

Activation Events during Thymic Selection

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Summary

During their differentiation in the mouse thymus, CD4⁺8⁻ cells undergo several of the sequential changes observed upon normal activation of mature, peripheral CD4⁺ lymphocytes. Expression of CD69, an early activation marker, is first observed on a minority of cells at the T cell receptor (TCR)^{lo/med} double-positive stage, is maximal (50–90%) on heat-stable antigen (HSA)^{hi} TCR^{hi} double-positive, HSA^{hi} TCR^{med} CD4⁺8^{lo}, and HSA^{hi} TCR^{hi} CD4⁺8⁻ cells, and is downmodulated at the mature HSA^{lo} CD4⁺8⁻ stage. In contrast, CD44, a late activation marker, is selectively expressed at the HSA^{lo} stage. The set of lymphokines that CD4⁺8⁻ thymocytes can produce upon stimulation also characteristically expands from mainly interleukin 2 (IL-2) at the HSA^{hi} stage, to IL-2 and very large amounts of IL-4, IL-5, IL-10, and interferon γ (IFN- γ) at the HSA^{lo} stage. 1 in 30 HSA^{lo} CD4⁺8⁻ adult thymocytes secrete IL-4 upon stimulation through their TCR. This frequency is 25% of the frequency of IL-2 producers, about 100-fold above that of peripheral (mainly resting) CD4⁺ T cells. With time after their generation in organ culture, CD4⁺8⁻ thymocytes lose their capacity to secrete IL-4, IL-5, and IFN- γ , but not IL-2. Similarly, the frequency of IL-4, but not of IL-2, producers progressively decreases after emigration to the periphery as judged by direct comparison between thymic and splenic CD4⁺ cells in newborns, or by following the fate of intrathymically labeled CD4⁺8⁻ cells in adults after their migration to the spleen. This sequence suggests that thymic selection results from an activation process rather than a simple rescue from death at the double-positive stage, and shows that the functional changes induced after intrathymic activation, although transient, are still evident after export to the periphery.

When stimulated, mature peripheral CD4⁺ T lymphocytes secrete two different sets of lymphokines depending on their previous state of activation. Thus, resting peripheral CD4⁺ T cells mainly secrete the lymphokine IL-2 for a few days before reverting to the resting state, whereas preactivated cells produce a large panel of other lymphokines, including IL-4, IL-5, and IFN- γ (1–6). This secondary set of lymphokines is transiently observed at high levels upon reactivation, for 3–7 d after the initial stimulation, i.e., during the blast stage, and seems to be rapidly downmodulated after resting, both in vitro and in vivo (3, 7–8).

We have previously reported that new CD4⁺8⁻ cells generated in the thymus behave like recently activated cells (9). In their early, immature phase, characterized by high surface expression of the heat-stable antigen (HSA),¹ CD4⁺8⁻ thymocytes secrete mainly IL-2 after stimulation by the combination of ionomycin and PMA (TCR crosslinking failed

to induce lymphokine secretion at that stage). Mature (HSA^{lo}) CD4⁺8⁻ cells, however, secrete a wide range of lymphokines, including IL-2 and large amounts of the secondary set of lymphokines, particularly IL-4, and IL-5, IL-10, and IFN- γ upon stimulation by either TCR crosslinking or the combination of ionomycin and PMA. The magnitude of IL-4 secretion is on average 240-fold greater than that elicitable from other lymphoid organs, including peripheral and mesenteric lymph nodes, spleen, blood, and thoracic duct. Since the same phenotype was found in adult and in newborn thymuses, as well as in fetal thymic organ culture, we argued that the thymic environment itself generates this activation-like process.

Here, we have traced the origins and the fate of the IL-4 producer CD4⁺8⁻ cells generated in the thymus, by measuring their frequency in different in vivo and in vitro systems. We show that they follow the mainstream cell production and export by the thymus. We provide further evidence that the IL-4 producer state results from an intrathymic activation process by showing that CD69 and CD44, early and late ac-

¹ Abbreviation used in this paper: HSA, heat-stable antigen.

tivation markers, respectively, are sequentially expressed during the maturation of these IL-4 producer CD4⁺8⁻ thymocytes. Finally, we speculate on the relationship between these activation events and the thymic selection process.

Materials and Methods

Mice. 5-wk-old, specific pathogen-free BALB/c, C57BL/6, BALB/c × C57BL/6, and AKR mice were obtained from C. Reeder (National Cancer Institute, Frederick, MD) and housed in our specific pathogen-free facility for 1 wk before use. Day 13 fetuses were extracted from timed-pregnant C57BL/6 mice 13 d after vaginal plugs were observed.

Fetal Thymic Organ Cultures. Thymic lobes from day 13 fetuses were cultured on microporous membranes on top of 1.8 ml of Iscove's modified DMEM (IMDM; Biofluids, Rockville, MD) enriched with 10% heat inactivated FCS, glutamine, antibiotics, and 5×10^{-5} M 2-ME in six-well plates (Transwells; Costar, Cambridge, MA), at a density of 10 lobes per well, at 37°C, in a 5% CO₂ atmosphere. Half the medium was changed at day 6 and twice a week thereafter.

FACS[®] Analysis and Sorting of Thymic and Peripheral T Cell Subsets. Spleen or thymic cell suspensions were panned at 4°C for 30 min on plates coated with the anti-CD8 mAb 2.43 at 10 µg/ml, and CD8-depleted preparations were further purified, after CD4 staining with allophycocyanin (APC)-conjugated GK1.5 mAb and CD8 staining with 53.6.7-FITC by sorting using a dual laser instrument (FACStar Plus[®]; Becton Dickinson & Co., Mountain View, CA). HSA^{lo} thymocytes were identified by additional staining with biotinylated M1/69 mAb (10) followed by streptavidin-PE, and they were sorted within a gate set to include 95% of peripheral CD4⁺ T cells. They represented 20–30% of all CD4⁺8⁻ thymocytes in adult mice. We have previously shown that the majority of thymocytes that are able to secrete lymphokines upon TCR crosslinking are contained in this gate (9). The activation process on developing thymocytes was followed by four-color staining and analysis on the FACStar Plus[®] instrument using excitation wavelengths of 488 and 600 nm for the argon and dye lasers, respectively. Cells were stained with IM.78-FITC anti-CD44 (11) or H1.2F3-FITC anti-CD69 (12) (Pharmingen, San Diego, CA), and GK1.5-APC anti-CD4, 53.6.7-biotin anti-CD8 (Becton Dickinson & Co.), revealed with streptavidin-Texas red (Molecular Probes, Eugene, OR), H57-PE anti-TCR-α/β (13) (Pharmingen) or M1/69-PE anti-HSA, in the presence of anti-Fc receptor 2.4G2 antibodies to block nonspecific binding. The whole staining procedure was performed in ice-cold PBS containing 1% BSA and 0.1% sodium azide. As specificity controls, staining for CD69-FITC and CD44-FITC were completely abolished by a 100-fold excess of unlabeled purified anti-CD69 or anti-CD44 antibody, respectively.

