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Immunomodulatory Effects of Transforming Growth Factor-β on T Lymphocytes

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy at
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Dedication

This dissertation is dedicated to the memory of my father, Thomas Harris Inge, Sr. I know now that his early encouragement of intellectual pursuit, hard work, and personal role model demonstrating perserverence and struggle against all odds had a profound, although at the time inconspicuous, effect on my personal development. I am hopeful that my future endeavors continue to reflect his own uncompromising morals and work ethic.

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List of Abbreviations

BSA	Bovine serum albumin
C	Complement
CD	Cluster of differentiation (cluster designation)
CD8α	Cluster of differentiation 8, Lyt-2 determinant
CD8β	Cluster of differentiation 8, Lyt-3 determinant
CD25	IL-2Rα
cDMEM	Complete Dulbecco's modified Eagles medium
CTL	Cytotoxic T lymphocyte
CTLL-2	Cytotoxic T lymphocyte line 2
DN	Double negative, a $CD4^-8^-$ thymocyte
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
IL-2	Interleukin-2
IL-2R	Interleukin-2 receptor
IL-2Rα, CD25	Low affinity interleukin-2 receptor; p55
IL-2Rβ	Intermediate affinity interleukin-2 receptor; p75
Io	Ionomycin
LU	Lytic unit
MHC	Major histocompatibility complex

MFI	Mean fluorescence intensity
MLTC	Mixed lymphocyte tumor culture
PBS	Phosphate buffered saline
PDBu	Phorbol dibutyrate
RDES	arginine-glycine-glutamate-serine tetrapeptide
RGDS	arginine-glycine-asparagine-serine tetrapeptide
TBH	Tumor bearing host
TCR	T cell antigen receptor
[^{3}H] TdR	Tritiated thymidine
Th	T helper cell
TGFβ	Transforming growth factor-β_1
TNFα	Tumor necrosis factor-α

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ABSTRACT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Transforming growth factor-β1 (TGFβ) is a peptide cytokine implicated in control of growth, adhesion, and differentiation of cells in numerous tissues. TGFβ has potent inhibitory effects on many lymphocyte responses; we also found that TGFβ could inhibit *in vitro* generation of tumor-specific CTL. Suppression was largely reversed with exogenous IL-2. Proliferation of memory-stage CTL clones was also inhibited by TGFβ, while the upregulation of cytotoxicity was not inhibited by TGFβ. These studies suggested that TGFβ limited anti-tumor CTL responses via both an indirect effect on IL-2 production, and by a direct effect on proliferation of memory CTL.

In CTLL-2 cells, TGFβ inhibited IL-2-dependent DNA synthesis and cell

growth as early as 24h after addition. TGFβ inhibited IL-2-dependent surface IL-2Rα expression 24h after treatment, while cells remained 100% IL-2R$^+$ up to 48h after treatment. TGFβ inhibited c-myc mRNA expression as early as 1h after treatment, suggesting that TGFβ may inhibit T cell growth by either altering signal transduction through the IL-2R, or by otherwise inhibiting early gene expression events triggered by IL-2 binding.

TGFβ treatment also resulted in morphologic changes and increased adherence in 50% of CTLL-2 cells. Adherence required the presence of fetal calf serum and could be largely blocked by RGDS peptides. Under these conditions, *de novo* surface expression of CD8α and CD8β was observed and these cells rapidly accumulated mRNA encoding both CD8α and β chains, to a level 4-fold greater than control.

Treatment of CD4$^-$CD8$^-$ thymocytes with IL-2 + TGFβ similarly induced high levels of *de novo* CD8α expression on one-third of cells, while few thymocytes treated with IL-2 alone became CD8α$^+$. CD8 expression in mature, peripheral CD8$^+$ cells was not influenced by TGFβ. These data suggest that TGFβ has both positive and negative regulatory effects on the expression of molecules important for T lymphocyte growth, differentiation and function, and TGFβ may be a physiologically important cytokine for initial expression of CD8 in the thymus.

Introduction

Mammalian organisms are composed of complex networks of cells that, allied as tissues, work in concert to perform intricate tasks that are, in most cases, poorly understood. For any group of individuals to unite and advance their cause, a highly structured plan must exist and be rigorously followed. We have learned that the nucleic genome is such a plan. The genes (and the mechanisms which organize and regulate their expression) provide cells with the raw materials to accomplish the plan. Fundamental biological tenets have held that the expression of our genes is conservatively gaurded in a cell-type specific manner and restrained in order to lend precise identity to particular cells. In this way, neurons and hepatocytes need not concern themselves with acid secretion, any more than pancreatic acinar cells should be troubled to construct globin molecules. This sounds very logical, and as a paradigm to understand general aspects of biology, is quite sufficient. However, flaws in this model have emerged during the study of cell-cell communication, because so many different cell types express identical receptors for, and respond to, the same hormonal factors. This is especially true when considering the factors produced by cells of the immune system.

Over several millennia our immune system has evolved into an militant, and

almost intelligent force to accomplish an assortment of tasks, including the eradication of invading microorganisms and viruses, and repression of neoplastic upheavals from within. The immune system is endowed with highly evolved mechanisms of intercellular communication--an attribute which provides important and ingenious humoral links in the chain of command. This communication is made possible by the production of various cytokines, which are small glycoproteins that are analogous to hormones of the endocrine system. These glycoproteins act in an antigen-nonspecific manner to send signals from one cell to another over short distances. Thus, many of the cytokines elaborated by leukocytes in the immune system were dubbed "*inter*leukins," since they functioned *between* leukocytes. However, since the identification of numerous interleukins, it has become evident that the expression of receptors for most of these factors is not restricted to the surface of leukocytes, but receptors for interleukins exist on many different somatic cells; this fact makes evident the inaccuracy in the term "interleukin" (and thus the preference of the general term *cytokine*) and exposes the most flaws in the dogma of cell specific gene expression. A few examples will illustrate this point. Interleukin-1 (IL-1) is produced by antigen presenting cells and macrophages of the reticuloendothelial system but has important actions on cellular proliferation, prostaglandin and protein synthesis, and metabolism in general via interaction with IL-1 receptors that are expressed in cells of the lymphoid system, nervous system, liver, bone, synovium, muscle and connective tissue, endothelium, and epithelium. Other cytokines for which non-tissue-specific receptor expression is seen include tumor necrosis factor-α (TNFα), the interferons, other interleukins, and the colony stimulating factors. It has also been noted that cytokine receptors are organized into families, based on structural similarities. Granulocyte-

macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3), and interleukin-5 (IL-5) have multiple unique and common effects on various cells of the hematopoeitic system, but also regulate inflammation, cell adherence, and allergic reactions in part because these cytokines share a common receptor β subunit, capable of crossreacting with these different cytokines (Lopez et al., 1992). Perhaps the best example of this "one cytokine, multiple effects" concept is demonstrated by transforming growth factor-β (TGFβ). Some have likened this factor to a "switch," since the effects of TGFβ on cells can be stimulatory or inhibitory, depending on the particular context (Sporn and Roberts, 1990). Factors that have been shown to govern this context include the physiologic state of the target cell (activated or dormant), the other cytokines that the target cells is exposed to, as well as the nature of the TGFβ receptors that are being expressed by the target cell. Thus, numerous examples attest to the fact cytokines act broadly on many distinct tissue types, and can therefore have many different effects within the body. A paradox arises when we consider that although the expression of cytokine receptor genes is not restricted to particular tissue-types, the response to engagement of a cytokine receptor is often quite specific and restricted to a particular tissue or context. Therefore, the challenge now is the elucidation of cytoplasmic biochemical signalling pathways that receptors are plugged into in any particular cell type. The response to the interaction of any particular cytokine with its cell surface receptor then depends in large part on the downstream elements which are triggered by the receptor in the particular target cell, as well as the quantities and particular combination of other cytokines in the extracellular milieu.

The work that follows will illustrate a number of interesting new roles of regulating T lymphocyte physiology that can be assigned to TGFβ.

Literature Review

Transforming growth factors-β

Transforming growth factors-β (TGFβs) refers to a group of closely related peptide factors that are involved in the regulation of cellular proliferation, differentiation, and adhesion. TGFβ was originally described as a product of virally transformed cell lines capable of causing reversible anchorage-independent growth ("transformation") of normal rat kidney fibroblasts (Ellingsworth, 1990; Roberts et al., 1981). However, since these original observations, the vast majority of studies have shown that the effect of TGFβ on cell proliferation is inhibitory, revealing that TGFβ is in most cases, a misnomer. There are 3 distinct genes coding for 25kD heterodimeric TGFβ peptides that show highly conserved primary sequences. Mature TGFβ consists of two peptide subunits of 112 amino acids each, bonded together via disulfide bridges, a structure which is necessary for TGFβ's biologic activity. TGFβ is found in highest quantities in bone and platelets (>400ng/g) but is also produced by a variety of activated cells including macrophages, platelets, tumor cells, neutrophils, and activated T helper lymphocytes (Kehrl et al., 1986b; Grotendorst et al., 1989). Amino acid sequence analysis demonstrates that this rather ubiquitous cytokine has been highly conserved evolutionarily with only one amino acid difference in primary

sequence between mouse and man and only a two percent difference between man and chicken. From the above information it is not difficult to imagine that this peptide might have multiple functions and effects on different tissues; indeed the thousands of manuscripts related to TGFβ that have been published over the last decade support this premise.

TGFβ is a highly potent immunomodulatory peptide for T lymphocyte responses and has antiproliferative effects at doses as low as 1ng/ml (Stoeck et al., 1989a; Wahl et al., 1988; Ellingsworth et al., 1988; Stoeck et al., 1989b; Kehrl et al., 1986b). TGFβ has been shown to inhibit the proliferation of hematopoietic progenitors (Keller et al., 1990; Cashman et al., 1990), as well as the activation and proliferation of CTL (Ranges et al., 1987; Fontana et al., 1989; Shalaby and Amman, 1988; Wallick et al., 1990; Tada et al., 1991), lymphokine activated killer cells (Kasid et al., 1988; Kuppner et al., 1988; Mulé et al., 1988; Hirte and Clark, 1991), and B lymphocytes (Quere and Thorbecke, 1990; Shalaby and Amman, 1988; Coffman et al., 1989). It has also been shown recently that TGFβ inhibits the appearance of perforin mRNA in T cells stimulated with anti-CD3 mAb and IL-2 (Smyth et al., 1991). Many studies have examined mitogenic or antigenic responses of heterogeneous populations of naive lymphocytes (Kono et al., 1990; Stoeck et al., 1989b; Quere and Thorbecke, 1990; Fontana et al., 1989) in which the inhibition of T cell activities may occur at a number of points. The suppressive effects of TGFβ on responsiveness to IL-1 (Wahl et al., 1988; Morris et al., 1989) and IL-4 (Ruegemer et al., 1990) have been well documented and may account indirectly for the antiproliferative effects of TGFβ on some T cell populations. There is mixed opinion as to the effect of TGFβ on IL-2 production, with some reports suggesting suppression (Van Norstrand et al., 1990) and

some no effect (Wahl et al., 1988).

It has been shown that TGFβ has no effect on the rise in intracellular Ca^{++} seen immediately following T lymphocyte stimulation with anti-CD3 (Stoeck et al., 1989b) or Concanavalin-A (Con-A) (Morris et al., 1989). However, c-myc expression is inhibited by TGFβ in some cell lines (Ruegemer et al., 1990) but not in normal peripheral T cells (Morris et al., 1989). The effect of TGFβ on IL-2R expression is also controversial. Kehrl, investigating the effect of TGFβ on mitogenic responses of human tonsillar T cells, showed a 60-80% suppression of IL-2-dependent DNA synthesis of ConA activated T cells (Kehrl et al., 1986b). In this study, TGFβ seemed to inhibit IL-2-dependent up-regulation of surface IL-2Rα. In experiments done with two constitutively activated T cell clones, Ruegemer *et al.* found that proliferation was inhibited in both HT-2 ($CD4^+$) and CT6 ($CD8^+$) cell lines by TGFβ, but high affinity IL-2R expression was decreased by TGFβ only on HT-2 cells (Ruegemer et al., 1990). Conversely, growth factor dependent IL-2Rα mRNA levels were unaffected by TGFβ in HT-2 cells but were decreased by 80% in CT6. IL-2Rα expression on the cell surface was not altered by TGFβ in either of these cell lines. Wahl has shown that the proliferative response of peripheral blood mononuclear cells (PBMC) to lectin was inhibited by simultaneous administration of TGFβ, but that IL-2Rα mRNA and surface levels were not affected (Wahl et al., 1988). Smyth *et al.* reported a slight enhancement of surface IL-2Rα expression when $CD8^+$ T cells were cultured in TGFβ and high dose IL-2 (Smyth et al., 1991). There are contrasting results regarding the anti-proliferative effects of TGFβ on the widely used IL-2-dependent CTLL-2 T cell line. Interestingly, Ellingsworth reported that TGFβ did not inhibit proliferation of his CTLL-2 line (Ellingsworth et al., 1988), whereas Stoeck found that TGFβ at 1-5ng/ml

inhibited CTLL-2 cell cycle progression by 75-85% on day 1; however, surface IL-2Rα was not decreased in the treated group until d3 (Stoeck et al., 1989a). Ortaldo *et al.* have recently described the effects of TGFβ on human large granular lymphocytes in which IL-2Rα levels were decreased but surface expression of the p75 chain of IL-2R were unchanged (Ortaldo et al., 1991). They reported that TGFβ inhibited IL-2 induced LGL functions mediated by IL-2Rβ and that the inhibition was at a post-receptor binding level that appeared to involve regulation of initial signalling events.

As noted above, most of TGFβ's effects on cells of the immune system have been inhibitory, leading most authors to refer to TGFβ as immunosuppressive. However, TGFβ stimulates cellular functions in many different tissue types, including fibroblasts and osteoblasts. For instance, osteoblast lines responded to TGFβ with increased alkaline phosphatase activity and collagen synthesis (Mundy and Bonewald, 1990). With respect to cells of the immune system, studies of murine B cells demonstrated that TGFβ inhibits proliferation and total immunoglobulin synthesis, while increasing both class II expression (Cross and Cambier, 1990) and differentiation toward an IgA-producing lineage (Coffman et al., 1989). The enhancement of both class II expression and isotype switching to IgA were accompanied by increases in steady state levels of mRNA for these genes (Lebman et al., 1990a; Lebman et al., 1990b).

In summary, TGFβ is a product of both normal and neoplastic cells and probably plays a permissive role in progression of tumor growth. The mechanism by which this effect is accomplished is unknown but may relate to the fact that TGFβ inhibits proliferation of antitumor T cells. Finally, TGFβ has been described as a differentiation promoting "switch" for numerous cell types but as yet, little is known

about the effects of TGFβ on T lymphocyte differentiation.

Cluster of Differentiation 8

CD8 is expressed on mature T lymphocytes, NK cells, and the CD8 glycoprotein has been described as both a T cell accessory molecule and as a differentiation antigen (Parnes, 1989). Non MHC-restricted NK cells can express CD8 as αα homodimers, whereas CD8 that exists on MHC class I-restricted T cells is expressed as αβ heterodimers. On T cells, CD8β must form heterodimers with CD8α in order for surface expression of β chains to occur. Considerable data support the hypothesis that CD8 molecules on T lymphocytes act as receptors for non-polymorphic determinants of MHC class I molecules on accessory and target cells (Parnes, 1989; Meuer et al., 1982; Rosenstein et al., 1989; Norment et al., 1988; Swain, 1981). These CD8-class I interactions appear to increase the avidity of T cell-target cell contact and also positively influence transmembrane signal transduction in T cells via an interaction between the intracytoplasmic domain of CD8 and the protein tyrosine kinase $p56^{lck}$ (Gilliland et al., 1991; Miceli et al., 1991; Letourneur et al., 1990). Murine CD8 was originally defined by the monoclonal antibodies (mAB)s specific for Lyt2 and Lyt3 determinants that reside on the CD8α and CD8β chains respectively. It is now known that the CD8α gene can give rise to 2 forms of peptide, designated as α or α', that differ only by the absence of the cytoplasmic region in the α' form. The gene encoding CD8β is located 5' of the CD8α gene, and these are linked. Both are members of the immunoglobulin supergene family and are closely linked to the Igκ light chain locus on chromosome 6.

In the thymus, CD8 molecules are first expressed after transient expression of

IL-2Rα and prior to TCR expression. Later, interaction between class I MHC and CD8 appears to play an important role in positive selection of MHC class I-restricted, αβTCR$^+$ T cells (Nikolic-Zugic, 1991; Boyd and Hugo, 1991; MacDonald et al., 1988). The events or mediators that trigger initial expression of CD8 molecules on T lymphocytes are unknown.

Two fundamental classes of effector function have been ascribed to CD8$^+$ cells: antigen specific cytotoxicity and immunoregulation. We have been actively involved in investigating the role of CD8$^+$ T cells as 1) mediators of antitumor cytotoxicity *in vitro* (Inge et al., 1992a; Tuttle et al., 1992; Hoover et al., 1990b) and *in vivo* (McKinnon et al., 1990; Hoover et al., 1990b) and 2) as suppressors of immune responses (Bear, 1986; Bear, 1987; Hoover et al., 1990a). Although the mechanism of T cell mediated immunosuppression is not known, several possibilities exist, including secretion of immunoregulatory cytokines or other suppressor factors (Weiner et al., 1990; Damle and Engleman, 1990). Another interesting possibility for which there are compelling data is that the CD8 peptide itself is important in mediating antigen specific immunosuppression (Kaplan et al., 1989; Hambor et al., 1988; Hambor et al., 1990).

In summary, although many structural and functional details about CD8 have been elucidated, thus far the physiologic signals for induction of CD8 expression have not been described and therefore molecular analysis of this process has not been possible. Therefore, learning more about the expression of the gene for this peptide and its regulation by TGFβ will be important for understanding normal differentiation of CTL and for understanding thymic ontogeny.

Interleukin-2-Dependent T Cell Growth

"T cell activation" describes all of the events that occur which drive a resting, G_0 phase T lymphocyte into mitosis. These events can be broadly divided into two distinctly regulated stages. First is the signalling that is achieved by antigen, other mitogens (lectins), or phorbol ester/calcium ionophore (P/I) which stimulates the movement of a G_0 cell into G_1 of the cell cycle. This action has been termed competence, to denote one result of these events, namely cellular receptiveness to the T cell growth factor, interleukin-2 (IL-2), manifested physically as IL-2R surface expression. Antigen, in association with self MHC molecules (Ag/MHC), is the physiologic stimulus for competence. When a T cell receptor (TCR) encounters and binds a particular Ag/MHC complex for which it has high affinity, a number of well characterized responses occur within a few minutes, including the generation of reactive phosphoinositol species and diacylglycerol, translocation of protein kinase C from cytosol to membrane, and an increase in cytosolic free calcium ion ($[Ca^{2+}]_i$). Within the next few hours, nuclear responses predominate which yield production of mRNA coding for various activation associated genes, as well as IL-2 and IL-2 receptor (IL-2R). The production of high affinity (HA, K_d=10 pM) IL-2R and the interaction of IL-2 with these receptors results in the progression phase of T cell activation which is manifested by entry of G_1 phase cells into mitosis. Thus, the interaction of TCR with Ag/MHC determines the specificity of the response and IL-2 regulates the magnitude of the response.

IL-2, a well characterized glycoprotein growth factor, is critical to the generation of most immune responses. The presence of IL-2R on a wide variety of cells is evidence for the broad effects of IL-2 on immune responses. Proliferation,

differentiation, and other activation related events are triggered by IL-2/IL-2R interactions in T and B lymphocytes, macrophages, NK cells, LAK precursors, eosinophils, and probably cells of the central nervous system (Ruscetti, 1990; Smith, 1980; Okamoto et al., 1990). It is apparent from a number of studies that signal transduction pathways from the IL-2R are distinct from those that are coupled to the TCR (Albert et al., 1985; Nau et al., 1988). There is agreement from a number of systems that the extent of the proliferative response to IL-2 is dependent upon the interaction between IL-2 concentration, HA IL-2R density, and the duration of the IL-2/IL-2R interaction (Cantrell and Smith, 1984; Lowenthal et al., 1985; Malek and Ashwell, 1985). Stimulation of the TCR activation pathway induces HA IL-2R expression, but once expressed, IL-2 itself enhances the expression of HA IL-2 binding sites. At high concentrations, IL-2 alone can also induce HA IL-2R sites via binding to intermediate affinity (β chain) receptors (Bich-Thuy et al., 1987).

Currently, it is known that the participation of at least two peptides is necessary for HA IL-2 binding to T cells (Robb et al., 1984; Fujii et al., 1986; Wang and Smith, 1987; Fung et al., 1988). Both human and murine IL-2Rα or p55 (Leonard et al., 1984; Nikaido et al., 1984; Cosman et al., 1984; Miller et al., 1985) and IL-2Rβ or p70-75 (Kono et al., 1990; Hatakeyama et al., 1989b) have been cloned. The IL-2Rα chain alone can bind IL-2, but at an affinity far too low (K_d=10-20 nM) to be of significance physiologically; from a number of studies it appears unlikely that IL-2Rα plays an important role in IL-2 signal transduction (Kuziel and Greene, 1990). IL-2Rα is expressed at very low levels in resting T cells and these surface levels increase dramatically when resting T cells are activated with Ag or mitogen (Cantrell and Smith, 1983; Wilkinson and MacLeod, 1988; Meuer et al., 1984; Lowenthal et al.,

1985). IL-2 itself (Jankovic et al., 1989; Plaetinck et al., 1990; Smith and Cantrell, 1985; Lowenthal et al., 1989) plays an important role in amplifying the induction and maintenance of IL-2Rα and the mechanisms involved in regulating IL-2Rα gene expression have been studied extensively. Transcriptional regulation of this gene involves complex elements in the region 5' to the transcription start site (Smith and Greene, 1989; Plaetinck et al., 1990). In addition, there are important DNA sequence motifs (Greene et al., 1989; Pomerantz et al., 1989) and DNA binding proteins that enhance (Lenardo and Baltimore, 1989; Freimuth et al., 1989) or inhibit (Smith and Greene, 1989) the promoter of this gene.

IL-2Rβ, in contrast to IL-2Rα, is constitutively expressed on the surface of resting T cells. However, recent reports have shown that the mRNA is constitutively produced in some lymphoid cells but not others (Kono et al., 1990; Hatakeyama et al., 1989b). The genomic organization and promoter analysis of the human IL-2Rβ gene have recently been described (Shibuya et al., 1990). In comparison to IL-2Rα, little is known of the Ag and IL-2 dependent regulation of IL-2Rβ transcription in either resting or activated cells. To define the role of IL-2Rβ for IL-2 binding, a number of transfection-reconstitution studies with T cell and fibroblast cell lines and work done with IL-2Rβ$^+$ NK cells have shown that expression of IL-2Rβ is absolutely necessary for the production of intermediate affinity (IA) or HA IL-2 binding sites and for growth signal transduction (Hatakeyama et al., 1989b; Okamoto et al., 1990; Hatakeyama et al., 1989a; Kono et al., 1990; Tsudo et al., 1989; Lotze et al., 1987; Bich Thuy et al., 1987; Aribia et al., 1989; Minamoto et al., 1990).

Until recently, very little was known about IL-2R mediated signal transduction, and the pathways triggered by IL-2 binding to its receptor are still not entirely defined.

It is apparent that IL-2Rβ chain plays an important role in IL-2 signalling, as was suspected when the prominent 286 amino acid intracellular portion was identified (Hatakeyama et al., 1989b). Although neither the IL-2Rα or IL-2Rβ peptide have enzymatic domains (Hatakeyama et al., 1989b; Saltzman et al., 1990), β chain is required for the phosphorylation of multiple protein substrates (M_r 32 through 116) (Ferris et al., 1989). IL-2Rβ is itself phosphorylated on ser, thr, and tyr residues in lymphoid but not fibroblast IL-2Rβ$^+$ cells (Sugamura et al., 1990), and its intracytoplasmic signal may be transmitted through an association with $p56^{lck}$ (Hatakeyama et al., 1991). Increases in $[Ca^{2+}]_i$ are not required for IL-2-dependent cell growth. IL-2 binding to HA IL-2R is associated with phosphoinositol breakdown and in some cells PKC translocation (Redondo et al., 1988). Protein tyrosine kinases (PTK) of 97 and 57kD were purified from the IL-2R complex in an active form (Michiel et al., 1990), and anti-PTK immunoprecipitates from IL-2 stimulated cells contained high levels of a phospholipid kinase that may be involved in IL-2R signal transduction (Augustine et al., 1990). IL-2 dose-dependent tyrosine phosphorylation of various MW substrates (MW 100 to 57) has also been detected (Yamada et al., 1987; Ferris et al., 1989; Ishii et al., 1988).

Thymic Differentiation

T lymphocytes mature intrathymically from bone marrow or fetal liver precursors (Metcalf and Moore, 1971). Among the earliest phenotypic markers expressed by prothymocytes are Thy1 and Lyt1. Thy1 identifies lymphocytes as "T cells" for the duration of their existence in the body. The thymus contains many other cellular elements including epithelial cells, "nurse" cells, and bone marrow derived

accessory cells, that are crucial for proper T cell differentiation and development of self-tolerance. Additionally, there is increasing evidence that hormonal microenvironments in the thymus influence T cell development (Nikolic-Zugic, 1991; Van Ewijk, 1991).

Thymocytes are divided into 4 major groups based on surface staining for CD4 and CD8: $CD4^-8^-$ (double negative, DN), $CD4^+8^+$ (double positive, DP), $CD4^+8^-$ (single positive, SP), and $CD4^-8^+$ (single positive, SP). Lineage relationships between these subsets have been defined, but the factors that influence the differentiation of DN thymocytes are largely unknown. It is known that prothymocytes that arrive from the bone marrow to populate the thymus are of DN phenotype. These cells can reconstitute all thymocyte populations and mature into either CD4 or CD8 SP peripheral T cells (MacDonald et al., 1988). Moreover, it has also been reported that an immature, $CD3^-CD8^+$ SP cell can give rise to DP thymocytes *in vitro* (Nikolic-Zugic et al., 1989), and this subset is capable of reconstituting DP and SP thymocyte subsets as well as peripheral T cell populations (Nikolic-Zugic and Bevan, 1988; Guidos et al., 1989). It is thought that these cells arise directly from DN thymocytes (Guidos et al., 1989; Mizushima et al., 1989). To date, the factor(s) that promote the initial expression of CD8 on DN thymocytes has not been specifically identified. A recent series of reports has described a thymic stromal cell derived T cell growth factor (TSTGF) that stimulated the growth of thymic cells and synergized with phorbol esters and interleukins to promote the growth of immature thymocytes (Mizushima et al., 1989; Sato et al., 1988). The semipurified TSTGF from supernatants of a thymic stromal cell line promoted *in vitro* differentiation of a small percentage of DN thymocytes to a CD8 single positive stage. Progression to the more mature DP and CD4 SP thymocytes did

not occur with the supernatant factor alone, but required the presence of the stromal cell monolayer (Fujiwara et al., 1990; Tatsumi et al., 1990). Although a number of interleukins might be involved in thymocyte maturation (MacDonald et al., 1988; Shimonkevitz et al., 1987; Jenkinson et al., 1987; Gotlieb et al., 1990; Okazaki et al., 1989), this group has recently ruled out IL-1 through IL-7 as the critical differentiation factor present in TSTGF. Therefore, the exact nature of the factors which cause DN thymocytes to initiate production of surface CD4 or CD8 peptides is still unknown.

Materials and Methods

Mice. Virus-free DBA/2 and (C57BL/6 X DBA/2)F1 (BDF_1) mice were obtained from Harlan-Sprague-Dawley Laboratories, Indianapolis, IN. Mice were housed in a virus-free environment, given food and water *ad libitum*, and screened regularly for pathogenic viruses using standard serological tests.

Tumors. The P815 mastocytoma ($H\text{-}2^d$) was obtained and passaged as described (Bear, 1986). Briefly, P815 was passaged as ascites in syngeneic DBA/2 mice and cryopreserved in RPMI-1640: 10% FCS: 10% dimethyl sulfoxide in liquid N_2. Cells from ascites were used to inoculate TBH, to immunize mice, for *in vitro* experiments, and for passage. PHS-5 is a spontaneous mutant selected from P815 in 8-azaguanine as described previously (Bear, 1987), and is killed in the presence of hypoxanthine, aminopterin and thymidine (HAT). P815 and PHS-5 are antigenically indistinguishable, but PHS-5 was used for TBH to avoid outgrowth of metastatic tumor cells *in vitro*. PHS-5 cells were passaged as described for P815.

Inoculation of TBHs and Immunization. PHS-5 cells from ascites were washed in Hanks' balanced salt solution (HBSS) and injected i.d. on the abdomen at 10^6

cells/mouse in 0.05ml for tumor bearing hosts (TBH). After 8-12 days of tumor growth, mice were designated "early TBH," while mice bearing 18 day tumors were designated "late TBH." For immunization, 2 X 10^6 P815 cells were mixed with 50 μg of formalin-killed *Corynebacterium parvum* (Burroughs Wellcome, Research Triangle, NC) and inoculated i.d. in a total volume of 0.05 ml.

Mixed Lymphocyte Tumor Cell Cultures (MLTC). For establishment of MLTC, spleens were removed aseptically and pressed through 100-mesh screens in HBSS. Erythrocytes were lysed using Gey's hemolytic medium. Cells were then washed 3X in HBSS, and viable cells were counted by trypan blue exclusion and resuspended in complete medium (RPMI-1640 + 100 units/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 0.1mM non-essential amino acids, 10mM HEPES, $5x10^{-5}$M β-mercaptoethanol, 10% fetal bovine serum). Fifteen million spleen cells per well from P815 immune mice or PHS-5 TBH were cultured with $5x10^5$ mitomycin-C treated PHS-5 tumor cells in 24 well plates at 37°C in 5% CO_2. All cultures included 1X hypoxanthine-aminopterin-thymidine (HAT) (Sigma Chemical Co., St. Louis, MO) to prevent outgrowth of occult metastatic PHS-5 cells in the TBH splenocyte preparations.

Cytotoxicity Assays. After 6d in culture, MLTC cells were harvested, washed, resuspended in complete medium, and counted. Cytotoxic activity was assayed against ^{51}Cr-labelled target cells as described (Bear, 1986). Percentage of specific release (%SR) was calculated as:

$$\%SR = 100 \times \frac{\text{(cpm effector cells) - (cpm spontaneous release)}}{\text{(cpm maximum release) - (cpm spontaneous release)}}$$

Spontaneous release was determined by culturing target cells in medium alone. Maximum release was determined by detergent lysis of labelled target cells. %SR values for triplicate samples were averaged, standard deviations obtained, and data were plotted as a function of effector:target ratio.

Lytic units (LU) were calculated from cytotoxicity data, and a lytic unit was defined as the number of effector cells capable of causing lysis of 15% (LU_{15}) or 30% (LU_{30}) of radiolabelled target cells. Cytotoxicity data reduction was performed using a nonlinear, least-squares fitting program (NOTLIN) (Pross et al., 1981). Lytic units per culture represent the product of the $LU/10^6$ cells and the total viable cells harvested per culture well.

CTL Clones: GD11.10 CTL were derived and maintained as described (Hoover et al., 1991). Rested GD11.10 CTL clones were obtained by washing CTL extensively prior to 3 days' culture in medium lacking IL-2. Rested CTL demonstrated <5% lysis of PHS-5 at effector:target (E:T) ratio=5:1. Quiescent GD11.10 cells were obtained 10 days after PHS-5 stimulation and 2 days after the last IL-2 feeding (IL-2 was not washed out). Quiescent CTL demonstrated >70% lysis of PHS-5 at E:T=5:1, but proliferated well only when both tumor cells and IL-2 were

added, not in IL-2 alone.

Proliferation assay. To assess the DNA synthesis and cellular proliferation, $5x10^4$ cells were plated in each well of a 96-well flat-bottom tissue culture plate in complete medium. For stimulation of CTL clones, $2.5x10^4$ mitomycin-C treated PHS-5 (PHS-5mc) tumor cells ± cytokines were added to each well. For assay of CTLL-2, $5x10^4$ cells were added to each well with various concentrations of cytokines. All wells were pulsed with 37kBq of ^{3}H-thymidine 24h before harvest. At various timepoints, wells were harvested on a PHD cell harvester (Cambridge Technologies, Inc., Cambridge, MA). Liquid scintillation counting was performed on a Beckman LS 5000TD instrument. Statistical significance was determined using a two-tailed Students' t-test for unpaired data.

Cytokines. Unless otherwise specified, purified porcine platelet $TGF\beta_1$ (R&D Systems, Minneapolis, MN) was used and prepared per manufacturer's instructions. Accordingly, 1μg TGFβ was dissolved in a solution of 0.1% bovine serum albumin in 4mM HCl and stored for no longer than 2 months at 4°C. For use, this stock was diluted into complete medium and added to MLTC. Human recombinant $TGF\beta_1$ and murine recombinant TNFα were kindly provided by Genentech, San Francisco, CA. Human recombinant interleukin-2 was kindly provided by Hoffmann-LaRoche, Nutley, NJ.

CTLL-2 culture. CTLL-2, an IL-2 dependent cytotoxic T cell line, was

originally derived from C57BL/6 mice immunized against allogeneic (DBA/2, H-2^d) Friend leukemia cells (Baker et al., 1979). CTLL-2.TH, a non-cytolytic subclone obtained from Dr. Frank Fitch (University of Chicago, Chicago, IL), was used in these studies and will hence be referred to simply as CTLL-2. CTLL-2 were incubated in 24-well plates (Costar, Cambridge, MA) at $5x10^5$/well and cultured at 37°C in a humidified atmosphere of 5% CO_2:air in 2ml/well of complete medium (DMEM + 100 units/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 0.1mM non-essential amino acids, $5x10^{-5}$M β-mercaptoethanol, 10% FCS) + 20U/ml recombinant human IL-2. Cultures were split every 2 days to prevent overgrowth, at which time fresh medium and IL-2 were added. For experiments, CTLL-2 were used 2 days after the previous addition of IL-2, unless otherwise indicated.

