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Development And Implementation Of Knock-In And Bac-In Il-2 Reporter Mouse Models To Characterize Il2 Gene Regulation In Cd4 T Cells

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DEVELOPMENT AND IMPLEMENTATION OF KNOCK-IN AND BAC-IN IL-2 REPORTER MOUSE MODELS TO CHARACTERIZE *Il2* GENE REGULATION IN CD4 T CELLS

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DEVELOPMENT AND IMPLEMENTATION OF KNOCK-IN AND BAC-IN IL-2 REPORTER MOUSE MODELS TO CHARACTERIZE *Il2* GENE REGULATION IN CD4 T CELLS

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ABSTRACT

Maintenance of immune homeostasis involves a balance between T cell effector responses to antigen stimulus and reciprocal downregulation of this response through peripheral tolerance mechanisms. Upon exposure to pathogen, cytokine production and signaling serve to tightly coordinate cell-mediated clearance of antigen followed by contraction of the immune response. Interkeukin-2 (IL-2) is a type I family cytokine critical for expansion of activated T cells *in vitro* and enhancement of T cell memory responses *in vivo*. Deficiency of IL-2 *in vivo* also revealed a critical role for IL-2 in immune tolerance through the maintenance of T regulatory cell populations (Treg) in peripheral lymphoid tissues. Thus, regulation of *Il2* gene expression and autocrine signaling are central to the balance of immune homeostasis.

Defining the regulatory mechanisms associated with *Il2* gene expression is crucial for defining the integral role for IL-2 in tolerance and effector immunity. In order to evaluate the role of IL-2 production during the immune response we have engineered a BAC transgenic as well as a gene-targeted knock-in reporter mouse model system to mark *Il2* gene activation. Our studies indicate that both mouse models exhibit reporter expression with high fidelity to endogenous *Il2* expression patterns while stably marking IL-2-producing cells. Using our *Il2* BAC transgenic system (2BiT) system to model inhibition of IL-2 production in responder CD4 T cells by Tregs we describe a model in which Tregs prevent initial activation of *Il2* transcription that also subsequently

associates with increased responder cell death of non-IL-2 producers. Suppression of naïve activated CD4 T cells by Tregs followed a mechanism consistent with competition for co-stimulation and preventing a threshold of activation rather than cytokine deprivation. Utilizing an *Il2* GFP knock-in model we show evidence that IL-21 enhances proliferation and the frequency of IL-2-producing cells in activated naïve CD4 T cells. These results are suggestive of a coordinate relationship between *Il2* expression and IL-21 production and signaling in effector T cell function and fate. Future *Il2* expression studies using our novel mouse reporter systems will enable a more in-depth understanding of the role of this cytokine in effector function and immune tolerance.

Keywords: Interleukin-2, T regulatory cells, immune tolerance, T cell activation, gene transcription, BAC transgenic and knock-in reporter mice

DEDICATION

This work is dedicated in honor of my family whose sacrifices, perseverance, and unconditional support gives me the resolve to keep pushing forward.

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THE ROLE OF IL-21 IN THE GENERATION OF IL-2-PRODUCING CD4 T CELLS

LIST OF ABBREVIATIONS

2BiT	*Il2* BAC-in Transgene
7AAD	7-Aminoactinomycin D
AICD	Activation Induced Cell Death
AKT	Ak transforming
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cell
ARE	AU-rich Element
BAC	Bacterial Artificial Chromosome
Bcl6	B Cell CLL/lymphoma 6
Blimp-1	B lymphocyte-induced
BTLA	B and T Lymphocyte Attenuator
CD_	Cluster of differentiation
CHORI	Children's Hospital Research Center Institute
CREM	CAMP Response Element modulator
CXCR5	Burkhitt Lymphoma Receptor 1
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ES cell	Embryonic stem cell
FasL	Fas ligand
FBS	Fetal Bovine Serum
FLP*e*	Flippase *e*
Foxp3	Forkhead box 3
Frt	Flippase recognition target
GFP	Green Fluorescent Protein
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome
HMG1 (Y)	High Mobility group 1 (Y)
HSV-TK	Herpes Simplex Virus Thymidine Kinase
ICER	Inducible cAMP Early Repressor
ICOS	Inducible T Cell Co-stimulator
Idd3	Insulin-dependent diabetes susceptibility 3
IFN-	Interferon-
Il	Interleukin- (gene)
IL-	Interleukin- (RNA or protein)
Il2 GFP knock-in	*Interleukin 2* Green Fluorescent Protein knock-in
IRES	Internal Ribosomal Entry Sequence
Kan^r	Kanamycin resistance

