Title

Genetic and biochemical analyses of the role of CD8[beta] and CD28 in the differentiation and activation of T lymphocytes

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Publication Date

1996

Peer reviewed|Thesis/dissertation

Genetic and Biochemical Analyses of the Role of CD8β and CD28 in the Differentiation and Activation of T Lymphocytes.

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Immunology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

This work is dedicated to

my beloved wife Vickie

Acknowledgments

During the last six years and the previous twenty-six I've had the fortune to benefit from the efforts of a great many others. For many reasons, both good and bad, a dissertation seems to be treated more like winning an Oscar than winning the Lottery in that it carries a sociological import of the sort that a formal opportunity for giving thanks and recognizing material and spiritual contributions is the norm. Implied in this is the premise that a doctoral dissertation is inherently more significant or valuable than the multitude of other victories that litter the landscape of our lives. Whether this is ultimately the case, I do not know. Nevertheless, it a rare treat that one is given a moment to formally acknowledge and embrace those who have helped so much in that for which they have worked so hard. So here I go.

I'd like to begin by thanking those that got the ball rolling, my family. My mom and dad are likely quite pleased with my reaching this milestone, and they deserve to be, as it is they who made this process seem so simple and natural to me. In hindsight, I don't necessarily feel that any of this has been particularly simple or natural, but the illusion has been very settling. However, it is most likely the presence and example of my siblings that encouraged me to travel down this academic path. For most of my childhood I was the youngest in our family and from my hindmost position I could see what appeared to be a clear division between arduous work and the apparently pleasant lack of work. An obvious venue for not working, while maintaining some semblance of legitimacy and credibility, was school. Not surprisingly my experience has since shown my perception of the comparative comforts of a job versus school to be entirely untrue. This impression nevertheless, on a moment by moment basis, secured my fate.

When reminiscing about the various people that have made me do and be what I am, I'm inclined to recognize a single idea that each individual has shown to me. From my parents I gained confidence, Mike challenged me, Jeff inspired me, Shelly brought me compassion, Rick showed unerring loyalty, and Terri demonstrated that in the face of considerable adversity one can conduct themselves with style and grace. I am fortunate to have such a wonderful family. I'm also grateful for the friendship, encouragement and advice of my dear friends the Squillaces: Ellen, Big and Little Ralph, and Rachel, and also the transient member of the clan Craig Dupler.

As is commonly shown in public service advertisements these days, a number of educators had a lasting positive impact on me (I'll skip the negative impact folks). Notable among those are Mrs. Kobbs, Virgil Rayton, Mr. Christenson, Mrs. Fulton, Ken Davis, Joan Teachman and a Chemistry professor at the University of Washington whose name I will probably never remember (an unsung hero of sorts).

Had my initial laboratory experience been a poor one I most likely would have never entered graduate school. For that I am grateful to Gerry Cangelosi and Gladis Martinetti with whom I worked as an undergraduate in Eugene Nester's lab. I am also fortunate that Dr. Nester directed me towards UCSF for my graduate studies, as otherwise things may have worked out entirely differently for me. I was consistently lucky in my laboratory assignments and had good experiences in my rotations in both Tony DeFranco's and Art Weiss' labs. I am particularly grateful to the members of the Littman lab and particularly Craig Davis, Susan Stuart, and Nigel Killeen. They provided me with an invaluable amount of scientific and personal advice for which I will always be indebted. I also thank Nigel and the members of his lab for the use of their space and their friendship and advice

during the period since Dan has moved to New York. Finally, I wish to thank my aforementioned committee members Tony and Art and my advisor Dan. Dan gave me the freedom to explore, the desire to produce, and served as a talented and ethical example for me to follow.

Any acknowledgment would be incomplete without mentioning the members of the community in which I've now lived for the last six years. Those that have helped me get through the daily drudgery include Berouse at Val de Cole, Al at Peking, all of my friends at Finnegan's, our friends and kittysitters Michael, Shira, Kevin, and Guy, and all of the wonderful people that helped with our wedding, Roger, Kim, Tina, Ken, Clabe, Steve, Guy, and Ralph. I am most grateful to my wife, Vickie, ... for everything.

I know that this is all hopelessly incomplete, but such is the nature of everything.

I must also mention that much of the work contained herein includes previously published and copywritten material. The studies described in Chapter 2 are derived almost entirely from the following reference:

> **Crooks, M. E. C., and Littman D. R.** 1994. Disruption of T lymphocyte positive and negative selection in mice lacking the CD8 β chain. Immunity **1:**277-85.

Similarly, the work described in Chapter 3 is essentially derived from the reference:

> **Crooks, M. E. C., Littman, D. R., Carter, R. H., Fearon, D. T., Weiss, A., and Stein, P. H.** 1995. CD28-mediated costimulation in the absence of phosphatidylinositol 3-kinase association and activation. Molecular and Cellular Biology **15:**6820-8.

Furthermore, many of the figures in Chapter 3 are the result of a collaborative effort. Specifically, the work shown in Figures 9 & 10 was performed entirely by Dr. Peter Stein in Dr. Arthur Weiss' laboratory using reagents supplied by Dr. R. Carter and Dr. D. Fearon. The work shown in Figure 11 was performed entirely by myself, while the remainder of the work in Chapter 3 was performed jointly.

Genetic and Biochemical Analyses of the Role of CD8β and CD28 in the Differentiation and Activation of T Lymphocytes.

Merritt Evan Casey Crooks

Abstract

The CD4 and CD8 coreceptors have been shown to play significant roles in the differentiation and activation of helper and cytotoxic T lymphocytes, respectively. Coordinate binding of coreceptor and T cell antigen receptor (TCR) to the same major histocompatibility complex (MHC) molecule as well as interaction of coreceptor with the tyrosine kinase p56^{lck} are required for effective signal transduction. In the absence of ligand, CD4 is a monomer, whereas CD8 consists of either αα homodimers or αβ heterodimers. Signaling properties of CD8 have been ascribed to the α chain, whose ectodomain and cytoplasmic tail bind to the nonpolymorphic α3 domain of MHC class I and to p56^{lck}, respectively. To specifically study CD8β, we have generated mice defective in its expression. We observe a significant reduction in the numbers of CD8$^+$ T cells present in the thymus and periphery of CD8β-deficient mice but, in contrast to mice lacking CD8α, these animals possess normal cytolytic activity. By breeding CD8β-null mice with animals expressing a class I-specific TCR transgene, we show that CD8β is essential for positive selection and plays a significant role in negative selection of thymocytes expressing this receptor.

In addition to antigen-specific signaling events initiated by TCR-coreceptor recognition of peptide-MHC molecules, costimulatory signals are also required for optimal T cell activation and for overcoming the induction of anergy. These signals can be provided by the homodimeric T cell

glycoprotein CD28 through its interaction with the counterreceptors B7-1 and B7-2 on antigen presenting cells. Ligation of CD28 results in its phosphorylation and the subsequent recruitment of phosphatidylinositol 3-kinase (PI 3-kinase), which has been suggested to be required for costimulation. We report here that ligation of CD19, a heterologous B-cell receptor that also associates with PI 3-kinase upon ligation, failed to costimulate interleukin-2 production. Moreover, pharmacological inhibition of PI 3-kinase activity failed to block CD28-mediated costimulation in Jurkat T cells. By mutational analysis, we demonstrate that disruption of PI 3-kinase association with CD28 also did not abrogate costimulation. These results argue that PI 3-kinase association with CD28 is neither necessary nor sufficient for costimulation of interleukin-2 (IL-2) production in Jurkat cells. Finally, we identify specific amino acid residues required for CD28-mediated costimulatory activity.

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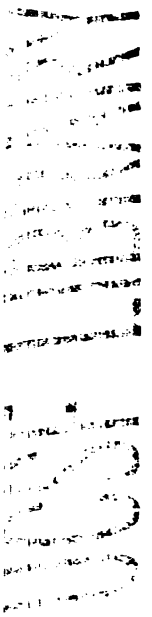
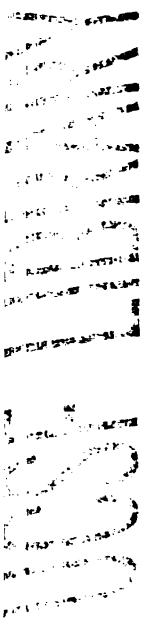
Chapter 1: Prologue

Immune responses can generally be considered to fall into two categories. The first, innate immunity, performs well in responding to challenges "anticipated" for by the coevolution of host and pathogen. This innate immunity, based on a comparatively limited number of genetically fixed determinants, generally lacks the ability to mount a more aggressive response to a secondary challenge from a given immunogen, and frequently fails to respond to novel and unexpected challenges. In higher organisms, innate immunity is augmented by a system of adaptive immunity. A fundamental tenet of adaptive immunity is the requirement for the expression on lymphocytes of remarkable antigen receptor diversity, beyond the reasonable coding capacity of a genome. A consequence of this randomly generated polyclonal population of lymphocytes is the production of a significant number of undesirable nonreactive and potentially autoreactive precursors. Such heterogeneity in the precursor population necessitates a mechanism for selecting potential responders on the basis of their reactivity. This process of generating diversity, selecting the useful, and eliminating the dangerous is perhaps best illustrated in the intrathymic generation and shaping of the T cell repertoire.



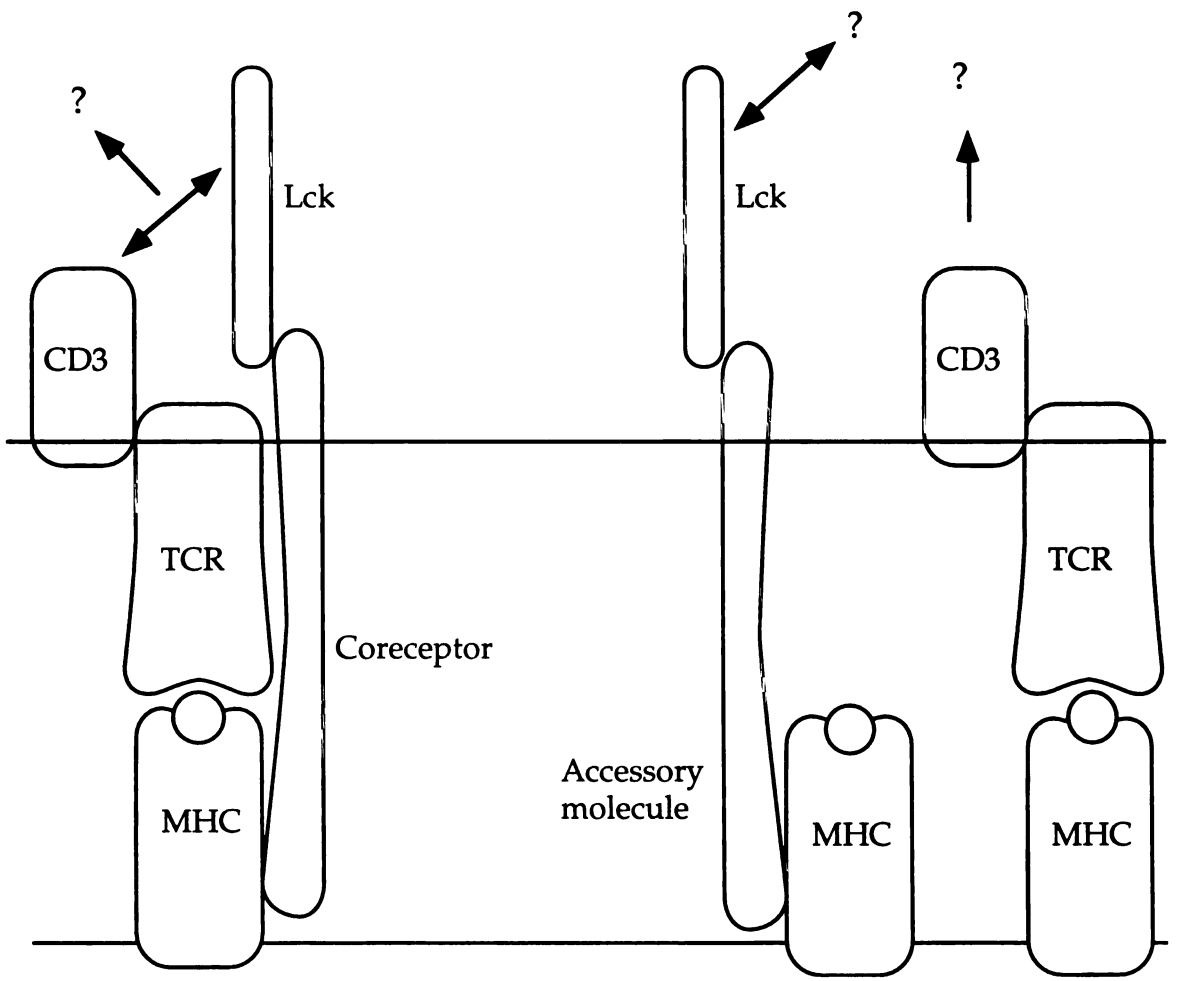
Positive selection of T cells has been shown to involve the specific recognition of peptide-MHC molecules by TCRs expressed on $CD4^+CD8^+$ double positive (DP) thymocytes. Initial observations of positive selection were made by analyzing the MHC restriction patterns in radiation bone marrow chimeras (10, 36, 156, 195). Donor bone marrow from heterozygous (A x B) haplotype animals was introduced into homozygous A haplotype hosts. The T cell specificities that emerged in these animals were observed to be restricted to the host MHC haplotype (A) rather than both the A and B haplotypes of the donor. These results indicated an active mechanism of

positive selection existed that was specific for the host haplotype. These phenomena were further defined with the advent of TCR transgenic mice (178). In a series of experiments using a transgenic T cell receptor specific for a Y chromosome encoded male antigen (H-Y) presented by MHC class I H-2D^b molecules, von Boehmer and colleagues confirmed the working paradigm of positive and negative selection. These experiments showed that the H-Y-specific clonotype was positively selected in female mice of the H-2^b background, deleted in the autoreactive H-2^b males, and absent from the periphery in animals of an irrelevant, or nonselecting, H-2^d haplotype (79, 165). This determination of cell fate driven by TCR specificity for peptide-MHC is a central component in determining the self-tolerance and utility of the T cell repertoire. In addition to the TCR, other coreceptor and accessory molecules play a critical role in the differentiation and activation of T lymphocytes.

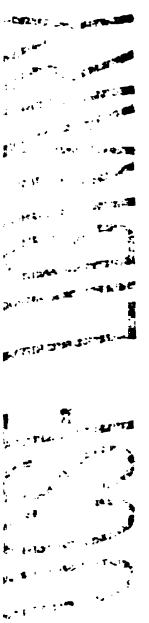


In a series of antibody blocking and transfection studies, the CD4 and CD8 molecules were observed to play important roles in the development and activation of T cells. Initially it was observed that antibodies against either CD4 or CD8 could block the effector function of helper and cytotoxic T cells respectively (72, 110, 113, 115). This suggested that these molecules play an important role in the activation of their respective T cell subsets. These observations coupled with the known segregation of CD4 to MHC class II-specific helper and CD8 to MHC class I-specific cytotoxic T cells suggested that CD4 and CD8 might bind directly to their respective MHC molecules. This hypothesis was confirmed in transfection studies, which led to the development of the *accessory molecule* and *coreceptor* hypotheses for CD4 and CD8 function (Figure 1)(33, 67, 121).

Figure 1. The coreceptor and accessory molecule models of CD4 and CD8 function. The coreceptor model (left) argues that interactions between CD4 or CD8 are with the same MHC molecule recognized by the TCR. The accessory molecule model (right) proposed that CD4 or CD8 interact with MHC independently of TCR recognition. The coreceptor model allows for stabilization of TCR-MHC binding and cooperative interactions between coreceptor-associated and TCR-associated molecules.



The accessory molecule model argued that CD4 or CD8 interact with MHC molecules independently from the TCR, while the coreceptor model hypothesized that the interaction is with the same MHC molecule recognized by the TCR. The accessory molecule hypothesis was supported by observations using somatic hybrid T cell lines that expressed CD4 and could respond to MHC class I molecules (47, 50). The ability of these cells to respond to MHC class I was enhanced if the APCs also expressed MHC class II molecules. Moreover, this enhanced response could be blocked by antibodies against either CD4 or class II MHC. These observations were consistent with the notion that CD4 could function independently from the TCR in potentiating a T cell response. This hypothesis did little, however, to explain the tight association between MHC class II-specificity and CD4 expression.



Meanwhile, evidence rapidly accumulated that supported a coreceptor model for CD4 and CD8 function. Although transfection studies showed that coexpression of coreceptor with the TCR was necessary to confer MHC-specific reactivity in a number of T cell hybridomas, these experiments did not distinguish between an accessory or coreceptor function for CD4 and CD8 (32, 46, 49). More direct evidence was provided using a panel of antibodies specific for distinct epitopes of a defined TCR and CD4. It was observed that some antibodies directed towards the TCR resulted in co-capping of CD4 into the TCR complex, indicating a physical association between the TCR and CD4 (72, 73, 139). Furthermore, it was observed that certain anti-CD4 Fab fragments could inhibit responses to certain anti-TCR antibodies although these cells would still respond to the lectin PHA. Similar results were obtained in fluorescence energy transfer experiments and with observations of CD4-TCR comodulation, supporting the idea that the coreceptor physically associates with the TCR upon antigen recognition. Formal demonstration for the

coreceptor model for both the differentiation and activation of T lymphocytes was shown in transgenic mice. It was shown in mice transgenic for both the H-Y-specific TCR and a selecting H-2D^b molecule that a mutant MHC molecule unable to bind CD8 failed to support positive selection or peripheral activation of cells bearing this receptor, even though wild-type H-2^d molecules were available for CD8 ligation (1, 62, 78).

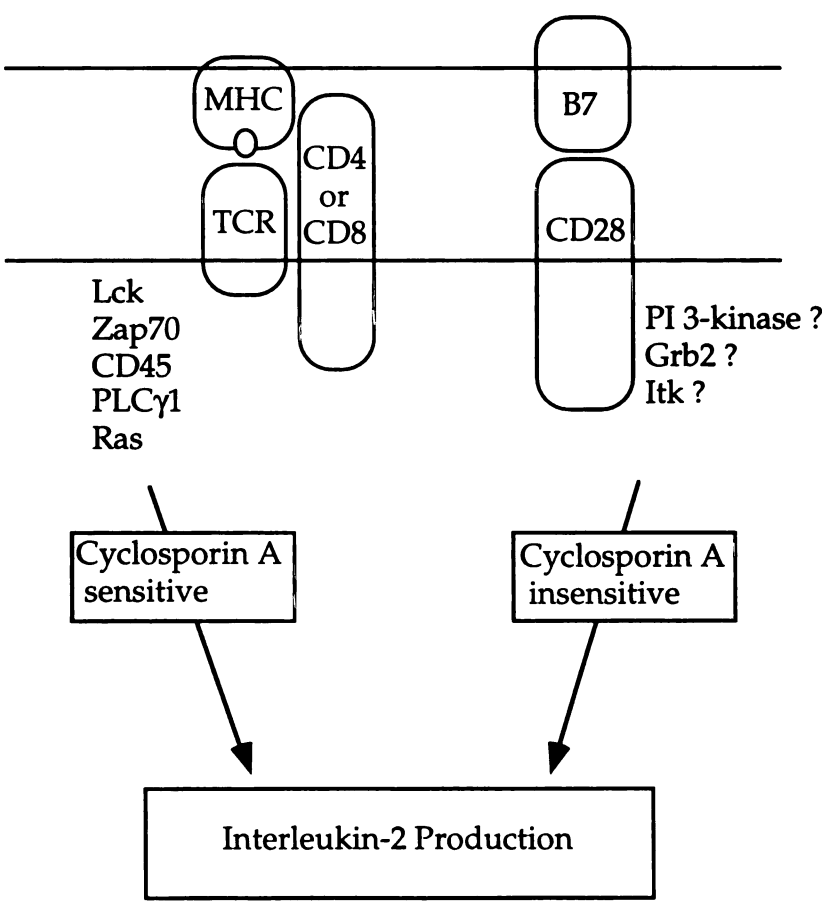
In addition to TCR-coreceptor interactions with MHC, other accessory molecules appear to be required to potentiate T cell effector function. In a series of experiments in the 1980's Schwartz and colleagues demonstrated that antigen receptor stimulation alone was not adequate to induce T cell responses (68, 116, 134, 144). Using a T cell clone specific for pigeon cytochrome c presented by B10.A APCs it was shown that the T cells could be rendered persistently unresponsive upon exposure to antigen by chemically fixed APCs or by planar lipid membranes. This unresponsive state, called clonal anergy, can persist for more than a week. This effect was clonally specific and appeared to reflect a defect in IL-2 production, as the IL-2 receptor signaling pathway still functioned in these cells. It is important to note, however, that the presence of exogenous IL-2 in these cultures did not prevent the induction of anergy, indicating that additional events are also involved in the generation of anergy. Injection of antigen and chemically fixed APCs into mice was similarly found to be toleragenic, suggesting that clonal anergy may serve as an important mechanism for peripheral T cell tolerance.

To explain these phenomena, it was proposed that two distinct signals provided by APCs were required to induce T lymphocyte effector function. While the primary signal is provided by TCR recognition of peptide-MHC, the secondary signal may be delivered by distinct molecular interactions

between T cells and APCs. In other experiments, it was shown that ligation of the T cell glycoprotein CD28 could significantly augment the production of IL-2 mediated by TCR stimulation (6, 51, 71, 187). It was subsequently shown that CD28 ligation triggers a distinct cyclosporin A(CsA)-insensitive signal transduction pathway that synergizes with TCR-derived signals in potentiating a T cell response (Figure 2)(2, 133, 141). Because of the distinct nature of the signals and the potent augmentation of interleukin-2 biosynthesis mediated by CD28, it is believed to play an important costimulatory role in T cell activation.