Assay of cell culture density by MTT. To assess cell culture density, lymphocytes were plated in each well of a 96-well flat bottom plate and cultured in 0.2ml CM. For assay, 0.1ml of medium was removed and 0.025ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 5mg/ml in saline; Sigma Chemical Co., St. Louis, MO) was added per well. Plates were incubated in the dark for 2h at 37°C prior to addition of 0.04N HCl in isopropanol (0.1ml per well). Optical density values were obtained by absorption of light of wavelength 570nm.

Assay of cell adhesion. To assess adherence of cells to the tissue culture plate, $5x10^4$ lymphocytes were added to each well of 96-well plates and cultured in 0.2ml CM for 24h. For assay, another 0.25ml HBSS was added to completely fill wells, and plates were wrapped tightly with cellaphane. Plates were inverted, and centrifugation

was done at 50 x g for 2min. Thus, cells were spun from the bottom to the top of each well. Following centrifugation, cellaphane was removed and nonadherent cells were decanted off with the medium. Wells were washed with 0.1ml HBSS and then 0.1ml CM was added to each well and MTT assay was performed to estimate the number of adherent cells remaining.

Monoclonal antibodies (mAb), anti-sera, and immunofluorescence reagents:

Phycoerythrin (PE) conjugated mAb against murine CD8α (rat IgG_{2a}, 53-6.7) and fluorescein isothiocyanate (FITC) conjugated mAb against murine CD8β (rat IgG_1, 53-5.8), CD3ε (hampster IgG, 145-2C11), αβ-TCR (hampster IgG, H57-597), γδ-TCR (hampster IgG, GL3), ICAM-1 (hampster IgG, 3E2) and LFA-1 (rat IgG_{2b}, 2D7) were obtained from Pharmingen, San Diego, CA. FITC conjugated mAb against murine IL-2Rα (rat IgG_{2a}, AMT-13), CD3ε (rat IgG_{2b}, YCD3-1) and CD4 (rat IgG_{2b}, YTS 191.1.2) were obtained from Gibco/BRL, Grand Island, NY. FITC conjugated rat mAb unreactive with murine splenocytes were obtained from Pharmingen and used as control reagents. These included rat IgG_1, 11014C; rat $IgG_{2a}κ$, R35-95; rat $IgG_{2b}κ$, R35-38.

Mouse anti-rat κ chains (mouse IgG_{2a}, MAR 18.5), anti-CD8α (rat IgG_{2a}, 53-6.7), anti-CD8α (Lyt2.1 specific rat IgG_{2a}, 116-13.1), anti-CD8α (Lyt2.2 specific rat IgG_{2b}, 2.43), anti-CD4 (rat IgG_{2b}, GK1.5), and anti-FcRγ (rat IgG_{2b}, 2.4G2) was obtained from the American Type Culture Collection (ATCC), and grown as ascites in pristane-primed nude mice.

Purified anti-ICAM-1 (rat IgG_{2a}, MK-1) was obtained from Dr. John Tew, Medical College of Virginia, Richmond, VA. Lyophilized rabbit-anti-rat Ig antiserum

was obtained from Cappell/Organon Teknika, Malvern, PA and reconstituted with distilled water.

Cell surface phenotyping by immunofluorescence with monclonal antibodies. Cells were incubated with fluorochrome-conjugated mAb (1μg/10^6 cells) in 50μl phenotyping solution (1% BSA, 0.1% NaN_3 in PBS) on ice for 20-30 min. Cells were then washed twice in phenotyping solution. Fluorescence intensity of viable cells was analyzed on a Becton-Dickinson FACScan instrument equipped with a 15mW 488nm argon laser and appropriate excitation filters (Becton-Dickinson Immunocytochemistry Division, San Jose, CA). Background green channel fluorescence was determined after incubating cells with isotype-matched, irrelevant FITC-conjugated mAb obtained from Pharmingen. Background orange channel fluorescence was determined after incubating cells with an excess of unconjugated anti-CD8 (53-6.72) prior to staining with PE-conjugated anti-CD8. Dead cells were excluded from analysis based on propidium iodide staining and forward angle light scatter. Data were collected on 5,000 or 10,000 cells with log amplification and analyzed on a Hewlett Packard series 300 computer (Portland, OR).

Staining of cells with IL-2 ligand. Biotinylated IL-2 was obtained from R&D Systems, Minneapolis, MN. Cells were incubated with biotinylated IL-2 on ice for 30 min prior to being washed twice in phenotyping solution. Next, cells were incubated with 5μl PE-conjugated streptavidin (Tago Immunologicals, Burlingame, CA) for 30 min on ice, and washed twice. Fluorescence intensity of viable cells was analyzed on a Becton-Dickinson FACScan instrument equipped with a 15mW 488nm argon laser

and appropriate excitation filters (Becton-Dickinson Immunocytochemistry Division, San Jose, CA). Background orange channel fluorescence was determined after incubating cells with PE-conjugated streptavidin alone. Dead cells were excluded from analysis based on propidium iodide staining and fluorescence in channel 3. Data were collected on 5,000 or 10,000 cells with log amplification and analyzed on a Hewlett Packard series 300 computer (Portland, OR).

cDNA probes and Northern analysis. cDNA probes used in these studies were kindly provided by the following investigators: IL-2Rα from Dr. R. Germain (Miller et al., 1985), IL-2Rβ from Dr. T. Tanaguchi (Okamoto et al., 1990), Granzyme B from Dr. R.C. Bleackley (Lobe et al., 1986), CD8α (Zamoyska et al., 1985) and CD8β (Gorman et al., 1988) from Dr. Jane Parnes, and β-actin from Dr. L. Kedes (Gunning et al., 1983). DNA was nick translated with α^{32}P- labelled deoxynucleotide triphosphates (ICN Biomedicals Corp., Costa Mesa, CA). Total RNA was prepared using RNAzol B (Biotecx Laboratories, Houston, TX). Accordingly, $5x10^7$ lymphocytes were harvested and washed x1 in HBSS in 50cc polypropylene tubes. Cell pellets were disrupted by vortexing (high speed, 5sec) and RNA extracted by addition of 0.2ml of RNAzol B (a guanidine isothiocyanate + phenol preparation) per 10^6 cells. This lysate was transferred to 1.5ml snap-cap tubes and to this 0.1ml ice-cold chloroform (per 1ml lysate) was added. Samples were vortexed (high speed) for 15sec and then incubated on ice for 5-15min. Samples were next centrifuged at 14,000g (Eppendorf Micro Centrifuge 5415, Brinkman Instruments Co., Westbury, N.Y.) at 4°C for 15min. After centrifugation, the homogenate consisted of two phases: a lower blue organic phase (phenol/chloroform) and an upper clear aqueous phase.

The organic phase and interface contained DNA and protein, whereas the RNA remained soluble in the aqueous phase. The aqueous phase was pipetted off, and transferred to a clean microfuge tube, an equal volume of ice-cold isopropyl alcohol was added and this solution was vortexed for 15sec. Samples were incubated on ice for 15-60min and then spun (14,000g, 4°C) for 15min. The RNA pellet was identified as a white/opaque streak on the side of the tube near the bottom. Supernatant fluid was decanted or aspirated off and the pellet was washed once with cold 75% ethanol. Ethanol was aspirated and RNA was dried under vacuum for 10-15min. Dried RNA was re-dissolved in 0.050-0.2ml of diethylpyrocarbonate (DEPC)-treated, RNAase-free dH_2O. Incubation (10min) at 60°C was sometimes required to completely re-dissolve the RNA. A 10- or 20-fold dilution of the sample was made into water and the RNA concentration measured by spectrophotometry and routinely had a 260/280 ratio of 1.8-2.0. [RNA] in $\mu g/\mu l = OD_{260} \times 0.04 \times$ dilution. The RNA was prepared for electrophoresis by adding the following to a microfuge tube: 10µg RNA, 25µl formamide, 2.5µl of 20X 3-N-morpholino propane sulfonic acid (MOPS; 0.4M MOPS prepared as 100mM Na Acetate + 20mM EDTA, pH to 7.0 with NaOH or acetic acid), 8.9µl formaldehyde, QS to 50µl with dH_2O, 5µl bromphenol blue (BPB; 0.25% stock: 0.25g in 100ml 1X MOPS), 5µl 0.5mg/ml ethidium bromide (optional). This mixture was heated to 60°C for 5min and then quick-chilled.

The gel was prepared as follows: One gram electrophoresis grade agarose + 5ml of 20X MOPS + 77ml milli-Q dH_2O + stir bar in 500ml Erlenmeyer flask. This mixture was brought to a boil while stirring on a stir/heat plate (10min) then allowed to cool to 60°C. Just prior to pouring, 18ml of formaldehyde (37%, Fisher) was added. The agarose solution was added to the level gel plate under the exhaust hood.

The agarose was allowed to to polymerize, incubated at 4°C (15-30min) then placed in the gel rig. 1X MOPS (made up in bulk as 200ml 20X MOPS in 4L of milli-Q dH_2O) was added until the gel was covered by 0.5-1cm buffer. The RNA sample was added and electrophoresed for 4-18h at 15 to 50V. Electrophoresis was complete when the BPB dye had migrate 3/4 of the length of the gel. The gel was photographed (with a ruler along one edge) on a UV light box with black & white polaroid film (f-stop = 5.6, shutter speed 1/8 sec). The gel was next washed with 20X SSC, with agitation at 25C for 20min and then RNA was transferred overnight to a nitrocellulose membrane. Equal RNA loading of lanes was confirmed by ethidium bromide staining of gels before and after transfer. RNA was immobilized to the nitrocellulose by baking at 80°C under vacuum. Specific mRNA were detected by hybridization with radiolabelled cDNA, prepared using the Bethesda Research Labs (BRL) Nick Translation Kit. Briefly, 5µl of solution A2 (cold dATP, dGTP, and dTTP) + 5µl of solution C (DNA pol I + DNAse I), 7µl [α^{32}P]dCTP (specific activity 10mCi/ml), and 0.5µg cDNA was brought to 50µl total volume with soln E (dH20) and allowed to incubate 1h at 15°C. After nick translation, radioactive DNA was separated from unincorporated [^{32}P]dCTP using Elutip-D columns (Schleicher & Schuell). Double stranded nucleic acids (DNA or RNA) binds to the cellulose acetate in the column during low salt conditions (0.2M NaCl) and is eluted in high salt buffer (1M NaCl), as binding affinity between the DNA and cellulose is lower at higher ionic strenghts. Blots were hybridized with 10^6 cpm/ml of α^{32}P-labelled cDNA in hybridization medium (50% formamide, 10X SSC, 5X Denhardt's solution, 100µg/ml denatured salmon sperm DNA, and 15% dextran sulfate). After washing under high stringency conditions, radioactivity was detected by autoradiography on Kodak XO-MAT AR film

(Eastman Kodak Co., Rochester, NY) with intensifying screens at -80°C. Before re-probing, blots were placed in 0.1X SSC, 0.1% SDS at 90-100°C for 3-5min, and autoradiography performed to ensure complete removal of the previous probe. Hybridization was quantitated by densitometric scanning of appropriately exposed autoradiograms (Shimadzu CS-9000, Shimadzu Scientific Instruments, Columbia, MD).

Inhibition of DNA and RNA synthesis. To arrest cell growth, DNA synthesis was inhibited by incubation of CTLL-2 in hydroxyurea (Young et al., 1967) (HU; Sigma, St. Louis, MO). To determine the optimal conditions for inhibition of CTLL-2 cell growth, an HU dose-response curve was constructed by incubating cells with various doses of HU prior to ^{3}H-TdR addition. In this system, 1mM HU reliably and completely inhibited growth of CTLL-2. Loss of cell viability was only evident after 48h. To further confirm the efficacy of this treatment, DNA content of propidium iodide-stained nuclei was analyzed on an EPICS 753 cell sorter (Coulter Electronics, Hialeah, FL). Eighteen h after HU treatment, >95% of cells had accumulated in G_0/G_1. RNA synthesis was inhibited by 8h treatment of cells with 5μg/ml Actinomycin-D (Sigma) dissolved in ethanol (final [EtOH]= 0.5%). Cell viability after Actinomycin-D treatment was $\geq$ 71%.

CTLL-2 subset enrichment (panning). CTLL-2 cells were treated with 20U/ml IL-2 + 1ng/ml TGFβ on days 0 and 2. On day 3, cells were harvested, incubated with a 1/50 dilution of anti-CD8 (53-6.72) ascites, washed, and incubated at 4°C on a rabbit anti-rat Ig (Capell/Organon Technika, Malvern, PA; 4°C, 45min) coated bacteriologic

grade petri dish (Baxter Healthcare Corp., McGaw Park, IL). Non-adherent cells were harvested by gently swirling and treated with rabbit C obtained from Accurate Chemical and Scientific Corp., Westbury, NY (CD8-depleted). Adherent cells were harvested by vigorous pipetting (CD8-enriched). To determine the effectiveness of separation, CD8-depleted and CD8-enriched populations were incubated with anti-CD8α mAb, washed, stained with FITC-conjugated goat anti-rat Ig and analyzed by flow cytometry.

Activation and culture of thymocytes. Mice were sacrificed by CO_2 inhalation and after thymectomy, a thymocyte suspension (10^6/ml) was prepared in complete medium with 50 nM PDBu (Sigma, St. Louis, MO), 1 μM Io (Calbiochem, San Diego, CA) ± 20U/ml rIL-2 ± 1ng/ml porcine platelet TGFβ in 50ml polypropylene tubes (Costar, Cambridge, MA). After incubation at 37°C in humidified air with 5% CO_2 for 18h (on day 1), cells were washed 3X in warm HBSS to remove PDBu (McCrady et al., 1988) and re-cultured in complete medium ± 20U/ml rIL-2 ± 1ng/ml TGFβ at a cell density of $2.5x10^5$ cells/well in 48-well plates (Costar). One ng/ml TGFβ was chosen because it was optimal for the induction of CD8 in CTLL-2 cells. Cultures were split as necessary to prevent overgrowth; fresh medium, IL-2, and TGFβ were added every 2-3 days.

Preparation of DN and CD8 SP thymocytes. Single cell suspensions of thymocytes were incubated in cytoxicity medium (Accurate Chemical and Scientific Corp.) with a 1/50 dilution of anti-CD4 (GK1.5) ascites, washed, incubated with 1/50 dilution of mouse anti-rat Igκ chain (MAR18.5) ascites, washed, and treated with

rabbit C (1/12 dilution, Accurate Chemical and Scientific Corp.). Next, thymocytes were incubated with 1/50 dilution of anti-CD8α (116-13.1), washed, and incubated on a rabbit anti-rat Ig (which is cross reactive with mouse Ig) coated plastic petri plate at 4°C for 45min. Non-adherent cells were removed by gentle swirling of the plate; adherent cells were removed only after vigorous washing. Adherent cells were >96% CD8 SP and were used without further treatment. Non-adherent cells were further treated with a 1/50 dilution of GK1.5 and 116-13.1, followed by a second treatment with rabbit C. Typically, 0.5 to 3% of input cells remained, and were approximately 95% $CD4^-CD8^-$ as determined by immunofluorescence using anti-CD8α (53-6.72) and anti-CD4 (YTS 191.1.2). These conjugated antibodies do not cross-react with sites recognized by the cytotoxic antibodies used for C depletions (data not shown).

Separation of $CD8^+$ cells from DN thymocyte cultures treated with TGFβ. DN thymocytes were harvested after 6 days in culture with IL-2 + TGFβ, incubated with 1/50 dilution of anti-CD8 (53-6.72) ascites, washed and incubated on a rabbit anti-rat Ig coated petri dish (4°C, 45min). Nonadherent ($CD8^-$) cells were removed by gentle swirling of the plate; adherent ($CD8^+$) cells were removed only after vigorous washing with cytotoxicity medium.

Chapter 1

Inhibition of Tumor-Specific Cytotoxic T Lymphocyte Responses by Transforming Growth Factor-β_1

Author's note: The first chapter of this dissertation is derived in part from a published manuscript authored by myself, Dr. Shelley Hoover, Dr. Brian Susskind, Ms. Sandra Barrett, and Dr. Harry Bear (Inge et al., 1992b). Many of the figures in this chapter contain data derived from experiments that were designed by myself with assistance from my graduate advisor, and performed with the technical assistance of Laboratory Specialist Sandra Barrett (Tables 1.01-1.04 and Figures 1.01-1.02). Tumor-specific CTL clones used in this work were laboriously generated by Dr. Shelley Hoover during her post-doctoral fellowship in the lab (Hoover et al., 1991). Further, Dr. Hoover's contribution of the data shown in Figure 1.06 is gratefully appreciated. These data are presented here with the knowledge and consent of these co-authors.

Introduction

TGFβ has been shown to inhibit the activation of lymphokine activated killer (LAK) cells by IL-2 (Kuppner et al., 1988; Mulé et al., 1988; Jin et al., 1989), as well as the generation of allospecific cytotoxic T lymphocytes (CTL) in primary mixed lymphocyte reactions (Ranges et al., 1987; Wallick et al., 1990; Fontana et al., 1989; Shalaby and Amman, 1988). Inhibition of T cell responses and LAK activation by TGFβ, along with its secretion by malignant cells (Tada et al., 1991; Arrick et al., 1990; Hirte and Clark, 1991), has led to the proposal that this cytokine may play a key role in the *in vivo* immunosuppression associated with tumor progression, and may severely limit successful immunotherapy of cancer. It has been shown, for example, that transfection of a regressor tumor line with the gene for TGFβ caused it to grow progressively *in vivo* (Torre-Amione et al., 1990). There are convincing data showing that malignant cells produce TGFβ and that TGFβ inhibits primary immune responses, but studies showing that TGFβ directly inhibits the responses of host T lymphocytes to syngeneic tumor cells are lacking. The data presented in this chapter demonstrate that TGFβ inhibits *in vitro* generation of antitumor CTL. This observation may in part be attributed to an indirect effect on IL-2 production and/or by a direct effect on proliferation of memory CTL.

Results

TGFβ Suppresses CTL Generation from P815 Immune Mice

Splenocytes from mice immunized with P815 + *C. parvum* contain helper T lymphocytes (Th) and memory CTL that can be re-activated *in vitro* when stimulated with autologous tumor cells during MLTC (Bear et al., 1988). Since TGFβ has been implicated in suppression of anti-tumor immunity, we sought to determine whether exogenously added TGFβ would inhibit anti-tumor CTL generation. When purified TGFβ was added to MLTCs of P815 immune splenocytes from DBA/2 or BDF_1 mice, marked suppression of the CTL response was seen (Figure 1.01 and Table 1.01). With doses ranging from 0.675 to 10ng/ml TGFβ, we consistently observed >80% inhibition of lytic activity (Table 1.02). Similar results were obtained with recombinant human TGFβ (data not shown).

In addition to the effect on cytolytic activity, the addition of TGFβ to MLTC typically resulted in a moderate (30%) reduction in the number of viable cells per well at the end of the culture period, especially at higher doses of TGFβ. Similar to previous reports for primary allogeneic MLRs (Ranges et al., 1987), we only observed inhibition of CTL activity when TGFβ was added at the initiation of the cultures. Delay of 1, 2, or 4 days resulted in CTL activity similar to control, untreated groups

(Figure 1.02).

Effect of IL-2 on TGFβ Treated MLTC

Next, we sought to determine whether TGFβ might be acting to limit the availability of IL-2, as suggested by others (Tada et al., 1991). In two separate experiments, when 5 or 20 U/ml rIL-2 was added with 2 ng/ml TGFβ, inhibition was reversed by 50-80% (Table 1.03). However, cytolytic activity was not restored completely to control levels by 5 U/ml IL-2, and groups treated with IL-2 + TGFβ were less cytotoxic than groups treated with IL-2 alone, especially at low E:T ratios. To rule out the possibility that rIL-2 was inducing LAK activity in our system, rather than rescuing the CTL response, we also treated normal DBA/2 splenocytes with 5, 20, or 1,000 U/ml rIL-2. Only at 1,000 U/ml was significant LAK activity against P815 detected. In this same experiment (and others (McKinnon et al., 1990)), effector cells from MLTC supplemented with 5 or 20 U/ml IL-2 did not lyse the LAK sensitive B16 melanoma, indicating that low dose IL-2 was insufficient to generate LAK cells from DBA/2 early TBH splenocytes. Finally, in our hands, P815 cells are generally quite resistant to lysis by DBA/2 LAK cells.

Effect of TGFβ on Tumor-Specific CTL Clones

The above data suggest that TGFβ inhibits the *in vitro* activation of anti-tumor effector CTL, at least in part, by reduction of cytokine availability. However, since addition of low doses of exogenous IL-2 to TGFβ-treated MLTC did not result in complete restoration of cytotoxic activity to control levels, TGFβ might also act directly on the proliferation or maturation of memory CTL populations. We derived

several highly lytic, homogeneous CTL lines and clones from the spleens and axillary draining lymph nodes of PHS-5 TBH (Hoover et al., 1991) and the salient characteristics of these cells deserves brief mention. CTL clone GD11 was derived from the spleen of a PHS-5 TBH and is Thy 1.2^+, $CD8^+$, $CD4^-$, IL-$R\alpha^+$ and specifically cytolytic for P815 mastocytoma cells (Hoover et al., 1991). Subclones of GD11 were maintained by weekly stimulation with PHS-5 tumor cells and were fed 20U/ml rIL-2 every 3 days; as long as IL-2 was provided, CTL activity remained high (80-90% lysis at E:T=5:1). Five to seven days after tumor stimulation, while being fed rIL-2 every 3d, GD11 CTL lose proliferative responsiveness to rIL-2 (Figure 1.03), but remain >90% IL-2Rα positive (Figure 1.04) and highly cytolytic (>70% lysis at E:T=5:1); thus, such cells are termed "quiescent-lytic." These cells require tumor cells (antigen source) and exogenous IL-2 to trigger maximal proliferative responses to IL-2. In contrast, if rIL-2 is washed out of GD11 cultures and these CTL are maintained in complete medium alone for two to four days, the cells lose the ability to proliferate in response to IL-2 (Figure 1.05), become <5% IL-2Rα^+ (data not shown), and are essentially non-cytolytic (Figure 1.06)--a stage termed "rested."

We sought to determine what effect TGFβ had on the proliferation of rested, GD11 CTL clones in response to rIL-2 and tumor cell antigen. When rested GD11.10 subclones were stimulated with tumor cells and IL-2, treatment with 5 or 10 ng/ml TGFβ markedly inhibited short-term proliferation (Figure 1.07). In contrast to the effect on cell proliferation, the IL-2-dependent reactivation of cytotoxic activity was *not* diminished by TGFβ treatment (Figure 1.08). Interestingly, when TGFβ (5 or 10ng/ml) was added to quiescent-lytic GD11.10, neither proliferation (Figure 1.07) nor cytotoxicity (data not shown) was inhibited.

Discussion

TGFβ is a multifunctional cytokine made by numerous normal tissues, including T lymphocytes, and also by many tumors. Most tissues, including cells of the immune system, have receptors for TGFβ. Many studies to date have reported the negative modulatory effects of TGFβ during primary T lymphocytes responses to antigen or mitogen (Ranges et al., 1987; Mulé et al., 1988; Morris et al., 1989; Kehrl et al., 1986b). We have examined the effects of TGFβ on secondary *in vitro* anti-tumor CTL responses. P815-immune splenocytes contain memory CTL that, when cultured with mitomycin-C treated P815, proliferate and differentiate into tumor-specific effector CTL that can be detected by cytotoxicity assay (Bear et al., 1988). The generation of class I-restricted CTL in MLTC depends on the presence of tumor cells bearing MHC class I, syngeneic accessory cells, and MHC class II-restricted $CD4^+$ helper T cells (Bear et al., 1988). When TGFβ was added in nanogram quantities at the initiation of MLTC from tumor immune mice, we consistently saw >80% inhibition of CTL activity. If addition of TGFβ was delayed for 24h or more, the CTL response was not inhibited, favoring the hypothesis that T memory cells, like naive T cells, are more sensitive than activated T cells to the inhibitory effects of TGFβ.

It has been suggested that the generalized suppression of lymphocyte responses in tumor bearing hosts and the mediation of this immunosuppression by TGFβ is primarily the result of inhibiting $CD4^+$ T helper cell activation and IL-2 production (Tada et al., 1991; Tada et al., 1990; Kuppner et al., 1988). To determine what role limiting IL-2 availability in MLTC may play in the effect of TGFβ, we added recombinant IL-2 to TGFβ-treated MLTC; 5 U/ml IL-2 largely but still incompletely restored CTL activity; 20U/ml restored activity to near control levels. Parallel cultures of normal splenocytes treated with the same doses of IL-2 were incapable of lysing P815 or allogeneic LAK sensitive targets, thus ruling out LAK induction as an explanation for the increased killing in IL-2 supplemented MLTC (furthermore, LAK are TGFβ sensitive). In sum, these data indicate that one point at which TGFβ blocks the secondary CTL response of P815-immune splenocytes to tumor cells is at the level of lymphokine production by Th cells. This is in agreement with recent studies (Tada et al., 1991) showing that tumor cell supernatants containing TGFβ inhibited the CTL response to haptenated self which, like the response to P815 (Bear et al., 1988), requires $CD4^+$ Th cells. The CTL response to alloantigen, in contrast, can proceed without $CD4^+$ cells, using IL-2 from $CD8^+$ Th cells (Mizuochi et al., 1985) and was relatively resistant to inhibition by TGFβ (Tada et al., 1991).

However, since the inhibition of MLTC was not completely reversed by addition of low dose IL-2, it was still possible that TGFβ inhibited CTL by additional mechanisms other than inhibition of cytokine production. To measure direct effects of TGFβ on memory CTL, we employed cloned, homogeneous populations of P815-specific CTL. Rested CTL clones are analogous to pre-effector CTL as both are non-cytolytic, and display low levels of IL-2Rα until re-activated by tumor stimulator cells

and IL-2, at which time they proliferate and become specifically cytolytic for tumor cells. The proliferation of rested CTL clones was inhibited by 80-90% in the presence of 2.5 to 10ng/ml TGFβ. In contrast to rested cells, the proliferation of quiescent-lytic CTL was not inhibited by TGFβ. TGFβ blocks the exit of T cells from G_1 (Stoeck et al., 1989a; Morris et al., 1989), and therefore it is possible that the rested population is more sensitive to TGFβ because more cells in this group exist in the G_0/G_1 phase of the cell cycle than quiescent-lytic cells. These data are also congruent with our findings that CTL activity was inhibited only when TGFβ was added at the start of MLTC but not 24, 48 or 96h after initiation of the *in vitro* secondary response.

IL-2 is necessary for both the up-regulation of cytotoxicity and the proliferation of rested CTL clones. The fact that TGFβ inhibited tumor + IL-2 dependent growth of CTL but not the up-regulation of cytotoxicity indicates that TGFβ can discriminate between two distinct signalling pathways that are triggered by IL-2 binding to these CTL. This inability of TGFβ to inhibit activation of killing activity is in agreement with Shalaby *et al.* who found that TGFβ inhibited the generation of allospecific CTL but did not affect cytolytic function of such CTL generated in the absence of TGFβ (Shalaby and Amman, 1988). The fact that IL-2-dependent development of cytotoxicity was resistant to TGFβ suggests that TGFβ may discriminate between two distinct pathways triggered by IL-2R ligation. Such a hypothesis would predict that all responses to IL-2 may not be targets for the inhibitory action of TGFβ. Current themes in the cytokine receptor literature portray single surface receptor types communicating with multiple second messenger pathways, with much molecular "crosstalk" occurring intracytoplasmically. Thus, it is not difficult to imagine that surface receptors that have multiple functions (such as growth and differentiation in the case of

IL-2R) may feed into multiple divergent signalling pathways which might be regulated differently by cytokines such as TGFβ.

In summary, these studies demonstrated that the immunosuppressive activities of TGFβ observed by others in primary T cell responses can be extended to secondary *in vitro* CTL responses directed against syngeneic tumor cells. In experiments designed to identify the mechanism(s) of inhibition, our data suggest that TGFβ acts at multiple steps leading to the activation of anti-tumor CTL, including inhibition of IL-2 production, as has been shown for primary responses (Ranges et al., 1987; Tada et al., 1991; Espevik et al., 1987). Additionally, a direct anti-proliferative effect on resting CTL clones stimulated with IL-2 and tumor cells was observed. Investigation of the effects of TGFβ on mitotic responses of T cells would add to our understanding of TGFβ-mediated immunosuppression.

Table 1.01: Suppression of MLTC by TGFβ. Splenocytes from P815-immune DBA/2 (Experiments 1 and 2) or BDF_1 (Experiments 3-5) mice were used to establish MLTC. TGFβ was added to cultures on day 0 and after 6 days chromium release assay was performed. Cytotoxic activity was compared between groups and LU_{30} per 10^6 cells and per culture were calculated. LU/Cx = Lytic units per culture, calculated as the product of $LU/10^6$ cells and viable cell counts per well in each group.

Treatment:	PHS-5mc	TGFβ	% Lysis 40:1	% Lysis 20:1	$LU_{30}/10^6$±SD	LU/Cx
Expt 1	-	-	20	14	1.8±0.4	5
	+	-	59	50	11.3±0.1	33
	+	10ng/ml	24	17	2.0±0.2	6
Expt 2	-	-	5	6	0.1±0.1	0
	+	-	31	27	32.6±4.5	78
	+	5ng/ml	15	6	2.4±0.3	5
Expt 3	-	-	8	5	0.6±0.1	2
	+	-	78	58	23.4±4.3	56
	+	5ng/ml	35	24	3.6±0.5	7
Expt 4	-	-	3	3	0.2±0.1	1
	+	-	56	55	15.3±2.9	41
	+	5ng/ml	12	11	1.0±0.2	3
Expt 5	-	-	4	1	0.2±0.0	1
	+	-	61	74	71.0±21	284
	+	5ng/ml	46	34	5.3±0.3	15

Table 1.02: TGFβ dose response for inhibition of MLTC. MLTC cultures were established with P815 immune DBA/2 splenocytes. The first row shows results without tumor stimulator cells. 0-10ng/ml TGFβ was added to MLTC on day 0. Percent lysis is shown at E:T=40:1. LU_{15} or LU_{30} per 10^6 cells were calculated from % lysis data and are shown ± SD. LU/Cx = Lytic units per culture, calculated as the product of LU/10^6 cells and viable cell counts per well in each group.

PHS-5mc	TGFβ	%Lysis (40:1)	$LU_{15}/10^6$	LU/Cx
-	0	-1	<0	<0
+	0	25	5.1±0.5	16
+	0.68	4	0.7±0.1	2
+	1.25	2	0.4±0.2	3
+	2.5	8	1.2±0.1	3
+	5	6	0.9±0.1	1
+	10	6	0.9±0.1	2

Table 1.03: IL-2 reversal of TGFβ-mediated suppression of MLTC. Splenocytes from P815-immune DBA/2 mice were used to establish MLTC. 10^7 splenocytes were either unstimulated or stimulated with $5x10^5$ PHS-5mc ± 2ng/ml TGFβ ± 5 or 20U/ml recombinant human IL-2. Normal spleen cells were cultured with various doses of IL-2. On day 6, cultures were assayed for cytotoxic activity against ^{51}Cr labelled P815 cells, and these data are shown as % specific lysis. Lytic units per 10^6 cells and LU per culture well (LU/Cx) are also shown for each group. A parallel assay against ^{51}Cr labelled B16 cells yielded no significant lysis (<3% lysis at E:T=40:1 or 20:1) by these same experimental groups. However, the normal splenocytes cultured in 1,000 U/ml IL-2 (last row) did lyse these LAK sensitive targets (25% and 18% lysis at E:T=40:1 and 20:1). Imm, immune; NS, normal splenocytes

	Treatment(s)			%Lysis (E:T Ratio)				$LU_{30}/10^6$	LU/Cx
	PHS-5	TGFβ	IL-2	40	20	10	5		
Imm	-	-	-	0	0	-1	-1	<1	<1
	+	-	-	82	81	64	48	33± 4	86
	+	2	-	42	33	24	13	5± 1	11
	+	2	5	69	63	42	28	14± 2	35
	+	2	20	75	79	65	39	28± 5	84
	+	-	5	85	72	78	68	60±18	162
	+	-	20	86	72	62	57	34± 8	85
NS	-	-	5	-2	-2	-1	1	<1	<1
	-	-	20	1	1	-1	-1	<1	<1
	-	-	1000	14	11	10	6	1±0.3	2

Figure 1.01: TGFβ inhibits CTL generation from P815-immune splenocytes. MLTC established with splenocytes from P815-immune mice were either "unstimulted" = cultured in media alone; "stimulated" = cultured with mitomycin-C treated PHS-5 cells; "stimulated + 5ng/ml TGFβ" = PHS-5mc + 5ng/ml TGFβ added at initiation of culture. The means of triplicate determinations are shown ± SD.

