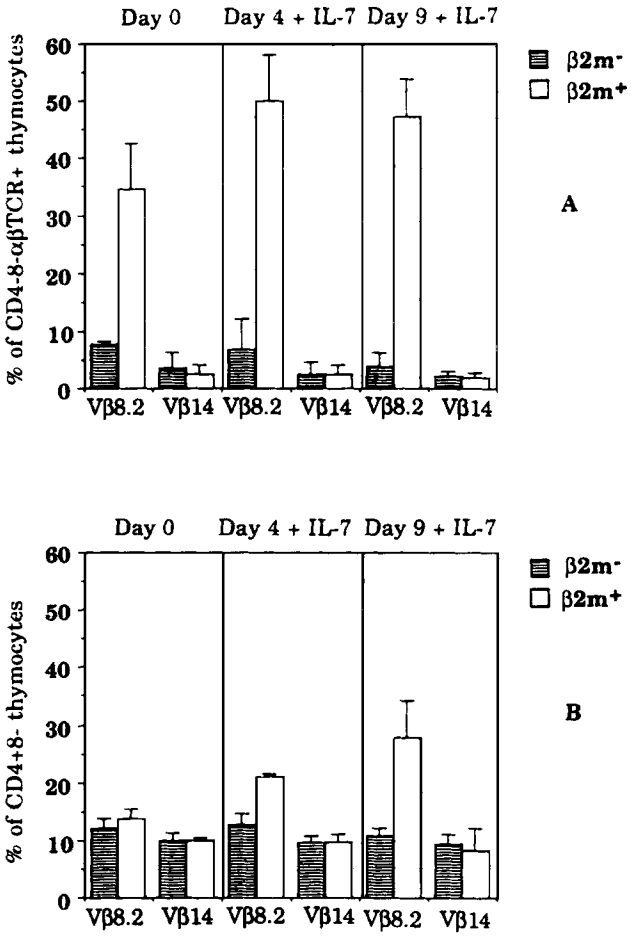

Figure 2. Vβ-TCR repertoire of $CD4^-8^-\alpha\beta$ TCR^+ and $CD4^+8^-$ thymocytes in $\beta2m^+$ and $\beta2m^-$ mice before and after 4- and 9-d IL-7 stimulation. The Vβ-TCR repertoire was analyzed by three-color flow cytometry in total thymocytes from β2-microglobulin-deficient mice (*β2m⁻, hatched*) and control littermates (*β2m⁺, solid*) before (*Day 0*) or after IL-7 stimulation (*Day 4 + IL-7*). This was done in $CD4^-8^-$ $\alpha\beta TCR^+$ (*A*) and $CD4^+8^-$ (*B*) thymocytes. The Vβ-TCR repertoire is represented as the percentage of positive cells. Results are mean percentages ± SD of three separate experiments.