In providing a costimulatory signal for T cell activation, it has been shown that CD28 functionally interacts with the counterreceptors B7-1/B7-2 (9, 41, 42), which are differentially expressed on APCs (97). A number of experiments have demonstrated the importance of costimulation through CD28 in T cell activation. Anti-CD28 Fab fragments can block the costimulatory effects of accessory cells on T cell clones activated in vitro with submitogenic doses of anti-CD3 antibodies (54). In contrast, intact anti-CD28 mAbs can prevent the induction of anergy in T cell clones, and can provide a costimulatory signal to purified T cells in the induction of lymphokine production and proliferative responses. Interfering with CD28 ligation in vivo, using a soluble CTLA-4 molecule that binds B7-1/B7-2 with high affinity, can block or dramatically delay tissue transplant rejection as well as suppress humoral responses (98, 103). Mice lacking CD28, due to targeted gene disruption, show significant immune system defects consistent with disrupted T cell function including depressed T cell responses to lectins, low total serum Ig, and markedly altered concentrations of serum Ig isotypes (149). It has also been shown that tumors transfected with the CD28 ligand

Figure 2. Two signal model of CD28-mediated costimulation of IL-2 production. A primary signal is generated by T cell antigen receptor recognition of peptide-MHC. TCR stimulation is known to involve the rapid induction of PTKs, activation of PLC and the cyclosporin A-sensitive induction of IL-2 production. A secondary signal provided by CD28 can augment IL-2 production in a cyclosporin A-insensitive manner, although the specific mechanisms of CD28 signaling are poorly understood.

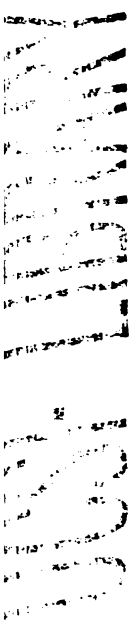


B7-1, but not untransfected parent tumors, are rapidly cleared from mice (24, 170). CD28, therefore, clearly plays an important role in the induction of T lymphocyte mediated immune responses. CD28 shares ligand specificity and a considerable degree of homology with the T cell glycoprotein, CTLA-4 (55).

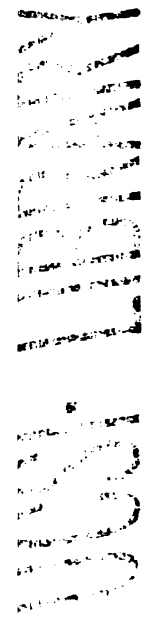
The expression of CTLA-4, which is induced upon T cell activation, has been shown to mediate inhibitory signals (180). Blocking CTLA-4 ligation in vivo with the use of monoclonal antibodies allows for the efficient clearance of otherwise unmanipulated tumors in mice (91). CTLA-4 stimulation in vitro has been shown to have a dominant effect over CD28 ligation in the induction of IL-2 production and cell cycle progression (85, 86). That the role of CTLA-4 in T cell activation is an inhibitory one is most clearly demonstrated in mice defective for its expression (168, 184). These animals exhibit massive lymphoproliferation leading to considerable tissue destruction and death. The shared ligand specificities, opposing regulatory roles, and differential temporal expression of CD28 and CTLA-4 indicate that these molecules cooperate in maintaining a dynamic regulation of T cell responses. A biochemical basis for the negative role of CTLA-4 has been suggested by recent experiments that show that CTLA-4 can interact with the tyrosine phosphatase Syp, which can act on Shc and thus negatively regulate Ras activity (112).

Collectively, the coreceptors and costimulators work to restrict the circumstances for T cell differentiation and activation. While the CD4 and CD8 coreceptors participate in the specific recognition of MHC molecules, CD28 participates in the specific recognition of activated antigen presenting cells. While both CD8 and CD28 serve to restrict and regulate the outcome of TCR encounter with MHC, the mechanism and roles of each are quite distinct. These studies will initially focus on the role of the β chain of the

CD8 coreceptor on the development and activation of cytotoxic T cells. Subsequently, the molecular requirements for the coupling of the CD28 molecule to the intracellular signaling machinery in peripheral T cells will be examined.

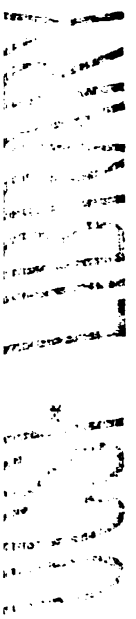


Chapter 2: Genetic and Biochemical Analysis of the Role of CD8β in the Differentiation and Activation of Cytotoxic T Lymphocytes



Introduction

T cell development can be considered to involve two major phases: 1) the generation of novel T cell receptor clonotypes through gene rearrangement and template-independent joint modification, and 2) the subsequent screening of this diverse repertoire of TCRs by mechanisms of positive and negative selection. During the first phase, RAG gene expression leads to the rearrangement of the TCRβ chain and its export to the surface with unrearranged surrogate pre-Tα molecule(s). Upon successful β chain rearrangement and expression, allelic exclusion is exerted upon the β locus, and TCRα gene rearrangement progresses as CD4 and CD8 expression is induced (4, 66). Cells that have successfully rearranged TCRα gene segments, and thereby express a novel TCR clonotype, may then participate in the second phase of development involving interaction with peptide-MHC complexes in the combined presence of CD4 and CD8 .



Experiments in organ culture systems monitoring the development of TCRs of defined specificity have shown that the overall avidity of the TCR interaction with peptide-MHC can determine the developmental fate of thymocytes (7, 58, 145). This avidity model of selection argues that TCRs with an avidity above a certain threshold are deleted, those with an intermediate degree of avidity are positively selected, and programmed cell death via neglect awaits thymocytes bearing TCRs of inadequately low avidity. In addition to the TCR-MHC based determinants of selection, the CD4 and CD8 coreceptors have been shown to play a pivotal role in this process by affecting both the clonality and functional phenotype of the peripheral repertoire (44, 96, 136, 138, 163). Although both CD4 and CD8 are expressed during the

$CD4^+CD8^+$ (double positive or DP) stage of development, they are expressed individually on mature CD4 single positive (SP) helper and CD8 SP cytotoxic T cells (76, 125, 179).

A considerable amount of structural data is available for the molecules involved in TCR-MHC-coreceptor interactions. Both CD4 and CD8 are type I transmembrane glycoproteins and members of the immunoglobulin superfamily (130). In the absence of ligand, CD4 is expressed as a monomer consisting of four extracellular immunoglobulin-like domains that extend approximately 130Å from the cell membrane (92, 182). The extracellular domain of CD4 binds to a membrane-proximal nonpolymorphic region of MHC class II proteins, while that of CD8 binds to an analogous region of MHC class I (82, 121, 141). The cytoplasmic domains of both CD4 and CD8α interact with the cytoplasmic protein tyrosine kinase $p56^{lck}$ through a shared Cys-X-Cys motif in their cytoplasmic domains (150, 173).

Although CD8 is functionally analogous to CD4 , it is structurally quite different. CD8 is generally expressed as either disulfide linked αα homodimers or αβ heterodimers with each chain containing a single immunoglobulin-like domain that extends approximately 30Å (92, 93). As CD4 and CD8 bind to analogous membrane proximal regions of their respective MHC molecules, it is believed that the membrane proximal region of CD8 must achieve an extended configuration in order to approximate the size of CD4 and span the length of the TCR-MHC interaction. This is consistent with the observation that this region of CD8 contains significant O-linked glycosylation which may serve to protect or stabilize the extended region (21). Transfection studies have shown that homodimers of CD8α are sufficient for binding to MHC class I and for interacting with $p56^{lck}$. Furthermore, in a CD8-dependent T cell hybridoma CD8α homodimers have

been shown to be sufficient for coreceptor function and to enable CD8-Lck dependent responses to suboptimal doses of antigen (150, 173, 194).

While the function of CD8αα homodimers is thought to be relatively well-understood, little is known of the function of CD8αβ heterodimers. Since surface expression of CD8β is dependent on the coexpression of CD8α, the function of CD8αβ may primarily reflect the properties of the α chain. That CD8αβ heterodimers have a specific function has been suggested by the finding that they are more effective that αα homodimers in facilitating signal transduction in a T cell hybridoma (188). Moreover, a chimeric molecule consisting of the extracellular domain of CD8β and the transmembrane and cytoplasmic domains of CD8α also functioned as a coreceptor, suggesting that CD8β may interact with MHC class I molecules in the absence of the α chain. CD8β has not yet been shown to physically interact with any molecules other that CD8α, but the strong cross-species conservation of its 19 amino acid cytoplasmic domain suggests that this region may have an important function in protein-protein interactions.

Interestingly, thymus-derived T cells express predominantly CD8αβ heterodimers whereas extrathymically-derived $CD8^+$ cells, such as gut-derived intraepithelial T lymphocytes and natural killer cells, express only CD8αα homodimers (53, 120, 169). The significance of CD8 expression patterns in thymus-dependent versus thymus-independent T lymphocytes is not known. One possibility is that CD8αβ heterodimers have a specific role in the differentiation and activation of thymus-derived T cells. This would suggest that CD8αα homodimers may have different signaling functions than CD8αβ heterodimers.

Genetic approaches to the study of the CD4 coreceptor have shown that targeted disruption of CD4 gene expression results in a sharp reduction in the

appearance of $CD8^-$ T cells in the periphery (136). Although some helper activity is observed in these animals, there is a significant overall defect (105). The helper function observed in CD4-null mice is likely derived from the significantly increased number of $CD4^-CD8^-$ (double negative or DN) T cells in these animals. The helper lineage can be completely restored in these animals by expressing elevated amounts of a CD4 molecule that cannot bind the tyrosine kinase $p56^{lck}$ (77). Considering that both CD4-null mice and those overexpressing a truncated CD4 molecule successfully generate CD4 lineage cells, it is clear that the interaction of CD4 with Lck is not obligate for CD4-lineage T cell development.

Similar genetic studies concerning the role of the CD8 coreceptor in development have also been performed. Mice lacking CD8 expression due to the targeted disruption of the CD8α gene show a severe block in cytotoxic T cell (CTL) development (44). Indeed, these studies have indicated that there is a more stringent requirement for CD8 expression in CTL differentiation than that for CD4 in helper T cell development. CD8-null mice lack any functional cytotoxic T lymphocytes as determined by a number of criteria including: no significant increase in $CD4^-CD8^-$ T cell populations as observed in CD4-null mice, loss of class I-specific alloreactivity, failure to clear viral pathogen, and failure to develop CD8-dependent responses in parasitic disease models (44, 128). Reconstitution of CD8 expression with a mutant form of CD8 unable to bind Lck rescues the development of CD8 lineage cells in a manner similar to that observed for the CD4 lineage (22, 43). Therefore, although the requirement for CD8 expression or CTL development appears more stringent than that for CD4 in helper development, it can nevertheless dispense with the Lck binding domain as is the case in CD4 lineage development.

Additional experiments examining the role of the CD8 coreceptor in T cell development have shown that CD8 expression levels can determine the fate of thymocytes bearing TCRs of defined specificity. In thymocytes expressing the 2C TCR allospecific for H-2L^d, increased expression of CD8 resulted in the deletion of this receptor in an otherwise positively selecting H-2^b background (96, 138). Interestingly, it was observed that although 10-fold overexpression of CD8α molecules was necessary for deletion of this receptor , coexpression of CD8α and CD8β at levels 2-fold greater than wild-type similarly resulted in 2C receptor deletion. This suggests that the β chain of the CD8αβ heterodimer may play a significant role in the ability of CD8 to affect cell fate.

In addition to participating in the positive and negative selection of developing thymocytes, the potential role for coreceptors in lineage determination is a topic of great interest. It has been proposed that CD4 and CD8 transmit distinct instructional signals that contribute to the lineage commitment of DP thymocytes (14, 137). A considerable amount evidence has accumulated, however, indicating that there is a stochastic component to T cell development. In mice lacking MHC class I, class II, or both, it has been shown that the presence of either class of MHC molecules is sufficient to generate intermediate populations of either CD4^{hi}CD8^{lo} or CD4^{lo}CD8^{hi} cells (23). However, in the absence of MHC, these populations do not develop, arguing that encounter with MHC of either class is sufficient to allow partial progression towards either lineage. A prediction of the stochastic model is that some thymocytes bearing, for example, MHC class II-specific TCRs will stochastically downregulate CD4 and perish due to inappropriate coreceptor expression. A number of experiments utilizing the ectopic expression of either CD4 or CD8 to rescue cells bearing these mismatched coreceptor-TCR

specificities have been performed. It has been shown that both $CD4^+$ class I-specific and $CD8^+$ class II-specific T cells can be rescued in this manner indicating that, at some level, cells bearing mismatched coreceptor-TCR-MHC specificities occur in a manner consistent with a stochastic mechanism for lineage determination (30, 132). It is generally the case, however, that efforts to rescue the development of stochastically derived cells expressing a mismatched endogenous coreceptor results in recoveries lower than would be expected with an entirely stochastic mechanism. This has resulted in the persistence of the notion that some instructional component of lineage determination may exist. As CD8α is more directly analogous to CD4 in both MHC and Lck binding, the discriminating factor between CD4 and CD8 coreceptor function in any instructional event may possibly reside with the CD8β chain.

Recent experiments using a novel coreceptor re-expression assay have cast doubts on the presumptive fate of the coreceptor-intermediate populations of thymocytes observed in the MHC-deficient mice described above (160). Investigators fractionated intermediate populations of thymocytes, stripped them of surface coreceptor expression by pronase treatment, and examined their coreceptor re-expression profiles after in vitro culture. It was observed in these experiments that there was a differential degree of lineage commitment between $CD4^{hi}CD8^{lo}$ and $CD4^{lo}CD8^{hi}$ populations. While all of the intermediate populations of cells could be derived from $CD4^{hi}CD8^{lo}$ cells, only $CD8^+$ cells could be recovered from $CD4^{lo}CD8^{hi}$ cells. Moreover, it was observed in mice bearing a class I-specific TCR and defective for MHC class I expression that $CD4^{hi}CD8^{lo}$ cells could not differentiate into CD8 SP cells, whereas they could in mice expressing the appropriate MHC class I ligand. CD4 SP cells, however, did not appear to

share this requirement, suggesting that a default pathway towards the CD4 lineage may exist and function in parallel with an instructional mechanism for CD8 lineage determination. Similar results have recently been obtained in vivo by following the fate of sorted populations after intrathymic transfer, indicating that these results are not likely to be an artifactual result of pronase treatment and in vitro culture (109). If an instructional event is required for CD8 lineage commitment then such a property may well reside with the CD8β chain. If so, one would predict that in the absence of CD8β, CTL development would be blocked.

In order to directly assess the role of CD8β in the development and activation of cytotoxic T lymphocytes, we have generated mice defective for its expression. We observed a sharp reduction in the number of CD8 SP cells in the thymus and periphery. Although there was a significant reduction in cellularity, we observed no differences in Vα and Vβ gene segment expression in these animals. To follow the fate of a specific receptor, we bred these CD8β-deficient animals to mice bearing a defined MHC class I-specific TCR and observed a complete block in positive selection and a less severe disruption of negative selection of this receptor. We analyzed the function of the CD8 lineage cells that do develop in these mice by assaying their ability to respond to allogeneic targets. We found that both the proliferative and cytolytic properties of CD8 SP cells in these mice appeared normal. As the CTLs that developed in the absence of CD8β may have compensated for this defect during selection, the actual role of CD8β in peripheral responses remains unclear.

Results

Generation of CD8β-deficient Mice.

CD8β-deficient mice were generated by standard techniques using the 129/Sv embryonic stem (ES) cell line, J1 (16, 99). A targeting construct that replaces most of exon 2 and part of intron 2 in the CD8β locus with the gene encoding neomycin resistance was introduced into the genome of ES cells by homologous recombination (Figure 3A and Experimental Procedures). This modification results in the deletion of 37% of the coding sequence of the mature protein including most of the immunoglobulin-like domain and all of the J-like region (119). Of 120 G418 resistant clones that were analyzed by southern and PCR analysis, two were found to contain the targeted mutant allele and were used for blastocyst injection. The chimeric animals generated were subsequently back-crossed to C57BL/6J mice and agouti progeny bearing the targeted allele were interbred. Southern analysis of tail biopsies from wild-type, heterozygous and CD8β-null animals showed the predicted conversion of a 16 kb wild-type BamHI fragment to a 10 kb mutant fragment, as detected by an external probe derived from the CD8β cDNA (Figure 3B). Additional PCR experiments were performed to confirm the fidelity of recombination. PCR analysis using primers specific for sequences internal to neomycin, internal to the deleted portion of exon 2, and spanning the 3' arm of homology all yielded results consistent with a unique double-reciprocal recombination event (data not shown). Cytofluorometric analysis of lymph node cells showed a consistent reduction of approximately 15% in CD8β fluorescence intensity in $CD8\beta^{+/-}$ as compared to wild-type mice (Figure 3C). Null animals, as expected, expressed no immunoreactive CD8β.

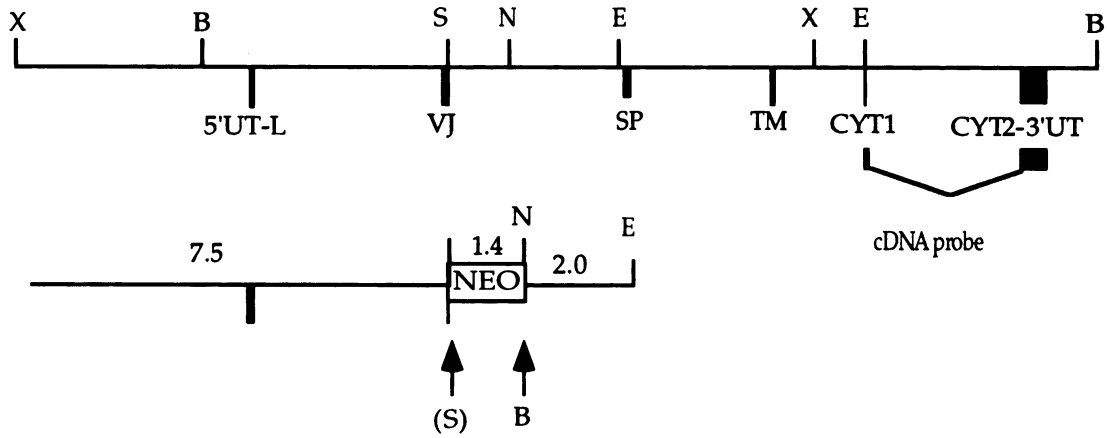
Figure 3. Targeted Disruption of the CD8β Gene by Homologous Recombination

(A) Schematic showing the genomic organization of the murine CD8β gene and the targeting strategy. A ScaI-NcoI fragment containing most of exon 2 of the CD8β gene was replaced with the gene encoding neomycin resistance. Closed boxes represent exons with abbreviations denoting structural domains within each exon: 5'UT-L, 5' untranslated and leader sequence; VJ, IgV-like and J-like regions; H, hinge-like region; TM, transmembrane domain; CYT1, first cytoplasmic exon; CYT2-3'UT second cytoplasmic exon and 3' untranslated region. X, XhoI; B, BamH1; S, ScaI; N, NcoI; E, EcoRI; NEO, pgkNeo in same transcriptional orientation.

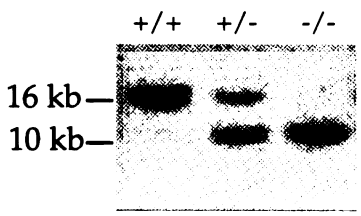
(B) Southern blot of DNA from tail biopsies of wild-type, heterozygous and homozygous mutations at the CD8β locus using a cDNA probe shown above. The introduction of a BamHI site at the 3' side of neo results in a reduction of a wild-type 16 kb fragment to a mutant 10 kb fragment.

(C) Cytofluorometric analysis showing CD8β immunoreactivity on lymph node cells from wild-type, heterozygous and CD8β-null mice. Data shown was electronically gated on $CD8\alpha^+$ cells.

