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CD4+ T Cell Responses: A Complex Network of Activating and Tolerizing Signals as Revealed by Gene Expression Analysis: A Dissertation

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CD4+ T Cell Responses: A Complex Network of Activating and Tolerizing Signals as Revealed by Gene Expression Analysis

A Dissertation Presented

By

David Spaulding Brown

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Abstract

Immunologic self-tolerance is maintained by both central and peripheral mechanisms. Furthermore, regulation of mature lymphocyte responses is governed by inhibitory as well as stimulatory signals. TCR recognition of cognate peptide bound to MHC molecules provides the initial stimulus leading to T lymphocyte activation and determines the antigen specificity of any subsequent response. However, lymphocytes must discriminate between foreign and self antigens presented by self-MHC molecules to maintain self tolerance and avoid pathological autoimmunity. Consequently, TCR ligation alone is reported to result in abortive activation, T cell anergy, apoptosis, and tolerance. Under normal physiological conditions, costimulatory signals modify lymphocyte responsiveness to TCR ligation to prevent autoimmunity while enabling robust responses to foreign antigen. Members of the CD28/B7 superfamily provide the critical secondary signals essential for normal immune cell function.

CD28 is an essential positive costimulatory molecule with critical functions in thymic development, lineage commitment, and regulation of peripheral lymphocyte responses to antigenic stimuli. CD28 ligation by APC-expressed B7 molecules alters proximal signaling events subsequent to MHC/TCR interactions, and initiates unique signaling pathways that alter mRNA stability and gene transcription. Furthermore, CD28 signaling is required for regulatory T cell development and function. Thus, CD28 has a central role in both potentiating lymphocyte activation mediated by TCR engagement and regulating peripheral tolerance. In contrast, Ctla-4 mediates an inhibitory signal upon binding B7 molecules on an antigen-presenting cell. Its importance in governing lymphocyte responses is manifested in the fatal

lymphoproliferative disorder seen in Ctla-4$^{-/-}$ mice. The lymphocyte proliferation is polyclonal, antigen and CD28 dependent, and arises from defects in peripheral CD4+ T cell regulation. The high percentage of peripheral T lymphocytes expressing activation markers is accompanied by lymphocyte infiltration into numerous non-lymphoid tissues and results in death by 3-4 weeks. While still controversial, Ctla-4 signaling has been reported to be essential for induction of peripheral T lymphocyte tolerance in vivo and in some model systems is proposed to regulate both T lymphocyte anergy induction and the immune suppressive effects of some regulatory T cells in the prevention of autoimmunity.

Signaling pathways activated by TCR ligation and CD28 costimulation have been extensively characterized. In contrast, the mechanisms mediating Ctla-4 maintenance of tolerance remain largely unknown. Ctla-4 gene expression is tightly controlled during T cell development and activation, and its intracellular localization and expression on the cell surface is regulated by numerous pathways and intermediates. While a tailless Ctla-4 mutant is capable of inhibiting T cell activation, recent studies have shown that a ligand independent form of Ctla-4 is also capable of providing an inhibitory signal to T lymphocytes. In conjunction with the strictly controlled expression kinetics and the perfect amino acid homology between the intracellular domains of mouse and human Ctla-4, this data suggests that Ctla-4 may participate in the modulation or initiation of intracellular signaling pathways.

Positive and negative costimulatory receptors on the T cell modify lymphocyte responses by altering both quantitative and qualitative aspects of the lymphocyte response including threshold of activation, cytokine secretion, and memory responses.

Positive costimulation augments T cell responses, in part, by downregulating the expression of genes that actively maintain the quiescent phenotype. This study was initiated to determine the role of Ctla-4 ligation in modifying the global gene expression profile of stimulated T cells and to determine if the Ctla-4 mediated maintenance of T cell tolerance was achieved, in part, by altering the transcription of quiescence genes necessary for the prevention of T cell activation subsequent to TCR and CD28 stimulation.

Previous studies investigating the influence of Ctla-4 ligation on transcriptional profiles of activated lymphocytes detected only quantitative alterations in the transcriptional regulation initiated by CD28 signaling. In contrast, our data suggests that quantitative effects of Ctla-4 ligation that differentially influence pathways acting downstream of stimulatory receptors results in a stable and qualitatively unique phenotype detectable at the level of the transcriptome. Thus, the cumulative effect of Ctla-4 signaling is unique and not constrained to reversing alterations in expression initiated by CD28. In addition, Ctla-4 ligation can be shown to influence T lymphocyte responsiveness and the resulting global expression profile within 4 hours after stimulation and prior to detectable Ctla-4 surface expression. In a subpopulation of T cells, TCR stimulation activates pathways that result in commitment to activation with 2-6 hours. In contrast, CD28 signaling must be maintained for 12-16 hours to ensure maximal responses at the population level. The period of sensitivity to Ctla-4 inhibition of activation is more constrained and does not extend beyond 12 hours. Together, these data support a potential role for Ctla-4 in modification of the early transcriptional response

and may explain various alterations in phenotype resulting from Ctla-4 ligation that have been reported in secondary responses.

Identification of genes involved in lymphocyte activation, maintenance of self-tolerance, and attenuation of immune responses opens the door to therapeutic manipulation of the pathways implicated. CD28 costimulation results in general amplification of TCR-initiated transcriptional responses, and specifically alters the expression profile of a subset of genes. In contrast, Ctla-4 ligation directly and specifically alters the expression of a select group of genes when ligated, and results in minimal suppression of the global CD28-mediated costimulatory transcriptional response. Ctla-4 regulated genes comprise a heterogeneous family, but include known quiescence factors, transcriptional regulators, and various determinants of cell cycle progression and senescence. The role of Ctla-4 in maintaining self-tolerance indicates that targeted manipulation of these gene products presents a novel therapeutic opportunity, and suggests that the mechanisms involved in Ctla-4-mediated maintenance of peripheral T cell tolerance and regulation of immune responsiveness is more nuanced than previously thought. In addition, this study provides the most comprehensive description of global gene expression during primary lymphocyte activation yet available. The integration of statistical and bioinfomatics analyses with large scale data mining tools identifies genes not previously characterized in lymphocytes and can direct future work by predicting potentially interacting gene products and pathways.

Table of Contents

List of Tables

List of Figures

Attributions

Purified T cell populations were sorted and prepared by the UMass Flow Cytometry Core Facility

Retroviral constructs were prepared by Dr. Hai Nguyen.

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CHAPTER I

INTRODUCTION

Introduction:

Central and peripheral mechanisms of tolerance in the immune system

Broad specificity within the adaptive immune system enables potent immunological defenses against a wide range of foreign pathogens. The repertoire of the peripheral T lymphocyte compartment encompasses an estimated $25x10^6$ T-cell receptors (TCRs) enabling recognition of both foreign and self-antigens(1). While the diversity of the TCR repertoire is necessary for effective immune surveillance against a range of potential pathogens, the processes that generate diverse TCR specificities also result in the generation of antigen receptors specific for self-peptides. Mechanisms of central tolerance serve to eliminate potently self-reactive lymphocytes during thymic maturation by induction of apoptosis in thymocytes that recognize self-peptide bound to Major Histocompatibility Complex (MHC) with high affinity, in a process termed "negative selection". Deletion of overtly autoreactive thymocytes is dependent on intra-thymic presentation of diverse peripheral tissue antigens to developing lymphocytes. Impaired self-peptide presentation results from defects in the regulation of transcription of peripheral antigens in the thymus and leads to autoimmune pathology with broad specificity for peripheral tissues(2, 3). Negative selection potently suppresses the generation of autoreactive lymphocytes, but mature peripheral lymphocytes with TCRs specific for self-antigen are nonetheless detectable in healthy individuals(4, 5). Mechanisms of peripheral tolerance serve to prevent or attenuate pathological activation of autoreactive lymphocytes that avoid thymic deletion. Nonetheless, positive selection of thymocytes ensures that all peripheral lymphocytes express TCRs with low inherent affinity for self-peptide/MHC complexes. Consequently, avoidance of inadvertent

lymphocyte activation in the periphery is dependent on additional receptor-ligand interactions that regulate lymphocyte responsiveness to TCR engagement, coordinate numerous cellular responses, and modulate the scope of any resulting immune activation.

T Lymphocytes and the Immune Response:

The Two Signal Model of T Cell Activation

Peripheral T cell responses to a given antigen involve the clonal expansion of a small subset of T cells that possess unique TCRs but have common antigen specificity. As a result, the overall response to antigen encounter is a direct consequence of factors influencing the activation, expansion, and regulation of individual T cells. TCR engagement of peptide/MHC (pMHC) ligands on professional antigen presenting cells (APCs) is critical for the initiation of T cell mediated immune responses(6). TCR recognition of cognate pMHC, referred to as signal one, is required for T cell activation and determines the antigen specificity of T cells involved in normal immune responses. In addition, the TCR is differentially responsive to subtle changes in its ligand and can mediate signals that result in diverse biological responses(7, 8).

TCRs possess inherent affinity for self-pMHC as a result of positive selection during thymic development. Central tolerance mechanisms restrict the affinity of interactions between TCRs and self-pMHC that are involved in peripheral immune homeostasis, and serve to prevent unwarranted lymphocyte activation. In the absence of additional signals, TCR engagement by a MHC-antigen complex is reported to result in

anergy, apoptosis, or inefficient T cell activation(9). The requirement for an additional signal to optimize T cell activation has proven critical for the maintenance of peripheral self-tolerance and the initiation of productive immune responses.

The *two-signal model* of lymphocyte activation states that optimal lymphocyte responses require an initial signal resulting from TCR interaction with APC-expressed pMHC complexes, coupled with a secondary antigen-independent signal termed costimulation(10)(Figure 1.1). CD28 is the archetypal costimulatory molecule and is constitutively expressed by >90% of human CD4+ T cells, and >50% of human CD8+ T cells. CD28 interaction with B7 molecules on APCs provides signal two and results in enhanced activation, accelerated cell cycle progression, T cell survival, and clonal expansion(11, 12). The hallmark of CD28 costimulation is enhanced interleukin-2 (IL-2) production and bcl-x_L upregulation, which promote T cell expansion and support the development of an effective immune response(13). CD28 enhances T cell activation by diverse mechanisms that serve to enable TCR signaling and promote prolonged T cell-APC interaction.

CD28 engagement amplifies membrane-proximal signaling initiated by the TCR, but also transduces a unique signal reportedly necessary for the stabilization of some RNA transcripts and efficient upregulation of numerous genes including IL-2 and bcl-X_L(11, 14-18). In addition, CD28 signaling potentiates the macromolecular reorganization of the cell membrane initiated by TCR ligation, resulting in aggregation of numerous multimolecular signaling complexes at the site of T cell/APC contact(19-21). In fact, recent work suggests that CD28:B7 interactions in the absence of TCR triggering are sufficient for cell polarization and lipid raft aggregation.(22) The resulting structure,

Figure 1.1: The two signal model of T cell Activation

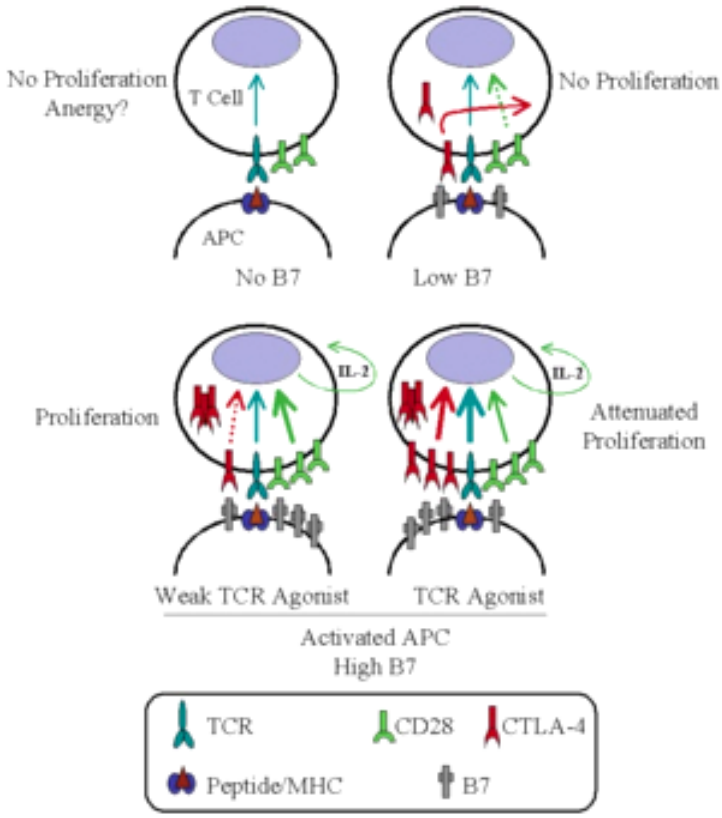


Figure 1.1: The two-signal model of T cell activation. In addition to antigen receptor triggering, T cells require second antigen-independent signals via CD28:B7 interaction in order to respond efficiently. CD28 ligation enhances TCR-induced signals, and also transmits a unique signal that results in increased survival, facilitates cell-cycle entry, and leads to efficient cytokine production. Ctla-4 ligation interferes with proximal TCR signaling and suppresses distal effects of CD28 costimulation. The relative balance of CD28 and Ctla-4 signaling may dictate the outcome of antigen-receptor engagement in a manner dependent on B7 ligand availability.

termed the *immunological synapse* (IS), stabilizes the intercellular contact for as much as 24 hours, and is reportedly necessary for full commitment to activation(23). However, the temporal parameters governing CD28 costimulation have not been well defined.

While CD28 functions primarily to enhance lymphocyte activation, it also plays a prominent role in the maintenance of peripheral tolerance. CD28 signaling is obligatory for the development and maintenance of regulatory T cells(24), up-regulates immune attenuators including Ctla-4, and skews T cell responses towards an autoimmune protective Th2 phenotype(24, 25). CD28-mediated effects on TCR signaling pathways have been well characterized, but the molecular and biochemical mechanisms mediating qualitative effects of CD28 costimulation are poorly understood and the distal effectors of CD28 signaling have not been fully elucidated.

The B7/CD28 family of immomodulatory receptors:

Members of the B7/CD28 family of receptors regulate diverse aspects of the adaptive immune system and are key determinants of T cell responsiveness and effector function (Table 1.1). Signaling pathways initiated by B7/CD28 family members are essential components of mechanisms that simultaneously promote and sustain T cell responses to pathogens and serve to maintain tolerance to self-antigen by inhibition or attenuation of T cell responses. CD28 and Ctla-4 are the critical costimulatory receptors regulating antigen responsiveness of mature peripheral T cells, and provide positive and negative second signals to T cells,

Table 1.1: The B7/CD28 family of immunomodulatory receptors

A: Comparison of CD28 family members.

Comparison of CD28 family of receptors

	CD28	**CTLA-4**	**ICOS**	**PD-1**	**BTLA**
% identity	100%	30%	27%	23%	23%
Chromosome					
Human	2q33	2q33	2q33	2q37	3q13.2
Mouse	1C1, 30.1 cM	1C2, 30.1 cM	1C2, 32 cM	1D, 55 cM	16A1
Structure					
Ligand binding motif	MYPPPY	MYPPPY	FDPPPF	?	?
Cytoplasmic domain	PI3K motif, PP2A	PI3K motif, PP2A, SHP2	PI3K	ITIM motif, SHP2ITSM motif, SHP1	Two ITIM motifs
Expression					
Cell type	T	T	T, NK	T, B, M	T, B

B:

Comparison of B7 family of costimulatory molecules

	B7-1 (B7.1)	B7-2 (B7.2)	ICOSL (B7h, B7-H2; B7RP-1)	PD-L1 (B7-H1)	PD-L2 (B7-DC)	B7-H3	B7-H4 (B7x; B7S1)
% identity of extracellular domain	100%	27%	27%	25%	23%	29%	21%
Chromosome							
Human	3q13	3q21	21q22	9p24	9p24	15q24	1p13.1
Mouse	16B, 32.8	16B, 26.9	10C1	19B	19B	9A	3F2.2
Expression[a]							
Lymphoid[b]	B, M, DC, T	B, M, DC, T	B, M, DC, T	B, M, DC, T	DC, M	B, T, M, DC, NK	T, B, M, DC
Non-lymphoid	Rare; including podocyte	Rare	FibroblastEndothelialEpithelial	Endothelial, Tissues (including placenta);Tumors (including many T cell lymphomas, carcinomas, melanomas, glioblastoma	Some B cell lymphomas	Bone Marrow	Lung and ovarian tumors
Receptor	CD28 CTLA-4	CD28 CTLA-4	ICOS	PD-1	PD-1	?	?

[a]Protein, but not mRNA expression is summarized; see text for regulation of expression.

[b]B=B cell; M=macrophage; DC=dendritic cell; T=T cell; NK=natural killer cell.

Table 1.1: Comparison of CD28 (Panel A) and B7 (Panel B) family members.

respectively. CD28 transmits signals important for T cell survival and proliferation, while Ctla-4 inhibits T cell responses and regulates peripheral tolerance. Ctla-4 and B7 share B7 ligands, B7.1 and B7.2. Regulation of Ctla-4 and CD28 signaling is effected by precise control of surface expression, availability of ligands, differential ligand-binding characteristics, and unique signaling properties. Additional members of the CD28 family include ICOS, PD-1, and BTLA. While the ligand for the inhibitory receptor BTLA remains undiscovered, both ICOS and PD-1 are known to bind B7 family members. ICOS and PD-1 result in positive and negative regulation of T cell responses respectively. However, CD28 and Ctla-4 are unique in their ability to regulate primary T cell responses and provide dominant signals controlling T cell activation in naïve lymphocytes.

Biology of CD28 Costimulation:

CD28 costimulation is essential for normal immune function. CD28-deficient mice or mice treated with antagonists of CD28:B7 interaction manifest profoundly defective responses to allograft antigens(11), infectious pathogens(26-29), GVHD(30), and induction of contact hypersensitivity(31). Similarly, absence of CD28 costimulation reduces T cell proliferation in vitro and in vivo in response to TCR stimulation(32-34). More globally, CD28 deficiency results in diminished germinal centre formation and defective isotype class switching in B cells, and diminished CD4-dependent CD8+ T cell responses(35-37).

CD28 is uniquely capable of synergizing with TCR-mediated signals to activate transcription factors controlling proliferation, differentiation, and cell death including NFκB(18), NFAT(15, 38), and AP-1(39). While receptor-ligand interactions mediating costimulatory signals are diverse, CD28 is unique in its ability to integrate signals with the TCR and to centrally determine the outcome of antigen receptor engagement in naïve T cells(40, 41). Moreover, CD28 is unique in its ability to provide signals required for cellular activation, cell cycle entry, and efficient cytokine production in lymphocytes, and results in greater enhancement of immune responses than other costimulatory receptor interactions(42, 43). An essential component of CD28 costimulation is regulation of distal determinants of cell death, proliferation, and differentiation including cytokines interleukin 2 (IL-2), interferon gamma (ifn-γ), and interleukin 4 (IL-4)(44); chemokines Mip-1alpha/CCL3(45); cytokine receptors CD25, IL-12R, and CXCR5(26, 46, 47); and APC counter-receptors Ctla-4/CD152, CD154/CD40L, ICOS, CD134/OX40, and 4-1BB(47, 48). Thus, CD28 orchestrates the regulation of genes that shape the phenotype of responding cells in ways that determine not only their immediate response, but also their ability to respond to and provide intercellular signals in subsequent days.

CD28 regulates key signaling intermediates that control the initiation and character of an immune response

CD28-ligand interactions determine proximal regulation of signaling thresholds as well as subsequent events leading to gain of effector function. Quantitatively, CD28 costimulation functions primarily to potentiate signals initiated by TCR engagement. CD28 costimulation directly alters intrinsic properties of TCR signaling by facilitating

TCR/pMHC interaction and enhancing ZAP70 phosphorylation(49-51). In addition, CD28 enhances TCR-induced glycolipid-enriched membrane microdomain (GEM) aggregation following TCR triggering and amplifies early TCR-signaling events mediated by PLC-γ(21, 51-53). Distally, CD28 alters the global expression profile of antigen receptor activated lymphocytes by amplifying gene regulations that result from TCR stimulation(15, 54). At the single-cell level, altered expression of CD28 target genes define cellular differentiation, burst size(55), and long-term cytokine production(34). Thus, CD28-mediated signals modulate the evolving character of an immune response via mechanisms distinct from its membrane-proximal enhancement of TCR signaling.

CD28 signaling modifies the activity of proteins involved in the determination of cellular commitment to proliferation prior to significant changes in transcription. The hallmark of CD28 function is the upregulation of Il-2 production and CD25 expression subsequent to antigen receptor engagement(26, 33, 44, 55). In vivo, however, common γ-chain cytokines, including IL-2 are not essential for antigen-driven cellular proliferation(56, 57) and CD28 enhancement of cell cycle entry and progression is at least partially independent of IL-2 production(58).

CD28 directly modulates cell cycle regulators prior to IL-2 production. Repression of E2f regulated genes is central to the ability of inactive pRB to maintain cells in the G0 stage of the cell cycle(59). CDK4/CDK6 mediated phosphorylation of pRB allows entry into the cell cycle, and progression through late G1 and S phases is dependent on cyclin-E/CDK2 phosphorylation of pRB as well(59). CD28 signaling upregulates D cyclin expression (60, 61) and leads to the activation of CDK4/CDK6 and

the consequent phosphorylation of pRB within 3-4 hours of activation, and prior to IL-2 production(62). Inhibition of CDK protein activity by CDK-interaction protein (CIP), and INK and cyclin-dependent Kinase inhibitors(KIP) protein families prevents entry into G1(63). CD28 signaling results in degradation of KIP1 via the PI3K-AKT pathway, but KIP1-deficient mice maintain normal proliferative responses to activation signals initiated by CD3/CD28 probably as a result of redundant inhibition of cell cycle entry by INK4C, which is also antagonized by CD28 signaling(61).

Entry into the cell cycle is accompanied by increases in cellular metabolism, increased expression of genes involved in synthetic pathways, and increases in cell size(64) in a process enhanced by CD28(15, 65). The CD28 signaling pathways leading to enhancement of cellular metabolism and macromolecular synthesis implicates MTOR, (66) acting downstream of PI3K in a pathway commonly dysregulated in cancer(67, 68). Similarly, the pleiotropic transcription factor c-myc regulates genes controlling transcription initiation factors, ribosomal RNA synthesis, and determinants of cellular division (E2Fs and CDC25(64)), making it a potential downstream target of CD28(69).

CD28 regulates mechanism influencing peripheral self-tolerance

TCR-triggering without concomitant CD28 ligation is reported to result in the induction of anergy or apoptosis. TCR engagement in the absence of costimulation may enhance apoptosis induction by failing to sufficiently upregulate anti-apoptosis factors or via inhibition of cell cycle progression(70-72). CD28 signaling specifically induces expression of the anti-apoptotic factor bcl-xL (73) in a NF-kB dependent manner while simultaneously repressing pro-apoptotic factors including p73(74). Thus, CD28

signaling may critically determine the clonal diversity of an immune response by enhancing survival of antigen-receptor-stimulated T lymphocytes in a cell-specific manner.

CD28 signaling may also control peripheral tolerance by regulating anergy induction. Anergy is an acquired, antigen-specific state of hyporesponsiveness in which lymphocytes fail to respond to appropriate antigenic stimulation(Reviewed in (75)). Anergy induction in T cells is a tolerance mechanism that results in the persistence of lymphocytes that are functionally inactivated by antigen encounter and remain hyporesponsive to subsequent antigen receptor triggering even in the context of sufficient CD28 costimulation.(76) Therapeutic anergy induction has potential in treating autoimmune disease and has been used to prolong allograft survival in animal models(77). Two general forms of anergy have been described that represent distinct biological states resulting from distinguishable signaling events.(78) Clonal anergy is principally a state of growth arrest resulting from incomplete T cell activation and is most often seen in previously activated cells. Clonal anergy is reversible by addition of exogenous IL-2 or anti-Ox40 treatment, results from a defect in the Ras/MAP kinase pathway that may involve Egr-2(79), and generally does not result in suppressed effector function. In contrast, adaptive anergy generally develops in vivo following submitogenic antigen-receptor triggering on naïve CD4+ lymphocytes in the absence of CD28 mediated costimulation or the presence of significant coinhibitory signals(80). Adaptive tolerance induction occurs subsequent to an initial proliferative response and requires antigen persistence. Concomitant CD28 ligation prevents antigen-receptor-mediated induction of T cell anergy(81) through mechanisms dependent on CD28 enhancement of

cell cycle progression(80). The state of adaptive anergy results from an early block in tyrosine kinase activation with consequent inhibition of calcium mobilization and impaired signaling through the IL-2 receptor(82), and proximal signaling defects in anergic cells may be related to defective LAT activation(83). However, it has also been demonstrated that anergy avoidance subsequent to CD28 ligation utilizes PKC-theta-dependent pathways distinct from those that augment proliferation(84) supporting the notion that CD28 determines qualitative aspects of immune responsiveness.

Numerous genes identified as potential mediators of anergy induction have recognized roles in T cell activation and costimulatory pathways. In particular, Cbl-b is one of several E3 ubiquitin ligases involved in the induction and maintenance of an anergic phenotype((85), Reviewed in (86)) that has a key role in the integration of costimulatory signals originating with CD28 and Ctla-4(87). Identification of pathways and signaling determinants involved in anergy induction will necessitate a detailed understanding of T cell costimulatory pathways and may enable therapeutic manipulation of this endogenous mechanism of self-tolerance.

CD28 alters qualitative aspects of T cell differentiation

Cellular division in CD4+ lymphocytes is accompanied by irreversible acquisition of specific phenotypes defined by cytokine profile. Following activation, undifferentiated naïve CD4+ Th0 cells respond to costimulatory signals that direct development toward the Th1 and Th2 effector cell lineages that coordinate cell-mediated immunity and humoral responses respectively (Reviewed in (88)). Th1 effector cells secrete interferon-gamma, and regulate CD8+ T cell responses in response to intracellular

pathogens. In contrast, Th2 cells produce IL-4, promote allergic responses, and are critical for effective immunity against helminthes. Polarizing influences result in the expression of distinct transcription factors in Th1 and Th2 cells necessary for cellular differentiation, particularly T-bet and GATA3 respectively(89), and are partially influenced by cellular division(89-91). CD28 preferentially skews T lymphocyte differentiation towards a Th2 phenotype via NF-kB induction of GATA3(92-94). Concomitant Ctla-4 engagement counteracts CD28 mediated changes in gene transcription via direct inhibition of GATA3 but not T-bet mRNA expression(54, 95) and encourages Th1 differentiation of activated naïve T cells.(96) Moreover, Ctla-4 deficiency increases the efficiency of CD28 signaling and results in enhanced NF-kB activation, GATA3 expression, and Th2 skewing(97). Thus, integration of costimulatory signals is critical to lineage fate determination in naïve cells with resulting long-term consequences and differential susceptibility to infection and autoimmunity. While the critical determinants of lineage fate decisions have been identified, a precise understanding of the upstream regulatory mechanisms involved in costimulatory signal integration that precede lineage commitment will enhance our ability to design knowledge-based therapeutic intervention strategies.

Epigenetic regulation of gene expression by CD28

In Eukaryotic cells, the physical structure of genes influences their expression. DNA methylation and chromatin remodeling are critical determinants of gene accessibility for transcriptional machinery. CD28 signaling results in chromatin modification and epigenetic regulation of gene expression.(90, 98-105) Mechanisms of

CD28-mediated epigenetic regulation of gene expression involve posttranslational regulation of constitutively expressed proteins, as evidenced by the fact that cytosine demethylation at the IL-2 locus occurs within 20 minutes of TCR/CD28 ligation.(104) Similarly, modification of chromatin accessibility at gene loci involved in cellular differentiation (IL-4 and IFN-γ) have been detected within one hour of TCR/CD28 ligation, and reinforce the notion that proximal signal integration of the TCR and CD28 pathways is definitive soon after activation(89). CD28-mediated signals reinforce re-positioning of the SW1/SNF-related chromatin remodeling BAF complex within minutes of TCR/CD28 engagement in naïve T cells in a process that is critically dependent on both PKC/RAS pathways and calcium flux.(98) CD28 engagement results in robust expression of c-REL(102) through a CsA sensitive pathway(15), and results in chromatin modification of the IL-2 gene locus via interaction with the CD28-responsive regulatory element.(18, 102) Critically, recent observations suggest that stable chromatin remodeling may alter subsequent cellular responses to activating stimuli. Antibody ligation of CD3 and CD28 results in increased IL-2 expression following active demethylation of a specific CpG site, recruitment of Oct-1, and histone modification.(106) Oct-1 remains bound to the IL-2 enhancer following termination of stimulatory signaling resulting in accelerated and magnified gene transcription upon reactivation. Moreover, fully-activated CD4+ effector cells possessed stable histone acetylation and loss of cytosine methylation at the IL-2 promoter enhancer with concomitant chromatin remodeling. In contrast, CD4+ T cells rendered anergic by TCR ligation in the absence of costimulation showed no histone acetylation or cytosine demethylation in the IL-2 promoter/enhancer region, and an absence of chromatin

remodeling(107). CD28 signal-specific epigenetic modification of the IL-5 gene locus has also been implicated in Th2 lineage differentiation and appears dependent on NFκB activation and GATA 3 upregulation(108). Stable epigenetic modification of target genes as a consequence of costimulation may provide a historical record of prior signaling events that have the potential to influence future responses.

CD28- and TCR-signaling utilize unique and overlapping pathways:

The TCR and CD28 signal independently and display unique biophysical and biochemical properties. Relative to TCR interactions with peptide/MHC complexes on the APC, CD28 displays significantly higher on and off rates in its interactions with B7 ligands(42, 50). Moreover, APCs express many more B7 molecules than TCR ligands, suggesting that CD28 engagement by B7 ligands is more numerous, dynamic, and fluid than TCR-pMHC interactions. Unlike the TCR, which signals through ten immunoreceptor tyrosine-based activation motifs (ITAMs) located on CD3-ζ chains(109), CD28 signaling is dependent on non-ITAM tyrosine phosphorylation events and a proline rich region(110). While the mechanism by which TCR-pMHC interaction initiates signaling, a process termed receptor triggering, remains controversial, it may depend on the inherent biophysical properties of the receptor ligand pairs that results in size-based segregation of the relevant cell-surface molecules based on the dimension of their ectodomains(42, 111-113). CD28 ligation activates discrete signaling cascades that differentially depend on its association with the TCR and localization to the

immunological synapse(15-17). CD28 aggregation in the immunological synapse is driven by APC-expressed B7(114) in a process dependent on the B7 cytoplasmic tail(115) that may regulate the micrometer-scale colocalization of the TCR and CD28 that is necessary for efficient costimulation(116). Both CD28 and the TCR relocalize to glycolipid enriched membrane microdomains (GEMs) containing active LCK protein tyrosine kinase upon interaction with their cognate ligands(112, 117). Association with the PTKs LCK or FYN results in phosphorylation of tyrosine residues in the cytoplasmic domains of CD28 and the TCR(118). In contrast to their shared dependence on PTK activity, the TCR, but not CD28, ligation results in activation of ζ-chain associated ZAP70 and phosphorylation of the LAT-SLP76 (linker for activation of T cells-SH2 domain containing protein of 76 kDa) complex(51). LAT-SLP76 phosphorylation and recruitment provides the structural framework upon which various adaptors and enzymes aggregate to activate all of the main pathways responsible for T cell activation(109). The resultant signaling complex results in rapid activation of PLC-γ, cleavage of membrane lipids to produce PIP2, PIP3, and DAG, increased intracellular calcium, and activation of RAS (via GRP, a DAG-binding guanine exchange factor(GEF)) and most PKCs(119). Simultaneously, RAS-GRP and VAV1 activity produces activated RAS-GTP and RHO family GTPases, and PI3K modified lipid byproducts are generated, with the resultant effect of reorganizing the plasma membrane and cytoskeleton, and orientation of the MTOC towards the site of APC contact(120, 121). Secondary changes in chromatin accessibility at specific loci, accelerated intracellular vesicular trafficking, and nuclear translocation of constitutively expressed transcription factors are detectable within hours.

Under conditions of supraphysiological TCR ligation, as may occur with antibody binding, stable TCR aggregation in the absence of costimulation can result in activation of all the main signaling pathways implicated in T cell activation, cell cycle entry, and widespread gene regulation. In contrast, isolated CD28 ligation does not lead to cellular activation, results in limited transient gene regulations, and has no known biological effect(15, 17, 54). However, the gene regulations that occur subsequent to CD28 engagement in the absence of TCR ligation specify some of the pathways involved in CD28 costimulation of TCR signals. In particular, binding of the p85 regulatory subunit of PI3K by the phosphorylated tail of CD28 initiates a PtdIns-dependent kinase(PDK)- and AKT-dependent phosphorylation of glycogen synthase kinases 3α and 3β(15, 122, 123). Ablation of PI3K interactions with CD28 by mutation of the relevant tyrosine residue results in defective AKT activation(124), diminished IL-2 production(125), reduced bcl-xL expression(126, 127), and a general reduction in antigen-driven T cell responses in vivo(128). Activated AKT is a critical downstream mediator of CD28 signaling as AKT amplifies TCR mediated gene regulations by cooperative influences on NF-kB activity with TCR-induced PKC-θ, and GSK3 inhibits NFAT export(15, 129-131).

The ability of CD28 signaling to directly regulate the activity and nuclear localization transcription factors underlies its ability to influence cell cycle progression and metabolism in activated T cells. AKT in particular is hypothesized to regulate numerous transcription factors downstream of CD28 including Forkhead, c-myc, and the cyclin-d-CDK4/CDK6 complex.(122, 132) In addition to its influence on AKT activity, PI3K activation by CD28 is likely to influence additional pathways involved in mediating

the pleiotropic effects of costimulation, including those involving mTOR and PKC activation(123, 133, 134). The costimulatory mechanisms regulating these diverse pathways during T cell activation remain only partially characterized but imply a central role for specific signaling intermediates involved in the integration of signals resulting from upstream activation of the TCR and CD28.

The TEC family kinases TEC and ITK are crucial mediators of activating signals delivered through the TCR, and were thought to be differentially regulated by CD28-mediated costimulatory signals. More recently, ITK has been shown to operate independently of CD28 costimulation(135), but the central role TEC family kinases play in T cell responsiveness warrant further discussion of their role in T cell activation. TEC and ITK are regulated by LCK or FYN-dependent phosphorylation, PIP3, PIP3 independent VAV1 mediated mechanisms, and direct protein-protein interaction(65, 136). Positive regulation of PLC-γ by TEC and ITK is dependent on TCR-mediated phosphorylation of LAT-SLP76. TEC and ITK activate PLC-γ to increase intracellular calcium, increasing PKC by DAG-dependent mechanism, and activating RAS/RAF/ERK through RAS-GRP(51, 129, 137). Mutations in the tail of CD28 that prevent ITK activation abolish costimulatory enhancement of PLC-γ activity, calcium flux, and NFAT activation(51). Mutant NFATc that is constitutively localized to the nucleus allows full activation of T cells activated through the TCR in the absence of CD28 costimulation, highlighting the central role TEC family kinases play in mediating distal effects of CD28 signaling(15, 138).

Vav1 stabilizes the numerous protein-protein interactions required for productive proximal signaling by the TCR, and participates in nearly all TCR signaling

pathways(139). Identification of costimulation-defective mutants of CD28 in which PI3K binding is unaffected but Vav1 phosphorylation is abolished highlights the central importance Vav1 plays in mediating CD28 costimulatory signals(125, 140, 141). CD28 recruits Vav1 to the membrane by unknown mechanisms, and Vav1 phosphorylation by CD28 activates GEF activity by removing intramolecular inhibition of the DBL-homology domain by the pleckstrin-homology domain(142). Early reports indicated that membrane recruitment and activation of Vav1 by CD28 required TCR dependent phosphorylation of SLP76(50, 143, 144). However, recent work indicates that B7 driven accumulation of CD28 at T cell:APC interfaces results in Vav1 activation in the absence of TCR ligation and correlates with increased calcium flux and NFκB p65 translocation to the nucleus(22). In addition, CD28 signaling has been shown to result in posttranslational modification of Vav1 via a novel CD28-costimulatory pathway that results in Vav1 methylation and nuclear localization(145). Vav1 mediates CD28 induction of NFAT, Nf-kB, and AP1 activity,(146-148) and is crucial for Th2 differentation independent of GATA3 expression(149). Several reports have linked Vav1 activity to Cbl-b, an essential downstream regulator of CD28 costimulation(150, 151). Absence of Cbl-b restores defective Ig class switching and germinal center formation in Vav1 deficient mice(152). In addition to its role in peripheral T cell activation, Vav1 is critical for normal TCR and CD28 mediated signaling in lymphocyte development(153). The profound defects observed in Vav1-deficient thymocytes and mature lymphocytes(153-157) result in large part from the ability of Vav1 to regulate RAC-GTP production. RAC-GTP participates in regulation of PI3K, which then regulates the activity of TEC PTK, PLC-γ1, AKT, and more distal downstream effectors including

nuclear import of NFAT, and activation of NF-kB and RAS/RAF/ERK(139, 154, 158, 159).

The E3 ubiquitin ligase cbl-b proto-oncogene(160) is a key mediator of CD28 costimulatory signals capable of direct interaction with proteins involved in signaling pathways downstream of the TCR and CD28(161, 162). Moreover, cbl-b is a key negative regulator of T cell responsiveness in vivo(161, 163, 164), influences the CD28-dependence of in vivo immunity(152), and regulates the induction and maintenance of anergy(85, 86, 149, 165-168). Cbl-b deficiency results in spontaneous autoimmunity characterized by auto-antibody production, T and B lymphocyte activation, overproduction of IL-2, infiltration of peripheral tissues and parenchymal damage(167). Cbl-b deficiency bypasses CD28-costimulation requirements for upregulation of Il-2, and restores T-cell-dependent antibody responses in CD28-deficient mice(169). Cbl-b regulates the activity of receptor protein tyrosine kinases as well as antigen and cytokine receptors that signal via associated cytoplasmic protein tyrosine kinases. Cbl-b negatively regulates T cell responsiveness by numerous mechanism including inhibition of receptor clustering and lipid raft aggregation(163), proteolysis-dependent and independent regulation of PI3K(170, 171), promotion of ligand-induced TCR down-modulation(172) and upregulation of receptor endocytosis(173), and ubiquitination-dependent regulation of key receptors and signaling intermediates(174, 175). Cbl-b contains an amino terminal Ring finger motif and putative phosphotyrosine binding domain, and carboxy terminal proline-rich region, conserved tyrosine residues, and a leucine zipper(176). Cbl-b is rapidly tyrosine phosphorylated by Syk- (syk/Zap-70) and Src- (Fyn/lck) family kinases upon TCR engagement(177, 178) allowing direct regulation

of the RAC-1 GTPase GEF Vav1 in a trimeric complex involving ligand-stimulated tyrosine kinases, resulting in inhibition of TCR mediated Vav-1 activation(169, 172) and subsequent Vav-1-mediated activation of c-Jun N-terminal kinase(179). CD28 costimulation counteracts cbl-b-mediated inhibition of TCR signals by targeting cbl-b for ubiquitination-dependent downregulation(87, 180, 181). Thus, regulation of cbl-b activation by CD28 is critical to the integration of TCR and costimulatory signals and broadly influences in vivo immunity by altering proximal signaling events and distal mediators of T cell activation(182).

Integration of TCR and CD28 signals is a multifaceted process

Cellular responses to immunological stimuli reflect the convergence of numerous, often contradictory, signaling pathways that necessarily operate to regulate distal outcomes via regulation of gene expression. Shared targets of diverse pathways determine the qualitative responses manifested by integrating quantitative signal information. The elucidation of pathways responsible for cellular activation in lymphocytes has often depended on non-physiological stimuli that might mask subtle relationships that exist between various signaling pathways, including the TCR and CD28. The differential sensitivity of antigen receptor stimulated cells to CsA inhibition in the presence or absence of PMA or anti-CD28 antibody was interpreted as evidence of distinct pathways downstream of these two receptors.(183) However, the supraphysiological stimulus provided by either anti-CD28 or PMA results in non-physiological chromatin remodeling of the IL-2 gene locus and bypasses the requirement for c-Rel and the CD28 response element that are essential for IL-2 gene upregulation in

antigen receptor stimulated cells.(35, 102, 184) More recent work has demonstrated that CD28 is, in fact, CsA sensitive under more physiological stimulation conditions,(15, 146) consistent with the ability of CD28 to enhance TCR induction of calcium flux and NFAT activity(51, 185). Similarly, the apparent dependence of IL-2 production on CD28 costimulation was thought to result from unique signal transduction pathways distinct from that used by the TCR(186, 187). However, further characterization of transcription factors regulating IL-2 gene transcription, including NFAT, c-REL-AP1, and NF-kB, identifies them as common targets of both TCR and CD28 signaling(18, 48). Nonetheless, it is a common biological phenomenon that integration of complex quantitative signals can result in qualitatively different responses.

Ctla-4 and Negative Costimulation in the Adaptive Immune Response

Molecular genetics and expression of Ctla-4

In addition to positive costimulation provided by CD28 ligation, regulation of peripheral lymphocyte responsiveness to antigen encounter is achieved by coinhibitory signaling mediated by negative regulators of T cell activation. Ctla-4 provides an inhibitory signal that counteracts positive signals transmitted through the TCR and CD28, and is necessary for the maintenance of peripheral T cell tolerance(188). Like CD28, Ctla-4 is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily(189). The Ctla-4 gene is located on chromosome 1 in mice and chromosome 2 in humans within a locus containing both ICOS and CD28 (189-193). The Ctla-4 gene contains four

exons encoding a signal sequence, an extracellular IgV-like domain containing the B7-binding MYPPY motif, a transmembrane region, and a cytoplasmic tail(190, 194).(Figure 1.2) Human and murine Ctla-4 share 76% amino acid identity overall, and the cytoplasmic domain of Ctla-4 is 100% conserved across mammals(190). The Ctla-4 gene product encodes a protein of 223aa with a 35aa signal peptide that exists as a covalently linked homodimer on the cell surface(195, 196). Homodimerization is mediated by a conserved cysteine residue at position 122 in the stalk region and by N-glycosylation at position 78 and 110(196, 197). The 36aa long cytoplasmic tail of Ctla-4 lacks enzymatic activity but contains a membrane-proximal lysine-rich region, tyrosine residues at positions 165 and 182, and a proline-rich region starting at position 169(198).

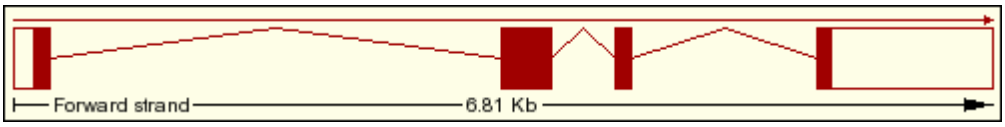
The Ctla-4 transcript undergoes alternative splicing(Figure 1.3). In humans, 3 transcripts can be detected: a full-length transcript composed of exons 1-4, a transcript coding for soluble Ctla-4 that excludes exon 3, and a transcript that includes only exons 1 and 4(191, 199-202) Mice express an additional transcript that excludes exon 2 and codes for a ligand-independent (li) form of Ctla-4(199).

In addition to thymocytes and mature T cells, Ctla-4 gene expression has been detected in a variety of cells including monocytes, B cells, CD34+ stem cells, fibroblasts, granulocytes, and mouse embryonic cells(203-208). While Ctla-4 may signal in these cells, or provide signals to other cells via B7 ligands, no function has yet been attributed to Ctla-4 in non-T cells.

Factors mediating transcriptional control of Ctla-4 gene expression in T cells have not been well defined, but are known to involve several signaling pathways. Transcriptional regulation of Ctla-4 is initiated 335bp upstream of the start codon, and the

Figure 1.2: Structure of Ctla-4 gene

A:



B: Protein Sequence

5'

```
MACLGLRRYKAQLQLPSRTWPFVALLTLLFIPVFSEAIQVTQPSVVLASSHGVASFPCEY
MACLGFQRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPAVVLASSRGIASFVCEY
*****::*:****:*.:**** . *: *******.:*::*:**:******:*:*** ***

SPSHNTDEVRVTVLRQTNDQMTEVCATTFTEKNTVGFLDYPFCSGTFNESRVNLTIQGLR
ASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTIQGLR
:.. :: *********::.*:*****:*:   * : *** .:*:** . .:*********

AVDTGLYLCKVELMYPPPYFVGMGNGTQIYVIDPEPCPDSDFLLWILVAVSLGLFFYSFL
AMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDFLLWILAAVSSGLFFYSFL
*:*****:***********::*:************************.*** ********

VTAVSLSKMLKKRSPLTTGVYVKMPPTEPECEKQFQPYFIPIN
LTAVSLSKMLKKRSPLTTGVYVKMPPTEPECEKQFQPYFIPIN 3'
:****************************************
```

C:

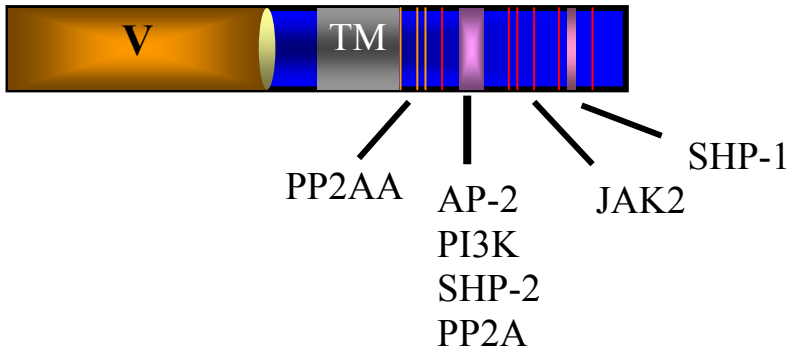


Figure 1.2: Structure of Ctla-4 gene and protein sequence. The Ctla-4 gene is located on mouse chromosome 1 and human chromosome 4. The full-length transcript encodes 4 exons corresponding to a gene length of 6.81Kb. (Panel A) Human and mouse protein are 76% conserved at the amino acid level. The cytoplasmic domain is 100% conserved. Potential phosphotyrosine residues are printed in yellow. (Panel B) Binding sites for molecules known to associate with the cytoplasmic tail of Ctla-4. (Panel C)

5' upstream region of Ctla-4 contains several transcriptional regulatory sequences including sites for NFAT, Nf-κB, AP-1, STAT, GATA-1, and Oct-1.(191, 192, 209) NFAT in particular may be central to transcriptional regulation of Ctla-4 as NFAT levels correlate directly with levels of Ctla-4 mRNA, and cyclosporine A mediated inhibition of NFAT activation reduces Ctla-4 gene transcription.(210, 211)

Ctla-4 mRNA expression is upregulated from nearly undetectable basal levels in naive T cells upon TCR-mediated activation, and its expression is further enhanced by CD28 signaling(25, 209, 211, 212). Levels of Ctla-4 mRNA are regulated, in part, by alterations in mRNA stability that occur as a result of TCR and CD28 signaling. Under conditions of TCR signaling alone Ctla-4 mRNA half-life is ~4.6 hours. CD28 costimulation increases the half-life to ~8.9 hours.(211, 213) Ctla-4 mRNA stabilization is likely mediated by three 3' UTR AUUUA motifs implicated as binding sites for proteins mediating mRNA degradation.(211, 214) Maximal Ctla-4 expression is achieved 24-48 hours after activation in both primary and secondary responses, and memory cells maintain a higher level of basal expression. (213). CD4+CD25+ regulatory T cells (Tregs) and anergized T cell clones express Ctla-4 constitutively(215) Regulation of Ctla-4 protein expression is complex and incompletely understood. Unlike CD28, which is constitutively expressed on T cells, Ctla-4 is undetectable on naïve T cells. Ctla-4 is quickly upregulated subsequent to TCR ligation and reaches maximal expression at the cell surface 24-48 hours following activation. In resting cells the vast majority of Ctla-4 protein is maintained intracellularly in vesicles located in the region of the microtubule organizing center (MTOC). TCR engagement initiates cytoskeletal

Figure 1.3: Ctla-4 undergoes alternative splicing

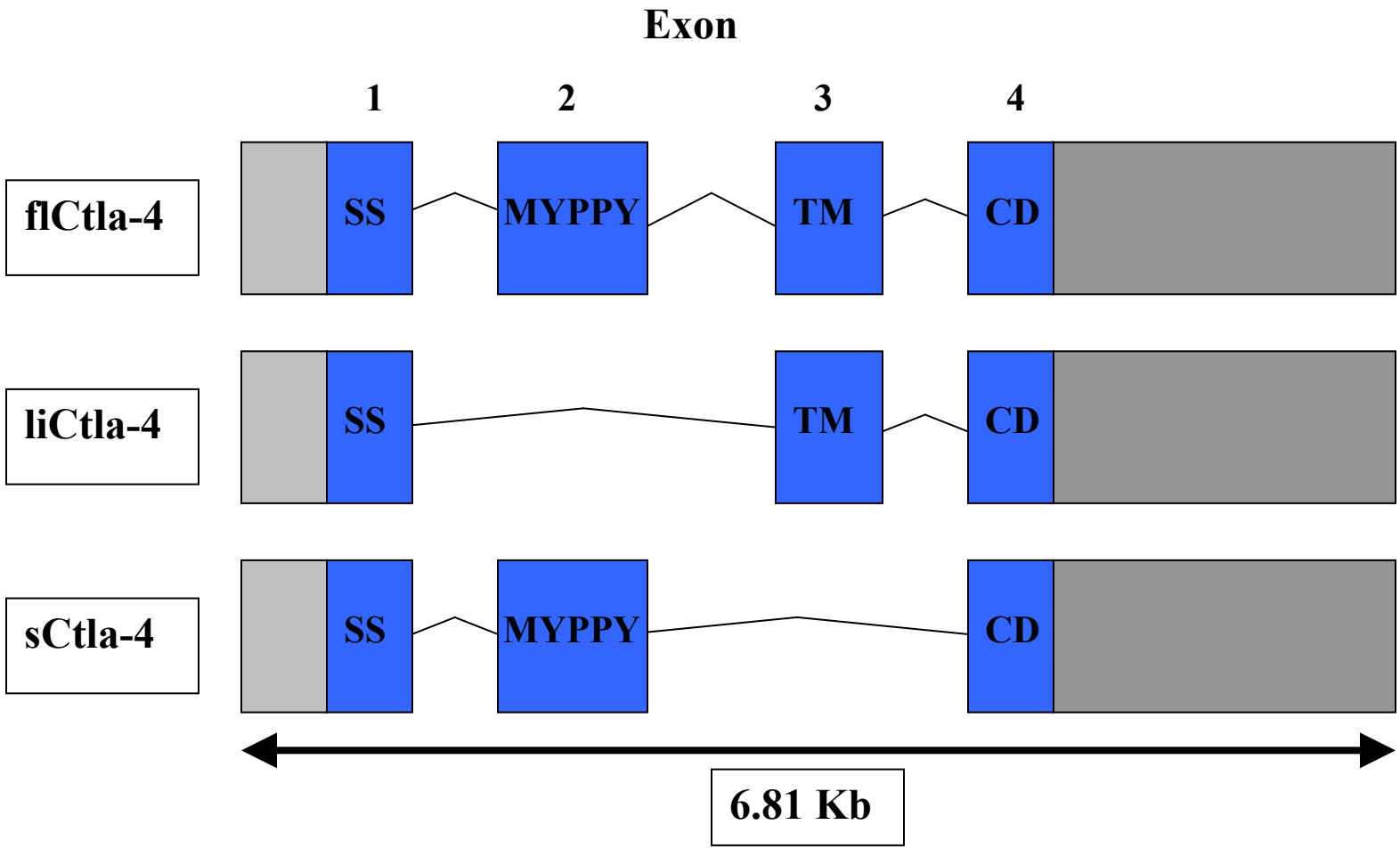


Figure 1.3: Ctla-4 undergoes alternative splicing. Ctla-4 contains four exons encoding a signal sequence, the ligand binding domain containing the B7-binding MYPPY motif, a transmembrane region, and the cytoplasmic domain. Single nucleotide polymorphisms in non-coding regions of the Ctla-4 gene in humans and mice result in altered splicing. In humans, common allelic variation results in reduced production of the soluble isoform of CTLA-4 which lacks the transmembrane region and is associated with increased risk of several common autoimmune diseases including Graves disease and type I diabetes. In mice, increased risk of type I diabetes is associated with lower mRNA levels for the ligand-independent form of Ctla-4 which has been shown to transmit an inhibitory signal in T cells. An alternative transcript lacking the cytoplasmic domain has also been reported in mice, but no function has yet been attributed to it.

rearrangement, reorientation of the MTOC towards the point of APC contact, and vesicular trafficking of CTLA-4 to a point proximal to the synapse where the majority of Ctla-4 is retained in vesicles throughout lymphocyte activation(216). In humans, only ~10% of total Ctla-4 is surface expressed following activation.(213, 217) However, translocation of Ctla-4 to the cell surface from intracellular vesicles and accumulation in the synapse is proportional to the strength of TCR signaling, suggesting that stronger TCR stimuli result in increased Ctla-4 mediated inhibition(216). Enhanced Ctla-4 inhibition may serve to moderate responses of T cells experiencing high-affinity antigen-receptor interactions as a means of maintaining clonal diversity in a polyclonal response(218). The half-life of Ctla-4 on the cell surface is regulated by the rate of internalization. Once internalized, Ctla-4 is rapidly degraded in lysosomal compartments, with a half-life of two hours.(213, 219, 220) Interestingly, TCR signaling can induce recycling of Ctla-4 from the lysosome to the cell surface, providing an alternative method of increasing surface expression of Ctla-4(221). Previously activated resting cells maintain higher levels of intracellular Ctla-4 and upregulate surface expression with faster kinetics.