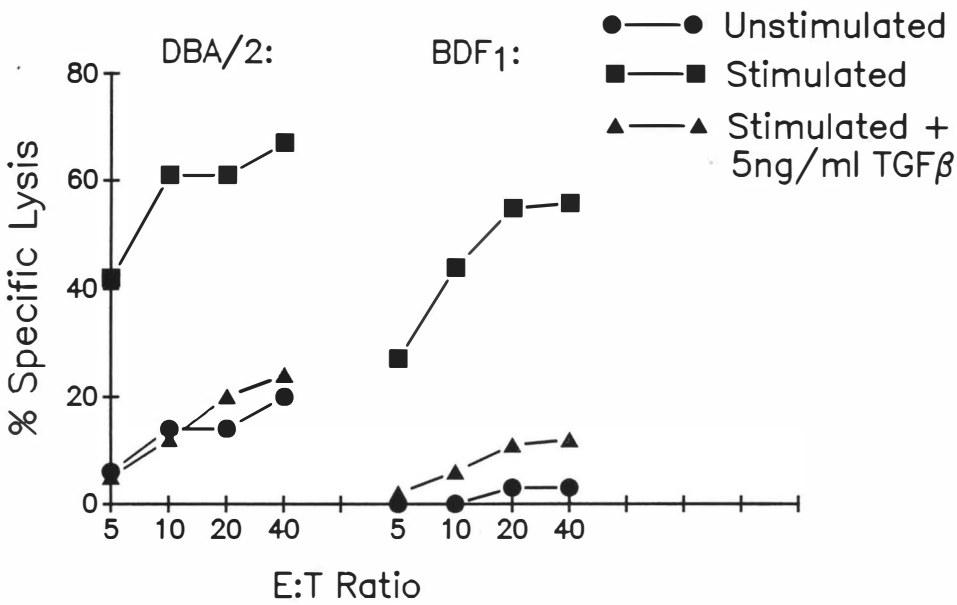

DBA/2:
BDF1:
Unstimulated
Stimulated
Stimulated +
5ng/ml TGFβ
% Specific Lysis
80
60
40
20
0
5
10
20
40
5
10
20
40
E:T Ratio

Figure 1.02: Effect of TGFβ added to MLTC on various days. MLTC were established with BDF_1 immune spleen cells. Five ng/ml TGFβ was added on days 0, 1, 2, or 4. Percent lysis at E:T=40:1 is shown ± SD; similar results were obtained at other E:T ratios and $LU_{30}/10^6$ cells are shown above each bar.

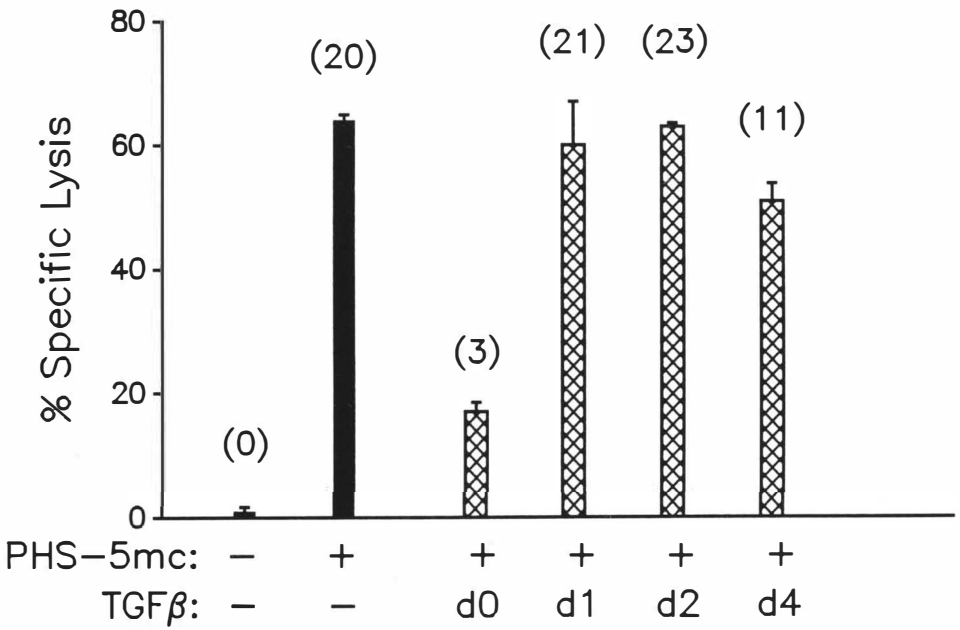

80
60
40
20
0
% Specific Lysis
(0)
(20)
(3)
(21)
(23)
(11)
PHS–5mc: – + + + + +
TGFβ: – – d0 d1 d2 d4

Figure 1.03: CTL clones lose proliferative responsiveness to IL-2 (ie., become quiescent) late after antigen exposure. GD11.17 clones were harvested from culture, washed, and stimulated in 24-well plates with PHS-5mc + 20U/ml IL-2. On days 3, 4, 6, 7, 8, and 9 after stimulation, cells were harvested from 24-well plates, washed, plated in 96-well plates with various concentrations of IL-2 + 37kBq of tritiated thymidine. Isotope incorporation was measured over a 24h period and plotted as a function of time after initial tumor stimulation. Points represent arithmetic mean cpm ± S.D. for quadruplicate samples.

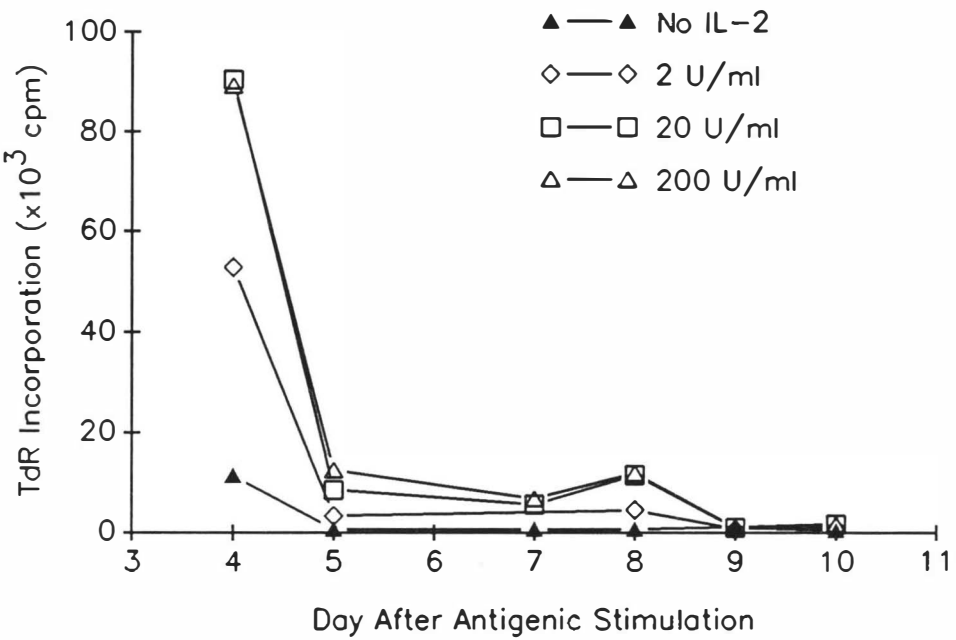
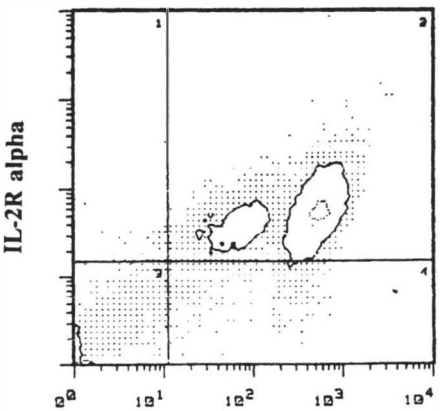

No IL-2
2 U/ml
20 U/ml
200 U/ml
TdR Incorporation (x10^3 cpm)
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
Day After Antigenic Stimulation

Figure 1.04: Quiescent CTL clones are IL-2Rα$^+$. GD11.17 cells were harvested from culture (5 days after previous stimulation with tumor cells) and treated with 20U/ml IL-2 with (A) or without (B) fresh tumor stimulator cells. Three days later, cells were stained for IL-2Rα and analyzed by FACS.

A.


IL-2R alpha
1
2
3
4
10^0
10^1
10^2
10^3
10^4

B.

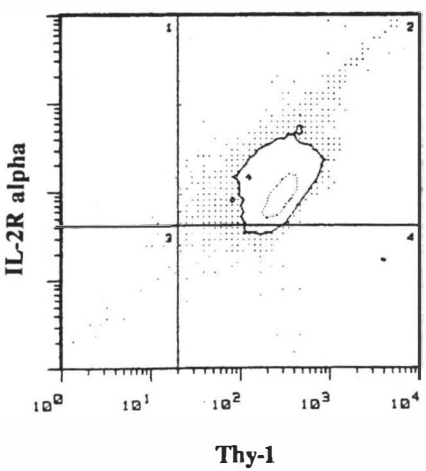

IL-2R alpha
1
2
3
4
10^0
10^1
10^2
10^3
10^4
Thy-1

Figure 1.05: Rested CTL clones do not proliferate in response to IL-2. GD11.17 cells were harvested from culture 5 days after previous stimulation with tumor cells and washed free of IL-2. These cells were "rested" by culture in the absence of IL-2 for an additional 4 days prior to stimulation with 0, 10, or 20U/ml IL-2 (BARS on right). The response of these rested cells was compared to that of cells that were activated by tumor stimulator cells 2 days prior to IL-2 challenge (BARS on left).

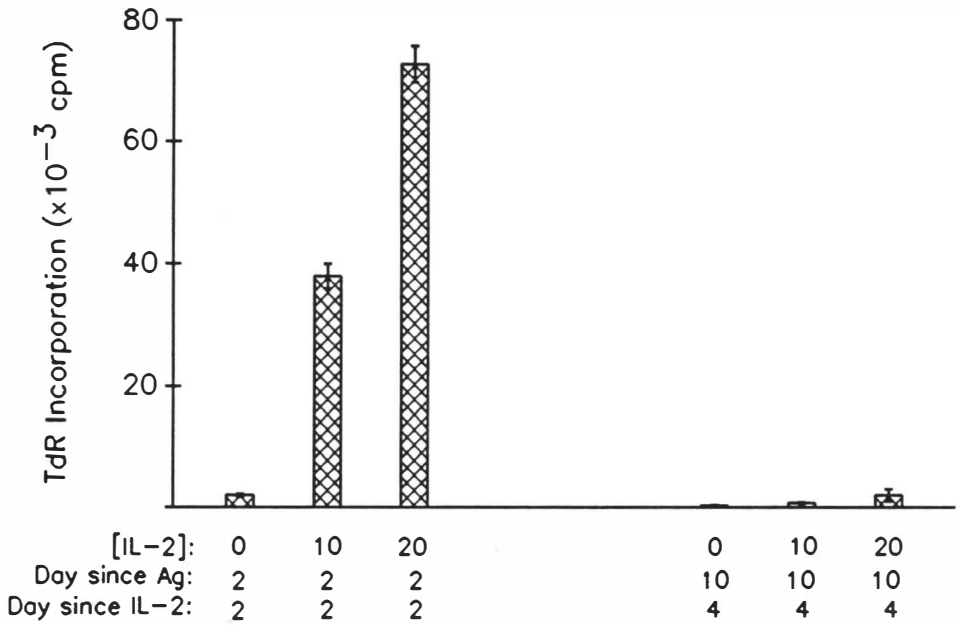

80
60
40
20
TdR Incorporation (x10^{-3} cpm)
[IL-2]: 0 10 20 0 10 20
Day since Ag: 2 2 2 10 10 10
Day since IL-2: 2 2 2 4 4 4

Figure 1.06: Rested CTL clones are non-cytolytic. Cytotoxic activity (against ^{51}Cr labelled P815) of rested GD11.17 CTL clones (9 days after tumor stimulation, 4 days after washout of IL-2) was compared to cytotoxic activity of active CTL clones (2 days after tumor stimulation, 2 days after previous feeding of IL-2).

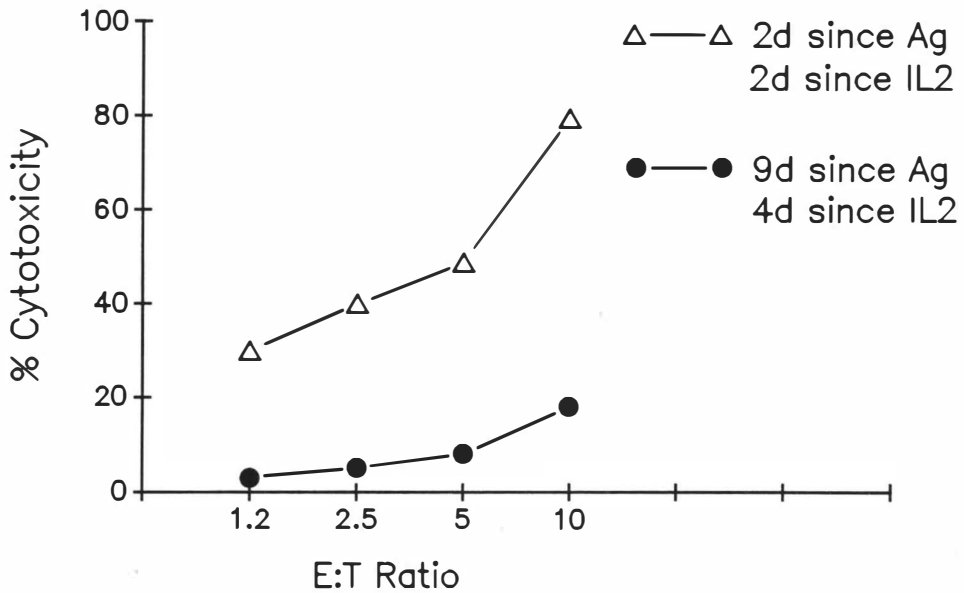

100
80
60
40
20
0
% Cytotoxicity
1.2
2.5
5
10
E:T Ratio
2d since Ag
2d since IL2
9d since Ag
4d since IL2

Figure 1.07: TGFβ inhibits proliferation of rested CTL clones. Fifty thousand rested or quiescent CTL clones were cultured ± 20U/ml IL-2 ± TGFβ ± $2.5x10^4$ PHS-5mc per well in 96 well flat-bottom plates. On day 2, 37kBq ^{3}H-thymidine was added per well and cells were harvested on day 3. The proliferation of rested GD11.10 + PHS-5 + IL-2 + 2.5ng/ml TGFβ was significantly different from the positive control group that did not receive TGFβ, $p<0.05$ by Students' t-test.

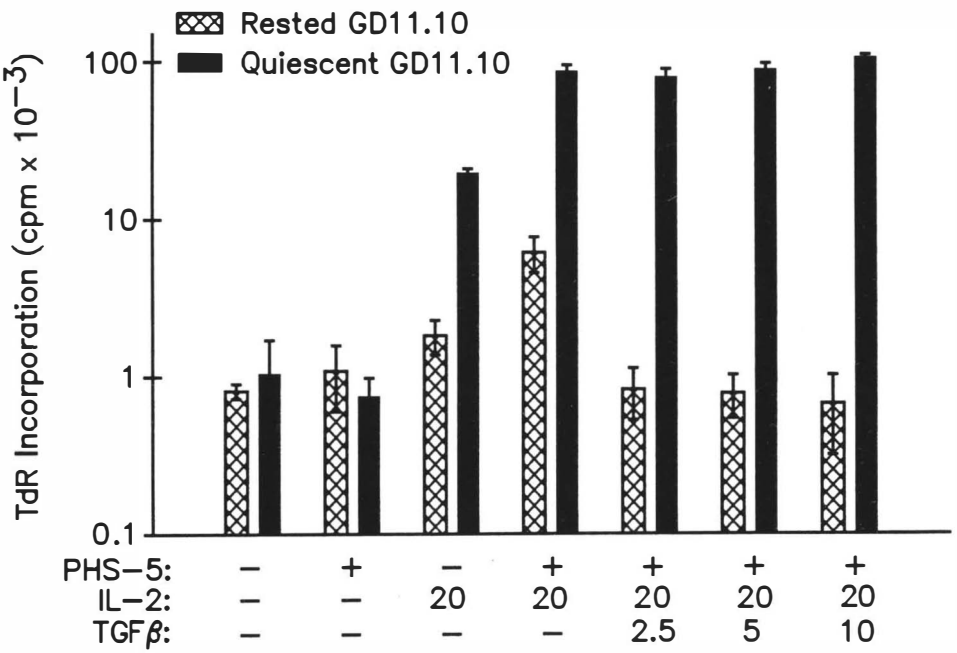
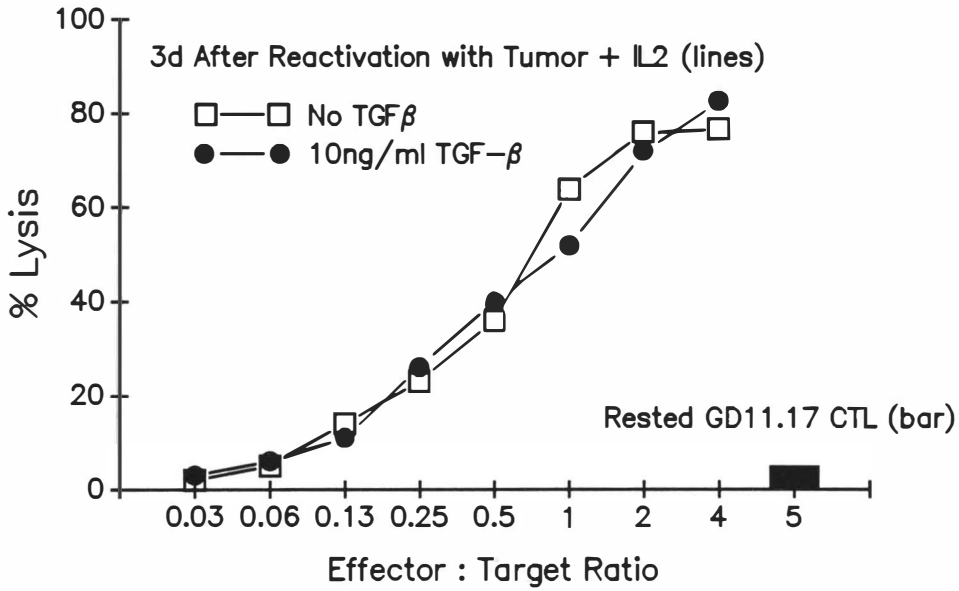

Rested GD11.10
Quiescent GD11.10
TdR Incorporation (cpm x 10^{-3})
100
10
1
0.1
PHS–5: – + – + + + +
IL–2: – – 20 20 20 20 20
TGFβ: – – – – 2.5 5 10

Figure 1.08: TGFβ does not inhibit cytolytic activation of rested CTL clones. $5x10^5$ rested GD11.10 CTL were added to each well of a 24 well plate with $2.5x10^5$ PHS-5mc and 20U/ml IL-2 ± 5ng/ml TGFβ. On day 2, cultures were assayed for cytolytic activity against ^{51}Cr labelled PHS-5 tumor cells. Baseline cytotoxic activity on day 0 is also shown ± SD (bar, E:T ratio = 5:1). LU_{30} ± SD values for control and TGFβ-treated cultures were 274±36 and 230±28, respectively.


100
80
60
40
20
0
% Lysis
3d After Reactivation with Tumor + IL2 (lines)
No TGFβ
10ng/ml TGF-β
Rested GD11.17 CTL (bar)
0.03 0.06 0.13 0.25 0.5 1 2 4 5
Effector : Target Ratio

Chapter 2

Effect of TGFβ on T Lymphocyte Growth

Introduction

The data presented in Chapter 1 above demonstrated that TGFβ inhibited the generation of CTL in MLTC culture and suggested that this effect was at least in part a result of decreased IL-2 production. However, the proliferation of cloned CTL was also inhibited by TGFβ in the presence of exogenous IL-2. Thus this inhibition appeared to be secondary to a direct antiproliferative effect on these cells. We chose next to investigate whether the antiproliferative effect of TGFβ on T lymphocytes might stem from inhibition of IL-2 responsiveness.

TGFβ regulates growth of numerous cancerous and non-neoplastic cell types and the inhibition of growth factor-dependent proliferation in epithelial and mesenchymal cells appears to be downstream of the interaction of growth factors with their receptors (Laiho et al., 1990a). In hematopoietic and lymphoid systems however, TGFβ has been shown to modulate cytokine receptor levels, suggesting the possible existence of more complex levels of cell cycle regulation in leukocytes (Jacobsen et al., 1991; Kehrl et al., 1986b; Kuppner et al., 1988). It was also suggested that TGFβ alters bio-

chemical events induced by intermediate affinity IL-2R ligation in NK cells (Ortaldo et al., 1991). With respect to T cells however, there are scant data concerning the effect of TGFβ on high affinity IL-2R-induced events, and the physiology of these two receptor types (intermediate and high affinity) may differ. There are, in fact, conflicting reports regarding the effect of TGFβ on IL-2Rα chain expression with some reports showing increases, some showing decreases, and others showing no change (Smyth et al., 1991; Kehrl et al., 1986b; Stoeck et al., 1989a; Ruegemer et al., 1990; Wahl et al., 1988).

The tumor-specific CTL clones described in Chapter 1 above grow relatively slowly, require much manipulation, and require the addition of live tumor cells for maintenance, which limits their usefulness for initial studies. CTLL-2 is an IL-2 dependent, non-neoplastic T cell line that was originally derived as a cytotoxic T lymphocyte clone against Friend leukemia cells. These Thy 1^+ cells constitutively display high levels of high affinity IL-2 binding sites, are not dependent upon antigen for IL-2 responsiveness, but are strictly dependent upon exogenous IL-2 for viability and growth (Baker et al., 1979). In fact, the growth of these cells has been commonly used as a bioassay for IL-2. Thus, CTLL-2 offers a model system to study the growth factor dependent phases of T cell activation without confounding effects of antigen presenting cells or TCR triggering. The results of the studies described here provide evidence that inhibition of IL-2 dependent T cell growth by TGFβ is not temporally associated with any major inhibition of IL-2R surface expression. However, inhibition of IL-2-induced synthesis of mRNA for various T cell associated molecules occurs in a matter of hours after TGFβ addition, suggesting that intracellular transduction of the IL-2 stimulus or early genetic events triggered by IL-2 may be

points at which TGFβ exerts an inhibitory effect on T cell growth.

Results

TGFβ inhibits proliferative responses to IL-2.

Treatment of CTLL-2 with TGFβ resulted in dose-dependent inhibition of tritiated thymidine incorporation in response to rIL-2. Maximal inhibition of proliferation (70-80%) was observed after addition of 1ng/ml TGFβ; half maximal inhibition was seen at 0.05 ng/ml (Figure 2.01). To determine whether this growth inhibition could be overcome with greater amounts of IL-2, proliferation was also examined as a function of IL-2 concentration at a fixed dose of 5ng/ml TGFβ. Control cultures of CTLL-2 incorporated TdR essentially as a function of IL-2 dose, with maximal incorporation observed at 25U/ml IL-2, while incorporation dropped off at higher doses of IL-2, presumably secondary to overgrowth of the cultures. When TGFβ was added to parallel cultures, the dose-response to IL-2 was flat even at the highest concentration of IL-2 (50U/ml), measured at 24 or 48h (Figure 2.02), indicating that DNA synthesis was significantly inhibited by addition of TGFβ. To ascertain whether the TGFβ-induced inhibition of DNA synthesis was also reflected in suppression of cell growth, cells were treated with optimal concentrations of IL-2 ± TGFβ and enumeration of viable cells was done. As shown in Figure 2.03, cell growth was not absolutely inhibited by TGFβ but rather was reduced by approximately 50% in a time- and dose-

dependent manner. These results demonstrate that the effect of TGFβ on DNA synthesis was associated with inhibition of CTLL-2 mitosis in response to IL-2. These data were essentially confirmed by analysis of DNA content in cells that were treated for 24h with IL-2 alone, IL-2 + TGFβ, or IL-2 + hydroxyurea. As shown in Figure 2.04, HU efficiently caused the accumulation of 95% of nuclei in G_0/G_1 as compared to 49% for TGFβ-treated cells and 38% for controls treated with IL-2 alone.

Effect of TGFβ on the IL-2R

The IL-2-dependent growth response of CTLL-2 is mediated by IL-2 binding to high affinity IL-2R sites (Malek and Ashwell, 1985). High affinity IL-2R binding sites are composed of three integral membrane proteins identified as IL-2Rα (p55), IL-2Rβ (p75), and the most recently cloned IL-2Rγ (p64) (Takeshita et al., 1992). We hypothesized that a reduction in the rate of proliferation and inhibition of cell cycle progression might result from a down-regulation of IL-2 receptor expression on CTLL-2 treated with TGFβ, as suggested by others (Ruegemer et al., 1990; Stoeck et al., 1989a; Kehrl et al., 1986b). To test this hypothesis, cultures were stimulated with 20U/ml IL-2 ± 1ng/ml TGFβ. IL-2Rα surface peptides were stained with FITC-conjugated anti-IL-2Rα Ab, and fluorescence intensity was quantitated and analyzed by FACS. At initiation of culture, cells were essentially 100% IL-2Rα$^+$. Analysis of IL-2Rα mean fluorescence intensity (MFI) values on day 1 showed in most cases that a modest up-regulation of the IL-2Rα antigen level occurred when CTLL-2 were treated with IL-2 alone; the cells also continued to score 100% IL-2Rα$^+$ (Table 2.01). In TGFβ-treated

cultures, although ≥93% of cells remained IL-2Rα$^+$, MFI values averaged only 66% that of controls on day 1 (Figure 2.05). Peak IL-2Rα immunofluorescence after IL-2 alone was detected on day 1 and by day 2 after treatment, IL-2Rα levels declined slightly. In the presence of TGFβ, this decline was more marked, as MFI values for TGFβ-treated cells averaged only 44% that of controls on d2. After 3 days of culture with IL-2 + TGFβ, IL-2Rα surface staining was consistently lower than for cells treated with IL-2 alone (Table 2.01 and Figure 2.06).

To determine whether TGFβ affected IL-2Rα at earlier times, CTLL-2 were treated with IL-2 ± TGFβ and IL-2Rα expression was measured after 1, 3, 6, 12, and 24h. As shown in Figure 2.07, a time-dependent increase in the accumulation of IL-2Rα chains occurred in response to IL-2, but no affect of TGFβ was seen until 24h, where TGFβ-treated cells demonstrated 40% less surface staining for IL-2Rα, as documented in other experiments shown above.

Although TGFβ modestly inhibited expression of IL-2Rα 24h after treatment, the overwhelming majority of cells were still IL-2Rα$^+$ at this time. Therefore it seemed unlikely that growth inhibition at 24h could be due solely to limiting numbers of IL-2Rα surface receptors. As other peptides are necessary for the assembly of functional IL-2R sites, we hypothesized that TGFβ might be acting to limit IL-2 binding by other mechanisms. To simultaneously correlate changes in IL-2Rα expression with IL-2 binding, cells were stained with both FITC-conjugated anti-IL-2Rα Ab and PE-conjugated IL-2-biotin. Cells demonstrated bright immunofluorescence with both stains, verifying that no significant competition occurred between these two reagents (Figure 2.08). When TGFβ treated cells

were compared to controls at 24h, IL-2Rα levels and biotinylated IL-2 staining were reduced by 25 and 43%, respectively, as scored by MFI. At 48h (Figure 2.09), when TGFβ-treated cells were compared to controls, IL-2Rα and biotinylated IL-2 staining were reduced by 73 and 75%, respectively. Thus, these results show that IL-2 receptor levels, as measured either by anti-IL-2Rα or by staining with biotinylated IL-2 ligand, were lower in groups treated with TGFβ, especially after 24h of treatment. TGFβ did not affect either IL-2Rα chain expression (Figure 2.07) or biotinylated IL-2 binding (data not shown) at times prior to 24h.

Effect of TGFβ on IL-2-induced gene expression

To determine whether TGFβ affects IL-2-induced gene expression, we first examined the effect of TGFβ on expression of mRNA for various T cell associated genes. In CTLL-2, we have found that intracellular levels of mRNA for IL-2Rα, IL-2Rβ, *c-myc*, and the cytolytic granule associated molecule Granzyme B are tightly regulated by exposure to IL-2. Several days after feeding cells with IL-2, these mRNA species are barely detectable and rise sharply (by 1-3h) in response to fresh IL-2 exposure. Thus, the cellular response to IL-2, as measured by the accumulation of RNA molecules, is very rapid. As shown in Figures 2.10-2.12, TGFβ inhibited IL-2-dependent accumulation of mRNA for IL-2Rα, and IL-2Rβ, Granzyme B, and IL-4R by 75%, 45-85%, 80%, and 55%, respectively at 12h. Marked inhibition of these mRNA species was also seen at times as early as 3h after TGFβ treatment (Figures 2.10 and 2.12). In two experiments, one hour after treatment of CTLL-2 with IL-2 ± TGFβ, *c-myc* mRNA expression was also

inhibited by 43 and 55% as measured by Northern analysis (Figure 2.13). These data suggest that a variety of very early T cell responses to IL-2 are inhibited by concomitant treatment with TGFβ.

TGFβ is known to inhibit the expression of many genes in many different tissue types and treatment of lymphocytes for 24h with TGFβ was shown to inhibit tritiated uridine incorporation by 50% (Bartlett et al., 1991). Thus, the inhibition of IL-2-induced mRNA expression seen in the above Northern analyses might be specific for the genes examined, or alternatively, may be secondary to a general inhibition of IL-2-dependent RNA synthesis. To determine whether TGFβ affected overall RNA synthesis in CTLL-2, CTLL-2 were treated with 20U/ml IL-2 ± 1ng/ml TGFβ for 4 or 18h and then nuclei were harvested for *in vitro* run-on transcription. Newly synthesized RNA transcripts were isolated and the incorporation of ^{32}P uridine triphosphate (UTP) was measured. Four hours after treatment with IL-2 vs. IL-2 + TGFβ, incorporation of $32x10^6$ and $16x10^6$ cpm (per $50x10^6$ nuclei) were measured, respectively, demonstrating a 50% inhibition of RNA synthesis for cells treated with TGFβ. For nuclei harvested 18h after addition of IL-2 vs. IL-2 + TGFβ, ^{32}P UTP incorporation was $16.4x10^6$ and $7.2x10^6$ cpm respectively (per $50x10^6$ nuclei), demonstrating 56% inhibition of *in vitro* RNA synthesis by nuclei from TGFβ-treated cells. The data from the Northern analyses and nuclear run-ons above demonstrate that TGFβ has a negative regulatory effect on very early responses that are signalled through pre-existing IL-2R sites which may be causally related to the inhibition of IL-2-dependent DNA synthesis (Figures 2.01 and 2.02) and the production of new surface IL-2Rα chains (Table 2.02) seen at 24h.

Discussion

The control of cellular proliferation in multicellular organisms is a complex process. Proper functioning and homeostasis in epithelial, endothelial, and lymphoid tissues, for example, is dependent upon strictly regulated cellular proliferation, and therefore a precise balance between proliferative and antiproliferative signals must be maintained. Much progress has been made in elucidating the nature of the growth stimulatory factors involved, and the identification of such cytokines as epidermal growth factor, platelet derived growth factors, and interleukins such as IL-2 and IL-4 and knowledge of their mechanisms of action attest to this fact. However, mechanisms by which inhibition of cell division (and thus avoidance of neoplasia) is achieved are only now being elucidated.

Antiproliferative effects of TGFβ.

A number of studies have shown that the transforming growth factors-β inhibit the proliferation of epithelial (Coffey et al., 1988), endothelial (Takehara et al., 1987), and lymphoid cell types (Kehrl et al., 1986b; Ruegemer et al., 1990; Stoeck et al., 1989a; Morris et al., 1989; Wahl et al., 1988). CTLL-2 cells are constitutively display an average of 4,000 high affinity and 40,000 low affinity IL-2R sites per cell (Robb et

al., 1984), and thus do not require antigenic stimulation for expression of IL-2 responsiveness. These cells therefore represent a useful system to study the antiproliferative effects of TGFβ on IL-2-dependent T cell growth, without consideration of the possibly confounding effects of TCR triggering on this process. Our data demonstrate that as early as 24h after addition of TGFβ, IL-2 driven proliferation of CTLL-2 cells was inhibited by 70-85%. Even though IL-2 could not overcome TGFβ-dependent inhibition of DNA synthesis, neither the inhibition of thymidine incorporation nor cell growth was absolute, suggesting that the effect of TGFβ was to modulate responsiveness to IL-2 rather than to completely block DNA synthesis or act as a cytotoxin. Differences in cell cycle progression were seen as early as 12h after TGFβ treatment and over the ensuing 48h, CTLL-2 steadily accumulated as a G_1 peak. In contrast, control cultures demonstrated normal cell cycle progression as indicated by an increased S phase fraction after IL-2 addition, prior to arrest in G_1, presumably due to exhaustion of IL-2 supplies after 2 days' culture. These data demonstrate that TGFβ is inhibitory to CTLL-2 proliferation and are congruent with other reports showing that TGFβ-treated cells are blocked in the G_1 phase of the cell cycle (Whitson and Itakura, 1992; Cross and Cambier, 1990; Lin et al., 1987).

The molecular details of TGFβ-dependent inhibition of epithelial cell growth has been elegantly described. In Mv1Lu cells, progression through G_1 into S phase requires the concerted action of numerous cellular proteins, including those encoded by the *c-myc* and retinoblastoma *RB* loci. The presence of a phosphorylated form of the retinoblastoma gene product RB is required for progression to S phase and thereafter, RB returns to a dephosphorylated form. As TGFβ was shown to inhibit progression of epithelial cells from G_1 to S, it was hypothesized that TGFβ may influence cell growth

via an effect on RB and/or on *c-myc* expression. Indeed, numerous studies have shown that TGFβ prevented G_1 progression by interrupting the appearance (Yan et al., 1992) or phosphorylation (Laiho et al., 1990a; Whitson and Itakura, 1992) of RB in late G_1. Inhibition of phosphorylation was accomplished by preventing the appearance of the serine-threonine kinase molecule responsible for the enzymatic phosphorylation of RB (Massagué et al., 1992) and TGFβ also blocked the activity of H1 kinase and $p34^{cdc2}$ kinase (Howe et al., 1991). Other studies have addressed the relationship between TGFβ, RB and *c-myc*. In keratinocytes that overexpress the underphosphorylated form of RB and in keratinocytes treated with TGFβ, the expression of *c-myc* was prevented; in both cases, a common 23 basepair element up-stream of *c-myc* was required for this inhibition of gene transcription (Pietenpol et al., 1991).