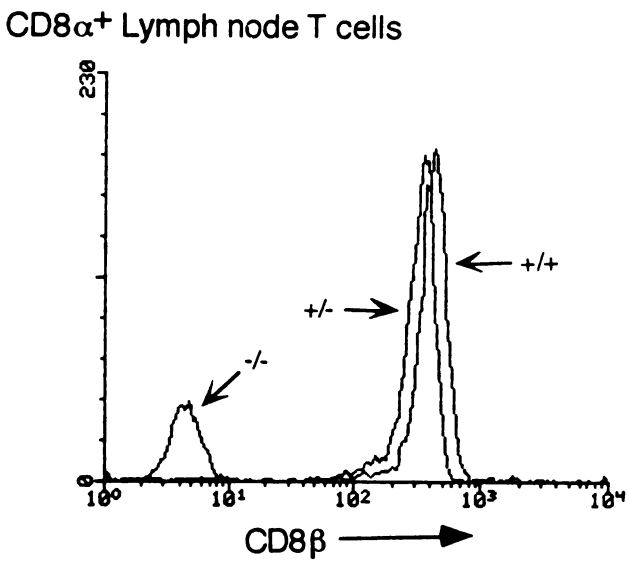
A


X
B
S
N
E
X
E
B
5'UT-L
VJ
SP
TM
CYT1
CYT2-3'UT
cDNA probe
7.5
N
1.4
E
2.0
NEO
(S)
B

B


+/+
+/-
-/-
16 kb
10 kb

C


CD8α+ Lymph node T cells
230
+/+
+/-
-/-
0
10⁰
10¹
10²
10³
10⁴
CD8β

Analysis of T cell Subsets in CD8β-null Animals

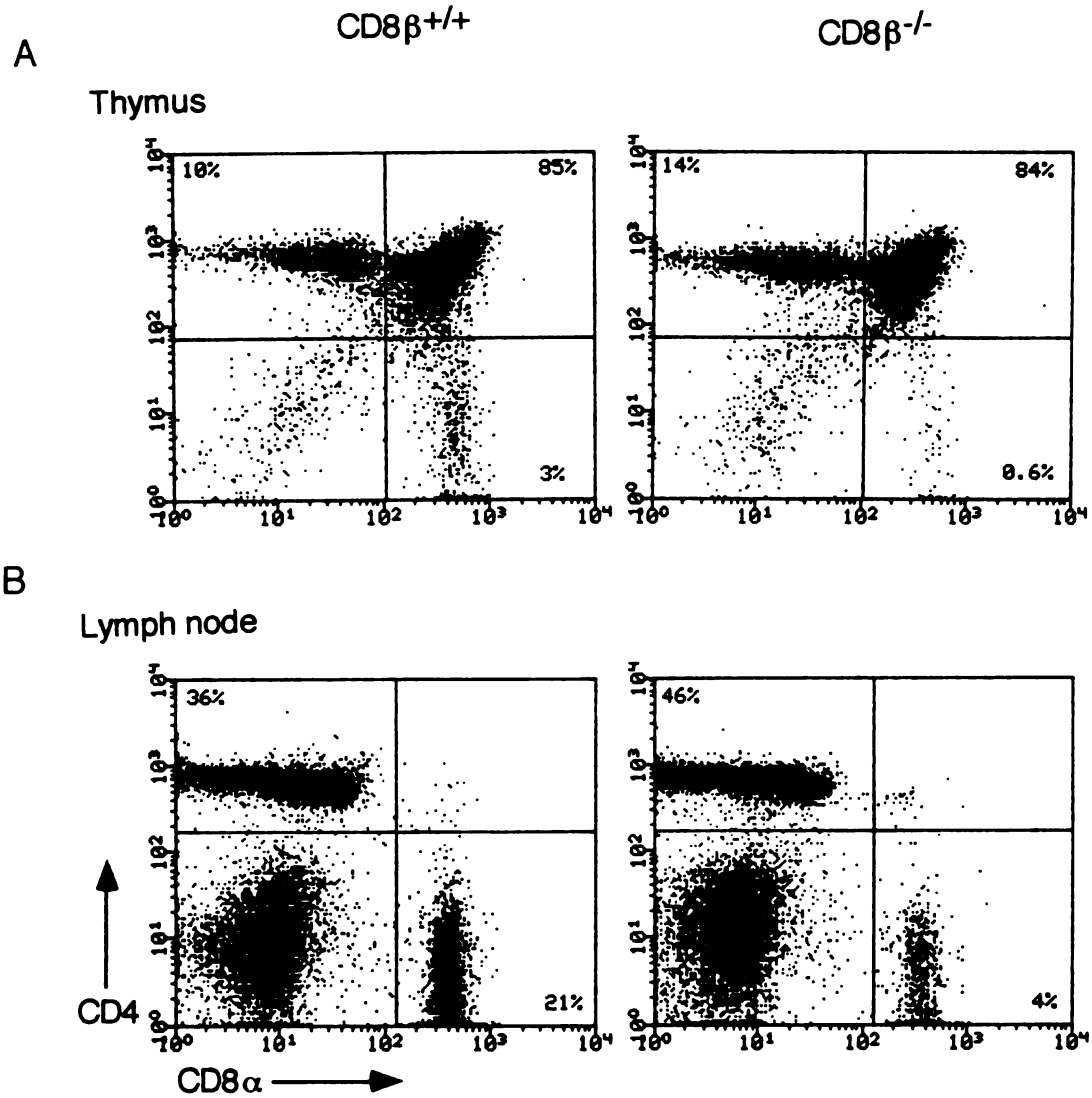
Thymocytes from CD8β-null mice and wild-type litter mates were analyzed by flow cytometry after staining with antibodies specific for CD4 and CD8α. There was a consistent 5-fold decrease in the relative number of mature $CD4^-CD8^+$ cells in the thymus of CD8β-null animals, with a corresponding increase in the numbers of $CD4^+CD8^-$ cells (Figure 4A). The $CD4^-CD8^+$ thymocytes present in the mutant mice expressed normal levels of CD69 and heat stable antigen (HSA) as well as high levels of TCRαβ, consistent with their having undergone positive selection (data not shown)(27, 127, 162). The immature $CD4^+CD8^+$ thymocytes from homozygous mutant mice had a mean fluorescence intensity of CD8α staining that was typically 35% less than that observed in heterozygous or wild-type litter mates. However, the CD8α level on $CD4^-CD8^+$ thymocytes was the same in mutant and wild-type animals.

The reduction in the number of mature $CD4^-CD8^+$ thymocytes was reflected in the periphery of the $CD8\beta^{-/-}$ mice, in which a similar 5-fold reduction in the relative number of $CD4^-CD8^+$ lymph node cells was observed (Figure 4B). The level of CD8α on the surface of peripheral T cells was the same in wild-type and CD8β-null animals. In an effort to determine if the disruption of $CD8^+$ T cell development was accompanied by skewing of T cell clonality, the TCR repertoire of $CD8^+$ T cells in the periphery of these animals was examined for any gross abnormality. No notable difference in TCR Vα3.2, Vα11, Vβ3, Vβ7, Vβ8, and Vβ11 was observed between CD8β-null and wild-type litter mates. Although these animals exhibit a significant block in $CD8^+$ T-cell development, the heterogeneity of the peripheral repertoire in this initial analysis appears to be intact.

Figure 4. Reduction in the Relative Numbers of CD8$^+$ Cells in CD8β-null Mice

(A) CD4 and CD8α expression on thymocytes of wild-type (left) and CD8β-null (right) mice. A 5-fold reduction in the relative number of CD8 SP cells (lower right quadrant) was observed.

(B) CD4 and CD8α expression on lymph node T cells from wild-type (left) and CD8β-null (right) mice.



Effect of CD8β-ablation on Positive Selection of a Defined MHC Class I-specific TCR

To further examine the requirements for CD8β in positive selection, we analyzed the fate of thymocytes expressing a single well-defined transgenic TCR in the CD8β-null animals. We chose to study development of T cells expressing a TCR specific for the male antigen H-Y in the context of the MHC class I molecule H-2D^b (11). This system permits analysis of positive selection in females and negative selection in males expressing the correct MHC molecule.

Thymocytes expressing the H-Y-specific TCR undergo positive selection and differentiate into CD4$^-$CD8$^+$ T cells in H-2^b female mice (165). This process was severely impaired in mice lacking CD8β. There was a 20-fold reduction in the number of CD4$^-$CD8$^+$ thymocytes in CD8β$^{-/-}$ versus CD8β$^{+/-}$ female mice expressing the transgenic TCR (Figure 5A). A similar effect was observed in peripheral lymphoid organs of CD8β$^{-/-}$ mice, with a 7-fold reduction in CD4$^-$CD8$^+$ T cells (Figure 5B). Since there is appreciable rearrangement of endogenous TCRα chain genes and usage of the protein product in TCR transgenic animals (14), we used the clonotype-specific monoclonal antibody T3.70 to assess expression of the H-Y-specific TCR in peripheral CD8$^+$ T cells. High levels of this TCR were present on CD8 single positive T cells from CD8β$^{+/-}$ animals, indicating that these cells had undergone positive selection mediated by this receptor (Figure 5C, left panel). In contrast, mice lacking CD8β had no CD4$^-$CD8$^+$ T cells that expressed a high level of the clonotypic TCR (Figure 5C, right panel). Thymocytes expressing the H-Y-specific TCR, therefore, were completely dependent on CD8β for successful completion of positive selection.

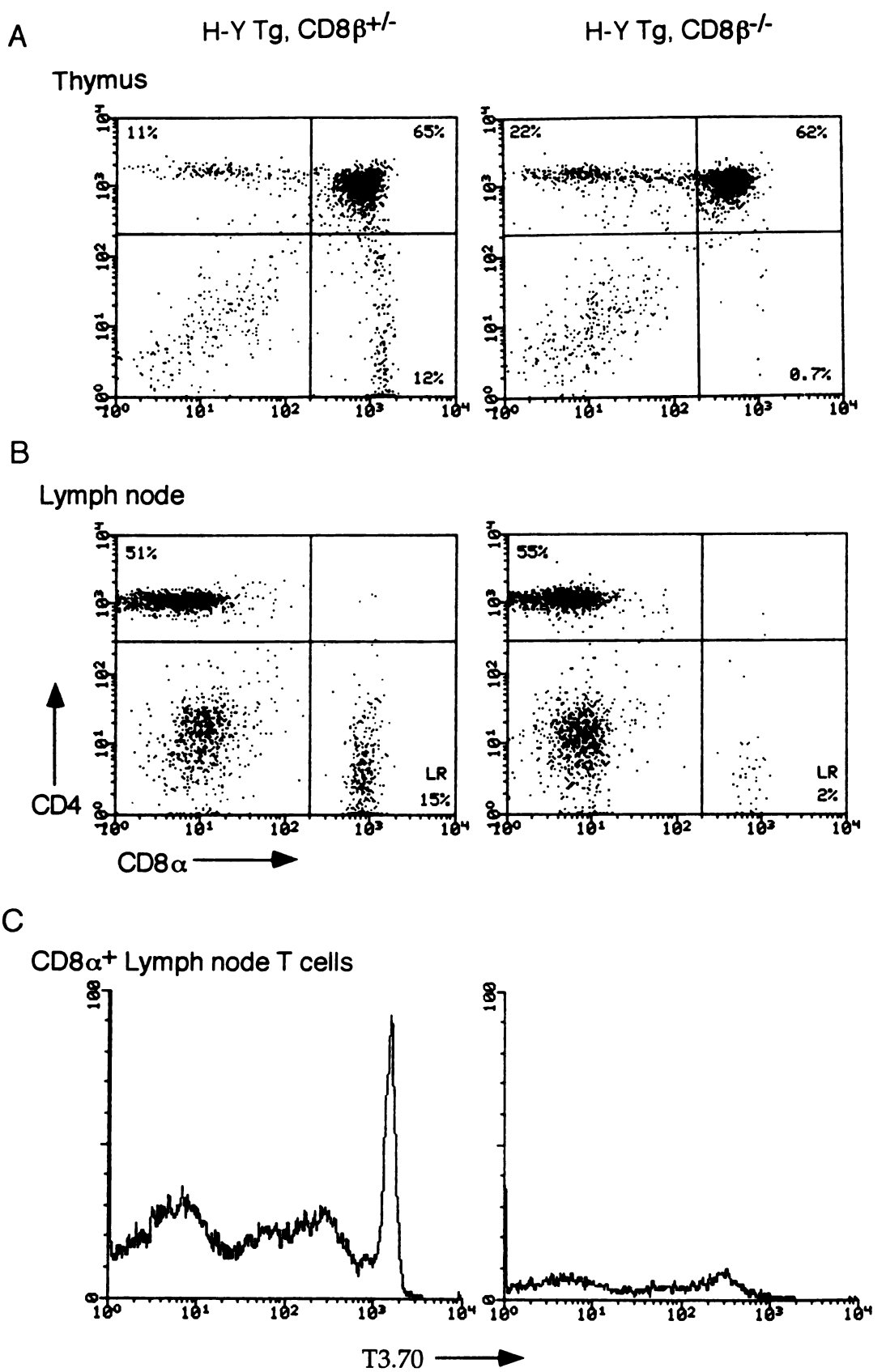
Figure 5. Requirement for CD8β in Positive Selection of the H-Y-specific TCR

(A) CD4 and CD8α expression on thymocytes from female $H2^b$ mice expressing the H-Y-specific TCR transgene. A reduction in the relative number of CD8 SP cells (lower right quadrant) was observed.

Total number of thymocytes ($x10^7$, n=3) in these animals were: $CD8\beta^{+/-}$, 12.4 +/- 3.4; $CD8\beta^{-/-}$, 6.6 +/- 0.5.

(B) CD4 and CD8α expression on lymph node cells from the female mice. Similar to the thymus, there was a 7-fold reduction in the relative number of CD8 SP cells (lower right quadrant).

(C) Expression of the clonotypic TCR on CD8 SP lymph node cells. Cells electronically gated for CD8α expression (LR in 5B) were stained with the T3.70 monoclonal antibody specific for the H-Y TCR.


A
H-Y Tg, CD8β+/-
H-Y Tg, CD8β-/-
Thymus
11%
65%
12%
22%
62%
0.7%
B
Lymph node
51%
LR
15%
55%
LR
2%
CD4
CD8α
C
CD8α+ Lymph node T cells
T3.70

Negative Selection in the Absence of CD8β

The role of CD8β in intrathymic clonal deletion was assessed by analyzing the fate of cells expressing the H-Y-specific transgenic TCR in male mice. In these animals, developing thymocytes interact with the male specific antigen presented by H-2D^b, resulting in early negative selection (79). Deletion in TCR transgenic animals is accompanied by a large reduction in the size of the thymus due to a sharp reduction in the total number of CD4$^+$CD8$^+$ thymocytes. Flow cytometric analysis of CD4 and CD8 expression in the thymus of male CD8β$^{+/-}$ H-2D^b H-Y-specific TCR transgenic animals showed a typical deletion profile with severe disruption in the relative and absolute numbers of CD4$^+$CD8$^+$ thymocytes (Figure 6A). In contrast, there was a significant recovery in the relative and absolute number of CD4$^+$CD8$^+$ thymocytes in H-Y-specific TCR transgenic male mice that lacked CD8β, suggesting that negative selection was impaired.

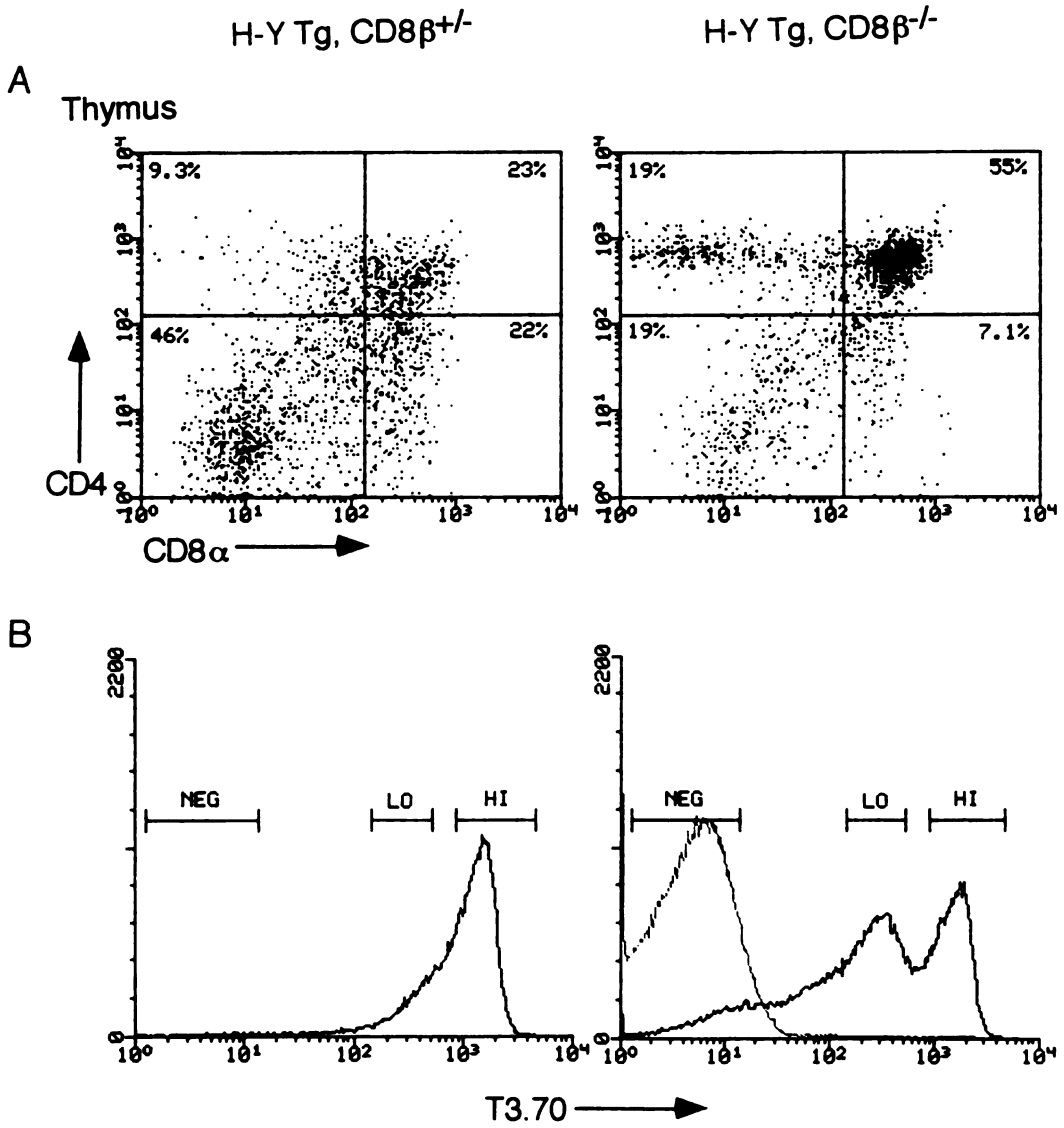
As previously observed, most thymocytes in the deleting environment expressed high levels of the transgenic TCRα, as shown by staining with the T3.70 monoclonal antibody (Figure 6B, left panel). In the CD8β-null mice, there were numerous T3.70^{lo} thymocytes that include the CD3^{lo} DP cells found in normal animals (153) (Figure 6B, right). Analysis of CD4 and CD8 expression on thymocytes electronically gated on T3.70^{hi} cells in CD8β-null animals recapitulated the deletion profile observed in mice expressing CD8β (Figure 6C). Gating on T3.70^{lo} cells, however, revealed a staining profile similar to that observed in non-TCR transgenic CD8β-null animals shown previously (Figure 4A) - mostly DP cells and a significant number of CD4 SP cells. Gating on T3.70^{neg} thymocytes showed a large increase in the CD4 SP mature thymocyte population. Thus, CD8β ablation results in a significant impairment of negative selection, allowing accumulation of

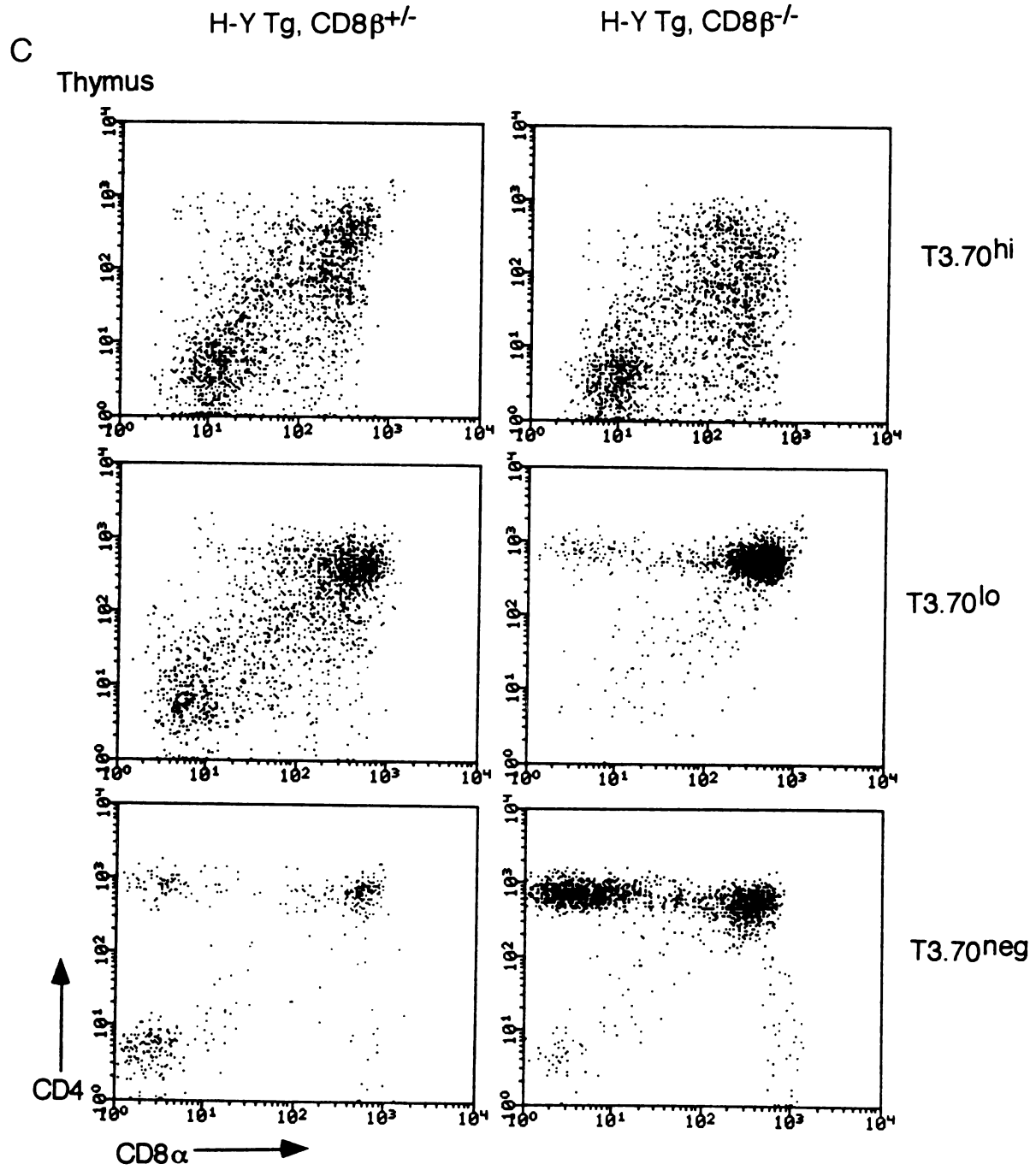
Figure 6. Clonal Deletion of the H-Y-specific TCR in the Absence of CD8β

(A) CD4 and CD8α expression on thymocytes from male $H2^b$ mice with the H-Y-specific TCR transgene. A significant recovery in the number of $CD4^+CD8^+$DP cells was observed in CD8β-null mice (compare percentages in upper right quadrants). Total number of thymocytes ($x10^7$, n=3) in these animals were: $CD8\beta^{+/-}$, 1.8 +/- 0.5; $CD8\beta^{-/-}$, 3.9 +/- 0.3.

