ANATOMICAL AND DEVELOPMENTAL PATTERNS OF INTERLEUKIN-2 GENE EXPRESSION IN THE MOUSE: ANALYSIS OF IL-2 EXPRESSING CELLS AND PARTIAL CHARACTERIZATION OF INTERACTIONS INVOLVED IN MEDIATING IL-2 GENE EXPRESSION *IN VIVO*

Thesis by Julia Ann Yang-Snyder

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ABSTRACT

Interleukin-2 (IL-2) is an important mediator in the vertebrate immune system. IL-2 is a potent growth factor that mature T lymphocytes use as a proliferation signal and the production of IL-2 is crucial for the clonal expansion of antigen-specific T cells in the primary immune response. IL-2 driven proliferation is dependent on the interaction of the lymphokine with its cognate multichain receptor. IL-2 expression is induced only upon stimulation and transcriptional activation of the IL-2 gene relies extensively on the coordinate interaction of numerous inducible and constitutive *trans*-acting factors. Over the past several years, thousands of papers have been published regarding molecular and cellular aspects of IL-2 gene expression and IL-2 function. The vast majority of these reports describe work that has been carried out *in vitro*. However, considerably less is known about control of IL-2 gene expression and IL-2 function *in vivo*.

To gain new insight into the regulation of IL-2 gene expression *in vivo*, anatomical and developmental patterns of IL-2 gene expression in the mouse were established by employing *in situ* hybridization and immunohistochemical staining methodologies to tissue sections generated from normal mice and mutant animals in which T-cell development was perturbed. Results from these studies revealed several interesting aspects of IL-2 gene expression, such as (1) induction of IL-2 gene expression and protein synthesis in the thymus, the primary site of T-cell development in the body, (2) cell-type specificity of IL-2 gene expression *in vivo*, (3) participation of IL-2 in the extrathymic expansion of mature T cells in particular tissues, independent of an acute immune response to foreign antigen, (4) involvement of IL-2 in maintaining immunologic balance in the mucosal immune system, and (5) potential function of IL-2 in early events associated with hematopoiesis.

Extensive analysis of IL-2 mRNA accumulation and protein production in the murine thymus at various stages of development established the existence of two classes of intrathymic IL-2 producing cells. One class of intrathymic IL-2 producers was found exclusively in the fetal thymus. Cells belonging to this subset were restricted to the outermost region of the thymus. IL-2 expression in the fetal thymus was highly transient; a dramatic peak of IL-2 mRNA accumulation

was identified at day 14.5 of gestation and maximal IL-2 protein production was observed 12 hours later, after which both IL-2 mRNA and protein levels rapidly decreased. Significantly, the presence of IL-2 expressing cells in the day 14-15 fetal thymus was not contingent on the generation of T-cell receptor (TcR) positive cells. The second class of IL-2 producing cells was also detectable in the fetal thymus (cells found in this class represented a minority subset of IL-2 producers in the fetal thymus) but persist in the thymus during later stages of development and after birth. Intrathymic IL-2 producers in postnatal animals were located in the subcapsular region and cortex, indicating that these cells reside in the same areas where immature T cells are consigned. The frequency of IL-2 expressing cells in the postnatal thymus was extremely low, indicating that induction of IL-2 expression and protein synthesis are indicative of a rare activation event. Unlike the fetal class of intrathymic IL-2 producers, the presence of IL-2 producing cells in the postnatal thymus was dependent on to the generation of TcR$^+$ cells. Subsequent examination of intrathymic IL-2 production in mutant postnatal mice unable to produce either $\alpha\beta$ or $\gamma\delta$ T cells showed that postnatal IL-2 producers in the thymus belong to both $\alpha\beta$ and $\gamma\delta$ lineages. Additionally, further studies indicated that IL-2 synthesis by immature $\alpha\beta$-T cells depends on the expression of *bona fide* TcR $\alpha\beta$-heterodimers. Taken altogether, IL-2 production in the postnatal thymus relies on the generation of $\alpha\beta$ or $\gamma\delta$-TcR$^+$ cells and induction of IL-2 protein synthesis can be linked to an activation event mediated via the TcR.

With regard to tissue specificity of IL-2 gene expression *in vivo*, analysis of whole body sections obtained from normal neonatal mouse pups by *in situ* hybridization demonstrated that IL-2 mRNA$^+$ cells were found in both lymphoid and nonlymphoid tissues with which T cells are associated, such as the thymus (as described above), dermis and gut. Tissues devoid of IL-2 mRNA$^+$ cells included brain, heart, lung, liver, stomach, spine, spinal cord, kidney, and bladder. Additional analysis of isolated tissues taken from older animals revealed that IL-2 expression was undetectable in bone marrow and in nonactivated spleen and lymph nodes. Thus, it appears that extrathymic IL-2 expressing cells in nonimmunologically challenged animals are relegated to particular epidermal and epithelial tissues in which characterized subsets of T cells reside and that

induction of IL-2 gene expression associated with these tissues may be a result of T-cell activation therein.

Based on the neonatal *in situ* hybridization results, a detailed investigation into possible induction of IL-2 expression resulting in IL-2 protein synthesis in the skin and gut revealed that IL-2 expression is induced in the epidermis and intestine and IL-2 protein is available to drive cell proliferation of resident cells and/or participate in immune function in these tissues. Pertaining to IL-2 expression in the skin, maximal IL-2 mRNA accumulation and protein production were observed when resident $V_\gamma 3^+$ T-cell populations were expanding. At this age, both IL-2 mRNA$^+$ cells and IL-2 protein production were intimately associated with hair follicles. Likewise, at this age a significant number of CD3ε^+ cells were also found in association with follicles. The colocalization of IL-2 expression and CD3ε^+ cells suggests that IL-2 expression is induced when T cells are in contact with hair follicles. In contrast, neither IL-2 mRNA nor IL-2 protein were readily detected once T-cell density in the skin reached steady-state proportions. At this point, T cells were no longer found associated with hair follicles but were evenly distributed throughout the epidermis. In addition, IL-2 expression in the skin was contingent upon the presence of mature T cells therein and induction of IL-2 protein synthesis in the skin did not depend on the expression of a specific TcR on resident T cells. These newly disclosed properties of IL-2 expression in the skin indicate that IL-2 may play an additional role in controlling mature T-cell proliferation by participating in the extrathymic expansion of T cells, particularly those associated with the epidermis.

Finally, regarding IL-2 expression and protein synthesis in the gut, IL-2 producing cells were found associated with the lamina propria of neonatal animals and gut-associated IL-2 production persisted throughout life. In older animals, the frequency of IL-2 producing cells in the small intestine was not identical to that in the large intestineand this difference may reflect regional specialization of the mucosal immune system in response to enteric antigen. Similar to other instances of IL-2 gene expression *in vivo*, a failure to generate mature T cells also led to an abrogation of IL-2 protein production in the gut. The presence of IL-2 producing cells in the

neonatal gut suggested that these cells may be generated during fetal development. Examination of the fetal gut to determine the distribution of IL-2 producing cells therein indicated that there was a tenfold increase in the number of gut-associated IL-2 producers at day 20 of gestation compared to that observed four days earlier and there was little difference between the frequency of IL-2 producing cells in prenatal versus neonatal gut. The origin of these fetally-derived IL-2 producing cells is unclear. Prior to the immigration of IL-2 inducible cells to the fetal gut and/or induction of IL-2 expression therein, IL-2 protein was observed in the fetal liver and fetal omentum, as well as the fetal thymus. Considering that induction of IL-2 protein synthesis may be an indication of future functional capability, detection of IL-2 producing cells in the fetal liver and fetal omentum raises the possibility that IL-2 producing cells in the fetal gut may be extrathymic in origin and IL-2 producing cells in these fetal tissues may not belong solely to the T lineage. Overall, these results provide increased understanding of the nature of IL-2 producing cells in the gut and how the absence of IL-2 production therein and in fetal hematopoietic tissues can result in the acute pathology observed in IL-2 deficient animals.

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INTRODUCTION

Background

The vertebrate immune system relies chiefly on the activities of functional B and T lymphocytes. B cells produce antibodies that constitute the humoral immune response, while T cells participate primarily in regulating antibody production, rejecting foreign tissue grafts, and eliminating virus-infected cells. B and T cells arise from pluripotent stem cells that are capable of giving rise to all blood cell lineages. These hematopoietic stem cells (HSCs) are derived from the blood islands of the yolk sac and fetal liver during embryonic life and the bone marrow during postnatal life. Unlike B cells, which develop at the site where HSCs are generated, T cells require a specialized microenvironment outside the fetal liver or bone marrow in order to develop. T-cell precursors immigrate from the fetal liver or bone marrow to the thymus, an organ that is ectodermal and endodermal in origin. Once in the thymic microenvironment, "prothymocytes" undergo a series of phenotypic changes that is accompanied by marked cell proliferation. During this period, genes encoding the T-cell receptor for antigen (TcR) rearrange in a highly ordered fashion. The ensuing expression of TcR on the cell surface results in the termination of proliferation. After this point, the majority of thymocytes are subject to programmed cell death (PCD), whereas only a small fraction of cells are "selected" to mature and become functional T cells. Mature T cells must be capable to carry out specific genetic programs including the synthesis of cytokines upon activation by foreign antigen and/or molecules utilized in cell-mediated cytotoxicity. Since this functional capacity must be acquired during development, it is crucial to determine the stage at which developing thymocytes are capable of expressing genes characteristic of a mature phenotype and whether immature cells express any of these genes during the course of development.

One such gene that has been studied extensively over the years, in terms of regulation in both mature T cells and developing thymocytes, is interleukin-2 (IL-2). IL-2 was one of the first lymphokines to be identified and was initially described as a soluble factor present in culture supernatants of phytohemagglutinin (PHA)-stimulated bone marrow cells capable of stimulating

T-cell proliferation (1). Interaction of IL-2 with its cell surface receptor (IL-2R) is crucial for the clonal expansion of activated T cells, resulting in amplification of the antigen specific T-cell immune response. Recently, a number of reviews have specifically addressed numerous aspects of IL-2 gene expression (2, 3), IL-2 function (4), and IL-2-induced signal transduction pathways (5).

Although extensive *in vitro* studies have been carried out to further understanding of IL-2 gene regulation, proportionally fewer studies to examine IL-2 gene expression and function *in vivo* have been performed. With the advent of transgenic and gene knockout technologies, as well as utilization of natural occurring mutations in the mouse yielding immunodeficient or immunocompromised animals, it has been possible to perturb T-cell development *in vivo* with relative ease to study the impact of such manipulation on the expression of T-cell "function" genes, such as IL-2, both *in vitro* and *in vivo*. The disruption of IL-2 gene expression *in vivo* produced several interesting results. In initial studies (6), it was shown that IL-2 was not necessary for the generation of the major thymocyte populations (i.e., the lack of IL-2 did not overtly affect T-cell development), and peripheral T cells isolated from homozygous knockout animals were unable to produce IL-2 upon activation. Later, it was reported that older mutant animals exhibit splenomegaly, lymphoadenopathy and severe anemia and/or develop ulcerative colitis with high penetrance (7). Thus, these results demonstrate that IL-2 is not required at any particular stage of T-cell development, but that an apparent lack of IL-2 expression subsequently results in defects in hematopoiesis and deregulation of the mucosal immune system which leads to inflammatory bowel disease (IBD).

Experimental Considerations

The IL-2 disruption studies are highly informative, yet they provide little information on the following issues that are vital to understanding IL-2 gene expression *in vivo*. First, even though IL-2 is not essential for in the generation of predominant thymocyte subsets, it is unclear if thymocytes are induced to express IL-2 during the course of development. This is of particular

interest for several reasons. The intrinsic competence to induce IL-2 gene expression is shared by both mature and very immature thymocytes, but the vast majority of T-lineage cells generated in the thymus refractory to signals that would otherwise result in the induction of IL-2 gene expression in competent cells. Hence during the generation of functional T cells, it is crucial that thymocytes, which are selected to mature, regain the functional competence. This would include the ability to express the IL-2 gene. Therefore, if IL-2 is indeed induced in thymocytes *in vivo*, this not only raises the possibility that IL-2 is present in the thymus to exert autocrine and paracrine effects, but also suggests that thymocytes may exercise functional competence during their development. The acquisition and/or maintenance of functional competence during thymocyte development as indicated by the ability to induce IL-2 gene expression technically cannot be answered by studying IL-2 -/- mice alone.

Second, the disruption studies do not address the question whether cell types other than those belonging to the T lineage express IL-2 *in vivo*. Induction of IL-2 gene expression appears to be a property exhibited exclusively by T cells *in vitro* and has classically been considered a functional marker for T-lineage cells. In addition, the induction of IL-2 gene expression requires the coordinate action of T-cell specific and ubiquitous transcription factors. However, it is uncertain that cell-type specificity of IL-2 gene expression is maintained *in vivo*. Identification of cell types that express IL-2 *in vivo* would provide insight into the regulation of IL-2 in the animal proper.

Finally, it is not known whether IL-2 is involved in the expansion of particular extrathymic populations of T cells or whether IL-2 is expressed in a nonimmunologically challenged animals. IL-2 mediates the rapid proliferation of antigen-specific T cells and clonal expansion of T cells is a crucial event for the success of the primary immune response. Notwithstanding, questions remain largely unanswered with regard to a potential role for IL-2 in the extrathymic expansion of T cells to populate the tissues in which they reside. Establishing the pattern of IL-2 gene expression in certain tissues where T cells are located as a function of development would help resolve these issues.

The body of work presented here focuses on experiments designed to answer these questions. In the following chapters, studies pertaining to IL-2 gene expression in the thymus, specificity of IL-2 gene expression *in vivo*, and extrathymic sites of IL-2 gene expression in nonimmunized animals will be described. Before proceeding further, a review of molecular and cellular aspects of IL-2 gene expression (including information on how T-cell activation influences the induction of IL-2 gene expression), as well as a summary of salient points and events during T-cell development will be given.

Molecular Aspects of IL-2 Gene Expression

Signal Transduction Pathways Crucial for the Induction of IL -2 Gene Expression

In general, the expression of specific genes involved in the functional T-cell response is contingent upon T-cell activation. IL-2 gene expression is completely dependent on T-cell stimulation, and this is one of the major ways that stimulation regulates the immune system. Antigenic stimulation of T cells initiates the appropriate signal transduction cascade that eventually results in the transient transcriptional activation of response genes, including IL-2. It should be noted that activation via the TcR can be bypassed using pharmacological agents that mimic activation by triggering the signal transduction pathways directly. Engagement of the TcR is crucial for T-cell activation. Specifically, T cells cannot be activated by native, intact antigen. The TcR exclusively recognizes small peptides generated from processed antigen (9-11 amino acids in length) embedded in the cleft of a major histocompatibility complex (MHC) molecule (8) on the surface of antigen presenting cells (APCs). The recognition of antigenic peptide/MHC by the TcR is further facilitated by the interaction of CD4 or CD8 coreceptors with invariant portions of MHC class II or MHC class I molecules, respectively. Finally, although engaging the TcR is crucial for T-cell activation, the interaction of other cell surface molecules, especially CD28, is necessary for the induction of IL-2 gene expression (to be discussed later).

The TcR complex itself is composed of numerous polypeptide chains which can be separated into antigen recognition and signal transduction subunits. Antigen (plus MHC)

recognition is specified by the disulfide-linked αβ or γδ heterodimer. Diversity of the α, β, γ, or δ TcR genes is generated by combinatorial joining of variable (V), diversity (D), joining (J), and constant (C) gene segments during T-cell development (9). The cytoplasmic domains of these chains are short (5 residues for α and β chains) and are not believed to be involved in signaling, *per se*. The αβ or γδ heterodimers are noncovalently linked to invariant CD3γ, δ, ε chains and a ζ chain homodimer or ζη heterodimer. Though the exact stoichiometry of these CD3 chains within the TcR complex remains unknown, each αβ or γδ heterodimer is thought to be associated with a CD3γε and a CD3δε dimer (10). The cytoplasmic domains of the CD3 chains are substantially longer (40 to 113 residues) than those of the TcRα, β, γ, and δ chains and are responsible for coupling the antigen-recognition subunits to the intracellular signal transduction machinery (10).

TcR-Dependent PTK Activation I: Participation of Src PTK Family Members

Protein tyrosine phosphorylation is vital for the initiation of cellular responses following the interaction of the TcR with antigen plus MHC. The CD3 subunits do not have intrinsic protein-tyrosine kinase (PTK) activity. However, the cytoplasmic domains of all CD3 subunits contain common sequences that are responsible for interacting with cytoplasmic PTKs of the Src and non-Src classes. A sequence motif, initially described by Reth (11), is characterized by paired tyrosine and leucine (or isoleucine) residues; the consensus sequence consists of (D/E)XXYXXL(X)6-8YXXL. This sequence is commonly referred to as the antigen recognition activation motif (AR-AM). ARAMs are found in single copies in the CD3γ, δ, and ε chains and are triplicated in the ζ chain. The multiplicity/redundancy of ARAMs with the TcR may serve to increase the efficiency of signal transduction by quantitatively increasing the ability of the TcR to bind and mobilize cytoplasmic PTKs (10).

Several Src family members have been shown to be expressed in T cells; two members, Lck and Fyn, have been studied extensively. Lck and Fyn are 56% homologous at the amino acid level and both exhibit increased PTK activity following TcR stimulation (12-14). Lck (MW, 56 kD) interacts at high stoichiometry with the cytoplasmic domains of either CD4 or CD8 (15, 16).

The association of Lck with CD4 or CD8 coreceptors could serve as a means by which Lck is brought into proximity of ARAMs within the TcR following TcR/coreceptor interaction with antigen/MHC. Lck can be activated solely by CD4 or CD8 crosslinking, but Lck activation in itself is insufficient to lead to T-cell activation (16, 17); in some cases, ligation of coreceptors alone frequently renders cells refractory to subsequent activation through the TcR (18). This may represent a form of regulation that necessitates coincident engagement of the TcR with antigen/MHC and coreceptor with MHC in order for T-cell activation to occur, thereby safeguarding against "spurious" activation should CD4 or CD8 interact with MHC alone in the absence of the appropriate TcR-antigen/MHC interactions. On the other hand, Fyn (MW, 59 kD) associates with the TcR at relatively low frequency. Of these two PTKs, the role of Lck in T-cell activation (and T-cell development) is more fully realized than that of Fyn.

Although Lck generally associates with CD4 and CD8, it has been shown that in the absence of coreceptor expression, Lck can directly associate with CD3 (19). This indicates that Lck may possess an intrinsic affinity for ARAMs in CD3. Additionally, Lck is able to interact with other cell surface receptors that lack ARAMs, suggesting that Lck may be utilized in signal transduction pathways other than those that are strictly TcR/CD3 dependent. For example, Lck may participate in IL-2 dependent signal transduction via interaction with the β-chain of the IL-2R; this notion is further supported by observations showing increased PTK activity and modification (serine/threonine phosphorylation) of Lck following IL-2 stimulation (20, 21).

The roles of Lck and Fyn in TcR signal transduction have been examined using genetic and biochemical approaches. Both *lck* and *fyn* genes have been disrupted in mice (22, 23). Whereas mice deficient in Lck have a block early in T-cell development, animals homozygous for a disrupted *fyn* gene exhibited no gross alterations in T-cell development (the function of Lck in T-cell development will be discussed later). Additionally, the few peripheral T cells that develop in animals lacking Lck activity have diminished responses to TcR stimulation (22). The *lck* knockout data are further supported by work done with T-cell lines and clones which lack Lck kinase activity, demonstrating an abrogation of early and late events associated with TcR-mediated signal

transduction, such as TcR-dependent intracellular calcium flux (24), and cytolytic effector functions (25), respectively. In contrast, the absence of Fyn activity has a less pronounced effect on TcR-dependent signaling; even though mature thymocytes (thymocytes that are phenotypically similar to peripheral T cells) isolated from Fyn deficient mice showed a TcR signaling defect, both immature TcR^+ thymocyte and peripheral T-cell populations were not profoundly affected (23). Nonetheless, Fyn does appear to be involved in TcR signal transduction, based on data obtained from overexpression of wild-type Fyn in transgenic mice (26) or overexpression of a constitutively activated form of Fyn in a T-cell hybridoma (27), resulting in the generation of altered cells that were hyperresponsive to TcR stimulation. Thus, Fyn appears to be involved in TcR-dependent signaling, but unlike Lck, its activity appears to be dispensable.

Lck and Fyn activation is also highly regulated by inhibiting PTPases. Src PTKs are negatively regulated by Csk, a widely expressed PTK that is itself structurally homologous to Src PTKs (28). Evidence suggests that dephosphorylation of the negative regulatory sites in Lck and Fyn is dependent upon the presence of the CD45 PTPase. This PTPase (MW, 180-220 kD) is differentially expressed on all cells of the hematopoietic lineage except fully differentiated erythroid cells; the various isoforms of CD45 PTPase are generated by alternative splicing (the regulation of differential splicing/cell type specificity and function of the different extracellular domains of CD45 isoforms has yet to be established). On T cells, two isoforms are predominantly utilized, CD45RB and CD45RO, which are expressed on resting/virgin and activated T cells, respectively. CD45 is also expressed on thymocytes, but its role in T-cell development is not well understood. The CD45 PTPase is involved in early events in T-cell signal transduction, including the induction of PTK activity as indicated by genetic evidence gathered from studies using T-cell lines deficient in CD45 expression (29,30). Likewise, in mice that are homozygous for a disrupted CD45 gene, T-cell development is blocked at the CD4/CD8 double positive stage, resulting in a reduced number of peripheral T cells; the T cells that manage to mature in these mutant animals exhibit a gross defect in TcR-mediated responses (31). Finally, *in vitro* reconstitution studies have shown that CD45 can dephosphorylate Lck, leading to the activation of PTK catalytic function by rendering

Lck accessible to phosphorylation substrates (32). Thus, dephosphorylation of Lck (and presumably Fyn) appears to be an important step in initiating the TcR signal transduction cascade.

TcR-Dependent PTK Activation II: Involvement of Syk/ZAP-70 Class PTKs

As mentioned previously, PTKs of the Src and non-Src families are believed to be crucial for TcR function. The second class, the Syk/ZAP-70 class, differs from the Src class in numerous ways. Unlike Src class PTKs that possess a myristilated N-terminus, Syk (expressed preferentially in B cells) and ZAP-70 (expressed exclusively in T cells) are not myristylated and are most likely not constitutively associated with the plasma membrane. Additionally, Syk/ZAP-70 family members contain two N-terminal SH2 domains, but lack an SH3 domain and C-terminal negative regulatory tyrosine phosphorylation site, indicating that this class of PTKs is not under identical regulation as the Src class PTKs (10).

ZAP-70 has been shown to be directly associated with the TcR upon stimulation. In the basal state, ZAP-70 is not associated with the TcR, but is rapidly bound to phosphorylated forms of ζ and CD3 chains following TcR stimulation (33). ARAMs are directly involved in recruiting ZAP-70 to the TcR (34); reciprocally, the SH2 domains of ZAP-70 are essential for the interaction of this PTK with phosphorylated ARAMs (35). These data also indicate that in order to ZAP-70 to interact with the TcR, the TcR must be modified by tyrosine phosphorylation prior to ZAP-70 association.

The likely candidates for PTKs that modify ζ and CD3 chains before ZAP-70 mobilization to the TcR are Lck and Fyn. The Lck/Fyn-ZAP-70 relationship has been examined using a heterologous Cos cell system in which specific components involved in T-cell activation can be studied in relative isolation. For example, Cos cells expressing ZAP-70 and a chimeric receptor containing a large portion of the ζ cytoplasmic domain must also be cotransfected with Lck or Fyn in order for ZAP-70 to associate with sequences following receptor crosslinking (36). The expression of either Lck or Fyn, but not ZAP-70 alone, in this system results in detectable ζ-domain phosphorylation. Based on these and other data, a sequential model of PTK activities

has been proposed (10). After receptor-ligand interaction, ARAMs in the TcR are phosphorylated by a Src family PTK, followed by ZAP-70 association with modified CD3 subunits. In addition, ZAP-70 is also modified by tyrosine phosphorylation following mobilization to the TcR (34). The mechanism by which ZAP-70 is modified is not known; it is unclear if ZAP-70 is subject to direct or indirect phosphorylation by Lck or Fyn or to autophosphorylation. Likewise, the effect of phosphorylation on ZAP-70 function is not understood (10). Nevertheless, the stepwise activation of PTKs, beginning with Lck (and Fyn) dephosphorylation and leading to ZAP-70 mobilization and modification is essential for TcR mediated signaling.

Finally, ZAP-70 has been shown to be essential for TcR-mediated signal transduction. It was reported recently that patients with selective T-cell deficiency (STD) were homozygous for an insertional mutation in the ZAP-70 gene (37). The lack of ZAP-70 had a profound effect on T-cell development (to be discussed later) and peripheral T cells isolated from afflicted individuals exhibited a marked decrease in tyrosine phosphorylation and a failure to produce IL-2 and to proliferate in response to T-cell receptor stimulation by mitogens and antigen. The decrease in PTK activity in STD patients led to a reduction of both PLC$_\gamma$1 phosphorylation (fivefold decrease composed to controls) and intracellular calcium flux (40% of normal) that have a direct bearing on IL-2 gene expression (see below). Thus, this genetic evidence indicates that ZAP-70 is indispensable for TcR signaling and T-cell function.

Generation of Second Messengers

Numerous events downstream of TcR-dependent PTK activation are implicated in specific T-cell responses, including transcriptional initiation of IL-2 gene expression. One such event is the activation of the phosphatidyl inositol (PI) pathway (38). Inositol phospholipid metabolism/turnover is contingent upon the activation of phospholipase C$_\gamma$1 (PLC$_\gamma$1) which catalyzes the hydrolysis of PI 4,5-bisphosphate yielding inositol 1,4,5-trisphosphate (IP3) and diaglycerol (DAG); for TcR-mediated signaling, activation of PLC$_\gamma$1 appears to occur in a G-protein-independent manner. These second messengers are responsible for the rapid rise and

sustained pulses in intracellular free calcium ([Ca^{2+}]i) and the activation of protein kinase C (PTK), respectively. Induction of TcR-dependent PLC activity relies on tyrosine phosphorylation of $PLC_{\gamma}1$ (39, 40). In this case, the manner by which $PLC_{\gamma}1$ becomes phosphorylated is not well understood. Although $PLC_{\gamma}1$ is mobilized to PTK growth factor receptors via interaction of its SH2-domain to a tyrosine phosphorylated receptor, the actual mobilization of $PLC_{\gamma}1$ to the TcR has not been readily detected (10). Likewise, it has been reported that $PLC_{\gamma}1$ can coimmunoprecipitate with Lck (41) and $PLC_{\gamma}1$ activation requires Lck function (24, 40), but it remains unclear whether Lck activity is directly or indirectly responsible for the activation of PLC1.

The Importance of $[Ca^{2+}]_i$ in Regulating Transcriptional Activation of the IL-2 Gene

Activation of $PLC_{\gamma}1$ leads to a release of [Ca^{2+}] from intracellular stores along with an influx of [Ca^{2+}] from the exterior, resulting in a rapid and sustained rise in [Ca^{2+}]i. Such an increase is thought to be responsible for regulating calcium/calmodulin-dependent events, including the induction of IL-2 gene transcription. Studies focusing on the action of immunosuppressive drugs on T-cell activation and function have been highly useful in dissecting the mechanism(s) by which increased [Ca^{2+}]i regulates events associated with T-cell activation, especially IL-2 gene expression. The mode of action of immunosuppressive drugs on T-cell function has been reviewed recently (42). Cyclosporin A (CsA), FK506, and rapamycin have been used extensively to prevent graft rejection following organ transplantation and exert their immunosuppressive activity by primarily inhibiting the T-cell specific expression of lymphokine genes, foremost of which is IL-2. FK506 and rapamycin possess considerable structural similarity, but are substantially dissimilar to CsA.. Nonetheless, CsA and FK506 exhibit similar effects on T-cell function (it should be noted that the effects of rapamycin on T-cell function is not identical to those of either CsA or FK506). The intracellular targets of CsA and FK506 are known as immunophilins; CsA and FK506. Cyclophilins and FKBPs share little sequence homology, but

both types of immunophilins are *cis-trans* peptidyl-prolyl isomerases and binding of CsA and FK506 inhibits isoserase activity (43-46). Immunophilins are widely distributed amongst various tissues and are believed to function *in vivo* by stabilizing nonnative conformations of proline-containing proteins. Biologically active drug-immunophilin complexes bind at high affinity to the Ca^{2+} and calmodulin-dependent serine phosphatase, calcineurin; calcineurin is activated as a result of increased [Ca^{2+}]; upon T-cell stimulation and its activity is crucial for the transcriptional regulation of IL-2 gene expression. Binding of drug-immunophilin complexes to a common site on the calcineurin molecule effectively inhibits PTPase activity (47). The resulting inhibition of calcineurin activity has a profound effect on IL-2 gene expression (see below). Significantly, overexpression of calcineurin renders T-cell activation more resistant to immunosuppressive drugs (48 and [Ca^{2+}]i; dependence following T-cell activation can be overcome by the expression of a deregulated subunit of calcineurin (49), both of which would have marked effects on the induction of IL-2 gene expression.

As mentioned above, the transcriptional activation of the IL-2 gene is highly dependent on the PTPase activity of calcineurin. Calcineurin is involved in the translocation of T-cell specific transcriptional activators that are essential for IL-2 gene expression from the cytoplasm into the nucleus. The vital target of calcineurin dephosphorylation in this process is the cytosolic component of nuclear factor of activated T cells (NFATc). The exact mechanism by which NFATc is translocated from the cytoplasm to the nucleus is not known, i.e., it remains speculative with regard to whether NFATc is directly or indirectly dephosphorylated by calcineurin.

To elaborate, NFAT is an inducible DNA-binding protein that recognizes two independent sites in the IL-2 promoter (NFAT-proximal and NFAT-distal binding sites; 50, 51). NFAT is a multisubunit transcription factor (52), consisting of at least three DNA-binding polypeptides: a pre-existing subunit, NFATc (as mentioned above), and a combination of minimally 2 components of AP-1 (52-61). The nuclear-associated/CsA-insensitive components of NFAT (i.e., Fos and Jun family members and Fos-related proteins) are apparently activated by a pathway involving Ras (outlined in 10). In contrast, NFATc is restricted to the cytosolic fraction of unstimulated T cells;

following T-cell activation, it is present in nuclear extracts and forms DNA-protein complexes with Fos and Jun family members and specific fos-related proteins (57-59). In addition, NFAT may be utilized in the transcriptional activation of other lymphokine genes, including the genes encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-4, and tumor necrosis factor-α (TNF-α) (62-65). It was reported recently that NFATc has been cloned; sequence analysis revealed that NFATc has a predicted MW of 97 kD and the isolation and sequencing of additional cDNA clones indicate that T cells express several forms of NFATc related to each other by alternative splicing and differing at their COOH-termini (60; as an aside, it would be highly interesting to determine the relative abundance of these NFATc isoforms in different populations of immature and mature T cells to see if a correlation between the ratio of different isoforms and the differential regulation of lymphokine gene expression).

NFAT binding activity is crucial for the transcriptional activation of the IL-2 gene. Indeed, multimers of NFAT-binding sites alone are sufficient to drive tissue-specific and TcR activation-dependent expression of a recorder gene (62). However, other inducible and constitutive *trans*-acting factors are involved in the coordinate transcriptional regulation of the actual IL-2 gene. Significantly the minimal IL-2 promoter in mouse and human (share 86% identity throughout approximately 580 bp of 5'-flanking region (63). Within this region are densely packed and frequently overlapping recognition sites for numerous DNA-binding proteins (reviewed in 64), including NF-κB or TcF-1 (65-67), AP-1 (53, 68), Oct-1 and other octamer-binding proteins (69), NFAT (as mentioned previously), and CD28RC or the CD28 response element (CD28RE) binding factor (70, 71; the importance of CD28 in T-cell activation and the regulation of IL-2 gene expression will be discussed later). The DNA-binding activities of several of these factors (NF-κB, CD28RC, NFAT, and AP-1) partitioned differentially in different subsets of developing thymocytes, as defined by electrophoretic mobility shift assays (72). The distribution of inducible DNA-binding factors in these subsets reflected the ability (or inability) of developing thymocytes to induce IL-2 gene expression *in vitro*. Additionally, *in vivo* footprinting of the IL-2 gene in an IL-2 expressing murine thymoma revealed several interesting aspects about the transcriptional activation

of the IL-2 gene (73). In these studies, additional inducible DNA-binding sites of (as of yet) unidentified factors were uncovered. Likewise, transcriptional activation of the IL-2 gene in stimulated cells was dependent on the coordinate binding of **all** *trans*-acting factors. In unstimulated or CsA-treated cells, the IL-2 promoter was completely unoccupied (i.e., no footprints were observed), thereby indicating that not only is translocation of NFATc to the nucleus CsA-sensitive, but formation of stable DNA-protein complexes at the remaining factor binding sites in the IL-2 promoter appears to be CsA-sensitive as well (72).

Negative Regulation of IL-2 Gene Transcription

The transcriptional regulation of IL-2 gene expression is also under stringent negative regulation. As indicated above, an increase in intracellular cAMP is able to suppress T-cell proliferation; suggestion is achieved by blocking IL-2 production (74-77). Besides being able to overcome cAMP-induced inhibition of IL-2 production by CD28 costimulation (see below; 78-80), IL-2 gene expression can be rendered insensitive to a forskolin-induced increase in intracellular cAMP by the expression of a dominant-negative mutant regulatory subunit of adenylate cyclase (81). Previous *in vivo* footprinting studies showed that complexes at the IL-2 promoter in stimulated IL-2 expressing cells are stable for more than 12 hours (73). Recently, it was reported that forskolin did not block the initial assembly of transcription-related complexes at the IL-2 promoter; however, forskolin treatment caused an accelerated destabilization of the transcription apparatus, as indicated by the disappearance of inducible footprints at the IL-2 promoter four to six hours following stimulation (81) (in cells expressing the mutant regulatory subunit of adenylate cyclase, inducible footprints were resistant to forskolin-induced destabilization). Hence, CsA and increased intracellular cAMP have different effects on the transcriptional regulation of IL-2 gene expression. Whereas CsA blocks the formation of active transcription-related DNA-protein complexes at the IL-2 promoter, intracellular cAMP appears to be involved in controlling the sustained stability of transcription complexes at the IL-2 promoter once they are established. Thus, the transcriptional activation of IL-2 gene expression is dependent on the coordinate regulation of

several signaling pathways which ultimately contribute to the highly regulated induction and maintenance of transcription factor-binding to the IL-2 promoter.

Post-Transcriptional Regulation of IL-2 Gene Expression

As mentioned previously, the cell surface molecule CD28 (MW: 44 kD) participates in the regulation of IL-2 gene activity (82). Even though little is known in terms of how CD28-costimulation influences the transcriptional activation of IL-2 gene expression, more is known about the effects of CD28 ligation on the post-transcriptional regulation of IL-2 gene expression. (The role of CD28 in antigen-induced T-cell responses is reviewed in 86.) With regard to T-cell activation and signal transduction, upon CD28-costimulation, CD28 is thought to recruit activated PTKs to the cell membrane, thereby facilitating the activation of $PLC_{\gamma}1$ and a Ca^{2+}-independent/CsA-resistant pathway (83-86). The CD28-induced/Ca^{2+}-independent signals have been implicated in controlling post-transcriptional effects on cytokine mRNA half-life by greatly increasing transcript stability (87). CD28 costimulation also confers substantial resistance to suppression of proliferation caused by an increase in intracellular cAMP in T cells (78-80). Thus, pathways activated by CD28 ligation provide additional regulation and amplification of T-cell activation and IL-2 gene expression.

CD28 costimulation relies on the interaction of CD28 with its cognate counter-receptor. CD28 is structurally related to another T-cell surface molecule, CTLA-4 (88). The counter-receptor for CD28 is a B7-related molecule (89, 90). B7 is a cell-surface glycoprotein expressed on activated B cells, dendritic cells, and macrophages. Inhibition of CD28 counterreceptor interactions blocks immune responses *in vitro* and *in vivo*. For example, anti-CD28 mAbs augmented proliferation of human T cells in the presence of treated APCs that were deficient in costimulatory activity (91). In addition, CD28 cross-linking blocked the induction of unresponsiveness (anergy) in murine $CD28^+$ T-cell clones (92), while blocking CD28/coreceptor interactions during a primary human mixed lymphocyte reaction (MLR) with CTLA-4Ig inducted antigen-specific unresponsiveness of $CD4^+$ T cells during subsequent stimulation (79;

unresponsiveness was reversed upon addition of anti-CD28 mAb or IL-2). Anergy/unresponsiveness is defined as a patial or incomplete activation state in which IL-2 is not induced. Thus, these data indicate a role for CD28/coreceptor interaction in the induction of T-cell responses and the prevention of anergy. However, CD28-deficient mice, though greatly compromised in the ability to mount adoptive immune responses, are not absolutely immunodeficient, suggesting that CTLA-4 may compensate for the absence of CD28 or other costimulatory pathways may be contributing to T-cell activation (93, 94). Notwithstanding, the dependence of T-cell activation on CD28/coreceptor interaction could be a mechanism by which T cells are stimulated only by appropriate cell types (those that are coreceptor+) and are not stimulated by the majority of inappropriate cell types that are coreceptor-, thereby insuring that T cells are, in large, unreactive to the plethora of cells that cannot (and should not) act as *bona fide* APCs.

Finally, events downstream of the transcriptional activation of the IL-2 gene provide an additional means of regulating IL-2 gene expression. IL-2 mRNA has a $t_{1/2}$ of 1 to 2 hours (95). IL-2 mRNA accumulation appears to be stringently regulated by RNase degradation; despite a relatively prolonged, high rate of synthesis, the intracellular level of IL-2 mRNA decreases rapidly, apparently due to the enhanced rate at which IL-2 mRNA is degraded (95). Interestingly, the addition of CsA following initial stimulation, while disrupting transcription of the IL-2 gene, led to an even faster disappearance of IL-2 mRNA compared to the natural decay of IL-2 message (98), suggesting that IL-2 mRNA degradation is enhanced in the presence of CsA and that CsA also affects the post-transcriptional regulation of IL-2 gene expression. It is not unreasonable to speculate that calcineurin or an immunophilin-regulated event controls some aspect of IL-2 mRNA degradation and exposure to immunosuppressive drugs also leads to deregulation of the degradation pathway. IL-2 mRNA is targeted for rapid degradation by the presence of an AU-rich domain in the 3'-untranslated region that is conserved between the mRNAs of many mediators of inflammation (96). It has been proposed that a specific, short-lived degradative RNase system is induced along with the coordinate expression of lymphokine genes (95). This degradation is

selectively sensitive to actinomycin D and cyclohexamide (CHX), inhibitors of RNA and protein synthesis, respectively (95). Specifically, CHX treatment results in the superinduction of IL-2 mRNA accumulation without affecting the transcriptional activity of the gene (95, 97). Hence, post-transcriptional mechanisms that appear to be induced simultaneously with the transcriptional activation of the IL-2 gene contribute to the highly transient nature of IL-2 gene expression upon induction.

Concluding Remarks

In conclusion, on a molecular level, IL-2 gene expression is stringently regulated, both in terms of transcriptional activation and post-transcriptional mRNA stability. The induction of IL-2 gene expression is contingent on the activation of numerous signaling pathways, beginning with TcR stimulation at the cell surface and leading to activation of Ca^{2+}/calmodulin-regulated events in the cytoplasm and transcriptional initiation at the IL-2 promoter in the nucleus. Post-transcriptionally, IL-2 is rapidly degraded by an apparently inducible but labile degradation system. All these factors contribute to the extreme transcience of IL-2 gene expression and have direct bearing on the function of IL-2 the inflammatory immune response.

Cellular Aspects of IL-2 Gene Regulation and Function

IL-2 Expression in Mature T cells Belonging to the αβ Lineage: Th1 versus Th2

In general, only a subset of mature T lymphocytes are capable of producing IL-2. T cells residing in peripheral lymphoid tissues that utilize αβ-heterodimers in the TcR complex (αβ-T cells) are traditionally divided into two main functional groups based on the mutually exclusive expression of CD4 and CD8 coreceptors (the differential regulation of these subsets is reviewed in 98). T cells that express the CD4 cell-surface glycoprotein are "amplifier" or "helper" cells that respond to the recognition of antigen by secreting lymphokines (Th cells; 99). In contrast, cells that express CD8 possess cytolytic activity and little helper activity (killer T cells). Furthermore, analysis of a series of T-cell lines suggests that functional heterogeneity exists within the Th subset

based on lymphokine production (100). Type 1 helper T cells (Th1 cells) produce IL-2, IL-3, interferon-γ (IFNγ), and TNF-α; these factors are involved in the regulation of cytotoxicity and delayed-type hypersensitivity. Type 2 clones (Th2 cells) synthesize factors that modulate B-cell growth and differentiation such as IL-4 and IL-5 (production of these factors augment humoral immunity), as well as IL-3, IL-6, and IL-10, but do not synthesize IL-2 or IFNγ. Interestingly, IL-10 has been implicated in the negative regulation of Th1-specific gene expression; exposure of APCs to IL-10 results in the down-regulation of B7 (and B7-related molecules) which, in turn, inhibits CD28 costimulation (101; reviewed in 102).

The differential induction of Th1 versus Th2-type responses may influence characteristics of some immune responses *in vivo*. The Leishmania major infections, mouse strains that are resistant to infection elicit predominantly Th1-type responses, whereas other strains that are susceptible to progressive disease elicit predominantly Th2-type responses (103). The Th1 versus Th2-type responses is directly correlated with the ability of resistant versus susceptible strains of mice to efficiently destroy this protozoan parasite. Protection against L. major infection is contingent upon the vigorous activation of macrophages; once activated, macrophages are able to effectively eliminate intracellular Leishmania organisms, while nonactivated macrophages are unable to do so. Since macrophage activation is preferentially regulated by IFNγ, whereas IL-4 and IL-10 abrogate the effects of IFNγ on microbial immunity (104), immune responses dominated by the production of IFNγ by $CD4^+$ T cells (Th1-type responses) would be protective against L. major infections. In contrast, responses dominated by the production of IL-4 (Th2-type responses) would not provide protection (105). Likewise, preferential induction of IL-4 (and IL-10) expression may contribute to susceptibility to infection by Listeria, Trichinella, and Scistosoma (106). Finally, Th1-versus Th2-type responses are observed when comparing patients with tuberculoid (resistant) and lepromatous (susceptible) leprotic lesions, respectively, with regard to cytokine mRNA expression and protein production (107, 108). Thus, the induction of a Th1-type response or Th2-type response against pathenogenic organisms is critical for establishing resistance to disease.

The Generation of Th Cells: Do Th1 and Th2 Cells Represent Common or Separate Lineages?

Until recently, the lineage relationship between Th subsets has not been well established. A considerable body of work, examining the induction and regulation of Th1 and Th2-type responses, exists, but does not shed a great deal of illumination on the subject of (1) whether Th1 and Th2 cells arise from a common precursor (the branched lineage model), (2) whether these subsets arise from different precursors (the separate lineage model), or (3) whether Th1 cells give rise to Th2 cells (the linear lineage model). Up to this point, it has been difficult to distinguish between these possibilities; however, several lines of investigation have addressed particular aspects related to these issues. For example, Firestein *et al.* (109) reported the isolation of murine CD4 T-cell clones that exhibited an "unrestricted" cytokine production profile, i.e., these cells secreted IL-2, IL-3, IL-4, IFNγ and TNFα. This new subtype was designated type 0 or Th0. Further observations supported the notion that Th0. Further observations supported the notion that the Th0 cell comprised a precursor subset. Following long-term culture, two independent Th0 clones lost the ability to secrete IL-2 and IFNγ, but not IL-4 upon mitogen activation (109). These results are compelling, but it is not known if these distinctions exist *in vivo*. In addition, it has been shown that IL-2 production is associated with a naive or virgin T-cell response. When stimulated by receptor engagement coincident with a costimulatory signal, naive T cells produce IL-2 but little IL-4 or IFNγ (110). On the other hand, IL-4 production is associated with T-cell memory (117; immunological memory has been the topic of several recent reviews, 112-114). Finally, a caveat to the Th1/Th2 distinction based solely on IL-2 and IL-4 production is presented by Harding *et al.* (115), as indicated by results showing that $CD4^+$ cells may respond to different mitogens by preferentially secreting IL-2, IL-4 or both, hence the segregation of Th cells into separate subsets or lineages may be somewhat artificial.

It has been proposed that Th0 cells arise from a precursor T-helper cell (pTh) that produced IL-2 exclusively (116), but it remains controversial as to whether a Th0-like state is obligatory for

the development of Th1 and Th2 effector cells (in this case, IFNγ production is the major indicator of a Th1-type response). Recent studies using a selective ablation technique has provided information pertinent to the resolution of this issue. In this system, transgenic mice that expressed the herpes simplex virus-1 thymidine kinase (HSV-TK) gene under the transcriptional control of the murine IL-2 promoter (117) or IL-4 promoter (118) were generated, thereby rendering IL-2 producing or IL-4 producing cells sensitive to the cytotoxic effects of the antiviral drug, gancyclovir (GANC), respectively. When $CD4^+$ T cells from IL-2:HSV-TK transgenic mice were stimulated with the superantigen, staphylococcal enterotoxin A (SEA) in the presence of GANC, proliferation and IL-2 production were inhibited and SEA-activated $CD4^+$ T cells were eliminated; on the other hand, when $CD4^+$ T cells from similar animals were stimulated with the lectin, conconavalin A (ConA), which mimics activation via the TcR, and GANC, proliferation was only partially blocked, even though IL-2 producing cells were efficiently eliminated (117). These data support the notion that an IL-2 producing state is not obligatory to elicit a (presumably IL-4 dependent) proliferative response to ConA. In contrast, exposure of naive $CD4^+$ T cells isolated from IL-4:HSV-TK transgenic animals to ConA and GANC *in vitro* under culture conditions that favored the expansion of cells that produce IL-4 or IFNγ not only resulted in the elimination of IL-4 expressing cells, but also of cells that principally made IFNγ (118). These results strongly suggest that effector cells producing either IL-4 or IFNγ have a common precursor which expresses the IL-4 gene. Based on these findings, a modified branch lineage model on the generation of IFNγ producing/Th1 cells and IL-4 producing/Th2 cells has been proposed (126). Thus, even though an-IL-2 expressing state is not an absolute requisite to progress to an IL-4 expressing state an IL-4 expressing precursor is able to give rise to both Th1 and Th2-type effector cells. The lineage relationship between the apparently more naive IL-2 expressing cells and IL-4 expressing cells requires further elucidation.

Functional Phenotype Plasticity *In Vitro*: Induction of IL-2 Gene Expression in CD8$^+$ T Cells

The phenotypic distinction that the ability to express IL-2 partitions exclusively with the CD4$^+$ T-cell population and not with the CD8 population can exhibit considerable plasticity *in vitro* (see Chapter 1). Whereas ConA plus the phorbol ester PMA (a direct activator of PKC) or anti-CD3 mAb plus PMA stimulation of virgin CD8$^+$ splenic T cells resulted in poor IL-2 production (as manifested by a failure to accumulate detectable levels of IL-2 mRNA), Ca^{2+}-ionophore (A23187) plus PMA stimulation (which altogether bypasses the TcR-dependent generation of second messengers) of fresh CD8$^+$ cells led to substantial IL-2 production (greater than 50% of CD8$^+$ splenic T cells were stimulated to express IL-2 under these conditions; Chapter 1). These results indicate that primary CD4$^+$ and CD8$^+$ cell do not differ so much in their intrinsic ability to initiate IL-2 transcription and that differences in signal transduction or post-transcriptional regulatory mechanisms (rather than the ability to induce IL-2 gene expression *per se*) may play a vital role in the segregation of CD4$^+$ and CD8$^+$ cells into distinct functional roles.

Inducibility of IL-2 Gene Expression in Immature T Cells

Not only can naive CD8$^+$ T cells be induced to express IL-2, but immature T cells are also inducible for IL-2 gene expression *in vitro* under the appropriate stimulation conditions, suggesting that IL-2 gene inducibility is an innate property of cells whose development is thymus dependent. Cells inducible for IL-2 gene expression are undetectable in unfractionated bone marrow (BM) which presumably contains T-cell precursors (BM cells were stimulated *in vitro* under a broad spectrum of conditions, including conditions sufficient to induce IL-2 gene expression in thymocytes and T cells; 119). However, it has been demonstrated that immature CD4$^-$CD8$^-$TcR$^-$ thymocytes are capable of expressing IL-2 upon stimulation with A23187 plus PMA, but unlike mature T cells, require an additional signal, provided by IL-1, in order to induce IL-2 gene expression (120). IL-2 gene inducibility is rapidly lost as developing thymocytes progress to the CD4$^+$CD8$^+$TcR^{lo} stage and this competence is not restored until thymocytes reach the CD4$^+$CD8$^-$

TcR^{hi} (or $CD4^-CD8^+TcR^{hi}$) stage. As alluded to previously, the ability of developing thymocytes to induce IL-2 gene expression is a reflection of the ability of different thymocyte subsets to mobilize or induce transcription factors involved in IL-2 gene transcription (72). Thus, IL-2 gene inducibility is not exclusively a Th "lineage" marker *per se*. Albeit has yet to be clarified whether all TcR^- thymocytes pass through an IL-2 inducible stage or only a "predetermined" TcR^- subset is inducible, IL-2 gene inducibility may potentially be a marker indicating commitment to the T lineage.

IL-2 Gene Expression in γδ T Cells

Finally, T cells belonging to the γδ lineage can also be induced to express the IL-2 gene. Whereas αβ T cells predominate in secondary lymphoid organs and the blood, γδ T cells predominate in epithelial tissues. With regard to specific populations of T cells for which the activating ligands have been characterized, within these subsets, IL-2 production can be induced *in vitro* and *in vivo*. For example, $V_\gamma3^+$ T cells (γδ cells that utilize the $V_\gamma3$ gene segment in the TcRγδ-chain) can be activated by keratinocytes. Following co-culture with the appropriate activating cell type, or stimulation by CD3ε or $V_\gamma3$ crosslinking, $V_\gamma3^+$ cells produce detectable amounts of IL-2 (121,122). Likewise, γδ cells that recognize Hsp60 of *Mycobactereum tuberculosis* (MT) (123) can be activated *in vivo* by immunization with MT and γδ cells isolated from draining lymph nodes of primed animals are inducible for IL-2 production (124). Strong circumstantial evidence exists with respect to γδ cells that express a certain V_δ or gene family (V_δM23) being spontaneous IL-2 producers; fusion of thymocytes isolated from newborn mice were fused with BW5417 cells yielded autoreactive γδ T-cell hybridomas that spontaneously produced IL-2 overnight in culture without stimulation, of which >80% expressed a member of the V_δM23 family (no correlation was found between V_γ usage and spontaneous IL-2 production) (131). In addition, splenic and intestinal γδ T cells stimulated with mobilized anti-CD3 mAb produced IL-2 and IFNγ but no IL-4 (125). Thus IL-2 production is a property of both αβ and γδ lineages (it would be interesting to determine if the mechanisms that regulate IL-2 gene expression

in particular subsets of γδ cells is identical to those acting in αβ cells).

Thymic Ontogeny and T-Cell Development

The development of functionally responsive T ells has been the subject of an extensive review (126). The body of work addressing this subject or any particular aspect therein is enormous. Taking this into consideration, even a cursory summary covering every area of T-cell development would be unduly long and perhaps, somewhat impractical. Therefore, only certain facets of T-cell development which have direct bearing to or are helpful for understanding the work that is presented in the subsequent chapters will be discussed.

The Thymus: Structure and Function

Unlike processes by which all other known hematopoietic cells are generated, the production of the majority of T cells is entirely contingent on the thymus, a specialized organ that is responsible for facilitating the expansion and rigorous selection of T-cell precursors. The steps involved in these processes are achieved via precursor interaction with the thymic microenvironment; the thymic microenvironment is a complex combination of epithelial cells and BM-derived cells such as dendritic cells and macrophages (127-130). Specifically, the nonlymphoid component of the thymus (the thymic epithelium) influences or controls the extensive proliferation and differentiation of early precursor cells, as well as the "education" of immature cells (the precise signals involved in these events are just beginning to be identified). As a consequence of the education process, properly educated cells are allowed to mature and exist the thymus, while all other inadequately or improperly educated cells are detained and eliminated.

In the postnatal animal, the thymic epithelium is separated into two distinct compartments, a cortex surrounding a medulla (the structure of the thymus is reviewed in 128 and 129). These epithelial domains are nonequivalent and are considerably heterogeneous in terms of cellular composition. The cortex is composed of a lattice of epithelial cells with long processes containing scattered macrophages and is densely packed with T-cell precursors (from this point onward, these

cells will be referred to as thymocytes). By contrast, the medulla contains a distinct population of epithelial cells of a more conventional form along with BM-derived cells that are found associated with the cortical-medullary junction and is a repository for thymocytes that display a mature T-cell phenotype. The cortex can be subdivided further into outer and inner cortical regions; the outer cortex, immediately beneath the fibrous capsule of the organ, provides a distinct microenvironment in which the majority of resident thymocytes are actively dividing (it is believed that the stroma delivers a powerful mitogenic signal) and the inner cortex serves as a holding area for the large number of thymocytes awaiting selection and/or elimination. There is also considerable evidence showing that the structural organization of the thymus is not only determined by the presence of the required nonlymphoid elements; indeed, it appears that the interaction of the thymic microenvironment with resident thymocytes influences the overall structural organization of the organ (131). Finally, T-cell development proceeds along a programmed intrathymic migration route (126). Precursor cells most likely enter the thymus at the cortical-medullary border and migrate to the outer region of the cortex, during which they commence to divide. Once in the subcapsular zone, the rate of proliferation reaches its maximum and this phase culminates in the surface expression of conventional TcR complexes. Soon afterwards, thymocytes cease to proliferate and move back into the cortex proper. Only positively selected cells are allowed to pass from the cortex to the medulla. Thus, in general, T-cell development relies on a complex set of cell-cell interactions which are determined by lymphoid and non-lymphoid cell types in the thymus.

Thymic Ontogeny

In mammals, the thymic epithelium is generated via the interaction of the third and fourth branchial pouch endoderm with the overlying ectoderm of the pharyngeal cleft, occurring at days 10-11 of gestation in the mouse and between weeks 7 and 8 in the human (126). The seeding of the thymus rudiment with precursors derived from HSCs occurs soon after its formation and continues up to birth. In mice and avian species, the first lymphoid precursors arise from the yolk sac (132). Derivatives from the yolk sac seed both the fetal liver (fL) and the thymus (126).

During embryogenesis, the fL is the predominant source of lymphoid precursors; additionally, the derivatives from the fL proceed to colonize the BM which becomes the major site of hematopoiesis after birth. There is substantial evidence from a variety of animals indicating that lymphoid precursors enter the thymus of several waves during gestation. These waves of immigrants have been observed in *Xenopus* (133, 134), chicken (135), quail (136), and mouse (137). A series of elegant experiments using reciprocal chick-quail chimeras with donor thymuses and recipients of different stages demonstrated that there are discrete periods in development when the thymus takes up cells separated by nonreceptive periods (136). Each cohort of precursors enters the thymus and remains quiescent as the cells from the previous wave proliferate; once the earlier cell types emigrate or die, the next cohort becomes active. Three distinct waves of precursors populate the embryonic thymus in chicken and quail (136), whereas only two are observed in mouse (137). As alluded to previously, the first wave of precursors in the mouse is derived from the fL and seeds the thymus between days 11 and 13 of gestation (137). Cells generated from this wave persist up to birth after which they gradually disappear by the first week of postnatal life. The second wave of precursors is derived from the BM and first enters the thymus around the time of birth and cells from this wave continue to populate the thymus for at least eight weeks postpartum. These results in the mouse indicate that the embryonic and postnatal T-lineage cells may be derived from different precursors. In addition, the architecture of the fetal thymus is substantially different than that of the adult thymus (128, 138). Thus, at least in the mouse, some of the steps involved in the generation of T cells in the fetus versus the postnatal animal are most likely unique to that particular period in development.

The Multipotential Hematopoietic Stem Cell

The characterization of the stem cell that ultimately gives rise to T-cell precursors has been a topic of extensive study over the past several years. Lymphoid cells (B and T lymphocytes), myeloid cells (macrophages, neutrophils, eosinophils, basophils, mast cells, platelets) and erythrocytes are derived from a common multipotent hematopoietic stem cell (MHSC). MHSCs

must maintain the ability for self-renewal **and** possess the capacity to give rise to all differentiated cell types, common features displayed by all multipotent progenitors. MHSCs are not actively cycling and can be distinguished from cycling HSCs that have lost the ability for self renewal based on the capacity of MHSCs to provide long-term repopulation of all hematopoietic compartments in irradiated recipients. Cycling HSCs give rise solely to short-term repopulation (139). MHSCs can be isolated from both fL (140, 141) and adult BM (142-143). In the mouse, MHSCs do not express most hematopoietic cell lineage markers (MHSCs are Lin^-, equivalent to $CD3^-CD4^-CD8^-B220^-Mac\text{-}1^-Gr\text{-}1^-$Erythrocyte Ag^-), but do express low levels of Thy-1 and high levels of stem cell antigen-1 (Sca-1) (142, 143). MHSCs isolated from fL versus adult BM differ in their ability to give rise to different subsets of T cells (144). Whereas fL MHSCs can give rise to both fetal and thymus-derived cell populations, adult BM MHSCs can only generate the adult T-cell pool. Again, these data support the notion that T-cell precursors derived from MHSCs isolated from fL versus adult BM represent nonequivalent cell types.

The Committed Lymphoid Precursor

A committed lymphoid precursor (CLP) that has lost the ability to give rise to myeloid and erythroid lineages but retains the ability to develop into T and B cells has been identified in the adult mouse thymus (145, 146). However, it has been difficult to identify a CLP at the site(s) where all other events associated with hematopoiesis are active or to determine what influences an HSC to become a CLP. The CLP differs in cell surface phenotype from HSC to become a CLP. The CLP differs in cell surface phenotype from HSCs in two respects; the more differentiated cell type expresses low levels of CD4 and high levels of Sca-2 (144, 146). When injected intravenously, the CLP gives rise to T and B cells only (likewise, when CLPs are injected directly into the thymus, they differentiate exclusively into T cells). Recently, a cell type similar to the adult thymic CLP has been detected in the fetal thymus (147). However, the fetal thymic CLP, while being able to reconstitute the B-lineage compartment, was unable to provide long-term repopulation of the T-lineage compartment in irradiated adult recipients. Additionally, CLPs

isolated from postnatal animals up to 4 weeks postpartum were unable to provide long-term repopulation of the adult thymus. The differences in repopulation capacities of fetal versus adult CLPs can be accounted for by potential inherent differences in the two types. Recall that fetal CLPs are adult CLPs are derived from the fL and BM, respectively, and that the first wave to repopulate the fetal thymus lasts for approximately 2 weeks and is completely displaced by the second wave one week after birth. Hence fetal CLPs may be "preprogrammed" to give rise only to short-term repopulation of the thymus). On the other hand, an alternative explanation can be formulated. Beginning at day 13 of gestation and continuing until approximately four weeks after birth, the thymus is expanding (i.e., cell numbers do not reach steady state until about four weeks after birth), thus CLPs during the period may be continuously forced to cycle. It is possible that being in active cycle versus being able to provide long repopulation are mutually exclusive routines.

The Generation of γδ T Cells

Two lineages of T cells are generated in the thymus and are distinguished by the expression of ab or gd heterodimers in the TcR complex (TcRγ- and δ-chains are not to be confused with CD3γ and δ chains). T cells represent a minority population in the postnatal thymus and peripheral lymphoid organs. It is still not clear at what point the γδ lineage diverges from the αβ lineage, but the development of γδ cells is not contingent on the development of αβ cells or vise versa. The elimination of αβ T cells by target mutation of the TcRα- or β-chain genes has no effect on the generation of γδ cells (148, 149) (likewise, αβ T-cell development is unperturbed in mice bearing mutant TcRδ genes, 150). Similar to their more prevalent $\alpha\beta^+$ counterparts, γδ cells appear to be subject to positive and negative selection. When mice that are transgenic for a defined γδ TcR with "nonconventional" class I MHC specificity were crossed with animals deficient in β_2-microglobulin (β_2m) expression (these mice are class I MHC^-), transgenic TcR^+ immature cells were abundant in the thymus, but transgenic cells were absent in peripheral lymphoid tissues, indicating that γδ cells are subject to a process analogous to positive selection, i.e., in the absence of selecting ligand,

transgene positive cells could not be positively selected and were blocked at an immature stage in the thymus (151, 152). In several defined cases, the targt antigen for a defined γδ TcR has appeared to be class I MHC-restricted. However, no gross abnormalities of γδ T cells were observed in β_2m-deficient mice that were not transgenic for a particular γδ TcR (153). Consequently, it has been proposed that γδ cells can be divided into two categories, one with β_2m-related specificities whose maturation is dependent on the presence of β_2m-associated proteins, and another with β_2m-unrelated specificity whose development is β_2m independent and can expand in β_2m-deficient mice to populate the γδ T-cell compartment (154). There may also be inherent differences in the positive selection of γδ versus αβ cells. Recent findings revealing that the general rules that define αβ T-cell specificity do not influence γδ T-cell specificity (155), therefore the process by which functional specificity is determined may be distinct for these two lineages. Finally, in terms of effector function, T cells exhibit cytolytic activity and are also capable of producing lymphokines, including IL-2. With regard to these aspects, γδ cells do not appear to be all that different from their αβ counterparts.

The development of γδ T cells has been reviewed recently (154, 156-158). Interestingly, γδ T cells can be divided into several subsets based on variable (V) gene usage, junctional diversity, and tissue distribution. In the fetal thymus, γδ T cells appear as a series of overlapping waves commending at approximately day 13 of gestation. The first two waves express $V_\gamma 3$ and $V_\gamma 4$, respectively, and both γ-chains are apparently paired with a δ-chain composed of $V_\delta 1/D_\delta 2/J_\delta 2$ segments. A striking feature of these early receptors is there is little junctional diversity of the rearranged genes, suggesting that the mechanisms by which junctional diversity is achieved in T cells derived from BM precursors are not active cells derived from fL precursors. Later in ontogeny, $V_\gamma 3$ and $V_\gamma 4$ cells disappear from the thymus and are replaced by cells expressing diverse receptors containing $V_\gamma 2$ and $V_\gamma 1$ and a variety of V_γ gene products. Up until approximately day 16 of gestation, γδ cells are the predominant $CD3^+$ population in the fetal thymus, after which the proportion of γδ cells drops and are displaced by αβ cells, which become the major lineage found in the thymus. The highly ordered appearance of γδ T cells is believed to

be regulated by programmed rearrangement at the γ-chain gene locus (159) as dictated by the thymic microenvironment and/or the γδ progenitor cell. There is a striking correlation between the transcription of unrearranged V_γ genes and the timing of their rearrangement (159). The sequential activation of V_γ genes may regulate the accessibility of the surrounding chromatin to the recombination machinery, resulting in the ordering of V_γ gene rearrangement during development. This correlation between transcriptional activation and targeted gene rearrangement is a general feature of TcR and Ig gene rearrangement. Notwithstanding, signals that induce transcriptional activation at the TcR gene loci is largely unknown. In normal adult mice, $V_\gamma3^+$ cells are associated exclusively in the skin, in which they are the predominant T-cell type, while $V_\gamma4^+$ cells are restricted to the tongue and female reproductive tract. $V_\gamma2^+$ and $V_\gamma1^+$ cells largely populate lymphoid tissues (spleen and lymph nodes) and the blood. Lastly, the intestinal epithelium contains a large number of γδ T cells, many of which possess diverse TcRs with V_γ-containing $V_\gamma5$ gene segments and δ-chains containing a variety of V_δ elements (these intestinal γδ cells apparently arise extrathymically; the extrathymic generation of T cells will be elaborated upon later.