The binding properties of the B7 molecules, CD28, and Ctla-4 support a role for Ctla-4 in the attenuation of T cell activation(42). Sequential expression of B7.2 and B7.1 on APCs favors the sequential binding of CD28 and Ctla-4 (CD28/Ctla-4 K_d are ~8 and 20 for B7,2 and B7.1 respectively). In fact, the earliest costimulatory complexes formed between CD28 and B7.2 are estimated to be 10,000 fold less stable than the later B7.1: Ctla-4 complexes. Complexes of B7.2:Ctla-4 and B7.1:CD28 are predicted to have

intermediate binding strengths and may allow fine tuning of the costimulatory or inhibitory signal. Ctla-4, despite its low level of expression in naïve cells, is capable of regulating both primary and secondary immune responses and inhibits early events in T cell activation(222). Temporal requirements of Ctla-4 mediated inhibition have not been defined, and it remains unknown how delayed or transient Ctla-4 ligation affects T cell activation.

Ctla-4 inhibits T cell activation: The Ctla-4 knockout phenotype

The central importance of Ctla-4 in the maintenance of self-tolerance is dramatically evident in the Ctla-$4^{-/-}$ mouse phenotype. Ctla-4 deficiency results in massive lymphoproliferation, multi-organ inflammatory infiltrates, and death by 3-5 weeks(223, 224). Thymocyte development is normal in Ctla-$4^{-/-}$ mice and the disease appears to result from a defect in peripheral T cell tolerance and homeostasis(225-227). Moreover, in vivo anti-Ctla-4 antibody blockade permits development of autoimmune disease in normally resistant mouse strains(228), hastens the onset while increasing the severity of diabetes in genetically predisposed mice(229), exacerbates disease in experimental autoimmune encephalitis or neuritis(230-232), and augments responses to tumor vaccines(218, 233-241). The clinical importance of such findings is already being investigated in clinical trials that utilize Ctla-4 blockade to augment endogenous anti-tumor responses(237, 239, 242-245).

Complementing the observations in Ctla-4 knockout mice and models utilizing Ctla-4 blockade, Ctla-4 ligation by B7 molecules has been shown to result in

decreased activation and inhibits both cell cycle progression through G_1 and IL-2 synthesis(222, 246). Investigators have shown alterations in the function of several known transcription factors following Ctla-4 ligation such as NFκB, IκB, and NFAT(95, 97, 247-251). Ctla-4 ligation also results in reduced phosphorylation of CD3(252) and differentially regulates mitogen activated protein kinases (MAPK) JNK and ERK(253). Targeted Ctla-4 engagement downregulates, or prevents, autoimmunity in animal models(254), and inhibits allograft rejection(230). In addition to the observed role of Ctla-4 in determining T lymphocyte activation upon primary stimulation, there is growing appreciation for the effects Ctla-4 signaling has on subsequent immune responses that are distinct in some ways from its ability to inhibit stimulatory signals originating with the TCR or with CD28. It has been shown that Ctla-4 signaling can affect the biology of cells in ways that determine susceptibility to subsequent infection by HIV(255), effector function(256), and threshold of activation upon restimulation(256). However, the mechanism by which Ctla-4 influences lymphocyte phenotype following activation is not known, and gene regulations that may be associated with enduring alterations in phenotype have not been investigated.

Ctla-4 undergoes alternative splicing

Recently, common allelic variation in the relative expression of alternative splice forms of Ctla-4 has been linked to autoimmune disease in both humans and mice(199). In mice a translationally silent polymorphism within exon 2 has been linked to altered

expression of a Ctla-4 isoform lacking the B7 binding domain. This ligand independent isoform (liCtla-4) is expressed in activated T cells from diabetes resistant strains at levels four-fold greater than that seen in the diabetes-prone NOD mouse and is proposed to be the Idd5.1 locus. In humans, susceptibility to the common autoimmune disorders of Graves' disease, autoimmune hypothyroidism, and type 1 diabetes was mapped to a 3' region of the Ctla-4 gene and the disease susceptible haplotype associated with a significant reduction in expression of a soluble isoform of Ctla-4 that lacks the transmembrane region encoded by exon 3(199). While these results indicate a central role for alternative Ctla-4 isoforms in determining susceptibility to autoimmune disease, the regulation of alternative isoform expression, target cells involved, and mechanism of action remain almost completely uncharacterized.

Ctla-4 signal integration

Ctla-4 inhibits some, but not all, signals generated by either TCR signaling or CD28 costimulation. Ctla-4 inhibits the CD28 mediated accumulation of NFAT, but not AP-1, in the nucleus, inhibiting NFAT-mediated gene regulations including IL-2 gene transcription (257). In contrast, Ctla-4 signaling does not influence CD28-mediated stabilization of IL-2 mRNA, consistent with its inability to prevent CD28 mediated Bcl-x_L accumulation(13, 257). In addition, Ctla-4 does not prevent trace amounts of IL-2 produced by CD28 costimulation from degrading the cell cycle inhibitor p27(257). Ctla-4 does not alter the transcriptional profile of human peripheral blood leukocytes stimulated through the TCR alone as assayed by microarray analysis(54), but prevents the

TCR-induced upregulation of cyclin D3(which is CD28 independent) as well as cdk4 and cdk6 as determined by western blotting(257).

Ctla-4 inhibits CD4+ lymphocyte activation by diverse mechanisms

Numerous models describing the mechanism of Ctla-4 function have been proposed and it is likely that Ctla-4 actively maintains T cell tolerance by acting at several levels to prevent dysregulated T cell activation. Ctla-4 competes with CD28 for B7 ligands(258), and a tailless mutant of Ctla-4 expressed on the surface is capable of down-regulating lymphocyte responses.(259) Furthermore, Ctla-4 forms a two-dimensional lattice with bound B7.2 that may disrupt molecular organization within the immunological synapse(197, 260), and recruits phosphatases to the lipid rafts which counteract kinase activity resulting from TCR and/or CD28 ligation(252)(Figure 1.4). More distally, Ctla-4 ligation inhibits changes in gene expression triggered by TCR and CD28 signaling,(54) but the significance of Ctla-4 mediated gene regulation in the maintenance of tolerance remains unclear.

A significant observation is that Ctla-4 mediates cell-extrinsic regulation. Transplantation of T cell depleted Ctla-$4^{-/-}$ bone marrow into RAG-deficient recipients reconstitutes the lymphoproliferation and multi-organ T cell infiltration characteristic of Ctla-4 deficiency. However, transplantation of a 1:1 mixture of Ctla-$4^{-/-}$ and Ctla-4^{+} T cell deleted bone marrow produces healthy mice, stable chimerism within the myeloid and lymphoid compartments, and normal cellular phenotype(261). Additionally, Ctla-$4^{-/-}$

CD4+ and Ctla-4$^{-/-}$ CD8+ population became activated, expanded, and contracted in a manner indistinguishable from their WT counterparts in response to lymphocytic choriomeningitis virus (LCMV), leishmania major, and mammary tumor virus which cause acute, chronic, and persistent infections, respectively(262). Together, results from chimeric bone marrow experiments indicate Ctla-4$^{-/-}$ lymphocytes can be non-autonomously regulated by Ctla-4 WT cells, implying that the pathology seen in Ctla-4$^{-/-}$ mice is likely to result from intrinsic defects in cell activation as well as extrinsic regulatory defects. Work in our lab has shown that depletion of WT cells in established bone marrow chimerae initiates the characteristic lymphocyte activation, proliferation and tissue infiltration seen in Ctla-4 deficient mice. However, depletion of either CD4+ or CD8+ WT cells alone fails to disrupt regulation of Ctla-4$^{-/-}$ cells. Moreover, current evidence fails to support a dominant role for Tregs in extrinsic regulation by Ctla-4 in bone marrow chimerae. To date the mechanism of regulation evident in mixed Ctla-4$^{-/-}$ /Ctla-4^{+} bone marrow chimerae has not been elucidated.

The differential effects of Ctla-4 ligation in the regulation of TCR and CD28 signaling pathways supports the notion that dynamic integration of Ctla-4 mediated signals is likely to occur at multiple points. In addition, the role of Ctla-4 in regulating T lymphocyte activation and progression of immune responses is strongly influenced by extrinsic factors. Published results support the notion that Ctla-4 regulation of T cell activation may depend on the relative availability of B7 ligands(114, 258, 263), TCR signal strength(264-266), and activational history(265, 267).

Figure 1.4: Ctla-4 lattice formation with bound B7.1

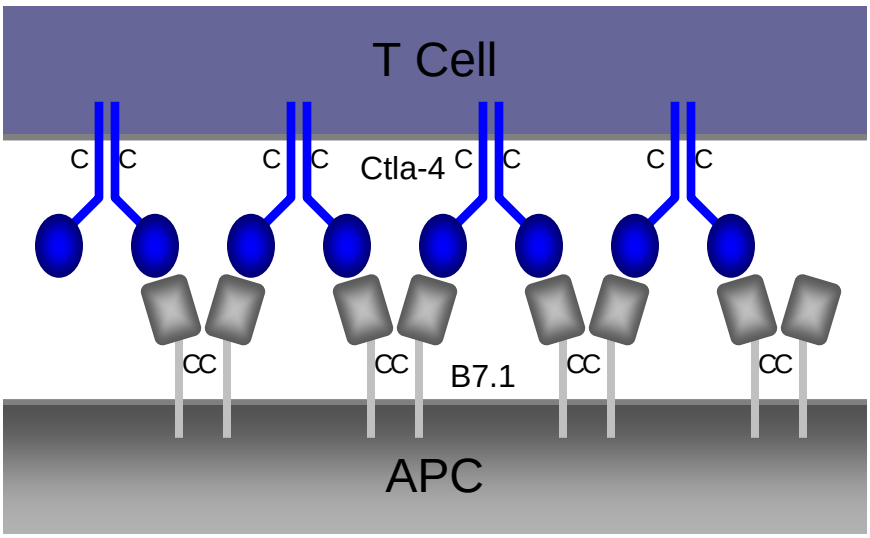


Figure 1.4: Ctla-4 interactions with B7.1 result in the formation of extended lattice arrays. Unlike B7.2 which exists as a monomer on the surface of cells, dimeric B7.1 is capable of binding two ligands. In addition, CD28 is most likely monomeric on the T cell surface, indicating that lattice formation is unique to Ctla-4:B7.1 interactions. The avidity enhanced binding that results from extended formation of Ctla-4:B7.1 interactions results in affinities of interaction more than 10^4-fold more stable than CD28:B7.2 interactions.

Mechanisms of Ctla-4 Mediated Inhibition of T Cell Activation

Several non-mutually exclusive mechanisms have been proposed in order to explain the observed phenomena that result from Ctla-4 manipulation or deficiency. The absence of pathological disease in Ctla-$4^{-/-}$ mice in the context of B7, CD28, class II MHC, or TCR deficiency implies a primary role for Ctla-4 in the attenuation or regulation of signals initiated by the TCR or CD28(268). Overexpression of Ctla-4 on CD4+ or CD8+ T lymphocytes inhibits both in vitro and in vivo T cell responses in a manner dependent on normal CD28 and B7 expression(269). Interestingly, Ctla-4 may initiate inhibitory pathways that utilize common signaling intermediates as indicated by the ability of alternative inhibitory receptors not normally expressed in CD4+ T cells to ameliorate disease in the context of Ctla-4 deficiency in CD4+ T lympohcytes(270). Moreover, observations in numerous experimental settings indicate that Ctla-4 operates to suppress or counteract TCR and CD28 mediated signals at several levels via distinct mechanisms.

Cell Intrinsic Regulation of T Cell Activation by Ctla-4

Ctla-4 inhibits CD28 signals by sequestering shared ligands

The recognition that CD28 and Ctla-4 share the ligands B7.1 and B7.2 suggest that Ctla-4 may compete with CD28 for ligands in situations where APC-expressed B7 is

limiting. Mice expressing a mutant form of Ctla-4 that lacks the cytoplasmic domain are long-lived and show no evidence of lymphocytic infiltration of peripheral tissues, but did exhibit lymphadenopathy, increased numbers of activated T cells, and a predominantly Th2 phenotype(271). The partial rescue was interpreted as evidence that ligand competition is a component of Ctla-4 action. In separate experiments, expression of Ctla-4 lacking the cytoplasmic domain resulted in defective IL-2 secretion, survival, and proliferation by naïve lymphocytes in response to antigen stimulation in vitro. Addition of exogenous IL-2 rescued the proliferative and survival defect suggesting that suppression of Il-2 secretion, the upregulation of which is a hallmark of CD28 costimulation, was the critical result of Ctla-4 surface expression in the absence of intracellular signaling that may be mediated by the cytoplasmic domain of Ctla-4. Moreover, adoptive transfer of lymphocytes expressing tailless Ctla-4 were compromised in their ability to expand when introduced into Rag-/- mice, a process at least partially dependent on CD28 signaling(259). Finally, memory CD4+ T cells, which are known to be less dependent on CD28-mediated signaling for activation and proliferation, that expressed tailless Ctla-4 showed normal proliferative capacity in a lymphopenic environment(259). As previously discussed, biochemical studies have shown that Ctla-4 has a much higher affinity for B7 ligands than CD28, further supporting the notion that ligand competition may be central to Ctla-4 mediated inhibition(42).

Ctla-4 disrupts the immunological synapse

T cell activation through the TCR results in cytoskeletal rearrangement, aggregation of lipid microdomains, and coordinated partitioning of receptors into discrete membrane compartments at the site of T cell:APC contact. The resulting immunological synapse stabilizes the intercellular interaction of multimolecular signaling complexes and may be essential for full cellular activation. Ctla-4 has been shown to cocluster with the TCR and the lipid raft ganglioside GM1 after T cell stimulation in a process that was dependent on the cytoplasmic tail of Ctla-4(272). Ctla-4 partitioning to lipid rafts was enhanced under conditions of TCR and Ctla-4 ligation, and could be forced by linking Ctla-4 to a GPI anchor. Forced localization of tailless Ctla-4 to the lipid rafts, although necessary, was not sufficient for inhibition of activation in the absence of Ctla-4 engagement by B7(272). Crystallization of a B7.1:Ctla-4 complex indicates that Ctla-4 homodimers bridge bivalent B7.1 homodimers in a periodic arrangement(273). The zipper-like oligomerization can potentially lead to the avidity-enhanced stabilization of extended lattice-like formations of B7:Ctla-4 complexes, potentially disrupting the ordered intercellular ligand-receptor interactions that characterize the immunological synapse. The potential ability of Ctla-4 ligand binding to result in mechanical disruption of activating receptor interactions within the synapse is compatible with the known characteristics of B7 ligation by CD28 and Ctla-4, and may function in parallel with ligand competition-mediated inhibition by Ctla-4.

Early reports suggested that B7.1 and B7.2 mediated similar costimulatory signals and suggested that unique roles for the related ligands was determined primarily by their

differential expression patterns.(274, 275) However, despite numerous structural similarities, B7.1 and B7.2 possess distinct biochemical features, affinities for CD28 and Ctla-4, and oligomeric states. Ctla-4 has higher affinity for both B7 ligands relative to CD28, and a relative preference for B7.1 as compared to B7.2 (CD28/Ctla-4 K_d are ~8 and 20 for B7.2 and B7.1 respectively)(42). B7.1 exists on the cell surface as a homodimer, while B7.2 exists as a monomer(111, 276, 277). B7.2 is constitutively expressed on mature APCs, while B7.1 is upregulated by activation stimuli via CD40:CD40L interactions(278). The binding properties, oligomerization states, and expression kinetics of CD28, Ctla-4, and the B7 molecules suggest that attenuation of activation signals may require a coordinated interplay between T cells and APC.

Ctla-4 sequesters signaling intermediates and recruits phosphatases to the IS

In some settings, Ctla-4 mediates inhibition of T cell responses in B7 independent manner. Mutant Ctla-4 incapable of B7 binding inhibits T cell proliferation, cytokine secretion, and ERK activation when expressed in Ctla-4 deficient T cells(279). Moreover, the naturally occurring ligand independent isoform of Ctla-4 suppresses T cell activation and delays the onset of lymphoproliferative disease when expressed in Ctla-4 deficient mice(200, 279). These data prove that Ctla-4 mediated inhibition of T cell activation is only partially dependent on ligand competition or disruption of the immunological synapse.

TCR and CD28 engagement initiates signaling cascades that result in cellular activation via coordinated regulation of protein activity and gene transcription. Ctla-4 is capable of modifying or attenuating signals initiated by TCR and CD28 engagement. The cytoplasmic tail of Ctla-4 and CD28 associate with common signaling intermediates regulating T cell activation. Association of the serine/threonine phosphatase PP2A with the CD28 cytoplasmic tail is negatively regulated by tyrosine phosphorylation of the CD28 binding domain following TCR triggering, and inhibition of PP2A activity enables potent CD28-mediated costimulation of T cell activation(280). Ctla-4 binds both the catalytic and regulatory subunits (PP2AA) of PP2A, and this association is abrogated by tyrosine phosphorylation of the Ctla-4 cytoplasmic domain resulting in enhanced Ctla-4 mediated inhibition of T cell activation as measured by IL2 gene transcription(198). These results indicate that PP2A negatively regulates the function of both CD28 and Ctla-4 via phosphotyrosine dependent association with their cytoplasmic domains. In addition to PP2A, Ctla-4 and CD28 also share binding infinity for Src homology domain-containing tyrosine phosphatases (SHP-1 and SHP-2) and PI3K. Early reports suggested that Ctla-4 recruited phosphatases to the immunological synapse resulting in reversal of TCR- and CD28-mediated phosphorylation events(281). The relative importance of these associations in the regulation of T cell activation remains controversial, but recent data suggest that they may mediate differential signaling based on their inclusion in multimeric signaling complexes whose organization is directed by CD28 and Ctla-4. Thus, association of Shp-2 or PI3K with Ctla-4 is not required for Ctla-4 mediated inhibition of T cell activation(282), but SHP-2 and PI3K associate with Gab2 to mediate

CD28 signaling that results in inhibition of TCR-mediated signaling events in a negative feedback loop(283). While not definitively shown, it remains possible that Ctla-4 functions in part by sequestering signaling intermediates required for efficient signal transduction by CD28. Alternatively, regulated phosphorylation of CD28 and Ctla-4 tyrosine residues by Src family tyrosine kinases(284-286) may alter the relative proportions of proteins associating with CD28 and Ctla-4, and thus change the balance of stimulatory and inhibitory signals they mediate.

Cell-Extrinsic Mechanisms of Ctla-4 Function

Wild-type T lymphocytes regulate Ctla-4$^{-/-}$ T cells in mixed bone marrow chimerae

Ctla-4 functions to maintain peripheral tolerance by cell extrinsic regulation of T lymphocyte responses by cell-contact-dependent and –independent mechanisms. The most dramatic example of cell-extrinsic regulation by Ctla-4 is seen in bone marrow chimerae reconstituted with donor cells from Ctla-4$^{-/-}$ and Ctla-4 wild-type mice. Bone marrow recipients reconstituted with T cell depleted Ctla-4$^{-/-}$ donor cells develop the lymphoproliferative disease and tissue infiltration characteristic of native Ctla-4 deficiency. The pathogenesis of the observed disease recapitulates all aspects of the native disease and is initiated only upon thymic emigration of donor T cells. In contrast, mixed bone marrow chimerae remain healthy and maintain a balanced ratio of naïve donor cells in the periphery(261). Moreover, Ctla-4-deficient cells in mixed bone

marrow chimerae respond to several pathogens in a manner indistinguishable from their wild-type counterparts(262). Numerous models have been proposed to explain cell-extrinsic regulation by Ctla-4, but the dominant mechanism remains undefined. Given the diversity of observations made about Ctla-4 function in various contexts, it is possible that numerous mechanisms mediated by Ctla-4 act cooperatively to maintain self-tolerance at the population level.

Regulation of cytokine production

Cytokines are critically important regulators of T cell activation, survival, differentiation, and effector function. Cytokine activity is determined by the expression pattern of appropriate receptors and intracellular proteins involved in mediating cytokine signaling. Thus, cytokines are capable of acting at a distance to coordinate varied responses in different cell types and serve to orchestrate immune responses at the population level. CD28 costimulation concomitant with TCR triggering results in potent upregulation of IL-2 gene transcription. IL-2 acts in both paracrine and autocrine fashion to enhance cell survival, proliferation, and effector function. Il-2 deficiency results in dramatically reduced T cell survival following antigen receptor stimulation, markedly reduced proliferation, and inefficient T cell mediated immune responses. Ctla-4 ligation inhibits CD28-mediated upregulation of IL-2 and IL-2R(287). Signals initiated by TCR triggering and CD28 ligation regulate the activity and expression of numerous other transcription factors that determine cellular differentiation, coordinate T cell-dependent humoral responses, and influence antiviral immunity through stimulation of the innate

immune system. In contrast, several cytokines result in potent immunosuppression of T cell responses and serve to maintain self-tolerance by inhibiting or attenuating unwarranted T cell activation. TGF-beta and IL-10 inhibit T cell responses and have been examined for their ability to mediate Ctla-4 function. Critically, no role for TGF-beta has been reliably discerned in the intrinsic regulation of T cell responses mediated by Ctla-4(288). However, a large body of evidence suggests that TGF-beta may mediate suppressive effects of regulatory T cells(289-294) and Ctla-4 has been shown to enhance the suppressive activity of CD4+CD25+ regulatory T cells(295). Ctla-4-deficient mice have functional CD4+CD25+ regulatory T cells that are generated in the thymus, indicating that suppressor function is at least partially independent of Ctla-4 action. In contrast, the generation of adaptive regulatory T cells in the periphery is dependent on TGF-beta induction of FoxP3(296-300) and may require permissive signaling through Ctla-4(301). Moreover, several lines of evidence support the notion that Ctla-4 does mediate some suppressive effects of regulatory cells(302, 303), but not all. The data suggest that Ctla-4 may regulate the development of a subset of adaptively generated regulatory T cells and contribute to specific mechanisms of suppression, thus explaining previous contradictions regarding the overlapping role of Ctla-4 and TGF-beta in maintaining immunotolerance. However, the critical downstream mediators of Ctla-4 signaling that control responses to TGF-beta and may result in phenotypic changes upon restimulation or long-term unresponsiveness to antigen receptor engagement have not been defined.

Ctla-4 maintains peripheral self-tolerance through tryptophan catabolism

Tryptophan catabolism and the consequent production of tryptophan catabolites mediates a suppressive effect on immune responses. In the short-term, tryptophan deprivation and exposure to tryptophan catabolites results in CD3 down-regulation and impaired lymphocyte effector function. Longer term, tryptophan catabolism results in the emergence of a regulatory phenotype in naïve CD4+CD25- T cells mediated through TGF-beta dependent upregulation of FoxP3(304). The $CD4^{+}CD25^{+}CD62L^{+}Ctla\text{-}4^{+}GITR^{+}CD69^{-}CD45RB^{Low}$ cells that develop in the context of increased tryptophan catabolism are capable of suppressing diabetogenic responses in vivo(304). Thus, tryptophan catabolism is an effector mechanism regulating peripheral self-tolerance through direct suppression of T cell effector responses and generation of suppressive T cell function in naïve CD4+ T cells. Ctla-4 signals through B7 on dendritic cells to upregulate the expression of indoleamineoxygenase resulting in increased tryptophan catabolism and suppression of immune responses(305). TGF-beta is crucial for the development of a regulatory phenotype in peripheral CD4+CD25- cells(300). Ctla-4 ligation soon after activation is critically required for TGF-beta-mediated FoxP3 induction, and Ctla-4 deficiency results in the inability to develop peripheral CD4+CD25+ suppressor cells(301). Furthermore, upregulation of IDO expression in dendritic cells is regulated by suppressor T cells constitutively expressing Ctla-4(306). Thus Ctla-4 enables TGF-beta-mediated generation of regulatory T cells in the periphery and provides a mechanism by which suppressor T cells acquire the ability to regulate both effector T cell responses and further generation of regulatory cells via modulation of

tryptophan catabolism. Interestingly, CD28 is also capable of signaling through B7 molecules expressed on dendritic cells, but induces immunostimulatory signals that counteract the induction of tolerogenic properties that result from Ctla-4-B7 interactions(307). Recent work also suggests that additional ligand-receptor interactions involving CD40 and CD40L, expressed on dendritic cells and lympohcytes respectively, may be crucially involved in regulating dendritic cell responses to B7 engagement(308). Cumulatively, these data highlight the complex interplay of costimulatory signaling pathways in the regulation and determination of peripheral tolerance mechanisms. Moreover, the cyclical interdependence of mechanisms underlying development of regulatory T cells and induction of tolerogenic properties in dendritic cells is dependent on CD28 and Ctla-4 signaling.

Ctla-4 regulates suppressor T cell function and signals through B7 on T cells

Ctla-4 enhances, and CD28 inhibits, the ability of regulatory CD4+CD25+ T cells to induce a tolerogenic phenotype in dendritic cells(295). In addition, this effect is differentially regulated by B7.1 and B7.2 and may reflect preferential binding to Ctla-4 and CD28 respectively(295, 309). A requirement for CD28 signaling in the generation of suppressor T cells is well established, but the role of Ctla-4 has been more controversial(310-312). Contradictory data regarding the role of Ctla-4 in regulatory T cell function may reflect differing requirements as a result of alternative mechanism of suppression. Thus Ctla-4 is reportedly necessary for the adaptive generation of regulatory cells developed in the periphery, but is not required during development of

thymic-derived regulatory T cells(313). Thus, Ctla-4 deficient mice maintain a population of CD4+CD25+ regulatory cells that possess potent suppressive activity in vitro, but are not sufficient to prevent the lymphoproliferative disease that develops in Ctla-$4^{-/-}$ mice.

B7-ligand interactions facilitate bidirectional signaling that serves to modulate immune responsiveness by various mechanisms. As indicated, Ctla-4 and CD28 initiate tolerogenic and immunostimulatory signaling pathways in dendritic cells upon binding B7(307, 314). Recent work has shown that T cell expressed B7 also regulates lymphocyte responses by engaging Ctla-4 in T-T interactions(315) in a manner analogous to Ctla-4 mediated inhibition by APC-expressed B7. However, recent data also suggest that CD4+CD25+ regulatory T cells can suppress T cell responses by Ctla-4 mediated ligation of B7 molecules expressed on alloreactive effector cells(303). Regulatory T cells are known to inhibit activation of effector T cells by altering the phenotype of dendritic cells and by secretion of inhibitory cytokines. Thus, the diversity of mechanisms utilized by suppressor T cells results in intercellular interactions that are differentially dependent on Ctla-4. Contact-dependent regulation of effector cells via B7 signaling represents a novel regulatory mechanism consistent with the known activity of B7 in dendritic cells, and may provide new opportunities for therapeutic manipulation. The mechanism by which B7 signals in T cells is not known. Our lab is currently in the process of exploring the role B7-mediated inhibition might play in various contexts of immune dysregulation.

Ctla-4 and immunotolerance

Costimulatory signals are critical determinants of T cell activation and influence the specificity, magnitude, character, and duration of immune responses. Alterations in CD28 and Ctla-4 function are associated with autoimmune pathology in humans, and therapeutic manipulation of costimulatory signals in the treatment of cancer is already being investigated in clinical trials(236, 316, 317). Altered Ctla-4 function in particular has been shown to result in increased susceptibility to several common autoimmune disorders. In humans, single nucleotide polymorphisms identified in the CTLA-4 gene influence alternative splicing and increase susceptibility to Hashimoto's thyroiditis, Graves disease, and type I diabetes (TID)(199). While definitive associations of CTLA-4 polymorphisms with susceptibility to numerous other diseases remains controversial, a central role for Ctla-4 in regulating progression of established autoimmune disease has been identified(318). In mice, Ctla-4 has been shown to control the severity and onset of numerous autoimmune diseases including experimental autoimmune encephalomyelitis(319, 320), autoimmune diabetes(200, 321), and chronic experimental colitis(322). In conjunction with the observed phenotype of Ctla-$4^{-/-}$ mice(323) and other reports suggesting subtle contributions of Ctla-4 function to the development and progression of numerous diseases, these data suggest that Ctla-4 might represent a general susceptibility gene for autoimmune disease(324, 325). The broad relevance of Ctla-4 to clinically relevant disease states makes it an ideal target for therapeutic manipulation.

TCR-triggering alters gene expression: CD28 amplification and Ctla-4 inhibition

Two reports have published the results of global gene expression profiling in T lymphocytes following activation(15, 54). According to both reports, TCR signaling alone results in the altered regulation of several thousand genes. CD28 signaling alone results in few transcriptional alterations, but is capable of substantially magnifying the response to TCR ligation when simultaneously engaged. For the vast majority of genes assayed, CD28 costimulation serves to enhance TCR initiated gene regulations although a number of genes are reported to be reciprocally regulated by CD28 costimulation in comparison with TCR ligation alone. In the one report that examined the influence of Ctla-4 ligation(54), Ctla-4 triggering was reported to inhibit gene regulations subsequent to CD28 costimulation but not TCR monostimulation. However, both studies used antibody-coated microbeads to stimulate human peripheral blood leukocytes (PBLs) heterogeneous for activational history and antigen specificity. Moreover, the stimulated cells were analyzed as bulk populations and undoubtedly contained cells with naïve phenotypes as well as cells undergoing both primary and secondary activation. No studies have been published that profile global gene expression in stimulated lymphocytes using naïve populations homogenous for phenotype and activation status. As a result, it remains unclear whether Ctla-4 transduces a unique signal that results in specific changes in gene expression, or whether Ctla-4 functions primarily by inhibiting signals generated by the TCR and/or CD28.

Ctla-4 functions to maintain quiescence in peripheral lymphocytes

Genes necessary for quiescence are necessarily downregulated upon T cell activation(326, 327). CD28 costimulation has been shown to reciprocally regulate gene expression and results in the simultaneous upregulation of genes necessary for proliferation and effector function while repressing genes associated with a quiescent phenotype(15, 54). Published reports also indicate nearly equal numbers of up and down-regulated genes in primary T cells stimulated ex vivo(15, 54). These reciprocal regulations evidence the complex choreography involved in gene regulation that occurs upon T lymphocyte activation and reinforces the notion that maintenance of a naïve phenotype is an active process. The balance between protective immunity and pathological autoimmunity is maintained by mechanisms that determine signaling thresholds of antigen and costimulatory receptors. Altered costimulatory pathways can result in enhanced activation of lymphocytes and autoimmunity, or antigen-specific tolerance induction through clonal anergy or active immunosuppression by regulatory T cells. The molecular mechanisms that underlie maintenance of immunotolerance in vivo and integrate co-stimulatory signals with antigen receptor signals in T cells are poorly understood.

The overall objective of this thesis work was to gain additional insight into the mechanisms underlying costimulatory regulation of T cell responses. Specifically, the present work sought to identify mediators of Ctla-4 function by assessing the genomic expression patterns that exist under varying conditions of Ctla-4 ligation and deficiency. To accomplish this necessitated a global characterization of the transcriptome of

activated naïve lymphocytes, thus providing additional insight into lympohocyte responses to antigen-receptor- and CD28-mediated signaling pathways. The central importance of costimulatory signals in immune regulation has been shown in numerous disease models, and a greater understanding of the mechanisms underlying signal integration in T cells will provide novel opportunities for knowledge based approaches to combating autoimmune disease and pathology. Thus, a second goal of this work was the creation of a database of gene regulations occurring subsequent to T cell activation that will enable the identification of novel genes central to the maintenance of peripheral tolerance.

CHAPTER II

MATERIALS AND METHODS

MATERIALS and METHODS:

Mice:

The phenotype and antigen specificity of 5C.C7 TCR-transgenic $Rag^{-/-}$ mice has been described previously(288, 328). In brief, the 5C.C7 TCR is specific for moth cytochrome C (MCC^{88-103}) oligopeptide presented in the context of the class II MHC molecule I-E^k. Expression of the 5C.C7 TCR transgene in developing thymocytes results in a predominantly (>95%) CD4+ peripheral T cell compartment. Ctla-4-deficient mice(323), B7.1-/-/B7.2-/- mice(329), $CD28^{-/-}$(26), and $Rag1^{-/-}$(330) mice have also been previously characterized. All mice were maintained in a specific-pathogen-free facility. Unless otherwise specified, lymphocyte donor mice were used at 6-12 weeks of age. All mice sacrificed were euthanized by CO_2.

T cell purification:

CD4+ T cells were isolated from lymph nodes of donor mice by dissection and tissue homogenization in RPMI supplemented with penicillin, streptomycin, beta-mercaptoethanol (2-ME), and 10% fetal bovine serum (FBS)(cRPMI). As indicated, CD4+ lymphocytes were isolated by positive selection using α-CD4 magnetic bead purification or by fluorescence activated cell sorting (FACS). 5C.C7 lymph node cells were >90% CD3+CD4+$CD44^{Low}$CD25- following homogenization and did not require further isolation steps prior to in vitro use. Magnetic bead purification utilized AutoMACS LS+ columns according to the manufacturer's protocols (Miltenyi Biotec,

Auburn, CA). FACS isolation was performed by the University of Massachusetts Medical School Flow Cytometry Core Facility under sterile conditions subsequent to fluorochrome-conjugated antibody labeling. Unless otherwise stated, purified populations were >95% pure by flow cytometry analysis.

Bone marrow isolation and transfer:

Femurs and tibiae from bone marrow donor mice were surgically dissected and cleaned of soft tissue. Marrow was flushed from dyaphyses with RPMI supplemented with 5% FBS using a 25 gauge needle and syringe after removal of the epiphyses. Marrow was homogenized by repeated aspiration through a 25 gauge needle before lysis of red blood cells in 1ml RBC lysis buffer at room temperature for 1 minute. Cells were washed in 5ml RPMI 5% FBS, counted, and resuspended in 3ml of sterile phosphate buffered saline (PBS). Lymphocyte depletion was achieved using magnetic beads recognizing CD4, CD8, and either the Thy1 or Ly5 congenic markers. Beads were washed twice in 4ml PBS to remove unconjugated antibody prior to use. Six beads total were used per target cell, divided evenly among appropriate molecular target identities. Ctla-4 wild-type (WT) and Ctla-4-deficient bone marrow typically consisted of <5% and 10-15% mature T cells respectively. Antibody-conjugated magnetic beads were incubated with RBC depleted bone marrow preparation for 30 minutes at 4 degrees Celsius with rotation. Magnetic separation utilized a stationary magnet for two minutes. Generally, lymphocytes were undetectable by FACS analysis following depletion protocols when analyzed for CD3, CD4, and CD8 expression. Lymphocyte depleted bone marrow cells

were resuspended at $33x10^6$ cells per ml in PBS. 150ul containing 5 $x10^6$ total cells was injected into the tail vein of recipient $Rag^{-/-}$ mice previously irradiated (within 36 hours) with 300 rads. Mature donor lymphocytes were typically detectable in blood samples 4-5 weeks after transplantation.

In vitro stimulation assays

Stimulation of isolated 5C.C7 $Rag^{-/-}$ lymph node cells by engineered antigen presenting cells (APCs) was accomplished as follows. Chinese Hamster ovary (CHO) cells expressing I-E^k , and differentially expressing synthetic ligands for CD28 and Ctla-4 have been described previously(288). CHO cells were grown in DMEM supplemented with penicillin and streptomycin, L-glutamine, 2-ME, 10% FBS, 300ug/ml hygromycin, and 300ug/ml zeocin. Prior to coculture with 5C.C7 $Rag^{-/-}$ lymph node cells, CHO cells were removed from culture using PBS with 5mM EDTA and 5% FBS to maintain the integrity of surface proteins, and incubated for 90 minutes at $1.5x10^7$ cells per milliliter at 37ºC in RPMI 5%FBS supplemented with 100ug/ml mitomycin C. Following mitomycin C treatment, CHO cells were washed 4x in cRPMI, resuspended at $5x10^6$ cells per milliliter of cRPMI containing the indicated concentrations of HPLC-purified moth cytochrome C (MCC^{88-103}) oligopeptide, and incubated for 5 hours in 96 well round-bottom plates at 37ºC with $5x10^4$ cells per well to allow equilibrated MHC binding of MCC. Excess MCC was removed after 5 hours, and 5C.C7 $Rag^{-/-}$ lymph node cells were added at a 1:1 ratio with CHO cells in 200ul cRPMI. Lymph node cells cocultured with CHO APCs were harvested at indicated times by gentle pipetting. ***Isolation of phenotypically pure***

in vitro activated lymphocytes was achieved by antibody-mediated staining and FACS isolation in the University of Massachusetts Medical School Flow Cytometry Core Facility as indicated. Isolated populations were always >90% pure, and generally >95% pure, for CD4 and the specified activation markers. ***Antibody-mediated stimulation of lymphocytes*** was accomplished using soluble, plate-bound, or polystyrene microbead-conjugated antibodies to CD3, CD28, and/or Ctla-4. Antibody-coated plates were prepared by incubating PBS containing antibody at the specified concentrations for 1 hour at 37ºC and washing the plates with cRPMI immediately prior to addition of cell preparations. Antibody-microbeads were prepared by incubation of $1.2x10^7$ polystyrene microbeads (Interfacial Dynamics Corporation, Eugene, OR) at 37ºC with rotation for 20 minutes in 1ml of PBS solution containing antibodies at the specified concentration. Conjugated microbeads were washed 3x in PBS, and resuspended in 1ml of cRPMI at room temperature a minimum of 1 hour before use in culture. Conjugated beads were used at a ratio of 12 beads per cell. ***Thymidine incorporation*** was measured in stimulated cells by the addition of 1uCi of Thymidine in 10ul of cRPMI per well in 96 well plates for 12-16 hours. Sample plates were frozen at -80ºC until analyzed.

Lklf Overexpression Construct, Transfection, and Retroviral Infection of NFCs

A retroviral expression construct was constructed that included a MSCV promoter, the protein coding region of Lklf, an internal ribosomal entry site (IRES), and the green fluorescent protein (GFP) gene. XL1 Blue ultra-competent E.coli cells were transfected using a standard heat-shock protocol, and grown on agarose. Selected colonies were used

to inoculate 5 ml cultures, and incubated overnight. Plasmid was purified using Qiagen MiniPrep protocol, and restriction enzyme digests confirmed the integrity and orientation of the gene insert. Large quantities of plasmid were prepared by bulk culture of transfected E.coli clones, and plasmid was isolated by Qiagen MaxiPrep protocol. Phoenix cells were transfected with the purified Lklf-construct or an empty MSCV plasmid using Lipofectamine. The Phoenix cell packaging line was grown overnight before replacing the media with a minimal volume of fresh media. NFCs, a double positive thymoma cell line capable of undergoing differentiation, were cocultured with Phoenix cells for several days and purified for positive GFP expression by FACS.

Microarray analysis:

RNA isolation was performed using either TriReagent (Molecular Research Center, Inc., Cincinatti, OH) or TRIzol (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's instructions. A 260/280 spectophotometric ratio of 1.8-2.0 was required before samples could be labeled for microarray analysis. ***Preparation of biotin-labeled cRNA for microarray analysis*** was performed using either single or double-amplification protocols, as indicated, that utilized either 4-8ug or 50-400ng of total RNA as the starting material respectively. When >4ug of total RNA was available, a "standard" labeling protocol was followed. Briefly, total RNA was converted into double-stranded cDNA using a two-step cDNA synthesis reaction utilizing a specific oligo$(dT)_{24}$ primer containing a T7 RNA polymerase promoter site located 3' of the poly T tract. Biotin labeled cRNA was generated from the cDNA sample by in vitro transcription driven by a

T7 RNA polymerase. When <4ug of total RNA was available, samples were amplified and labeled for microarray analysis using a two step protocol (Affymetrix SSvII Protocol) starting with 50-400ng of total RNA. Briefly, double-stranded cDNA was generated using a two-step reverse transcription protocol with the same oligo$(dT)_{24}$ primer used in the standard protocol. In vitro transcription was performed on the cDNA to produce cRNA. 400-800ng of first-cycle cRNA product was utilized as the starting material for a second reverse transcription reaction utilizing random hexamer primers that yielded cDNA. Amplified cDNA was used to generate biotin-labeled cRNA by in vitro transcription. Biotin-labeled cRNA was purified using RNeasy spin columns (Qiagen, Germany). ***Biotin-labeled cRNA samples were processed for microarray analysis*** by the University of Massachusetts Medical School Genomics Core Facility. Briefly, about 20ug of labeled cRNA were fragmented by mild alkaline treatment at 94ºC for 30 minutes, and the product used to prepare the hybridization cocktail. The hybridization cocktail includes 4 control cRNAs to inform subsequent image analysis and data normalization, and a biotinylated oligonucleotide marker to facilitate image orientation, microarray scanning, and probeset mapping. ***cRNA samples were hybridized to MgU74Av.2 microarrays and scanned*** according to the manufacturer's guidelines. Briefly, sample cocktails were heated to 99ºC for 5 minutes, equilibrated to 45ºc for 5 minutes, and centrifuged at room temperature for 5 minutes. A total of 10-15ug of each fragmented sample was hybridized to Affymetrix GeneChip according to the manufacturer's protocol. Arrays were washed with non-stringent buffered solution, stained with R-phycoerythrin streptavidin, washed again, and read by the GeneArray

Scanner. ***Array data normalization*** was achieved by maintaining global array intensity within the linear bounds of detector sensitivity, and subsequent normalization of measured values to signal intensities of spike-controls included in the hybridization cocktail. The resultant raw data was analyzed by MicroArray Suite 5.0 (MAS5.0) (Affymetrix), Genespring (Agilent Technologies), or Ingenuity Pathways Analysis (Ingenuity Systems). The determination of signal intensity significance in relation to background signal detection was performed according to Affymetrix MAS5.0 standard algorithms and resulted in "present", "absent", or "marginal" calls (i.e. "flags"). In order to allow rational determination of fold-change differences between sample conditions, sequential data normalization was applied as follows: measured probeset signal intensity values below 0.01 were set to 0.01; each measurement was divided by the 50.0th percentile of all measurements in that sample; when appropriate, specific samples were normalized to one another as indicated by dividing the median signal measurement of each gene in each experimental samples by the median values of that gene's measurement in the corresponding control samples. Measures of significance were obtained by calculating the standard deviation from the average expression value for each gene when sufficient replicates were available, or by use of the cross gene error model, as indicated. The cross-gene error model provides reliable estimation of significance when fewer than three replicates are available by combining measurement variation and between-sample varation information. For the analyses included herein, when two replicates were available the cross gene error model was configured to compute significance based on the assumption that variability between replicates is similar for all

genes with similar measurement levels. In contrast, in the few conditions assayed where only one chip was analyzed per condition, the cross-gene error model was configured based on the assumption that a minority of the genes measured were biological significance. The cross-gene error model calculates deviation for each gene based on the appropriate assumption as outlined above and orders the values based on increasing control strength. Medians of the deviation and control strength are calculated and the deviation on squared control strength is computed using the following equation expressed in terms of normalized data:

$$S(norm)^2=a^2/C^2+b^2$$

Where:

a = fixed (absolute) error (base)

b = proportional error (proportion), $\pm$ 5%

C = control value (also referred to as the control strength)

The control value is a synthetic value equal to the product of the values used in prior normalization steps. In analyses included herein where a per-chip and per-gene normalization was applied, the control value is defined as:

$$C=C(per\text{-}chip)*C(per\text{-}gene)$$

In experiments with two samples per condition where variability between replicates was assumed similar for genes with similar measurements, a separate curve for each group of replicates was applied. Where only one chip was analyzed per condition, and a minority of genes were considered to vary with biological significance, the error model applied a separate curve for each sample. The number of replicates per condition, specific

characteristics of the included samples, labeling protocols utilized, and error models applied for each experimental approach are presented in the corresponding chapter.

Lklf Real-time PCR

Oligonucleotide primers with the sequences 5':CTATCTTGCCGTCCTTTGCCACTTTCG and 3':ATGGAGAGGATGAAGTCCACACACG were made by Integrated DNA Technologies to amplify a 132 base pair product. Realtime protocol was: hot start 95°C for 8'30"; melt at 95°C, 30", anneal at 55.4°C, 30", extend 72° 25" for 43 cycles; melt curve recorded from 55°C at increments of 0.5°C increase every 30" over 80 cycles. LKLF was normalized to beta-2-microglobulin using the primer sequences 5':AATCCAGTTTCTAATATGCTA and 3':TATTGCTCAGCTATCTAGG. Realtime protocol was: 95°C for 3'; melt at 94°C, 20", anneal at 50°C, 25", extend 72° 25" for 40 cycles; melt curve recorded from 55°C at increments of 0.5°C increase every 10" over 80 cycles. Biorad real-time PCR Super mix reagent was used for all reactions. Data analysis, standard curve generation and copy number calculation was performed with MyiQ software. Time points including 0, 8, 12, 16, 20, 24, 35, 55, 60 and 65 hrs post-stimulation were looked at in both mechanical and biological replicates.

CHAPTER III

RESULTS

Global Expression Profiling of Naïve CD4+ Lymphocytes in Steady-State Ctla-4-Deficient Model Systems

Identification of genes differentially regulated by Ctla-4 deficiency in naïve peripheral CD4+ lymphocytes in the absence of B7 ligands:

To provide a contextual framework for the analysis of expression profiles subsequent to differential Ctla-4 ligation, we utilized in vivo models that allowed for steady-state analysis of naïve Ctla-4 deficient CD4+ cells. As an initial approach we utilized Ctla-4 deficient mice crossed onto the B7.1$^{-/-}$/B7.2$^{-/-}$ double knockout. As published, impaired CD28 costimulation subsequent to the absence of B7 ligand completely abrogates the lymphoproliferative disease that invariably results in Ctla-4 single knockout mice(268). The lack of positive costimulation in B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ mice results in healthy mice with naïve peripheral CD4+ lymphocytes capable of primary activation responses with normal kinetics and burst size, but heightened TCR sensitivity when stimulated by plate-bound or soluble antibody, pharmacologic activation by PMA $\pm$ ionomycin, or coculture with antigen and splenic or engineered APC (Data not shown). Alternate approaches to the study of naive Ctla-4-deficient CD4+ lymphocytes can be achieved by the use of TCR-transgenic Ctla-4 knockout mice, or CD28$^{-/-}$/Ctla-4$^{-/-}$ double knockout mice. However, analysis of the resultant expression profiles in these strains is complicated by the absence of physiologic interaction of the TCR with self-antigen/MHC and the inability to verify the normal responsiveness of the lymphocytes to numerous stimuli in a primary activation, respectively. In contrast, B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ lymphocytes remain sensitive to CD28 costimulation and reconstitute the lymphoproliferative disease seen in Ctla-4 deficient mice in Rag$^{-/-}$ bone-marrow

recipients upon thymic emigration of Ctla-4$^{-/-}$ donor lymphocytes (Figure 3.1). For this reason, B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ mice provide a good model to investigate the possibility that absence of Ctla-4 genetically predisposes lymphocytes to pathologic activation by altering the baseline expression of genes involved in the determination of lymphocyte responsiveness. Additionally, the global expression profile of naïve Ctla-4 deficient CD4+ lymphocytes can provide both a normative measure of gene expression for analysis of alternate models of Ctla-4 deficiency, and indicate a steady-state expression profile that will inform the analysis of any subsequent kinetic profile.

