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Precocious expression of T cell functional response genes *in vivo* in primitive thymocytes before T lineage commitment

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Abstract

The genes encoding effector molecules of mature T cells, IL-2, perforin and IL-4, were found to be expressed *in vivo* in the most primitive subsets of thymocytes of adult mice. These subsets have previously been identified by their cell surface markers and by their expression of other T lineage-associated genes. While IL-2, perforin and IL-4 are expressed in distinct patterns, all three are expressed before the induction of RAG-1 and pre-TCR α mRNA expression, and are confined to subsets of cells that apparently have not yet undergone commitment to the T lineage. Thus, expression of T cell response genes appears to be one of the earliest markers of lymphocyte differentiation. Activation events marked by CD69 induction occur in these early cell types, but the response gene expression by these cells is separable from CD69 expression. IL-2 and perforin are induced again much later in thymocyte development, during TCR-dependent repertoire selection. At those stages, IL-2 protein and RNA levels per cell are higher, but the fraction of cells expressing IL-2 appears to be much lower than in the most immature stages. In addition, a striking feature of the immature populations is the robust IL-2 expression by presumptive immature NK cells. These findings are discussed in terms of the developmental origins of lineage specificity in T cell response gene regulation.

Introduction

A fundamental question in T cell development is how changes in gene expression that give cells mature recognition and response properties are coordinated with cellular changes that commit a cell to the T lineage developmental fate. Different gene regulatory events are needed to provide recognition and response functions. The TCR and associated molecules that confer recognition are expressed constitutively by mature T cells, so that once the genes that encode them are turned on, the cell expresses them indefinitely. By contrast, 'response' genes, such as cytokines and cytolytic molecules, are expressed by mature T cells only in response to specific stimulation. Therefore, cells with the ability to express these response genes cannot usually be distinguished from those without it solely on the basis of their current transcriptional activity. Acquisition of response capability in T cell development involves not only an all-or-none ability to express the response genes themselves, but also the ways these genes are coupled to respond to appropriate activation pathways by processes that are not yet well understood (reviewed in 1). It is not known whether any common regulatory elements are used by both recognition and response genes to make their expression T cell specific. Critical to the understanding of this regulation is the question of whether the cells that acquire these properties are already committed to the T lineage or not at the time of their first expression.

The stages at which different T cell recognition genes are first activated provide a series of discrete molecular landmarks for murine T cell development (2–7). It has been more difficult to determine when early thymocytes first acquire response capability, in part because the activation requirements of the cells shift as a function of developmental stage (reviewed in 8; also see 9–12). However, several recent reports indicate

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that murine thymocytes can acquire functional potential very early, before any TCR gene rearrangement (13–17). A striking and related observation is that some of the cells can be seen to express cytokine genes in the thymus *in vivo* without any experimental intervention, particularly during fetal development when the thymus contains a substantial population of highly immature cells (15,18–21). Arguably, some of these fetal cells expressing the cytokine, IL-2, *in situ* could be immature enough to be still uncommitted to the T cell lineage. On the other hand, there has been reason to doubt whether this is the rule for all T cell precursors, because apparently different results have been obtained in analyses of postnatal thymocytes. *In situ* hybridization and immunohistochemistry suggested that IL-2 expression is restricted to TCR^+ thymocytes in adult mice (19,22), and RT-PCR analysis has linked IL-2 expression to cells undergoing particular stages of TCR-dependent positive selection in human thymocytes (9,23). Nevertheless, an outstanding issue to be resolved is whether functionally active immature cells of the 'fetal' type continue to be generated from the immature precursors seeding the adult thymus.

In this paper, we show that immature cells expressing IL-2, IL-4 and/or perforin RNA are indeed present in the adult murine thymus. Using immunodeficient mutants for enrichment and multiparameter flow cytometry for characterization, we demonstrate that these spontaneously active cells are found in the most immature of all known thymocyte subsets, with surface marker and molecular expression profiles that indicate that they are not yet committed to the T cell lineage. We suggest that this precocious response may differ in its general characteristics from a second, later peak of response gene expression, which occurs during positive selection. Finally, our results suggest that the T cell response gene IL-2 is expressed initially in the common precursors of T and NK cells, and then is extinguished by a repression mechanism that is conditional in the T lineage progeny, but definitive in the NK progeny. These data place response gene expression prior to T lineage commitment and among the earliest of all T lineage characteristics acquired by lymphoid precursors.

Methods

Animals

C57BL/6 and C57/BL6-*Tla*^{a} normal mice were bred in our facility and maintained under conventional clean conditions. All immunodeficient mice were bred in our facility and maintained under sterile conditions in Isotec flexible film isolators, with autoclaved food, bedding and acidified water, as previously described (14). RAG-2$^-$ homozygous mice, partially backcrossed onto a B6 background, were originally obtained from Drs Yoichi Shinkai and Frederick Alt (Harvard Medical School). C.B-17-*scid* mice were originally obtained from Dr Kenneth Dorshkind (UCLA). C57BL-6-*scid* mice and mutant mice with homozygous disruptions of the TCRβ (C57BL/6-*Tcrb*^{tm1Mom}), TCRδ (C57BL/6-*Tcrd*^{tm1Mom}), or TCRβ and TCRδ (C57BL/6J-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}) genes were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were regularly monitored for health and used for thymus tissue at 3–5 weeks of age.

Flow cytometry and FACS

Extensive characterization of the main populations of immature thymocytes has been reported previously (14). Methods used in the present work, flow cytometry conditions and most mAb sources were also essentially as previously described (7,14,17,24). Flow cytometric analysis for FITC, phycoerythrin (PE) and PE–Cy5 tandem conjugates (PE–Cy5, CyChrome or Red670) was performed using a Coulter Epics Elite flow cytometer/cell sorter (Coulter, Miami, FL) equipped with a 488 nm air-cooled argon laser (15 mW). Daily alignment and calibration for instrument drift and quality control were performed using Coulter DNA-Check beads, which were set in standard channel settings and recorded for mean, median and CV along with the voltage and gain settings necessary to achieve those values, as described elsewhere (25). Forward angle and side scatter light gating were used to exclude dead cells and debris. Spectral overlap between fluorochromes was compensated electronically using single-color biological controls. The optics design initially separated FITC from the other fluorochromes with a 550 nm long-pass dichroic mirror. PE was separated from the PE–Cy5 with a 600 nm long-pass dichroic mirror. Detectors used bandpass filters of 525df10, 575df40 and 675df20 respectively for FITC, PE and PE–Cy5. Data were generated in log mode for fluorescence (four-decade scale) and in linear mode for scatter, and were presented as dot-plots or histograms using the standard Elite software (version 4.2). This software allows amorphous gates to be used to define cell populations for sorting.

In addition to the CD24–FITC (HSA), Sca-1–PE and –biotin, c-kit–biotin (CD117), CD25–PE and –biotin, CD44–PE and –biotin, CD4–PE, and CD8–FITC antibodies already reported (14), the present studies employed CD69–FITC and CD69–biotin (H1.2F3), CD44–CyChrome (IM7), CD24–PE (M1/69), and CD4–CyChrome (RM4-5) antibodies, all from PharMingen (San Diego, CA). Briefly, thymocytes from age-matched normal or immune-deficient mice were pre-incubated with 2.4G2 antibody to block Fc receptors and then stained with titrated concentrations of fluorochrome- or biotin-conjugated mAb. One-step incubations were used for three-color staining with FITC, PE and CyChrome conjugates; two-step incubations were used for three-color staining with FITC, PE and biotin conjugates in which the biotinylated antibody was detected by subsequent incubation with streptavidin–Red670 (Life Technologies, Gibco/BRL, Gaithersburg, MD). When sorting cells for subsequent RT-PCR analysis, known numbers of cells (2×10^4 to 1×10^5 in most cases) from each population were collected in HBSS with 0.25% BSA.