The Generation of αβ T Cells

The bulk of work on T-cell development focuses on the generation of αβ cells. Discrete phenotypic changes are associated with particular events in αβ T-cell development and the mechanisms that control of influence these changes are currently being dissected. In highly abridged form, cells of the αβ lineage arising from $CD4^{lo}CD8^-TcR^-$ precursors progress from a $CD4^-CD8^-\alpha\beta TcR^-$ (triple negative/TN) "immature" stage (comprising 4% of thymocytes) to $CD4^+CD8^+TcR^{lo}$ (double positive/DP) "cortical stage" (representing the bulk population in the postnatal thymus at 80%). Potentially autoreactive cells are eliminated from the repertoire by the process of negative selection. Maturation beyond the cortical stage is dictated by positive selection, yielding TcR^{hi} cells that are either $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) and accumulate in the medulla. Of cells at the medullary stage, 10% are CD4-SP and 4% are CD8-SP. The cells that emigrate from the thymus to populate the periphery are primarily of the medullary SP type (126).

This simplified depiction provides useful landmarks, but it should be stressed that numerous "intermediate" phenotypes exist, indicating transitions between all three stages (for a detailed description of the phenotypic changes that accompany transitions throughout T-cell development, see 133). Specific intermediate phenotypes will be discussed but only in the context of transitions from one major stage to the next.

Transitions Prior to αβ-TcR Surface Expression

Prior to TcR gene rearrangement, TNs downregulate expression of Pgp-1 (CD44) and upregulate expression of Thy-1, heat stable antigen (HSA) and IL-2R α-chain (CD25). To reiterate the signals that induce this change in phenotype are not known. This is a particularly significant intermediate phenotype because the state that is associated with this phenotype appears to be an arrest point before TcRβ-chain gene rearrangement. Cells in which β-chain rearrangement or expression is perturbed or suppressed are frozen in the $CD44^{-}CD25^{+}$ TN state or in a $CD25^{-}$ immediately beyond. For example, thymi of mice homozygous for the severe combined immunodeficiency (SCID) mutation (manifestation of this defect results in an inability to rearrange TcR and Ig chain genes, successfully 160) are enriched for TN cells, especially for those expressing CD25, and are depleted for all other downstream cell types (161). Likewise, mice deficient in recombinase activating gene (RAG-1 or RAG-2 activity (162-163) or TcRβ-chain expression (149, 150) exhibit a block at the TN to DP transition, and the thymus isolated from these animals contain two orders of magnitude fewer cells than wild-type animals. On the other hand, unlike RAG-deficient or SCID mice that possess defects in the actual recombination process, TcRβ-deficient animals are not recombination defective. β-chain -/- mice are able to rearrange their α-chain loci to some extent; nevertheless, these mice do not produce DP thymocytes, indicating that α-chain rearrangement is irrelevant for induction of the TN or DP transition.

The role of TcRβ-chain expression in the absence of α-chain expression in the TN or DP transition has been defined in several genetic studies. Introduction of a rearranged β-transgene into RAG-deficient (164) or SCID animals (165) results in the generation of $CD4^{+}CD8^{+}$ cortical

thymocytes. However, the β+SCID thymus contains one tenth the number of cells found in the β+RAG-2 -/- thymus (166). It has been proposed that these differences may be caused by the inability of SCID thymocytes to properly ligate lethal DNA double-strand breaks (160) and the SCID-related lethality is not rescued by introduction of a rearranged β-chain (166); indeed, these cells may continue to attempt α-chain rearrangement with little success. As an aside, as extension of this argument implies that in nontransgenic RAG-deficient and SCID mice, RAG-2 -/- thymocytes are essentially held at a state in which TcR genes are completely unrearranged, whereas SCID thymocytes are actively attempting to rearrange their TcR genes, suggesting that SCID thymocytes may have progressed further along the developmental pathway than RAG-2 -/- thymocytes. To the contrary, thymocytes isolated from SCID animals appear to be blocked at the $CD44^+CD25^-$ stage prior to the upregulation of CD25 (126). However, SCID-related lethality may be responsible for the accumulation of $CD44^+CD25^-$ cells, since cells with potentially more mature phenotypes may be eliminated as they initiate rearrangement of β-chain genes. Recent findings revealed that RAG-2 -/- thymocytes are arrested further downstream at the $CD44^-CD25^-$ stage, representing the immediate progeny of $CD25^+$ immature cells (unpublished). Hence, the developmental block in RAG-2 -/- versus SCID mice do not appear to be identical. Likewise, the block in TcR β-chain deficient animals may not be identical to that in RAG-2 -/- animals, since β-chain -/- thymocytes can proceed with rearrangement at other TcR-chain gene loci and are not subject to SCID-related lethality.

The discovery that TcRβ-chain is expressed on the surface of immature thymocytes in the absence of α-chain has sparked substantial interest in identifying TcRβ-chain (in which TcR β-chain-complexes detected would be represented of complexes expressed during the TN to DP transition) revealed that TcRβ-protein complexes contained CD3γ, δ, ε and ζ or η chains as well as a glycosylated polypeptide identified as gp33 (166). gp33 is disulfide linked to TcRβ protein and acts as a putative TcRα surrogate chain. The association of TcRβ-chain with gp33 appears to be developmentally regulated, since TcRβ-chain/gp33-containing complexes are not detected in DP cells in which TcRβ-chains are associated exclusively with TcRα chains (167). The TcRβ/gp33

heterodimer function in T-cell development is believed to be analogous to that of the μ-heavy chain/Vpre-Bλ5 complex in B-cell development (168-170).

Interaction of the TcR/gp33 complex with its unidentified ligand is thought to generate an intracellular signal which promotes further development as indicated by the downregulation of CD25 expression, appearance of CD4 and CD8 coreceptors, explosive cell proliferation, and suppression of further TcR β-chain gene rearrangement (allelic exclusion). Recently, studies have demonstrated that CD3γ, δ, and ε but not ζ chains are detectable on the cell surface of RAG-2 -/- mice and that *in vivo* treatment of these recombination deficient animals with anti-CD3 mAb results in the generation of DP cells (171). Hence, even when TcRβ-chain cannot be expressed, CD3 complexes can be found on the surface and can mediate signals sufficient to induce coreceptor expression and extensive cell proliferation. The absence of CD3ζ-chain surface expression in RAG-2 -/- thymocytes can be reconciled with the result showing that ζ-chain is present on the cell surface of the SCID/TcR β-chain transfectant, because it is possible that surface expression of CD3ζ is dependent on TcRβ expression (recall that RAG-2 -/- thymocytes are still TcRβ-deficient). Additionally, circumstantial evidence suggests that CD3ζ-associated signaling events may not be vital for the TN to DP transition; in thymi removed from STD patients who are ZAP-70 -/-, the generation of cortical thymocytes is not blocked, implying that CD3 ζ/ZAP-70 mediated signaling events are not necessary with regard to T-cell development up to the DP stage. To the contrary, targeted disruption of the CD3ζ chain in mice resulted in a tenfold reduction of the DP population compared to wild type (172). Nevertheless, it has not been determined whether CD3ζ -/- mice still express other CD3 subunits along with TcRβ/gp33 on immature cells and if the DP population in these animals can be restored to normal levels following anti-CD3ε mAb treatment, or whether the TN to DP transition is simply inefficient in CD3ζ -/- mice. Perhaps CD3ζ influences the generation of DPs by facilitating the TN to DP transition but is not absolutely required in this process. Finally, Lck function has been implicated in controlling the TN to DP transition. In Lck-deficient mice or transgenic animals expressing a dominant-negative variant of Lck (22, 173), T-cell development is blocked at the TN stage. Progression to the DP stage in these animals is not

restored by the introduction of a rearranged TcRβ-chain gene (22). Conversely, overexpression of a wild-type *lck* gene prevents TcRβ-chain rearrangement, while leaving TcRα-chain rearrangement unimpaired (174). This implies that while Lck is required for initiating TcRβ-chain rearrangement, an increase in Lck activity may arrest this process, ergo the modulation of Lck activity is crucial for the proper regulation of β-chain rearrangement.

Overall, early transitions prior to the DP stage are determined by the induction of a series of signaling pathways in the absence of *bona fide* αβ-TcR surface expression. Using genetic approaches, facilitated by transgenic and targeted gene disruption technologies, many of these pathways are being dissected. Several components of the signaling apparati are shared between immature TN and mature SP thymocytes (CD3 components, TcRβ chain, Lck). However, the effects exerted by these components in immature versus mature cells are profoundly different. Crucial to the differences observed in TN thymocytes is the coexpression of gp33 with rearranged β chain. Additionally, Lck activity has differential effects in immature thymocytes. In conclusion, immature thymocytes are subject to a barrage of signals that are vital for the progression from an αβ-TcR$^-$ to an αβ-TcR$^+$ state. It remains largely known whether these signaling events in TN thymocytes *in vivo* are sufficient to induce the expressioln of genes characteristic of a mature T-cell response, first and foremost is the induction of IL-2 gene expression.

Transitions Following αβ-TcR Surface Expression

In a normal animal, once αβ TcR is expressed on the cell surface, the proliferation initiated by the putative engagement of TcRb/gp33 ceases, after which DP thymocytes decrease in size (these cells are classified as small cortical thymocytes). Small cortical thymocytes do not express high levels of αβ TcR on the cell surface, due to the degradation of an unusually high proportion of newly synthesized chains before they reach the cell surface (126). The vast majority of cortical thymocytes do not progress past the DP stage and undergo programmed cell death (the topic of PCD is reviewed in 175 and 176). The average life span of a TcRβ$^+$, TcRα-rearranging CD4$^+$CD8$^+$ thymocyte is 3.5 days, during which time a small proportion of thymocytes are

positively selected for further maturation (<5%) or after which the remaining cells die *in situ* (>95%; 177, 178). This death process is also known as apoptosis and is characterized by surface membrane "blebbing," condensation of chromatin, degradation of DNA into nucleosome-sized fragments of approximately 180 bp, and subsequent fragmentation of affected cells. The apoptototic suicide response is triggered by a rapid increase in $[Ca^{2+}]i$. Bcl-2, a nuclear encoded mitochondrial protein, is believed to be involved in rescuing cells from apoptosis. *bcl-2* is not expressed by cortical cells but is upregulated in medullary thymocytes (179, 180). Genetic evidence indicates that *bcl-2* is not the only gene responsible for limiting the extent to PCD in the thymus. Overexpression of *bcl-2* in normal mice, while increasing the resistance of thymocytes to apoptosis induced by glucocorticoids of ionizing radiation, does not result in the overpopulation of the thymus with DP cells nor rescue cells from negative selection or PCD induced by acute CD3e-crosslinking. Therefore, expression of *bcl-2* in cortical thymocytes expressing a transgenic TcR appears to delay apoptosis in nonselected DP thymocytes and reduce the efficiency of negative selection, but is not sufficient to enable further maturation of these cells (181, 182). The disruption of *bcl-2* expression in mice has little effect on fetal or early neonatal development, whereas after two weeks of postnatal life, both T and B lymphocytes and their precursors in the thymus and BM gradually disappear, suggesting that differential requirements for *bcl-2* exist in the generation of T and B cells derived from the fL versus those derived from the BM (183). Lastly, recent studies have revealed that PCD in the thymus may be controlled by the combined effects of *bcl-2* and *bcl-2*-like/related genes (184, 185).

Negative Selection

The progression from the DP stage to the SP stage is regulated by the negative and positive selection. Negative selection is the active process by which autoreactive cells are removed from the repertoire, and positive selection is the means by which cells bearing useful TcRs with the potential to recognize foreign antigen in the context of self-MHC products are rescued from PCD. Both processes resemble mature T-cell activation in ligand-receptor requirements. However, the next

results of negative selection, positive selection and mature T-cell activation are not equivalent (elimination versus survival versus induction of functional responses). Initial reports on negative selection described the arrested development and clonal deletion of superantigen-reactive $V\beta^+$ cells in the thymus of mice positive for superantigen expression (186, 189; overviews of negative selection and superantigen recognition can be found in 189, 190, respectively). Negative selection has been examined in detail using transgenic mice expressing rearranged TcRa and TcRβ-chain genes that encode receptors of defined specificities. When transgenes encoding class I-restricted receptors that recognize male H-Y antigen in the context of $H\text{-}2D^b$ in one case, or $H\text{-}2L^d$ in another, were bred onto genetic backgrounds in which the target antigen was a self-antigen (male $H\text{-}2^b$ or $H\text{-}2^d$, respectively), the resulting mice had thymi that were greatly depleted in both DP cortical and SP medullary cells (191-194). Similar observations were observed in mice bearing transgenic TcRs with class II-specificities (195, 197). Although the net result of negative selection in these transgenic systems are the same (i.e., the clonal deletion of autoreactive cells), particular details pertaining to this process may not be completely identical between class I and class II-specific T cells. For example, whereas class I MHC molecules are expressed more-or-less ubiquitously throughout the thymic microenvironment, class II^+ cells are found on only a subset of cells therein, hence resident thymocytes will potentially "see" class I molecules continuously as they develop and will encounter class II molecules only in specific areas of thymic microenvironment where class II^+ cells are localized. Introduction of TcRα- and β-chain transgenes causes premature expression of transgenic TcRs on resident thymocytes, thus many class I-specific transgenic TcR^+ thymocytes which would not be expressing TcR at the equivalent state in nontransgenic animals could be subject to "premature" negative selection soon after TcR surface expression; in contrast, premature expression of a transgenic class II-specific TcR may not grossly effect the kinetics of negative selection, since class II-specific transgenic TcR^+ thymocytes may not encounter negatively selecting ligand for sometime following TcR surface expression. In general, negative selection of class I-specific TcR^+ thymocytes may be more efficient than that of cells expressing class II-specific TcRs (the points discussed here also apply to the process of

positive selection). Such studies have revealed that two signals are required to induce negative selection, the first activated by TcR ligation, the second by an unidentified interaction that is not contingent on CD28 engagement.

Particular aspects of negative selection have been successfully studied *in vitro*. Clonal deletion of DP thymocytes isolated from transgenic mice expressing a class II-restricted αβ-TcR specific for cytochrome C was induced following co-culture with E^b-transfected fibroblasts and antigenic peptide in a dose-dependent manner (198). Further studies obtained from this *in vitro* culture system revealed that negative selection was insensitive to CsA and that substantial clonal deletion of DP thymocytes was induced at a relatively low concentration of antigenic peptide which, at the same concentration, was insufficient to stimulate detectable lymphokine production in isolated peripheral T cells. Albeit that injection of anti-CD3ε mAb induces acute depletion of cortical cells *in vitro*, exposure of transgenic TcR^+ DP thymocytes to anti-CD3 mAb *in vitro* did not induce clonal deletion. DP thymocytes remained viable but downregulated CD4 and CD8 coreceptors in a process defined as DP "dulling," (199). Subsequent deletion was induced upon addition of APCs, thereby indicating that two signals are required for negative selection. This second signal is not generated through interaction with B7, since antagonists of B7-CD28 signaling had no effect on clonal deletion (199). Taken together, these results reinforce the notion that signals crucial for inducing clonal deletion of potentially autoreactive cells may be quantitatively and qualitatively different than those for activating mature T cells.

Positive Selection and TcR Ligand Interactions: Affinity Versus Avidity

As alluded to previously, though interactions required for negative selection, positive selection and mature T-cell activation are similar if not identical (TcR-peptide-MHC and coreceptor engagement), the resulting signals generated by these interactions in immature versus mature cells may be quantitatively and qualitatively different. Transgenic and targeted gene disruption technologies have been applied to understand the nature of interactions involved in positive selection. Positive selection has been reported in both class I and class II MHC-restricted TcR

transgenic mice (191-197), as evinced by the generation of mature transgenic TcR$^+$ SP thymocytes in the appropriate positively selecting genetic background. However, a controversial aspect of positive selection has been the nature of positively selecting ligands in the thymus. The most widely accepted model for positive selection maintains that the process requires the presentation of peptides in conjunction with MHC molecules; however, until recently, the properties of positively selecting peptides have remained undefined. Unlike negative selection which can be examined *in vitro*, positive selection has not been adequately reconstituted in the absence of a thymus using defined cell populations. Hence, in order to examine and define the character of positively selecting ligands *in vitro*, several investigators have adapted a modified fetal thymic organ culture (FTOC) system in which thymi from class I MHC-restricted TcR transgenic mice on a mutant class I-deficient background were used and both MHC surface expression and presentation of different defined peptides were manipulated in culture and the effects of such manipulation on the generation of mature CD8$^+$ SP thymocytes were analyzed (200, 201). Positive selection of CD8$^+$ SP thymocytes from FTOC of thymi isolated from transgenic mice expressing a TcR with specificity for an octamer peptide from ovalbumin ($OVA_{257-264}$) in the context of the H-2K^b molecule bred onto a β_2m -/- background was reconstituted upon addition of peptide plus exogenous β_2m (the addition of β_2m stabilized the empty class I heavy chains that had made their way to the cell surface in the absence of β_2m). Under these conditions, the peptides that were able to induce positive selection were all variants of the antigenic peptide and had previously been defined as being antagonistic for this receptor, whereas addition of antigenic peptide caused clonal deletion (200). Based on these results alone, low affinity interactions appear to mediate positive selection.

In contrast, a similar set of experiments using FTOC of thymi from animals expressing a transgenic TcR recognizing a lymphocytic choriomeningitis virus (LCMV) peptide in the context of H-2D^b and deficient in transporter associated with antigen processing (TAP) activity demonstrated that avidity as well as affinity influences positive selection (in the TAP -/- background, a low number of unstable empty class I molecules is expressed on the cell surface and molecules are stabilized upon addition of exogenous peptide; 201). In the second case, even though antigenic

LCMV peptide does not stabilize H-2D^b molecules efficiently, the TcR-specific peptide was the only one capable of inducing positive selection of CD8$^+$ SP cells, but only at low concentration (again, higher concentrations of antigenic peptide induced negative selection). Thus, antigenic peptide is capable of inducing positive selection (similar results were obtained in yet another FTOC system, 202). The findings generated from these two studies are not completely at odds with one another. Taken together, these findings show that positive selection occurs on low affinity ligands when ligand density is high (OVA-specific TcR plus β_2m -/- case); on the other hand, positive selection is induced only upon interaction with high affinity ligands when ligand density is low (LMCV-specific TcR plus TAP -/- case). Hence both affinity and avidity are parameters that affect positive selection.

Signals Induced by Positive Selection

Although these seminal studies shed considerable light on the molecular interactions involved in positive selection at the cell surface, considerably less is known about the specific signals generated by these interactions that rescue positively selected cells from PCD. Since the dual affinity/avidity model of positive selection mandates that both low TcR occupancy/little TcR clustering and high TcR occupancy/extensive TcR clustering (as determined by ligand affinity for TcR) cause positive selection, signals generated by these different interactions may not be equivalent. Additionally, there is no guarantee that signaling mechanisms involved in the positive selection of CD4$^+$ SP cells are identical to those of CD8$^+$ SP cells. This is supported by recent findings obtained from STD patients who are deficient in ZAP-70 activity (37). Somewhat unexpectedly, thymi taken from afflicted individuals were greatly depleted for CD8$^+$ SP cells, while there was no evident effect on the numbers of DP and CD4$^+$ SP cells. The defect in the production of CD8$^+$ SP cells was also manifested in the periphery. CD8$^+$ cells were absent in secondary lymphoid compartments but CD4$^+$ cells were easily detected, also indicating that a deficiency in ZAP-70 activity leads to the production of nonfunctional CD4$^+$ cells. Thus, these observations suggest that ZAP-70 activity is required for the DP to CD8$^+$ SP transition yet is

nonessential for the DP to $CD4^+$ SP transition and that signals transduced in DP thymocytes following TcR-ligand interactions that induce positive selection of $CD8^+$ SP cells are not identical to those that induce positive selection of $CD4^+$ SP cells. The question of whether these potential differences are dictated by the specificity of the TcR alone or in a cell autonomous manner prior to TcR-ligand interaction cannot be answered at this time.

Commitment to the Helper Versus Killer Lineage: Instructive Versus Stochastic/Selective Models

It is not clear by what mechanism cells expressing class I or class II-specific TcRs become $CD4^-CD8^+$ killer or $CD4^+CD8^-$ helper cell precursors, respectively. Two models have been proposed to explain CD4/CD8 lineage determination (203, 204). In the instructive model, coengagement of a class I molecule generates a different signal from class II-restricted TcR/CD4 and class II MHC interaction, thereby directing the differentiation into either killer or helper cell precursors. In the stochastic/selective model, commitment occurs stochastically by the random downregulation of either CD4 or CD8 surface expression, and cells with a complementary coreceptor combination, i.e., class I-restricted TcR plus CD8 or class II-restricted TcR plus CD4, interacting with the appropriate MHC molecules would be selected for survival and maturation. In both cases, cells with mismatched TcR-coreceptor combinations are not selected and coreceptors play essential roles in positive selection. Evidence showing that $CD4^+CD8^+$ thymocytes upregulate TcR levels prior to downregulation of coreceptor support the instructive model of positive selection (205, 206) (the instructive model mandates that thymocytes expressing both coreceptors are the primary targets of positive selection). On the other hand, the existence of a significant population of $CD4^+$ SP cells in class II-deficient mice that is intermediate in maturity between $CD4^+CD8^+$ and end-stage $CD4^+CD8^-$ thymocytes as a result of having interacted with class I MHC (these cells are TcR^{hi} $CD69^+$ and have downregulated RAG-1 and TdT activities) implies that TcR-MHC engagement may provoke random downmodulation of either CD4 or CD8 and a certain degree of differentiation, concepts consistent with the stochastic model (a reciprocal

intermediate population is found in class I-deficient mice) (207). Likewise, the rescue of $CD8^+$ cells in β_2m -/- mice or in animals expressing a class II-restricted TcR by overexpression of a CD4 transgene (rescued cells express both CD8 and transgenic CD4, are class II-restricted, and exhibit cytotoxic activity) (208) support the notion that a two-step process is involved in thymocyte maturation in which initial TcR interaction with restricting MHC causes the stochastic downregulation of CD4 or CD8 followed by selection based on compatible TcR and remaining coreceptor interaction with MHC. Additional studies will be required before it can be determined whether the instructive or stochastic/selective model more accurately describes the process of positive model more accurately describes the process of positive selection. Finally, neither of these models take into account whether commitment to the helper or killer lineage occurs prior to the DP stage, thus more information must be gathered in order to understand lineage commitment in the thymus.

Extrathymic T-Cell Development

The events and processes described up to this point have focused on the thymus-dependent generation of $\alpha\beta$ and $\gamma\delta$ T cells. However, cells belonging to both lineage can be generated extrathymically. Mice bearing the nude (*nu*) mutation are unable to generate thymus-dependent subsets of T cells due to defects associated with the thymic epithelium, but contain low numbers of $CD8^+$ T cells which increases as the animal ages. Extrathymic $CD8^+$ cells can be distinguished from their thymus-processed counterparts by expression of CD8α-homodimers rather than CD8$\alpha\beta$-heterodimers and by the expression of a surface antigen encoded by the Ly-6 complex (126). A large portion of both extrathymically derived $\alpha\beta$ and $\gamma\delta$ cells are associated with the gut and there is some evidence that the intestinal epithelium is the site where these subsets develop (209). Relatively little is known about the processes that control extrathymic T-cell development and whether T cells developing extrathymically are subject to any of the processes that control development of T cells in the thymus. Recent findings have shown that T-lineage cells developing in the intestinal epithelium are subject to negative selection (210). In thymectomized radiation

chimeras reconstituted with fetal liver, T cells expressing potentially autoreactive TcRs were eliminated from the resulting donor T-cell population. It is still not clear if positive selection plays a role in shaping the extrathymic T-cell repertoire.

Concluding Remarks

In conclusion, all the information presented here indicates that the generation of functionally responsive T cells is highly dependent on a series of highly complex processes. On a descriptive level, much is known about the phenotypic states through which developing T-lineage cells progress while in the thymus. However, until recently, little was known about the molecular nature of interactions involved in specific transitions as hallmarked by specific phenotypic changes. The application of transgenic and targeted gene disruption techniques has provided a wealth of information, providing details of several crucial transitions on a molecular level. Even so, much work remains to produce a complete portrait of T-cell development, as defined by both cellular and molecular interactions.

Finally, based on data generated within the past two years, many of the cell surface and intracellular signaling components involved in mature T-cell activation are also utilized in pathways regulating specific transitions as immature T cells progress from one developmental stage to another. As in the development of any cell lineage, "naive" immature cells must acquire the capacity to execute specific genetic programs characteristic of the mature phenotype. With regard to T-cell development, one of these programs is the induction of IL-2 gene expresion. As shown *in vitro*, induction of IL-2 gene expression is a property exhibited by both immature TcR^- and mature SP thymocytes (211), whereas cells in the immediate transition from the TN to DP stage as well as the majority of thymocytes represented by the DP/cortical population are refractory to signals that would otherwise be sufficient to induce IL-2 gene expression in competent cells. The inability of cortical thymocytes to express the IL-2 gene is manifested on a molecular level, since numerous transcription factors essential for transcriptional initiation of IL-2 gene expression are apparently missing in DP cells (72). Thus, the ability to induce IL-2 gene expression must be

"reacquired" as thymocytes progress from a functionally incompetent state to a mature/functionally competent one. This reacquisition process is not well understood. However, if IL-2 is indeed expressed in the thymus in regions where developing T cells reside, this suggests that the thymic microenvironment is able to induce proper signaling pathways in immature thymocytes resulting in IL-2 gene expression. Likewise, the induction of IL-2 expression in developing thymocytes may be indicative of a commitment to function and/or of function reacquisition. Viewed in this light, tantalizing possibilities are raised with regard to whether IL-2 gene expression is induced during any of the developmental transitions just described. Hence, identification of IL-2 expressing cells in the thymus and characterization of interactions by which IL-2 expression is controlled is of particular interest. This topic is the subject of Chapter 5.

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CHAPTER 1

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Influence of activating stimulus on functional phenotype: Interleukin 2 mRNA accumulation differentially induced by ionophore and receptor ligands in subsets of murine T cells

(*in situ* hybridization/RNA probe protection/CD4, CD8/anti-CD3)

KATHLEEN L. MCGUIRE*, JULIA A. YANG, AND ELLEN V. ROTHENBERG

Division of Biology, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **We have investigated the linkage between CD4/CD8 phenotype and programing for specific responses in primary T-cell populations. *In situ* hybridization has been used to determine the frequency of cells competent to express the interleukin 2 (IL-2) gene after short-term stimulation with various polyclonal activators. The effects of the T-cell receptor ligands Con A and anti-CD3 monoclonal antibody were compared with those of a calcium ionophore that bypasses membrane receptors altogether. Induction with a calcium ionophore and phorbol ester revealed that potential IL-2 producers not only constitute >85% of the cells with a $CD4^+$ "helper/inducer" phenotype but also constitute over half of the cells with a $CD8^+$ "killer/suppressor" phenotype. There is no defect in the ability of these $CD8^+$ cells to accumulate IL-2 transcripts under these conditions. By contrast, in response to phorbol ester and either Con A or anti-CD3, the $CD8^+$ cells show an abortive IL-2 production response with rapid disappearance of IL-2 mRNA. This results in substantially lower yields of IL-2 per cell than is made by $CD4^+$ cells in response to the same stimuli. The extent to which these populations appear to have diverged in function thus depends on the stimulus used to trigger the response. The results suggest that differences in signal transduction or posttranscriptional regulatory mechanisms, rather than effector gene inducibility *per se*, may initially underlie the commitment of $CD4^+$ and $CD8^+$ cells to distinct functional roles.**

Mature T cells are functionally specialized in their responses to recognition of antigen. In general, T-cell lines that express the CD4 cell-surface glycoprotein are "helper" or "amplifier" cells that respond to the recognition of antigen by secreting lymphokines, often including the major T-cell growth factor interleukin 2 (IL-2) (1, 2). Cells that express CD8 include most killer T cells and show little helper activity (3–6). This suggests that the constitutive CD4/CD8 phenotypes of T cells are correlated with the inducibility of different, limited sets of functional response genes. Although the correlation is frequently observed in memory T cells and T-cell lines, it is not known how it is established during T-cell differentiation. When fresh $CD8^+$ cells are activated and cloned, ≤10% of the clones respond to stimulation by expression of IL-2 (5, 6). This would suggest an intrinsic difference between $CD8^+$ cells and $CD4^+$ cells in programing for gene expression. However, in a variety of other studies, $CD8^+$ cells appear to make IL-2 as well as $CD4^+$ cells (7–11). In the work reported here, we used *in situ* hybridization and quantitative probe protection analyses to measure the accumulation of IL-2 mRNA in individual splenic T cells responding for the first time to different stimuli. We demonstrate that the majority of $CD4^+$ and ≈50% of $CD8^+$ splenocytes are competent to accumulate similarly high levels of IL-2 mRNA when treated with calcium ionophore and phorbol ester. However, the two cell types differ markedly in their kinetics of message accumulation when stimulated by means of the T-cell receptor with concanavalin A (Con A) or an anti-CD3 monoclonal antibody (mAb). The results suggest that primary $CD4^+$ and $CD8^+$ cells differ not so much in their intrinsic abilities to initiate IL-2 transcription but by a mechanism that limits IL-2 message accumulation in the $CD8^+$ cells following stimulation by means of the T-cell receptor.

MATERIALS AND METHODS

Animals. C57BL/6 or C57BL/6-Tla^a mice were bred in our facilities and used at 6–8 weeks of age.

Cell Preparation and Culture. Cell suspensions were prepared from spleen, and $CD4^-$ and $CD8^-$ populations were isolated as described (12). Cultures were incubated with or without 0.1 μM A23187 (Sigma) and 17 nM phorbol 12-myristate 13-acetate (PMA). Where indicated, Con A (Pharmacia) at 6 μg/ml was used to replace A23187. The mAb against murine CD3, clone 145-2C11 (13), was kindly provided by Jeffrey Bluestone (University of Chicago, Chicago) via Linda Sherman (Research Institute of Scripps Clinic, La Jolla, CA) and was added to the cells as a 1:10 dilution of hybridoma supernatant. Note that our culture medium contains 10% fetal bovine serum and that under these conditions we (12) and others (14) find IL-2 RNA expression to be restricted to T cells. The amounts of IL-2 mRNA accumulated per cell in these experiments are higher than we previously reported, due, at least in part, to the use of a different lot of fetal calf serum, which supported higher levels of IL-2 production by all T-cell populations tested (data not shown). Enriched T-cell populations were obtained by two rounds of panning using affinity-purified goat anti-mouse IgG (Boehringer Mannheim) as described (15). In our hands, nylon wool purification resulted in less B-cell contamination but with significant and biased losses of T cells (data not shown).

***In Situ* Hybridization.** Fixed splenocytes were hybridized with the "antisense" IL-2 probe as described (12). Grain counts were obtained from at least 500 cells per slide. The number of cells above background with only one grain was extremely variable between experiments and has not been included in the "positive" cell calculation. To analyze the distribution of IL-2 RNA in responding cells, grain counts from ≥1-month exposures were used. Under these conditions, most uninduced cells still show no grains (12) and cells that fail to respond to induction account for most of the

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Abbreviations: IL-2, interleukin 2; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; nt, nucleotide.

*Present address: Dana–Farber Cancer Institute, Jimmy Fund Building, 44 Binney Street, Boston, MA 02115.

zero-grain cells in an induced population (n_i^0). The observed percentage of cells in each grain count category in the induced population, n_i, is the percentage of "true" responding cells, n_i(corr), with specific hybridization above background, plus the percentage of residual nonresponding cells, with all grains due to background. Nonresponding cells are assumed to show the same grain distribution as uninduced cells (n_u percent in the corresponding grain count category). Overall, they should constitute a fraction of the induced population, which, at most, is given by the ratio of the percentages of zero-grain cells in the induced and uninduced populations (n_i^0/n_u^0). Thus, for each grain count category, $n_i(\text{corr}) = n_i - n_u(n_i^0/n_u^0)$. Distributions of n_i(corr) are plotted in Fig. 2. For the percentage of positive cells given in Table 1 (column D), the sum of all n_i(corr) for grain count categories ≥ 2 was subjected to a further slight correction for potential nonspecific sticking of the probe to the induced cells, by subtracting the sum of n_i(corr) obtained after hybridization with a "sense" strand probe. The sense strand probe gave a higher background than the antisense probe (although <10% cells with ≤2 grains) but showed little difference between induced and uninduced samples. We have previously reported the *in situ* hybridization technique to detect levels as low as 10 molecules per cell in our hands (12).

Ribonuclease Probe Protection. Probe protection measurements of IL-2 RNA have been described in detail elsewhere (12). For quantitation, the 400-nucleotide (nt) *Hin*dIII–*Acc* I probe for the 3′ half of the IL-2 mRNA was used as described. The integrity of the RNA was confirmed by electrophoresis in a 50% urea/5% polyacrylamide gel. The average number of molecules per cell was determined by liquid scintillation spectrometry of protected probe retained on DE81 paper by the following equation: pg of RNA (i.e., pg of probe protected) × (1 pmol/1.2 × 10^5 pg) (i.e., molecular weight of probe = 400 nt × 300 pg/pmol of base) × (1/cell equivalents) × (1 pmol/10^{-12} mol) × (6.03 × 10^{23} molecules per mol). The sensitivity of this technique is <0.1 copy per cell in our hands (12), and no IL-2 mRNA is ever detected in uninduced samples. To map the 5′ end of the message as shown in Fig. 2, a 5′ probe was derived from a genomic 3.0-kilobase (kb) *Eco*RI–*Hin*cII fragment from the 5′ end of the IL-2 gene. This was cloned into pGEM-2, cleaved with *Acc* I, and transcribed to give the 220-nt antisense cRNA probe shown in Fig. 2. Riboprobe control template size markers (Promega Biotec, Madison, WI) were used to determine fragment sizes after electrophoresis. To determine the poly(A) sites used, the 0.8-kb genomic *Eco*RI fragment including the fourth exon of the IL-2 gene (16) was subcloned into pSP65 (T. J. Novak and E.V.R., unpublished results), digested with *Rsa* I, and transcribed with SP6 polymerase. The resulting 456-nt antisense transcript was used as a 3′ probe (data not shown).

RESULTS

We previously demonstrated that mature splenic T cells accumulate large amounts of IL-2 mRNA when stimulated for 24 hr in the presence of the calcium ionophore A23187 and PMA (12). The induction of IL-2 RNA expression in response to these stimuli is not influenced by the presence of accessory cells and is independent of antigenic stimulation (12, 17). Production of IL-2 mRNA is decreased by >95% when all $CD4^+$ and $CD8^+$ T cells are eliminated from the splenocyte population (12). Therefore, even with stimuli that do not require a functional T-cell receptor, IL-2 gene inducibility is a T-cell-specific response under these conditions. As shown in column D of Table 1, after 24 hr of treatment with A23187 and PMA, an average of 20% of surviving spleen cells contain cytoplasmic IL-2 mRNA detectable by *in situ* hybridization analysis. The population of T cells in these splenocyte samples was initially 25–30% and usually declined slightly during culture, suggesting that the large majority of surviving T cells in unseparated splenic cell preparations can activate the IL-2 gene under these stimulation conditions. This interpretation was supported by two additional experiments analyzing responses of enriched splenic T cells. A population with 47% Thy-1^+ cells (27% $CD4^+$, 16% $CD8^+$) at the start of culture yielded 37% with IL-2 RNA and a population with 78% Thy-1^+ cells (42% $CD4^+$, 27% $CD8^+$) responded to stimulation by IL-2 RNA expression in 47%. Quantitative RNase probe protection analysis demonstrated that splenic IL-2-producing cells accumulate, on average, about 650 IL-2 transcripts per positive cell.

To determine whether both $CD4^+$ and $CD8^+$ cells participated in this response, we used mAbs against CD8 and CD4 to mediate complement elimination of mature splenic T cells bearing these cell-surface markers. Over 90% of the susceptible population was eliminated when cells were subjected to two rounds of elimination with the appropriate antibody. This treatment routinely allows recovery of ≈85% of the expected number of total spleen cells in our hands. Additionally, after correcting for viability and total cell recovery, we calculate that >80% of the input spleen cells scoring as IL-2 producers could be recovered in one or the other eliminated population.

Table 1. IL-2 mRNA accumulation by splenic T cells

Spleen	A Viability after culture, % input	B % T cells in initial sample*	C % T cells after culture†	D % positive cells for IL-2 mRNA‡	E Average no. of IL-2 RNA molecules per viable cell§	F Average no. of IL-2 RNA molecules per positive cell¶	G IL-2, units/ml‖
Total	54 ± 4	26 ± 2	20–23	20 ± 4 (3)**	133 ± 33 (5)	665	830
$CD8^-$	61 ± 3	22 ± 0.5	13–15	12.6 ± 2 (3)	100 ± 28	794	670
$CD4^-$	61 ± 1	10.5 ± 0.5	10–13	6.8 ± 0.7	33 ± 3	485	290

All values are the average of two experiments with 24-hr stimulations by A23187 and PMA, except where noted in parentheses. Standard deviations are given.

*As determined by flow cytometry using the rat mAbs 3.155 (anti-CD8) and GK1.5 (anti-CD4) (18) and fluorescein-conjugated anti-rat κ-chain mAb MAR18.5 (Becton Dickinson) on an Ortho system 50 H cytofluorograph with a 5-W argon laser.

†Determined by staining with anti-Thy-1 antibody T24/31.7 (a gift of R. Hyman, Salk Institute, La Jolla, CA) using propidium iodide to exclude dead cells (R. J. Hill and E.V.R., unpublished results). CD4 and CD8 antigens modulate after stimulation.

‡Determined by *in situ* hybridization analysis from 4-month exposures.

§Determined by quantitative RNase probe protection analysis. Quantitation is done by using a ^{32}P-labeled cRNA transcript (12) identical to that used for *in situ* hybridization, which exhibits a lower background than seen in Fig. 1. Values given were derived from two or more independent RNA preparations as indicated.

¶Values in column E divided by those in column D.

‖Units of IL-2 per ml produced in 24 hr by 10^6 cells per ml of culture.

**Two previously reported *in situ* hybridization experiments yielded 17% and 24% of total spleen positive for IL-2 mRNA at 4 weeks exposure (12), which included all cells with ≥1 grain above background.

The levels of IL-2 RNA accumulated by the eliminated populations similarly account for 85% of the IL-2 RNA expressed in the unfractionated spleen cells. Therefore, complement treatment does not appear to interfere with the ability of the surviving cells to express the IL-2 gene.

Quantitative RNase protection analysis (Fig. 1 *A* and *B*) demonstrated that strikingly similar amounts of IL-2 RNA could be induced in eliminated splenocyte populations, whether the cells depleted were $CD8^+$ or $CD4^+$ (compare 4^- and 8^- in lanes marked A23187 + PMA). Table 1 (column E) shows that populations from which all $CD4^+$ cells had been removed accumulated only three times less IL-2 RNA than populations from which only $CD8^+$ cells were removed. As we have previously noted (12), removal of $CD4^+$ and $CD8^+$ cells resulted in >95% loss of inducible IL-2 RNA expression under these conditions. Assuming that B cells and nonlymphoid cells survive equally in both eliminated populations, this implies that a significant fraction of the IL-2 RNA inducible in total spleen is present in $CD8^+$ cells. Indeed, both induced populations included significant fractions of cells with IL-2 transcripts detectable by *in situ* hybridization (Table 1, column D). In at least five experiments with eliminated or unfractionated populations, we have noted that $CD8^+$ cells are slightly enriched and $CD4^+$ cells are slightly depleted following stimulation (Table 1, column C, and unpublished results). Hence, the 12.6% positive cells in the $CD8^-$ population presumably reflect responses from among the 13–15% $CD4^+$ cells remaining, and the 6.8% positive cells in the $CD4^-$ population were drawn from the 10–13% $CD8^+$ cells (Table 1, compare columns C and D). This suggests that at least 80% of $CD4^+$ cells and about 50% of $CD8^+$ cells can express IL-2 RNA, as discussed further below.

The accumulation of IL-2 RNA in response to A23187 and PMA was similar in individual $CD4^+$ and $CD8^+$ cells. As demonstrated by the representative *in situ* hybridization analysis shown in Fig. 2, the grain distributions for IL-2 producers within the $CD4^-$ and $CD8^-$ depleted populations were hardly distinguishable, except in the overall percentage of cells involved. This result agrees with calculations from the quantitative RNase protection analysis, which implied that $CD8^+$ IL-2 producers accumulate high levels of IL-2 RNA (≈500 molecules per cell), although somewhat lower, on average, than individual $CD4^+$ IL-2 producers (≈800 molecules per cell; Table 1, column F). As shown in column G of Table 1, IL-2 protein was secreted by all populations in relative amounts consistent with the amount of IL-2 RNA present at 24 hr (compare columns D and F). RNase mapping of the IL-2 RNA accumulated by these cells demonstrated that all three populations use identical 5′ start and 3′ poly(A) sites (Fig. 1 and data not shown), further indicating that the IL-2 RNA present is in the form of the mature, functional message. Taken together, these measurements indicate that about half of the surviving $CD8^+$ T-cell population can respond to A23187 and PMA by accumulation of *bona fide* IL-2 mRNA to levels comparable to those in $CD4^+$ cells.

To test further whether such a high fraction of $CD8^+$ cells is inducible, we have used two other sets of results to calculate the expected frequencies of potential IL-2 producers, x and y, in the $CD8^+$ and $CD4^+$ populations, respectively. One set of results is that for total spleen (Table 1) in which about 23% of surviving cells after stimulation were T cells. The relative survival rates of $CD4^+$ and $CD8^+$ cells (column C) imply that 10% were $CD8^+$ and 13% were $CD4^+$. The second set of results is from the analysis of enriched T cells described above, in which 42% of input cells were $CD4^+$

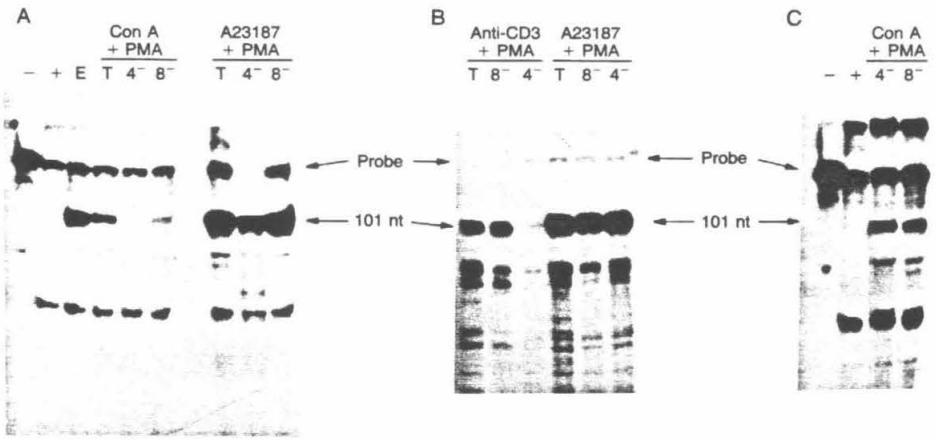

FIG. 1. Quantitation and mapping of 5′ ends of IL-2 mRNA accumulated by splenic T cells in response to various stimuli. (*A*) RNase protection of an *Acc* I–*Hin*cII cRNA probe spanning the IL-2 cap site, using cytoplasmic RNA isolated after 24 hr in culture with A23187 or Con A and PMA. The migration of the fragment protected by RNA with the reported IL-2 cap site is marked as 101 nt. − or +, Probe incubated with 20 μg of yeast tRNA and carried through the analysis as described below without (−) or with (+) RNase A and T1 treatment, respectively. E, 20 μg of RNA isolated from the thymic lymphoma EL4.E1, stimulated for 4 hr with 10 ng of PMA per ml, as a positive control. T, Total spleen; 4^-, $CD4^-$ spleen; 8^-, $CD8^-$ spleen. For all samples stimulated with A23187, 5×10^6 cell equivalents (16–21 μg of RNA) were used, and for the total, $CD4^-$, and $CD8^-$ Con A-stimulated samples, 10^7, 1.3×10^7, and 9×10^6 cell equivalents (20–28 μg of RNA) were used, respectively. The exposure time shown was 2 days. IL-2 RNA levels in these Con A-stimulated samples were measured to be 6 molecules per cell for total spleen, 2 molecules per cell for $CD8^-$ spleen, and 0.2 molecule per cell for $CD4^-$ spleen. (*B*) Total, $CD4^-$, and $CD8^-$ splenocytes were stimulated for 24 hr with PMA and either A23187 or a 1:10 dilution of anti-CD3 hybridoma supernatant, and 1×10^7 cell equivalents of cytoplasmic RNA from each was used to protect the IL-2 probe. The exposure time was 20 hr. In these samples, the 5′ probe was used for quantitation. The levels of IL-2 RNA per cell were calculated to be 207, 173, and 206 molecules per cell for the total, $CD8^-$, and $CD4^-$ cells stimulated with A23187 and PMA, respectively, and 26, 23, and 1 molecule per cell for total, $CD8^-$, and $CD4^-$ cells stimulated with anti-CD3 and PMA, respectively. (*C*) RNase protection as in *A*, using 2×10^7 cell equivalents (30–44 μg) of cytoplasmic RNA isolated from cells stimulated for 6 hr. This film was exposed for 6 days. The levels of IL-2 RNA quantitated in these samples were 10 molecules per cell for total spleen, 5 molecules per cell for $CD8^-$ spleen, and 3 molecules per cell for $CD4^-$ spleen.

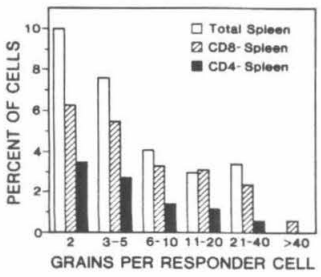

FIG. 2. Distribution of IL-2 mRNA accumulated in different classes of splenic IL-2 producers in response to A23187 and PMA. Total, CD4-eliminated, and CD8-eliminated spleen cells were cultured with or without inducers and probed for IL-2 RNA by *in situ* hybridization. The histograms plot the corrected percentages of cells in induced populations [n_i(corr)] that exhibited the indicated numbers of grains in a representative experiment. Higher grain numbers are associated with higher numbers of transcripts per cell (12). The grain counts for each population, induced and uninduced, were determined from a 4-week exposure (29 days) of a representative experiment, and the corrected histograms were derived by subtracting background values. The similarity between the distributions of IL-2 RNA among producing cells in all three populations was observed in three experiments.

and 27% were $CD8^+$. Here, about 90% of the input cells were viable 24 hr after stimulation, suggesting only minor population changes. At most, if all dying cells were $CD4^+$, the stimulated cells were 35% $CD4^+$ and 30% $CD8^+$. These values can be used to solve for x and y with two independent equations. (*i*) The fraction of cells positive by *in situ* hybridization is

$$\text{Fraction inducible} = (CD4^+ \text{ poststimulation})y + (CD8^+ \text{ poststimulation})x. \quad [1]$$

(*ii*) As shown in Table 1, column E, CD4-eliminated spleen cells make one-third as much IL-2 RNA as CD8-eliminated spleen cells (i.e., 33 ± 3 vs. 100 ± 28). Then, if an average $CD8^+$ responder has 500 copies of IL-2 RNA and an average $CD4^+$ responder has 800 copies, it follows that ($CD8^+$ poststimulation)(500x) = 1/3($CD4^+$ poststimulation)(800y), or

$$(CD8^+ \text{ poststimulation})x = 8/15\ (CD4^+ \text{ poststimulation})y. \quad [2]$$

In the total spleen analysis, the average fraction inducible is 0.2 (Table 1, column D). By using our estimates for $CD4^+$ and $CD8^+$ subsets after stimulation, $y \approx 1.0$ and $x = 0.7$, with almost all $CD4^+$ cells and over half of $CD8^+$ cells responding. Similarly, in the experiment with enriched T cells, 0.47 of the cells could express IL-2 RNA. Depending upon whether $CD4^+$ cells are assumed to have died preferentially, values for y range from 0.73 to 0.9 and those for x range from 0.54 to 0.61. These are in good agreement with the previous values and those estimated from CD4- or CD8-eliminated populations (Table 1). Altogether, these calculations indicate the consistency of the *in situ* hybridization analyses and support the competence of at least half of splenic $CD8^+$ cells to make IL-2.

To test whether the mode of triggering influenced responsiveness, we replaced A23187 with either of two polyclonal stimuli that could mimic more physiologic triggering by binding to the T-cell receptor—i.e., the lectin Con A or a mAb against the ε subunit of CD3. In all cases, PMA was added to obviate the requirement for accessory cells. RNase probe protection analysis demonstrated that 24 hr of culture with 6 μg of Con A per ml stimulated spleen cells to express IL-2 mRNA, although at lower levels (by a factor of ≈10–20) than in cultures induced with A23187 (Fig. 1 legend). Consistent with the levels of RNA detected, Con A-stimulated cultures also secrete, on average, fewer units of IL-2 activity per ml (by a factor of 10–30) than A23187-stimulated cultures (data not shown). However, the effects of Con A on $CD4^+$ and $CD8^+$ cells were not the same. As shown in Fig. 1*A*, probe protection analysis revealed that $CD4^-$ splenocytes expressed only about 1/10th as much IL-2 mRNA as $CD8^-$ cells after 24 hr of stimulation with Con A and PMA. They correspondingly secreted lower titers of IL-2 (by a factor of 8–10) than $CD8^-$ splenocytes during the incubation (not shown). The poor IL-2 response of $CD8^+$ cells did not result from an overall inability to respond to Con A and PMA, for $CD8^+$ cells were fully activated to express IL-2 receptors (J.A.Y., data not shown). As shown in Fig. 1*B*, $CD8^+$ cells also expressed less IL-2 mRNA (by a factor of at least 10) than $CD4^+$ cells when stimulated for 24 hr with anti-CD3 and PMA. Thus, although many or most $CD8^+$ cells can accumulate IL-2 mRNA similarly to $CD4^+$ cells when stimulated with a calcium ionophore, they do not appear to do so when stimulated by Con A or by anti-CD3.

The differences in overall accumulation of IL-2 protein are consistent with a prolonged disparity between the levels of translatable IL-2 mRNA in $CD4^+$ and $CD8^+$ cells, but they do not rule out a brief response in the $CD8^+$ cells. This was of concern because time course analysis of IL-2 RNA accumulation in total spleen revealed that the kinetics of the accumulation were different in response to Con A and PMA than for A23187 and PMA (Fig. 3). In response to Con A, an initial peak was seen at 6 hr of stimulation, then a decline and a second peak at 24 hr, instead of the continued increase through 24 hr of stimulation induced by A23187. This is consistent with previous reports of a peak in IL-2 mRNA expression ≈5 hr after induction with mitogen (9, 10, 19). To analyze the initial activation process, we compared the levels of IL-2 mRNA in splenic subpopulations after only 6 hr of stimulation with Con A (Fig. 1*C*). In three experiments with either Con A or anti-CD3 stimulation, we found that $CD8^+$ cells contribute at least half as much accumulated IL-2 mRNA as $CD4^+$ cells at this time point. This result suggests

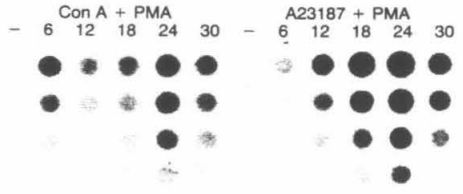

FIG. 3. Time course of IL-2 mRNA production by total spleen cells in response to different stimuli. Cell cultures consisting of 4×10^7 cells supplemented with Con A and PMA and 2×10^7 cells supplemented with A23187 and PMA were incubated for the indicated times (given in hours). One-half of the resulting cytoplasmic RNA isolated from each culture was blotted in descending 2-fold serial dilutions as described (12). Viable cell equivalents ranged from 13 to 20×10^6 cells for Con A and 6.5 to 10×10^6 cells for A23187, with the lowest numbers of viable cells in the 6-hr Con A and 24-hr A23187 cultures and the highest numbers in the uninduced controls. The autoradiograph shown is an overnight exposure of the filter hybridized with an IL-2 cDNA probe.

that $CD8^+$ T cells may initially activate the IL-2 gene when stimulated by means of the T-cell receptor for antigen, but that under these conditions, $CD8^+$ and $CD4^+$ cells differentially regulate subsequent IL-2 mRNA accumulation.

DISCUSSION

We have used *in situ* hybridization to measure the competence of individual splenocytes to express the IL-2 gene prior to any antigen-driven clonal expansion. This approach has revealed a strikingly broad phenotypic distribution of cells with intrinsic competence to make IL-2, for many or most $CD8^+$ cells as well as $CD4^+$ cells are fully competent under certain stimulation conditions. A difference between $CD4^+$ and $CD8^+$ IL-2 producers emerged, however, in their responses to different polyclonal activators. Though the calcium ionophore induced sustained IL-2 RNA accumulation in both, the membrane receptor ligands Con A and anti-CD3 provoked a limited IL-2 production response in the $CD8^+$ cells as compared to the $CD4^+$ cells. Thus, many $CD8^+$ splenocytes appear to differ from $CD4^+$ cells not so much in their potential to express the IL-2 gene as in their interpretations of particular activation stimuli.

Previous work (9–11, 20, 21), using bulk culture and limiting dilution analysis, has established that some $CD8^+$ cells have the ability to secrete IL-2, but it did not reveal the prevalence of potential IL-2 producers in the $CD8^+$ population that we observe upon stimulation with calcium ionophore and PMA. Several methodological differences may explain our findings. We have looked within the first 24 hr of stimulation, which may be important for detecting IL-2 production by $CD8^+$ cells (cf. refs. 20 and 21). In particular, *in situ* hybridization analysis allows us to look at the primary T-cell response prior to clonal expansion. Recent studies using the Pgp-1 marker to identify memory cells (22) support the interpretation that most (70%) of our splenic $CD8^+$ cells are indeed virgin T cells. By contrast, A23187 and PMA do not induce IL-2 RNA in many long-term killer cell lines (T. J. Novak and E.V.R., unpublished data), suggesting either that $CD8^+$ cells may lose this capacity after exposure to antigen (20, 21), or that those that proliferate extensively *in vitro* come primarily from the noninducible fraction. In any case, the inducibility of IL-2 in about half of the $CD8^+$ cells after stimulation with calcium ionophore and phorbol ester clearly shows that in virgin $CD8^+$ cells the IL-2 gene need not be locked in an inactive chromatin configuration. The gene itself may always be competent to be expressed in mature primary T cells.

Although there appears to be little difference between $CD4^+$ and $CD8^+$ cells in the potential inducibility of their IL-2 genes by A23187 and PMA, there is a clear difference in their abilities to accumulate IL-2 mRNA following prolonged stimulation with PMA and either Con A or mAb directed against CD3. Similarly, in response to antigen, IL-2 production is apparently limited to certain responding T cells dominated by, though not restricted to, cells of the $CD4^+$ phenotype (4–6). Our results from the 6-hr stimulation with T-cell receptor ligands, as well as previous reports in the literature (9, 10), support the hypothesis that transcription may initially be comparable in the two cell types but that RNA accumulation is limited at a later step in $CD8^+$ cells. Such a mechanism might affect the accumulation of IL-2 transcripts in individual cells or decrease the frequency of responding cells. Several laboratories have reported that cycloheximide, which blocks protein elongation, enhances the lectin-induced accumulation of IL-2 mRNA (9, 10, 23) without affecting the transcriptional activity of the gene (24). These data suggest that a protein product may be responsible for limiting IL-2 mRNA accumulation after stimulation with lectin. $CD4^+$ and $CD8^+$ T cells might differentially express this regulatory protein product, with its expression being particularly prominent in $CD8^+$ lymphocytes. The accumulation of high levels of IL-2 mRNA in both subpopulations of mature T cells and the kinetics of that response suggest that ionophore and phorbol ester may bypass this proposed regulatory mechanism altogether. Further work is required to test this possibility directly. However, the results presented here persuasively suggest that the difference between cells committed to "IL-2-producing" and "non-IL-2-producing" lineages may not be attributed either to the chromatin structure of the IL-2 gene or to the initial availability of specific transcription factors for IL-2. Particularly in these primary T-cell populations, signal transduction and/or RNA stabilization mechanisms may play a vital role in regulating a surprisingly plastic set of functional responses.

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CHAPTER 2

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Developmental and Anatomical Patterns of IL-2 Gene Expression *In Vivo* in the Murine Thymus

JULIA A. YANG-SNYDER and ELLEN V. ROTHENBERG*

Division of Biology, 156-29, California Institute of Technology, Pasadena, California 91125

Interleukin-2 (IL-2) is a potent growth factor that mature T lymphocytes synthesize and use as a proliferation signal. Much controversy has arisen concerning whether it is used to drive the extensive proliferation of immature pre-T cells in the thymus. Immature thymocytes acquire the competence to express IL-2 at an early stage, but it has remained uncertain whether they are activated to exercise this competence *in vivo*. Therefore, we have used *in situ* hybridization and immunohistochemistry on serial sections obtained from fetal and adult thymuses of normal C57BL/6 mice and of mice bearing the *scid* defect to determine where, when, and whether IL-2 is expressed *in vivo*. Our results show a striking spatial and temporal pattern of IL-2 expression in the normal fetal thymus. We detected a burst of IL-2 mRNA accumulation at day 14.5 of gestation, which rapidly decreased by day 15. At day 15, we observed maximal IL-2 protein production that subsequently decreased by day 16 of gestation. Both *in situ* hybridization and immunohistochemical staining revealed an unexpectedly strict localization of IL-2 expressing cells to patches around the periphery of the fetal thymus, creating a previously unrecognized compartment of high IL-2 protein content. IL-2 production in the day-15 fetal thymus appeared to be unaffected by the *scid* mutation, indicating that this response is likely to be T-cell receptor (TcR)-independent. Several features distinguish the IL-2 induction pattern in the adult thymus from that in the fetal thymus. In the normal adult thymus, IL-2-expressing cells are extremely rare (found at a frequency of 10^{-7}), but they are reproducibly detectable as isolated cells in the outer cortex and subcapsular region of the thymus. Unlike the fetal thymic IL-2 producers, the IL-2 producers in the adult thymus are completely eliminated in mice homozygous for the *scid* mutation. This suggests that the IL-2-expressing cells in the normal adult thymus are of a more mature phenotype than the immature, TcR-negative cells that accumulate in the *scid* adult thymus. Thus, our work demonstrates that two developmentally distinct types of cell interactions induce IL-2 expression *in vivo:* one, a broadly localized interaction in day 14–15 fetal thymus that is unaffected by the *scid* mutation; the other, a rare event that occurs asynchronously from late fetal through adult life, but which is completely eliminated by the *scid* defect. These results imply that significant differences exist between the physiological processing of thymocytes in the fetal and postnatal thymic microenvironments.

KEYWORDS: Interleukin-2 (IL-2), interleukin-2 receptor (IL-2R), thymus, *in situ* hybridization, immunohistochemistry, severe combined immunodeficiency *(scid)*, T-cell receptor (TcR).

INTRODUCTION

T cells, unlike other cell types of the hematopoietic lineage, differentiate in the thymus, where differentiation is accompanied by extensive proliferation, apparently triggered by mitotic signals from the thymic stroma. Thymus-specific lymphostromal interactions are critical for driving T-cell development, yet both the nature of the signals and the molecular basis of their impact remain unknown. Among the

*Corresponding author.

properties acquired and modulated as thymocytes develop is the competence to express "response" genes that drive proliferation in mature T cells, such as the gene encoding the potent growth factor interleukin-2 (IL-2) (McGuire and Rothenberg, 1987; Howe and MacDonald, 1988; Rothenberg et al., 1988; Tentori et al., 1988b; Fischer et al., 1991; Chen and Rothenberg, 1993). Although the inducibility of these genes at certain stages of differentiation is easily demonstrable under artificial conditions *in vitro*, it is not known whether intrathymic signals provide inductive stimuli for these genes *in vivo*. Thus, it is unclear to what extent growth factors such as IL-2 are normally available to contribute to the hormonal microenvironment of the thymus. Similarly, it has remained uncertain whether the characteristic signaling pathways that activate IL-2 expression *in vitro* are utilized in normal thymocyte development *in vivo*.

Recent gene-disruption experiments have formally proven that IL-2 is not required for the generation of normal cell populations in the thymus (Schorle et al., 1991). However, severe perturbations can be induced in thymocyte development, *in vivo* or in organ culture, by anti-IL-2R antibody treatment (Jenkinson et al., 1987; Tentori et al., 1988a; Zuñiga-Pflücker and Kruisbeek, 1990; Zuñiga-Pflücker et al., 1990), by the addition of excess IL-2 (Skinner et al., 1987; Plum and de Smedt, 1988; Waanders and Boyd, 1990), or by the introduction of a transgenic IL-2Rα chain with inappropriate species specificity (Kroemer et al., 1991). Thus, the possibility remains that IL-2 production *in vivo* plays some developmental role. Furthermore, studies from our own laboratory and from others have shown that the intrinsic competence to express the IL-2 gene is shared by both mature and immature thymocytes (Howe and MacDonald, 1988; Rothenberg et al., 1990; Fischer et al., 1991). However, it is unknown whether the thymic microenvironment can actually provide signals to induce IL-2 expression in either or both populations of thymocytes. Therefore, IL-2 production (1) could potentially be induced at any of several stages *in vivo* and (2) might cause autocrine or paracrine effects, either early or late in T-cell development. These issues make it instructive to determine the conditions under which developing thymocytes actually exercise their competence to express IL-2 *in vivo*.

Several laboratories have analyzed cytokine expression during fetal thymocyte development *in vivo* as an approach to determine which factors might be present in the thymus during T-cell development. Von Boehmer and coworkers initially reported that IL-2 is secreted as fetal thymocytes mature in organ culture (Kisielow et al., 1985). Subsequent studies have shown that murine embryonic thymocytes harvested at different times during gestation express a variety of cytokine mRNAs, using either *in situ* hybridization to cytocentrifuged preparations of freshly isolated cells (Carding et al., 1989, 1990; Zuñiga-Pflücker et al., 1990) or reverse transcription/polymerase chain reaction (rtPCR) analysis of total RNA samples obtained from fetal thymuses (Montgomery and Dallman, 1991). Most of these studies have focused exclusively on dissociated cell suspensions, making localization and identification of the IL-2-expressing cells within the thymus impossible. On a more general level, these studies have failed to distinguish between the developmental events involved in the fetal ontogeny of the thymus itself and those involved in the maturation of T-cell precursors processed therein. In adult animals, thymocyte development generates a different spectrum of T-cell subtypes than that generated during thymocyte development in the fetus (reviewed in Rothenberg, 1992). This could be attributable either to differences in the thymic environment or to the difference in hematopoietic origins between the precursors that seed the fetal and the postnatal thymus (Ikuta et al., 1990). Thus, it is not clear whether IL-2 production is a universal feature of T-cell development or a peculiarity of the cell types present in the fetal thymus.