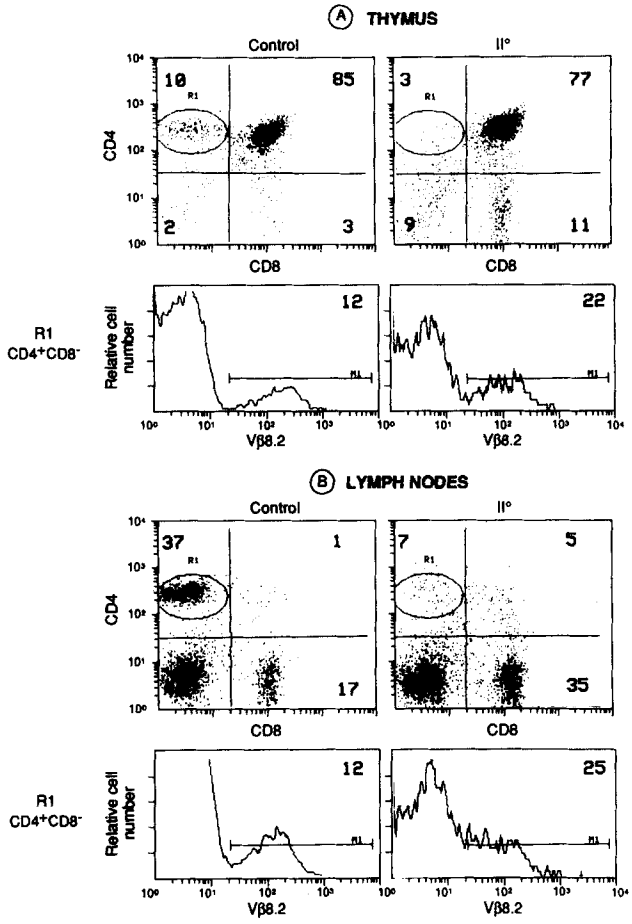

Figure 3. Vβ8.2 expression in thymic and peripheral $CD4^+8^-$ lymphocytes from class II-deficient mice. Three color flow cytometry was used to analyze Vβ8.2 expression among $CD4^+8^-$ lymphocytes (corresponding to the gated R1 population in CD4 versus CD8 dot plots) in the thymus (*A*) and lymph nodes; (*B*) of class II-deficient (*II°, right*) and wild-type control mice (*left*). Vβ8.2 expression is indicated in each log scale histogram as the percentage of positive cells compared to negative control.

rived cells (11). Moreover, $CD4^-8^-\alpha\beta TCR^+$ thymocytes from $\beta2m^-$ mice can overexpress Vβ8.2 in $\beta2m^-$ + $\beta2m^+$ mixed chimeras (11). For this reason, we verified whether the preferential expansion of $V\beta8.2^+$ cells was due to IL-7 rather than an in vitro selection process (13). We thus performed mixed cultures of $\beta2m^+$ and $\beta2m^-$ total thymocytes in equal numbers and stimulated them with IL-7 for up to 9 d (Fig. 4). If $V\beta8.2^+$ thymocytes were selected in vitro, we would have expected to generate the Vβ8.2 bias in $\beta2m^-$ thymocytes co-cultured with $\beta2m^+$ thymocytes. We assessed class I expression on thymocytes by examining anti-$H\text{-}2K^b$ labeling in flow cytometry. After 9-d IL-7 stimulation, all thymocytes from $\beta2m^+$ cultures were $H\text{-}2K^{b+}$, whereas all thymocytes from $\beta2m^-$ cultures were $H\text{-}2K^{b-}$ (data not shown). Thymocytes from mixed cultures were ~40% $H\text{-}2K^{b+}$ (data not shown). When we examined Vβ8.2 expression among $H\text{-}2K^{b-}$ ($\beta2m^-$ derived) and $H\text{-}2K^{b+}$ ($\beta2m^+$ derived), $CD4^-8^-\alpha\beta TCR^+$ and $CD4^+8^-$ thymocytes, we observed no difference between mixed and separate cultures (Fig. 4). These experiments establish that IL-7 preferentially stimulates $V\beta8.2^+$ thymocytes previously selected by class I molecules in vivo.

Discussion

In the thymus, a small population of $CD4^+8^+$ immature thymocytes gives rise to mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes. During this development process, the vast majority of the TCR repertoire is positively selected so that $CD4^+8^-$ and $CD4^-8^+$ thymocytes express TCRs capable of interaction with self MHC class II and I molecules, respectively (18). However, the striking finding that distinct, although rare, populations of $CD4^+8^-$ thymocytes and peripheral T lymphocytes are present in mice rendered deficient in MHC class II expression supports the notion that not all $CD4^+8^-$ thymocytes are positively selected by class II MHC