Kb	kilobase
KIAA1109	Gene of unknown function within the *Idd3* locus
lacZ	β-galactosidase
LCMV	Lymphocytic choriomeningitis
LCR	Locus Control Region
MAPK	Mitogen-activated protein kinase
MFI	Mean Fluorescent Intensity
mRNA	messenger ribonucleic acid
Neo^r	Neomycin resistance
NF-90	Nuclear Factor 90
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NOD	Non-obese diabetes
PAC	Plasmid Artificial Chromosome
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
PEV	Position Effect Variegation
PMA	Phorbol 12-Myristate 13-Acetate
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RAD50	Protein involved in DNA double-strand break repair
RAG1/RAG2	Recombination activating gene 1/Recombination activating gene 2
RUNX1	Runt-related transcription factor 1
SNP	Single nucleotide polymorphism
SOCS2	Suppressor of cytokine signaling 2
STAT	Signal transducers and activators of transcription
SV40	Simian Vacuolating virus 40
T-bet	T-box expressed in t cells
TCR	T cell receptor
TENR	Testis nuclear RNA-binding protein
Tfh	Follicular helper T cell
Th	T helper
TID	Type I diabetes
TNFα	Tumor necrosis factor α
Treg	T regulatory cell
TTP	Tristetraprolin
UC	Ulcerative colitis

UT	Untranslated region
VDJ	Variable Diversity Joining
VISTA	Visualization Tool for Alignment
YAC	Yeast Artificial Chromosome
YB-1	Y Box Protein 1
ZEB1	Zinc finger E-box homeobox 1

INTRODUCTION

Our current understanding of cytokine biology encompasses an expanding list of soluble regulators. Cytokine signals help to orchestrate the complex events involved in an immunological response triggered by a wide variety of environmental insults. Interleukin-2 (IL-2) is a type I cytokine that is primarily produced by CD4 and CD8 T cells that have undergone antigen activation. To a lesser extent, IL-2 is produced from NK cells, NKT cells, and dendritic cells (DCs) stimulated with microbial products (Granucci, et al., 2001; Jiang, Game, Davies, Lombardi, & Lechler, 2005; Setoguchi, Hori, Takahashi, & Sakaguchi, 2005; Yui, Hernandez-Hoyos, & Rothenberg, 2001; Yui, Sharp, Havran, & Rothenberg, 2004). In addition, detection of IL-2-producing cells in the cortical medullary junction and medulla of the thymus, in the epidermis surrounding hair follicles, and in the murine intestine during various stages of mouse embryonic development suggest that the function of IL-2 is also integral to a diverse array of developmental processes (De Creus, et al., 2002; Kawai, et al., 1998; Porter & Malek, 1999; Yang-Snyder & Rothenberg, 1998; Ye, et al., 2001). A better understanding of the complex role of IL-2 in T cell homeostasis and development will require the use of tools that report on *Il2* gene expression with a high degree of accuracy and sensitivity in cell lineages specific for expression of this critical cytokine mediator.

Interleukin-2

T Cell Growth Factor

IL-2 was first isolated and characterized over three decades ago based on its functional role as a T cell growth factor (TCGF). The growth-promoting properties of IL-2 were first observed in the supernatants of activated T cells and later several studies showed that IL-2 was critical for inducing T cell proliferation *in vitro* (Gillis, Ferm, Ou, & Smith, 1978; Gillis & Smith, 1977; Morgan, Ruscetti, & Gallo, 1976; Smith, Favata, & Oroszlan, 1983; Smith, Gillis, & Baker, 1979; Taniguchi, et al., 1983). Collectively, many of these earlier studies validated IL-2 as a legitimate growth hormone due to its saturable high affinity binding to a unique receptor while also showing a direct correlation with ligand binding and generation of a proliferative response (Robb, Munck, & Smith, 1981). Studies by Cantrell and Smith, et al using activated human T cells and murine IL-2-dependent cytolytic T cell lines showed that lectin-activated T cells undergo rapid accumulation of IL-2 in the supernatant followed by upregulation of IL-2 receptor on the cell surface. In addition, an increase in IL-2 concentration as well as IL-2 receptor density on the cell surface upon activation coincided with an increased rate of T cell proliferation. These studies were the first to establish the importance of IL-2 autocrine signaling in DNA replication by facilitating a G1→S phase transition and allowing further expansion of activated T cells *in vitro* (Cantrell & Smith, 1983, 1984; Smith, 1988b). Once IL-2:IL-2 receptor binding takes place on the cell surface, ligand-mediated internalization of this complex acts as a negative feedback signal, another characteristic of growth hormones, to downregulate further T cell proliferation (Smith, 1988a; Sojka, Bruniquel, Schwartz, & Singh, 2004). Thus, the IL-2:IL-2 receptor signaling loop serves

to ensure both the transitioning of antigen-experienced cells into an actively proliferating state responsive to immune invasion, but further internalization acts to safeguard against aberrant proliferative signals that may lead to detrimental immune consequences. Later studies combining mathematical modeling and *in vitro* assays evaluating the effect of IL-2 on proliferation of antigen-activated CD4 T cells further confirmed the earlier findings of Smith, et al. These analyses also more specifically indicated that exogenous IL-2 increased the number of precursor cells that enter cell cycle in the first division. Furthermore, addition of IL-2 resulted in an increase in the rate of proliferation and most notably a decrease in the rate of cell death after the first round of division (Deenick, Gett, & Hodgkin, 2003; Ganusov, Milutinovic, & De Boer, 2007). Thus, early studies evaluating the effect of IL-2 on lymphocyte growth indicated a critical role for IL-2 production and autocrine signaling through the IL-2 receptor for *in vitro* T cell expansion through the induction of proliferation upon antigen exposure.