(B) T3.70 (anti-H-Y-specific TCR) expression on thymocytes from male H-Y-specific TCR transgenic mice. The gray trace in the right panel represents background staining in a non-TCR transgenic mouse.

(C) CD4 and CD8α expression on thymocytes electronically gated on T3.70 expression levels as shown in (B): $T3.70^{hi}$ (upper panel), $T3.70^{lo}$ (middle panel) or $T3.70^{neg}$ (lower panel).


H-Y Tg, CD8β+/-
H-Y Tg, CD8β-/-
A
Thymus
9.3%
23%
46%
22%
19%
55%
19%
7.1%
CD4
CD8α
B
NEG
LO
HI
NEG
LO
HI
T3.70


C
Thymus
H-Y Tg, CD8β+/-
H-Y Tg, CD8β-/-
T3.70hi
T3.70lo
T3.70neg
CD4
CD8α

$CD4^+CD8^+T3.70^{lo}$ thymocytes that, due to rearrangement of endogenous TCRα chains, can subsequently be positively selected on the basis of other novel TCR specificities (131). The mature thymocytes were mostly CD4 single positive, presumably due to the additional requirement for CD8β in positive selection of CD8 lineage cells. The disruption of negative selection in mice lacking CD8β was incomplete however, as the relative and absolute numbers of $CD4^+CD8^+$ thymocytes were only partially restored (Figure 6A).

Analysis of the lymph nodes in H-Y-specific TCR transgenic CD8β-null male mice confirmed the observations in the thymus. The relative number of $CD3^+$ lymphocytes was increased by approximately 50% (Figure 7A). Examination of CD4 and CD8 expression on $CD3^+$ cells showed a substantial increase in the relative number of $CD4^+$ lymphocytes, whose development was presumably enhanced due to the reduced efficiency of deletion (Figure 7B). By gating on $T3.70^{hi}$ cells, it was demonstrated that these $CD4^+$ T cells do not express high levels of the H-Y-specific TCR (Figure 7C). Furthermore, there were no $CD8^{hi}$ $T3.70^{hi}$ cells in these animals, although there were many $T3.70^{hi}$ cells that were $CD8^{lo}$ or double negative, as previously found in the periphery of deleting male TCR transgenic mice (164). Hence, deletion of thymocytes expressing the autoreactive H-Y-specific TCR is occurring in CD8β-null animals, but it is sufficiently impaired to allow the positive selection of cells that utilize endogenous TCR α chains.

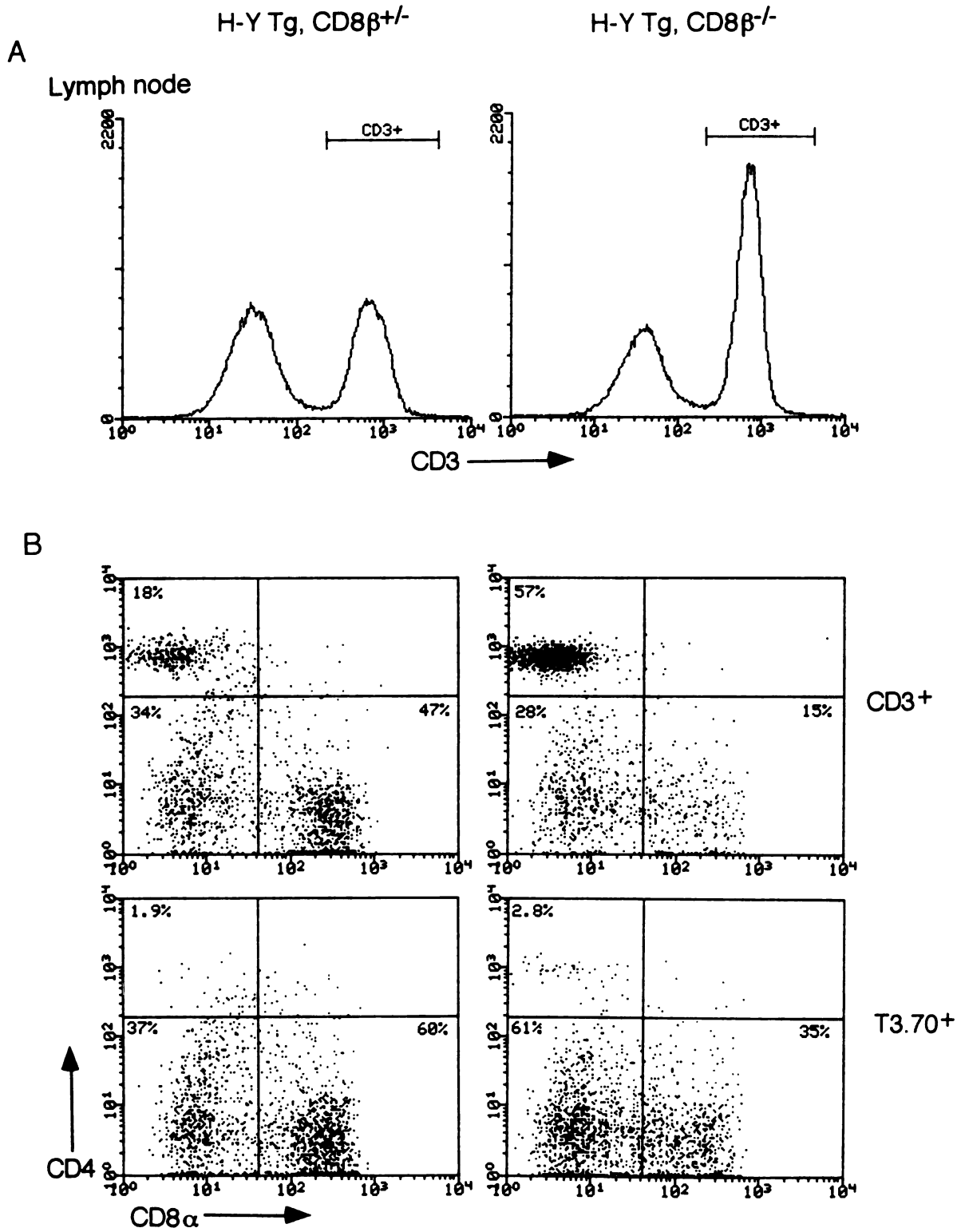
Function of Peripheral $CD8^+$ T cells in Mice Lacking CD8β

To characterize the function of peripheral $CD8^+$ cells in mice lacking CD8β, we tested the ability of these cells to mount a cytolytic response to allogeneic stimuli. Splenocytes from $CD8\beta^{+/-}$ and $CD8\beta^{-/-}$ mice ($H\text{-}2^b$) were

Figure 7. Peripheral T Cells in Male TCR Transgenic Mice Lacking CD8β

(A) CD3 expression levels on lymph node cells from male H-Y-specific TCR transgenic $CD8\beta^{+/-}$ (left) and $CD8\beta^{-/-}$ (right) mice.

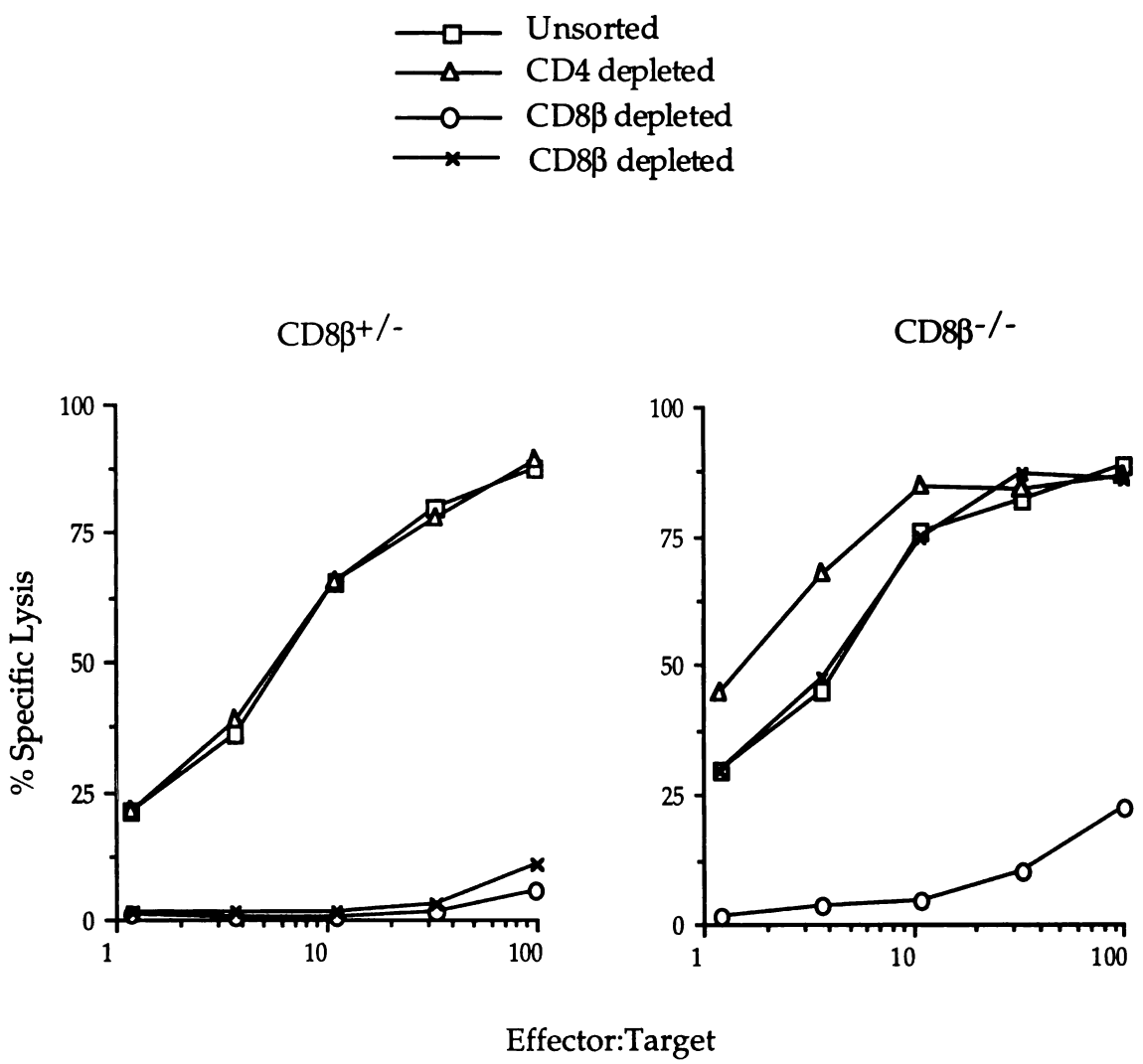
(B) CD4 and CD8α expression on lymph node T cells electronically gated on $CD3^+$ cells (top). CD4 and CD8α expression on lymph node T cells electronically gated on $T3.70^+$ cells (bottom).


H-Y Tg, CD8β+/-
H-Y Tg, CD8β-/-
A
Lymph node
CD3+
CD3+
CD3
B
18%
34%
47%
57%
28%
15%
CD3+
1.9%
37%
60%
2.8%
61%
35%
T3.70+
CD4
CD8α

stimulated with H-2^d-expressing cells and tested for their cytolytic activity on appropriate targets. Despite having less than 20% of the number of $CD8^+$ T-cells found in normal animals, $CD8\beta^{-/-}$ mice mounted a normal CTL response, comparable to that of $CD8\beta^{+/-}$ litter mates (Figure 8). To determine which lymphocyte population was responsible for the allogeneic cytolytic response, we depleted from pools of stimulated splenocytes those cells that expressed CD4, CD8α, or CD8β and assessed the cytotoxic activity of the remaining cells. In $CD8\beta^{+/-}$ mice, cytolytic activity was eliminated by removing cells with antibodies against either CD8α or CD8β. Similarly, cytolytic activity was eliminated from splenocytes of $CD8\beta^{-/-}$ mice by depleting with anti-CD8α antibody. Depletion of cells expressing CD4 had no significant effect on killing of target cells. Hence, as in normal mice, the substantial CTL activity observed in mice lacking CD8β can be attributed to the $CD8^+$ T-cell subset.

Figure 8. Cytolytic Activity in Mice Lacking CD8β

Splenocytes from CD8β$^{+/-}$ and CD8β$^{-/-}$ mice (H-2^{b}) were stimulated with γ-irradiated splenocytes from C57BL/6J x DBA/2 F1 mice (H-2$^{b/d}$) after which they were depleted of the cell type indicated in the legend and tested for their ability to lyse P815 (H-2^{d}) target cells. No appreciable cytolysis was observed against EL-4 (H-2^{b}) targets. Results are shown as the mean of triplicate samples.



Discussion

The function of the CD8β polypeptide has been a long-standing puzzle because in vitro experiments have shown that CD8α alone can provide MHC class I-specific coreceptor function. In this study, we have demonstrated that CD8β is required for efficient selection processes during thymocyte maturation, but not for antigen-specific responses by mature $CD8^+$ cytotoxic T cells. CD8αβ heterodimers thus appear to have an important role in thymus-dependent development of the MHC class I-restricted cytotoxic T cell lineage.

The Role of CD8β in Repertoire Selection

The requirement for CD8αβ heterodimers during development is not absolute, since as many as 20% of the normal level of CD8 lineage cells develop in the absence of the β chain. In contrast, development of thymocytes bearing the H-Y-specific transgenic TCR was completely dependent on the expression of CD8β. Negative selection of cells with this receptor was also notably impaired in the absence of CD8αβ heterodimers. These observations are consistent with previous studies showing that the H-Y-specific TCR is particularly dependent on CD8-class I interaction for both positive and negative selection, suggesting that it has relatively low affinity for $H\text{-}2D^b$ (45, 78). Class I-restricted T cells that develop in the absence of CD8β may thus have receptors with relatively high affinity for host peptide-MHC.

These results are in many ways analogous to those observed in CD4-null animals. In these mice, there is a significant population of $CD4^-CD8^-$ cells that have apparently normal helper activity and are transcriptionally active for CD4 subset-specific gene expression (105). Therefore, in both CD4-

null and CD8β-null mice positive selection is reduced to about 20% of normal levels and results in the appearance of functionally competent helper and cytotoxic T cells, respectively.

The phenotype of CD8β-null mice differs in some potentially important aspects from that reported for CD8α knock-out mice (44). In the absence of CD8α and, hence, of any CD8 molecules on the cell surface, there was no detectable cytolytic T cell activity. This may simply reflect the complete absence of CD8 lineage cells in these mice, but may also be due to a requirement for CD8 homodimers or heterodimers in the function of peripheral T cells. Recently, it has been shown that CTLs with a $CD4^-CD8^-$ $TCR\alpha\beta^{lo}$ phenotype can be generated in CD8α-deficient animals by in vivo priming, consistent with experiments showing a residual allogeneic cytotoxic activity in mice lacking both CD8α and CD4 (29, 142). As the existence of these cells is comparatively difficult to demonstrate, the requirement for CD8α expression in normal T-cell development still appears to be more stringent than that observed for CD4 and CD8β.

Recent studies have provided strong support for the notion that positive and negative selection signals are dependent on the relative avidities of interaction between TCR complexes and peptide-MHC on thymic epithelial cells (7, 145). The level of coreceptor expression can clearly contribute to this process. For example, overexpression of CD8α or both CD8α and CD8β resulted in conversion from positive selection to negative selection of thymocytes expressing a class I-restricted transgenic TCR (96, 138). Comparison of these earlier studies suggests that clonal deletion occurs at lower levels of CD8 overexpression if CD8β is expressed in addition to CD8α. Consistent with these observations, we have shown here that loss of CD8β diminished the efficacy of negative selection of cells expressing an

autoreactive TCR. In the transgenic TCR system, this permitted rescue of T cells that would otherwise have been deleted before reaching the $CD4^+CD8^+$ stage in the thymus. These thymocytes thus had the opportunity to rearrange their endogenous TCRα chains, allowing them to be selected by MHC class II and to mature into CD4 lineage cells.

A prediction of the avidity model is that a reduction in avidity that would result in a deletional signal would instead result in positive selection of the same cell. This was not observed in the CD8β-null mice expressing the autoreactive transgenic TCR. In these animals, negative selection of cells expressing the transgenic TCR may still occur, but may be delayed to a later stage of development. This raises the possibility that the signal for clonal deletion may have different avidity requirements at different stages of thymic development. A very high avidity interaction may thus be required for deletion of cells as they begin to express CD8, but an interaction of lower avidity may be sufficient to deliver a similar signal once cells have reached the double positive stage and have begun to rearrange their TCRα genes. It is noteworthy that although there is ample evidence to show that positive selection of a particular clonotype can be converted into negative selection, the opposite has not been observed. For example, although CD8α is required for the positive selection of all defined class I-specific TCRs examined, it is differentially required for deletion of these receptors. In the case where deletion is blocked by the lack of CD8α (as judged by thymus size and subset distribution), these receptors still do not reach the periphery (45). Similar results were obtained when coreceptor contribution was reduced by selecting on a chimeric MHC molecule bearing a human α3 domain (151). This suggests that either a significant safety margin exists between avidities required for positive and negative selection or that negative selection may

involve a qualitatively distinct signal. This possibility is supported by pharmacological and biochemical evidence suggesting that the signals mediating positive and negative selection are distinct (161, 181).

In view of the fact that the level of CD8 expression determines the nature of the selection signal, it is important to note that the CD8β$^{-/-}$ mice exhibited an approximate reduction of 35% from wild-type in the relative fluorescence intensity of CD8α staining of immature CD4$^+$CD8$^+$ thymocytes. This observation is difficult to interpret, since we do not know the stoichiometry of monoclonal antibody binding to CD8αα versus αβ dimers. Nevertheless, this finding raises the possibility that disruption of CD8 lineage differentiation in these mice is a consequence of a decrease in the level of surface CD8 dimers rather than a CD8β-specific effect. Two points argue to the contrary: in CD8α heterozygous animals an even greater reduction, approximately 50%, in CD8 fluorescence intensity on CD4$^+$CD8$^+$ thymocytes had no significant effect on positive selection (44); and mice expressing a transgenic CD8β lacking its cytoplasmic domain had reduced positive selection of CD4$^-$CD8$^+$ cells expressing the H-Y-specific TCR, even though the level of CD8α expression in CD4$^+$CD8$^+$ thymocytes remained unchanged (64). Together these observations imply a specific role for CD8β in positive selection and suggest that part of this function is mediated via its cytoplasmic domain.

Role of Coreceptors in Lineage Commitment

In addition to their role in selection, coreceptors may also function in determining lineage commitment of bipotential DP thymocytes to CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ functionally differentiated single positive T cells (31). As discussed above, some helper and cytotoxic lineage cells can develop in the

absence of CD4 or CD8β, consistent with the notion that lineage commitment occurs by a stochastic process. It is still possible, however, that instructional signals can be superimposed on the stochastic process to bias the decision towards a particular lineage. The finding that CD8αβ heterodimers have an important role in positive selection raises the intriguing possibility that they, rather than αα homodimers, provide discriminatory signals that function to bias $CD4^+CD8^+$ thymocytes toward the CD8 lineage. Alternatively, the CD8 heterodimers may function more effectively than homodimers in facilitating T cell maturation after a stochastic event that results in down-regulation of one of the coreceptors; this may occur through enhancement of the avidity of TCRs for peptide-MHC. Recent experiments that argue for an asymmetric commitment model suggest that CD8 lineage development requires an instructional event while CD4 lineage development does not. While an instructional component for CD8 lineage development may exist, our results indicate that either CD8β is not necessary for such a process or that a stochastic process may function in the absence of instructional signals that allow for the development of some CD8 lineage cells. Although CD8β may contribute to an instructional event, there does not appear to be an obligate qualitative contribution of CD8β to lineage commitment.

CD8 Function in the Periphery

The CD8α and β genes are closely linked and are coordinately regulated during development of the thymus-dependent TCRαβ lineage (130). In thymus-independent lineages, such as natural killer cells and gut-derived intraepithelial lymphocytes bearing αβ and γδ TCRs, only CD8α is expressed. The difference in regulation of the CD8 locus in the thymus-dependent and -independent lineages suggests that CD8β may have a specific role in

thymocyte maturation. The finding in CD8β-null mice of CD8 lineage cells that have normal CTL activity after migration to the periphery is consistent with this notion.

Our results on the role of CD8β in positive selection are qualitatively similar to those recently observed in an analysis of chimeric mice (118). However, the results differ quantitatively, as we observe a less severe reduction in CD8 lineage cells. This difference may reflect a competitive disadvantage of mutant cells developing in parallel to wild-type cells in the chimeric mice. This raises important questions regarding the ability to accurately assess the absolute requirements for immune system development in a chimeric environment.

Experimental Procedures

Construction of the Targeting Vector

A λDASH II genomic library derived from 129/Sv mice (a gift of Dr. P. Soriano) was screened using the murine CD8β cDNA. A number of overlapping clones comprising the entire CD8β gene were isolated. One such clone was used for generating a targeting construct. A 2.0 kb NcoI-EcoRI fragment internal to intron 2 of the CD8β gene was blunt end ligated onto the 3' end of pgkNeo and a 7.5 kb fragment encompassing the 5′ end of the CD8β gene and extending from a NotI site in the polylinker to a ScaI site in exon 2 was subsequently ligated onto the 5′ end of pgkNeo. The resulting construct was named pLyt3.Targ1 and used for subsequent targeting experiments (Figure 4A).

Generation of CD8β Mutant ES Cells.