Recent Thymic Migrant Assay. Adult C57BL/6 mice were anesthetized by intraperitoneal injection of 0.2 ml of a mixture containing 0.2 mg of ketamine (Parke-Davis, Morris Plains, NJ) and 2 mg of Xylazine (Haver, Shawnee, KS), and each thymic lobe was injected with 10 µl of a solution of FITC (Research Organics Inc., Cleveland, OH) at 0.5 mg/ml in PBS as described by Scollay et al. (14). Groups of 10–15 mice were killed 10 h later, and their spleen cells were pooled and enriched in CD4⁺ cells by incubation with anti-CD8 mAb 3.155 followed by panning on anti-mouse Ig-coated plates to remove CD8⁺ cells and B cells, then passed on a lympholyte solution (Lympholyte-M; Cedarlane Laboratories, Hornby, Ontario, Canada) to remove red cells and dead cells, before staining with GK1.5-APC anti-CD4 and FACS[®] sorting for

CD4⁺ lymphocytes positive or negative for FITC. To follow the fate of the recent thymic migrants with time, some mice were thymectomized 48 h after intrathymic injection of FITC, and the FITC-positive cells present in the spleen were FACS[®] sorted 3 or 6 d after thymectomy.

Limiting Dilution Assays. Specific indicator cell lines were used to detect single cells producing lymphokines as modified from Rocha and Bandeira (15) and Seder et al. (16). FACS[®] purified CD4⁺ T cells were plated at various numbers per well, at 30 replicates per concentration point, in round-bottomed microwells containing 1,000-rad irradiated, dendritic cell-enriched accessory cells (200 cells per well), and Con A at 3 µg/ml (similar results were obtained with plate-bound anti-CD3 antibody), in a final volume of 30 µl of medium composed of a 1:1 mixture of Click's medium and RPMI (Biofluids, Rockville, MD) enriched with 10% heat-inactivated FCS, glutamine, antibiotics, and 5×10^{-5} M 2-ME. The dendritic cell-enriched preparation was made from spleen cells adhered for 2 h at 2×10^8 cells in 10 ml in plates (Falcon 3003; Becton Dickinson, Lincoln Park, NJ) with a cocktail of anti-T cell mAbs (J1J.10 anti-Thy-1, 172.4 anti-CD4, and 3.155 anti-CD8) and guinea pig complement at 1:10 added during the last 45 min of incubation. Nonadherent cells were removed by strong and repeated washings and the preparations reincubated for an additional 6 h, before collecting the detached cells and centrifuging them over a 50% percoll solution (Pharmacia-LKB, Uppsala, Sweden). Low density cells prepared in this way contained 80–95% dendritic cells specifically stained with biotinylated 33D1 antibody (17) and streptavidin-PE. Assay wells destined to measure the frequency of IL-4 producers were supplemented with a source of exogenous human rIL-2 at 10 U/ml (30 pM) (Hoffmann-La Roche, Basel, Switzerland). This is because, in our culture conditions, IL-4 secretion requires the presence of IL-2 (2–3, 16). After 48 h of culture, the plates were irradiated (1,000 rad) and the indicator cells were added in situ in a volume of 10 µl. IL-2-producing wells were detected by adding 100 CTLL cells (American Type Culture Collection, Rockville, MD) with the anti-IL-4 mAb 11B11 (18) at 30 µg/ml, and IL-4-producing wells by adding 500 IL-4-specific CT.4S cells (19) with a cocktail of anti-IL-2 (S4B6 [20], 20 µg/ml), and anti-IL-2R antibodies (PC 61 [21] and 7D4 [22] at 10 µg/ml and 1:100 final dilution of an ammonium sulfate semi-purified supernatant, respectively). In these conditions, addition of antibodies specific for the lymphokine assayed always resulted in a 100% inhibition of detection. Positive wells were scored 48 h later, usually by visual scoring of the wells containing live indicator cells. This method was shown to give excellent correlation with scoring based on thymidine incorporation by CT.4S cells at 48 h and by CTLL cells at 96 h. Control wells where T cell responders were omitted always scored negative. These assays can detect a frequency of one in one IL-4 producers in two different Th2-type CD4⁺ T cell clones, and 1 in 1.8 IL-2 producers in one Th1-type CD4⁺ T cell clone, with a single hit Poisson distribution (16, and data not shown). Frequencies and their 95% confidence interval limits were calculated according to the χ^2 minimization method of Taswell (23), on a computer program designed by Dr. Steve Stringfellow (NOAA Pacific Fleet, Seattle, WA).

Bulk In Vitro Stimulation of Lymphokine Secretion. CD4⁺ cells at 10^5 /well (except as otherwise stated) were plated on round-bottomed microwells coated with 10 µg/ml of anti-TCR-α/β mAb H57 (13), in the presence of $2-3 \times 10^4$ dendritic cell-enriched accessory cells, and supernatants were collected at 48 h and kept frozen at -20°C until assayed. The dose of stimulating antibodies and the time for collection of the supernatants had previously been de-

terminated to be optimal for the detection of maximal levels of all the lymphokines assayed.

Lymphokine Assays. IL-2 was measured by the CTLL bioassay run in the presence of the anti-IL-4 mAb 11B11 at 30 $\mu\text{g/ml}$, and IL-4 by the IL-4 specific CT.4S bioassay run in the presence of the cocktail of anti-IL-2 and anti-IL-2R antibodies described above. The specificity of the two bioassays in these conditions was demonstrated by the complete blocking of the indicator cell proliferation by the homologous neutralizing antibody, and is attested in many experiments where cell subsets were identified that secreted very high levels of one lymphokine without detectable or with only traces of the other lymphokine. IFN- γ and IL-5 were measured by two-site sandwich ELISAs (24, 25). Results are expressed in picomolar equivalents, by comparison with recombinant standards run for each assay. IL-2 was recombinant human protein from Hoffmann-La Roche (Basel, Switzerland), IL-4 was mouse recombinant purified from a baculovirus expression system (C. Watson; NIH, Bethesda, Maryland), IL-5 was mouse recombinant from DNAX (Palo Alto, CA), mouse recombinant IFN- γ was from Amgen (Thousand Oaks, CA).

Results

The Large Amounts of IL-4 Secreted by HSA^{lo} CD4⁺ 8⁻ Thymocytes Reflect a High Producer Cell Frequency. As thymocytes mature from the double-positive to the single-positive stage, they progressively express lower levels of HSA. We used this marker to purify mature (HSA^{lo}) CD4⁺ 8⁻ thymocytes, and compared their ability to secrete various lymphokines with that of peripheral inguinal and axillary CD4⁺ lymph node cells upon stimulation by plate-bound anti-TCR- α/β antibodies. As previously shown (9), peripheral CD4⁺ cells produce a pattern of lymphokines that is radically different from that of their presumptive immediate precursor, HSA^{lo} (mature) CD4⁺ 8⁻ thymocytes (Fig. 1). LN CD4⁺ cells produce mainly IL-2 and only traces of other lymphokines, such as IL-4 or IFN- γ , whereas mature CD4⁺ 8⁻ thymocytes produce low levels of IL-2, and high levels of IL-4 and γ -IFN- γ (370- and 60-fold more than LN cells, respectively). To de-