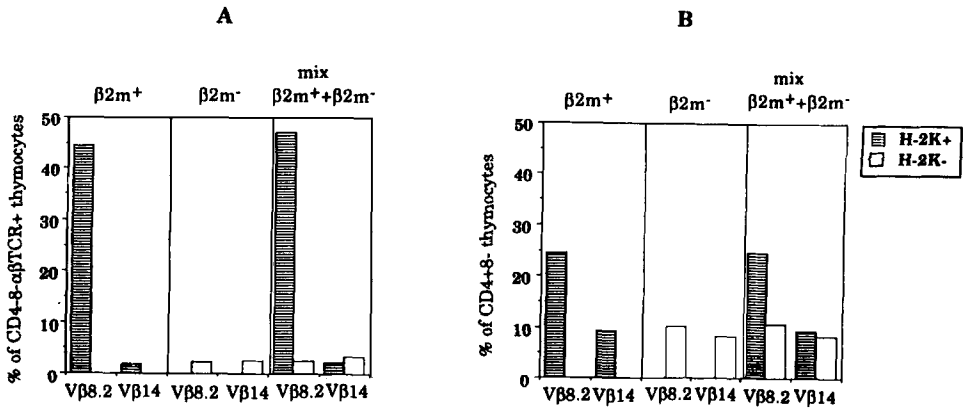

Figure 4. Vβ-TCR repertoire of $CD4^-8^-\alpha\beta$ TCR^+ and $CD4^+8^-$ thymocytes in mixed cultures of $\beta2m^+$ and $\beta2m^-$ thymocytes after a 9-d IL-7 stimulation. The Vβ-TCR repertoire was investigated by three-color flow cytometry in $CD4^-8^-\alpha\beta TCR^+$ (*A*) and $CD4^+8^-$ (*B*) thymocytes from cultures of $\beta2m^+$, $\beta2m^-$ and mixed $\beta2m^+$ + $\beta2m^-$ thymocytes after 9-d IL-7 stimulation. In mixed cultures, thymocytes from $\beta2m^+$ and $\beta2m^-$ were discriminated by the use of anti-H-$2K^b$ mAb, as all $\beta2m^+$ stimulated thymocytes were H-$2K^{b+}$ and all $\beta2m^-$-stimulated thymocytes were H-$2K^{b-}$ in independent cultures (data not shown). Results are mean percentages of two separate experiments.

molecules (15, 19). Class I selection of a minute fraction of $CD4^+8^-$ thymocytes could explain this phenomenon. However, it is not known if such cells exist in normal mice.

We observed preferential expansion of normal $CD4^+8^-$ thymocytes expressing the Vβ8.2 gene family in a culture system of total thymocytes stimulated by IL-7. This preferential expansion generated a bias in the Vβ-TCR repertoire of $CD4^+8^-$ thymocytes towards overexpression of Vβ8.2. The Vβ8.2 bias did not exist among both $CD4^+8^-$ and $CD4^-8^-\alpha\beta TCR^+$ IL-7–stimulated thymocytes from β2m-deficient mice, despite a similar response to IL-7 stimulation. This finding strongly suggests that the fraction of $CD4^+8^-$ thymocytes overexpressing Vβ8.2 that expand in our system have previously been selected by class I molecules in vivo. Furthermore, we found that $CD4^+8^-$ thymocytes and peripheral T lymphocytes from class II–deficient mice were enriched in $V\beta8.2^+$ cells. Taken together, these data provide evidence that, in normal mice, a fraction of $CD4^+8^-$ thymocytes may be selected by class I molecules. Moreover, it has been reported that rare subsets of $CD4^+8^-$ thymocytes from normal mice overexpress Vβ8.2. These cells were found in the $Ly6C^+$, $NK1.1^+$, and HSA-3G11–defined subsets of $CD4^+8^-$ thymocytes, the latter being referred to as the Thy0 population (12, 20, 21). Thy0 thymocytes share other properties with $CD4^-8^-\alpha\beta TCR^+$ thymocytes, including an enrichment in $CD44^+$ cells (22). We therefore conclude that class I–positive selection may contribute to the development of the Thy0 $CD4^+8^-$ thymocyte subset.