In order to examine the spatial and temporal pattern of IL-2 gene expression as induced naturally during T-cell ontogeny *in vivo*, we have used a combination of *in situ* hybridization and immunohistochemical staining of mouse thymus sections isolated at different stages of development. Our findings show different and distinctive patterns of IL-2 gene expression in fetal versus adult thymuses. Additionally, IL-2 gene expression appears to be normal in the fetal thymus but perturbed in the adult thymus of animals that cannot generate mature T cells as a result of the severe combined immunodefficiency *(scid)* mutation.

RESULTS

Developmental Pattern of IL-2 Gene Expression in the Fetal Thymus as Determined by *In Situ* Hybridization

Figure 1 shows the patterns of IL-2-specific hybridization in the murine fetal thymus at different stages of gestation as defined using 590 nt antisense or sense riboprobes. At day 14, a few highly positive cells were found, mainly in the outer region of the thymus (Fig. 1A). At day 14.5, the outer region of the thymus contained a dramatically increased number of highly positive cells, clustered in distinctive, large patches of hybridization one cell deep that "outlined" the periphery of the tissue (Fig. 1C). At this stage of gestation, no corticomedullary boundary was observed in the thymus; cells appeared equally distributed throughout the organ (Figs. 1C and 1D). Although the majority of sections of day 14.5 fetal thymus did not include any regions of strong hybridization, every thymus examined contained sections exhibiting this striking localization of highly positive cells. By day 15, the percentage of IL-2 mRNA positive cells decreased, and only an occasional highly positive cell was found (6–8 per half lobe equivalent; Fig. 1E). Isolated positive cells were also observed later, at day 18 of gestation, and distinct cortical and medullary regions were identifiable by this time by DAPI staining (data not shown). The remaining IL-2 mRNA positive cells in the fetal thymus at later stages of gestation were still restricted to the cortex and not to the medulla. Thus, highly positive IL-2 mRNA-expressing cells were present at day 14 of gestation in the fetal thymus and some highly positive cells persisted through day 18 of gestation. However, a dramatic peak of highly localized IL-2 gene expression was observed at day 14.5, which decreased abruptly by day 15.

Pattern of IL-2 Expression in the Fetal Thymus at Different Stages of Development by Immunohistochemistry

To confirm the pattern of IL-2 expression as determined from the *in situ* hybridization data, fetal thymuses isolated at various stages of gestation were also stained with an anti-IL-2 mAb (Fig. 2). At day 14–14.5, weak immunoreactivity was observed (Fig. 2A; data not shown). At day 15, strong staining appeared in the outer region of the tissue (Fig. 2C). This staining pattern was not seen when normal rat IgG was substituted for the anti-IL-2 mAb (Fig. 2D); the IL-2 specificity of the staining reagent was further confirmed by control experiments with excess rmuIL-2 (see below). At day 16, immunoreactivity was still present around the periphery of the thymus, albeit at a reduced level (Fig. 2E), and at day 18, only isolated immunoreactive cells were found in the cortical region. A background of endogenous peroxidase-positive cells became detectable by day 18 (data not shown), but these cells could be distinguished from IL-2 immunoreactive cells by prior blocking and peroxidase quenching (for additional controls, see below). Nonetheless, based on these characteristics, the pattern of IL-2 protein expression, detected by immunohistochemistry, recapitulated the pattern of IL-2 mRNA accumulation as determined by *in situ* hybridization. Additionally, maximum IL-2 protein expression was observed 12 hr following maximum IL-2 mRNA accumulation.

Vγ3 and CD16 Expression versus IL-2 Expression in Day 15 Fetal Thymus as Determined by Immunohistochemistry

IL-2 has been implicated in the growth of V$\gamma3^+$ T cells and natural killer (NK) cells from the fetal thymus *in vitro* (Skinner et al., 1987; LeClerq et al., 1990). Therefore, we were interested in determining whether intrathymic IL-2 was associated with the generation or expansion of particular subsets of T cells or NK cells in the fetal thymus *in vivo*. By day 15 of gestation, almost all of the rare thymocytes expressing surface TcR specifically utilize the Vγ3 segment in TcR gene rearrangements (Havran and Allison, 1988). Likewise, the majority of cells in the fetal thymus at day 15 of gestation express CD16 (FcRII/III; Rodewald et al., 1992), a marker that persists preferentially on pre-NK and NK cells. We used a Vγ3-specific (536) or CD16-specific (2.4G2) mAb along with the IL-2-specific mAb for immunohistochemical staining of adjacent serial sections of day 15 fetal thymus to determine the relative location of V$\gamma3^+$ and CD16$^+$ cells with respect to IL-2-producing cells (Fig. 3). At this stage of gestation, the majority of the cells in the fetal thymus were cytoplasmic CD3ε^+ (data not

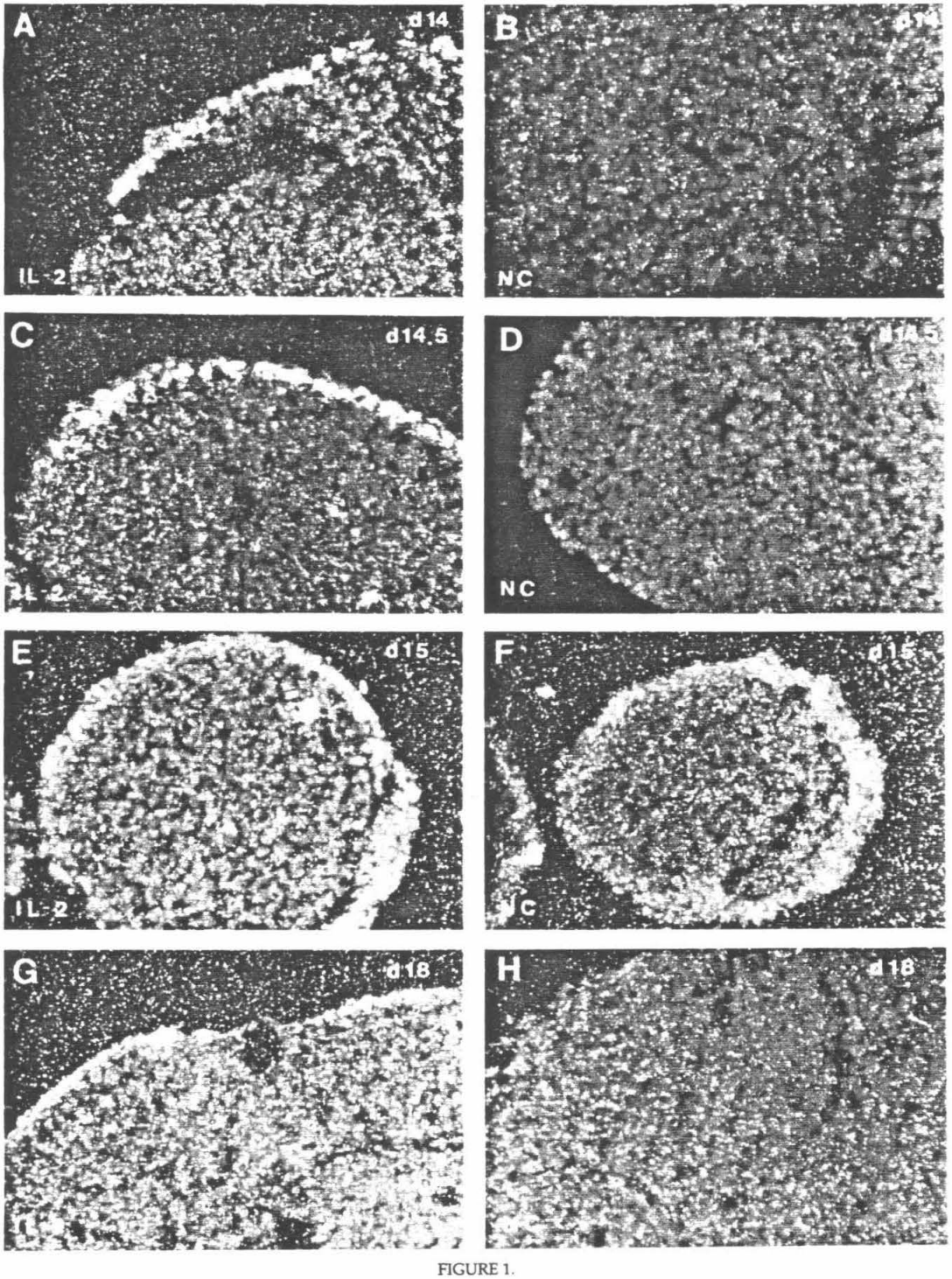


FIGURE 1.

shown). CD16$^+$ cells were detected in a heterogeneous distribution throughout the fetal thymus. Vγ3$^+$ cells were also detectable in a similar distribution (Fig. 3), but were substantially less abundant than CD16$^+$ cells. Neither cell type was concentrated in the peripheral region of the lobe. Thus, these results gave no clear evidence for colocalization of IL-2-producing cells with either Vγ3 or CD16 expression (see Discussion).

IL-2 Expression in the *Scid* Day 15 Fetal Thymus

Mice homozygous for the *scid* mutation fail to rearrange their TcR genes normally, causing a developmental arrest that blocks the production of all classes of TcR$^+$ thymocytes (Bosma and Carroll, 1991). In order to determine whether the *scid* defect affected IL-2 expression in the fetal thymus, we used immunohistochemistry to detect IL-2-producing cells in the *scid* day 15 fetal thymus (Figs. 2I to 2L). *Scid* day 15 fetal thymuses appeared normal in terms of the intensity and localization of IL-2 immunoreactivity (Fig. 2I). This staining was also shown to be IL-2-specific. When an excess of exogenous recombinant IL-2 was added, IL-2 immunoreactivity was abolished (Figs. 2K and 2L); as a control, addition of recombinant IL-4 had little effect (data not shown). Likewise, the gross architecture and size of the *scid* day 15 fetal thymus appeared to be normal in terms of cell density and number (data not shown). *In situ* hybridization of *scid* day 15 fetal thymus sections revealed that a similar number of highly labeled IL-2 mRNA positive cells were present as compared to the normal counterpart (data not shown). Hence, at this stage of gestation, TcR rearrangement and expression are not prerequisites for IL-2 expression *in vivo*.

Analysis of IL-2Rα expression confirmed the lack of gross perturbation of thymocyte development in *scid* mice at this age. When sections were stained with a rat anti-mouse IL-2Rα mAb, normal and *scid* day 15 fetal thymuses were similar with regard to the number and distribution of IL-2Rα^+ cells in the thymus (Figs. 4A and 4B). By contrast, differences were readily apparent when comparing the IL-2Rα staining pattern of the normal versus *scid* adult thymus. The overwhelming majority of the cells in all regions of the *scid* adult thymus are IL-2Rα^+, as compared with the irregular clusters of IL-2Rα^+ cells in the cortex of the normal tissue (Figs. 4C and 4D). This aberrant IL-2Rα staining pattern of the *scid* adult thymus convincingly showed the developmental arrest-blocking progression beyond the IL-2Rα^+ immature stage. These staining results indicated that the *scid* mutation did not severely affect the control of IL-2 or IL-2Rα expression in the fetal thymus, in spite of its severe developmental effects observed in the adult thymus.

In Situ Hybridization of Normal Adult Thymus Sections

In previous analyses of cytocentrifuged preparations of adult thymus cell suspensions, we had failed to detect any cells expressing IL-2 mRNA (McGuire and Rothenberg, 1987; Rothenberg et al., 1990). Ribonuclease protection assays failed to detect any IL-2 transcripts in samples of up to 100 μg of adult thymus total RNA, roughly equivalent to the amount of RNA isolated from half a thymic lobe (400 μg total RNA/thymus; data not shown). However, the results of four independent experiments confirmed the presence of extremely rare, highly labeled IL-2 mRNA-positive cells in the thymus of 4-week-old mice. Approximately 160 sections hybridized alternately with each strand of the 430-nt probe were analyzed per experiment (approximately 320 sections examined overall, corresponding to a thymus lobe; i.e., a half lobe equivalent for each probe). Highly labeled cells were found only in sections that had been hybridized with the antisense riboprobe. These IL-2-positive cells were reproducibly found in the outer cortex and subcapsular region of the thymus (Fig. 5A); the cortical versus medullary regions of the thymus were distinguished by higher or lower cell density within the section, as shown by DAPI staining (Figs. 4A and 4B). Surprisingly, of all the IL-2-

FIGURE 1. (See Colour Plate VI at the back of this publication). *In situ* hybridization to sections of normal thymuses from days 14 to 18 of gestation for IL-2 expression, using either a ^{35}S-labeled, 590-nt IL-2 antisense RNA probe (IL-2; Figs. 1A, 1C, 1E, 1G) or the complementary sense strand (NC; Figs. 1B, 1D, 1F, 1H). Samples were counterstained with DAPI and observed under dark field microscopy. 14-day exposure; magnification ×200.

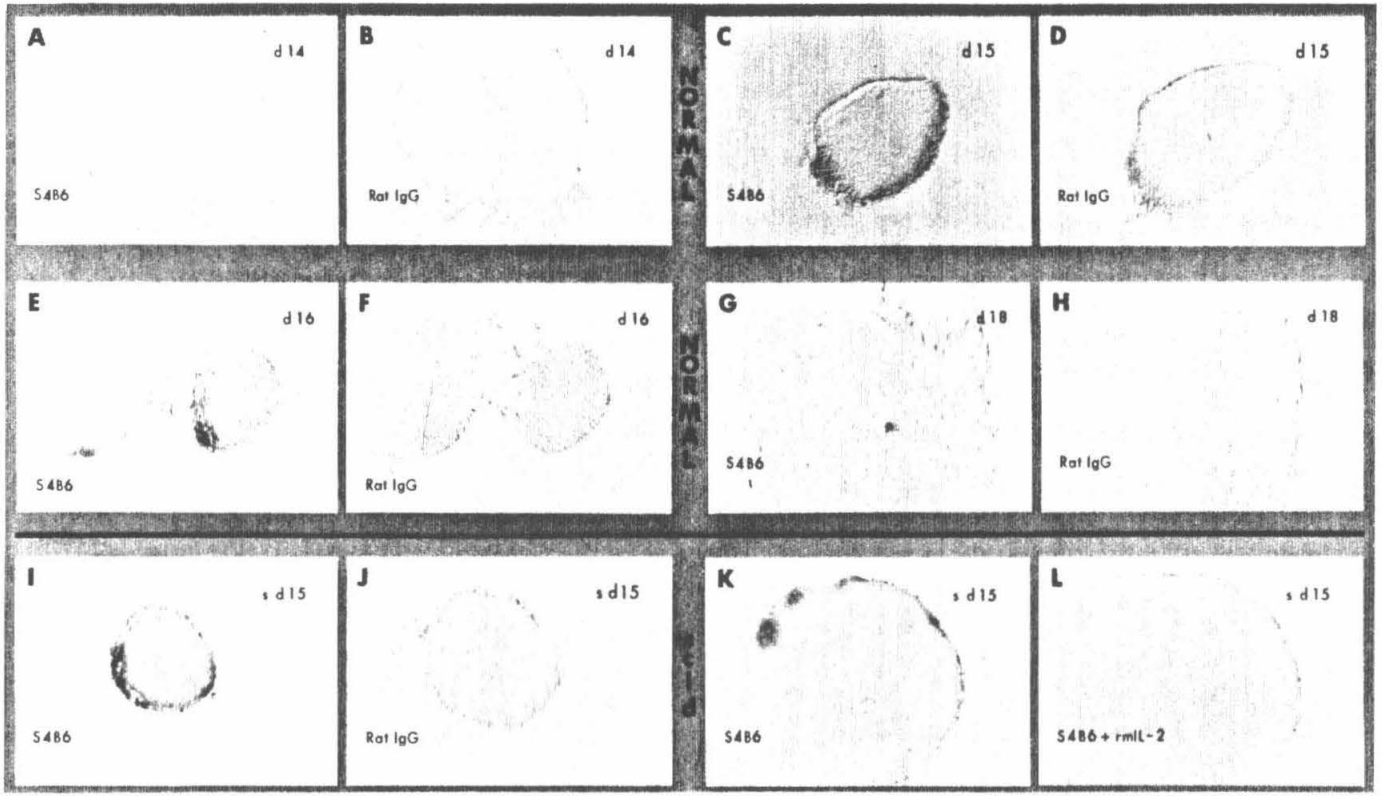


FIGURE 2. (See Colour Plate VII at the back of this publication). Immunoperoxidase staining for IL-2 immunoreactivity of normal and *scid* fetal thymus sections from various stages of gestation. Adjacent serial sections were stained with anti-IL-2 mAb (S4B6; Figs. 2A, 2C, 2E, 2G, 2I, 2K) or with normal rat IgG (Figs. 2B, 2D, 2F, 2H, 2J). To show specificity of the IL-2 staining in the day 15 *scid* fetal thymus, the anti-IL-2 mAb was preincubated at room temperature for 1 hr with 1000 U/ml recombinant murine IL-2 prior to staining (Fig. 2L). Variation in staining intensity around the periphery of immunopositive sections can be attributed to concentration effects that are frequently observed at the very edge of sections. Magnification ×100.

expressing cells detected, none was ever located in the medullary regions, where mature cells are expected to reside.

The "intensity" of specific hybridization shown for the cortical IL-2 mRNA-positive cells was similar to that observed using the same probe in parallel to analyze EL4 thymoma cells that had been induced for 5 hr with calcium ionophore and phorbol ester to serve as a calibration standard/sensitivity control (data not shown). Roughly 70% of these cells accumulated IL-2 mRNA following induction, and a positive cell contained approximately 220 IL-2 transcripts (Table 1). An average of 10 positive cells per half lobe was determined (Fig. 6) and, based on the assumption that an average thymic lobe contained 1.5×10^8 cells (3×10^8 cells per thymus total, data not shown), the frequency of IL-2 mRNA positive cells among thymocytes was estimated to be 1.3×10^{-7}. Finally, assuming that there were roughly 200 IL-2 transcripts per positive cell in the thymus (based on the similarity to induced EL4 cells; data not shown and Table 1), the number of IL-2 transcripts per average cell in the adult thymus was calculated to be on the order of 3×10^{-5} copies per cell, or a total of 2000 transcripts in a 100 μg RNA sample (equivalent to half a thymic lobe). This amount of RNA is two orders of magnitude lower than the threshold of detection for ribonuclease probe protection analysis under our conditions (see Materials and Methods).

Immunohistochemical Staining of Normal Adult Thymus Sections Using Anti-IL-2 Antibody

The presence of rare IL-2 producers in the adult thymus was confirmed by immunohistochemical staining. Figure 7A shows a typical, isolated IL-2

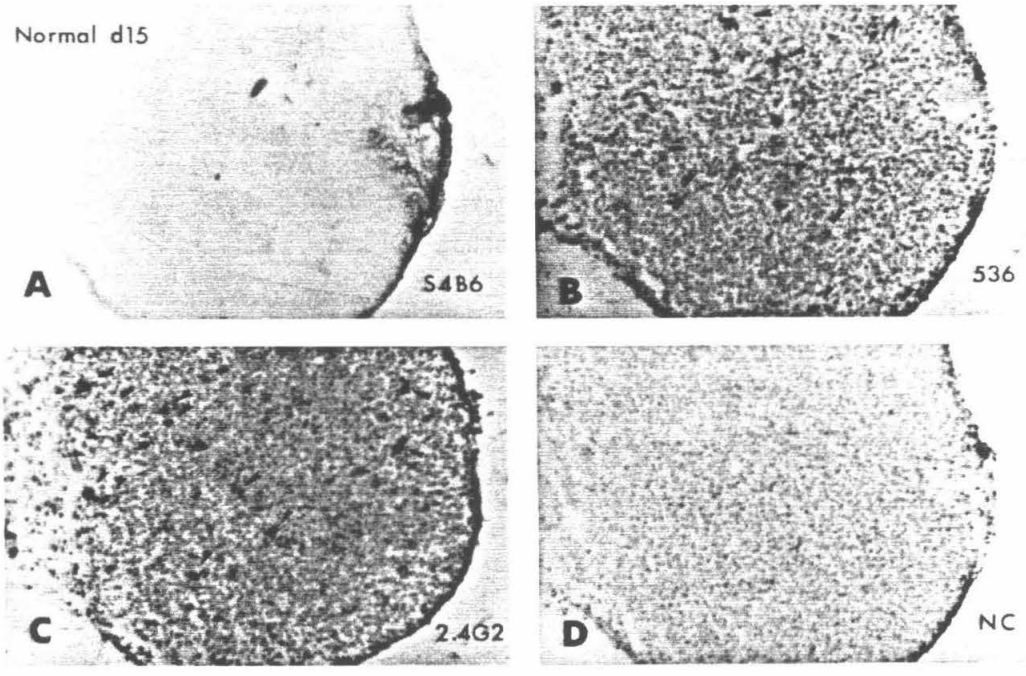


FIGURE 3. (See Colour Plate VIII at the back of this publication). Immunoperoxidase staining of normal day 15 fetal thymus, comparing localization of IL-2 producing cells, Vγ3$^+$ and CD16$^+$ cells. Adjacent serial sections were stained with either S4B6 (anti-IL-2 mAb; Fig. 3A), 536 (anti-Vγ3 mAb; Fig. 3B), 2.4G2 (anti-CD16/FcRII mAb; Fig. 3C), or normal rat IgG (NC; Fig. 3D). The S4B6 sample was stained as in Fig. 2, and 536, 2.4G2, and NC samples were developed with nickel/cobalt-enhanced diaminobenzidine. Arrowheads denote representative areas of immunoreactivity.

immunoreactive cell found in the normal adult thymus. As for the IL-2 mRNA-positive cells detected by *in situ* hybridization, the IL-2 immunoreactive cells were exclusively restricted to the cortex. About 25–32 immunopositive cells were observed per half lobe (data not shown). Nonspecific, peroxidase-positive cells were present in the adult thymus (possibly macrophages or other bone marrow-derived cells), but this background was drastically reduced when sections were avidin-biotin blocked prior to staining and quenched for exogenous peroxidase activity. Under these conditions, sections stained with normal rat IgG did not contain immunoreactive cells (Fig. 7B). The staining seen in the thymus was similar in intensity to that observed in a positive staining control containing mature T cells activated *in vivo* (Fig. 7D). Sections of lymph nodes isolated 8 days following immunization showed characteristic parafollicular staining with the anti-IL-2 mAb, in good agreement with the pattern of IL-2 immunoreactivity reported in the literature (Bogen et al., 1991). Addition of exogenous recombinant IL-2 abrogated the appearance of these immunoreactive cells (Fig. 7C), confirming that reactivity seen with this mAb was IL-2-specific.

IL-2 mRNA Positive Cells Are Absent in the Adult Thymus of *Scid* Mice

Because the IL-2 mRNA-positive/immunoreactive cells were restricted to a region in the adult thymus where rapidly proliferating immature cells are found, it was important to establish whether the IL-2 positive cells in the adult thymus were immature. Therefore, we examined thymuses of adult *scid* mice that are enriched at

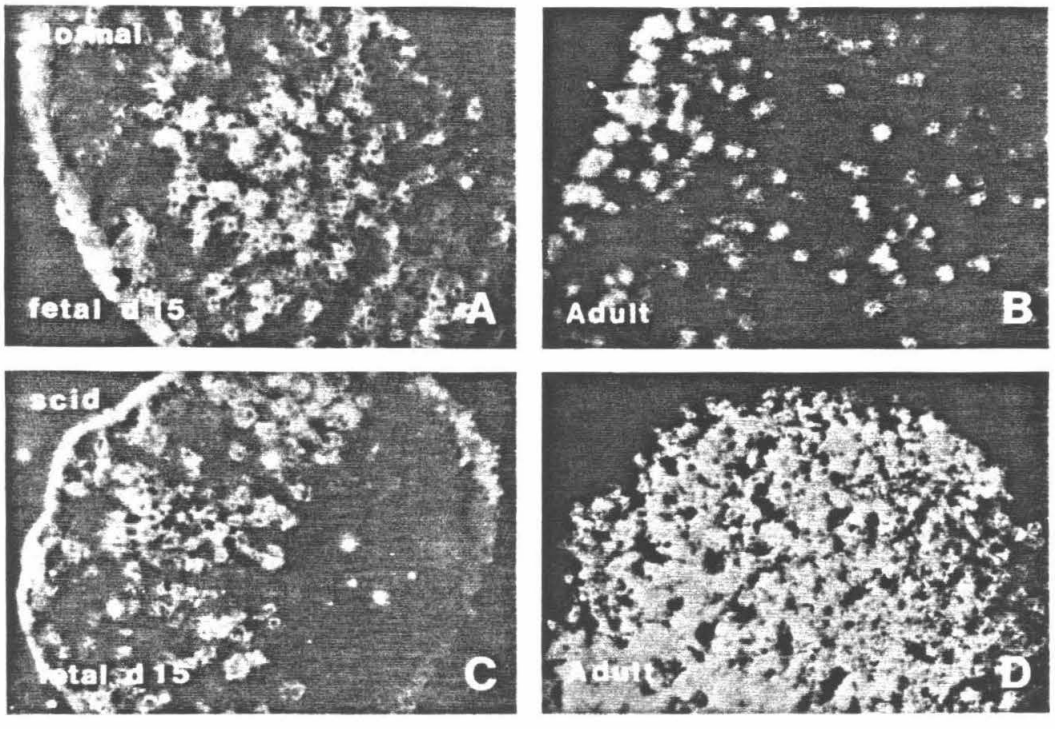


FIGURE 4. (See Colour Plate IX at the back of this publication). Direct immunofluroescence staining of normal and *scid* thymus sections using a phycorerythrin-conjugated rat anti-mouse CD25 (IL2Rα) mAb. Figures 4A and 4B show day 15 fetal and young adult thymus sections obtained from normal mice, respectively, and Figs. 4C and 4D show comparable sections obtained from *scid* mice. Magnification ×200.

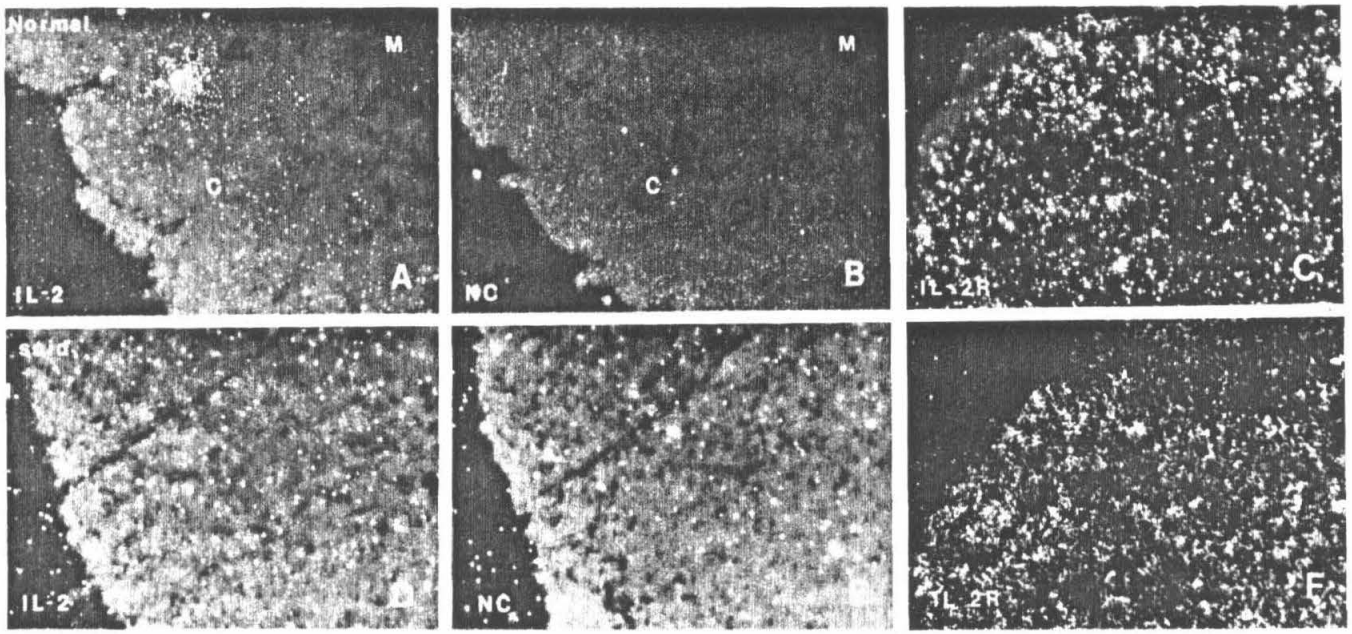


FIGURE 5. (See Colour Plate X at the back of this publication). *In situ* hybridization of adult thymus sections obtained from normal and *scid* mice for IL-2 and IL-2Rα expression. Thymuses were isolated from 4-week-old normal and *scid* animals. Adjacent serial sections were hybridized with either a 430-nt ^{35}S-labeled IL-2 antisense RNA probe (Figs. 5A and 5D) or the complementary sense strand (Figs. 5B and 5E). Additionally, normal and *scid* thymus sections were hybridized with ^{35}S-labeled IL-2Rα antisense RNA probes (Figs. 5C and 5F, respectively). Figure 5A shows a representative field containing a typical IL-2-positive cell located in the cortex of the normal adult thymus; other positive cells were found in the subcapsular region (data not shown). In panels 5A and 5B, cortex (C) and medulla (M) are indicated. The differences in grain size between normal and *scid* samples in this experiment are anomalies presumably due to variation in emulsion quality. 21-day exposure; magnification ×200.

TABLE 1
Titration of 430-nt 3'-IL-2 Probe by Ribonuclease A, T1 Protection Using Total RNA Isolated from Induced EL4 Cells

% induced EL4[a]	% IL-2 mRNA$^+$ by *in situ*[b]	No. copies IL-2 protected[c]	No. copies IL-2 per avg. cell[d]	No. copies IL-2 per induced cell[e]	No. copies IL-2 per mRNA$^+$ cell[f]
1	0.64	1.5×10^6	1.5	150	234
2	1.4	2.9×10^6	2.9	145	207
5	3.4	7.1×10^6	7.1	140	206
10	8.1	1.8×10^7	18	180	222
20	13.9	3.3×10^7	33	165	240
50	35.0	7.9×10^7	79	160	229
100	74.0	1.7×10^8	170	170	230

The average number of copies IL-2 per IL-2 mRNA$^+$ cell=224±18

[a] EL4 cells induced for 5 hr with calcium ionophore and phorbol ester were mixed with uninduced EL4 cells at the percentages indicated (a total of 5×10^6 viable cell equivalents per sample).

Total RNA was extracted according to the method described by Chomczynski and Sacchi (1987).

One-fifth of each sample (or RNA from 1×10^6 cell equivalents) was used per protection. No protection was observed above background using total RNA isolated from uninduced EL4 cells.

[b] Identical samples of cells were processed as described in Materials and Methods and analyzed by *in situ* hybridization. Signals from at least 5000 cells were analyzed per sample.

[c] $$\text{No. copies protected} = \text{conversion factor} \times \frac{\text{phosphor imager value for protected product}}{\text{cpm undigested probe used per protection}} \times \frac{\text{ng probe used per protection}}{\text{molec. wt. protected product}} \times 6\times10^{23} \text{ molecules per mole}$$

where the conversion factor $= \frac{(\text{liquid scintillation cpm}}{\text{phosphor imager value})}$, determined for known amounts of undigested probe.

[d] $$\frac{\text{No. copies IL-2 protected}}{1\times10^6}$$

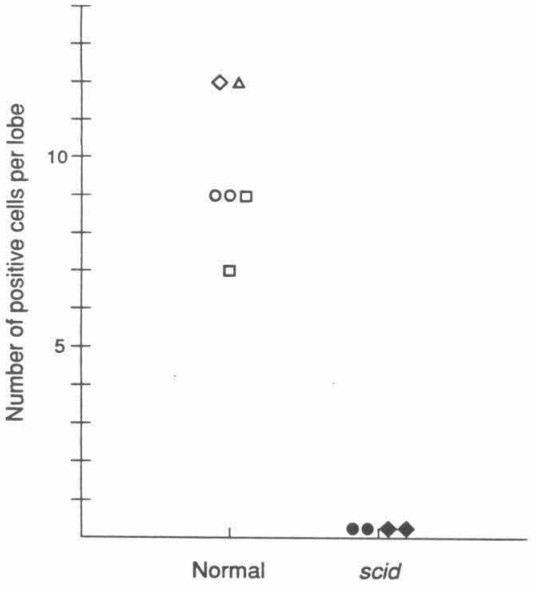
[e] $$\frac{\text{No. copies IL-2 per avg. cell}}{\%\text{ ind. EL4}\times10^{-2}}$$

[f] $$\text{No. copies IL-2 per ind. cell} \times \frac{\%\text{ induced EL4}}{\%\text{IL-2 mRNA}^+\text{ by } \textit{in situ}}$$

least 100-fold for immature thymocytes at all stages leading up to the $CD3^-CD4^-CD8^-$ (TN) IL-2Rα^+ stage of development (see Fig. 4) (Bosma and Carroll, 1991). If the IL-2 mRNA-positive/immunoreactive cells in the thymus of normal animals were of an immature phenotype, they should be present in greater numbers in the *scid* adult thymus than in the normal counterpart. On the other hand, if these cells had matured past the stage of TcR gene rearrangement, they should be depleted in the *scid* adult thymus. *In situ* hybridization revealed that IL-2 mRNA-positive cells were conspicuously absent from the *scid* adult thymus; both antisense and sense probes gave identical results in all sections analyzed (Figs. 5D and 5E). The absence of hybridization in the *scid* adult thymus samples was not due to artifactual degradation of probes hybridized to the *scid* thymus sections, because alternate sections hybridized with IL-2Rα antisense probes yielded strong signals (Fig. 5F), consistent with the results obtained from direct immunofluorescent staining (Fig. 4D).

For direct quantitative comparison, hybridization of *scid* thymus sections were carried out in parallel with hybridization of normal thymus sections (Fig. 6). On average, fewer sections per *scid* thymus were analyzed than per normal thymus because *scid* adult thymuses were substantially smaller than normal adult thymuses (*scid* thymuses, which lack all TcR$^+$ cell types, contain approximately 1/100 the number of viable cells found in normal thymuses; data not shown). Due to the reduced cell numbers in the *scid* adult thymus, the frequency of IL-2-expressing cells could not be proven to be lower in the *scid* adult thymus than in the normal adult thymus on a per cell basis. Nonetheless, the depletion of IL-2 mRNA-positive cells per lobe in the *scid* thymus was highly significant (Fig. 6).

The absence of cells producing IL-2 protein in the *scid* adult thymus was difficult to confirm by immunohistochemistry due to a high background of endogenous, peroxidase-positive cells that were "reactive" in the absence of specific antibody, even after extensive H_2O_2/peroxidase quenching (data not shown). Nevertheless, based on the unambiguous *in situ* hybridization data, it



Average Number of Sections Hybridized with Either Strand: Normal=162
scid =80
Average Number of Sections Analyzed per Experiment: Normal=324
scid =160

FIGURE 6. Compiled data of *in situ* hybridization for IL-2 expression in adult thymus from normal and *scid* mice. Adult thymus sections from normal and *scid* animals (open and shaded points, respectively) were analyzed by *in situ* hybridization with the 430-nt antisense or sense IL-2 RNA probe. Approximately half a thymic lobe was hybridized with each probe. The results using the antisense probe were compiled from four independent experiments. No signals were detected with the sense probe (data not shown). Normal and *scid* sections hybridized and analyzed in parallel are denoted with identical shapes (circles, diamonds). Positive cells were considered as having greater than 10 silver grains. The average, total numbers of sections analyzed per normal and *scid* thymus were 320 and 80, respectively, corresponding to complete thymic lobes. Slides were exposed for 21 days.

appeared that IL-2 mRNA-positive cells in the normal adult thymus were very likely to be of a more mature phenotype than the developmentally arrested thymocytes found in the *scid* adult thymus.

DISCUSSION

Our results show that IL-2 expression is induced in thymocytes by intrathymic signals *in vivo*. This expression is subject to complex developmental regulation that constrains the possible roles of IL-2 as a mediator of signals. On the other hand, the induction of IL-2 itself serves as a probe for specific cell–cell interactions, which reveal surprising differences between the fetal and adult thymus.

Restricted Activation of IL-2 Expression in the Fetal Thymus

Our results show that the day 15–16 fetal thymus includes distinct, substantial zones of low and high regional IL-2 protein concentration, in good agreement with the results of Waanders (1990). Twelve hours prior to maximal protein production, a burst of IL-2 message accumulation is observed in cells restricted to the identical area where IL-2 protein will be found, namely, the periphery of the fetal thymus. The combined *in situ* hybridization and immunohistochemical staining data reported here for day 14.5–15 fetal thymus show that only a minority of the cells in the fetal thymus were expressing IL-2 message or protein at the given time points. This interpretation is not in complete agreement with that of Zuñiga-Pflücker et al. (1990), who reported that 50–60% of fetal thymocytes expressed IL-2 mRNA at day 15 gestation, based on *in situ* hybridization to cytocentrifuged samples. The disparity in percentage of IL-2 mRNA-positive cells could be due to any of several factors. Because the kinetics of IL-2 expression are clearly transient, our time points might have missed the true peak. Alternatively, the timing of the actual impregnation of the mice could be affected by the setting of the 12-hr light–dark cycle in the respective mouse colonies, leading to apparent shifts in kinetics. Also, in principle, there could be a lower threshold of detection for *in situ* hybridization to cytocentrifuged cell suspensions than to tissue sections. Finally, as Zuñiga-Pflücker et al. (1990) did not subdivide categories of IL-2 mRNA-positive cells based on silver grain count, they might have included cells that we would not consider to be unequivocally IL-2-positive. However, our immunohistochemical staining data support our *in situ* hybridization results, indicating that only a highly localized minority population express IL-2 protein that is detectable at days 15–16 of gestation. Thus, according to these staining results, if 50–60% of cells do express an IL-2 message at the time of

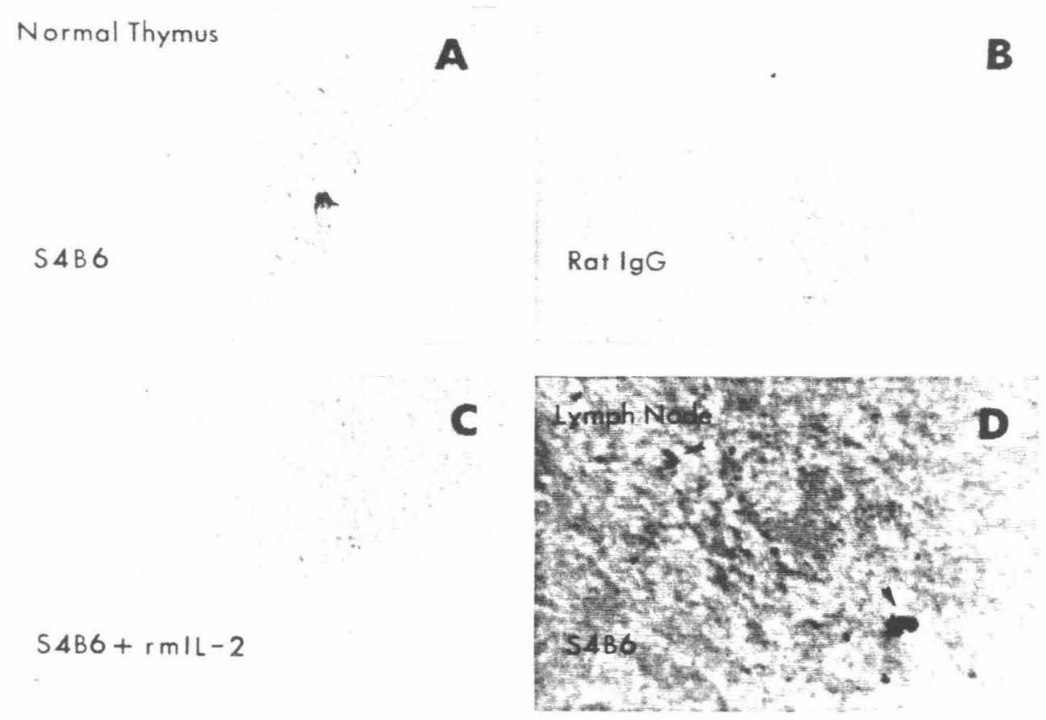


FIGURE 7. (See Colour Plate XI at the back of this publication). Immunoperoxidase staining of normal adult thymus and activated lymph node (removed 8 days after immunization) for IL-2 immunoreactivity. Figures 7A, 7B, and 7C show adjacent serial sections from a normal adult thymus. Figure 7A shows a section containing an isolated immunoreactive cell located in the cortex following staining with the anti-IL-2 mAb, S4B6. No immunoreactive cells were found in the same region in an adjacent section following staining with normal rat IgG (Fig. 7B). Specificity of staining was shown by staining the remaining adjacent section with anti-IL-2 mAb preincubated with rmuIL-2 (Fig. 7C; also see legend for Fig. 2). Activated lymph node sample was counterstained with methyl green (Fig. 7D). Magnification ×200.

maximal IL-2 mRNA accumulation in the fetal thymus, then only a fraction of those cells (corresponding to the highly positive cells we detect) ultimately synthesize IL-2 protein.

IL-2 as an Effector: Constraints on the Intrathymic Role of IL-2

The nonuniform distribution of IL-2 raises the possibility that the zones of high IL-2 expression may represent specific sites for expansion and differentiation of particular subsets of thymocytes. Initial candidates for such IL-2-regulated subsets that we have considered here are the $V\gamma3^+$ subset of $\gamma\delta$ thymocytes and fetal thymic NK cells, both of which are highly responsive to IL-2 *in vitro*. The first wave of surface $CD3^+$ cells in the fetal thymus at day 15 of gestation specifically utilize the $V\gamma3$ gene segment in TcR γ-chain gene rearrangement (Havran and Allison, 1988; Allison and Havran, 1991). These $V\gamma3^+$ cells leave the thymus soon afterwards and are undetectable in the thymus by the time of birth (Allison and Havran, 1991), and are exclusively found associated with the epidermis in postnatal mice (Havran and Allison, 1988). *In vitro* exposure of late fetal thymocytes to high concentrations of IL-2 resulted in the preferential expansion of $V\gamma3^+$ cells relative to other TcR^+ lineages (LeClerq et al., 1990). IL-2 also influences the production of NK cells; recent findings revealed that cells exhibiting substantial NK activity can be generated *in vitro* from cultures of $CD16^+$ cells isolated from day "15.5" fetal thymus (equivalent to day 14.5 in

our terminology) grown in moderate amounts of IL-2 (Rodewald et al., 1992). Furthermore, transgenic mice constitutively expressing IL-2 and IL-2Rα chain show great preferential expansion of NK cells (Ishida et al., 1989). Based on these data, the generation of either of these cell types in the fetal thymus *in vivo* could be positively regulated by the local concentration of IL-2. However, when we examined Vγ3, CD16, and IL-2 expression in the day 15 fetal thymus by immunohistochemical staining, we were unable to show a relationship between the localization of V$\gamma3^+$ or CD16$^+$ cells and IL-2 producers (Fig. 4). Although we cannot formally disprove the possibility that V$\gamma3^-$ or CD16$^-$ precursors of either cell type respond to IL-2, these results make it unlikely that IL-2 is limiting for the generation of V$\gamma3^+$ T cells or CD16$^+$ cells (presumptive T/NK precursors) in the fetal thymus *in vivo*. The targets for the burst of fetal IL-2 production therefore remain undefined.

In contrast to the results obtained from day 14.5–15 fetal thymuses, IL-2-expressing cells are extremely rare in the adult thymus with a frequency on the order of 10^{-7}, as determined by both *in situ* hybridization and immunohistochemical staining. Any cells responding to IL-2 in the adult thymus *in vivo* would most likely need to be in close proximity to these IL-2 producers, based on the minimal diffusion of IL-2 protein observed in the cortex. Unlike IL-2 production in the fetal thymus, no continuous zones of high IL-2 concentration are found in the adult organ. Furthermore, IL-2 may be available in these local sites for only a limited time. Our data suggest that the steady-state frequency of IL-2-expressing cells reflects the asynchronous activation of different rare cells. In the adult thymus, cells producing IL-2 protein were detected at an approximately threefold higher frequency than cells expressing IL-2 mRNA at any one time. The IL-2 mRNA is known to be labile (Shaw et al., 1988; Lindsten et al., 1989), and our results with the fetal thymus indicate that IL-2 protein can persist in the thymus at least 1 day longer than IL-2 mRNA. Thus, we interpret the excess of IL-2 immunoreactive cells over IL-2 mRNA-positive cells as evidence that IL-2 gene induction in the adult thymus is also transient but asynchronous; that is, that most cells synthesizing IL-2 protein have already shut off synthesis of the short-lived IL-2 mRNA. When the resulting limited availability of IL-2 is compared with the 10–20% of thymocytes that are actively in cycle (Penit, 1986; Boyer et al., 1989), it is clear that thymocyte-derived IL-2 is grossly inadequate to drive the extensive proliferation in this organ.

Our results support the finding that transgenic mice homozygous for an IL-2 gene disruption did not make IL-2 and yet generated all major thymocyte populations in normal numbers (Schorle et al., 1991). However, the extreme rarity of IL-2-rich "niches" in the normal adult thymus raises a caveat about the interpretation of the gene disruption results. If IL-2 were normally required as a growth factor for a particular subset of cells in the thymus, then its targets must represent a very minor fraction of cells in the cortex, the deletion of which might not drastically affect thymocyte population dynamics as a whole. Thus, an effect of IL-2 on some minor or transient branch of the T-cell lineage cannot be ruled out.

Developmental Significance of IL-2 Induction *In Vivo*: IL-2 as an Indicator of a Rare Activation Event

What may be the most telling aspect of our data is the access they provide into the intrathymic activation events that are detected as the induction of IL-2 gene expression in the murine thymus *in vivo*. These activation events depend both on the delivery of the appropriate signal from the thymic microenvironment and on the developmental status of the responding thymocyte (Howe and MacDonald, 1988; Rothenberg et al., 1990; Fischer et al., 1991). Little is known about the biochemical basis of thymic lymphostromal interactions, and key events in which activation signals are delivered have been more commonly inferred than demonstrated. On the other hand, the IL-2 induction response *in vitro* is relatively well characterized, both in terms of cytoplasmic signaling cascade and the transcription factors that are mobilized in the activated cells. Thus, our results provide strong evidence that this particular cascade of responses is indeed triggered *in vivo* in particular cells in the fetal and postnatal thymus.

As both immature (TcR$^-$) and mature (TcR^{high}) thymocytes can in principle make IL-2, the intrathymic IL-2 production observed could represent a response either of an immature cell, presum-

ably to differentiating signals, or of a mature cell (possibly to antigen). We have utilized two complementary kinds of evidence to reach a provisional conclusion. One is the intrathymic location of IL-2-expressing cells: In postnatal mice, the cortex is a zone of differentiation and precursor expansion, and the medulla is a domain where mature thymocytes accumulate and encounter circulating cells and antigen. The other relates to the effect of the *scid* mutation on intrathymic IL-2 expression. By preventing correct TcR gene rearrangement, this mutation blocks all TcR-expression-dependent events in T-cell development while permitting those that precede TcR gene rearrangement. The results of our analysis suggest that both fetal and postnatal cells that make IL-2 in the thymus are responding to developmental signals, rather than environmental antigen, but that distinct, nonequivalent cell types are responding to these signals in the fetal versus postnatal thymus.

To elaborate, the fetal thymocytes that make IL-2 are unaffected by the *scid* mutation, even though the *scid* mutation effectively blocks the generation of fetal TcR^+ thymocytes. Therefore, the activation signal that induces IL-2 expression in fetal thymocytes is likely to be delivered to cells at a stage upstream of the *scid* arrest point, that is, prior to TcR expression. This interpretation is supported by the failure of IL-2-expressing cells to colocalize with TcR-expressing cells in the day 15 fetal thymus (i.e., $V\gamma3^+$ cells). By contrast, since the *scid* mutation does block the appearance of all IL-2-expressing cells in postnatal thymus, it is probable that postnatal thymocytes normally receive an IL-2-inducing signal *downstream* of the *scid* arrest point, as discussed further below. Thus, in the context of their own developmental lineage, the postnatal IL-2 expressors are likely to be more advanced than the fetal IL-2 expressors. However, the cortical location of the postnatal IL-2 producers argues against any possibility that they have completed their intrathymic processing. Instead, the signal that induces IL-2 expression is apparently delivered while cells are still in the differentiative domain of the thymus.

Successful induction of IL-2 expression *in vivo* indicates not only that thymocytes have the capacity to express IL-2 and that the thymic stroma has the capacity to deliver triggering signals, but also that the signal is matched to satisfy the activation requirements for the particular classes of thymocytes present. The failure of adult *scid* thymocytes to activate IL-2 expression could be caused by defects in any of these aspects. However, our *in vitro* studies (Chen and Rothenberg, 1993; Rothenberg et al., submitted) clearly show that the *scid* thymus is significantly *enriched* for cells with the competence to express IL-2, provided that a certain set of triggering conditions are used *in vitro*. Therefore, either a lack of inductive signal or a mismatch between the available signals and the requirements of the developmentally arrested cells seems to be implicated. One possible explanation for the failure of cells in the *scid* adult thymus to express IL-2 *in vivo* would be that the *scid* thymic microenvironment (i.e., the nonlymphoid component of the thymus) does not develop properly, due to the lack of feedback interactions from mature thymocytes. Indeed, there is some evidence for architectural abnormality of the *scid* thymus (Shores et al., 1990, 1991; van Ewijk, 1991). However, the main components reported to be reduced or absent in the *scid* thymus are medullary epithelium, and as previously noted, the induction of IL-2 expression that we detect in the normal postnatal thymus is never associated with the medulla. Thus, another explanation for the inability of cells in the *scid* adult thymus to express IL-2 *in vivo* seems more plausible, namely, that the *scid* mutation directly prevents the maturation of thymocytes themselves to the stage where they can respond to those IL-2 inductive signals generated by the postnatal thymic cortex. In this case, responsiveness to IL-2-inducing signals in the adult thymus *in vivo* would be at least indirectly dependent on successful TcR gene rearrangement. Whether the TcR itself is implicated in the triggering event remains to be determined. We are currently in the process of defining the signals that induce IL-2 gene expression in the postnatal thymus *in vivo*.

Conclusions

To summarize, our work shows that IL-2 is expressed in the fetal and adult thymus of normal mice *in vivo*. Although the pattern of IL-2 gene expression differs in the fetal versus adult thymus, IL-2 expression is highly localized during both times in development. In the fetal thymus, high expression of IL-2 is synchronous and

transient. Surprisingly, fetal thymic expression of IL-2 *in vivo* is not disrupted by the *scid* mutation, suggesting that normal TcR expression is not a requisite for IL-2 gene expression in the cell types present in the fetal thymus. On the other hand, IL-2 gene expression appears to be dependent upon TcR gene expression in the adult thymus, because the *scid* mutation abrogates the appearance of IL-2-expressing cells in the adult thymus. Finally, our results indicate that IL-2 is available to cells in highly restricted areas of the thymus during development. Taken together, this work implies that TcR-independent and TcR-dependent interactions are made within the thymus at different stages of development that, in turn, provide the proper inducing signal(s) in resident cells for IL-2 expression *in vivo*. Likewise, differences between the fetal and postnatal thymic microenvironments were clearly evident, both in terms of IL-2 protein content and in terms of the subsets of thymocytes induced to express IL-2 therein. The nature of the responsive and responding cells, and the criteria by which they are distinguished from others in the same stage of differentiation are intriguing issues for further investigation.

MATERIALS AND METHODS

Mice

C57BL/6 Tla$^+$ and C.B-17-*scid* animals were bred and maintained in our own facility. Thymuses from embryos at various stages of gestation were obtained from females using timed matings. The appearance of a vaginal plug was designated as day 0 and on this day the females were separated from the males. Young "adult" animals were used at 4 weeks of age. All *scid* animals were maintained in an Isotec flexible film isolater (Indianapolis, Indiana) without antibiotic treatment, except those used for timed matings that were kept outside the isolator.

Removal and Sectioning of Tissues

Fetal thymuses were removed from embryos every 12 hr on and following day 14 of gestation (pregnant females were sacrificed at 10:00 A.M. and 10 P.M.) and were immediately embedded in Tissue-Tek O.C.T. compound (Miles Inc., Kankakee, Illinois). Thymuses removed from adult animals were frozen in n-pentane/dry ice and stored at −70°C and embedded in O.C.T. prior to sectioning. For *in situ* hybridization, 6–8-μm serial cryosections were collected onto poly-L-lysine-coated glass slides, dried briefly, fixed in freshly made 4% paraformaldehyde in PBS for 1–4 min, rinsed in PBS and stored in 70% ethanol at 4°C. Alternatively, rinsed sections were stored with desiccant at −70°C and transferred to ethanol at 4°C, 3–7 days prior to hybridization. For sensitivity controls/hybridization standards, the murine thymoma EL4 was induced to express IL-2 by culturing the cells in the presence of the calcium ionophore A23187 (70 nM) and phorbol myristate acetate (PMA; 17 nM, 10 ng/ml) for 5 hr in RPMI 1640 (Irvine Scientific, Santa Ana, California) based complete medium supplemented with 5% fetal bovine serum (v/v) as described elsewhere (Chen and Rothenberg, 1993). Following induction, cells were harvested, mixed with uninduced cells at a known ratio of induced-to-uninduced cells, embedded in O.C.T., sectioned, fixed, and stored accordingly. For immunofluroescence and immunohistology, 6–8-μm cryosections were collected onto 3-aminopropyltriethoxysilane (Fluka, Ronkonkoma, New York) coated slides, air dried for approximately 1 hr, fixed in cold acetone for 10 s and stored with desiccant at −20°C. For activated lymph node samples, adult mice (8–12 weeks) were immunized by hind foot pad injection using Freund's complete adjuvant. Popliteal, inguinal, and pericardial lymph nodes were removed 8 days after immunization and tissues were processed as described before.

Hybridization Probes

All IL-2 probes used for *in situ* hybridization were IL-2 cDNA fragments cloned into the polylinker of the pGEM-1 and -2 plasmids (Promega, Madison, Wisconsin) using standard procedures. To ensure IL-2-specific hybridization, RNA probes did not include the CAG-repeat located in the 5'-region of the IL-2 cDNA. Two IL-2 RNA probes were used; a 590-nucleotide (nt) probe gave a strong signal, and a 430-nt AT-rich probe (contained within the 590-nt probe) gave extremely low backgrounds. For the 590 nt probe, a 750-base pair (bp) PstI-AccI fragment derived from the full-length IL-2 cDNA (DNAX, Palo

Alto, California) was subcloned into pGEM-1 and -2 (pCmIL2-1, -2). For the 430-nt probe, a 430-bp HindIII-AccI fragment derived from the same full-length cDNA clone was also subcloned into pGEM-1 and -2 (pmIL2.3-1, -2; McGuire and Rothenberg, 1987). The pCmIL-2 plasmids were linearized with PvuII and the pmIL2.3 plasmids were linearized with HindIII. The IL-2Rα probes were derived from two IL-2Rα cDNA fragments (Miller et al., 1985), a 5' 410-bp PstI fragment and an internal 460-bp TaqI-PvuII fragment. Both were subcloned into pGEM-1 and were linearized with HindIII. ^{35}S-labeled sense or antisense transcripts were synthesized using T7 RNA polymerase as described previously (McGuire and Rothenberg, 1987; McGuire et al., 1988). Transcription products were resuspended in 200 μl of 100 mM DTT and 0.1% SDS in 10 mM Tris, 1 mM EDTA, pH 7.4, and stored at −70°C prior to use. All steps in the procedure were carried out using solutions made with diethylpyrocarbonate-treated water and RNase-free glass- and plasticware.

In Situ Hybridization

For each *in situ* hybridization experiment, alternate serial sections were hybridized with antisense and sense (negative control, "NC" in figures) riboprobes. Cells were only considered positive where the signal with the antisense probe clearly exceeded the sense-strand background. Hybridization was carried out with modifications to a protocol described previously (Rothenberg et al., 1990). The hybridization buffer described previously was modified by the addition of 4 mM vanadyl ribonucleoside complexes, and hybridization was carried out for 8–12 hr. Following hybridization, cover slips were soaked off in 4×SSC. Unhybridized probe was removed with three washes in 50% formamide, 2×SSC, 10 mM DTT, and 1% 2-ME at 50°C (1 hr each). Slides were briefly rinsed in 2×SSC, transferred to digestion buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) containing 40 μg/ml RNase A and 40 U/ml RNase T1 and digested at 37°C for 30 min, after which slides were transferred to digestion buffer with 20 mM DTT and 1% 2-ME and incubated at 37°C for an additional 30 min. Slides were subsequently washed overnight in 50% formamide, 2×SSC, 10 mM DTT, 1% 2-ME at 45°C and/or in 2×SSC, 1 mM DTT, 1% 2-ME at 45°C for 30 min. In each case, the final wash was in 0.1×SSC, 0.1% 2-ME at room temperature for 20 min. Slides were dehydrated in 30, 50, 70, and 95% ethanol containing 0.3 M ammonium acetate, dried for 1 hr and dipped in NTB-2 emulsion (Eastman Kodak Co., Rochester, New York) diluted at a ratio of 2 to 1 with 0.9 M ammonium acetate. Slides were exposed for 12–21 days, developed at 13°C for 3 min in GBX developer (Eastman Kodak Co.), briefly rinsed in 2% acetic acid, and fixed for 7 min in Ektaflo fixer (Eastman Kodak Co.). Counterstaining was done with 250 μg/ml 4,6-diamidino-2-phenylindole (DAPI) at room temperature for 1–2 hr or with hematoxylin and eosin for 2 min and cover slips were mounted using Permount (Fisher Scientific, Fair Lawn, New Jersey). Dual dark field and fluorescence microscopy were used for analysis.

Antibodies

The rat anti-mouse IL-2 mAb, S4B6, was obtained commercially at a concentration of 1 mg/ml (Pharmingen, San Diego). Normal rat IgG was reconstituted at a concentration of 20 mg/ml (Miles Inc., Kankakee, Illinois). Immunoselect phycoerythrin (PE)-conjugated rat anti-mouse CD25 was purchased from Gibco BRL (Gaithersburg, Maryland), and biotinylated hamster anti-mouse CD3ε (145-2C11) and fluorescein (FITC)-conjugated Vγ3 TcR (536) mAbs were purchased from Pharmingen. Biotinylated rabbit anti-rat IgG or goat anti-hamster IgG (both mouse-absorbed) were obtained from Vector (Burlingame, California) or CALTAG Laboratories (San Francisco), respectively. The hybridoma 2.4G2 (rat anti-mouse CD16; FcRII/III) was grown in Fetal Clone II (Hyclone Sterile Systems, Logan, Utah) and pooled culture supernatants were used neat for staining.

Immunohistochemistry

The immunohistochemical staining protocol was adapted from procedures described by Farr et al. (1990) and Bogen et al. (1991). Sections were warmed to room temperature, fixed in cold acetone for an additional 2 min, transferred to 1% formaldehyde in PBS for 1 min, and washed in PBS for 5 min. Sections were blocked with 50 μg/ml avidin in PBS, followed by treatment with

250 μg/ml D-biotin in PBS, each for 20 min. Sections were then washed in PBS/0.025% sodium azide (NaN_3) and incubated with 2% normal mouse serum (Cappel/Organon Teknika Co., Durham, North Carolina) and 2% normal rabbit serum in PBS/1% BSA/0.025% NaN_3 in a humidified chamber at room temperature for 30 min. After every subsequent incubation, sections were washed with PBS/NaN_3. Following blocking steps, sections were incubated with 10 μg/ml primary antibody or normal rat IgG in PBS/BSA/NaN_3 at room temperature for 1 hr. Sections were then incubated with 10 μg/ml biotinylated secondary staining reagent (mouse absorbed) in PBS/BSA/NaN_3 at room temperature for an additional hour. Subsequently, sections were refixed in 2% paraformaldehyde in PBS for 10 min and quenched with 0.6% H_2O_2 in methanol for 15 min. Following extensive washing in PBS, staining was detected using an avidin-biotin-horseradish peroxidase complex (Vectastain Elite ABC; Vector Laboratories) and diaminobenzidine (DAB) as the enzymatic substrate (as described in the Vectastain protocol). Blocking experiments were performed using recombinant murine IL-2 or IL-4 purchased from Genzyme (Boston) and were carried out by preincubating the primary antibody with an excess of recombinant lymphokine (1000 U/ml) at room temperature for at least 1 hr prior to staining. Nickel/cobalt-enhanced staining was achieved using 30 mg $NiCl_2$ and 30 mg $CoCl_2$ per 10 ml DAB: H_2O_2 developing solution.

Direct Immunofluorescence

Staining procedure was modified from a protocol provided by Calbiochem (La Jolla, California). Sections were warmed to room temperature, fixed in cold acetone and briefly in 1% formaldehyde in PBS, and washed in PBS. Sections were then blocked with 2% normal mouse serum in PBS/BSA/NaN_3. Following a PBS/NaN_3 wash, sections were incubated with 6–10 μg/ml primary antibody in PBS/BSA/NaN_3 in a humidified chamber for 1 hr. Sections were washed again with PBS/Na azide. Stained sections were covered with 2–4 drops of Fluorosave Reagent (Calbiochem, La Jolla) and cover slips were mounted and sealed with clear nail polish.

Ribonuclease Probe Protection

Total RNA from samples containing known percentages of induced and uninduced EL4 was isolated using the technique published by Chomczynski and Sacchi (1987). Probe-protection measurements of IL-2 mRNA using the 430-nt IL-2 probe have been described elsewhere (McGuire and Rothenberg, 1987; McGuire et al., 1988). The average number of molecules per cell was determined using a Phosphor Imager (Molecular Dynamics, Sunnyvale, California) and assuming complete recovery of protected products from each sample of 10^6 cell equivalents. The sensitivity of this technique was 0.2 copies per cell (or a total of 1×10^5 copies per RNA sample isolated from 10^6 cells; data not shown), and no IL-2 mRNA was detected in uninduced samples.