J1 ES cells (a gift of Drs. E. Li and R. Jaenisch) were maintained, transfected, and selected as described (16, 99). Briefly, 20 μg of linearized pLyt3.Targ1 was introduced into $2x10^7$ ES cells in 0.8 mls by electroporation in a Bio-Rad Gene Pulser at 250 mV, 250 μF. The cells were plated onto four 10 cm tissue culture dishes containing $4x10^6$ mitomycin C treated neo^r primary embryonic fibroblasts. After 36 hours the cultures were placed under G418 selection at a final active concentration of 180 μg/ml. Selection was maintained for 9 days after which individual colonies were picked with a 20 μl micropipet and trypsinized for 5 minutes at 37°C in 25 μl 25 mM HEPES buffered (pH 7.3) Hank's Balanced Salt Solution containing 0.5% trypsin and 1 mM EDTA. The ES cells were then dispersed into 24 well plates after which

G418 selection was removed. After 3 days, one-half of the cells were harvested and frozen and the other half were used for DNA preparation as described (89)].

Southern and PCR Analysis.

Southern analysis of putative recombinant clones was performed using a 450 bp EcoRI fragment of the CD8β cDNA that corresponded to 18 bp of exon 5 and all of exon 6 of the CD8β gene (119). This probe hybridizes to a 16 kb wild-type and a 10 kb mutant BamHI fragment (Figure 4A, B). Additional PCR analysis using primers internal to the deleted region (sense 5'-AAGTACTTTGAGTTCCTGGCC-3', antisense 5'-CACAGTCAGCTTCGTCCCTG-3'), internal to neo (sense 5'-ATTGAACAAGATGGATTGCAC-3', antisense 5'-CGTCCAGATCATCCTGATC-3'), and external to the 3' arm of homology (neo sense 5'-CTTGACGAGTTCTTCTGAGGGGA-3' and exon 3 antisense 5'-GTTGGGGCAGTTGTAGGAAGGACATCAA-3') was performed to insure that no abnormal rearrangements had occurred. PCR specific for neo and the deleted region was performed in the same reaction for 35 cycles at 94°C 1', 60°C 1', 72°C 1'. Conditions used for PCR across the 3' arm of homology were 94°C 1', 58°C, 1', 72°C, 2.5'.

Generation of CD8β Deficient Mice.

Generation of mice defective for CD8β was performed as described (16, 99). C57BL/6J blastocysts were isolated 3.5 days post coitus, injected with 15-20 ES cells, and 6 blastocysts were transferred into each uterine horn of 2.5 day pseudopregnant foster mothers. Offspring were judged chimeric on the basis of agouti coat color and male animals were back crossed onto C57BL/6J mice.

Progeny mice with germline transmission as identified by agouti coat color, were screened for the presence of the mutant CD8β allele by southern analysis of DNA from tail biopsies. These animals were interbred to homozygosity. All animals were maintained in specific pathogen free facilities.

Flow Cytometric Analysis and Antibodies.

Flow cytometry was performed by staining 10^6 freshly isolated thymocytes or lymphocytes (from cervical, axillary, brachial and mesenteric lymph nodes) in 100 µl of PBS supplemented with 0.3% BSA and 0.01 % sodium azide on ice for 30 minutes. For three color analysis, cells were first incubated with a cocktail of antibodies including normal mouse serum and subsequently with Streptavidin-Tricolor (Caltag Laboratories). Antibodies used were: anti-CD4 (YTS 191.1-phycoerythrin (PE)) and anti-CD8α (YTS 169.4-fluorescein isothiocyanate (FITC)) from Caltag Laboratories, anti-CD69 (H1.2F3-biotin), anti-HSA (M1/69-biotin), anti-TCRαβ (H57-597-biotin), anti-Vα3.2 (RR 3-16-biotin), anti-Vα11 (RR 8-1-biotin), anti-Vβ3 (KJ-25-biotin), anti-Vβ7 (TR310-biotin), anti-Vβ8 (MR5-2-biotin), anti-Vβ11 (RR3-15-biotin), anti-CD8β (53-5.8-FITC) and anti-CD3ε (145-2C11-PE), all from Pharmingen. Anti-H-Y-specific-TCR (T3.70-biotin) was a gift of Dr. H.-S. Teh. Data was collected on a Becton-Dickinson FACScan equipped with LYSYS II software.

CTL Assays

Splenocytes from 8-10 week old CD8β$^{+/-}$ (H-2$^{b/b}$) or CD8β$^{-/-}$ (H-2$^{b/b}$) mice were pooled and incubated at a 1:1 ratio with γ-irradiated (2800 rad) splenocytes from C57BL/6 x DBA/2 (H-2$^{b/d}$) F1 mice as described (191). After 5 days cells were harvested and isolated by a one-step gradient method (84) (Nycoprep, Gibco). Responders were then incubated with anti-CD4 (GK1.5

hybridoma supernatant), anti-CD8α (53-6.7 hybridoma supernatant) or anti-CD8β (53-5.8 from Pharmingen) antibodies and depleted by two rounds of immunomagnetic depletion with sheep anti-rat magnetic beads according to the manufacturer's instructions (Dynal). Responders were then counted and incubated at the indicated ratios with 10^4 ^{51}Cr-loaded P815 (H-2^d) or EL-4 (H-2^b) targets for 4 hours. Specific release of ^{51}Cr was determined by the following formula: % Specific Release = (experimental release - spontaneous release)/(maximum release - spontaneous release).

Chapter 3: Functional Analysis of the Molecular Requirements for the Initiation of CD28-mediated Signal Transduction

Introduction

The signal transduction events that result in T cell activation following interaction between T lymphocytes and antigen presenting cells are not fully understood. Antigen-specific interactions between the TCR and processed antigenic peptide bound to MHC molecules on APCs initiate a signaling cascade that includes the rapid induction of protein tyrosine kinase activity and subsequent phosphatidylinositol hydrolysis (185, 186). A number of experiments have shown that a second, or costimulatory, signal is required for maximal T cell activation and effector function. Such a costimulatory signal can be provided by the homodimeric T cell glycoprotein CD28.

Biochemical analysis has shown that CD28 ligation results in a significant increase in lymphokine gene transcription and mRNA stability (37, 38, 100, 102, 167). It has been shown that the CD28 signal transduction pathway is distinct from that of the T cell receptor. Unlike TCR mediated signaling, signal transduction through CD28 is insensitive to the immunosuppressants cyclosporin A and FK-506 (70, 71), which act by inhibiting the activity of the Ca^{+}/calmodulin dependent cytoplasmic phosphatase, calcineurin. CD28 ligation synergizes with TCR or protein kinase C (PK-C) activation and, by acting through regions of the interleukin-2 promoter distinct from TCR response elements, increases IL-2 gene transcription 4-7 fold (38, 177, 187). Hence, T cell activation appears to involve a bipartite signal transduction mechanism with calcineurin-dependent and calcineurin-independent components.

Analysis of receptor-proximal events in CD28 signal transduction has shown that the cytoplasmic tail of CD28 is sufficient for mediation of costimulatory signals and that it rapidly associates with PI 3-kinase activity

following ligation (107, 129, 133, 158). A number of growth factor receptors containing a conserved YMXM motif are known to functionally interact with PI 3-kinase through interactions between SH2 domains in the p85 regulatory subunit of PI 3-kinase and a phosphorylated YMXM motif (18, 35, 74, 80). CD28 also contains a YMXM motif that is highly conserved across a number of species. Similarly, the B cell membrane glycoprotein CD19, which can enhance activation through membrane-Ig (20), has also been shown to recruit PI 3-kinase through a YMXM motif upon ligation (174). In view of these results, we have examined the functional significance of CD28 association with PI 3-kinase in costimulation of IL-2 synthesis.

Our results demonstrate that CD28-mediated recruitment and activation of PI 3-kinase is neither necessary nor sufficient for the costimulation of IL-2 production in Jurkat cells. Moreover, using mutational analysis, we define specific requirements for CD28-mediated signal transduction which suggest that other factors, acting through regions within or overlapping the consensus p85 binding motif, are required for costimulation of IL-2 production.

Results

The Heterologous Receptor CD19 Binds to and Activates PI 3-Kinase in Jurkat T Cells.

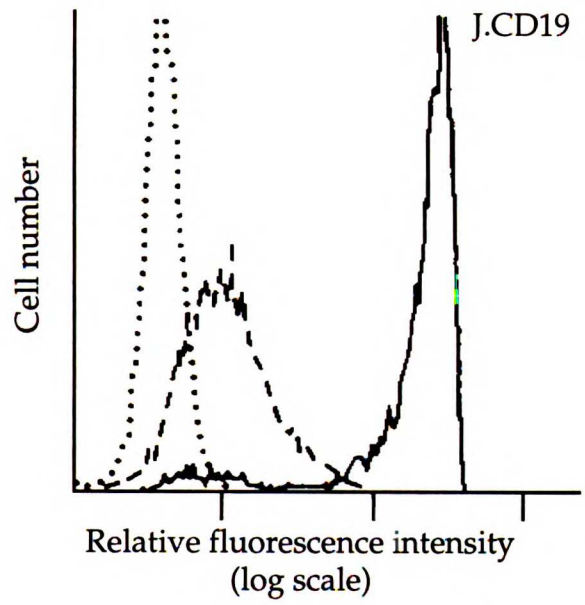
SH2 domains interact with proteins containing phosphorylated tyrosines (18). The specificity of these interactions is determined by the amino acids flanking the phosphotyrosine residue (35, 108). The SH2 domain of the p85 subunit of PI 3-kinase has been shown to bind to phosphotyrosine residues within a YMXM motif (35, 80). Previous studies have shown that CD28-mediated costimulation correlates with CD28 binding and activation of PI 3-kinase (107, 129, 133, 158). As the cytoplasmic domain of CD28 contains a YMXM motif, we examined whether PI 3-kinase activation is sufficient for costimulation.

CD19 is a B lymphocyte surface molecule which is not normally expressed on the surface of T lymphocytes. The cytoplasmic region of CD19 contains tandem YMXM motifs which fit the consensus binding site for the p85 subunit of PI 3-kinase. In response to anti-Ig or anti-CD19 stimulation of B cells, CD19 binds p85 and recruits PI 3-kinase activity (174). To determine whether such recruitment of PI 3-kinase activity is sufficient to mediate costimulatory activity, we used the previously described Jurkat subclone, J.CD19, that has been stably transfected with CD19 (174). This clone expressed surface CD19 at high levels (Figure 9A), although CD28 expression was very low compared to typical Jurkat clones (158).

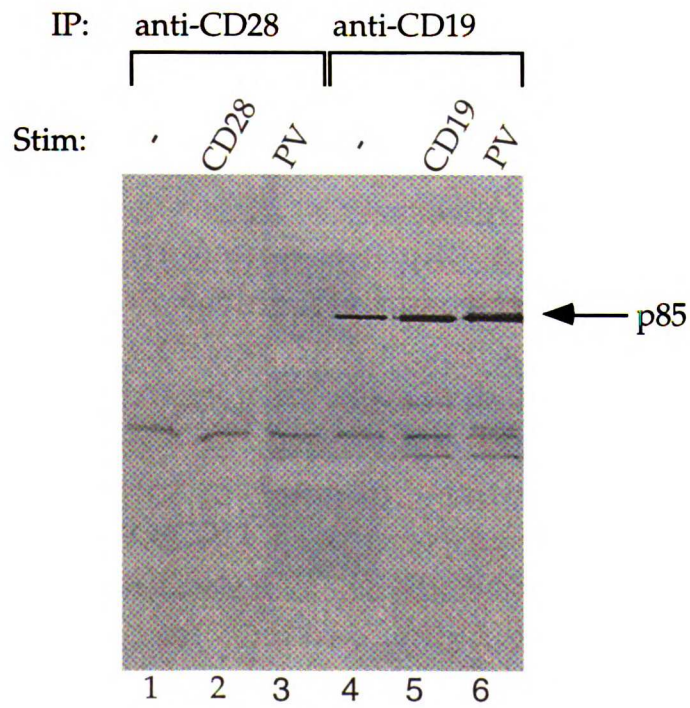
We confirmed previous results regarding the association of CD19 and the p85 subunit of PI 3-kinase by stimulating J.CD19 with anti-CD19 mAb and analyzing anti-CD19 immunoprecipitates by immunoblotting with anti-p85 antiserum (Figure 9B). In unstimulated cells some p85 was constitutively

Figure 9. CD19-mediated recruitment of p85 and PI 3-kinase activity are insufficient for costimulation of IL-2 secretion in Jurkat T cells. (A) The Jurkat subclone J.CD19, which has been stably transfected with CD19, was stained with a control mAb (dotted line), anti-CD19 (solid line) or anti-CD28 (dashed line) followed by FITC-conjugated goat anti- mouse antibody and analyzed by flow cytometry. (B) J.CD19 cells were unstimulated (lanes 1&4), stimulated with anti-CD28 (lane 2), anti-CD19 (lane 5) or pervanadate (lanes 3&6). Lysates were immunoprecipitated with anti-CD28 (lanes 1-3) or anti-CD19 (lanes 4-6) and the p85 subunit of PI 3-kinase was detected with anti-p85 mAb followed by goat anti-mouse alkaline phosphatase. (C) J.CD19 cells were stimulated and immunoprecipitated as in panel B and the immunoprecipitates were analyzed for PI 3-kinase activity. The arrow indicates the position of phosphatidylinositol 3-phosphate. Lanes 1-6 correspond directly to those in panel B. (D) J.CD19 cells were transiently transfected with the IL2-luciferase reporter plasmid and stimulated 40 hours later with PMA (P; 50 ng/ml), ionomycin (I; 1 μM), and mAb (1:2500 ascites) as indicated. After 6 hours, cell lysates were assayed for luciferase activity.

A


J.CD19
Cell number
Relative fluorescence intensity
(log scale)

B


IP:
anti-CD28
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CD28
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CD19
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p85
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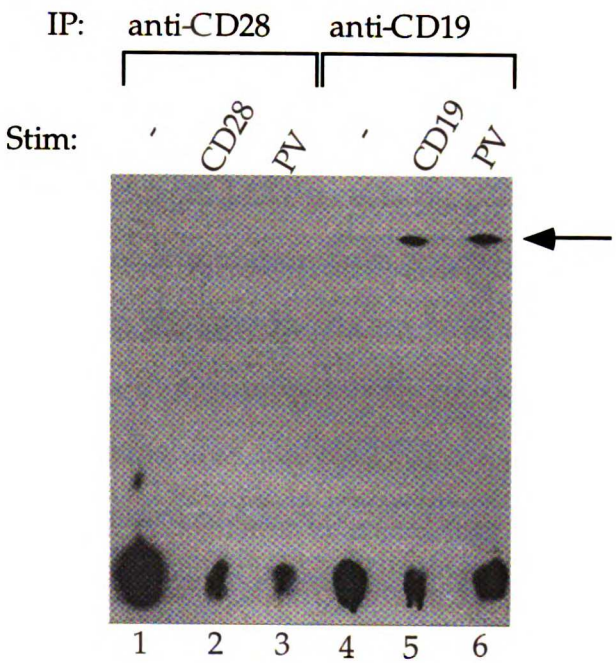
C


IP:
anti-CD28
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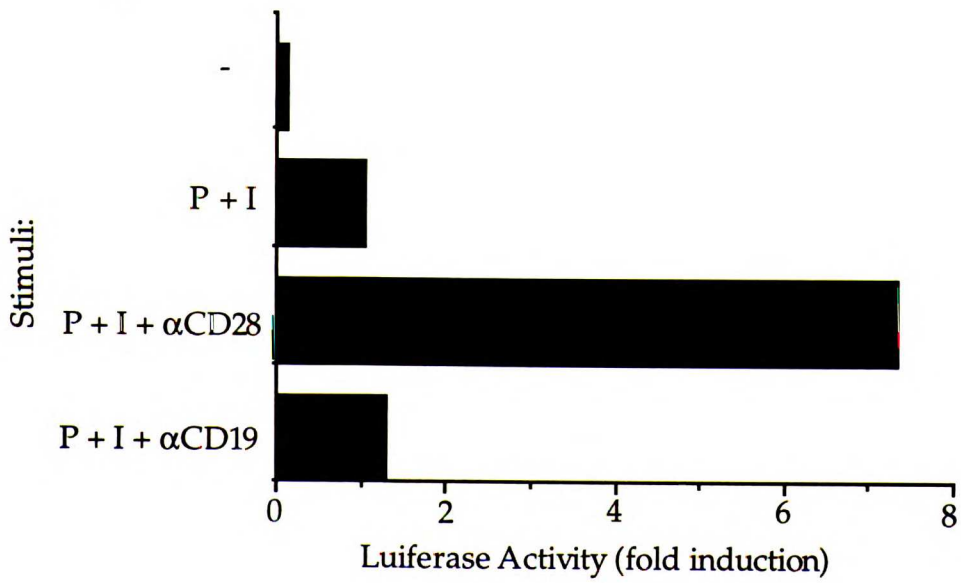

Stimuli:
-
P + I
P + I + αCD28
P + I + αCD19
0
2
4
6
8
Luiferase Activity (fold induction)

associated with CD19 (Figure 9B, lane 4), and association increased substantially after stimulation with anti-CD19 mAb (Figure 9B, lane 5). Treatment of cells with the phosphatase inhibitor pervanadate results in the hyperphosphorylation of tyrosine residues on many cytoplasmic proteins (146), and has been shown to induce the association of p85 with CD28 (158). Similarly, pervanadate treatment of J.CD19 resulted in increased association of p85 with CD19 (Figure 9B, lane 6). When CD28 molecules were immunoprecipitated from J.CD19 cells, no p85 observed associated with CD28 either before or after stimulation with anti-CD28 (Figure 9B, lane 2) or pervanadate (Figure 9B, lane 3). As we have observed p85 association with CD28 upon ligation in a number of other Jurkat clones, the most likely explanation for our inability to detect the association of p85 with CD28 was the very low expression of CD28 in this clone and/or possible competition with the substantially more abundant CD19 molecule.

We tested the CD19 and CD28 immunoprecipitates for PI 3-kinase activity (Figure 9C). In unstimulated cells, there was very little PI 3-kinase activity in CD19 immunoprecipitates, but stimulation with anti-CD19 or pervanadate resulted in a substantial increase in PI 3-kinase activity associated with CD19. Hence, in this particular cell line, CD19 binds and activated PI 3-kinase to a much greater extent than CD28.

CD19-mediated Activation of PI 3-Kinase Does Not Result in Costimulatory Activity.

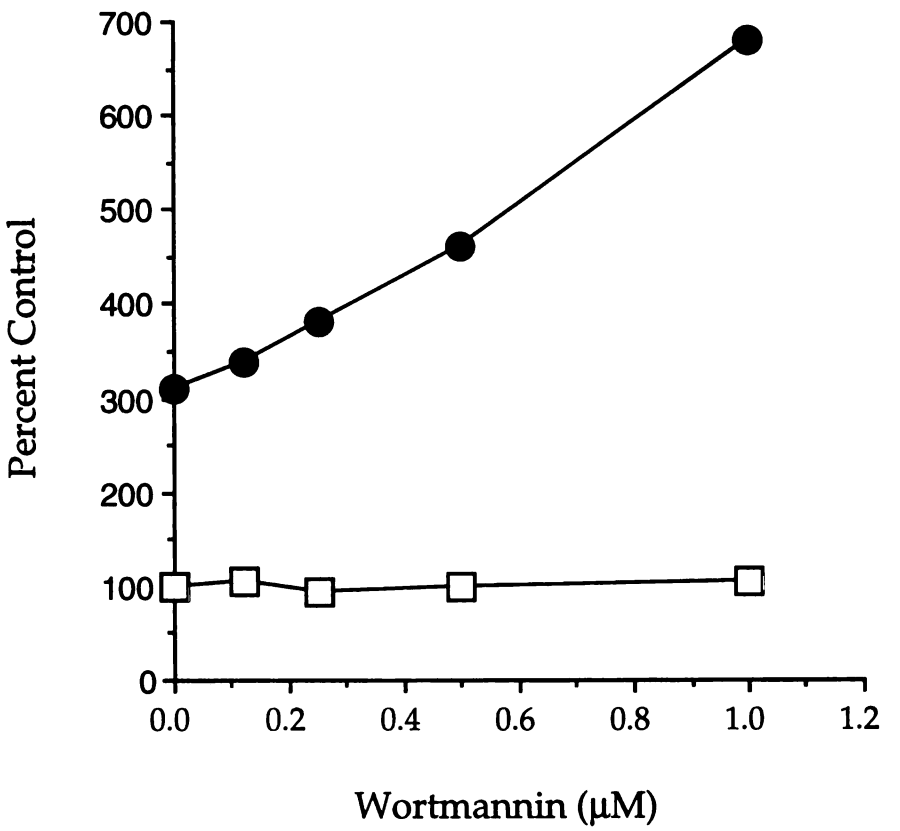
Previous studies have demonstrated that stimulation through CD28 significantly enhances the secretion of IL-2 in response to PMA and ionomycin (37, 158). This costimulatory effect can also be detected using a transcriptional reporter construct, IL-2-luciferase, which contains the IL-2

regulatory region (-326 to +47) upstream of a luciferase reporter gene. J.CD19 cells were transfected with IL2-luciferase and stimulated with PMA and ionomycin, resulting in induction of luciferase activity (Figure 9D). When anti-CD28 mAb was used in combination with PMA and ionomycin, there was approximately a seven-fold enhancement of luciferase activity. Stimulation with anti-CD19 mAb, however, did not increase the response to PMA and ionomycin, although the same concentration of anti-CD19 mAb did stimulate association and activation of PI 3-kinase in CD19 immunoprecipitates. It appears, therefore, that binding and activation of PI 3-kinase is insufficient for costimulation of IL-2 production.

Pre-treatment of Jurkat Cells with Wortmannin, a PI 3-kinase Inhibitor, Enhances CD28-Mediated Costimulation of IL-2 Production.