Several mechanisms could account for the preferential IL-7–induced growth of class I–selected $CD4^-8^-\alpha\beta TCR^+$ and $CD4^+8^-$ thymocytes in our culture conditions. We showed that this phenomenon was not due to an in vitro selection process. It is also not explained by overexpression of IL-7R on $V\beta8.2^+CD4^+8^-$ thymocytes before culture, though it cannot be excluded that an undetectable fraction of these $V\beta8.2^+CD4^+8^-$ thymocytes are enriched in $IL\text{-}7R^+$ cells in vivo. The same conclusion applies to the $V\beta8^+CD4^-8^-$ population since the vast majority of $CD4^-8^-\alpha\beta TCR^+$ cells were IL-7R before culture, whatever the Vβ expressed. It is thus possible that $CD4^-8^-\alpha\beta TCR^+$ thymocytes bearing other Vβs are also selected by class I molecules and are therefore sensitive to IL-7 stimulation. Indeed, Vβ8.2 overexpression among IL-7–stimulated $CD4^-8^-\alpha\beta TCR^+$ thymocytes was only slightly enhanced. In addition, our method of IL-7R detection does not discriminate between IL-7 receptors of different affinities. Finally, $CD4^+8^-$ or $CD4^-8^-$ $\alpha\beta TCR^+$ thymocytes selected by class I molecules could have expanded preferentially because they were more mature or in an activated state before culture. Indeed, it has been shown that both $CD4^-8^-\alpha\beta TCR^+$ and Thy0 $CD4^+8^-$ thymocytes are mature cells that respond better to various stimuli, including CD3 cross-linking and mitogens, than other thymocyte subsets (23, 24). Moreover, it has been suggested that the Thy0 subset is composed of activated cells (22).

The particular sensitivity of class I–selected $CD4^+8^-$ and $CD4^-8^-\alpha\beta TCR^+$ thymocytes to IL-7 stimulation leads us to speculate on the possible physiological roles of IL-7. It has been proposed that murine $CD4^-8^-V\beta8^+$ T cells recognize and are selected by a specific set of nonclassical class I molecules, such class Ib or class I–like CD1 molecules (11, 25). If so, one possible role for these $CD4^-8^-V\beta8^+$ T cells would be to recognize stress- or inflammation-induced autologous antigens bound preferentially to class I–like molecules, as originally proposed for various subsets of $CD4^-8^-$ $\gamma\delta TCR^+$ T cells (11, 26–28). It is thus conceivable that a particular subset of T cells, including class I–selected $CD4^-8^-\alpha\beta TCR^+$ and possibly $CD4^+8^-$ T cells, recognize a defined set of peptides related to inflammation. Interestingly, we have previously found a synergistic effect of IL-1 on IL-7–dependent thymocyte proliferation (13). IL-7 is mainly expressed by thymic stromal cells (2), but its level is probably low in healthy animals and this could explain why these populations are not expanded in normal mice. In contrast, the release of IL-7 cofactors like IL-1 during inflammation may promote their preferential expansion in other organs, together with the release of IL-7 by particular cells, e.g., keratinocytes (29). Another function of $CD4^-8^-\alpha\beta TCR^+$ and Thy0 $CD4^+8^-$ thymocytes is related to their ability to produce IL-4 upon stimulation (12, 23, 24). IL-4 is a major

regulatory molecule of peripheral immune responses, modulating Th2 versus Th1 responses (30). Interestingly, IL-4 itself seems to be necessary to induce IL-4 production by conventional peripheral $CD4^+8^-$ T cells (31). IL-4–producing thymocytes could therefore be a source of IL-4 which may influence the peripheral Th1/Th2 balance. If so, the role of IL-7 in the proliferation of thymic IL-4 producers, which remains to be fully demonstrated, would be of major relevance.

We thank E. Schneider, B. Rocha, S. Ezine, and A. Zlotnik for helpful discussions and advice and F. Vasseur for expert technical assistance. We are particularly indebted to Dr. Gillis (Immunex Research and Development Corporation, Seattle, WA) and to Sterling Winthrop Inc. (Collegeville, PA) for providing us with IL-7.