As an initial approach in the identification of genes regulated by Ctla-4 ligation, we used microarray analysis to compare the global expression profile of ex vivo CD4+ lymphocytes from B7.1$^{-/-}$/B7.2$^{-/-}$ (B7KO) double knockout mice and B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ (B7CT) triple knockout mice. CD4+ lymphocytes were MACS purified to >90% from lymph nodes, and analyzed for expression of the activation markers CD44, CD69, and CD62L. All mice except one showed identically low expression of CD44, an absence of CD69 expression, and similar CD62L profiles. Donor cells from two mice were pooled for each sample analyzed by microarray, with biological duplicates performed for both strains. One B7CT mouse had a relatively higher CD44 expression (15% vs ~5-7%) and this sample was processed separately for later analysis.

Microarray analysis utilized the Affymetrix MGu74Av2 Genechip covering 12,422 gene probesets. Results were normalized as follows: values below 0.01 were set to 0.01; each measurement was divided by the 50th percentile of all measurements in that sample; each gene was divided by the median of its measurements in all samples; If the

Figure 3.1: **B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ bone marrow recipients recapitulate immune pathology of Ctla-4-deficient mice and die by 8-10 weeks**

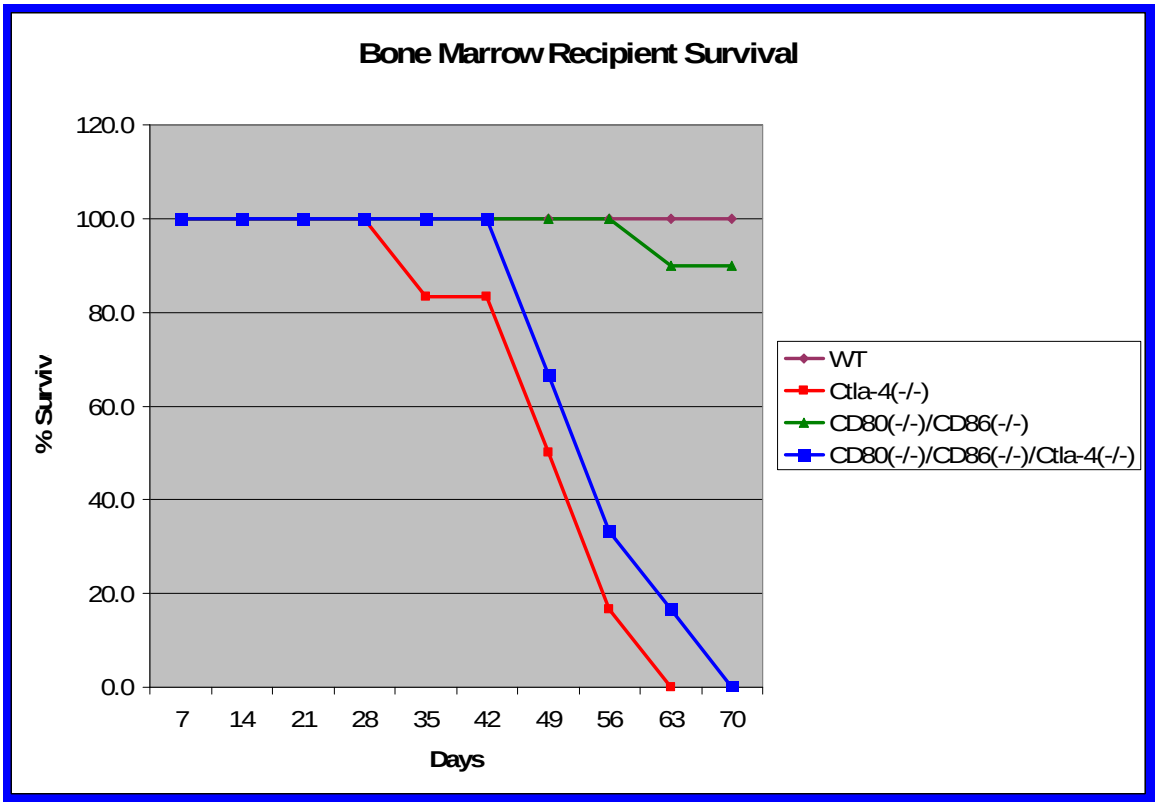


Figure 3.1: $Rag^{-/-}$ mice receiving $B7.1^{-/-}/B7.2^{-/-}/Ctla\text{-}4^{-/-}$ bone marrow succumb to a lymphoproliferative disease with similar kinetics as mice receiving $Ctla\text{-}4^{-/-}$ marrow. $5x10^6$ T cell depleted bone marrow cells from $Ctla\text{-}4^{-/-}$, $B7.1^{-/-}/B7.2^{-/-}$, $B7.1^{-/-}/B7.2^{-/-}/Ctla\text{-}4^{-/-}$, or wild-type B6 mice were transferred to minimally irradiated $Rag^{-/-}$ recipients. The percentage of surviving recipients with respect to time is shown. Recipients of wild-type B6 or $B7.1^{-/-}/B7.2^{-/-}$ bone marrow remain healthy indefinitely and successfully reconstitute their peripheral lymphocyte compartment. In contrast, recipients of Ctla-4-deficient bone marrow succumb to a lymphoproliferative autoimmune disease upon thymic emigration of T cells that is identical to the disease seen in native Ctla-4-deficient mice. $B7.1^{-/-}/B7.2^{-/-}/Ctla\text{-}4^{-/-}$ bone marrow recipients also develop disease in contrast to $B7.1^{-/-}/B7.2^{-/-}/Ctla\text{-}4^{-/-}$ mice which remain healthy. Disease in $B7.1^{-/-}/B7.2^{-/-}/Ctla\text{-}4^{-/-}$ bone marrow recipients recapitulates all aspects of disease seen in native Ctla-4 deficiency including a high percentage of activated peripheral lymphocytes, lymphocytic infiltration of most tissues, and death within 3-5 weeks of initial thymic emigration.

median of the raw values was below 10 then each measurement for that gene was divided by 10 if the numerator was above 10, otherwise the measurement was thrown out. 5475 genes were flagged as "present" or "marginal" in 2 or more samples by Affymetrix MAS5.0, representing 44% of the included genome. Global comparison of the chips showed segregation of the sample conditions (B7KO or B7CT) as expected, but the B7CT sample with a greater percentage of $CD44^{high}$ lymphocytes was a relative outlier, and further analyses were performed twice to allow analysis with and without this sample.

Globally, the expression profiles of naïve CD4+ lymphocytes in B7KO and B7CT mice are very similar, as expected from their phenotype and responsiveness to activating signals. However, slight but consistent variation in the expression of nearly 100 genes was detected at a greater than two-fold difference. Further analysis of significance, normalized expression, and flag calls resulted in the identification of 79 genes differentially regulated in naïve CD4+ cells in the absence of Ctla-4 with a statistically significant fold-changer greater than 1.5-fold. 84% of the identified genes were downregulated in the absence of Ctla-4, expressed at an average of 39% (~2.6 fold change) of expression in B7KO samples. Upregulated genes in Ctla-4 deficient samples were, on average, more slightly altered at 215% of expression in B7KO samples. As expected, Ctla-4 was dramatically altered, with the second highest calculated fold change, flagged as present and absent in B7KO and B7CT samples respectively with a signal strength 25-fold greater than background on the B7KO chips in spite of the minimal expression known to exist in naïve T cells. Only four genes were altered more than four

fold, including Mela, Ctla-4, Ccr9, and Sel1h. However, 64% of genes downregulated more than two-fold in B7CT cells were consistently flagged as present to absent calls.

Most genes identified as differentially regulated have not been extensively studied in lymphocytes but have been at least partially characterized in other cell types, and several of them are known to regulate T lymphocyte activation. Genes differentially regulated in the absence of Ctla-4 that are known to regulate T cell responses to antigen receptor ligation include slfn4, an uncharacterized member of the schlafen family of putative quiescence factors, recognized for their role in regulating lymphocyte development and activation(331-333); lag-3(CD223), a CD4 homologue known to inhibit primary T lymphocyte activation localizes to the c-smac within the immunological synapse(334) and negatively regulates T cell expansion following antigen stimulation(335), influences susceptibility to multiple sclerosis(336), and appears to be a Treg marker involved in active suppression of effector T cells(337); the antiapoptotic Src regulator, ptpn13/FAP-1, is a putative tumor suppressor known to regulate NFκB(338) and is negatively regulated by activated p53(339) and IL-2(340, 341) ; the proto-oncogene and transcriptional activator Mybl1 is known to upregulate Bcl-2(342) and influence proliferation in leukemic cells(343); the proto-oncogene and cell-cycle regulator, rb1; the retinoblastoma binding protein, Rbap46, is a tumor suppressor, a member of the WD-repeat protein family, and component of histone modifying and remodeling complexes, but has no known function in lymphocytes(344); the tumor suppressor and growth inhibitor Sel1L(345) has not been characterized in lymphocytes

but is associated with autoimmune-diabetes(346); TGF-beta inducible protein BigH3 is a suspected growth inhibitor not characterized in lymphocytes.

Genes altered more than 1.5-fold in the absence of Ctla-4 with p-values less than 0.05 are shown in Table 3.1. Importantly, a non-biased search for putative regulatory sequences failed to identify over-represented sequences in the 1kb upstream regions of the identified genes when limited to elements 10bp or less in length. The preponderance of genes identified as differentially regulated in the absence of Ctla-4 in the context of B7 deficiency that have known functions relating to growth regulation, signal transduction in lymphocytes, or tumor suppression are altered in ways that would be expected to increase the threshold of activation and resistance to cell cycle entry. This may reflect a non-specific, generalized down-regulation of genes in the absence of Ctla-4, consistent with the observed ratio of up- and down-regulated genes. Alternatively, the maintenance of a naïve phenotype in Ctla-4-deficient lymphocytes may require, or result from, compensatory changes in basal expression of additional determinants of T cell activation thresholds. However, the controversial role of Ctla-4 in thymocyte development(203, 226, 347-349) and the paucity of data regarding the function of genes differentially expressed in B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ CD4+ lymphocytes requires additional characterization of their potential relationship to Ctla-4 function.

The identification of genes differentially expressed in naïve Ctla-4 deficient cells will allow comparative analyses with expression profiles in primary Ctla-4 sufficient cells subsequent to Ctla-4 ligation. The data identifies a small number (<80) of genes displaying statistically significant changes in expression in the absence of Ctla-4. Genes

Table 3.1: Genes differentially expressed in naïve B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ CD4+ lymphocytes as a result of Ctla-4 deficiency

Category	Common Name	Fold Change	Characterization
Cell Cycle	Sugt1	-1.64	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)
	Nbn	-1.57	nibrin
	Cdc6	1.59	cell division cycle 6 homolog (S. cerevisiae)
Anti-apoptotic	**Thy28**	1.54	thymocyte protein thy28
Pro-apoptotic	Ifi16	-1.81	interferon, gamma-inducible protein 16
	Bad	1.60	ES cell Mus musculus cDNA clone 2410088E21, mRNA
	Sgpp1	1.94	sphingosine-1-phosphate phosphatase 1
Signaling	**Lancl1**	-2.24	LanC (bacterial lantibiotic synthetase component C)-like 1
	Usp18	-1.98	ubiquitin specific protease 18
	Stat5b	-1.92	signal transducer and activator of transcription 5B
	Stard7	-1.76	START domain containing 7
	Gna11	-1.73	Mus musculus guanine nucleotide-binding protein (Gna11)
	Nr4a2	-1.70	
	Cops3	-1.69	COP9 (constitutive photomorphogenic) homolog, subunit 3
	Rhoc	-1.62	ras homolog gene family, member C
	Dtx1	-1.55	deltex 1 homolog (Drosophila)
	Ccr2	-1.55	chemokine (C-C) receptor 2
	Il6st	-1.54	interleukin 6 signal transducer
	Cd3g	-1.51	Mouse CD3-gamma (T3-gamma) gene, exon 7.
	Pik3cd	1.54	phosphatidylinositol 3-kinase catalytic delta polypeptide
	Fzd3	1.64	frizzled homolog 3 (Drosophila)
	Itm2a	-1.69	integral membrane protein 2A
	Ctla4	-17.67	cytotoxic T-lymphocyte-associated protein 4
Transcription	**Trp63**	-1.88	transformation related protein 63
	Rnf20	-1.76	ring finger protein 20
	AW547477	-1.60	thymopoietin
	Nr3c1	-1.54	nuclear receptor subfamily 3, group C, member 1
	Tceb1	1.67	transcription elongation factor B (SIII), polypeptide 1
Translation	**Ebna1bp2**	-2.86	EBNA1 binding protein 2
	Eif5b	-2.24	vs58d02.r1 Stratagene mouse skin (#937313
	Styx	-2.19	
	Ncbp2	-1.80	nuclear cap binding protein subunit 2
	Eif1a	-1.69	eukaryotic translation initiation factor 1A
	Pa2g4	1.76	proliferation-associated 2G4

Category	Common Name	Fold Change	Characterization
Immune	Ifit1	-2.05	interferon-induced w/ tetratricopeptide repeats 1
	G1p2	-1.78	interferon, alpha-inducible protein
	Ctsb	-1.52	
	Kcnq1	1.51	3' similar to U70068 Mus musculus (KvLQT1)
Cytoskeleton	Cd9	-1.91	CD9 antigen
And Adhesion	Tuba1	-1.71	tubulin, alpha 1
	Slmap	-1.54	sarcolemma associated protein
	Plec1	-1.52	plectin 1
Metabolism	Idh1	-1.86	isocitrate dehydrogenase 1 (NADP+), soluble
	Atp11a	-1.58	ATPase, class VI, type 11A
	Arsa	-1.57	arylsulfatase A
	Dhrs1	-1.57	dehydrogenase/reductase member 1
	Mrpl12	-1.55	mitochondrial ribosomal protein L12
	Glud	-1.54	glutamate dehydrogenase
Miscellaneous	Slc29a1	-2.98	solute carrier family 2, member 1
	Mrpl44	-2.35	mitochondrial ribosomal protein L44
	Tnfaip1	-2.11	Mus musculus Edp1 protein (Edp1) gene,
	1110004L07Rik	-1.94	exportin, tRNA
	G22p1	-1.84	thyroid autoantigen
	B230312B02Rik	-1.74	RIKEN cDNA B230312B02 gene
	Rcl1	-1.72	RNA terminal phosphate cyclase-like 1
	Ensa	-1.69	endosulfine alpha
Unknown	Mela	-52.36	melanoma antigen
	Wt1	1.88	EST: 3' similar to M55512 Mouse Wilms' tumor
	Gtrgeo22	-1.79	gene trap ROSA b-geo 22
	Rnf138	-1.67	ring finger protein 138
	Pabp4i1	-1.66	Similar to Poly(A) binding protein, cytoplasmic 4, isoform 1 (LOC216817)
	Rnf149	-1.59	ring finger protein 149
	Glo1	-1.58	CDNA clone IMAGE:4037400
	DXImx46e	-1.53	vw47f05.x1 Soares_mammary_gland_
	1500001L20Rik	2.17	WD repeat domain 40A

Table 3.1: Genes whose expression is significantly altered in the absence of Ctla-4. Peripheral CD4+ lymphocytes from were purified by FACS from B7.1$^{-/-}$/B7.2$^{-/-}$ and B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ mice. RNA was isolated from purified population and analyzed by Affymetrix MgU74Av2 microarray following single-step cRNA amplification and labeling starting with 5-8ug of total RNA. Individual samples were obtained from starting populations representing pooled lymphocytes from 2-3 mice. Biological replicates were isolated and processed at separate times. Microarray sample processing and scanning was performed as described utilizing the Affymetrix MAS5.0 software and yielded "present" or "absent" flag calls based on signal intensity relative to background. Further analysis using Genespring allowed for identification of genes differentially expressed. Briefly, data was normalized by setting values below 0.01 to 0.01 and dividing each measurement by the 50.0th percentile of all measurements in that sample. Average measurements in each condition were compared and genes differentially expressed at least 1.5 fold are listed in table 3.1. Genes without statistically different expression when compared using the cross-gene error model were excluded. Genes without statistically significant expression in at least one condition were excluded. The majority of genes (84%) with detectable expression in both conditions were downregulated in the absence of Ctla-4.

involved in signal transduction are the most commonly affected, and several identified genes have known roles in regulating T cell activation. The majority of genes identified, however, are relatively uncharacterized in lymphocytes. The finding that a majority (>80%) of the genes identified were down-regulated may result from one of several mechanisms. It is possible that experimental error introduced bias and suppressed expression levels as a whole. This is unlikely given the standardization procedures involved in setting detector thresholds, the use of global expression patterns to detect outlying samples, and normalization steps that provide statistical data of specific genes relative to the whole transcriptome. Biologically, several other mechanisms may explain the preponderance of down-regulated genes. While B7 ligands are not required for thymocyte development, interactions between B7:CD28, and perhaps B7:Ctla-4, do influence aspects of thymic selection processes and are required for the development of specific subsets of peripheral T cells. Thus, the absence of B7 may alter both central and peripheral tolerance mechanisms. However, the decreased threshold of activation that accompanies Ctla-4 deficiency in $B7^{-/-}$ mice strongly suggests that Ctla-4 plays a critical role in regulating naïve T cell responsiveness in a manner analogous to that seen in Ctla-4 wild-type mice. Our data suggest that minor alterations in basal transcription may result from Ctla-4 absence allowing further investigation of the role these genes play in T cell activation.

Chapter III

Results

Global Gene Expression Analysis of Naïve, Extrinsically Regulated, Ctla-4-Deficient Cells in Bone Marrow Chimerae

Genomic expression patterns in extrinsically regulated Ctla-4 deficient T cells

Ctla-4 deficiency results in intrinsic defects in lymphocyte activation and self-tolerance that are manifested in the CD28 costimulation dependent lymphoproliferative disease in Ctla-4 knockout mice(268). Moreover, despite contradicting reports(203, 347, 349), previous work, supported by results generated in our lab, indicates that Ctla-4-deficient thymocyte development is normal(226), and that disease pathogenesis originates with uncontrolled CD4+ lymphocyte activation in the periphery as a consequence of TCR interaction with self-p/MHC in the context of CD28 costimulation. Overt disease resulting from the lack of Ctla-4 is avoided in the context of B7 and/or CD28 deficiency, depletion of CD4+ peripheral lymphocytes(224, 268), or inactivating mutations of TCR signaling or MHC presentation pathways(224, 227, 323). Similarly, limitation of the TCR repertoire in TCR-transgenic Ctla-$4^{-/-}$ mice prevents or delays autoimmune disease(227). In conjunction with in vitro stimulation assays showing a decreased threshold of activation in Ctla-$4^{-/-}$ CD4+ lymphocytes, and biochemical evidence of alterations in proximal TCR signaling pathways in the absence of Ctla-4, these observations provide compelling evidence of an intrinsic regulatory defect. However, published results have also indicated that defective regulatory mechanisms intrinsic to Ctla-4 deficient lymphocytes can be regulated extrinsically in the presence of Ctla-4 WT cells(261, 262).

When transferred to $Rag^{-/-}$ recipients, T cell depleted Ctla-$4^{-/-}$ bone marrow reconstitutes the lymphoproliferation and multiorgan infiltration seen in Ctla-$4^{-/-}$ mice at 4-8 weeks after transplantation. However, when co-transferred with Ctla-4 WT bone

marrow, the recipient displays a stable, balanced chimerism of Ctla-4 WT and KO peripheral lymphocytes. Bone marrow chimerae remain healthy, and Ctla-4$^{-/-}$ peripheral lymphocytes display none of the autoimmune reactivity characteristic of isolated Ctla-4 deficiency. Thus, Ctla-4 maintenance of peripheral tolerance is not limited to the down-regulation of specific T cell responses in a T cell-autonomous fashion. Work in our lab has demonstrated that depletion of WT lymphocytes using antibody to Ly5 or Thy1 congenic markers in the chimera initiates the autoimmune phenotype seen in Ctla-4$^{-/-}$ mice, indicating a persistent and active regulation of the Ctla-4$^{-/-}$ cells by Ctla-4 sufficient cells is necessary for the maintenance of balanced chimerism (Data not shown). Moreover, the rapidity with which lymphoproliferation and pathology is observed following depletion of wild-type T cells suggests that altered thymic development in mixed chimerae does not underlie the tolerant phenotype of Ctla-4$^{-/-}$ cells in the periphery.

To date, the phenotype of wild-type cells that mediate regulation of Ctla-4-deficient T cells in mixed bone marrow chimerae has not been established. Either CD4 or CD8 WT lymphocytes provide sufficient regulation of Ctla-4$^{-/-}$ cells in the periphery. Moreover, the Ctla-4 deficient lymphocyte population maintains its ability to respond to antigenic stimuli, and becomes activated, expands, and contracts indistinguishably from WT cells in response to lymphocytic choriomeningitis virus, Leishmania major and mouse mammary tumor virus, which cause acute, chronic and persistent infections, respectively(262). Thus, regulation of Ctla-4-deficient cells restores normal immune responsiveness and does not require an antigen-independent, generalized suppression of

their function. However, the mechanism by which Ctla-4^{+} T cells regulate their Ctla-$4^{-/-}$ counterparts remains unknown. Again, using microarray analysis, we examined the global expression profiles of $CD4^{+}$ Ctla-$4^{-/-}$ and $CD4^{+}$Ctla-4^{+} cells from stably reconstituted bone marrow chimera.

Generation of mixed bone marrow chimera and microarray sample preparation:

Minimally irradiated (300 rads) $Rag^{-/-}$ mice were injected with $5x10^{6}$ T cell depleted bone marrow cells at a 1:1 ratio of Ctla-4^{+}: Ctla-$4^{-/-}$ or Ctla-4^{+}:Ctla-4^{+}. Donor cells were distinguishable by the expression of Ly5 or Thy1 congenic markers. Thymic emigration, indicated by the presence of peripheral lymphocytes, was first detected at 3-4 weeks after transfer. In most cases, stable reconstitution is achieved by 12 weeks with nearly a 1:1 ratio of donor cells in the periphery. Recipient mice were sacrificed at 12-16 weeks, CD4+$CD44^{Low}$ lymphocytes were isolated by FACS, and RNA was prepared using either Trizol or Ultraspec reagents. $CD44^{Low}$ isolated cells were CD69- and CD25- by flow cytometry. In one experiment, RNA samples from numerous mice were pooled to allow 5ug of total RNA to be labeled in a single-step amplification, and the resuling cRNA was analyzed on one chip per condition. In two subsequent experiments, 1-2 mice were pooled to provide sufficient RNA for two-step amplification. Affymetrix MgU74Av2 Genechips were utilized, representing 12,488 genes. Identical labeling, sample preparation, and Genechip loading were used for the two-step amplification analyses, and samples were analyzed as replicates using the Genespring software package. Samples prepared from 5ug of pooled RNA were analyzed separately and the

results of the two experiments cross-referenced for validity, taking into account differences in amplification magnitude and spectrum that result from the two protocols.

Ctla-4$^{-/-}$CD4+ lymphocytes in mixed bone marrow chimera display a unique transcriptional profile:

Because single step amplification of total RNA has shown more consistent and robust differential expression profiles in past experiments, initial analyses of global expression in Ctla-4$^{-/-}$:Ctla-4^{+} bone marrow chimera was performed using pooled RNA from several mice analyzed on single Genechips. 6449 genes had detectable expression in at least one of the two samples, representing 52% of the included genome. Overall, the expression profiles between Ctla-4$^{-/-}$ and WT samples correlated at 93 and 96 percent with and without weighting for signal intensity respectively. In contrast, samples processed via two-step amplification correlated with single-step amplified products at only 46-52% when weighted for expression intensity, and 77-81% when unweighted, supporting the use of separate analyses.

Among genes with measurable expression in both 5ug samples labeled in a single-step protocol, 317 genes, 75 genes, 33, and 21 genes evidenced a minimum of 2-, 3-, 5-, or 10-fold differences in expression respectively. While there were almost identical numbers of genes up- and down-regulated more than two-fold in Ctla-4 deficient CD4+ lymphocytes (49% and 51% respectively), there was positive and consistent correlation between the magnitude of the differential expression and the likelihood of being

upregulated in Ctla-4$^{-/-}$ cells (60% and 71% of genes altered more than 5- and 10-fold respectively were upregulated in Ctla-4$^{-/-}$ cells). A summary of genes regulated more than 5-fold in extrinsically regulated Ctla-4-deficient cell, their expression profiles, and known functional ontology are listed in Table 3.2.

While 1117 genes were identified as having mixed calls in the two samples (P to A changes, or vice versa), only 225 of those genes had measurable signals more than 3-fold over background and constitute reliable measurements (Figure 3.2). However, a list of genes without detectable expression in one genotype can be ranked by the expression of the gene in the alternate sample (Table 3.3). Together with genes identified as reliably altered in the absence of Ctla-4, these genes constitute a steady-state profile that can be reliably cross-referenced with replicative experiments using the less-sensitive two-step amplification. However, 280 genes identified in the high-stringency screen of single-step amplified samples did not have consistently detectable expression in either Ctla-4$^{-/-}$ or WT samples that underwent double-amplification. Among genes identified in our initial screen with sufficient expression in our second experiment, 35 of the 102 downregulated genes and 56 of 160 upregulated genes showed expression patterns confirming our initial screens. In summary, using pooled samples in a high-stringency labeling protocol identified 542 genes differentially regulated in the absence of Ctla-4 as detected in naïve peripheral CD4+ lymphocytes from mixed bone marrow stably reconstituted with a 1:1 ratio Ctla-4$^{-/-}$:Ctla-4^{+} T cell depleted bone marrow cells. 282 identified genes were screened against the results of subsequent experiments using a lower sensitivity protocol, confirming the expression pattern in 91 genes, and leaving 280 genes unvalidated.

Table 3.2: Genes differentially regulated in naïve Ctla-4-deficient CD4+ T cells in mixed bone marrow chimerae

Fold Change	Common Name	Description & Characterization
Up-regulated Genes		
479.5	0610008N23Rik	RIKEN cDNA 0610008N23 gene
97.5	Pou2f1	Transcription Factor
61.2	Hrb2	EST
52.3	ORF6	EST
27.6	Pigt	Ribosomal Subunit
26.7	Itsn	intersectin (SH3 domain protein 1A)
26.0	102130_f_at	EST
24.9	Hnrph3	EST; Diiferentially expressed in normal and neoplastic cells
21.9	Mafk	Transcription Factor
17.3	2010200I23Rik	cytochrome c oxidase, subunit VIIa 2
16.4	161085_r_at	EST
14.1	Dnajc4	DnaJ (Hsp40) homolog, subfamily C, member 4
12.6	Mfn1	EST
11.1	Ttc1	tetratricopeptide repeat domain 1
11.0	D3Ertd789e	tetratricopeptide repeat domain 14
8.6	Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked
8.3	Elovl1	EST
7.8	Samhd1	EST
6.6	160934_s_at	Murine (DBA/2) mRNA fragment for gag related peptide.
5.4	Akp5	Mouse embryonic alkaline phosphatase gene, complete cds.
Down-regulated		
-189.0	Ercc1	excision repair cross-complementing rodent repair deficiency, complementation group 1
-71.9	Il4	Intereukin4
-66.2	5430432M24Rik	EST
-64.5	Prss11	protease, serine, 11 (Igf binding)
-13.7	Limk1	LIM-domain containing, protein kinase
-12.0	Slc30a4	zinc transporter; Mus musculus zinc transporter (ZnT4) gene, fragment 4, and partial cds.
-7.2	4930517K11Rik	ribosomal subunit
-7.0	Pard6a	cell cycle; cytokinesis; par-6 (partitioning defective 6,) homolog alpha (C. elegans)
-6.0	Spock2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2
-6.0	Kcnj9	EST
-5.8	Iga	secreted form; Mouse Ig germline D-J-C region alpha gene and secreted tail.
-5.5	Homer3	EST
-5.2	Gpr132	G protein-coupled receptor 132

Table 3.2: Genes with detectable expression in all samples regulated more than 5-fold in Ctla-4-deficient naïve CD4+ T cells relative to their wild-type counterparts in mixed bone marrow chimerae. CD4+ Ctla-4 deficient and wild-type populations were isolated from stably reconstituted mixed bone marrow chimerae and analyzed by Affymetrix MgU72Av2 microarray. Samples analyzed represent pooled RNA from populations isolated from >5 mice per condition. Only one microarray per condition was analyzed utilizing a single-step amplification protocol. The cross gene error model was applied to allow for statistical comparison in the absence of biological replicates. Genes in Table 3.2 were detectable in Ctla-4-deficient and wild-type CD4+ lymphocyte populations as determined by MAS5.0 software analysis of the microarray data. Further analysis using Genespring allowed for identification of genes differentially expressed. Briefly, data was normalized by setting values below 0.01 to 0.01 and dividing each measurement by the 50.0th percentile of all measurements in that sample. 33 unique genes meeting these criteria displayed 5-fold or greater changes in expression in the absence of Ctla-4. Differential expression was significant at $p<0.05$ as determined by the cross-gene error model. Red highlighted genes were called present in all samples but possessed extremely low signal strength in at least one sample that required transformation to a minimal baseline value (0.01), potentially skewing calculated fold-change values.

Table 3.3: Genes with mixed calls that possess the highest signal intensity levels in samples with detectable expression

Calculated Fold Change	Common Name	Description and Characterization
		Upregulated Genes
638.5	C330006A16Rik	ES cells cDNA, RIKEN full-length enriched library, clone:C330006A16 product:unknown
413.5	Nfatc2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2; regulation of transcription
214.6	Dag1	dystroglycan 1; morphogenesis of an epithelial sheet
178.9	Prkcz	AV367375 RIKEN full-length enriched, similaro to protein kinase C zeta mRNA
88.5	Cbl	Casitas B-lineage lymphoma
7.3	Rn18s	Mouse gene for 18S rRNA.
5.2	Eif1a	eukaryotic translation initiation factor 1A
4.6	Hccs	holocytochrome c synthetase
4.1	Gpam	glycerol-3-phosphate acyltransferase gene, nuclear gene encoding mitochondrial protein
3.8	Cdon	cell adhesion molecule-related/down-regulated by oncogenes
3.7	Kcna3	potassium voltage-gated channel, shaker-related subfamily, member 3
3.6	160799_at	UI-M-BH1-ann-g-07-0-UI.s1 NIH_BMAP_M_S2 Mus musculus cDNA clone
3.1	2510010F15Rik	RIKEN cDNA 2510010F15 gene
		Down-regulated Genes
-204.9	D1Bwg1363e	DNA segment, Chr 1, Brigham & Women's Genetics 1363 expressed
-164.5	Pcbp4	UI-M-AQ1-aee-h-05-0-UI.s1 NIH_BMAP_MHI_N Mus musculus cDNA clone.
-61.0	Prss15	protease, serine, 15
-15.4	Arl4	ARF-like 4 protein; intracellular protein transport; small GTPase mediated signal transduction
-11.9	102155_f_at	EST
-11.2	Ctsh	cathepsin H; proteolysis and peptidolysis
-10.4	Snai2	snail homolog 2;development; regulation of survival gene product activity; regulation of transcription
-9.3	Kdap	napsin A aspartic peptidase
-4.5	Igl-V1	variable and contstant regions; V-J; Mus musculus immunoglobulin lambda-1 light chain precursor
-3.8	2410015N17Rik	RIKEN cDNA 2410015N17 gene
-3.8	Bcl2l2	Bcl2-like 2; apoptosis; regulation of apoptosis
-3.6	Tyk2	member of Jak (Janus) family; contains Jak homology regions 7 to 4; non-receptor tyrosine kinase.
-3.6	Ptprv	protein tyrosine phosphatase, receptor type, V
-3.5	Dnalc4	dynein, axonemal, light chain 4
-3.5	Cct2	Mus musculus Cctb gene for chaperonin containing TCP-1 beta subunit, complete cds.
-3.4	Il1r2	interleukin 1 receptor, type II
-3.1	Mela	melanoma antigen
-3.0	Itgb1bp1	integrin beta 1 binding protein 1

Table 3.3: Differentially expressed genes flagged as absent in one condition (Ctla-4-deficient or wild-type) were ranked by their level of expression in the remaining genotypic sample as determined by normalized signal intensity. 31 genes with mixed calls ("present" to "absent" changes or vice versa) with the highest normalized signal intensities in the sample with detectable expression are shown. Fold change values reflect approximate fold-increases in differential expression based on a comparison of signal strength value in the sample with detectable expression to background signal strength detected in the sample flagged "absent" by MAS5.0 software analysis of signal to noise ratios. Fold change numbers are demonstrative only and can not be construed as true ratios of expression due to the high signal:noise ratio in one of the samples. Genes with known effects in lymphocytes are highlighted red.

Figure 3.2: Scatter plot of gene expression intensity in mixed bone marrow chimerae

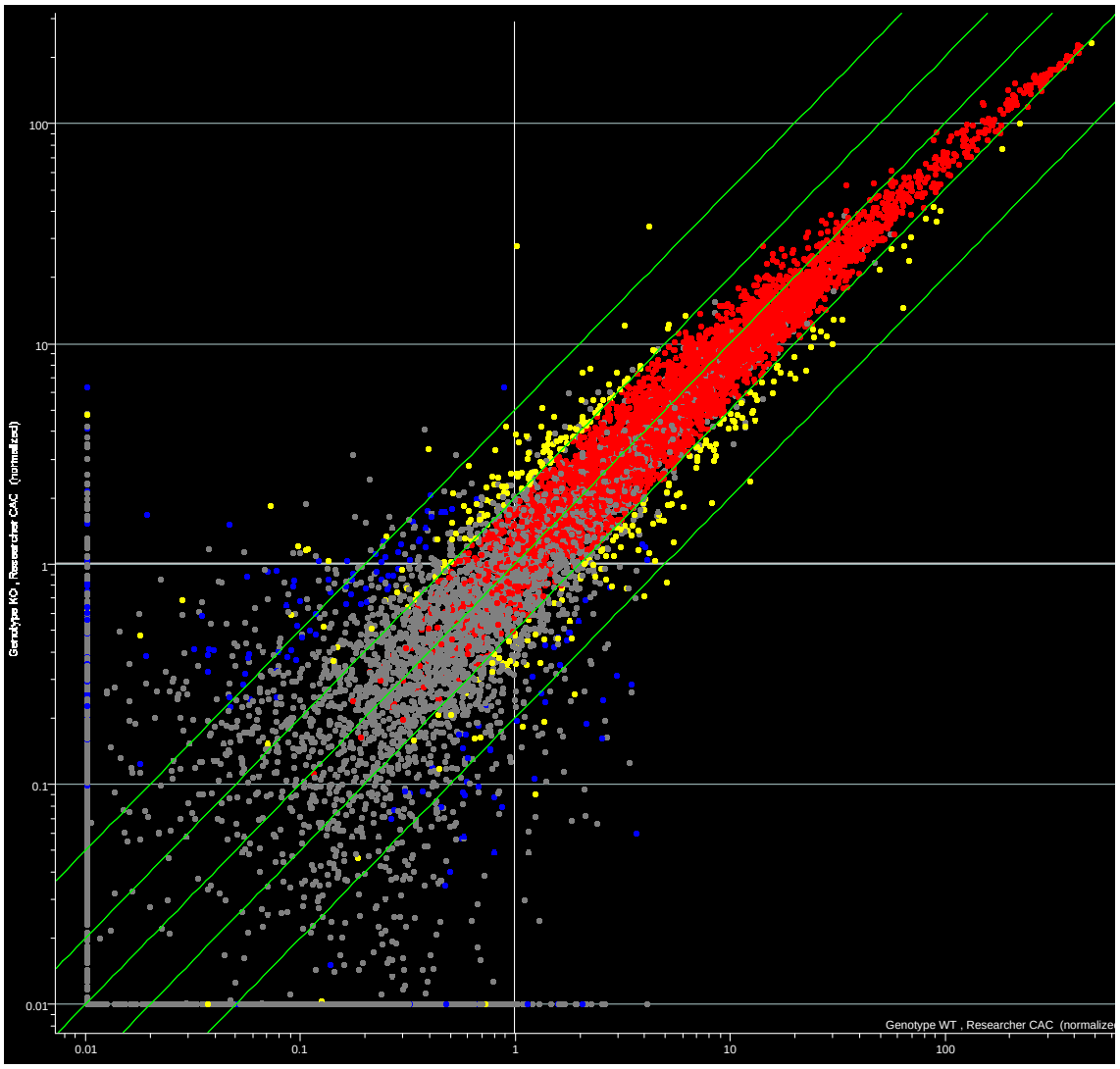


Figure 3.2: Scatter plot of gene expression level in Ctla-4-deficient and-wild-type naïve CD4+ T cells from mixed bone marrow chimerae. Minimally irradiated $Rag^{-/-}$ mice were transfused with $5x10^6$ bone marrow cells consisting of a 1:1 mixture of T cell-depleted wild-type and Ctla-4-deficient donor cells. Recipient mice successfully reconstituted their peripheral lymphocyte compartment with an approximate 1:1 representation of donor cells. Recipient mice remained healthy and display no symptoms of lymphoproliferative disease or autoimmunity. Recipient mice were sacrificed 12 weeks after bone-marrow transfer and donor $CD4+CD44^{Low}$ cells purified by FACS isolation using Ly5.1 or Thy1.1 congenic markers to discriminate Ctla-4-deficient and wild-type populations. $CD4+CD44^{Low}$ populations represented 85-95% of peripheral CD4+ T cells and were also CD69-, $CD62L^{High}$, and CD25-. RNA from $CD4+CD44^{Low}$ populations of wild-type and Ctla-4-deficient donor cells was isolated and analyzed by oligonucleotide microarray using Affymetrix MgU74Av2 GeneChips. Chip scanning, intensity normalization, and determination of "present" and "absent" calls were performed using Affymetrix MAS5.0 software. Further data analsysis was performed using Genespring software and data normalization was achieved by setting values below 0.01 were to 0.01 , and dividing each measurement by the 50.0th percentile of all measurements in that sample. The cross gene error model was applied to allow statistical comparison in the absence of biological replicates amplified by similar single-step labeling protocols. Each dot in the scatter plot represents one gene where X-axis and Y-axis values correspond to the average level of expression in wild-type and Ctla-4-deficient CD4+ $CD44^{Low}$ cell populations respectively according to normalized data values. Gray colored genes have

levels of expression not statistically above background signal intensity in any sample. Red genes are present in all samples but are not changed more than two-fold. Yellow genes (317) are present in both samples with measurable fold-changes greater than two fold. Blue genes (225) are expressed in one sample at 3-times the control signal intensity, but are absent in the alternate genotype. The central diagonal line indicates equivalence of expression. The most proximal and distal pairs of parallel lines around the line of equivalence indicate 2-fold and 5-fold changes in expression respectively.

A comparison of genes differentially regulated in B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ CD4+ lymphocytes versus B7.1$^{-/-}$/B7.2$^{-/-}$ CD4+ lymphocytes against genes differentially regulated in Ctla-4$^{-/-}$ cells in Ctla-4$^{-/-}$:Ctla-4^{+} bone marrow chimerae could potentially reveal a mechanism by which Ctla-4$^{-/-}$ cells are regulated in the presence of Ctla-4 WT cells provided that alterations in gene regulation result from the interaction. In total, only 9 genes identified as differentially expressed in B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$ versus B7.1$^{-/-}$/B7.2$^{-/-}$ CD4+ cells ex vivo showed similar expression patterns in Ctla-4$^{-/-}$ versus Ctla-4^{+} CD4+ cells from stably reconstituted bone marrow chimera relative to Ctla-4 expression. Among these, only the decoy receptor Il1R2 and Ctla-4 have known regulatory function in lymphocytes. Similarly, only 11 genes were inversely regulated in the bone marrow chimera relative to B7.1$^{-/-}$/B7.2$^{-/-}$/Ctla-4$^{-/-}$:B7.1$^{-/-}$/B7.2$^{-/-}$ comparisons. Of those, only Xlra3 and CD2ap are significant as determined by the cross-gene error model (See Materials and Methods).

Discussion

Ctla-4 maintains peripheral T cell tolerance through cell-intrinsic and -extrinsic mechanisms that are only partially understood. As previously discussed, intrinsic regulation of T cell responses by Ctla-4 involves potentially diverse mechanism that are likely to include ligand competition, mechanical disruption of activating interactions within the immunological synapse, and regulation of membrane proximal signaling events. Mechanisms of cell-extrinsic regulation of T cell responses are not as well

studied, but may involve either cell contact-dependent or –independent interactions with Ctla-4 expressing cells.

Regulatory T cells (Tregs) are attractive candidates for mediators of non-autonomous immune regulation by Ctla-4 given their well-established role in regulating lymphocyte responses to foreign and self-antigens both in vitro and in vivo(350). Mechanisms of Treg function are not well understood, but may involve regulation of APC function(304-306), secretion of regulatory cytokines(351), or direct suppression of effector T cells via cell-cell contact(352). Ctla-4 is constitutively expressed on regulatory T cells and was initially considered to be essential for Treg function(353, 354) or development(301, 355). However, published results(356) and work in our lab indicate that naturally occurring CD4+CD25+ peripheral lymphocytes in Ctla-4-deficient mice evidence potent immunoregulatory phenotypes indistinguishable from wild-type regulatory T cells in vitro. In conjunction with the observation that CD8+ Ctla-4 wild-type cells are capable of extrinsic regulation of Ctla-4 deficient lymphocytes in mixed bone marrow chimerae, the data suggest that non-autonomous regulation of Ctla-4 deficiency can not be attributed solely to CD4+CD25+ suppressor T cell function. However, multiple lines of evidence suggest that regulatory T cells may utilize multiple mechanism to suppress effector T cells, some of which may require Ctla-4 expression(301, 351, 356, 357).

A role for B7-mediated signaling in immunological tolerance was first recognized in dendritic cells. Ctla-4 acts as ligand for B7 receptor molecules that transduce intracellular signals resulting in upregulation of inoleamine-oxygenase, increased

tryptophan catabolism, and suppression of lymphocyte responses(305). In contrast, simultaneous ligation of B7.1 and B7.2 on dendritic cells by soluble CD28 enhances T cell-mediated immunity by upregulating IL-6 and Ifn-gamma production(307), suggesting that B7 signaling may control functionally distinct effector responses as a result of differential ligand engagement. Engagement of B7 expressed on effector T cells by regulatory T cells is also capable of inhibitory signaling, and may have a central role in autoimmune disease(303). Together, these data suggest that Ctla-4 engagement of B7 may provide a generalized mechanism of immunosuppression via bidirectional signaling. In the context of Ctla-$4^{-/-}$:Ctla-4^{+} mixed bone marrow chimerae, inhibitory signaling through B7 on effector cells may explain numerous, previously unexplained, findings. For example, both CD4+ and CD8+ cells can express Ctla-4, potentially explaining the apparent sufficiency of either population in the regulation of Ctla-4-deficient cells. While B7-mediated inhibition of effector cells may operate independently of gene transcription, the regulation of tryptophan catabolism in dendritic cells indicates that, in some systems, B7 ligation regulates gene expression. Genes identified in our system can be verified in vitro and in vivo to determine their regulation subsequent to B7 ligation on T cells. Likewise, the importance of B7 signaling in the regulation of Ctla-4 deficient cells can be ascertained by transplantation of mixed Ctla-4^{+} and B7.$1^{-/-}$/B7.$2^{-/-}$/Ctla-$4^{-/-}$ bone marrow. These experiments are already underway in our lab, and will inform our understanding of the gene expression patterns identified in extrinsically regulated Ctla-$4^{-/-}$ CD4+ T cells.

Cytokines are important immunomodulatory signaling molecules involved in the regulation of autoimmune disease whose function is determined by the expression pattern and molecular associations of their respective receptors. The role of cytokines, particularly, Il-10 and TGF-beta, in Ctla-4-mediated regulation of T cell responses has been controversial(288, 358), and a role for cytokine-mediated immunosuppression in extrinsic regulation of Ctla-$4^{-/-}$ T cells has not been elucidated. Ctla-$4^{-/-}$ T cells actively regulated by wild-type cells maintain tolerance to self-antigen while maintaining normal antigen-specific responses to pathogens(262). Consequently, indiscriminate immunosuppression by cytokines is an unlikely mechanism by which Ctla-$4^{-/-}$ T cells could be regulated. Moreover, cytokine signaling regulates gene transcription via characterized pathways and activation of transcription factors with known target specificities. Over-represented regulatory sequences consistent with these pathways were not detectable in genes differentially regulated in Ctla-$4^{-/-}$ CD4+ cells suggesting that suppression of autoimmune disease development in mixed bone marrow chimera does not result from canonical signaling pathways operating downstream of cytokine receptors.

Vesicular transport of active protein constituents between cells of the immune system has been described(359-362). The transfer of Ctla-4 between lymphocytes has not been demonstrated, and is being investigated in our lab. While Ctla-4 has not been detected on Ctla-$4^{-/-}$ cells in bone marrow chimerae, uptake of exogenous Ctla-4 could restore tolerance to self-antigen without detectable surface expression in a manner consistent with the native function of Ctla-4. It is likely that such a mechanism would

not result in appreciable changes in gene expression as Ctla-4 functionsto suppress T cell activation, in part, by inhibiting membrane proximal signaling events that do not require regulation of gene transcription. Despite growing evidence that intercellular protein exchange occurs in the immune system, there is currently no evidence to support models of immunoregulation involving intercellular vesicular transport of native Ctla-4 protein.

We have characterized the global expression pattern of Ctla-4-deficient CD4+ lymphocytes extrinsically regulated by wild-type T cells in order to elucidate the mechanism involved. Numer genes involved in intracellular signaling, transcriptional regulation, or cell-cycle control are differentially regulated in Ctla-4-deficient CD4+ T cells. Table 3.4 summarizes genes with known functions related to these processes that are differentially expressed between Ctla-4-deficient or wild-type cells in mixed bone marrow chimerae displaying either a statistically significant two-fold change in expression or detectable expression in only one condition of either Ctla-4-deficiency or wild-type Ctla-4 exprssion. Notably, NFATc is upregulated to a greater relative degree than any other gene. NFAT is centrally involved in the regulation of genes mediating T cell survival, activation, cytokine production, and anergy induction. Thus, NFAT may be centrally involved in the regulation of Ctla-4-deficient responses by wild-type cells. The majority of differentially regulated genes identified have not been characterized in T cells. However, their role in T cell activation can be examined within the context of a kinetic study of T cell activation.

Table 3.4: A selected list of genes involved in T cell immune responses that are differentially regulated in Ctla-4 KO/WT mixed bone marrow chimerae.

Fold Change	Common Name	Description and Characterization
413.5	Nfatc2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2
152.8	Xlr3a	X-linked lymphocyte-regulated 3b
97.5	Pou2f1	POU domain, class 2, transcription factor 1
88.5	Cbl	Casitas B-lineage lymphoma
62.8	Zfp326	zinc finger protein 326
29.4	Ncoa6	nuclear receptor coactivator 6
26.1	Rpo2tc1	RNA polymerase II transcriptional coactivator
24.8	MVA5T	T cell receptor alpha chain AV20S1 precursor, mRNA, partial cds.
22.1	Ptk2	PTK2 protein tyrosine kinase 2
21.9	Mafk	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein K
19.5	Sox6	AV246999 RIKEN full-length enriched, 0 day neonate head Mus musculus cDNA clone
17.0	Meox1	mesenchyme homeobox 1
15.8	Cdc7	gene for muCdc7
12.2	Rock2	Rho-associated coiled-coil forming kinase 2
9.9	Hoxc5	homeobox protein (Hoxc-5) gene
9.3	Ccr1	chemokine (C-C motif) receptor 1
7.0	Zfx	M.musculus Zfa gene.
5.3	Il9	P40 T-cell and mast cell growth factor precursor;
4.7	Ptpra	protein tyrosine phosphatase, receptor type, A
4.3	Cnot7	CCR4-NOT transcription complex, subunit 7
4.1	Prrx1	paired related homeobox 1
3.9	Tmpo	thymopoietin
3.3	Trp53bp1	transformation related protein 53 binding protein 1
3.1	Eomes	eomesodermin homolog
2.8	Tbx6	T-box 6
2.8	Btrc	beta-transducin repeat containing protein
2.6	Pnn	pinin
2.6	Il11ra1	interleukin 11 receptor, alpha chain 2
2.5	5730497N03Rik	trans-acting transcription factor 4
2.4	Zfp148	zinc finger protein 148
2.4	Zfp95	zinc finger protein 95
2.4	Itpr1	inositol 1,4,5-triphosphate receptor 1
2.3	Plk2	polo-like kinase 2 (Drosophila)
2.2	Nfyb	nuclear transcription factor-Y beta
2.2	Cdk4	cyclin-dependent kinase 4
2.2	Pou6f1	POU domain, class 6, transcription factor 1
2.2	Zfp68	zinc finger protein 68
2.1	Tec	cytoplasmic tyrosine kinase, Dscr28C related (Drosophila)
2.1	Sox10	SRY-box containing gene 10

Fold Change	Common Name	Description and Characterization
-112.6	Figla	factor in the germline alpha
-82.0	Gadd45b	growth arrest and DNA-damage-inducible 45 beta
-71.9	Il4	interleukin 4
-70.9	Fem1a	Mus musculus sex-determination protein homolog Fem1a gene, complete cds.
-64.9	Csf1r	colony stimulating factor 1 receptor
-45.5	Ccnb1	cyclin B1
-32.1	Rgnef	Rho-guanine nucleotide exchange factor
-26.1	Hck	hemopoietic cell kinase
-12.2	Gna12	guanine nucleotide binding protein, alpha 12
-10.4	Snai2	snail homolog 2 (Drosophila)
-9.0	Creg	cellular repressor of E1A-stimulated genes
-6.5	Snx5	sorting nexin 5
-5.2	Gpr132	G protein-coupled receptor 132
-4.3	Ctla4	cytotoxic T-lymphocyte-associated protein 4
-4.2	Cd44	CD44 antigen
-3.6	Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1
-3.6	Tyk2	member of Jak (Janus) family; Mus musculus non-receptor tyrosine kinase (Tyk2) gene.
-3.6	Ptprv	protein tyrosine phosphatase, receptor type, V
-3.5	Ptprk	protein tyrosine phosphatase, receptor type, K
-3.4	Il1r2	interleukin 1 receptor, type II
-3.3	Nek2	NIMA (never in mitosis gene a)-related expressed kinase 2
-2.9	Cd79a	CD79A antigen (immunoglobulin-associated alpha)
-2.7	Ccr6	Mus musculus CCR6 gene.
-2.6	Tnfsf5	tumor necrosis factor (ligand) superfamily, member 5
-2.6	Tnfrsf4	tumor necrosis factor receptor superfamily, member 4
-2.6	Nfyc	nuclear transcription factor-Y gamma
-2.6	Cd79b	CD79B antigen
-2.6	Itga4	integrin alpha 4
-2.5	Bcl3	B-cell leukemia/lymphoma 3
-2.5	Batf	basic leucine zipper transcription factor, ATF-like
-2.5	Ly6d	lymphocyte antigen 6 complex, locus D
-2.4	Il2rb	interleukin 2 receptor, beta chain
-2.4	Pglyrp1	peptidoglycan recognition protein 1
-2.4	Rras	Harvey rat sarcoma oncogene, subgroup R
-2.4	Maf	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog
-2.4	Bcl2l1	Bcl2-like 1
-2.3	Gadd45a	Mus musculus GADD45 protein (gadd45) gene, complete cds.
-2.3	Maz	MYC-associated zinc finger protein (purine-binding transcription factor)
-2.2	Cd19	Mus musculus cell surface protein CD19 gene, complete cds.
-2.1	Smarcb1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
-2.1	Blr1	Burkitt lymphoma receptor 1
-2.0	Inpp5d	inositol polyphosphate-5-phosphatase D
-2.0	Itgal	integrin alpha L

Table 3.4: Genes differentially expressed in Ctla-4-deficient cells in mixed bone marrow chimerae. Minimally irradiated $Rag^{-/-}$ mice were transfused with $5x10^6$ bone marrow cells consisting of a 1:1 mixture of T cell-depleted wild-type and Ctla-4-deficient donor cells. Recipient mice successfully reconstituted their peripheral lymphocyte compartment with an approximate 1:1 representation of donor cells. Recipient mice remained healthy and display no symptoms of lymphoproliferative disease or autoimmunity. Recipient mice were sacrificed 12 weeks after bone-marrow transfer and donor $CD4+CD44^{Low}$ cells purified by FACS isolation using Ly5.1 or Thy1.1 congenic markers to discriminate Ctla-4-deficient and wild-type populations. $CD4+CD44^{Low}$ populations represented 85-95% of peripheral CD4+ T cells and were also CD69-, $CD62L^{High}$, and CD25-. RNA from $CD4+CD44^{Low}$ populations of wild-type and Ctla-4-deficient donor cells was isolated and analyzed by oligonucleotide microarray using Affymetrix MgU74Av2 GeneChips. Chip scanning, intensity normalization, and determination of "present" and "absent" calls were performed using Affymetrix MAS5.0 software. Further data analsysis was performed using Genespring software and data normalization was achieved by setting values below 0.01 were to 0.01 , and dividing each measurement by the 50.0th percentile of all measurements in that sample. The cross gene error model was applied to allow statistical comparison in the absence of biological replicates amplified by similar single-step labeling protocols. Genes with a minimum 2-fold differential expression in the absence of Ctla-4 in mixed bone marrow population or displaying detectable expression in only one genoptypic condition were screened for known functions relating to cell-cycle regulation, transcriptional regulation, or T cell

activation and survival. The differential regulation observed is statistically significant as determined by the cross-gene error model.