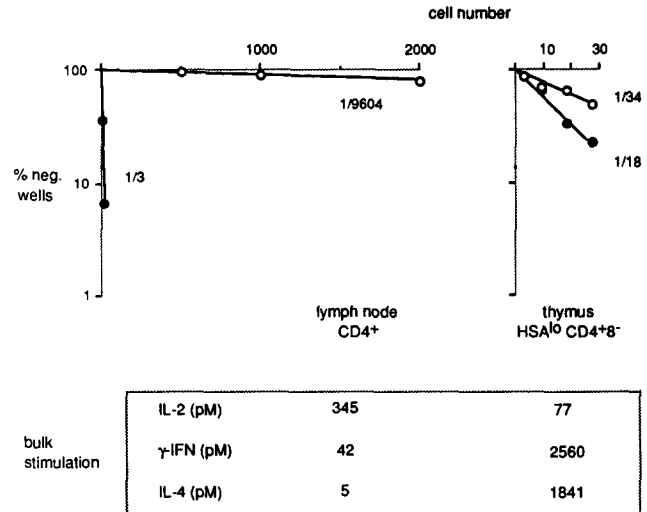


Figure 1. Frequencies of IL-2 and IL-4 producers in mature (HSA^{lo}) CD4⁺ 8⁻ thymocytes and peripheral CD4⁺ lymphocytes. (○) IL-4 producers; (●) IL-2 producers. The 95% confidence limits of the frequencies measured were: LN IL-2, 1/2–1/4; IL-4, 1/5,930–1/14,056; thymus IL-2, 1/14–1/22; IL-4, 1/26–1/41. For comparison, the table shows the amounts of lymphokines obtained in bulk stimulation of the same purified populations.

termine whether the large production of IL-4 was related to a high frequency of producers or to a rare cell type capable of secreting large amounts of this lymphokine, we measured the frequencies of cells secreting IL-2 or IL-4 in limiting dilution assays. We found that the differences in bulk stimulation corresponded to differences in producer frequencies. Thus, in the experiment shown in Fig. 1, 1/3 of LN CD4⁺ cells produced IL-2 vs. 1/18 of mature CD4⁺ 8⁻ thymocytes. Conversely, 1/34 of thymic CD4⁺ 8⁻ cells produced IL-4 vs. only 1/9,600 of LN CD4⁺ cells (Fig. 1). Results from several experiments performed in C57BL/6 or BALB/c mice are

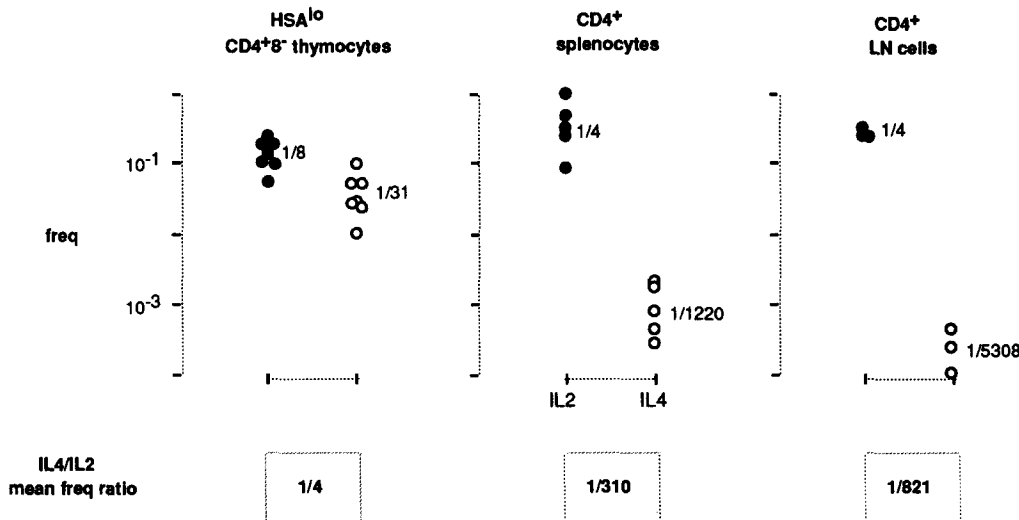


Figure 2. Frequencies of IL-2 and IL-4 producers in mature CD4⁺ cell populations. (○) IL-4 producers; (●) IL-2 producers. Experiments in which the frequency of IL-4 producers and that of IL-2 producers were simultaneously measured are displayed in the figure. The geometric mean of the frequencies is written on the right of each group of symbols. The geometric means of the individual IL-4/IL-2 frequency ratios are displayed inside boxed areas at the bottom.

summarized in Fig. 2. On average, there is one mature CD4⁺8⁻ thymocyte able to secrete IL-4 for four that can produce IL-2, whereas the proportions in spleen and lymph nodes have dropped to 1 for 300 and 1 for 800, respectively. It is not yet clear whether IL-4-producing cells are distinct from or included in the IL-2-producing cell population. We will refer however to the ratio of IL-4/IL-2 frequencies in this paper, because it provides a way to normalize the levels of mature, lymphokine-secreting cells in CD4⁺ populations obtained from different in vivo and in vitro systems.

CD4⁺8⁻ Thymocytes with the Capacity to Secrete IL-4 and IFN- γ Are Exported to the Spleens of Adult Mice. Two possible general explanations could be proposed for the difference between the frequency of IL-4 producers in the thymus and in the peripheral lymphoid organs. The first is that IL-4 producers are not exported out of the thymus, and therefore represent either resident cells, cells on their way to die, or even cells leaving the CD4⁺ pool for the CD4⁻8⁻ compartment (26–29). The second is that IL-4 producers are exported but they may have special circulation properties that exclude them from the spleen and the peripheral lymphoid organs, or have a very short lifespan, or perhaps convert to IL-2-only producers soon after reaching peripheral lymphoid organs. To discriminate between these alternatives, we analyzed the pattern of lymphokines secreted by recent thymic migrants in the spleen. Thymocytes were labeled in situ by an intrathymic injection of FITC, and FITC⁺ CD4⁺ cells were recovered in the spleen by electronic cell sorting 10 h after the injection (Fig. 3). In three different experiments, 0.4–0.5% of the spleen CD4⁺ cells were FITC⁺ at that time point, a result close to that obtained by others (14). However, because the proportion of thymic cells labeled by this technique was ~50% for all subpopulations including HSA^{lo} CD4⁺8⁻ cells (not shown), the actual proportion of migrants may be closer to 0.8–1%. As shown in Fig. 4, bulk stimulation of recent thymic migrants (FITC⁺) showed that they produced 20-fold more IL-4 than FITC⁻ cells (a mixture of nonlabeled migrants, activated/memory cells and the majority of periph-

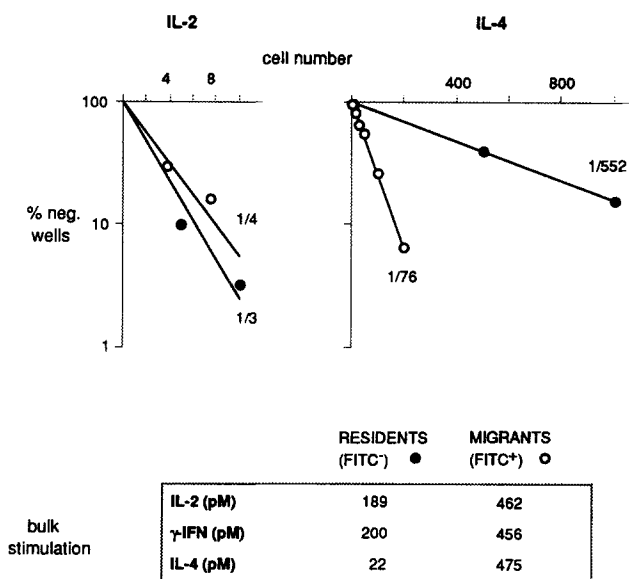


Figure 4. Frequencies of IL-2 and IL-4 producers among recent thymic migrants in the spleen. The frequencies of IL-2 and IL-4 producers were determined in the purified populations of migrants (○) and residents (●) shown in Fig. 3. The 95% confidence limits of the measured frequencies were: resident IL-4 producers, 1/417–1/662; resident IL-2 producers, 1/2–1/3; migrant IL-4 producers, 1/61–1/86; migrant IL-2 producers, 1/3–1/4. For analysis of lymphokine secretion in bulk, 6×10^4 cells were stimulated by TCR crosslinking, as described in Materials and Methods. Results are shown in the table at the bottom.