RT-PCR analysis of RNA expression

Samples of predetermined numbers of thymocytes were lysed, their RNA extracted in acid guanidinium thiocyanate and the RNA used as a template for reverse transcription with random hexamer primers as previously described (7,14). PCR reactions were carried out for 20–40 cycles, with the inputs corrected to normalize to a constant signal from HPRT control primers, as previously described (7). All primer pairs were chosen to cross an intron–exon boundary, to disfavor and exclude from analysis any amplification from contaminating genomic DNA. The primers and cycling parameters used to detect response gene expression were taken from the follow-

ing references: IL-2 and IL-4 (26), IL-7Rα (27), and perforin (14). Amplified IL-2 and HPRT products were detected in some experiments by Southern blot hybridization using internal oligonucleotide probes as described (26). Conditions and primers used for detection of RAG-1, pre-TCR α (pTα), CD3ε, terminal deoxynucleotidyl transferase (TdT), TCR C_β, Igα and HPRT were exactly as previously reported (7). Samples were analyzed by electrophoresis on agarose gels and detected by ethidium bromide staining. Radioactive signals were quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemistry and tissue preparation

Thymus glands were fixed, frozen, sectioned and processed for immunohistochemistry with antibody against murine IL-2 (S4B6; PharMingen) exactly as described previously (22). For negative controls, excess recombinant murine IL-2 was added or the primary antibody was replaced with normal rat IgG.

Results

Unexpected enrichment of IL-2 RNA-expressing cells in TCRβ⁻ δ⁻ thymocytes

Using *in situ* hybridization and immunohistochemistry to trace sites of IL-2 expression *in vivo*, we recently showed that there are rare cells in the adult murine thymus which express IL-2 RNA and secrete IL-2 protein (19,22). The most highly active cells are likely to be TCR^+ because they are missing in animals that cannot express γδ or αβ TCR complexes (22). If such cells were the only IL-2-expressing cells in the thymus, the overall level of IL-2 RNA, as detected by reverse transcriptase-dependent PCR analysis, should be far lower in thymocytes of TCR-deficient, doubly mutant TCRβ⁻ δ⁻ mice than in thymocytes of wild-type or singly mutant mice. Instead, in comparisons designed to identify the IL-2 producing cells in the present study, we obtained the opposite results, as shown in Fig. 1.

The cell numbers per thymus and representation of developmental subsets differ greatly in wild-type, TCRδ⁻, TCRβ⁻ and TCRβ⁻ δ⁻ genotypes (see Methods), due to the massive proliferation and differentiation that are triggered by TCR β chain expression (28). While the most immature precursors are present in similar absolute numbers in all cases, they are extensively diluted with more advanced cells in the wild-type and TCRδ⁻ cases. Conversely, mature TCRαβ thymocytes and certain medullary types of stromal cells are only present in the wild-type and TCRδ⁻ thymus samples. Therefore, in order to detect IL-2 RNA irrespective of the subset that produces it, two complementary approaches were used to measure IL-2 RNA expression. First, equal cell numbers from single-cell thymocyte suspensions were analyzed from each genotype (Fig. 1A). Second, RNA was prepared from whole thymus organs without dissociating the lymphocytes and samples representing equal fractions (though very different cell numbers) of the thymus populations of the four genotypes were compared directly (Fig. 1B). To establish a basis for quantitation, all samples were submitted to a PCR cycle titration in which the signals were analyzed by blot hybridization after 20–35 cycles and the results were taken from the

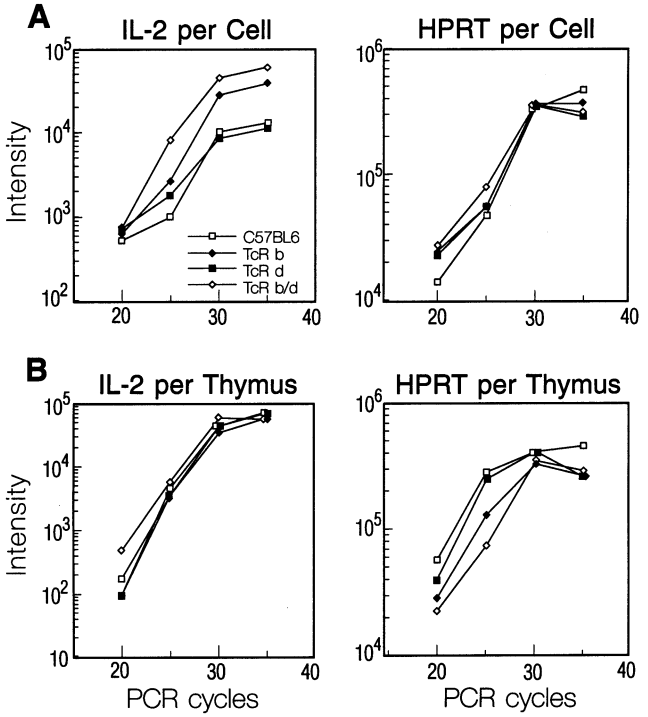

Fig. 1. Quantitation of IL-2 expression in wild-type and TCR-deficient thymus. Expression of IL-2 mRNA *in vivo* was measured by semiquantitative RT-PCR using different numbers of amplification cycles, from 20 to 35, and detecting the product by hybridization with an internal IL-2 oligonucleotide probe. Wild-type C57BL/6 thymus samples are compared with samples from TCRβ-deficient (TcR b), TCRδ-deficient (TcR d), and double-mutant TCRβ, TCRδ-deficient animals (TcR b/d). The signal intensity is plotted as a function of amplification cycle number, comparing IL-2 expression with expression of a control gene (HPRT). In (A), the samples consist of thymic lymphocytes only, normalized to equal cell numbers. In (B), the samples consist of whole-thymus homogenates, normalized to equal fractions of the whole organ.

portions of the curves in which product accumulated with parallel kinetics in the different samples (Fig. 1). The comparison suggested, unexpectedly, that the total amount of IL-2 RNA per thymus is similar in all four genotypes (Fig. 1B). This implies that IL-2 is expressed predominantly either by some non-lymphoid component of the thymus or by some very immature lymphoid cell type. Consistent with the second interpretation, in the single-cell lymphocyte suspension samples of constant cell number, IL-2 RNA was found to be most enriched in the TCRβ⁻ δ⁻ thymocytes as compared to all the other genotypes (Fig. 1A).

These results indicate that, although not previously detected by *in situ* hybridization (19,22), some TCR-independent, TCR^- cells express IL-2 RNA in the adult thymus as well as in the fetus. The high enrichment of IL-2 RNA in TCR^- stages of thymocyte development was separately confirmed with $RAG\text{-}2^-$ and *scid/scid* thymocyte samples compared to wild-type (data not shown).

IL-2 expression in the earliest T cell precursors from immunodeficient mouse thymus

To identify the cells that might be responsible for this early IL-2 expression, we used multiparameter flow cytometric cell

sorting of subsets of immature thymocytes, with combinations of markers that we have described previously in detail (14,17). To remove any possibility of contamination with more mature (TCR^+) subsets, we obtained the thymocytes from three strains of immunodeficient homozygous mutant mice, i.e. C.B17-*scid*, B6-*scid* and RAG-2^- (see Methods), all of which block TCR gene rearrangement and arrest development at or prior to the β-selection checkpoint (14). Lymphoid differentiation markers were chosen to distinguish between lymphoid and non-lymphoid elements in the thymus, and to subdivide stages of lymphoid differentiation prior to TCR expression, based on our extensive investigations of these cell types (7,14,17). Especially useful markers are CD24 (HSA), which is expressed in a pattern that correlates very highly with CD25; CD44, which subdivides the $CD24^+$ population according to developmental progress; and Sca-1 (Ly-6A/E), which splits the $CD24^-CD44^{++}$ population into two very distinct classes. Elsewhere we have described in detail how the distribution of cells among these subsets differs between *scid* and RAG-2^- thymocytes (14). A typical analysis is shown in Fig. 2(A and B).