IL2 Receptor Signaling

Discovery of the individual subunits of the IL-2 receptor have revealed further insights into the functional significance of receptor:ligand affinity. IL-2, like IL-4, IL-7, IL-9, IL-15, and IL-21 are members of the type I family of cytokines. A common feature of this family of cytokines is the shared use of the cγ chain subunit, CD132, within their corresponding receptor binding complexes. In addition to the cγ chain, the IL-2 receptor employs the use of the IL-2 receptor β chain, CD122, also shared by the IL-15 receptor as well as the IL-2 receptor α chain, CD25 (Leonard, 2001; Nelson & Willerford, 1998). These three receptor chains have been reported to generate two forms of the IL-2 receptor. The dimeric receptor has a weaker binding affinity for IL-2 cytokine and

consists of a complex between CD22 and the CD132 subunits. The low affinity IL-2 receptor has a dissociation constant of [Kd] ≈ 10^{-9} mol/L and is present on $CD4^+$ T cells at low levels. Due to its weaker affinity for IL-2 cytokine, the low affinity IL-2 receptor does not facilitate significant IL-2 signaling. Alternatively, the high affinity receptor consists of a trimer between the CD25, CD122, and the CD132 subunits with a dissociation constant of [Kd] ≈ 10^{-11} mol/L (Letourneau, Krieg, Pantaleo, & Boyman, 2009). The stronger affinity of the trimeric IL-2 receptor complex for IL-2 cytokine imparts IL-2 signaling competency to cell populations expressing the high affinity IL-2 receptor such as $Foxp3^+$ T regulatory cells (T regs), which show more stable expression of the high affinity IL-2 receptor and conventional T cells that show transient upregulation shortly after TCR-mediated antigen activation (Letourneau, et al., 2009; Malek, 2008). The cell-type specific expression of this high affinity receptor on T cell subsets is imparted primarily by the differential expression of the CD25 subunit as both CD22 and CD132 are more uniformly expressed on T cells (Malek, 2008). Formation of the high affinity receptor complex with IL-2 cytokine is initiated by the binding of IL-2 cytokine to the CD25 subunit, which subsequently recruits the CD22 and CD132 subunits. Complex formation upon IL-2 signaling also facilitates rapid turnover of receptor:cytokine components through ligand mediated endocytosis and lysosomal degradation of CD122:CD132:IL-2 subunits and recycling of CD25 onto the cell surface. Consequently, the CD25 subunit has a longer half-life than CD122 and CD132 receptor subunits (Hemar, et al., 1995; Letourneau, et al., 2009). Thus, differences in induction and duration of CD25 subunit expression and turnover of high affinity IL-2 receptor components among conventional and regulatory T cells may contribute to distinct

functional outcomes as a result of unique IL-2 signaling patterns within these T cell subset populations.

Tregs and IL-2 signaling

$CD4^+Foxp3^+$ T regs, which also express CD25 on their cell surface, function in immune tolerance by suppressing autoreactive T cells that have escaped thymic deletion measures (Suri-Payer, Amar, Thornton, & Shevach, 1998). In addition to its presumed role in effector T cell function, IL-2 has been shown to play a significant role in immune tolerance through the maintenance of T regs (Fontenot, Rasmussen, Gavin, & Rudensky, 2005). *In vitro* studies originally indicated a role for IL-2 signaling in inducing T cell proliferation and expansion of the effector response (Gillis, et al., 1978; Gillis & Smith, 1977; Morgan, et al., 1976; Smith, 1988b; Smith, et al., 1979; Taniguchi, et al., 1983). Based on the founding role of IL-2 as a T cell growth factor it was predicted that a deficiency of this cytokine would lead to immunodeficiency. However, nearly two decades after the initial discovery of IL-2, generation of IL-2-deficient mice using gene-targeting technology revealed a phenotype of lethal autoimmunity (Sadlack, et al., 1995; Sadlack, et al., 1993; Schorle, Holtschke, Hunig, Schimpl, & Horak, 1991; Willerford, et al., 1995). This observation was later attributed a decreased frequency of T regs in the peripheral lymphoid tissues (Sakaguchi, 2004). The importance of IL-2 in the maintenance of T regs was further shown as mice treated with anti-IL-2 monoclonal antibody also had a reduced frequency of $Foxp3^+$ T regs (Bayer, Yu, Adeegbe, & Malek, 2005; Setoguchi, et al., 2005). Moreover, deficiency of other cγ chain cytokines did not significantly effect the frequency of $Foxp3^+$ T regs indicating that the maintenance of T regs by IL-2 is functionally non-redundant among other type I cytokines family members

(Burchill, Yang, Vogtenhuber, Blazar, & Farrar, 2007; Peffault de Latour, et al., 2006). Taken together, these studies emphasize the functional significance of IL-2 in immune tolerance by promoting the presence of critical regulatory T cell populations. A mechanistic understanding of an IL-2 target in T reg homeostasis has begun to be elucidated in studies evaluating molecular targets of IL-2 autocrine signaling. Similar to mice with a targeted deletion in *Il2*, initial studies with CD25-deficient mice also showed a phenotype of lethal autoimmunity due to a diminished T reg population (Willerford, et al., 1995). Furthermore, adoptive transfer of wild-type $CD4^+CD25^+$ cells into CD25-deficient mice rescued this autoimmune phenotype (Almeida, Legrand, Papiernik, & Freitas, 2002; Antov, Yang, Vig, Baltimore, & Van Parijs, 2003; Malek, Yu, Vincek, Scibelli, & Kong, 2002; Wolf, Schimpl, & Hunig, 2001). As mentioned previously, CD25 was found to be expressed stably on T regs *in vivo*, unlike the transient expression seen in conventional T cells, further suggesting a role for this IL-2 receptor subunit expression in T reg homeostasis (Letourneau, et al., 2009). These studies further implicated the importance of the high affinity IL-2 receptor and IL-2 autocrine signaling in the maintenance of T regs.