ipheral, resting, IL-2-only-producing cells). The migrant cells also secreted IFN- γ , although not significantly more than the resident cell population, and their secretion of IL-2 was comparable with that of resident cells. In limiting dilution assays, the frequency of IL-2 producers was similar among migrants and residents but the frequency of IL-4 producers among migrants (1/76) far surpassed that among the residents (1/552). Thus, the ratio of IL-4/IL-2 producers is ~10-fold higher in migrants (1/19) than in residents (1/184). When

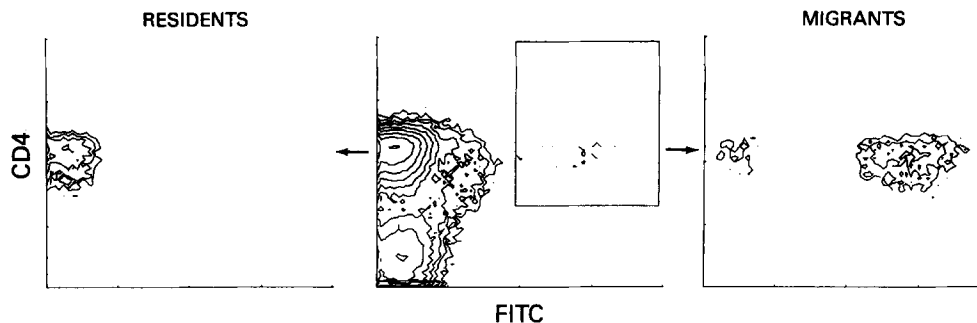


Figure 3. Thymic migrants in the spleen. Intrathymically FITC-labeled migrants were identified within the splenic CD4⁺ compartment. (Middle) Logarithmic display of fluorescence intensities in a CD4⁺-enriched population prepared from the pooled spleens of 15 C57BL/6 mice. The gate includes the CD4⁺ cells that migrated out of the thymus during the last 10 h: 0.4% of the splenic CD4⁺ pool. This percentage was calculated from the analysis of 10^5 CD4⁺ cells; only 1 of 10^5 (0.001%) CD4⁺ cells from control uninjected mice was found in the same gate. Resident- and migrant-enriched CD4⁺ cell populations were obtained by FACS[®] sorting (left and right, respectively).

compared with the average among mature $CD4^+8^-$ thymocytes, however, the IL-4/IL-2 ratio in thymic migrants was found to be decreased fivefold (compare with Fig. 2). This was due to both an increase of the IL-2 producer frequency (twofold) and a decrease in the IL-4 producer frequency (2.5-fold). Similar results were obtained in two additional experiments shown in Fig. 5 (see the minus 38-h time points). To follow the fate of the thymic migrants over time, mice were intrathymically injected with FITC, and then divided into three groups. The first group was analyzed at 10 h. Mice from the other two groups were thymectomized at 48 h, and one group was analyzed after 3 d, and the other after 6 d. Fig. 5 shows the evolution of the frequencies of IL-4 and IL-2 producers and their ratio in both the migrants and the control residents. The frequency of IL-2 producers was similar in both populations and did not change significantly. In contrast, a major reduction was observed over time in the frequency of IL-4 producers among the thymic migrants. The frequency had already decreased an order of magnitude by 3 d, down to 1:1,745, threefold lower than that of the peripheral residents assayed at the same time. After an additional 3 d, the frequency was almost undetectable at 1:15,612, 30-fold lower than the resident cells. Similar conclusions were obtained from bulk stimulation cultures (not shown). These kinetic studies establish that phenotypically activated thymic migrants are exported to the spleen, and they show that the frequency of the activated phenotype drops by 100-fold within a period of 6 d. From calculations based on the measured rate of export, the frequency of IL-4 producers in the spleen and in the migrant cell population, and their functional half-life, we estimate that $\sim 10\%$ of the cells able to produce IL-4

Table 1. Frequencies of IL-2 and IL-4 Producers in Newborn $CD4^+8^-$ Thymocytes and $CD4^+$ Splenocytes

	Exp. 1		Exp. 2	
	Newborn HSA ^{lo} $CD4^+8^-$ thymocytes	Newborn HSA ^{lo} $CD4^+8^-$ thymocytes	Newborn HSA ^{lo} $CD4^+8^-$ thymocytes	Newborn $CD4^+$ splenocytes
IL-2	1/12	1/6	1/6	1/3
IL-4	1/183	1/95	1/95	1/358
IL-4/IL-2	1/15	1/16	1/16	1/119

Exp. 1, 1-d-old C57BL/6 newborns; Exp. 2, 3-d-old C57BL/6 newborns.

in the spleen of unimmunized, specific pathogen-free mice are in fact recent thymic migrants.

$CD4^+8^-$ Thymocytes Capable of Secreting IL-4 Are Exported to the Spleen of Newborn Mice. We have previously shown, using neonatal mice and fetal thymic organ culture, that a significant proportion of IL-4 producer cells in the thymus are not likely to be recirculating cells (9). We now asked the same question of the migrants. At 1 d after birth, $CD4^+$ cells are undetectable in the spleen, and, by day 3, they reach a level of only 1% of the total splenic population. We therefore purified HSA^{lo} $CD4^+8^-$ cells from C57BL/6 newborn thymuses at days 1 and 3 after birth and compared them with $CD4^+$ spleen cells sorted at day 3. Table 1 shows that the ratio of IL-4 to IL-2 producers is the same ($\sim 1/15$) for thymocytes of both ages. Thus, at a time when the periphery is