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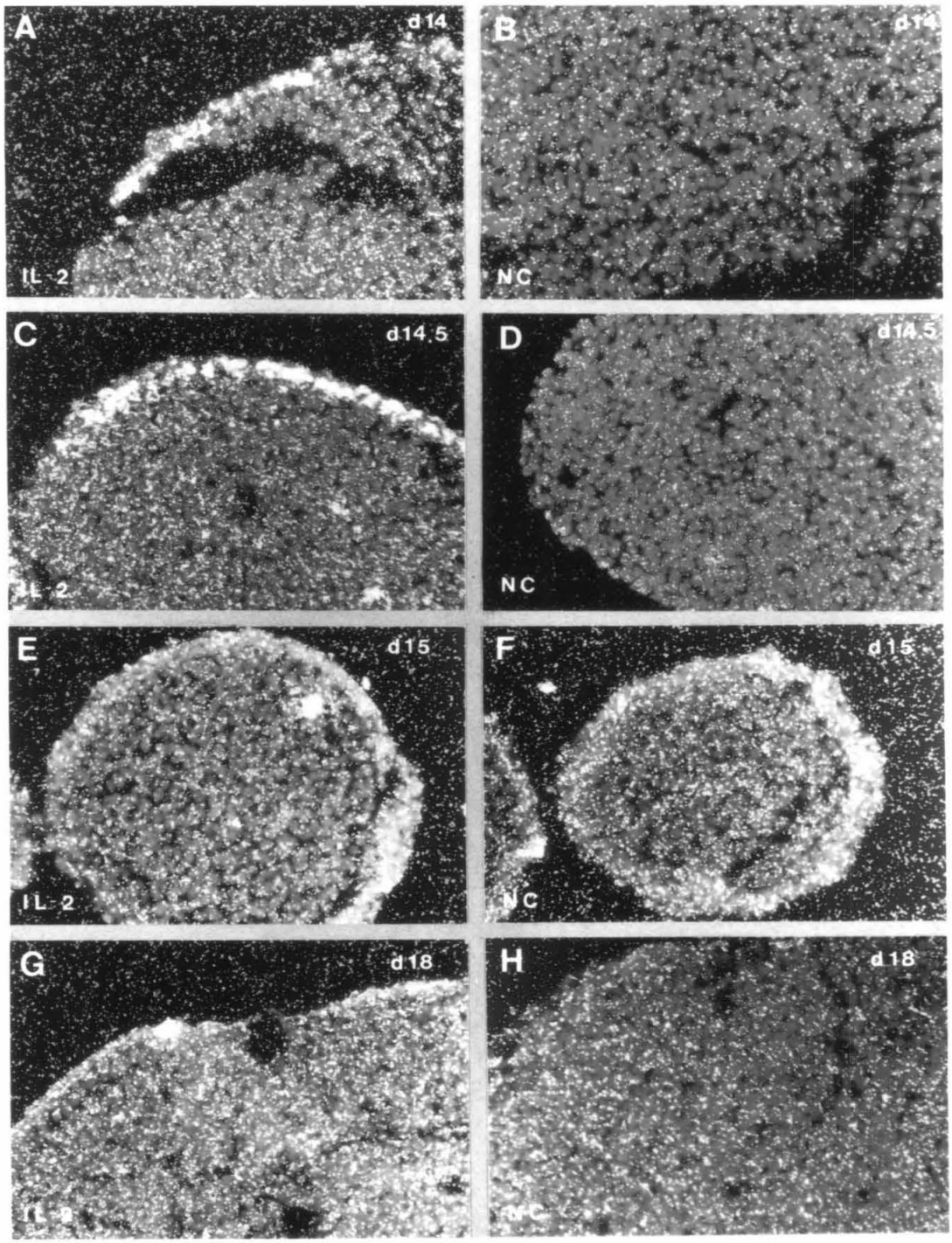
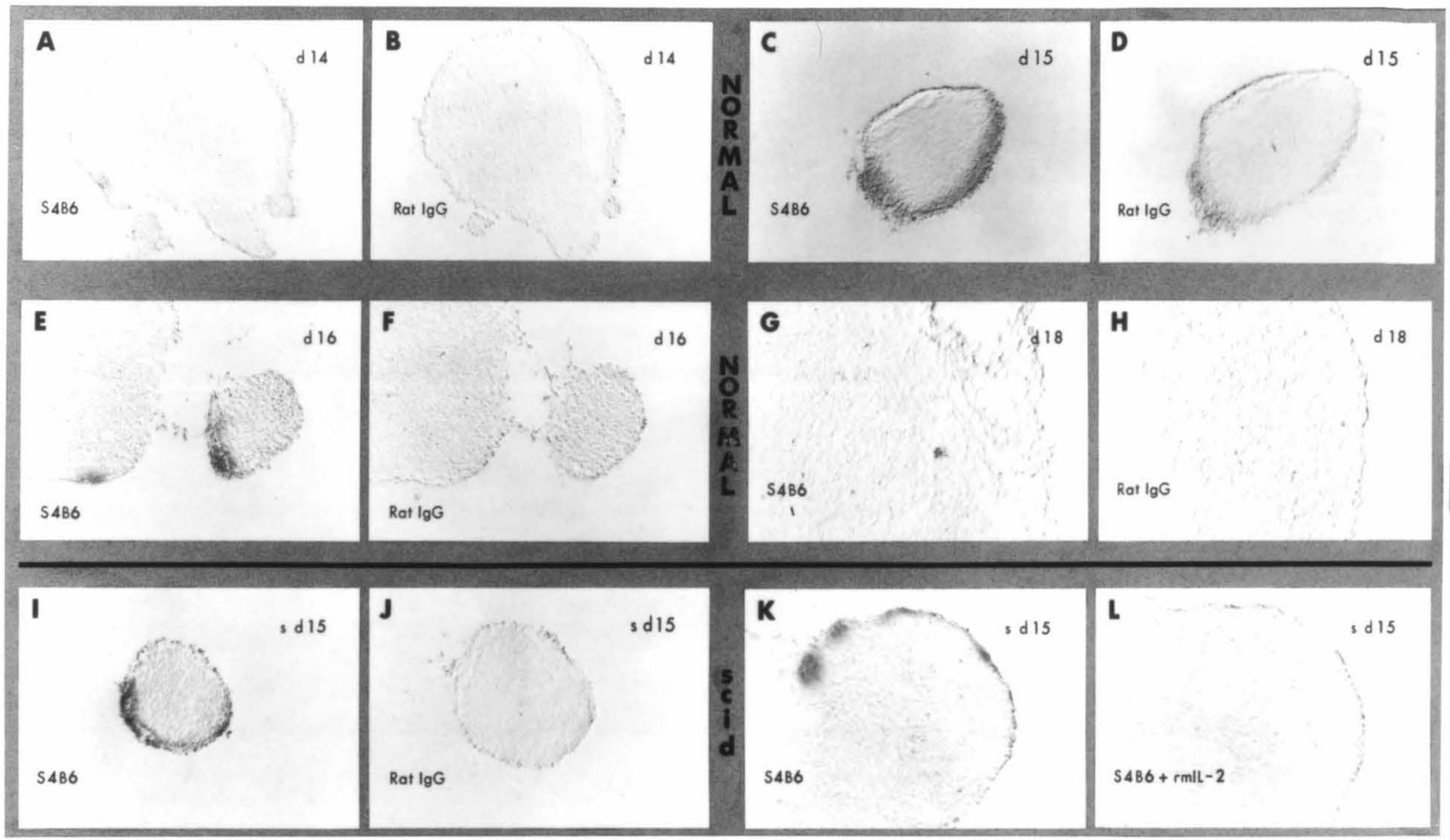
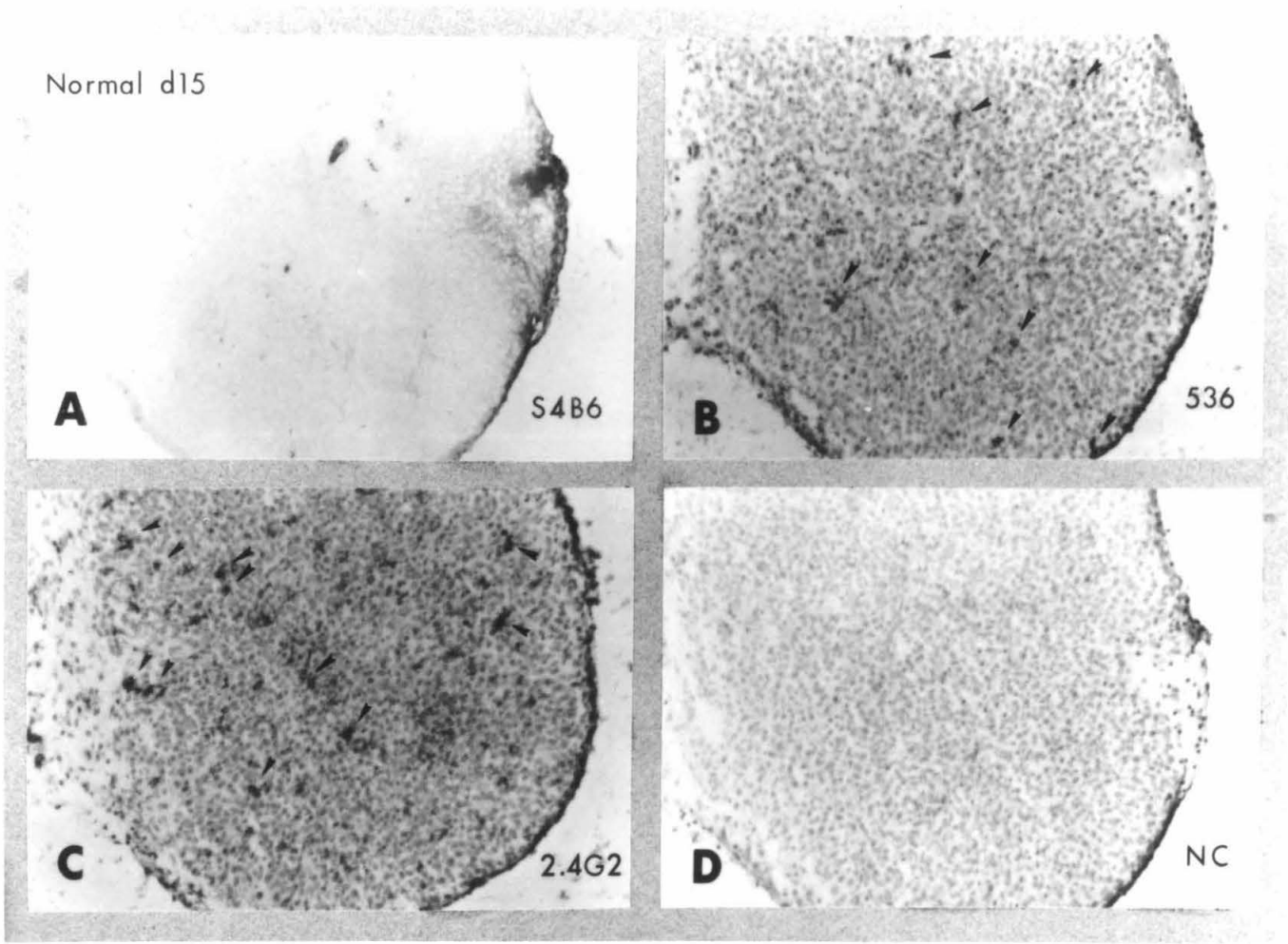


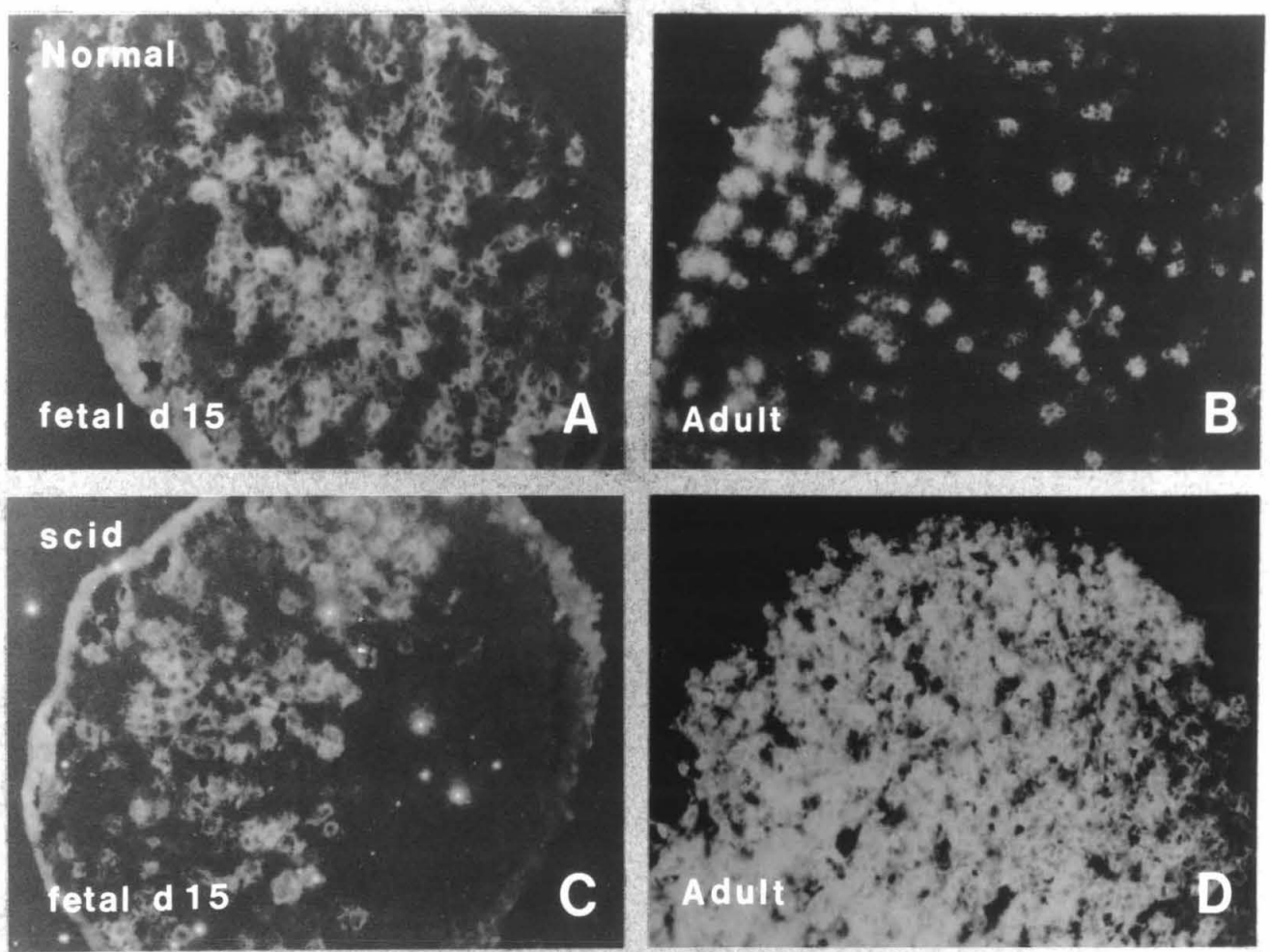
Colour Plate VI (see page 89, Figure 1). *In situ* hybridization to sections of normal thymuses from days 14 to 18 of gestation for IL-2 expression, using either a ^{35}S-labeled, 590-nt IL-2 antisense RNA probe (IL-2; Figs. 1A, 1C, 1E, 1G) or the complementary sense strand (NC; Figs. 1B, 1D, 1F, 1H). Samples were counterstained with DAPI and observed under dark field microscopy. 14-day exposure; magnification ×200.



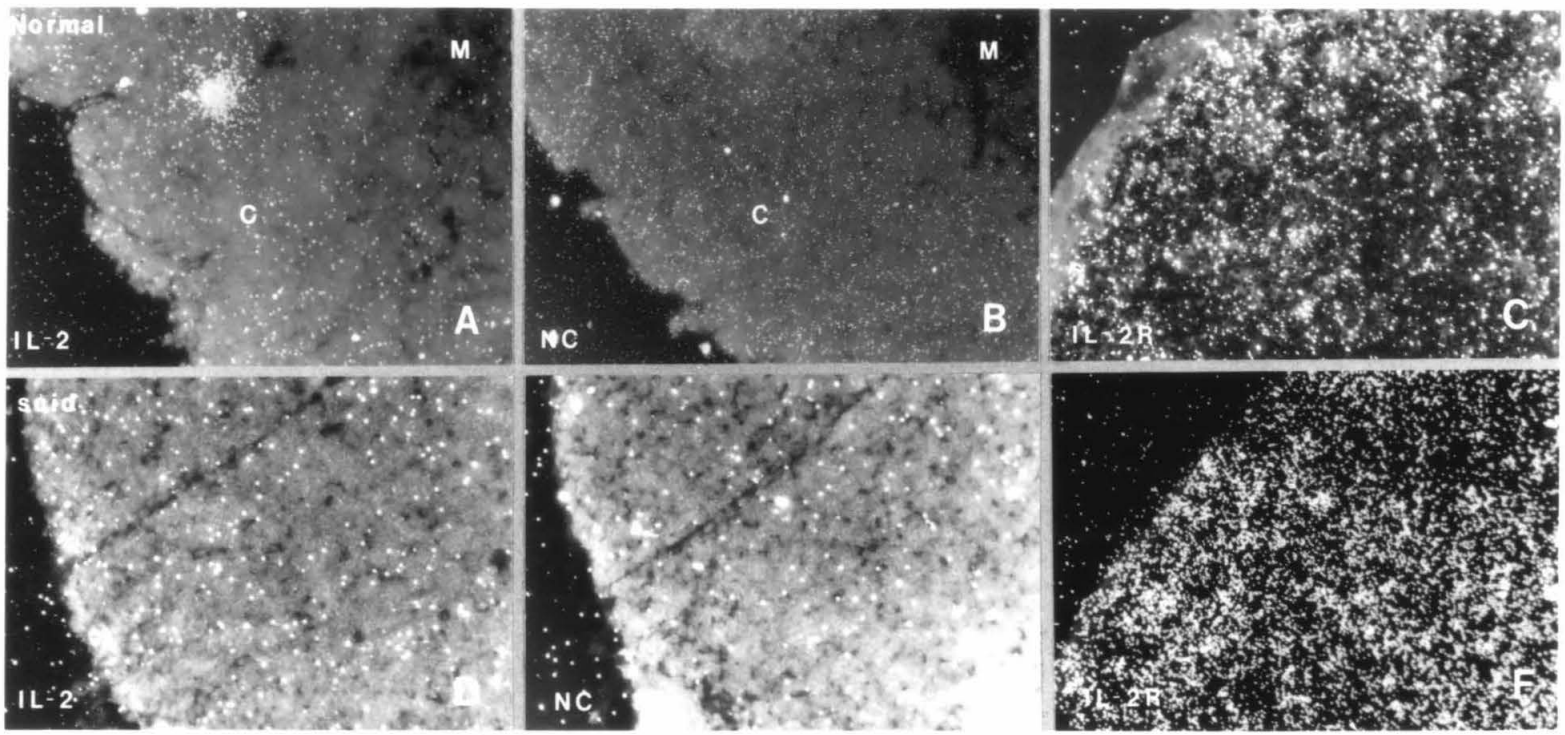
Colour Plate VII (see page 90, Figure 2). Immunoperoxidase staining for IL-2 immunoreactivity of normal and *scid* fetal thymus sections from various stages of gestation. Adjacent serial sections were stained with anti-IL-2 mAb (S4B6; Figs. 2A, 2C, 2E, 2G, 2I, 2K) or with normal rat IgG (Figs. 2B, 2D, 2F, 2H, 2J). To show specificity of the IL-2 staining in the day 15 *scid* fetal thymus, the anti-IL-2 mAb was preincubated at room temperature for 1 hr with 1000 U/ml recombinant murine IL-2 prior to staining (Fig. 2L). Variation in staining intensity around the periphery of immunopositive sections can be attributed to concentration effects that are frequently observed at the very edge of sections. Magnification ×100.



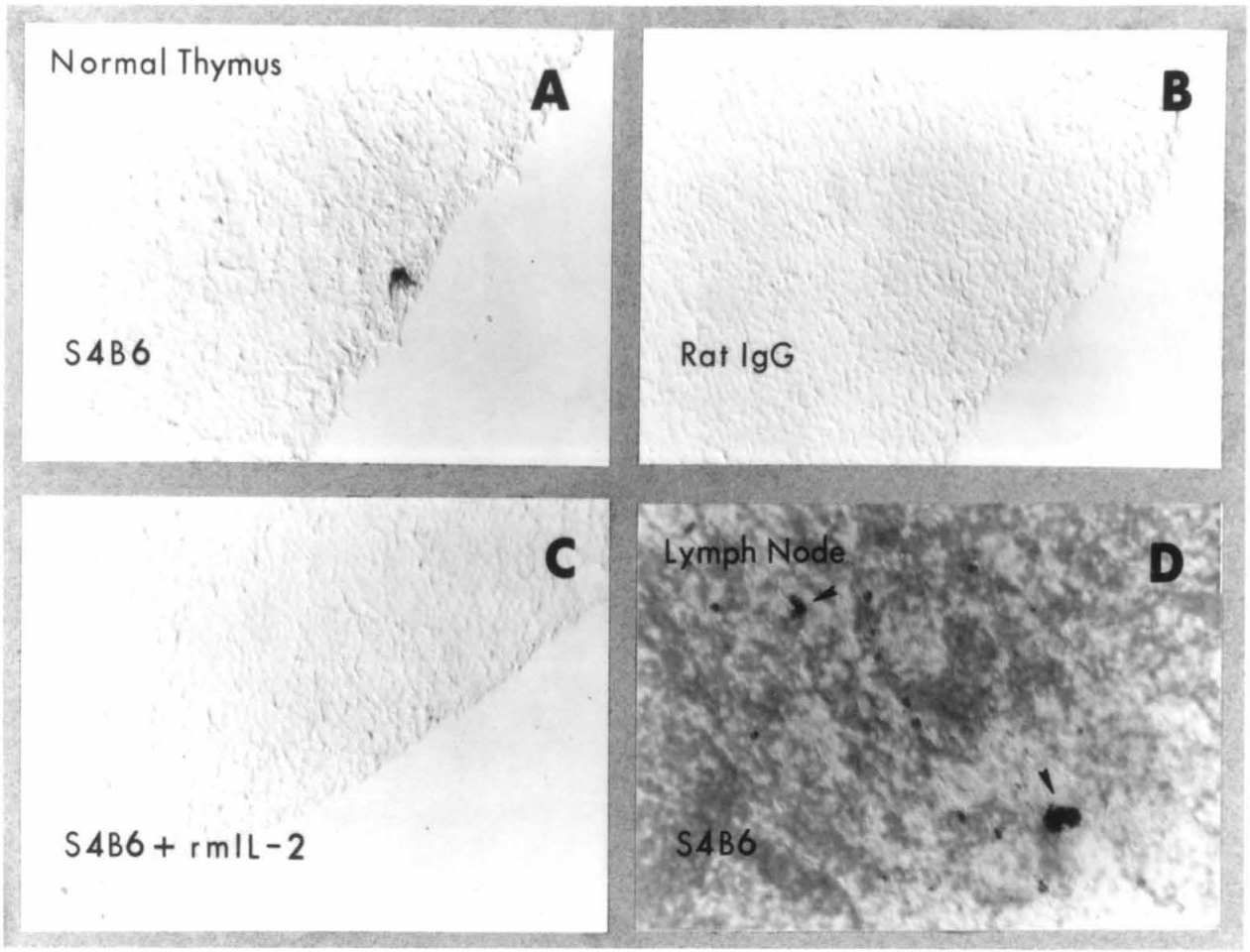
Colour Plate VIII (see page 91, Figure 3). Immunoperoxidase staining of normal day 15 fetal thymus, comparing localization of IL-2 producing cells, Vγ3$^+$ and CD16$^+$ cells. Adjacent serial sections were stained with either S4B6 (anti-IL-2 mAb; Fig. 3A), 536 (anti-Vγ3 mAb; Fig. 3B), 2.4G2 (anti-CD16/FcRII mAb; Fig. 3C), or normal rat IgG (NC; Fig. 3D). The S4B6 sample was stained as in Fig. 2, and 536, 2.4G2, and NC samples were developed with nickel/cobalt-enhanced diaminobenzidine. Arrowheads denote representative areas of immunoreactivity.



Colour Plate IX (see page 92, Figure 4). Direct immunofluroescence staining of normal and *scid* thymus sections using a phycorerythrin-conjugated rat anti-mouse CD25 (IL2Rα) mAb. Figures 4A and 4B show day 15 fetal and young adult thymus sections obtained from normal mice, respectively, and Figs. 4C and 4D show comparable sections obtained from *scid* mice. Magnification ×200.



Colour Plate X (see page 93, Figure 5). *In situ* hybridization of adult thymus sections obtained from normal and *scid* mice for IL-2 and IL-2Rα expression. Thymuses were isolated from 4-week-old normal and *scid* animals. Adjacent serial sections were hybridized with either a 430-nt ^{35}S-labeled IL-2 antisense RNA probe (Figs. 5A and 5D) or the complementary sense strand (Figs. 5B and 5E). Additionally, normal and *scid* thymus sections were hybridized with ^{35}S-labeled IL-2Rα antisense RNA probes (Figs. 5C and 5F, respectively). Figure 5A shows a representative field containing a typical IL-2-positive cell located in the cortex of the normal adult thymus; other positive cells were found in the subcapsular region (data not shown). In panels 5A and 5B, cortex (C) and medulla (M) are indicated. The differences in grain size between normal and *scid* samples in this experiment are anomalies presumably due to variation in emulsion quality. 21-day exposure; magnification ×200.



Colour Plate XI (see page 96, Figure 7). Immunoperoxidase staining of normal adult thymus and activated lymph node (removed 8 days after immunization) for IL-2 immunoreactivity. Figures 7A, 7B, and 7C show adjacent serial sections from a normal adult thymus. Figure 7A shows a section containing an isolated immunoreactive cell located in the cortex following staining with the anti-IL-2 mAb, S4B6. No immunoreactive cells were found in the same region in an adjacent section following staining with normal rat IgG (Fig. 7B). Specificity of staining was shown by staining the remaining adjacent section with anti-IL-2 mAb preincubated with rmuIL-2 (Fig. 7C; also see legend for Fig. 2). Activated lymph node sample was counterstained with methyl green (Fig. 7D). Magnification ×200.

CHAPTER 3

Prepared for Publication

Title: Identification of IL-2 Expressing Cells in the Skin: A Potential Role of IL-2 in the Extrathymic Expansion of T cells in the Murine Epidermis

SUMMARY

Upon activation by foreign antigen, a subset of mature T lymphocytes synthesize interleukin-2 (IL-2), a potent T-cell growth factor. IL-2 is an important mediator of the primary immune response. It is responsible for pushing resting T cells into cycle, resulting in the clonal expansion of antigen-specific T cells. Although it is unlikely that IL-2 drives the extensive proliferation of immature pre-T cells in the thymus, it is not known whether IL-2 participates in the autocrine or paracrine expansion of certain cell types belonging to the T lineage to populate the tissues in which they are found. For example, in order for fetally-derived $V_\gamma3^+$ $\gamma\delta$ TcR^+ cells to populate the epidermis, the tissue with which they are associated, extensive extrathymic expansion is required. Several *in vitro* findings suggest that $V_\gamma3^+$ T cells may use IL-2 as a proliferative signal. We have used *in situ* hybridization and immunohistochemical staining on epidermal samples obtained from mice of different ages to determine the extent of IL-2 expression in the skin. Our results revealed that maximal IL-2 mRNA and protein accumulation was observed in the epidermis at a time when the $V_\gamma3^+$ population is still expanding. Cells that express IL-2 mRNA have a distinctive dendritic morphology, characteristic of resident T cells found associated with the skin. Likewise, striking similarities between the pattern of IL-2 expression and distribution of T cells exist at this point are no longer detectable in the epidermis, indicating that IL-2 expression in the skin is temporal and transient. IL-2 expression is absent in the epidermis of SCID mice. The SCID mutation also inhibits the appearance of T cells in the skin. Taken together, the results obtained in SCID mice further reinforces the notion that IL-2 expressing cells associated with the skin associated with the epidermis belong to the T lineage. In addition, IL-2 is expressed by $\alpha\beta^+$ T cells in the epidermis of $\alpha\beta$ TcR transgenic mice in which $\gamma\delta$ TcR expression in the skin is suppressed, thereby indicating that IL-2 expression in the skin is not specified by TcR expression. The results presented here demonstrate that IL-2 may play a role in the post-thymic expansion of a particular population of T cells in the absence of an active immune response.

KEYWORDS: Interleukin-2 (IL-2), αβ and γδ T-cell receptor (TcR), *in situ* hybridization, immunohistochemical staining, severe combined immunodeficiency (SCID), dendritic epidermal cell (DEC).

INTRODUCTION

The proliferation of antigen-specific cells is pivotal for the success of the vertebrate immune response. In a health animal, the frequency of any particular antigen-specific mature cell is low in lymphoid organs or in blood, thus clonal expansion of antigen reactive effector cells is necessary following immunologic challenge. This amplification process is regulated by the synthesis of specific lymphokines. One such factor that regulates antigen-induced T-cell proliferation is interleukin-2 (IL-2). The production of IL-2 is triggered in particular subsets of T lymphocytes only upon activation as a result of T-cell receptor (TcR) engagement with cognate MHC molecules and foreign antigen. IL-2 acts as a mediator to push resting T cells into cycle. Once T cells are stimulated to produce IL-2, the lymphokine is available to induce both autocrine and paracrine effects (1-3). IL-2 is clearly essential for antigen-driven T-cell proliferation in the primary immune response, but it remains largely unknown if IL-2 participates in the expansion of T-cell populations in certain tissues that is not related to exposure to foreign antigen.

Targeted disruption of the IL-2 gene in mice has shown that IL-2 is not essential for the generation of normal cell populations in the thymus, indicating that IL-2 is not required for driving the extensive proliferation of immature pre-T cells (4). However, even though IL-2 -/- animals have normal numbers of T cells in lymphoid organs, these phenotypically mature cells were immunologically incompetent (4). Albeit that these studies reveal a great deal about the function of IL-2 *in vivo*, they do not disprove the possibility that IL-2 may play a role in the autocrine or paracrine expansion of particular T-cell subsets to populate the tissues in which they reside.

One candidate for a target T-cell type that could potentially utilize IL-2 for expansion and maintenance of cell numbers in the periphery *in vivo* was initially chosen on a number of results obtained *in vitro*. To be specific, T cells of the gamma/delta ($\gamma\delta$) lineage that incorporate the $V_\gamma 3$ gene segment in γ-chain rearrangements ($V_\gamma 3^+$ T cells) are generated during fetal ontogeny (5). Preferential expansion of $V_\gamma 3^+$ cells relative to other lineages has been observed upon culture of fetal thymic lobes (6) or cell suspensions of fetal thymocytes (7) in high concentrations of IL-2.

In normal mice, fetally-derived $V_\gamma3^+$ cells comprise the vast majority of T cells in the skin and are found nowhere else. In their target tissue site, $V_\gamma3^+$ cells have a distinctive dendritic morphology and are often referred to as Thy-1^+/TcR^+ dendritic epidermal cells (DECs). In all likelihood, $V_\gamma3^+$ cells recognize self-antigens expressed on keratinocytes. DEC clones can be activated by keratinocytes or fibroblasts treated with tryptic digests of keratinocytes to produce detectable amounts of IL-2 and IL-2 production is blocked by the addition of anti-CD3ε or anti-$V_\gamma3$ mAbs.. Similarly, DEC clones can be stimulated to produce IL-2 and upregulate IL-2 receptor (IL-2R) α-chain in response to the lectin, concanavalin A (ConA) or to CD3 crosslinking (9). At the time in gestation when $V_\gamma3^+$ cells are generated, the fetal thymus contains only about 0.5-1.0 x 10^5 cells. Hence, in order to populate the epidermis at a density of approximately 6-8 x 10^2 cells /mm^2 in the adult animal, extensive extrathymic expansion of $V_\gamma3^+$ cells is required. As suggested by the findings described above, one growth factor that could potentially regulate this process is IL-2.

To determine whether IL-2 is produced in the skin of normal mice, we have used a combination of *in situ* hybridization and immunohistochemical staining of epidermal samples obtained from mice of varying ages. Our findings reveal several striking features about IL-2 gene expression in the skin. IL-2 mRNA and protein are easily detected in the epidermis at a time when the $V_\gamma3^+$ cell population is expanding. Once $V_\gamma3^+$ cells have reached steady-state numbers in the skin, IL-2 gene expression is downregulated. IL-2 producing cells are also found in the skin of mice expressing transgenic TcRα- and β-chain genes in which ab T cells and no $\gamma\delta^+$ cells are associated with the epidermis. Finally, the presence of IL-2 expressing cells in the skin is contingent upon the generation of mature T cells, since IL-2 expression is not detected in epidermis removed from mice that are unable to generate mature T cells as a result of the severe combined immunodeficiency (SCID) mutation.

MATERIALS AND METHODS

Mice

C57BL/6 Tla$^+$, αβ-TcR specific for H-2L^d transgenic (10, 11), and C.C.-17-SCID mice were bred and maintained in our own facility. αβ-TcR transgenic animals were rederived to be virus-free, backcrossed to either B10 (Jackson Laboratories, Bar Harbor, Maine) or B10.S (Charles River Laboratories, Willmington, MA) genetic backgrounds, and were kept on sterile food and bedding. All SCID animals were maintained in an Isotec flexible film isolator (Indianapolis, IN) without antibiotic treatment. Day 15 fetal thymuses were obtained from fetuses removed from pregnant BL/6 Tla$^+$ and SCID mothers using timed matings. The appearance of a vaginal plug was designated as day 0.

Removal and Processing of Tissues

Mice were killed by cervical dislocation. For preparation of ear epidermal sheets, ears were collected and hair was removed by shaving with a single edge razor blade or by using a hair removal wax strip kit (Sally Hansen/Del Laboratories Inc., Farmingdale, NY). Shorn ears were then floated on 0.5 M ammonium thiocyanate (SIGMA, St. Louis, MO) in 0.1 M phosphate, pH 6.8, outer surface not submerged, and incubated at 37°C for 10-15 min. The outer epidermis was separated from the underlying tissue and immediately washed in cold phosphate buffered saline (PBS). For *in situ* hybridization using ^{35}S-probes, ear epidermal sheets were air dried onto poly-L-lysine coated slides, fixed in freshly made 4% paraformaldehyde in PBS for up to one hour on ice, rinsed in cold PBS and stored in 70% ethanol at 4°C. Samples were used within seven days after processing. For unmounted digoxygenin (dig) *in situ* hybridization, ear epidermal sheets were blotted dry on paper towels and fixed in 4% paraformaldehyde solution for up to one hour on ice, rinsed in cold PBS, dehydrated in graded ethanols and also stored in 70% ethanol at 4°C. For immunohistochemical and immunofluorescence staining, epidermal sheets were fixed in acetone for up to one hour at room temperature, after which they were rehydrated in PBS and stained on the same day. Activated lymph node and fetal thymus samples

were prepared as described in (12). Neonatal whole body samples for *in situ* hybridization were generated by quickly decapitating newborn pups, washing bodies in PBS and embedding immediately in Tissue-Tek O.C.T. (Miles Laboratories, Kankakee, IL). 8 μm sections were collected onto poly-L-lysine coated slides, briefly air dried, and fixed in 4% paraformaldehyde solution for 10 minutes on ice. Following a wash in cold PBS, slides were air dried completely and stored with desiccant at -80°C prior to hybridization. At least 12 hours before hybridizing, slides were thawed and transferred to 70% ethanol and kept at 4°C.

Hybridization Probes

All probes used have been described previously (12). Details for the synthesis of ^{35}S-labeled transcripts are given elsewhere (13, 14). *In vitro* transcription of labeled RNAs using dig-11-UTP was carried out according to the manufacturer's specifications (Boehringer Mannheim Biochemicals, Indianapolis, IN; 15). Transcription products were resuspended in 200 μl of 100 mM DTT and 0.1% SDS at -80°C prior to use. ^{35}S-labeled transcripts were used within a week, while dig-labeled probes were used up to six months after synthesis.

***In situ* Hybridization**

Whole body section were hybridized to ^{35}S-probes as given in (12). Ear epidermal sheets isolated from the same animal were used per single hybridization experiment, as one sheet was hybridized to a labeled IL-2 antisense RNA and the other to the corresponding sense probe. Hybridization using ^{35}S-probes was performed with slight modifications to a protocol described previously (12). Following rehydration of samples in PBS, epidermal sheets immobilized on glass slides were digested with 2 μg/ml proteinase K in PBS for 5-10 min. at room temperature. Digestion was stopped by transferring samples to 0.2% glycine in PBS for 2-3 min. Samples were rinsed in PBS, refixed in 4% paraformaldehyde in PBS for 20 min. at room temperature, and rerinsed in PBS. Prehybridization and hybridization treatment, removal of unhybridized probe and high stringency washes were carried out as detailed in (12). However, prior to RNase

digestion, hybridized samples were first washed in 2.5X SSC, 50 mM DTT, and 1% 2-ME for 1-2 hours with gentle agitation, and then in 2.5X SSC for 30 min. at 37°C. For unmounted *in situ* hybridization using dig-labeled probes, a procedure was adapted from a published protocol (15). Briefly, fixed, unmounted epidermal sheets were rehydrated in graded ethanols, washed in PBST (PBS containing 0.1% Tween-20) and briefly digested in 2 μg/ml proteinase K in PBS for 2-4 min. at room temperature. Digestion was stopped upon transfer to 0.2% glycine in PBS for 2-3 min. Samples were then rinsed in PBST, refixed in 4% paraformaldehyde solution for 20-30 min., and rinsed again in PBST. Samples were acetylated with 0.3% acetic anhydride in 0.1 M triethanolamine, pH 7. Following another PBST wash, samples were soaked in 0.1 M Tris-Glycine, pH 7.4 for 30 min. and transferred back into PBST. All steps following refizzation were performed at room temperature with agitation. Samples were prehybridized in hybridization buffer without probe (50% deionized formamide, 10% PEG-8000, 600 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 500 μg/ml yeast tRNA, 2X Denhardt's, 0.1% Tween-20, 0.1% CHAPS, and 10 mM vandyl ribonucleoside complexes) for 3-4 hours at 37°C. In the mean time, for every 1 μg dig-labeled RNA used for hybridization, probes were hydrolyzed in carbonate buffer (60 mM Na_2CO_2, 40 mM $NaHCO_3$, pH 10.2) for 2-3 min. at 60°C. Hydrolyzed probes were quickly transferred to an ice bath and neutralized with an equal volume of 200 mM sodium acetate, 1% acetic acid (v/v), pH 6.0. Probes were then precipitated and then resuspended in a solution of 20 mg/ml yeast tRNA (500 μg tRNA per μg probe). Nucleic acids were denatured by heating for 7-10 min. at 95°C and added directly to hybridization buffer. Following prehybridization, samples were washed briefly in PBST and transferred to hybridization buffer containing 0.05-0.1 ng/ml hydrolyzed probe. Hybridization was carried out for 12-15 hours at 42°C. The next day, samples were washed once in PBST at room temperature and once in PBST at 37°C, both for 30 min. with agitation. To remove unhybridized probe, samples were digested in 100 μg/ml RNase A in PBS for 20 min. at 37°C. After RNase digestion, samples were washed in PBS at 37°C, 1X SSC at 45°C, 0.1X SSC at room temperature, and finally in PBST at room temperature, 30 min. per wash with agitation. For

detection, samples were blocked in 5% goat serum in PBST for 30-60 min. at room temperature and then incubated in PBST containing a 1:200 dilution of alkaline phosphatase conjugated anti-dig Ab, Fab fragment (Boehringer Mannheim Biochemicals), 5% goat serum and 5% mouse serum for 1-3 hours. Samples were subsequently washed several times in PBST for at least 2 hours, twice briefly in Tris buffered saline + 0.1% Tween-20 (TBST) and twice in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20, 1 mM levamisole). Stained samples were developed in alkaline phosphatase buffer containing 338 μg/ml nitro blue tetrazolium (NBT) and 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (X-phosphatase), both purchased from Boehringer Mannheim Biochemicals, for 3-4 hours at room temperature in the dark. Colorimetric reactions were monitored periodically and stopped by transferring samples to PBST + 1 mM EDTA. Detection was highly dependent on the amount of time in which samples were developed. Overdeveloping led to a dramatic increase in background staining. Samples were finally dehydrated in graded ethanols, transferred to 2-propanol, cleared in xylene, and mounted into glass slides under coverslips and observed under bright field microscopy.

Antibodies

Rat anti-mouse IL-2 mAb (S4B6), phycoerythrin (PE)-conjugated antimouse αβ TcR mAb (H57), and biotinylated hamster-anti mouse CD3ε (500.AA2) and γδ TcR (GL3) mAbs were purchased from Pharmingen (San Diego, CA). Normal rat IgG was reconstituted at a concentration of 20 μg/ml (Miles Laboratories, Inc.). Fluorescein (FITC)-conjugated hamster anti-mouse CD3ε (145-2C11) and PE- and biotinylated rat anti-mouse IL-2Rα (AMT-13) were obtained from Boehringer Mannheim Biochemicals and GIBCO/BRL (Gaithersburg, MD), respectively. Lastly, biotinylated secondary reagents, rabbit anti-rat IgG was purchased from Vector Laboratories (Burlingame, CA) and goat anti-hamster IgG from CALTAG Laboratories (San Francisco, CA).

Immunohistochemistry and Direct Immunofluorescence Staining

Protocols for immunohistochemical and immunofluorescence staining were essentially unchanged from those described in (12). It should be noted that for immunohistochemistry, the avidin-biotin blocking step was omitted, all antibody incubations were carried out overnight at 4°C, and washes were increased in 1-2 hours at 4°C. Histochemical detection was performed using an avidin-biotin-horseradish peroxidase complex (Vectastain *Elite* ABC, Vector Laboratories) and metal enhanced diaminobenzidine (DAB + 30 μg/ml Ni- and $CoCl_2$).

RESULTS

Developmental Pattern of IL-2 Gene Expression in the Mouse Epidermis as Determined by *In Situ* Hybridization.

We initially identified IL-2 mRNA positive cells in the dermis following *in situ* hybridization to IL-2-specific ^{35}S-labeled RNA probes (Figure 1). These positive cells were clearly not associated with the epidermal layer. However, the reproducible detection of IL-2 mRNA positive cells associated with the dermis raised the possibility that these IL-2 mRNA expressing cells were perhaps migrating to the epidermis to populate the skin. Subsequent analysis of IL-2 mRNA accumulation in the epidermis of older mice revealed several interesting results (Figure 2). At three weeks of age several large areas of IL-2 expression was found in ear epidermal sheets (Figure 2A), as revealed by ^{35}-S *in situ* hybridization. The high concentration of silver grains observed in 3-week old samples with the antisense strand were not detected in similar samples hybridized with the complementary sense control strand (Figure 2B). At this age, $V_\gamma3^+$ cell populations are still expanding in the skin. In contrast, by 5 weeks of age when $V_\gamma3^+$ cell numbers have reached steady state (16, 17), zones of IL-2 expression were not observed (data not shown). Likewise, IL-2 expression was not detected in samples isolated from 14-week old mice (data not shown). These results indicate that IL-2 expression is induced at stages in life when $V_\gamma3^+$ cells are presumably still increasing, whereas once $V_\gamma3^+$ cells are no longer proliferating, IL-2 expression appears to be downregulated.

In the hope of increasing the sensitivity of detection, we employed a modified "whole mount" *in situ* hybridization procedure using dig-labeled probes to examine IL-2 message in unmounted, free-floating epidermal samples. In accord with the results described above, at 3 weeks of age, extensive areas of IL-2 mRNA accumulation was observed using a dig-labeled IL-2 antisense RNA probe, as indicated by large zones of deep purple staining along with distinct, positively staining cells with dendritic morphology (Figure 2C). No significant staining was observed using a dig-labeled sense RNA probe (Figure 2D). Moreover, this pattern of expression was not detected in older animals, in good agreement with the hybridization results

generated using ^{35}S-labeled probes (Figure 2E-H). A compilation of the hybridization data is given in Figure 3. The combined results indicate that IL-2 expression drops off and is undetectable by 5 weeks of age. Thus IL-2 mRNA expression in the skin is transient and may be correlated with the period during which $V_\gamma3^+$ cell numbers are increasing to populate the skin.

Distribution of IL-2 Expressing Cells in the Skin in Similar to that of CD3$^+$ Cells at 3 Weeks of Age

After having established the pattern of IL-2 expression in the skin, we were interested in defining the distribution of CD3ε^+ cells in the skin at a time when IL-2 was expressed therein. At 3 weeks of age, the pattern of IL-2 gene expression as revealed by dig-*in situ* hybridization was strikingly similar to that of CD3$^+$ cells as shown by immunohistochemical staining (Figure 4). Using both techniques on ear epidermal sheets isolated from the same animal, we found extensive reactivity associated with hair follicles as well as with scattered cells in the proximity of follicles at this age and the significance of the association will be discussed later. CD3ε staining concentrated in follicles in ear epidermal sheets isolated from normal mice did not appear to be artifactual. Comparable staining was not seen in samples isolated from age matched SCID mice which do not have mature T cells populating peripheral tissues (see Figure 7A) and hair follicles did not stain in samples taken from older wild-type animals; by 5 weeks of age, only a "confluent" distribution of scattered positively staining cells was observed (data not shown). A strong correlation between IL-2 expression and CD3ε^+ cells associated with hair follicles can be drawn, as shown in Figure 5. It is evident that CD3ε^+ cells were distributed throughout the skin in such a manner that closely reflected the distribution of areas where IL-2 expressing cells were detected. The similarities between IL-2 expression and the distribution of CD3$^+$ cells provides tantalizing circumstantial evidence that IL-2 expressing cells are indeed CD3ε^+. It should be mentioned that we could not detect high levels to $\gamma\delta$ TcR expression at this time. Nevertheless, by 5 weeks of age, $\gamma\delta$ cells were easily detected (see Figure 8C), hence it is

possible that these T-lineage cells are not expressing high levels of TcR at a time when they are proliferating.

Pattern of IL-2 Protein Production in the Skin at Different Ages as Determined by Immunohistochemistry

To confirm the pattern of IL-2 expression as revealed by *in situ* hybridization, we stained ear epidermal sheets from age matched littermates with an anti-IL-2 mAb (Figure 6). At 3 weeks of age, distinctive patches of IL-2 reactivity were observed (Figure 6). Occasionally, isolated IL-2 reactive cells were also detected (data not shown). These areas of immunohistochemical staining were similar in appearance to those revealed by *in situ* hybridization (compare Figure 1 with Figure 5). Slight differences were noted between IL-2 mRNA versus protein accumulation as a function of age. While IL-2 mRNA accumulation in the skin was not observed beyond 5 weeks of age, weakly staining zones of IL-2 reactivity associated with follicles were still detected (Figure 5C). However, by 5 weeks, there was an approximately five to tenfold reduction of IL-2 positive zones compared to the number seen at 3 weeks (data not shown), and about 14 weeks, a twenty- to fiftyfold reduction relative to that seen at adolescence was observed (Figure 5E). Moreover, in younger animals, IL-2 mRNA positive zones appeared to be slightly more prevalent than IL-2 protein positive zones (1.5 to 2 times more abundant; data not shown). Thus in 3 week old mice, even though IL-2 mRNA accumulation is apparently synchronous, this phenomenon may not be reflected at the level of protein production. It is also possible that detection of IL-2 mRNA positive cells by dig- *in situ* hybridization is more sensitive than that of IL-2 protein positive cells by immunohistochemistry.

IL-2 Expressing Cells Are Not Found in the Epidermis of SCID Mice

Due to a defect that interferes with productive TcR gene rearrangement, mice homozygous for the SCID mutation are unable to produce mature T cells (18). Manifestation of the SCID defect is evident in the skin in which both CD3ε and γδ TcR$^+$ cells are absent (Figure

7A, B; data not shown). To determine if the SCID mutation affects IL-2 expression in the skin, *in situ* hybridization and immunohistochemical staining were performed in parallel on ear epidermal sheets isolated from 3-week old SCID mice and epidermal sheets taken from age-matched normal animals. The results obtained using SCID mice were significantly different than those obtained using normal animals. Whereas *in situ* hybridization of normal ear epidermal sheets to a dig-labeled antisense IL-2 RNA resulted in extensive zones of staining (as described in a previous section), no appreciable hybridization was detected above background in the SCID epidermis, i.e., there were no observable differences in hybridization patterns, using antisense or sense probes (Figure 7, C, D). Likewise, immunohistochemical staining of epidermal sheets isolated from 3-week old SCID mice yielded results that recapitulated those obtained by *in situ* hybridization (Figure 7, E, F). IL-2 protein in the epidermis was not detected in SCID animals at this age, nor was IL-2 protein detected in the skin of older animals (data not shown). The SCID mouse findings indicate that IL-2 expression in the skin requires the presence of $CD3\varepsilon^+$ cells therein, suggesting that either $CD3\varepsilon^+/TcR^+$ cells themselves are producing IL-2 or that the induction of IL-2 expression in an unidentified cell type is dependent on the presence of $CD3^+$ cells.

Detection of IL-2 Protein in the Skin of αβ-TcR Transgenic Mice

Most T cells found in the skin express an invariant γδ TcR and are defined by the usage of the $V_\gamma 3$ gene segment in γ-chain gene rearrangements. It was recently reported that mice that do not generate γδ T cells due to targeted disruption of at the δ-chain gene locus still retain a small number of $\alpha\beta\text{-}TcR^+$ cells associated with the epidermis (19). Although these rare αβ T cells found in the skin are detectable in wild-type animals, we were unable to detect this subset under our staining conditions (Figure 8A). Nevertheless, it was still of interest to establish whether there was a relationship between IL-2 expression in the epidermis and the expression of γδ- versus αβ-TcR in resident T-lineage cells. Using mice expressing a transgenic αβ TcR (10, 11), ample number of $\alpha\beta^+$ T cells were detected in the skin (Figure 8B). There were no significant

differences observed in H-2^s (nonselecting) versus H-2^b (positively selecting) backgrounds (data not shown). Albeit that cells expressing γδ TcR were detected in the thymus both immunohistochemical staining and fluorescence activated cell sorting analysis (data not shown), γδ TcR^+ cells were undetectable in the skin of αβ TcR transgenic mice, suggesting that the T cells observed here only use the αβ TcR (Figure 8D). In terms of CD3ε expression (Figure 8E, F), IL-2 protein production (Figure 8G, H) the distribution of $CD3\varepsilon^+$ cells and the pattern of IL-2 protein accumulation in the skin of TcR transgenic mice was indistinguishable compared to that seen in a wild-type littermate. Therefore, IL-2 is produced in the skin even in the apparent absence of γδ/$V_\gamma 3$ expressing cells, indicating that αβ TcR^+ T cells in the epidermis are capable of producing IL-2 and induction of IL-2 expression in the skin may not require a specific TcR-antigen interaction.

DISCUSSION AND CONCLUSIONS

The results presented here provide new insights into the potential function of IL-2 *in vivo* in mediating the expansion of T-cell populations in the epidermis. *In vitro* findings suggest that IL-2 influences the generation of $V_\gamma3^+$ cells (6, 7). Significantly, this lineage is found in strict association with the murine epidermis (γδ T-cell development has been recently reviewed; 20, 21). Moreover, IL-2 is expressed in the fetal thymus at the point in gestation when $V_\gamma3^+$ cells are being generated (12). Although an absolute correlation between IL-2 expression and $V_\gamma3$ cells in the fetal thymus was not established (12), this is not the case in the postnatal skin. Even at birth, isolated IL-2 expressing cells are reproducibly found in the dermis in a similar location to that in which $CD3\varepsilon^+$ cells are found (data not shown). By three weeks after birth, IL-2 expressing cells are readily detected and IL-2 and CD3ε expression appear to colocalize. At this age, the majority of IL-2 and CD3ε expressing cells were found associated with hour follicles. A few individual IL-2 mRNA positive cells not in direct contact with hair follicles were detected, but as a general rule, the number of IL-2 expressing cells found in the skin was inversely proportional to the distance from particular hair follicles with which IL-2 expressing cells were associated. The association of both IL-2 and CD3ε expressing cells with hair follicles is age dependent. By the time IL-2 expression was no longer detected at 5 weeks of age, $CD3\varepsilon^+$ cells were no longer found associated with hair follicles and were evenly dispersed throughout the skin. Assuming that IL-2 expressing cells are also $CD3\varepsilon^+$, we propose a dynamic model for the mechanism by which IL-2 gene expression is induced in the skin at the time when T cells are still proliferating therein. Rare IL-2 expressing $CD3\varepsilon^+$ cells move through the dermis and home to hair follicles. Following contact with follicles, IL-2 expression is upregulated and $CD3\varepsilon^+$ cells proliferate in the basal layer. As $CD3\varepsilon^+$ cells migrate up through and away from hair follicles to populate the epidermis, IL-2 expression is downregulated. This implies that an unidentified cell type found in hair follicles may be responsible for upregulating IL-2 gene expression in this particular T-cell population. Once T cells are no longer in contact with resident cells in the follicle, T-lineage cells do not receive the necessary stimulus to induce IL-2 gene expression.

IL-2 protein is also detected in the skin at three weeks of age, showing that IL-2 is available to resident cells to use as a proliferation signal. A slight discrepancy between the distribution of IL-2 mRNA positive zones and IL-2 protein rich areas in the skin was noted. Extensive areas of IL-2 reactivity associated with follicles were detected, but unlike the pattern of staining obtained by dig-*in situ* of epidermal sheets taken from 3-week old mice showing individual IL-2 mRNA positive cells in between areas of high IL-2 mRNA expression, rare, individual cells producing IL-2 protein not in direct association with follicles were detected. This suggests that the immunohistochemical staining method employed may no have been sensitive enough to stain individual cells producing IL-2 protein and that areas in which a gross excess of IL-2 protein was being synthesized were reactive under these staining conditions. Another possibility exists to account for the variation in expression patterns generated by *in situ* hybridization and immunohistochemical staining. The individual IL-2 mRNA positive cells not in direct contact with hair follicles could be migrating away from follicles to populate the epidermis and the IL-2 message detected in migrant cells may represent residual accumulated mRNA that was synthesized during the period in which IL-2 inducible cells were in contact with follicles. Like most cytokine messages, IL-2 mRNA is unstable (22), therefore upon dissociation from follicles, IL-2 inducible cells would no longer be receiving stimulus to transcribe the IL-2 gene and the remaining IL-2 transcripts could be rapidly degraded prior to translocation into protein. Additionally, this explanation would also account for the transcience of IL-2 gene expression observed in the skin.

The generation of mature T cells and their presence in the skin appears to be essential for IL-2 gene expression observed therein. In SCID mice, neither CD3ε^+ cells not IL-2 expressing cells were found associated with the epidermis, strengthening the notion that IL-2 expressing cells in the skin are also CD3ε^+ T cells. We cannot formally dismiss the possibility that an unknown non-T lineage cells type is induced by T cells to produce IL-2, but based on the cell-type specificity of IL-2 gene expression that only cells of the T lineage are inducible for IL-2

gene expression, and on studies showing that Thy-1/CD3ε$^+$ DECs are inducible for IL-2 gene expression *in vitro* (8, 9), we regard this explanation to be unlikely.

In terms of signals involved in inducing IL-2 gene expression in the skin, our findings in αβ TcR transgenic mice indicate that IL-2 expression in the skin does not absolutely depend on the expression of $V_\gamma3^+$ γδ TcR *per se*. Previous studies have demonstrated that $V_\gamma3$ expression was not requisite for the generation of T cells in the skin, since introduction of an irrelevant transgenic γδ TcR has no obvious effects on the development of γδ T cells associated with the epidermis (2, 5). Indeed our studies using αβ TcR transgenic mice indicate that T cells were still found associated with the skin, though they expressed αβ rather than γδ TcRs. It is unclear whether the αβ T cells in the αβ TcR transgenic animals are actually cells of the γδ lineage that expressed αβ TcR in lieu of endogenous γδ TcR or whether CD3ε$^+$ DECs in these mice represented an expanded population of *bona fide* αβ T-lineage cells. Irrespective of the origin/lineage of αβ$^+$ DECs cells in αβ TcR transgenic mice, resident cells were still induced to express IL-2, suggesting that IL-2 is expressed by T cells in the skin independent of αβ or γδ TcR expression and that induction of IL-2 gene expression in the skin may not be specified by engagement of a particular TcR (in this case $V_\gamma3$-containing γδ TcRs) with an undislased ligand.

Since the evidence indicates that DECs expressing proper $V_\gamma3^+$ γδ-TcRs or "inappropriate" αβ-TcRs can be induced to express IL-2 independent of TcR specificity *in vivo*, this raises important questions with regard to the activation of DECs resulting in the induction of IL-2 gene expression in the apparent absence of an overt inflammatory/primary immune response. Several provisional pieces of evidence suggest that under normal circumstances, cells in the $V_\gamma3^+$ lineage may not be expressing multisubunit form of high affinity IL-2R at a stage when they are proliferating in response to IL-2. Both fetal thymic organ and fetal thymocyte suspension culture showed preferential expansion of $V_\gamma3^+$ subsets only in high doses of IL-2. If $V_\gamma3^+$ cells at this stage were expressing high affinity receptors for IL-2, they should have expanded *in vitro* in response to much lower concentrations of IL-2. Likewise, while IL-2 is abundantly expressed in the skin at 3 weeks of age, little high affinity IL-2R expression was

detected as evinced by the lack of staining by an anti-IL-2Rα mAb (Figure 9A). As a positive staining control, fetal thymus sections were subjected to immunohistochemical staining for IL-2 and IL-2Rα expression (Figure 9B). IL-2Rα was readily detected in these samples (Figure 9B). No good staining reagents exist for the rapid identification of moderate affinity IL-2R, thus we cannot unequivocally show that DECs are expressing functional IL-2R at a time when IL-2 expression in the skin is maximal. Nevertheless, induction of IL2Rα is indicative of the activation of mature T cells to foreign antigen. IL-2Rα expression is crucial for the formation of the high affinity IL-2R that mediates the rapid clonotypic expansion of antigen-specific T cells that is vital for the success of the vertebrate immune response. The lack of IL-2Rα expression while IL-2 itself is expressed in the skin indicates a potential uncoupling of high affinity receptor and ligand expression. This implies that high affinity IL-2Rα expression may not be involved in the gradual, IL-2 driven expansion of T-cell populations in the tissues where they reside, as demonstrated by the differential regulation of IL-2 and IL-2Rα in DECs during a period in which this population is still expanding in the skin.

In conclusion, we have shown that IL-2 is expressed in the epidermis of mice at a time when resident T cells are still expanding. IL-2 expression appears to be induced in the absence of an immune response against antigen and IL-2 may participate in the post-thymic expansion of T-cell subsets *in vivo* in the absence of acute inflammation. Although IL-2 is not necessary for the expansion of pre-T cells in the thymus, IL-2 may function as a general growth factor for T cells in the periphery. IL-2 may be required for achieving and maintaining steady-state numbers of T cells in peripheral tissues. In IL-2 -/- mice, defects were observed in peripheral lymphoid organs in which high numbers of T cells are normally found as manifested as a decrease in cellularity, implying that IL-2 may be involved in T-cell homeostasis of the nonactivated immune system (23). Finally, our studies using normal and SCID mice strongly suggest that IL-2 expressing cells in the skin belong to the T lineage. Up to this point, little is known about the regulation of IL-2 expression in DECs. NFAT, an inducible transcription factor in T cells, is absolutely required for transcriptional activation of the IL-2 gene. A recent study using an

oligomerized NFAT binding site to drive SV40 T-antigen expression in transgenic mice revealed that constitutive NFAT binding activity present in the skin (24). Phenotypic analysis of the cells in the epidermis displaying this characteristic was not performed, but a tantalizing possibility exists that DECs cells in their resident tissue may be constitutively expressing high levels of functional NFAT and this may facilitate the coordinate induction of IL-2 gene expression in DECs as they proliferate in the skin, contribute to the regulation of IL-2 expression in DECs. Likewise, potential constitutive NFAT binding activity in DECs may be peculiar to this lineage. Identification of additional cell types able to induce IL-2 expression in DECs and detailed examination of the molecular mechanisms involved in controlling IL-2 gene expression in this T-cell subset will provide new avenues for further research and increase understanding of the function, role and regulation of IL-2 expression *in vivo*.

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FIGURE LEGENDS

FIGURE 1. Identification of IL-2 mRNA positive cells in the dermis of neonatal mice following *in situ* hybridization of whole body mouse sections to a 410 nt IL-2 specific ^{35}S-labeled antisense RNA probe. Representative field showing an isolated IL-2 mRNA positive cell. Highly positive cells were not detected in serial sections hybridized to the complementary sense-strand RNA (data not shown). Hair follicle (fol).

(A): Bright field, 200X.

(B): Dark field, 200X.

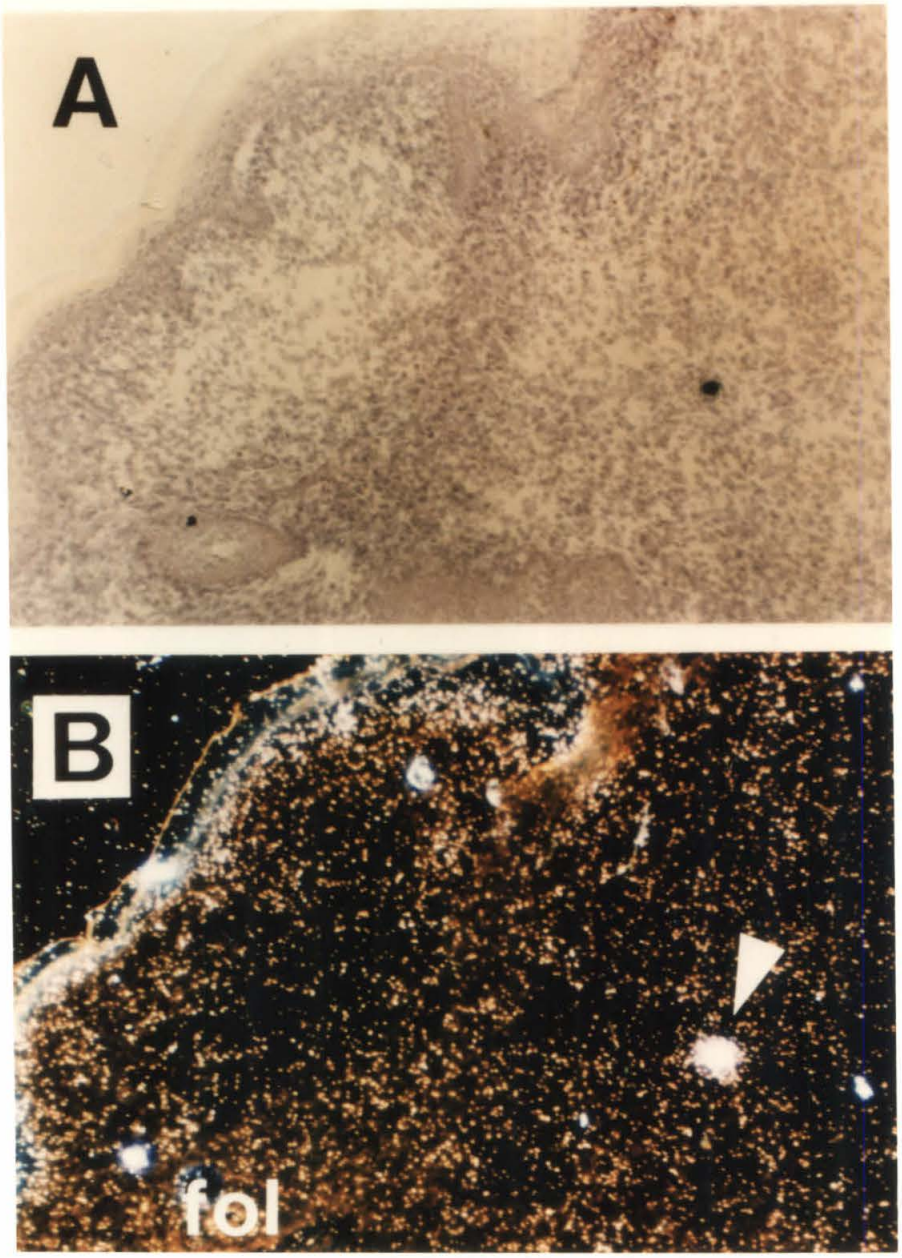

A
B
fol

FIGURE 2. *In situ* hybridization of ear epidermal sheets to 410 nt IL-2 specific ^{35}S- or digoxygenin-labeled RNA probes. (A) and (B) hybridized to ^{35}S-labeled antisense and negative control sense-strand RNAs, respectively. (C), (E), and (G) hybridized to digoxygenin-labeled antisense RNA probes; (D), (F), and (H) to negative control sense-strand RNAs. Representative fields shown. Arrows indicate representative hair follicles.