STAT5, a known transcription factor downstream target of IL-2 high affinity receptor signaling, has been shown to target Foxp3 expression in T regs (Burchill, et al., 2008; Burchill, et al., 2007). More specifically, T cell-specific deficiency in the STAT5a and STAT5b isoforms prevents the production of $Foxp3^+$ T regs generating an autoimmune phenotype reminiscent of IL-2-deficient mice (Snow, et al., 2003). In addition, studies have shown that IL-2 receptor signaling is necessary for sustained Foxp3 expression and suppressive function of T regs (Burchill, et al., 2007). Taken

together, these results indicate a critical role for IL-2 signaling through the high affinity IL-2 receptor in T regs for the maintenance, development, and suppressive function of this regulatory population. This is in contrast to the effect of STAT5 signaling in effector T cells. Prior studies suggested that IL-2 autocrine signaling stimulates proliferation of effector T cells, however other studies indicate that IL-2 signaling can induce negative feedback regulation of *Il2* gene expression through a STAT5-dependent mechanism (Gong & Malek, 2007; Villarino, et al., 2007). Several studies implicate a role for Blimp-1 in suppression of IL-2 production (Gong & Malek, 2007; Kallies, et al., 2006; Martins, et al., 2006). Furthermore, Blimp-1 induction during IL-2 autocrine signaling suppresses IL-2 production (Gong & Malek, 2007). While IL-2 autocrine signaling may induce events that promote proliferation in conventional T cells *in vitro*, more recent studies indicate that the IL-2-induced autocrine signaling pathway imposes negative feedback regulation on further IL-2 production within this population. These studies indicate that IL-2 signaling leads to distinct downstream signaling consequences depending on the T cell subset in which it occurs.

While T regs can become committed in the absence of Foxp3 expression, IL-2 expression by bystander cells and subsequent IL-2 signaling through the high affinity IL-2 receptor are critical for Foxp3 expression and suppressive function of this regulatory lineage (Burchill, et al., 2008; Burchill, et al., 2007; Gavin, Rasmussen, Fontenot, Vasta, Manganiello, & al, 2007; Lin, et al., 2007). Unlike their conventional T cell counterparts, T regs do not express IL-2. Studies have indicated that the IL-2 minimal promoter does not undergo chromatin remodeling upon TCR activation in T regs (L. Su, et al., 2004). In addition, the forkhead transcription factor Foxp3, known to be a unique transcriptional

regulator of the T reg lineage has been shown to bind directly to the transcription factor NFAT, which promotes *Il2* transcription in conventional T cells and together with AML/Runx1 factors can direct transcriptional repression of *Il2* within the T reg lineage (Ono, et al., 2007; Wu, et al., 2006). Other repressive factors known to inhibit *Il2* transcription, such as Blimp-1 and Eos (Gong & Malek, 2007; Kallies, et al., 2006; Martins, et al., 2006; Pan, et al., 2009) may also function to drive chromatin silencing of the *Il2* locus. Despite the depth of knowledge that we now have regarding the role of IL-2 in regulatory T cell responses, the significance of how *Il2* transcriptional silencing in T regs relates to maintenance of suppressive function still remains to be determined. In addition to showing intrinsic suppression of IL-2 within the T reg lineage, T regs have also been shown to suppress *Il2* transcription in activated conventional CD4 T cells *in vitro* (Duthoit, Mekala, Alli, & Geiger, 2005; Thornton & Shevach, 1998; Yui, et al., 2001). In fact, the primary readout of T reg suppression is inhibition of not only T cell proliferation, but also inhibition of IL-2 transcript and protein production. However, despite several proposed mechanisms of T reg suppression, the process by which T regs facilitate inhibition of effector responses and associated *Il2* transcription remains undefined. *In vitro* studies by Duthoit, et al attempted to clarify the suppressive effects imparted on conventional T cells by T regs. These studies determined that T regs acted to uncouple the mitogenic and IL-2-producing capacity of conventional cells from their ability to undergo STAT5 phosphorylation and receive survival signals from exogenous IL-2 autocrine signaling (Duthoit, et al., 2005). Contrary to an active mechanism of transcriptional suppression, other studies have suggested that T regs inhibit the effector response by sequestering and binding to available IL-2 in the supernatant thus starving

the effector T cell of necessary survival signals and leading to subsequent apoptosis rather than active suppression of *Il2* transcription in a living cell population (Paniyan, Zheng, Ishihara, Reed, & Lenardo, 2007). Despite attempts to clarify molecular events within the effector cell that result from suppression of this population, a clear understanding of T reg-induced inhibition of the conventional T cell is still lacking. Future studies will be required to further define the role of IL-2 in mechanisms of tolerance.