Currently, it is uncertain whether similar mechanisms underlie growth inhibition in lymphoid cells. Kehrl *et al.* found that lymphocytes have high affinity TGFβ surface receptors, that TGFβ inhibited T (Kehrl et al., 1986b) and B (Kehrl et al., 1986a) cell proliferation in response to IL-2, and further demonstrated that TGFβ was produced by these cells during immunologic responses. These data suggested that TGFβ may play an autocrine or paracrine role in the regulation of growth factor dependent proliferation of T lymphocytes. Although it is possible that TGFβ inhibits the growth of normal T cells via interference with initial signals emanating from the TCR, such an effect would not fully explain the marked growth inhibition seen when TGFβ is added to cultures of T lymphocytes that are polyclonally stimulated with mitogenic lectins, which are thought to act by multivalently crosslinking numerous

surface structures. Furthermore, Lee and Rich demonstrated that TGFβ stimulated IL-2-independent proliferation of murine splenic T cells in response to anti-CD3 stimulation (Lee and Rich, 1991). Thus, an inhibition of TCR signalling alone cannot account for data showing that TGFβ inhibits IL-2-dependent T cell growth; consequently the investigation of antiproliferative effects of TGFβ has focussed on growth factor receptor expression and signal transduction.

IL-2Rα is slowly downregulated after TGFβ treatment.

Competence for IL-2-stimulated proliferation requires adequate surface densities of functional high affinity (HA) IL-2 receptor sites. In T cells, the limiting chain for formation of such HA sites is IL-2Rα and triggering of the TCR complex by antigen/MHC is sufficient for limited expression of IL-2Rα chains. Subsequently, IL-2/IL-2R interactions serve to amplify the expression of IL-2Rα levels further (Malek and Ashwell, 1985; Smith and Cantrell, 1985; Plaetinck et al., 1990). Although CTLL-2 cells are not dependent upon antigenic stimulation for competence, increases in IL-2Rα density are seen in response to IL-2 treatment. Similar to a previous report (Stoeck et al., 1989a), we found that TGFβ inhibited the IL-2-induced rise in IL-2Rα levels and caused a more rapid fall in IL-2Rα expression at later time-points. Because the inhibition of IL-2Rα upregulation on day 1 was of small magnitude and because IL-2Rα density was high regardless of TGFβ treatment, it seemed unlikely that the early inhibition of IL-2Rα upregulation was responsible for growth inhibition seen at 24h. Results of IL-2 binding analysis were similar; minimal inhibition of the IL-2-dependent upregulation of IL-2 binding sites was seen after TGFβ addition. Again,

>90% of cells stained positive with the biotinylated IL-2 reagent in the presence or absence of TGFβ. In summary then, TGFβ partially inhibits the transient rise in IL-2R density seen 24h after IL-2 treatment but TGFβ does not markedly change the total quantitiy of surface IL-2 receptors or IL-2 binding at the time (24h) when growth inhibition can be first documented. These data are consistent with a site of action for TGFβ at a point downstream of the IL-2/IL-2R interaction. We cannot, however, exclude the possibility that TGFβ affects the affinity of IL-2R sites, as affinity cannot be measured using the methodology employed in these studies. Of note, in other IL-2 responsive murine T cell lines (HT2 and CT6) TGFβ interferes with proliferation without an effect on the affinity of IL-2 binding (Ruegemer et al., 1990).

TGFβ inhibits early genetic events induced by IL-2

At the time when inhibition of proliferation was documented (ie, 24h) IL-2R surface levels did not appear to be limiting. Therefore, the effect of TGFβ on earlier IL-2-induced processes was investigated in greater detail by using early genetic responses as a convenient parameter to monitor rapid cellular responses to IL-2 and TGFβ. Prior to addition of IL-2, mRNA for components of the IL-2R and for Granzyme B was barely detectable. By 3h after IL-2 addition, 4- to 10-fold increases in mRNA level were seen, suggesting that transcription was affected. These experiments demonstrated very early (by 1 and 3h) inhibition of the IL-2-induced upregulation of *c-myc*, IL-2Rα, IL-2Rβ, Granzyme B, and IL-4R mRNA by TGFβ. Thus, TGFβ similarly inhibited accumulation of mRNA from several non-linked loci rapidly after IL-2 treatment. One explanation for this observation could involve an effect of TGFβ on IL-2R signalling. The biochemical sequelae of engagement of HA

IL-2R by IL-2 are poorly understood, but recently more information has become available regarding early cytoplasmic and nuclear responses mediated through the cytoplasmic domain of IL-2Rβ. Within 5-15min, multiple protein substrates (ie. the tyr kinase $p56^{lck}$ and the ser/thr kinase p72-74 Raf-1) become enzymatically phosphorylated on tyr and ser/thr residues (Horak et al., 1991; Turner et al., 1991). By 10-30min, marked stimulation of *c-jun*, *jun*-B, *c-fos*, and *c-myc* protooncogene mRNA occurs in response to signalling through IL-2Rβ, and at least some of these are required for IL-2-induced S phase entry (Shibuya et al., 1992; Hatakeyama et al., 1992). Therefore multiple points have been identified in IL-2R signal transduction that may be targets for TGFβ. Ortaldo recently demonstrated that 16h preincubation with TGFβ inhibited the tyr phosphorylation of multiple proteins in $CD3^-$ NK cells 15min after treatment with high-dose (1,000 U/ml) IL-2 (Ortaldo et al., 1991), which supports the hypothesis that TGFβ may inhibit T cell growth by an effect on events immediately triggered by IL-2 binding. Whether such findings are also true for T cells awaits further study.

TGFβ also affects the expression and/or activity of protooncogene products that function as DNA binding proteins. In this way, it is possible that TGFβ may regulate RNA transcription from many distinct loci. It was shown that TGFβ inhibited the expression of mRNAs encoding products such as albumin and transin/stromelysin, and some have found that these effects correlated with increased *jun*-B and *fos*-B mRNA expression (Ohtsuki and Massagué, 1992; Beauchamp et al., 1992). Kerr *et al.* demonstrated a 10 basepair Fos binding element in the transin promotor which was required for TGFβ's inhibitory effect. This element was also found in the upstream regions of other genes inhibited by TGFβ including *c-myc* (Kerr et al., 1990). Further evidence

of regulation of transcription factor expression by TGFβ stems from the finding that autoinduction of the TGFβ gene in TGFβ-treated lung carcinoma cells is mediated by the (Fos-Jun) AP-1 complex (Kim et al., 1990). As more becomes known about the normal biochemistry of IL-2 receptor signalling of nuclear responses, the role of TGFβ in modulating these processes can be further scrutinized.

In summary, inferences that may be made based on these data are: 1) Reduced expression of IL-2R is probably secondary to inhibition of IL-2-dependent maintenance of IL-2Rα expression, rather than a direct cause of growth inhibition. 2) Decreased proliferation is likely a result of TGFβ's effect on IL-2-induced gene expression in general and not simply an isolated effect on IL-2-induced proliferation related events. 3) Sites that could formally be implicated as targets of the antiproliferative effect of TGFβ include IL-2R transmembrane signalling, biochemical events in the cytoplasmic components of IL-2R signal transduction, and/or modulation of broadly acting factors (such as *c-myc*) which regulate the genetic response to IL-2.

Table 2.01: IL-2Rα expression in CTLL-2 cells treated with TGFβ. CTLL-2 were treated with 20U/ml IL-2 ± 1ng/ml TGFβ for various periods prior to FACS analysis of IL-2Rα.

		IL-2 alone		**IL-2 + TGFβ**		**%C[a]**
Exp #	Day	%[+]	MFI[b]	%[+]	MFI	
1	0	100	198			
	1	100	444		100	32473
2	0	100	106			
	1	100	91		100	7684
3	0	98	47			
	1	100	176		100	13376
	2	99	45		99	3680
4	0	97	176			
	1	98	181		93	8547
	2	97	158		84	11774
5	1	97	250		93	13253
	2	95	100		46	2727
6	0.5	100	461		100	36880
	1	98	300		88	18461
	2	98	246		67	8233
7	0	99	65			
	2	100	419		99	12530
	3	99	170		91	6337
	4	100	112		83	1715
8	2	100	128		77	2520

[a] %C, Percent control=MFI of IL-2+TGFβ divided by MFI of IL-2 alone
[b] MFI, mean fluorescence intensity

Figure 2.01: TGFβ dose-dependent inhibition of proliferation. CTLL-2 cells were treated for 24h with 20U/ml IL-2 ± various concentrations of TGFβ. Concurrently, cultures were treated with 37kBq of [^{3}H] TdR. TdR incorporation was measured and expressed as the arithmetic mean (± S.D.) of quadruplicate determinations.

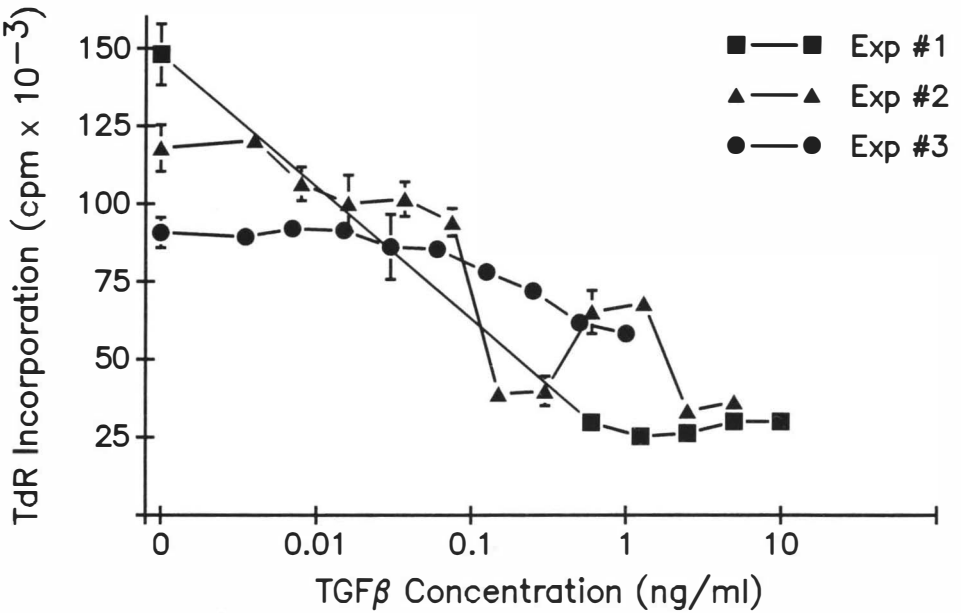

TdR Incorporation (cpm x 10^{-3})
150
125
100
75
50
25
0
0.01
0.1
1
10
TGFβ Concentration (ng/ml)
Exp #1
Exp #2
Exp #3

Figure 2.02: TGFβ inhibits proliferation of CTLL-2. CTLL-2 were treated with various concentrations of IL-2 ± 5ng/ml TGFβ for 24 or 48h. For the final 24h of culture [^{3}H]-TdR was added and incorporation of this isotope was quantitated and expressed as the arithmetic mean (± S.D.) of quadruplicate determinations. rh IL-2, recominant human IL-2

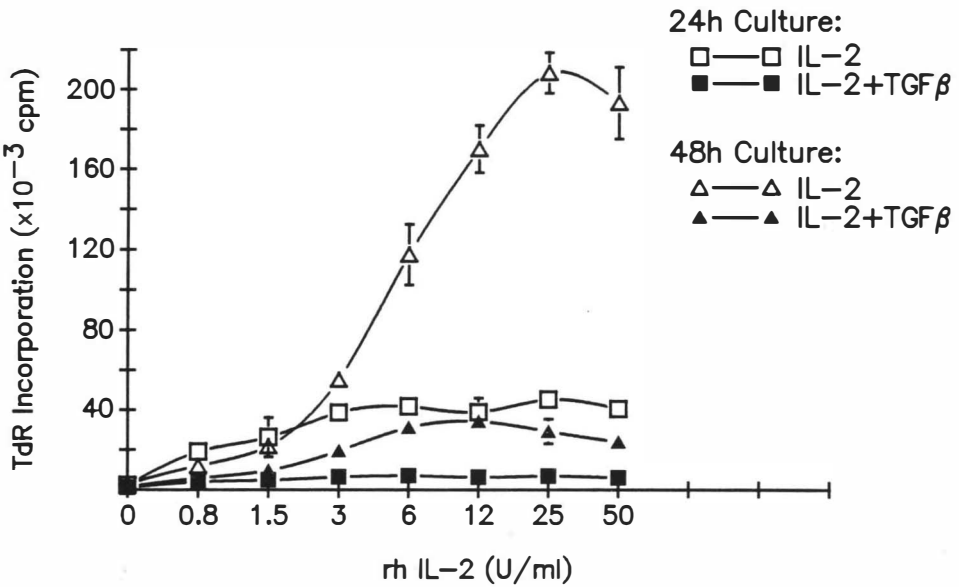

24h Culture:
IL-2
IL-2+TGFβ
48h Culture:
IL-2
IL-2+TGFβ
TdR Incorporation (x10⁻³ cpm)
200
160
120
80
40
0
0.8
1.5
3
6
12
25
50
rh IL-2 (U/ml)

Figure 2.03: TGFβ inhibits CTLL-2 growth. 1) Line graph: CTLL-2 cells were incubated with 20U/ml IL-2 ± 1ng/ml TGFβ and harvested at various times for enumeration of viable cells by hemocytometer after trypan blue treatment. Open squares, 20U/ml IL-2 alone; filled squares, IL-2 + 1ng/ml TGFβ. 2) Bar graph: In a different experiment, CTLL-2 were treated for 24h with 20U/ml IL-2 and various concentrations of TGFβ (ng/ml) prior to counting viable cells.

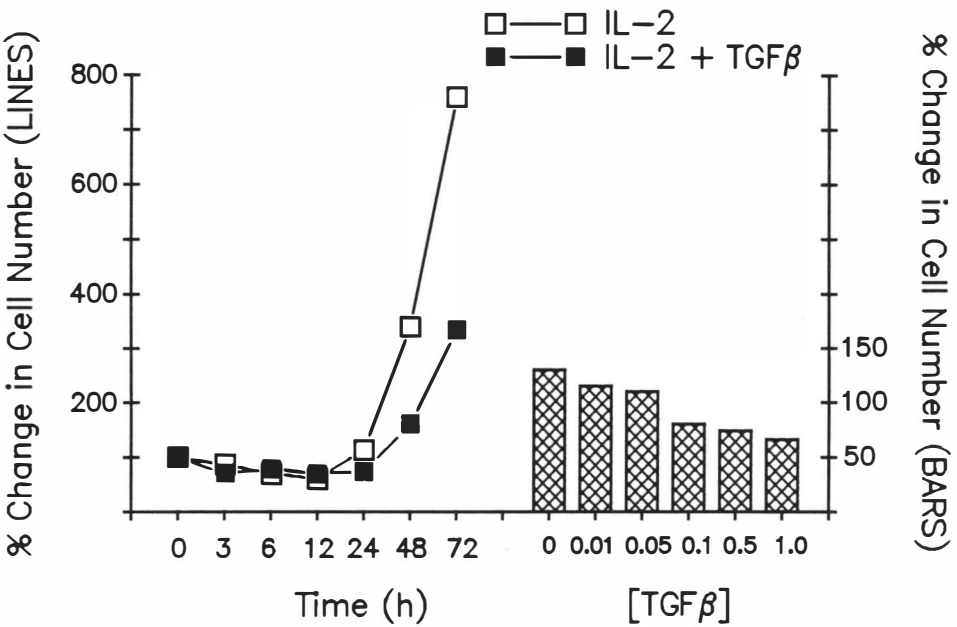

IL-2
IL-2 + TGFβ
% Change in Cell Number (LINES)
% Change in Cell Number (BARS)
800
600
400
200
150
100
50
0 3 6 12 24 48 72
Time (h)
0 0.01 0.05 0.1 0.5 1.0
[TGFβ]

Figure 2.04: Cell cycle analysis of TGFβ-treated CTLL-2. CTLL-2 were treated with 20U/ml IL-2 ± 1ng/ml TGFβ ± 1mM hydroxyurea (HU) for 24h prior to cell lysis and staining of nuclei with propidium iodide. DNA fluorescence was then analyzed by FACS and DNA histograms were plotted.

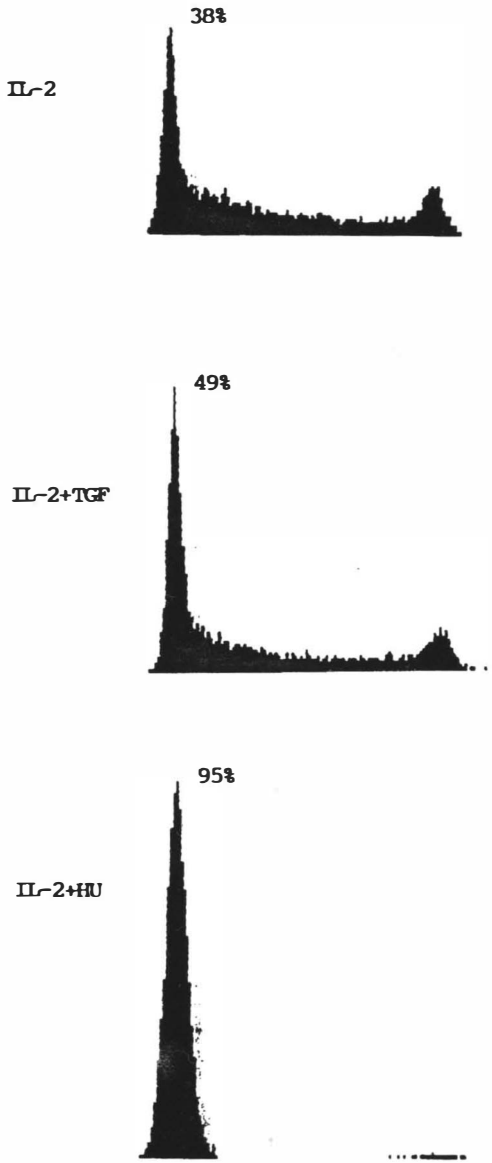

38%
IL-2
49%
IL-2+TGF
95%
IL-2+HU

Figure 2.05: Effect of TGFβ on IL-2Rα. Graphic representation of data in Table 2.02.

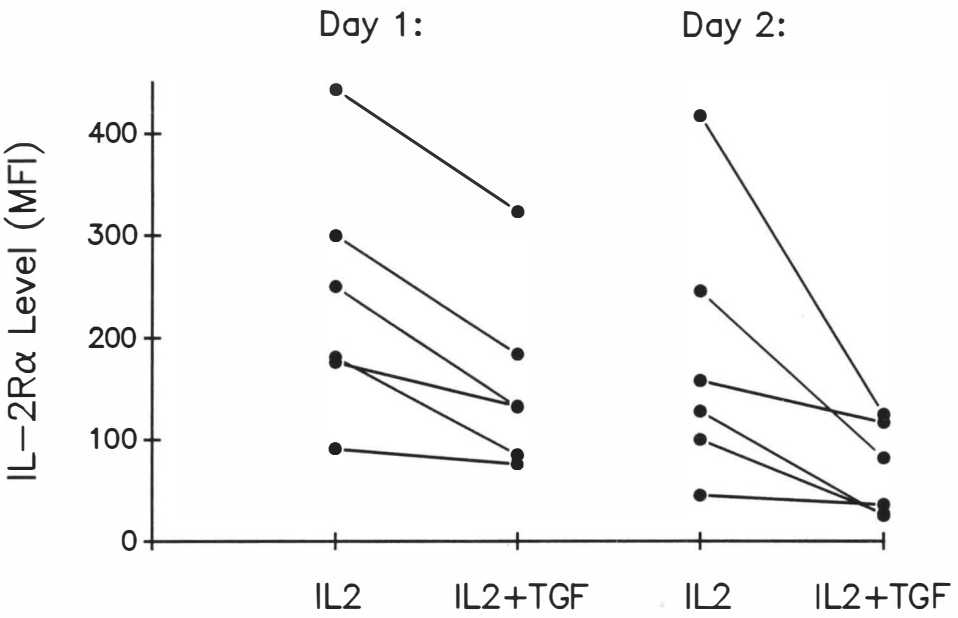

Day 1:
Day 2:
IL−2Rα Level (MFI)
400
300
200
100
0
IL2
IL2+TGF
IL2
IL2+TGF

Figure 2.06: IL-2Rα expression falls late after TGFβ treatment. CTLL-2 were treated with 20U/ml IL-2 ± 5ng/ml TGFβ for various days prior to FACS analysis of IL-2Rα surface expression. IL-2Rα immunofluorescence data obtained from 10,000 cells are expressed on a four decade abscissa as a function of cell number (ordinate). Fresh cytokines were added on day 2.

IL-2 **IL-2 + TGFβ**

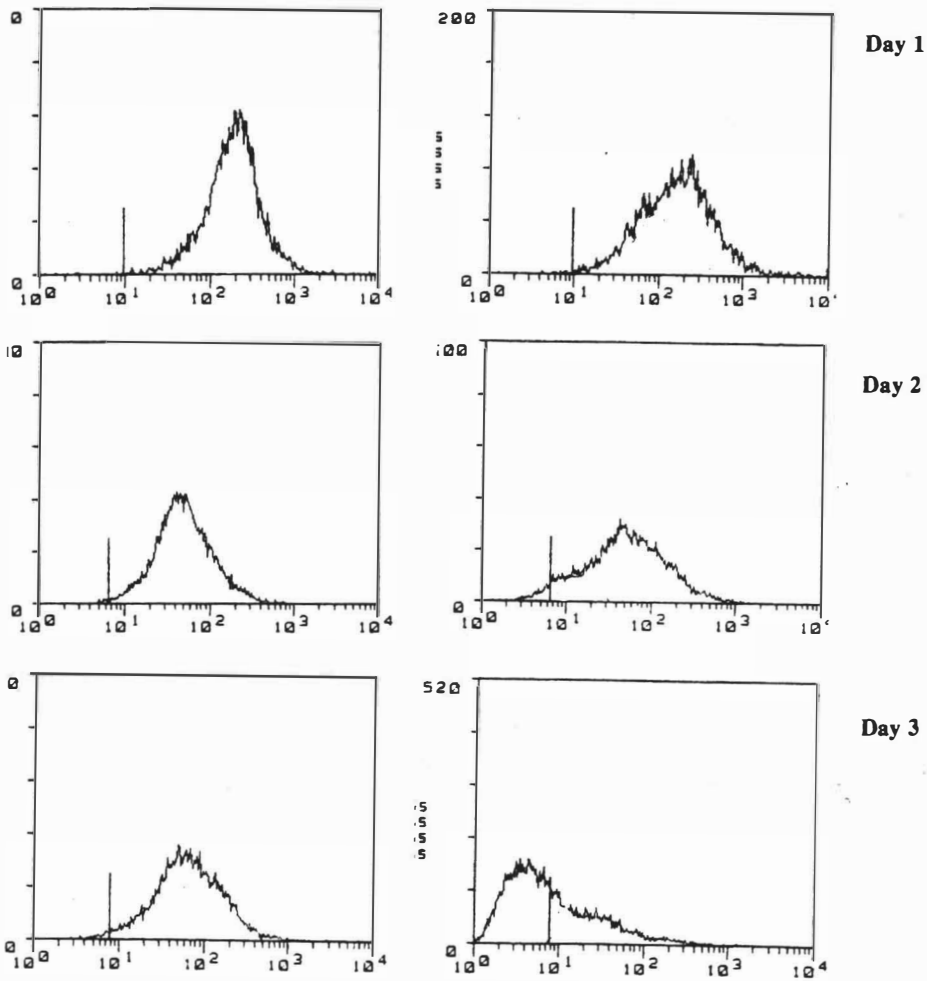


Figure 2.07: TGFβ does not affect early expression of surface IL-2Rα. CTLL-2 cells were harvested from culture 2d after previous IL-2 feeding and stimulated with 20U/ml IL-2 ± 5ng/ml TGFβ prior to FACS analysis of IL-2Rα expression. Mean fluorescence intensity (LINES, left axis) was plotted as a function of time. Percent IL-2Rα positive cells (BARS, right axis) was also plotted for these samples.

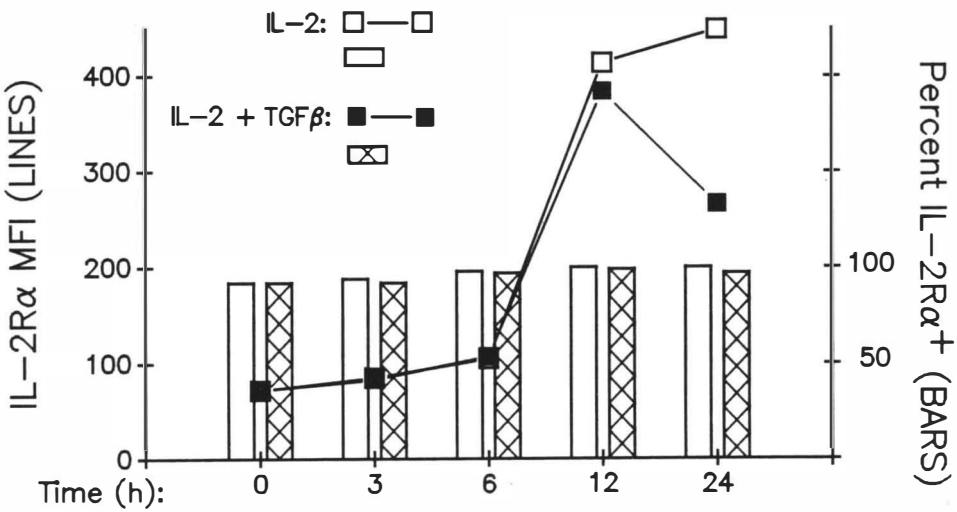
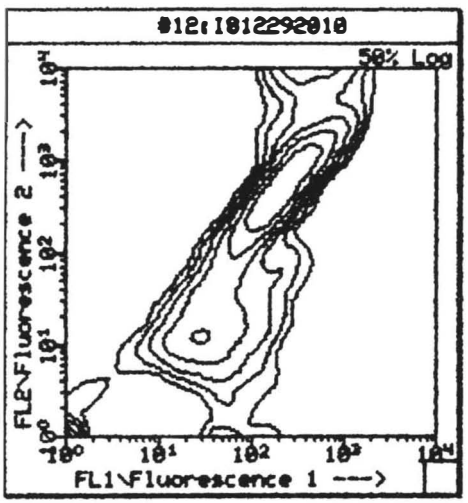

IL−2:
IL−2 + TGFβ:
IL−2Rα MFI (LINES)
Percent IL−2Rα+ (BARS)
400
300
200
100
0
100
50
Time (h):
0
3
6
12
24

Figure 2.08: Coordinate expression of IL-2Rα and IL-2 binding (24h). CTLL-2 were treated for 24h with 20U/ml IL-2 (left panel) or IL-2 + 1ng/ml TGFβ (right panel) prior to analysis. FACS analysis after staining cells with FITC-conjugated Ab recognizing IL-2Rα (abscissa) and PE-labelled IL-2 (ordinate) is shown. These data were subsequently analyzed and plots generated using Lysys computer software.

24h:

IL-2



IL-2 + TGFβ

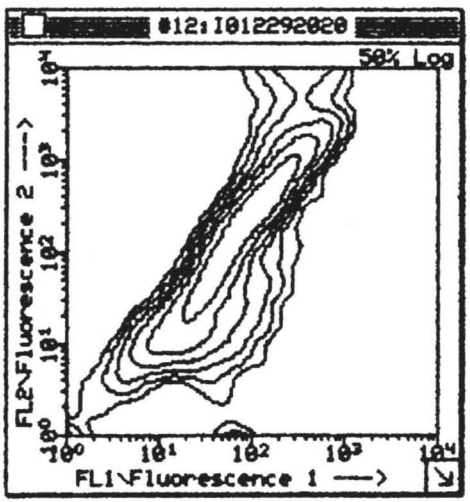


Figure 2.09: Coordinate expression of IL-2Rα and IL-2 binding (48h). Cells from the experiment described in Figure 2.09 above were analyzed at 48h.

48h:

IL-2 **IL-2 + TGFβ**

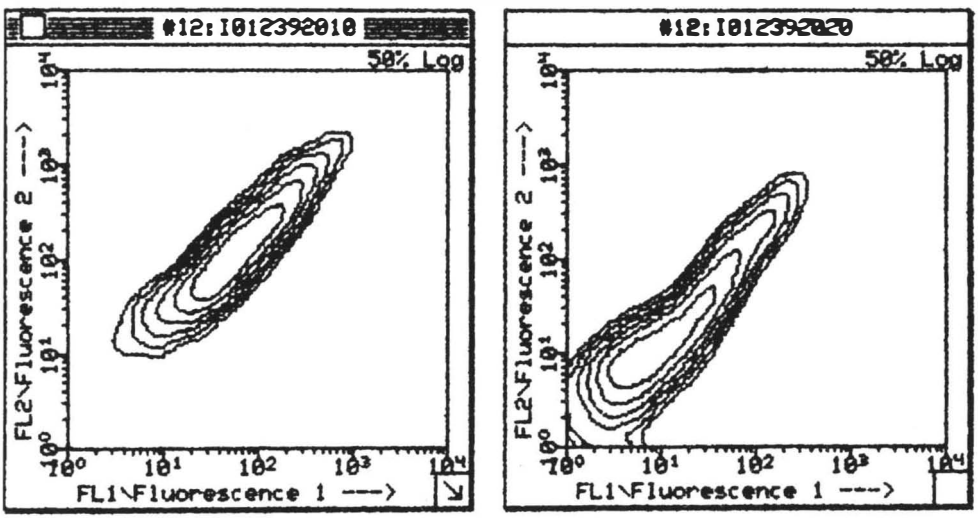
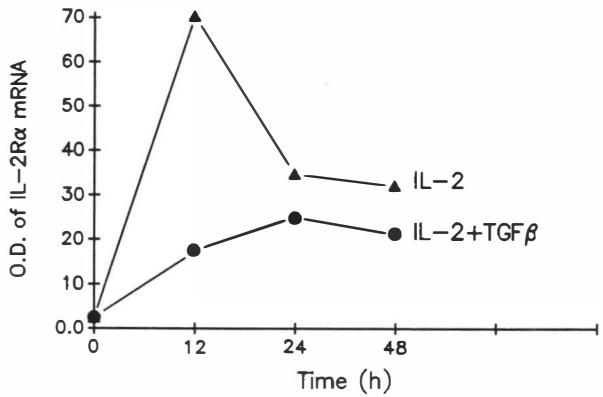
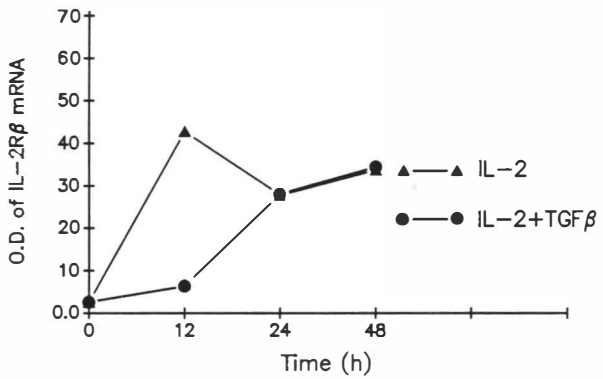


Figure 2.10: Northern analysis of CTLL-2, Experiment #1. CTLL-2 were cultured for 0, 12, or 48h with 20U/ml IL-2 in the absence (triangles) or presence (circles) of 1ng/ml TGFβ. The mRNA signal intensity (ordinate) from appropriately exposed autoradiograms was quantitated as described in Materials and Methods and plotted as a function of time (abscissa). Legend: A) IL-Rα, B) IL-2Rβ, C) β-actin.

A.


O.D. of IL-2Rα mRNA
70
60
50
40
30
20
10
0.0
0
12
24
48
Time (h)
IL-2
IL-2+TGFβ

B.


O.D. of IL-2Rβ mRNA
70
60
50
40
30
20
10
0.0
0
12
24
48
Time (h)
IL-2
IL-2+TGFβ

C.

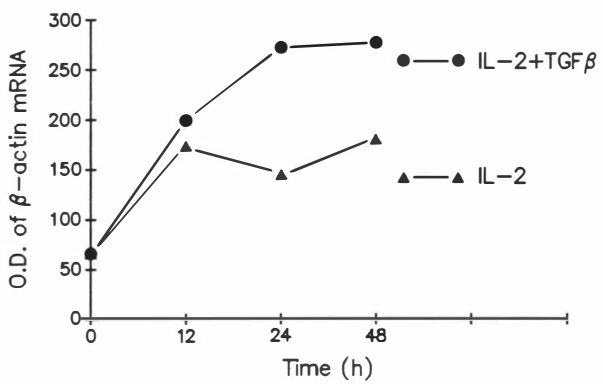
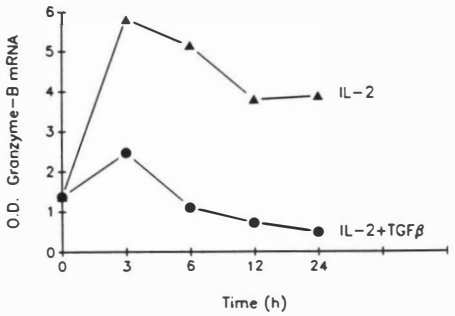
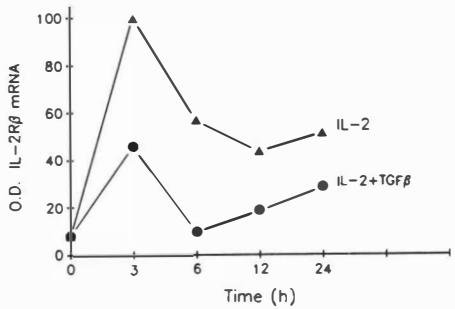

O.D. of β-actin mRNA
300
250
200
150
100
50
0
0
12
24
48
Time (h)
IL-2+TGFβ
IL-2

Figure 2.11: Northern analysis of CTLL-2, Experiment #2. In this experiment, CTLL-2 were cultured for 0, 3, 6, 12, or 24, with 20U/ml IL-2 in the absence (triangles) or presence (circles) of 1ng/ml TGFβ. Northern analysis was performed and blots were sequentially probed with cDNA corresponding to A) Granzyme-B, B) IL-2Rβ, C) IL-4R, and D) β-actin.