The characteristics of each population were verified and placed in a likely developmental series by correlation with the cell-transfer results of others (29,30). On this basis the $CD24^+$ cells can be provisionally identified as T lineage committed, with the possible exception of the $CD44^+CD24^+$ minority (equivalent to the $CD44^+CD25^+$ 'pro-T cells' of ref. 31) which may still be able to give rise to dendritic cells (32). The $CD24^-$Sca-1^- and $CD24^-$Sca-1^+ cells correspond to subsets which are thought to be, as yet, uncommitted to the T cell lineage. The $CD24^-$Sca-1^- cells appear to be 'immature NK-like cells' because of their unique expression of the NK surface markers NK1.1, CD122, CD16/32 and DX5 along with c-kit (14) (Fig. 2G and H; and data not shown) and because of their constitutive expression of perforin (see below). Finally, we refer to the $CD24^-$Sca-1^+ cells as 'precursor-like' because they share numerous aspects of pluripotent precursor surface phenotype, including c-kit expression (14), and because they include the $CD4^+$ immature cells which have been described as a common lymphoid precursor subset within the thymus (Fig. 2C and D) (33).

These populations were analyzed for expression of response genes as well as genes associated with recognition (Fig. 3). Cells at the point of undergoing T lineage commitment, i.e. $CD44^+CD24^+$ ($CD25^+$) cells, were separated from stages before and after this commitment point by three-color sorting of RAG-2^- thymocytes for CD24, Sca-1, and CD44 (Fig. 3A and B). Four clearly distinguishable populations were sorted, representing immature NK-like, precursor-like, pro-T like, and committed T lineage cells, as shown in Fig. 3(A and B, populations 1–4 respectively). Figure 3(C) shows that immature NK-like (Fig. 3C, lane 1), precursor-like (Fig. 3C, lane 2), pro-T like (Fig. 3C, lane 3) and committed T lineage (Fig. 3C, lane 4) cells can be readily distinguished by their relative expression of RAG-1, TdT, CD3ε, TCR C_β and pTα. Expression of these genes was consistent with stepwise activation as T lineage differentiation progressed, with TCR C_β expressed first, then CD3ε, then TdT, then pTα and finally RAG-1, in agreement with our previous studies (7). Expression of IL-7Rα, shared by all fractions, supports the interpretation that

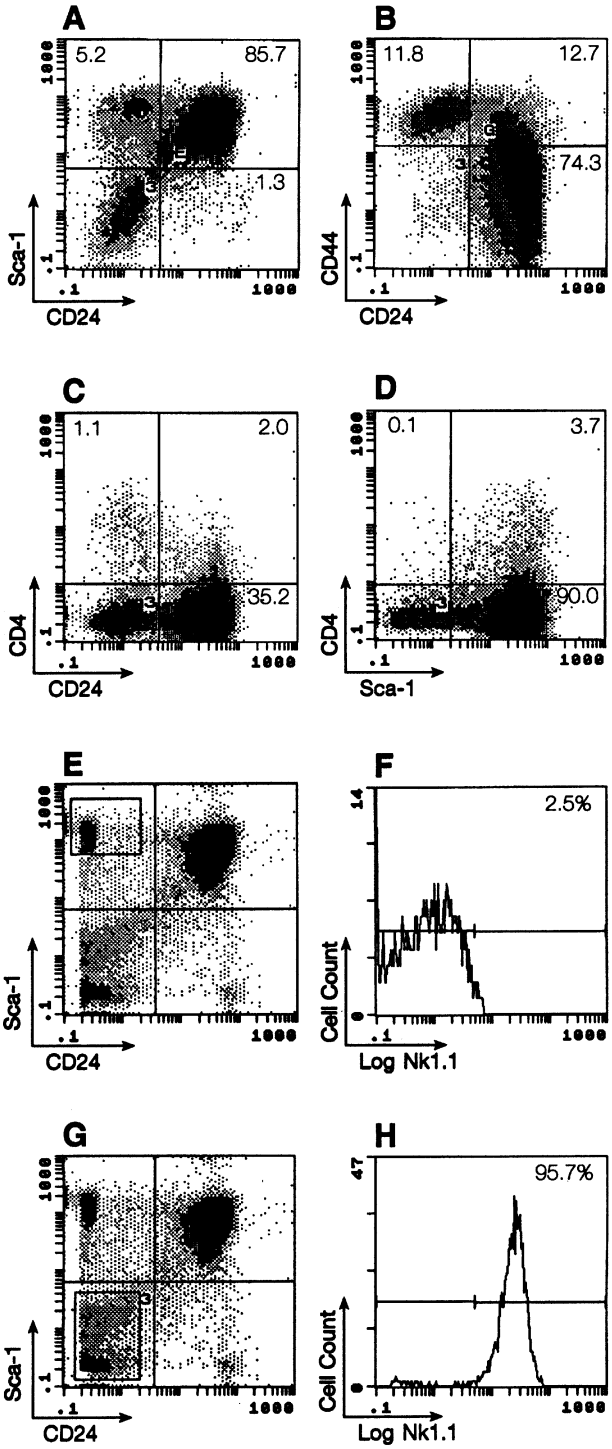

Fig. 2. Flow cytometric dissection of thymocyte subsets in immune-deficient scid mice. Three-color analyses of immature thymocytes from B6-*scid* mice are shown, using antibodies against Sca-1, CD24 and CD44 (A and B), against Sca-1, CD24 and CD4 (C and D), and against Sca-1, CD24 and NK1.1 (E–H). The regions indicated in (E) and (G) were used as gates to define the populations analyzed for NK1.1 expression in (F) and (H) respectively. Numbers represent the percentage of the population in each quadrant. Note that all the $CD4^+$ cells (including '$CD4^{lo}$' precursors) are Sca-1^{high} (D) but low to negative for CD24 expression (C). Also note that the Sca-1^+CD24^- cells (E) are all low or negative for NK1.1 expression (2.5% positive, F), while the Sca-1^-CD24^- cells (G) are almost uniformly NK1.1-bright (95.7% positive, H).