CHAPTER IV

RESULTS

Kinetic Analysis of Global Expression Profiles in TCR-Transgenic CD4+ Lymphocytes During Primary Activation

Kinetics of T Cell Activation, Division, and Commitment

Temporal constraints on costimulatory signal integration

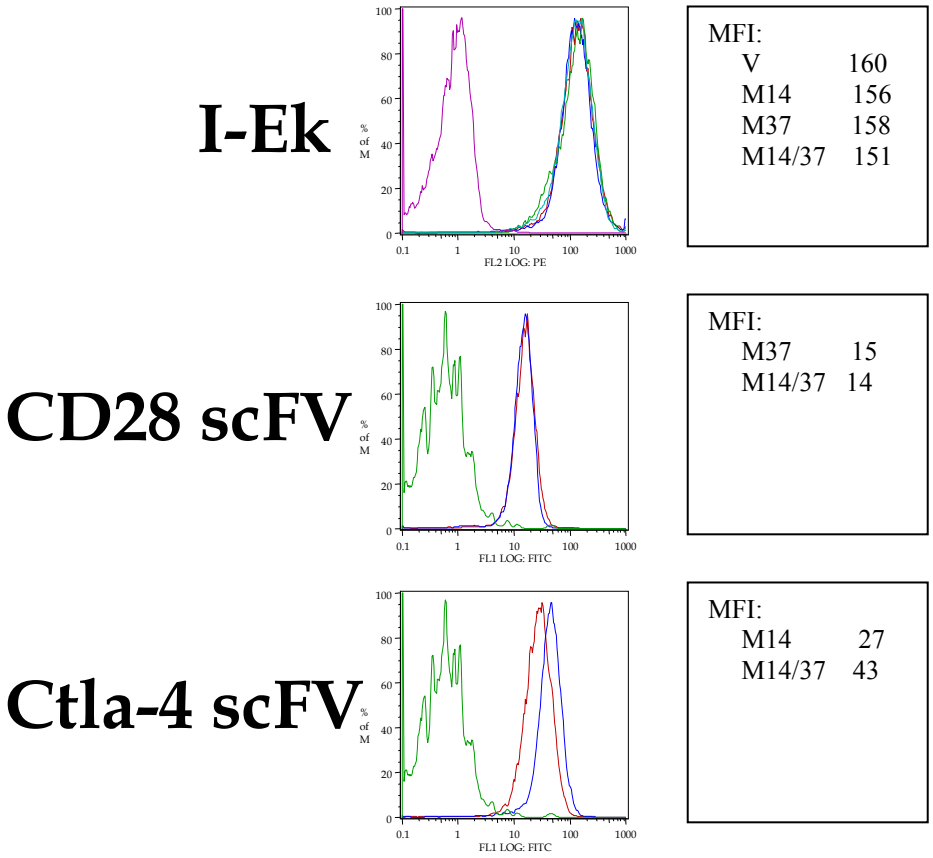
Although it is well established that T cells require antigen-independent costimulation in addition to TCR triggering for efficient activation, the temporal and spatial parameters of signal integration that occur in lymphocytes are incompletely understood. Rapid increases in intracellular calcium(363) and regulated phosphorylation of signaling intermediates occur within minutes of TCR engagement(364). Transcriptional, post-transcriptional, and post translational regulation of gene transcription, mRNA stability, and protein activity and degradation respectively are detectable within hours of T cell activation. However, in vivo, commitment to activation and integration of costimulatory signals influencing T cell differentiation and effector function are postulated to require formation of stable intercellular contact via the immunological synapse and extended periods of receptor engagement(365-369). However, the precise temporal constraints affecting costimulatory signal integration following TCR engagement have not been well defined. Moreover, the effect of transient or delayed CD28 or Ctla-4 engagement on T cells stimulated through the TCR has not been clearly examined. A greater understanding of parameters governing costimulatory signal integration will aid the development of strategies manipulating T cell signaling pathways for therapeutic benefit. In addition, a greater understanding of the mechanisms involved in costimulatory signaling will facilitate the identification of key signaling intermediates involved in the determination of lymphocyte respnsiveness.

Optimization of an in vitro, antigen-specific stimulation protocol

To better understand how costimulatory signals delivered by CD28 or Ctla-4 ligation are temporally integrated with TCR-mediated activation signals, we utilized an established in vitro stimulation system that allows discriminatory signaling through CD28 and Ctla-4(288). Synthetic ligands composed of the transmembrane region of B7.2 linked to the scFV region of antibodies specific for CD28 or Ctla-4 were stably expressed in CHO cells capable of presenting MCC^{88-103} in the context of $I\text{-}E^k$, either singly or in combination. Expression of T cell ligands were determined by staining with anti-$I\text{-}E^k$, CD28Ig, or Ctla-4Ig. Repeated purification of populations matched for ligand expression by FACS yielded stable lines expressing equivalent levels of their respective ligands. (Figure 4.1) 5C.C7 $Rag^{-/-}$ lymph node cells were cocultured with peptide pulsed, mitomycin C treated CHO cells under conditions of TCR monostimulation, CD28 costimulation, or Ctla-4 ligation. Increasing responder frequency correlated with increasing APC:T cell ratios below 1:1 suggesting that APC ligands could be limiting (data not shown). All subsequent experiments utilized a 1:1 ratio of APC:T cell cocultured in 96 well round-bottom plates containing 10^5 cells total. The stability of ligand expression on CHO cells in culture following mitomycin C treatment was assessed. Downregulation of surface ligands was insignificant over the first 36 hours (data not shown). Increasing the duration of peptide preincubation with APC also increased responder frequency significantly up to 5 hours (data not shown), and all subsequent experiments were performed after a 5 hour peptide prepulse with removal of

excess peptide. The stability of peptide presentation in culture was assessed by prepulsing APCs with varying concentrations of peptide and culturing them for intervals

Figure 4.1: Engineered APCs express equivalent T cell ligands



V: I-E^k
M14: I-E^k + Ctla-4 scFV
M37: I-E^k +CD28 scFV
M14/37: I-E^k +CD28 scFV+ Ctla-4 scFV

Figure 4.1: Engineered APCs expressing I-E^k and a combination of synthetic ligands for CD28 and Ctla-4 were repeatedly FACS purified for stable, homogenous expression of surface-expressed T cell ligands. Synthetic ligands for CD28 and Ctla-4 were stained with CD28-Fc orCtla-4-FC as appropriate followed by a goat anti-human FITC conjugated secondary antibody. Relative to CD28 scFV, higher Ctla-4 scFV levels were obtained in order to maximize Ctla-4-mediated inhibition. The biological relevance of CD28 ligation by the chimeric CD28 ligand expressed on the CHO cells is evident in the enhancement of TCR-mediated T cell stimulation assessed by responder frequency. The binding affinities of both chimeric ligands for their T cell-expressed ligands are expected to be similar based on the affinities of the antibodies from which they were derived. Preincubation of the CHO cells with CD28-Fc or Ctla-4-Fc failed to significantly affect the apparent enhancement or inhibition of T cell responses seen after stimulation with CHO cells expressing CD28 and/or Ctla-4 ligands respectively. Thus, the low median fluorescent intensity (MFI) detected for the CD28 and Ctla-4 ligands may reflect poor binding characteristics of the chimeric Fc linked molecules in culture as revealed by their inability to reverse the stimulatory and inhibitory properties induced in T cells by their cognate receptors on engineered CHO cells.

up to 48 hours prior to addition of responder cells. Minimal decreases in responder frequency were observed over the first 36 hours of coculture, reflecting stable antigen presentation in culture for extended times (data not shown).

Parameters of activation: dose response, kinetics, and costimulation

Because ex vivo 5C.C7 $Rag^{-/-}$ T cells are homogenous for activational history and TCR specificity, responder frequency reflects the average strength of signals received by lymphocytes in a given system. The magnitude of the responder frequency correlated with peptide prepulse concentration over a wide range of concentrations. (Figure 4.2) Kinetics of early activation marker CD69 expression, upregulation of CD4, and cell division following activation was characterized in cells receiving TCR monostimulation, CD28 costimulation, and Ctla-4 ligation. (Figure 4.3) Critically, maximal responder frequencies at most antigen concentrations were generally achieved by 8-12 hours, suggesting a high-degree of synchronicity existed in activated populations. TCR monostimulation resulted in markedly diminished responder frequencies relative to CD28 costimulated cells over the entire range of antigen concentration tested as measured by CD69 upregulation. Amplification of TCR-mediated activation signals by CD28 ligation was most pronounced at lower and intermediate antigen concentrations when measured as percentage increase in responder frequency. A reduction in thymidine incorporation at very high doses of antigen following CD28 costimulation was inconsistent with the expression of CD69 at early times and could be explained by either increased cell death or reduced burst size in CD28 costimulated cells at high antigen concentrations (Figure

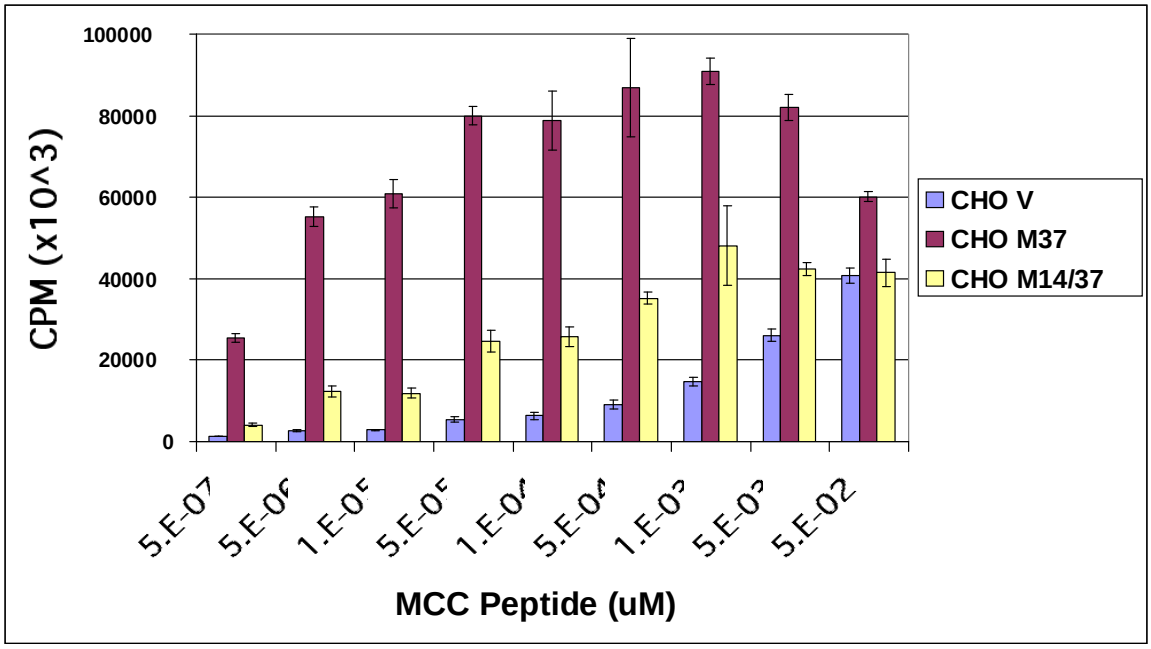
4.2). TCR monostimulation resulted in slightly delayed kinetics with regards to CD69 expression, CD4 upregulation, cell cycle entry, and rates of cellular division. Ctla-4 ligation resulted in a reduction in responder frequency that was greatest at lower antigen concentration, and became negligible at the highest concentration tested. Dose response curves were only marginally shifted by Ctla-4 ligation, and the kinetics of activation marker expression and cellular division at the population level were unchanged relative to cells receiving only CD28-mediated costimulation. Maximal CD28 costimulation, measured as an absolute increase in responder frequency relative to TCR monostimulated cells, was greatest at intermediate antigen concentration. Ctla-4 mediated inhibition of CD28 costimulation was also greatest at an intermediate antigen concentration of $5x10^{-4}$ uM MCC^{88-103}. Because Ctla-4 only marginally inhibited activation induced by TCR monostimulation, we chose to quantify Ctla-4 mediated inhibition relative to CD28-mediated enhancement of TCR activation signals according to the following equation:

$$[(RF_{CD28})-(RF_{Ctla-4})]/[RF_{CD28})-(RF_{TCR})]$$

where RF denotes "responder frequency" and TCR, CD28, and Ctla-4 indicate conditions of TCR monostimulation, TCR+CD28 ligation, and TCR+CD28+Ctla-4 ligation respectively.

Temporal constraints on costimulatory signal integration are not well known. To define the critical time period in which CD28 and Ctla-4 must be engaged to respectively enhance and inhibit T cell responses in our system, we utilized a cell transfer protocol that allowed for quantitative and qualitative changes in stimulation conditions over time. T cells were cultured under varying conditions of TCR monostimulation, and CD28 or

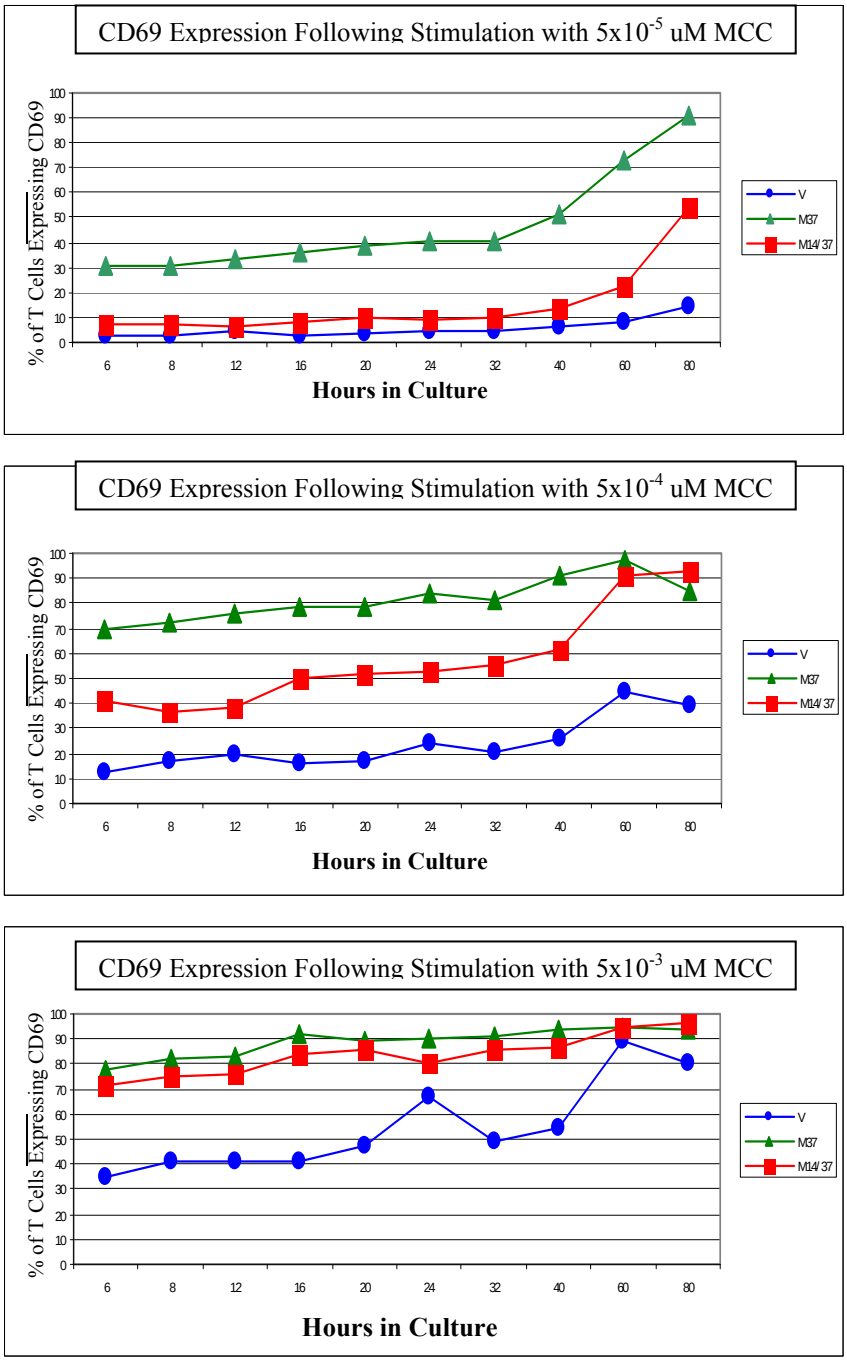
Figure 4.2: Dose response of curve of TCR-tg T cells stimulated with engineered APCs prepulsed with antigen



V: I-E^k
M37: I-E^k +CD28 scFV
M14/37: I-E^k +CD28 scFV+ Ctla-4 scFV

Figure 4.2: TCR-transgenic CD4+ lymphocytes respond to TCR stimulation, CD28 positive costimulation, and Ctla-4 mediated inhibition over a wide range of antigen concentrations. $5x10^4$ 5C.C7 TCR-transgenic CD4+ T cells were co-cultured in 96-well round-bottom plates with $5x10^4$ mitomycin C-treated Chinese hamster ovary (CHO) antigen presenting cells (APCs) engineered to express surface ligands for TCR, CD28 and Ctla-4 in varying combination that were prepulsed for five hours with the indicated concentration of moth cytochrome C oligonucleotide (MCC^{88-103}). 56 hours after the start of co-culture, 1μCi of tritiated Thymidine was added per well. Culture plates were frozen rapidly at -80º Celsius 16 hours after the addition of Thymidine. Individual culture conditions were performed in triplicate, and measured Thymidine incorporation represents the average of replicate wells. Data shown is representative of numerous experiments. CD28 resulted in the greatest relative enhancement of TCR-mediated activation at low doses of antigen. Similarly, Ctla-4 ligation was most effective in counteracting CD28 costimulatory signals at low doses of antigen. Bulk culture stimulations were performed with $5x10^{-4}$ uM MCC prepulse concentration to improve yields in both activated and naïve populations while ensuring adequate inhibition by Ctla-4.

Figure 4.3: TCR-tg T cells exhibit consistent responder frequency and reliable kinetics of activation and division when stimulated by engineered APCs.



V: I-E^{k}
M37: I-E^{k} +anti-CD28 scFV
M14/37: I-E^{k} +anti-CD28 scFV+ anti-Ctla-4 scFV

Figure 4.3: TCR-tg T cells stimulated in vitro by engineered APCs reveal antigen-dose-dependent costimulation responses to CD28 and Ctla-4 ligands. $5x10^4$ 5C.C7 TCR-transgenic CD4+ T cells were co-cultured in 96-well round-bottom plates with $5x10^4$ mitomycin C-treated Chinese hamster ovary (CHO) antigen presenting cells (APCs) engineered to express surface ligands for TCR, CD28 and Ctla-4 in varying combination that were prepulsed for five hours with the indicated concentration of moth cytochrome C oligonucleotide (MCC^{88-103}). CD4+ T cells were harvested from triplicate wells at specific times after the start of co-culture, pooled, and analyzed for the expression of the early activation marker CD69. CD69 expression is predictive of commitment to activation and cell-cycle entry. The top, middle, and bottom panels display the percentage of CD69+ CD4+ T cells at serial timepoints after stimulation with engineered APCs prepulsed with $5x10^{-5}$, $5x10^{-4}$, or $5x10^{-3}$ μM MCC respectively. Responses to CHO APCs expressing ligands for the TCR, the TCR+CD28, or the TCR+CD28+Ctla-4 are shown in blue (clone V), green (clone M37), and red (clone M14/37) respectively. $5x10^{-4}$ uM MCC prepulse concentration results in roughly 50% inhibition of CD28-mediated costimulation by Ctla-4. In addition, irreversible commitment to activation, as indicated by CD69 upregulation, is determined early after TCR triggering as reflected in relatively stable responder frequencies from 6 hours post-stimulation until first division. First division in the presence of CD28 occurs around 32-36 hours. TCR monostimulation results in delayed entry into cell-cycle and the majority of activated cells do not divide until 40-50 hours. Values shown are from pooled triplicates. Representative experiment

is shown.

Ctla-4 costimulation for defined intervals, removed from culture, and cocultured with APCs providing all possible combinations of TCR, CD28, and Ctla-4 ligation in the presence or absence of antigen. Flow cytometry analysis revealed that less than 10% of transferred cells were APCs from initial cultures (data not shown). Internal controls were provided by T cells that remained in initial cultures, and by transfer of T cells between identical culture conditions. The influence of costimulatory signals received by T cells was measured by CD69 expression 20-24 hours after initial stimulation, or after 16 hours in secondary culture conditions. No difference was seen between measurements taken at those times. Moreover, responder frequency in samples transferred between identical conditions was similar to that seen in unmanipulated cultures.

As shown, T cells cultured under conditions of TCR monostimulation became increasingly refractory to activation by CD28 costimulation over time(Figure 4.4). T cells were consistently and maximally responsive to CD28 ligation at any time up to 6 hours following TCR monostimulation. However, CD28 ligation occurring later than 6 hours potentiated TCR activation signals in a decreasing manner over time, and CD28 ligation after 16 hours of TCR monostimulation failed to increase responder frequency at all. Decreased responsiveness resulted from interaction with APC ligands. T cells initially cultured with APC in the absence of antigen did not become significantly refractory to stimulation over the initial 12 hours in culture (data not shown). Ctla-4 coligation with CD28 following TCR monostimulation inhibited CD28-mediated costimulation, but did not appear to produce independent effects on responder frequency apart from CD28. Importantly, maximal T cell responses to TCR monostimulation were

evident after 1 hour when transferred between identical culture conditions, or transferred to cultures lacking antigen and costimulation. This suggests that, in this system, T cell activation as a result of isolated TCR stimulation utilizes mechanisms that commit cells to become activated within one hour of TCR triggering. In contrast, CD28 and Ctla-4 costimulation can be integrated over an extended period.

Responder frequencies in cultures initially receiving TCR and CD28 signals also suggest that persistent costimulatory signals influence T cell responsiveness for several hours after TCR triggering (Figure 4.5). Our results show equivalent responses to TCR monostimulation and the absence of TCR ligation following one hour of combined TCR and CD28 stimulation, supporting the notion that TCR ligation activates all relevant pathways within one hour. Moreover, increasing the duration of CD28 ligation increases responder frequency up to 12 hours, at which point, no difference is seen in comparison to cultures maintained under conditions of CD28 ligation throughout. This suggests that CD28 engagement is stable for at least 12 hours and provides persistent enhancement of TCR signals. Abrogation of CD28 ligation at any time prior to 12 hours reduced responder frequency. Likewise Ctla-4 ligation inhibits T cell responses to TCR and CD28 coligation up to 12 hours after stimulation, supporting the idea that mechanisms of Ctla-4 inhibition of T cell activation are not relevant in the absence of CD28 costimulation. Moreover, the similar temporal constraints seen with CD28 and Ctla-4 signal integration as assessed by responder frequency support models of Ctla-4 function that emphasize shared signaling pathways with CD28.

Figure 4.4: Submitogenic TCR signaling results makes T cells increasingly refractory to costimulatory signals over time.

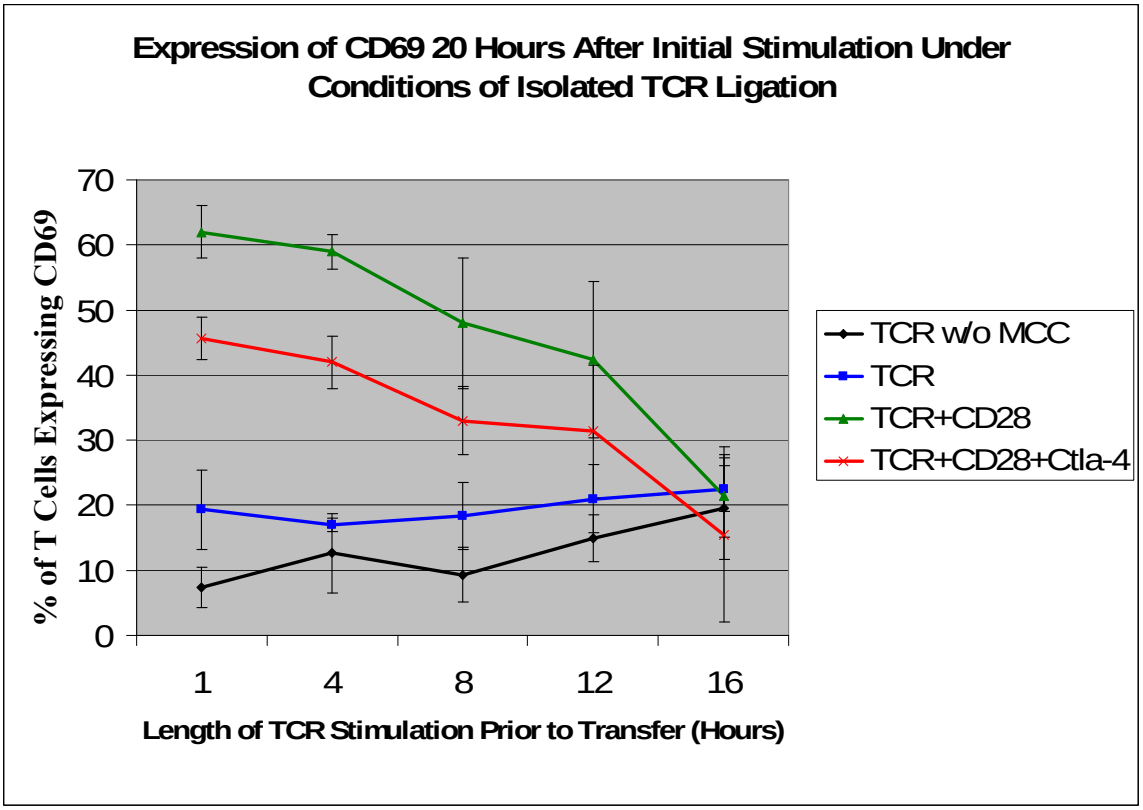


Figure 4.4: TCR monostimulation makes T cells increasingly refractory to costimulatory signals. Serial–coculture conditions under varying condition of TCR, CD28, and Ctla-4 ligation conditions define the temporal parameters of TCR monostimulation and CD28 and Ctla-4 costimulatory signal integreation as assessed by CD69 expression. $5x10^4$ 5C.C7 TCR-transgenic CD4+ T cells were co-cultured in 96-well round-bottom plates with $5x10^4$ mitomycin C-treated Chinese hamster ovary (CHO) antigen presenting cells (APCs) engineered to express I-E^k. APCs were prepulsed for five hours with $5x10^{-4}$ μM moth cytochrome C oligonucleotide (MCC^{88-103}) prior to start of coculture. T cells were removed from initial co-culture conditions at indicated times (X-axis) by gentle pipetting and co-cultured with CHO APCs prepulsed with $5x10^{-4}$ μM MCC under varying conditions of TCR monostimulation, CD28 costimulation, and CD28+Ctla-4 costimulation (blue, green, and red curves respectively). In addition, T cells were removed from initial culture conditions and co-cultured with CHO APCs expressing I-E^k in the absence of MCC peptide (black curve). T cells were generally greater than 93% pure after removal from initial culture conditions. T cells were analyzed for CD69 expression 20 hours after start of co-culuture in initial conditions. Cells stimulated by the TCR alone displayed maximal responder frequency by 1-2 hours and were not activated further by sustained TCR stimulation. In contrast, CD28 costimulation could fully restore maximal responder frequencies at any time up to 6-8 hours. In addition, Ctla-4 was capable of inhibiting CD28-mediated costimulation to a degree that paralleled the magnitude of CD28 enhancement of TCR activation. Costimulatory signals produced no

alteration in responder frequency beyond 16 hours. Results reflect average of three experiments performed in triplicate.

Figure 4.5: CD28 costimulatory signals are integrated over a period of 12 hours

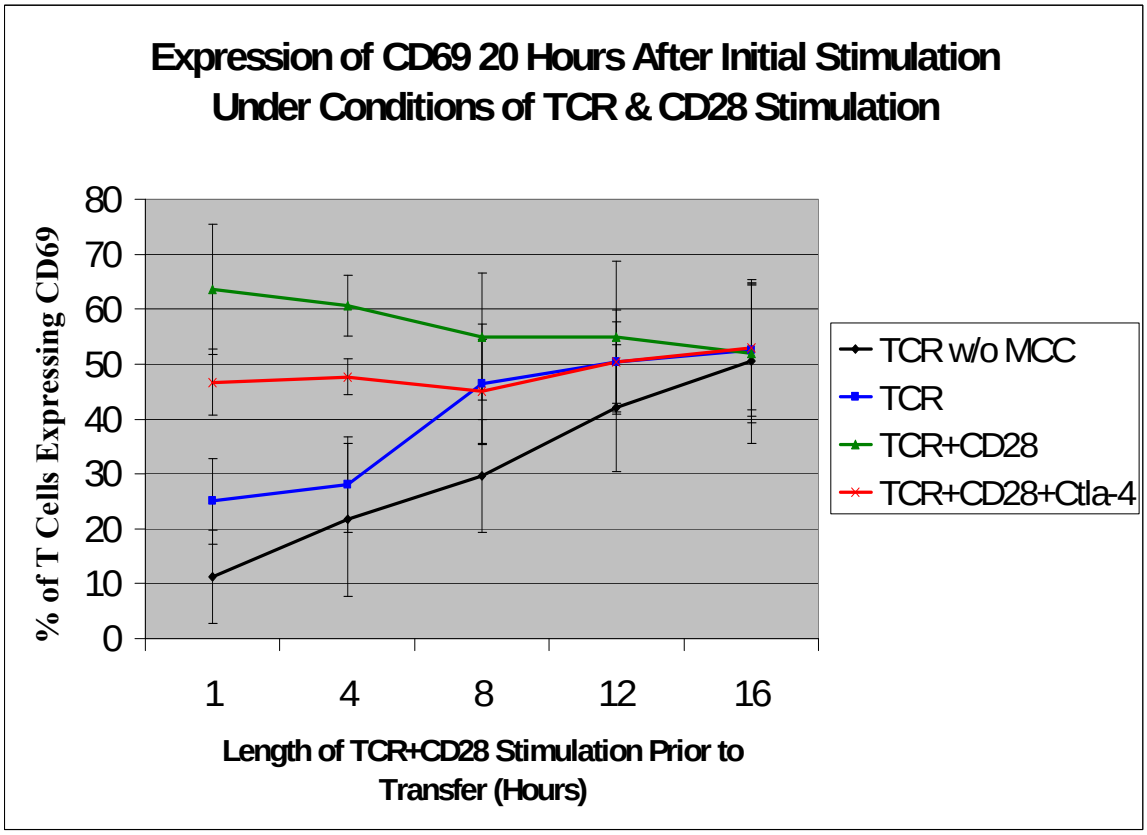


Figure 4.5: CD28-mediated signals are incorporated over a period 12 hours after initial T cell stimulation. Serial–coculture conditions under varying condition of TCR, CD28, and Ctla-4 ligation conditions define the temporal parameters of TCR monostimulation and CD28 and Ctla-4 costimulatory signal integreation as assessed by CD69 expression. $5x10^4$ 5C.C7 TCR-transgenic CD4+ T cells were co-cultured in 96-well round-bottom plates with $5x10^4$ mitomycin C-treated Chinese hamster ovary (CHO) antigen presenting cells (APCs) engineered to express I-E^k and a CD28 ligand. APCs were prepulsed for five hours with $5x10^{-4}$ μM moth cytochrome C oligonucleotide (MCC^{88-103}) prior to start of coculture. T cells were removed from initial co-culture conditions at indicated times (X-axis) by gentle pipetting and co-cultured with CHO APCs prepulsed with $5x10^{-4}$ μM MCC under varying conditions of TCR monostimulation, CD28 costimulation, and CD28+Ctla-4 costimulation (blue, green, and red curves respectively). In addition, T cells were removed from initial culture conditions and co-cultured with CHO APCs expressing I-E^k in the absence of MCC peptide (black curve). T cells were generally greater than 93% pure after removal from initial culture conditions. T cells were analyzed for CD69 expression 20 hours after start of co-culuture in initial conditions. Abrogation of CD28 ligation at any time prior to 12 hours reduces responder frequency. Similarly, Ctla-4 ligation is capable of inhibiting CD28-mediated over a similar time frame in support of models emphasizing shared signaling pathways downstream of CD28 and Ctla-4. Results reflect average of 3 experiments performed in triplicate.

Figure 4.6: Ctla-4 signals are integrated over a period of 8-12 hours

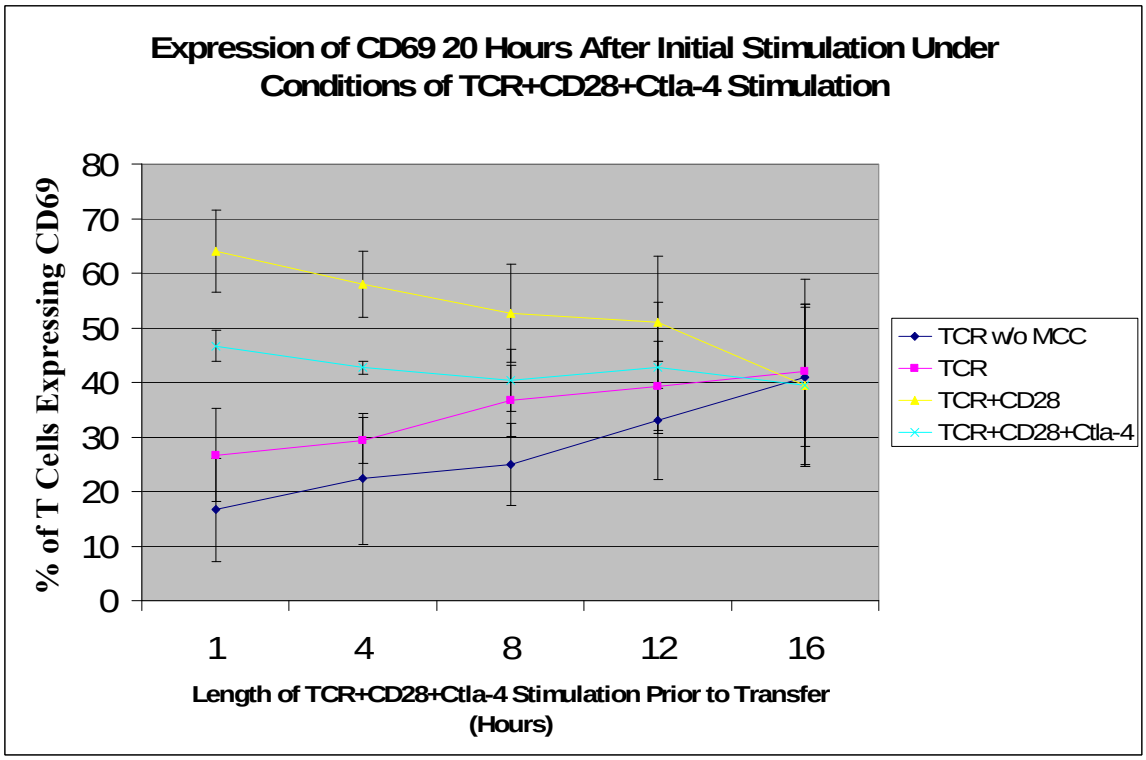


Figure 4.6: Ctla-4 signals are integrated over a period of 8-12 hours. Serial–coculture conditions under varying condition of TCR, CD28, and Ctla-4 ligation conditions define the temporal parameters of TCR monostimulation and CD28 and Ctla-4 costimulatory signal integreation as assessed by CD69 expression. $5x10^4$ 5C.C7 TCR-transgenic CD4+ T cells were co-cultured in 96-well round-bottom plates with $5x10^4$ mitomycin C-treated Chinese hamster ovary (CHO) antigen presenting cells (APCs) engineered to express I-E^k as well as ligands for CD28 and Ctla-4 APCs were prepulsed for five hours with $5x10^{-4}$ μM moth cytochrome C oligonucleotide (MCC^{88-103}) prior to start of coculture. T cells were removed from initial co-culture conditions at indicated times (X-axis) by gentle pipetting and co-cultured with CHO APCs prepulsed with $5x10^{-4}$ μM MCC under varying conditions of TCR monostimulation, CD28 costimulation, and CD28+Ctla-4 costimulation (blue, green, and red curves respectively). In addition, T cells were removed from initial culture conditions and co-cultured with CHO APCs expressing I-E^k in the absence of MCC peptide (black curve). T cells were generally greater than 93% pure after removal from initial culture conditions. T cells were analyzed for CD69 expression 20 hours after start of co-culuture in initial conditions. Abrogation of Ctla-4 ligation prior to 8 hours results in CD28-mediated increases in responder frequency. However, elimination of both CD28 and Ctla-4 signaling prior to 8 hours results in decreased responder frequency reflecting ongoing positive costimulation by CD28. Collectively, the data suggest that TCR-mediated and costimulation-mediated signals use distinct mechanism and signaling pathways. Results reflect average of three experiments performed in triplicate.

Ctla-4 inhibition of T cell activation can be reversed by attenuation of Ctla-4 ligation prior to 8-12 hours after initial TCR stimulation (Figure 4.6). In conjunction with previous results, this observation suggests that Ctla-4 alters the threshold of activation by mechanisms that primarily counteract CD28 mediated signals. However, there is a slight difference between the temporal parameters of signal integration seen with CD28 and Ctla-4. The critical period for ligation in the determination of activation threshold appears to be somewhat more constrained for Ctla-4 relative to CD28 suggesting that Ctla-4 may initiate unique signaling pathways that are independent of CD28 and can not be overridden by CD28 ligation at later times despite evidence supporting a continued role for CD28 signaling.

Discussion

The mechanisms underlying signal integration from antigen and costimulatory receptors is only partially understood. CD28 functions primarily to enhance signaling through the TCR(12, 50, 54). However, CD28 also mediates signaling that is independent of TCR triggering and results in qualitatively different immunological T cell responses(370). The immunological synapse may provide the necessary biophysical environment for prolonged signaling through receptors on the T cell surface, and may be essential for costimulatory signal integration and gain of effector function(371). In support of this model, our data suggests that TCR monostimulation leading to activation requires only transient (<1 hour) TCR engagement. In contrast, CD28 alters the threshold of activation by persistent signaling over several hours, indicating a

fundamentally different mechanism leading to cell activation is involved. Relative to TCR signaling, CD28 is unique in its ability to selectively stabilize mRNA transcripts(15, 17, 186, 372). T cell activation results in transcriptional upregulation of genes necessary for coordinated regulation of diverse processes involved in survival, cell growth, intercellular communication, and effector function. The ability of CD28 signaling to amplify expression of key regulatory molecules involved in these processes by coordinated transcriptional, post-transcriptional, and post translational regulation is, in some instances, distinct from TCR signaling pathways and may serve to explain why continued CD28 ligation is an important determinant of threshold of activation. Similarly, the ability of Ctla-4 and CD28 to influence responder frequency profiles over similar timeframes and with opposing results is suggestive of a shared mechanism of regulation that is operative over extended periods of time. Costimulatory signals are critical regulators of immunity and tolerance, with roles in T cell differentiation and effector function. The diversity of lymphocyte responses that can result from TCR triggering may require additional time, beyond that required for TCR ligation and, perhaps, commitment to activation, to sufficiently integrate the array of costimulatory signals that are often temporally and spatially distinct. Importantly, the consistency of our findings with current models of T cell activation and receptor signaling paradigms supports the use of engineered APCs as a physiologically relevant model system that recapitulates some published findings related to T cell activation, and may provide clarity in answering questions obscured by the use of supraphysiological stimuli and heterogeneous lymphocyte populations.

Our in vitro system provides stringent control of TCR signal strength and differentially signals through CD28 and Ctla-4. Moreover, the kinetics of activation indicates that cells identified by activation marker expression are highly synchronized for activation status and provide ideal samples for the characterization and isolation of phenotypically homogenous populations.

Chapter IV

Results

Kinetic Analysis of Global Expression Profiles in TCR-Transgenic CD4+ Lymphocytes During Primary Activation

Kinetic Analysis of Genomic Expression Patterns in Activated TCR-tg CD4+ Lymphocytes

TCR-tg lymphocytes and engineered APCs allow tight control of activation stimuli

Ctla-4 and CD28 utilize the same B7 family members, B7.1 and B7.2, as ligands, complicating the characterization of the two opposing costimulatory pathways. Using an established in vitro stimulation system, we optimized a protocol of primary stimulation in CD4+ cells that allowed tight control of TCR signal strength, synchronous activation of TCR-tg lymphocytes, and differential signaling through either CD28 or Ctla-4. Numerous experiments delineated the parameters of T cell activation using this system, and allowed us to specify a degree of TCR stimulation that allowed for moderate activation in the absence of costimulation, significant positive costimulation by CD28, and apparent inhibition by Ctla-4.

To facilitate the identification of genes potentially regulated by Ctla-4, it was crucial that the kinetics of T cell commitment to activation and integration of CD28- or Ctla-4-mediated costimulatory signals be well defined. Using expression of the very early activation marker CD69, upregulation of CD4, and the division marker CFSE, we established a normative profile of T cell activation with reproducible kinetics and responder frequency. Importantly, maximal responder frequency was routinely achieved by 12 hours, implying a high degree of synchronicity in the activated populations.

To determine the predictive value of CD69 expression in T cell activation, we stimulated ex vivo 5C.C7 lymphocytes with CHO APC prepulsed with $5x10^{-4}$ uM MCC, harvested the cells at 4, 8, and 12 hours, and sorted the cells by FACS for expression of CD69. Sorted cells were then cocultured with CHO APC expressing I-E^k without MCC and analyzed at 24 and 72 hours for expression of activation markers and division status. CD69 expression was highly predictive of commitment to activation and eventual cell division. It was noted that CD69+ cells isolated at earlier timepoints were more susceptible to cell death as indicated by Trypan blue staining and cell yield at subsequent timepoints. While it was clear that a small fraction of sorted CD69- lymphocytes were capable of becoming activated when put back into culture, there was no evidence that interruption of stimulation ever reversed the activation status of cells as evidenced by the absence of naïve cells at late timepoints in cultures sorted for positive CD69 expression. In addition, while it is possible that sparse cell recovery at 4-8 hours after TCR stimulation alone skewed the results of subsequent analyses, the trend towards longer times to commitment in the absence of CD28 costimulation are consistent with activation marker expression data indicating a more prolonged and gradual achievement of maximal responder frequency with TCR stimulation alone. These results again reflect a high degree of synchronicity in lumphocyte activation kinetics when using this system.

Having established the kinetics of activation in our system, the predictive and sequential nature of activation marker expression, and the temporal parameters of costimulatory signal integration, we scaled our activation assay up to allow purification of homogenous populations by FACS as indicated by CD69 expression, CD4expression,

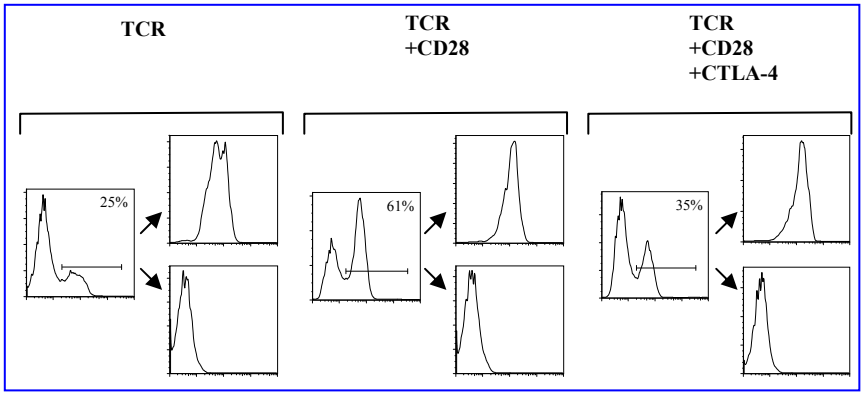
and the cell-division marker, CFSE. Figure 4.8 shows the sort protocols for samples isolated prior to 32 hours, 32-48 hours, and 60 hours and beyond. As shown previously, the populations identified are expected to be highly synchronized and homogenous for activation history, activation status, and commitment to division.

As an initial screen for the utility of this protocol, we sorted a large quantity of cells and performed the standard single step amplification and labeling procedure available at the time, using 5-8ug of starting total RNA. Microarry analysis using Affymetrix MgU74aV2 Genechips showed robust changes in global expression profiles. RT-PCR verification of select gene expression levels verified the results seen on microarray (Data not shown). Because the amount of RNA isolated was limiting for most experiments, a single sample was amplified in both a single- and double-step amplification procedure, and the results compared. Figure 4.9 summarizes the results seen with both labeling protocols, and suggests that despite some flattening of signal intensity over the entire expression profile relative to single-step amplification, the limits of detection and linearity of amplification were not sufficiently altered with double amplification to significantly alter the profile seen. As a result, all subsequent experiments were performed using the double amplification protocol.