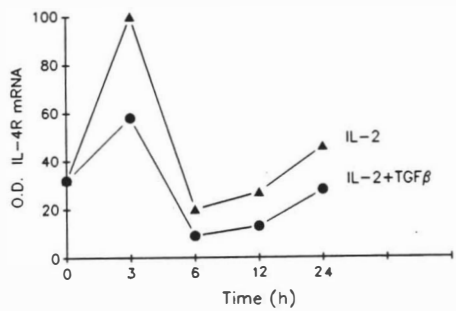
A.


O.D. Granzyme-B mRNA
6
5
4
3
2
1
0
0
3
6
12
24
Time (h)
IL-2
IL-2+TGFβ

B.


O.D. IL-2Rβ mRNA
100
80
60
40
20
0
0
3
6
12
24
Time (h)
IL-2
IL-2+TGFβ

C.


O.D. IL-4R mRNA
100
80
60
40
20
0
0
3
6
12
24
Time (h)
IL-2
IL-2+TGFβ

D.

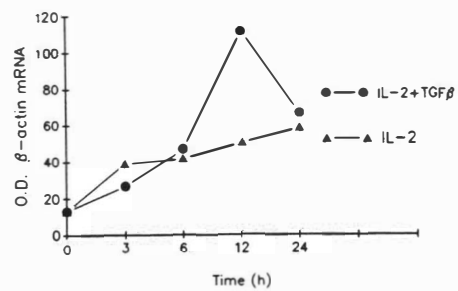

O.D. β-actin mRNA
120
100
80
60
40
20
0
0
3
6
12
24
Time (h)
IL-2+TGFβ
IL-2

Figure 2.12: Autoradiogram data from Northern analysis of CTLL-2. This figure is a composite of data shown in Figures 2.10 (IL-2Rα from Experiment #1) and 2.11 (IL-2Rβ, Granzyme-B, β-actin, from Experiment #2). CTLL-2 were cultured for 0, 3, 6, 12, or 24h with 20U/ml IL-2 in the absence ("-") or presence ("+") of 1ng/ml TGFβ.

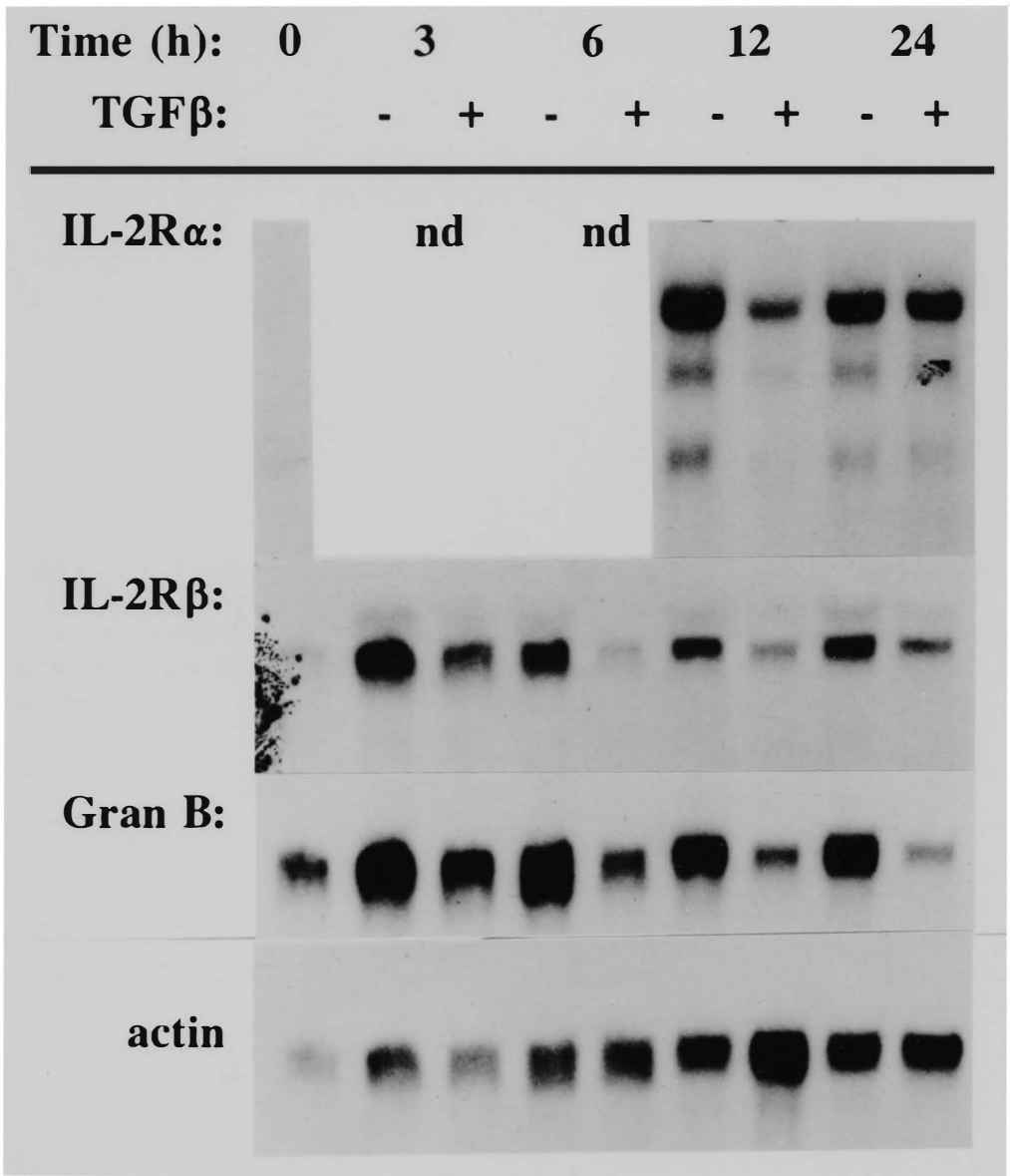
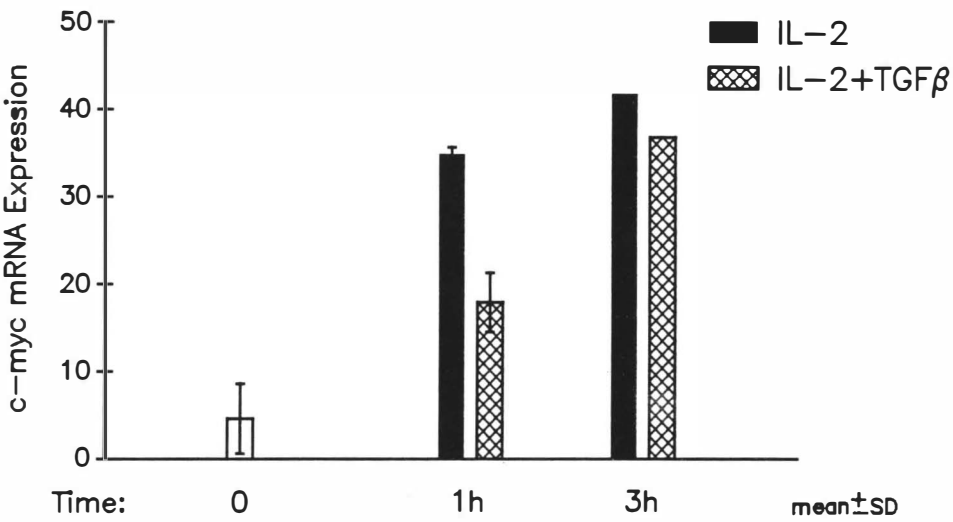

Time (h): 0 3 6 12 24
TGFβ: - + - + - + - +
IL-2Rα: nd nd
IL-2Rβ:
Gran B:
actin

Figure 2.13: Northern analysis of c-myc expression. CTLL-2 cells were cultured for 1 or 3h in IL-2 with (crosshatched bars) or without (solid bars) 1ng/ml TGF. Northern analysis was performed as described in Materials and Methods and the arithmetic means of autoradiogram optical densities from two experiments were plotted.


50
40
30
20
10
0
c-myc mRNA Expression
IL-2
IL-2+TGFβ
Time:
0
1h
3h
mean±SD

Chapter 3

Effect of TGFβ on Morphologic and Phenotypic Characteristics of T Lymphocytes

Introduction

There is a great deal of interest in the effects of TGFβ on lymphocyte growth and differentiation, and numerous reports of TGFβ having stimulatory and inhibitory effects on differentiation in lymphoid compartments have recently emerged. TGFβ inhibited LPS-stimulated B cell growth and secretion of most Ig isotypes. In contrast, under the same condition, TGFβ caused preferential isotype switching to IgA resulting in a 5-10 fold enhancement in the amount of IgA secreted. In contrast, secretion of all other Ig isotypes was decreased (Coffman et al., 1989; Lebman et al., 1990a; Kim and Kagnoff, 1990). Also, surface expression of MHC class II antigens on LPS-stimulated B cells was shown to be modestly up-regulated by TGFβ (Cross and Cambier, 1990). Finally, TGFβ inhibited growth of $CD4^+$ T cells stimulated with Con-A while favoring the maturation of cells with a Th1 cytokine secretion profile (Swain et al., 1991). Taken together, these data provide evidence that TGFβ has multiple regulatory effects on lymphocytes, and suggest that in addition to inhibition of cell growth, differentiation of lymphoid cells may be influenced by low concentrations of this cytokine.

Indeed, this probably reflects a more generalized phenomenon--that cell growth must be halted for functional differentiation to occur.

While studying the anti-proliferative effect of TGFβ on the CTLL-2 murine T cell line, we noted striking morphologic and phenotypic changes in these cells after treatment with TGFβ. In this chapter, we provide evidence that TGFβ up-regulates CD8 expression in CTLL-2 cells, and we also show that TGFβ has a similar effect on normal, immature thymocytes. These data suggest a role for TGFβ in thymocyte differentiation, and these models offer a way to study the molecular regulation of CD8 gene expression.

Results

TGFβ treatment results in morphologic and phenotypic changes in T cells and increased substrate adherence.

In addition to the growth inhibition described in Chapter 2, TGFβ markedly altered the morphology of CTLL-2 cells. As shown in Figure 3.01, compared to the uniformly round appearance of CTLL-2 grown in IL-2 alone, many of the cells exposed to IL-2 + TGFβ took on a stellate and spread morphology. Differences in morphology were also documented by FACS analysis, in that TGFβ-treated cells demonstrated reduced forward angle light scatter (FSC), indicative of a smaller cell size. Side angle light scatter (SSC), which reflects intracellular complexity, was essentially unaffected by TGFβ treatment (Figures 3.02).

In addition to the altered morphology described above, a large percentage of CTLL-2 exposed to TGFβ became firmly adherent to the plates, requiring vigorous pipetting with ice-cold media for efficient harvest; cells which received IL-2 alone were non-adherent. To quantitate this phenomenon, nonadherent cells were removed by centrifugation and MTT (tetrazolium) reagent was used stain viable cells remaining in the adherent population. Reduced MTT was then solubilized and the optical density of cell lysates was measured. As with ^{3}H-thymidine incorporation (Figure 2.01), a

TGFβ inhibited cell growth over 24h (Figure 3.03). Simultaneous analysis of adherent cells demonstrated a TGFβ-dose dependent increase, with plateau levels of adherence (50-60%) essentially being reached at 5ng/ml of TGFβ. When the kinetics of adherence were investigated, maximal adherence was seen at 24h (Figure 3.04).

The requirements for T cell adhesion in response to TGFβ were investigated by first determining whether fetal calf serum (FCS) was required for the effect. In the absence of FCS, no increased adherence (above background levels seen in cells cultured in IL-2 alone) was detected in response to TGFβ (Figure 3.05). However, a direct relationship was seen between the concentration of FCS and adherence of CTLL-2 measured after 24h of culture in TGFβ. Of the enumerable biochemical components contained in FCS, several peptides have been shown to be important for T lymphocyte-substrate adhesion. Among these, fibronectin and vitronectin are extracellular matrix components for which T cells express specific receptors of the integrin family. We next sought to determine whether CTLL-2 cells, incubated in the absence of FCS, would adhere to plates when fibronectin (FN) was added in purified, soluble form to the medium. Adhesion in response to TGFβ was not seen when FN or control proteins were added (Figure 3.05). Since the repeating RGDS motif on substrate peptides is recognized by β-subunits of numerous heterodimeric integrin receptors, we next questioned whether the TGFβ-dependent adherence of CTLL-2 could be antagonized by addition of soluble RGDS sequences. As seen in Figure 3.06, adherence was reduced to baseline when 10 or 50μg/ml RGDS was added to groups that received a suboptimal concentration of FCS (1%). The control tetrapeptide RGES was without effect on adherence.

To determine whether TGFβ was triggering other surface phenotypic changes in

CTLL-2, we examined the expression of T lymphocyte surface markers. Figure 3.07 represents a profile of expression of various surface determinants 24 or 48h after treatment with IL-2 alone or IL-2 + TGFβ. The expression of Thy1, CD3, CD4, αβTCR, γδTCR, LFA-1, NK1.1, Pgp-1 (CD44), and HSA were essentially unaffected by TGFβ treatment. TGFβ-treated cells demonstrated reduced IL-2Rα levels. In some experiments, there were modest elevations in ICAM-1 levels. Most striking, however, was the induction of high levels of CD8α (Lyt2 antigen) expression in a distinct subpopulation of TGFβ-treated CTLL-2. During routine passage, less than 10% of CTLL-2 cells were $CD8\alpha^+$, whereas 20 to 40% of cells expressed this antigen when treated with IL-2 + TGFβ. Notably, the proportion of $CD8\alpha^+$ cells was not different for adherent versus non-adherent TGFβ-treated cells (Figure 3.08). To determine whether the $CD8\alpha^+$ cells differed morphologically from $CD8\alpha^-$ cells, FSC and SSC as a function of CD8 expression were examined. As shown in Figure 3.09, the $CD8^+$ cells were homogeneously distributed throughout the population, with respect to cell size (FSC) and cytoplasmic complexity (SSC). To determine whether the $CD8^+$ and $CD8^-$ populations of TGFβ treated CTLL-2 differed with respect to any other surface markers, cells were stained for CD8α and for IL-2Rα, LFA-1, Thy-1, ICAM-1, and CD8β (Figure 3.10). For most markers, $CD8\alpha^+$ cells were not different from $CD8\alpha^-$ cells, with both subpopulations expressing identical levels of these other surface antigens. The only difference between $CD8\alpha^+$ and $CD8\alpha^-$ subsets was regarding CD8β expression, where it was found that only the $CD8\alpha^+$ subpopulation was capable of CD8β expression (Figures 3.10D). On average, 20% of $CD8\alpha^+$ cells also expressed the CD8β antigen.

Dose response and kinetics of CD8 expression induced by TGFβ.

Various concentrations from 0.01 to 5 ng/ml of TGFβ were titrated into CTLL-2 cultures. After 24h, dose-dependent increases in expression of CD8α and CD8β were seen, with plateau levels of both chains (40% and 8%, respectively) essentially being reached by the addition of 0.5 ng/ml TGFβ (Figure 3.11). Thus, 1 ng/ml TGFβ was selected for subsequent experiments. Similar effects on CD8 were seen when 1ng/ml recombinant human TGFβ (Biomedical Technologies, Inc., Stoughton, MA) was used (data not shown), thus excluding the possibility that the porcine platelet derived TGFβ contained contaminating factors that were responsible for the effect on CD8 expression. The relationship between CD8α and CD8β expression in response to TGFβ was similar in all experiments, with only 20-40% of $CD8\alpha^+$ cells also expressing CD8β. Cells expressing CD8β without CD8α were not detected under any culture conditions.

The kinetics of TGFβ-induced CD8 expression were analyzed over a four day period. At culture initiation, which was 2 days after the last addition of IL-2, ≤5% of CTLL-2 cells were $CD8\alpha^+$ (Figure 3.12). When treated with IL-2 alone, no sustained increase in CD8 expression was noted. However, in the presence of TGFβ, 30-40% of the cells rapidly became $CD8\alpha^+$ by 24h and maintained their expression during the 4-day culture. When expression of this antigen was analyzed in cells cultured long-term in IL-2 + TGFβ (10-14d), again 30-40% of cells were $CD8\alpha^+$. Similar to the data presented in Figure 3.11, 10-20% of cells treated with TGFβ during the time-course became $CD8\beta^+$, CD8β was only detected on a subset of $CD8\alpha^+$ cells, and maximal expression of the β chain routinely lagged 24-48h behind CD8α (Figure 3.13).

Appearance of CD8$^+$ cells is neither secondary to selective outgrowth of a minor CD8$^+$ subset nor a result of growth inhibition.

A rise in the percentage of CD8$^+$ cells might result from *de novo* expression of the determinant in cells previously negative for this marker, and/or from selective survival or outgrowth of cells expressing this antigen. After 30% of cells became CD8$^+$ by 24h, the percentage did not increase further over time beyond this plateau value. Thus, it seemed unlikely that the increase in CD8$^+$ cells could occur by a selective growth advantage of CD8$^+$ cells in TGFβ. To test the alternate possibilities more directly, experiments were performed to determine whether cell growth was required for CD8$^+$ cells to appear in TGFβ-treated cultures. CTLL-2 were treated for 24h with IL-2 ± TGFβ ± hydroxyurea (HU, 1mM; at this concentration, HU inhibited proliferation and cell cycle progression by >95%, shown in Figures 3.14 and 2.04, respectively). The results of two separate experiments showed identical proportions of CD8$^+$ cells in cultures treated with TGFβ in the presence (41%) or absence of HU (41%). Thus, these data demonstrate that CD8 can be induced by TGFβ in the virtual absence of cell division.

To determine whether CD8-bearing cells were selectively resistant to the growth inhibiting effect of TGFβ, CD8 was induced in CTLL-2 by three days' culture in IL-2 + TGFβ, after which time CD8$^+$ and CD8$^-$ cells were separated by "panning." Both subsets were then re-cultured in IL-2 ± TGFβ for an additional 2 days prior to performing cell counts. The growth of CD8$^+$ and CD8$^-$ cells re-cultured in IL-2 alone was similar, with cell recoveries of 168% and 186%, respectively. The growth of both groups was inhibited by 1ng/ml TGFβ added during re-culture, with recoveries of 48% and 76% for CD8$^+$ and CD8$^-$ respectively. Thus, CD8$^+$ cells were, if anything, more

sensitive to the anti-proliferative effect of TGFβ, suggesting again that selective outgrowth or survival of this subpopulation would not occur.

CD8 expression on cells in TGFβ-treated cultures may be a specific response to this cytokine, or could merely be secondary to growth inhibition. To distinguish between these two alternatives, CTLL-2 cells were treated for 24h with 1) IL-2 alone, 2) IL-2 + HU, or 3) IL-2 + TGFβ. The percentage of $CD8^+$ cells for these three groups was 9, 11, and 30%, respectively; similar results were obtained in three such experiments. Thus, despite complete inhibition of growth, the addition of HU alone caused no change in expression of CD8 as compared to cells cultured in IL-2 alone.

TGFβ is required for maintenance of $CD8^+$ phenotype.

To determine whether CD8 expression was a stable phenotypic change, CTLL-2 were first treated with IL-2 and TGFβ for three days, then washed free of TGFβ and re-cultured in IL-2 alone or IL-2 + TGFβ. As shown in Figure 3.15, when re-cultured in IL-2 alone, the percentage of $CD8^+$ cells declined over the next four days, as compared to cells re-cultured in IL-2 + TGFβ, where the percentage of $CD8^+$ cells doubled. This experiment suggested that continued CD8 expression was dependent on the presence of TGFβ. To examine the stability of TGFβ-induced CD8 expression more directly, CTLL-2 were cultured for three days in IL-2 + TGFβ, washed, and separated into $CD8^+$ and $CD8^-$ subsets by panning. Each fraction was then re-cultured for an additional eight days in IL-2 ± TGFβ. CD8 expression in the CD8-enriched group remained high in IL-2 + TGFβ but dramatically declined in IL-2 alone (Figure 3.16), indicating that in most of the separated $CD8^+$ cells, CD8 expression required the continuous presence of TGFβ. Interestingly, the group depleted of $CD8^+$ cells did not

express this determinant when treated with IL-2 + TGFβ for an additional eight days.

Induction of CD8 expression by TGFβ requires RNA synthesis and correlates with increased steady-state levels of CD8α/β mRNA.

To determine whether TGFβ-induced CD8 expression was dependent on new RNA synthesis, CTLL-2 were incubated for 8h in IL-2 ± TGFβ in the presence or absence of 5μg/ml actinomycin-D. The viability of cells treated for 8 hours with medium alone, EtOH vehicle, and actinomycin-D was 95%, 78%, and 71%, respectively. Beyond 8h, the viability in actinomycin-D declined rapidly, and FACS data could not be interpreted. FACS analysis at the 8h timepoint showed that in IL-2 alone, 7% of cells were $CD8^+$. Treatment with IL-2 + TGFβ in the absence or presence of the EtOH vehicle resulted in 17 and 18% $CD8^+$ cells, respectively, compared to only 4% for cells treated with IL-2 + TGFβ + actinomycin-D. Such inhibition of TGFβ-induced CD8 expression by actinomycin-D was consistently seen in three separate experiments (Table 3.01). These data suggested that new RNA synthesis was required for the increased percentage of $CD8^+$ cells in TGFβ-treated cultures.

The hypothesis that TGFβ treatment induced the *de novo* expression of CD8 was also tested using Northern blot analysis. As shown in Figure 3.17, CD8α and CD8β mRNA accumulated rapidly in response to TGFβ and IL-2. Only transient, low-levels of CD8 mRNA were detected in response to IL-2 alone, in agreement with the FACS data. However, in the presence of TGFβ, heightened levels of both mRNA species detected with the CD8α cDNA (1.7 and 3.5 Kb) were seen as early as 3-6h after treatment with TGFβ. By 12-24h, 4- to 5-fold higher steady-state CD8α mRNA levels

were seen in TGFβ-treated cells, as compared to cells cultured in IL-2 alone (Figure 3.18). Again, consistent with the FACS data, CD8α message peaked more rapidly (by 12-24h) than CD8β, which was still increasing at 24h after addition of TGFβ. In most cases, mRNA for CD8 fell to baseline by 48h. Upon re-addition of fresh IL-2 and TGFβ, a striking rise in mRNA for CD8α was seen. When comparison was made between CD8α mRNA levels 3h after initial treatment with IL-2 + TGFβ and 3h after re-addition of fresh IL-2 + TGFβ to cultures that had been previously treated for 48h with IL-2 + TGFβ, a 55-fold increase in signal intensity was seen (Figure 3.19). The mechanism by which this enhanced "secondary response" occurs is currently being investigated. These data clearly demonstrate that the appearance of CD8 mRNA in this cell line is tightly coupled to treatment with TGFβ. To control for equal RNA loading, ethidium bromide stained gels were inspected and β-actin was also probed. Equivalent 18S and 28S rRNA bands were seen for all timepoints.

To further define the response of CD8 mRNA to TGFβ, cells were treated with 20U/ml IL-2 and various doses of TGFβ. Analysis of CD8α and CD8β mRNA showed increasing accumulation of these molecules up to the highest dose of TGFβ used (5ng/ml). Thus, no plateau was seen over the dose interval used (Figure 3.20). Interestingly, when this blot was re-probed with a β-actin specific cDNA, a TGFβ-dose dependent stimulation of this message was observed, rising approximately 5-fold above control at this 24h timepoint. When ethidium bromide stained rRNA bands were compared between lanes, equal loading of total RNA was confirmed.

CTLL-2 are non-cytolytic.

In general, when cellular differentiation programs are activated, cells cease to

proliferate, while changes in morphology and phenotype are commonly observed. During antigen-dependent sensitization of $CD8^+$ T lymphocytes in the presence of IL-2, maturation of cytolytic function occurs. As TGFβ-treated CTLL-2 demonstrated some characteristics of differentiating cells, it was important to ascertain whether differentiation of functional characteristics also occurred in response to TGFβ. CTLL-2 were treated with TGFβ for 48h and then harvested for cytotoxicity assay. Neither nonspecific nor anti-CD3 redirected killing of the Fc receptor bearing P815 mastocytoma was seen with TGFβ-treated CTLL-2 effectors; $CD3^+$, PHS-5-specific CTL clones that were used as positive controls appropriately lysed targets (Figure 3.21). In light of the inhibition of Granzyme B by TGFβ, and the predominant lack of surface CD3/TCR on CTLL-2, this result was not unexpected.

Intracellular signalling involved in TGFβ-dependent CD8 expression.

Currently, intracellular signalling mechanisms by which mammalian cells respond to TGFβ are incompletely understood. We sought to determine whether PKC or intracellular calcium played a role in the CD8 response to TGFβ in CTLL-2 cells. We hypothesized that if TGFβ triggered CD8 expression via a PKC- or calcium-dependent pathway, incubating cells with PDBu or ionomycin (Io) may also result in CD8 expression. Cells were incubated with IL-2 ± PDBu ± Io for 24h prior to CD8 immunofluorescent analysis. We confirmed that PDBu had some effect on the cells by measuring a nominal increase in cell size of the populations exposed to PDBu. However, as shown in Table 3.02, CD8 expression was not seen when cells were treated with PDBu (100nM or 1,000 nM) or PDBu + Io (10^{-6} M), indicating that neither PKC activation nor intracellular calcium elevation could replace the TGFβ

signal.

Although direct PKC activation was not *sufficient* for CD8 expression, the possibility that PKC was *necessary* for this effect still existed. It was shown that incubation of CTLL-2 with 500ng/ml (990nM) PDBu for 48h resulted in rapid and sustained depletion of PKC (Redondo et al., 1988). Therefore, CTLL-2 were incubated with IL-2 ± 1,000 nM PDBu for 48h then washed and cultured ± PDBu ± TGFβ for an additional 24h prior to CD8 analysis (washing T cells in warm medium reverses effects of PDBu on T lymphocytes (McCrady et al., 1988)). In cells depleted of PKC, CD8 expression only reached 50-60% of control level in response to TGFβ, suggesting that PKC indeed played a role in optimal signalling of CD8 expression.

TGFβ does not increase CD8 expression in mature T cells or other T cell lines.

Next, we sought to determine whether CD8 expression can also be manipulated by TGFβ in mature splenic T lymphocytes. Using a well established model of cytotoxic T lymphocyte generation in response to tumor cells, we showed in Chapter 1 that tumor-sensitized T cells from P815 tumor immune mice generate cytotoxic T cells when incubated *in vitro* with P815 tumor cells and that this response was inhibited by TGFβ. Splenocytes from P815-bearing mice were stimulated with P815 tumor cells and cultured in complete medium ± 2.5 or 5 ng/ml TGFβ for a period of 7 days. As shown in Table 3.04, CTL generation was markedly inhibited in TGFβ-treated groups. However, no change in the proportions of CD4- or CD8-positive cells was seen. Additionally, no change in the CD8 fluorescence intensity was detected in cells treated with TGFβ (Table 3.04 and Figure 3.22). To further assess the effect of TGFβ on CD8 expression in normal, mature T cells, splenocytes from C57BL/6J mice were

harvested and stimulated to grow with soluble anti-CD3 (145-2C11) and cultured in 20U/ml IL-2 or IL-2 + 5ng/ml TGFβ. After 2 days' culture, cell numbers in both groups had tripled and the expression (percent positive @ MFI) of CD8α, CD8β, and IL-2Rα was assayed by FACS. For cells cultured in αCD3 + IL-2, results were: CD8α=43% @ 279, CD8β=45% @ 507, IL-2Rα=55% @ 188. For cells treated with αCD3 + IL-2 + TGFβ results showed: CD8α=41 @ 350, CD8β=42% @ 460, IL-2Rα=46% @ 234. We also determined that the expression of CD8 in $CD4^-CD8^+$ P815-specific CTL clones was not altered by TGFβ. When GD11.10 cells were treated with 20U/ml IL-2 or IL-2 + 5ng/ml TGFβ, cells from both groups stained >90% $CD8\alpha^+$ and mean CD8α immunofluorescence was 12.6 and 13 for these two groups respectively. These data demonstrate that expression of CD8 in populations of mature, $CD4^+$ and/or $CD8^+$ cells was not changed by TGFβ.

To determine whether other $CD8^-$ cell lines were capable of CD8 expression in response to TGFβ, EL-4, BW5147, and an independent CTLL-2 line obtained from the American Type Culture Collection were tested. When treated with IL-2 ± TGFβ, *de novo* CD8 expression was only noted in CTLL-2 derived from ATCC and the CTLL-2.TH line used throughout this manuscript (data not shown), demonstrating that independently maintained CD8-negative CTLL-2 lines respond to TGFβ by CD8 expression, and that these cells may represent a unique cell line model of this phenomenon.

Effect of TGFβ on CD8 expression in unfractionated thymocytes.

We sought to determine whether TGFβ would also induce CD8 expression in normal murine thymic T lymphocytes. Thymocytes from 12-week-old DBA/2 mice

were stimulated by overnight culture in PDBu and Io and then cultured in IL-2 alone or IL-2 + TGFβ. As previously described (Ellingsworth et al., 1988) TGFβ had a marked antiproliferative effect on thymocytes (Figure 3.23). To determine whether CD8 expression was affected by TGFβ in this population, FACS analysis of CD4, CD8α and CD8β was performed. Prior to culture, thymocytes were 8% $CD4^-8^-$, (DN, double negative) 5% $CD4^+8^-$, 2% $CD4^-8^+$, and 86% $CD4^+8^+$ (DP, double positive) (Figure 3.24A). When cell surface phenotypes were compared at day 2 (data not shown) and day 4, it was noted that in both treatment groups the proportion of DP cells had dropped markedly (Figure 3.24B, C). CD4 expression was similar for controls and TGFβ-treated thymocytes. However, the percentage of $CD8^+$ cells decreased when thymocytes were cultured in IL-2 alone, with 24% of IL-2-treated thymocytes being $CD8^+$ on culture day 4 (Figure 3.24B). In contrast, more than twice as many (56%) $CD8^+$ cells were detected in cultures treated with IL-2 + TGFβ (Figure 3.24C), and approximately 80% of these $CD8^+$ cells were $CD4^-CD8^+$. Thymocytes treated with IL-2 + TGFβ also demonstrated brighter CD8α fluorescence, with an MFI of 723 compared to 334 in IL-2 alone. Enhanced CD8β expression was also seen in IL-2 + TGFβ-treated cultures, with 40% (MFI=223) of cells $CD8\beta^+$ compared to 21% (MFI=136) in IL-2 alone. Notably, thymocyte growth and viability in this culture system required exogenous IL-2, and enhanced CD8 surface expression was not seen when thymocytes were cultured in TGFβ alone. The enhancement of surface CD8 expression in thymocytes treated with IL-2 + TGFβ also correlated with 2- and 5-fold increases in CD8α mRNA levels at 18 and 48h, as compared to thymocytes treated with IL-2 alone (Figure 3.25). These data indicate that treatment with IL-2 + TGFβ resulted in enhanced *de novo* CD8 surface and mRNA expression in cultures of

unfractionated, normal thymocytes as compared to cultures treated with IL-2 alone.

Effects of TGFβ on double negative thymocytes.

The DN thymocyte subset contains cells that can directly give rise to $CD3^-CD4^-8^+$ cells, which are thought to give rise in turn to the DP subpopulation (MacDonald et al., 1988; Guidos et al., 1989; Shortman et al., 1988; Nagamine et al., 1991; Tatsumi et al., 1990; Nikolic-Zugic et al., 1989; Nikolic-Zugic and Bevan, 1988). To determine whether TGFβ was influencing CD8 expression in immature thymocytes, DN cells were isolated and then distributed into four treatment groups prior to being pulsed with PDBu + Io: 1) medium alone, 2) IL-2, 3) TGFβ, and 4) IL-2 + TGFβ. Surface markers were analyzed by immunofluorescence over 12 days of culture. At culture initiation, <7% of cells stained positive for either CD4 or CD8α. Substantial cell growth occurred only in cultures containing IL-2. Regardless of treatment, <2% of cells were $CD4^+$ at any timepoint (Figure 3.26). Less than 13% of cells cultured with IL-2 alone were $CD8^+$ on day 2, whereas 30% of cells treated with IL-2 + TGFβ were $CD8^+$ (Figure 3.26 and Table 3.05). As with CTLL-2 cells, a clearly identifiable and strongly $CD8^+$ subpopulation emerged only in cultures treated with IL-2 + TGFβ, and this subpopulation demonstrated a dramatic increase in CD8 fluorescence intensity, with MFI values of 295, 507, and 716 on days 2, 4, and 6, respectively. In contrast, the proportion of cells expressing CD8 was constant in cultures that received IL-2 alone, with a maximal MFI of only 490 on day 6. For comparison, the MFI of CD8 on normal DBA/2 double positive thymocytes was 1300. After initial expression, the proportion of $CD8^+$ cells in cultures with IL-2 + TGFβ remained approximately 30% throughout the 12 day culture period. DN thymocytes cultured in medium alone or

TGFβ without IL-2 were 5 and 10% $CD8^+$, respectively.