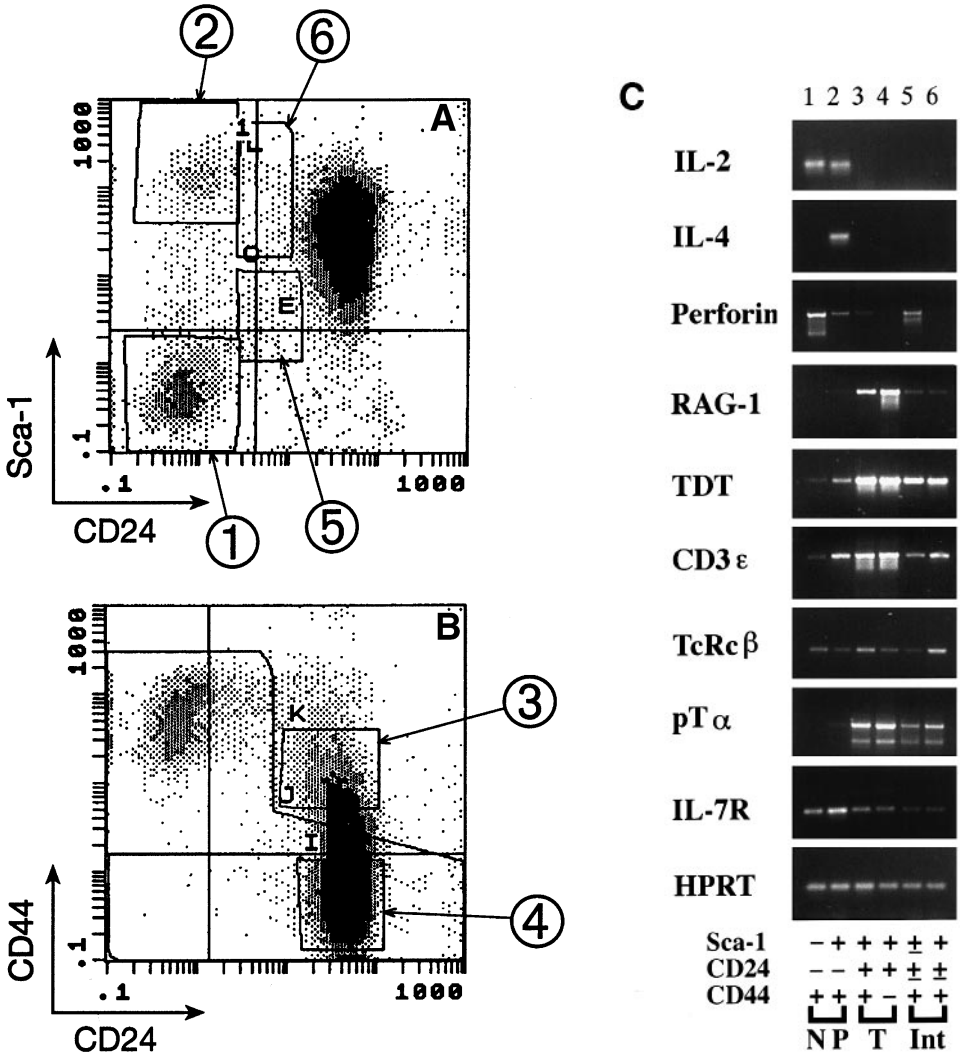

Fig. 3. Response gene expression confined to stages of immature thymocyte development after IL-7Rα expression but before RAG-1 or pTα expression. RAG-2^{-} thymocytes were separated on the basis of expression of Sca-1, CD24 and CD44 using the sorting gates shown in (A) and (B). They were then analyzed by RT-PCR for their expression of multiple developmentally regulated genes and the expression of response genes IL-2, IL-4 and perforin (C). The amorphous sorting gates (1–6) indicated in (A) and (B) were used to isolate the samples characterized for gene expression in the corresponding lanes (lanes 1–6) in (C). Upon reanalysis, population 1 was 98.9% pure (within the initial sorting gate), population 2 was 96.7% pure, population 3 was 88.3% pure (96.2% within the CD44^{high}CD24^{+} quadrant), and population 4 was 96.5% pure. Population 5 was only 64.4% within the original sorting gate, but it formed a unimodal distribution trailing toward population 1 (23.5% within population 1), with <1% Sca-1^{high} cells and <5% CD24^{high} cells. Population 6 could not be reanalyzed on this occasion because too few cells were recovered. The surface phenotypes of the different subsets are indicated at the bottom of each lane in panel C. Lane 1: immature NK-like population. Lane 2: precursor-like population. Lane 3: early pro-T cells (definitive T lineage). Lane 4: T lineage cells awaiting β-selection (definitive T lineage). Lanes 5 and 6 show fractions enriched for intermediates between the CD24^{-} and CD24^{+} cells. Lane 5: intermediates with low Sca-1 expression, possible NK → pro-T transitional cells. Lane 6: intermediates with high Sca-1 expression, possible precursor-like → pro-T transitional cells. Sample inputs were approximately normalized by their levels of HPRT expression (though lanes 1–3 yield slightly more HPRT product than lanes 4–6). Equal aliquots of cDNA were amplified with primers for each of the genes indicated at the left of each panel. RAG-1 and TdT: VDJ recombinational components. CD3ε, pTα and germline TCR C_{β}: components of the pre-TCR. IL-7Rα: growth factor receptor, earliest marker of lymphoid lineage specification.

even the most immature populations are highly enriched for lymphoid precursors (34).

IL-2 expression was readily detected by RT-PCR in sorted subpopulations of these freshly isolated, immature thymocytes. The light scatter properties and c-kit expression of the sorted fractions (data not shown) verified that the expressing cells were hematopoietic and not stromal. However, as shown in Fig. 3, the IL-2 expression was confined to the two most primitive cell types, the only two populations that are not thought to be committed to the T lineage. The two populations that express IL-2 were found to be the immature NK-like cells (Fig. 3C, lane 1) and the precursor-like cells (Fig. 3C, lane 2). The immaturity of cells in these two populations was shown by their lack of expression of the recombinase RAG-1 compared to their high expression of IL-7Rα. The NK-like cells, in particular, also showed low or undetectable

expression of CD3ε, TdT and pTα (Fig. 3C, lane 1). However, in the populations of cells that had reached the $CD24^+$ stages when RAG-1 and pTα are turned on, IL-2 expression was no longer detectable (Fig. 3C, lanes 3 and 4).

The expression of IL-2 by NK-like cells was noteworthy because mature NK cells do not express IL-2. Although they appear immature, because they all express c-kit, the $Sca\text{-}1^-$ $CD24^-$ cells also appear to be firmly within an NK differentiation pathway because of their nearly homogeneous cell-surface expression of CD122, NK1.1, DX5 and CD16/32 (14) (Fig. 2H; and E. V. Rothenberg *et al.*, unpublished results), as noted above. Yet in repeated independent experiments, these cells were consistently found to express almost as much IL-2 as the cells in the precursor-like population, which consists of cells with a completely different spectrum of phenotypes (14) (Fig. 2C–F; and H. Wang *et al.*, unpublished results). This makes it unlikely that the expression seen is coming from cross-fraction contaminants.

Although the actual arrest points in *scid* and RAG-2^- thymocytes are somewhat different, and the mechanisms of cell death at the arrest points also appear to be distinct (14), these basic features of IL-2 regulation in early thymocyte development were shown in multiple analyses to be conserved in *scid* mice on two genetic backgrounds and in RAG-2^- mice (*cf.* Fig. 5 and data not shown).

Shutoff of IL-2 expression prior to T lineage commitment

Both $CD4^{lo}$ cells, included in our $Sca\text{-}1^+CD24^-$ population, and NK-like cells, included in our $Sca\text{-}1^-CD24^-$ population, could include some cells capable of differentiating into T cells, based on cell transfer studies (13,29). To examine whether the IL-2 expression seen in these populations is linked with a transition to T lineage commitment, two minor $CD24^-$intermediate populations, potentially enriched for developmental intermediates between $CD24^-$ and $CD24^+$ stages, were also analyzed. One such population (Fig. 3C, lane 5) was the Sca-1 intermediate, $CD24^-$ intermediate smear of cells appearing to link the immature NK-like cells with the $CD24^+$ cells (Fig. 3A, population 5); this was shown on reanalysis to represent a discrete subset (data not shown). The other set of putative intermediates(Fig. 3C, lane 6) was the $Sca\text{-}1^+CD24^-$ intermediate smear linking the precursor-like cells to the $CD24^+$ subset (Fig. 3A, population 6), of particular interest because $CD4^+$ immature precursors are preferentially found with this phenotype (*cf.* Fig. 2C and D, and data not shown). In the experiment shown, too few cells of population 6 were obtained to allow both reanalysis and RT-PCR, but as shown in lanes 5 and 6 of Fig. 3(C), the gene expression patterns of these two populations are consistent with their identification as developmental intermediates. Their expression of pTα clearly separates them from the $CD24^-$ immature NK-like (Fig. 3C, lane 1) and precursor-like (Fig. 3C, lane 2) populations; whereas most importantly, their near-absence of RAG-1 expression shows that they are not substantially contaminated either with pro-T cells or with committed cells (*cf.* Fig. 3C, lanes 3 and 4). These differences are consistent with both populations 5 and 6 being dominated by actual developmental intermediates.