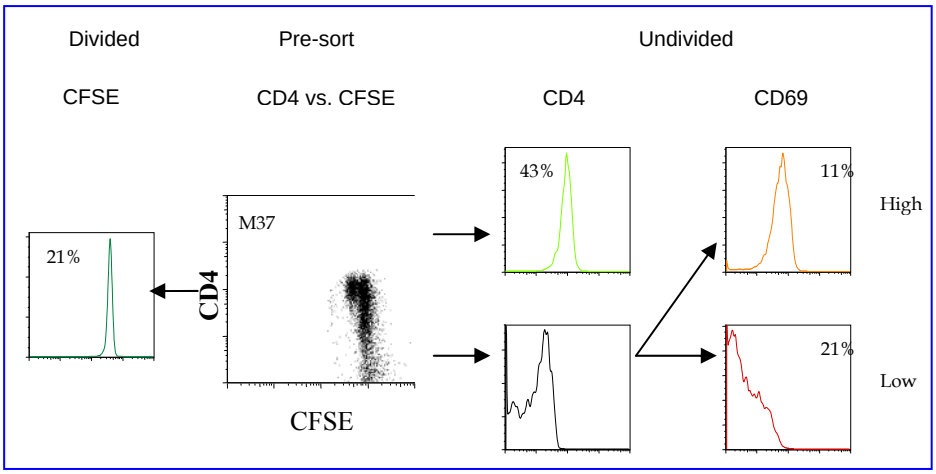
Samples were isolated at 4 hour intervals up to 24 hours based on CD69 expression. At 36 hours, 4 population were isolated per stimulation condition based on CD4 expression and CFSE staining, allowing additional characterization of the changes that occur during cell-cycle entry. At 60 hours, populations were sorted based on division status. It is noteworthy that despite a high degree of consistency in the responder

Figure 4.8: Sort protocols for in vitro-stimulated T cells at varying times after activation

A:



B:



C:

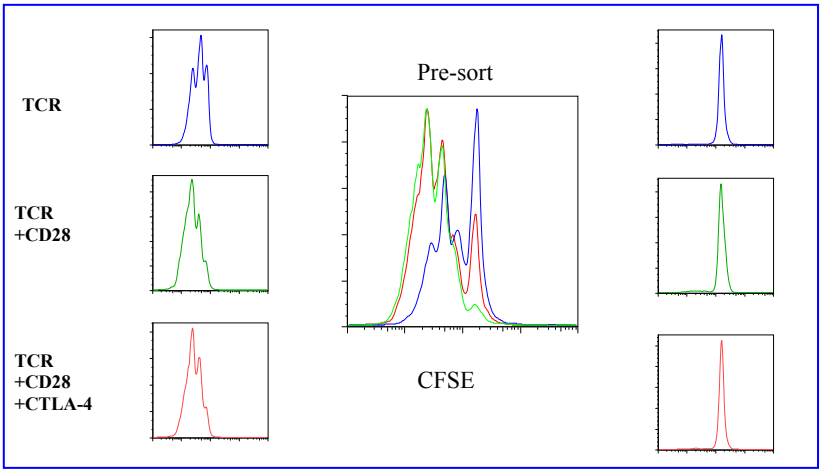


Figure 4.8: Sorting protocols used to isolate CD4+ populations homogenous for activation status. $5x10^4$ 5C.C7 TCR-transgenic CD4+ T cells were co-cultured in 96-well round-bottom plates with $5x10^4$ mitomycin C-treated Chinese hamster ovary (CHO) antigen presenting cells (APCs) engineered to express surface ligands for TCR, CD28 and Ctla-4 in varying combination that were prepulsed for five hours with $5x10^{-4}$ μM moth cytochrome C oligonucleotide (MCC^{88-103}). 5C.C7 lymphocyte activation, as measured by responder frequencey (CD69 upregulation) correlates with antigen prepulse concentration and is enhanced or inhibited by CD28 and Ctla-4 ligands respectively. A) At timepoints up to 24 hours CD4+ T cells co-cultured with CHO APCs under varying conditions of TCR, CD28 a, and Ctla-4 ligation were sorted for activation status based solely on expression of the early activation marker CD69. Representative sort protocol shown. Responder frequency (percent of CD69+ lymphocytes) is indicated in the pre-sort histogram. B) At timpoints from 32-48 hours, CD4+ T cells were sorted into 4 populations based on the sequential upregulation of CD69 and CD4 and subsequent division. Division status was determined by staining the cells with the cytoplasmic dye CFSE prior to stimulation. Populations isolated were CD69-, CD69+$CD4^{Low}$, $CD4^{High}$-undivided, and divided. C) At timepoints beyond 48 hours, CD4+ T cells were isolated based solely on their division status as determined by CFSE intensity, and sorted into undivided and divided populations.

frequency seen with TCR and TCR+CD28 stimulation, TCR+CD28+Ctla-4 ligating condition produced a more variable activation response. Because the greatest influence of the Ctla-4 binding M14 molecule is seen in the context of simultaneous CD28 stimulation, the degree of Ctla-4 mediated inhibition was defined as ($M37_{RF}$-$M14/37_{RF}$)/($M37_{RF}$-V_{RF}), where "RF" indicates the responder frequency indcated by CD69 expression seen with the corresponding CHO APC. Or more generally, "Ctla-4-mediated inhibition" was defined as the percentage of CD28-mediated increase in responder frequency apparently reversed by Ctla-4 ligation. To increase the likelihood of detecting consistent changes in gene expression subsequent to Ctla-4 ligation, lymphocytes stimulated in the presence of TCR, CD28 and Ctla-4 ligands were selected to maintain a roughly consistent 50% inhibition by Ctla-4 for samples analyzed by microarray. A summary of in vitro 5C.C7 CD4+ T cell samples isolated by FACS for the production of RNA and subsequent microarray analysis is documented in Table 4.1

TCR-Mediated T cell Activation Results in a Dynamic Regulation of Several Thousand Genes

TCR-signaling in the absence of positive costimulation has been reported to result in anergy, abortive activation, cellular death via apoptosis induction, and tolerance induction. As shown previously, our in vitro stimulation system utilizing engineered APCs presenting MCC to 5C.C7 TCR-tg lymphocytes is capable of initiating T cell activation by TCR monostimulation, or T cell signaling with positive and negative

Figure 4.9: Comparison of amplification protocols

A:

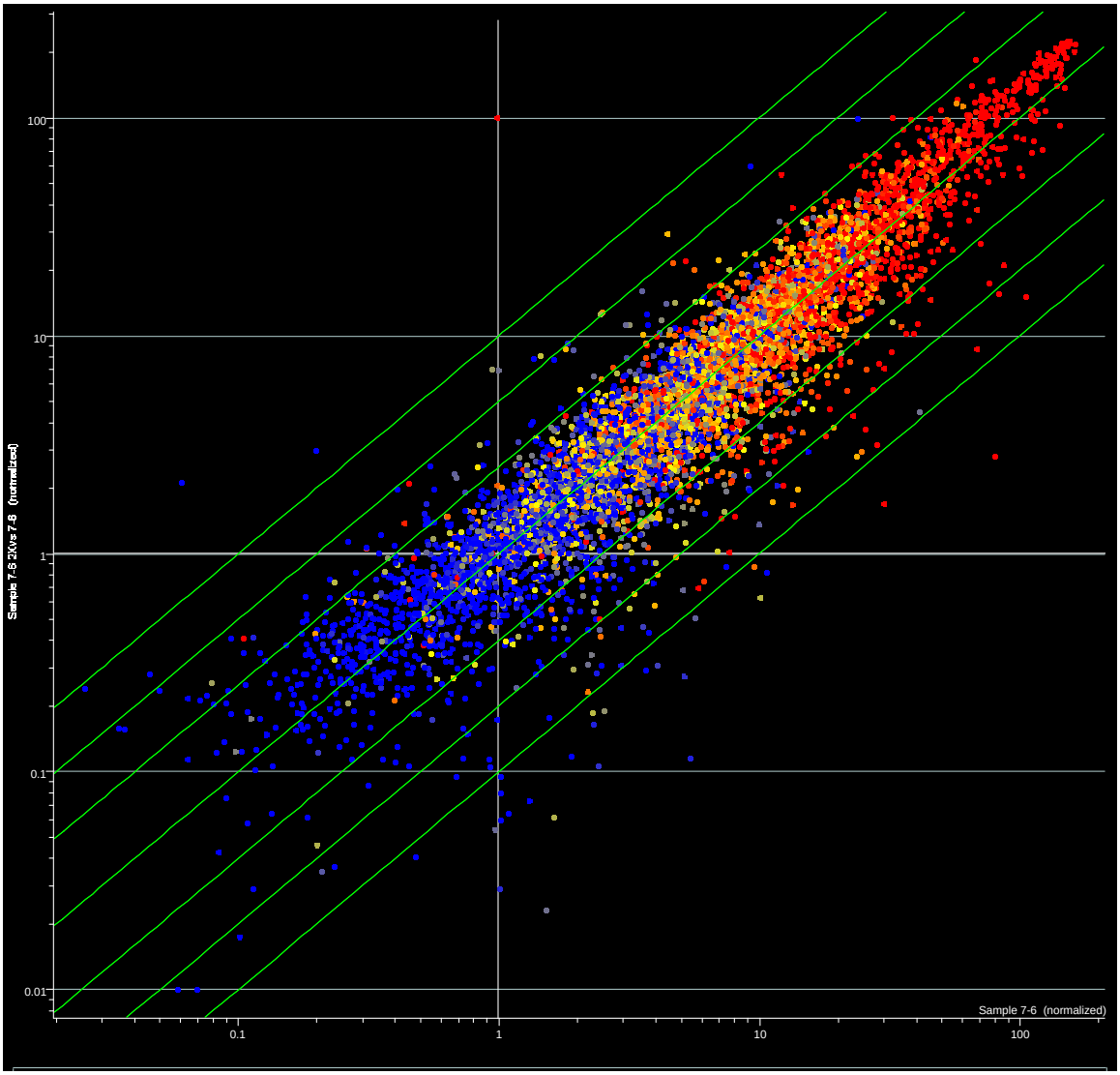


Figure 4.9: Expression values obtained from alternate amplification protocols that utilized the same starting RNA sample were analyzed. 5C.C7 $Rag^{-/-}$ T cells were activated in vitro by co-culture with peptide pulsed APCs for 16 hours in the presence of ligands for TCR, CD28, and Ctla-4. Non-activated CD4+CD69- lymphocytes were isolated by FACS and used to prepare total RNA. RNA was labeled for microarray analysis by both single- and two-step amplification and labeling protocols, and products of both protocols analyzed on Affymetrix MgU74Av2 oligonucleotide microarrays. One chp per condition was analyzed. Microarray scanning and qualitative determination of expression ("present" or "absent") based on signal to noise ratios was performed using Affymetrix MAS5.0 software. Further analysis was performed using Genespring analysis software. Data was transformed and normalized by setting signal intensity values below 0.01 were to 0.01 and dividing each measurement by the 50.0th percentile of all measurements in that sample. The cross-gene error model was applied to provide statistical measures of significance in the absence of replicates. 6480 transcripts, representing 52% of the total probeset array utilized, were detectable in at least one of the two conditions (amplification protocols) examined. 5653 genes out of 6480, representing 88% of the identified transcriptome were flagged as present on both samples. This indicates that only 0.6% of all probesets were differentially flagged on the microarrays. Of the 5653 genes present in both samples 550, 147, and 39 genes indicated fold differences of 2, 3, or 5 respectively, corresponding to 10, 3, and 0 percent of genes expressed.

costimulation. T cell responses to TCR monostimulation in our system, while diminished with respect to responder frequency and displaying slightly delayed kinetics, still results in full commitment to activation, cell division, and did not result in increased cell death in culture. In vitro blockade of potential T-T costimulation via anti-B7 antibody blockade did not alter responder frequency or entry into the cell cycle during primary stimulation as assessed by CD69 expression, CD4 upregulation, and CFSE staining. (Data not shown) Moreover, naïve lymphocytes activated by TCR monostimulation, TCR ligation plus CD28 costimulation, or the combined ligation of the TCR, CD28 and Ctla-4 did not display dramatically different responses upon restimulation, and differences observed upon restimulation appeared to correlate with the overall strength of activation signals in the primary activation as assessed by responder frequency, and not the specific combination of TCR and costimulatory signals received. (Dat not shown) Together, these data indicate that TCR ligation alone is capable of initiating a robust T cell response when sufficiently strong, and this observation correlates with the global expression profile assayed by microarray.

2129 genes were identified as present in a minimum of 4 of the samples receiving monostimulation alone and as having undergone a 3-fold change in expression in at least 2 samples relative to ex vivo naïve cells. Figure 4.10 shows a box plot representation of these genes in TCR-monostimulated samples segregated according to time and activation status. Perhaps surprisingly, unactivated populations evidenced numerous significant gene regulations in culture despite receiving sub-mitogenic signals. Although this could be attributed to bystander effects in the presence of activated cells, attrition of normal

Table 4.1: Summary of in vitro stimulated 5C.C7 CD4+ lymphocyte sample analyzed by microarray

Hours Stimulated	Stimulation Conditions	Activation Phenotypes Isolated per Stimulation Condition	# of Replicates	Total Number of Samples per timepoint	Average Inhibition in Presence of Ctla-4 Ligands
0	NA (Ex Vivo)	CD69-CD25-CD44^{low}	2	2	NA
4	TCR TCR+CD28 TCR+CD28+Ctla-4	CD69- CD69+	2	12	
8	TCR TCR+CD28 TCR+CD28+Ctla-4	CD69- CD69+	2	11 (Missing one TCR stimulated CD69-)	44%
12	TCR TCR+CD28 TCR+CD28+Ctla-4	CD69- CD69+	2	11 (Missing one TCR stimulated CD69-)	47%
16	TCR TCR+CD28 TCR+CD28+Ctla-4	CD69- CD69+	2	12	40%
20	TCR TCR+CD28 TCR+CD28+Ctla-4	CD69- CD69+	2	10 (Missing one CD69+ sample from TCR+CD29 and TCR+CD28+CTLA-4)	76%
24	TCR TCR+CD28 TCR+CD28+Ctla-4	CD69- CD69+	1	6	53%

32	TCR TCR+CD28 TCR+CD28+Ctla-4	CD69- CD69+CD4^{Low} CD69+CD4^{High}Undivided Divided	1	11	~50% prior to start of division
60	TCR TCR+CD28 TCR+CD28+Ctla-4	Undivided Divided	1	6	~55% prior to start of division

Table 4.1: Summary of in vitro stimulated 5C.C7 CD4+ lymphocyte sample analyzed by microarray. 5C.C7 $Rag^{-/-}$ CD4+ T cells were stimulated in vitro by APCs engineered to differentially express ligands for the TCR, CD28, and Ctla-4. APCs were prepulsed with $5x10^{-4}$ MCC for 5 hours prior to coculture with T cells. At the indicated times, T cells were harvested, sorted for the indicated activation and division markers, and their transcriptome analyzed by microarray subsequent to RNA isolation, amplification, and labeling. All isolated populations were greater than 93% pure by flow cytometry analysis after FACS purification. Two ex vivo CD69-CD25-$CD44^{Low}$ samples were isolated to provide baseline gene expression measurements. From 4-60 hours, samples were isolated at 8 timepoints under all three conditions of TCR, TCR+CD28, and TCR+CD28+Ctla-4 ligand stimulation. From 4-24 hours, naïve and activated populations were purified based on CD69 expression. At 32 hours, 4 populations were isolated including CD69-, CD69+$CD4^{Low}$, CD69+$CD4^{High}$, and divided. At 60 hours, cells were isolated into undivided and divided populations. Biological replicates were produced for the majority of sample conditions isolated prior to 24 hours. From 24-60 hours, only one sample per condition was processed for microarray analysis. TCR monostimulation induced responder frequencies (assessed by CD69 expression) of 11-22%. CD28 costimulation increased responder frequencies up to ~45-75%. T cells stimulated in the presence of Ctla-4 ligands displayed varying responder frequencies between those seen with TCR monostimulation and CD28 costimulation. Samples processed for microarray analysis were chosen to maintain a roughly 50% inhibition of CD28-mediated costimulation as

determined by responder frequencies. In total, 78 stimulated samples and two ex vivo samples were analyzed by microarray.

signals received in a more physiological environment, or unanticipated effects of the specific culture conditions irrespective of activating stimuli, it is also a potential result of penetrant but sub-mitogenic signaling. This is an important possibility as sub-mitogenic signaling has been reported to confer a lasting phenotype on naïve cells. These possibilities will be more fully explored in the comparison of expression profiles between stimulation conditions. As expected, activated conditions displayed a greater amplitude and diversity of gene regulation across all timepoints relative to unactivated populations. Interestingly, while activated populations showed a mean increase in gene expression at all other timepoints, samples isolated 8 hours after activation displayed a large generalized downregulation of numerous genes, suggestive of active gene suppression. This phenomenon was unlikely to be due to spurious effects of microarray sample processing as it was unique to the activated populations and consistent across duplicates. The nearly 1200 genes downregulated at this time were characterized by an oscillatory pattern of regulation occurring over the initial 20 hours of activation. Genes involved in metabolic processes were highly overrepresented in this group. In contrast to the general downregulation observed at this timepoint, cell surface molecules and signaling intermediates were predominantly upregulated to a great degree. The majority of known transcription factors were upregulated at 8 hours, but a significant minority was transiently downregulated. A quick overview of genes identified as key regulators or participants in T cell activation indicated that most were highly regulated over numerous timepoints. As a result, genes were further segregated into those showing a >10-fold change in expression over a minimum of 3 samples, and those showing a 5- to 10-fold

change over at least 3 samples. A combined condition tree and gene tree of a combination of these groups indicated a linear divergence of the expression profile of unactivated populations from ex vivo samples over time. Among activated samples, the 12 hour and 20 hour sample segregated in a non-linear fashion with respect to time, being found most similar to each other as a result of distinct upregulation of a cluster of 120 genes. When a similar analysis is performed on the 122 most regulated genes, known to include numerous key mediators of T cell activation, the linearity of progression is improved and only the 16 hour sample mis-segregates, being identified most closely with the 24 hour sample. (Figure 4.11)

Visual editing of genes identified as significantly regulated (>5-fold change in three timepoints in either activated or unactivated populations) yielded 418 genes. Hierarchical clustering of these genes in the activated samples provides a clear indication of the grouping of coordinately regulated genes over time, and K means clustering identified the 19 most significantly related groups of coordinately regulated genes in TCR activated samples over 60 hours of activation (Figure 4.12).

A search for putative regulatory sequences within the 19 gene clusters identified by K-means clustering indicated a heterogeneous pattern of overly represented sequences in the 500bp upstream sequences of included genes. Overall, 168 sequences 5-10bp in length were over-represented ($p<0.05$) in the upstream sequences of the 360 most highly regulated genes with known sequence relative to the rest of identified genes in the genome. Moreover, principal component 1 of the K-means clustering includes only 36 genes, but statistical analysis identified 304 over-represented putative regulatory

Figure 4.10: Box-plot representation of gene regulation in TCR-monostimulated populations

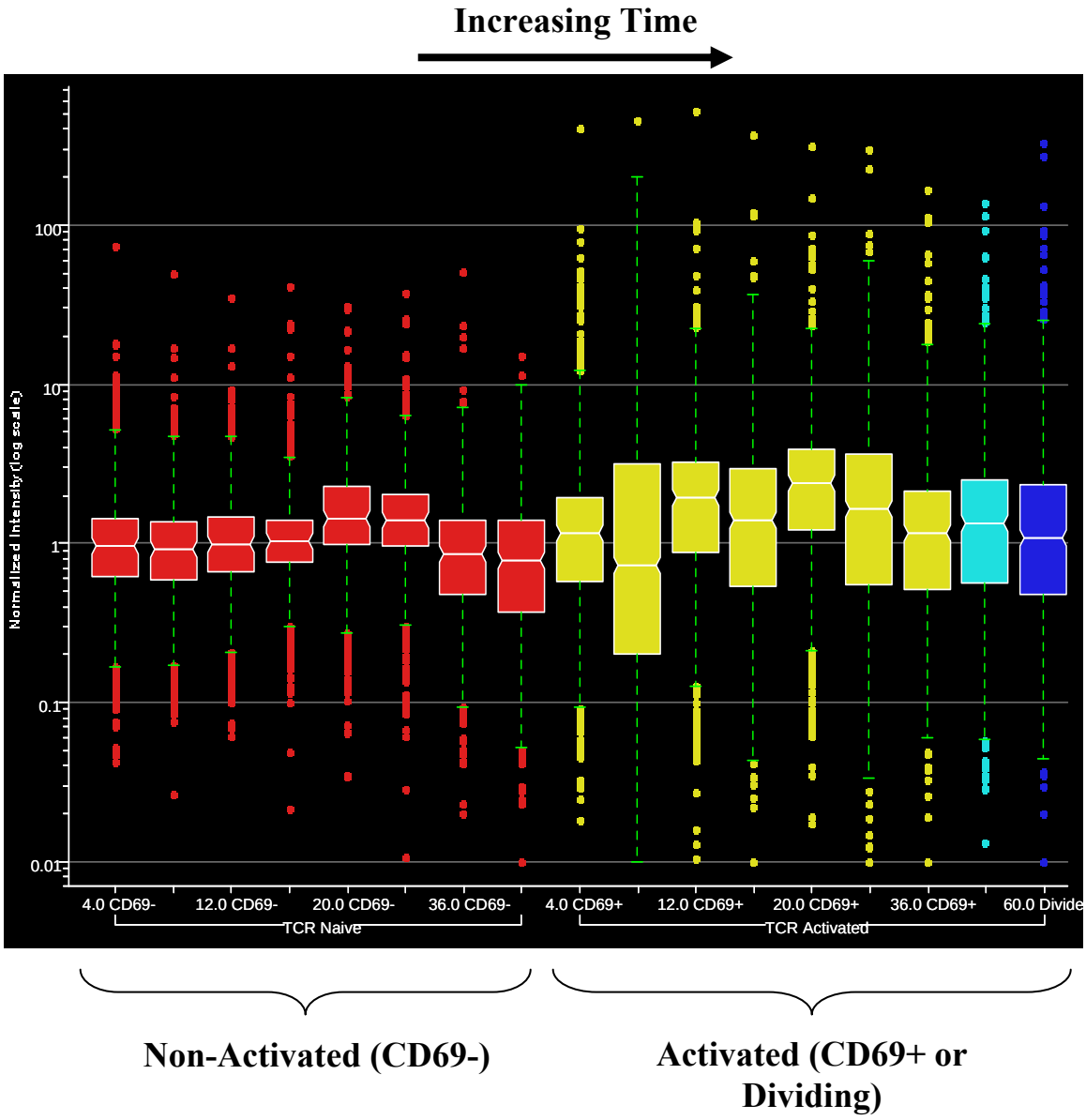


Figure 4.10: Box plot diagram showing magnitude of regulation in genes expressed in TCR monostimulated cells that remain naïve or become activated. 5C.C7 TCR-tg lymphocytes were stimulated in vitro by CHO APCs expressing I-E^k and prepulsed with $5x10^{-4}$ μM MCC. Lymphocytes were purified based on expression of activation markers (CD69, upregulation of CD4) and division status at numerous intervals from 4 to 60 hours. Total RNA isolated from the purified populations was labeled in a two-step amplification and labeling protocol, and subsequently analyzed on Affymetrix MgU74Av2 microarrays using the Affymterix MAS5.0 sorftware. Duplicate biological samples were obtained for all timepoints prior to 24 hours. Single samples were analyzed at 24, 36, and 60 hours. 6041 genes had detectable expression in at least 4 of the 23 samples included in this analysis. Further analysis utilizing Genespring analysis software was performed. Unactivated samples are color-coded red, activated samples are green. Median gene expression is indicated by indents, boxed regions indicate quartile gene distributions, and feathered extensions equal 1.5 times the interquartile distance. Data was transformed and normalized by setting signal intensity values below 0.01 were to 0.01 and dividing each measurement by the 50.0th percentile of all measurements in that sample. Measurements for each gene in each sample were divided by the average value of that gene's measurement in two samples of untreated and purified 5C.C7 $Rag^{-/-}$ CD4+ lymphocytes isolated ex vivo. Thus, expression values (Y-axis) are equivalent to fold-change values relative to untreated ex vivo samples. Box plots indicate the magnitude and scope of gene regulation occurring in naïve and activated populations in vitro from 4-60 hours in culture. Indents indicate the median gene expression value for genes detected

in at least 4 of 23 samples relative to its ex vivo expression value. The length of the upper and lower segments of the boxes reflect interquartile distances among all genes analyzed. The dotted line segments are arbitrarily set to 1.5 times the interquartile distance. Outlying highly regulated genes are indicated by individual dots. Thus, 5C.C7 CD4+ lymphocytes activated by TCR monostimulation display broad gene regulations. More than one-third of all detectable genes display at least 3-fold changes in expression in two of 17 conditions (involving time after stimulation and activation status). 504 genes display 5-fold or greater changes in expression in at least 3 of the 17 conditions. At eight hours, the general trend is towards downregulation of gene expression in activated cells. At all other timepoints assayed, gene upregulation predominates. The greatest global change in expression occurs ~20 hours after stimulation.

sequences ($p<0.05$), with 264 sequences significant at $p<9e-5$. Longer upstream sequences tended to be the most statistically significant with 40 of the 50 most significant sequences (all $p<9.1e-27$) being 10bp in length. In contrast, only 3 sequences were identified in component 10 (15 genes) and only 36 in component 14 (31 genes). No overrepresented sequences were found in any of the other components (4-33 genes). The search for putative regulatory sequences is independent of the clustering analysis performed. Thus, the uniquely large number of over-represented sequences identified in the upstream region suggestive of shared mechanisms of regulation may reflect a common role for these genes in T cell activation. The analysis is made more robust however by the inclusion of costimulated samples, and will be reconsidered in that context.

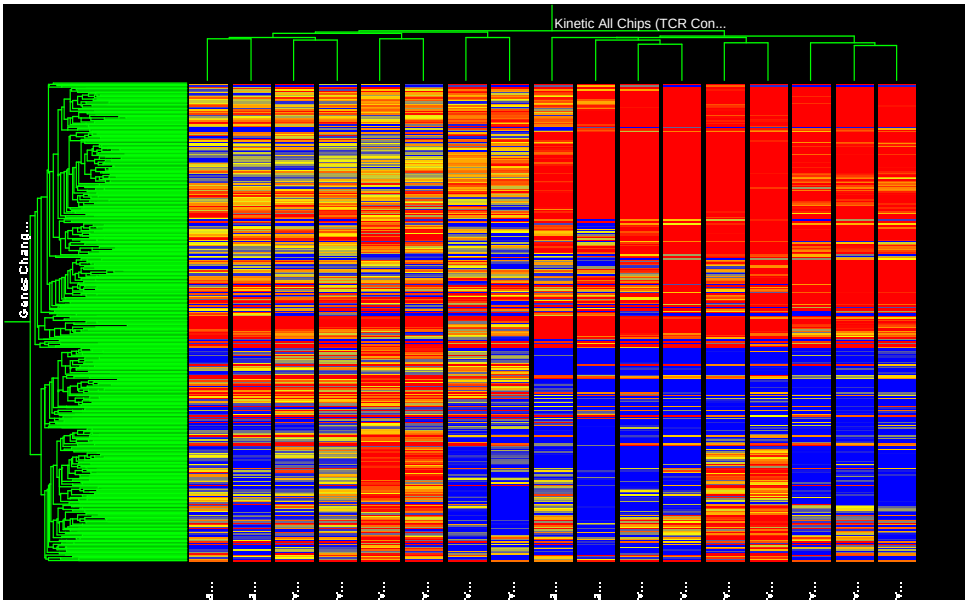
TCR-Mediated Stimulation Results in Robust T Lymphocyte Activation

Global expression profiling of naïve CD4+ TCR-activated TCR-tg lymphocytes in vitro reveals a coordinated pattern of global gene regulation involving several thousand genes (Figure 4.13). TCR monostimulation has previously been reported to result in abortive activation, anergy, or cell death. While a severe reduction in responder frequency was observed following TCR monostimulation relative to CD28 costimulated cells, no defect in subsequent responsiveness or increased cell death was observed in our system. These results are suggestive of a primarily quantitative effect of costimulation in setting the threshold for activation.

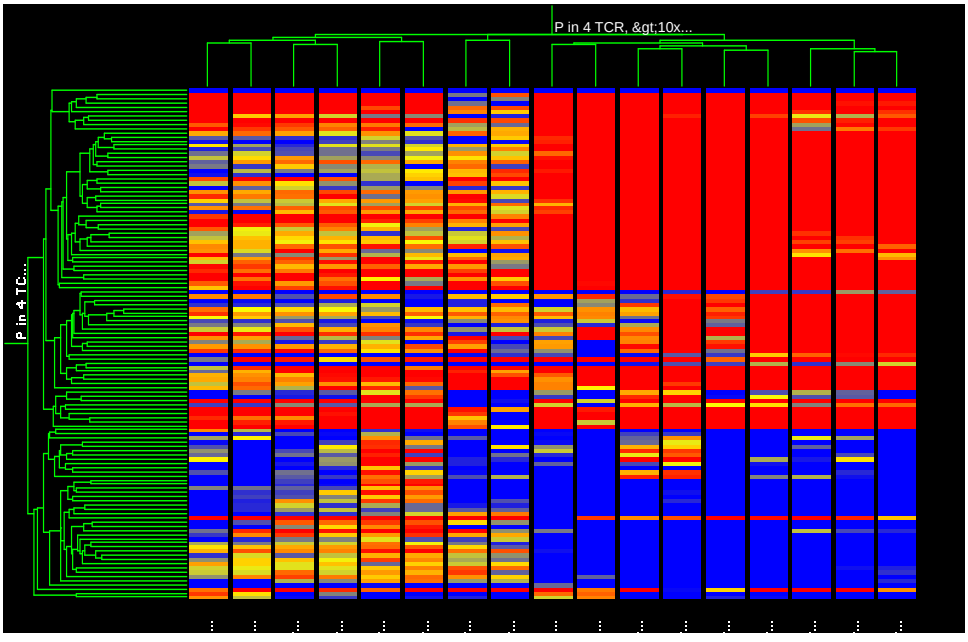
Figure 4.11: Combination gene and condition tree of TCR monostimulated samples

Increasing Time

A:



B:



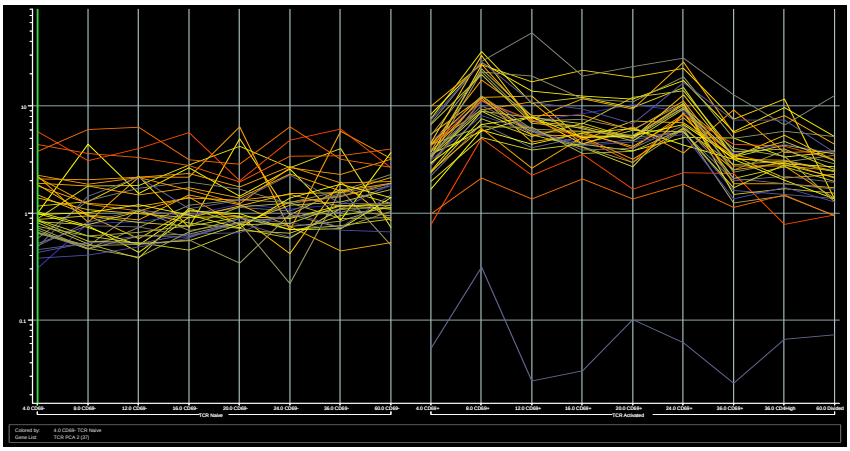
Non-Activated (CD69-)

Activated (CD69+ or Dividing)

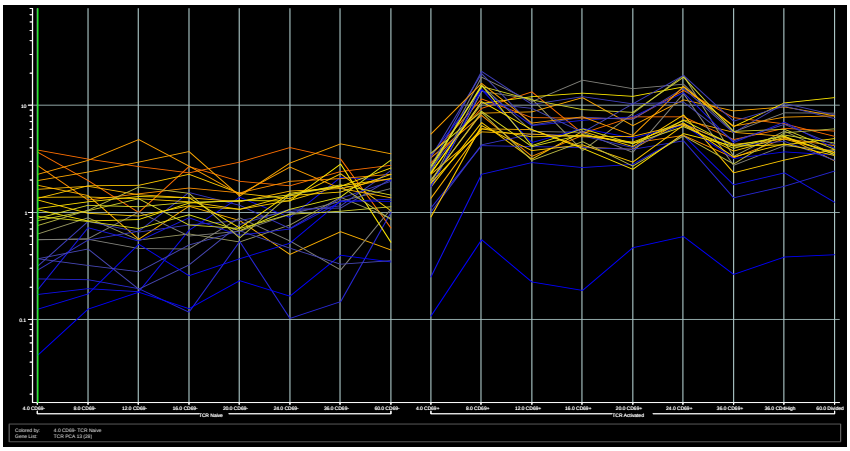
Figure 4.11: A combination gene and condition tree reveals complete segregation of activated and unactivated population stimulated by TCR ligation alone across all timepoints. Hierarchical clustering was performed on all naïve and activated samples isolated from 5C.C7 CD4+ T cell activated in vitro by engineered APCs expressing I-E^k for 4-60 hours. Global gene expression data was normalized to naïve, unstimulated CD4+ peripheral 5C.C7 ex vivo samples based on average values in replicates when available. 504 genes consistently up- or down-regulated more than five-fold in activated populations for three of the eight timepoints examined were used to generate the cluster analysis and condition tree in Panel A. The majority of genes reported to regulate lymphocyte activation, cell-cycle entry, and apoptosis are included in this analysis. Statistically, the global expression patters reveal almost perfect linear segregation of activated population and identifies coordinately regulated genes. Restricting the clustering analysis to genes displaying a minimum ten-fold change in expression in 3 of the eight timepoints analyzed from 4-60 hours improves the linearity of segregation according to (Panel B).

Figure 4.12: Prinicpal components analysis identifies coordinately regulated genes in TCR monostimulated populations

A:



B:



C:

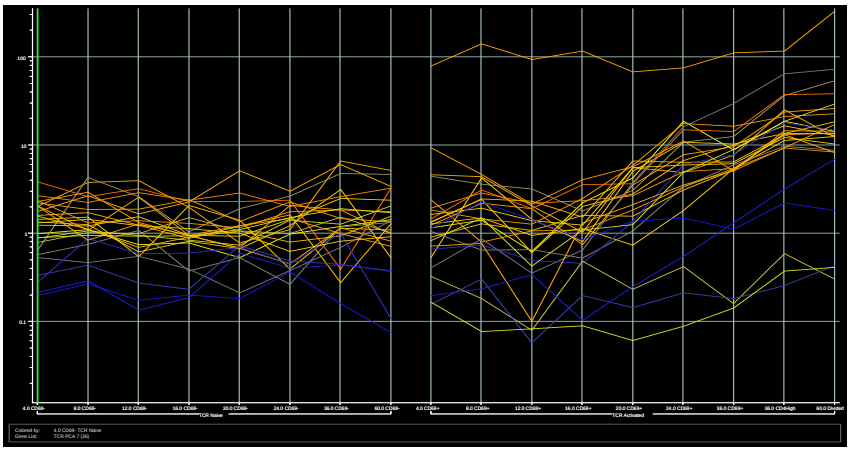


Figure 4.12: A K-means clustering analysis identified the 19 most determinative expression profiles contributing to the overall patterns identified across all relevant samples stimulated by TCR ligation alone. The analysis was performed using normalized values for all genes displaying a minimum 5-fold change in expression in at least 3 timepoints when normalized to naïve unstimulated 5C.C7 CD4+ lymphocytes isolated ex vivo. Parameters used in the analysis: 19 clusters, maximum 100 iterations, Pearson correlation similarity measure. The clusters converged after 22 iterations and 5 randomly chosen cluster failed to improve on the initial clustering results. The 19 clusters contained from 8-44 genes each. Panels A, B and C display the 3 dominant expression patters determinig the global expression profile of TCR stimulated 5C.C7 CD4+ T cells. Panel A contains the dominant component in TCR activated lymphocytes. Searches for putative regulatory sequences identified numerous overrepresented elements in the 1kb upstream region of genes in 3 of the 19 clusters identified, validating their classification as coordinately regulated genes (Panels A, B, and C). Genes comprising these clusters were analyzed further to identify signaling pathways involved in their regulation using Ingenuity Pathways Analysis software.

Costimulatory Signal Integration

CD28 alters the threshold of activation and serves to potentiate TCR-mediated signals. By itself, CD28 ligation is reported to transiently alter the expression level of several genes, primarily by mechanisms that serve to stabilize mRNA transcripts transcribed at a basal level(15). In the presence of simultaneous TCR ligation, CD28 has been shown to dramatically enhance the TCR-mediated alterations in gene expression, and is reportedly necessary for the expression of a subset of genes not reliably regulated by TCR monostimulation. In contrast, Ctla-4 inhibits signals transmitted through the TCR and CD28 and reduces T cell responsiveness through mechanisms that reportedly alter the magnitude of transcriptional response but result in no qualitative changes. Using our in vitro stimulation system, CD4+ TCR-tg lymphocytes were provided with either TCR monostimulation alone or in conjunction with CD28 ligation and/or Ctla-4 ligation. The CD28 and Ctla-4 ligands expressed on engineered APCs consisted of the scFV region of the relevant antibody linked to the B7 transmembrane region, were stably expressed, and at our chosen concentration of antigenic peptide resulted in a 3-5 fold increase in responder frequency by CD28 costimulation and a 50% Ctla-4-ligation-mediated inhibition of CD28-mediated costimulation as assessed by responder frequency. Consistent with its known role, CD28 ligation served to enhance the magnitude of CD4+ lymphocyte responsiveness in a manner dependent on the strength of TCR signaling, with relatively greater effects on responder frequency evident at lower doses of antigenic peptide.

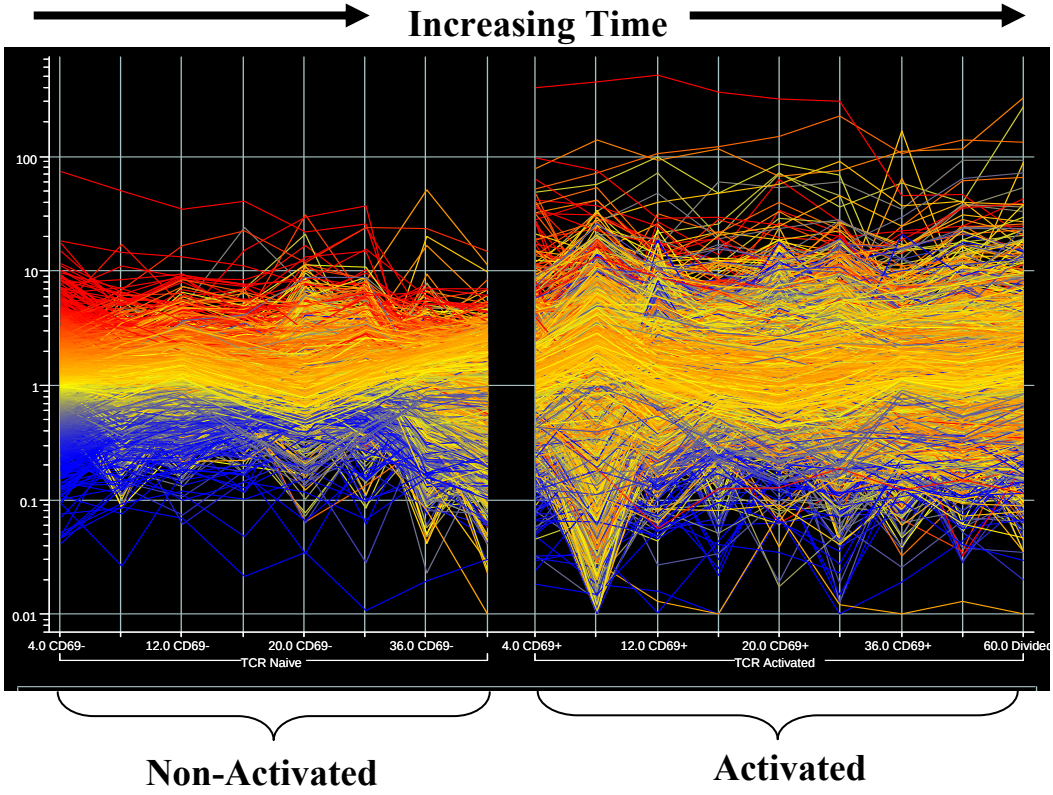
CD28 ligation initiates a signaling cascade that is, relatively speaking, well established and defined. However, the numerous points of interaction between signaling pathways makes it likely that downstream effectors of CD28 function are not all known, and the distal mediators of CD28 costimulation remain to be defined. Ctla-4 has not been reported to regulate a unique subset of genes independent from those involved in TCR and CD28 signaling. Using global expression profiling, we attempted a characterization of the transcriptome subsequent to varying condition of TCR,CD28 and Ctla-4 ligation. Using expression profiles of TCR-monostimulated cultures over several days as our baseline, we defined the magnitude and scope of changes in gene expression that result from CD28 costimulation. We then characterized changes in global expression patterns that result from simultaneous Ctla-4 ligation concomitant with CD28. Keeping in mind the results of our in vitro stimulation assays defining the temporal limitations of costimulatory signal integration, we sought to identify genes specifically regulated by CD28 and Ctla-4 ligation that can account for lasting phenotypic changes reported in the literature, and attempted to characterize subsequent alterations in transcriptome profiles that may be causally related.

Serial timepoint analysis of the relative magnitude and similarity of gene regulation subsequent to TCR and costimulatory signal integration

The following pages comprising Figure 4.14 detail several facets of signal integration as they pertain to global expression patterns that result in the first twenty hours of T cell activation. For each timepoint from 4-20 hours, screens were used to

Figure 4.13: TCR monostimulation results in robust gene regulation

A:



B:

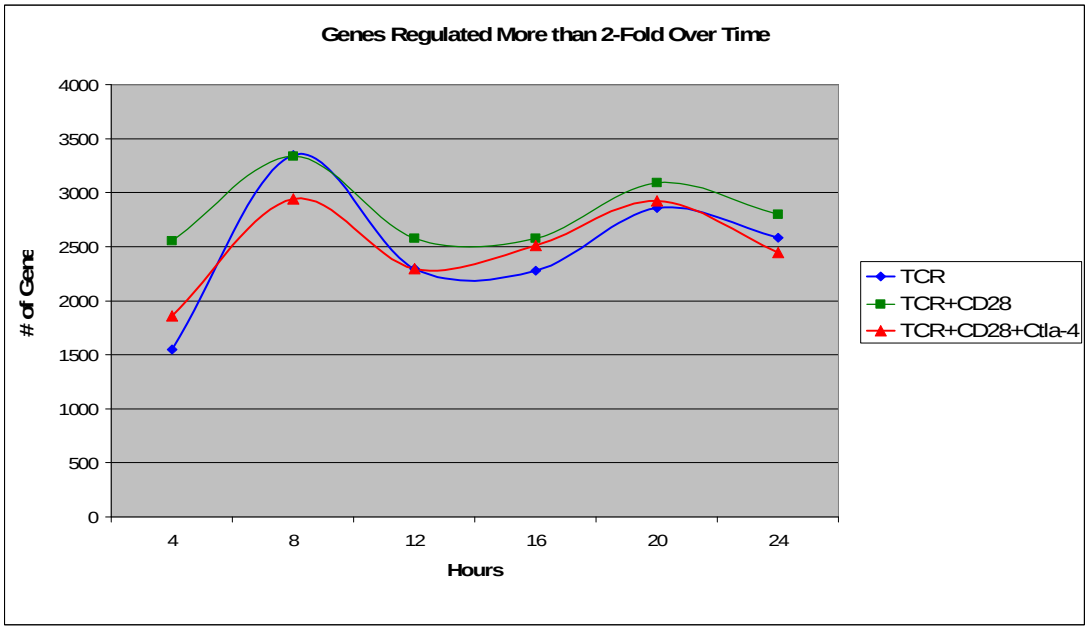


Figure 4.13: TCR stimulation results in robust gene regulation in activated cells in the absence of costimulation. 5C.C7 CD4+ lymphocytes were stimulated in vitro with engineered APCs prepulsed with MCC and expressing I-E^k. At discrete intervals from 4-60 hours after initial stimulation, CD4+ lymphocytes were sorted for activation status based on expression of CD69, upregulation of CD4, and division status as described. Microarray analysis of RNA from populations purified for activation status was performed and the data normalized to naïve, unstimulated, peripheral CD4+ lymphocytes from 5C.C7 $Rag^{-/-}$ mice. Biological replicates were obtained for all samples isolated prior to 24 hours. Single samples were isolated at 24, 32, and 60 hours. The cross gene error model was apllied (see Materials and Methods). 26 samples representing 17 conditions of activated and naïve populations stimulated in vitro for 4-60 hours were obtained. A) Kinetic profile of 6041 genes with detectable expression in a minimum of 4 TCR-stimulated samples is shown. TCR ligation in the absence of costimulation results in a minimum two-fold regulation of more than 1500 genes within 4 hours after activation. B) TCR triggering in the absence of costimulation delays gene expression changes over the first four hours, but results in comparable numbers of genes regulated more than two fold at all subsequent time points relative to lymphocytes stimulated in the presence of Ctla-4 and/or CD28 ligands that results in enhance responder frequency.

identify genes regulated two-fold or more under varying conditions of stimulation. Genes were grouped by the pattern of responsiveness they showed to TCR, CD28 and Ctla-4 ligation as shown in the Venn diagrams. Box plots demonstrate the predominant quality (up or down) and the relative magnitude of gene regulation. Scatter plots, colored by the respective categories identified in the Venn diagram, illustrate the relative influence of CD28 and Ctla-4 signaling on the global expression pattern. Together, these data indicate a predominantly quantitative influence of costimulation, but also indicate that subtle differences do result from differential ligation of costimulatory receptors.

CD28-signaling accelerates changes in gene transcription mediated by the TCR

Differences in gene transcription that result from CD28 costimulation are most significant at earlier times. As indicated in the box plots, CD28 results in robust gene regulation by four hours that is significantly greater than is seen with TCR signaling alone. From 8-20 hours, however, there is no substantial difference in the overall magnitude of regulation occurring. TCR stimulated and CD28 costimulated T cells both regulate the greatest number of genes at 8 hours. CD28 signaling is unique in that it produces a transient, but significant downregulation of several hundred genes by 4 hours that is not recapitulated at any other timepoint regardless of activation condition. CD28 signaling also maintains numerous transcripts at higher levels at late timepoints relative to TCR ligation alone as indicated by the relative interquartile distances observed in the box plot at 12-24 hours.