TGFβ also inhibited the proliferation of DN thymocytes stimulated with phorbol ester and ionophore. Thus, as with CTLL-2, the observed increase in the proportion of $CD8^+$ cells might have resulted either from *de novo* expression of CD8 in previously negative cells, or from selective growth or survival of residual $CD8^+$ cells in the DN cell preparation. In our experiments, TGFβ inhibited the growth of DN thymocytes by 40% during 4 days in culture. During this period, the percentage of $CD8^+$ cells increased from <2% to 30%, and the total cell number rose by 16-fold. Therefore, the absolute number of $CD8^+$ cells increased by >240-fold during 4 days in culture. Thus, $CD8^+$ cells present on day 1 would have had to divide every 9 hours to achieve these results by growth alone, which is an unlikely possibility. To determine directly whether cells in the CD8 SP thymic subpopulation were capable of such growth, CD8 SP cells were isolated from murine thymi and stimulated with PDBu + Io + IL-2 ± TGFβ. With or without TGFβ, CD8 SP thymocytes had a doubling time of 22h and actually grew more slowly than DN cells (doubling time=18h). Thus, selective outgrowth of $CD8^+$ contaminants present on day 0 was unlikely under these conditions.

An increased proportion of $CD8^+$ cells might also have been seen if the replication rate of $CD8^+$ cells emerging in TGFβ-treated cultures was greater than that of DN cells. However, proliferation of DN cells and CD8 SP cells positively selected after 6 days in culture with IL-2 + TGFβ were not significantly different when re-cultured in IL-2 + TGFβ (Table 3.06). Taken together with the results described above, these data demonstrate that, as with the CTLL-2 line, the emergence of a $CD8^+$ population from DN thymocytes cannot be attributed to the selective outgrowth or survival of $CD8^+$ cells.

To characterize further the subpopulation of DN thymocytes that became $CD8^+$ in response to TGFβ, dual parameter analysis was performed with antibodies specific for Thy1.2 or monomorphic determinants on either αβ- or γδ-TCR. Regardless of TGFβ treatment, ≥95% of all cells remained $Thy1.2^+$ for the duration of the culture. Sixty percent of $CD8^+$ cells were $TCR/CD3^-$, demonstrating that the major effect of TGFβ was to promote the appearance of $TCR/CD3^-CD4^-CD8^+$ T cells (Figure 3.27). Even after 12 days in culture, these $CD8^+$ cells did not express increased levels of CD3 or CD4, indicating that further maturation did not occur.

Discussion

CD8, a novel target for TGFβ action.

After treatment of CTLL-2 cells with TGFβ and IL-2, 30% of cells became $CD8\alpha^+$, and CD8β was detected only on $CD8\alpha^+$ cells. As CD8 expression was not seen when either TGFβ or IL-2 were added alone, it is possible that either a synergistic relationship exists between these two cytokines, or, as IL-2 is necessary for viability of these cells, the absence of CD8 expression in response to TGFβ alone might be explained by the fact that most CTLL-2 are dying after 24h without IL-2. In this model, CD8 surface expression was tightly coupled with TGFβ exposure, with CD8 surface levels falling rapidly after removal of the cytokine. Interestingly, when $CD8^+$ cells were removed from TGFβ-treated CTLL-2 cultures, the remaining $CD8^-$ cells could not express the CD8 antigen even after prolonged culture in TGFβ demonstrating that not all CTLL-2 were sensitive to this effect of TGFβ; this may suggest heterogeneity in this CTLL-2 clone and perhaps a finite number of non-renewable CD8 precursor cells existed in the population before TGFβ treatment. It should be noted that, although none of the $CD8^-$ cells (that were panned from TGFβ-treated cultures) expressed CD8 when subsequently cultured with TGFβ, these cells *were* growth inhibited by TGFβ to a similar degree as $CD8^+$ cells. However, it is also

possible that other mechanisms, such as secretion of soluble CD8 peptide (Norment et al., 1989), or non-uniform distribution of TGFβ receptor(s) may account for the fact that only a portion of cells in this CTLL-2 line expressed surface CD8 antigens in response to TGFβ. In this regard, it is possible that the different types (I, II, and III) of TGFβ receptor have distinct, unshared functions (ie. CD8 expression vs growth inhibition) and the expression of these receptors may be regulated independently.

In our studies, treatment of CTLL-2 cells with TGFβ also resulted in rapid growth inhibition concurrent with an increase in the proportion of $CD8^+$ cells, whereas inhibiting CTLL-2 growth with HU caused no significant change in CD8 expression. Thus, we can conclude that the increased CD8 expression was not merely a side-effect of inhibiting growth. It might have been argued that we did not see increased CD8 expression after HU treatment because some other processes required for this to occur were inhibited or because the cells did not survive long enough in the presence of HU. However, CTLL-2 viability was maintained for two days after addition of HU, and CD8 expression was induced by TGFβ in HU-treated cells within 24h, which also demonstrates that the increased number of $CD8^+$ CTLL-2 cells was not a result of selective growth of a $CD8^+$ subset in the presence of TGFβ.

Although expression of CD8α/β polypeptides must depend in part upon RNA transcription, the relative contributions of transcriptional and post-transcriptional mechanisms to the regulation of CD8 are unclear. It was reported that intracellular CD8β peptide levels are controlled at the mRNA level, although surface expression of CD8β is also regulated by post-translational mechanisms (Parnes, 1989). Wilkinson *et al.*, using a lymphoma model, showed that CD8α mRNA increased when protein synthesis was inhibited (Wilkinson et al., 1991). No change in transcriptional rate was

detected, suggesting that post-transcriptional mechanisms were responsible for the increased CD8 mRNA level. Only a transient shift in the peak CD8 immunofluorescence was detected in those cells. In contrast, our studies using the non-neoplastic CTLL-2 line demonstrated the emergence of a discrete $CD8^+$ subpopulation as long as TGFβ was provided. We are currently in the process of determining the role of labile regulatory proteins in TGFβ-dependent CD8 expression in CTLL-2.

Routinely, less RNA is recovered per cell after treatment of CTLL-2 with IL-2 + TGFβ for >12-24h. Thus, the augmentation of CD8 mRNA level was a specific effect of TGFβ, and steady-state levels of other genes, such as Granzyme B and IL-2R, were decreased in the same populations of TGFβ-treated cells (Figure 2.11). Thy-1 immunofluorescence was unchanged by TGFβ treatment, indicating that indiscriminate down-regulation of other pre-existing surface antigens was not occurring. In CTLL-2, TGFβ-dependent CD8 surface expression was sensitive to Actinomycin-D, which demonstrated that new CD8 expression required RNA synthesis. By Northern analysis, CD8 mRNA was practically undetectable at time zero and increased >4-fold by 6h compared to IL-2 alone, demonstrating that TGFβ was indeed effecting *de novo* CD8 expression in CTLL-2 and not merely favoring outgrowth of $CD8^+$ cells.

When changes in the steady-state level of a specific mRNA are seen during Northern analysis, it is formally possible that transcriptional and/or post-transcriptional mechanisms account for these changes. Preliminary *in vitro* nuclear run-on assays have shown no increase in the rate of CD8α transcription after treatment of CTLL-2 with TGFβ. Further work is necessary to identify the precise mechanism by which IL-2 and TGFβ increase CD8α mRNA levels. Whatever the mechanism, it is clear that the metabolism of CD8 mRNA is changed in TGFβ-treated cells as evidenced by the

dramatic enhancement (>50-fold in 24h) of CD8α mRNA level upon re-addition of fresh IL-2 + TGFβ 48h after the initial treatment with IL-2 + TGFβ. Mechanistically, one could speculate that the primary (time zero) addition of TGFβ resulted in the production of stable transcriptional components that were, at 48h still poised to continue rapid synthesis of CD8α mRNA in response to fresh IL-2 and TGFβ. It is also formally possible that the enhanced response to TGFβ 48h after primary treatment with TGFβ results from an increase in the number or affinity of cell surface receptors for TGFβ, thus resulting in a quantitatively or qualitatively stronger signal upon secondary addition. In any event, it is interesting that no greater number of $CD8^+$ cells emerged after secondary treatment with IL-2 + TGFβ, nor did the level of CD8 antigen increase on a per cell basis when MFI values were compared before and after re-addition, suggesting that in this model, CD8α surface expression may be regulated post-transcriptionally, as has been reported for CD8β (Parnes, 1989).

TGFβ affects cytoskeletal components and adhesion receptors

Treatment of CTLL-2 cells with TGFβ resulted in a striking change in morphology, causing these cells to become spindle-shaped or stellate and causing a large percentage of cells to adhere to the culture plate. The expression of several molecules (CD44, LFA-1, and ICAM-1) known to be involved in the interaction of lymphocytes with the extracellular matrix were examined and shown to be minimally affected in by TGFβ in this CTLL-2 model. Further efforts are underway to ascertain the identity of the molecules involved in adherence in TGFβ-treated cultures. Stoeck, *et al.* also described changes that occurred in the microscopic appearance of their CTLL-2 line after treatment with TGFβ, but noted that the cells became more clustered, ie. adherent

to each other, rather than to the plate (Stoeck et al., 1989a). Nonetheless, it is likely that these two observations are secondary to similar changes that are triggered by TGFβ in CTLL-2 cells. T lymphocytes can attach to protein-coated tissue culture plates via surface integrin molecules (Utsumi et al., 1991), and it is known that TGFβ can cause up-regulation of multiple components of the integrin family of adhesion molecules, can modulate extracellular matrix organization, and enhance the attachment of thymocytes to a fibronectin substratum (Heino et al., 1989; Ignotz et al., 1989; Merwin et al., 1990; Ignotz and Massagué, 1987). Therefore, the expression of an adherent phenotype in CTLL-2 and the antagonism of this adherence with RGDS peptide essentially confirms the premise that TGFβ up-regulates integrin β chains on T cells. Further, the presence of TGFβ in thymus tissue sections (Ellingsworth et al., 1986) allows the speculation that this cytokine may be involved in adherence of differentiating thymocytes to the selecting epithelium.

In several experiments presented in Chapter 2, it appeared that increases in β-actin mRNA level were seen in CTLL-2 treated with TGFβ. These increases were seen at the same timepoints when TGFβ inhibited accumulation of other mRNA species, and ethidium stained rRNA revealed little gross difference in total RNA loaded. In this chapter, TGFβ-dose response Northern analyses were performed which clearly showed that CTLL-2 accumulated β-actin mRNA in a TGFβ-dose dependent manner. It was previously shown that TGFβ induces cytoskeletal changes in epithelial cell lines (Garbi et al., 1990) and some of these changes have involved TGFβ-dependent increases in actin polymerization (Lomri and Marie, 1992). Further, it was demonstrated that TGFβ elevates steady-state β-actin mRNA levels (but without effect on mRNA coding for glyceraldehyde-6-phosphate dehydrogenase) in epithelial cell lines (Suemori et al.,

1991) and in cardiac fibroblasts (Eghbali et al., 1991) and synergises with epidermal growth factor for increasing the rate of transcription of β-actin in AKR fibroblasts (Ranganathan and Getz, 1990). Thus, our data suggest that T lymphocyte β-actin is also a target of TGFβ action which may be causally related to the microscopic changes seen in CTLL-2 morphology.

TGFβ receptor signalling

The serine and threonine specific protein kinase-C (PKC) is involved in TCR signalling (Rao, 1991; Albert et al., 1985; Valge et al., 1988), but is not required for signalling through the IL-2R (Tigges et al., 1989; Mills et al., 1988; Valge et al., 1988). As neither viability or growth of CTLL-2 cells requires TCR signals and since IL-2 signalling is independent of PKC activation, CTLL-2 cells represent a suitable model to study the role of PKC in TGFβ-induced CD8 expression. Our results showed that when CTLL-2 were depleted of PKC by continuous exposure to high-dose PDBu, only 50-60% of cells became $CD8^+$, even when challenged with 5ng/ml of TGFβ. Since treatment of CTLL-2 with PDBu does not interfere with IL-2-induced proliferation (data not shown), these data suggest that PKC participates in TGFβ receptor signalling in CTLL-2. These data are congruent with results obtained in epithelial cell lines where it was found that although TGFβ responses could be blocked by serine/threonine kinase inhibitors H7 and H8, treatment with PKC-specific staurosporine or depletion of PKC with high-dose PMA was without effect on TGFβ responses (Ohtsuki and Massagué, 1992).

Cytokine regulation of thymocyte ontogeny

Intrathymic T cell differentiation undoubtably involves complex interrelationships between stromal elements in the thymic microenvironment (Van Ewijk, 1991). It is also clear that cytokines such as IL-1 through IL-7, CSFs, TNF-α, IFN-γ, TGF-α and -β play important roles in T cell ontogeny (Carding et al., 1991), although precise cause and effect relationships between the various cytokines and T cell differentiation processes are unclear. Many studies (including those described herein) of T cell development must then be interpreted with caution, since the major shortcoming of cell differentiation models is that they often cannot accurately replicate microenvironmental conditions that are present *in vivo*.

Thymic lymphocytes are a heterogeneous population of cells; distinct subpopulations of thymocytes, each having its own microheterogeneity have been noted (MacDonald et al., 1988) and the small size of some of these subpopulations have limited extensive investigation of functional potential. Herein, we have demonstrated that when unseparated thymocytes are briefly incubated with an activator of protein kinase C and a calcium ionophore and subsequently cultured in IL-2, TGFβ treatment causes higher percentages of $CD8\alpha^+$ cells to emerge. On average, these cells also exhibited higher surface densities of CD8α and CD8β antigens as compared to controls, while CD4 expression was unaltered by TGFβ treatment. Since elevated CD8 mRNA levels were seen at 18 and 48h after TGFβ addition, it is likely that TGFβ is effecting *de novo* expression of surface CD8 on these thymocytes rather than promoting passive acquisition of the CD8 antigen from other cells, as described by others (Shores et al., 1991).

The most immature thymocytes are contained in the DN subpopulation. In this system, treatment of DN thymocytes with IL-2 + TGFβ induced progressively higher

levels of CD8 surface density in 30% of cells, whereas IL-2 or TGFβ alone was an insufficient stimulus for CD8 expression. Although DN thymocyte growth was inhibited by TGFβ, approximately a 240-fold increase in the number of $CD8^+$ cells was observed during 4 days in culture. Thus, this culture system may be valuable for investigating CD8 precursor potentials in populations that have low starting cell numbers, such as subsets of DN thymocytes. Most of the $CD8^+$ cells that emerged from the DN population were immature, $Thy1^+TCR/CD3^-CD4^-CD8^+$, thus providing further evidence for the existence of such cells as a discrete phenotypic stage in thymocyte development. These data also implicate TGFβ as a possible mediator of the transition from the DN to the immature CD8 SP stage, which is thought to precede the DP stage of development (Nikolic-Zugic et al., 1989). During these studies, cells with a more mature phenotype did not emerge, even after prolonged culture in IL-2 or IL-2 + TGFβ. Perhaps additional cytokine(s) and/or cell-to-cell contact between lymphocytes and stromal cells are required for CD4 expression in immature thymocytes. In this regard, co-culture of DN thymocytes with a thymic stromal cell clone (MRL104.8a), or a semi-purified T cell growth factor released from these cells, resulted in the emergence of a small subpopulation (10-20%) of $CD3^-CD4^-CD8^+$ cells (Tatsumi et al., 1990; Nagamine et al., 1991). Interestingly, DP cells emerged only after prolonged co-culture with the MRL104.8a monolayer but not after treatment with the supernatant factor alone (Tatsumi et al., 1990). There is currently little information available regarding the exact identity of cytokines that are required for intrathymic T cell maturation processes.

There are abundant data implicating TGFβ as a regulatory molecule in cellular differentiation processes. TGFβ inhibits differentiation of pre-adipocytes (Ignotz and

Massague, 1985), myoblasts (Massague et al., 1986; Olson et al., 1986), and inhibits the terminal differentiation of CTL precursors into CTL effectors (Ranges et al., 1987; Fontana et al., 1989; Shalaby and Amman, 1988; Wallick et al., 1990; Tada et al., 1991). Conversely, there are numerous reports that TGFβ stimulates differentiation of epithelial and certain mesodermal cells (Seyedin et al., 1986; Seyedin et al., 1987; Potts and Runyan, 1989; Kurokowa et al., 1987). Our results further suggest that TGFβ may be a positive regulator of early differentiation-related events in immature T cells and are in agreement with recent reports by Suda and Zlotnick demonstrating that TGFβ as well as TNFα stimulate CD8 expression in normal, $CD3^-CD4^-CD8^-CD25^+$ thymocytes (Suda and Zlotnik, 1992a; Suda and Zlotnik, 1992b). TGFβ mRNA is expressed in the thymus during murine embryogenesis (Millan et al., 1991) and immunohistochemical analysis of bovine thymus has shown that TGFβ can be detected in Hassall's corpuscles and in medullary reticuloepithelial cells--anatomic sites thought to be involved in critical steps of T cell differentiation and selection--but not in thymocytes, cortical accessory cells, or thymic capsule (Ellingsworth et al., 1986). Despite a large body of knowledge regarding the regulation of expression of TCR components during thymocyte ontogeny (Clevers and Owen, 1991), very little is known about the physiologic signals and genetic regulatory mechanisms that result in initial CD8 expression in T lymphocytes. This area has not been fully explored largely due to the lack of suitable models in which CD8 expression can be manipulated. The fact that both CTLL-2 and normal immature thymocytes show enhanced *de novo* expression of CD8 chains in response to IL-2 and TGFβ indicates that these will be useful models to study the molecular regulation of CD8 expression, and supports the hypothesis that these cytokines may be important immunoregulatory factors during

the differentiation of normal T lymphocytes.

TGFβ: Physiological regulator of T lymphocyte proliferation and memory T lymphocyte production?

It is known that T cell expansion after activation is tightly regulated in a paracrine and/or autocrine fashion by the production of IL-2 and the expression of IL-2R. However, the factors regulating two other important processes that occur after T cells are clonally expanded remains elusive: restoration of the state of IL-2 unresponsiveness and long-term survival of the subset of T cells that has proven the value of its particular TCR paratope (memory induction). TGFβ may be pivotal in this sense. In response to antigen/MHC in the context of APC-derived costimulating factors, antigen specific clones of Th cells become activated and begin to produce a battery of cytokines to "help" the proliferation and functional differentiation of pre-effector CD8 T lymphocytes. Among these cytokines are IL-2 and IL-4, which are responsible for the well characterized autocrine proliferative loop. TGFβ may be also involved in a parallel autocrine loop, one which may function to both counteract (in terms of proliferation) and alter cellular differentiation programs in the presence of other cytokines such as IL-2 and TNFα. Th cells synthesize TGFβ mRNA within several hours of being activated, however the protein product is not secreted until days afterward (Kehrl et al., 1986b). Coincident with the general timeframe of TGFβ production (5-7 days post activation), effector T cells that had been IL-2 responsive and proliferative for several days become IL-2 resistant (Gullberg and Smith, 1986). This IL-2 resistant state is characterized by the inability of the cells to grow in response to exogenous IL-2 even though IL-2Rα levels may remain high; this condi-

tion is mirrored by functional CTL clones *in vitro* (Figures 1.03 and 1.04). Interestingly, it was recently shown that this IL-2 resistant phenotype was lost in T cell clones that had lost TGFβ receptors (Siepl et al., 1991). The observed antiproliferative effect of exogenous TGFβ on T cells supports the hypothesis that TGFβ is a factor which could account for the phenomenon of IL-2 resistance that occurs in the ensuing days after antigenic stimulation, and further predicts that TGFβ that is produced physiologically in T cell microenvironments may modulate IL-2 responsiveness.

There are now several lines evidence implicating TGFβ in alteration of T cell differentiation programs. We and others have shown that TGFβ stimulates differentiation of thymocytes of the CD8 lineage (Inge et al., 1992c; Suda and Zlotnik, 1992a), but TGFβ also affects expression of other differentiation markers. When normal T cell are treated with exogenous TGFβ a high percentage of cells begin to express isoforms of CD45 that are characteristic of memory T cells (Swain et al., 1991). T cells treated with TGFβ have been shown to more aggressively engage antigenic stimuli in an encephalitis model, again suggesting that TGFβ may be important for the development of memory T cells (Weinberg et al., 1992). Thus, some of the observed effects of TGFβ on T cells make it a good candidate to explain some of the yet poorly understood phenomena that occur in the life of a T lymphocyte. Future work will surely elucidate complex relationships between the many cytokines that together regulate T cell differentiation, T-dependent immune responses, and ensure the survival of relevant clones for future reactivation. In summary, this information supports the hypothesis that TGFβ plays a key role in T lymphocyte homeostasis, participating in the regulation of cellular differentiation, proliferation, and memory induction.

Table 3.01: CD8 surface expression requires new RNA synthesis

	%CD8$^+$		
	Expt #1	Expt #2	Expt #3
IL-2	7	9	3
IL-2 + TGFβ	18	16	7
IL-2 + TGFβ + Act-D	4	2	4

Table 3.02: PKC activation does not substitute for TGFβ signal

	Percent $CD8^+$ Cells	
Tx	Expt 1	Expt 2
IL-2	18	4
IL-2 + TGFβ	33	12
IL-2 + 100nM PDBu	6	3
IL-2 + 1,000nM PDBu	8	3
IL-2 + 10^{-6}M Io		4
IL-2 + 100nM PDBu + Io		2

Table 3.03: Effect of PKC depletion on CD8 expression

Incubate 48h in:	Wash, then another 24h in:	%CD8$^+$	
	Expt:	#1	#2
IL-2	IL-2	4	3
	IL-2 + TGFβ (1ng/ml)	25	18
	IL-2 + TGFβ (5ng/ml)		18
IL-2 + PDBu	IL-2	7	1
	IL-2 + TGFβ (1ng/ml)	16	10
	IL-2 + TGFβ (5ng/ml)		10
	IL-2 + PDBu	5	1
	IL-2 + PDBu + TGFβ (1ng/ml)	13	6
	IL-2 + PDBu + TGFβ (5ng/ml)		7

Concentrations used: IL-2, 20U/ml; PDBu, 1μM

Table 3.04: CD8 expression in mature, peripheral T cells

Responder[a]	Stim[b]	TGFβ	Phenotype				Lysis[c]	
			CD4		CD8		10:1	5:1
			%	MFI	%	MFI		
TBH spleen	-	-	66	115	24	77	2	2
TBH spleen	+	-	52	116	26	77	44	33
TBH spleen	+	2.5	49	105	23	73	26	15
TBH spleen	+	5	45	108	25	75	23	15

[a] Splenocytes were obtained from early tumor bearing hosts (TBH, day 12 after i.d. inoculation of PHS-5 tumor cells

[b] Stimulator (Stim) cells were P815 cells treated with mitomycin-C

[c] Lysis refers to % specific lysis of ^{51}Cr labelled P815 target cells

Table 3.05: CD8α expression in TGFβ-treated double negative thymocytes

	% CD8α$^+$ Cells (MFI)		
	Exp 1	Exp 2	Exp 3[a]
Day 4			
IL-2	7 (23)	7 (38)	12 (291)
IL-2+TGFβ	32 (22)	36 (50)	31 (506)
Day 6 (or 8)			
IL-2	7 (23)	11 (38)[b]	10 (490)
IL-2+TGFβ	26 (49)	48 (53)[b]	29 (716)

[a] Cells from experiment 3 were stained with a phycoerythrin-conjugated anti-CD8α, resulting in higher MFI values than in experiments 1 and 2, where FITC-conjugates were used

[b] Immunofluorescence performed on day 8

Table 3.06: Proliferation of $CD8^+$ and $CD8^-$ subpopulations of TGFβ-treated DN thymocytes

	Mean ^{3}H-Thymidine Incorporation in cpm (SD)	
Subset	IL-2	IL-2+TGFβ
$CD8^+$	25,788 (3,582)[a]	19,646 (2,280)[b]
$CD8^-$	55,167 (3,059)	24,805 (3,556)[c]

[a] $p<0.001$ compared to $CD8^-$ cells in IL-2 alone by Students' t-test

[b] $p<0.03$ compared to $CD8^+$ cells in IL-2 alone

[c] $p>0.05$ compared to $CD8^+$ cells in IL-2 + TGFβ; $p<0.0001$ compared to $CD8^-$ cells in IL-2 alone

Figure 3.01: Photomicrograph of TGFβ-treated CTLL-2. CTLL-2 were cultured in 24-well tissue culture plates in 20U/ml IL-2 in the absence (panel A) or presence (panel B) of 1ng/ml TGFβ for 2 days prior to phase contrast photomicroscopy. Bar length is 100μm.

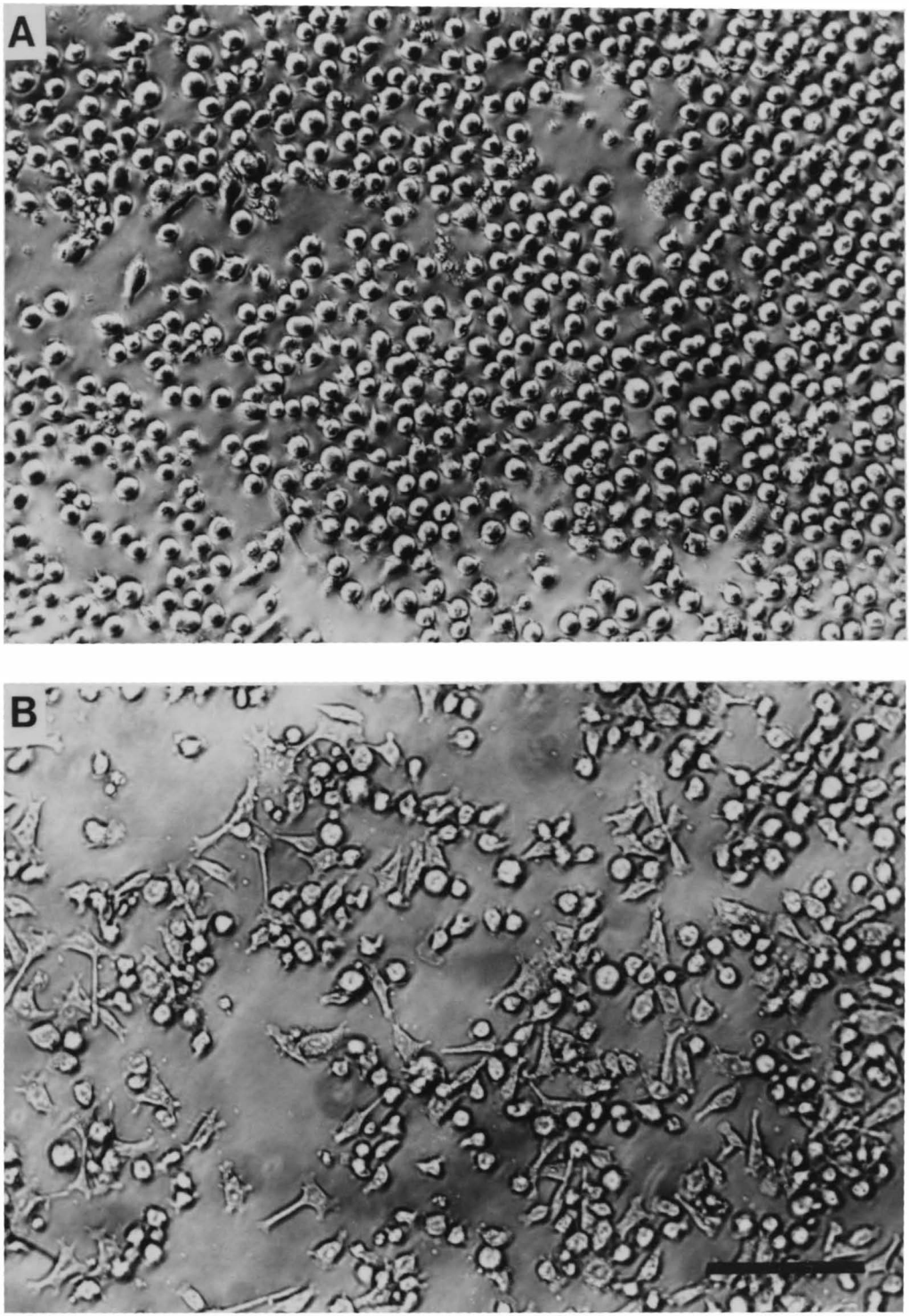

A
B

Figure 3.02: Morphologic characteristics of CTLL-2 by FACS analysis. CTLL-2 were treated with 20U/ml IL-2 ± 1ng/ml TGFβ for 24h prior to measurement of forward angle (FSC, left panel) and side angle (SSC, right panel) light scattering characteristics. Light scatter is shown along the abscissa, and cell number along the ordinate.

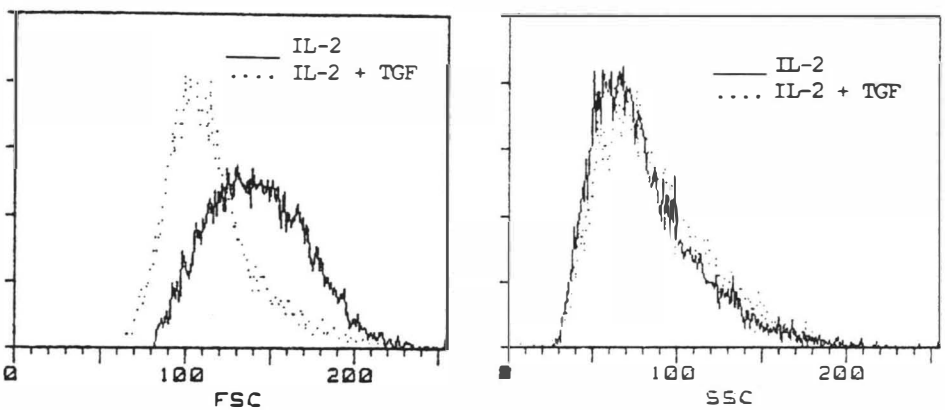
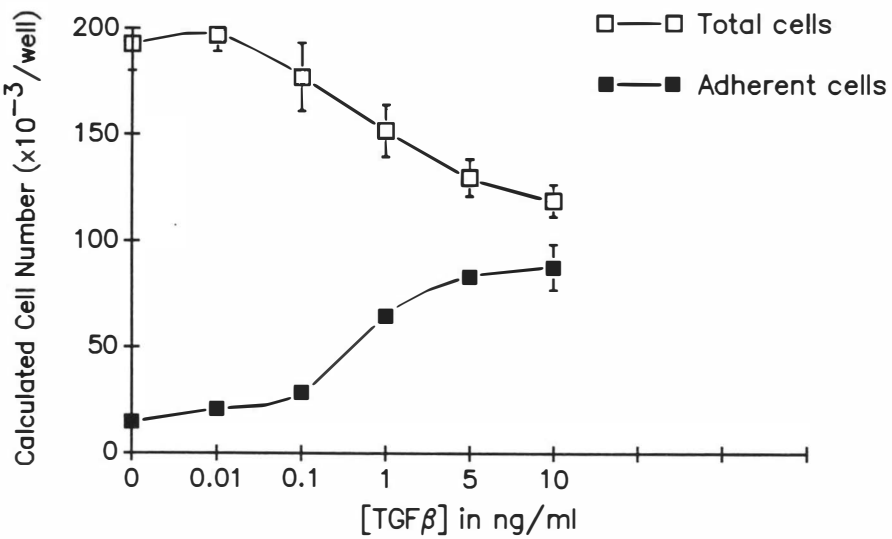

IL-2
IL-2 + TGF
0
100
200
FSC
IL-2
IL-2 + TGF
100
200
SSC

Figure 3.03: CTLL-2 adhere to culture plate after TGFβ treatment. A) Experiment #1; B) Experiment #2. In each experiment, CTLL-2 were cultured at $5x10^4$ cells/well in duplicate 96-well plates with 20u/ml IL-2 and various concentrations of TGFβ. After 24h, one plate was filled with medium, inverted, and centrifuged as described in Materials and Methods. Adherent cell mass was then measured by MTT, and cell numbers calculated by comparison of optical density values to a standard curve. Cells in the duplicate plate were not centrifuged, but were similarly stained with MTT to obtain "total" cell numbers.

A.


Total cells
Adherent cells
Calculated Cell Number (x10^{-3}/well)
200
150
100
50
0
0
0.01
0.1
1
5
10
[TGFβ] in ng/ml

B.

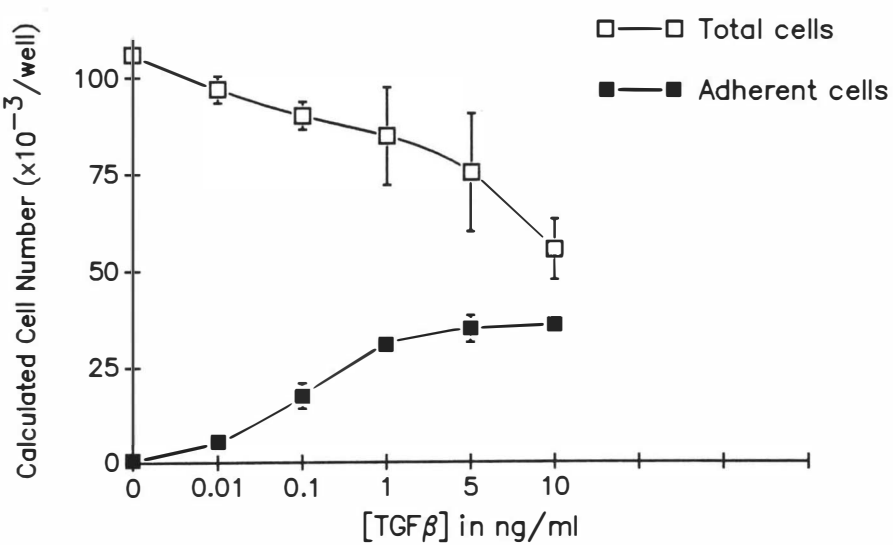

Total cells
Adherent cells
Calculated Cell Number (x10^{-3}/well)
100
75
50
25
0
0
0.01
0.1
1
5
10
[TGFβ] in ng/ml

Figure 3.04: Maximal adherence occurs at 24h. CTLL-2 were cultured for various periods of time in 20U/ml IL-2 ± 1 or 5ng/ml TGFβ. Adherence was measured as described in Figure 3.03 and in Materials and methods.