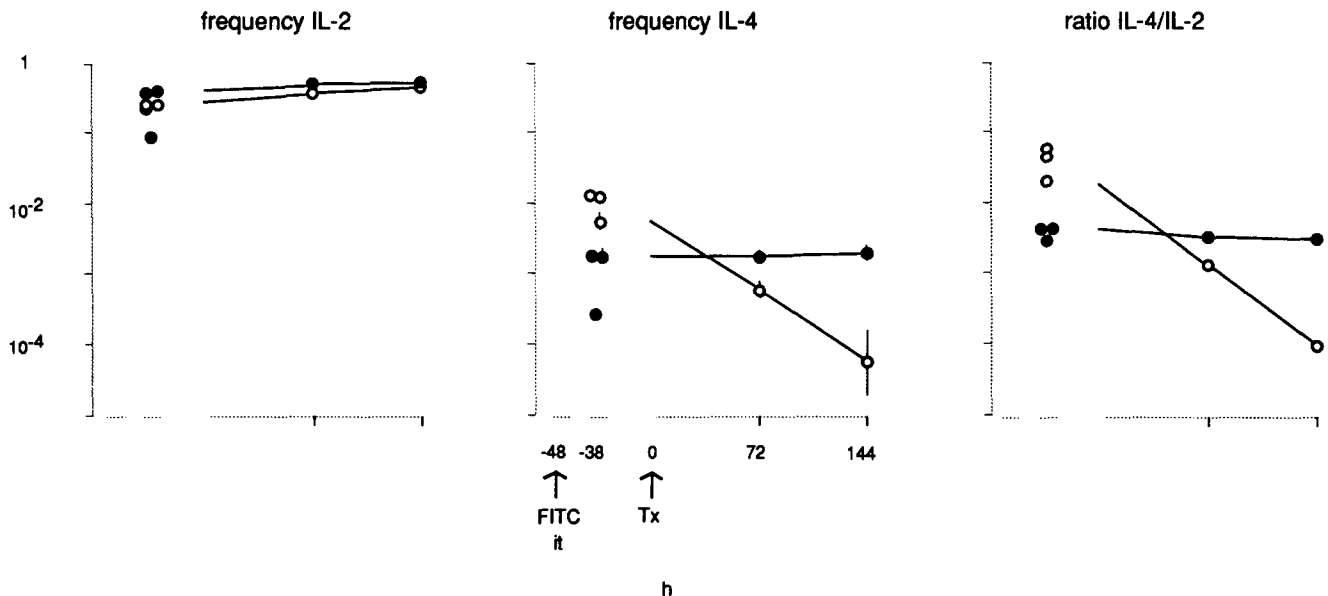


Figure 5. The fate of thymic migrants. Pools of C57BL/6 mice were injected intrathymically with a solution of FITC and the frequencies of lymphokine producers among thymic migrants (O) and residents (●) in the spleen were measured 10 h later in three experiments. In one of these experiments, mice from the same pool assayed at 10 h were thymectomized at 48 h. 3 or 6 d later, FITC⁺ $CD4^+$ cells in the spleens (respectively, 2.2 and 1.8% of the $CD4^+$ cells, over backgrounds of 0.004 and 0.001% measured on 10^5 cells) were FACS[®] sorted and analyzed. The 95% confidence intervals of the measured frequencies of IL-4 producers among migrant or resident cells over time in this experiment are represented by the bars in the middle figure.

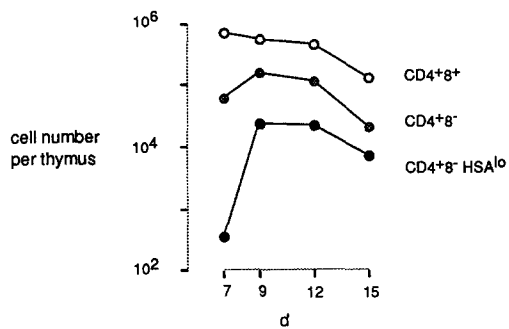


Figure 6. Kinetics of generation of $CD4^+8^-$ cells in fetal thymic organ culture. Day 13 fetal thymuses were cultured as described in Materials and Methods. At the indicated time points, pools of five to six thymuses were collected and analyzed to measure the numbers of cells belonging to the various cell subsets. The number of double-positive thymocytes peaks at day 7 (not shown).

virtually devoid of lymphocytes, a significant proportion of the lymphokine-secreting cells in the thymus are IL-4 producers, reinforcing our previous conclusion that IL-4 producers are generated in the thymus. When we compared 3-d-old splenic $CD4^+$ cells with their thymic precursors, we found that the frequency of IL-4 producers had decreased fourfold while the frequency of IL-2 producers had increased twofold, to a final ratio of 1/119, eightfold lower than in the thymus. This is similar to what was observed in the adult thymic migration assay, where the ratio decreased fivefold, and supports the contention that the IL-4 producer $CD4^+8^-$ population generated in the thymus is exported to the spleen, where it soon begins to lose its capacity to secrete IL-4.

Kinetics of Expression of the Potential to Secrete IL-4, IL-5, and IFN- γ during Fetal Thymic Organ Culture. In the periphery, the acquisition of the potential to secrete IL-4, IL-5, and IFN- γ by $CD4^+$ T cells occurs by 2–4 d after activation (1, 3, 4, 7, 8). We used fetal thymic organ cultures to study the timing of appearance of IL-4 producers, their rate of accumulation, and their ultimate fate as compared with IL-2 producers. Fig. 6 shows the kinetics of the generative phase in fetal thymic organ culture. The number of double-positive cells peaks at 7 d in culture (day 20 of fetal life) and $CD4^+8^-$ reach their maximum number 2 d later. This sequence is similar to that found in fetal and newborn thymuses followed in vivo (30). Although HSA^{lo} $CD4^+8^-$ cells were first detected at day 7, neither day 7 nor day 8 HSA^{lo} -enriched $CD4^+8^-$ cells produced detectable lymphokines (including IL-2) when stimulated through the TCR (data not shown). This is presumably because these very minor subpopulations identified as HSA^{lo} in fact belong to the overlapping HSA^{hi} major population at these time points. Day 9 marked the appearance of the earliest functionally mature HSA^{lo} $CD4^+8^-$ cells and, in two separate experiments, the ratio of IL-4/IL-2 frequencies at this time was 1/11 and 1/20 (Fig. 7), a result in the same range as that measured in newborn thymus, and 2.5–5-fold lower than the ratios observed in adult thymuses. With time, the IL-4/IL-2 ratio decreased (Fig. 7, right). Thus, it appears that the development and fate of IL-4 producers in the organ culture system in vitro is the same as in vivo. The mature $CD4^+8^-$ population first emerges enriched in IL-4 producers and then reverts to a mainly IL-2 producer population. This is similar to what was observed a few days after migration of thymocytes to the spleen, and suggests that the disappearance of IL-4 producers in the spleen is not simply

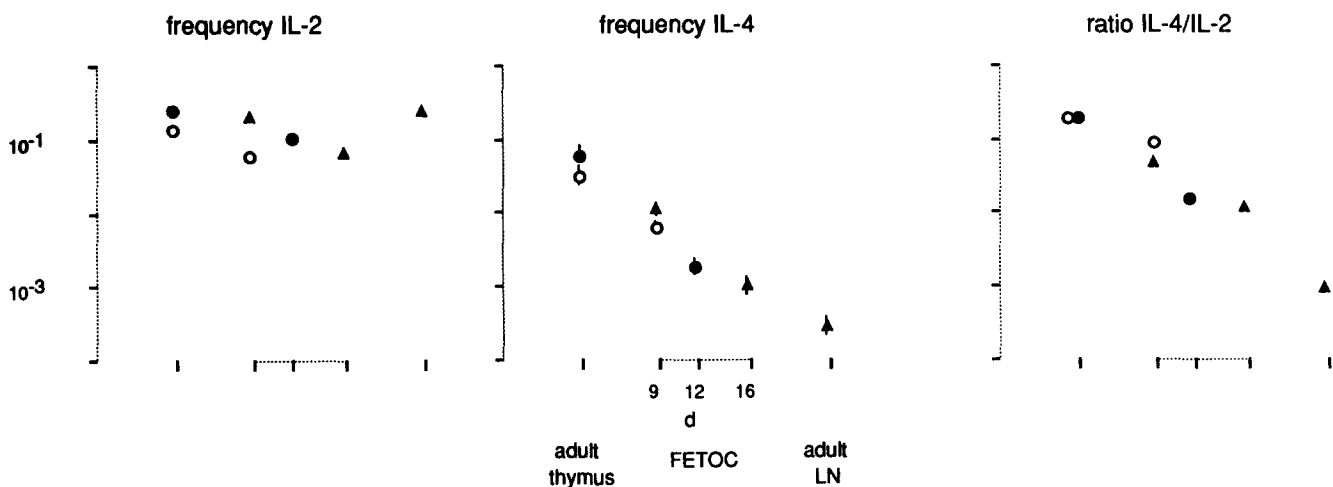


Figure 7. Frequencies of lymphokine producers among HSA^{lo} $CD4^+8^-$ cells during fetal thymic organ culture. The frequencies of lymphokine producers among purified HSA^{lo} $CD4^+8^-$ cells in fetal thymic organ culture were measured at days 9, 12, and 16, and compared with those of adult HSA^{lo} $CD4^+8^-$ thymocytes and $CD4^+$ LN cells. In one experiment (circles), HSA^{lo} $CD4^+8^-$ cells were purified at day 9 (open circles) and day 12 (closed circles) of the same fetal thymic organ culture, and compared each time to their homologue from fresh adult thymuses, represented in open and closed circles, respectively, at the left part of each graph. In another experiment (triangles), HSA^{lo} $CD4^+8^-$ thymocytes were purified the same day from two distinct fetal thymic organ cultures set up 1 wk apart (days 9 and 16), and they were compared with fresh adult $CD4^+$ LN cells (presented on the right part of each graph). Results are expressed as frequency values for IL-2 and IL-4 producers (left and middle, respectively), or as the IL-4/IL-2 frequency ratios (right). The 95% confidence intervals are shown for the IL-4 producer frequencies (middle).