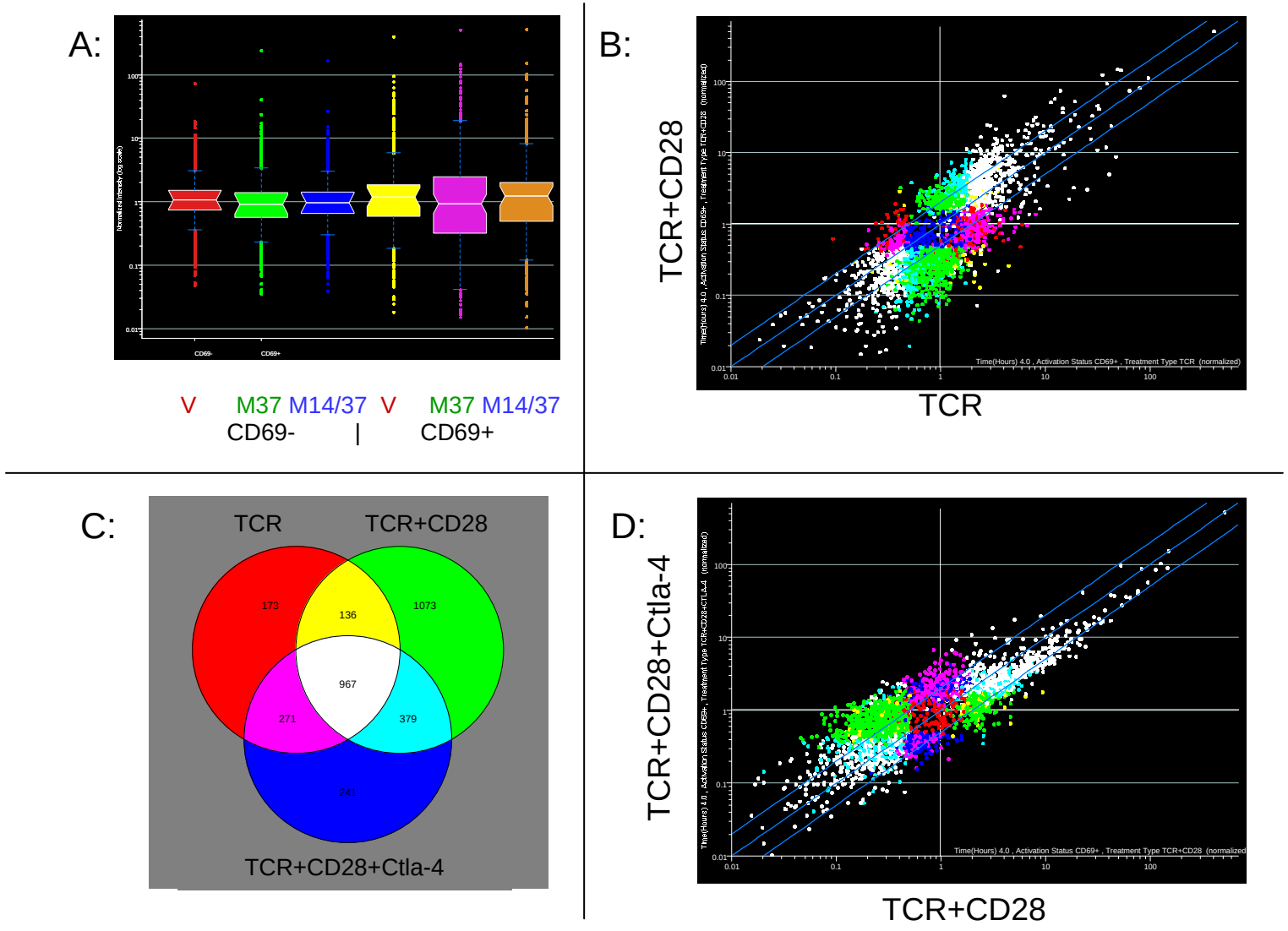


Figure 4.14.1: Global Expression Patterns 4 Hours After Activation

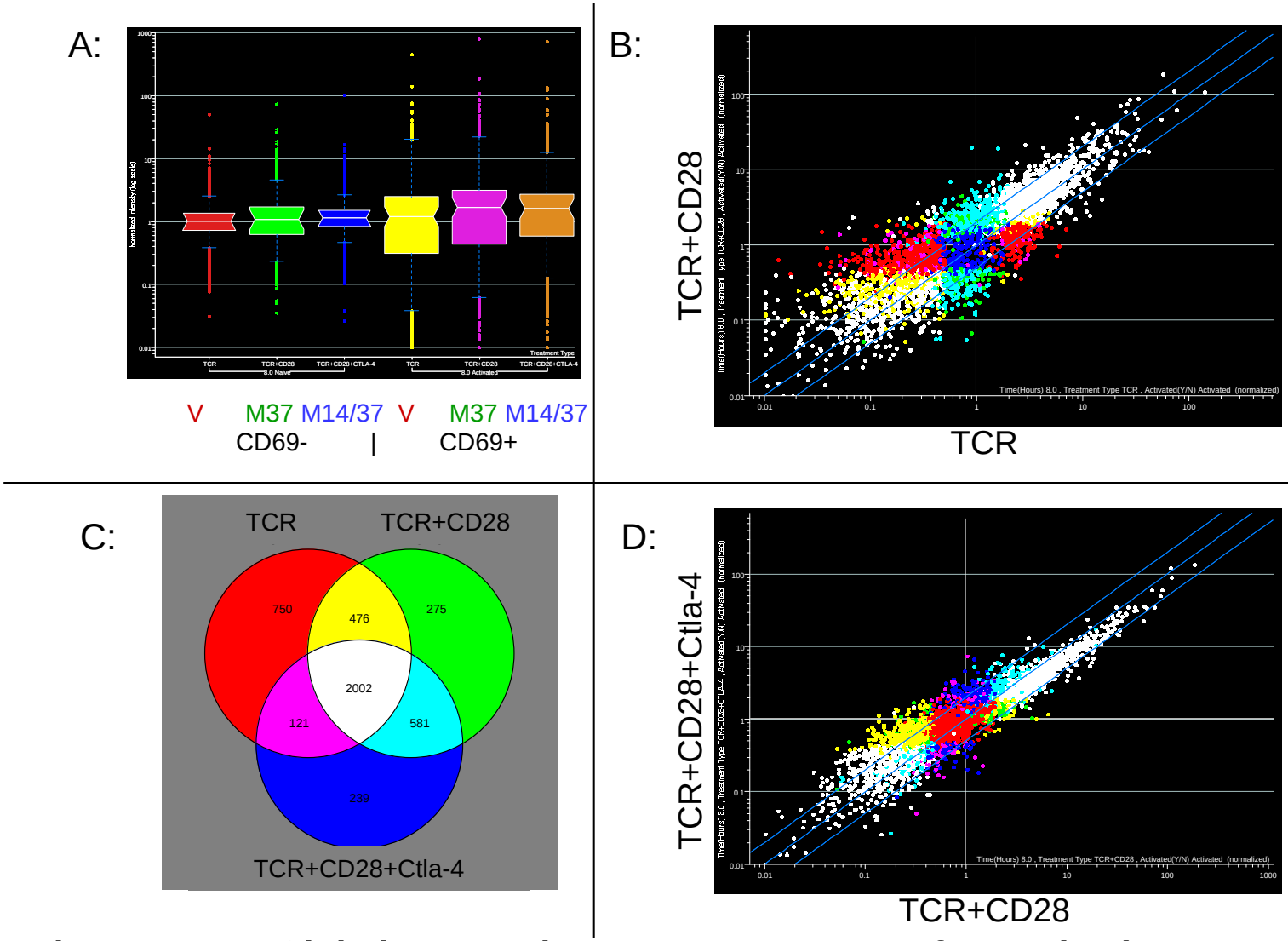


Figure 4.14.2: Global Expression Patterns 8 Hours After Activation

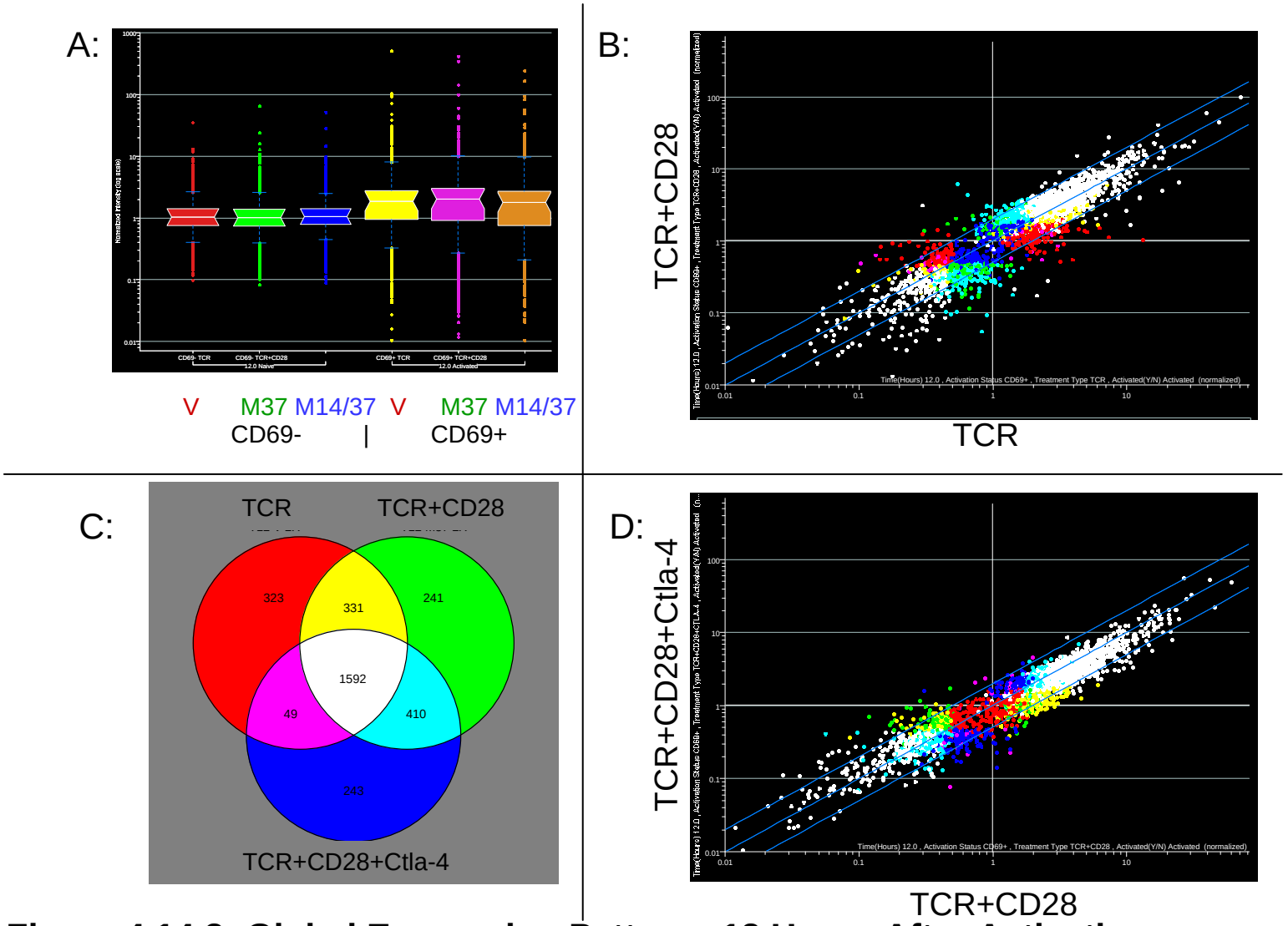


Figure 4.14.3: Global Expression Patterns 12 Hours After Activation

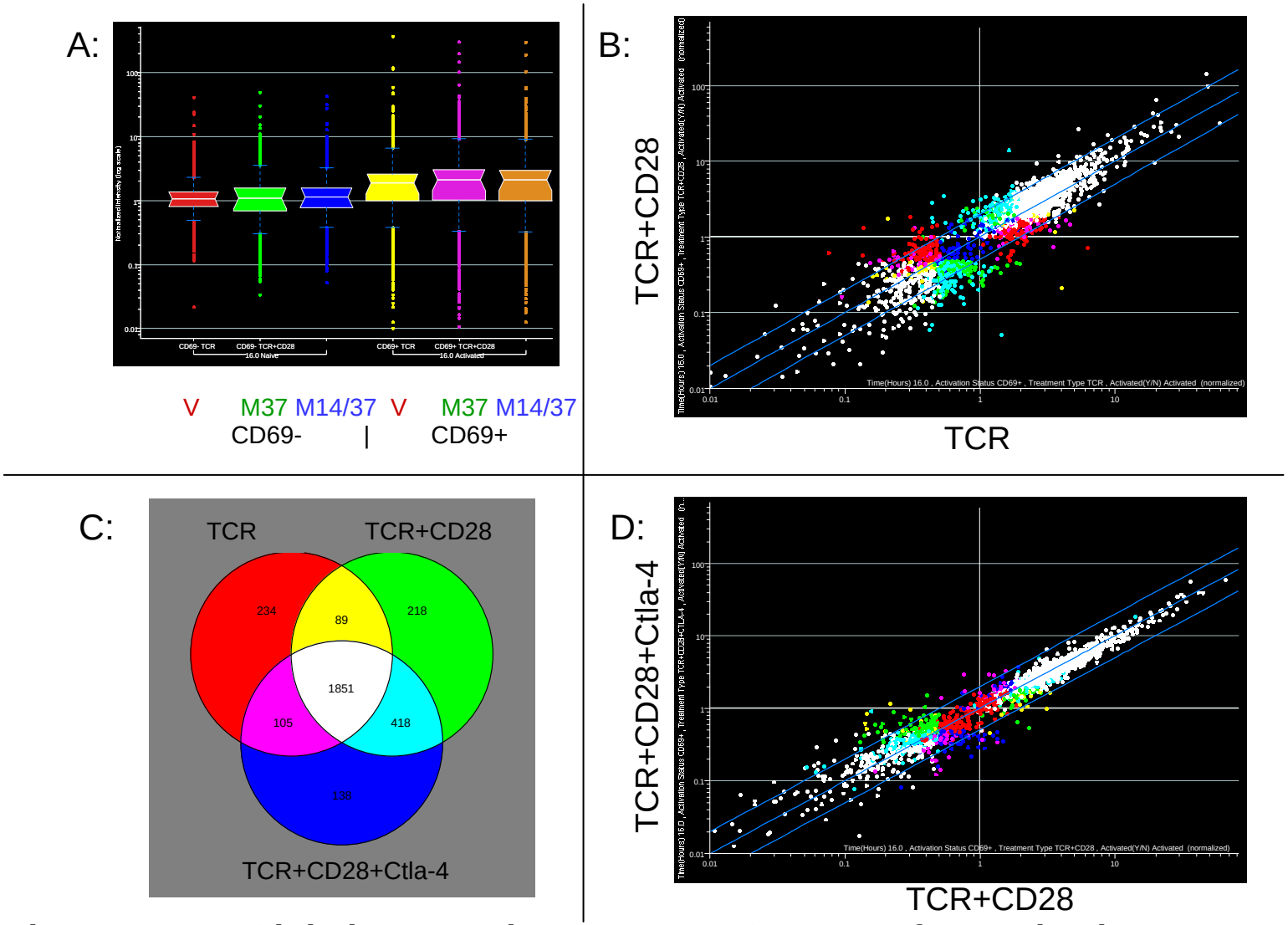


Figure 4.14.4: Global Expression Patterns 16 Hours After Activation

Figure 4.14: Comparisons of global expression patterns in 5C.C7 CD4+ lymphocytes stimulated in vitro by engineered APCs differentially expressing ligands for the TCR, CD28, and Ctla-4 were performed. Figures 4.14.1-4 show comparisons of global expression profiles at 4 hour intervals from 4-16 hours respectively. V, M37, and M14/37 designations correspond to the use of engineered APC clones expressing ligands for the TCR, TCR+CD28, and TCR+CD28+Ctla-4 respectively during stimulation. All gene measurements reflect the average of two biological replicates normalized to expression in unstimulated, naïve, peripheral 5C.C7 CD4+ lymphocytes. Panel A shows a box plot representation of the overall magnitude of gene regulations that occur in naïve and activated populations under the three conditions of stimulation in the presence of TCR, CD28, and Ctla-4 ligand expression. The analysis included all genes with detectable expression in at least one of the six conditions displayed. Box indents indicate the median change in gene expression. Upper and lower box segments reflect the interquartile distance of all genes analysed. Dashed extensions are set to 1.5 times the interquartile distance. Highly regulated genes are represented by individual dots. Panel C is a Venn diagram showing the number of genes regulated uniquely or in common by lymphoyctes stimulated in the presence of varying combinations of ligands for the TCR, CD28, and Ctla-4. Panel B and D show scatter plots of genes regulated at the corresponding time under conditions of TCR+CD28 vs. TCR monostimulation or TCR+CD28+Ctla-4 vs. TCR+CD28 respectively. The average expression values of individual genes are indicated by colored dots. The color of gene dots corresponds to colors in the Venn diagram. The medial blue line represents equivalent expression in the

two conditions displayed. Outer parallel lines reflect two-fold changes in gene expression.

Figure 4.15: Increasing convergence of target genes regulated by TCR and CD28 with time

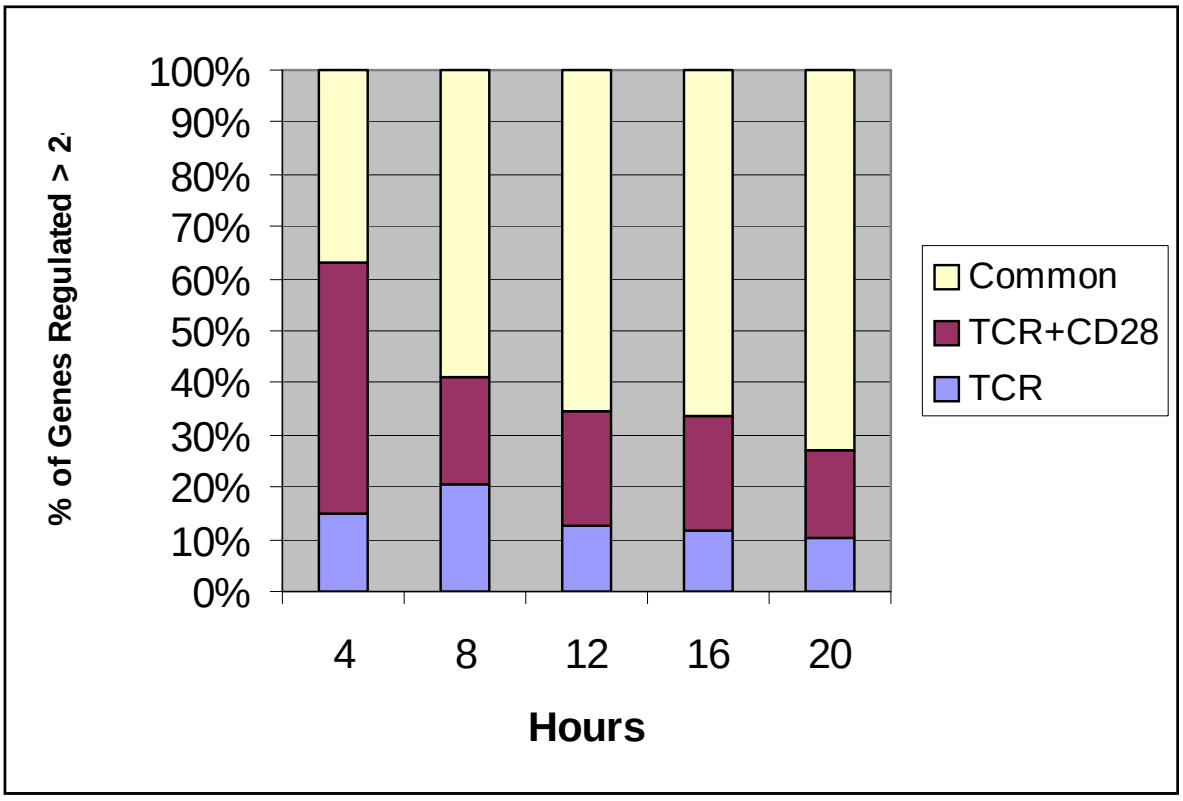


Figure 4.15: Expression patterns initiated by TCR and TCR+CD28 signaling converge over time. 5C.C7 CD4+ lymphocytes were stimulated with engineered APCs presenting MCC in the context of I-E^k in the presence or absence of CD28 costimulation. Populations purified for the positive expression of CD69 allowed for subsequent microarray analysis of the transcriptome and identification of gene regulation occurring over time in activated lymphocyte populations. Biological duplicates were obtained and average gene expression at each time interval normalized to expression in naïve, unstimulated, peripheral CD4+ 5C.C7 lymphocytes. Genes regulated more than two-fold were identified in both TCR monostimulated and CD28 costimulated samples. Of all genes regulated more than two-fold at a a given timepoint, the percentages of genes regulated uniquely or in common by TCR and CD28 signaling are shown. At all timepoints the majority of genes regulated at least two-fold are regulated under conditions of either TCR monostimulation or CD28 costimulation. CD28 signaling uniquely regulates the greatest number of genes at early times after stimulation. However, subsets of genes remain uniquely regulated by TCR and CD28 signals beyond 20 hours, suggesting that long-term differences in phenotype or responsiveness may result from altered costimulatory signaling during primary activation.

Additional evidence for accelerated regulation by CD28 is also evident in the trend towards increasing commonality of regulated genes over time. At 4 hours, TCR and TCR+CD28 signaling resulted in a minimum 2-fold regulation of 1547 and 2555 genes respectively. 1103 genes were regulated in common, representing 71% and 43% of all genes regulated by TCR monostimulation or TCR+CD28 costimulation, respectively. As indicated in Figure 4.15, there is an increasing convergence of target genes regulated by TCR and CD28 signaling from 4-20 hours. This trend is quantitative as well as qualitative, as scatter plots of regulated genes reveal reduced numbers of genes differentially regulated by CD28 costimulation over time relative to their level of expression in TCR monostimulated cells. Thus, CD28 signaling results in a qualitatively different phenotype at early times by regulating the expression of genes not altered by TCR triggering. In addition, CD28 mediates a quantitative effect on TCR-induced gene transcription by enhancing the level of expression of TCR regulated genes. However, these differences in expression pattern and magnitude become increasingly less significant over time.

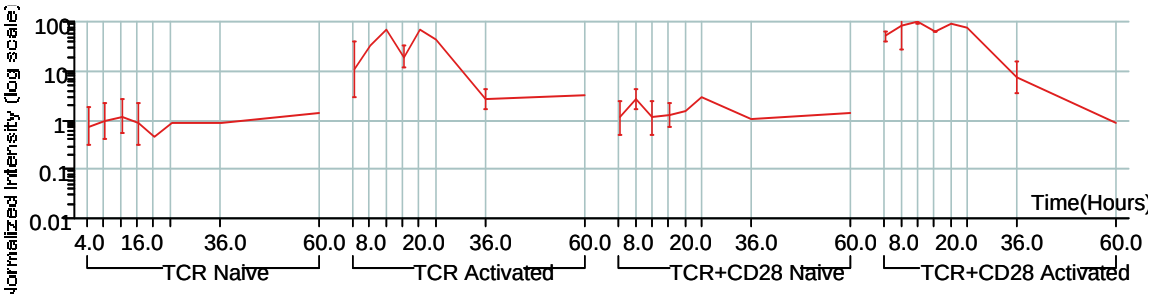
Delayed gene regulation in the absence of costimulation is a general trend that is also seen in the expression pattern of individual genes that are regulated upon T cell activation. It has been proposed that CD28 costimulation is essential for the upregulation of genes that are necessary for cell-cycle entry and T cell survival. Il-2 upregulation, in particular, is considered a hallmark of CD28-mediated costimulation and was considered a primary mechanism by which CD28 augments T cell responses. Our

data suggests that IL-2, and other known CD28 target genes are only affected by the absence of CD28 signaling to a relatively small degree. (Figure 4.16)

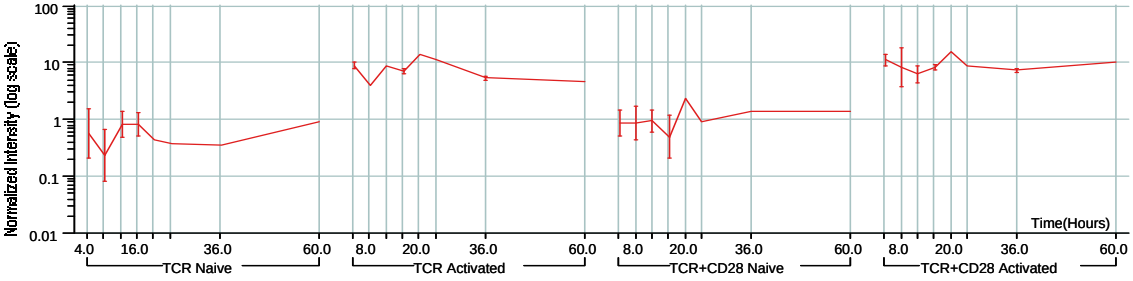
CD28 has modest effects on the expression of numerous genes regulated by TCR ligation. In contrast CD28 uniquely regulates only a very small subset of genes. Identification of genes differentially expressed upon CD28 ligation over numerous timepoints indicated that very few genes were consistently altered in a CD28-specific manner or inversely regulated relative to isolated TCR stimulation. Only 4 genes were more than 2-fold altered by CD28 ligation at all six timepoints from 4-24 hours. 20 genes, 83 genes, and 315 genes were differentially expressed following CD28 ligation in 5, 4, and 3 timepoints respectively. A search for putative regulatory sequences identified 33 elements ($p<0.05$) 5-10bp in length that were over-represented to a significant degree in the 1kb upstream of the 424 genes identified after multiple testing correction. Thus, these genes are likely regulated in a coordinated manner by specific mediators of CD28 signaling.

Altered gene expression subsequent to CD28 signaling is dependent on transcription factor activity. While it is possible that CD28 alters transcriptional profiles exclusively by post-translational control of transcription factor activity, CD28 may also mediate its pleiotropic effects on T cell phenotype via targeted regulation of transcription factor expression. In addition, the rapidity with which CD28 regulates gene transcription suggests that specific mediators of CD28 function are active at early times. To identify potential mediators of CD28 function, we screened genes differentially regulated by CD28 over at least three timepoints for known or potential transcription factors based on

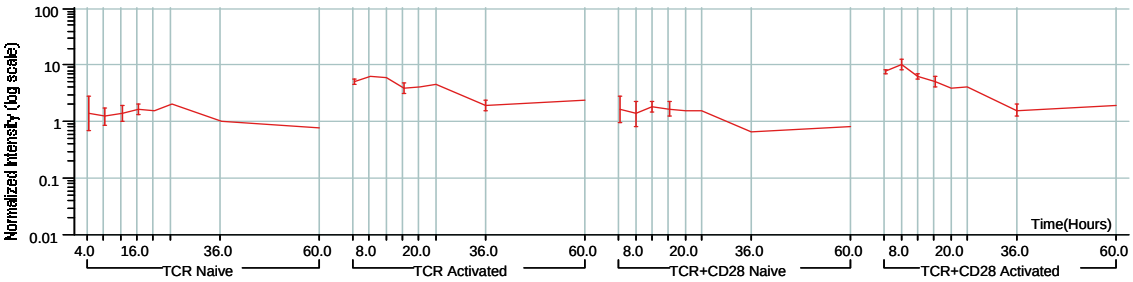
Figure 4.16: Isolated TCR ligation potently upregulates genes necessary for activation and survival



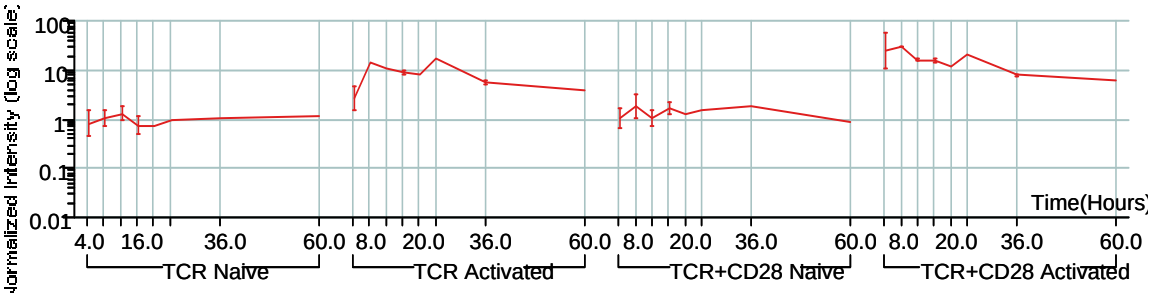
Interleukin 2



CD25



NFkappa-B



Bcl2sl1

Figure 4.16: TCR signaling is capable of potently upregulating numerous genes involved in T cell activation and survival. 5C.C7 CD4+ lymphocytes were activated in vitro by APCs engineered to express TCR ligands alone or in conjunction with CD28 ligands. Purified populations of naïve and activated T cells were obtained at time intervals from 4-60 hours and analyzed by microarray. Expression data was normalized to unstimulated, naïve, peripheral CD4+ 5C.C7 lymphocytes. Expression profiles for genes reportedly regulated in a primarily CD28-dependent manner are shown on a log scale. These include interleukin-2, CD25, NFκ-B, and the Bcl2 family member Bcl2sl1. Expression profiles in TCR-stimulated naïve, TCR-stimulated activated, CD28 costimulated naïve, and CD28-costimulated activated population are shown left to right as indicated. Error bars indicate standard deviation across biological replicates. The absence of error bars indicates only one sample was analyzed for the corresponding condition of time, stimulation condition, and activation status. In general, TCR monostimulated cells achieve peak expression of highly regulated genes at levels greater than 80% of maximal expression in CD28-costimulated cells. The majority of genes show little appreciable difference in peak expression in the absence of CD28 signaling, but a significant minority of genes show consistently lower levels of expression across numerous timepoints. Broad screens for genes uniquely, or inversely, regulated by CD28 reveal few CD28-speciific targets.

published information, homology to known proteins, and domain identity. Using a non-stringent screen intended to identify proximal regulators of transcription in addition to DNA-dependent transcription factors resulted in the identification of 57 unique genes. Hierarchical clustering of these genes based solely on their expression in naïve and activated populations under condition of TCR or TCR+CD28 stimulation shows several unique patterns of expression and identify subsets of genes displaying coordinate regulation (Figure 4.17). The 57 genes identified as transcriptional regulators specifically controlled by CD28 costimulation are shown in Table 4.2.1 and Table 4.2.2. Only 27 of the 57 identified transcriptional regulators potentially mediating CD28 signaling are known to bind DNA. These 27 genes display unique temporal expression patterns suggesting a potential hierarchy of regulation may exist between some of them.

An examination of the stability and magnitude of expression changes mediated by CD28 upon differentially regulated transcription factors revealed that Lklf/Klf2 was the most significantly altered target over the first 24 hours of activation. Because Lklf expression is downregulated by CD28 costimulation and maintained at low levels for the duration of activation, restricting a similar analysis to earlier intervals yields slightly different results. However, across all early intervals (4-12 hours, 4-16 hours, and 4-20 hours) the same five TF genes are ranked as the most highly regulated. (Table 4.3) These include Myb, Zfp46, Batf, Ahr, and Lklf. Of these five only Myb and Lklf are downregulated by CD28 costimulation, implying that they may serve to restrict processes involved in T cell activation. Expression profiles further reveal that Lklf is unique in its

Figure 4.17: Hierarchical clustering reveals coordinated expression of CD28-induced transcriptional regulators

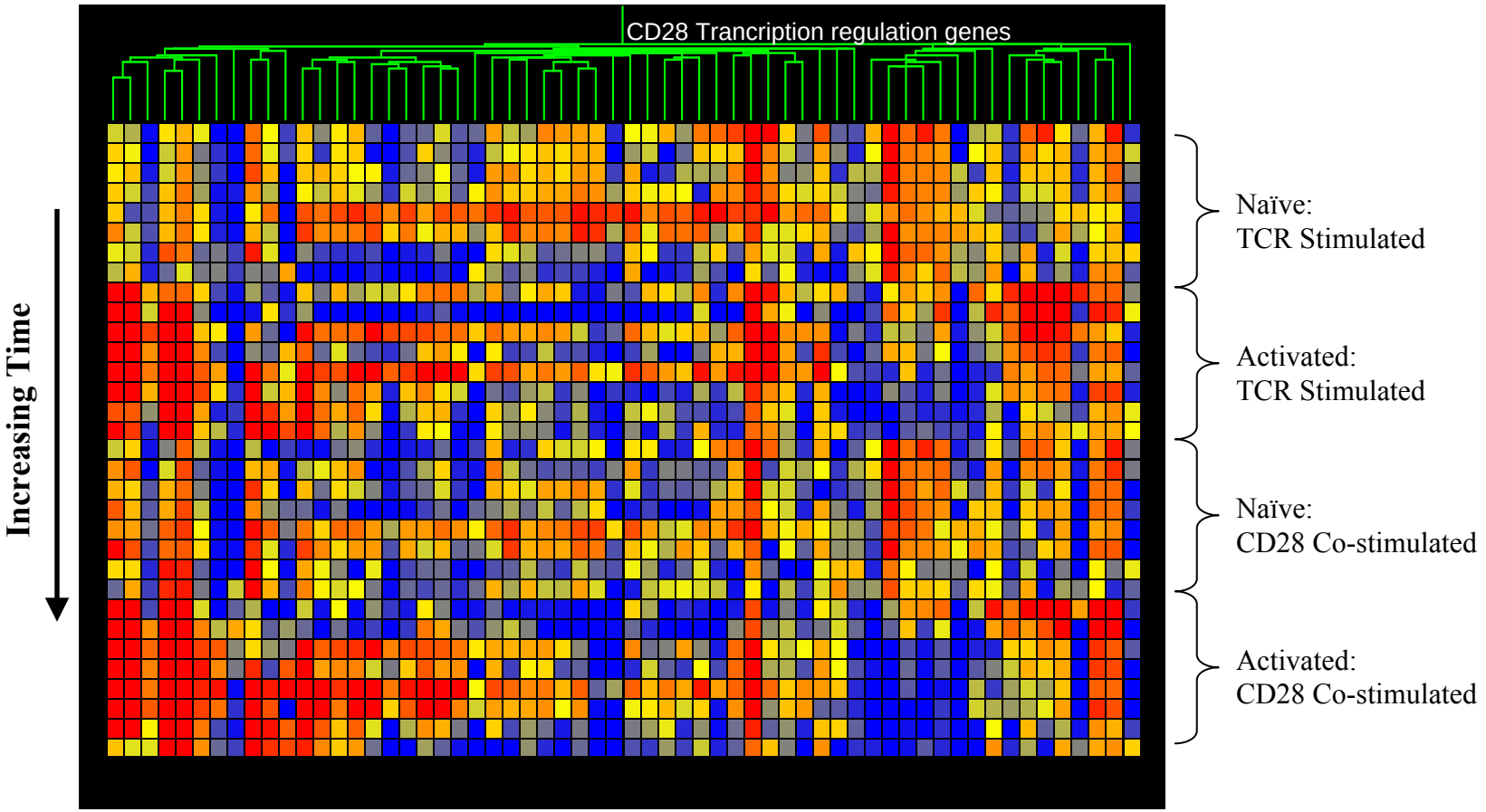


Figure 4.17: Hierarchical clustering of 60 transcriptional regulators differentially expressed by CD28 ligation. Microarray analysis was performed on TCR monostimulated and CD28 costimulated 5C.C7 CD4+ lymphocytes stimulated in vitro and purified for expression of activation markers at eight imepoints from 4-60 hours. Biological replicates were obtained for samples isolated prior to 24 hours in culture. Average expression values were normalized to unstimulated, naïve, peripheral CD4+ 5C.C7 lymphocytes. 423 genes were regulated more than two fold by CD28 costimulation at at least 3 of 6 timepoints analyzed prior to 24 hours relative to TCR monostimulation. 60 of the 423 genes are known or putative quiescence factors. Hierarchical clustering of the transcription factor expression profiles under condtions of TCR monostimulation and CD28 costimulation over time is shown. The expression pattern of transcriptional regulators reflects the gene programs initiated upon T cell activation. In this instance, clustering revealed several coordinately regulated patterns temporally offset over a period of 12 hours suggesting that causal relationships between them might exist. Public database mining was used to guide predictions about relevant pathways that might relate observed expression via known associations, both direct and indirect.

Table 4.2.1: Transcriptional regulators potentially mediating CD28 signaling effects

Table4.2.1: DNA-dependent/binding transcriptional regulators

Probeset ID	Common Name	Description
92703_at	**2310032M22Rik**	RIKEN cDNA 2610016F04 gene
97438_r_at	**4631416I11Rik**	Mus musculus cDNA clone UI-M-BH2.2-aou-f-01-0-UI 3', mRNA
97437_f_at	**4631416I11Rik**	Mus musculus cDNA clone UI-M-BH2.2-aou-f-01-0-UI 3', mRNA.
160495_at	**Ahr**	Mouse (strain C571) Ah-receptor (Ah) mRNA
93444_at	**Batf**	basic leucine zipper transcription factor, ATF-like
101475_at	**Bmi1**	minor transcript; Mouse zinc finger protein (bmi-1) gene, complete cds.
95755_at	**Csda**	cold shock domain protein A
99917_at	**Ezh2**	enhancer of zeste homolog 2 (Drosophila); vertebrate polycomb-group
93250_r_at	**Hmgb2**	high mobility group box 2
98465_f_at	**Ifi16**	interferon, gamma-inducible protein 16
92440_at	**Irf6**	interferon regulatory factor 6
96109_at	**Klf2**	Kruppel-like factor 2 (lung)
99024_at	**Mad4**	Max dimerization protein 4
92644_s_at	**Myb**	myeloblastosis oncogene
102209_at	**Nfatc1**	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
99076_at	**Nr1d2**	nuclear receptor subfamily 1, group D, member 2
104070_at	**Pcaf**	p300/CBP-associated factor
94319_at	**Rab18**	RAB18, member RAS oncogene family
97948_at	**Rb1**	Mouse retinoblastoma susceptibility protein (pp105 Rb) mRNA,
104476_at	**Rbl1**	retinoblastoma-like 1 (p107)
98335_at	**Recc1**	replication factor C 1
92399_at	**Runx1**	runt related transcription factor 1; AML1 homologue
92199_at	**Stat5b**	signal transducer and activator of transcription 5B
95536_at	**Tceb3**	transcription elongation factor B (SIII), polypeptide 3
102882_at	**Zfp46**	zinc finger protein 46
102872_f_at	**Zfp51**	zinc finger protein 51
95521_s_at	**Zfp68**	zinc finger protein 68

Table 4.2.2: Genes regulating transcription via DNA-independent mechanisms

Probeset ID	Common Name	Description
93055_at	1110054N06Rik	RIKEN cDNA 1110054N06 gene
103071_at	2810429C13Rik	topoisomerase (DNA) II beta binding protein
93204_r_at	3230402J05Rik	RIKEN full-length enriched library, clone:E030006G18
92992_i_at	5730497N03Rik	trans-acting transcription factor 4
93104_at	Btg1	M.musculus btg1 mRNA.
101583_at	Btg2	TIS21; Mouse TIS21 gene, complete cds.
101878_at	Cd72	CD72 antigen
100533_s_at	Crem	
94395_at	D3Ertd330e	DNA segment, Chr 3, ERATO Doi 330, expressed
160248_at	D3Ertd330e	DNA segment, Chr 3, ERATO Doi 330, expressed
93493_at	Ddx5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
103532_at	Eomes	eomesodermin homolog (Xenopus laevis)
94332_at	Ets1	cDNA clone IMAGE:1396445 5', mRNA sequence.
160662_r_at	Gata6	GATA binding protein 6
102705_at	Il2	interleukin 2
103657_i_at	Mtf2	Mus musculus cDNA clone UI-M-AP0-abe-e-08-0-UI 3', mRNA
96152_at	Narg1	NMDA receptor-regulated gene 1
92248_at	Nr4a2	
93915_at	Pou2af1	POU domain, class 2, associating factor 1; BOB1/OBF1; coactivator
160793_at	Pou6f1	Mus musculus cDNA clone UI-M-BH0-akf-h-09-0-UI 3', mRNA sequence.
92356_at	Ptpn8	protein tyrosine phosphatase, non-receptor type 8
104125_at	Rnf12	ring finger protein 12
100974_at	Ssbp4	musculus cDNA clone UI-M-AL1-ahk-g-10-0-UI 3', mRNA sequence.
94292_at	Strap	serine/threonine kinase receptor associated protein
97238_at	Tacc3	transforming, acidic coiled-coil containing protein 3
94008_at	Tceb1	transcription elongation factor B (SIII), polypeptide 1
102681_at	Tnfrsf4	tumor necrosis factor receptor superfamily, member 4
94809_at	Tsg101	tumor susceptibility gene 101
102263_at	Zfp143	zinc finger protein 143
103753_at	Zzz3	zinc finger, ZZ domain containing 3

Table 4.2: 57 transcription factor were identified as stably and differentially regulated upon CD28 ligation. 5C.C7 CD4+ lymphocytes were stimulated with engineered APCs presenting MCC in the context of I-E^k in the presence or absence of CD28 costimulation. Populations purified for the positive expression of CD69 allowed for subsequent microarray analysis of the transcriptome and identification of gene regulation occurring over time in activated lymphocyte populations. Biological replicates were obtained, and average gene expression levels were normalized to unstimulated, naïve, peripheral CD4+ 5C.C7 lymphocytes. 57 known or suspected transcriptional regulators displayed a minimum two-fold change in expression in at least 3 of the six timepoints analyzed between 4 and 24 hours upon CD28 costimulation. Table 4.2.1 lists the 27 transcription factors differentially regulated by CD28 signaling that are known to operate via DNA-dependent mechanisms. Table 4.2.2 lists the 30 transcriptional regulators known to function by DNA-independent mechanism that are differentially expressed subsequent to CD28 costimulation.

Table 4.3: Lklf is the most significantly and stably altered transcription factor regulated by CD28

					Timepoint					
Probeset ID	**Common Name**	**Average**	**FC**	**Abs FC**	**T4**	**T8**	**T12**	**T16**	**T20**	**T24**
96109_at	**Klf2**	0.19	**-5.40**	5.40	0.696	0.215	0.0681	0.0353	0.0681	0.029
92644_s_at	**Myb**	0.22	**-4.62**	4.62	0.0586	0.451	0.0797	0.117	0.238	0.353
160495_at	**Ahr**	3.46	**3.46**	3.46	0.406	8.743	2.589	4.14	2.853	2.03
102882_at	**Zfp46**	3.28	**3.28**	3.28	0.611	12.77	0.297	3.081	0.747	2.166
97438_r_at	4631416I11Rik	2.95	**2.95**	2.95	0.564	4.914	0.448	1.198	0.519	10.03
99076_at	Nr1d2	2.80	**2.80**	2.80	0.349	5.78	0.874	1.127	0.932	7.724
93444_at	**Batf**	2.77	**2.77**	2.77	7.104	2.892	2.184	1.618	1.658	1.183
97437_f_at	4631416I11Rik	2.23	**2.23**	2.23	0.33	7.331	0.666	1.363	0.942	2.745
95521_s_at	Zfp68	2.20	**2.20**	2.20	0.409	7.048	0.823	1.058	0.833	3.036
99024_at	Mad4	0.46	**-2.17**	2.17	0.572	0.39	0.512	0.453	0.439	0.405
98335_at	Recc1	2.06	**2.06**	2.06	0.417	1.508	1.127	2.91	2.062	4.359
92199_at	Stat5b	1.96	**1.96**	1.96	3.566	2.218	2.319	1.189	1.657	0.834
95536_at	Tceb3	1.88	**1.88**	1.88	0.805	2.978	2.619	2.351	1.481	1.04
95755_at	Csda	1.85	**1.85**	1.85	2.486	2.274	1.434	1.45	2.164	1.318
102872_f_at	Zfp51	1.82	**1.82**	1.82	2.513	3.13	0.759	0.809	0.709	2.984
102209_at	Nfatc1	0.56	**-1.77**	1.77	0.953	0.432	0.339	0.467	0.52	0.678
99917_at	Ezh2	1.67	**1.67**	1.67	0.678	0.783	2.003	2.464	1.806	2.275
92440_at	Irf6	0.63	**-1.59**	1.59	0.943	0.923	0.781	0.442	0.322	0.362
104070_at	Pcaf	0.66	**-1.51**	1.51	0.125	1.29	0.385	0.551	0.474	1.157
97948_at	Rb1	1.50	**1.50**	1.50	0.141	1.125	0.66	2.802	1.325	2.921
104476_at	Rbl1	1.41	**1.41**	1.41	0.48	2.373	0.748	1.113	1.001	2.766
101475_at	Bmi1	1.39	**1.39**	1.39	0.101	2.615	0.935	1.64	0.758	2.314
93250_r_at	Hmgb2	1.39	**1.39**	1.39	0.386	0.465	1.288	0.828	1.993	3.379
92703_at	2310032M22Rik	1.39	**1.39**	1.39	0.458	0.174	1.351	2.218	1.202	2.926
98465_f_at	Ifi16	1.29	**1.29**	1.29	1.665	0.592	0.155	0.422	0.505	4.401
94319_at	Rab18	1.27	**1.27**	1.27	0.193	2.014	0.896	1.362	0.984	2.173
92399_at	Runx1	1.25	**1.25**	1.25	0.339	0.31	0.444	0.893	2.647	2.871

Table 4.3: Analysis of the magnitude and stability of CD28 mediated transcriptional regulation of transcription factors. 27 DNA-dependent transcription factors (TFs) were differentially expressed following CD28 signaling in activated 5C.C7 CD4+ lymphocytes stimulated by engineered APCs in vitro. (minimum two-fold change in expression relative to TCR-monostimulated cells for $\geq$3 timepoints from 4-24 hours). Only 6 TFs were, on average, downregulated during the first 24 hours of T cell activation, and only two, Myb and Lklf/Klf2, were stably downregulated more than two-fold. However, Lklf and Myb were the most significantly regulated transcription factors upon CD28-costimulation over the first 24 hours of activation. Bolded common names denote the 5 genes that are the most highly regulated over any interval from 4-20 hours.

FC: Fold change.

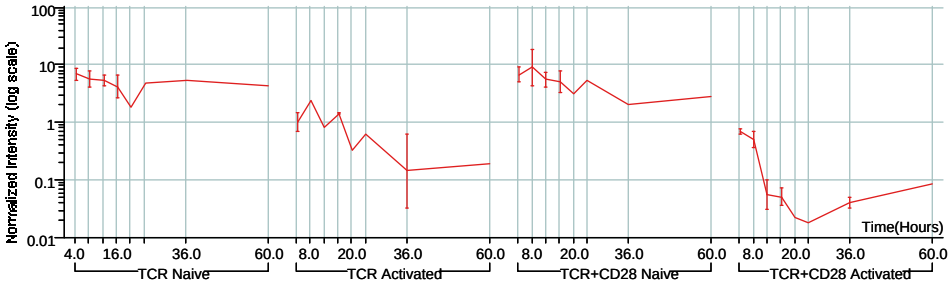
Abs FC: Absolute Fold Change

ability to maintain differential expression by CD28 signaling over the long term. (Figure 4.18)

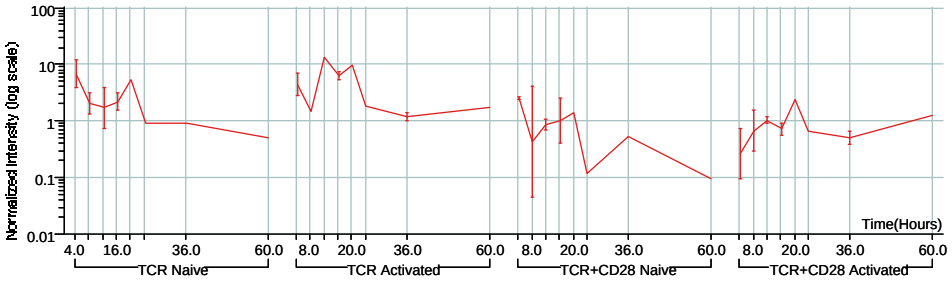
CD28 enhances T cell activation by amplifying proximal signaling events and by induction of gene transcription leading to enhanced survival, quickened cell-cycle entry, and more efficient effector function. Moreover, CD28 signaling may abrogate mechanisms of tolerance such as anergy induction. The identification of genes specifically regulated by CD28 in the context of antigen receptor stimulation provides novel opportunities for therapeutic manipulation of the pathways involved and will lead to a greater understanding of T cell tolerance. It is an intriguing possibility that the

the ability of CD28 to modestly enhance the expression of numerous genes may have unrecognized biological significance. It is clear that TCR stimulation is capable of activating all pathways necessary for activation, cell cycle entry, and survival. Moderate increases in these processes, along with the increase in responder frequency that results from CD28 engagement, could potentially lead to physiologically significant changes in the context of a clonally expanding population of T cells. Thus, modest changes in gene expression resulting from CD28 ligation can be significant if the differential expression is maintained long term. Identifying factors that might mediate such an effect may reveal the mechanisms by which CD28 influences numerous processes in immunoregulation.

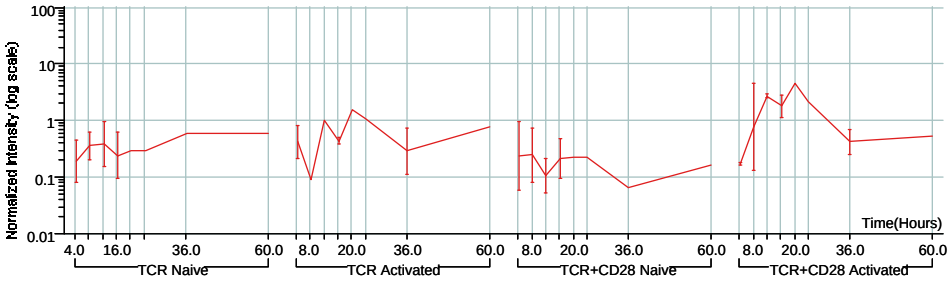
Figure 4.18: Stable, long-term CD28 mediated regulation of Lklf



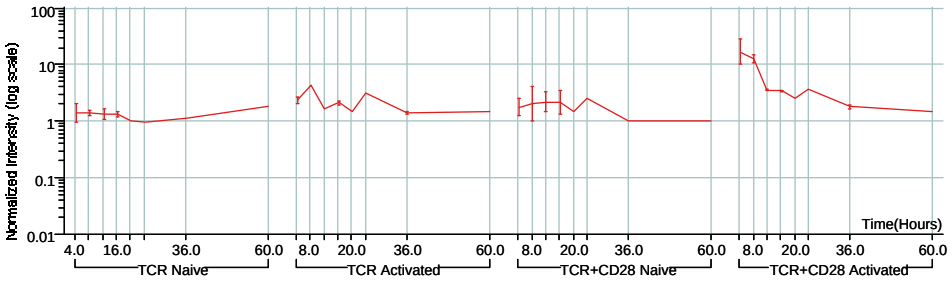
Lklf



Myb



Ahr



Batf

Figure 4.18: Expression profiles reveal that CD28-mediated regulation of Lklf provides stable changes in gene expression relative to TCR monostimulation. 5C.C7 CD4+ lymphocytes were activated in vitro by APCs engineered to express TCR ligands alone or in conjunction with CD28 ligands. Purified populations of naïve and activated T cells were obtained at time intervals from 4-60 hours and analyzed by microarray. Expression data was normalized to unstimulated, naïve, peripheral CD4+ 5C.C7 lymphocytes. 60 known or suspected transcriptional regulators were differentially expressed at least two-fold by CD28 signaling in a minimum of 3 of 6 timepoints analyzed between 4 and 24 hours. Expression profiles for the four most highly and consistently regulated transcription factors are shown. Expression profiles in TCR-stimulated naïve, TCR-stimulated activated, CD28 costimulated naïve, and CD28-costimulated activated population are shown left to right as indicated. Error bars indicate standard deviation across biological replicates. The absence of error bars indicates only one sample was analyzed for the corresponding condition of time, stimulation condition, and activation status. The apparent decline of Lklf in TCR monostimulated samples at late timepoints could result from activation-induced downregulation independent of CD28, or may reflect inappropriate data normalization as a result of the magnitude of expression changes that occur in dividing cells relative to naïve cells or activated cells at early timepoints.

Ctla-4 Influences the Global Expression Profile of CD28-costimulated T Cells

One report has investigated the role of Ctla-4 in modifying the global transcriptional response to activating stimuli delivered throught the TCR and CD28(54). The results suggested that Ctla-4 ligation in the absence of CD28 costimulation did not alter the genomic expression pattern subsequent to TCR ligation. In addition, the effect of Ctla-4 ligation was limited to a partial suppression of CD28-mediated gene regulation and did not result in qualitative differences in gene expression. Our system of in vitro stimulation has been designed to allow tight control of stimulating and inhibitory signaling in TCR-tg T cells while allowing for physiologically relevant interactions with APC-expressed ligands. Moreover, purification of naïve and activated cells increases the likelihood of detecting subtle changes in gene expression that might be masked by the homogeneity of unsorted cells under varying conditions of costimulation. In vitro, cells become activated despite the absence or presence of of CD28 and and Ctla-4 respectively. Moreover, some cells remain naïve by ignorance in the absence of Ctla-4 signaling resulting in identical phenotypes maintained by different mechanisms. We propose that Ctla-4 maintains peripheral tolerance, in part, by up-regulating factors necessary for the maintenance of self-tolerance and quiescence. In the context of T cell activation, the activity of such factors may be manifested in the maintenance of a naïve phenotype, altered parameters of T cell activation and division, or long-term phenotypic changes. Thus, detecting transcriptional regulation subsequent to Ctla-4 ligation may require higher stringency protocols than have been used previously.

Ctla-4 inhibits CD28 mediated changes in gene transcription

The influence of Ctla-4 on the magnitude of CD28-mediated transcriptional regulation is seen in Figures 4.14.1 to 4.14.4. Box plots reflect the ability of Ctla-4 to inhibit transcriptional regulation to much greater degree at early times after activation. At the genomic level, the ability of Ctla-4 to influence the scope of gene regulations is abolished by 12 hours. This is reflected graphically in Figure 4.13 panel B. As indicated by the Venn diagrams in Figure 4.14, the majority of gene expression changes occurring subsequent to TCR+CD28 ligation are not qualitatively affected by Ctla-4, suggesting that Ctla-4 functions to inhibit the progressive nature of CD28 transcriptional regulation. Over time, this is reflected in the convergence of target gene identities regulated in the presence or absence of Ctla-4 (Figure 4.19). The decreasing relevance of Ctla-4 ligation over time, as it relates to the global expression profile is best seen in the serial scatter plots comparing gene expression levels in samples experiencing different combinations of CD28 or CD28+Ctla-4 costimulation. (Figure 4.14) In contrast to the broadly distributed levels of relative expression seen at 4-8 hours, later times reveal a predominant 1:1 expression ratio reflected in the close grouping of genes around the axis. However, a subset of genes remains differentially regulated by Ctla-4 at every time point investigated.