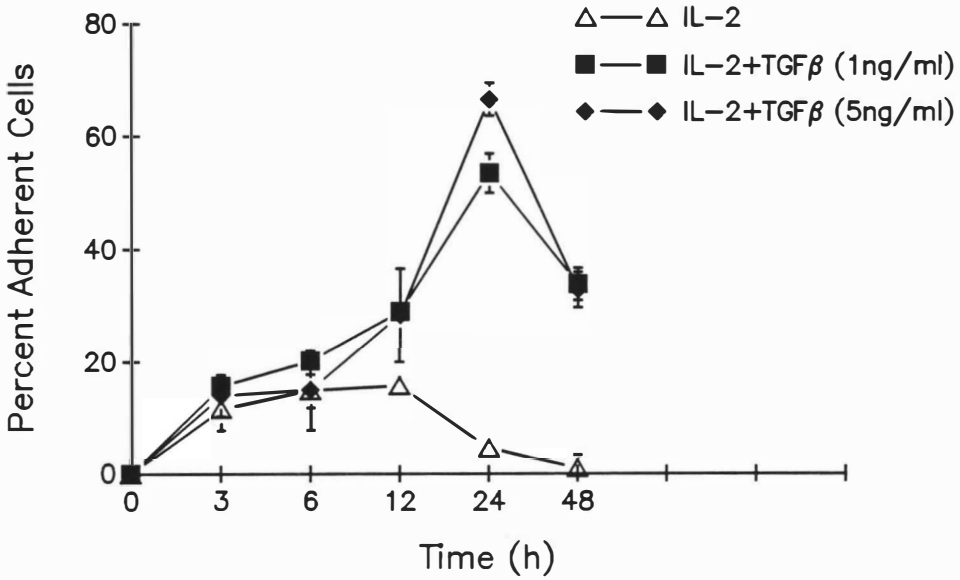

Percent Adherent Cells
80
60
40
20
0
0
3
6
12
24
48
Time (h)
IL-2
IL-2+TGFβ (1ng/ml)
IL-2+TGFβ (5ng/ml)

Figure 3.05: FCS is required for adherence. CTLL-2 were cultured in DMEM without fetal calf serum (FCS) with IL-2 + 5ng/ml TGFβ for 24h prior to adherence assay. The following soluble ingredients were added to groups as designated along the abscissa: fetal calf serum (FCS) at various % concentrations (vol/vol) bovine serum albumin (BSA) at 1% (wt/vol), rat tail collagen type I (COLL) at 10μg/ml, or bovine serum fibronectin (FN) at 10 or 50μg/ml. Simultaneous assay of groups treated with IL-2 without TGFβ (data not shown) demonstrated that the high background adherence in Expt #1 in the absence of FCS was equivalent between the groups, and therefore was not attributable to TGFβ.

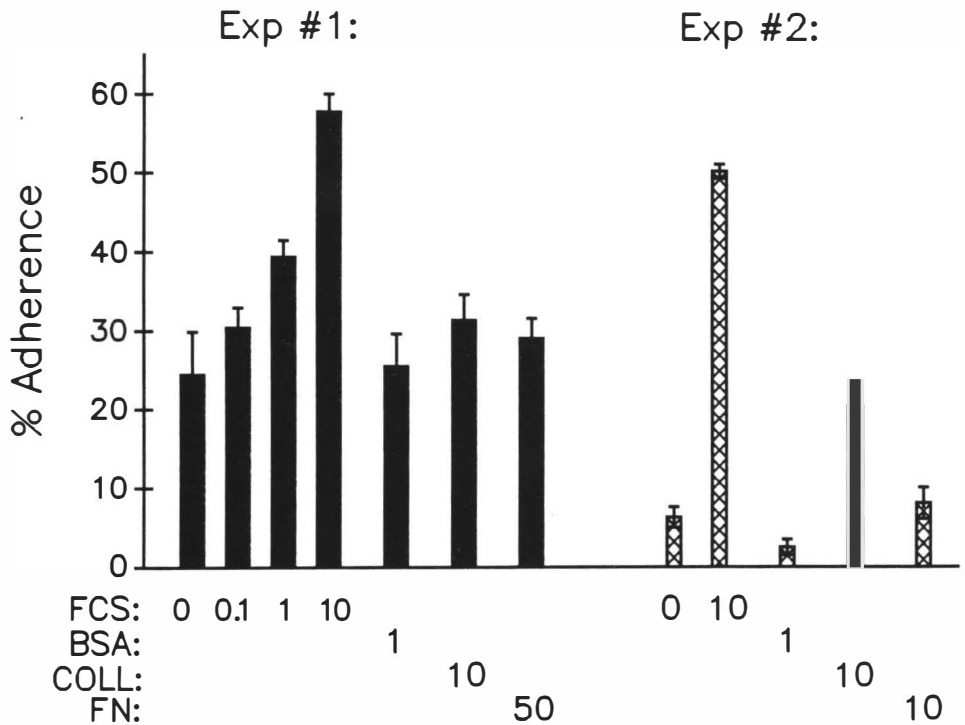

Exp #1:
Exp #2:
% Adherence
60
50
40
30
20
10
0
FCS: 0 0.1 1 10 0 10
BSA: 1 1
COLL: 10 10
FN: 50 10

Figure 3.06: RGDS peptide blocks adherence. CTLL-2 were treated with 20U/ml IL-2 + 5ng/ml TGFβ in medium containing various concentrations of FCS and tetrapeptides. RGDS (Arg-Gly-Asp-Ser) was added at 10μg/ml (Experiment #1) or 50μg/ml (Experiment #2). Control tetrapeptide RGES (Arg-Gly-Glu-Ser) was added at 50μg/ml.

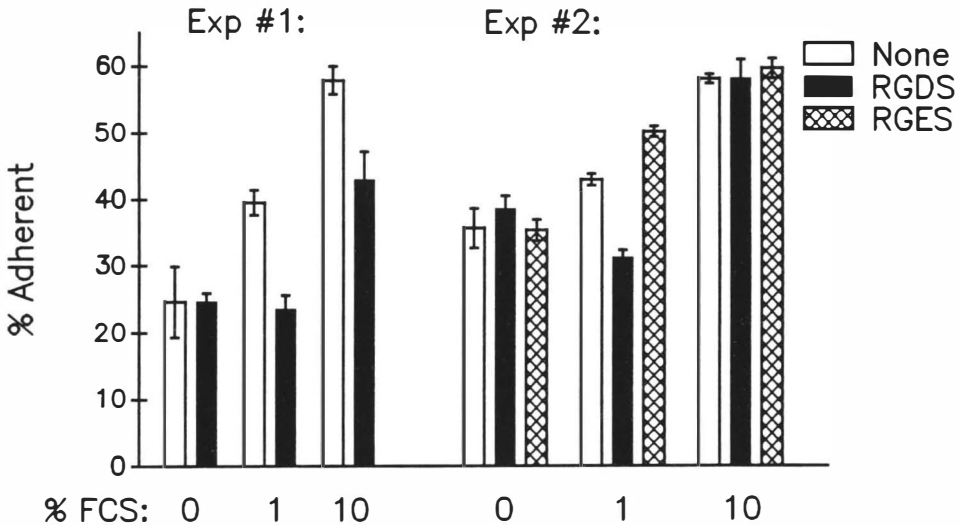
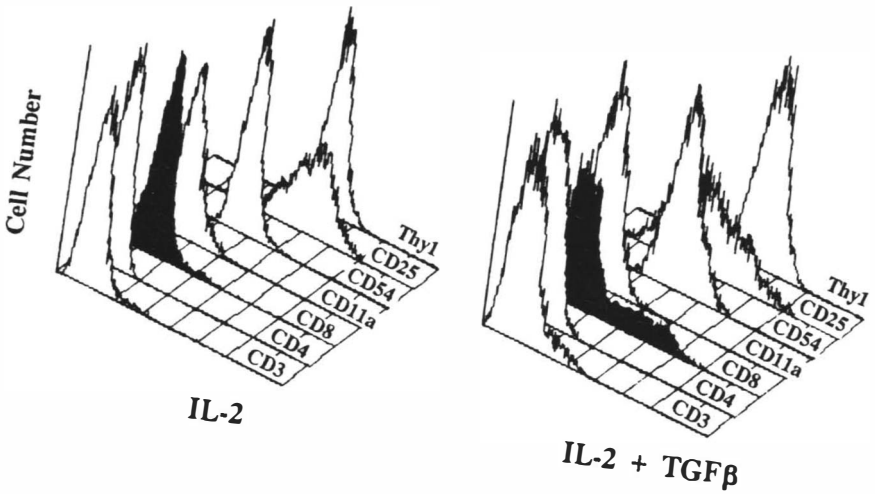

Exp #1:
Exp #2:
None
RGDS
RGES
% Adherent
60
50
40
30
20
10
0
% FCS: 0 1 10 0 1 10

Figure 3.07: Surface phenotype of TGFβ-treated CTLL-2. CTLL-2 were cultured for two days (A) or one day (B) in 20U/ml IL-2 alone (left) or IL-2 + 1ng/ml TGFβ (right) prior to immunofluorescence analysis. Fluorescence intensity (abscissa) was quantitated by flow cytometry using four decade log amplification. These data are representative of 3 separate experiments. In panel A, CD8 was detected with PE-conjugated Ab, in panel B with a FITC-conjugate.

A.


Cell Number
Thy1
CD25
CD54
CD11a
CD8
CD4
CD3
IL-2
Thy1
CD25
CD54
CD11a
CD8
CD4
CD3
IL-2 + TGFβ

B.

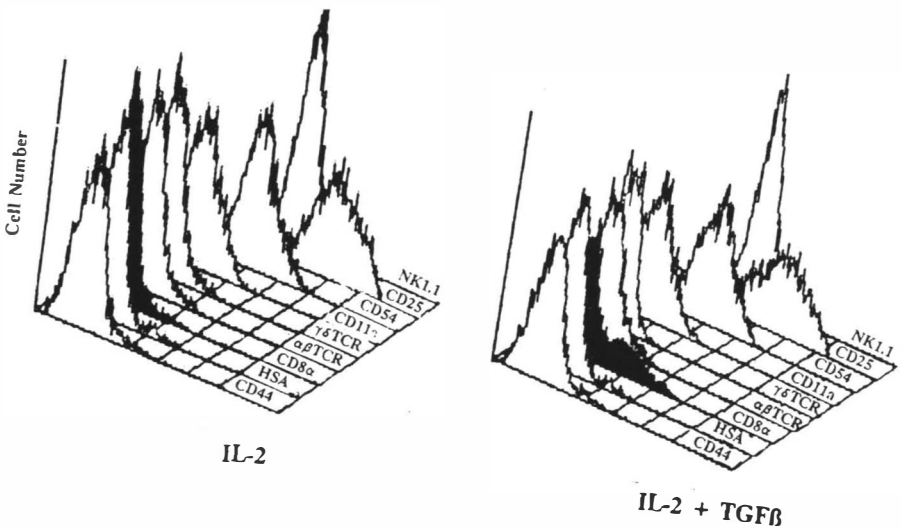

Cell Number
NK1.1
CD25
CD54
CD11a
γδTCR
αβTCR
CD8α
HSA
CD44
IL-2
NK1.1
CD25
CD54
CD11a
γδTCR
αβTCR
CD8α
HSA
CD44
IL-2 + TGFß

Figure 3.08: CD8 expression in adherent and non-adherent CTLL-2. CTLL-2 were cultured for 48h in DMEM + 10% fetal calf serum + 20U/ml IL-2 + 1ng/ml TGFβ in 24-well plates. Non-adherent cells (NonAdh) were harvested by gently pipetting, while adherent (Adh) cells required vigorous pipetting with ice-cold media for efficient harvest. Plots are shown for isotype control staining (Control) and TGFβ-treated cells stained for CD8α.

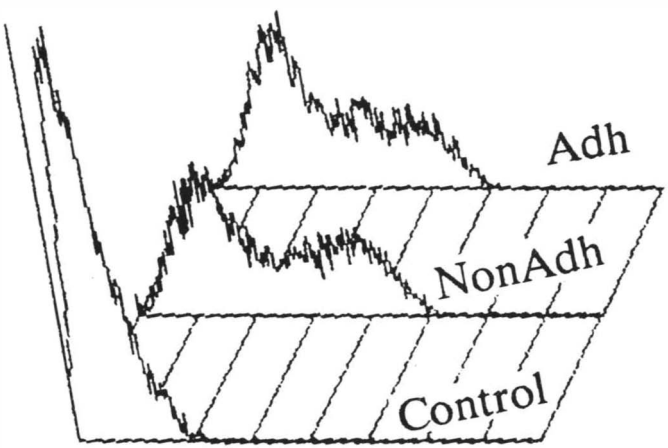

Adh
NonAdh
Control

Figure 3.09: CD8 expression is uniform throughout the population. CTLL-2 were treated for 24h with 20u/ml IL-2 + 1ng/ml TGFβ prior to staining for CD8α. Fluorescence data were then displayed as follows: A) CD8α immunofluorescence (abscissa) as a function of cell number (ordinate); B) contour plot of CD8α (ordinate) as a function of forward angle light scatter (FSC, abscissa); C) contour plot of CD8α (ordinate) as a function of side angle light scatter (SSC, abscissa).

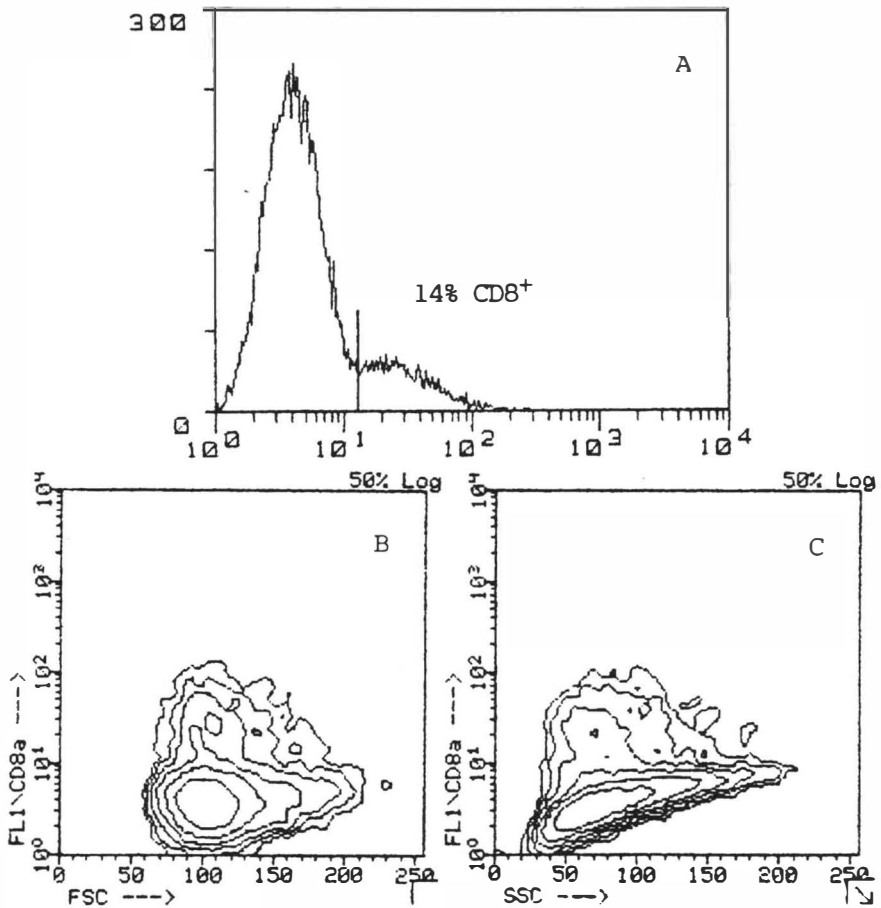

300
A
14% CD8+
0
10^0
10^1
10^2
10^3
10^4
50% Log
B
FL1\CD8a --->
FSC --->
0
50
100
150
200
250
50% Log
C
FL1\CD8a --->
SSC --->
0
50
100
150
200
250

Figure 3.10: Coordinate analysis of CD8α and other markers. CTLL-2 cells were treated for 24h with 20U/ml IL-2 + 1ng/ml TGFβ. Cells were stained with PE-conjugated CD8α (ordinate) and either no additional stain (panel A), or the following FITC-conjugated Ab: B) IL-2Rα, C) LFA-1, D) CD8β, E) Thy-1, F) ICAM-1. 10,000 events were collected and displayed as contour plots.

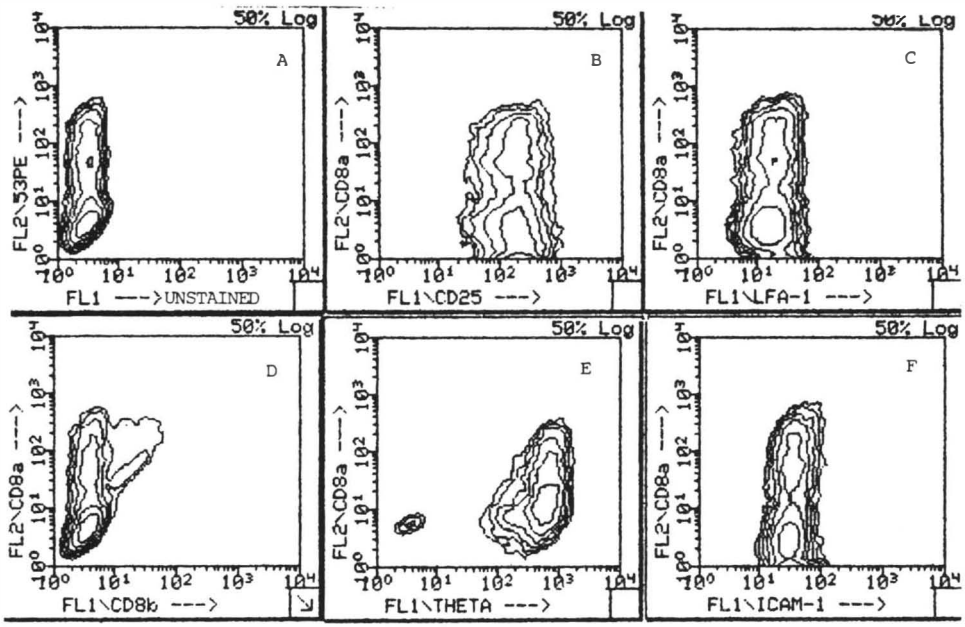

50% Log
A
FL2\53PE --->
FL1 --->UNSTAINED
B
FL2\CD8a --->
FL1\CD25 --->
C
FL2\CD8a --->
FL1\LFA-1 --->
D
FL2\CD8a --->
FL1\CD8b --->
E
FL2\CD8a --->
FL1\THETA --->
F
FL2\CD8a --->
FL1\ICAM-1 --->
10⁰ 10¹ 10² 10³ 10⁴

Figure 3.11: Coordinate expression of CD8α and CD8β. CTLL-2 were treated with IL-2 alone or IL-2 + various concentrations of TGFβ. After 1 day, cells were analyzed by immunofluorescence. These data are representative of 2 separate experiments.

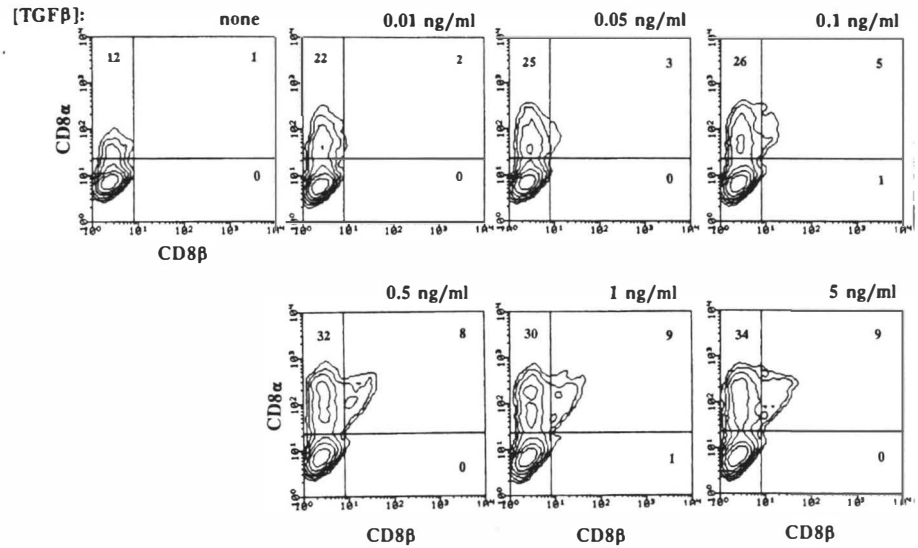

[TGFβ]:
none
0.01 ng/ml
0.05 ng/ml
0.1 ng/ml
12
1
0
22
2
0
25
3
0
26
5
1
CD8α
CD8β
0.5 ng/ml
1 ng/ml
5 ng/ml
32
8
0
30
9
1
34
9
0
CD8α
CD8β
CD8β
CD8β

Figure 3.12: Kinetics of CD8α expression on TGFβ-treated CTLL-2. CTLL-2 were treated with IL-2 ± 1ng/ml TGFβ and CD8α chain was analyzed over time. Multiple experiments were pooled to obtain mean %$CD8^+$ values ± SEM. The number of determinations (n) for days 0, 1, 2, 3, and 4, was 5, 6, 5, 3, and 2, respectively.

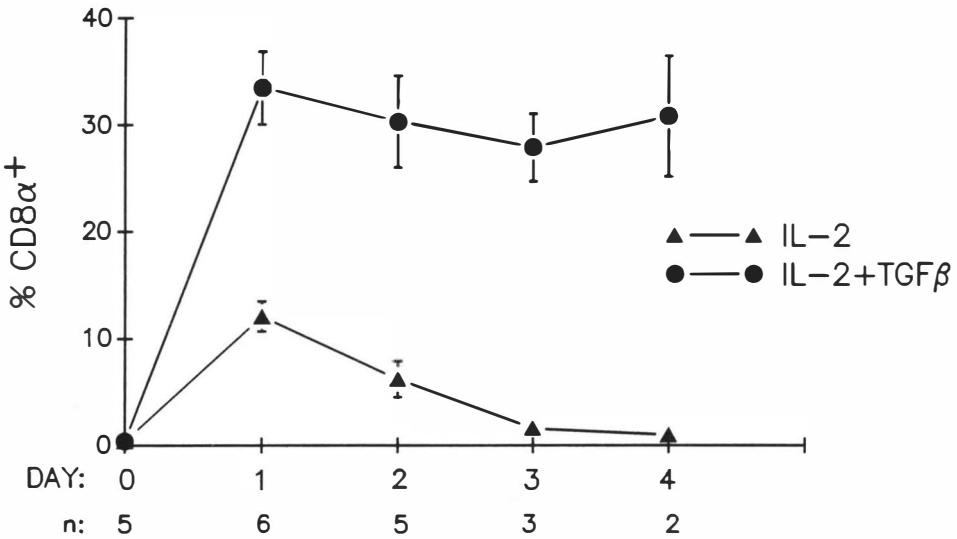

40
30
20
10
0
% CD8α+
DAY: 0 1 2 3 4
n: 5 6 5 3 2
IL−2
IL−2+TGFβ

Figure 3.13: Kinetics of CD8β expression on TGFβ-treated CTLL-2. CTLL-2 were treated with IL-2 ± 1ng/ml TGFβ and CD8β chain expression was analyzed over time.

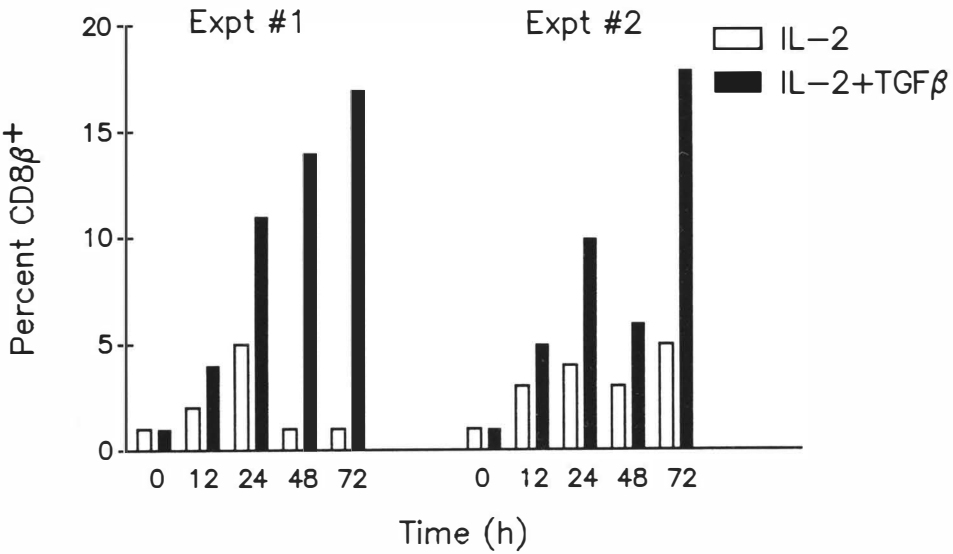

Expt #1
Expt #2
IL−2
IL−2+TGFβ
Percent CD8β+
20
15
10
5
0
0 12 24 48 72
0 12 24 48 72
Time (h)

Figure 3.14: Inhibition of CTLL-2 proliferation with hydroxyurea. CTLL-2 were treated with medium alone, 20U/ml IL-2, or IL-2 + various concentrations of hydroxyurea (HU). Thirty seven kBq of ^{3}H-thymidine was added at initiation of cultures and incorporation was measured 24h later. Points represent the arithmetic mean cpm (± S.D.) of quadruplicate samples for each treatment group.

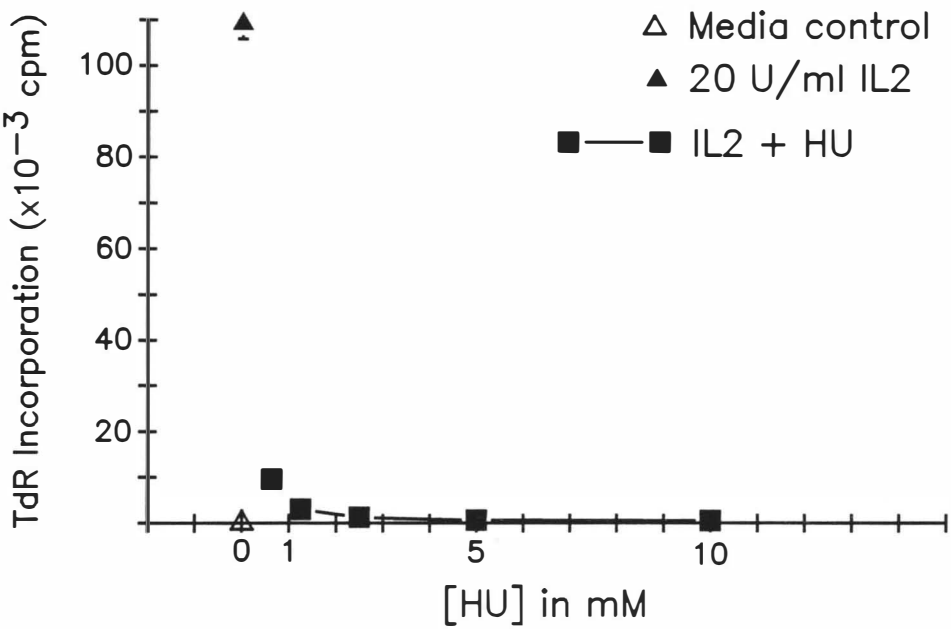

Media control
20 U/ml IL2
IL2 + HU
TdR Incorporation (x10^{-3} cpm)
100
80
60
40
20
0
1
5
10
[HU] in mM

Figure 3.15: Stability of CD8α expression after TGFβ washout. CTLL-2 were treated with IL-2 + 1ng/ml TGFβ. One day later, cultures were harvested and washed prior to re-culture in IL-2 ± TGFβ. On various days thereafter, CD8 immunofluorescence was determined.

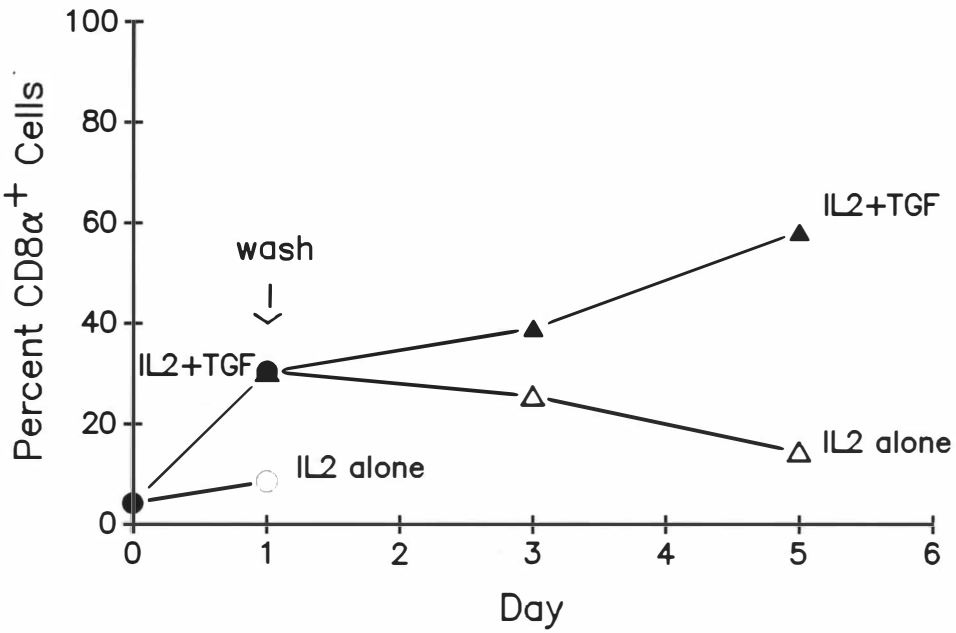

Percent CD8α+ Cells
100
80
60
40
20
0
wash
IL2+TGF
IL2+TGF
IL2 alone
IL2 alone
0
1
2
3
4
5
6
Day

Figure 3.16: Stability of CD8α expression after subset enrichment. CTLL-2 were treated with IL-2 + 1ng/ml TGFβ on days 0 and 2. On day 3, panning was performed to separate $CD8^+$ from $CD8^-$ cells, and each subset was then cultured in IL-2 ± TGFβ. On days 2, 5, and 8 thereafter, CD8 immunofluorescence was determined. These data are representative of 2 separate experiments.

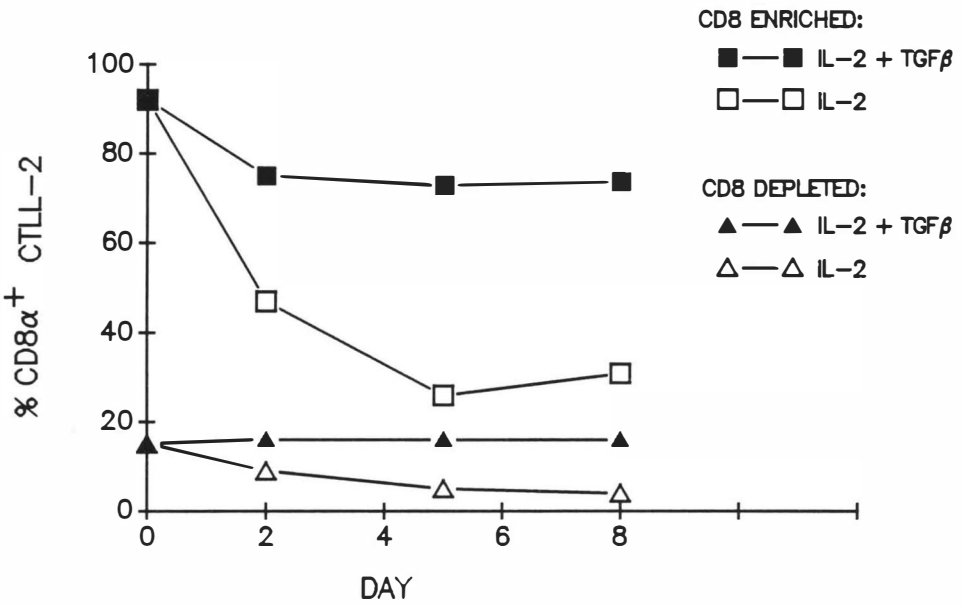

CD8 ENRICHED:
IL-2 + TGFβ
IL-2
CD8 DEPLETED:
IL-2 + TGFβ
IL-2
% CD8α+ CTLL-2
100
80
60
40
20
0
0
2
4
6
8
DAY

Figure 3.17: Northern analysis of TGFβ-treated CTLL-2. CTLL-2 were cultured for various periods in 20U/ml IL-2 ± 1ng/ml TGFβ prior to Northern analysis of CD8α and β-actin.