In view of our finding that PI 3-kinase activation was not sufficient for costimulatory activity, we sought to determine whether PI 3-kinase activation is necessary for costimulation. For this purpose, we used the fungal product wortmannin, which inhibits several kinases, including PI 3-kinase (166). Jurkat cells that had been stably transfected with the IL2-luciferase reporter construct were pre-treated for 30 minutes with various doses of wortmannin prior to stimulation with PMA and ionomycin with or without anti-CD28 mAb (Figure 10). Wortmannin treatment alone had no effect on the response to PMA and ionomycin. If PI 3-kinase is necessary for costimulation, wortmannin would be expected to block any enhancement of IL-2-luciferase activity induced by stimulation of CD28. However, the opposite result was observed: wortmannin enhanced the response to anti-CD28 in a dose-dependent manner. In experiments not shown, the 0.25 to 1.0 mM doses of wortmannin used could inhibit the CD28-associated PI 3-kinase activity (data

Figure 10. Wortmannin treatment fails to inhibit CD28-mediated costimulation of IL-2 transcription. Jurkat cells which were stably transfected with the IL2-luciferase reporter gene were preincubated in 50 µl cultures of 10^5 cells with the indicated amount of wortmannin. The cells were then activated with PMA (P; 50 ng/ml), ionomycin (I; 1 µM), with (solid circles) or without (open squares) anti-CD28 mAb (1:2500 ascites) and luciferase activity was determined. Data are expressed as the percent of PMA-plus-ionomycin (P + I) response.



not shown), consistent with recent studies in Jurkat cells and normal T cells (108, 183). These results indicate that PI 3-kinase is not necessary for the costimulatory activity mediated by CD28, although a more complicated interpretation of this data cannot be ruled out, such as the possibility that an inhibitory effect of wortmannin on some other kinase relieves a PI 3-kinase requirement for costimulation under normal physiological conditions.

Mutational Analysis Reveals Specific CD28 Residues Required for Costimulation of IL-2 Gene Transcription.

Since binding and activation of PI 3-kinase with the heterologous receptor CD19 and the inhibition of PI 3-kinase activity with wortmannin failed to provide supportive evidence of a role for PI 3-kinase in the costimulation of IL-2 production, we performed a mutational analysis of the cytoplasmic domain of CD28 in order to identify regions necessary for costimulatory activity.

Using a chimeric molecule containing the extracellular portion of human CD8α fused to the transmembrane and cytoplasmic regions of murine CD28 (CD8-28WT), we introduced a number of mutations in the cytoplasmic domain of CD28 (Figure 11A). We assayed the ability of these constructs to mediate costimulatory activity by measuring their ability to transactivate an IL2-luciferase reporter gene. Jurkat-TAG cells were transiently cotransfected with the mutant constructs and the IL2-luciferase reporter. Cells expressing comparably high levels of the chimeric proteins at the cell surface were then used for analysis (Figure 11B). Activation was performed by treating cells with anti-CD8 and the phorbol ester, PMA. Antibody crosslinking of the CD8-28WT construct resulted in a significant enhancement of IL-2 transcription,

whereas similar treatment of the tailless chimera failed to enhance reporter gene activity, consistent with previous results (Figure 11C&D).

We then analyzed the effect of mutations within and near the consensus YMXM p85 binding motif (Figure 11C). Surprisingly, a mutation of the tyrosine residue at position 189, the putative site of p85 binding upon phosphorylation, to phenylalanine resulted in the retention of significant reporter gene activity. In contrast, the D188A mutation at the -1 position (relative to Y189) reduced reporter gene activity to that observed with the tailless molecule, while a more conservative mutation (D188E) retained a significant amount of activity. As an acidic residue is highly conserved at the -1 to -5 position in SH2 binding motifs (18, 111), it is noteworthy that the SD187-188TG mutant functioned similarly to the D188E mutant, demonstrating that an acidic residue at the -1 position in the p85 binding motif was not necessary to generate reporter gene activity. Results consistent with those observed in the transient assays were obtained in experiments with Jurkat cells that stably expressed the CD8-28WT, Y189F, and D188A chimeras (data not shown). Mutation at the +2 position (N191A) of the YMXM motif, which is the most degenerate residue within SH2 binding motifs and is unlikely to interfere with p85 binding, resulted in complete abrogation of costimulatory activity (6, 18, 111).

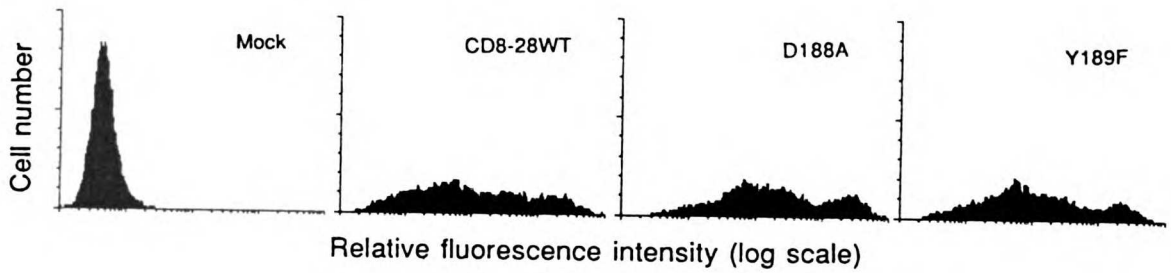
In most cases in which the YMXM motif has been shown to functionally interact with PI 3-kinase, this motif occurs in tandem. Although no other consensus p85 binding motif exists in the cytoplasmic tail of CD28, we considered the possibility that the three downstream tyrosine residues at positions 204, 207, and 216 might be involved in facilitating costimulation. However, mutation of each of these tyrosines (Y204F, Y207F and Y216X) had no effect on the ability to induce reporter gene activity (Figure 11C).

Figure 11. Mutational analysis of the cytoplasmic region of CD28 within the CD8-28 chimera. (A) The wild-type sequence of murine CD28 from amino acid 175-218 (relative to translational start site) is at the top with residues conserved between mouse, human, rat and chicken shown in bold. The position of numerous single (and one double) point mutations are indicated immediately below and the full sequence of the three truncations and one internal deletion shown at the bottom. The point mutants A195+ and R196- refer to an insertion of an alanine between 195-96 and a deletion of the arginine at 196, respectively. (B) Mutant chimeras, coded as wild-type(position)substitution, were transiently cotransfected with the IL2-luciferase reporter gene in Jurkat-TAG cells as indicated and analyzed by flow cytometry as described above. The data shown are from a representative experiment. Similar results were obtained with all of the constructs analyzed. (C) Jurkat-TAG cells transfected as in panel B and after 24 hours $2x10^6$ cells were treated with PMA (P; 20 ng/ml) and anti-CD8 mAb (1:1000 ascites). After 7 hours cell lysates were analyzed for luciferase activity as described above. The mean and standard deviation of four representative experiments is expressed as the percent of CD8-28WT activity. (D) Chimeric proteins bearing truncations, deletions, and multiple mutations as indicated were analyzed as described in panel C.

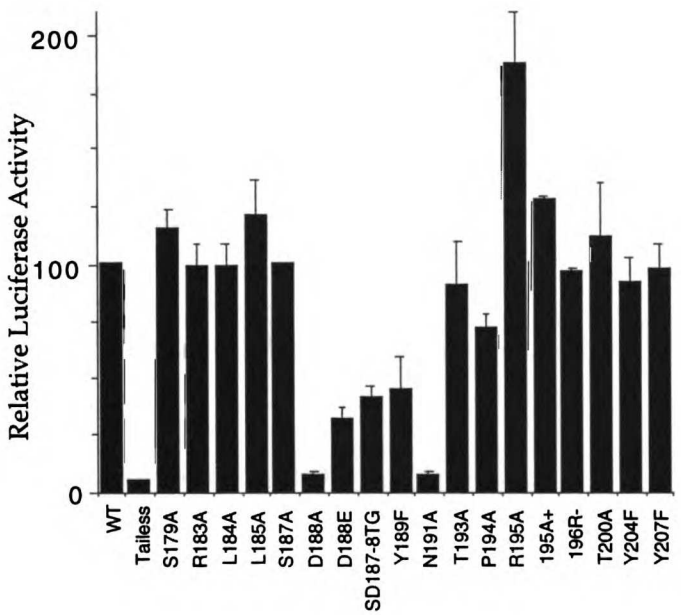
A

```
     180          190          200          210
      |            |            |            |
IWTNSRRNRLLQSDYMNMTPRRPGLTRKPYQPYAPARDFAAYRPX
     A    AAA AAF A AAA     A   F   F
               E
              TG
                    +A
                    -R
IWTNSRR-------------------------------------X tailless
IWTNSRRNRLLQSDYMNMTPRRPGL-------------------X T200X
IWTNSRRNRLLQSDYMNMTPRRPGLTRKPYQPYAP---------X A210X
IWTNSRRNRLLQSDYMNMTPRRPGLTRKPYQPYAPARDFAA---X Y216X
IWTNA----------------------RKPYQPYAPARDFAAYRPX 179-200
```

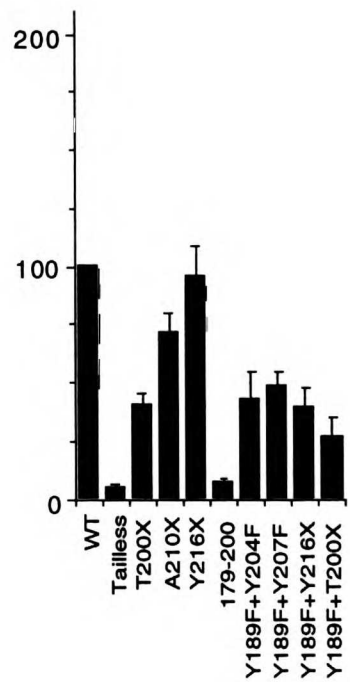
B



C



D



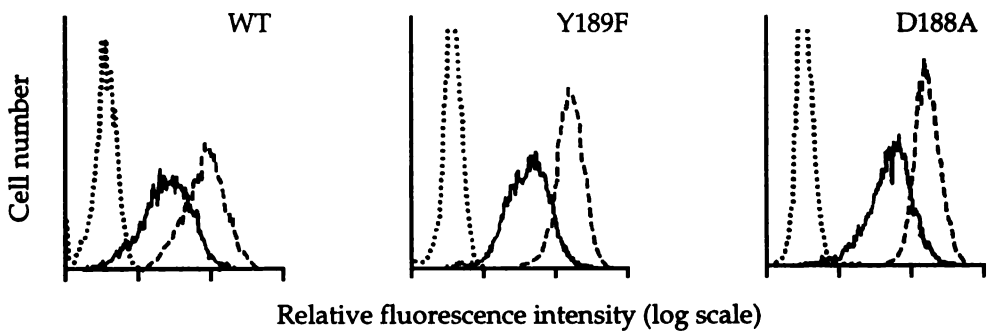
Additionally, combining each of these three mutants with the Y189F mutation (Y189F+Y204F, Y189F+Y207F, and Y189F+Y216X) still failed to completely eliminate IL-2 reporter gene activity (Figure 11D). Chimeric molecules with a C-terminal truncation at position 200 (T200X) also retained a significant amount of costimulatory activity, comparable to that of the Y189F mutant (Figure 11C). A combination of the Y189 mutation and the T200X truncation also did not eliminate the enhancement of IL-2 transcription (Figure 11D), although there was consistently decreased activity as compared to either mutation alone, suggesting that the C-terminal region of the protein may play some role in CD28-mediated costimulation. The C-terminal segment of the protein (179-200 deletion) could not function alone to mediate any costimulatory activity (Figure 11D), although this result may reflect the consequences of gross structural perturbation or changes in membrane proximity of potentially relevant C-terminal domains.

A number of other single amino acid substitutions, including alteration of the spacing between the highly conserved prolines at positions 194 and 197 (A195+, R196-), had no effect on IL-2 reporter gene activity (Figure 11C). In summary, these results indicate that the minimal elements required for costimulatory activity reside in or near the consensus p85 binding motif.

Mutation of the PI 3-Kinase Binding Motif in CD28 Abolishes Binding of p85 by CD28.

Previous investigations demonstrated a correlation between CD28-mediated costimulatory activity and the binding and activation of PI 3-kinase (107, 129, 133, 158). Therefore, we examined whether mutation of the tyrosine residue in the YMXM motif abolished both PI 3-kinase binding

Figure 12. Jurkat cells stably transfected with the CD8-28WT, Y189F, and D188A constructs express similar levels of chimeric protein at the surface. Transfectants were analyzed by flow cytometry as described above using control mAb (dotted line) anti-CD8 (dashed line) or anti-CD28 (solid line).

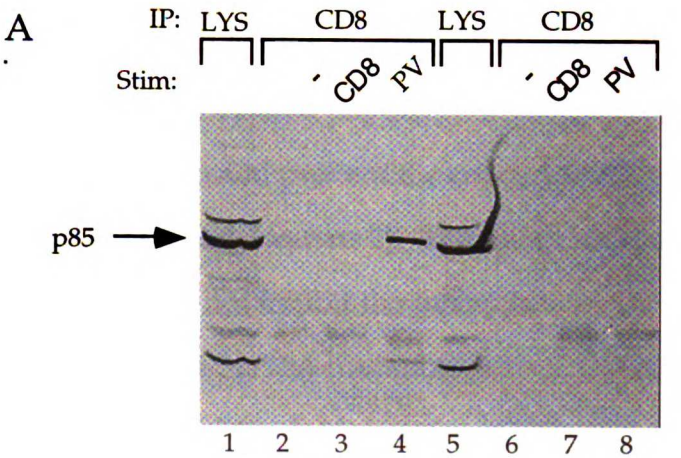


and costimulation. Wild-type and mutant (Y189F, D188A) chimeras were stably expressed in Jurkat cells. FACS analysis was performed to identify clones which had equivalent surface expression levels of the three constructs (Figure 12). These three clones also expressed approximately equivalent levels of endogenous CD28. Cells expressing these three constructs were then stimulated with anti-CD8 or anti-CD28 mAb and lysates were analyzed for the association of the p85 subunit of PI 3-kinase with the chimeric molecules. Whole cell lysates of the three clones contained approximately the same amount of p85 (Figure 13A, lanes 1&5, Figure 13B, lane 1). When cells expressing the CD8-28WT chimera were stimulated with anti-CD8 mAb or pervanadate, p85 was found to associate with the chimeric molecule (Figure 13A, lanes 3&4). In contrast, stimulation of the Y189F chimera failed to recruit immunoprecipitable p85 (Figure 13A, lanes 7&8). Similarly, stimulation and immunoprecipitation of the D188A chimera failed to coprecipitate p85 (Figure 13B, lanes 3&4).

When the cell line stably expressing the Y189F mutation was stimulated with antibodies to endogenous CD28 or with pervanadate, p85 was observed to coprecipitate with CD28, indicating that the negative result for the mutant chimera did not reflect an anomalous function of p85 in this cell line (Figure 13B, lanes 6&7). Similar association of p85 with endogenous CD28 was observed after anti-CD28 treatment of the CD8-28WT and D188A clones (data not shown). Thus, mutation of either the tyrosine in the YMXM motif (Y189F) or the upstream aspartic acid (D188A) results in the abrogation of p85 binding upon stimulation.

Finally, to provide further evidence that PI 3-kinase is not responsible for the CD28-mediated costimulatory activity, we focused on whether the N191A mutation in the PI 3-kinase p85 consensus binding site, which

Figure 13. Jurkat cells stably expressing the Y189F and D188A mutant chimeras are unable to bind the p85 subunit of PI 3-kinase. (A) Cells expressing CD8-28WT (lanes 1-4) or Y189F (lanes 5-8) were stimulated with anti-CD8 mAb or pervanadate as indicated, immunoprecipitated with anti-CD8 mAb, and p85 was detected in the immunoprecipitates by immunoblotting with anti-p85 antisera. (B) Cells expressing the D188A chimera (lanes 1-4) were analyzed as described in panel A. Cells expressing the Y189F chimera (lanes 5-7) were stimulated with antibody against endogenous CD28 or with pervanadate as indicated, immunoprecipitated with anti-CD28 mAb, and p85 was detected in the immunoprecipitates as described above.


A
IP:
LYS
CD8
LYS
CD8
Stim:
-
CD8
PV
-
CD8
PV
p85
1
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3
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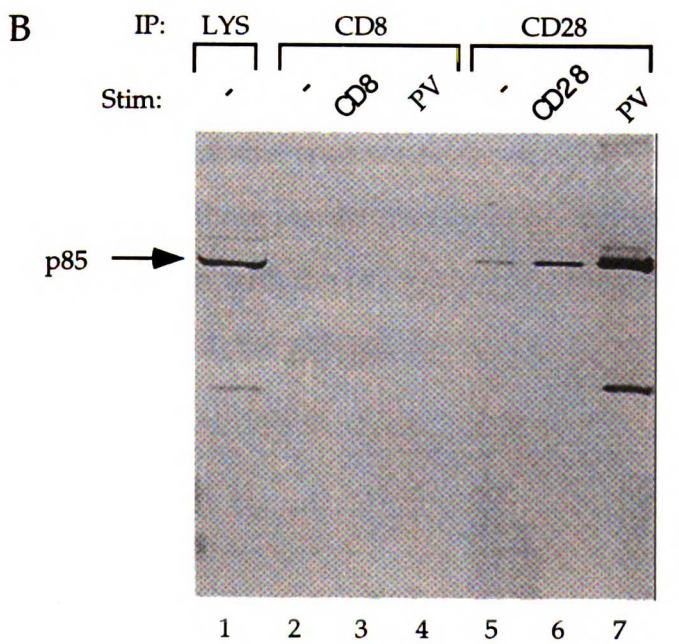
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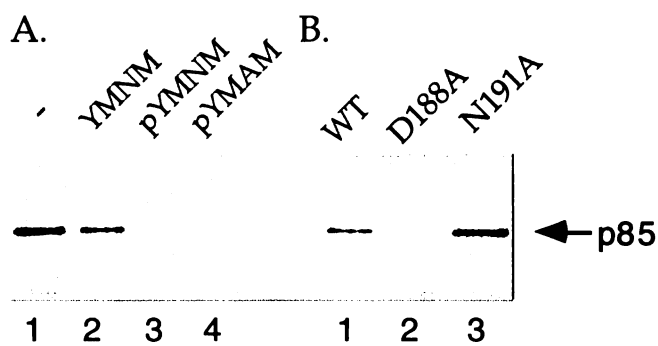
abolished costimulatory activity (Figure 11C), still allowed binding of p85. First, we mixed synthetic tyrosine-phosphorylated wild-type (pYMNM) or N191A mutant (pYMAM) peptides (10 amino acids long) encompassing the p85 binding site with lysates from pervanadate-stimulated Jurkat cells to determine whether the N191A mutant could competitively bind p85. Like the wild-type phosphorylated peptide, the phosphorylated peptide containing the N191A mutation could prevent the coprecipitation of p85 with CD28 (Figure 14A, lanes 3 and 4 versus 1). The nonphosphorylated wild-type peptide (YMNM) did not inhibit the interaction of p85 with CD28 (Figure 14A, lane 2). Moreover, the N191A mutation did not prevent p85 binding to the CD8-28 chimera in pervanadate-stimulated cells that had been transiently transfected (Figure 14B). The binding of the p85 subunit of PI 3-kinase to the chimera with the N191A mutation was comparable to the chimera with the wild-type sequence (Figure 14B, lanes 1 and 3) and is in marked contrast to the abrogation of binding to the chimera with the D188A mutation (Figure 14B, lane 2). Collectively, these results demonstrate that the loss of CD28 costimulatory function with the N191A mutation does not result from the loss of interactions with the p85 subunit of PI 3-kinase.

Figure 14. N191A mutant of CD28 binds to the p85 subunit of PI 3-kinase. (A) Lysates from pervanadate-stimulated Jurkat cells were mixed with the indicated phosphorylated or nonphosphorylated synthetic phosphopeptides at 50 μM prior to immunoprecipitation of CD28 and blotting for associated p85. Lane 1, no peptide (-); lane 2, wild-type CD28 residues 185-194 (LQSDYMNMTP; peptide YMNM); lane 3, tyrosine-phosphorylated residues 185-194 (LQSDpYMNMTP; YpMNM); lane 4 tyrosine-phosphorylated residues 184-194 containing the N191A mutation (LQSDpYMAMTP; YpMAM). (B) Jurkat-TAG cells transiently transfected with the CD8-28 chimeras indicated were stimulated with pervanadate. CD8 immunoprecipitates were probed by Western blotting for associated p85. Lane 1, wild-type chimera (WT); lane 2, the chimera containing the D188A mutation (D188A); lane 3, the chimera containing the N191A mutation (N191A).



Discussion

The cytoplasmic tail of CD28 provides a potent costimulatory signal for IL-2 production by T cells (70, 158). Crosslinking CD28 results in the rapid recruitment of PI 3-kinase activity. In this study, we have addressed the functional significance of the association between CD28 and PI 3-kinase.

First, we explored whether recruitment of PI 3-kinase activity to the cytoplasmic aspect of the plasma membrane was sufficient for costimulatory activity. We employed a previously described Jurkat clone, J.CD19, that had been stably transfected with the B cell surface molecule CD19 (174). Confirming previous results, we found that CD19 ligation resulted in the association of p85 and PI-3 kinase activity in CD19 immunoprecipitates. Nevertheless, the heterologous CD19 molecule failed to costimulate transcription of IL-2, demonstrating that recruitment and activation of PI 3-kinase alone was not sufficient to generate costimulatory activity. However, this does not rule out a necessary role for PI 3-kinase in IL-2 costimulation, and may be a consequence of the heterologous molecule failing to couple appropriately to other T cell-specific signaling mechanisms.