(A), (B), (C), and (D): Epidermal sheets isolated from 3-week old mice.

(E) and (F): Epidermal sheets isolated from 5-week old mice.

(G) and (H): Epidermal sheets isolated from 14-week old mice.

Bright field, 200X.

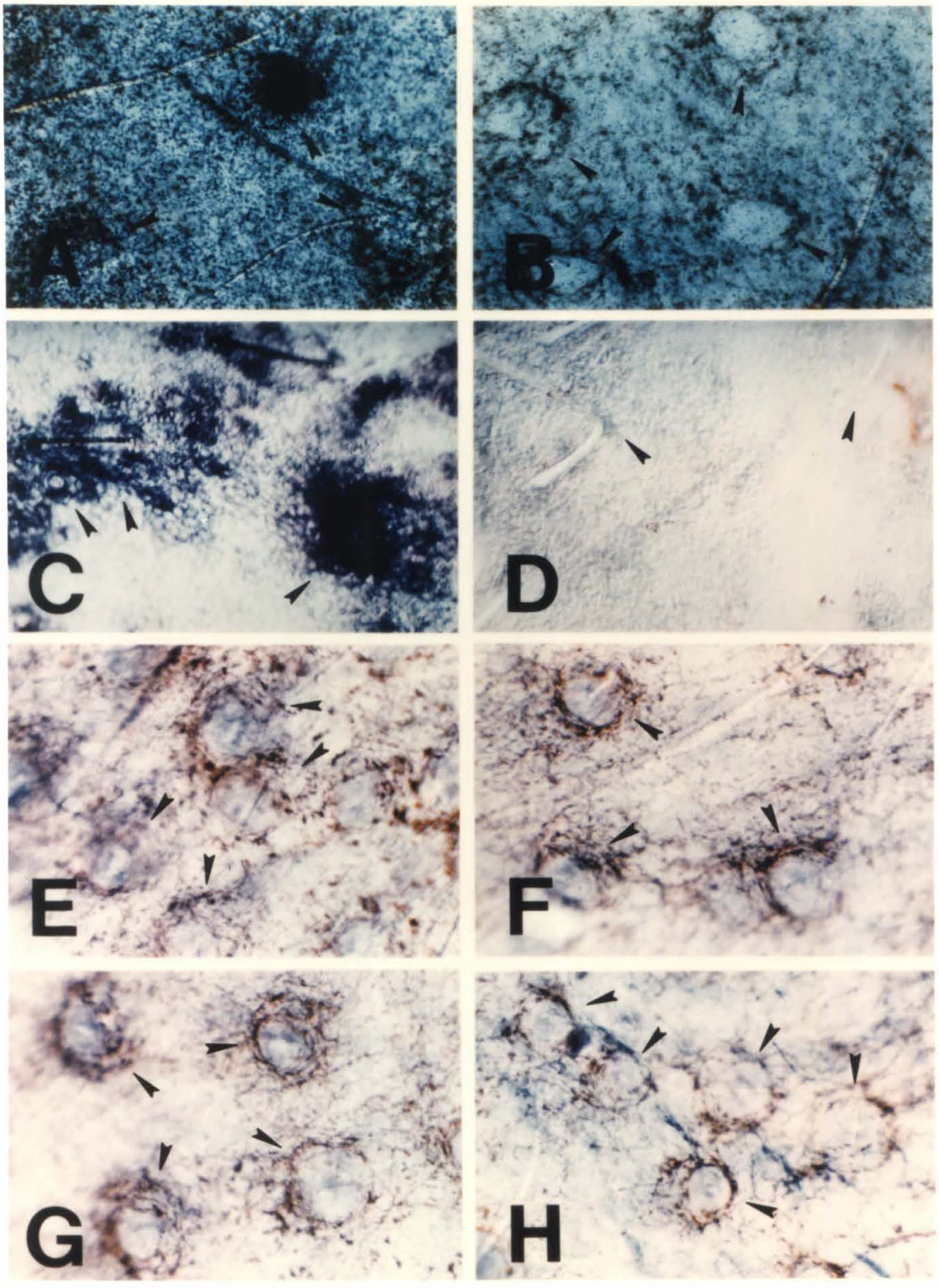

A
B
C
D
E
F
G
H

FIGURE 3. Compilation of ^{35}S- and digoxygenin *in situ* hybridization data using 410 nt IL-2 specific RNA probes to show the development progression of IL-2 gene expression associated with hair follicles in the epidermis. Hybridization to follicles was not detected when epidermal sheets were hybridized to negative control sense-strand RNAs.

IL-2 *IN SITU* HYBRIDIZATION OF EAR EPIDERMAL SHEETS
ON AVERAGE, 500 FOLLICLES FROM RANDOM FIELDS ANALYZED

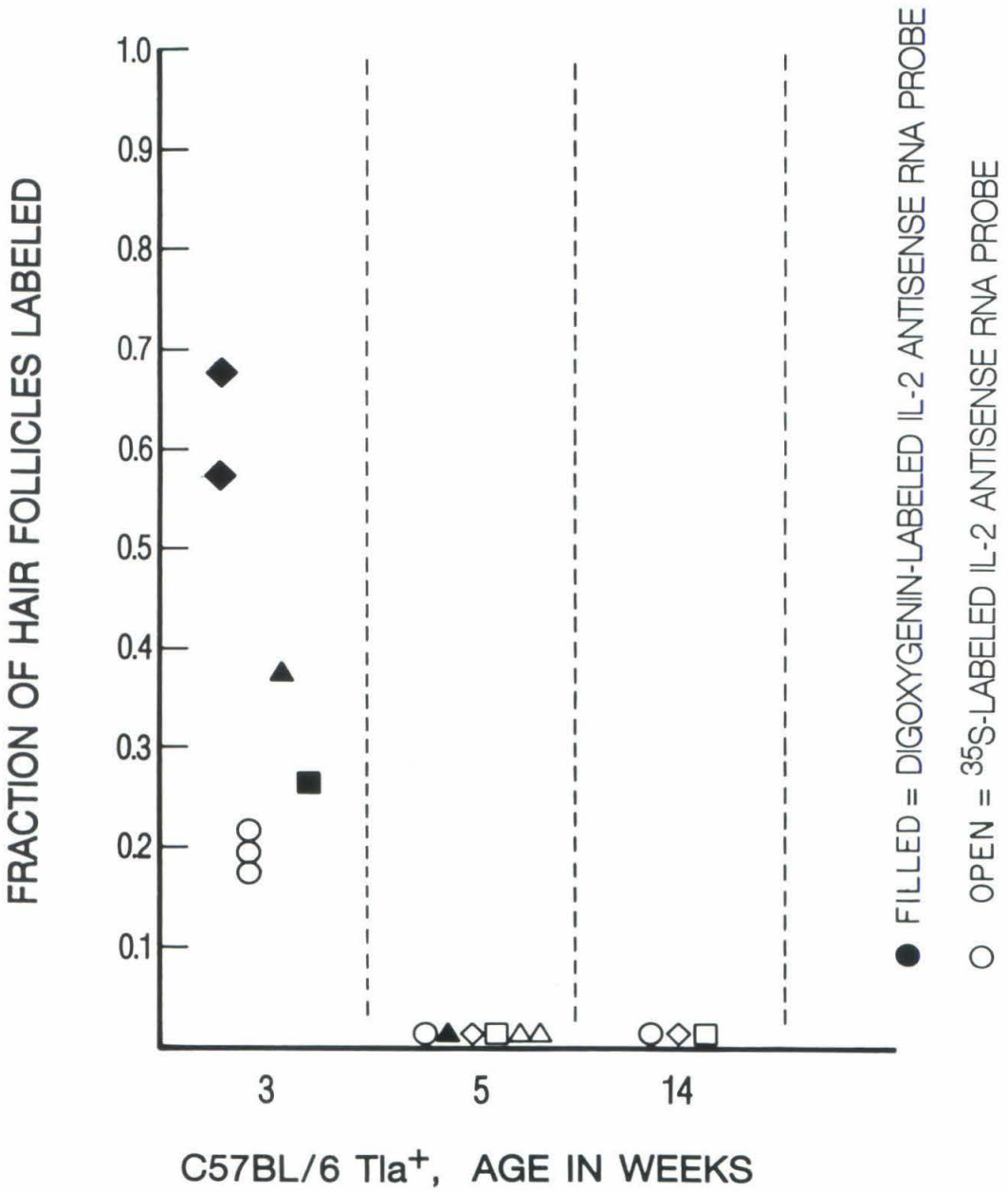


FIGURE 4. Comparative dioxygenin-*in situ* hybridization using 410 nt IL-2 specific RNA probes and immunohistochemical staining for CD3ε expression in epidermal sheets isolated from 3-week old mice. Smaller arrows indicate individual positive cells with dendritic morphology; larger arrows indicate representative hair follicles.

(A): *In situ* hybridization to the antisense RNA probe.

(B): *In situ* hybridization to negative control sense-strand RNAs.

(C): Immunohistochemical staining for CD3ε expression.

(D): Negative control staining, determined by omission of primary antibody.

Bright field, 200X.

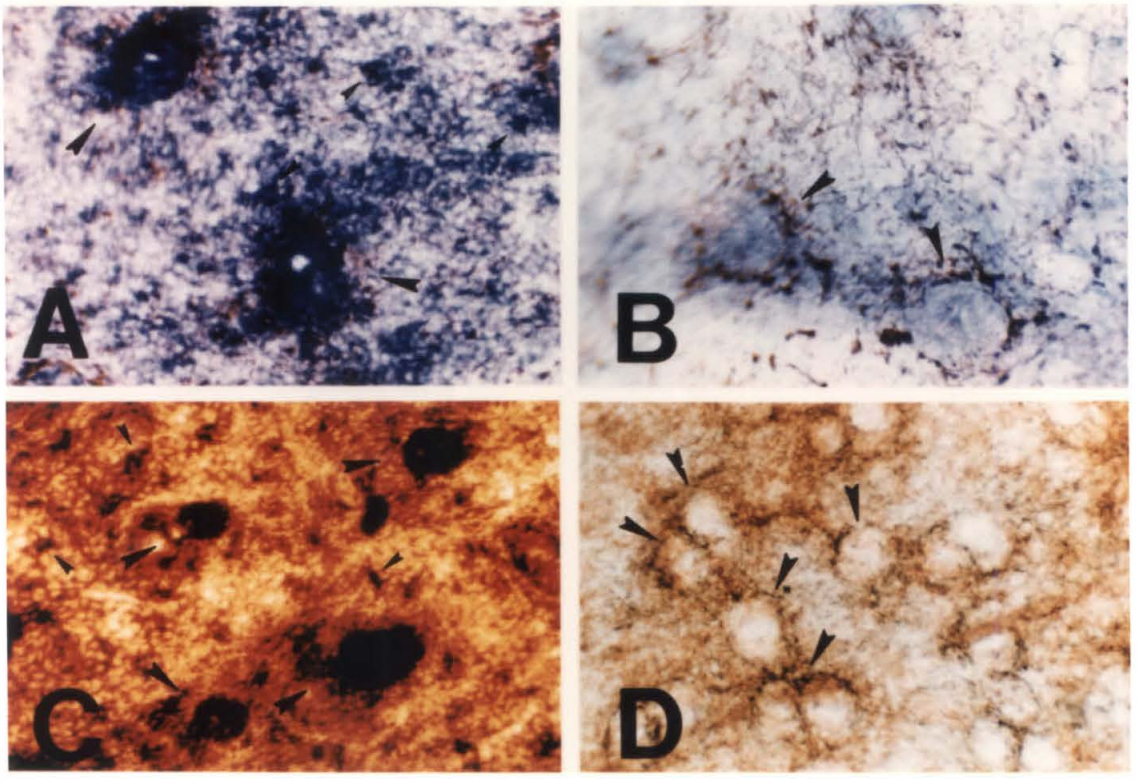

A
B
C
D

FIGURE 5. Diagram demonstrating the correlation between IL-2 mRNA positive hair follicles detected by digoxygenin *in situ* hybridization and CD3ε^{+} stained follicles detected by immunohistochemical staining of ear epidermal sheets isolated from 3-, 5- and 14-week old normal mice and 3-week old SCID mutant mice. Multiple random fields at 100X magnification were analyzed to reduce bias to positively staining regions and an average number of 600 follicles per experimental condition was examined.

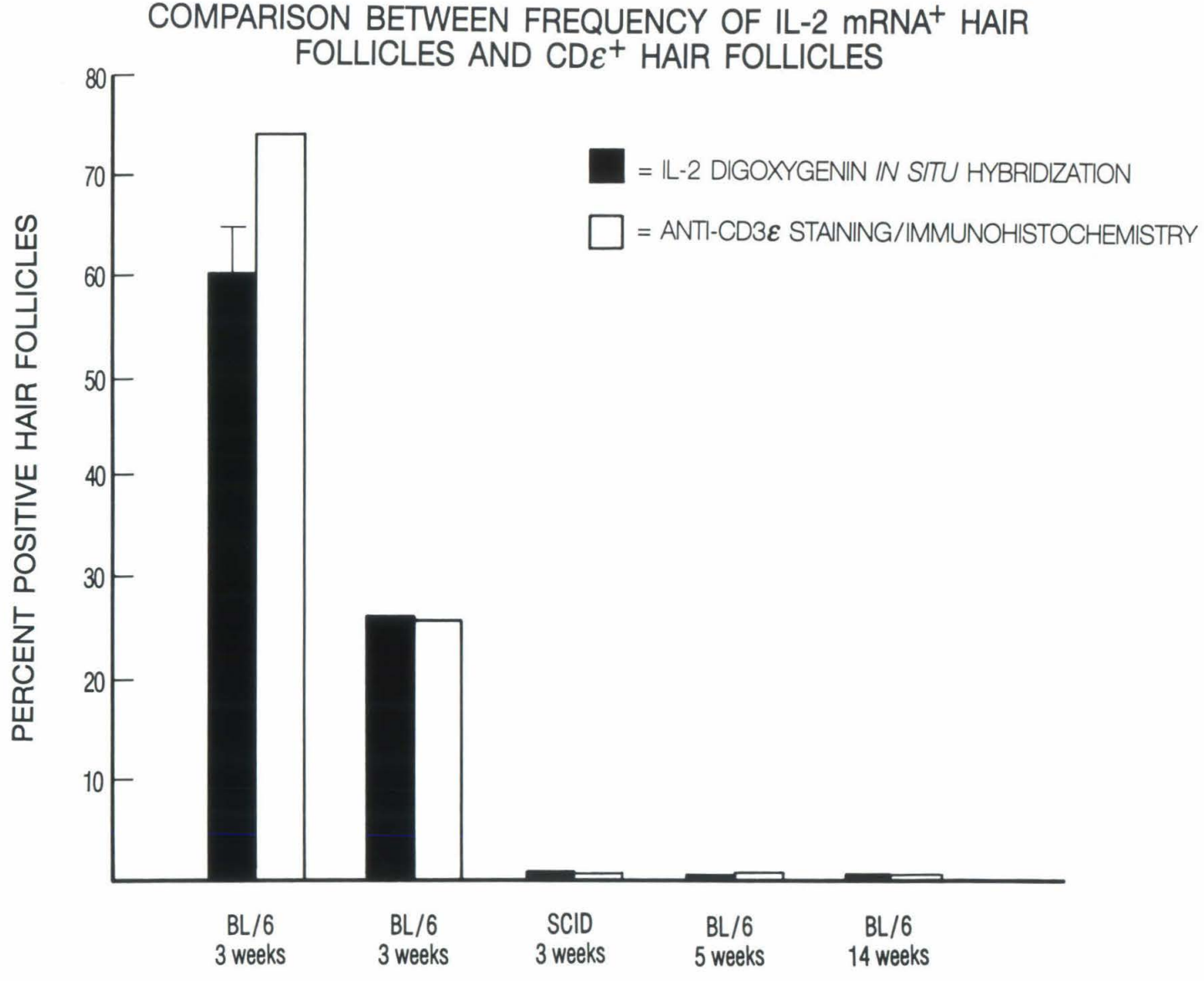

COMPARISON BETWEEN FREQUENCY OF IL-2 mRNA+ HAIR FOLLICLES AND CDε+ HAIR FOLLICLES
PERCENT POSITIVE HAIR FOLLICLES
80
70
60
50
40
30
20
10
= IL-2 DIGOXYGENIN IN SITU HYBRIDIZATION
= ANTI-CD3ε STAINING/IMMUNOHISTOCHEMISTRY
BL/6 3 weeks
BL/6 3 weeks
SCID 3 weeks
BL/6 5 weeks
BL/6 14 weeks

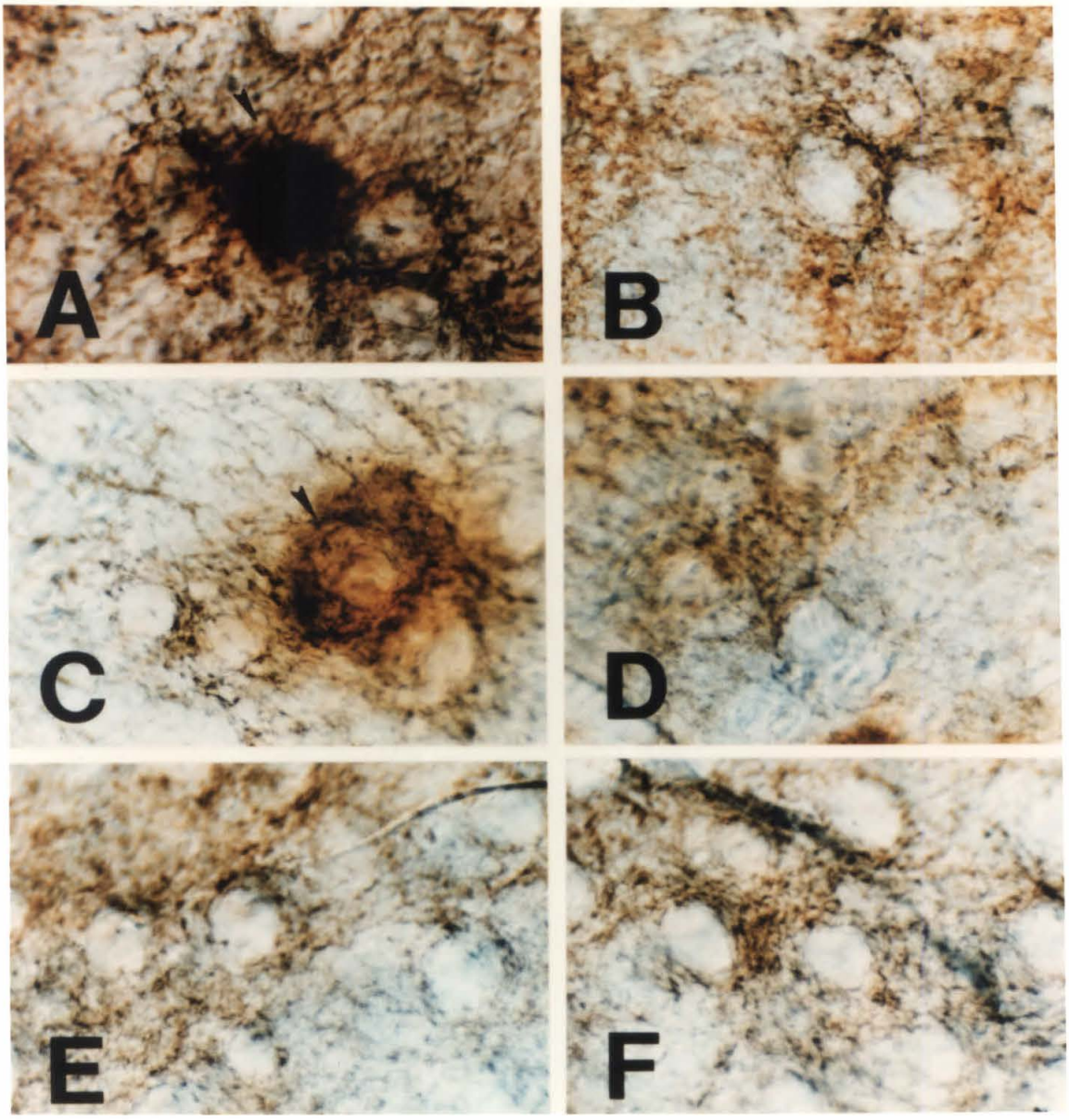
FIGURE 6. Immunohistochemical staining for IL-2 protein in ear epidermal sheets isolated from mice of different ages. (A), (C), and (E) stained with a rat anti-mouse IL-2 mAb. (B), (D), and (F) stained with normal rat IgG. Specificity of the primary antibody was shown previously (12). Arrows indicate reactive follicles positive for IL-2 protein.

(A) and (B): Epidermal sheets isolated from 3-week old littermates of mice shown in Figure 2C-D.

(C) and (D): Epidermal sheets isolated from 5-week old littermates of mice shown in Figure 2E-F.

(E) and (F): Epidermal sheets isolated from 14-week old littermates of mice shown in Figure 2G-H.

Bright field, 200X.


A
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C
D
E
F

FIGURE 7. Absence of CD3ε-specific staining, IL-2 mRNA positive areas, and IL-2 protein production in ear epidermal sheets isolated from 3-week old SCID mutant mice. Data were generated in parallel with those shown in Figure 4. Arrows indicate representative hair follicles.

(A): Immunohistochemical staining for CD3ε expression.

(B): Negative staining control.

(C): Digoxygenin-*in situ* hybridization for a 410 nt IL-2 specific antisense RNA probe.

(D): Hybridization to negative control 410 nt IL-2 sense-strand RNAs.

(E): Immunohistochemical staining for IL-2 protein.

(F): Rat IgG negative staining control.

Bright field, 200X.

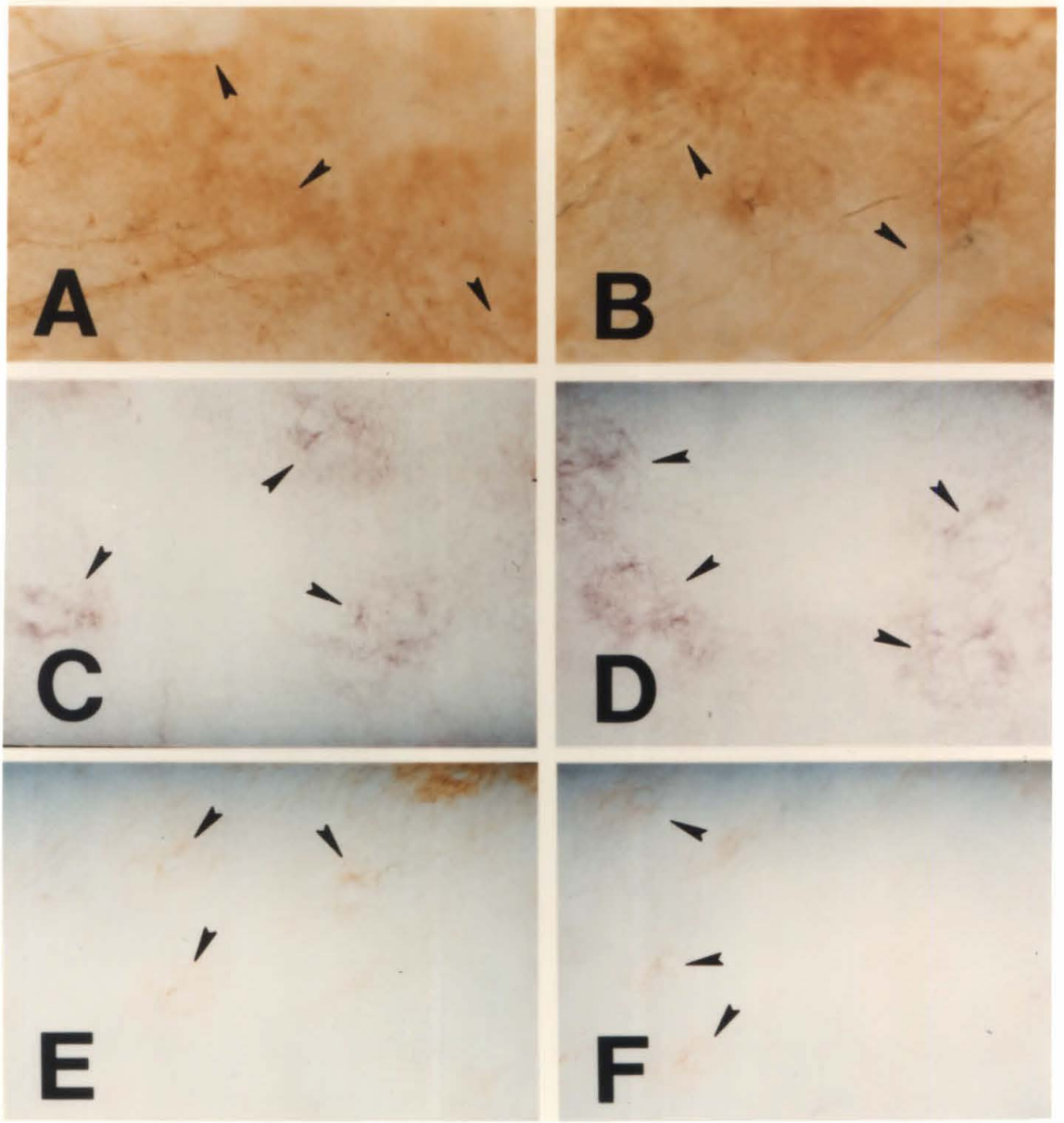
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FIGURE 8. Comparative staining for αβ-TcR, γδ-TcR, CD3ε expression and IL-2 protein of ear epidermal sheets isolated from normal and αβ-TcR transgenic mice. Negative controls are not shown.

(A): Direct immunofluorescence staining for αβ-TcR expression in an epidermal sheet isolated from a 5-week old wild-type littermate.

(B): Direct immunofluorescence staining for αβ-TcR expression in an epidermal sheet isolated from a 5-week old αβ-TcR transgenic mouse.

(C): Immunohistochemical staining for γδ-TcR expression in an epidermal sheet isolated from the same wild-type mouse shown in (A).

(D): Immunohistochemical staining for γδ-TcR expression in an epidermal sheet isolated from the same transgenic shown in (B).

(E): Immunohistochemical staining for CD3ε expression in an epidermal sheet isolated from a 3-week old littermate.

(F): Immunohistochemical staining for CD3ε expression in an epidermal sheet isolated from a 3-week old ab-TcR transgenic mouse.

(G): Immunohistochemical staining for IL-2 protein in an epidermal sheet isolated from the same wild-type mouse shown in (E).

(H): Immunohistochemical staining for IL-2 protein in the epidermal sheet isolated from the same transgenic mouse shown in (F).

(A) and (B): Fluorescence microscopy, 200X.

(C) - (H): Bright field, 200X.

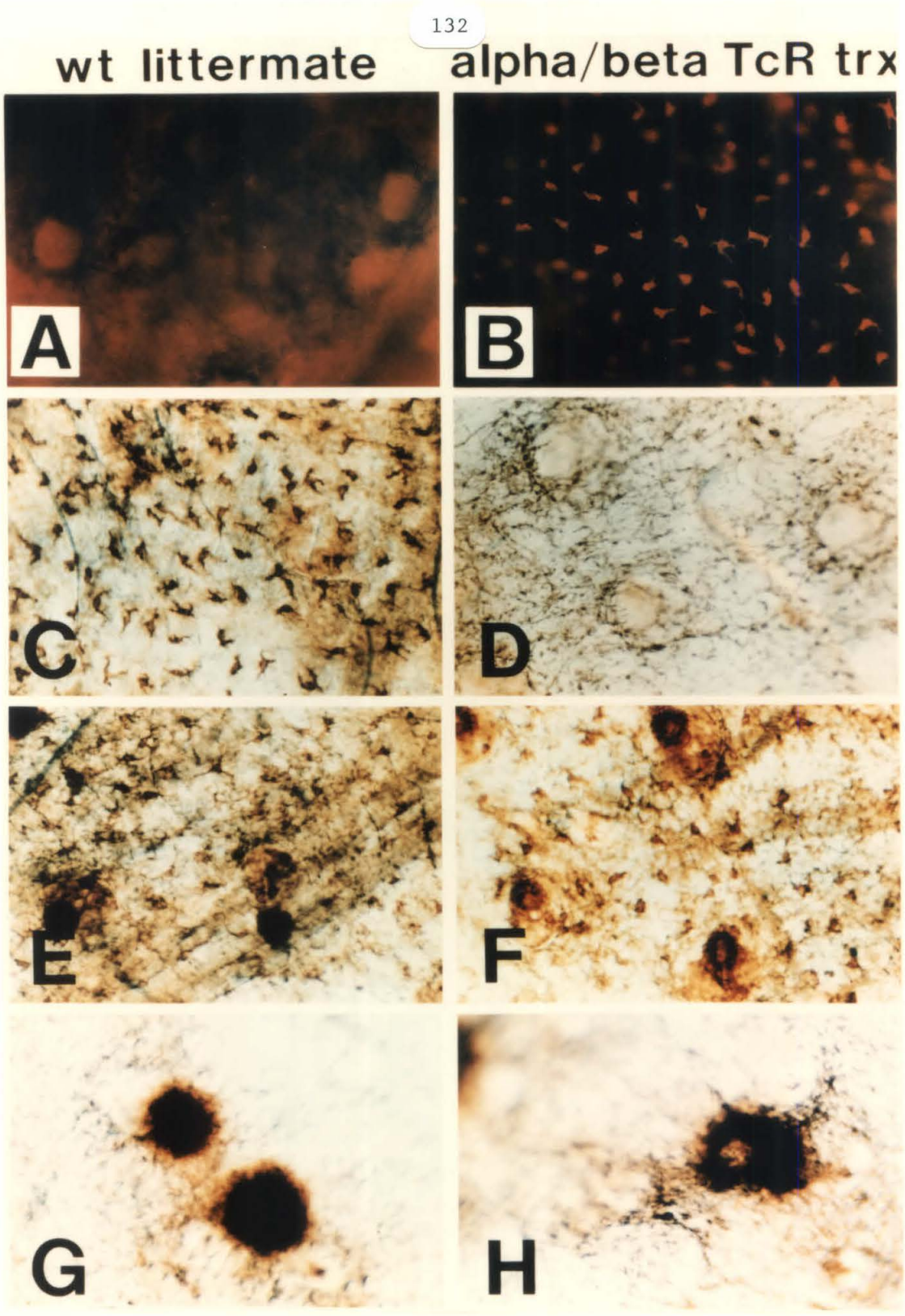

wt littermate
alpha/beta TcR trx
A
B
C
D
E
F
G
H

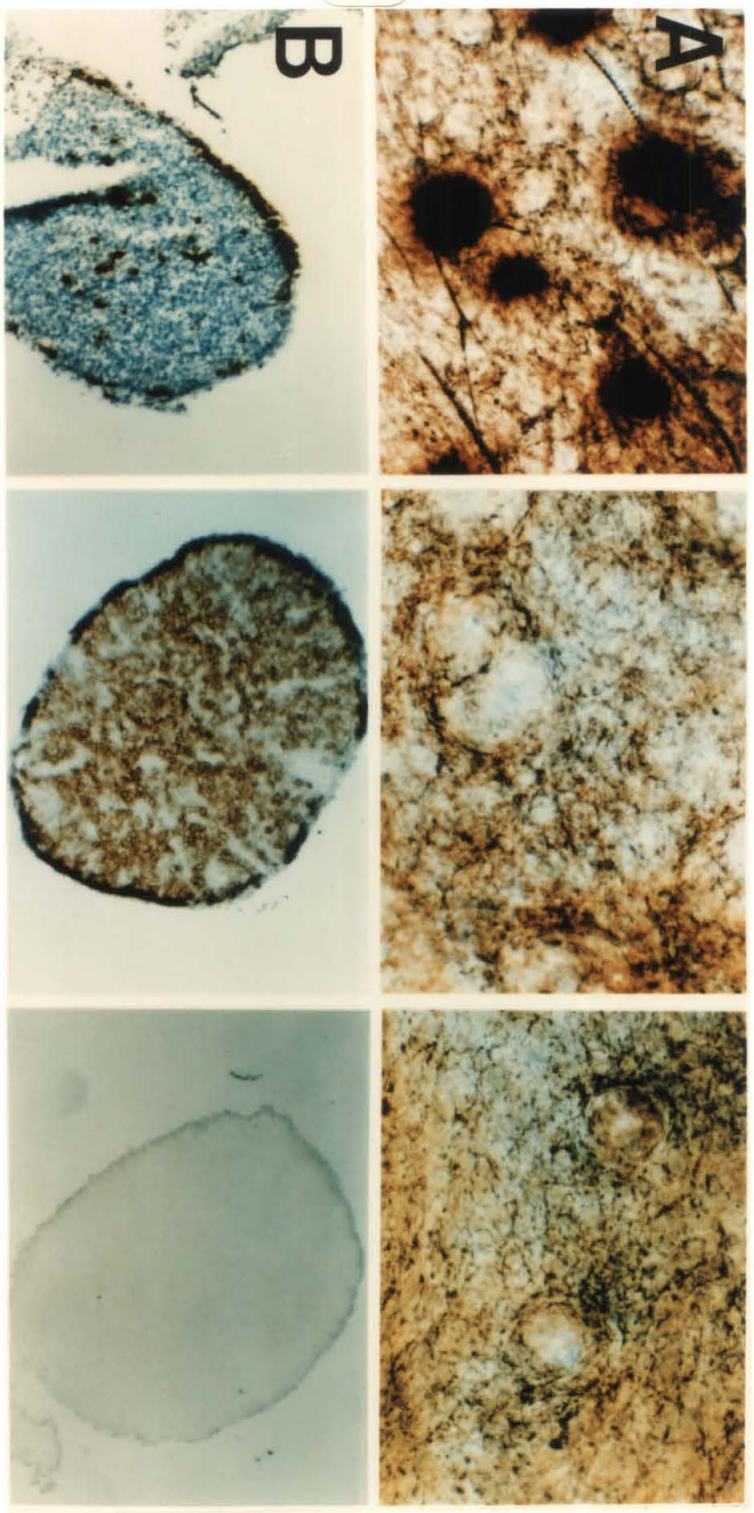
FIGURE 9. Immunohistochemical staining for IL-2 protein and IL-2Rα expression in ear epidermal sheets isolated from normal 3-week old mice and in 8 μm day 15 fetal thymus sections.

(A), left to right: Staining for IL-2, IL-2Rα, and rat IgG control in ear epidermal sheets isolated from 3-week old mice.

Bright field, 200X

(B), left to right: Staining for IL-2, IL-2Rα, and rat IgG control in day 15 fetal thymus sections (positive staining control).

Bright field, 100X.


B
A

CHAPTER 4

Prepared for Publication

Detection of IL-2 Expressing Cells in the Gut of Normal Mice: Evidence that IL-2 is Involved in Controlling Dysfunction in the Mucosal Immune System

SUMMARY

Interleukin-2 (IL-2) is a potent growth factor that mature T lymphocytes produce upon activation by foreign antigen and is used as a proliferation signal, resulting in the clonal expansion of antigen-specific T cells. Analysis of thymocyte populations in IL-2 deficient mice indicated that IL-2 is not necessary for the generation of the major subsets of pre-T cells in the thymus. On the other hand, IL-2 appears to play a role in suppressing detrimental immune responses to intestinal microflora, since the absence of IL-2 causes a disease state resembling ulcerative colitis in humans. Although highly informative, these findings provide only circumstantial evidence that IL-2 is expressed in the gut of healthy animals. We have used *in situ* hybridization and immunohistochemical staining to identify IL-2 expressing cells in the gut of normal mice at various ages. Initially, IL-2 mRNA positive cells were detected in the neonatal gut. Subsequent analysis of IL-2 protein accumulation revealed that numerous IL-2 producing ells were found associated with the gut of newborn animals. IL-2 producing cells were also detected in the lamina propria of the small and large intestine but not in Peyer's patches or intestinal epithelium of older mice and IL -2 production appeared to persist throughout life. Differential distribution of IL-2 producing cells in the small and large intestine was observed, indicating that IL-2 producers in the gut may be involved in regional specialization of the mucosal immune system. Gut-associated IL-2 expressing cells were not found in mice homozygous for the SCID mutation, demonstrating that the generation of IL-2 producing cells is contingent on the generation of mature T and B cells. Finally, a substantial number of IL-2 producers were present in the prenatal gut, hence a large portion of gut-associated IL-2 producing cells are generated during fetal life. Examination of fetal tissues that may be potential sources of IL-2 producing cells in the gut revealed that IL-2 protein positive cells were not found exclusively in the thymus, but were also abundant in the fetal liver and omentum at a stage in gestation when gut-associated IL-2 producing cells were scarce. These data suggest that a subpopulation of intestinal IL-2 producing cells may be extrathymic in origin and raise the possibility that cells other than those belonging to the T lineage may express IL-2 in fetal tissues,

including the gut. Taken altogether, the work we describe here demonstrates that IL-2 expression is indeed induced in the intestine of healthy animals and supports the notion that IL-2 producing cells are available to participate in the prevention of inflammatory bowel disease (IBD).

KEYWORDS: Interleukin-2 (IL-2), T cells, Peyer's patch associated lymphocytes (PPLs), lamina propria associated lymphocytes (LPLs), intestinal epithelial lymphocytes (IELs), severe combined immunodeficiency (SCID), targeted gene disruption, fetal liver, fetal omentum, inflammatory bowel disease (IBD).

INTRODUCTION

The gut can be considered the only region in an organism that is under constant antigenic stimulus. The gastrointestinal tract is extensively populated by T lymphocytes. Not only are T cells found in gut associated lymphoid tissues (Peyer's patches in the small intestine and lymphoid aggregates in the large), but T-lineage cells are also disseminated all along the mucosa and are located in both the lamina propria and the epithelium. T lymphocytes from the Peyer's patches or lymphoid aggregates (PPLs/LALs), in the lamina propria (LPLs) and within the intestinal epithelium (IELs) represent three distinct populations which differ in origin, cellular composition, and functional properties. In terms of ontogeny, the generation of PPLs is generally thymus dependent. On the other hand, development of the majority of IELs is believed to be thymus independent (1, 2), while, the LPL population may be a composite of thymus-dependent and independent cell types (3). Phenotypically, detailed analysis of PPL/LAL, LPL and small and large intestine IEL populations showed that the subsets contained within these populations are not equivalent (4). Finally, with regard to function, PPLs and LPLs are thought to regulate B-cell isotype responses by producing lymphokines, whereas in addition to being able to produce lymphokines, IEL exhibit cytotoxic activity.

Lymphokine production by gut associated T cells has been chiefly characterized using purified T lymphocyte populations *in vitro*. Far less is known about the regulation of lymphokine production by different T-cell populations in the gut and how this contributes to mucosal immunity and immunologic balance of relevant cells therein in normal animals. The immunobiology of gut-associated T cells and the role of specific lymphokines *in vivo* have been examined indirectly by characterization of several pathologic states of disease in humans (5-8) and by genetic analyses of mutant mice rendered deficient in lymphokine production by targeted gene disruption (9, 10).

The absence of interleukin-2 (IL-2) has striking effects on the regulation of the mucosal immune response to intestinal microflora in humans (6, 11) and mice (9). IL-2 is a major mediator of the vertebrate immune response (12, 13). It is a potent T-cell growth factor that

particular subsets of mature T cells produce upon antigenic stimulation. IL-2 mediated events are controlled by interaction of the lymphokine with its cognate multichain receptor (14). IL-2 also participates in B-cell differentiation (15) and promotes macrophage, NK and LAK cell activation. Mice homozygous for a disrupted IL-2 gene, while being immunocompetent, develop normally for the first 3-4 weeks of age, and produce all major populations of thymocytes and have apparently normal proportions of T cells in peripheral lymphoid tissues (16). However, profound defects are observed in older animals (9). Shortly after one month of age, roughly half of IL-2 -/- mice become severely compromised, as manifested by splenomegaly, lymphoadenopathy, and severe anemia, and die within 9 weeks of age. The remaining animals contract inflammatory bowel disease (IBD) resembling ulcerative colitis in humans with 100% penetrance. Characteristics of the disease state include spurious activation of T and B cells, elevated immunoglobulin secretion, and production of anti-colon antibodies. Hence, IL-2 may play a role in suppressing an abnormal immune response to a normal antigenic stimulus in the gut.

Although the results obtained using the IL-2 -/- mice are highly informative, they do not conclusively demonstrate that IL-2 is expressed in normal healthy animals. Likewise, as with regard to whether IL-2 is expressed in the gut, little is known about the distribution of expressing cells therein. Therefore, we have used both *in situ* hybridization to and immunohistochemical staining of gut sections obtained from appropriate tissues of normal and T-cell deficient mice of various ages. Our findings reveal numerous striking features with regard to IL-2 gene expression in the gut. IL-2 mRNA positive cells were first identified in the gut by *in situ* hybridization of neonatal "whole body" sections to an IL-2 specific probe. Additionally, IL-2 protein is readily detected in the newborn gut. IL-2 protein production persists in older animals; IL-2 producing cells were observed in 3-week and 3-month old animals. In older animals, there was conspicuous regional specialization of IL-2 producing cells in the lamina propria of the small and large intestine, and few IL-2 producing cells were found in Peyer's patches, lymphoid aggregates, and in the intestinal epithelium. were reportedly localized in the colon (9), IL-2

producing cells were more prevalent in the small intestine of healthy animals. IL-2 expression in the gut is contingent upon the development of mature T cells, since IL-2 expression was not detectable in gut tissues taken from mice that cannot generate mature T and B cells as a result of the combined immunodeficiency (SCID) mutation. Finally, IL-2 producing cells are abundant in the gut prior to birth, indicating that a high proportion of IL-2 producing cells may be generated during fetal life. Prior to extensive population of the fetal gut with IL-2 expressing cells, IL-2 expression is detected in the fetal thymus and, surprisingly, throughout the fetal liver, the major site of hematopoiesis, and in the fetal omentum. Considering that the induction of IL-2 expression may be a hallmark of future function of cells derived from these particular tissues, these observations suggest that the generation of IL-2 producing cells in the fetal gut may be predominantly thymus-independent and raise the possibility that cell types other than those belonging to the T lineage may express IL-2 in the gut.

MATERIALS AND METHODS

Mice

C57BL/6 Tla$^+$ and C.B.-17-SCID mice were bred and maintained in our own facility. All SCID animals were maintained in an Isotec flexible film isolator (Indianapolis, IN) without antibiotic treatment. Regular monitoring of our colony verified that animals used were pathogen-free (data not shown). Neonatal animals were taken within 12 hours after birth. Fetal tissues were taken from fetuses removed from pregnant BL/6 Tla$^+$ and SCID mothers using timed matings. The appearance of a vaginal plug was designated as day 0. "Germ free" normal animals were weaned from mothers bred under conventional housing and were kept on sterile bedding and food for at least 8 weeks.

Removal and Processing of Tissues

Pre- and neonatal mice were killed by decapitation. Older animals were killed by cervical dislocation. For *in situ* hybridization, whole bodies or separated tissues were washed thoroughly in cold phosphate buffered saline (PBS), blotted dry and immediately embedded in Tissue-Tek O.C.T. compound (Miles Laboratories, Kankakee, IL). 8 μm cryosections were collected and processed according to a published procedure (17). Fixed and washed sections were air dried and stored with desiccant at -80°C prior to use. At least 12 hours before hybridizing, sections were thawed and transferred to 70% ethanol at 4°C. For combined IL-2 and cell surface staining of sections generated from tissues of older animals, pieces of intestine approximately 0.5 cm in length were extensively flushed with cold PBS to remove debris. Flushed segments were cut in half; one half was directly embedded in O.C.T., and the other was fixed in freshly made 4% paraformaldehyde in PBS overnight at 4°C. The following day, fixed tissues were washed in cold PBS for at least 2 hours and then saturated in 30% sucrose in PBS at 4°C. Once saturated (samples descended to the bottom in solution), processed tissues were embedded in O.C.T. Following cryosectioning, both fixed and unfixed samples were soaked in acetone for 20-30 min.

at room temperature. Fixed/refixed sections were air dried and stored with desiccant at -80°C prior to use.

In Situ Hybridization

All probes used have been described previously (17). Either 410 or 590 nucleotide (nt) IL-2 antisense or sense ^{35}S-labeled RNA probes were used. Hybridization was performed essentially as described previously (18). Hybridized sections were dehydrated in graded ethanols containing 0.3 M ammonium acetate, dipped twice in NT-2B Nuclear Track Photography Emulsion (Eastman Kodak, Rochester, NY), and were air dried in a light-tight box. Slides were then transferred to slide boxes containing drierite, sealed with parafilm, wrapped twice in aluminum foil and exposed for 14-21 days. Slides were developed at 12-13°C, coverglasses were mounted, and sections were counterstained with hematoxylin and eosin and analyzed by bright and dark field microscopy.

Antibodies and Staining Reagents

Rat anti-mouse IL-2 mAb (S4B6), and biotinylated hamster-mAbs mouse against CD3ε (145-2C11) and γδ TcR (GL3) were obtained commercially (Pharmingen, San Diego, CA). Biotinylated rat anti-mouse CD4 (H129.19) was purchased from GIBCO/BRL (Gaithersburg, MD) and biotinylated rat anti-mouse CD8α from Becton-Dickinson (Mountain View, CA). Normal rat Ig was reconstituted at a concentration of 20 μg/ml (Miles Laboratories, Inc.). Biotinylated secondary reagents, rabbit anti-rat IgG and goat anti-hamster IgG, both mouse absorbed, were obtained from Vector Laboratories (Burlingame, CA) and CALTAG Laboratories (San Francisco, CA), respectively. Recombinant mouse IL-2 was purchased from Genzyme (Boston, MA).

Immunohistochemistry

Protocol for immunohistochemical staining of non-prefixed sections was similar to that described in (17) except that the avidin-biotin blocking step was omitted, all antibody incubations were carried out for 16-20 hours at 4°C, and washes were increased to 1-2 hours at 4°. For staining of prefixed sections to detect IL-2 protein, several modifications were adapted. Prefixed sections were thawed to room temperature and rehydrated in PBS. Following a brief refix in 1% formaldehyde in PBS (1-2 min.), sections were washed in PBS. Prior to blocking in PBS/1% BSA Fraction V/NaN_3 at 4°C, sections were soaked in PBS + 0.1% Tween-20 (PBST) for 30 min. at room temperature. Blocked sections were incubated in 2-5 μg/ml anti-IL-2 mAb for 16-20 hours at 4°C. For the remaining staining steps, prefixed sections were treated identically to non-prefixed sections. Histochemical detection was carried out using an avidin-biotin-horseradish peroxidase complex (Vectastain *Elite* ABC, Vector Laboratories) and metal-enhanced diaminobenzidine (600 μg/ml DAB + 30 μg/ml 1:1 $NiCl_2$ and $CoCl_2$). For blocking experiments, the primary antibody was incubated with an excess of recombinant mouse IL-2 for at least 12 hours at 4°C before use in staining. Potential aggregates were cleared from the solution by centrifugation.

RESULTS

Identification of IL-2 mRNA positive Cells in the Gut by *In Situ* hybridization of Neonatal Whole Body Sections to an IL-2 Specific Probe

To understand the overall pattern of IL-2 gene expression in the mouse, we generated neonatal whole body mouse sections and hybridized these sections to IL-2 specific RNA probes (Figure 1). Most tissues wre consistently negative for IL-2 expression, including heart, lung, stomach, bladder, kidney, liver, esophagus, spinal cord, and brain (tissues were considered as being negative when no significant hybridization to the IL-2 antisense RNA probe was observed above background as determined by hybridization to the corresponding sense probe). However, we reproducibly found highly positive IL-2 expressing cells in the dermis (Figure 1) and in the gut (Figure 1). IL-2 mRNA positive cells in the gut were predominantly located in the small intestine and associated with villi (Figure 1J-M). These results indicated that IL-2 expressing cells were detectable in the gut of very young postnatal animals.

IL-2 Protein is Readily Detected in the Gut of Neonatal Animals

To reinforce the finding obtained in *in situ* hybridization showing the presence of IL-2 mRNA positive cells in the neonatal gut, we stained sections of isolated neonatal guts with an anti-IL-2 mAb. The results are shown in Figure 2. In our initial attempts to identify IL-2 protein in the neonatal gut, we used acetone fixed sections generated from fresh frozen tissues. This treatment of sections was routinely used for the detection of cell surface antigens. We were unable to reproducibly detect IL-2 protein in the gut when tissue sections were treated in the manner just described (data not shown). However, when immunohistochemical staining was performed on sections obtained from tissues prefixed in 4% paraformaldehyde, IL-2 protein was readily detected without an increase in nonspecific staining using rat IgG in lieu of rat anti-mouse IL-2 mAb (Figure 2). Again, IL-2 producing cells were mostly confined to the villi of the small intestine, but scattered IL-2 reactive cells were also found in the large intestine (data not shown). Finally, the cells expressing IL-2 were characteristically large in size.

When *in situ* hybridization and immunohistochemical staining were carried out in parallel on tissue sections of intestines removed from neonatal littermates, the IL-2 protein producing cells outnumbered IL-2 mRNA positive cells by a factor of at least 20 (data not shown). We can account for this discrepancy in several ways. First, IL-2 mRNA accumulation may be asynchronous relative to IL-2 protein production. The asynchrony in IL-2 message versus protein accumulation was observed in the day 14-15 fetal thymus in which maximal IL-2 mRNA accumulation was observed 12 hours prior to maximal IL-2 production and in the adult thymus where IL-2 protein positive cells are more prevalent than IL-2 message positive cells (17). Another explanation for the difference in numbers between IL-2 mRNA versus IL-2 positive cells focuses on stability of IL-2 mRNA relative to IL-2 protein *in vivo*. It is well documented that the IL-2 message, like many other cytokine mRNAs, is highly unstable ($t_{1/2}$ = 1-2 hours; 19). Therefore, IL-2 message may be degraded rapidly while IL-2 protein may not, resulting on a net decrease in detectable IL-2 mRNA positive cells. This how the number of mRNA positive and protein positive cells sould differ by twentyfold in steady state (i.e., for every cell tht is degrading IL-2 mRNA, another is accumulating message). In addition, this inequivalency is also observed in lymph nodes activated by foreign antigen in which IL-2 mRNA accumulation is not readily apparent but IL-2 protein production is (20). Finally, a rather mundane account for the elevated numbers of IL-2 protein positive cells relative to IL-2 mRNA positive cells is that, in our hands, immunohistochemical staining is more sensitive than *in situ* hybridization in detecting positive cells. Nevertheless, the combined observations made following analysis of *in situ* hybridization and immunohistochemical staining of neonatal gut samples examining IL-2 gene expression indicate that IL-2 is expressed in the intestine of newborn animals and IL-2 protein is present therein.

IL-2 Protein Production in the Gut of Older Mice: Restriction of IL-2 Producing Cells to the Lamina Propria

We were interested in determining whether IL-2 protein was present in the gut of older animals. Therefore, we generated small and large intestine sections treated for immunohistochemical detection of IL-2 protein obtained from tissues of normal 3-week and 3-month old BL/6 Tla^+ mice. Since the morphology of the gut was better defined in tissue sections taken from older animals, it was clear that IL-2 producing cells were restricted to the lamina propria (Figure 3, left panels). Very rare IL-2 reactive cells were found in gut-associated lymphoid tissues or within the epithelium. In general, IL-2 producing cells appeared to be smaller than those observed in the neonatal gut. As a control to demonstrate the specificity of staining, the anti-IL-2 mAb was preincubated with an excess of recombinant IL-2 prior to use. This resulted in a marked decrease in IL-2 reactivity (Figure 3, right panels).

Percentages of IL-2 protein positive cells in the small and large intestine for 3-week and 3-month old animals are given in Table I. At three weeks of age, IL-2 reactive cells represent 2.3% of all the cells in the small intestine villi and 1.2% in the large. By 3 months of age, IL-2 producing cells in the small intestine had doubled, while the number of IL-2 producing cells in the colon remained unchanged. Hence, IL-2 producing cells represent a detectable and defined population in intestinal villi, especially in the small intestine. It was somewhat unexpected that the number of IL-2 producing cells in the small intestine was approximately two- to fourfold greater than that in the large intestine, given that the colon and <u>not</u> the small intestine is largely affected in IL-2 -/- animals (9).

For comparison, the percentages of IL-2 reactive cells found in Peyer's patches and mesenteric lymph nodes are included in Table I. IL-2 producers represent a small subset of cells in these lymphoid tissues. Moreover, IL-2 producing cells were at least 5 times more prevalent in the villi of the small intestine than in Peyer's patches. It should be noted that the percentage of IL-2 producing cells in the small intestine villi represents a lower boundary figure, because the data listed in Table I was based on the number of IL-2 producers detected by

immunohistochemical staining relative to all cells in the villi (this would include a substantial number of epithelial cells). Thus, if cells contained only in the lamina propria were used in these calculations, the number of IL-2 producing cells in the LPL population would be significantly higher. The difference between the number of IL-2 reactive cells in the Peyer's patches and in the lamina propria could reflect the regulation of B-cell Ig production in the gut. It is possible that the decreased number of IL-2 producers in Peyer's patches is an indication that IL-4 expressing cells rather than IL-2 expressing cells predominate in Peyer's patches in which B cells would be chiefly producing IgA and IgG_1. On the other hand, increased numbers of IL-2 expressing cells associated with the lamina propria suggest that IL-2 propria suggest that IL-2 producers may participate in the primary line of defense in the immune response in the mucosa.

To correlate the frequency of IL-2 producing cells relative to defined subpopulations of cells in the gut, we performed parallel immunohistochemical staining on sections generated from the same animal to detect IL-2 production and surface expression of CD3ε, CD4, CD8α and γδ T-cell receptor (TcR). A comparison between IL-2 and CD3ε expression in the small and large intestine of a 3-month old animal is shown in Figure 4. The distribution of IL-2 producing cells in regions of intestine where they reside was highly similar to of the distribution of $CD3\varepsilon^+$ cells in a comparable area. A high concentration of $CD3\varepsilon^+$ cells was found associated with the lamina propria and $CD3\varepsilon^+$ cells were distributed therein in a pattern highly reminiscent to that of IL-2 producing cells in the small and large intestine (Figure 4). $CD3\varepsilon^+$ cells outnumbered IL-2 producing cells (Table II), thus if IL-2 producing cells express CD3ε, they are only a subset of all CD3ε-expressing cells in the gut. Although these observations do not conclusively show that IL-2 expressing cells also express CD3ε, these combined staining results do reveal that IL-2 producing cells can be found in areas of the gut where $CD3\varepsilon^+$ cells are located.

A compilation of staining results to determine the frequencies of different subsets of T cells is given in Table II. As shown previously by fluorescence activated cell sorting analysis of isolated small and large intestinal IELs (4), our immunohistochemical staining data also indicated that the composition of the T-lymphocyte pool in the small and large intestine is not

identical. Additionally, we were able to distinguish between IEL, LPL and PPL/LAL populations (data not shown). In general, CD8α^+cells represented a higher percentage of CD3ε^+ cells in the small intestine compared to the large intestine. On the other hand, CD4$^+$ cells are more highly represented in the large intestine CD3ε^+ population. $\gamma\delta$ T cells are also less prevalent in the large intestine versus the small intestine. Unfortunately, we did not determine the frequency of B cells in our analysis of gut-associated lymphocyte populations, thus it is not certain if CD3ε^+ cells represent a higher proportion of all lymphocytes in the small intestine. With regard to IL-2 producing cells and the expression of T-cell surface markers in the small intestine, IL-2 producers cannot be accounted for by the CD4$^+$ population alone, since 5-6 times as many IL-2 producing cells are found associated with villi compared to CD4$^+$ cells (4.5% versus 0.8%). This provisionally suggests that other T-cell subsets (CD8$^+$ and $\gamma\delta$TcR$^+$ cells) may produce IL-2 in the small intestine. In contrast, the frequency of IL-2 producing cells in the large intestine was roughly equivalent to that of CD4$^+$ cells, thus it is possible that IL-2 producers are also CD4$^+$ in the large intestine. Without double staining for T-cell markers in conjuction with IL-2 protein production, no definitive conclusions can be drawn with respect to IL-2 production and T-cell subsets in the gut. Nevertheless, the nonequivalent distribution of T-cell populations in the small and large intestine as determined by immunohistochemistry supports the notion that regional specialization of T-cell function may exist in the mucosal immune system (4).

IL-2 Expressing Cells are Absent in Mutant Mice That Do Not Generate Mature T and B Cells

Mice homozygous for the SCID mutation are unable to productively rearrange their TcR and Ig genes, resulting in a failure to generate mature T and B cells. The results from simultaneous *in situ* hybridization in isolated neonatal gut sections obtained from the tissues of normal and SCID mouse pups are shown in Figure 5A-D. As described above, in normal neonatal animals, IL-2 mRNA positive cells were consistently found in the gut following hybridization of tissue sections to an IL-2 specific antisense RNA probe (Figure 5A). In contrast, no hybridization above

to an IL-2 specific antisense RNA probe (Figure 5A). In contrast, no hybridization above background was observed in sections of SCID neonatal gut (Figure 5B). In addition, the morphology of SCID neonatal gut appeared to be slightly disorganized as a result of a decrease in cellularity (compare Figure 5A with 5B). Hence, IL-2 mRNA positive cells were not detectable in the gut of newborn mice that do not generate functional T and B lymphocytes.

To strengthen the findings obtained by *in situ* hybridization, immunohistochemical staining was carried out on prefixed normal and SCID neonatal gut sections (Figures 5C, D). Staining of identically treated SCID neonatal gut sections revealed that IL-2 producing cells were conspicuously missing in the SCID neonatal gut (Figure 5D, H). It is evident that these staining results were in good agreement with the *in situ* hybridization results. Taken together, these data support the conclusion that IL-2 producers associated with the gut are absent in SCID neonatal animals, thereby indicating that the SCID defect affects IL-2 expression in the gut.

In general, we were unable to achieve satisfactory staining for CD3ε in neonatal gut sections regardless of the source of tissue, hence we could not verify that T cells were missing in the SCID neonatal gut. To demonstrate that $CD3\varepsilon^+$ cells were indeed absent in the SCID gut, we stained 3-week old SCID small and large intestine sections for IL-2 protein production and CD3ε expression. Neither CD3ε staining (data not shown) nor IL-2 staining (Figure 6) were observed on the older SCID gut sections, indicating that IL-2 production in the gut was undetectable in the gut when gut-associated T cells were also absent. At this point, we cannot formally disprove that the SCID mutation affects some unidentified non-T IL-2 producing cell type which is dependent on T cells for its development or for the induction to produce IL-2. Likewise, we cannot completely dismiss the possibility that B cells make IL-2 in the gut, since SCID mice exhibit a deficiency in the production of both T and B lymphocytes (the possibility that B cells are able to produce IL-2 will be discussed later). Nevertheless, the staining results indicate that there is a correlation between IL-2 expression in the small and large intestine and the presence of gut-associated T cells.

IL-2 Producing Cells are Found in the Fetal Gut

With regard to expression in the neonatal gut, it was possible that IL-2 expression was induced upon feeding in neonatal mouse pups. To explore this possibility, we sacrificed pregnant mice to obtain prenatal tissues to look for IL-2 protein production in the day 20 fetal gut (Figure 7). IL-2 producing cells were easily observed in the prenatal gut (Figure 7A). Similar to IL-2 producers in the neonatal gut, IL-2 reactive cells in the prenatal gut appeared to be large blast cells. Interestingly, "nests" of IL-2 reactive cells were found in the lamina propria. This observation suggests that IL-2 producers may be using IL-2 as a proliferation signal (experiments are under progress to determine whether IL-2 producing cells express IL-2 receptor). The identification of IL-2 producing cells in the prenatal gut demonstrates that IL-2 expression is not induced upon nursing and that a very high proportion of IL-2 producing cells arise during fetal life. Additionally, this prenatal result suggests that IL-2 expression in the gut is induced by exposure to an unknown self antigen and not to ingested stimuli.

The findings in the prenatal gut led us to examine at what point n gestation is IL-2 production first detected in the fetal gut. In the day 16 fetal gut, we detected occasional IL-2 reactive cells by immunohistochemical staining (Figure 7B). IL-2 producing cells were ten times less abundant in the day 16 fetal gut compared to the prenatal gut (3.3% IL-2 reactive cells versus 0.3% positive cells, respectively). Therefore, between days 16 and 20-21 of gestation, there is a dramatic increase in IL-2 producing cells in the fetal gut. It is still undetermined whether the increase in IL-2 producers associated with the gut is due to expansion of cells *in situ* or to immigration of cells coming from a source outside the fetal gut.