Il2 Gene Expression and Effector T Cell Function and Fate

Studies to date have uncovered a functional role for IL-2 as a central soluble regulator bridging both effector responses and immune tolerance. As described, production of IL-2 from conventional T cells upon an antigenic stimulus results in secretion of IL-2 and further IL-2 signaling either in an autocrine or paracrine fashion among bystander T cells. Analysis of downstream events as a result of IL-2 signaling have given new insights as to the functional significance of this pathway as it relates to regulation of the effector responses and T reg function. Alternatively, an understanding of the lineage-dependent and temporal patterns of *Il2* gene expression is critical to gain a broad understanding of the integral role of IL-2 in T cell homeostasis.

Intraclonal Heterogeneity of IL-2 Production. *Il2* transcription arises shortly after antigen activation in CD4 T cells. Kinetic analysis of IL-2 mRNA production revealed a transcript that is short-lived, declining just prior to the onset of effector cell differentiation *in vitro* (Sojka, et al., 2004). Similarly, IL-2 protein production is also transient and tightly coupled to transcriptional regulation of the *Il2* locus (Lindsten, June, Ledbetter, Stella, & Thompson, 1989; Sojka, et al., 2004). Despite the proposed roles for

IL-2 as a growth factor *in vitro* or in tolerance *in vivo*, single cell analysis of cytokine-producing T cells revealed that only a small proportion of activated CD4 T cells actually generate cytokine (Bucy, et al., 1995; Bucy, et al., 1994; Sadlack, et al., 1995; Sadlack, et al., 1993; Schorle, et al., 1991; Willerford, et al., 1995). A majority of cytokine-producing cells, however, do express IL-2 and do so with an all-or-none activation profile (Saparov, et al., 1999; Smith & Popmihajlov, 2008). This is in accordance with the proposed quantal theory of immunity, which states that in order for a CD4 T cell to produce IL-2 a T cell must incur a certain threshold of TCR signals (Smith & Popmihajlov, 2008). This explains previous studies indicating that once CD4 T cells exceed this critical threshold of activation at a given antigen dose that the degree of activation and IL-2 production is unaffected by increasing concentrations of antigen (V., et al., 2003). Studies using CD4 T cells from an IL2 promoter-green fluorescent protein (GFP) reporter mouse model showed that previously activated GFP^+ and GFP^- cells were able to produce IL2 as well as Th1 and Th2 responses during secondary polarization. Production of IL-2, albeit to a lesser extent from GFP^- cells, during secondary stimulation suggests the presence of cells within the population with varying competency to activate *Il2* gene expression upon TCR engagement (Saparov, et al., 1999). Furthermore, additional studies have indicated that IL-2 (GFP) production from CD4 T cells arises from only a small proportion of total activated cells that make long-term contacts with their APC counterparts. Variable IL-2 activation may result from inherent antigen-induced chromatin accessibility differences within the *Il2* locus of individual cells. An effect due to variations within the antigen presenting cell population was previously excluded suggesting *Il2* expression from sufficiently activated T cells was due to

intraclonal heterogeneity within the CD4 T cell population (V., et al., 2003). These intrinsic differences within CD4 T cells that allow for variable *Il2* activation potential may be manifested by prior antigen exposure within the thymus or peripheral lymphoid tissues. This possibility may fit with the quantal theory of immunity whereby individual T cells in the peripheral lymphoid tissues have experienced a variable number of TCR signals during either thymic development or routine peripheral trafficking that are not sufficient to produce a fully activated T cell but are capable of incrementally modifying the chromatin architecture of the *Il2* locus toward a more "open" configuration poised for future activating signals.

Il2 Expression in Effector and Memory T Cell Responses. An understanding of the functional significance of the intrinsic T cell variation seen in IL-2 expression capacity may help to shed light on the role of IL-2 in primary and secondary immune responses. Originally, it was predicted that IL-2 played a critical role in the expansion of activated naïve T cells with kinetics that preceded T cell lineage commitment (Cantrell & Smith, 1983, 1984; Smith, 1988b). However, several studies have yielded conflicting results concerning the dependency of CD4 and CD8 primary effector responses on *Il2* expression and signaling *in vivo*. Some reports indicate reductions in the frequency of $CD8^{+}IFN\gamma^{+}$ T cells and anti-viral responses in IL-2-deficient models of LCMV infection (Cousens, Orange, & Biron, 1995; H. Su, et al., 1998). Yet, additional studies using IL-2-deficient, CD25-deficient, and CD122-deficient mice display normal primary responses to infection from a variety of viral and bacterial pathogens with minor impairments to cytotoxic T cell activity and IFNγ production in response to LCMV infection (Bachmann, Wolint, Walton, Schwarz, & Oxenius, 2007; D'Souza & Lefrancios, 2003; D'Souza,