In this experiment and others like it, however, IL-2 expression was clearly shown to be confined to populations (Fig. 3C, lanes 1 and 2) that are neither pro-T cells (Fig. 3C, lane 3) nor enriched for developmental intermediates in progress toward T lineage commitment (Fig. 3C, lanes 5 and 6). IL-2 expression in fractions 5 and 6 was not artifactually missed due to dilution with contaminating committed cells, since in other experiments an IL-2 RT-PCR product was readily detected in samples from impure fractions containing a minority of $CD24^-$ cells in a majority of $CD24^+$ cells (data not shown). Thus the results indicate that the spontaneous IL-2 RNA expression not only begins, but even terminates, prior to T lineage commitment.

IL-2, IL-4 and perforin expression in distinct developmental patterns in the most immature thymocyte subsets

To determine whether other response genes are expressed *in vivo* at these early stages, IL-4 and perforin were similarly examined, as examples of T_h2 and cytotoxic T cell effector genes respectively. Representative results are included in Fig. 3(C). Like IL-2, IL-4 was spontaneously expressed in the precursor-like population (Fig. 3C, lane 2), but in contrast to IL-2, it was not detected in the immature NK-like population (IFig. 3C, lane 1). Conversely, perforin was expressed reproducibly in the immature NK-like population, but at lower levels in the precursor-like population. Only perforin, of the three response genes, was detectably expressed in any of the putative intermediate populations (Fig. 3C, lane 5) or in the pro-T cells (Fig. 3C, lane 3). Thus, all three T cell response genes can be activated in cells that are not (yet) committed to the T lineage, although the distinctions they preserve among their expression patterns show that these genes are not simply turned on through a promiscuous hyperactivation mechanism.

CD69 expression in early thymocyte development unlinked to response gene expression

In mature T cells, IL-2, IL-4 and perforin are normally induced as parts of the response to an acute activating signal. CD69 expression is generally co-induced in such responses of lymphocytes and other hematopoietic cells via protein kinase C, Ras pathway and other signals (35). Several important checkpoints in thymocyte development are known to involve activation events that are marked by CD69 induction (36–39). We therefore examined the frequency of CD69 expression in the major subsets and transitional cell types present in the immunodeficient thymi (Fig. 4). Both the $Sca\text{-}1^+CD24^-$ precursor-like population and the $Sca\text{-}1^-CD24^-$ immature NK-like population consistently include many $CD69^+$ cells, from 60 to 70% of the populations (Fig. 4B). However, the frequency of $CD69^+$ cells in the more advanced $CD24^+$ population drops to $<4\%$ (Fig. 4B). Similar results were seen in RAG-2^-, C.B-17-*scid* and B6-*scid* thymocytes (Fig. 4 and data not shown). All the $CD69^+$ cells are positive for c-kit expression (data not shown), suggesting that they are no more mature than the early pro-T compartment. However, there is no preferential expression of CD69 by putative transitional cells with intermediate levels of CD24 or CD25 (Fig. 4, panel C versus A and data not shown). These data and analysis of the expression of developmentally regulated genes in these subsets (Fig. 5 and data not shown) shows that CD69

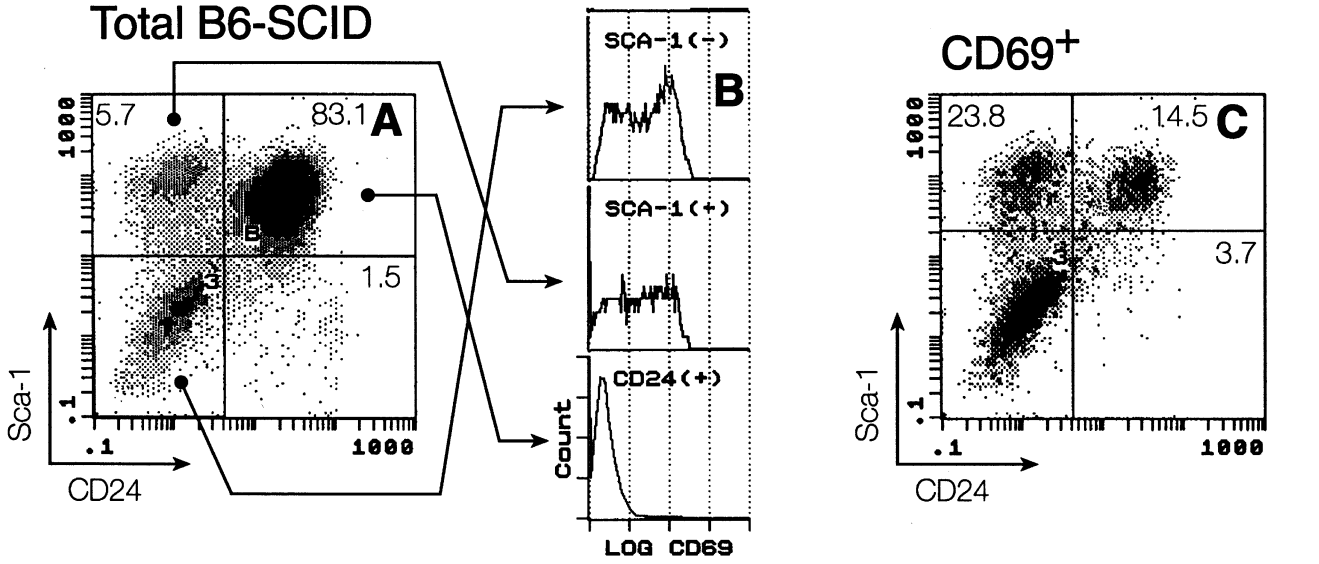

Fig. 4. Distribution of activated cells in immature thymocyte subpopulations, measured by expression of CD69. The figure shows three-color analyses of thymocytes from B6-*scid* mice, correlating CD69 expression with Sca-1 and CD24. (A) Sca-1 and CD24 expression of total thymocytes. (B) shows individual CD69 expression histograms of the populations of thymocytes lying in three main quadrants of (A): top, Sca-1$^-$CD24$^-$; middle, Sca-1$^+$CD24$^-$; and bottom, Sca-1$^+$CD24$^+$. (C) Sca-1 and CD24 expression of the CD69$^+$ subset of thymocytes. Both CD24$^+$ and CD24$^-$ populations of CD69$^+$ thymocytes uniformly express c-kit (data not shown).

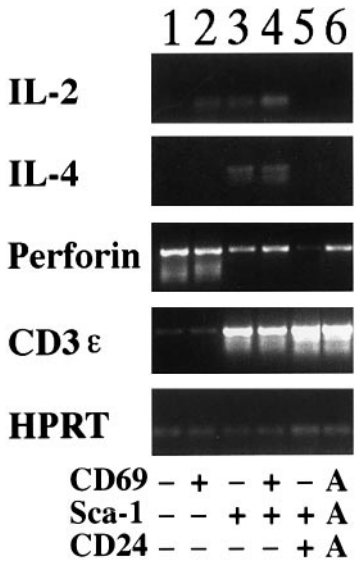

Fig. 5. Response gene expression is not tightly linked to CD69 expression in the primitive CD24$^-$ populations. B6-*scid* thymocytes were separated on the basis of CD69, Sca-1 and CD24 staining and analyzed for expression of response genes as described for Fig. 3. For each major population defined by Sca-1 and CD24 expression, the CD69$^-$ (lanes 1, 3 and 5) and CD69$^+$ subsets (lanes 2 and 4) were analyzed in parallel. All samples shown were >95% pure on reanalysis; a CD69$^+$ Sca-1$^+$CD24$^+$ fraction was omitted because of impurity. The cell-surface phenotypes of the samples are indicated at the bottom of each lane. Lanes 1 and 2: immature NK-like cells. Lanes 3 and 4: precursor-like cells. Lane 5: CD69$^-$ definitive T lineage cells. Lane 6: (A) = all, unfractionated thymocytes. Response gene expression is virtually absent from the CD69$^-$Sca-1$^+$CD24$^+$ population, which includes ~85% of the thymocytes.

induction is not correlated with the transition to a 'pro-T' or committed T lineage state.