To address whether PI 3-kinase is, nevertheless, necessary for costimulation of IL-2 production, we utilized the fungal product wortmannin, which inhibits the enzymatic activity of PI 3-kinase and other kinases (26). Pretreatment of Jurkat cells with wortmannin failed to inhibit costimulatory activity, consistent with recent studies by others who used Jurkat cells (108). These results are not consistent with the studies of Ward et al., who observed an inhibitory effect of wortmannin on CD28-dependent T cell IL-2 production and proliferation (183), but this may reflect the assay conditions (72 hr), in which excessive toxicity of wortmannin may have been

a factor. Alternatively, this disparity may reflect the different cells used in these studies. Interestingly, we observed that wortmannin treatment resulted in a dose-dependent increase in CD28-mediated costimulation. One possible explanation for this result may be that PI 3-kinase may play an inhibitory role in CD28-mediated signaling. It has been observed that ligation of CD28 results in down-regulation of surface CD28 expression and a refractory period of subsequent signaling through CD28 (101). Furthermore, mice that constitutively expressed a transgenic B7-1 molecule capable of binding CD28 have depressed immune responses (148). PI 3-kinase is known to be involved in a number of vesicular trafficking and membrane transport mechanisms including: ligand induced rapid internalization of the PDGF receptor (69), EGF and IGF-1 induced membrane ruffling (19, 75, 83), and vacuolar protein sorting in yeast (157). Hence, it is possible that the association of PI 3-kinase with CD28 may be required for receptor internalization and may therefore serve as a desensitization mechanism in CD28-mediated signaling that can be blocked by wortmannin. Alternatively, the pleiotropic effects of wortmannin may inhibit other kinases that may regulate the costimulatory signaling pathway in a manner which is independent of PI 3-kinase. In any event, these results suggest that PI 3-kinase activity is not necessary for costimulation of IL-2 production.

We examined the ability of the CD8-28WT, Y189F, and D188A chimeras stably expressed in Jurkat cells to bind and activate PI 3-kinase and costimulate CD28-mediated IL-2 secretion. We found that the D188A mutant was incapable of associating with p85 upon ligation, consistent with the apparent requirement for an acidic residue in the -1 to -5 position of SH2 binding motifs which is believed to be important for phosphorylation of the tyrosine. The D188A chimera also failed to costimulate IL-2 production,

consistent with results obtained in transient transfections. The Y189F mutant failed to interact with p85 or PI 3-kinase activity upon ligation, as expected. However, the Y189F mutant was nevertheless capable of significant costimulatory activity. Therefore, the association of PI 3-kinase with CD28 is not necessary for CD28-mediated costimulation of IL-2 production in Jurkat T cells. Determination of the functional significance of PI 3-kinase association with activated CD28 molecules will require further investigation.

Our observation of the dispensability of PI 3-kinase in CD28-mediated costimulation of IL-2 production differs markedly from that in a recent study by Pagès *et al* (129). In that system, the function of a mutant analogous to Y189F was assessed by antibody crosslinking of mutant CD28 molecules expressed in a T cell hybridoma. In those experiments, a mutation of the tyrosine within the consensus p85 binding motif eliminated CD28-mediated IL-2 production, suggesting that association with PI 3-kinase is a critical event in CD28 signal transduction. We offer a number of explanations for this apparent discrepancy: 1) In the experiments by Pagès *et al.* no primary stimulus was used to activate the cells. In our experiments and those of others (37, 177, 189), signal transduction through CD28 is dependent on synergy with a primary stimulus such as crosslinking of CD3 or treatment with phorbol ester. With all of the mutants we describe, treatment with antibody alone had no appreciable effect on IL-2 production or reporter gene activity (data not shown). 2) Pagès *et al.* also used high concentrations of antibody coupled with extensive secondary antibody crosslinking to achieve IL-2 production. We and others have shown that secondary crosslinking of CD28 molecules is not normally necessary to activate CD28-mediated signal transduction (9, 37). As such, the IL-2 production observed by Pagès *et al.* may have been a consequence of massive membrane perturbation rather than

CD28-specific signaling events. 3) Other studies have suggested that although signaling through CD28 involves a distinct calcineurin-independent pathway, CD28 may also contribute to signaling pathways common with the TCR. However, when we maximally activated the cell line stably expressing the Y189F chimera with PMA and ionomycin and then measured the ability of the chimeras to mediate IL-2 production beyond this level we continued to observe costimulation (data not shown). Hence, as we do observe decreased costimulatory activity in the Y189F chimera, it may be that the system we employed is more sensitive for detecting costimulatory activity than the system employed by Pagès *et al.*

Mutational analysis of the cytoplasmic domain of CD28 revealed specific requirements for costimulatory activity. We found that CD28-mediated costimulation of IL-2 production was particularly sensitive to mutations in and near the consensus p85 binding motif. In both transiently and stably transfected cell lines, mutation of the tyrosine residue which disrupts PI 3-kinase association (Y189F) failed to abolish costimulatory activity. Interestingly, the Y189F chimera, although less active than the wild-type chimera upon ligation, consistently induced significantly higher basal levels of reporter gene activity in unstimulated or PMA treated cells compared to the wild-type chimera (data not shown). As it is possible that the CD8 extracellular region of the chimeras can interact functionally with endogenous MHC class I molecules, the higher basal levels of activity observed with the Y189F chimera may be a result of disrupted PI 3-kinase binding, which is consistent with the enhanced CD28-mediated costimulatory activity observed in cells treated with the PI 3-kinase inhibitor, wortmannin.

Mutation of the aspartic acid at the -1 position (relative to tyrosine in the YMXM consensus motif) to alanine (D188A) completely eliminated

costimulatory activity. However, replacement of aspartic acid 188 with either glutamic acid (D188E) or glycine (SD187,188TG) resulted in retention of a significant amount of activity. Hence, conservation of an acidic residue within the -1 to -5 positions, which is highly conserved in p85 binding motifs and necessary for binding of PI 3-kinase in CD28 (18, 35, 111), is dispensable for costimulatory activity. Interestingly, a single substitution at the degenerate +2 position within the p85 binding motif from an asparagine to an alanine completely abrogated costimulatory activity. A number of other single amino acid substitutions at highly conserved residues throughout the cytoplasmic domain of CD28 had no effect on costimulation, suggesting that the critical region for CD28-mediated costimulation is in or near the YMXM motif, independent of the association of PI 3-kinase with CD28. This is consistent with our observation that a CD8-28 chimera truncated at threonine 200 (T200X) maintained significant costimulatory activity, and that combining that mutant with Y189F also failed to completely abolish costimulatory activity. The requirement for this region is further suggested by the absence of costimulatory activity observed with the 179-200 deletion, although other factors could explain the loss of function in this large deletion mutant. These results strongly suggest that other, as yet unidentified, molecules are required for CD28-mediated costimulation of IL-2 production. It appears likely that interactions of these molecules with the highly conserved p85 binding region of CD28 will be critical for costimulatory activity. This raises the possibility that factors that mediate costimulatory activity compete with a desensitizing PI 3-kinase for the same or overlapping binding sites, thereby maintaining tight control over CD28-mediated costimulation of the immune response. Use of the chimeras bearing mutations within this region will facilitate

Experimental Procedures

Recombinant DNA Constructs

The transmembrane and cytoplasmic domains of murine CD28 were fused to the extracellular domain of human CD8α by PCR using overlapping primers that introduced a silent mutation giving rise to a SphI site at the fusion junction (51, 104). The resulting chimeric construct was subcloned into pBlueScript. Single-stranded template was generated for use in oligonucleotide directed site-specific mutagenesis by standard techniques. Mutant constructs were verified by DNA sequencing and subcloned into the eukaryotic expression vector pCDNA/Amp (Invitrogen). Plasmid DNA for use in stable transfections was generated by replacing the SV40 and polyoma origins (Avr II- Kpn I) of the pCDNA/Amp vector containing the relevant mutants with a puromycin-resistance expression cassette. Sequences for the oligonucleotides used to generate the wild-type and mutant chimeras are available upon request.

Cell lines and transfections.

Transient transfections were performed using the SV40 large T antigen-expressing cell line Jurkat-TAG as previously described (26). In brief, Jurkat-TAG cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin and 10 mM HEPES (pH 7.2). Exponentially growing cells were harvested, washed in serum free RPMI 1640, and resuspended at 3.3×10^7 cells per ml. 10^7 cells (0.3 ml) were combined with 45 μg of effector construct and 15 μg of the IL2-luciferase reporter gene, pIL2-Luc (34, 38), in a 0.4 cm cuvette and electroporated using a BioRad Gene Pulser at

220 mV, 960 μF. The cells were allowed to rest for 10 minutes at room temperature and then resuspended in 20 ml of culture medium. Transfected cells were harvested 24 hours later and used for activation studies. Expression level of each construct was determined by FACS analysis.

Stable transfectants were derived by electroporating 10^7 Jurkat cells with 10-20 μg of plasmid DNA at 250 mV, 960 μF. After 48 hours, the cells were aliquoted into 96 well plates at limiting dilution and subjected to puromycin selection at 1 μg/ml. Two to three weeks later, puromycin resistant colonies were expanded and screened for expression of the relevant construct by FACS analysis using the anti-CD8 mAb, OKT8. Subclones were maintained in RPMI 1640 supplemented with 5% FCS, penicillin, streptomycin and puromycin. Transient IL2-luciferase transfections into stable cell lines were performed using the DEAE-dextran method as previously described (158).

FACS Analysis.

Cell surface expression of stably and transiently transfected cells was determined by incubating 10^6 cells with the relevant primary antibody on ice for 30 minutes, followed by washing and incubation with FITC-conjugated goat anti-mouse antibody (Caltag). Data were collected and analyzed using a FACScan (Becton Dickinson) using LYSYS I and LYSYS II software as previously described. Antibodies used were: anti-CD28 (9.3, Oncogene), anti-CD8 (OKT8, American Type Culture Collection), anti-CD19 (B43, Pharmingen) and the negative control mouse mAb MOPC 195 (Litton Bionetics). Dead cells were identified with propidium iodide staining and were electronically excluded from the analysis.

IL-2 bioassays.

Triplicate samples of 10^5 Jurkat cells expressing the relevant construct were stimulated in 200 µl cultures with 10 ng/ml phorbol myristic acetate (PMA, Sigma) and 1 µM ionomycin (Calbiochem), with or without saturating amounts of mAbs as indicated. After 24 hours, culture supernatants were harvested and frozen, and subsequently assayed for IL-2 activity using the CTLL-20 indicator line and the MTT colorimetric assay (169).

IL-2 promoter activity assays.

Jurkat-TAG cells, transiently transfected with effector and reporter constructs, were harvested 24 hours post-transfection. Activation was performed using $2x10^6$ cells in 1 ml of complete media with 20 ng/ml PMA, with or without anti-CD8 mAb (OKT8, 1:1000 ascites). Stable cell lines transiently transfected with the IL2-reporter construct were harvested 48 hours post-transfection and stimulated at $2x10^6$/ml in 2.5 ml with 50 ng/ml PMA and 1 µM ionomycin, with or without anti-CD28 mAb (9.3), anti-HLA mAb (w6/32), or anti-CD19 mAb (B43). After 6 to 8 hours, the cells were harvested, washed in 100 mM KPO_4 (pH 7.8), 1 mM DTT and either lysed in wash buffer containing 1% Triton X-100, or subjected to hypotonic lysis as described (34). Luciferase activity was determined as previously described (158). In the PI 3-kinase inhibition experiments using wortmannin, 10^5 Jurkat cells which had been stably transfected with the pIL2-luciferase reporter gene were incubated for 10 minutes in 50 µl cultures with the indicated concentrations of wortmannin (Sigma), or DMSO alone. After wortmannin treatment, the cells were activated and luciferase activity determined as described above.

Western blotting for the p85 subunit of PI 3-kinase.

Jurkat subclones expressing the relevant chimeric constructs were activated by suspending $5x10^7$ cells in 0.5 ml PBS and incubating in the presence of anti-CD28, anti-CD8, or anti-CD19 mAbs (1:250 ascites) or pervanadate as previously described (158) for 2 minutes at 37°C. The cells were then lysed in a buffer consisting of 1% NP-40, 150 mM NaCl, 1 mM PMSF, 1 mM sodium vanadate, 1 μg/ml leupeptin, 18 μg/ml aprotinin, 0.4 mM EDTA, and 10 mM NaF. Nuclear and cytoskeletal material was removed by centrifugation after which the lysates were incubated for 2 hours at 4°C with protein A sepharose beads coated with anti-CD28, anti-CD8, or anti-CD19 mAb. For peptide inhibition studies, lysates from pervanadate treated cells were reconstituted with the indicated synthetic phosphorylated or nonphosphorylated peptides at 50 μM for 2 hr at 4°C prior to immunoprecipitation. The antibody-coated beads were then washed five times with 1% NP-40, 0.5 M NaCl, 10 mM Tris, pH 7.4, 1 mM PMSF, and 1 mM sodium vanadate. The proteins were eluted from the beads by boiling in SDS sample buffer and separated on 9% SDS-polyacrylamide gels and transferred to nitrocellulose. Immunoblotting was performed with antiserum specific for the p85 subunit of PI 3-kinase (Upstate Biotechnology) and visualized with alkaline phosphatase-conjugated goat anti-mouse Ig antisera as described (158).

PI 3-kinase assay.

Jurkat cells were stimulated, lysed, and the relevant proteins immunoprecipitated as described above. PI 3-kinase activity was then determined as described (81).

Chapter 4: Epilogue

The development of a system of adaptive immunity yields considerable advantages to an organism in defending against pathogenic challenge. The hallmark to an adaptive immune system is the generation of tremendous diversity in the pool of potential responders. As the generation of this diversity entails the creation of novel antigen specificities, there are a number of hazards associated with adaptive immunity. Any selective advantage provided by acquired immunity would rapidly be lost if responses against self-antigens or innocuous antigens were not sharply curtailed or eliminated. The molecules studied here contribute to specificity and control of T cell differentiation and activation upon encounter with presented antigen.

The role of CD8β in the development of cytotoxic T cells.

We have shown that the targeted disruption of the CD8β gene results in a five-fold reduction in the numbers of CD8 SP thymocytes and peripheral T cells. Furthermore, we have observed this defect to involve the complete failure of particular TCR clonotypes to develop in the absence of the CD8β chain. Hence, CD8β contributes a critical feature to CD8 molecules necessary for the development of most CD8 lineage cells. The developmental defect resulting from the loss of CD8β expression may either involve reduced adhesion of CD8 molecules to MHC class I or defective signaling by CD8 molecules lacking the β chain.

Perhaps the simplest explanation for the developmental defects observed is that CD8αα homodimers have an intrinsically lower affinity for MHC than CD8αβ heterodimers. However, experiments involving both CD4 and CD8 suggest that mechanisms for avidity enhancement may be important for coreceptor function. It has recently been proposed that the CD4 coreceptor forms an intricate multimeric lattice upon recognition of MHC ligand (140).

This phenomena is believed to be mediated by specific interactions between the structurally defined membrane proximal D3 and D4 immunoglobulin-like domains of CD4. Unlike CD4, the membrane proximal region of CD8 is believed to exist in an extended conformation that is unlikely to maintain a specific structure. This raises the question of whether an alternative mechanism for avidity enhancement exists for CD8 molecules.

In experiments using purified MHC molecules immobilized to solid surfaces Mescher and colleagues have shown that CD8 undergoes a significant enhancement in avidity for class I molecules upon fluid phase TCR ligation (114, 125, 126). This effect is rapid and specific to CD8 and murine class I MHC (although not haplotype specific) as antibodies to either block the interaction. This phenomenon has been shown to require tyrosine kinase activity. Furthermore, at the levels of TCR stimulation used, there was no appreciable phosphatidylinositol hydrolysis observed in these cells. In agreement, treatment with PMA and ionomycin failed to "activate" CD8, indicating that the relevant events are upstream or independent of PLC. Moreover, it was demonstrated that PMA treatment inhibited the inducible binding of CTL clones and accelerated the dissociation of cells prebound to MHC coated surfaces. Hence it appears that early pre-PLC (or PLC-independent) events may serve to increase adhesion, and subsequent post-PLC events serve to lower adhesion. This may reflect the cyclic nature of a CTL recognizing and killing a target and then disengaging to seek a new target. In these experiments, cytolytic degranulation correlated directly with CD8 avidity enhancement giving strong support to the physiological relevance of these observations. A similar activation-dependent increase in avidity for MHC has not been observed for CD4 (124). As CD4 lattice formation is observed in the absence of TCR ligation, it is possible that CD4 and CD8 differ

fundamentally in their respective mechanism for enhancing avidity for MHC. Considering the homologous nature of CD4 and CD8α, one possibility is that the CD8β chain plays a specific role in the activation-dependent increase in binding to class I molecules.

Experiments in a CD4-dependent T cell hybridoma have shown that CD4-associated Lck can play an important role in T cell activation independent of the kinase activity of Lck (192). It was proposed that interactions between the SH2 and SH3 domains of Lck with TCR-associated molecules could facilitate an inside-out mechanism for recruiting CD4 into activated TCR complexes. This raises the question of whether defective Lck recruitment by CD8 molecules lacking the β chain may provide a molecular basis for a potential loss in activation-induced adhesion. In recent experiments by Burakoff and colleagues using a T cell hybridoma stably transfected with CD4 and CD8 molecules, it was shown that CD8αβ heterodimers were much more efficient that αα homodimers in recruiting Lck (63). This raises the possibility that reduced CD8-Lck interactions in CD8β$^{-/-}$ thymocytes may be responsible for the reduced numbers of CD8-lineage cells observed in these mice. This possibility is supported by the observation that overexpression of CD4 in thymocytes results in reduced CD8-Lck interactions and disrupted development of certain TCR clonotypes (163). We have examined the relative association of Lck with CD8 in thymocytes in wild-type and null mice and have observed a reduction in CD8-Lck association (data not shown). At this point it is unclear whether the reduced Lck association is a consequence of the loss of CD8β or a reflection of the apparently lower levels of CD8 expression in DP thymocytes observed in these mice (refer to Figure 4).

As CD8α is known to interact with Lck, it is important to note that 50% of the CD8α molecules expressed in the thymus are of a truncated isoform that is unable to bind Lck (130). This may either reflect a diminished significance for CD8-Lck interactions in selection, or alternatively may render CD8 molecules particularly sensitive to changes in the stability of the CD8-Lck interaction. Some insight into this may be provided by the results of experiments where CD8α-null mice were reconstituted with mutant forms of CD8α unable to bind Lck. In one set of experiments a truncated form of CD8α was found to be very inefficient at rescuing CD8 lineage development (43). Interestingly, in a separate set of experiments it was observed that a CD8α transgene bearing point mutations that disrupted Lck association appeared to be much more effective at rescuing development (22). This indicated that the cytoplasmic domain of CD8α may be important for reasons other that the association with Lck. This is supported by experiments in transgenic mice overexpressing a CD8β molecule lacking its cytoplasmic domain (64). As CD8β is dependent on CD8α for surface expression, the high levels of expression of this truncated CD8β molecule effectively exclude >85% of the wild-type CD8β molecules from the surface of thymocytes in a dominant negative fashion. It was shown that this truncated form of CD8β disrupted the development of the H-Y-specific TCR, suggesting that the cytoplasmic domain of CD8β plays an important role in its function. When combined with the observation that a CD8α molecule bearing point mutations that disrupt Lck binding is more effective in rescuing CD8-lineage development than a truncated CD8α molecule, it appears possible that although the cytosolic aspect of CD8 appears important for T cell differentiation, it may depend on interactions with molecules other than Lck.

The apparently different mechanisms of avidity enhancement for CD4 and CD8 molecules is noteworthy when considered in the context of the recently proposed asymmetric model of lineage commitment. As discussed above, there is evidence that CD8-lineage development may involve an instructional event while CD4-lineage development may not (109, 160). While CD4 appears to form high avidity lattices independently of TCR ligation, CD8 avidity enhancement appears to require TCR stimulation. Experiments supporting the asymmetric commitment model have shown that CD8-lineage development depends on specific TCR-MHC interactions (160). Hence the differential requirements for lineage commitment may reflect the differential requirements for coreceptor involvement rather than a distinct instructional event -- i.e. while CD4 molecules may form high avidity aggregates independently of TCR ligation, CD8 molecules are functionally available only after TCR stimulation. This would result in CD8 appearing to require an instructional event (TCR ligation-dependent) to achieve a high avidity state while CD4 would appear to be constitutively available (TCR ligation-independent). Although this notion cannot resolve the discrepancy between stochastic and instructional models for lineage commitment, it can explain the differential requirement for appropriate MHC expression for the appearance of the various coreceptor intermediate populations of thymocytes.

Although seemingly less likely, it is still possible that TCR stimulation induces a modification of the extracellular domains of CD8 resulting in its enhanced affinity for MHC. Results consistent with this have been obtained in experiments addressing the glycosylation state of $CD8^+$ cells in vivo (21). It was observed that CD8β was differentially glycosylated in thymocytes compared to naive peripheral cells. Furthermore, activation of peripheral cells resulted in the acquisition of a glycosylation state similar to that

observed in thymocytes. Hence, it is possible that TCR stimulation induces changes in the glycosylation state of CD8 that increases its affinity for MHC. As the change in glycosylation was found to be specific for CD8β, this may provide a molecular basis for the developmental defects observed in CD8β-null mice. It seems probable, however, that the activation-dependent increase in binding and changes in glycosylation represent different aspects of CD8 function as the kinetics of these two phenomena are not likely to be the same.

In summary, our current understanding of CD8 biology suggests three possible explanations for the developmental defects observed in CD8β-deficient mice. First, CD8 is known to undergo an activation-dependent increase in adhesion to MHC molecules. Second, CD8β is known to become differentially glycosylated in the course of thymic development and upon the activation of peripheral cytotoxic T lymphocytes. And third, CD8αβ heterodimers are known to be more effective than CD8αα homodimers in recruiting Lck in T cell hybridomas. These phenomena may be related, or they may represent different aspects of CD8β function. As protein tyrosine kinase activity is known to be required for inducible CD8-mediated adhesion, the defective recruitment of Lck into CD8 molecules lacking the β chain may provide a basis for a reduction in activation-dependent binding to MHC. Assuming the cytoplasmic domain of CD8β is involved in Lck recruitment, this is then consistent with the disrupted development observed in animals expressing the truncated CD8β transgene. If so one would predict that both CD8β-null cells and those reconstituted with a tailless CD8β molecule would be defective in activation-dependent binding. This possibility could be tested by generating T cell clones from transgenic mice that express a tailless CD8β

molecule and assaying the ability of these cells to inducibly bind to purified MHC molecules.