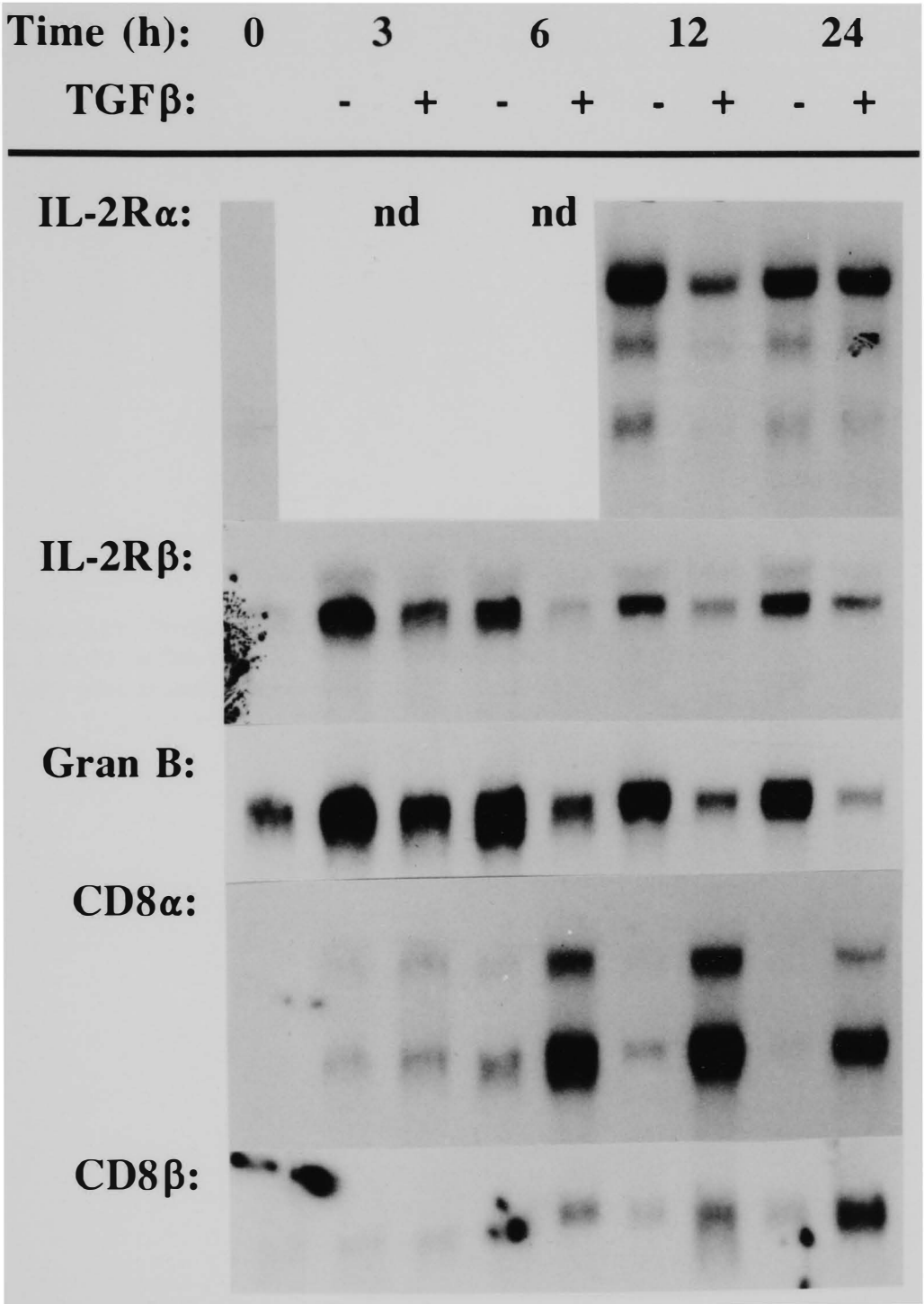
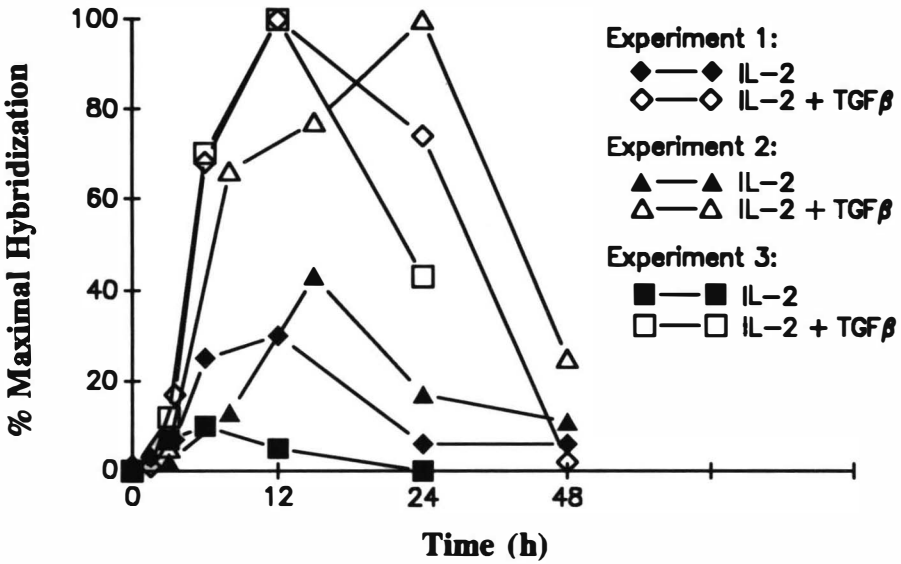

Time (h): 0 3 6 12 24
TGFβ: - + - + - + - +
IL-2Rα: nd nd
IL-2Rβ:
Gran B:
CD8α:
CD8β:

Figure 3.18: Northern analysis of TGFβ-treated CTLL-2. CTLL-2 were cultured for 0, 3, 6, 12, or 24h with 20U/ml IL-2 in the absence ("-") or presence ("+") of 1ng/ml TGFβ prior to analysis of mRNA.

A.


% Maximal Hybridization
100
80
60
40
20
0
0
12
24
48
Time (h)
Experiment 1:
IL-2
IL-2 + TGFβ
Experiment 2:
IL-2
IL-2 + TGFβ
Experiment 3:
IL-2
IL-2 + TGFβ

B.

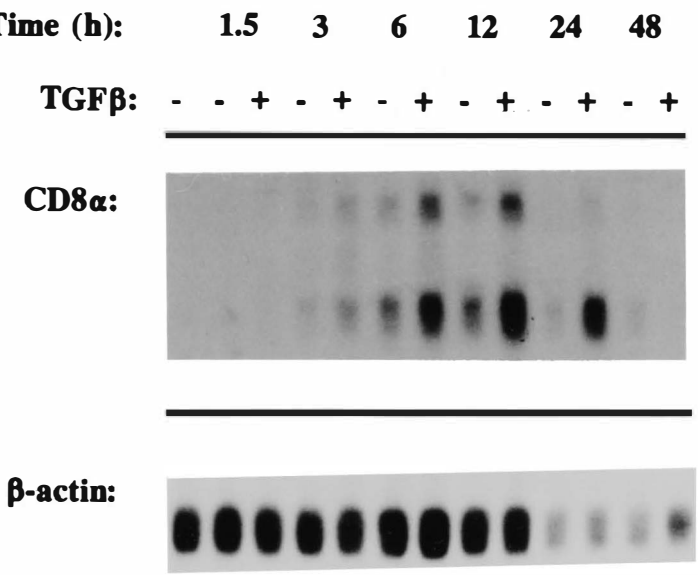
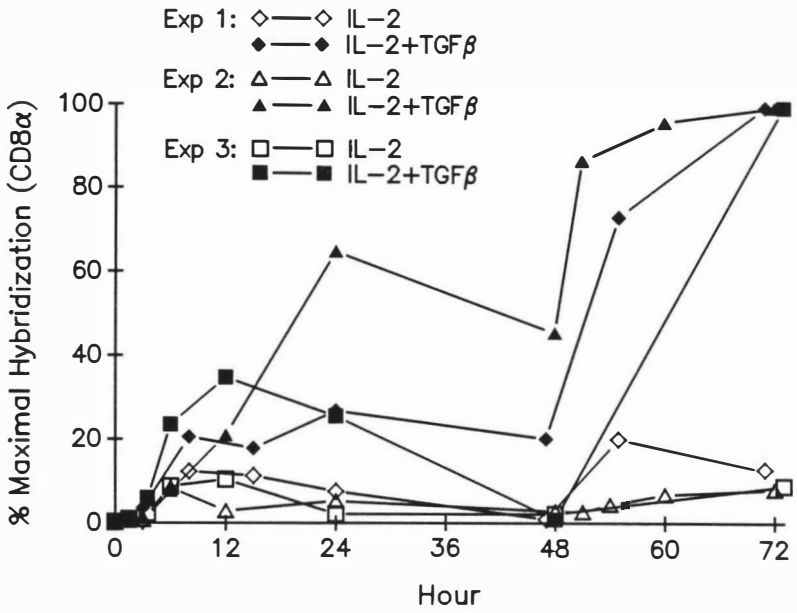

Time (h): 1.5 3 6 12 24 48
TGFβ: - - + - + - + - + - + - +
CD8α:
β-actin:

Figure 3.19: Secondary response of CD8α mRNA after TGFβ treatment. A) CTLL-2 were cultured for various periods in 20U/ml IL-2 ± 1ng/ml TGFβ prior to Northern analysis of CD8α. B) Rehybridization of blot from experiment 3 with β-actin probe.

A.


Exp 1: IL−2
IL−2+TGFβ
Exp 2: IL−2
IL−2+TGFβ
Exp 3: IL−2
IL−2+TGFβ
% Maximal Hybridization (CD8α)
100
80
60
40
20
0
0
12
24
36
48
60
72
Hour

B.

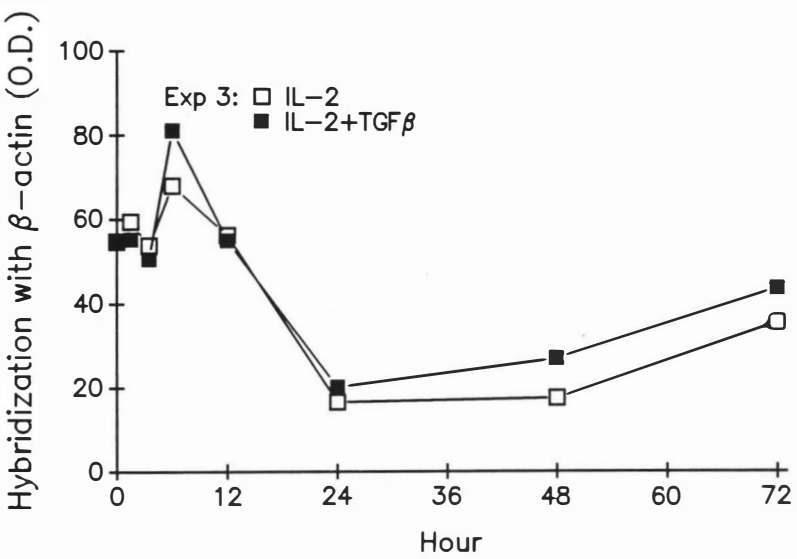
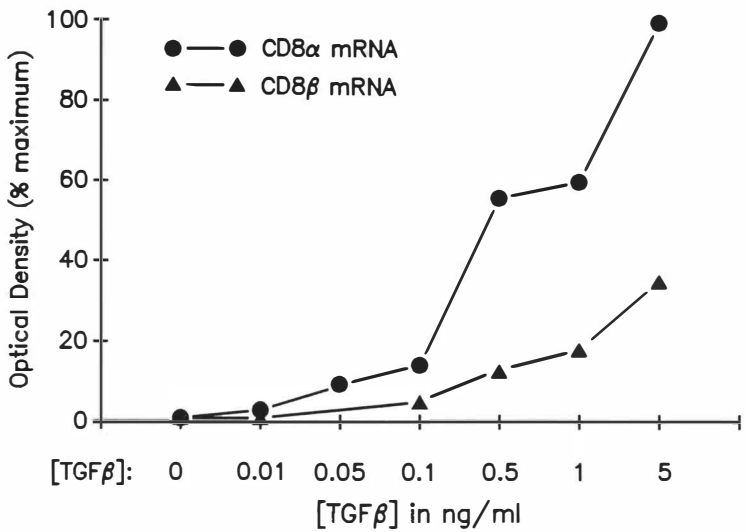

Exp 3: IL−2
IL−2+TGFβ
Hybridization with β−actin (O.D.)
100
80
60
40
20
0
0
12
24
36
48
60
72
Hour

Figure 3.20: TGFβ-dose response Northern analysis of CD8. CTLL-2 were treated with 20U/ml IL-2 ± various concentrations of TGFβ for 24h prior to Northern analysis of CD8α and CD8β. After probing for CD8, the blot was stripped and reprobed for β-actin. Data shown represent relative signal intensities as measured by densitometric scanning of autoradiograms.


100
80
60
40
20
0
Optical Density (% maximum)
CD8α mRNA
CD8β mRNA
[TGFβ]: 0 0.01 0.05 0.1 0.5 1 5
[TGFβ] in ng/ml

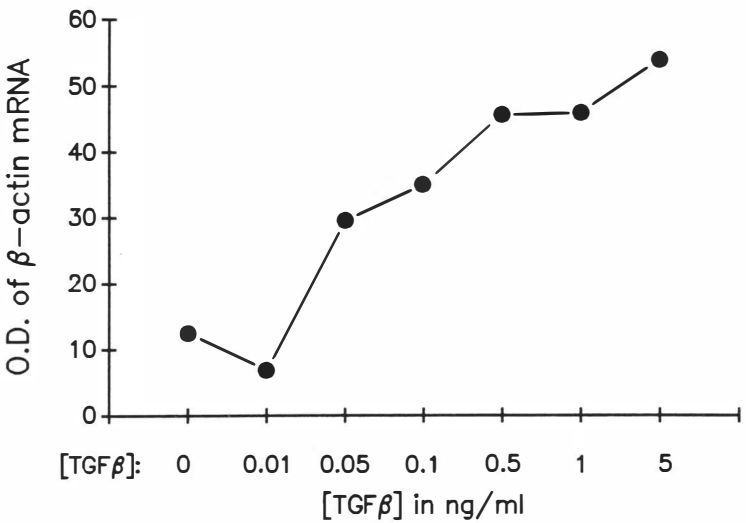

60
50
40
30
20
10
0
O.D. of β-actin mRNA
[TGFβ]: 0 0.01 0.05 0.1 0.5 1 5
[TGFβ] in ng/ml

Figure 3.21: CTLL-2 cytolytic activity. CTLL-2 and control tumor-specific CTL were treated with 20U/ml IL-2 ± 1ng/ml TGFβ for 2 days prior to cytotoxicity assay against ^{51}Cr-labelled PHS-5 or F45 tumor cells. "PHS-5 Specific CTL" was a control CTL line generated by repeated rounds of *in vitro* sensitization culture against the PHS-5 mastocytoma as described (McKinnon et al., 1990). "TGFβ" along the abscissa of this graph refers to the presence or absence of this cytokine during the 2d culture period prior to assay. "2C11" along the abscissa refers to the presence or absence of the anti-CD3 Ab 145-2C11 added during the 4h cytotoxicity assay at a concentration of 4μg/ml.

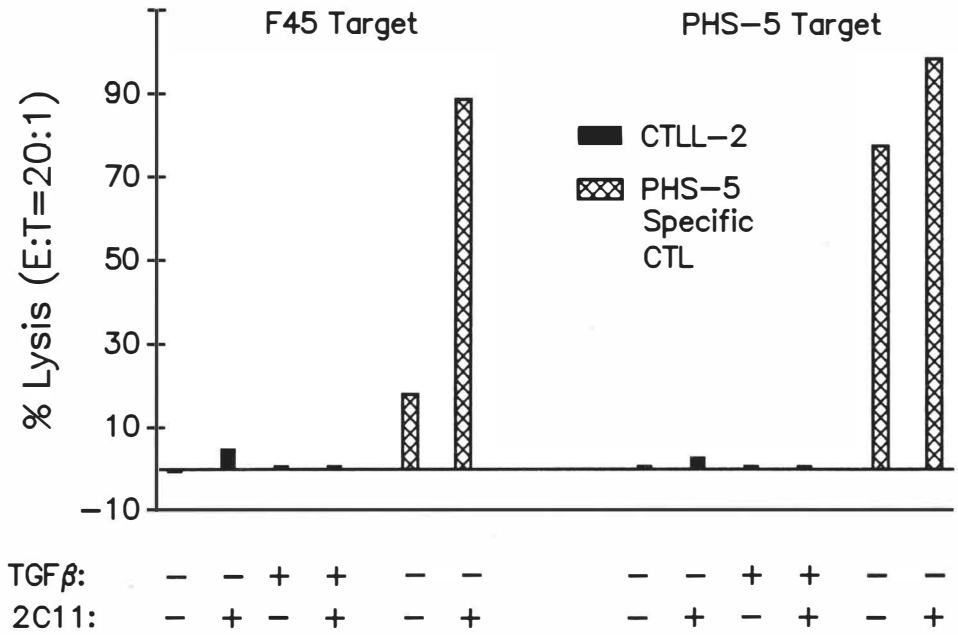

F45 Target
PHS–5 Target
% Lysis (E:T=20:1)
90
70
50
30
10
–10
CTLL–2
PHS–5 Specific CTL
TGFβ: – – + + – – – – + + – –
2C11: – + – + – + – + – + – +

Figure 3.22: TGFβ does not affect CD8 expression in MLTC. MLTC established with splenocytes from day 10 P815-tumor bearing mice were either: A) unstimulted = cultured in media alone; B) stimulated = cultured with mitomycin-C-treated PHS-5 cells; C) stimulated + 2.5ng/ml TGFβ added at initiation of culture; or D) stimulated + 5ng/ml TGFβ added at initiation of culture. After six days of culture, CD8α expression was ascertained by FACS. See Table 3.04 for numerical analysis of FACS data for this experiment.

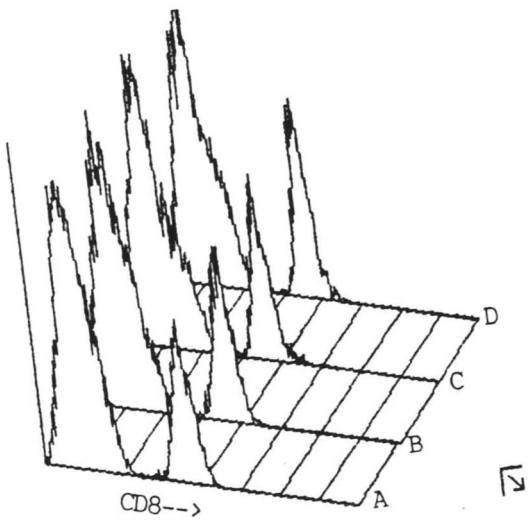

D
C
B
A
CD8-->

Figure 3.23: TGFβ inhibits thymocyte proliferation. Thymocytes were stimulated overnight with PDBu + Io and cultured with 20U/ml IL-2 ± 1ng/ml TGFβ. At various timepoints [^{3}H] TdR incorporation was measured and is shown as the arithmetic mean ± S.D.

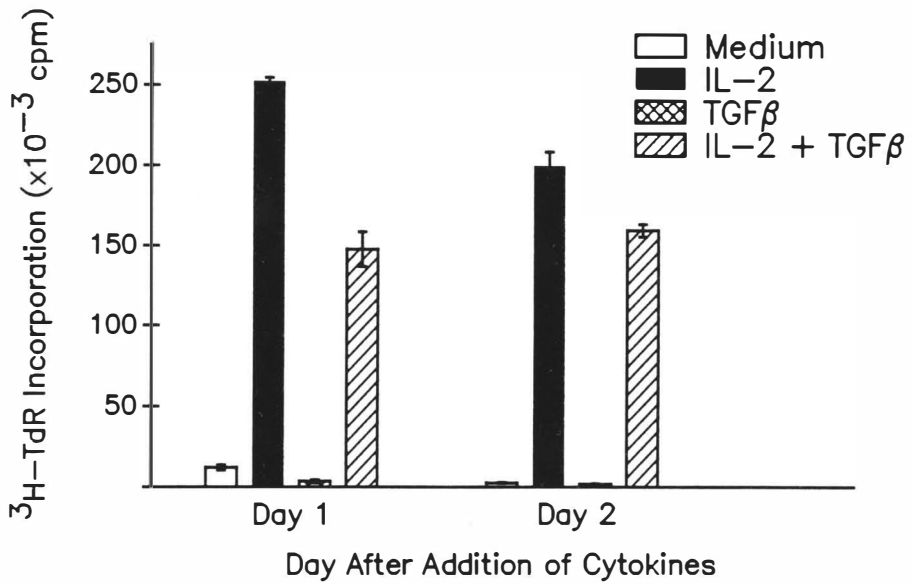

Medium
IL−2
TGFβ
IL−2 + TGFβ
^{3}H−TdR Incorporation (x10^{-3} cpm)
250
200
150
100
50
Day 1
Day 2
Day After Addition of Cytokines

Figure 3.24: FACS analysis of CD8α and CD4 or CD8β on normal thymocytes. Panel A. Fresh DBA/2 thymocytes. Panel B. Thymocytes stimulated overnight with PDBu + Io and cultured for 4 days with 20U/ml IL-2 alone. Panel C. Thymocytes stimulated overnight with PDBu + Io and cultured for 4 days with 20U/ml IL-2 + 1ng/ml TGFβ. These data are representative of 2 separate experiments.

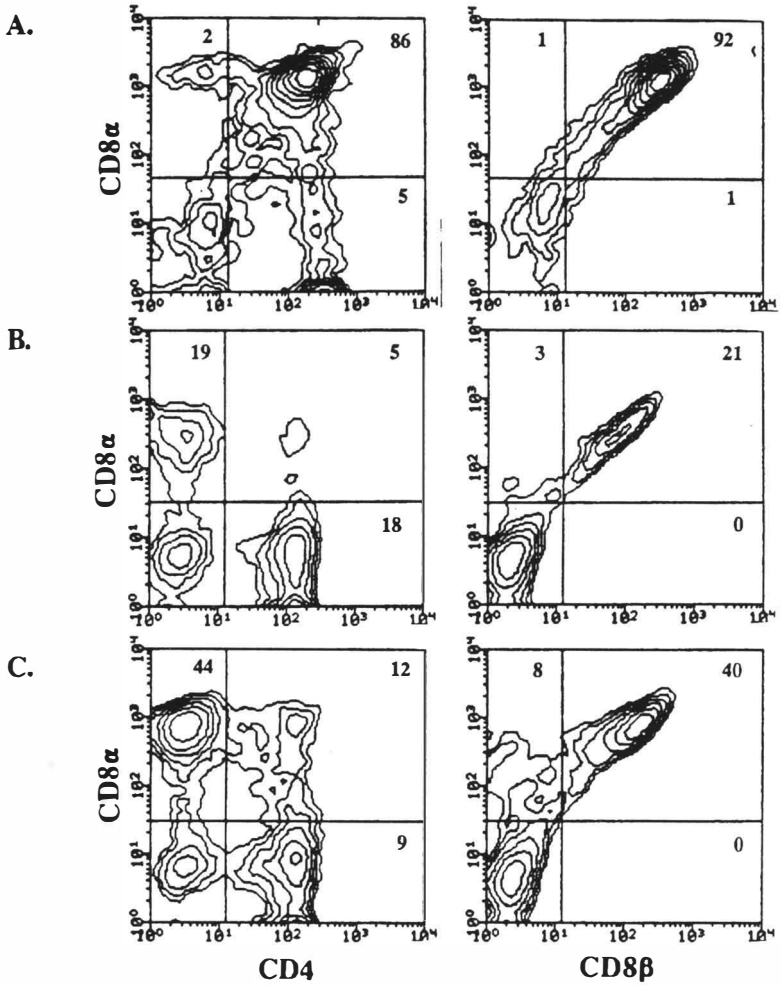

A.
B.
C.
CD8α
CD4
CD8β
2
86
5
1
92
1
19
5
18
3
21
0
44
12
9
8
40
0

Figure 3.25: Northern analysis of TGFβ-treated thymocytes. DBA/2 thymocytes were stimulated overnight with PDBu + Io + 20U/ml IL-2, then cultured for 2 additional days in 20U/ml IL-2. On day 3, cells were washed and re-cultured in either medium alone, 20U/ml IL-2, 1ng/ml TGFβ, or IL-2 + TGFβ for 18 or 48h prior to Northern analysis of CD8α. For cells that did not receive IL-2, (lanes 1 & 3) viability rapidly decreased after 18h, and were thus not analyzed at 48h. Despite equal loading of RNA by ethidium bromide staining, cells cultured without IL-2 also demonstrated lower levels of β-actin mRNA, congruent with the fact that most of these cells were dead after 24h of IL-2 deprivation. Comparison of β-actin signals between groups that received IL-2 or IL-2 + TGFβ did not reveal any differences in expression of this mRNA.

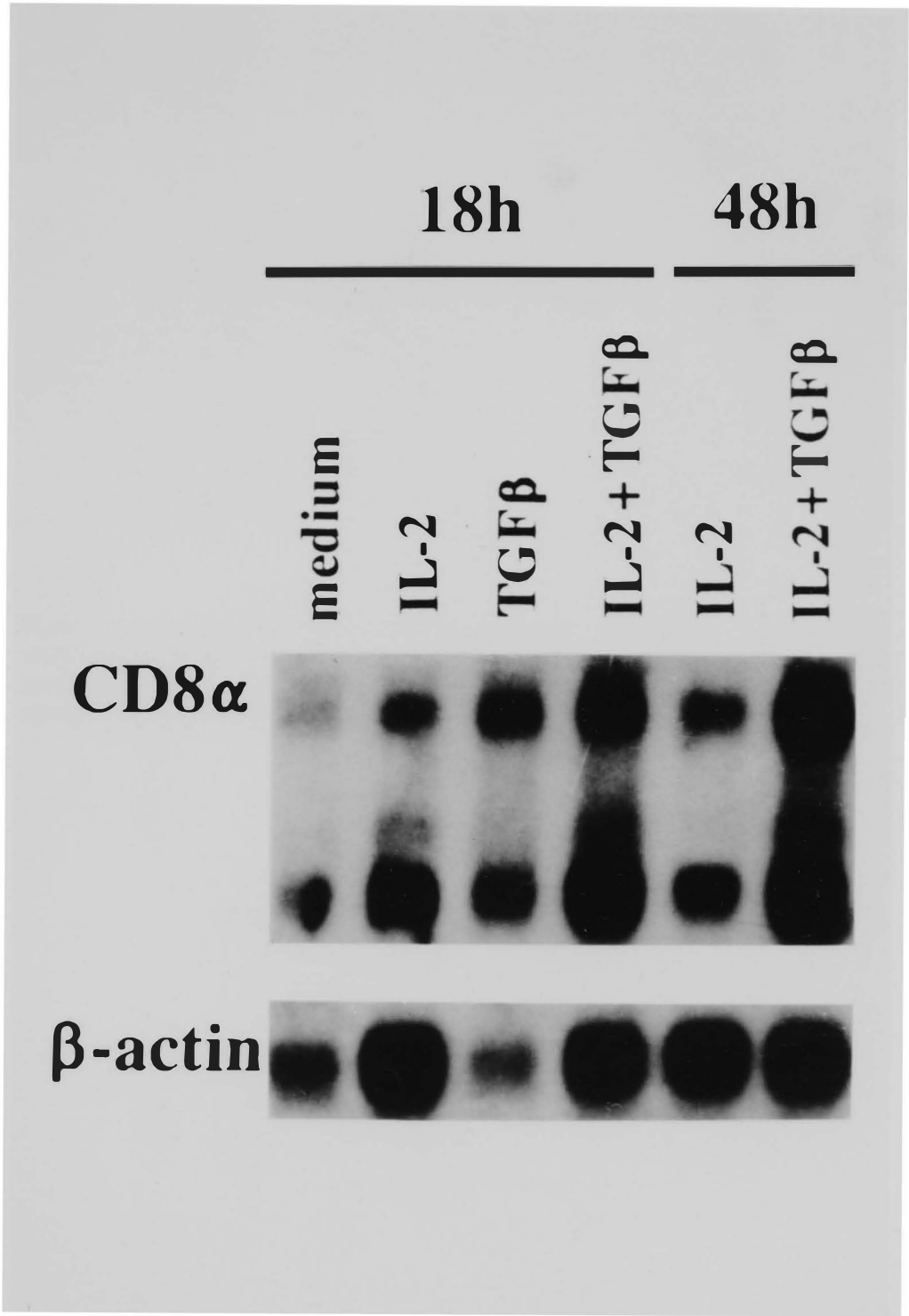

18h
48h
medium
IL-2
TGFβ
IL-2+TGFβ
IL-2
IL-2+TGFβ
CD8α
β-actin

Figure 3.26: CD4 and CD8 expression on cultured DN thymocytes. DN thymocytes were cultured in 20U/ml IL-2 alone (upper panel) or IL-2 + 1ng/ml TGFβ (lower panel) and stained on days 2, 4, and 6 with anti-CD8α and anti-CD4. These data are representative of 5 separate experiments.

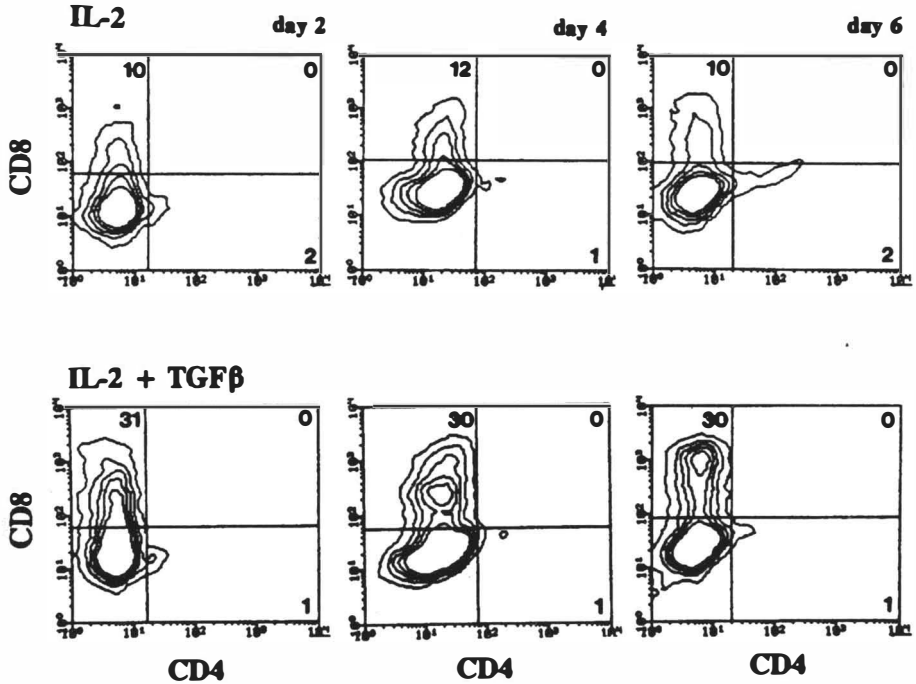
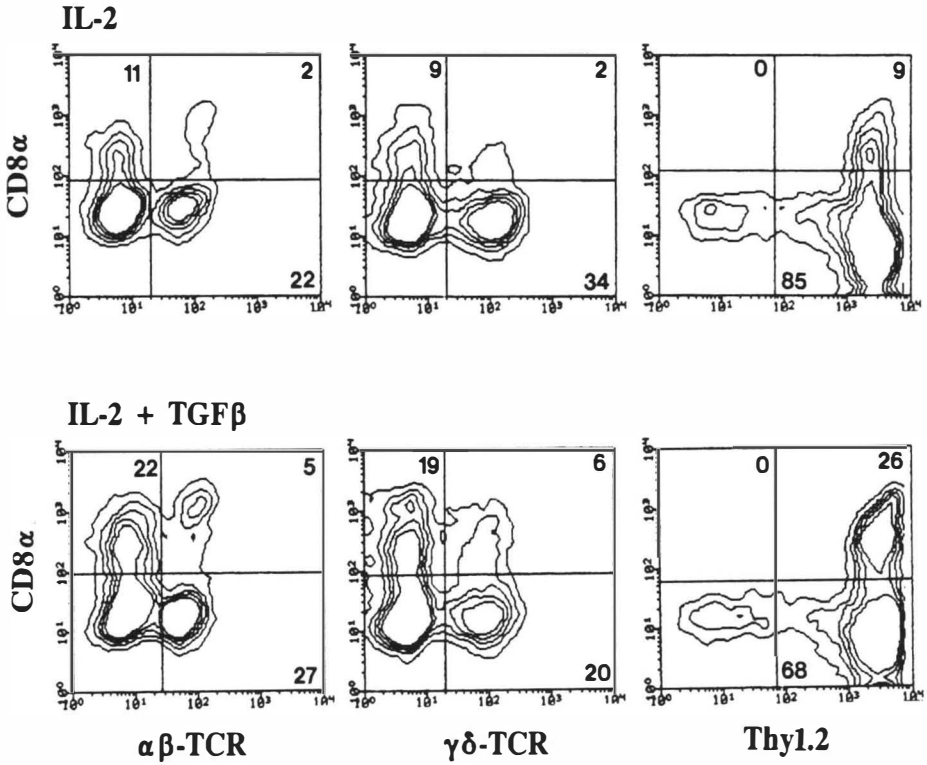

IL-2
day 2
day 4
day 6
CD8
10
0
2
12
0
1
10
0
2
IL-2 + TGFβ
CD8
31
0
1
30
0
1
30
0
1
CD4
CD4
CD4

Figure 3.27: TCR expression on TGFβ-treated DN thymocytes. DN thymocytes were cultured in 20U/ml IL-2 alone (upper panel) or IL-2 + 1ng/ml TGFβ (lower panel) and analyzed on day 4 for surface expression of Thy1, αβ-TCR, or gamma delta-TCR as a function of CD8α immunofluorescence.


IL-2
CD8α
11
2
22
9
2
34
0
9
85
IL-2 + TGFβ
CD8α
22
5
27
19
6
20
0
26
68
αβ-TCR
γδ-TCR
Thy1.2

Literature Cited

Literature Cited

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Bich-Thuy, L.T., Dukovich, M., Peffer, N.J., Fauci, A.S., Kehrl, J.H., and Greene, W.C. (1987). Direct activation of human resting T cells by IL 2: The role of an IL 2 receptor distinct from the Tac protein. J. Immunol. *139*, 1550-1556.

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