the result of migration to other organs but a change of state of the migrant cells. Table 2 shows that in addition to IL-4, other lymphokines characteristic of the activated stage, such as IL-5 and IFN- γ , are also produced at day 9 by CD4⁺8⁻ fetal thymocytes, and the production of IL-5 decreases with time, similar to IL-4. Thus, by analogy with the sequence of events reported during the activation of peripheral CD4⁺ lymphocytes (1, 3, 7, 8), the transient acquisition by HSA^{lo} CD4⁺8⁻ thymocytes of the potential to secrete IL-4 and IL-5 suggests that activation events have occurred 2–4 d earlier, between days 5 and 7 of fetal organ culture, at the double-positive or early immature (HSA^{hi}) CD4⁺8⁻ stage.

Sequential Expression of CD69 and CD44 during the Differentiation of CD4⁺8⁻ Thymocytes. The activation/memory marker CD44 is present on most peripheral CD4⁺ T cells capable of secreting IL-4, IL-5, and IFN- γ (4, 9, 31). CD44 is a late activation marker that is maximally expressed within 2–3 d after activation (32). It is also expressed on a variable proportion of the CD4⁺8⁻ thymic subset in low expressor strains such as C57BL/6 and AKR (33). Fig. 8 shows that it is selectively expressed on the HSA^{lo} cells in C57BL/6 mice. This was also observed in AKR and BALB/c \times C57BL/6 mice (not shown). To test whether CD44 expression would overlap with the potential to secrete IL-4 by HSA^{lo} CD4⁺8⁻ thymocytes, we sorted CD4⁺8⁻ thymocytes from C57BL/6 mice into HSA^{lo} CD44^{hi} and HSA^{lo} CD44^{lo} cells. The table at the bottom of Fig. 8 shows that the CD44^{hi} population indeed contains threefold more IL-4 producers and fivefold fewer IL-2 producers than the CD44^{lo} population. Thus, the ratio of IL-4 to IL-2 producers in the CD44^{hi} subpopulations (1/3) is much higher than that in the CD44^{lo} subpopulation (1/50). In this and another experiment, analysis of the lymphokines secreted after bulk stimulation showed that IFN- γ production was exclusively found in the CD44^{hi} population (not shown).

The chronology of events leading to the activated functional phenotype of the HSA^{lo} CD4⁺8⁻ thymocytes was further investigated, using four-color cytofluorometry to

Table 2. Lymphokines Produced by HSA^{lo} CD4⁺8⁻ Thymocytes from Fetal Thymic Organ Culture

	day 9	day 12
	<i>pmol</i>	
IL-2	133	199
IFN- γ	233	193
IL-4	247	88
IL-5	390	53

After 9 or 12 d in organ culture, cells were purified out of the same pool of 200 thymuses from day 13 fetuses as used in Fig. 7 (experiment represented by circles) to measure IL-4 and IL-2 producer frequencies. 3×10^4 (day 9) or 5×10^4 (day 12) CD4⁺ cells were stimulated with plate-bound anti-TCR- α/β mAb as described in Materials and Methods.

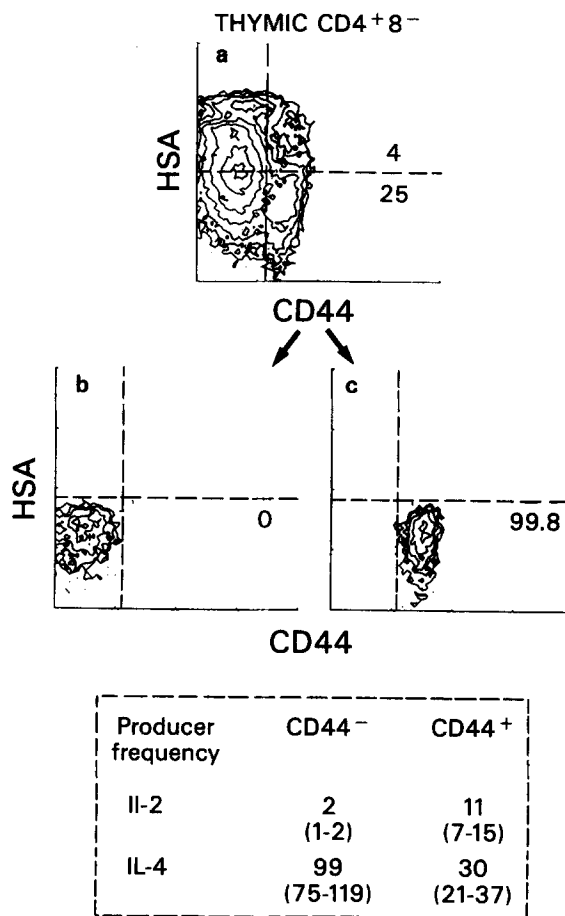


Figure 8. CD44⁺ CD4⁺8⁻ thymocytes are HSA^{lo} and are enriched in IL-4 producers. CD4⁺8⁻-enriched thymocytes from 6-wk-old C57BL/6 mice were stained with CD4-APC, HSA-biotin revealed by streptavidin-PE, and CD44-FITC (a), before sorting into HSA^{lo} CD4⁺ cells negative (b) or positive (c) for CD44. The frequency of IL-2 and IL-4 producers (and its 95% confidence limits) was then measured in each population (table).

follow the levels of expression of CD69, an early activation marker that is induced within a few hours after activation (12, 34, 35). For each thymic subset, we compared CD69 expression with thymocyte maturation markers such as membrane levels of TCR and HSA (Fig. 9). High levels of TCR were defined by reference to the CD4⁺8⁻ subset (f), absence of detectable TCR by reference to double-negative cells (c), and low/medium levels are in between. Low vs. high levels of HSA were defined by reference to splenic CD4⁺ cells (h). Fig. 9 shows that CD69 expression is directly correlated to TCR levels among double-positive cells. CD69 is first expressed on a minor subset (3%) of TCR^{lo/med} double-positive cells and is then present on 50% of the TCR^{hi} double-positive population (d). The majority of a presumptive transitional (36) TCR^{med} CD4⁺8^{lo} subset is CD69 positive (e), as is the immature HSA^{hi} CD4⁺8⁻ population (g). CD69 expression drops at the HSA^{lo} CD4⁺8⁻ stage (g), thus showing a reciprocal distribution with CD44 (compare with Fig. 8), and remains low on the vast majority of pe-