Schluns, Masopust, & Lefrancios, 2002; Jin, et al., 2006; Teague, Tempero, Thomas, Murali-Krishna, & Nelson, 2004; M. A. Williams, Tyznik, & Bevan, 2006; A. Yu, et al., 2003). Collectively, these data suggest that additional signals independent of IL-2 are necessary for T cell expansion *in vivo*. An additional layer of complexity is the role of IL-2 in CD4 T helper cell differentiation. Depending on the type of pathogen present and the downstream cytokine profile generated during an initial effector response, CD4 T cells undergo a distinct differentiation program specific for targeting the immune challenge. T helper 1 cells (Th1), which respond to intracellular pathogen, have been shown to be key producers of IL-2 in addition to IFNγ and the pro-inflammatory cytokine TNFα (Letourneau, et al., 2009). In addition, IL-2 has been shown to promote the T helper 2 (Th2) lineage, which respond to extracellular parasites, through signaling activation of the IL-4 locus (Ansel, Djuretic, Tanasa, & Rao, 2006; Liao, et al., 2008). On the other hand IL-2 has been shown to inhibit differentiation of the T helper 17 lineage that respond to extracellular bacteria and some fungi (Laurence, et al., 2007; Mathur, et al., 2007). Thus, IL-2 may not have a significant impact in initial effector cell expansion *in vivo*, yet effects on T cell lineage commitment are duly noted. In addition, there is evidence to support a significant role for IL-2 in secondary responses. Studies using IL-2-deficient and IL-2 receptor-deficient mice with LCMV and *Listeria monocytogenes* infection models indicate that secondary responses are largely inhibited while primary immunity remains intact (Bachmann, et al., 2007; M. A. Williams, et al., 2006). Furthermore, IL-2-deficient CD4 T cells show poor survival and yield during a memory response. This deficit during the recall response is associated with defective IL-7 receptor expression, which is critical for CD4 memory cell responses (Dooms,

Wolslegel, Lin, & Abbas, 2007; Z., Konieczny, & Lakkis, 2000). Thus, *Il2* gene expression occurs early after antigen-induced activation. While the requirement for IL-2 early after T cell activation may be limited, IL-2 in the effector response seems to function in the priming of initial effector populations for subsequent CD4 and CD8 recall responses. IL-2 may also play a role in limiting the immune response and possibly affecting differentiation through activation induced cell death (AICD) (Malek, 2008). Under conditions of excessive TCR signaling, some reports have indicated that IL-2 can signal FasL-mediated cell death (Lenardo, 1991; Malek, Yu, Scibelli, Lichtenheld, & Codias, 2001). However, other studies suggest little effect on contraction of the immune response in IL-2-deficient and CD25-deficient mice (Dooms, et al., 2007; Leung, Morefield, & Willerford, 2000). Additional studies will be necessary to substantiate a role for IL-2 in promoting activation induced cell death. In sum, production of IL-2 has multiple roles in antigen-activated T cell responses from priming the effector response, T cell differentiation, to promoting secondary immunity.

Il2 Gene Regulation

Promoter Regulation

Molecular regulation of *Il2* gene expression has been studied extensively and continues to reveal mechanistic insights into signaling events and locus regulation that are important to the expression of this cytokine and subsequent functional effects on T cell homeostasis. Promoter elements proximal to the *Il2* transcriptional start site and essential in the regulation of *Il2* gene expression have been well characterized within a ~300-500 bp region (Jain, Loh, & Rao, 1995). Binding of inducible or constituitive

transcription factors like NFAT, OCT-1, NFκB, HMGI (Y) and the AP-1 complex to the minimal *Il2* promoter region has been shown to be necessary for initiation of *Il2* gene expression (Jain, et al., 1995). The CD28 response element located between -154 and -162 is an absolute requirement for *Il2* transcription (Jain, et al., 1995). Additional transcription factors such as ZEB1, ICER/CREM, and T-bet have been shown to bind to the *Il2* promoter within a-230 bp region and suppress *Il2* transcription (Bodor, Fehervari, Diamond, & Sakaguchi, 2007; Hwang, Hong, & Glimcher, 2005; Wang, et al., 2009; Yasui, et al., 1998).

Chromatin Remodeling and Epigenetic Modifications

Chromatin remodeling and epigenetic events have been shown to be involved in locus control and regulation of gene expression. The NFκB family member, c-Rel, has been shown to bind to the *Il2* minimal promoter and rapidly induce chromatin remodeling events of this region early after antigen activation allowing for locus accessibility (Attema, et al., 2002; Rao, Gerondakis, Woltring, & Shannon, 2003; Rao, Procko, & Shannon, 2001; Ward, et al., 1998). In addition, several studies have characterized epigenetic modifications that likely cooperate to accompany changes in *Il2* transcription. Several modifications within a -800 to +1 minimal *Il2* promoter such as demethylation of CpG residues, acetylation, and phosphorylation have been documented to parallel transcriptional alterations within the *Il2* locus (Bruniquel & Schwartz, 2003; X. Chen, Wang, Woltring, Gerondakis, & Shannon, 2005; Murayama, et al., 2006; Rothenberg & Ward, 1996; Thomas, Gao, & Wells, 2005). Schwartz, et al have further characterized these modifications in the context of a primary and secondary response and show that *Il2* expression is "biphasic" where a primary response effectively "primes" the *Il2* locus in a

T cell intrinsic manner altering the modification requirements necessary for further locus activation. Thus, during subsequent rechallenge responses the threshold of activation for *Il2* expression is lowered and induced more rapidly (McKarns & Schwartz, 2008).