Crudely, the populations with a high frequency of CD69$^+$ activated cells correlate with the populations that express response genes *in vivo*. However, cells expressing CD69 were not the only immature cells expressing the response genes. As shown in Fig. 5, the CD69$^-$ (Fig. 5, lane 1) and CD69$^+$ (Fig. 5, lane 2) subsets of NK-like cells were found to express indistinguishable levels of perforin, and the CD69$^-$ (Fig. 5, lane 3) and CD69$^+$ (Fig. 5, lane 4) subsets of precursor-like cells expressed equal levels of perforin and IL-4. IL-2 expression alone was higher in the CD69$^+$ subsets of each of these populations (Fig. 5, lanes 2 and 4), but it was always readily detected in the CD69$^-$ subsets as well (Fig. 5 and data not shown). Thus in the most immature cells, there was no systematic restriction of response gene expression to the cells with the CD69$^+$ phenotype of recently activated cells. These results raise the possibility that the expression of response genes in immature cells may be less tightly coupled to specific activation stimuli than it is in definitive T lineage cells.

Response gene re-induction by positive/negative selection signals late in thymocyte development

Wild-type cells that progress in T lineage development beyond the *scid* or RAG knockout arrest points down-regulate response gene expression, throughout the CD4$^-$CD8$^-$ [double negative (DN)] to CD4$^+$CD8$^+$ (double positive (DP)] transition and the DP stage, when cell surface TCR complexes are first expressed. However, as previously described, there are cells that express IL-2 in a TCR-dependent way *in vivo* (19,22), and these can be identified as a subset of the TCR$^+$ cells undergoing positive and/or negative selection. Figure 6 shows

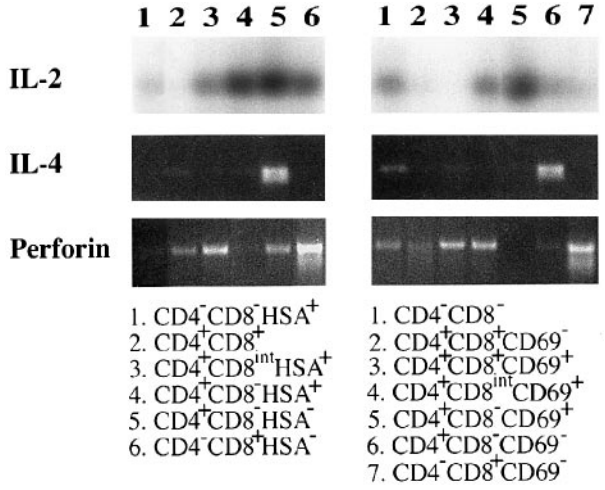

Fig. 6. Response gene expression late in normal thymocyte development. Normal thymocytes from C57BL/6-*Tla*^{a} mice were fractionated by their expression of CD4, CD8 and either CD24 (lanes 1–6, left) or CD69 (lanes 1–7, right), to resolve CD4$^-$CD8$^-$ cells (DN), CD4$^+$CD8$^+$ cells (DP), CD4$^-$CD8$^+$ cells (CD8 SP), and a series of positive selection intermediates between DP and CD4$^+$CD8$^-$ (CD4 SP) mature thymocytes. The figure shows the intermediate fractions arranged in developmental order. All these subsets except the DN cells are TCR$^+$, relatively 'mature' cells in contrast to those shown in Figs 2–5. Sorting gates and the relationship between the populations were exactly as described previously (7). Equal aliquots of the cells were then analyzed for expression of IL-2, IL-4 and perforin by RT-PCR. For each primer set, the PCR products are displayed by ethidium bromide staining, except in the case of IL-2. In contrast to its expression in immature thymocyte subsets (data not shown; *cf.* Figs 3 and 5), the amplified IL-2 product from the 'mature' TCR$^+$ subsets is not detectable by ethidium bromide staining and is therefore displayed by Southern blotting and hybridization with an internal oligonucleotide probe. Although IL-2 RNA levels during selection are thus substantially lower than in the immature cells, perforin RNA levels during selection are intermediate between those seen in Sca-1$^-$CD24$^-$ immature NK-like cells and those in Sca-1$^+$CD24$^-$ immature precursor cells, as shown in Figs 3 and 5.

that perforin as well as IL-2 is turned on in some cells among the selection intermediates between TCR^{low} DP and TCR$^+$ CD4$^+$CD8$^-$ or TCR$^+$ CD4$^-$CD8$^+$ 'single positive' (SP) cells. Landmarks in the progression through selection were defined by the induction of CD69 (Fig. 6, right-hand lanes), the shutoff of RAG-1 expression (7) (data not shown) and finally the down-regulation of CD24 in response to positive selection signals (Fig. 6, left-hand lanes), as described in detail elsewhere (7). As shown in Fig. 6, transcripts of perforin and IL-2 could be detected in a broad range of selection intermediates, especially in CD69$^+$ stages, but poorly if at all in the starting CD69$^-$ DP population. Both RNAs are also expressed in CD4 and CD8 SP thymocytes themselves, with IL-2 expressed somewhat more in the CD4 SP and perforin expressed more highly in the CD8 SP cells, in qualitative agreement with previous work in the human system (9,23). This continuum of IL-2 and perforin expression into the latest stages of maturation indicates that many of the cells actively transcribing functional response genes are undergoing positive selection. Response gene expression in these TCR$^+$ stages is signal dependent, as demonstrated by immunohistochemical staining analyses which show that high-level secretion of IL-2 protein by TCR transgenic thymocytes is blocked on a non-selecting MHC background (J. A. Yang-Snyder, unpublished results).

In contrast to this broad span of IL-2 and perforin expression, most IL-4 expression is enriched in DN cells and in cells within the CD4 SP population that have already reached a fully mature CD24$^-$CD69$^-$ state (Fig. 6). This expression could be due to the activity of mature 'NK T' or 'natural T' cells which are found in the DN and CD4 SP populations (40). Substantially stronger than the expression of IL-2 in the same samples, this IL-4 expression complicates the analysis of any expression, at much lower levels, that may be induced by selection events proper.

IL-2 protein expression in early and late stages of thymocyte differentiation

While expression of IL-2 in late thymocyte differentiation results in profuse secretion of IL-2 protein (22), the early expression is not associated with such pronounced staining features. However, carefully controlled immunohistochemistry indicates that the immature cells do express some IL-2 immunoreactive protein. While less intense than the signals in the normal thymus sections, sections from immunodeficient *scid* and RAG-2$^-$ mice do indeed show some specific IL-2 staining over background (Fig. 7 and data not shown). Whereas the IL-2 expressed by more mature thymocytes is generally found associated with thymocyte cell bodies (22), much of the IL-2 immunoreactivity in immunodeficient *scid* or RAG-2$^-$ thymus sections is found associated with vessels and connective tissue (Fig. 7). This suggests that the protein has been secreted. The lack of mature T cells in these animals makes the cells of origin most likely to be the resident highly immature thymocytes expressing IL-2 RNA, as described above. Thus the precocious response gene expression by immature cells, like the expression by cells undergoing selection, appears to reflect expression of functional protein-coding RNAs that can result in release of immunoreactive protein to the environment.

Discussion

This paper shows that the functional response genes that will be used by mature T cells in immune responses are already accessible and activated in the earliest T cell precursors. The acquisition of these response capabilities significantly precedes the onset of TCR gene rearrangement and, based on current understanding, also precedes commitment to the T cell developmental lineage. IL-2, perforin and apparently IL-4 as well are thus among the earliest T cell genes activated in lymphoid progenitors.