Subsequently, we were interested in determining the potential origin(s) of gut-associated IL-2 producers in the developing fetus. Considering that induction of IL-2 expression during the development of a particular cell type may be indicative of future function as an IL-2 producing cell, sites at which IL-2 expression is induced can be regarded as a potential source of IL-2 producers. We examined three embryonic sites at which development of T cells or blood cells takes place during ontogeny, the fetal thymus, the fetal liver and the fetal omentum, for IL-2

expression by immunohistochemical staining, the results of which are shown in Figure 8. With increased detection of IL-2 protein by staining prefixed sections, our staining results revealed two populations of IL-2 producers in the day 15 fetal thymus (Figure 8A). The first subset was restricted to the subcapsular region of the organ. This subset was also observed in the SCID fetal thymus, indicating that induction of IL-2 expression in this population does not require productive TcR gene rearrangement and expression (17). On the other hand, a second subset of IL-2 producers were found in the interior of the normal fetal thymus. These intrathymic IL-2 expressors were also detected by *in situ* hybridization (17; data not shown). However, this subpopulation of IL-2 expressors was not found in the SCID fetal thymus, as determined by both *in situ* hybridization and immunohistochemical staining (data not shown). Hence a SCID-sensitive population of IL-2 expressing cells were defined in the fetal thymus and the appearance of this subset in the fetal thymus is contingent upon TcR expression. It should be noted that at this stage of gestation, T cells expressing a specific invariant TcR are being generated that home exclusively to the skin (21). Ergo, we do not know for certain which fetal lineage of T cells is induced. This T-cell type represents the major lineage developing in the day 15 fetal thymus, it is unlikely that it is the sole cell type of T-lineage developing therein to express IL-2 intrathymically at this point in gestation. The uncertainty about lineage and IL-2 expression not withstanding, the fetal thymus must still be considered a source of IL-2 expressing cells that could migrate to the fetal gut. Additionally, at day 15 of gestation, the fetal thymus contains only one 5 x 10^5 cells. Therefore, in order for a thymus-derived IL-2 producing cell to seed the gut, further expansion of this cell type in the gut would be required.

Immunohistochemical staining of day 15 fetal omentum and liver for the presence of IL-2 protein yielded unexpected results (Figure 8B, C). IL-2 protein was abundant in both these tissues. Specificity of staining was demonstrated by preincubating the anti-IL-2 mAb in an excess of recombinant murine IL-2 prior to staining. This blocking treatment totally inhibited IL-2 reactivity in fetal omentum and liver samples. In addition, the immunohistochemical staining results in the fetal liver were reconfirmed by *in situ* hybridization. Similar to the

staining results, IL-2 mRNA positive cells were found throughout the day 15-16 fetal liver (data not shown). This finding is in good agreement with other published results (25). However, in the earlier published accounts of IL-2 expression in the fetal liver, only IL-2 mRNA accumulation was examined. Here, we verify that not only is the IL-2 gene being transcribed in the fetal liver, but IL-2 protein is present and available to resident cells in the fetal liver. Finally, the findings just described suggest not only that IL-2 producers associated with the gut may be extrathymic in origin, but they also raise the possibility that a cell type other than that belonging to the T lineage may be producing IL-2 in the gut. The fetal liver is the primary site of B-cell development during embryonic life and the omentum is a documented source for a specialized population of B cells. Viewed in this light, B cells in particular may also express IL-2 within and outside the fetal liver and omentum.

DISCUSSION AND CONCLUSIONS

Our results presented here show that IL-2 is indeed expressed in the gut of normal mice. IL-2 mRNA and protein are detected in the neonatal gut and IL-2 producers persist in the small and large intestine apparently throughout life. In newborn mice, IL-2 producing cells appeared to be blast cells, since these cells are distinctively large and round in shape. This blasted appearance suggests that neonatal IL-2 producers may still be dividing. In older animals, IL-2 producing cells were smaller in size than their neonatal counterparts and clearly restricted to the lamina propria of the small and large intestine. It is not known whether IL-2 producing cells in older mice are the progeny of neonatal IL-2 producers or represent an independent population in the gut.

In the small intestine, IL-2 producers were reproducibly 2-4 times more prevalent than IL-2 producers in the large intestine. This finding was surprising taken in the context of results obtained with IL-2 -/- mice (9). In IL-2 -/- animals, no profound effects were seen in the small intestine, while pronounced abnormalities were observed in the large intestine. The defects in the large intestine of IL-2 -/- deficient mice reflected a generalized deregulation of T- and B-cell responses. Again, these results are significant, but the pattern of IL-2 gene expression in the gut of wild-type mice was never defined. We can reconcile our results with the IL-2 -/- results in one of two ways. First, IL-2 producers in the small intestine may influence responses downstream in the large intestine that would normally be suppressed, hence the absence of IL-2 production in the gut, especially in the small intestine, may result in indesirable immune responses in the large intestine which cause IBD. On the other hand, IL-2 producers in the small intestine may be dispensable to terms of regulating the immune response to intestinal microflora (11), whereas those in the large intestine are absolutely required for the suppression of harmful activation of the mucosal immune system. This second account also takes into consideration the potential segregation of gut-associated lymphocyte function, especially in terms of IL-2 expression. At this point, neither our studies nor the IL-2 -/- studies can distinguish between

these two explanations. Regardless, we establish that IL-2 is produced in the gut and a lack of IL-2 protein in the gut presumably results in IBD.

The difference between the number of IL-2 reactive cells in the lamina propria and Peyer's patches may reflect how Ig production by B cells is regulated in the gut. It is possible that the decreased number of IL-2 producers in Peyer's patches is an indication that IL-4 expressing cells rather than IL-2 expressing cells predominate therein, hence Peyer's patch B cells would chiefly produce IgA and IgG1. On the other hand, an increased number of IL-2 expressing cells associated with the lamina propria suggests that IL-2 producers may participate in the primary line of defense in the mucosal immune response against foreign antigen. Experiments to determine the pattern of IL-4 production in the gut relative to that of IL-2 production are in progress.

With regard to observations made from the study of IL-2 -/- mice kept under GF or SPF conditions, it is not likely that keeping animals in such a manner would have affected IL-2 production *per se*, as demonstrated by our findings on IL-2 production in normal animals kept in GF conditions. Perhaps keeping IL-2 deficient animals under controlled conditions affects T- and B-cell activation as a result of a commensurate decrease in intestinal microflora, thereby eliminating the need for IL-2 dependent control of the mucosal immune response. Hence in conventionally housed animals, IL-2 may play a role in suppressing responses to normally nonpathogenic microflora. On the other hand, when intestinal microflora is controlled or limited under GF and SPF conditions, IL-2-related suppression of the mucosal immune response may be redundant, as indicated by the studies of IL-2 -/- animals in which mutant mice do not contract IBD when housed under GF or SPF conditions.

Data on the identification of IL-2 producing cells in the lamina propria *in vivo* are in good agreement with recent findings obtained *in vitro* with regard to the characterization of cell types present in the LPL population. It has been shown that freshly isolated LPLs produced substantial amounts of IL-2, IL-4 and IFN-γ, but decreased levels of IL-5 (22). Likewise, $CD4^+$ lines established from LPLs secreted IL-2, IL-4, IFN-γ upon stimulation using anti-CD3 mAb and

phorbol myristate acetate (22). These data indicate that CD4+ IL-2 producers are contained within the LPL pool, and this subset may represent a population induced to express IL-2 in the gut *in vivo*.

Production of IL-2 *per se* may not be sufficient to protect animals from IBD. As demonstrated by the studies using IL-2 -/- mice, IL-2 appears to be necessary for the maintenance of nonreactivity of resident lympyhocytes to endogenous microflora in the intestine. However, injection of CD4+ purified T cells that produce IL-2 and IFNγ and little IL-4 induced IBD in congenic SCID mice (23). On the other hand, identical treatment with a defined population of CD4+ cells that produces both IL-2 and IL-4 did not cause immune related pathology (24). Significantly, wasting disease and colitis was prevented when both CD4+ populations were cotransferred into immunodeficient animals (24). These studies show that IL-2 alone cannot protect animals susceptible to IBD and that the coordinate interactions of cytokines is important for the maintenance of immunologic balance in the mucosa. Any disruption of these interactions cause lethal inflammatory responses that, under normal circumstances, would be held in check. Disruption of IL-10 gene expression also led to chronic enterocolitis in mutant mice (10). IL-10 has been classically defined as a negative regulator of IL-2 expression, thus putative deregulation of IL-2 production in the gut is likewise detrimental. The IL-10 -/- studies further support the notion that the proper regulation of cytokine expression is vital for preventing IBD and any perturbation in the balance between regulatory pathways dictated by cytokine production has pathologic consequences.

The studies demonstrating that peripheral T-cell subsets cause or prevent colitis in *SCID* mice show that mature splenic T cells can home to the gut and infiltrate the colon. This indicates that thymus-dependent T cells can migrate to the gut and contribute to immune regulatory interactions therein. Additionally, phenotypic characterization of lymphocytes obtained from congenic parabionts revealed that peripheral T cells home rapidly to the Peyer's patches and lamina propria but not to the intestinal epithelium (3). Therefore, at least a subset of IL-2 producers in the gut may be initially thymic in origin. Nevertheless, not all IL-2 producers in the

gut may require on the thymus for their development. Analysis of fetal liver radiation chimeras using adult thymectomized recipients revealed that T cells of donor origin were found in both the intestinal epithelium and lamina propria and were absent in secondary lymphoid tissues such as the spleen and Peyer's patches (3). Subsequently, it was postulated that extrathymic development of T cells occurs in the intestinal epithelium, after which phenotypically mature T cells migrate to the lamina propria. Thus, the LPL population is heterogeneous, composed of both thymic-dependent and -independent T-cell types. It is possible that both lineages of T cells contribute to the IL-2 producing pool in the lamina propria. It would be informative to determine if IL-2 producing cells appear in the donor population of mature T cells in the lamina propria of thymectomized irradiation chimeras reconstituted with fetal liver.

Our results analyzing IL-2 production in the fetal gut revealed that a significant population of IL-2 producers is present therein gut prior to birth. It is not known whether a comparable population of gut-associated IL-2 producers exist in humans. Regardless, several questions remain unanswered with regard to the development of IL-2 producers associated with the fetal gut. Immunohistochemical staining of the fetal liver and omentum for the presence of IL-2 protein revealed that IL-2 producers in the fetal gut may be derived from either of these sources of IL-2 producing cells. As discussed above, fetal liver is able to give rise to LPL lymphocytes in the absence of thymus-dependent T-cell development. In addition, rearranged γ- and δ-chain TcR gene rearrangements can be detected in the fetal liver (25), suggesting that T and B lymphopoiesis occurs in the fetal liver. These observations strengthen the notion that fetal-gut associated IL-2 producing cells may be generated directly from progenitors in the fetal liver. Albeit that the fetal liver cells can populate the LPL compartment, it may not be the sole contributor of lymphocytes (and potential IL-2 producers) in the gut. The close association of IL-2 producing cells in the fetal omentum with the gut at day 15 of gestation is provocative in terms of raising the possibility that IL-2 producers in the fetal gut may also be derived from the omentum. The fetal omentum is a highly specialized hematopoietic tissue. Not only is the fetal omentum an embryonic source of T and B lymphocytes in the mouse (26), it is the principal

tissue from which Ly1$^+$/CD5^{lo} B cells arise (27). Ly1$^+$/CD5^{lo} B cells represent a separate lineage that can be distinguished from conventional B cells with regard to several characteristics (28-32). Ly1$^+$ B cells are derived only from fetal tissues (33) but not adult bone marrow, and are associated with the peritoneal and plural activities of normal adult mice (34, 35). More importantly, fetal omentum has been shown to give rise to IgA-producing cells in the gut (27). Due to the marked production of IL-2 in the omentum, we cannot overlook the likelihood that B cells (especially those of the Ly1$^+$ lineage) may express and/or utilize IL-2 during their development. Likewise, the fetal omentum is able to contribute to the lymphocyte pool associated with the gut and may also be a putative source of intestinal IL-2 producers. We are currently in the process of determining whether the SCID mutation affects IL-2 expression in fetal tissues. Since IL-2 expressing cells are not detected in the SCID neonatal gut, it is likely that the SCID mutation may also affect processes that influence the development of fetally-derived, gut-associated IL-2 producers, one of which may be IL-2 expression at these extrathymic sites.

Examination of SCID mice revealed that IL-2 producing cells are not detected in neonatal and adult mutant animals. SCID mice cannot generate mature T and B cells (36) and are not susceptible to IBD. It is apparent that animals in which both T and B-cell development is disrupted do not contract IBD (37) and that IBD is induced only following alterations that affect T- or B-cell function. With regard to gut-associated IL-2 production, the SCID results do not clarify whether cells belonging to the T or B lineage or both produce IL-2 in the small and large intestine. Based solely on the information described here, we cannot determine the exact identity of IL-2 producing cells *in vivo*. Importantly, mice that are unable to produce T cells as a result of targeted disruption of TcR genes develop IBD (37). In these immunodeficient mouse strains, B-cell development is unperturbed. Analysis of gut-associated IL-2 production in TcRα -, β- and β-xγ-chain mutant mice would clearly demonstrate whether T cells and/or B cells synthesize IL-2 in the small and large intestine.

It was observed that when IL-2 deficient mice are kept under germ-free conditions (GF) or in a defined microbial (specific-pathogen free/SPF) environment, they do not contract disease or that symptoms of IBD were delayed significantly (9). We were interested in examining how IL-2 production in the gut is affected when animals are kept under GF conditions. Analysis of IL-2 production in gut tissues of mice kept on sterile bedding, food and water and showed that there was essentially no difference in the number of IL-2 producing cells in the small and large intestine or the distribution of IL-2 producers associated with the lamina propria therein of animals kept under GF conditions (n=4) and littermates kept in conventional housing (data not shown). However, a tenfold increase in IL-2 producing cells was observed in Peyer's patches taken from animals kept under GF conditions compared to conventionally housed mice (2.6% versus 0.2% IL-2 producing cells, respectively; see Figure 9). These results suggest that IL-2 producers are responding to ingested antigenic and raise the possibility that IL-2 producers are stimulated by endogenous antigen found in the intestine. Indeed, the response of potential IL-2 producers in the Peyer's patches may be inhibited by ingested antigen. In addition, the results obtained using mice maintained under GF conditions demonstrated that the lack of IL-2 expression in the gut of SCID mice was not due to the manner by which immunodeficient animals were housed.

In conclusion, the results presented here provide new insights into how a deficiency in IL-2 production contributes to dysfunction of the mucosal immune system resulting in IBD. Additional work is still required to determine if IL- 2-producing cells are absent in IL-2 -/- animals or if potential IL-2 producing cells are still present in the gut but are simply nonfunctional. Although IBD in humans is a disease of multigenic etiology, some cases of IBD are related to IL-2 deficiency. The identification of cells associated with the gut that produce IL-2 *in vivo* will undoubtedly increase understanding of the pathologic mechanisms involved in initiating and perpetuating IBD as a result of IL-2 deficiency. Further characterization of IL-2 producing cells in normal mice would provide information on cell types that could prevent IBD

in IL-2 -/- mice, thereby aiding in the development of new approaches to the prevention and therapeutic intervention of IBD caused by a defect in IL-2 gene expression.

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TABLE 1. Frequencies of IL-2 reactive cells detected by immunohistochemical staining of normal small and large intestine sections.

Sample	Number IL-2$^+$ Cells	Number Nuclei Counted	Percentage IL-2$^+$ Cells
Small Intestine 3 weeks	220	9544	2.3
Large Intestine 3 weeks	112	9451	1.2
Small Intestine 3 months	429	9629	4.5
Large Intestine 3 months	121	9378	1.3
Peyer's Patch 3 months	47	5883	0.7
Mesenteric Lymph node 3 months	22	3505	0.6

Following immunohistochemical staining, sections were counterstained with DAPI. Nuclei were visualized under fluorescence microscopy. Random fields at 200X magnification were analyzed to remove bias toward positively staining areas. For small intestine analysis, villi were

predominantly analyzed. Immunoreactive cells were not observed in negative control stainings of serial sections.

TABLE II. Frequencies of CD3ε, CD4, CD8α and γδ-TcR positive cells determined by immunohistochemical staining of small and large intestine sections (3-month old animals)

SMALL INTESTINE

Antigen	Number Positive Cells	Number Nuclei Counted	Percentage Positive Cells
CD3ε	510	6951	7.3
CD4	73	9036	0.8
CD8α	321	6256	5.1
γδ-TcR	208	7646	2.7

LARGE INTESTINE

CD3ε	360	10398	3.5
CD4	144	11002	1.3
CD8α	192	10769	1.8
γδ-TcR	102	12031	0.8

Analysis was carried out as described in legend for Table I. Samples stained in this experiment were identical to those stained in Figure 4.

FIGURE LEGENDS

FIGURE 1. ^{35}S-*in situ* hybridization of longitudinal neonatal whole body and saggital head sections to a 410 nt IL-2 specific antisense RNA probe. Representative fields were taken from 3 separate hybridization experiments. Extensive hybridization was not detected in any region in serial sections hybridized to the complementary sense-strand RNA (data not shown). Counterstained with hematoxylin or alcian blue. Bright and dark fields shown.

(A): Valves of the heart, 40X.

(B): Lung, 40X.

(C): Liver (LI), ans stomach (S, lumen), 40X.

(D): Bladder, 100X.

(E): Spine, spinal cord, 40X.

(F): Midbrain, 100X.

(G): Right ventricle, 40X.

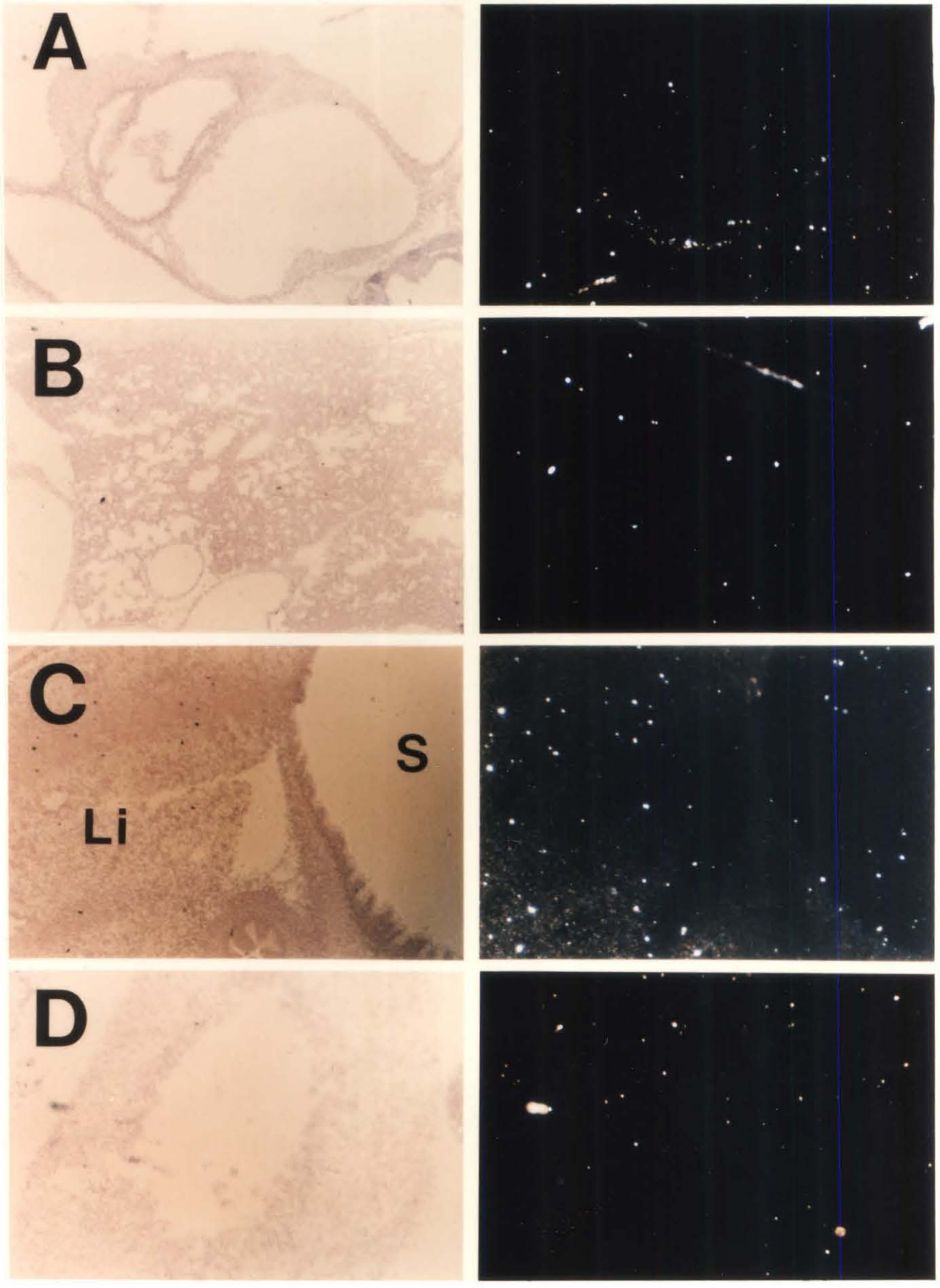
(H): Left olfactory bulb, 40X.

(I): Thymus, 40X.

(J): Dermis (D), epidermis, 100X. Arrow points to positive cells.

(K): Small intestine, 40X.

(L): Small intestine, 100X.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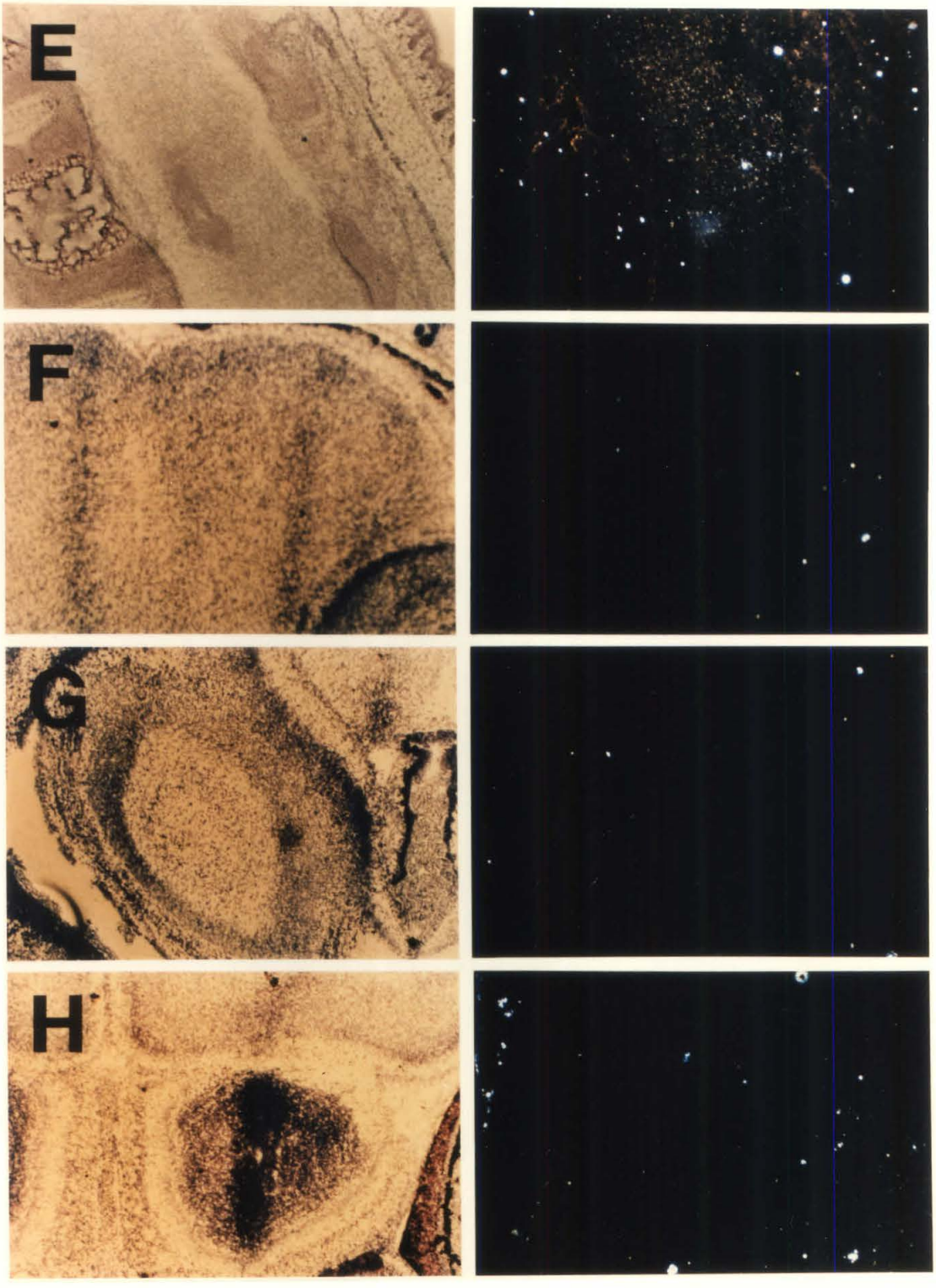
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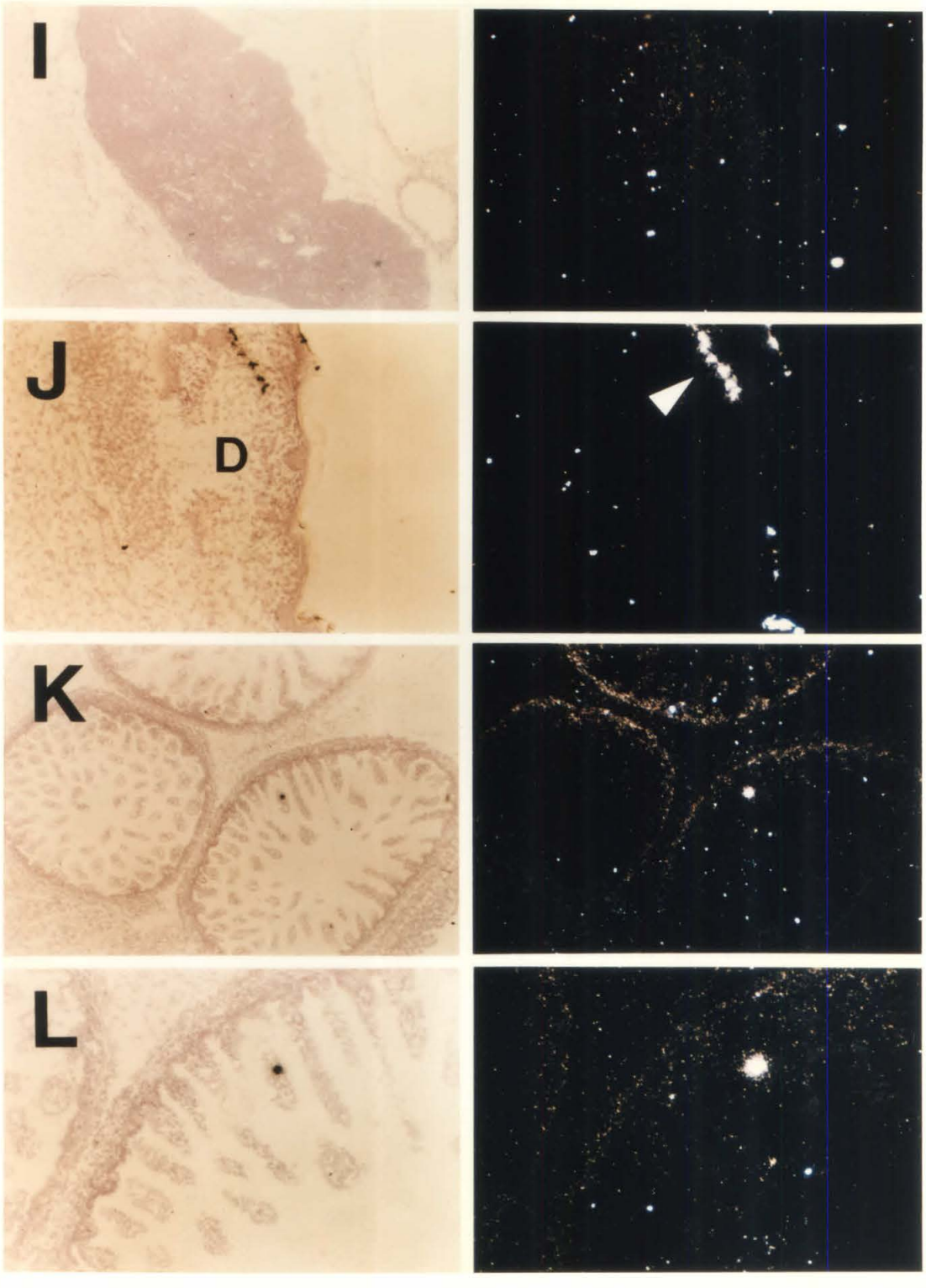
I
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FIGURE 2. Immunohistochemical staining for IL-2 protein in the neonatal gut. Bright field, 280X.

(A): Staining using a rat anti-mouse IL-2 mAb.

(B): Staining using irrelevant rat IgG.

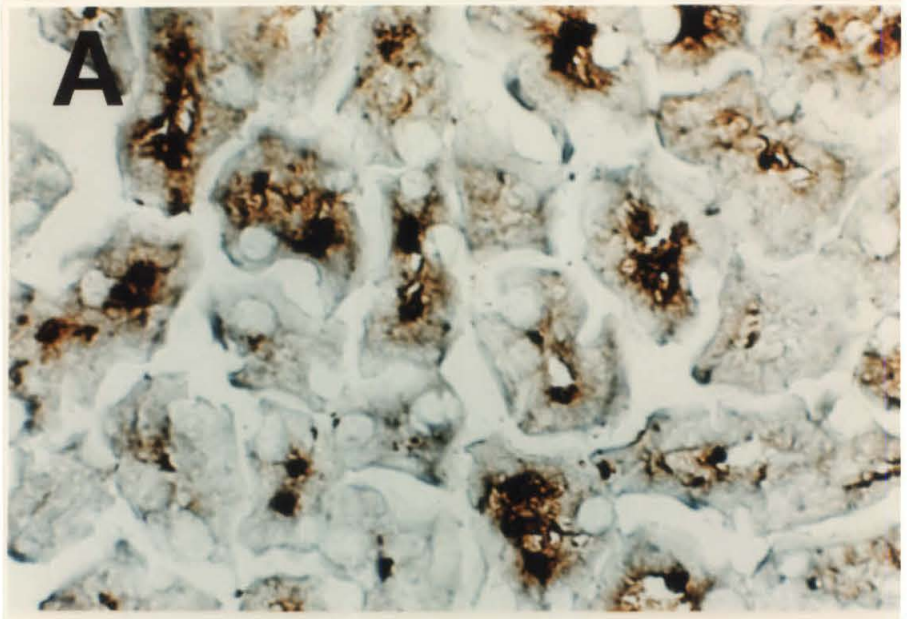
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FIGURE 3. Immunohistochemical staining for IL-2 protein in small and large intestine sections generated from tissues of normal mice and demonstration of staining specificity. Left panels show staining pattern using a rat anti-mouse IL-2 mAb. Right panels show staining pattern using the same antibody preincubated with 1000u of recombinant mouse IL-2. Bright field, 200X.

(A): Small intestine, 3 weeks of age.

(B): Large intestine, 3 weeks of age.

(C): Small intestine, 3 months of age.

(D): Large intestine, 3 months of age.

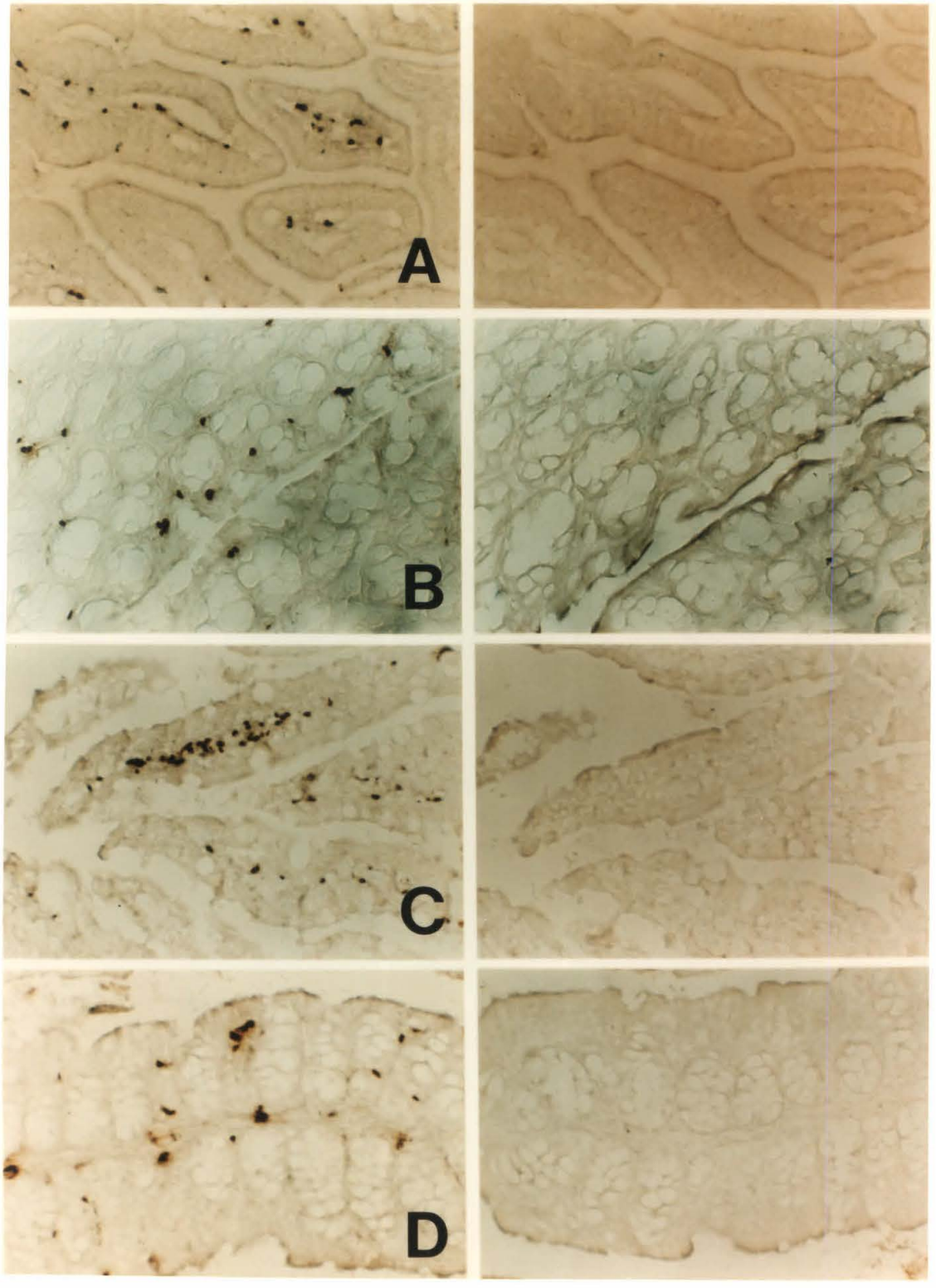
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FIGURE 4. Parallel immunohistochemical staining of normal small and large intestine sections generated form tissues taken from 3-month old mice. Left panels show experimental staining, right panels show negative control staining. Bright field, 200X.

(A): Small intestine, IL-2 staining plus negative control.

(B): Small intestine, CD3ε staining plus negative control.

(C): Large intestine, IL-2 staining plus negative control.

(D): Large intestine, CD3ε staining plus negative control.

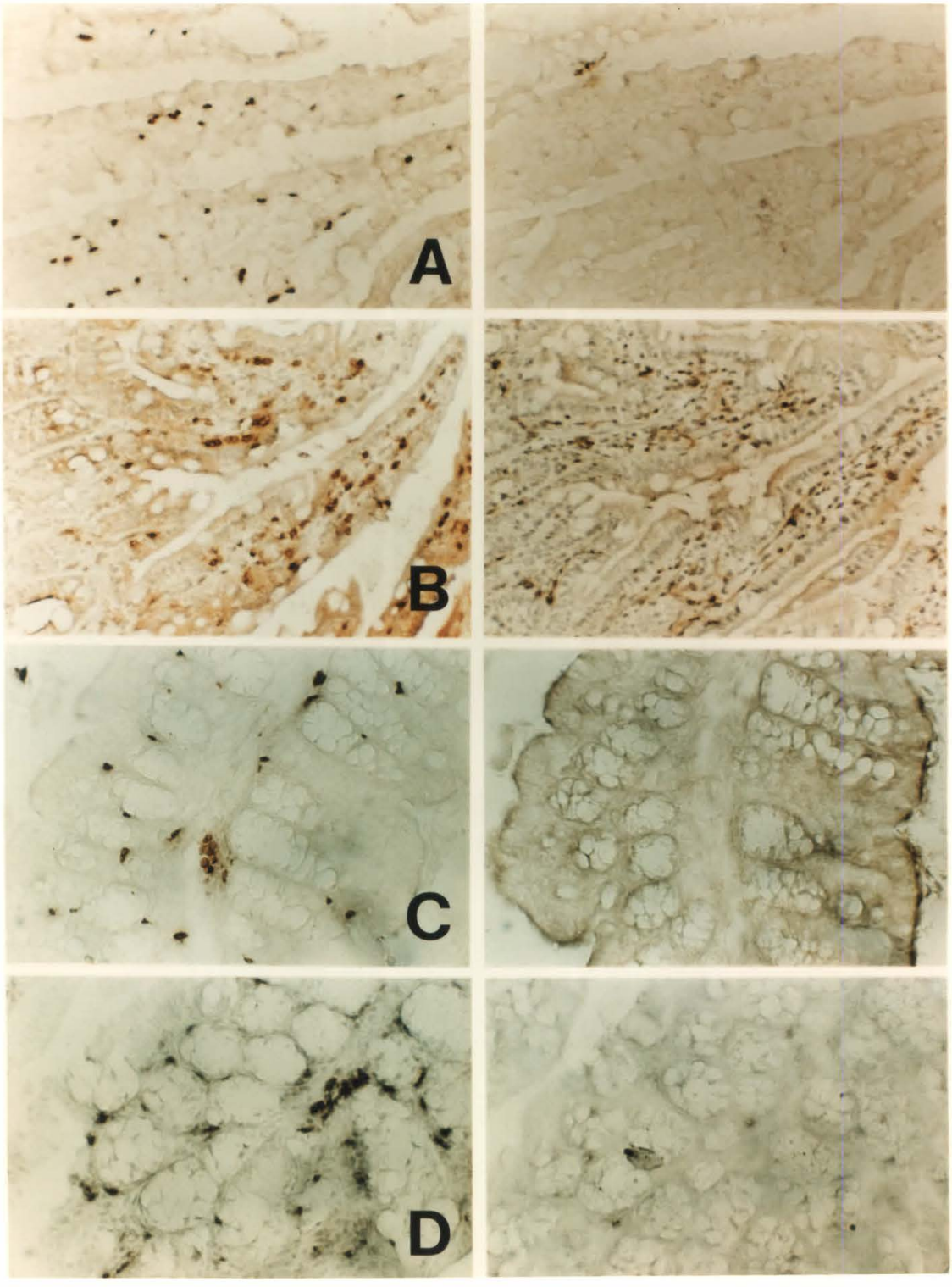
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FIGURE 5. Comparison of IL-2 gene expression and protein production in the normal versus SCID neonatal gut. Panels included in (A) and (B) show *in situ* hybridization of neonatal gut sections hybridized to a ^{25}S-labeled 410 nt IL-2 specific antisense RNA probe. No appreciable hybridization was detected in adjacent serial sections hybridized to the negative contol sense-strand RNA (data not shown). Sections were counterstained with hematoxylin and eosin. (C) and (D) show immunohistochemical staining of comparable sections. Normal and SCID sections were collected onto the same slides. 200X magnification.

(A): Normal neonatal gut section hybridized to a radiolabeled IL-2 specific antigense RNA probe, bright and dark field.

(B): SCID neonatal gut section hybridized to the same probe in (A), bright and dark field.

(C): Normal neonatal gut section stained for IL-2 protein production (left panel) and negative control staining (right panel).

(D): SCID neonatal gut section stained for IL-2 protein production (left panel) and negative control staining (right panel).

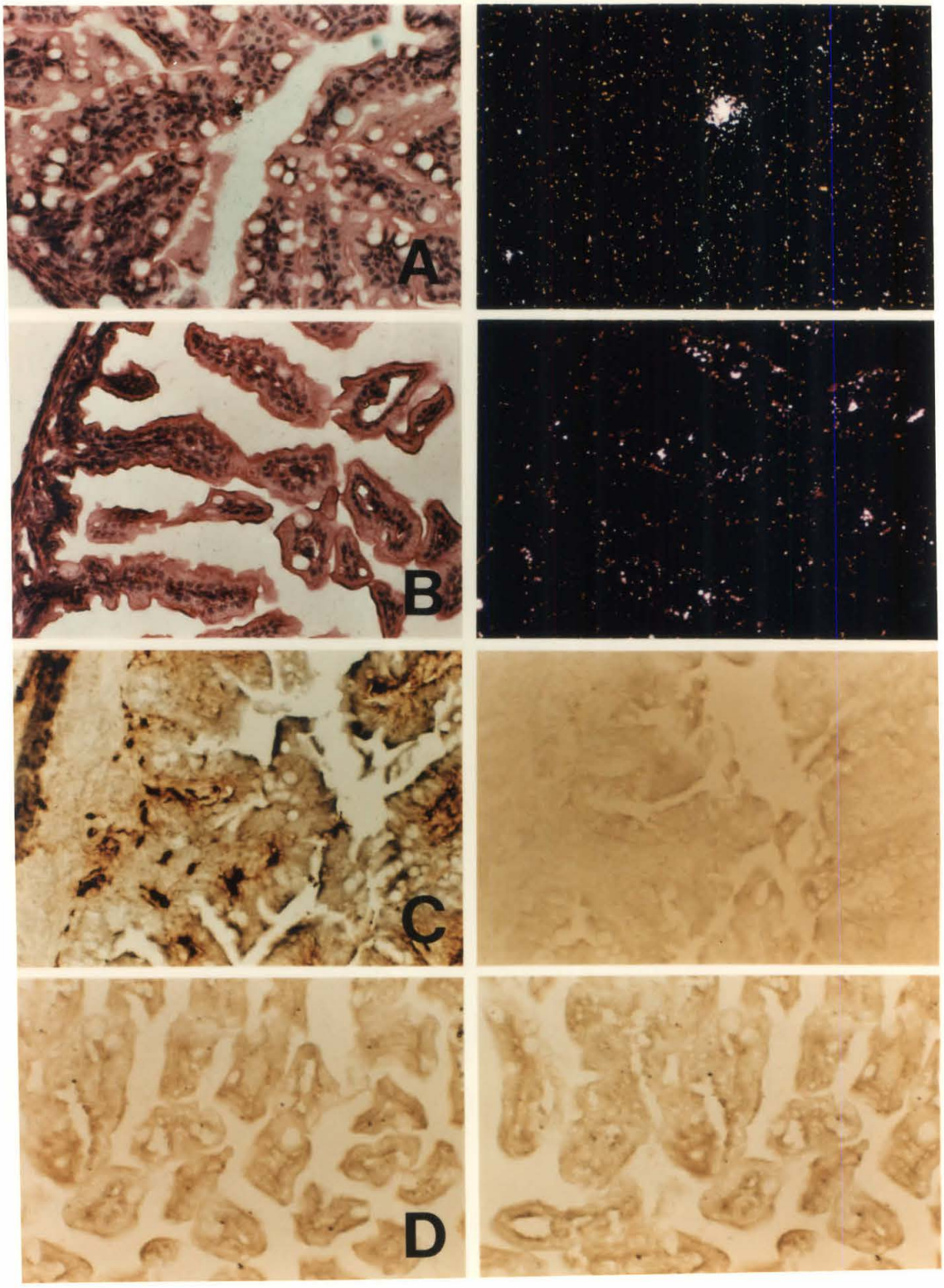
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FIGURE 6. Immunohistochemical staining for IL-2 protein production in SCID 3-week old small and large intestine. Bright field, 230X.

(A): Small intestine section stained with anti-IL-2 mAb (left panel) or negative control staining (right panel).

(B): Large intestine section stained with anti-IL-2 mAb (left panel) or negative control staining (right panel).

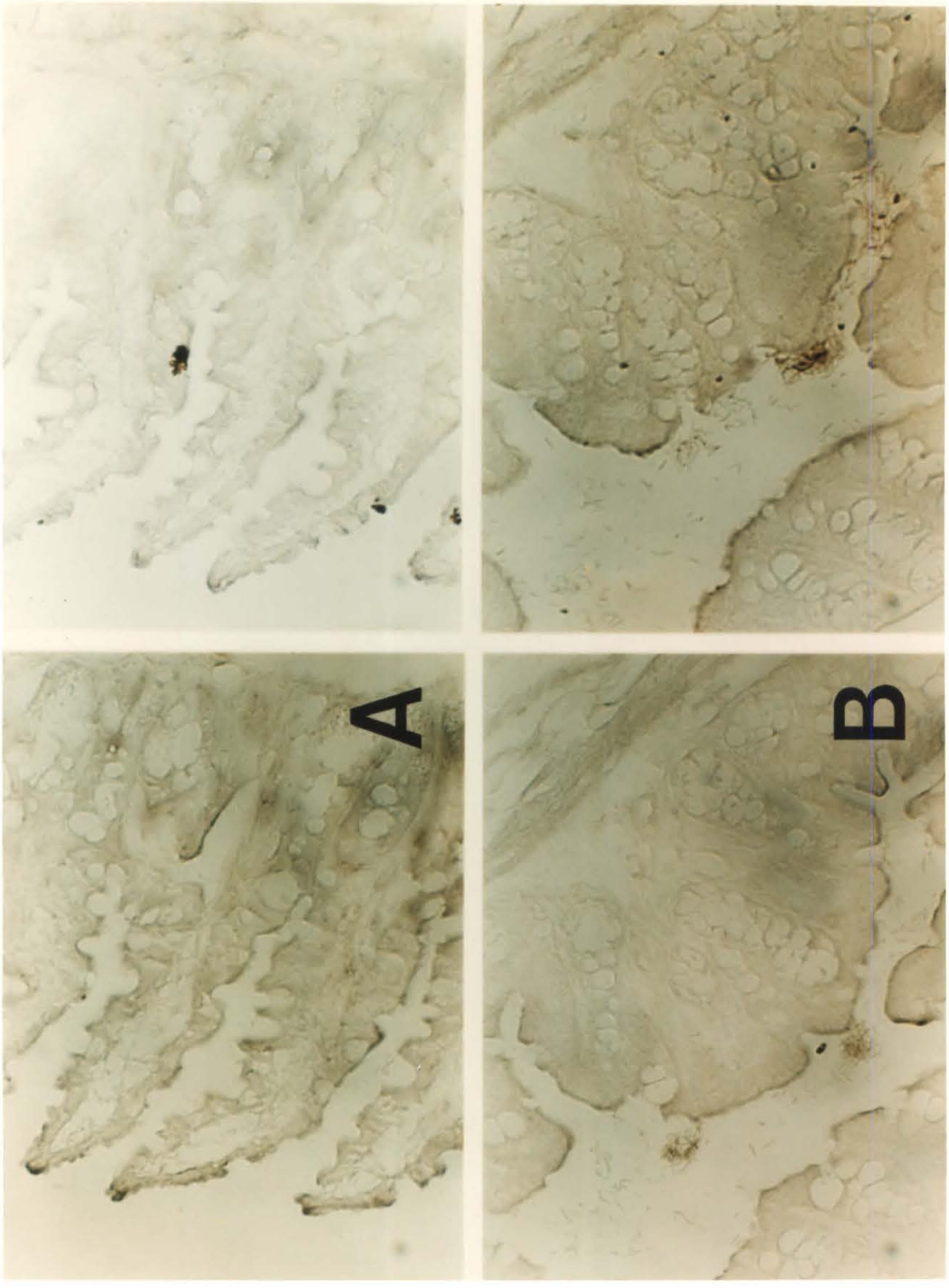
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FIGURE 7. Immunohistochemical staining for IL-2 protein production in prenatal and day 16 fetal gut of normal mice. Bright field, 230X.

(A): Prenatal gut (day 19-20 of gestation) section stained with anti-IL-2 mAb (left panel) or negative control staining (right panel).

(B): Day 16 fetal gut section stained with anti-IL-2 mAb (left panel) or negative control staining (right panel).

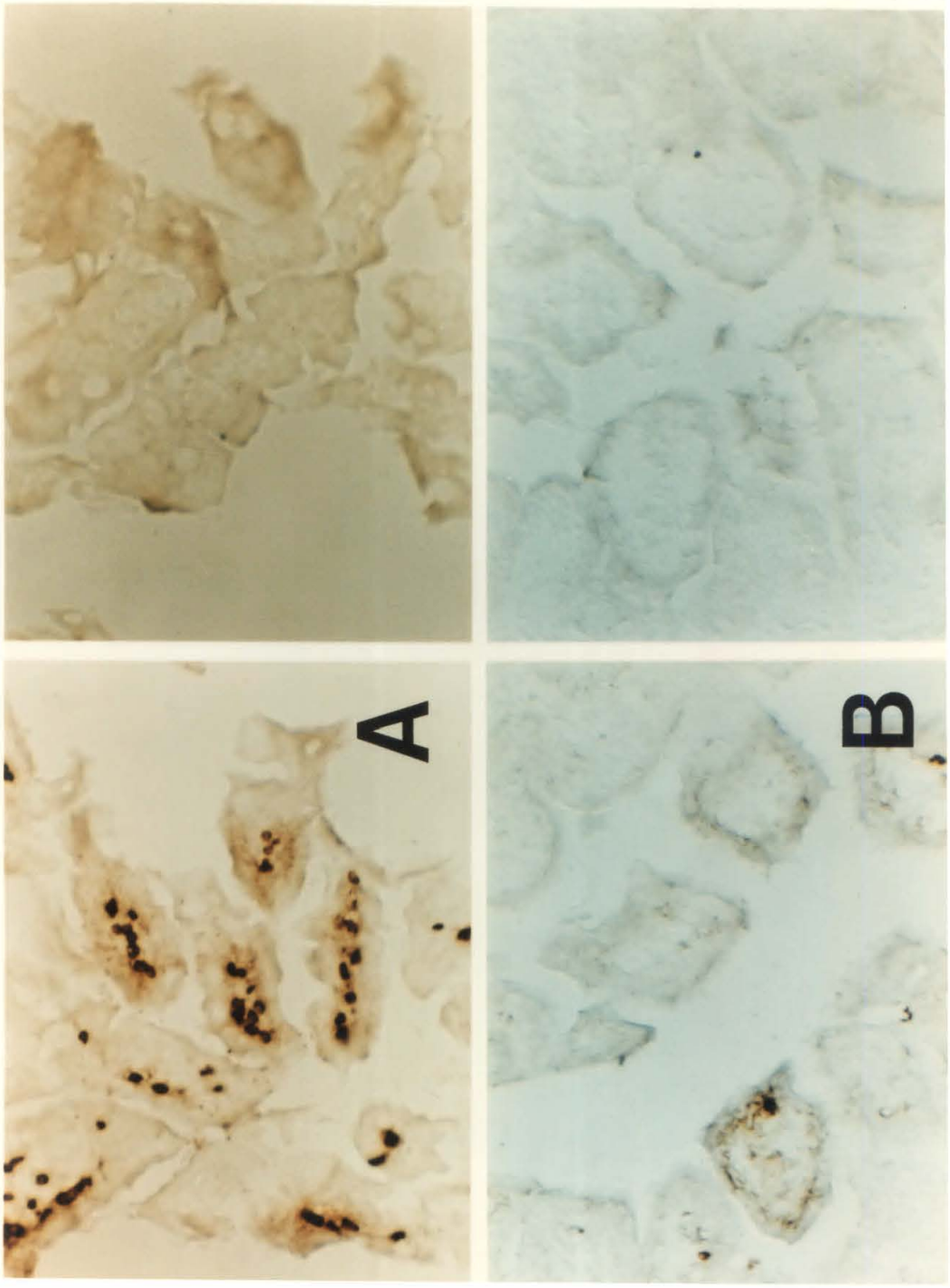
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FIGURE 8. Pattern of IL-2 protein production in day 15 fetal tissues. Specificity of staining observed in (B) and (C) was demonstrated by preincubation of primary mAb with 1000u recombinant mouse IL-2 prior to use in staining. Bright field, 40X.

(A): Fetal thymus section stained with anti-IL-2 mAb (left panel) or negative control staining (right panel). Sections were counterstained with methyl green.

(B): Fetal omentum section stained with anti-IL-2 mAb (left panel) or mAb preincubated with recombinant IL-2 (right panel).

(C): Fetal liver section stained with anti-IL-2 mAb (left panel) or mAb preincubated with recombinant IL-2 (right panel).

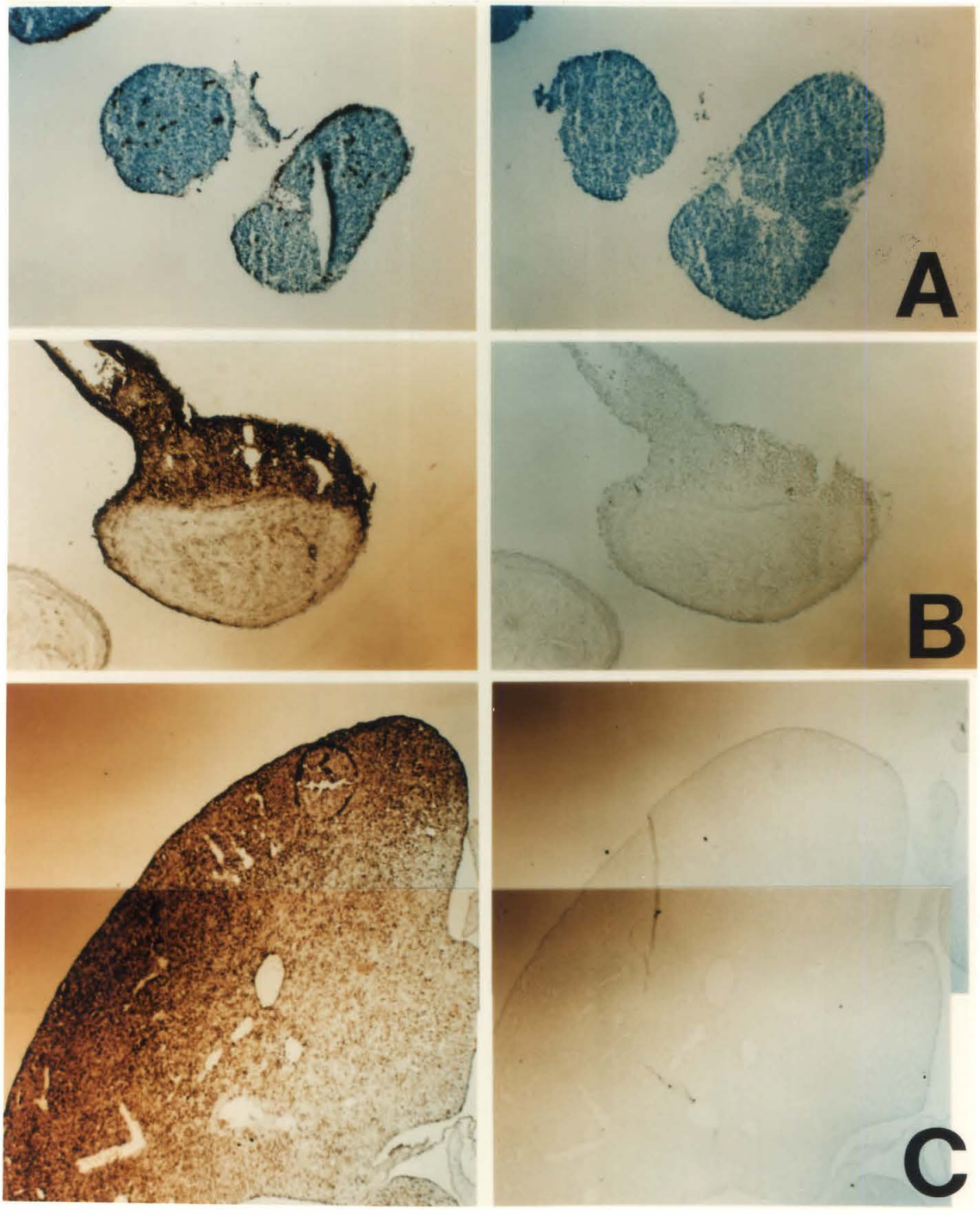
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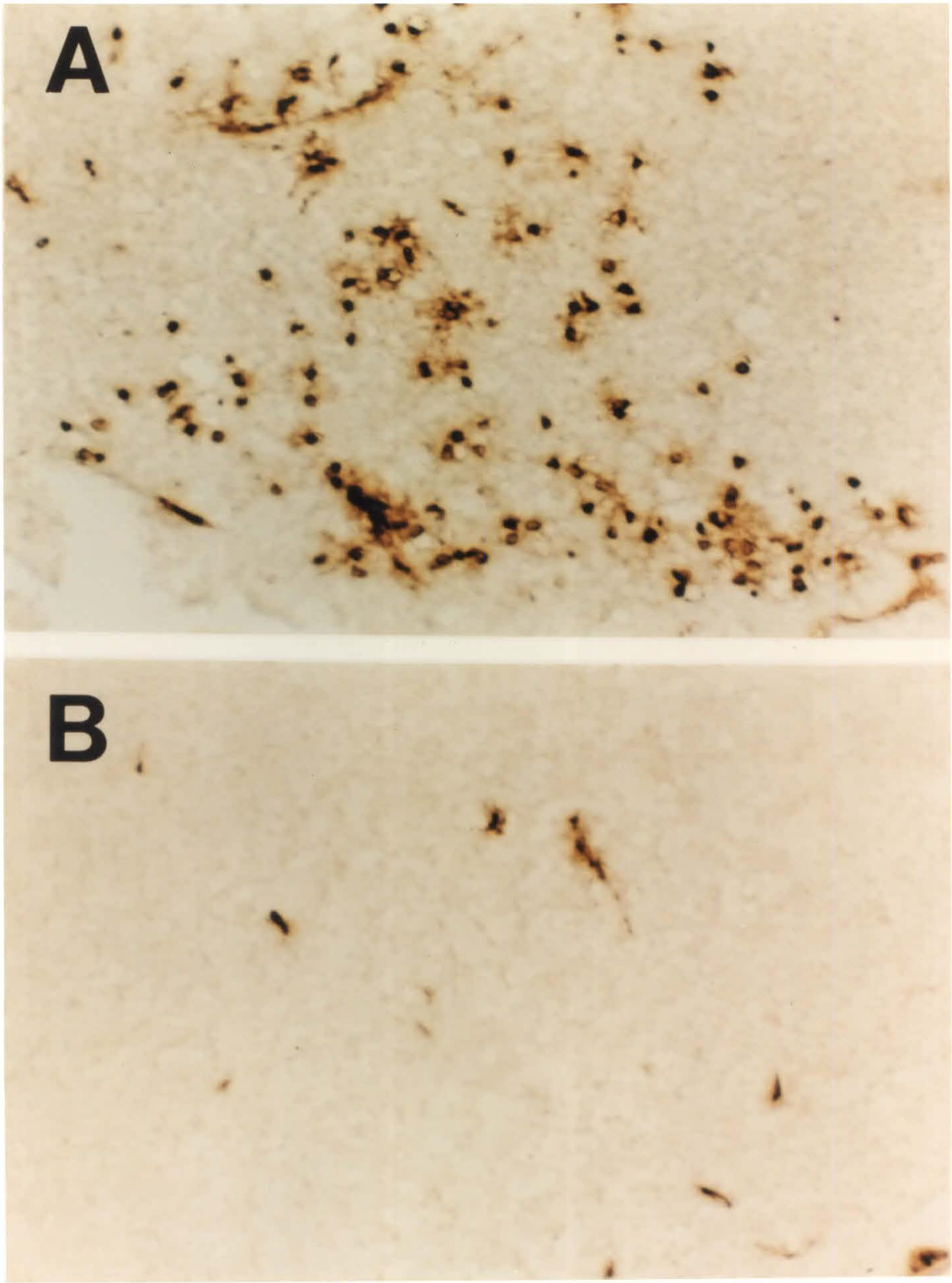
FIGURE 9. Comparison of IL-2 protein production in Peyer's patches of mice kept under germ-free conditions and littermates maintained in conventional housing. Bright field, 140X.

(A) Peyer's patch section generated from a mouse kept under GF conditions stained with anti-IL-2 mAb.

(B) Peyer's patch section generated from conventionally housed littermate stained with anti-IL-2 mAb.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CHAPTER 5

INDUCTION OF IL-2 GENE EXPRESSION IN THE MURINE THYMUS: EXERCISE OF FUNCTIONAL COMPETENCE IN IMMATURE T CELLS AS REVEALED BY INTRATHYMIC IL-2 PROTEIN PRODUCTION IS DEPENDENT ON FUNCTIONAL TcR EXPRESSION

ABSTRACT

Induction of interleukin-2 (IL-2) gene expression resulting in IL-2 protein synthesis is a functional response that a major subset of T lymphocytes must exercise following contact with foreign antigen. Although a great deal is known about the regulation of IL-2 gene expression in the mature T-cell population *in vivo* and *in vitro*, proportionally less is known about events and interactions controlling induction of IL-2 gene expression in developing T cells. *In vitro* studies have shown that the competence to express IL-2 is acquired early on in T-cell development, before surface expression of the T-cell receptor (TcR). However, once cell surface TcR is detectable, immature T cells lose the capacity to induce IL-2 gene expression and this capability must be regained prior to the completion of the maturation process. Previous work demonstrated that induction of IL-2 gene expression represents a rare activation event in the murine thymus. Additional examination of intrathymic induction of IL-2 gene expression in mutant mice exhibiting a block in T-cell development resulting in the enrichment of immature TcR cells in the thymus revealed that immature T cells which are inducible for IL-2 gene expression *in vitro* do not exercise the competence to express IL-2 *in vivo*. Hence the stage in development when competence to induce IL-2 gene expression is initially acquired and that when competence is exercised intrathymically are most certainly not equivalent. Using an improved staining method to detect IL-2 protein in the thymus as a function of the induction of IL-2 gene expression therein, we report the following. First, intrathymic IL-2 is synthesized by both immature αβ- and γδ-TcR$^+$ cells as shown by detailed analysis of IL-2 protein accumulation in the thymus of mice deficient in the expression of a single chain of the TcR. In the αβ lineage, intrathymic IL-2 production is dependent on αβ TcR-heterodimer expression, since TcRβ-chain expression alone is insufficient to induce IL-2 gene expression/protein production. Finally, increased IL-2 protein synthesis in the thymus was not observed in αβ-TcR transgenic animals for which a bias toward positive selection was displayed. Taken together, the demonstration of functional competence

and responsiveness as indicated by intrathymic production of IL-2 protein in developing T cells *in vivo* occurs after TcR-heterodimer expression and before export into the medulla.

INTRODUCTION

T (thymus-derived) lymphocytes play pivotal roles in the vertebrate immune system and in the regulation of cell growth and differentiation in numerous hematopoietic lineages. The coupling of specific antigen recognition with a diverse array of effector functions is integral to cells in the T lineage. The processes by which developing T cells gain functional responsiveness in conjunction with recognition specificity have been under the scrutiny of cell, developmental and molecular biologists for many years (1). As immature T cells progress from one developmental state to another, transitions are accompanied by well-documented changes in cell-surface phenotype. In contrast, relatively little is known with regard to the cellular and molecular events that control and influence the majority of these transitions. However, enormous strides are being made in understanding the regulation of many important transitions in T-cell development. This has been greatly facilitated by the use of naturally occurring mouse mutants and genetically engineered animals with the aid of transgenic and targeted gene disruption technologies.