While several arms of regulation have been characterized within the context of a short span of *Il2* endogenous regulatory sequence, additional studies indicate that regulatory elements further upstream of the *Il2* transcriptional start site are necessary for lineage-specific control of *Il2* gene expression *in vivo*. IL-2 GFP transgenic mouse models generated with varying lengths of *Il2* promoter sequence controlling expression of a GFP reporter showed that a 2.0 kb promoter sequence was insufficient to drive reporter expression in an IL-2 lineage-specific manner. However, an 8.4 kb promoter fragment on the other hand was sufficient to report with more accurate *Il2*-like gene regulation (Yui, et al., 2001). These studies suggested that previously uncharacterized distal regulatory elements are required to provide specific *Il2* locus control *in vivo*.

Post-transcriptional Regulation

Il2 gene regulation however is not limited to proximal transcriptional initiation events at the level of promoter control. Post-transcriptional regulation of the IL-2 transcript can rapidly alter the stability of message and thus serve as a key point in cytokine gene regulation (Lindsten, et al., 1989). Like IL-2, many cytokine mRNAs subject to rapid turnover contain cis-acting regulatory elements within the 3'UTR of the transcript known as AU-rich elements (AREs) (Caput, et al., 1986; C. Y. Chen & Shyu, 1995; Yang, et al., 2003). These regulatory sequences act as targets for RNA binding proteins capable of stabilizing or destabilizing these transcripts via recruitment of exosomal components for degradation (C. Y. Chen, et al., 2001). Several studies have

indicated that IL-2 transcript stabilization occurs via a TCR-independent CD28 signaling pathway mediated by AKT (Sanchez-Lockhart, et al., 2004; Sanchez-Lockhart & Miller, 2006) and the RNA binding protein NF-90 (Pei, et al., 2008; Shi, Godfrey, Lin, Zhao, & Kao, 2007). Other RNA binding proteins such as YB-1, Nucleolin, TTP, and HuF are also known to target the ARE sequence elements within the 3'UTR of the IL-2 transcript and affect mRNA stability (C. Y. Chen, et al., 2000; Ogilvie, et al., 2005). Additional studies have shown that while cis-regulatory sequences throughout the remainder of the IL-2 transcript cooperate in transcript stability mechanisms such as in the case of coding sequences, the ARE element within the 3'UTR is required for appropriate post-transcriptional control of the IL-2 transcript (C. Y. Chen, Del Gatto-Konczak, Wu, & Karin, 1998). Thus, *Il2* transcription is subject to multiple levels of control necessary to maintain T cell homeostasis.

Reporter Mouse Models

Conventional Transgenes

Accurate reporting of *Il2* gene expression is vitally important for tracking effector:tolerance decisions among T cell populations. Conventional transgenic mouse models typically consist of small recombinant transgenes containing coding sequence for a traceable marker under the control of minimal promoter region. Use of smaller transgenic constructs provides the advantage of short generation time, and relatively simple molecular cloning expertise. In addition, transgene integration into the genome occurs randomly. Thus, if transgene expression aims to report on transcriptional regulation of inserted regulatory elements, this goal can be achieved without disrupting

endogenous loci and normal function (Krulewski, Neumann, & Gordon, 1989; Miao, et al., 1994; Rachel, Wellington, Warburton, Mason, & Beermann, 2002; Ross, et al., 1998). However, several caveats exist when employing small transgenes. First, random integration of transgenes leads to head-to-tail concatamerization. The degree of concatamer formation and copy-number-dependent expression typically depends on the size of the transgene where smaller transgenes integrate more copies (~50+ copies) than larger transgenes (~5 copies) (Antoch, et al., 1997; Caron, et al., 2002; Constantini & Lacy, 1981; Giraldo & Montoliu, 2001; Hatada, Kuziel, Smithies, & Maeda, 1999; Karpen, 1994). In addition, smaller transgenes are limited by stable and silencing position effects due to local cis-regulatory sequences and the degree of chromatin silencing, respectively. This limitation is due to a lack of sufficient regulatory sequence containing insulator elements that act to ensure autonomous transgene regulation independent of position variegation effects (PEV) from neighboring endogenous chromatin terrain. Resulting misregulation of transgenes may yield suboptimal or undetectable expression levels or ectopic transgenic expression disrupting appropriate spatial transgene regulation (Heaney & Bronson, 2006). IL-2 transgenic reporter mouse models containing transgenes with minimal *Il2* promoter regulatory sequence have historically lacked fidelity of endogenous gene expression patterns making it difficult to precisely track the effector response from IL-2-producing lineages (Brombacher, et al., 1994; Yui, et al., 2001). A conventional transgenic model generated previously using a -583 bp *Il2* minimal promoter driving lacZ expression produced 40 founder lines where only four generated lacZ mRNA. Two of these four lacZ mRNA positive founder lines showed lacZ enzymatic activity where only one displayed inducible expression of reporter in an

IL-2 lineage-specific manner. The low frequency of founder lines that present activation-induced reporter expression in this study illustrates the lack of sufficient locus control activity in the -583 bp minimal *Il2* promoter to maintain consistent transcriptional regulation of reporter expression in all founder lines (Brombacher, et al., 1994). These studies exemplify the need for transgenes harboring sufficient cis-regulatory sequence by generating reporter transgenic models containing either sufficient insulator elements or a broader base of endogenous regulatory sequence from which to drive a more reliable expression of target genes. As a means to circumvent position-dependent effects using a transgenic approach, many transgenes have employed the use of additional regulatory elements such as locus control regions, insulators, or additional distal endogenous regulatory sequence to ensure inducible, lineage-specific expression.