Frequent, low-level response gene expression in immature thymocytes

The novel features of these findings are the extreme immaturity of the response gene-expressing cells with respect to T cell development, the termination of this expression as the cells progress to defined T lineage phenotype, and the appearance of this transient expression in postnatal lymphoid precursors. Earlier studies had found abundant evidence for response gene expression in primitive fetal thymocytes and precursors

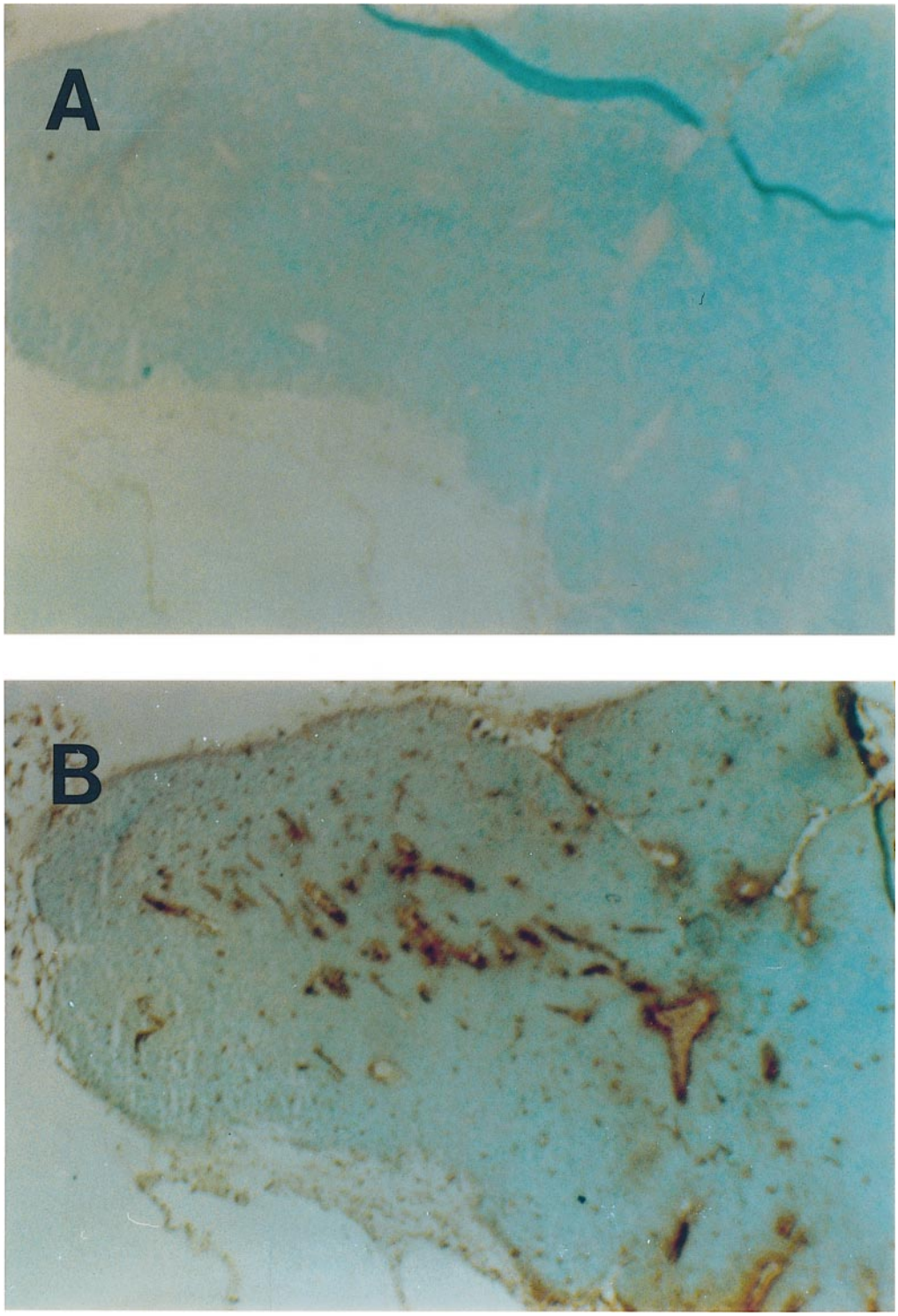

Fig. 7. Low-level IL-2 immunoreactivity in sections of the thymus from *scid* mice. Prefixed thymus sections taken from 4- to 5-week-old *scid* mice stained for IL-2 protein accumulation (brown). Sections were counterstained with methyl green. Bright field, ×50. (A) Negative control staining with normal rat IgG. (B) Stained with S4B6 anti-IL-2 mAb.

immigrating into the thymus, but not in postnatal precursors (15,18,19,21). In those studies, using *in situ* hybridization and immunohistochemistry, it appeared that response gene expression in postnatal thymocytes was mainly associated with positive or negative selection of mature thymocytes (9,22,41). Here, to reconcile those results with the population