Although much is being revealed about how particular events determine the progression to a defined phenotypic state, it is not clear how these events may or may not influence the acquisition, "reacquisition" or maintenance of functional responsiveness during T-cell development. In addition, a great deal of uncertainty exists with respect to the possibility that cells, once having acquired functional responsiveness, exercise this capacity prior to leaving the thymus. For example, induction of interleukin-2 (IL-2) gene expression in the mature T-cell population, resulting in the synthesis of IL-2, is vital for mediating the clonal expansion of antigen-specific T cells during the vertebrate immune response. As demonstrated *in vitro*, the competence to induce IL-2 gene expression is acquired early on in T-cell development (2-4). However, as immature cells develop, they lose this competence as indicated by an inability to induce IL-2 gene expression under conditions that are otherwise sufficient to induce IL-2 expression in competent cells. The ability to induce IL-2 gene expression is reacquired by the time developing T cells display a mature cell surface phenotype. Up to this point, it is not known

if immature T cells display the competence to express the IL-2 gene *in vivo* and if this is true, whether signals produced during specific transitions in development are adequate to induce IL-2 gene expression in developing T cells.

It is widely accepted that IL-2 is not essential for the generation of mature T cells, as demonstrated by the production of normal numbers and proportions of thymocyte subsets in the thymus of IL-2-deficient mice (5). Nevertheless, mature T cells generated in these mutant animals are not completely functional, since they cannot produce IL-2. From a technical standpoint, it is virtually impossible to study the exercise of functional competence in developing T cells *in vivo*, as evinced by the actual intrathymic induction of IL-2 gene expression in mice incapable of demonstrating functional competence by synthesizing IL-2. Thus the results obtained from the study of IL-2 -/- mice are informative, but they provide little information as to whether IL-2 gene expression is induced in immature T-cell populations *in vivo*, the significance of which has already been explained.

In a previous report, we have shown that IL-2 is induced in a minority population in the postnatal thymus of normal mice (6). Not only was IL-2 mRNA detected reproducibly in the thymus, but IL-2 protein was also present therein. The absence of IL-2 expressing cells in the thymus of mice homozygous for the SCID mutation indicated that induction of IL-2 gene expression requires functional T-cell receptor (TcR) gene rearrangement and expression (6). Importantly, the localization of IL-2 expressing cells predominantly in the subcapsular region and cortex indicated that cells induced to express the IL-2 gene have not completed their intrathymic processing. In order to further characterize IL-2 expressing cells in the thymus and the intrathymic interactions required for the induction of IL-2 gene expression, we examined IL-2 expression as indicated by IL-2 protein synthesis by employing an improved immunohistochemical staining technique on thymus sections generated from mice in which surface expression of TcR components has been specifically perturbed (7, 8) or positive selection can be manipulated (9, 10). Our results reconfirmed that IL-2 expression in the thymus of normal mice was found throughout the cortex but not in the medulla. In mice rendered deficient

for expression of a particular chain of the TcR, we demonstrate that cells belonging to both the αβ and γδ lineage were induced to express IL-2 in the thymus. Significantly, IL-2 expressing cells of the γδ lineage were restricted to the outer cortex and those of the αβ lineage were relegated to the inner cortex and corticomedullary junction. Induction of IL-2 gene expression in the thymus appears to be dependent on *bona fide* αβ or γδ TcR expression, since IL-2 expression was not readily detected in the thymus for which only rearranged TcRβ-chain was expressed (11). Finally, in terms of positive selection, IL-2 expressing cells were <u>not</u> enriched in αβ-TcR transgenic animals bred onto a positively selecting background, implying that should IL-2 expression be correlated with positive selection, positive selection "niches" are perhaps already saturated (12). Taken altogether, these results show that induction of IL-2 gene expression in the thymus is strictly contingent upon functional TcR gene rearrangement and/or heterodimer expression and suggest that the exercise of functional responsiveness *in vivo* does not occur prior to TcR surface expression.

Materials and Methods

Mice

Pathogen-free C57Bl/6 Tla^{+}, B6PLThy1A/Cy, αβ-TcR transgenic mice specific for H-2L^{d}, and C.B.-17-SCID animals were bred and maintained in our own facility. αβ-TcR transgenic mice were rederived by embryo transfer and kept on sterile bedding and food. For positive selection and "nonselection" *in vivo* , transgenic mice were backcrossed to B10. (Jackson Laboratories, Bar Harbor, ME) and B10.S (Charles River Laboratories, Inc., Wilmington, MA) genetic backgrounds, respectively. All SCID mice were maintained in an Isotec flexible film isolator (Indianapolis, IN) without antibiotic treatment.

Treatment and Sectioning of Tissues

Thymuses were removed from 3-5 week old animals. For detection of cell surface antigens, organs were immediately embedded in Tissue-Tek O.C.T. compound (Miles Laboratories, Inc., Kankakee, IL). For detection of IL-2 protein, thymuses were fixed in freshly made 4% paraformaldehyde in phosphate buffered saline (PBS) for 12-16 hours at 4°C. Following fixation, organs were thoroughly washed in PBS at 4°C, saturated in 30% sucrose in PBS, and embedded in 0.C.T. If samples were not sectioned immediately, they were stored at -80°C. 8 μm serial cryosections collected onto poly-L-lysine plus glutaraldehyde coated slides. Sections were air-dried, dehydrated in acetone for 20-30 min. at room temperature and stored with desiccant at -80°C prior to staining.

Antibodies and Staining Reagents

The rat anti-mouse IL-2 mAb, S4B6, was purchased from Pharmingen (San Diego, CA) at a concentration of 0.5 mg/ml. Biotinylated hamster anti-mouse CD3ε and γδ TcR or rat anti-mouse CD4 and CD8 antibodies were also purchased from either Pharmingen, Gibco-BRL (Gaithersburg, MD) or Becton-Dickinson Immunocytometry (Mansfield, MA). Normal rat IgG was reconstituted at a concentration of 20 mg/ml (Miles Laboratories, Inc.) and normal mouse

serum at 60 mg/ml (Cappel/Organon Teknika Corp., West Chester, PA). Biotinylated secondary staining reagents (rabbit anti-rat or goat anti-hamster IgG, mouse-absorbed) were obtained from Vector (Burlingame, CA) or CALTAG Laboratories (San Francisco, CA) and recombinant mouse IL-2 from Genzyme (Cambridge, MA). Antigen-antibody-biotin complexes were detected using an avidin-biotin-horseradish peroxidase complex (Vectastain *Elite*, Vector Laboratories).

Immunohistochemistry

Sections were thawed and rehydrated in PBS and briefly post-fixed in 1% formaldehyde in PBS for 1-2 min. at room temperature. For detection of IL-2 protein, sections were washed in PBS and incubated in PBS + 0.1% Tween-20 for 30 min at room temperature (this step was omitted for detection of cell surface antigens). Sections were blocked in PBS/1% BSA, Fraction V/0.1% NaN_3 for 1-2 hours at 4°C, after which they were incubated with primary antibody at a concentration of 2-20 μg/ml in PBS/BSA/NaN_3 for 18-20 hours at 4°C in humidified chambers. Following a PBS/0.1% NaN_3 wash, sections were treated with a secondary staining reagent (5-10 μg/ml) in a similar manner and rinsed again in PBS/NaN_3 at 4°C. Samples were then fixed in 2% paraformaldehyde in PBS for 20-30 min., and washed in PBS only. Endogenous peroxidase activity was quenched using 1% H_2O_2 in methanol for 40-50 min. Detection of antibody-biotin complexes were detected as specified by the manufacturer (Vector Laboratories). Treated sections were developed with diaminobenzi-(0.6 mg/ml) with 0.002% $NiCl_2$ and $CoCl_2$ in 50 mM Tris-HCl, pH 7.5, and were counterstained with methyl green. Sections were cleared in xylenes and coverglasses were mounted onto slides.

Analysis and Quantitation

Sections stained with relevant primary antibody, equivalent to half a thymus lobe were analyzed under 100X magnification. Every experimental section was examined to exclude any bias to

non-random distribution of positively staining regions. Unique staining regions as determined after analysis of all sections were counted once.

RESULTS

Pattern of IL-2 Expression in the Normal Postnatal Mouse Thymus as Determined by IL-2 Protein Accumulation

With the prefixation of tissues prior to sectioning, we were able to increase the sensitivity of the immunohistochemical staining technique for the detection of IL-2 protein (Figure 1). Whereas a single IL-2 protein positive cells were observed in sections taken from fresh frozen tissues (Figure 1A, 1B), extensive zones of IL-2 expression were readily detected in thymus sections generated from prefixed samples (Figure 1C, 1D). Treatment of tissues prior to sectioning may have prevented the diffusion of IL-2 protein away from the local in which it was produced once sections were generated and stained. Zones of IL-2 reactivity in prefixed tissue sections localized exclusively to regions where IL-2 producing cells in tissue sections from thymi that were not pretreated (Figure 1, data not shown). As demonstrated by previous staining results obtained from sections of freshly isolated thymi (6), regions of IL-2 staining in tissue sections generated from prefixed organs were relegated to the cortex and subcapsular region and not to the medulla (Figure 2A). Comparing the data generated by both tissue treatments, we determined that the number of unique IL-2 reactive zones observed in prefixed tissue sections was equivalent to the number of IL-2 producing cells detected in fresh frozen tissue sections (data not shown). This correlation suggests that a single IL-2 producing cell contributes to a particular IL-2 protein-rich area. Specificity of staining in pretreated tissue sections in shown in Figure 2B. Preincubation of the primary antibody with an excel of recombinant mouse IL-2 (1000u) totally abolished IL-2 staining, thus the increased reactivity observed in pretreated tissue sections was indeed IL-2 specific.

IL-2 Expression is Still Not Observed in the SCID Adult Thymus Under Improved Staining Conditions

Due to a generalized defect in DNA repair of double-strand breaks, mice bearing the severe combined immunodeficiency (SCID) mutation cannot productively rearrange TcR or

immunoglobulin chain genes, resulting in the accumulation of immature T and B cells arrested at a stage in development prior to TcR and Ig chain gene rearrangement (13). As demonstrated previously, IL-2 mRNA expressing cells were not found in the SCID postnatal thymus (6). However, the absence of IL-2 protein in the SCID postnatal thymus was not confirmed, since detection of IL-2 protein was hampered by a high background of endogenous, peroxidase positive cells in sections collected from freshly isolated tissues (6). Significantly, with prefixation treatment not only was detection of IL-2 protein enhanced, but background peroxidase activity was decreased dramatically (data not shown). Therefore, to verify that IL-2 protein was absent in the SCID postnatal thymus, we stained prefixed SCID thymus sections for the presence of IL-2 protein therein. The results are shown in Figure 3. No significant IL-2 reactivity was observed in the SCID thymus (Figure 3). However, nonspecific crossreactivity to vessels in the SCID thymus was detected. Nonspecific staining of vessels by this particular rat anti-mouse IL-2 mAb has also been observed in other independent studies (15). Additionally, recombinant IL-2 was unable to abrogate crossreactive staining of vessels, showing that IL-2 protein was not responsible for the residual staining seen in SCID thymus sections. More importantly, these results reveal that even with improved detection methodology, IL-2 expression was absent in the SCID thymus. Hence functional TcR rearrangement and surface expression is an absolute requirement for the induction of IL-2 expression in the postnatal thymus.

Patterns of Intrathymic IL-2 Protein Accumulation in TcR-Mutant Mice

To investigate whether cells belonging to the αβ or γδ lineage are responsible for IL-2 protein synthesis in the thymus, we examined IL-2 protein accumulation in thymi taken from mice carrying different mutant-TcR genes (Figure 4). Detection of intrathymic IL-2 protein accumulation in these animals permitted analysis of IL-2 gene expression in the thymus when either αβ or γδ T-cell development is specifically inhibited. The deletion of a particular TcR chain is sufficient to block development of cells belonging to the relevant lineage (7, 8). In TcRα- or TcRβ-deficient mice, IL-2 protein was still detected in the thymus. Since the

development of γδ T cells does not depend on TcRα- or TcRβ-expression (7) and viewed in the context of the SCID data indicating that IL-2 protein is observed in the thymus only when functional TcR rearrangement occurs, the intrathymic IL-2 staining results obtained from TcRα and TcRβ -/- animals imply that IL-2 producing cells in these mutant thymic belong to the γδ lineage. In αβ-lineage mice, IL-2 reactivity in the thymus was constrained to the cortex and subcapsular region; this is dramatically demonstrated by the IL-2 protein staining pattern observed in the thymus of TcRβ-deficient mice (Figure 4C).

In comparable staining studies to examine intrathymic IL-2 protein accumulation in the thymus of mice rendered TcRδ-deficient by targeted gene disruption, IL-2 protein was also detected. Again, the generation of αβ T cells is independent of γδ T-cell development (8), cells induced to express IL-2 in the TcRδ -/- thymus must belong to the αβ lineage. Surprisingly, the pattern of IL-2 protein accumulation in the TcRδ -/- thymus revealed that IL-2 protein was limited to the inner cortex and cortical-medullary junction (Figure 4E). Thus, the patterns of IL-2 protein accumulation in the thymus of αβ- and γδ-lineage mice are apparently nonoverlapping. The combined intrathymic IL-2 staining data from TcRα-, TcRβ-, and TcRδ-deficient mice are given in Table I and Figure 5. These results indicate that the intrathymic pattern of IL-2 staining in normal mice is a composite of patterns generated in αβ TcR^- and γδTcR^- animals. This finding is completely consistent, since the normal thymus contains both αβ and γδ lineages. In detail, the number of intrathymic IL-2 reactive zones in TcR-deficient mice was routinely smaller than that in wild-type animals (Table I). However, when added together, the number of IL-2 protein-rich areas in TcRαβ$^-$ and TcRγδ$^-$ mice was generally equal to the number of unique reactive zones in wild-type mice in which both αβ- and γδ lineages are generated (Figure 5). As a control, IL-2 protein was not detected in TcRβ/TcRδ-double mutants, confirming that IL-2 expression in the thymus is dependent on expression of either αβ and γδ TcR. These combined data demonstrate that the intrathymic IL-2 expressing population contains both αβ and γδ TcR^+ cells.

TcRβ-Expression Alone is Insufficient to Induce IL-2 Gene Expression in the Thymus

Although the results from the study of intrathymic IL-2 protein accumulation in TcRδ-deficient mice indicate that cells of the lineage are inducible for IL-2 gene expression in the thymus, it was not possible to determine whether TcRαβ-expression or TcRβ-expression alone induces intrathymic IL-2 protein synthesis. To address this issue, IL-2 protein accumulation was analyzed in RAG-2-deficient mice expressing a TcRβ-chain transgene (16). Animals deficient in recombination activating gene (RAG) activity cannot initiate V(D)J rearrangement (17, 18). The RAG-2 -/- block in T-cell development is comparable to that observed in SCID mice. However, the expression of a functional TcRb-transgene in the RAG-2 mutant background allows cells to progress from a TcR-CD4-CD8- state to a $CD4^+CD8^+$ state (16). This transition is presumably regulated by the interaction of TcRβ and its "pre-TcRα" partner, gp33 (19). However, these cells are never subject to conventional positive or negative selection because they never express a complete TcRαβ heterodimer. As expected, IL-2 protein was not detected in the RAG-2 -/- thymus (Figure 6A). More importantly, IL-2 staining was not observed in the $TcR\beta^+$, RAG-2 -/- thymus (Figure 6C). Thus, these findings reveal that signals transduced by TcRβ/gp33 cannot induce IL-2 gene expression *in vivo*, and that intrathymic IL-2 gene expression is induced cells of the αβ lineage upon αβ-TcR surface expression.

Induction of IL-2 Gene Expression in αβ-TcR Transgenic Thymus Bred Onto Positively Selecting and Nonselecting Backgrounds

The dependence of intrathymic IL-2 gene expression on αβ-TcR expression raises the possibility that intrathymic IL-2 gene expression in developing $\alpha\beta\text{-TcR}^+$ cells is linked to a gain in function, induced by the process of positive selection. Therefore, the pattern of intrathymic IL-2 protein accumulation was determined in animals expressing a transgenic αβ-TcR specific for $H\text{-}2L^d$ bred onto H-2B (positively selecting) and H-2S (nonselecting) genetic backgrounds (9, 10). The representative staining patterns are shown in Figure 7. IL-2 protein was detected in the αβ-TcR transgenic/H-2B thymus (Figure 7A), but was also detected in the αβ-TcR transgenic/H-

2S thymus (Figure 7B). Likewise, the number of unique IL-2 reactive zones was similar in both cases (data not shown). These results show that background and frequency of positive selection do not appear to affect the frequency of IL-2 protein producers in the thymus.

DISCUSSION

In this study, we examined the effects of particular TcR mutations and positive selection on the induction of IL-2 gene expression in the thymus, as monitored by the accumulation of IL-2 protein therein. These new findings help resolve several issues with regard to the identity of cells induced intrathymically to express the IL-2 gene and the nature of the interactions that induce IL-2 gene expression in the IL-2 expressing cell population. In addition, the data presented here open new possibilities with regard to mechanisms by which developing T cells acquire functional competence and respond to fate-determining intrathymic signals.

By using an enhanced immunohistochemical staining technique, we were able to improve detection of IL-2 protein accumulation in the thymus. As mentioned previously, the comparison between the frequency of individual IL-2 producing cells detected in sections generated from fresh frozen tissues was comparable to that of unique IL-2 reactive zones seen in sections of prefixed tissue, supporting the notion that IL-2 protein synthesized in a specific area can be attributed to a single IL-2 producer. Additionally, earlier *in situ* hybridization data revealed that cells induced to express IL-2 mRNA in the postnatal thymus were found at relatively the same frequency as single IL-2 producing cells (6) and were highly IL-2 mRNA$^+$; qualitatively, the amount of accumulated IL-2 mRNA in intrathymically induced IL-2 expressors was comparable to that routinely observed in EL4 cells, a high IL-2 producing thymoma cell line, stimulated to express IL-2 with calcium ionophore and phorbol ester (6). Thus, it is likely that IL-2 producing cells in the thymus are synthesizing large amounts of protein. Finally, with regard to the location of IL-2 production in the thymus, the are in which an intrathymically induced cell produces IL-2 protein may <u>not</u> be identical to that in which the cell was initially induced to express IL-2 mRNA. In general, maximal IL-2 mRNA expression precedes maximal IL-2 protein synthesis by 10-12 hours (6). Hence, IL-2 producing cells restricted to a particular "zone," especially at the cortical-medullary junction, may have originally been induced to express IL-2 mRNA elsewhere and migrated to the site of protein production.

Our studies using immunocompromised mice to identify interactions essential for the induction of IL-2 gene expression leading to IL-2 protein synthesis in the thymus demonstrated that there are three independent ways to perturb TcR expression which result in the elimination of intrathymic IL-2 producing cells. Our data show that the disruption of TcR expression by targeted gene disruption, introduction of SCID mutation, or deficiency in RAG-2 activity results in either the partial or complete abrogation of IL-2 protein production in the thymus. Consequently, we can rule out any basis for blocking IL-2 expression/protein production in the thymus other than the absence of TcR expression and the cells on which TcRs are expressed.

Induction of IL-2 gene expression in the thymus is contingent upon TcR surface expression. Mutations that block TcR gene rearrangements or interfere with αβ and γδ TcR surface expression inhibit intrathymic IL-2 gene expression. This is supported by results demonstrating that intrathymic accumulation of IL-2 protein was not detected in SCID mice and animals deficient in RAG-2 activity of TcRβ along with TcRδ chain expression. However, expression of a rearranged TcRβ-chain is insufficient to induce IL-2 gene expression in thymocytes *in vivo*. It has been shown that TcR chain can be expressed on the cell surface of immature thymocytes in the absence of TcRα-chain expression (19-21). Prior to TcRα-chain surface expression in conjunction with TcRβ-chain, TcRβ-chain is disulfide linked to a newly identified polypeptide, gp33 (19). TcRβ/gp33 dimers apparently associate with CD3γ, δ, and ε, but only weakly with CD3ζ (16, 19). Expression of gp33 may be developmentally regulated; once functional TcRα-chain is expressed, TcRβ-chains preferentially associate with TcRα-chains and TcRβ/gp33 dimers are no longer detectable (19). Partial characterization of intracellular signals transduced by TcRβ/gp33 revealed that TcRβ/gp33 crosslinking leads to a rapid calcium flux (21). Altogether, the signals transduced by TcRβ/gp33 with its undisclosed ligand(s) are able to initiate the explosive expansion of cell numbers, expression of CD4 and CD8 coreceptors, and suppress further TcRβ-chain gene rearrangement (22). Presumably, activation of the *src*-family protein tyrosine kinase, Lck, is involved in TcRβ/gp33-mediated signaling, since upregulation of Lck mediates immature thymocyte proliferation and suppresses TcRβ-chain gene

rearrangement (23, 24). A recent report details that Lck is directly involved in regulating these events (25). Thus, many of the signals transduced by TcRβ/gp33 in immature thymocytes are shared with those transduced by αβ TcR in mature T cells. Nevertheless, TcRβ/gp33 interactions cannot induce intrathymic IL-2 protein production, indicating that signal transduction pathways mediated by TcRβ/gp33 interactions may be quantitatively and qualitatively different than those mediated by αβ TcR.

Although results obtained in SCID mice demonstrate that induction of IL-2 gene expression resulting in IL-2 protein synthesis in the thymus relies on productive TcR gene rearrangement and expression, they provide little information on whether cells induced intrathymically for IL-2 gene expression belong to the αβ or γδ lineage. Detailed analysis of the intrathymic patterns of IL-2 protein accumulation in TcRα -/-, TcRβ -/-, TcRδ -/-, and TcRβ -/-δ-/- mice yielded evidence indicating that both αβ TcR$^+$ and γδ TcR$^+$ cells are included in the thymocyte population inducible for IL-2 gene expression *in vivo*. A multitude of studies *in vitro* and *in vivo* have demonstrated that subsets mature αβ and γδ TcR$^+$ cells can be induced to express IL-2, thus induction of IL-2 gene expression is a functional response that subsets of both αβ and γδ lineage T cells must possess and acquire during development. Should induction of IL-2 gene expression in the thymus be regarded as the exercise of functional competence, then, according to prediction, intrathymic induction of IL-2 gene expression would be detected in both lineages, and this is indeed the case. Interestingly, the partitioning of IL-2 inducible cells belonging to the αβ and γδ lineages in the thymus suggests that demonstration of functional competence may occur in different regions in the thymus and perhaps at different times following *bona fide* TcR expression in immature αβ TcR$^+$ versus γδ TcR$^+$ cells. On the other hand, based on arguments presented earlier, the apportionment of IL-2 producers belonging to the αβ- or γδ lineage to specific sites of IL-2 protein production may be somewhat artificial.

The correlation between functional TcR expression and induction of IL-2 gene expression in the thymus alludes to the possibility that intrathymic induction of IL-2 gene expression may be coupled to the process of positive selection. It is common knowledge that positive selection is

involved in determining specificity and function of the αβ TcR repertoire, yet less is known with respect to whether positive selection shapes the γδ TcR repertoire. Nevertheless, aspects of positive selection in the development of cells in the αβ lineage provide clues into the unanswered question regarding the role that positive selection might play in inducing IL-2 gene expression in thymocytes *in vivo*. Recent studies have demonstrated that cognate peptide for a specific transgenic αβ TcR can induce both positive and negative selection (26 27). The decision as to whether positive or negative selection is induced in the presence of peptide is highly dose dependent. These studies show that several participants in cell-surface interactions determining negative selection, positive selection, and mature T-cell activation are more or less identical-TcR, peptide, and MHC molecules in particular. Although the signals transduced by these processes are most likely nonequivalent, since the net result of TcR engagement under these sets of circumstances are death, survival, and induction of effector functions, respectively, it is still tantalizing to speculate that TcR-specific interactions in immature T cells may be sufficient to induce genetic programs characteristic of the functionally mature state. In terms of induction of IL-2 gene expression manifested by the production of IL-2 protein and positive selection in the thymus, no direct correlation could be drawn. Regardless, this does not formally disprove that intrathymic induction of IL-2 gene expression may be coupled to positive selection.

We did not observe an increase in the number intrathymic IL-2 producers represented by unique zones of IL-2 reactivity in animals for which positively selected T cells is enriched. These results can be accounted for in numerous ways. First, γδ T-cell development is not suppressed in these transgenic animals, since γδ TcR^+ cells were detected in αβ-TcR transgenic animals, since γδ TcR^+ cells were detected in αβ-TcR transgenic thymi as determined fluorescence activated cell sorting analysis (data not shown). Hence IL-2 protein production in the αβ-TcR transgenic/nonselecting thymus may be due to intrathymic induction of IL-2 gene expression in γδ TcR^+ cells. On the other hand, cells induced to express IL-2 in the transgenic/nonselecting thymus could belong to the αβ lineage, but may express nonidiotypic, endogenous TcR α-chains coupled with transgenic TcRβ-chains. In addition, kinetic studies of

thymocyte population dynamics in αβ-TcR transgenic mice have shown that there may be a limiting number of selecting "niches" available to immature thymocytes (12). Therefore, it is not entirely unexpected that an enrichment of IL-2 inducible cells in an αβ-TcR transgenic/positively selecting thymus, since the process of positive selection in itself may be rate limiting. Finally, an increase in IL-2 production may not have been observed using this ab-TcR transgenic system because positive selection is biased toward the generation of $CD8^+$/class I MHC-restricted T cells. Canonically, $CD8^+$ T cells exhibit a "killer" functional phenotype and participate directly in cell-mediated cytotoxicity. The generation of $CD4^+$ T "helper" cells that respond to foreign antigen by producing lymphokines, including IL-2, is perturbed in mice expressing transgenic ab TcRs with class I specificity (8, 10, 28, 29). Hence the failure to detect on an increase in intrathymic IL-2 production in positively selecting class I-restricted αβ TcR transgenic mice may have resulted from a failure to bias positive selection toward the generation of $CD4^+$ helper cells.

In conclusion, induction of IL-2 gene expression in the thymus can be regarded as an exhibition of functional responsiveness that occurs as developing thymocytes mature. It remains to be proven or disproven that this inductive event is linked to positive selection, the process by which immature T cells are "chosen" to mature, based on recognition specificity. Likewise, the dependence of intrathymic induction of IL-2 gene expression on functional TcR expression suggests that there is a coupling between the exercise of functional competence and recognition specificity within the thymus. However, additional research is necessary to show that this is true. Finally, intrathymic induction of IL-2 gene expression as hallmarked by intrathymic IL-2 protein production is a rare activation event which may occur independent of the positive selection process. These issues, along with further characterization of precise interactions involved in inducing IL-2 gene expression in the thymus, provide continued interest and expanded areas of research in the processes involved in the generation of functionally responsive T cells.

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TABLE I: Regions of IL-2 Staining in Thymus Lobes of Different TcR-Mutant Mice

		No. of Discrete IL-2 Staining Zones in:			
Genotype	No. Sections Examined	Outer Cortex	Inner Cortex	Cortico-Medullary	Total No. + Regions
TcRα -/-, #1	136	11	11	1	23
TcRα -/-, #2	126	13	15	0	28
TcRβ -/-, #1	72	13	5	0	18
TcRβ -/-, #2	72	8	10	0	18
TcRβ -/-, #3	74	7	10	0	17
TcRδ -/-, #1	126	2	17	22	41
TcRδ -/-, #2	126	2	22	16	40
TcRβ-/-δ-/-	62	0	0	0	0
TcRα+/-	136	13	18	18	49
TcRβ+/+ w.t.	132	12	19	20	51

Total number of sections examined for each sample represents half a thymic lobe in each case. IL-2 staining regions identified by immunohistochemistry of serial sections, and corrected for regions detected in more than one section. No staining was observed in the medulla proper.

FIGURE LEGENDS

FIGURE 1. Fresh frozen versus prefixed tissue sections of normal thymuses generated from 4-5 week old mice stained with a rat antimouse IL-2 mAb. Tissues prefixed prior to sectioning were incubated in 4% paraformaldehyde/PBS for 16-18 hours, washed thoroughly in PBS, saturated in 30% sucrose/PBS and then embedded in O.C.T. Negative control stainings were carried out using normal rat IgG in lieu of the primary antibody. No apprecial reactivity was observed in negative control samples (data not shown). Bright field, 200X. Medulla (M).

(A): IL-2 reactive cell associated with cortex (arrow) in fresh frozen thymus section.

(B): IL-2 reactive cell associated with cortical-medullary junction (arrow) in fresh frozen thymus section.

(C): Zone of IL-2 reactivity associated with cortex in prefixed thymus section.

(D): IL-2 reactive zones associated with cortical medullary junction in prefixed thymus section.

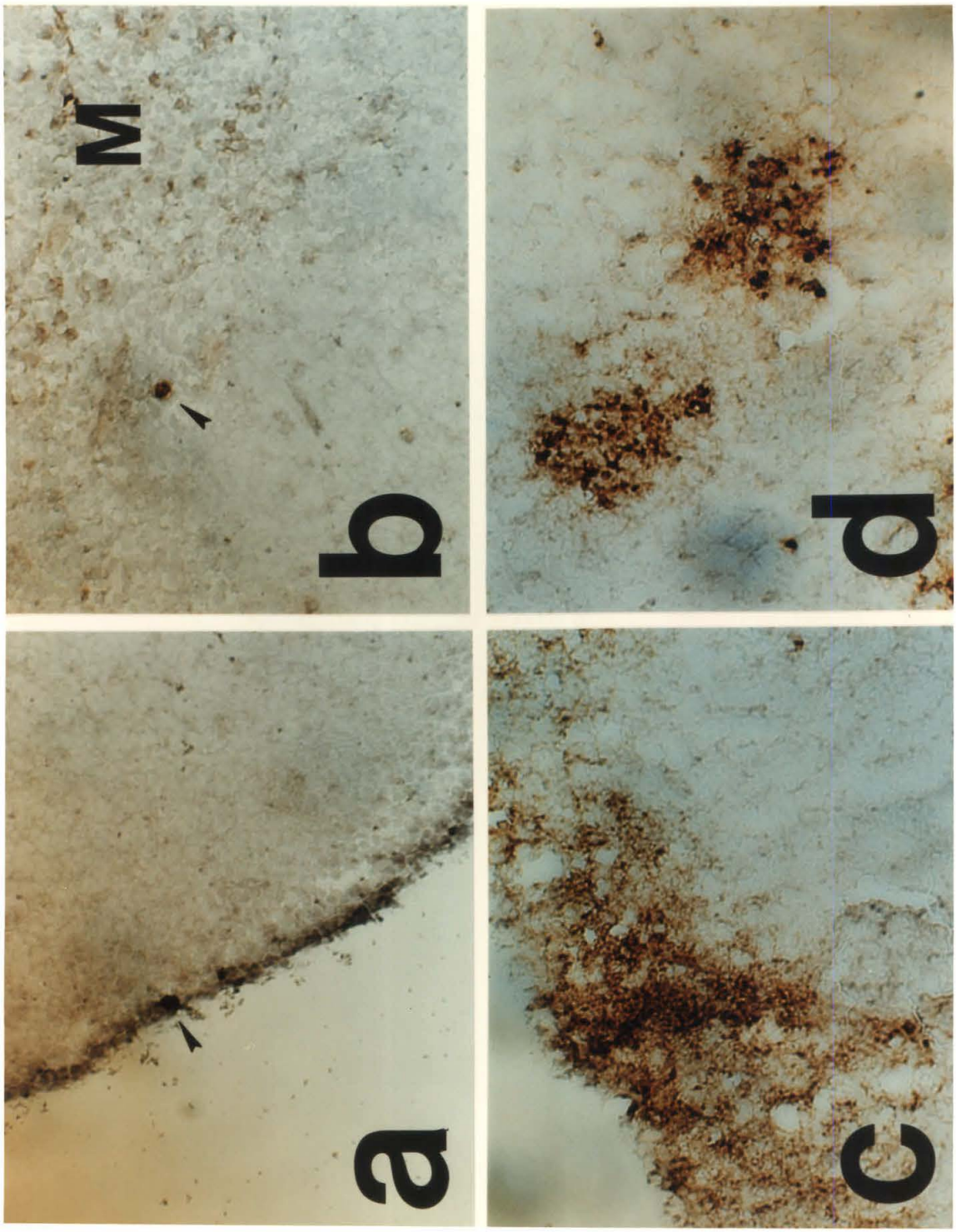

a
b
M
c
d

FIGURE 2: Blocking experiment to demonstrate specificity of staining in prefixed normal mouse thymus sections. Blocking treatment is described in Materials and Methods. Arrows point to "islands" of cortex in the medulla. Counterstained with methyl green. Bright field, Upper panels 15X, lower panels 30X. Bar = 1 mm.

(A): Stained with anti-IL-2 mAb.

(B): Stained with primary antibody after preincubation with recombinant mouse IL-2.

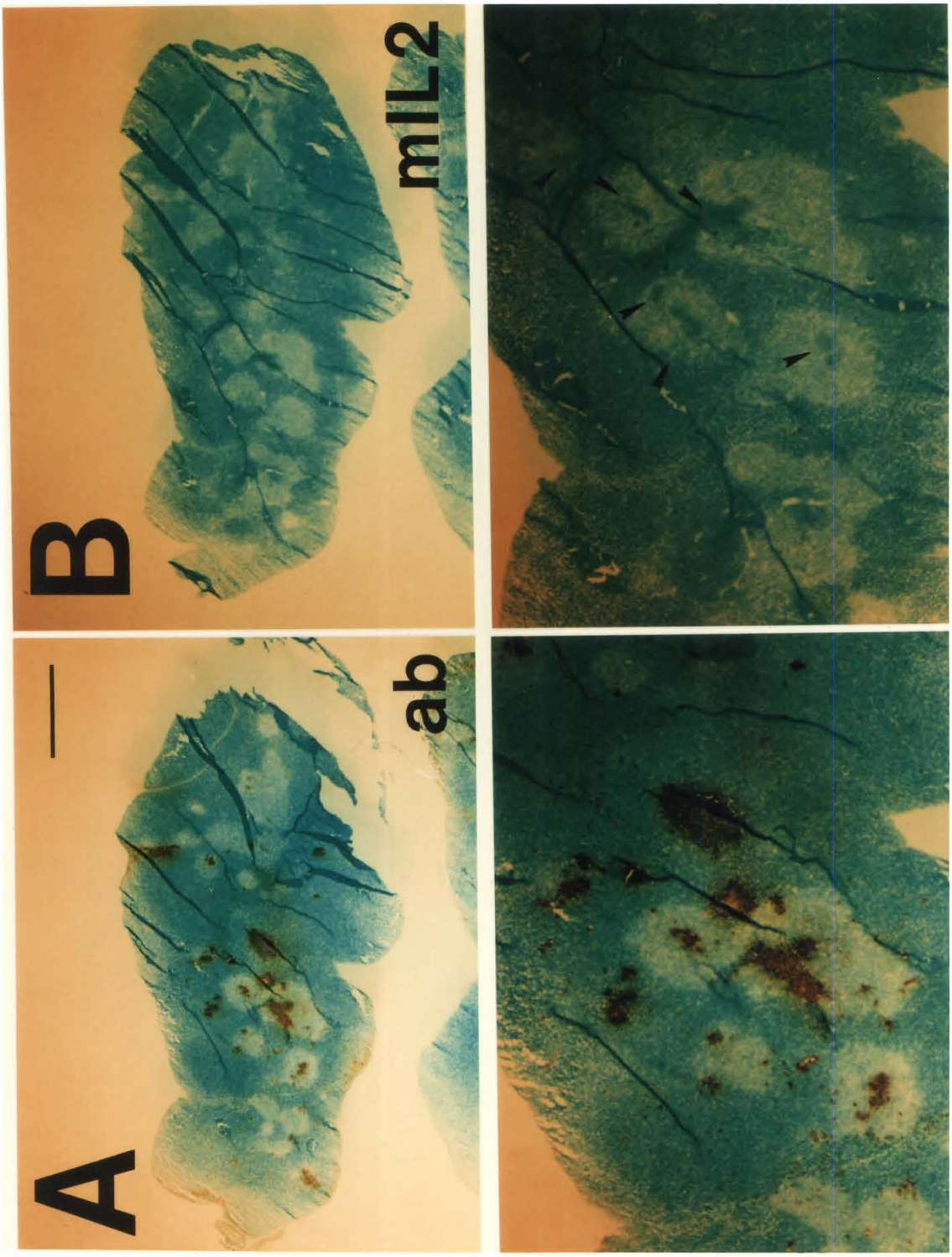

A
ab
B
mIL2

FIGURE 3. Prefixed thymus sections taken from 4-5 week old SCID mice stained for IL-2 protein accumulation. Counterstained with methyl green. Bright field, 15X. Bar = 1 mm.

(A): Stained with anti-IL-2 mAb.

(B): Negative control staining.

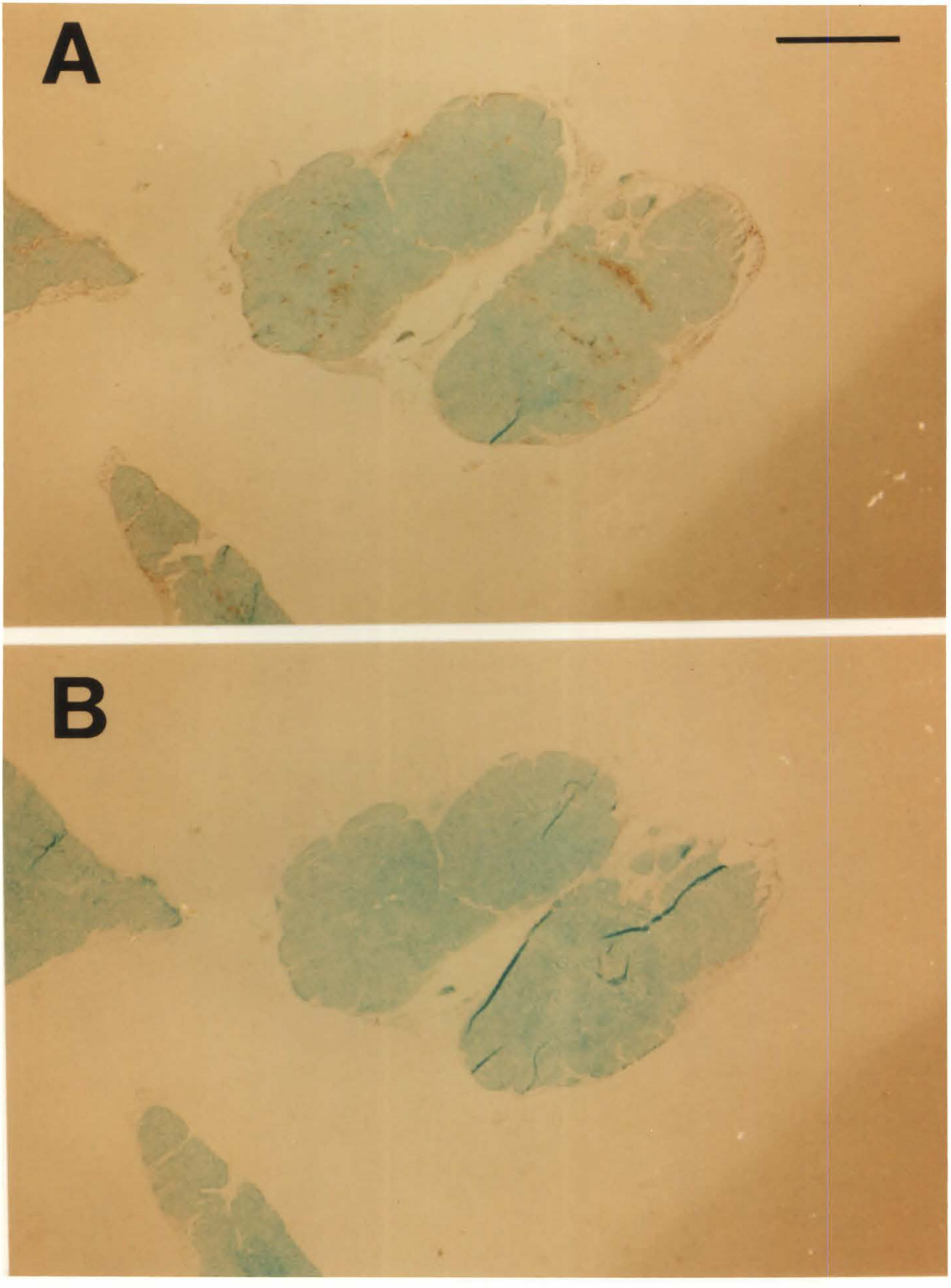

A
B

FIGURE 4. Staining of prefixed thymus sections generated from TcR-deficient mutant mice for IL-2 protein accumulation. Counterstained with methyl green. Bright field, 15X. Left panels: staining with primary antibody. Right panels: Negative control staining. Bar = 1 mm.

(A): TcRα -/- thymus sections.

(B): TcRβ -/- thymus sections.

(C): TcRγ -/- thymus sections.

(D): TcRβ-/-δ-/- thymus sections.

Medulla is identified by less intensely stained areas following visualization by counterstaining. Arrows point to reactive zones.

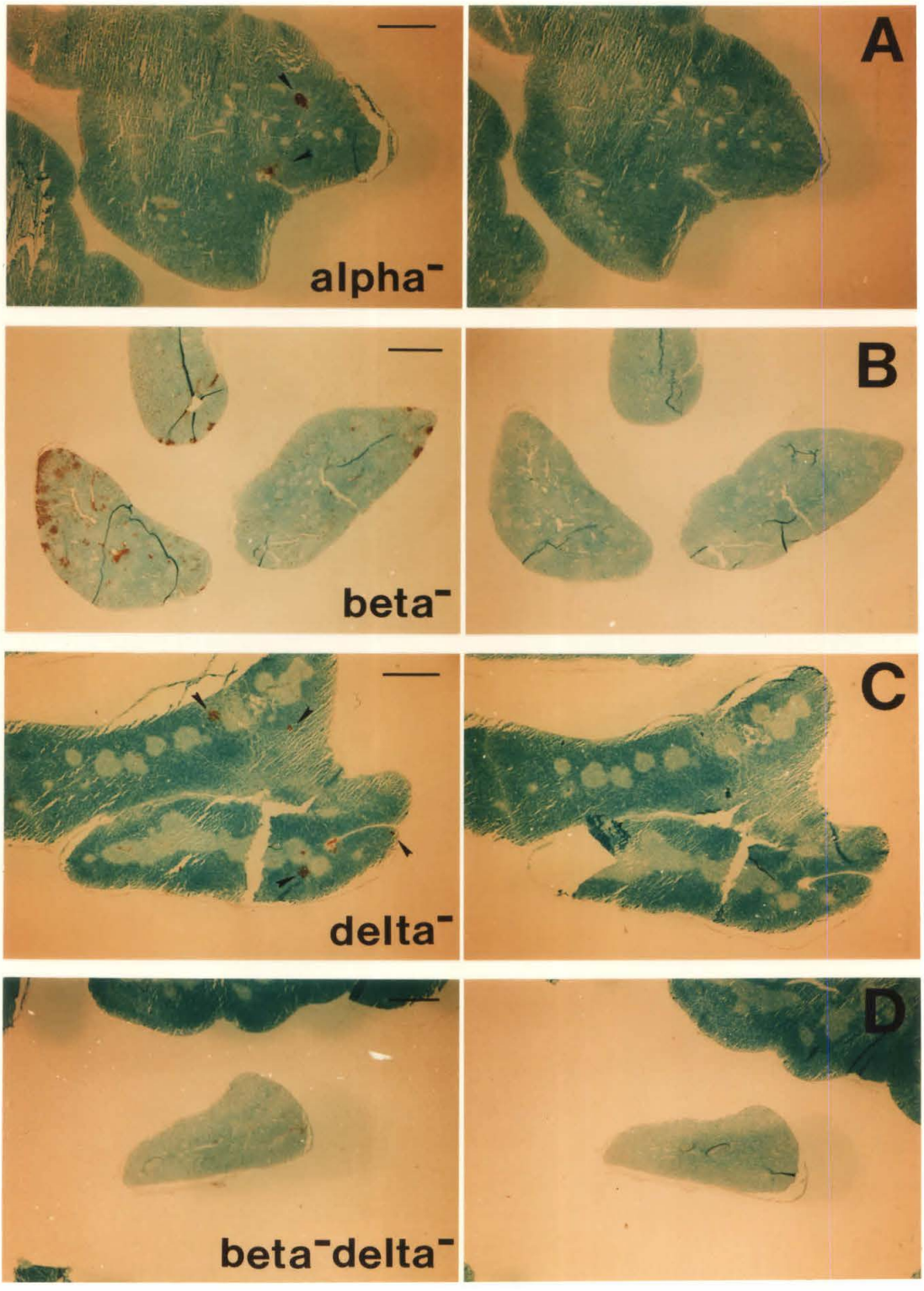

alpha⁻
A
beta⁻
B
delta⁻
C
beta⁻delta⁻
D

FIGURE 5. Bar graph showing frequency and distribution of unique IL-2 reactive zones in the thymus of TcRα -/-, β -/-, δ -/-, β -/- δ -/-, and wild-type mice. Analysis was performed on thymi isolated from at least two different animals.

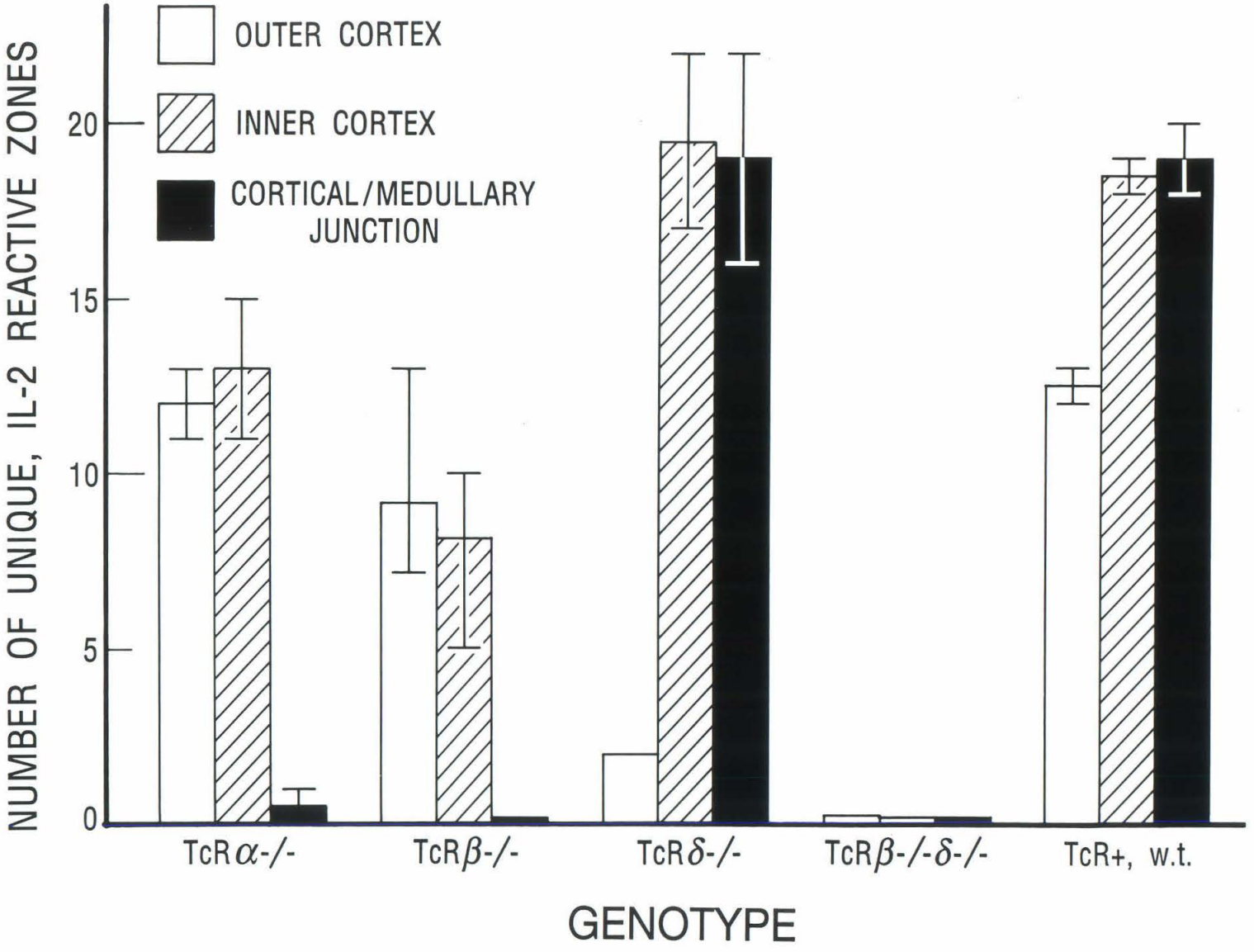

OUTER CORTEX
INNER CORTEX
CORTICAL/MEDULLARY JUNCTION
NUMBER OF UNIQUE, IL-2 REACTIVE ZONES
0
5
10
15
20
TcRα-/-
TcRβ-/-
TcRδ-/-
TcRβ-/-δ-/-
TcR+, w.t.
GENOTYPE

FIGURE 6: Expression of TcRβ does not restore intrathymic IL-2 production in rearrangement-deficient mice. Left panels: staining using anti-IL-2 mAb. Right panels: negative control staining. Positive control staining (normal thymus sections) are not shown. Counterstained with methyl green. Bright field, 15X.

(A): Prefixed thymus sections generated from 4-week old RAG-2 -/- mice.

(B): Prefixed thymus sections generated from a 5-week old TcRβ-transgenic RAG-2 -/- mouse.

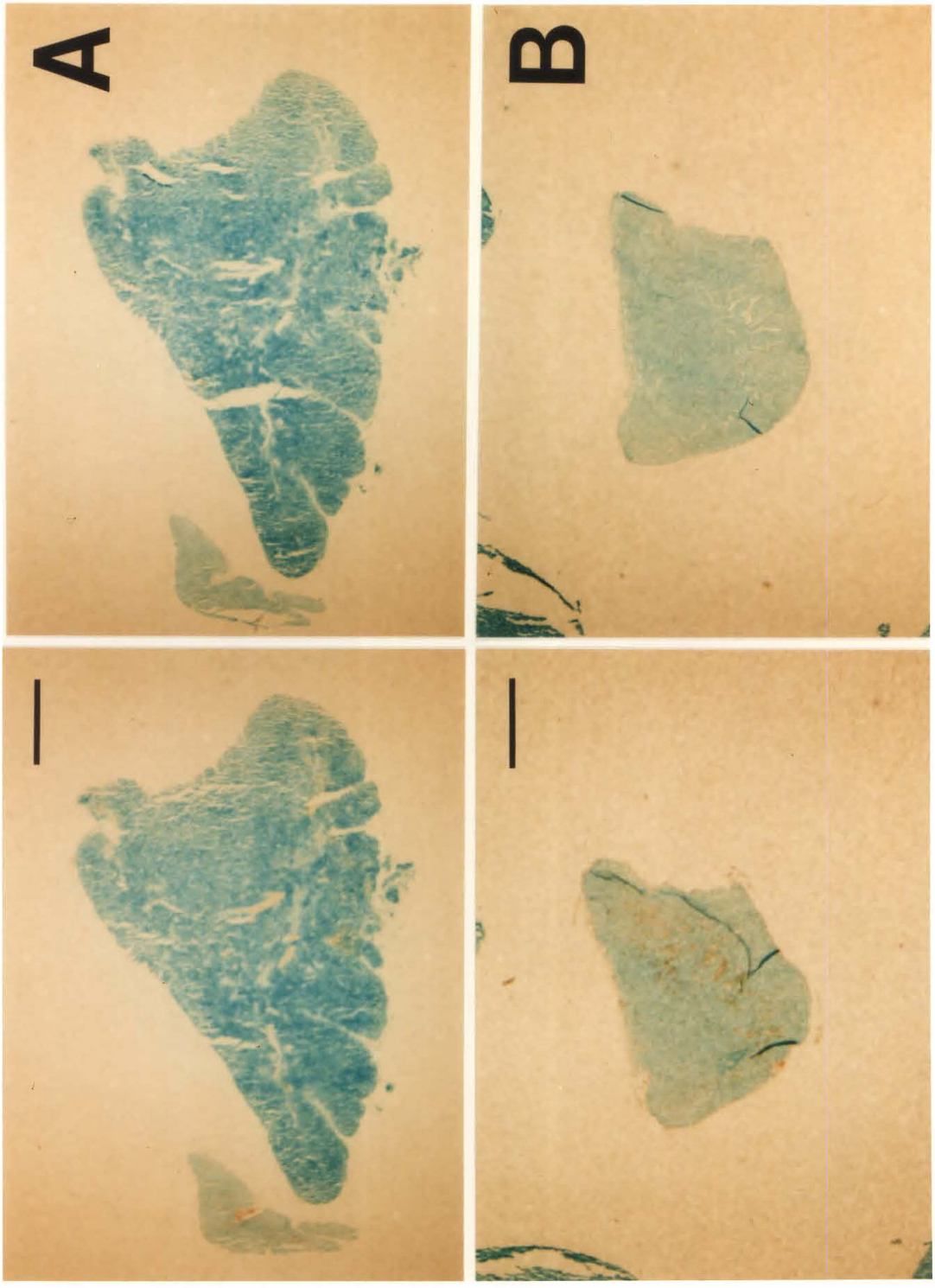
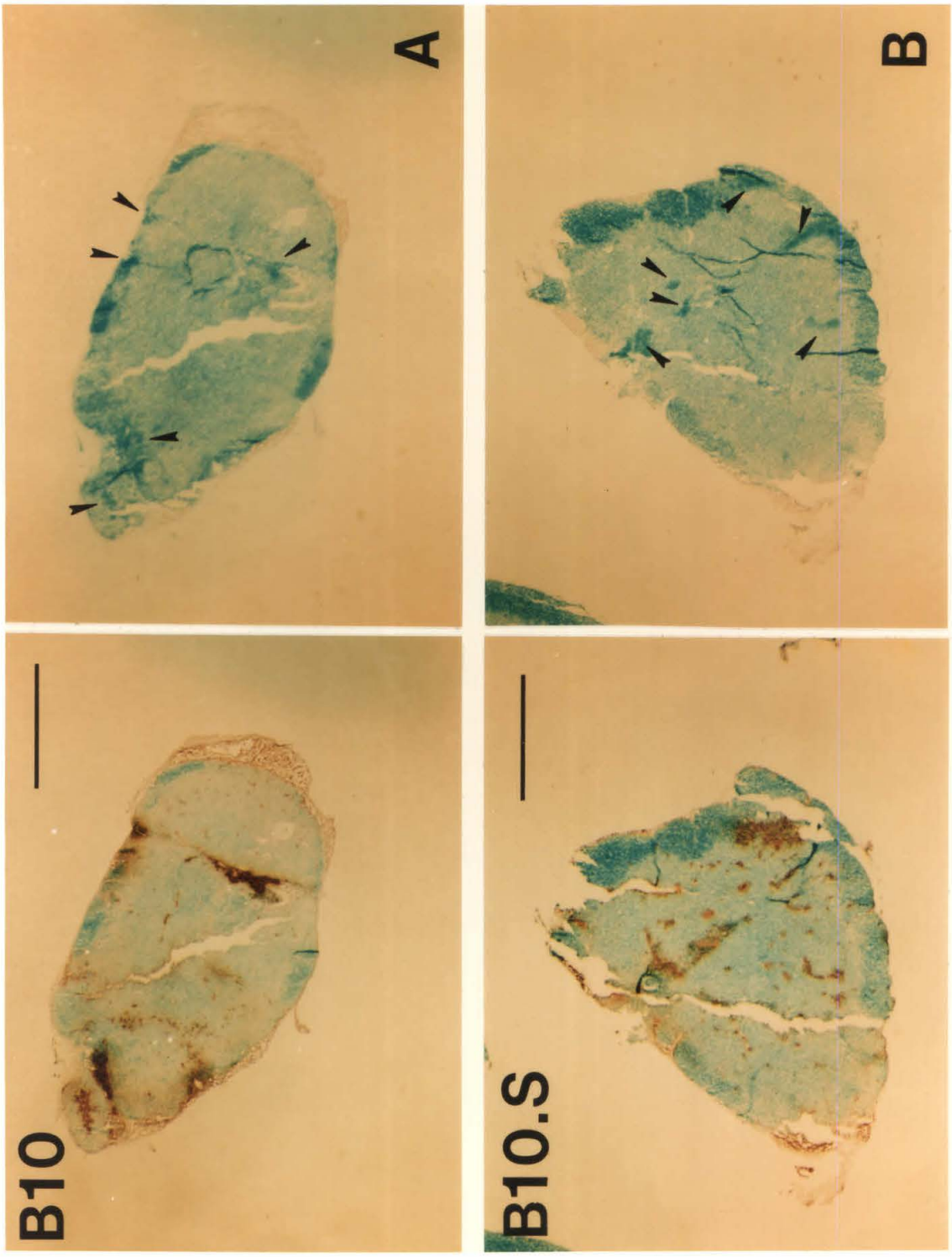

A
B

FIGURE 7: Staining of prefixed tissue sections generated from 3-5 week old αβ-TcR transgenic mice using a rat anti-mouse IL-2 mAb. Left panels: staining with primary antibody. Right panels: negative control staining. Counterstained with methyl green. Arrows indicate correponding regions of cortex in negative control staining that are immunoreactive in the adjacent serial section. Bright field, 25X. Bar = 1 mm.

(A): H-2B αβ-TcR transgenic thymus.

(B): H-2S αβ-TcR transgenic thymus.


A
B
B10
B10.S

CONCLUSION

The body of work presented here sheds new light on several aspects of IL-2 gene expression *in vivo* . First, IL-2 expression is detected in the thymus, the paramount site of T-cell development. Cells induced to express IL-2 intrathymically can be separated into two main groups as defined by the dependence of IL-2 expression on functional TcR rearrangement and surface expression. One subset is found in the day 14-15 fetal thymus. IL-2 expressing cells belonging to this subset are restricted to the periphery of the fetal thymus. Peak IL-2 mRNA accumulation in this fetal thymic IL-2 expressing cells is detected at a day 14.5 of gestation and maximal protein synthesis is observed 12 hours later. Induction of IL-2 gene expression in this subset is extremely transient; extensive IL-2 mRNA accumulation and IL-2 protein synthesis cannot be detected in the outer region of the thymus by day 16 of gestation. Significantly, this population of IL-2 expressing cells is not eliminated by the SCID mutation, thus induction of IL-2 gene expression in these cells occurs independently from the generation of function TcRs. These results raise the possibility that this fetal subset of intrathymic IL-2 producers may not belong to the T lineage (see below).

The second class of intrathymic IL-2 expressing/producing cells is also present in the fetal thymus. However, IL-2 expressing/producing cells of this class are also found in the postnatal thymus. Unlike the first class of IL-2 producers, induction of IL-2 gene expression and IL-2 protein synthesis in cells belonging to this class requires functional TcR expression. In the day 14-15 fetal thymus, IL-2 expressing cells of this class represent a minor population of intrathymic IL-2 expressing cells and reside in the inner region of intrathymic IL-2 expressing cells and reside in the inner region of the organ (data not shown). In the postnatal thymus, IL-2 expressing/producing cells are extremely rare and are found throughout the cortex and at the cortical-medullary junction. The absence of IL-2 expressing cells in the medulla demonstrates that these cells are not mature thymocytes nor recirculating T cells that have homed back to the thymic medulla. This class of IL-2 expressing/producing cells is not generated in TcR gene

recombination-defective mice, since the early block in T-cell development which eliminates all subsets of TcR$^+$ cells also results in the abrogation of IL-2 expression and IL-2 protein production in the postnatal thymus.

Although results obtained in SCID (and RAG-2 -/-) mice suggest that intrathymic IL-2 production is contingent on productive TcR-gene rearrangement and expression, Ig-chain gene rearrangement is equally affected in these animals, thus it is conceivable that intrathymic IL-2 producing cells represent a population of non-T lineage cells in the thymus (i.e., could belong to the B lineage). B-cell development is not observed in the thymus; in fact, the thymus may even suppress development of B cells derived from the bipotential lymphoid precursor found within. However, bone marrow-derived cells are found in the thymus and putative non-T-lineage IL-2 producing cells could be associated with this population. Nonetheless, results from the examination of IL-2 production in mutant mice deficient in TcRβ- and TcRδ-chain expression indicated that IL-2 producing cells in the thymus belong to the T lineage. In these animals, the development of αβ and γδ T cells is impaired without affecting B-cell development. The complete block in the generation of TcR$^+$ cells had a profound effect on intrathymic IL-2 production. Intrathymic IL-2 producing cells were not found in TcRβ-/-TcRδ-/- double mutant mice, thereby demonstrating that intrathymic protein synthesis requires the exclusive generation of TcR$^+$ cells. Finally, further characterization of intrathymic protein accumulation in mutant mice deficient in the expression of a single TcR chain resulting in the elimination of either αβ- or γδ-TcR$^+$ cells showed that intrathymic IL-2 producing cells were found irrespective of the absence of a single T-cell lineage, hence both αβ- and γδ-TcR$^+$ cells are found in the second class of intrathymic IL-2 producers.

Identification of immature T cells capable of producing IL-2 *in vivo* reveals subtle differences between the acquisition of functional competence defined *in vitro* and the exercise of functional responsiveness *in vivo*. Whereas a high proportion of TcR$^-$ immature cells are competent to induce IL-2 gene expression *in vitro*, they are not induced to do so *in vivo*. Likewise, TcR$^+$ immature cells that are induced to express/produce IL-2 in the thymic cortex are

functionally competent by definition, unlike the vast majority of cortical thymocytes that are functionally incompetent. It is not clear what other properties delineate functionally responsive TcR^+ immature cells from functionally incompetent ones other than the display of IL-2 protein synthesis *in vivo*. Due to the extreme infrequency of the activation event involved in inducing IL-2 expression *in vivo*, additional characterization of cells exercising functional competence by producing IL-2 and interactions involved in inducing expression in these cells is technically daunting. Lastly, since TcR^+ intrathymic IL-2 producing cells have theoretically not completed the maturation process, the developmental fate of these cells is still unknown. With the advent of *in vivo* cell lineage tracing techniques that permanently label cells that have transiently expressed a particular gene during the course of development with a heritable genetic marker (1-3), it may be possible to follow the fate of immature cells that have been induced to express IL-2 in the thymus. Hence, the initial identification of functionally responsive cells in the thymus lays the foundation for new studies to examine the relationship between the exercise of functional competence during T-cell development and the actual generation of functionally mature cells.