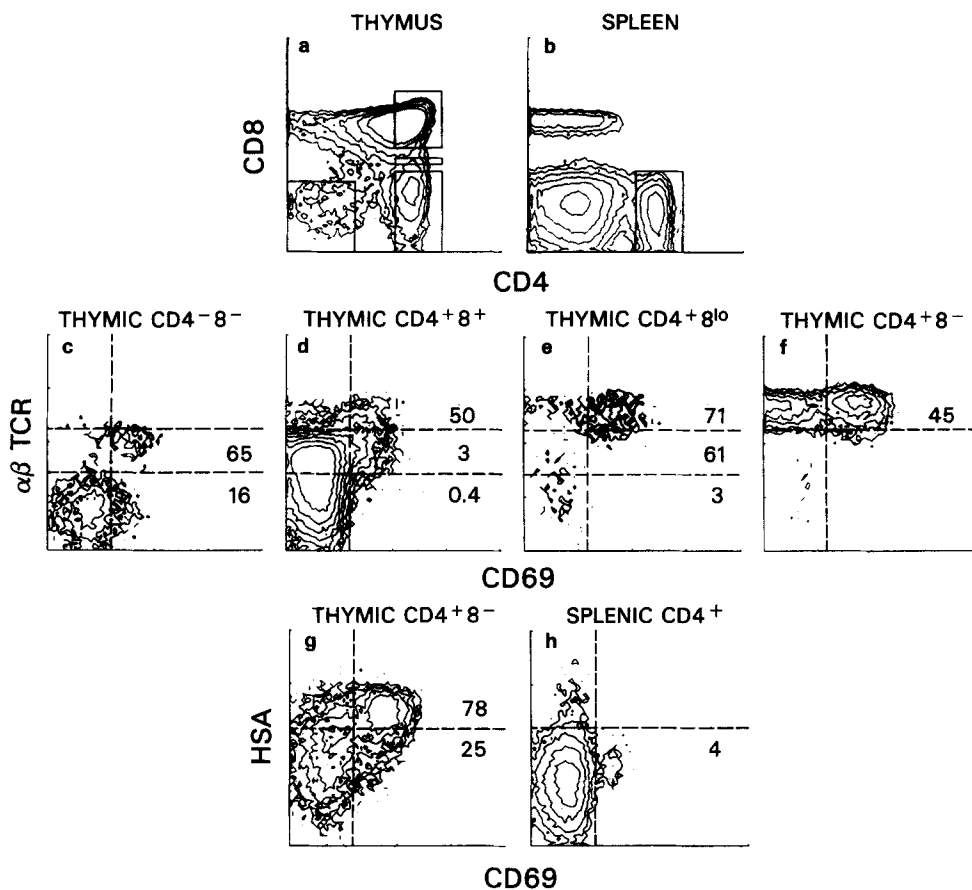


Figure 9. CD69 expression during thymocyte development. Four-color analysis of thymocytes and splenocytes from a 6-wk-old AKR mouse. *a* (thymus) and *b* (spleen) show the gates used for analysis of the different subpopulations. The gate defining CD69-positive vs. negative cells is set by reference to the background staining in the presence of a 100-fold excess of unlabeled CD69 antibody. This gate does not apply for the TCR⁻ CD4⁻8⁻ cells (*c*), which have a higher level of autofluorescence than the other subpopulations, and are all CD69 negative (not shown).

peripheral CD4⁺ lymphocytes (*h*). The faint apparent labeling of TCR⁻ double-negative thymocytes (*c*) is in fact non-specific, due to a higher level of autofluorescence of this population, because it is not modified by a 100-fold excess of unconjugated anti-CD69 antibody (not shown). Double-negative thymocytes expressing intermediate levels of TCR were consistently found to contain 60–70% CD69⁺ cells (*c*). Similar results were found in all strains tested, including AKR, C57BL/6, BALB/c, and BALB/c × C57BL/6. In particular, the highest proportions of CD69⁺ cells (50–90%) were always found among “transitional” TCR^{med} CD4⁺8^{lo} cells, HSA^{hi} TCR^{hi} double-positive cells and HSA^{hi} CD4⁺8⁻ cells. Because the CD69 antigen is expressed within a few hours after stimulation of peripheral cells (12, 34, 35), these results suggest that the thymic activation events occur at the TCR^{lo/med} double-positive stage.

Discussion

The experiments described in this paper were initiated to explore the intrathymic pathway leading to the generation of IL-4 producer CD4⁺8⁻ thymocytes, in terms of rates of production and export, and to understand the origin of this unexpected phenotype. By most criteria examined, this pathway appears to identify the normal process of thymic selection and export to the periphery of CD4⁺ T lympho-

cytes. The IL-4 producer stage of the HSA^{lo} CD4⁺8⁻ population correlates with the expression of the CD44 late activation marker, and follows a stage where HSA^{hi} CD4⁺8⁻ thymocytes transiently express the early activation marker CD69 and are IL-2-only producers. By the time when HSA^{lo} CD4⁺8⁻ thymocytes get exported to the periphery, they progressively revert to the resting phenotype (CD44^{lo}, IL-2-only-producer) observed for the majority of peripheral CD4⁺ T cells. These sequential events are reminiscent of changes observed in peripheral CD4⁺ T cells after activation and suggest the hypothesis that thymocyte selection is also an activation process.

We found that the frequency of cells able to secrete IL-4 is extraordinarily high among mature CD4⁺8⁻ thymocytes. On average, one IL-4 producer was found for every four IL-2 producers. Such a high proportion of IL-4 producers has no known equivalent in lymphoid organs: the bulk production as well as the frequency of mature CD4⁺ cells capable of secreting IL-4 in the thymus is 30–800-fold above that found in other lymphoid compartments in the body, including spleen, peripheral and mesenteric lymph nodes, blood and thoracic duct (9), and also peri-thymic lymph nodes (our unpublished observations), or Peyer’s patches (2).

We next showed that these CD4⁺8⁻ thymocytes are exported to the periphery: first by labeling the thymocytes *in vivo* with FITC and collecting the recent thymic migrants

in the spleen 10 h later, and second by analyzing the rare CD4⁺ cells present in the spleens of unmanipulated, 3-d-old newborns, that is, 2–3 d after the first mature CD4⁺8⁻ thymocytes become detectable in the thymus. IL-4 producer cells were present in both types of migrating population in great excess over what would be expected from a population of normal virgin, resting peripheral cells. In both cases, however, it was found that the frequency of recently migrated cells with this phenotype was lower than in the thymus. Soon after export to the spleen, the ratio of IL-4/IL-2 frequencies had decreased ~5 fold. 3 d after export, the frequency of IL-4 producers had dropped by another ~10-fold in adult thymectomized mice, and it dropped 10-fold further after an additional 3 d so as to leave a prototypical resting, naive peripheral population such as the one described, for example, in human cord blood (5, 6) (where the immune system is mature at 14–15 wk of gestation and the exported T cells are secluded from environmental antigens). The possibility that the IL-4-secreting cells had in fact left the spleen for another compartment during the time of the study was argued against by the thymic organ culture experiments. In these experiments, a decrease in the frequency of IL-4 producers was also observed with time, while the frequency of IL-2 producers remained stable. These results suggest that the drop in the frequency of IL-4 producers is related to their conversion to the resting, IL-2-only secretor phenotype, although the formal possibility remains that the IL-4 producers generated in the thymus constitute a short-lived, separate lineage.