This work was supported by institute funds from the INSERM and the CNRS, and by a grant to A. Vicari from the Association pour la Recherche sur le Cancer, and a grant to M. do C. Leite de Moraes and to J. M. Gombert from the Fondation pour la Recherche Médicale.

Address correspondence to Dr. Alain Vicari, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304-1104.

Received for publication 25 January 1994 and in revised form 1 April 1994.

References

1. Boyd, R.L., and P. Hugo. 1991. Toward an integrated view of thymopoiesis. *Immunol. Today* 12:71.
2. Sakata, T., S. Iwagami, Y. Tsuruta, H. Teraoka, Y. Tatsumi, Y. Kita, S.I. Nishikawa, Y. Takai, and H. Fujiwara. 1990. Constitutive expression of interleukin-7 mRNA and production of IL-7 by a cloned murine thymic stromal cell line. *J. Leukocyte Biol.* 48:205.
3. Rich, B.E., J. Campos-Torres, R.I. Tepper, R.W. Moreadtih, and P. Leder. 1993. Cutaneous lymphoproliferation and lymphomas in Interleukin 7 transgenic mice. *J. Exp. Med.* 177:305.
4. Samaridis, J., G. Casorati, A. Traunecker, J.C. Guttierez, U. Müller, and R. Palacios. 1991. Development of lymphocytes in interleukin 7-transgenic mice. *Eur. J. Immunol.* 21:453.
5. Godfrey, D.I., J. Kennedy, T. Suda, and A. Zlotnik. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of $CD3^-CD4^-CD8^-$ triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J. Immunol.* 150:4244.
6. Suda, T., and Z. Zlotnik. 1991. IL-7 maintains the T cell precursor potential of $CD3^-CD4^-CD8^-$ thymocytes. *J. Immunol.* 146:3068.
7. Murray, R., T. Suda, N. Wrighton, F. Lee, and A. Zlotnik. 1989. IL-7 is a growth and maintenance factor for mature and immature thymocyte subsets. *Int. Immunol.* 1:526.
8. Suda, T., R. Murray, C. Guidos, and A. Zlotnik. 1990. Growth-promoting activity of IL-1α, IL-6, and tumor necrosis factor-α in combination with IL-2, IL-4 or IL-7 on murine thymocytes. Differential effects on CD4/CD8 subsets and on $CD3^+/CD3^-$ double-negative thymocytes. *J. Immunol.* 144:3039.
9. Folwkes, B.J., A.M. Kruisbeek, H. Hon-That, A. Weston, J.E. Coligan, R.H. Schwartz, and D.M. Pardoll. 1987. A novel population of T-cell receptor $\alpha\beta$-bearing thymocytes which predominantly express a single Vβ family. *Nature (Lond.).* 329:251.
10. Takahama, Y., A. Kosugi, and A. Singer. 1991. Phenotype, ontogeny and repertoire of $CD4^-CD8^-$ T cell receptor α/β^+ thymocytes: variable influence of self-antigens on T cell receptor Vβ usage. *J. Immunol.* 146:1134.
11. Bix, M., M. Coles, and D. Raulet. 1993. Positive selection of $V\beta8^+CD4^-8^-$ thymocytes by class I molecules expressed by hematopoietic cells. *J. Exp. Med.* 178:901.
12. Hayakawa, K., B.T. Lin, and R.R. Hardy. 1992. Murine thymic $CD4^+$ T cell subsets: a subset (Thy0) that secretes diverse cytokines and overexpresses the Vβ8 T cell receptor gene family. *J. Exp. Med.* 176:269.
13. Herbelin, A., F. Machavoine, E. Schneider, M. Papiernik, and M. Dy. 1992. IL-7 is requisite for IL-1 induced thymocyte proliferation. Involvement of IL-7 in the synergistic effects of granulocyte macrophage colony-stimulating factor of tumor necrosis factor. *J. Immunol.* 148:99.