Alternatively, CD8β may facilitate interactions with proteins other that Lck that serve to cluster CD8 molecules in a manner analogous to that proposed by the D3 and D4 domains of CD4. If an intracellular mechanism is to explain the increase in adhesion observed upon activation, then the regulated changes is CD8β glycosylation may represent other features of CD8β function, such as the acquisition of a memory phenotype. However, as the glycosylation state of activated CTLs is similar to that in the thymus, the role of an extracellular modification of CD8β in differentiation cannot be ruled out. Indeed, the regulated and reversible changes in CD8 avidity for class I molecules may be more important to the function of peripheral CTLs. In this case the disruption of positive selection observed with a truncated CD8β molecule may reflect a requirement for the cytoplasmic domain in targeting CD8β for differential glycosylation in the thymus and upon activation of peripheral T cells. Answers to these questions will require examination of the biochemical consequences of the loss of CD8β with regards to binding to MHC, Lck association, and CD8 glycosylation. As the H-Y-specific TCR is known to require CD8β for positive selection, this receptor can serve as an indicator of CD8β function. The use of a truncated CD8β transgene in CD8β-null animals and CD8α-β chimeric constructs should allow the mapping of the critical regions of CD8β required for normal development. Biochemical analysis of cells derived from these reconstituted animals will allow the clarification of the features of CD8β that are important in development and peripheral function.

Proximal requirements for coupling CD28 to the intracellular signal transduction machinery.

Upon T cell activation by antigen presenting cells at least two signal transduction pathways are induced. A primary signal that appears dependent on p56^{lck} and the transmembrane tyrosine phosphatase CD45 is induced by interactions between the TCR complex and peptide-MHC (186). A second signal can be provided by the ligation of CD28 by the B7 counterreceptors (2). Because of the distinct characteristics of these signals and the synergistic nature of their interaction, the identification of where these signals converge and what components are unique to each is of considerable interest. Experiments focusing on IL-2 gene transcription have shown that a component of CD28-mediated signaling converges with TCR-derived signals at the level of the IL-2 promoter by inducing the association or activation of factors within a distinct CD28-responsive element (25, 37, 177). This component of CD28-mediated signal transduction appears to be CsA-insensitive, consistent with primary observations of CD28-mediated signal transduction (71).

Recent experiments, however, have shown that an earlier point of signal integration may occur in CD28-mediated signal transduction (159). In these experiments it was observed that anti-CD3 and anti-CD28 crosslinking resulted in the synergistic activation of JNK. JNK phosphorylates and increases the transcriptional activity of c-jun (57). It was also shown that ERK1,2, which play an important role in c-fos induction, were maximally activated by a primary stimulus alone (48). As Jun and Fos are components of AP-1 which participates in the formation of the NF-AT complex, the regulation of the these proteins is an important indicator of T cell activation (5, 65, 122). Curiously, it was shown in these experiments that the CD28-

dependent activation of JNK was CsA-sensitive while the CD28-independent activation of ERK was CsA-insensitive, in contradiction to the pharmacological characteristics of CD28-mediated costimulation. Hence, although CD28 may synergize with TCR signals in the induction of JNK activity, there are possibly other significant CsA-insensitive aspects of CD28-mediated costimulation. Alternatively, these results may reflect the somewhat irregular characteristics of Jurkat cells regarding cyclosporin A sensitivity.

It has been proposed that CD28 ligation induces at least two signal transduction pathways, one of which is CsA-insensitive and another CsA-sensitive (70). Recent analysis of proximal events in CD28 signal transduction showing multiple interactions induced upon CD28 ligation certainly allows for this possibility. CD28 has been shown to inducibly interact with PI 3-kinase, Grb2, and Itk, while more distal events in CD28-mediated signaling have been reported to include the activation of PLC, raf-1, p21^{ras}, and sphingomylinase, the induction of tyrosine kinase activity, and increased generation of cGMP (8, 15, 56, 60, 94, 95, 106, 133, 143, 154, 176). The relationship between the proximal interactions of CD28 with PI 3-kinase, Grb2, and Itk and these various downstream events is not understood. Our studies were designed to examine the role of CD28-associated PI 3-kinase in the costimulation of IL-2 production, and to describe the molecular requirements in the CD28 cytoplasmic domain for costimulating IL-2 production.

We observed in these experiments that PI 3-kinase was dispensable for the costimulation of IL-2 production in Jurkat cells, but that the asparagine within the YMNM p85-SH2 binding motif was required for activity. We also showed that mutation of this asparagine residue did not disrupt p85 binding

to CD28, which is consistent with the current understanding of this being a degenerate residue within the consensus YXXM motif for p85-SH2 association. Recent experiments have shown that association of Grb2 with CD28 is also mediated by this region (17, 143). Interestingly, the Grb2-SH2 consensus binding motif appears to be YXNX, indicating that our N191A loss of function mutant may interfere with Grb2 binding (155). It has been shown that mutation of the tyrosine in the YMNM motif still allows association of Grb2 with CD28, albeit at reduced levels (143). Hence it is quite possible that the PI 3-kinase independent costimulation of IL-2 production observed in our experiments may result from the CD28-mediated recruitment of Grb2 which could serve to couple the T cell surface to the regulation of $p21^{ras}$ which is important in the activation of ERK1&2 and JNK and subsequent IL-2 induction (123, 159).

Although a number of laboratories have also reported that PI 3-kinase is dispensable for CD28-mediated costimulation of IL-2 production , others have reached the opposite result -- that PI 3-kinase was necessary for CD28-mediated IL-2 production (17, 28, 88, 108, 129, 171, 183). Most noteworthy of these are the experiments of Rudd and colleagues using a murine T cell hybridoma transfected with mutant forms of human CD28 (14). In these experiments, association of PI 3-kinase with CD28 was required for IL-2 production when these cells where stimulated with anti-CD3 and B7 transfected CHO cells. This was found to be true by disrupting either the tyrosine or the methionine in the YXXM motif. Importantly, while the methionine mutation blocked PI 3-kinase association, it did not disrupt Grb2 association. Hence, our observation of costimulatory activity in the absence of PI 3-kinase association mediated by Grb2 is inconsistent with the results of Rudd and colleagues. The most likely reason for the difference between our

results and those of Rudd and colleagues is the use of different cellular systems: CD28-mediated signaling in Jurkat cells is independent of PI 3-kinase association and activation, whereas some primary T cells, T cells clones and T cell hybridomas do exhibit a PI 3-kinase requirement. Another important experimental variable is the use of either receptor ligation, or pharmacological activation as the primary stimulus. Of note, it has been recently reported that PMA treatment of Jurkat cells resulted in hypophosphorylation of PI 3-kinase, reduced PI 3-kinase activity, and an inhibition of PI 3-kinase association with CD28 upon ligation (61). Hence, the activation protocol used in these studies may have predisposed us and others towards the recognition of PI 3-kinase independent events. In a comparison of Jurkat cells and primary human T cells it was shown that, while CD28 responses in Jurkat were insensitive to wortmannin treatment, primary cells were sensitive (175). Furthermore, when PMA was used as a stimulus for primary cells they too became insensitive to the effects of wortmannin. It appears that the method of activation as well as the cell line used may account for the discrepancies observed in these experiments.

In addition to its role in augmenting IL-2 production, it has been shown that ligation of CD28 can prevent the induction of T cell apoptosis (13, 135, 152, 172). Interestingly, it has been reported in both the NGF and PDGF receptor systems that PI 3-kinase may play an important role in the enhancement of survival mediated by these receptors (59, 193). This raises the possibility that PI 3-kinase may play an important role in CD28-mediated cell survival. Ligation of CD28 is known to cause upregulation of the survival promoting factor bclxL (12, 117). One possibility is that PI 3-kinase regulates cell survival and receptor expression as discussed above, while Grb2 mediates the costimulation of IL-2 production. The conflicting results

described above may thus reflect differential dependencies of Jurkat, T cell clones, and primary cells on PI 3-kinase regulation of cell survival in the assays used. Indeed in experiments by Rudd and colleagues, wortmannin treatment induced apoptosis in the T cell line used, suggesting that the conflicting results observed after wortmannin treatment may reflect properties other than the role of PI 3-kinase in IL-2 production (17). It is noteworthy that the mutation of the tyrosine in the YXXM motif reduces Grb2 association by approximately 80%, which may well reduce costimulatory signaling below the sensitivity of many of the systems used (143). This combined with the loss of costimulation observed in the N191A mutation make a strong argument for Grb2, not PI 3-kinase, being important for increased IL-2 production in Jurkat cells. It appears likely, however, that the disparate and reproducible results regarding the necessary interactions in CD28-mediated signal transduction may reflect multiple aspects of CD28 function that are differentially highlighted by the various systems and methods used.

As mentioned above, CD28 has also been shown to inducibly associate with Itk upon ligation. Neither the site of interaction nor the functional role of this association is understood. Given the multiple biological consequences of CD28 ligation, it is becoming increasingly apparent that CD28 interactions with a number of proteins may serve to initiate multiple signal transduction events in a manner analogous to the PDGF receptor. It is interesting to note that TCR-mediated signals are also known to involve Grb2-SOS. This raises the question of whether CD28 signals mediated through Grb2 represent a cyclosporin A-insensitive pathway. In the Jurkat line we used for our transient studies, we found CD28-mediated costimulation of the full-length IL-2 reporter to be sensitive to the effects of CsA (data not shown). Hence, it is

possible that other aspects of CD28-mediated signaling are responsible for a CsA-insensitive pathway. One possibility may reside with the tyrosine kinase Itk, which is also know to interact inducibly with CD28 upon ligation. As mice defective for Itk expression have recently been described, it will be interesting to see if the CsA-insensitive component of CD28-mediated signaling is intact in these animals. CD28-mediated augmentation of IL-2 production and proliferative responses does appear to be intact in itk-deficient mice (X. Liao and D. Littman, personal communication), but it is still possible that some aspects of CD28-mediated signaling are disrupted in these animals. The mutants we've described will be valuable tools in allowing for the dissection of these various signaling pathways and their relative role in the apparently multi-component CD28 signal transduction apparatus.

Finally, CD28 is known to share ligand specificity with the homologous protein CTLA-4. CTLA-4 has recently been shown to be a negative regulator of T cell activation as demonstrated by antibody blocking and gene targeting experiments (91, 168, 180) Although the function of CD28 and CTLA-4 appear to be opposite with regards to T cell activation, the cytoplasmic domains of these proteins are nevertheless quite similar. Not only does CTLA-4 appear to negatively regulate T cell activation, it appears to function dominantly over CD28 mediated signals. One possibility is that CTLA-4-mediated signals intersect with and block CD28-mediated signals. Alternatively, CTLA-4 signals may be entirely separate from CD28 mediated signals, but nevertheless function dominantly. Recent experiments have shown that CTLA-4 interacts with the tyrosine phosphatase Syp which may serve as a mechanism for differential signaling through CTLA-4 (112). Future experiments that examine the nature of CTLA-4 regulation of T cell responses will give important insights into the mechanisms for positive and negative regulation

Appendix 1: Targeted Disruption of B7-1 in Embryonic Stem Cells.

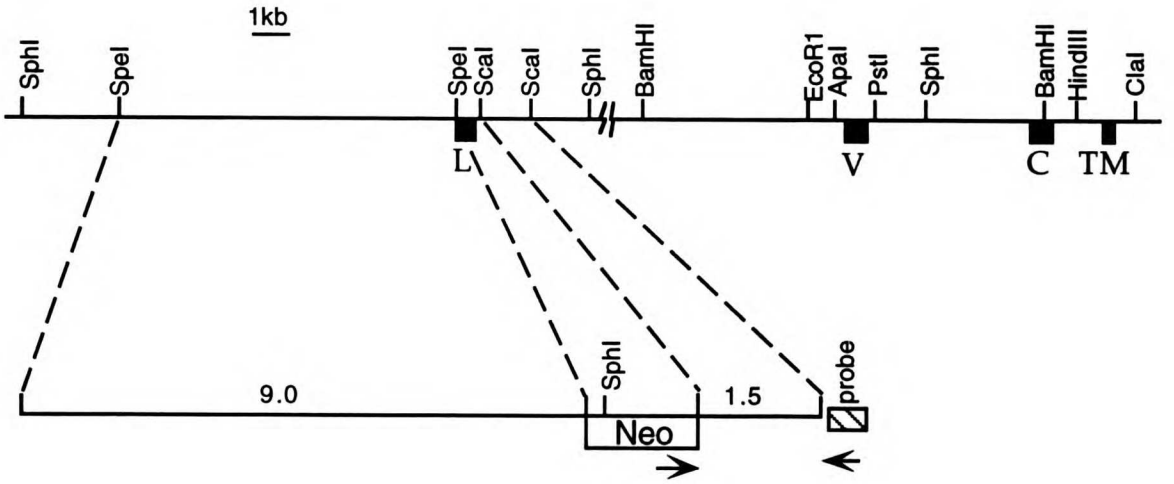
The B7 family members are differentially expressed on a variety of antigen presenting cells (9, 41, 42, 97). Both B7-1 and B7-2 can serve as a functional ligand for CD28 and participate in the activation of T cells in this manner (39, 90). While B7-2 is constitutively expressed on some cells of hematopoietic origin, B7-1 is an activation antigen. It has been shown in tumor transfection and transgenic experiments that expression of B7-1 can result in enhanced tumor clearance, increased autoimmunity, and alteration in the type of T-mediated responses (24, 52, 87, 170, 190). This indicates that the B7 molecules can serve as important regulators of T-mediated immune responses. While CD28 and CTLA-4 appear to function differentially as positive and negative regulators of T cell activation respectively (3), the relative functions of the B7 family members to T cell and APC activation is not well understood. In order to understand the role of B7-1 in development and activation of T cells we endeavored to examine the consequences of the targeted disruption of B7-1 on these processes.

We obtained genomic clones of murine B7-1 by screening a λDASH II 129/Sv library with oligonucleotides specific to the predicted leader and IgV-like exons. We recovered three independent genomic clones the comprise a significant portion of the B7-1 locus (Figure 15A). Two of these contained the putative leader exon of B7-1. The third genomic clone contained the putative second (IgV-like), third (IgC-like), and fourth (TM) exons and possible additional exons. Our understanding of the genomic organization of B7-1 is consistent with the information since published on this locus (147).

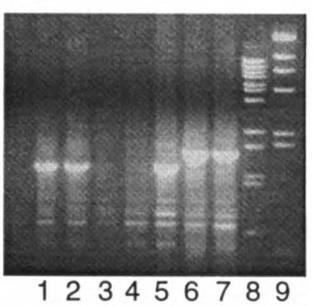
To create a null allele of the B7-1 locus we developed a targeting construct designed to delete all of the coding sequence and the splice donor of the leader exon. The targeting construct was designed to allow for rapid PCR analysis across the 3' arm of homology (Figure 15A). As a positive control for

Figure 15. Targeted Disruption of B7-1 in Embryonic Stem Cells. (A) Genomic organization of a portion of the B7-1 locus indicating the location of exons containing the 5′ untranslated and leader region (L), the IgV-like domain (V), the IgC-like domain (C), and the transmembrane domain (TM). The arrows indicate the position of the PCR primers used in panel B. The hatched region indicates the location of the 0.25 kb XbaI-PstI fragment used as a probe in panel C. (B) PCR analysis of ES clones transfected with the targeting construct shown in panel A. Positive clones are shown in lanes 1,2&5, negative clones are shown in lanes 3&4, and control reactions are shown in lanes 6&7. Molecular weight markers are shown in lanes 8 (λ-BstEII) and 9 (λ-HindIII). (C) Southern analysis of a wild-type (lane 1) and recombinant (lane 2) ES cell clones showing the predicated conversion of the 16 kb endogenous SphI fragment to a recombinant 3.5 kb fragment due to the introduction of an SphI site in the neomycin cassette.

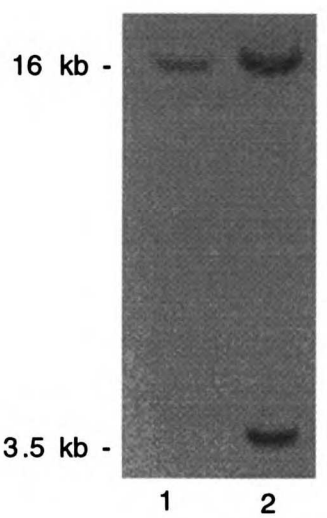
A


1kb
SphI
SpeI
SpeI
ScaI
ScaI
SphI
BamHI
EcoR1
ApaI
PstI
SphI
BamHI
HindIII
ClaI
L
V
C
TM
9.0
SphI
1.5
probe
Neo

B


1 2 3 4 5 6 7 8 9

C


16 kb -
3.5 kb -
1
2

the PCR reaction in the genomic environment we developed a second slightly larger control construct that contained the 3' external primer sequences of the targeting construct. This construct was transfected in parallel with the targeting construct and handled identically throughout the experiment.

PCR analysis of individual clones of a typical experiment revealed that the construct gave rise to homologous recombinants at approximately 30% efficiency (Figure 15B). Southern analysis using an external probe confirmed the results of the PCR analysis (Figure 15C). An internal probe specific for the neomycin resistance marker was used to confirm that single integration event had occurred (data not shown). Several individual clones were used for blastocyst injection, but in all cases the degree of chimerism as judged by coat color was disappointingly low.

Mice lacking B7-1 have since been successfully generated (40). These animals appear normal in most aspects of immune development and responses but do show a reduced ability to serve as stimulators for allogeneic responses. Future experiments that examine the role of the family member B7-2 should help elucidate the relative contribution of the B7 family members to the development and activation of T cells.

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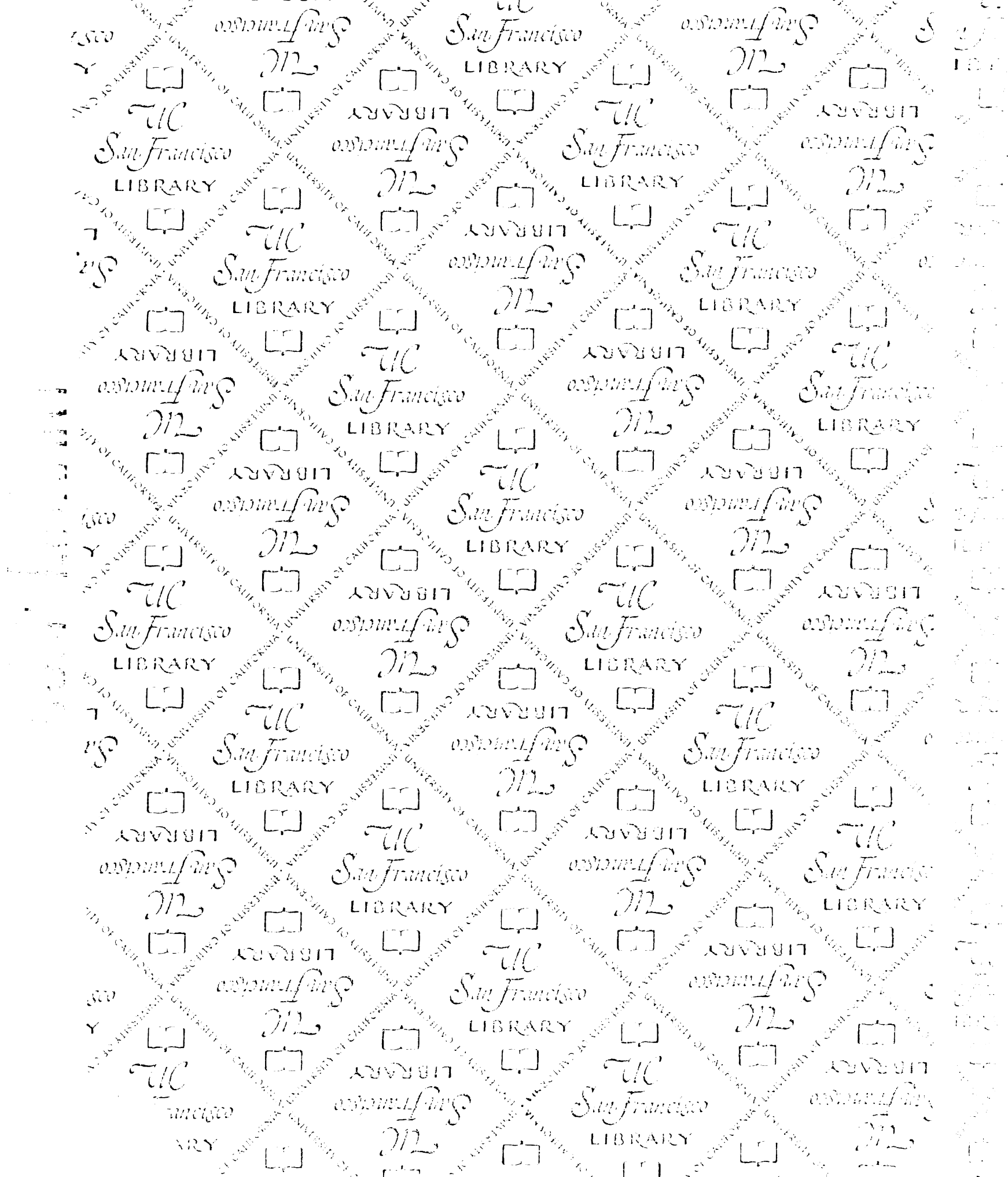
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