We have proposed that Ctla-4 maintains immunotolerance, in part, by inducing the expression of transcriptional regulators that impose lasting phenotypic changes. Ctla-4 coligation with CD28 resulted in a minimum 2-fold change in expression of 11, 23, 102, and 322 genes in 6, 5, 4, or 3 of the six timepoints between 4 and 24 hours. Of these

Figure 4.19: Ctla-4 differentially regulates a small subset of genes

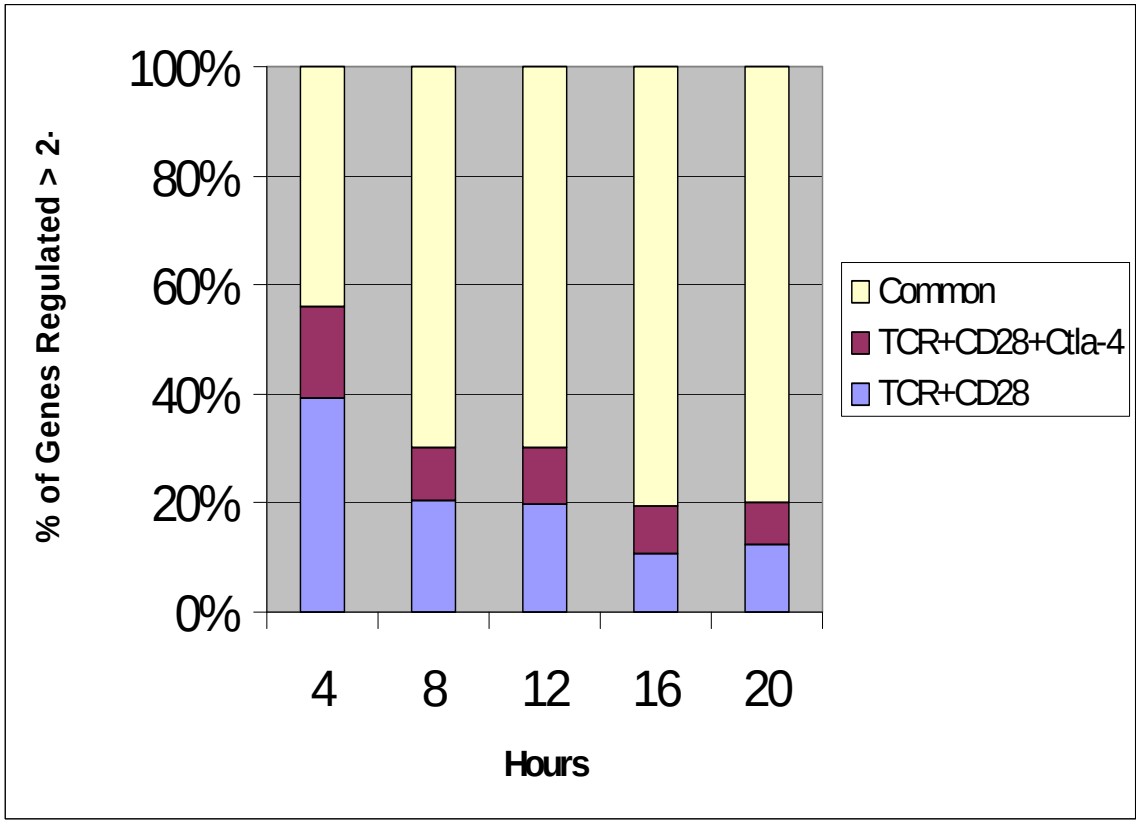


Figure 4.19: Ctla-4- and CD28-signaling pathways converge on the same set of target genes. 5C.C7 CD4+ lymphocytes were stimulated with engineered APCs presenting MCC in the context of I-E^k in the presence or absence of Ctla-4 ligands in conjunction with CD28 costimulation. Populations purified for the positive expression of CD69 allowed for subsequent microarray analysis of the transcriptome and identification of gene regulation occurring over time in activated lymphocyte populations. Biological duplicates were obtained and average gene expression at each time interval normalized to expression in naïve, unstimulated, peripheral CD4+ 5C.C7 lymphocytes. Genes regulated more than two-fold were identified lymphocytes activated in the presence of Ctla-4 and/or CD28 ligands. Of all genes regulated more than two-fold at at a given timepoint, the percentages of genes regulated uniquely or in common by Ctla-4 and CD28 signaling are shown. At all timepoints the majority of genes regulated at least two-fold are regulated under conditions of either CD28 costimulation or combined CD28 and Ctla-4 costimulation. CD28 signaling uniquely regulates the greatest number of genes at early times after stimulation. However, subsets of genes remain uniquely regulated by Ctla-4 and CD28 signals beyond 20 hours, suggesting that long-term differences in phenotype or responsiveness may result from altered costimulatory signaling during primary activation. Over time there is increasing convergence of genes regulated more than two-fold under isolated CD28 costimulation or combined CD28/Ctla-4 ligation. Ctla-4 suppresses CD28 mediated transcriptional regulation to a greater degree at very early times after activation.

Table 4.4: Transcriptional factors stably regulated by Ctla-4 ligation

Probeset ID	Timepoints Observed	Common Name	Description
92344_at	6	Smarca3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3
99076_at	**5**	**Nr1d2**	**nuclear receptor subfamily 1, group D, member 2**
96109_at	**5**	**Klf2**	**Kruppel-like factor 2 (lung)**
92992_i_at	**5**	**5730497N03Rik**	**trans-acting transcription factor 4**
101528_at	5	Tcea1	
92934_at	4	Zfp90	zinc finger protein 90
104669_at	4	Irf7	interferon regulatory factor 7
102959_at	4	Tle4	transducin-like enhancer of split 4
101919_at	4	Zfx	zinc finger protein X-linked
99001_at	4	Zfp292	zinc finger protein 292
95530_at	4	6330549H03Rik	general transcription factor II A, 1
99950_at	3	Tbp	TATA box binding protein
99558_at	3	Ccnc	cyclin C
98628_f_at	3	Hif1a	hypoxia inducible factor 1, alpha subunit
98465_f_at	3	Ifi16	interferon, gamma-inducible protein 16
98030_at	3	Trim30	tripartite motif protein 30
95617_at	3	Rbl2	retinoblastoma-like 2
94467_at	3	Cebpz	CCAAT/enhancer binding protein zeta
93611_at	3	Tbx6	T-box 6
93250_r_at	**3**	**Hmgb2**	**high mobility group box 2**
92991_at	3	Sp4	trans-acting transcription factor 4
92468_at	3	Gbif	globin inducing factor, fetal
104476_at	**3**	**Rbl1**	**retinoblastoma-like 1 (p107)**
101180_at	3	Atm	ataxia telangiectasia mutated homolog (human)
100130_at	3	Jun	Jun oncogene
99462_at	3	Top2b	topoisomerase (DNA) II beta
98855_r_at	**3**	**A930001N09Rik**	**RIKEN cDNA A930001N09 gene**
98629_f_at	3	Hif1a	
95879_at	3	Asf1a	ASF1 anti-silencing function 1 homolog A
94325_at	3	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
103074_f_at	3	Taf9	TAF9 RNA polymerase II, (TBP)-associated factor
101529_g_at	3	Tcea1	
101439_at	**3**	**Arl6**	**myc induced nuclear antigen**
100533_s_at	**3**	**Crem**	

Table 4.4: TCR and CD28-mediated stimulation of 5C.C7 CD4+ lymphocytes in thepresence of Ctla-4 ligands results in the differential expression of known transcription factors. 5C.C7 CD4+ lymphocytes were stimulated with engineered APCs presenting MCC in the context of I-E^k in the presence or absence of Ctla-4 ligands in conjunction with CD28 costimulation. Populations purified for the positive expression of CD69 allowed for subsequent microarray analysis of the transcriptome and identification of gene regulation occurring over time in activated lymphocyte populations. Biological duplicates were obtained and average gene expression at each time interval normalized to expression in naïve, unstimulated, peripheral CD4+ 5C.C7 lymphocytes. Table 4.4 lists transcription factors displaying more than two-fold differential expression identified in lymphocytes activated in the presence of Ctla-4 and/or CD28 ligands in the first 24 hours of in vitro stimulation. Six timepoints from 4-24 hours after stimulation were examined, and the number of timepoints at which a minimum two-fold differential expression was detected in the presence of Ctla-4 ligands is indicated for each transcription factor identified.

genes, 34 are known or suspected transcriptional regulators. (Table 4.4) In addition, 8 of these genes were identified as specific targets of CD28 signaling in previous screens, suggesting that the balance of stimulatory and inhibitory costimulation may define genomic expression patterns through the action of these transcription factors. When weighted by the average change in expression seen over increasing intervals from 4-12 hours up to 4-24 hours, the same 8 genes are ranked highest. These include Lklf, Rbl2, Hmgcs1, Hif1a, cyclin C, Zfx, Tcea1, and a Riken clone (probeset ID 92992_i_at).

In contrast to the rapid downregulation of Lklf that occurs with unopposed CD28 costimulation, Lklf appears to be downregulated in activated cells at later timepoints (beyond 32 hours) under conditions of Ctla-4 ligation (Data not shown). However, the statistical significance of Lklf differential regulation by Ctla-4 increases over time, in apparent contrast to its expression profile. This may reflect the difficulty involved in choosing appropriate normalization standards when comparing vastly different biological samples. This analysis makes use of data normalized to expression levels in ex vivo CD4+ lymphocytes. The magnitude and scope of gene regulation that occurs during T cell activation makes standard microarray normalization steps insufficient over long periods of time. Screens used to identify differentially regulated genes in samples processed at the same timepoint are internally controlled. In contrast, gene expression profiles normalized to the ex vivo sample are not internally controlled. Thus, the trends observed, and the relative expression observed under varying stimulation conditions at any given time remains reliable, but the magnitude of long-term trends in gene expression will not necessarily correlate with direct measurements of fold change.

Discussion

Transcriptional changes during lymphocyte activation have been previously characterized. Our system improves on previous reports by utilizing phenotypically pure sample populations homogenous for activational history and stimulated by engineered APCs capable of differentially signaling through the TCR, CD28, and Ctla-4. In addition, we have investigated genomic-scale expression patterns at numerous timepoints prior to cell-cycle entry allowing for identification of downstream mediators of costimulatory function that may determine distal outcomes of proximal integration of TCR, CD28, and Ctla-4 signals. Our results provide the most comprehensive and stringently controlled kinetic study of genomic expression patterns in activated lymphocytes yet available. While an exhaustive categorization of observed changes is beyond the scope of this work, our data does provide numerous findings that inform our understanding of signal integration as it occurs during lymphocyte activation.

The temporal parameters governing antigen-receptor and costimulatory signal integration have not been well defined. In our system, TCR triggering activates all relevant pathways within the first two hours and can result in commitment to activation as early as one hour after stimulation. In contrast, signals through CD28 and Ctla-4 are persistent over 12-16 hours and are integrated accordingly. This suggests a distinctly different mechanism of signaling is utilized. However, CD28 is also capable of significantly enhancing responder frequency to TCR engagement as early as one hour after stimulation. Thus, CD28 functions to lower the threshold of activation in response

to antigen receptor engagement over an externded period, accelerates the rate of transcriptional regulation leading to cell-cycle entry at the population level, and marginally amplifies the transcriptional response of the majority of genes regulated by TCR signaling in activated cells. However, alterations in gene regulation occurring as a result of CD28 ligation are heterogeneous, and a subset of genes is specifically sensitive to CD28-mediated regulation. In contrast, the availability of Ctla-4 ligands serves to counteract CD28-mediated costimulation primarily by raising the threshold of activation without shifting the peak of the antigen-dose response curve or altering the kinetics of activation or cell-cycle entry. In addition, the availability of Ctla-4 ligands has minor effects on the global expression profile of naïve or activated CD4+ T cells, and only a small number of genes are differentially regulated in the presence of Ctla-4 ligands for any appreciable amount of time. Like CD28, Ctla-4 modifies the transcriptional response to the greatest degree at very early times after stimulation, and the effect is largely abrogated by twenty hours post-stimulation. Thus, both CD28 and Ctla-4 appear to regulate membrane-proximal signaling events controlling T cell activation prior to one hour, yet also serve to modify the clonal response to TCR engagement over prolonged periods in a TCR-independent manner. Numerous models have been proposed to explain the disparate effects of costimulation on T cell activation, and our results support the notion that CD28 and Ctla-4 function at multiple levels to regulate T cell resonses.

TCR ligation results in rapid changes in the phosphorylation status of numerous proteins and triggers rapid fluxes in intracellular calcium with the net effect of initiating changes in gene transcription through the activation of specific transcription factors(373-

375). Using in vitro stimulated TCR-transgenic CD4+ lympohcytes we have shown that TCR ligation alone results in antigen dose-dependent activation of primary T cells leading to broad transcriptional regulation and commitment to cell-cycle entry and division. In contrast to published reports, TCR ligation in the absence of CD28 costimulation resulted in robust upregulation of genes essential for cell survival, division, and effector function including interleukin-2 and Bcl-xL. Moreover, the lack of CD28-mediated signaling did not result in altered secondary responses or increased cell death. Thus, TCR signaling was capable of activating all pathways necessary for CD4+ T cell activation within 1-2 hours resulting in a minimum two-fold regulation of nearly 50% of all genes with detectable expression by 8 hours after stimulation suggesting that TCR engagement is sufficient for productive activation in CD4+ lymphocytes.

CD28 ligation lowers the threshold of activation and results in increased responder frequency within one hour of TCR-mediated signaling. In addition, CD28 signaling potentiates TCR-mediated activation signals over a prolonged period of 12-16 hours and enhances the expression patterns initiated by TCR engagement. However, In contrast to published reports, our data indicates that CD28 does not enhance expression of TCR-induced genes to the degree reported. The observed difference is likely a result of the use of purified populations in our analysis. In our system, TCR signaling is generally capable of regulating gene expression to 80% or more of the maximal level observed with CD28 costimulation. Moreover, CD28 has the greatest qualitative influence on genomic expression patterns in activated CD4+ lymphocytes at very early times after TCR stimulation, with diminishing effect over time. Thus, the primary effect

of CD28 is to lower the threshold of activation by influencing membrane proximal signaling events while sustaining TCR-mediated activation signals over an extended period. In addition, our results suggest that CD28 uniquely regulates a very small subset of genes not altered by isolated TCR engagement. The functional result of CD28 ligation is increased responder frequency at the population level, accelerated kinetics and amplified magnitude of transcriptional regulation at the cellular level, and specific regulation of a small subset of genes such as Lklf and Batf. Thus, CD28 has a primarily quantitative effect on T cell responsiveness and clonal activation, but does induce qualitatively unique gene programs of unknown significance via regulation of genes that are, generally, not well characterized in lymphocytes.

CD28 is postulated to increase T cell activation by numerous mechanisms. CD28 ligation has been shown to enhance phosphorylation of key signaling intermediates operating downstream of the TCR, to activate specific signaling pathways involving PI3K independent of TCR ligation, and to stabilize T cell interaction with APCs resulting in enhanced activation and gain of effector function by TCR-dependendent and –independent mechanisms. The ability of CD28 to enhance responder frequency to TCR stimulation within one hour of antigen encounter supports the notion that CD28 functions to potentiate membrane proximal signaling events. However, we have also shown that CD28-signaling is capable of enhancing responder frequency when ligated up to 12 hours after initial TCR stimulation. These results suggest that CD28 ligation permits T cell activation in response to otherwise sub-mitogenic TCR signaling. In conjunction with the identification of genes uniquely regulated by CD28 in activated CD4+ lympohocytes,

these data also suggest that CD28 transmits a signal that operates independently from TCR signaling pathways. While our system can not address the relevance of additional costimulatory signals that may result from prolonged T cell interaction with an APC, the extended kinetics of CD28 signal integration support a model of costimulation in which rapid sub-mitogenic signaling resulting from TCR triggering allows for the relatively slow development of an unresponsive state that can be rescued by subsequent costimulatory signals. Thus, prolonged low affinity TCR interactions may program T cells to become unresponsive with kinetics that allow for extended integration of costimulatory signals.

Ctla-4 ligand-expressing APCs suppressed CD28-mediated amplification of TCR-induced transcriptional changes and counteracted CD28-mediated increases in responder frequency. In addition the availability of Ctla-4 ligands delayed the rapid increase in gene regulation enabled by CD28 following antigen recognition. The delay in trancscriptional responsiveness may allow time for the integration of additional costimulatory signals prior to commitment. However, the availability of Ctla-4 ligands did not shift the antigen-specific dose response curve of TCR-tg CD4+ T cells, alter the kinetics of transcriptional regulation beyond eight hours, delay cell-cycle entry, or change the kinetics of CD28-signal integration. Thus, the critical effect of Ctla-4 was to increase the threshold of activation in a primarily CD28-dependent manner. Interestingly, four hours after stimulation CD4+ T cells activated in the presence of Ctla-4 ligands displayed a global suppression of transcriptional regulation that was more pronounced than TCR monostimulation when compared with cells receiving CD28

costimulation. This suggests that Ctla-4 is capable of differentially regulating transcriptional responses to TCR ligation and CD28-mediated effects on threshold of activation. Moreover, while expression patterns resulting from CD28 and Ctla-4 ligation converge over time, a small subset of genes remains differentially expressed at late timepoints. Thus, it remains likely that Ctla-4 differentially influences CD28 signaling pathways and may provide unique signals independent from CD28. It is tempting to speculate that the stable differential expression of these genes may mediate a long-term phenotypic change reflecting costimulatory conditions. However, during secondary stimulations in vitro, we did not detect changes in activation responses that correlated with the presence or absence of costimulation in the primary stimulation. Instead, T cell recall responses were altered to reflect the overall strength of signals they received in the primary stimulation as measured by responder frequency (Data not shown). Thus. we have failed to identify a lasting phenotype reflective of primary stimulation conditions with regards to CD28 and Ctla-4 ligation. However, our results suggest that Ctla-4 alters the threshold of activation soon after TCR triggering by counteracting CD28-mediated signals, and that this effect persists as long as CD28 signaling is maintained.

At the transcriptional level, we were able to detect significant and stable differential regulation of a small subset of genes by both CD28 and Ctla-4. Interestingly, the most significantly altered transcription factor identified is also a putative quiescence factor. Lklf was specifically downregulated by CD28 ligation in activated CD4+ T cells, and it's expression was maintained in the presence of available Ctla-4 ligands. Thus,

Lklf regulation may reflect the sum of signals received through CD28 and Ctla-4, and the stability of regulation over time indicates that it may mediate long-lasting effects.

CHAPTER V

RESULTS

Initial Characterization of Lklf-Mediated Regulation of Gene Expression

Lklf is putative T cell quiescence factor

Cellular quiescence is a state characterized by decreased cell size and metabolic activity characteristic of unstimulated, mature peripheral lymphocytes. While quiescence was previously considered a default state for lymphocytes in the absence of activating stimuli, it is now clear that active process of gene regulation are involved in the maintenance of the resting state characteristic of peripheral lymphocytes. The gene program regulating quiescence operates through constitutive activity of specific transcription factors that maintain survival while preventing unwarranted cellular activation. Similarly, the induction of clonal anergy restricts T cells to a state of quiescence in the absence of specific signals. Moreover, inhibition or loss of T cell-expressed quiescence factors results in immune activation and lymphoproliferation. Thus, quiescence factors are key regulators of gene programs controlling peripheral self-tolerance and autoimmunity, and are potential candidates for therapeutic manipulation.

Lung Kruppel-like factor (Lklf) is a zinc-finger transcription factor(376) that regulates T cell quiescence and survival(377). The Lklf gene is composed of three exons and is predominantly regulated by a single upstream promoter element (-138/-111bp) that does not correspond to known transcription factor consensus sequences(378). Lklf expression is high in naïve T cells, reportedly down-regulated upon antigen stimulation, and re-expressed in memory T cells as a result of IL-7 stimulation(379) where its expression correlates with long-term survival(380). Lklf expression in naïve T cells is IL-7 independent and is upregulated by ERK5 activation of MEF2 transcription factors(381). Lklf is upregulated two to three fold in Jurkat cells when SHIP-1 is

overexpressed, and correlates with significant growth inhibition(382). Recently, numerous factors critical for Lklf expression have been identified including GRp-78, hnRNP-U and –D, CBF, PCAF, CREB, and SWI/SNF(383). However, the mechanism and signaling pathways responsible for Lklf regulation in lymphocytes remain incompletely understood.

The Lklf protein contains a transactivation domain and an autoinhibitory domain that mediates negative regulation of Lklf transactivation upon binding the E3 ubiquitin ligase, WWP1(384). Lklf-deficiency is embryonic lethal and results in widespread growth and developmental defects in numerous tissues(385). Moreover, Lklf overexpression in certain transformed cell lines is sufficient for the induction of a quiescent phenotype characterized by decreased proliferation, reduced cellular metabolism and cell size, and reduced expression of activation markers on the cell surface(386). Lklf is reported to maintain cellular quiescence by negatively regulating expression of the proto-oncogene, c-myc, and a dominant negative form of c-myc recapitulates many of the phenotypic changes that result from Lklf deficiency(386). Lklf negatively regulates expression of the proto-oncogene Vav in some tissues, a critical determinant of T cell activation and membrane proximal signaling in antigen receptor stimulated T cells(387). Lklf is also reported to reciprocally regulate genes involved in cellular division (p21WAF1/CIP1)(388)and apoptosis (WEE1)(389) Despite its central role in the maintenance of T cell quiescence, little is known about the role Lklf plays during T cell activation.

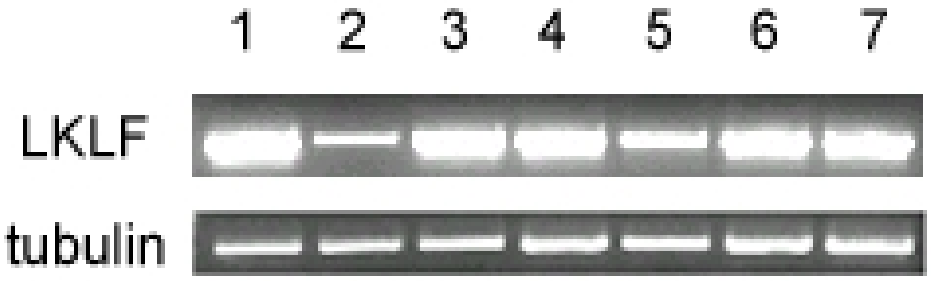
Ctla-4 functions to prevent T cell activation to self-antigen in the periphery, and preserves the quiescent phenotype of naïve T cells. Thus, we proposed that Ctla-4 operated to maintain peripheral self-tolerance by regulating the expression of quiescence factors. An initial screen for genes differentially regulated by Ctla-4 ligation in actively dividing cells identified Lklf as positively regulated by Ctla-4 engagement (Figure 5.1). Inspection of Lklf expression within the context of a kinetic study of global expression profiles in antigen-stimulated TCR-tg T cells under varying conditions of costimulation indicated a central role for CD28 and Ctla-4 signals in the regulation of Lklf expression (Figure 5.2). Moreover, numerous genes regulated by antigen receptor stimulation were coordinately regulated with Lklf upon costimulation, suggesting that integration of costimulatory signals originating with CD28 and Ctla-4 may operate via Lklf to regulate distal events in T cell activation. Thus, we sought to define Lklf-regulated genes as a means of better understanding the genetic programs that result from TCR, CD28, and Ctla-4 ligation.

Expression pattern of Lklf resulting from CD28 and Ctla-4 costimulation

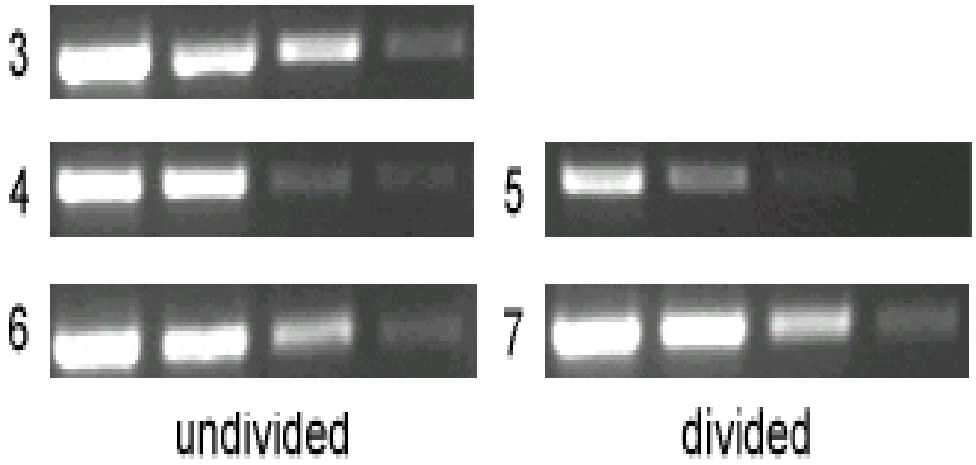
The observed expression of Lklf in our kinetic analysis of genomic expression profiles in activated T cells is internally controlled, ensuring that relative measurements and the trends that result are reliable. However, appropriate data normalization is dependent on certain assumptions that may be invalidated by the high percentage of genes being regulated in activated or dividing lymphocytes. In order to more precisely determine the expression of Lklf in stimulated cells sorted for activation status under

Figure 5.1: Ctla-4 ligation maintains high Lklf expression in dividing CD4+ T cells

A:



B:



1: Ex Vivo
2: Previously stimulated resting cells
3: TCR monostimulated cells, undivided at 60 hours
4: TCR+CD28 stimulated cells, undivided at 60 hours
5: TCR+CD28 stimulated cells, dividing at 60 hours
6: TCR+CD28+Ctla-4 stimulated cells, undivided at 60 hours
7: TCR+CD28+Ctla-4 stimulated cells, dividing at 60 hours

Figure 5.1: Ctla-4 ligation during activation influences Lklf expression in dividing 5C.C7 TCR-tg CD4+ T cells stimulated with TCR and CD28 ligands. 5C.C7 CD4+ lymphocytes were activated in vitro by coculture with engineered APCs expressing varying combinations of ligands for the TCR, CD28 and Ctla-4. 60 hours after stimulation, naïve and dividing lymphocytes were purified by FACS based on dilution of the cytoplasmic dye CFSE. Unstimulated, naïve, peripheral CD4+ 5C.C7 lymphocytes were purified ex vivo. Previously activated cells were obtained by stimulating 5C.C7 CD4+ T cells in the presence of engineered APCs expressing TCR and CD28 ligands. 10 U/ml of IL-2 was added at day 3 after stimulation, and resting previously activated cells were harvested at day 7 after stimulation. RNA was isolated from all samples and RT-PCR was performed to provide semi-quantitative measurement of Lklf and tubulin gene expression. Lklk is highly expressed in naïve peripheral CD4+ lymphocytes, its expression is maintained in non-dividing cells culture in stimulating conditions, and it is downregulated during T cell activation following TCR and CD28 stimulation. Cells activated in the presence of Ctla-4 ligands, in addition to TCR and CD28 stimulation, maintain Lklf expression despite entry into cell-cycle (Panel A). 4-fold serial dilutions indicate that Lklf is maintained at levels nearly 16-fold greater in dividing cells following stimulation in the presence of Ctla-4 ligands relative to TCR and CD28 stimulation (Panel B). Panel B samples were normalized to tubulin expression as seen in panel A.

Figure 5.2: Ctla-4 ligation reverses CD28-mediated Lklf downregulation

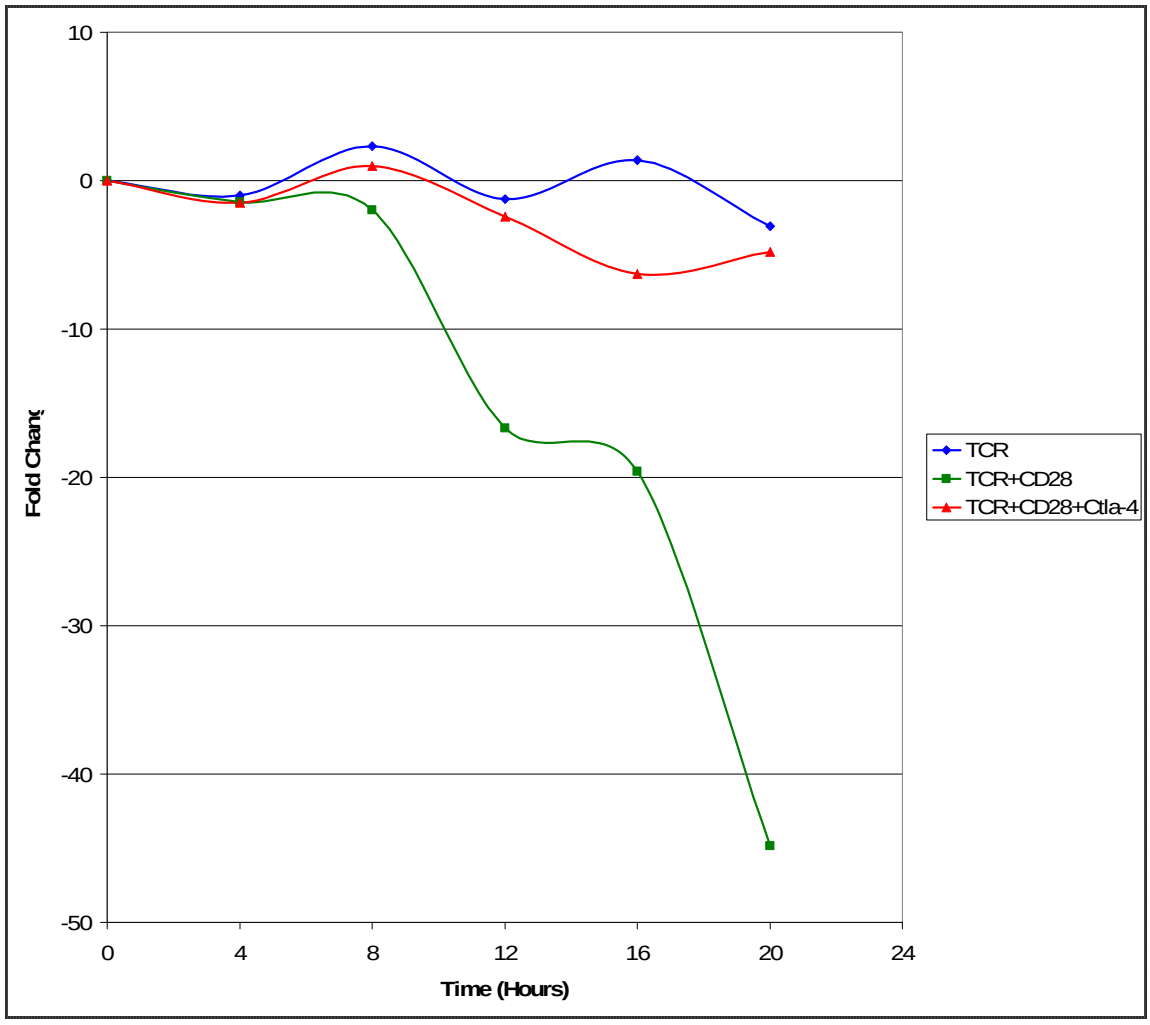


Figure 5.2: Ctla-4 ligation reverses CD28-mediated Lklf downregulation over the critical period of costimulatory signal integration in activated CD69+ CD4+ T cells. 5C.C7 CD4+ lymphocytes were stimulated with engineered APCs presenting MCC in the context of I-E^k in the presence or absence of Ctla-4 ligands in conjunction with CD28 costimulation. Populations purified for the positive expression of CD69 allowed for subsequent microarray analysis of the transcriptome and identification of gene regulation occurring over time in activated lymphocyte populations. Biological duplicates were obtained and average gene expression at each time interval normalized to expression in naïve, unstimulated, peripheral CD4+ 5C.C7 lymphocytes. Measurements represent the average of two experiments per condition, per timepoint. As indicated, costimulatory signals delivered by CD28 and Ctla-4 ligation in this sytem are integrated over a period of 12-16 hours. Lklf expression remains steady in TCR monostimulated cells despite expression of the early activation marker CD69 and commitment to cell-cycle entry as previously shown. CD28 costimulation results in rapid down regulation of Lklf more than 44-fold by 20 hours. T cell activation in the presence of Ctla-4 ligands prevents CD28-mediated Lklf downregulation and maintains Lklf expression at levels comparable with cells activated by TCR monostimulation.

varying condition of costimulation, we performed real-time RT-PCR on the same samples analyzed by microarray.

Lklf primers were designed and tested in our lab. Biological replicates were processed in triplicate, and repeated (Figure 5.3). The observed expression pattern was consistent with the general trend observed by microarray analysis, but indicated that levels of Lklf gene expression detected by microarray were artificially suppressed to an increasing degree beyond 24 hours (data not shown). The divergence of the two datasets over time reflects both the difficulty involved in normalizing microarray data from dramatically different biological samples, and may also reflect a dilution of stably expressed transcripts leading to underestimation of expression and decreased signal intensity and significance.

Genomic Expression Patterns Resulting from Overexpression of Lklf in NFC cells

As a first approach towards understanding the role of Lklf in activated lymphocytes we sought to identify genes regulated by Lklf using microarray analysis. A retroviral expression construct was constructed that included a MSCV promoter, the protein coding region of Lklf, an internal ribosomal entry site (IRES), and the green fluorescent protein (GFP) gene. NFC cells, a double positive thymoma cell line capable of undergoing differentiation, were cocultured with the Phoenix cell packaging line transfected with the Lklf-MSCV or control construct for several days and purified for positive GFP expression by FACS. RNA was isolated from purified Lklf-MSCV- and control MSCV-infected GFP+ populations for microarray analysis. Lklf overexpression

was confirmed in Lklf-MSCV transfected cells by RT-PCR (Data not shown). RNA from FACS-purified Lklf-transfected and control samples was labeled for microarray analysis using 5-8ug of total RNA in a single-step amplification and labeling protocol. Labeled samples were subsequently processed for, and analyzed on, Affymetrix MgU74Av2 microarrays. Three independent experiments were performed starting with transfection of the Phoenix cell packaging line yielding triplicate RNA samples for microarray analysis. Biological replicates were processed for microarray hybridization and scanning at separate times and data analysis was performed using the Genespring expression analysis software.

To allow for the identification of Lklf target genes, the microarray data was normalized as follows. Data transformation was performed such that signal values below 0.01 were set to 0.01. Per chip normalization was accomplished by dividing each measurement by the 50.0th percentile of all measurements in that sample. Specific samples were normalized to one another: values obtained in Lklf transfected samples were normalized to MSCV control samples. Each measurement for each gene in Lklf transfected samples was divided by the median of that gene's measurements in the corresponding MSCV control samples.

Screens to identify differentially expressed genes were performed as follows. An initial analysis was applied to identify genes whose expresseion was altered 2-fold following Lklf overexpression using a Student's T test with p-value cutoff of 0.05 and utilizing the Benjamini and Hochberg multiple testing correction. 766 genes were identified in this initial screen. Genes with detectable expression in at least 3 samples for

Figure 5.3: Real-time quantitative PCR of Lklf in sorted population.

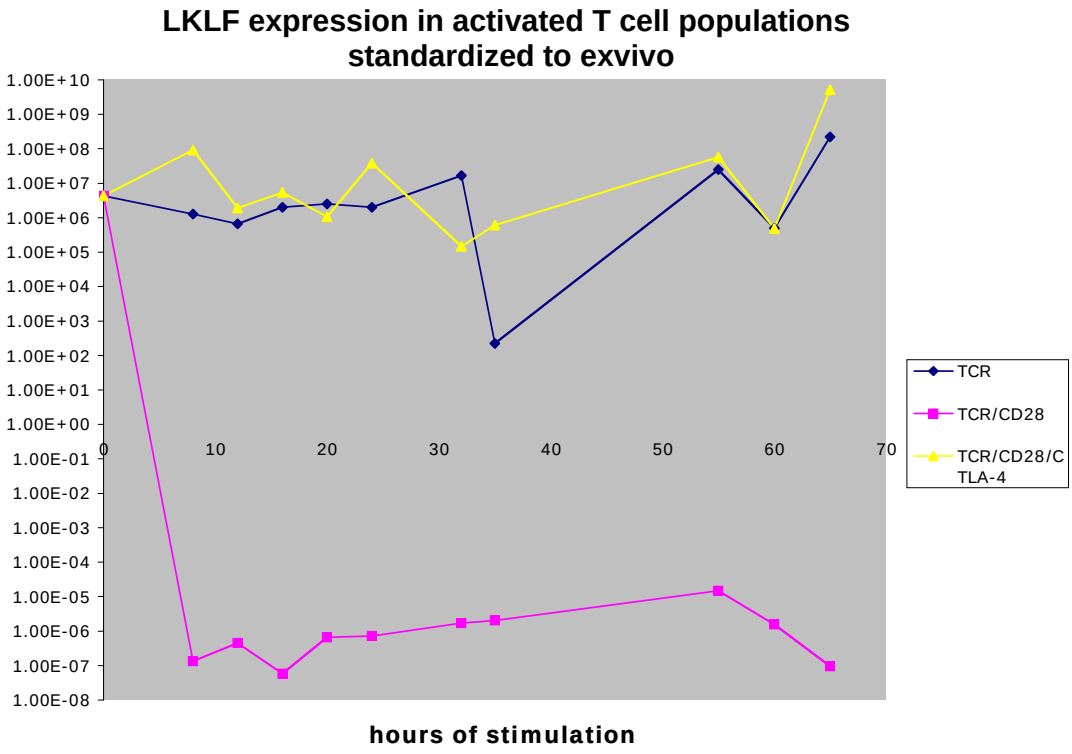


Figure 5.3: Quantitative real-time PCR verifies inhibition of CD28-mediated Lklf downregulation by Ctla-4. 5C.C7 CD4+ lymphocytes were stimulated with engineered APCs presenting MCC in the context of I-E^k in the presence or absence of Ctla-4 ligands in conjunction with CD28 costimulation. Activated lymphocyte populations were purified based on expression of CD69 at early times after stimulation and dilution of the cytoplasmic dye CFSE at late timepoints (>32 hours post-activation). Biological duplicates were obtained for all conditions prior to 24 hours. Real-time PCR reactions specific for Lklf were performed in triplicate on all samples used in the kinetic microarray analysis of expression patterns in activated TCR-tg CD4+ T cells. The results indicate that Ctla-4 ligation provides long-term modification of CD28-induced changes in gene expression despite similarities in activation marker expression, commitment to cell-cycle entry, and division status. Moreover, the stability of Lklf expression detected in TCR monostimulated and Ctla-4 costimulated populations beyond 24 hours exceeds that seen by microarray analysis, suggesting that data normalization at late time-points is inadequate as a result of large-scale alterations in the global expression profile.

both MSCV control and Lklf-transfected samples were screened for fold-changes greater than 3 with p values <0.05 assuming equal variance, and ranked by the magnitude of observed regulation. 176 genes were identified in this screen. Genes detectable in at least three samples in either condition of MSCV control or Lklf-transfected samples but not in the alternate condition were considered present to absent calls and ranked by the magnitude of expression in the condition with detectable expression. An additional 151 genes with significant expression in one condition only were detected in this manner. Thus 766 genes displayed statistically significant ($p<0.05$) regulation resulting in 2-fold or greater differences in expression. Moreover 327 genes were regulated at least 3-fold by Lklf expression or displayed consistent mixed calls (present to absent, or vice versa) between MSCV control and Lklf-transfected samples.

Discussion

Lklf is essential for the maintenance of peripheral T cell quiescence in vivo, and Lklf deficiency result in a predominantly activated phenotype in peripheral T cells. Moreover, Lklf is dramatically downregulated during T cell activation, suggesting that it may serve to restrict or prevent cellular processes leading to cell-cycle entry and gain of effector function. However, a precise understanding of Lklf function is still lacking. Our data suggests that Lklf is differentially regulated by engagement of the TCR or CD28, and may potentially be regulated by Ctla-4. Thus, Lklf expression and subsequent transcriptional regulation of target genes may initiate gene programs resulting in unique phenotypes depending on the specific combination of stimulatory signals received.

A single report has been published describing microarray analysis of Lklf – regulated genes detected in Jurkat cells expressing a tetracycline inducible form of LKLF(386, 390). Induction of LKLF in Jurkat cells generates a quiescent phenotype resembling memory CD4+ T lympohcytes. Approximately 200 transcripts were reportedly altered more than 1.5 fold by LKLF induction with nearly equivalent numbers up and down regulated. Based on a perceived over-representation of cell-surface molecules involved in the initiation or propagation of signaling cascades, the authors conclude that LKLF expression primes quiescent cells for responses to specific extracellular stimuli. However, in our kinetic study of genomic expression patterns in CD4+ T cells undergoing primary activation, the majority of published Lklf target genes identified in Jurkat cells were not significantly regulated, and none of the identified genes showed significant coregulation with Lklf (Data not shown). Thus, it seems likely that genes regulated by Lklf in Jurkat cells may not correlate with Lklf-mediated gene regulation in primary CD4+ T cells. To characterize Lklf-mediated gene regulation, we identified genes differentially regulated in NFC cells, a cell line that may be more physiologically relevant to primary CD4+ T cells than Jurkat cells. In general, the results we obtained upon over-expressing Lklf in NFC cells do not correlate with those published by Buckley et.al(386). Moreover, the amplitude and scope of transcriptional regulation by Lklf detected in NFC cells is significantly greater than those reported to occur in Jurkat cells. Lklf regulates transcription of genes involved in diverse cellular process and is critical for development of numerous tissues. Thus Lklf transcriptional regulation is likely to depend on the concomitant action of numerous other factors

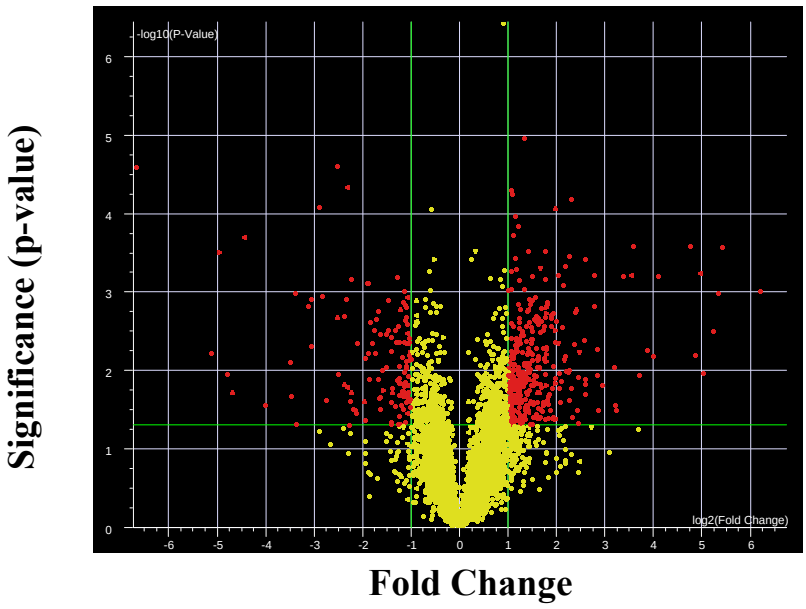
enabling coordinated gene programs in a context-dependent manner. It is possible that the undifferentiated state of NFC cells is more permissive for Lklf activity than transformed leukemic cells derived from mature peripheral lymphocytes. Consequently, the relevance of Lklf target genes identified in cell lines is best assessed by inspection of their regulation in primary cells following antigen receptor mediated stimulation.

Microarray analysis of Lklf regulated genes was performed in triplicate and measures of sample similarity show consistent segregation of MSCV and Lklf-MSCV when compared across all probesets present or across probesets with significant signal. More than six thousand genes had significant signal intensity in 3 of the 6 samples (3 MSCV control and 3 Lklf-transfected samples) analyzed, which is consistent with our earlier results using various cell lines and tissue samples. We detected 1863, 568, and 151 genes with fold changes greater than 2, 3, and 5 respectively based on average expression values. A screen for differential expression with p values less than 0.05 yielded 766, 327, and 111 genes with fold changes of 2, 3, and 5 respectively (Figure 5.4). Nearly equivalent numbers of genes were up- and down-regulated by Lklf.

Lklf-mediated gene transcription is dependent on the cellular context in which it operates and genes identified by enforced overexpression may not recapitulate physiological expression patterns. A screen for Lklf target genes regulated during T lymphocyte activation revealed a subset of genes that may mediate Lklf function following T cell stimulation. Significant expression during in vitro T cell stimulation was defined as detectable expression in 10 of 75 samples processed, and a 3-fold change in expression in at least 5 of 48 conditions. Among the 327 most significantly regulated

Figure 5.4: Identification of gene regulated by Lklf in NFC cells

A:



B:

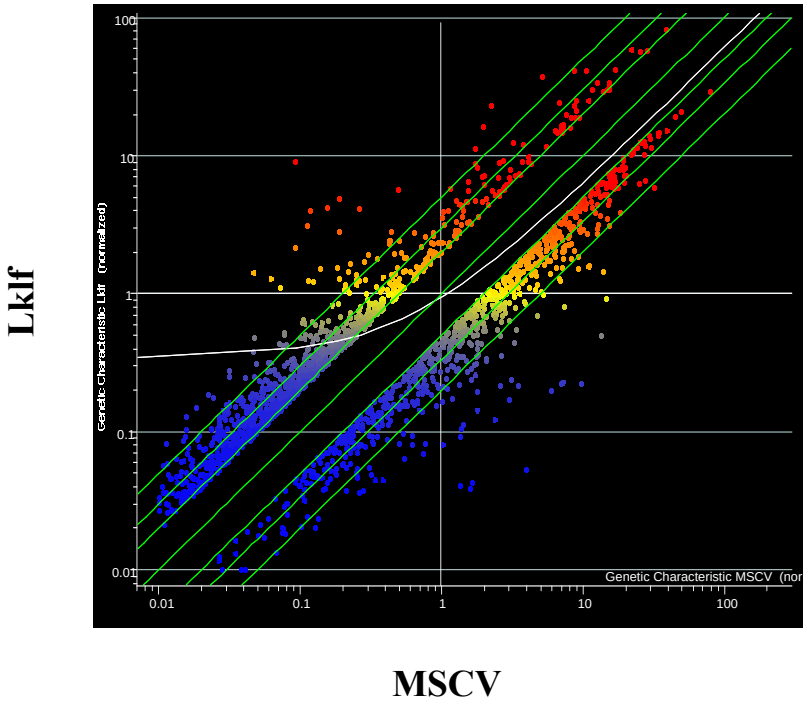


Figure 5.4: Identification of genes differentially regulated by Lklf expression in NFC cells. A MSCV retroviral construct encoding GFP was used to overexpress Lklf in the double-positive thymoma NFC cell line. RNA was isolated from GFP positive NFC cells infected with a control MSCV vector or a Lklf-MSCV vector, and microarray analysis of the global expression profiles that resulted was performed. Triplicate biological samples of both control-MSCV and Lklf-MSCV infected NFC cells were obtained. Data was normalized by settting signal values below 0.01 to 0.01 and dividing each measurement by the 50.0th percentile of all measurements in that sample. In some analyses (Panel A), gene expression values in Lklf expressing samples were normalized to control samples by dividing the measurement for each gene in Lklf-expressing samples by the median of that gene's measurements in the MSCV control samples. Genes whose expression was significantly altered by Lklf expression were identified. A volcano plot (Panel A) shows the relative significance (Y-axis p-value) and fold change distribution (X-axis) of genes in Lklf-expressing NFC cells when compared to control MSCV-infected samples. Individual genes are indicated by dots. Average fold-change expression (log-base 2) is shown on the X-axis. Genes in red display a minimum two-fold change in expression in Lklf-expressing NFC cells with a p-value less than 0.05. A scatter-plot of these genes indicates that Lklf results in widespread positive and negative gene expression changes (Panel B). The average magnitude of signal intensity for specific genes is depicted on a log scale in MSCV control (X-axis) and Lklf-expressing (Y-axis) samples. Indicvidual genes are indicated by dots. The central line indicates equivalent expression. Inner, middle, and outer sets of parallel lines surrounding the line of equivalence indicate 2-, 5-,

and 10-fole changes in expression respectively. Genes displaying less than two-fold changes in expression in Lklf expressing samples relative to controls have been excluded for clarity. The greatest number of significant regulation involved enhanced expression of genes expressed at low level in MSCV control samples (see line of best fit).

genes (>3-fold change in expression, $p<0.05$, assuming equal variances or consistent mixed "present"/"absent" calls) altered by overexpression of Lklf in NFC cells, 92 were considered to be relevant to T cell activation in vitro based on signal detection and expression pattern. 80 of the 92 genes correspond to unique transcripts, and these are listed in bold print in Table 5.1. Manual editing of genes regulated in both systems, revealed 10 Lklf-upregulated target genes with expression patterns consistent with regulation by Lklf during primary cell activation (Listed in red). These included the cell cycle regulator Cdkn1c, the pro-apoptotic transcription factor mad4, and cell surface signaling molecule Cipp/Patj. In contrast, six genes displayed expression patterns that were inversely correlated with results from Lklf overexpression in NFC cells (Listed in blue). These included the transcription factor BatF, the immunomodulatory surface protein Gadd45B/MyD118, and the Bcl2-associated athanogene Bag2. In addition, several genes positively regulated by Lklf in NFC cells were expressed by T cells following activation but displayed expression patterns that failed to coregulate with Lklf expression.

Among genes inhibited in NFC cells following enforced Lklf expression, 14 displayed negative co-regulation with Lklf in activated T cells as expected (Listed in red). These include critical regulators of cell signaling, division, and survival including Cyclin C, the src-like adaptor SLA, and antiapoptotic genes Bcl2a1a and Bcl2a1b. 15 genes showed positive co-regulation with Lklf, in direct contradiction of results in NFC cells (Listed in blue). These include retinoblastoma 1, caspase 8-associated protein 2, Map3K3, Map4K2, and the tyrosine kinase Txk.

Using microarray analysis we have identified genes regulated by Lklf overexpression in the NFC cell line and examined their expression and coregulation with Lklf in a kinetic profile of genomic expression patterns in primary CD4+ cell activated in vitro. A relatively small number of genes with known function identified as Lklf target genes showed significant regulation during primary CD4+ lymphocyte activation. However, the results suggest that a primary mechanism of Lklf maintenance of quiescence may involve upregulation of Mad4. Expression of a dominant-negative MadMyc protein recapitulates many of the effects seen in Jurkat cells overexpressing Lklf, and overexpression of c-Myc reverses the phenotype that results from Lklf overexpression(386). Member of the Mad family of basic-helix-loop-helix/leucine-zipper proteins heterodimerize with Max and function as transcriptional repressors. The balance between Myc-Max and Mad-Max complexes is postulated to control cellular proliferation and differentiation(391). Mad4 forms complexes with Max more efficiently than other Mad family members or Myc (392) suggesting that Mad4 may have a dominant influence on Myc activity. Moreover, overexpression of Mad4 has been shown to induce a replicative senescence-like state in human fibroblasts(393) and Mad4 may mediate some effects of TGF-β in multiple tissues (394, 395). However, to date no specific role for Mad4 has been shown in T lymphoyctes. Our results suggest that Lklf may inhibit unwarranted lymphocyte activation in naïve peripheral CD4+ lymphocytes by suppressing Myc activity via upregulation of the Max dimerization protein Mad4.