13a. Herbelin, A., F. Machavoine, A. Vicari, E. Schneider, M. Papiernik, H. Ziltener, C. Penit, and M. Dy. 1994. Endogenous granulocyte macrophage-colony stimulating factor (GM-CSF) is involved in IL-1 and IL-7-induced murine thymocyte proliferation. *J. Immunol.* In press.

14. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in β2-Microglobulin, MHC class I proteins, and $CD8^+$ T cells. *Science (Wash. DC).* 248:1227.
15. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. *Cell.* 66:1051.
16. Armitage, R.J., S.F. Ziegler, D.J. Friend, L.S. Park, and W.C. Fanslow. 1992. Identification of a novel low-affinity receptor for human interleukin-7. *Blood.* 79:1738.
17. Penit, C., and F. Vasseur. 1993. Phenotype analysis of cycling and postcycling thymocytes: evaluation of detection methods for BrdUrd and surface proteins. *Cytometry.* 14:757.
18. Folwkes, B.J., and D.M. Pardoll. 1989. Molecular and cellular events in T cell development. *Adv. Immunol.*. 44:207.
19. Viville, S., J. Neefjes, V. Lotteau, A. Dierich, M. Lemeur, H. Ploegh, C. Benoist, and D. Mathis. 1993. Mice lacking in the

MHC class II-associated invariant chain. *Cell.* 73:635.
20. Arase, H., N. Arase, K. Nagakawa, R.A. Good and K. Onoe. 1993. NK1.1$^+$CD4$^+$CD8$^-$ thymocytes with specific lymphokine secretion. *Eur. J. Immunol.* 23:307.
21. Takahama, Y., S.O. Sharow, and Singer. 1991. Expression of an unusual T cell receptor (TCR)-Vβ repertoire by Ly-6C$^+$ subpopulations of CD4$^+$ and/or CD8$^+$ thymocytes. *J. Immunol.* 147:2883.
22. Bendelac, A., P. Matzinger, R.A. Seder, W.E. Paul, and R. Schwartz. 1992. Activation events during thymic selection. *J. Exp. Med.* 175:731.
23. Zlotnik, A., D.I. Godfrey, M. Fischer, and T. Suda. 1992. Cytokine production by mature and immature CD4$^-$8$^-$ T cells. *J. Immunol.* 149:1211.
24. Bendelac, A., and R. Schwartz. 1991. CD4$^+$ and CD8$^+$ T cells acquire specific lymphokine secretion potential during thymic maturation. *Nature (Lond.).* 353:68.
25. Stroynowoski, I. 1990. Molecules related to class-I major histocompatibility complex antigens. *Annu. Rev. Immunol.* 8:501.
26. O'Brien, R., M.P. Happ, A. Dallas, E. Palmer, R. Kubo, and W.K. Born. 1989. Stimulation of a major subset of lymphocytes expressing T cell receptor $\gamma\delta$ by an antigen derived from myobacterium tuberculosis. *Cell.* 57:667.
27. Janeway, C.A., B. Jones, and A. Hayday. 1988. Specificity and function of T cells bearing $\gamma\delta$ receptors. *Immunol. Today.* 9:73.
28. Asarnow, D.M., W.A. Kuziel, M. Bonyhadi, R.E. Tigelaar, P. W. Tucker, and J.P. Allison. 1988. Limited diversity of $\gamma\delta$ antigen receptors genes of Thy-1$^+$ dendritic epidermal cells. *Cell.* 55:837.
29. Matsue, H., P.R. Bergstresser, and A. Takashima. 1993. Keratinocyte-derived IL-7 serves as a growth factor for dendritic epidermal T cells in mice. *J. Immunol.* 151:6012.
30. Trinchieri, G. 1993. Interleukin-12 and its role in the generation of TH1 cells. *Immunol. Today* 14:335.
31. Le Gros, G., S.Z. Ben-Sasson, R. Zeder, F.D. Finkelman, and W.E. Paul. 1990. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J. Exp. Med.* 172:921.