With regard to extrathymic sites of IL-2 gene expression, the identification of IL-2 expressing cells in the gut and fetal hematopoietic tissues facilitates the interpretation of results obtained in studies of IL-2 -/- mice. IL-2 producing cells are found throughout the gut and can contribute to mucosal immune responses and immunologic balance in mucosal tissues. The association of IL-2 producing cells with the lamina propria suggests that LPL populations are involved in the prevention of inflammatory bowel disease. The absence of IL-2 producing cells and/or the absence of IL-2 protein leads to a debilitating immunoreactive state. A more thorough characterization of intestinal IL-2 producing cells will increase the understanding of how IL-2 regulates important immune-related processes in the gut. Since a significant number of gut-associated IL-2 producing cells are generated during fetal ontogeny, the susceptibility to contract ulcerative colitis could be a congenital defect resulting from a failure to produce functionally competent cells found in the gut prior to birth. Treatment of IL-2 -/- mice with fetally-derived gut-associated IL-2 producing cells or potential fetal precursors of these cells would certainly

define the role that these cells play in immunity and maintenance of immunologic balance in the mucosa.

Since IL-2 -/- mice also display defects in hematopoiesis, it is of particular interest that IL -2 protein synthesis was detected in fetal hematopoietic tissues. Although problems related to hematopoiesis are observed in older postnatal animals, it is particularly provocative to speculate that the disruption of IL-2 production in the fetal liver, a major site of hematopoiesis, somehow contributes to abnormalities manifested later in postnatal life. It is known that precursors from the fetal liver seed the bone marrow. Again, it would be fascinating to investigate the possibility that cells which produce IL-2 in fetal tissues with which hematopoiesis is associated contribute to processes involved in regulating balance of immune responses in the mucosa and hematopoiesis in postnatal animals. This issue can also be addressed using cell lineage tracing techniques to examine the developmental fate of IL-2 producing cells in the fetal liver and omentum, an additional embryonic site of lymphopoiesis. Based on the IL-2 knockout data, it can be postulated that descendants of cells induced to synthesize IL-2 protein in the fetal liver and omentum would be found later in the fetal gut and postnatal hematopoietic and secondary lymphoid tissues. In addition, detection of IL-2 protein in the fetal liver and omentum indicate that induction of IL-2 expression and protein synthesis also occurs extrathymically in tissues actively participating in lymphopoiesis, thus signals exist outside the thymus to induce IL-2 gene expression in subsets of fetal cells that may or may not belong to the T lineage; a more in depth characterization is required to determine the actual identity of IL-2 producing cells in fetal hematopoietic tissues.

Additional analysis of extrathymic IL-2 expression *in vivo* revealed that IL-2 can be implicated in the extrathymic expansion of T cells associated with the skin of normal mice. Temporal expression of IL-2 in the skin depends on the presence of T cells therein. The distribution of IL-2 expressing cells in the dermis and epidermis of young mice is strikingly similar to that of resident CD3 + dendritic epidermal cells. The absence of both IL-2 expressing cells and DECs in the skin samples taken from SCID mice provide strong circumstantial

evidence that these IL-2 expressing cells belong to the T lineage. At a time when DEC populations are expanding, IL-2 mRNA and protein are readily detected in the skin. The association of IL-2 expressing cells in the skin with hair follicles in young mice indicates that inductive signals that regulate IL-2 expression in these cells are found in follicles. Once DECs migrate away from follicles to populate the epidermis in older animals, they are no longer induced to express IL-2. Hence transience of IL-2 gene expression in DEC populations appears to be regulated by the interaction of DECs with hair follicles. In normal mice, the majority of CD3 + DECs express invariant γδ TcRs that recognize keratinocyte specific antigen(s). However, interaction with hair follicles of DECs expressing completely "inappropriate" TcRs nevertheless resulted in IL-2 protein synthesis, suggesting that expression of a particular TcR does not specify IL-2 expression in expanding DEC populations. Dissection of the regulation of IL-2 gene expression in DECs on a molecular level may provide insight into the apparent "flexibility" regarding TcR expression and induction of IL-2 gene expression in DECs.

The studies described here have primarily focused on IL-2 expressing cells and IL-2 production in particular tissues. The precise identities of cells responding to IL-2 have not been determined. Expression of the high affinity IL-2 receptor as defined by the presence of the inducible component (CD25/IL-2Rα) was superficially examined in tissues where IL-2 producing cells are found (data not shown). The absence of IL-2Rα expression is not necessarily indicative of nonresponsiveness to IL-2, since cells expressing lower affinity IL-2Rs (forms of IL-2R that do not include the α-chain) are also functional and cannot be readily detected at this time. Detailed characterization of IL-2 responding cells *in vivo* is an extensive undertaking in itself. However, the identification of IL-2 producing cells *in vivo* provides a sound foundation on which studies to characterize cells responding to IL-2 in specific tissues can be based.

Overall, the results described in previous chapters pertaining to the patterns of IL-2 gene expression *in vivo* reveal vital information regarding the regulation of IL-2 gene expression and the function of IL-2 in the mouse. Data presented here provide new opportunities to understand differential regulation of IL-2 gene expression in specific populations of IL-2 producing cells

identified initially *in vivo*. Likewise, the fate of cells induced to synthesize IL-2 protein *in vivo* is a fascinating issue with respect to the development of cells in the T lineage and precursor "potential" associated with particular IL-2 production found in fetal tissues. Finally, additional characterization of IL-2 producing cells induced *in vivo* and responding cells therein will generate a more complete picture of the function of IL-2 in the mouse. Hence this body of work provides a basis for fruitful areas of research for many years to come.

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IL-2 GENE INDUCIBILITY IN T CELLS BEFORE T CELL RECEPTOR EXPRESSION

Changes in Signaling Pathways and Gene Expression Requirements during Intrathymic Maturation[1]

ELLEN V. ROTHENBERG,[2] ROCHELLE A. DIAMOND, KAREN A. PEPPER, AND JULIA A. YANG[3]

From the Division of Biology, 156-29, California Institute of Technology, Pasadena, CA 91125

The ability to express the growth hormone IL-2 upon stimulation gives T lymphocytes one of their major effector functions in the immune system. IL-2 is apparently synthesized only by T cells, and only by a subset of T cells which constitutes a "helper" class. It remains unknown how and when the IL-2-producing lineage becomes distinct from other functional effector lineages. We have therefore examined immature T cell precursors to determine when IL-2 inducibility is acquired in relation to other maturation events, such as expression of an Ag-binding TCR, which is suspected to play an influential role in the determination of subclass commitment. In mature T cells, IL-2 is inducible via agonists of the phosphoinositide pathway, a network of signaling mediators shared by a wide variety of metazoan cell types. The universality of this activation pathway makes it seem less likely, a priori, to be a target of developmental change than the intrinsic susceptibility to induction of the IL-2 locus. However, our results presented here refute this expectation. In this report, we show that both TCR$^+$ cells and pre-T cells too immature to express TCR can be induced to express IL-2 at high levels. The induction requirements for IL-2 expression, however, are different in TCR$^-$ and TCR$^+$ cells. Even by using Ca^{2+} ionophore and phorbol ester to bypass the requirement for the TCR in cell activation, the TCR$^-$ cells also require the presence of the polypeptide hormone IL-1. By contrast, TCR$^+$ mature cells not only can express IL-2 without IL-1, but also show no response to IL-1 when Ca^{2+} ionophore and phorbol ester are present. IL-1-dependent IL-2 producers appear in the thymus of repopulating radiation chimeras before "mature" (TCR$^+$) T cells, whereas IL-1-independent IL-2 production is found only afterward. Thus, IL-2 inducibility per se apparently precedes TCR expression and all TCR-associated fate determination events. However, developmental alteration of signal transduction pathways may play a vital regulatory role in the later allocation of particular functional responses to appropriate lineages of T cells.

T lymphocytes are diverse in two essential features: their Ag-recognition specificities, and their functional responses to activation. These features are controlled by independent molecular mechanisms. Recognition specificity is determined by the particular combination of gene segments that a given T cell randomly selects for rearrangement during assembly of its TCR transcription units. Functional responses are defined by the battery of transiently expressed genes that a given T cell will activate, reproducibly, upon stimulation, for example after Ag binding. In the best-studied case, that of competence to express the important T cell growth factor IL-2, the response is regulated by the availability of *trans*-acting transcription factors, some of which are cell type specific and/or activation dependent (reviewed in Ref. 1). A striking but unexplained observation in mammalian T cell populations is that separate T cell lineages distinguished by criteria of Ag recognition specificity ("MHC restriction") are also largely distinct in functional response capabilities. That is, T cells whose Ag receptors bind class II MHC glycoproteins tend to be much better IL-2 producers, but poorer killers, than those whose receptors bind class I MHC targets (reviewed in Ref. 2). The correlation is strong but imperfect, reinforcing the interpretation that target recognition and response are mechanistically independent. To shed light on how these separate lineage characteristics become linked in T cell development, we have investigated whether competence to make specialized functional responses is acquired in a process that depends on expression of Ag-binding receptors.

Developing T cells in the thymus undergo a programmed sequence of gene rearrangements at the loci encoding the Ag-binding chains of the TCR complex, coordinated with other changes in cell surface phenotype (reviewed in Ref. 3). It appears that just before expression of assembled TCR complexes, most thymocytes acquire the glycoprotein markers CD8 and then CD4 (4–7). Soon thereafter, a selection event based on TCR recognition specificity appears to catalyze permanent separation of the TCR$^+$ cells into CD8-only and CD4-only lineages (8, 9). Those rare thymocytes which lack both CD4 and CD8,

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[2] To whom correspondence and reprint requests should be addressed.
[3] Supported by a predoctoral training grant from the U.S. Public Health Service.

i.e., "DN"[4] cells, are predominantly immature cells that have not yet finished rearranging their TCR loci. The DN population thus provides the test case for whether particular functional responses are only acquired as a result of events dependent on TCR specificity.

We have previously reported that there are cells in the DN population that could be induced to express IL-2, provided that a chemical stimulus was used that bypassed the need for a cell-surface TCR (10, 11). Recent work, however, has delineated minority subpopulations within the DN thymocyte set which have apparently retained a $CD4^-CD8^-$ phenotype in spite of having already acquired potentially functional TCR. The present work was undertaken, therefore, to test directly whether IL-2 gene inducibility was confined to cells already expressing TCR. We have found that the IL-2 locus is highly inducible even in immature, TCR^- cells, but that the biochemical triggering requirements for its inducibility shift dramatically during T cell maturation. Thus, the alteration in signal transduction pathways provides a distinct tier of regulation in the developmental acquisition of competence to make IL-2.

MATERIALS AND METHODS

Animals. C57BL/6J and C57BL/6Boy-Tla[a] mice were bred and maintained in our own colony and used in most experiments between the ages of 4 and 6 wk. For radiation chimeras, 7 to 9-wk-old C57BL/6J mice were used as recipients and B6.PL-Thy-1.1/Cy mice (Jackson Laboratories, Bar Harbor, ME) were used as donors of marrow. In most experiments, splenocytes were taken from the same young animals as the thymocytes. For isolation of purified splenic T cells, however, in Table I, experiment 3, splenocytes were isolated from older animals, 3 to 4 mo old.

Reagents and antibodies. Human rIL-1α and murine rIL-2 and rIL-4 were obtained from Genzyme (Boston, MA). A 1000 U/ml stock of IL-1 in Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS was added to cultures to a final concentration of 50 U/ml. The calcium ionophore A23187 and TPA were obtained from Sigma (St. Louis, MO) and prepared as stock solutions in Me_2SO. Aliquoted stocks were stored at −20°C. The concentration of the A23187 stock was determined spectrophotometrically to be 0.7 mM. TPA was dissolved at 10 μg/ml. Antibodies used for cytolysis and to verify cell-surface phenotype by flow cytometry were as described previously in detail (12). Briefly, directly coupled fluorescein-conjugated 53.6.7 and phycoerythrin-conjugated GK1.5 detected CD8 and CD4, respectively, and 500A2 with fluorescein-conjugated goat-anti-hamster Ig detected CD3/TCR complexes. In some experiments, fluorescein-conjugated Mar-18.5 (Becton Dickinson, Mountain View, CA) detected residual antibody on C-treated cell populations. For cytolytic elimination, RL172.4 (anti-CD4) and 3.155 (anti-CD8) were used, with the addition of CG-16 anti-CD5 (Cedarlane, via Accurate Chemical Co., New York, NY) to eliminate $CD5^{high}$ cells (10, 13). Rabbit complement was from selected lots (#2728 and #3069, Pelfreez, Deer Park, MI), and used in one-step incubations with antibody at 1/25 final dilution.

Preparation of cell populations. DN thymocytes and splenocytes were prepared essentially as described previously (12, 14). In general, one round of cytolysis with anti-CD8 or anti-CD4 and two with both anti-CD8 (1/2000 ascites) and anti-CD4 (1/100 ascites or 1/8 dilution of a 10-fold concentrated supernatant) sufficed to prepare pure DN cells. $CD4^+$ contaminants survived in some cases where cells were exposed only twice to anti-CD4 (RL172.4) hybridoma supernatant. Antibodies currently available against murine CD3 are not cytolytic and could not be used for complement-mediated lysis of TCR^+ cells. Therefore, the correlated marker CD5 was used as a target of cytolysis instead. For $CD5^{low}$ cells, CG-16 ascites was added in the second and third rounds of elimination, at 1/20 final dilution. The high concentrations necessary for this antibody did show some interference with the efficacy of anti-CD4 killing. All populations were depleted of dead cells by centrifugation over a Ficoll-metrizoate cushion after each round of elimination. The entire procedure took about 8 h and resulted in cells of high viability and responsiveness. Samples were taken and stained for flow cytometric analysis immediately. We have noted that slight variations in the densities of different Ficoll-metrizoate preparations result in variable recovery of small, nondividing DN cells, which may include many of the TCR^+, IL-1-independent IL-2 producers.

Enriched splenic T cell populations were prepared as described by Julius et al. (15). Briefly, after RBC lysis in Tris-buffered ammonium chloride (0.16 M), splenocyte cell suspensions in PBS/5% FCS/10 mM HEPES (pH 7.0), at a concentration of 1 to 5×10^8 viable cells in 2 ml medium, were loaded onto nylon wool columns with warm medium (37°C). These columns (scrubbed nylon wool fibers obtained from Cellular Products, Inc., Buffalo, NY) had previously been washed and equilibrated overnight with the medium described above in a 37°C humidified incubator. Columns were sealed with parafilm and left for 1 h at 37°C. Nonadherent cells were then slowly washed from the columns with warm medium. Dead cells were depleted by centrifugation over a Ficoll-metrizoate cushion. This procedure resulted in cells >95% viable, as determined by eosin exclusion, that were >93% Thy-1.2^+ as determined by flow cytometric analysis (data not shown).

Radiation chimeras. Bone marrow cells were obtained by flushing tibiae and femora of four to five B6.PL-Thy-1.1/Cy mice with RPMI 1640 containing 0.25% BSA, 1 mM HEPES + 5 μg/ml DNAse. Cells were treated with 1/10 anti-Thy1 hybridoma supernatant T24/31.7 and lysed with 1/25 C to clear bone marrow of circulating T cells. Dead cells were removed by centrifugation over Ficoll-metrizoate. Bone marrow cells were washed twice with RPMI 1640 containing 10 mM HEPES and 0.25% BSA before resuspending in PBS for i.v. tail injection into 25 to 35 C57BL/6 lethally irradiated (900 rad) mice, at 1×10^7 bone marrow cells/mouse.

Thymuses were harvested along a time course varying from 10 to 35 days postinjection. In all cases host Thy-1.2 thymocytes were twice eliminated by addition of anti-Thy-1.2 (HO13-4) hybridoma ascites at 1/10,000 dilution and C at 1/25 dilution. Dead cells were removed as described above between each round of elimination. Thymocytes with and without elimination were analyzed by flow cytometry as described above. Thy-1.2 eliminated thymocytes were stimulated with A23187 and TPA and cultured for 20 h with and without IL-1 as described below and supernatants assayed for IL-2 production.

Culture conditions. Cells were cultured for 5 to 20 h in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% (v/v) FCS (Hyclone, Sterile Systems, Logan, UT), 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (= complete medium), with or without 50 U/ml rIL-1 and other additives as indicated. TPA (17 nM, 10 ng/ml) and A23187 (70 nM) were diluted freshly from stocks in Me_2SO at the initiation of the cultures. The final concentration of diluent, 0.11% Me_2SO, gave no detectable effects alone.

IL-2 bioassay. Supernatants from stimulated cells were collected sterilely and stored at −70°C until assay. To detect IL-2 activity, a subline of CTLL-2 cells (16) was used in a modification of the colorimetric cell viability assay described by Denizot and Lang (17), which was originally developed by Mosmann (18). Before the assay CTLL-2 cells were grown to a density of 3×10^5 cells/ml in complete medium containing 19 U/ml of IL-2. The medium used for the assay consists of phenol red-free RPMI 1640, 5% (v/v) FBS, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were harvested and resuspended in the phenol red-free RPMI 1640 medium to a density of 1×10^5 cells/ml. Doubling dilutions of the test samples were prepared to final volumes of 50 μl in the wells of 96-well, flat bottom microtiter plates (Corning 25860, Corning, NY) using the assay medium as diluent. To each well 50 μl of CTLL-2 cells were then added. After 36 h of incubation at 37°C, 20 μl of MTT, prepared as a 5 mg/ml stock in PBS, was added. The plates were incubated for 3 h at 37°C. Next, 100 μl of isopropanol were added to each well and mixed vigorously to ensure the solubilization of the blue formazan product. The OD of each well was measured within the next 2 h by using a Molecular Devices (Palo Alto, CA) V_{max} Kinetic Microplate Reader with a 590 nm test wavelength and a 650 nm reference wavelength.

In each assay, an activity standard was included for purposes of comparison between assays. The standard, a single preparation of conditioned medium from EL4.E1cells treated with phorbol ester, routinely gave 30% maximal stimulation (defined as 1 U/ml here) at dilutions from 1/500 to 1/1100 in different assays. This corresponds to a titer of 300 standard U/ml by comparison with a 200 U/ml commercial source of murine rIL-2 (Genzyme). The titers reported in Table I are raw assay units, not converted to commercial units, to emphasize the difference between activities seen and the threshold of detection. The minimum level of IL-2 detectable in a given assay is 0.5 U/ml.

In situ hybridization analysis of IL-2 RNA. cRNA transcripts from the 3′ half of the IL-2 mRNA were prepared essentially as

[4] Abbreviations used in this paper: DN, double negative ($CD4^-CD8^-$) thymocyte; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

previously described (11, 14), except that [^{35}S]UTP (850 Ci/mM, Amersham) was used carrier-free at 17.5 to 19 μM in place of [^{3}H]UTP and [^{3}H]ATP. Premeasured amounts of probe were subjected to limited hydrolysis for 10 min on ice in 0.1 N NaOH before being used for hybridization to cell samples.

The protocol for cell fixation and hybridization was modified in several ways from our previous methods (11, 14, 19). 1) Cells were centrifuged onto slides alive before fixation, at 1500 rpm at 25°C. They were not extensively washed to remove protein. This promoted better adhesion and quantitative cell recovery on slides. 2) Cells were fixed on slides with cold 4% paraformaldehyde in PBS, for 1.5 to 2 min, and then stored at 4°C in 70% ethanol. This gave lower backgrounds and much higher signals than fixation with glutaraldehyde. 3) The proteinase K digestion step was omitted, as it reduced retention of RNA in these lightly fixed cells. 4) Rehydrated slides were acetylated in 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8), then washed in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na_3 citrate) and soaked for 30 min in 0.1 M Tris, 0.1 M glycine (pH 7.0). 5) To block reactive sulfhydryl groups on the slides, the slides were washed in 2 × SSC with 1% (v/v) 2-ME, then prehybridized for 1 to 3 h at 42°C in 50% formamide, 0.9 M NaCl, 20 mM 2-[*N*-morpholino]ethanesulfonic acid (pH 5.8), 1 mM EDTA, 1% 2-ME, and 2× Denhardt's solution (1× Denhardt's = 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll). They were not dehydrated at any time before adding the probe. Prehybridization resulted in stronger signals and no increase in background, compared with slides to which probe was added when dry. 6) Hybridization buffers contained 50 mM dithiothreitol and 0.8% (v/v) 2-ME. The pH of the hybridization buffer (11) was adjusted to 5.8 by addition of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.8). The probe was denatured at 80 to 85°C in hybridization buffer immediately before loading on slides. 7) Hybridization proceeded for 3 to 6 h at 42 to 45°C. To minimize background, further processing was carried out immediately. 8) Buffer for soaking off the cover slips after hybridization (4 × SSC) and the RNase digestion buffer (0.5 M NaCl, 10 mM Tris, 1 mM EDTA, with 20 μg/ml boiled pancreatic RNase A) were not supplemented with 2-ME, to avoid interfering with the RNase. However, all subsequent washes 1) included 1% (v/v) 2-ME. The final wash, 0.1 × SSC at 45°C (30 min), was supplemented with 0.1% (v/v) 2-ME. 9) To assure efficient detection of the energetic ^{35}S-β-emissions over the tops of the cells, a thick emulsion was prepared with two parts Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) to one part 0.9 M NH_4 acetate in distilled water. 10) Slides were developed after 5 to 10 days instead of after 4 to 18 wk.

Data analysis. No positive cells were detected in any sample cultured without stimulation. This background, with the "antisense" probe, was always lower than the background with a negative control "sense" probe, which yielded 0 to 1% of cells with over 5 but less than 10 grains on stimulated and unstimulated cells alike.

The use of ^{35}S-labeled probes resulted in significantly higher backgrounds than with ^{3}H probes. This was partly due to the long path length of ^{35}S emissions rather than from nonspecific sticking, because unstimulated cells or populations with few responders showed backgrounds almost as low as those with ^{3}H probes (i.e., the majority of cells had no grains over them). By contrast, samples with many high level IL-2 producers (e.g., immature populations stimulated in the presence of IL-1) had regions with high numbers of grains in the emulsion between cells. In these cases, grain counts of 1 to 5 grains per cell often did not represent a concentration of radioactivity significantly higher than in the intercellular spaces. Figure 3 shows typical examples. To minimize the counting of false positives, we therefore adopted a stringent cutoff for positivity (≥8 grains/cell, ≥10 grains/cell on highly expanded blast cells), and excluded from the analysis cells located close to high level IL-2 producers (>30 grains/cell) unless they clearly constituted a second, comparably intense center of hybridization. In practice, this led to the exclusion of about 2 to 4% of cells from the tally. To equalize variations in probe diffusion on the slides, counts were taken from at least 12 different fields photographed from randomly distributed regions over a cell sample. To allow low frequencies of positive cells to be enumerated without observer bias, cell densities were set such that an average of 50 cells could be scored in each photographic field. Thus, e.g., with 2% responding cells, only about one field in three would lack positive cells altogether. Grain counts were made on phase-contrast pictures (e.g., Fig. 2) in which individual grains were better defined than in darkfield photographs. Grain counts represent grains within or touching the cell border, and do not include the significant number of trailing grains surrounding many of the cells with >30 grains.

RNase protection assay for IL-2 transcripts. To quantitate levels of IL-2 transcripts, the 5′ probe pJY.1, described in McGuire et al. (14) and Rothenberg et al. (19), was used. RNA from stimulated cells was extracted, hybridized, and analyzed in a modification of the method of Melton et al. (20), as previously described (14). After hybridization, the samples were digested with RNase, resolved in a denaturing 20% polyacrylamide gel, and exposed to x-ray film (Kodak XAR-5) with an intensifying screen at −70°C. In some experiments, radiation was quantitated directly with a planar β-counter developed by R. Britten and E. H. Davidson. The number of protected copies of IL-2 mRNA per average cell was calculated as follows:

$$\text{Average copy \#/cell} = \frac{\text{cpm protected (planar)}}{\text{\# of cell equivalents}} \times \frac{\text{(planar counter conversion factor)}}{\text{(dpm/ng probe)}} \times \frac{1 \text{ protected copy}}{5.5 \times 10^{-11}\text{ng}}$$

The probe specific activity (dpm/ng probe) was calculated from the known specific activity of the nucleotide precursors. The planar counter conversion factor [dpm/cpm(planar)] was measured empirically by loading a known amount of radioactivity as undigested probe in one lane of the analytical gel, then scanning the band with the planar counter. This method corrects not only for the geometry of radioactivity detection by the planar counter but also for general recovery during electrophoresis.

RESULTS

Effect of IL-1 on IL-2 secretion by DN cells. Our standard conditions of stimulation with A23187 and TPA were chosen to provide the cytoplasmic signaling intermediates that would normally be generated during TCR interactions with Ag, but in a manner independent of TCR (21). Dosages of A23187 and TPA that were optimal for IL-2 production from mature thymocytes were also optimal for IL-2 production from DN cells in our hands (10) (data not shown). However, several reports indicated that IL-1 could further potentiate IL-2 production from DN cells (22, 23). Table I reports the IL-2 activity titers induced in three independent experiments (representative of over six) with and without 50 U/ml of recombinant-derived human IL-1α (IL-1). In all cases, the addition of IL-1 elicited high titers of IL-2 from the DN population. The degree of enhancement over levels induced without IL-1 depended on the purity of the starting population, because low levels of CD4$^+$ cell contamination often led to increased IL-2 production in the absence of IL-1 (see below, and data not shown). Notably, IL-1 did not enhance IL-2 production from mature cells in response to A23187 and TPA. For example, in all experiments with spleen cells or purified splenic T cells (Expts. 2 and 3) (24) (data not shown), in each case the cells made virtually indistinguishable responses with and without IL-1. The effect of IL-1 on mature cells from total thymus and spleen will be discussed further below.

To ensure that the growth factor activity enhanced by IL-1 was due to IL-2, and not to some other lymphokine, control experiments were carried out as indicated in Figure 1. These data show that our IL-2 indicator cells fail to respond to IL-1 itself or to the alternative T cell growth factor IL-4, alone or in combination, and that neither IL-1 nor IL-4 gives a synergistic effect with IL-2 (data not shown). Thus it is most likely that this biologic assay measures IL-2 selectively, and the effect of IL-1 is indeed to increase production of IL-2 by the induced cell populations.

IL-1 permits TCR$^-$ cells to participate in IL-2 production. We reported previously that individual-cell accumulations of IL-2 RNA in DN cells activated with A23187 and TPA alone were lower than those achieved in mature T cells (11, 19, 24). Thus, IL-1 might either amplify the potential for IL-2 RNA accumulation in conventionally inducible DN cells, or it might allow the recruitment of

TABLE I

Effect of IL-1 on IL-2 production by immature T lineage cells

Expt.	Time of Stimulation (h)[a]	Sample	Cells ($\times 10^{-6}$/ml)	Percent DN	Percent CD4+[b]	IL-2 Biologic Activity[c]
1	16.7	DN, no IL-1	3.0	98.5	<1	22
		DN, +IL-1	3.0			243
2	20	CD5^{low} DN, no IL-1	3.0	94	6.0	26
		CD5^{low} DN, +IL-1	3.0			333
		DN, no IL-1	3.0	95.5	4.5	32
		DN, +IL-1	3.0			243
		Spleen,[e] no IL-1	4.0	<1	17	384
		Spleen,[e] + IL-1	4.0			332
3	20	Splenic T cells, purified, no IL-1	5.0	1	54	614
		+IL-1	5.0			717

[a] Cells stimulated with A23187 and TPA. Human rIL-1α added to 50 U/ml where indicated.

[b] Percent CD4+ = percent CD4+, CD8- as determined by two-color flow cytometry.

[c] Units/ml of IL-2 activity determined by colorimetric growth (MTT) assay with CTLL-2 cells. One unit/ml is here defined as giving 30% maximal stimulation, as described in *Materials and Methods*. This activity corresponds to 0.33 to 0.53 U/ml in standard commercial units, in different assays.

[d] Spleen cells were 17% CD4+, 8% CD8+, and 29.4% CD3+ (18.8% CD3-bright).

[e] In several experiments, IL-1 addition appeared to decrease the frequency of IL-2 producers in spleen by 20 h (Fig. 4). This effect was not seen at the level of IL-2 RNA in purified T cells, nor in the yield of supernatant IL-2 activity. It seems likely to reflect preferentially augmented survival of B cells under these conditions.

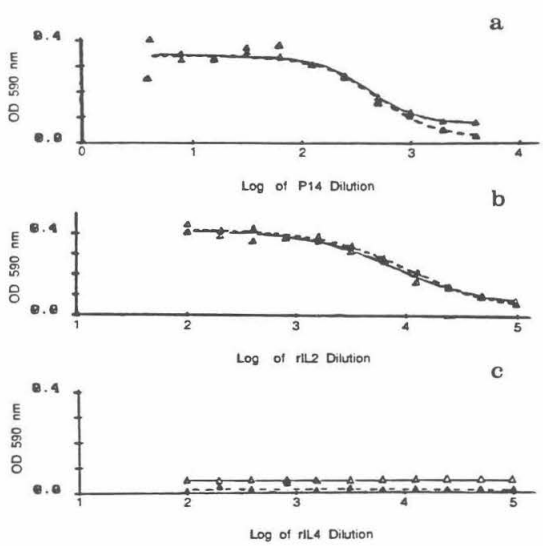

Figure 1. The IL-2 bioassay is insensitive to IL-4 and unaffected by IL-1. The panels show growth factor activity titrations on CTLL-2 indicator cells, measured by the colorimetric MTT assay as described in *Materials and Methods*. The A_{590} value is proportional to the number of mitochondrially active cells. *Panel a* shows the titration curve of our assay standard ("P14"), an IL-2-containing supernatant from TPA-induced EL4.E1 cells. *Panel b* shows the titration of murine rIL-2, 10,000 U/ml before dilution (i.e., 50 U/ml during culture in the most concentrated sample). *Panel c* shows the titration of murine rIL-4, 20,000 U/ml before dilution (i.e., 100 U/ml during culture in most concentrated sample). *Solid lines* (*open triangles*) show data with the indicated growth factors alone. *Broken lines* (*filled triangles*) show data obtained when the first dilution was supplemented with IL-1 to a final concentration of 12.5 U/ml. This is the titer that would be present in the most concentrated samples when assaying culture supernatants supplemented with 50 U/ml of IL-1. In other experiments, IL-1 also failed to affect the assay when added to the same final concentration to all dilutions of IL-2 or IL-4. Furthermore, IL-2 failed to synergize with IL-4 in the presence or absence of IL-1.

new IL-2 producers altogether.

Although DN cells represent only 2 to 5% of all thymocytes, they are heterogeneous in surface phenotype and developmental potential. About 10 to 15% of them express TCR and may therefore be relatively mature. To test whether immature thymocytes could express IL-2, we utilized two approaches. First, we sorted DN cells directly for expression of any CD3-associated TCR structure, preparing TCR+ and TCR- fractions. To avoid perturbing cellular responsiveness, anti-CD3 staining and sorting were carried out after stimulation. The second approach was based on negative selection before stimulation, thus rigorously defining the phenotype of the input cells before incubation with A23187 and TPA. To avoid artifactual modulation of the TCR, we used antibodies against the marker CD5, which is expressed at high levels on TCR+ DN cells but at low levels on most or all TCR- DN cells. It is the TCR- DN cells which include essentially all the immature precursors with the capacity to repopulate the thymus (25, 26). Therefore, CD5^{low} DN cells were isolated by antibody-dependent C elimination, sampled for analytical staining to verify lack of CD3/TCR expression, and then tested for their responses to stimulation with the Ca^{2+} ionophore A23187 and the phorbol ester TPA, as previously described (10, 11, 19). In situ hybridization was used to quantitate the frequency of cells with IL-2 mRNA at the indicated times after stimulation.

In the first approach, we activated highly purified DN cells (Table I, Expt. 1) with and without IL-1, incubated them for 17 h in culture, and then sorted them preparatively into TCR+ and TCR- fractions. These fractions were then analyzed for IL-2 RNA by in situ hybridization, as summarized in Table II. The results were strikingly different for the two populations. After stimulation with A23187 and TPA alone, the TCR+ fraction clearly included cells with IL-2 RNA, but the TCR- cells had essentially failed to respond, with <0.5% expressing detectable IL-2 RNA. In parallel experiments, when TCR+ cells were depleted from DN populations prior to culture by cytolysis

TABLE II

Recruitment of IL-2-inducible cells by IL-1 in TCR⁻ DN cells isolated by sorting after 17 h of stimulation

Cell Population[a]	First Hybridization		Second Hybridization	
	% >5 grains	n	% >5 grains	n
TCR⁺[b] no IL-1	7.1	239	ND	
TCR⁺[b] + IL-1	8.6	255	ND	
TCR⁻[c] no IL-1	0.26	385	0.39	768
TCR⁻[c] + IL-1	2.4	419	1.2	750

[a] Cells were stimulated with A23187 and TPA for 16 h 45 min in the presence or absence of IL-1 (Table I, Expt. 1). Cells were then stained with anti-CD3 and sorted to separate CD3⁺ (TCR⁺) cells from CD3⁻ (TCR⁻) cells. In the unsorted populations, cells were 98.5% DN. The percentage of cells "TCR⁺" after stimulation was about 11% for the samples stimulated +IL-1 and about 9% for the sample without IL-1. Thus, IL-1 did not cause artifactual collection of previously TCR⁺ cells in the TCR⁻ fraction.

[b] The brightest 14.5 to 16% of the cells. The window intentionally included marginal and TCR^{low} cells, to collect any cells that may have undergone TCR modulation. Resulting populations were 79.6% TCR⁺ (no IL-1) and 90.2% TCR⁺ (+IL-1).

[c] The dimmest 42% of the cells. The window excludes many of the blast cells, probably TCR⁻ (13), whose autofluorescence allows them to approach the brightness of the dimmest TCR⁺ cells. Resulting populations were <1% TCR⁺ in each case.

with anti-CD5 and C, the remaining cells failed to make IL-2 under these conditions (data not shown, and see below). However, the sorted, TCR⁻ population stimulated with the addition of IL-1 showed an appreciable frequency of positive cells, some with high grain counts. These seemed unlikely to represent TCR⁺ contaminants for two reasons. First, in the TCR⁺-sorted fractions, IL-1 addition had caused no significant enhancement, either in frequency of positive cells or in grain counts per positive cell (Table II). Second, only a minority of the TCR⁺ fraction expressed IL-2 RNA in either case, so that the number of TCR⁺ cells that might remain in the TCR⁻ fraction (<1% based on flow cytometry) is likely to be quantitatively insufficient to account for the IL-2 expressing cells there (~2%). A third possibility, that the TCR⁻ IL-2 producers simply modulated their TCR in response to stimulation, is ruled out by the confirmatory results using CD5 elimination (see below). Thus IL-1 appears to allow immature TCR⁻ DN cells to participate in IL-2 production.

Accelerated kinetics of IL-1-dependent IL-2 induction in TCR⁻ cells. The increase in IL-2 RNA elicited by IL-1 at 16 to 20 h (Table II and data not shown) was too modest to account for the dramatic enhancement of IL-2 secretion reported in Table I. It was possible, therefore, that IL-2 RNA might be synthesized early in response to stimulation with IL-1, translated, and then rapidly degraded. IL-2 RNA is highly unstable, and although it appears to accumulate in splenic CD4⁺ and CD8⁺ cells and DN thymocytes under our standard conditions of stimulation (11, 14), it may exhibit sharp transients of production and decay under various other conditions (27–30). Alternatively, an important population of IL-1 responsive cells may have been missed in the analysis shown in Table II, since the most highly activated blasts were autofluorescent and excluded to preserve the purity of the TCR⁻ fraction (data not shown). To sample the full TCR⁻ DN population, therefore, we used depletion with anti-CD5 and C and analyzed the appearance of cells with IL-2 RNA after 5 h or 18 to 20 h of stimulation. Results of two experiments are presented in Figures 2 to 4 and Table III.

Figures 2 and 3 show that although TCR⁻ DN cells do not accumulate IL-2 RNA after 5 h of stimulation without IL-1, about 10% of them do show a dramatic accumulation of IL-2 RNA to high levels at this time if IL-1 is included. The grain counts in these cells at 5 h significantly exceed those in responding splenic T cells at the same time, with or without IL-1 (Fig. 4; Table III). They are comparable to the grain counts in splenic T cells at 20 h of stimulation, when the mature cells contain an average of 500 to 800 copies of IL-2 mRNA per cell (14, 24). Thus, if IL-1 is provided, the immature cells make an accelerated response relative to mature cells, and not a delayed response that might be indicative of a requirement for some in vitro maturation event. Rough time courses for RNA accumulation, based on grain counts, are shown schematically in Figure 4. Note that, by 20 h of incubation, the immature IL-2 producers have lost most or all of their detectable IL-2 RNA, many of them now scoring as RNA-negative, whereas mature cells, with or without IL-1, continue to accumulate IL-2 transcripts. The TCR⁻ IL-2 producers thus differ from mature IL-2 producers both in their profound requirement for IL-1 as a costimulus, and in their explosive accumulation and swift loss of the IL-2 transcripts they synthesize under these conditions.

Subpopulation specificity of the IL-1-dependent response. Depletion of TCR⁺ cells, including CD4⁺ contaminants, is not always complete by using complement-mediated lysis. Comparison of the frequency of responding cells with the frequency of these contaminants, however, excludes the possibility that the IL-1 dependent responders are drawn exclusively from TCR⁺ subsets. In three independent experiments (Table III, Fig. 4, and data not shown), the frequency of "CD5^{low}" cells making IL-2 RNA in response to IL-1 (5-h point) clearly exceeded the frequency of TCR⁺ contaminants as determined by surface expression of the TCR-linked CD3 complex at the start of culture (Fig. 4). This is most readily seen in the case of Table III, where the CD5^{low} cells were 97% TCR⁻, but in all three cases the number of IL-2 producers was greater than the number of all TCR⁺ cells by a constant increment of 7 to 8% of the population. At these brief times of stimulation, input DN cells are recovered with >90% viability (with or without IL-1) and do not undergo significant population shifts. Therefore, the majority of the IL-1-dependent IL-2 producers could not be accounted for by cells expressing any CD3-linked TCR structure.

These data do not exclude the possibility that some TCR⁺ cells are also IL-1 dependent. The TCR⁺ contaminants represent two distinct populations: CD4⁺ cells surviving lysis, and a subset of DN cells. Most CD4⁺ cells are unlikely to require IL-1 under these conditions, because they respond in the absence of IL-1 (19), and because we

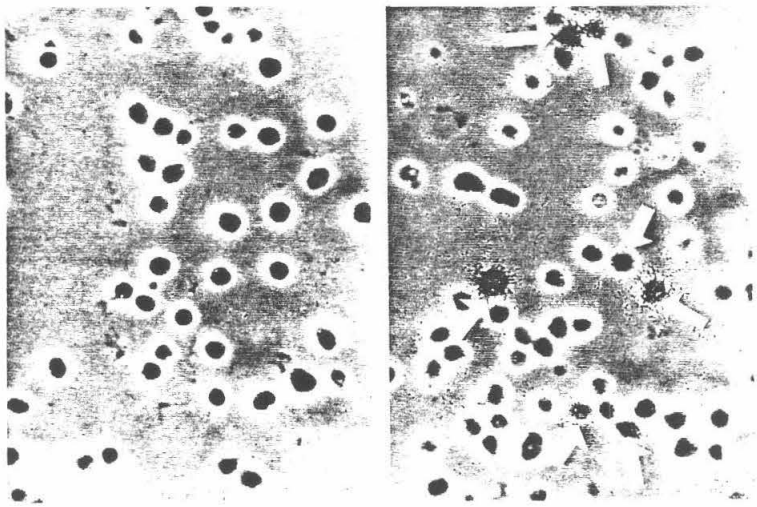

Figure 2. Rapid accumulation of IL-2 RNA in immature cells stimulated with IL-1. The panels show phase-contrast fields of $CD5^{low}$ DN cells hybridized with the antisense IL-2 probe before stimulation (left) or after 5 h of stimulation in the presence of IL-1 (right). The panels shown are representative of photographs from which grain counts were made. Note the absence of labeled cells before stimulation. At 5 h, cells with >15 grains are indicated with *white arrows*.

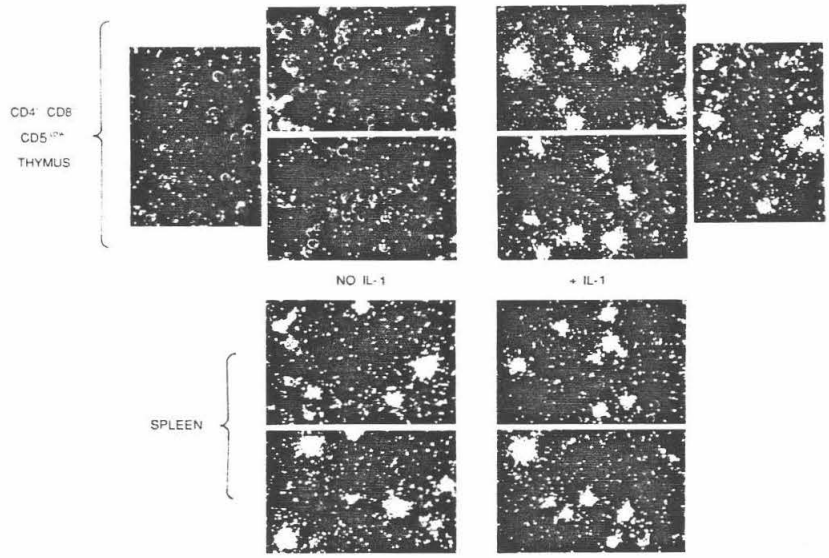

Figure 3. High level induction of IL-2 in immature cells dependent upon costimulation with IL-1. Data are from 5 h of stimulation of the indicated populations, as documented in Table III. The *upper six panels* show representative fields of $CD5^{low}$ DN cells, from cultures stimulated without IL-1 (*left*) or with IL-1 (*right*) as indicated. The *lower four panels* show corresponding fields from spleen cells at the same time points, with IL-1 absent (*left*) or present (*right*) as indicated.

have shown previously that they are responsible for most of the IL-2 production from "mature" thymocytes, which is unaffected by IL-1 (24) (data not shown). However, because of the sharp transience of the IL-1 effect on mRNA expression, we reexamined $CD4^+$ cell responses to IL-1 at an early time point. Figure 5 shows the levels of IL-2 RNA accumulated by total thymocytes and by a population comprising roughly equal numbers of $CD4^+$ thymocytes and DN cells, after 5 h of stimulation in the presence and absence of IL-1. Neither is responsive to the presence of IL-1. The presence of the $CD4^+$ cells almost completely obscures the effect of IL-1 on IL-2 RNA expression in the accompanying DN cells (cf. *lanes 5* and *6*), resulting in abundant but almost indistinguishable levels of IL-2 RNA in the presence or absence of IL-1. In *lanes 3* and *4*, both $CD4^+$ and DN cells are diluted with a 5- to 10-fold excess of cortical $CD4^+CD8^+$ thymocytes, many of them TCR^+ but unresponsive under conven-

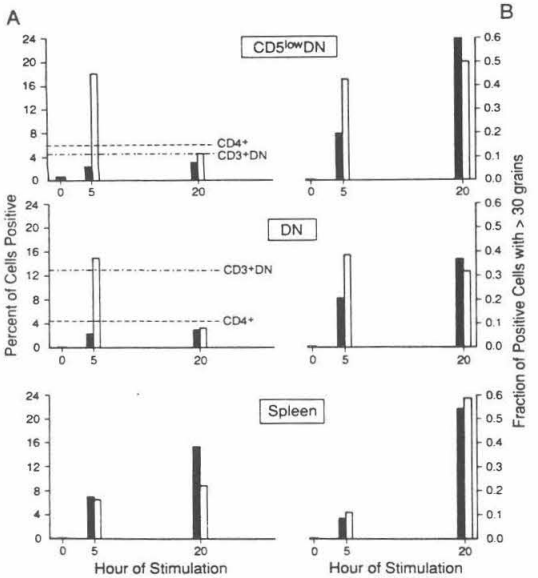

Figure 4. The IL-1-dependent immature cell response shows accelerated kinetics both in cell participation and in accumulation of RNA by individual cells. In *A* (*left-hand panels*) are shown the percentages of cells positive for IL-2 RNA (>7 grains associated) as a function of time of stimulation in the samples described in Table I, experiment 2. *Dark bars* represent samples stimulated without IL-1, and *open bars* samples with IL-1. The figure graphically compares the maximum frequency of IL-2 RNA-positive cells in the $CD5^{low}$ DN population (*top*) with the measured levels of contamination of this sample by two subsets of $CD3^+$ cells. The dashed line (- - - -) shows the fraction of the cells that is $CD4^+$, and (–·–) indicates the calculated fraction that is $CD3^+$ (TCR^+) but $CD4^-$ and $CD8^-$, i.e., TCR^+ DN. For comparison, the levels of $CD3^+$ and $CD4^+$ contaminants are indicated similarly for the total DN population (*middle*). Note that the IL-1-dependent response at 5 h does not increase with the level of $CD3^+$ cells (cf. *top* and *middle panels*). Note the slower kinetics of cellular response in the mature cells of the spleen (*bottom*). For discussion of the effect of IL-1 on this population, see Table I, note *e*. In *B* (*right-hand panels*), the kinetics of RNA accumulation in individual cells are indicated by plotting the fraction of the "positive" cells in each population that have high RNA levels (>30 grains). Note that while those cells able to respond without IL-1 in all populations show a progressive increase in high grain-count cells between 5 and 20 h (*solid bars*), the IL-1-dependent IL-2 producers in immature populations have already achieved these high levels of RNA in a maximal fraction of cells at only 5 h (*open bars*). These results have been confirmed in three independent experiments.

tional stimulation conditions (13, 19). Comparison of *lanes 3* and *4* indicates that this enigmatic population is also probably unresponsive in the presence of IL-1. Thus, neither $CD4^+$ contaminants nor other major thymocyte subsets appear likely to contribute to the IL-1-dependent IL-2 expression seen in TCR^- DN cells.

Although residual TCR^+ DN cells could not account for the magnitude of the transient IL-1-dependent response, we considered that they might participate in it, since their properties are less fully characterized. As noted above, most of them do not accumulate IL-2 RNA like mature cells over 17 h of incubation (Table II). However, the "DN" populations analyzed in Table III and Figure 4 contained about three times as many TCR^+ DN cells as the corresponding "$CD5^{low}$" populations, and yet contained no higher frequencies of IL-1-dependent IL-2 producers than the $CD5^{low}$ cells, when assayed at 5 h. Thus, it is unlikely that a major fraction of the TCR^+ DN cells contribute to the IL-1-dependent response, although it cannot be excluded that a minority of these cells are also IL-1 dependent.

In summary, in the presence of A23187 and TPA, we were consistently unable to detect any enhancement of IL-2 production, or any shift to more rapid kinetics of IL-2 expression, when IL-1 was added to mature (TCR^+) T cells from thymus or spleen. The results in Table III and Figure 4 and those of three other independent experiments (data not shown) (24) gave indistinguishable titers of secreted IL-2 and indistinguishable levels of IL-2 RNA in splenocytes, whether stimulated in the presence or absence of IL-1, analyzed either at 5 to 6 h or at 18 to 20 h of treatment. This was the case whether unfractionated splenocytes or purified splenic T cells were assayed, and whether they were stimulated under conditions which favored IL-2 expression from $CD4^+$ cells only or conditions in which both $CD4^+$ and $CD8^+$ cells respond (14, 24). At the opposite extreme, even in our "$CD5^{low}$ DN" populations where most IL-2 producers required exogenous IL-1, the presence of as few as 4 to 6% cells of mature phenotype always resulted in the appearance of 2 to 3% of cells making maximal levels of IL-2 in the absence of IL-1. Such contaminants also showed the slow induction kinetics typical of mature splenic IL-2 producers (Fig. 4). Isolated mature subsets of thymocytes and the total thymocyte populations whose response they dominate consistently showed no IL-1 effect (Fig. 5) (24) (data not shown). The similar responses of cells of mature phenotype *regardless of context*, whether highly purified or incubated in the presence of splenic non-T cells, of inert cortical thymocytes, or of nonlymphoid elements in the DN thymocyte population, indicate that IL-1 independence is not a result of the presence of particular accessory cell populations. Rather, it appears that in the mature T cells themselves, any signals required for IL-2 induction that IL-1 might supply are already provided by the combination of A23187 and TPA.

Maturation and IL-1 independence. The TCR^- DN cells that do respond to IL-1 clearly differ from mature T cells. It was formally possible, however, that lack of TCR expression in their case might not be a sign of immaturity, but rather of failure to make in-frame TCR gene rearrangements, or of differentiation along some alternative maturation pathway. To test whether IL-1 dependence was associated with immaturity per se, we determined the kinetics of appearance of IL-1-dependent and IL-1-independent IL-2 producers during the repopulation of thymus glands by donor cells in radiation chimeras. B6 (Thy-1.2) mice were lethally irradiated and reconstituted with congenic B6.PL-Thy-1.1 T-depleted bone marrow cells. At various intervals afterward, the animals were sacrificed, donor-type cells isolated from the thymus by cytolysis with anti-Thy-1.2 and C, and the resulting populations stimulated for IL-2 production in the presence or absence of IL-1.

Table IV shows that the $Thy\text{-}1^-$ (T cell depleted) bone marrow cells used as donors could not secrete detectable IL-2, with or without IL-1. Upon arrival and expansion in the recipient thymus, however, the earliest IL-2 producers to arise were highly IL-1 dependent. Similar results were obtained in two to five experiments with different time points, to be described in more detail elsewhere (data not shown) (R. A. Diamond, D. Chen, and E. V. Rothen-

TABLE III

IL-1 enhances IL-2 RNA expression in immature cells at early times of stimulation

Time of Stimulation (h)	Sample	Percent CD3+[a]	n	Overall Percent Positive for IL-2 RNA[b]	Overall Percent >15 Grains[b]	Overall Percent >30 Grains[b]
0	DN[c]	7.5	622	<0.16	0	0
	Spleen[d]	25	606	0.17	0	0
5	CD5^{low}[e]	3.2				
	No IL-1		434	1.4	1.2	0.7
	+IL-1		823	10.0	8.5	6.4
	DN	7.5				
	No IL-1		646	2.2	1.2	0.8
	+IL-1		844	10.7	8.5	4.5
	Spleen	25				
	No IL-1		510	9.4	5.7	2.4
	+IL-1		453	6.8	3.5	1.8

[a] Includes both CD4+ and CD3+ DN cells (and CD8+ cells in spleen only). Compare with the overall percent positive for IL-2 RNA in DN and CD5^{low} samples.

[b] Positive cells determined by in situ hybridization with the antisense IL-2 cRNA probe. Positive cells have >7 grains. Samples showing IL-1-dependent enhancement are boxed. To highlight the relative abundance of transcripts/cell under different conditions, data are also given for the subsets of cells with >15, >30 grains. Percentages of these highly labeled cells are calculated with respect to the whole cell population. Note that all cells with >30 grains are also included among the cells with >15 grains, which themselves are among the total fraction positive for IL-2 RNA.

[c] DN cells ≤0.7% CD4+, CD8 undetectable. CD3 expression on 7.5% of cells.

[d] Spleen cells were 14.9% CD4+, 7.5% CD8+, and 25% CD3+ (20% CD3-bright).

[e] CD5^{low} contained 1.4% CD4+ contaminants, CD8 undetectable. CD3 staining was detectable on only 3.2% of the cells, implying that 1.8% were CD3+ DN.

berg, in preparation). Only after full reconstitution were similar IL-2 titers elicited with and without IL-1. At the earliest time point (day 10), when less than 2% of thymocytes are of donor type, these are predominantly DN blasts undergoing exponential expansion. Less than 3% have acquired CD4 or CD8. The great enhancing effect of IL-1 on the ability of these cells to express IL-2 argues strongly that IL-1-dependent responses can be acquired early in T cell differentiation, not merely as a consequence of maturation in a variant lineage.

DISCUSSION

This work shows that the ability to express IL-2 upon induction is acquired relatively early in T cell differentiation, before expression of a cell-surface TCR complex. IL-2 inducibility presumably develops as a result of contact with the thymic microenvironment, for it is not detectable in hemopoietic cells generally as found in the T cell-depleted bone marrow. However, the requirements for induction of IL-2 in immature cells are significantly different from those in mature thymocytes and peripheral T cells, even as measured with the TCR-independent pharmacological stimuli of Ca^{2+} ionophore and phorbol ester. Unlike most mature IL-2 producers, the immature cells utilize a signaling pathway that depends on IL-1 even in the presence of TPA. It is not certain that all TCR$^-$ cells require IL-1 absolutely; however, the levels of TCR$^+$ contamination are always sufficient to account for the low responses seen without IL-1, and the TCR$^-$ DN population may contain cells capable of synthesizing IL-1 endogenously (31, 32). Thus, in spite of the precocious inducibility of the IL-2 locus in immature thymocytes, in practice IL-2 is not accessible to the same activating stimuli in TCR$^-$ as in TCR$^+$ cells. This makes it possible that changes in signaling pathways may be rate-limiting in the differentiation or lineage commitment of Th cells.

Distinctive signaling requirements. The signaling biochemistry of mature T cell activation has been studied intensively in recent years, and in large part antigen binding appears to exert its effects through the phosphoinositide breakdown pathway (reviewed in Ref. 21). Ca^{2+} ionophores mimic the rise in intracellular free Ca^{2+} normally elicited by inositol trisphosphate (and/or other inositol phosphates), whereas phorbol esters can optimally activate protein kinase C in place of diacylglycerol. The natural triggers of these signaling events may be quite complex, including protein interactions with the TCR complex, the accessory molecules CD4 and CD8, and other monomorphic accessory molecules, with possible participation of IL-1 or IL-6 (33–40). Nevertheless, in general, the mixture of A23187 and TPA in vitro elicits as least as strong a functional response as the natural ligands. IL-1 has a controversial status as an IL-2 activator. An important assay in its initial characterization relied on its synergy with mitogenic lectins to induce IL-2 production from thymoma cells (41–44). Yet recent analyses of nonmalignant Th cell lines have indicated that most IL-2 producers apparently lack IL-1R (45, 46), except for some relatively rare IL-2 producers in the CD8$^+$ population (47). In any case, even in those thymomas which can respond to IL-1 as a costimulus with TCR-binding lectins, A23187 (or ionomycin) and TPA elicit maximal IL-2 production whether IL-1 is present or not (48). Although neither A23187 nor TPA precisely mimics the effects of IL-1 in mature cells (49), the combination of these stimuli makes IL-1 superfluous.

Thus, two points are striking about IL-2 induction in the immature cells described here. One is the inability of A23187 and TPA to reveal the responsiveness of these cells in spite of their proliferation, blastogenesis, and high viability when treated with these compounds (12, 19). The other is their possibly unique possession of a distinct pathway for transducing signals from IL-1 that can synergize with the phosphoinositide pathway. A recent report described a human thymic lymphoma line with similar activation requirements (50). The work presented here shows that the joint requirement for IL-1 along with agents mimicking phosphoinositide pathway mediators is likely to be a characteristic property of normal immature cells.

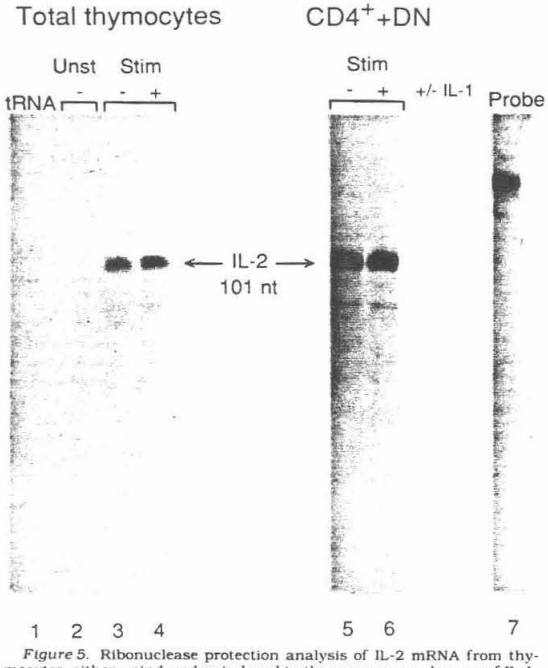

Figure 5. Ribonuclease protection analysis of IL-2 mRNA from thymocytes, either uninduced or induced in the presence or absence of IL-1. Cytoplasmic RNA was isolated from total thymocytes (*lanes 2* to *4*) or from CD8-eliminated ($CD4^+$ and DN) thymocytes (*lanes 5* and *6*) after 5 h of stimulation in the presence or absence of IL-1. Total thymocytes consist of 12 to 15% mature cells ($CD4^+$ or $CD8^+$) and only 2 to 5% DN cells, with an excess of nonresponsive cortical thymocytes. The CD8-eliminated population consists of ~60% $CD4^+$ cells and ~40% DN cells. The populations and their relative yields of IL-2 RNA at 20 to 24 h of stimulation are described in detail in Rothenberg et al. (19). The RNA was hybridized to the 220-nucleotide (nt) ^{32}P-labeled probe in conditions of probe excess. IL-2 RNA is detected by its stoichiometric protection from ribonuclease digestion of a 101-nt fragment. The lanes show hybridization with: *1*, tRNA as a negative control; *2*, total thymocyte RNA, cultured without stimulation; *3*, total thymocyte RNA, induced without IL-1; *4*, total thymocyte RNA, induced with IL-1; *5*, $CD8^-$ thymocyte RNA, stimulated without IL-1; *6*, $CD8^-$ thymocyte RNA, stimulated with IL-1; and *7*, undigested probe. RNA from 0.4 to 0.6 × 10^7 viable cell equivalents was used for each lane. The levels of IL-2 mRNA seen here correspond to the following copy numbers per average cell, calculated as described in *Materials and Methods*: *lane 2*, <0.1; *lane 3*, 3.0; *lane 4*, 3.3; *lane 5*, 20; and *lane 6*, 31 transcripts/cell.

Possible developmental significance. It is uncertain how many of the immature cells are competent to make this IL-2 induction response. At 5 h in the presence of IL-1, about 7 to 10% of the cells lacking TCR clearly contain IL-2 RNA. This low frequency of identifiable responders might define a discrete subpopulation in the process of acquiring functional competence. Alternatively, with the suddenness of IL-2 RNA accumulation and destruction in the immature cells, any asynchrony could markedly reduce the fraction of cells seen to be responding at any given time. Thus, this number represents a minimum estimate of cells that ultimately participate in the response. Furthermore, the extent of any particular response, even as integrated over time by measurement of secreted IL-2 protein, may still underestimate the number of cells potentially able to respond. For example, the stimulatory signal may not last long enough to reach cells that were in refractory cell cycle stages at the initiation of culture. Note that the DN population is enriched for cycling cells, a condition which is often associated with a constraint on IL-2 inducibility (51–53). Thus, our results leave open the possibility that a majority of the TCR^- DN cells could express IL-2. Further characterization will be required to determine which cells in the TCR^- population are in fact the IL-1-dependent IL-2 producers.

The early appearance of IL-1-dependent IL-2-inducible donor-type cells in radiation chimeras repopulated with bone marrow supports the interpretation that these cells are immature developmental intermediates. By contrast, another minor population of DN thymocytes, comprising cells that express TCR-$\alpha\beta$, does not appear for over a month in such animals (54), long after "mature" $CD4^+$ and $CD8^+$ thymocytes differentiate. Inducibility of the IL-2 locus is acquired in development before appearance of mature cell-surface phenotypic markers, including TCR complexes (R. A. Diamond, D. Chen, and E. V. Rothenberg, manuscript in preparation). Thus, expression of a TCR complex, and selection for an "appropriate" class II MHC binding specificity, *cannot* be a prerequisite for chromatin accessibility of the IL-2 gene or provision of necessary *trans*-acting transcription factors.

We may speculate, then, that the IL-1 dependent IL-2 inducible cells may continue their development, rearranging their TCR loci, and randomly acquiring recognition specificities for class I or class II MHC. Most cells (in steady state) that have completed their TCR gene rearrangements are neither IL-1 dependent nor IL-1 sensitive, when activated in vitro to express IL-2. An attractive hypothesis is, thus, that the IL-1-dependent signaling pathway needed to complement the phosphoinositide pathway in immature IL-2 inducible cells might become permanently activated in selected mature cells, through an interaction with the thymic stroma, at about the time of high level TCR acquisition. Such signal transduction alterations, by rendering the phosphoinositide pathway sufficient to induce IL-2 gene expression, could well be vital to the ability of a T cell to express IL-2 in vivo.

It remains to be proven that the IL-1 dependent immature cells can serve as precursors of IL-1 independent IL-2 producers. It is also unknown at what level the IL-1 signal may be integrated with the Ca^{2+} and protein kinase C signals, for example, whether exposure to intrathymic IL-1 alone (32) may convert a developing T cell to an IL-1-independent state. Several striking implications remain, nevertheless. First, the involvement of the phosphoinositide pathway in IL-2 induction might suggest that most of the difference between IL-2 producers and nonproducers would reside in the *accessibility* of the IL-2 locus to any activator, since the phosphoinositide pathway is virtually ubiquitous in animals. Yet our results show that at different stages of development, the same gene may require activation through *different* induction pathways. Second, our results show that the IL-2 locus may be open for induction before TCR specificity is acquired, and thus before all TCR-dependent fate determination processes. Thus, the bias toward association of IL-2 producer function with class II-restricted TCR specificity may not be established at the level of IL-2 inducibility per se. Rather, alterations in signal transduction pathways may play a central role in the events we perceive as functional lineage commitment.

TABLE IV

IL-1-dependent IL-2 producers appear before IL-1 independent IL-2 producers during thymic repopulation in radiation chimeras

Donor-Type Cells from[a]:	IL-2 Activity (U/ml)			
	Experiment 1		Experiment 2	
	−IL-1	+IL-1	−IL-1	+IL-1
Bone marrow (Thy-1⁻)[b]	<0.5	<0.5	<0.5	<0.5
Thymus, day 10[c]	<4 (8)[d]	58 (83)[d]		
Thymus, day 11[e]			<0.5	16
Thymus, day 35[f]	18 (27)[g]	27 (26)[g]		
Thymus, day 34[h]			54 (109)[d]	51 (218)[d]

[a] B6.PL/J-Thy-1.1 donor bone marrow cells, treated with allele-nonspecific anti-Thy-1 to deplete mature T cells, was used to reconstitute B6/J mice after lethal irradiation. Donor-type cells were recovered by anti-Thy-1.2 elimination of recipient thymocytes. Culture times for stimulation in each case were from 18 to 21 h.

[b] Donor marrow, cultured at 5×10^6/ml (not transferred).

[c] After elimination with anti-Thy-1.2, cells were cultured at 5×10^6/ml. Thy-1.1⁺ cells were 85% pure and constituted 1 to 2% of thymocytes.

[d] Values in parentheses were from the same cell preparations, but cultured at 10^7/ml.

[e] After elimination with anti-Thy-1.2, cells were cultured at 1×10^6/ml. Thy-1.1⁺ cells were 98% pure and constituted 1% of thymocytes.

[f] After elimination, cells were cultured at 4×10^6/ml. Cells were 98% pure and constituted ~95% of thymocytes.

[g] From this time point, values in parentheses are from parallel samples cultured with addition of 0.01% anti-IL-2R ascites (7D4) to down-regulate IL-2R expression and prevent adsorption of IL-2 to activated cells.

[h] After elimination, cells were cultured at 4×10^6/ml. Donor-type cells were 98% pure and constituted 95% of thymocytes.

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