Lklf also regulates expression of genes involved in cell survival. Bcl2a1A has been shown to regulate apoptosis induction in B cells after antigen receptor engagement

(396), promote CD8+ lymphocyte survival following 4-1BB stimulation (397), promote thymocyte survival after pre-TCR signaling (398), and is upregulated in CD4+ and CD8+ T cells after TCR engagement in a manner independent from cytokine receptor signaling, bcl-xL induction, or Bcl-2 upregulation (399). Upregulation of Bc2a1 in naïve lymphocytes treated with retinoid x receptor agonists is also independent of effects on Bcl-2 or Bcl-xL and results in decreased apoptosis (400). A specific role for Bcl2a1 in CD4+ lymphocyte activation has not been identified, but our data suggest that Lklf may serve to inhibit Bcl2a1 expression in naïve CD4+ T cells and may enhance apoptosis following antigen receptor triggering in the absence of sufficient CD28 costimulation. Conversely, CD28 costimulation-mediated downregulation of Lklf may result in enhanced Bcl2a1 expression, preventing induction of apoptosis in the short term while allowing sufficient time for additional survival signals delivered by cytokine receptor signaling and subsequent induction of Bcl-2 and Bcl-xL. Bcl2a1 induction is likely cooperative with the CD28-specific downregulation of the apoptosis facilitator Bcl2l11 (Bcl2-like 11), and TCR-mediated induction of Bcl2a1C, bcl2l1, and Bcl2l2.

In order to assess the inclusiveness of our screen for genes regulated by Lklf during primary T cell activation, we performed an analysis to identify genes whose expression pattern correlated with Lklf in our kinetic study of gene expression profiles. Among 505 genes with the greatest negative correlation to Lklf during T cell activation,

Table5.1.1: Genes upregulated by Lklf

Category	Common Name	Fold Change	
Cell Cycle Regulators	Tgf-alpha	8.4	Tranforming growth factor-alpha
	Cdkn1c	7.5	Cdk inhibitor
	Ros1	6.0	Protein kinase, proto-oncogene
	Cxcl12	4.5	Growth factor
	Cxcl1	4.5	Growth factor
	Pkd2	3.6	Jak-Stat pathway
	Fgf5	3.1	Growth factor
Transcriptional Regulation	Lklf	28.1	DNA-dependent TF
	Lyl1	11.2	DNA-dependent TF
	TgfB1i4	10.6	DNA-dependent TF
	Batf	6.8	DNA-dependent TF
	Hist1h1c	6.5	Nucleosome organization
	Klf12	5.6	DNA-dependent TF
	FoxC1	5.2	DNA-dependent TF
	Mad4	3.7	Mxd4, Max dimerization protein 4
	Hist1h2bc	3.6	Nucleosome organization
	Six5	3.2	DNA-dependent TF
	Etsrp71	3.0	DNA-dependent TF
Immune Defense	Cd2/Ly37	10.1	LFA-3 receptor, Protein kinase, adhesion
	Prf1	5.7	Perforin 1, Cytolysis, Ca sensitive
	Klra4	4.2	Heterophilic cell adhesion
	Litaf	3.7	LPS-induced TNF
	Gadd45B	3.3	Negative growth-regulatory protein MyD118
	Pik3cd	3.3	PI3K catalytic domain
Apoptosis Factor	Bag2	4.3 to 3.4	Chaperone, Bcl2-associated athanogene
	Casp4	3.9	Apoptosis induction
	Mad4	3.7	Mxd4, Max dimerization protein 4
Signaling Molecule	Cipp	8.3	INADL, Patj, Protein binding
	Gnat1	6.1	G-protein signaling
	Cer1	5.5	Inhibits BMP signaling
	Rasgrf2	4.7	GEF activity
	Scn9a	4.6	V-gated ion channel
	3830613022	4.5	G-protein signaling
	Map2k1	3.9	Mapk signaling
	Pkd2	3.6	Jak-stat pathway

Table5.1.1: Genes Upregulated by Lklf

Category	Common Name	Fold Change	
Cellular Adhesion And Cytoskeleton	Selenbp1	101.6	Selenium binding protein
	Tmsb4x	34.8	Actin Binding
	Agc1	27.8	Binds hyaluronic acid
	Mmp8	15.5	Collagen catabolism
	Gsn	8.4	Gelsolin, Actin binding
	Capg	6.9	Actin binding
	Asgr1	5.3	Endocytosis, adhesion
	Strm	3.9	Striamin
Metabolic/Synthetic Processes	Siat7a	11.2	Sialyltransferase 7A, Ganglioside biosynthesis
	Fabp4	8.7	Lipid transport
	Ptgs2	5.1	Prostaglandin synthesis, cyclooxygenase 2
	Aldh3a1	5.1	Metabolic pathway
	Tgm2	5.0	Proteolysis, peptidolysis
	Nqo1	4.6	NAD(P)H dehydrogenase quinone 1, Electron transport
	Tgm2	5.0	Transglutaminase2
	Sth2	3.8	soft tissue heal 2, Steroid Metabloism
	Txnip	3.7	VDUP1, thioredoxin interacting protein
	Tcn2	3.6	Transcobalamin 2
	Smpdl3a	3.1	Sphingomyelin phosphodiesterase

Category	Common Name	Fold Change	
Unknown	Mkrn3	4.4	Makorin-3, Zfp127
	Tfpi	3.6	Tissue factor pathway inhibitor
	Rere	3.4	Atr2, atrophin-2
	Gkap42	3.1	G kinase anchoring protein
	Drd3	3.1	EST
	937302	5.2	
	Eppb9	5.0	endothelial precursor protein B9

Table5.1.2: Genes down-regulated by Lklf

Category	Common Name	Fold Change	
Cell Cycle Regulation	Bin1	-5.0	Bridging Integrator, Myc box dependent interacting protein 1, Sh3p9
	Ccnc	-3.1	Cyclin C
	Rnf2	-3.3	Chromatin binding
	Rb1	-3.3	Retinoblasoma-1, Cell-Cycle Inhibitor
	Cops2	-3.7	Signalosome subunit
Transcriptional Regulation	Pola1	-3.4	DNA polymerase
	Pnn	-3.4	Pinin, DNA-dependent TF
	Cnot7	-3.1 to -5.8	DNA-dependent TF
	NP220	-3.4	Zfml, matrin-like protein, Zinc finger protein 638, Znf638
	Rora	-3.5	RAR-related orphan receptor alpha
	Smarca3	-3.6 to -6.0	SNF2/SWI2 family member
	Cops2	-3.7	COP9 signalosome complex subunit 2, Csn2, SGN2, Trip15
	Gabpa	-3.8	GA binding protein alpha chain
	Tcfap2c	-3.9	AP2 TF
	Zfpn1a2	-5.4	DNA-dependent TF
	Idb3	-6.0	Id3, inhibits Pol II
	Hells	-6.8	LSH, PASG, lymphoid-cpecific helicase
	Idb1	-12.5	Id1, inhibits Pol II
	HIVep1	-12.9	HIV enhancer binding
Immune Defense	Rasa1	-3.0	RasGAP, RAS p21 protein activator
	Cd2ap	-3.1	CD2-associated protein
	Rock1	-3.1	Rho-associated coiled-coil forming kinase
	CD99	-9.1	
	Ltb	-9.4	Lymphotoxin B
	CD48	-11.7	BCM1, BLAST1, OX-45
	Ctsw	-12.1	Cathepsin W
	Cst7	-18.8	Leukocystatin
	Gzma	-31.3	Granzyme A
Apoptosis Factors	Casp8ap2	-4.6	FLASH, caspase 8 associated protein 2
	Tnfaip3	-6.7	Tumor necrosis factor, alpha-induced protein 3, zinc finger protein A20
	Notch1	-6.7	
	Nr4a1	-7.2	DNA-dependent TF, Caspase inhibitor
	Bcl2	-7.5	
	Bcl2a1a	-15.9	Bfl1
	Bcl2a1b	-31.0	aka Bcl2a1c
Cellular Adhesion And Cytoskeleton	Itgb7	-4.0	Integrin-beta 7
	Kif5b	-3.9	Kinesin family member 5B

Table5.1.2: Genes down-regulated by Lklf

Category	Common Name	Fold Change	
Signaling Molecule	Acvrl1	-3.4	Activin receptor-like kinase 1, kinase activity
	Gem	-3.4	RAS-like protein KIR,G-protein signaling
	Arhgdig	-3.4	RHO GDI-gamma
	Rock1	-3.7	Rho-associated coiled-coil forming kinase 1
	Map3k3	-3.7	MAPKKK3, Mekk3
	Sla	-4.1	Slap, Src-like adaptor
	Ptprc	-4.7 to -6.8	Protein tyrosine phosphatases receptor
	Rock2	-4.8	Rho-associated coiled-coil forming kinase 2
	Ramp2	-4.8	G-protein signaling
	Map4k2	-5.5	MAPK signaling
	Sh2d1a	-6.1	Sap, Xlp, SLAM-associated protein
	Gpr65	-10.4	G-protein signalin
	Txk	-17.2	Tyrosine kinase
Metabolic/Synthetic Processes	Abcb7	-3.0	ATP-binding cassette transporter 7
	Edem1	-3.1	Mannosidase alpha-like1, ER degradation enhancer
	Slc30a4	-5.2	Zinc transporter
	Tfrc	-5.4	Transferrin receptor
	Kcna3	-6.0 to -32.8	Potassium channel
	Man1a	-7.6 to -40.5	Mannosidase 1 alpha

Table 5.1.2: Down-regulated by Lklf

Category	Common Name	Fold Change	
Unknown Activity	Ube2v2	-3.0	Ubiquitin-conjugating enzyme E2 variant 2
	1110013I07	-3.1	Riken cDNA
	261010N10	-3.1	Riken cDNA
	2010300G19	-3.2	Riken cDNA
	1700065A05	-3.2	Riken cDNA
	Bat9	-3.2	zinc finger and BTB domain containing 12
	Rbms1	-3.2	Mssp, RNA binding motif, single stranded interacting protein 1
	Adm	-3.2	Adrenomedullin
	97154_f_at	-3.2	EST
	4921518A06	-3.3	Riken cDNA
	Siat8d	-3.3	Sialyltransferase 8
	Cspg6	-3.3	Chondroitin sulfate proteoglycan 6
	Tbc1d15	-3.3	EST
	Rnf2	-3.3	Ring1B
	Sema4a	-3.4	Receptor
	Pnpt1	-3.4	polyribonucleotide nucleotidyltransferase 1
	D3Ertd330e	-3.4	94395_at
	2310005N03	-3.5	Riken cDNA, Hnrpu
	CD52	-3.5	CAMPATH-1 (Cdw52)
	Fndc3	-3.8	EST
	Smarca5	-3.9	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
	Narg1	-3.9	Nat1, NMDA receptor-regulated gene 1
	Serpin1	-4.1	serine (or cysteine) proteinase inhibitor
	Hrb2	-4.1	HIV-1 Rev binding protein 2
	Matr3	-4.1	matrin 3
	Lypla1	-4.2	lysophospholipase 1
	Rcbtb1	-4.4	regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1
	Tm4sf9	-4.5	TM-4 family member 9
	Crtam	-4.6	Surface protein
	Ptbp2	-4.6	polypyrimidine tract binding protein 2
	Atp1a1	-4.8	ATPase, Na+/K+ transporting, alpha 1
	Zfp146	-5.1	OZF, zinc finger protein 146
	Pscd1	-5.3	Pleckstrin homology, Sec7, coiled-coil domains 1
	Zfpn1a2	-5.4	zinc finger protein, subfamily 1A, 2 (Helios)
	Spred2	-8.8	sprouty-related, EVH1 domain containing 2
	Bub1	-12.5	Mitotic checkpoint serine/threonine-protein kinase
	Htatip2	-14.6	HIV-1 tat interactive protein 2, homolog
	1190002N15	-27.0	Riken cDNA
	Cd53	-37.6	Ox-44, Tspan25
	Emb	-42.7	EST
	Itm2a	-73	integral membrane protein 2A

Table 5.1: Overexpression of Lklf in NFC cells identifies potential target genes in primary CD4+ T cells. A retroviral vector encoding GFP was used to overexpress Lklf in double-positive thymoma NFC cells. Control-MSCV and Lklf-encoding MSCV vectors were used to infect NFC cells. Infected cells were purified by FACS based on expression of GFP. Triplicate samples were prepared and processed for microarray analysis. Genes differentially regulated upon Lklf overexpression in NFC cells were identified and their expression pattern in primary 5C.C7 CD4+ lymphocytes stimulated in vitro under varying conditions of TCR, CD28, and Ctla-4 ligation examined. 80 genes identified as differentially regulated by Lklf in NFC cells showed significant expression and/or regulation in 5C.C7 CD4+ primary cell activated in vitro. Genes coregulating with Lklf during primary CD4+ T cell activation in a manner consistent with their regulation by Lklf in NFC cells are listed in red print. Genes with expression patterns in primary cells that reflect an inverse relationship with Lklf relative to that seen in NFC cells are listed in blue. Genes identified as Lklf targets in NFC cells that have variable expression patterns without significant co-regulation with Lklf in primary CD4+ T cells stimulated in vitro are listed in black.

Figure 5.5: Hierarchical clustering of Lklf target genes based on kinetic expression profiles in activated CD4+ T lymphocytes

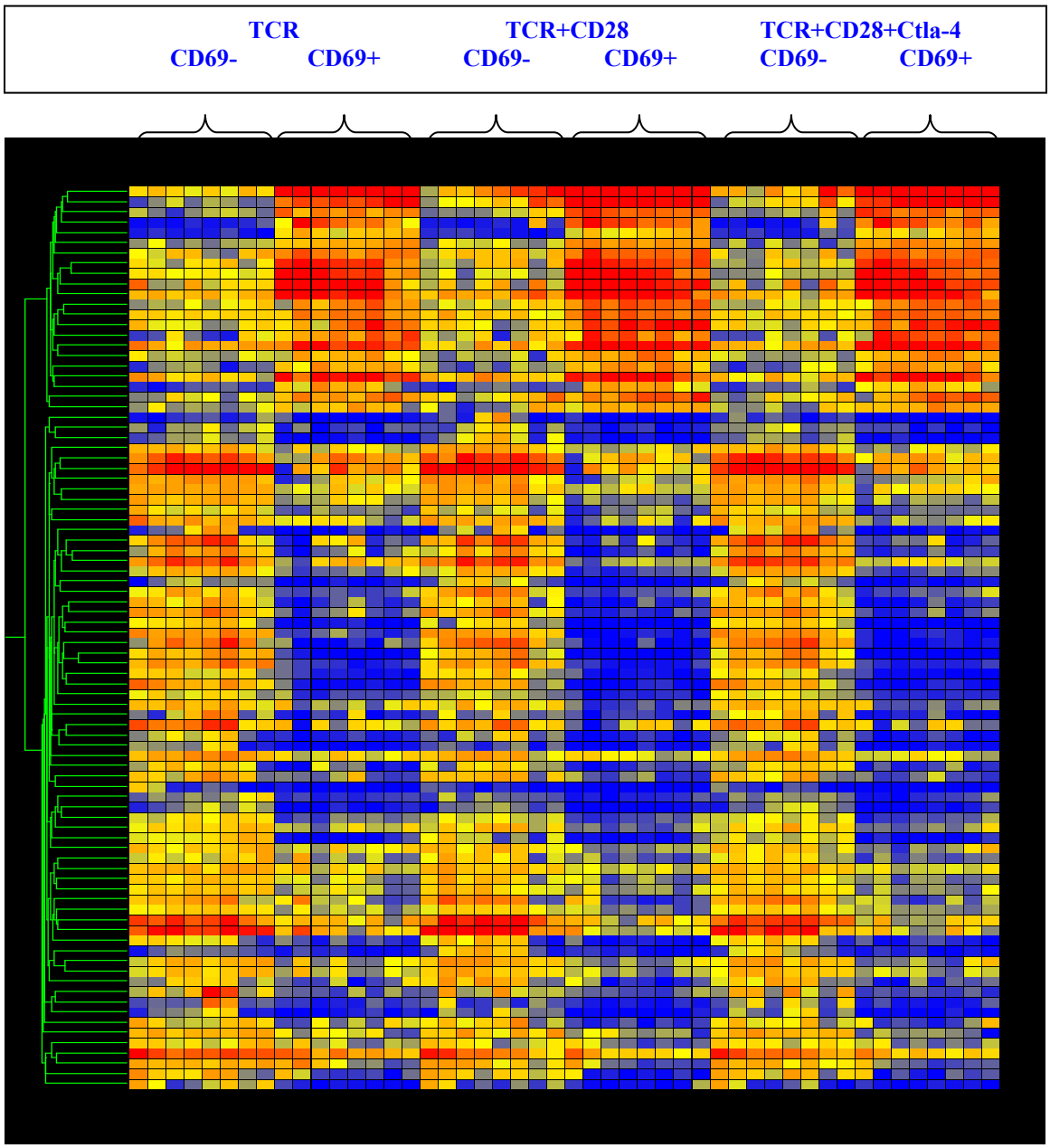


Figure 5.5: Hierarchical clustering of Lklf target genes in kinetic study of genomic expression patterns in CD4+ T cells undergoing primary activation in vitro. 5C.C7 CD4+ lymphocytes were stimulated in culture by engineered APCs expressing different combinations of ligands for the TCR, CD28, and Ctla-4. Populations of naïve and activated lymphocytes were purified by FACS at discrete times between 4 and 60 hours and their global expression profiles analyzed by microarray. Genes with detectable expression in at least 10 of 78 samples analyzed were ranked by their degree of positive or negative co-regulation with Lklf across all stimulation conditions, states of activation, and timepoints based on their Spearman correlation values. More than 1000 genes displaying some measure of co-regulation with Lklf were cross-referenced with Lklf target genes identified by retroviral overexpression of Lklf in the NFC cell line. All 88 genes represented displayed statistically significant ($p<0.05$) regulation upon enforced Lklf expression in NFC cells analyzed in triplicate on microarrays. Hierarchical clustering based on expression patterns in 5C.C7 CD4+ lymphocytes undergoing primary activation in vitro was performed to characterize the temporal and qualitative relationship of potential Lklf target genes significantly regulated in primary CD4+ T cells. Genes included in the above diagram are listed in Table 5.2 according to known function and degree of regulation following over expression in NFC cells. These genes had not been identified as significantly regulated by Lklf in prior screens that utilized search algorithms that biased results based upon the magnitude of regulation resulting from Lklf over-expression in NFCs. Moreover, genes identified by initial screens for co-regulation

in primary cells were twice as likely to predict qualitative regulation by Lklf following over-expression in NFC cells than analyses performed in the reverse order.

Table 5.2

Categoory	Fold Change	LkIf Correlation	Common Name	Description
Cell cycle	2.2	-0.751	Jun	Jun oncogenes
	-3.4	-0.698	Qscn6	Quiescin Q6
Apoptosis	2.4	-0.762	Btg2	B-cell translocation gene 2, anti-proliferative
Transciption	6.7	-0.782	Rere	
	6.5	-0.716	Hist1h1c	histone 1, H1c
	2.3	-0.803	Cebpd	CCAAT/enhancer binding protein delta
	2.1	-0.771	Hdac5	Histone deacetylase 5
	-2.7	-0.689	Pcaf	p300/CBP-associated factor
Translation	2.4	-0.732	Cpeb1	cytoplasmic polyadenylation element-binding protein
Immune Response	6.3	-0.832	Samhd1	SAM domain- and HD domain-containing protein 1
	4.3	-0.691	G1p2	interferon, alpha-inducible protein
	3.8	-0.827	Ian1	immune associated nucleotide 1
	2.8	-0.767	Imap38	immunity-associated protein
	2.1	-0.774	Ly6c	lymphocyte antigen 6 complex, locus C
	2.1	-0.68	Ctsl	Cathepsin L
	-2.2	-0.686	Ifit2	interferon-induced protein with tetratricopeptide repeats 2
	-3.2	-0.753	Tap2	transporter 2, ATP-binding cassette, sub-family B
Signaling	4.2	-0.797	Tgtp	T-cell specific GTPase
	3.4	-0.867	Btbd14a	BTB (POZ) domain containing 14A
	2.5	-0.782	Il7r	interleukin 7 receptor precursor
	2.4	-0.696	Gbp2	guanylate nucleotide binding protein 2
	2.3	-0.898	Il6ra	interleukin 6 receptor, alpha
	2.2	-0.8	Fln29	IFN and LPS inducible, negatively regulates TLR signaling
	2.1	-0.678	Frat2	frequently rearranged in advanced T-cell lymphomas 2
	-2.1	-0.895	Tec	cytoplasmic tyrosine kinase, Dscr28C related
	-2.1	-0.682	Sema4d	semaphorin 4D
	-2.2	-0.736	Sh3bp5	SH3-domain binding protein 5 (BTK-associated)
	-2.3	-0.8	Il27ra	interleukin 27 receptor, alpha
	-2.3	-0.721	Itpr5	inositol trisphosphate receptor type 2
	-2.4	-0.734	Map3k3	mitogen activated protein kinase kinase kinase 3
	-2.5	-0.695	Nsg2	neuron specific gene family member 2
	-3.5	-0.871	Txk	TXK tyrosine kinase
	-3.5	-0.712	Cd3d	CD3 antigen, delta polypeptide
Adhesion	23.9	-0.781	Cd9	CD9 antigen
Cytoskeleton	2.0	-0.848	Map1lc3b	microtubule-associated protein 1 light chain 3 beta
	-2.5	-0.75	Tubb2	tubulin, beta 2

Table 5.2

Categoory	Fold Change	Lklf Correlation	Common Name	Description
Metabolism	5.3	-0.88	Dhrs8	dehydrogenase/reductase (SDR family) member 8
	3.7	-0.917	Dntt	deoxynucleotidyltransferase, terminal
	2.5	-0.92	Dntt	Riken cDNA
	2.1	-0.831	Ctss	Cathepsin S
	2.1	-0.706	Kcnj8	potassium inwardly-rectifying channel J8
	2.1	-0.878	Cyp2s1	cytochrome P450, family 2, subfamily s, polypeptide 1
	2.0	-0.733	Cyp4f13	
	2.0	-0.798	Abca1	
	-2.1	-0.736	Hdc	histidine decarboxylase
	-2.5	-0.719	Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)
	-4.5	-0.794	Ugcg	UDP-glucose ceramide glucosyltransferase
	-5.5	-0.712	Cnga1	cyclic nucleotide gated channel alpha 1
Miscellaneous	2.3	-0.773	Daf1	decay accelerating factor 1
	2.3	-0.737	Dnahc8	dynein, axonemal, heavy chain 8
Unknown	26.0	-0.694	0610011I04 Rik	hepatocellular carcinoma-associated antigen 112
	6.2	-0.678	cDNA clone IMAGE:100 5369	
	4.7	-0.691	Sntb1	Riken cDNA
	2.3	-0.678		similarity to protein ref:NP_032607.1 (M.musculus) melanoma antigen
	2.2	-0.685	Itm2c	integral membrane protein 2C
	2.2	-0.685	ud35h06.r1	
	2.2	-0.743	Usp18	ubiquitin specific protease 18
	2.1	-0.801		EST
	2.1	-0.913	1500005K1 4Rik	Riken cDNA
	-2.0	-0.894	5830431A1 0Rik	
	-2.2	-0.776	Osbpl5	EST
	-2.2	-0.683	6330442E1 0Rik	Riken cDNA
	-2.6	-0.744	4930422J18 Rik	EST
	-2.6	-0.741	Gbp3	EST
	-2.7	-0.819	BC052328	EST
	-3.1	-0.855	2810052M0 2Rik	Riken cDNA

Table 5.2

Categoory	Fold Change	LkIf Correlation	Common Name	Description
Cell Cycle	2.6	0.829	Gfi1	growth factor independent 1
	-3.4	0.814	Arhgdig	Rho GDP dissociation inhibitor (GDI) gamma
Transcription	-2.0	0.807	Rorc	RAR-related orphan receptor gamma
	-2.2	0.803	Sap30	sin3 associated polypeptide
	-2.2	0.865	Ddx18	Myc-regulated DEAD-box protein
	-2.5	0.862	Gtf2h4	general transcription factor II H, polypeptide 4
	-2.8	0.913	Idb2	inhibitor of DNA binding 2
Signaling	4.3	0.809	Sdc4	syndecan 4
	-2.1 to -2.7	0.873	Pla2g12a	phospholipase A2, group XIIA
Immune	2.7	0.824	Tnfsf11	tumor necrosis factor (ligand) superfamily, member 11
	-2.8	0.938	Lta	lymphotoxin A
Metabolism	2.5	0.9	Alad	aminolevulinate, delta-, dehydratase
	2.2	0.853	Fabp5	fatty acid binding protein 5, epidermal: mal1; Fabpe gene.
	-2.1	0.806	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1
	-2.3	1	Hk2	hexokinase 2
	-2.8	0.878	Car12	carbonic anyhydrase 12
	-2.2	0.856	Tnnt1	troponin T1, skeletal, slow
Unknown	-2.2	0.922	AA408556	hypothetical protein LOC107094
	-2.2	0.811	D3Ertd330e	DNA segment, Chr 3, ERATO Doi 330, expressed
	-2.7	0.87	Dnmt3a	vv58e04.r1 Soares_thymus_2NbMT
	-3.0	0.836	1200007D18Rik	hypothetical protein LOC67458

Table 5.2: Identification of potential Lklf target genes in primary CD4+ lymphocytes based on initial measures of coregulation. Microarray analysis was performed to characterize the global expression profile of 5C.C7 CD4+ lymphocytes activated in vitro by APCs engineered to express varying combinations of ligands for the TCR, CD28 , and Ctla-4. Naïve and activated populations were isolated at discrete times from 4-60 hours after initial stimulation based on expression of activation markers and division status. Genes with detectable expression in at least 10 of 78 samples analyzed were ranked by their degree of coregulation with Lklf across all timepoints, activation states, and stimulation conditions. Genes with the greatest degree of positive or negative co-regulation with Lklf in primary cells stimulated in vitro were cross referenced with genes differentially expressed in NFC cells overexpressing Lklf. Co-regulation with Lklf in primary cells accurately predicted the qualitative nature of Lklf-mediated changes in gene expression in NFC cells. In contrast, the magnitude of Lklf-mediated regulation in NFC cells did not accurately predict qualitatitve (positive or negative) co-regulation with Lklf in primary cells stimulated in vitro. Genes initially identified by their coregulation with Lklf in primary CD4+ 5C.C7 lymphocytes that had not been previously identified as significant Lklf target genes are shown in Table 5.2. The magnitude of the fold change in expression seen with Lklf overexpression in NFC cells is listed. Correlation values for expression profiles detected in primary CD4+ 5C.C7 T cells is shown relative to Lklf. Correlation values less than 0 indicate positive coregulation, and correlation values greater than 0 indicate negative coregulation in primary cells. Genes displaying a qualitatively consistent relationship with Lklf expression in both NFC and primary CD4+

T cells are shown in red. Genes displaying inverse relationships in the two systems are shown in blue.

27 genes (5.3%) showed statistically significant changes in expression of at least two-fold upon Lklf overexpression in NFC cells. 22 of the 27 genes (81%) had not been previously identified in our analysis as important Lklf targets based on the magnitude of their regulation by Lklf in NFC cells. Similarly, among 503 genes that positively co-regulated with Lklf during T cell activation in vitro, 80 genes (16%) showed at least 2-fold changes that were statistically significant ($p<0.05$) following Lklf overexpression in NFC cells. 66 of the 80 genes (83%) had not been previously identified as important Lklf target genes as a result of the small fold-changes observed in NFC cells upon Lklf overexpression. These results strongly suggest that for genes undergoing dynamic regulation across a variety of conditions and sampled at numerous times, measures of correlation based on extended kinetic profiles provides greater predictive value of physiologically-relevant gene interaction than the magnitude of resultant gene expression changes detected in the context of isolated overxpression. Hierarchical clustering of genes regulated more than two fold by Lklf expression in NFCs displaying coregulation with Lklf in activated primary CD4+ T cells is shown in Figure 5.5.

Among genes altered more than two-fold by Lklf expression in NFC cells, the magnitude of regulation detected did not correlate with the likelihood of consistent gene co-regulation with Lklf in primary T lymphocytes following stimulation. However, measures of co-regulation in a kinetic study of T cell activation reliably predicted the qualitative regulation exerted by Lklf over-expression in NFC cells. Thus, 83% of genes evidencing negative co-regulation with Lklf in the kinetic study were depressed in NFC

cells over-expressing Lklf. A summary of Lklf regulated genes identified by statistical comparison of gene expression profiles in primary CD4+ T cells stimulated in vitro is presented in Table 5.2.

Our data suggest that Lklf expression is not sufficient for the maintenance of a naïve phenotype. Instead, Lklf expression is differentially regulated by costimulatory signals triggered by CD28 and Ctla-4 ligation and may mediate unique, long-term phenotypic changes as a result. The lack of overt autoimmunity and pathological disease in mice containing Lklf-deficient lymphocytes suggests that mechanisms of peripheral tolerance remain intact. The tendency of Lklf-deficient lymphocytes to display an activated phenotype may result from altered expression of only a few genes involved in regulating threshold of activation or cell cycle progression, consistent with its down-regulation by CD28 ligation. For example, in NFC cells Lklf up-regulates cyclin C while down regulating the cyclin-dependent kinase inhibitor Cdkn1c (p57KIP2). p57Kip2 is known to inhibit both cyclin A- and cyclin E-associated cdk2 kinase activities resulting in decreased cell proliferation and DNA synthesis(401). Similarly, cyclin C has been shown to act cooperatively with c-myc to induce cdc2 expression and promote G1/S and G2/M cell cycle transitions(402). Additional genes involved in cell cycle regulation (e.g. jun, Gfi1), signaling (e.g.Cipp, sla, Gpr65, IL7R, FRAT2, Tgtp, etc), and transcriptional regulation (e.g. Pola1, Hells, Cebpd, Hdac5) were identified as Lklf target genes co-regulating with Lklf in primary T cell activation. Literature supports the notion that altered expression of these genes may predispose lymphocytes to become activated in the absence of Lklf.

Several genes encoding important T cell signaling molecule that were regulated by Lklf in NFC cells possessed contradictory expression patterns in activated primary T cells. Many of these genes, including Tec, Txk, Map3k3, Map4k2, and CD3-delta are central to the propagation of antigen-receptor signals. All of these were downregulated by Lklf in NFC cells, and increased expression in Lklf-deficient lymphocytes could lead to increased antigen receptor sensitivity in the periphery and inappropriate activation. Similarly, Gadd45-beta, was upregulated by Lklf in NFCs but negatively co-regulated with Lklf in primary T cells. Gadd45-beta deficiency predisposes mice to the development of spontaneous autoimmune lymphoproliferation(403), and decreased expression in the context of Lklf-deficiency may partially explain the $Lklf^{-/-}$-phenotype. The positive co-regulation of these genes with Lklf in activated primary T cells committed to cell-cycle entry is likely to reflect the influence of dominant mechanisms of regulation that result from TCR triggering.

In summary, we have shown that Lklf is regulated by costimulatory signals mediated by CD28 and Ctla-4. Moreover, we have identified genes significantly regulated by Lklf expression in a thymoma cell line and characterized their expression pattern in a kinetic study of activated T cells under varying condition of costimulation. Our results indicate that a specific subset of dysregulated genes may explain the predominantly activated phenotype of Lklf-deficient peripheral T lymphocytes. Moreover, we have identified likely targets of Lklf in primary cells that could potentially explain observed differences in phenotype that result from varying costimulation conditions. Many of the identified Lklf target genes regulated during primary CD4_ T

cell activation, specifically Bcl2a1 and Mad4, have not been well characterized in this context. This work provides the basis for further exploration of mechanisms underlying the maintenance of peripheral tolerance, costimulation, and regulation of T cell responsiveness.

CHAPTER VI

DISCUSSION

The central importance of costimulatory signals in immune regulation has been shown in numerous disease models and is evident in the phenotype of $CD28^{-/-}$ and $Ctla\text{-}4^{-/-}$ mice. While CD28 signaling pathways have been relatively well defined, attempts to define the mechanisms by which Ctla-4 inhibits T cell activation and maintains peripheral tolerance have been hampered by the complexities of Ctla-4 expression, ligand binding, and signal integration. Although much has been learned about the proximal events associated with CD28 and Ctla-4 ligation, relatively little is known about distal effects of costimulatory signal integration that may underlie qualitative aspects of immunity. While CD28 has been shown to reciprocally regulate genes necessary for quiescence and T cell activation, a definitive role for Ctla-4 in this process has not been elucidated. Thus we sought to define the transcriptional targets involved in mediating Ctla-4 tolerance induction.

Progressive T cell activation is dependent on changes in gene transcription initiated by signals transmitted through the TCR. CD28 is reported to greatly enhance the TCR-induced transcriptional response in a primarily quantitative manner. Ctla-4 reportedly counteracts CD28 signaling and mediates a partial inhibition of gene transcription upon T cell activation. We have proposed that Ctla-4 ligation results in the specific regulation of genes necessary for the maintenance of a naive phenotype such as quiescence factors and that these changes result in altered responsiveness of the T cell to further stimulation. To provide a contextual framework for the identification of potential mediators of Ctla-4 function, we utilized an in vitro stimulation system capable of differentially signaling

through the TCR, CD28, and Ctla-4 to facilitate the generation of an extensive database of gene regulations occurring during primary activation of CD4+ T cells experiencing varying combinations of antigen receptor stimulation and positive and negative costimulation. Microarray analysis was performed on FACS-purified CD4+ T cells stimulated under varying conditions of costimulation and homogenous for activation hisotry, expression of activation markers, and division status. In addition, stringent control of activation conditions made possible by the use of engineered APCs and TCR-tg T cells facilitated further characterization of the temporal parameters of antigen receptor and costimulatory signal integration. The database of gene regulations permits extensive data mining and the application of combined bioinformatics approaches. Our results are relevant to our understanding of autoimmune disease, peripheral T cell tolerance, and T cell differentiation. Furthermore our results are relevant to numerous other model systems, such as those seeking to explain the phenomenon of anergy induction in lymphocytes. Understanding the mechanisms involved in costimulatory signal integration, the long term effects of CD28 and Ctla-4 ligation, and the intermediates bridging the two, will be essential for the productive manipulation of costimulation pathways in clinical applications.

In general, our results clearly indicate that TCR ligation in the absence of costimulation is capable of activating all signaling pathways necessary for commitment to activation and cell-cycle entry within 1-2 hours. TCR signaling induces a dynamic transcriptional response characterized by reciprocal regulation of several thousand genes, including key determinants of cellular activation and quiescence. In CD4+ T cell subpopulations

purified on the basis of activation marker expression, TCR signaling potently upregulates genes necessary for long-term survival and effector function to levels comparable with those seen subseqeuent to CD28 costimulation, including interleukin-2, CD25, and Bcl-xL. CD28 enhances proximal TCR-signaling events leading to CD4+ T cell activation as evidenced by dramatically enhanced responder frequency to a broad range of antigen concentrations within 1-2 hours. In addition, CD28 transmits unique signals that can be integrated with sub-mitogenic TCR signals over an extended period of 12-16 hours. Thus, CD28 functions to lower the threshold of activation in a manner that allows for rapid amplification of T cell responses to antigen receptor stimulation in the presence of CD28 ligands, but also allows for prolonged costimulatory signaling that is permissive for commitment to activation in CD4+ T cells that have received prior sub-mitogenic TCR signals. Consequently, CD28 signaling prevents the induction of an unresponsive phenotype in CD4+ T cells exposed to low levels of antigen receptor stimulation for an extended period. At the transcriptional level, CD28 differentially modifies TCR-mediated changes in gene expression. In general, CD28-signaling accelerates the rate of transcriptional regulation subsequent to TCR-triggering and results in modest changes in the overall scope and magnitude of genomic expression patterns observed during primary T cell activation. Globally, expression patterns of T cells activated by TCR triggering with or without CD28 costimulation converge over time and result in relatively few stably expressed transcripts displaying differential regulation by CD28 signaling. These data suggest a general overestimation of the role CD28 plays in determining the magnitude of gene expression changes in previous reports that failed to use

physiologically relevant ligand and did not examine T cell populations homogenous for activation status. Il-2 and bcl-xL, traditionally considered CD28-specific target genes, display less than two-fold changes in expression over extended intervals subsequent to CD28 costimulation. The magnitude of differential regulation is consistent with increased mRNA stabilization mediated by CD28, and may not require increased transcription. Critically, several genes reportedly expressed in activated T cells in a CD28-dependent manner were only marginally increased in expression by CD28 costimulation relative to TCR monostimulation. In contrast, numerous genes not previously identified as CD28-specific targets were more highly regulated following CD28-mediated costimulation, suggesting that novel pathways downstream of CD28 remain undiscovered. No genes were reliably regulated by CD28 costimulation in a manner reciprocal to that seen following TCR monostimulation. However, a very small number of genes appeared to specifically dependent on CD28 signaling for efficient regulation. The vast majority of genes differentially regulated by CD28(>2-fold average difference over a minimum of 12 hours) were associated with metabolic, cytoskeletal, or synthetic pathways and may partially explain the observed decrease in time to cell cycle entry observed with CD28 costimulation. A small subset of genes differentially regulated by CD28-signaling are transcription factors, and only one transcription factor, Lklf, maintained long-term differential expression subsequent to CD28 ligation suggesting that mechanisms underlying regulation of Lklf expression may utilize novel CD28-mediated signaling pathways.

We have shown that the expression of Ctla-4 ligands on engineered APCs providing TCR- and CD28-mediated stimulatory signals to TCR-tg CD4+ T cells correlates with changes in gene expression that serve to counteract transcriptional regulation by CD28. . However, the degree to which Ctla-4 suppressed CD28-mediated changes in gene expression was much less than has been reported previously and global patterns of expression were largely identical, both quantitatively and qualitatively, by 20 hours in cells activated under conditions of CD28 ligation with or without concomitant Ctla-4 ligation. A predominant preferential effect of Ctla-4 suppression of transcriptional regulation based on molecular function or process was not detected. However a small number of genes remained stably altered by Ctla-4 relative to TCR and CD28 coligation. Surpisingly, the most stably altered transcription factor, Lklf, was also the most significantly changed by CD28 signaling relative to isolated TCR ligation.

The central role of CD28 and Ctla-4 in determining the nature and extent of immune responses implies that identification of downstream mediators might provide useful targets for immunotherapeutic and pharmacological approaches to combating immunological disease. The strength and precision of tolerance induction that can be affected by costimulation dependent mechanisms is dramatically revealed in the cell-extrinsic regulation of Ctla-4 knockout cells in mixed bone marrow chimera. The stable chimerism that results from a 1:1 donor transfusion of Ctla-4-knockout and wild-type marrow is dependent on continual Ctla-4-dependent mechanisms of regulation that are sufficient for the restoration of normal pathogen-specific immunity, yet maintain immunotolerance to self-antigen in otherwise potently self-reactive lymphocyte

populations. Analysis of the genomic expression pattern in these regulated Ctla-4-deficient CD4+ T cells reveals numerous changes in gene expression that may indicate the pathways involved in extrinsic regulation.

Wild-type cells are capable of reversing the autoreactivity native to Ctla-4-deficient T cells while maintaining normal responsivness to foreign antigen. An understanding of mechanisms of cell-extrinsic regulation of autoimmune T cells operative in the context of mixed Ctla-4-gentoype bone marrow chimerae may lead to the development of therapeutic strategies. Transcriptional mechanisms that might potentially mediate the regulatory effect of wild-type cells would be expected to involve regulators of lymphocyte signaling pathways operating downstream of the antigen receptor and/or CD28. Expression of the E3 ubiquitin ligase c-Cbl is turned on in Ctla-4 –deficient cells and expressed at high levels. c-Cbl expression and activity also correlates with anergy induction and inhibition of peripheral T cell responsiveness, and c-cbl is known to regulate proximal signaling molecules downstream of the TCR including Rap1 and CrkL(86). The NFATc2/NFAT1 transcription factor is involved in the regulation of numerous processes in lymphocytes including gene regulation during activation and cooperative activity with FoxP3 during regulatory T cell development(404) . In Ctla-4 deficient cells extrinsically regulated by wild-type cells in mixed bone marrow chimerae, NFAT1 is severely upregulated. Very recently, NFAT1 was clearly identified as a critical component of the signaling pathway that results in anergy induction in B cells chronically exposed to self-Ag(405). In contrast, other work has shown that a constitutively active form of protein kinase B(PKB) reduced nuclear accumulation of

NFAT1 and NF-kappaB when introduced into T cells. Unexpectedly, the effect of the reduced shuttling and nuclear accumulation of NFAT and NF –kappaB was a reduction in activation threshold, enhanced cell cycle progression, and increased production of Th1 and Th2 cytokines similar to what was seen with CD28 costimulation(406) Cumulatively, these results suggest that in certain contexts NFAT1 can counteract signaling pathways mediated by CD28. In addition to c-Cbl and NFAT1 upregulation, Ctla-4 deficient cells upregulated the atypical protein kinase C, PKC–zeta. Interestingly, T cell:APC interaction via CD40:CD40L results in transient association of c-Cbl with the cytoplasmic tail of PI3K resulting in increased PIP_3 production. PIP_3 activates 3' (PI)-protein kinase 1 that activate PKC-zeta by phosphorylation. PKC-zeta increases nuclear NFAT1 accumulation and phosphorylates the catalytic domain of NFAT1. Thus a direct line of interactions from c-CBL to increased NFAT activity and subsequent inhibition of pathways normally regulated by CD28 is enhanced in extrinsically regulated Ctla-4-deficient T cells. The lymphoproliferative defect in Ctla-4 deficiency is CD28 dependent, suggesting that increased nuclear NFAT resulting from a combination of increased expression and enhanced upstream activation through c-Cbl and PKC-zeta may explain the tolerant phenotype of Ctla-4 deficient T cells in mixed bone marrow chimerae. To date, no other molecular mechanism mediating extrinsic regulation of Ctla-4 deficiency has been proposed.

The restoration of appropriate antigen-specific responses in Ctla-deficient T cells in the presence of wild-type cells suggests that generalized mechanisms of immunosuppression are not operative in this context. Instead, contact dependent

mechanisms seem likely to prevail. Cumulatively, the data available is consistent with a model in which Ctla-4 deficient cells are tolerized in a process similar to anergy induction by interaction with APCs rendered tolerogenic by interactions with wild-type cells. This model is in accord with numerous findings. First, Ctla-4 deficiency requires antigen receptor triggering and CD28 costimulation to initiate disease(268), suggesting that mechanisms of tolerance induction must precede or coincide with antigen recognition by the TCR. The regulation of Ctla-4-deficient cells is dependent on the continued presence of either CD4+ or CD8+ wild-type T cells indicating that Ctla-4 functions on a continuing basis to regulate the knockout T cells. The ability of Ctla-4 to provide extrinsic regulation is only known to occur through a limited number of distinct mechanisms. Ctla-4 can signal through B7 to induce a tolerogenic phenotype in dendritic cells characterized by enhanced tryptophan catabolism. Direct suppression of effector cells has also been achieved by T cell-expressed B7-mediated signaling upon binding Ctla-4 on regulatory T cells(303). However, this would represent an antigen non-specific mechanism if it did not occur concomitantly with lymphocyte:APC interaction. Moroever, PKC-zeta is known to be regulated by CD40/CD40L interaction. Thus I would propose a model in which a three-way interaction occurs between wild-type regulatory T cells specific for self-antigen, tolerogenic dendritic cells presenting self-antigen, and Ctla-4-deficient effector cells capable of responding to self-antigen. Tolerogenic dendritic cells provide altered signaling via accessory costimulation molecules such as CD40 potentially enabling the upregulation of c-Cbl and blockade of CD28-mediated signals by an NFAT1-dependent mechanism. Confirmation that NFAT1

functions as an anergy factor in T cells and is critical for tolerance induction in regulated Ctla-4-deficient cells will be an important first step in clarifying the validity of this model.

In contrast to the number and magnitude of changes seen in exrinsically regulated Ctla-4 deficient T cells, Ctla-4 deficiency in the context of absent B7 resulted in only insignificant changes in expression. With the exception of Ctla-4 and the melanoma antigen, Mela, there were no genes significantly altered more than four fold. Given the absence of pathology in Ctla-4 deficient mice in the presence of inactivating mutations of the B7:CD28 signaling pathway, it is likely that the slightly increased sensitivity of Ctla-4 deficient mice to isolated TCR triggering reflects a partial role for biophysical disruption of the immature immunological synapse or altered activity of proximal signaling intermediates as a result of Ctla-4 recruitment to the APC:T cell interface. Given the recent reemergence of reports suggesting a role for Ctla-4 in thymic development, it was important to establish that baseline gene expression is not altered in the absence of Ctla-4 prior to activation.

Similarities in the the phenotype of Lklf-deficient and Ctla-4-deficient mice suggest that they both function to maintain the quiescent phenotype of peripheral lymphocytes. RT-PCR and quantitative real-time PCR confirmed the differential regulation by CD28 and Ctla-4. Lklf is not a Ctla-4-specific target gene. However, Ctla-4 ligation abrogated the suppressive effect of CD28 signaling on Lklf expression completely, as assessed by quantitative PCR. Thus, regulation of Lklf transcription may represent a critical juncture in the integration of the opposing costimulatory pathways.

Lklf expression is necessary but not sufficient for the maintenance of a quiescent phenotype. Lklf expression is maintained in activated and divided cells stimulated by isolated antigen receptor triggering or in the presence of Ctla-4 ligation. In transformed cell lines, Lklf overexpression did not alter growth characteristics (Data not shown). Moreover, overexpression of Lklf in primary T cells infected with a retroviral construct encoding Lklf showed normal responses to antigen receptor ligation or costimulation through CD28 or Ctla-4 (Data not shown). The ability of forced Lklf expression in Ctla-4-deficient peripheral T cells to suppress or prevent the development of autoimmune disease is being investigated. The CD28-dependent nature of the lymphoproliferative disease in Ctla-$4^{-/-}$ mice suggests that transcriptional regulation mediated by CD28 may be important. Lklf

Lklf reproducibly regulates several hundred genes when overexpressed in a double positive thymoma cell line. In contrast, similar experiments with other putative immunoregulatory or quiescence factors did not provide reproducible results. These included FoxP3, TSC-22, and Tob. In an attempt to characterize targets of Lklf relevant to primary T cells, a comparison was made between all genes altered more than three fold by Lklf overexpression in NFC cells and the observed degree of coregulation with Lklf expression in the kinetic profile of T cell activation. Lklf expression remains unchanged in naïve or TCR monostimulated cells, is quickly downregulated by CD28 costimulation, and is maintained by Ctla-4 signaling despite concurrent CD28 ligation. As reported, nine genes identified as Lklf targets in NFC cells coregulated with Lklf expression in primary

cells. Two of the identified genes, Sh2d1a(SAP/SLAM-associated protein) and Sla(Src-like adaptore protein), are known to regulate T cell responses.

Sla is an adaptor protein containing Src homology 3 (SH3) and SH2 domains highly similar to those found in Lck and other Src family members. Initially characterized as an inhibitor of mitogenesis(407), Sla has been shown to inhibit TCR signaling by association with a multimolecular signaling complex containing CD3-zeta, ZAP-70, VAV, LAT, and SLP-76(408). Sla mediates downregulation of the TCR in thymocytes by targeting TCR-zeta for degradation in a c-cbl-dependent manner(409) and interferes wih TCR signaling in peripheral T cells leading to functional downregulation of NFAT and NF-kB(410). Similarly, Sh2d1a/SAP functions to fine-tune immune cell activation and disruption of Sh2d1a/SAP function results in X-linked lymphoproliferative syndrome (XLP)(411). XLP is often fatal and is characterized by hypogammaglobulinemia, fulminant infectious mononucleosis, and lymphoma. Sh2d1a/SAP is required for signaling through CD150/SLAM. Slam is upregulated on naïve T cells during primary activation and is constitutively expressed on memory cells where it mediates a positive costimulatory signal capable of enhancing TCR-dependent T cell responses independent from CD28. While most reports have focused on the role Sh2d1a/SAP plays in CTL and NKT function, a recent report has shown that memory and effector CD4+ T cells from XLP patients are specifically defective in IL-10 secretion(412). These observations suggest that Ctla-4 maintenance of Lklf expression may result in the direct downregulation of Sla and a consequent increase in TCR signal strength. Enhanced TCR signaling can induce anergy by altering the relative activity of

specific transcription factors including NFAT1. Thus Ctla-4 ligation may operate through Lklf to prime cells for anergy induction.

The relationships identified by cross-referencing expression profiles from in vitro overexpression data with kinetic expression patterns in primary T cell activation facilitate hypothesis-driven experiment design. Newly available bioinformatics tools allow mass-incorporation of published observations into statistical analyses defining coordinately regulated genes. Thus the dataset generated by the kinetic profiling of in vitro activated primary CD4+ T cells will enable efficient cross-referencing with observations made in more focused investigations of gene function.

The validity of the results presented above is dependent on the the integrity of the in vitro stimulation system we have utilized. The use of TCR-tg T cells and engineered APCs capable of differentially signaling through Ctla-4 and CD28 should allow precise control of stimulation conditions. However, apparent instability in the magnitude of inhibition mediated by the presence of Ctla-4 ligands raises concerns about the nature of the T cell/APC interaction that is occurring. While surface expression of T cell ligands has been matched across cell lines, and is stable over long periods of time, the apparent inhibition of T cell activation in the presence of Ctla-4 ligands has intermittently been observed to result from undefined variables that are Ctla-4 independent. Recent work in our lab has definitvely shown that the engineered APCs are capable of signaling through Ctla-4, and no explanation can be given for the apparent Ctla-4 independent suppression of T cell responses that has been observed at various times. Recent experiments suggest

that the CHO APCs maintained in culture for extended periods are more likely to result in spurious changes in T cell responsiveness. The experiments included in the above analyses were performed using CHO APCs that had been cultured for relatively shorter durations, and thus may have inhibited T cells in a purely Ctla-4 dependent manner. Some reassurance is provided by the stability of expression trends seen with the more highly expressed genes reportedly influence by costimulatory signals. However, the possibility remains that observed phenotypes may reflect non-specific properties of the stimulation system. Thus all observations and predictions derived from the dataset must be validated in independent systems.

CHAPTER VII

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