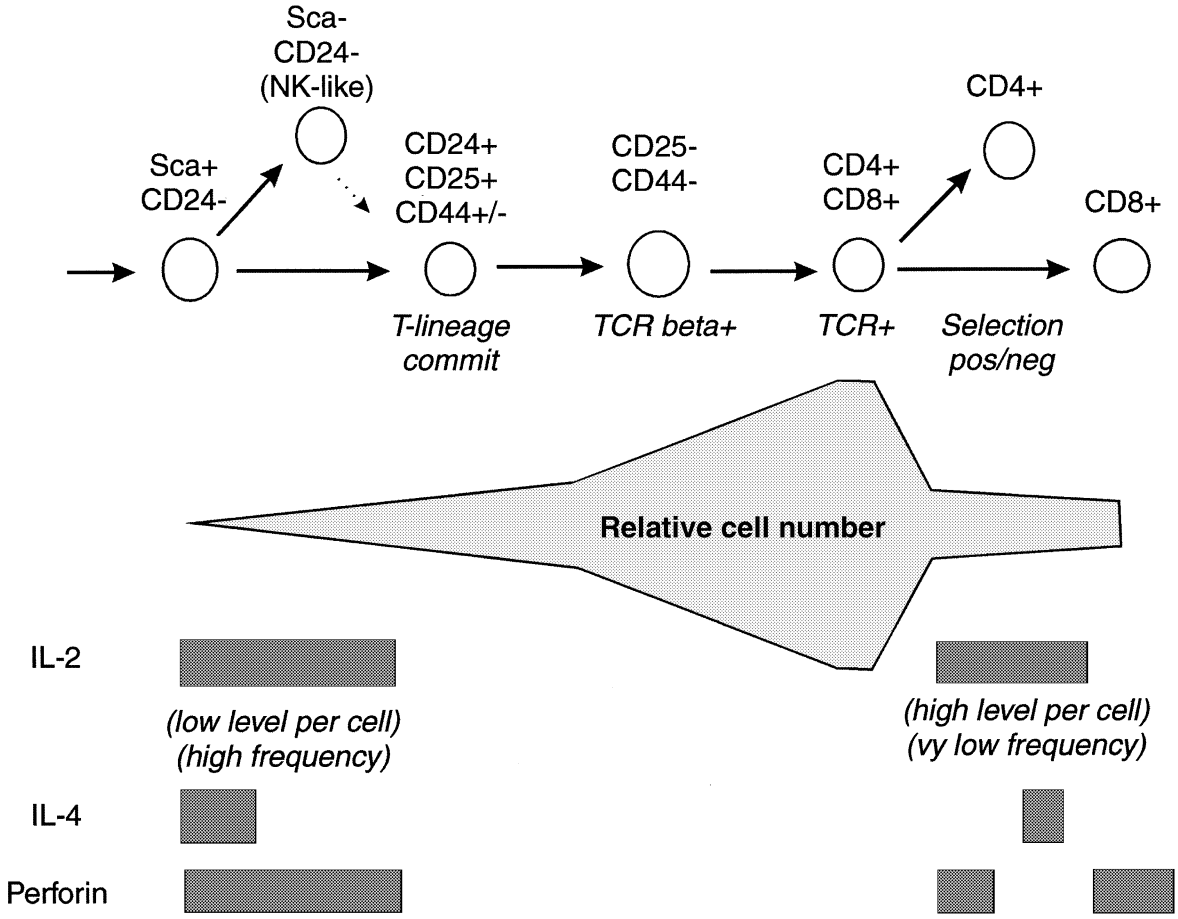

Fig. 8. Summary of response gene expression pattern in thymocyte development. The figure summarizes data for expression of IL-2, IL-4 and perforin RNA *in vivo* in different thymocyte populations. Major stages in T cell differentiation are indicated in the upper part of the figure, with the approximate timing of key events indicated in italic type. Also indicated are the stages during which normal thymocyte populations expand and contract. In the normal thymus, the fraction of the total population in the Sca-1$^+$CD24$^-$ subset is ~5×10^{-4} of the total cells and the fraction in the NK-like subset is ~8×10^{-4} (based on levels in immune deficient thymi). The normal equivalents of the CD24$^+$CD25$^+$ (CD44$^+$ to CD44$^-$) cells comprise ~0.02 of the normal thymus. CD25$^-$CD44$^-$ cells constitute 0.01–0.02. Of the later subsets, CD4$^+$CD8$^+$ cells make up 0.8 of normal thymocytes; CD4$^+$ cells make up ~0.13 of thymocytes (but fully mature, CD24^{low}CD69$^-$CD4$^+$ cells only ~0.05); and CD8$^+$ cells make up 0.05 of thymocytes. Shaded bars indicate the extent of stages in which particular response gene transcripts are detected. For IL-2, though not for IL-4 or perforin, data are available from previous studies to indicate that the level of RNA per average expressing cell is lower in immature cells than in the more mature stages (see Discussion).

RT-PCR data in this report, we propose that for postnatal thymocytes, the level of expression per cell may be higher for mature cells undergoing TCR-dependent selection, but the fraction of cells participating is much higher at the earliest stages of development. Thus, response gene expression is a general feature of the earliest lymphoid precursors at all stages of ontogeny. A summary of the overall pattern of expression of IL-2, IL-4 and perforin during postnatal thymocyte development, based on this report and previous data (19, 22), is presented in Fig. 8.

Distinct classes of activation events in early thymocyte development

IL-2, IL-4 and perforin expression by immature precursors could be a sensitive indication of responses to acute activation signals in the developmental context. The potential role of such signals is interesting in light of previous results that have linked the transcription factors used by activated mature T cells with early thymocyte development (42–44). One influential report has further argued that an activated state might be important to generate T lineage commitment, because CD25 expression, known to be induced in response to activating signals like tumor necrosis factor-α and IL-1, is induced when cells undergo commitment to the T lineage (44). Whatever its developmental role, however, the induction of CD25 (and CD24) cannot be equated with a 'normal' activation event in the terms of mature T cells; because our results show that it does not involve CD69 induction and it correlates with the shutoff, not the induction, of IL-2, perforin and IL-4 expression. The expression of CD69 by large subsets of the most primitive cells and NK-like cells does indicate that cells are exposed to activating stimuli at these earlier stages. Yet the expression of IL-4, perforin and a substantial fraction of IL-2 RNA by these cells is surprisingly uncoupled from their expression of

CD69. This indicates that response gene expression in these primitive cells either persists much longer after activation than does CD69 expression or else is turned on by a developmental mechanism that is separate from conventional activation altogether.

Lineage choice and T lineage 'specific' response gene expression

Rigorously, the timing of T lineage commitment and its molecular correlates still need to be confirmed by further studies of these populations at the single-cell level. However, if the molecular phenotypes of our primitive thymocyte subsets are representative of individual cells within those populations and if the developmental potentials of the populations, as established by others, are representative of these cells, then there are several implications.

The early response gene expression shows that the ability to activate the IL-2, perforin and IL-4 genes is already established in the cells before they are committed to the T lineage. If T cell response gene expression precedes T lineage commitment, then at least some of the cells expressing these genes may go on to become something else. Large subsets of T cells, if not all T cells, will later be able to use these response genes in an induction-dependent way. However, other potential derivatives of the uncommitted cells, such as dendritic cells, presumably never use the IL-2 and IL-4 genes once mature, while other potential derivatives, such as B cells, will not use perforin. Could precursors of such cells be expressing these response genes anyway?

Our NK-like population is a vivid example of a cell type that appears to be progressing homogeneously along a non-T pathway while expressing a T cell response gene, IL-2, that its mature descendants will not express. Unless all the IL-2 in this population is produced by an atypical minority, these cells appear to violate the 'rule' that NK cells cannot express IL-2. Interestingly, there are previous exceptions to this 'rule' in enriched populations of fetal NK cells (45). Because the NK-like thymocytes we describe uniformly express the immature cell marker c-kit (14), the ability to express IL-2 may be associated with the primitive state for some postnatal NK cells as well. We propose, therefore, that immature NK cells share with T cell precursors an ability to express IL-2, which they lose as their maturation proceeds. As we have recently discussed elsewhere (7,46), lineage-specific repression of genes expressed in multilineage progenitors appears to play a variety of roles in refining the lineage identity of thymocytes, as well as in other hematopoietic lineage choices (47,48). The regulatory sequences of IL-2 may help identify some valuable repressive factors involved in NK development.

The expression of the response genes IL-2, perforin and IL-4 precedes so many other events in T lineage differentiation that it could provide a new window into previously hidden stages. The next step will be to determine the developmental potential(s) of all the cells involved. We have been unable, so far, to detect any IL-2 or perforin expression in Sca-1^+c-$kit^+$$Lin^-$ putative stem cells in the fetal liver, under conditions in which other subsets of fetal liver cells can be shown to express these genes (H. Wang *et al.*, unpublished data). Thus it is possible that these genes are turned on only as cells become restricted to a lymphoid fate. Alternatively, in light of the ability of certain non-lymphoid cells to express these genes (49–53), the expression may begin earlier. With new methods to isolate common lymphoid progenitors from prethymic tissues (34), it should soon be possible to test these alternatives.

Implications for response gene regulation

In the long run, a particularly interesting aspect of this early response gene expression may be the definition of the gene regulatory mechanisms involved. The expression of these genes prior to T lineage commitment self-evidently cannot depend on factors which are only expressed as part of T lineage commitment. Therefore, the precocious expression of 'T cell-specific' genes like IL-2 is in harmony with the apparent lack of requirement for any T lineage-specific transcription factors interacting with the IL-2 minimal enhancer (1).

On the other hand, the identification of progenitor cells expressing response genes may ultimately be a useful step toward the definition of mechanisms that do restrict to particular cell types the ability to express these genes. On further analysis, we may find that the precursor-like population in the thymus (and/or the response-gene-expressing cells in fetal liver) might turn out to be primitive enough to contain progenitor activity for all the cell types that will ever express these genes. If so, it is interesting to speculate that the expression we detect might reflect the first time that a given precursor cell transcribes any of these three response genes. This would be of great interest for the analysis of gene regulatory mechanisms, since the initial opening of chromatin and/or the transfer of genes from a silenced to an accessible intranuclear state are poorly understood (54–56). New evidence indicates that the IL-2 gene may particularly require relief of silencing (57) in order to be available for signal-dependent induction (1). Thus, this early expression may involve chromatin-modifying components in a uniquely conspicuous role and offer a system in which their action can be studied.

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Abbreviation

DP	double negative
DN	double negative
PE	phycoerythrin
pTα	pre-TCR α

SP single positive
TdT terminal deoxynucleotidyl transferase

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