To verify that the thymic steady-state levels of IL-4 producers reflect a high production rate rather than a slow export rate, we followed the early generation of these cells in the sequestered environment provided by the *in vitro* fetal thymic organ culture system. We found that the frequency of IL-4 producers peaked as soon as mature cells were generated, both in absolute and in relative (compared with the IL-2 frequency) numbers, by day 9 in culture. The peak ratios were as high as one IL-4 producer for 10–20 IL-2 producers, similar to the ratios measured *in vivo* in day 1 and day 3 newborns. Altogether, these results suggest that the thymus generates and exports a significant proportion of IL-4 producer CD4⁺ cells.

What mechanism generates these IL-4 producer CD4⁺8⁻ thymocytes? In the periphery, the functional property to secrete IL-4 upon stimulation is tightly correlated to activation markers such as a low cell density (2) and the surface expression of activation/memory markers, in particular of CD44 (4, 9). We found that CD44^{hi} CD4⁺8⁻ thymocytes are indeed enriched in IL-4 producers (IL-4/IL-2 producer ratio of 1/3) as compared with their CD44^{lo} counterpart (ratio of 1/50). In addition, the forward scatter index and the density of HSA^{lo} CD4⁺8⁻ thymocytes are those of activated rather than resting cells (data not shown). Thus, by these criteria, IL-4 producer CD4⁺8⁻ thymocytes appear to be in an activated state.

What could be the origins of these activated cells in the mature thymic CD4⁺8⁻ compartment? Aside from the thymic selection process itself, which may account for all

the activation observed, two other potential sources of activated cells could contribute to some extent to the population described in this paper: mature peripheral cells recirculating back to the thymus (37–44), which may mainly consist of activated cells (42), and early cells that have been immunized in the medulla before leaving to reach peripheral organs (45, 46, and Guerder, S., and P. Matzinger, manuscript submitted for publication). Because both these cell populations would only exist in adult thymuses but not in newborn thymuses or in fetal thymic organ culture, they could only account for the threefold difference in the relative frequency of IL-4 producers at the fetal/newborn and the adult stages. An alternative possibility to explain the threefold difference is that the stem cells and/or the thymic microenvironment in the newborn are different from the adult, as has been suggested by many reports (30, 47–51).

Mature CD4⁺8⁻ thymocytes have a rather long period of residency in the medulla, between 1 and 2 wk (52). Since it takes only 2–4 d to prime a resting cell to become an IL-4 producer (1, 3, 7), thymocyte activation could in theory occur at various times, from the TCR^{med} double-positive stage to the HSA^{lo} CD4⁺8⁻ stage itself. To identify the precise timing of the activation event in the thymus, we first performed kinetic studies of the activation-related changes. In both fetal thymic organ culture and in newborn mice, the potential to secrete IL-4, IL-5, and IFN- γ was present and at peak levels as soon as mature HSA^{lo} cells were detectable. Thus, this property is already maximal at the early HSA^{lo} stage. In addition, because the CD69 early activation marker first appears on a minor subset of the TCR^{lo/med} double-positive thymic population, at the stage when the selection process is expected to operate (36, 53), and is expressed on 50–90% of the cells at the presumptive “transitional” HSA^{hi} TCR^{hi} double-positive and HSA^{hi} TCR^{med} CD4⁺8^{lo} stages as well as on a majority of the HSA^{hi} CD4⁺8⁻ cells, the simplest hypothesis is that the bulk of the activation was imparted by the thymic selection process itself.

The extent to which the CD4⁺8⁻ thymic population appears activated is different, depending on the markers studied: up to 70–90% express CD69 at the HSA^{hi} stage, whereas 3% have the functional capacity to produce IL-4 at the HSA^{lo} stage. Even after correction for possible suboptimal *in vitro* stimulation conditions for lymphokine production, for example, by considering the IL-4/IL-2 ratio rather than the absolute frequency measured for the IL-4 producers, there would be at most 25% of the HSA^{lo} CD4⁺8⁻ thymocytes that are IL-4 producers. CD44 expression is observed on 2–10% of the CD4⁺8⁻ thymocytes (and on an approximately threefold larger fraction of the mature HSA^{lo} CD4⁺8⁻ population) depending on age and on the mouse strain (32, 33, and data not shown), and CD44^{hi} cells are enriched (although only threefold by comparison to CD44^{lo}) in IL-4 producers. If every cell being selected goes through the same phenotypic sequence, these differences in expression could reflect different periods of retention for each of the phenotypic markers of activation. Alternatively, the CD44^{hi} IL-4 producer phenotype may result from high affinity interactions

between TCR/CD4 on one side, and self-ligand/MHC on the other during the selection process. It is also possible that the CD44^{hi} subpopulation is generated by a second stimulus encountered late in development, e.g., self-antigens selectively expressed in the medulla, but not in the cortex (54–56). Interestingly, there is an increased frequency of TCR-V β 8⁺ cells among those adult HSA^{lo} CD4⁺8⁻ thymocytes that have an activated phenotype, as defined by levels of expression of markers such as Ly-6C (27), or CD44, 3G11, and MEL-14 (Hayakawa et al., manuscript submitted for publication), and it has been proposed that these cells may belong to an intrathymic pathway leading to the generation of mature, TCR- α/β double-negative thymocytes, possibly as a way to impart tolerance (27, Hayakawa, K., B. T. Lin, and R. R. Hardy, manuscript submitted for publication). This raises the question of whether all IL-4 producer CD4⁺8⁻ thymocytes are destined to end up in this pathway. We think that their high rate of production and export, even in the newborn period, weeks before a significant increase in V β 8⁺ cells, is detected (27, Hayakawa et al., manuscript submitted for publication), argues against this hypothesis. CD44^{lo} IL-4 producers are likely to be the cells exported to the periphery, based on their lymphokine secretion potential which is similar to that of thymic migrants (compare Figs. 4 and 8), and the report that thymic migrants are mainly CD44^{lo} (57). Future experiments are needed to clarify the relationship between

CD44^{hi} and CD44^{lo} IL-4 producers and to determine the origin, rate of generation, and the fate of the V β 8 pathway of CD4⁺8⁻ thymocyte differentiation.

At present, the signals transduced by the TCR during thymic selection are unknown, as are their consequences. It is also unclear which one of several potential receptors, aside from the TCR/CD3 complex, including CD28, Thy-1, or CD2, will transduce the first activating signals. We have found that the TCR seems deficient in its ability to transduce signals leading to lymphokine secretion (9) or even lymphokine responsiveness (our unpublished observations) until the HSA^{lo} CD4⁺8⁻ stage, although other biochemical responses, such as calcium fluxes, can be elicited to some extent (58). Thus, although a full activation leading to lymphokine secretion and expansion probably does not occur, several well-defined activation markers can be identified as a result of the selection process, such as the expression of the surface activation antigen CD69 (previously reported on human TCR^{hi} thymocytes [34]), of CD44, and of the potential to secrete the lymphokines IL-4, IL-5, IL-10, and IFN- γ .

In conclusion, the sequence and the extent of induction of several activation markers during thymic ontogeny suggest that thymic selection is not simply a rescue from programmed cell death, but involves an activation process that can be followed by a series of markers, and that is still evident after export to the periphery.

We thank Kyoko Hayakawa for sharing information before publication and for discussion; Ron Germain and Claude Penit for discussions; Ron Germain and Ethan Shevach for reviewing the manuscript; Chuan Chen for technical help; and Jeff Hooley and David Stephany for expert assistance with electronic cell sorting and four-color FACS[®] experiments.

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Received for publication 8 November 1991.

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