Bacterial Artificial Chromosome Transgenic Models

Inclusion of insulator elements allows for position-independent reporter expression with IL-2 lineage-specificity (Saparov, et al., 1999). An IL2GFP transgenic model, which encodes a GFP reporter controlled by a 2.7 kb *Il2* promoter, was generated to track IL-2 producing effector T cells. The GFP reporter is expressed in a lineage-specific manner in effector T cell populations by virtue of the addition of a human CD2 locus control region located 3' to the reporter coding sequence (Saparov,et al). The inclusion of this cis-regulatory element ensures that local chromatin configurations are not able to hinder appropriate regulation of this transgene. In addition, larger fragments of endogenous sequence from artificial chromosomes (YACs, BACs, and PACs) have a high probability of containing critical locus control regions and have been employed to ensure faithful expression of transgenic mouse models (Giraldo & Montoliu, 2001;

Sparwasser & Eberl, 2007). Due to its more manageable size and simplicity of manipulation with the use of slightly modified standard cloning techniques gives BAC DNA a superior advantage for transgene generation as opposed to other artificial chromosome elements. In addition to position-independent expression, BAC transgenic technology also offers the advantages of a shorter generation time than targeting endogenous loci with standard knock-in approaches and low copy number insertion as opposed to high number tandem repeats created by smaller more conventional transgenes (Giraldo & Montoliu, 2001; Heaney & Bronson, 2006). Furthermore, BAC transgenic technology has been routinely used to study the regulation of numerous genes important for immunological processes including IFNγ (Hatton, et al., 2006), RAG1, RAG2, and the immunoglobin heavy chain constant region locus, as well as regulatory regions within rearranged VDJ genes, and the identification of the T helper 2 locus control region located within a downstream neighboring gene, RAD50 (Sparwasser & Eberl, 2007).

Gene Targeting Knock-in Strategies

Alternatively, knock-in gene targeting strategies have been utilized to introduce reporter coding sequences within the endogenous loci to evaluate regulation of gene expression (Naramura, Hu, & Gu, 1998). Unlike transgenic reporter methods that may be limited in the amount of critical distal regulatory sequence, knock-in reporter targeting approaches allow the monitoring of changes in gene expression within the context of all necessary regulatory elements required for appropriate gene regulation (Yui, et al., 2001). Targeting reporter sequences to the endogenous locus also has the added advantage of eliminating the need for introducing non-native boundary elements such as locus control regions and insulators to ensure position-independent expression (Saparov, et al., 1999).

Furthermore, in the case of knock-in strategies, the site of integration of the targeting sequence is known since reporter sequences are directly introduced into the endogenous loci, whereas transgenic approaches entail random integration of modifying sequences into an unknown genomic location. While the knock-in strategy poses several advantages over the use of transgenic reporter models, likewise this approach to targeting endogenous loci can have the potential caveat of affecting gene dosage. Knock-in techniques that involve ablation of endogenous gene expression from a single targeted allele leaving the second allele intact will alter the normal gene dosage of genes that require biallelic expression thus having potential physiological consequences (Heaney & Bronson, 2006). In summary, there are both advantages and caveats to either mouse model engineering approaches in developing effective reporter systems. Thus, the use of reporter models that contain endogenous-targeted sequences would complement the use of transgenic mouse models in validating lineage-specific gene expression.

Biological Significance of *Il2* Gene Regulation and Signaling

Integral Role for IL-2 in Effector T Cell Responses and Immune Tolerance

The role of IL-2 is central to mechanisms of tolerance and priming of the effector response. An understanding of the regulation of this cytokine is critical for the design of effective therapies to target multiple immune-compromising conditions. Contrary to the originally described role for IL-2 as a T cell growth factor, IL-2 was also paradoxically found to be essential for immune tolerance through the maintenance of $Foxp3^+$ T regs (Fontenot, et al., 2005; Sadlack, et al., 1995; Sadlack, et al., 1993; Schorle, et al., 1991; Willerford, et al., 1995). IL-2 signaling may help to explain this contradiction firstly

because signaling through the high affinity IL-2 receptor is thought to lead to T cell growth via activation of cell cycle machinery and subsequent DNA replication (Cantrell & Smith, 1983, 1984; Smith, 1988b). However, IL-2 signaling in T regs promotes Foxp3 expression through STAT5 signaling (Bayer, Yu, & Malek, 2007) as well as suppressive function (Gavin, Rasmussen, Fontenot, Vasta, Manganiello, & al, 2007; Wan & Flavell, 2007; L. M. Williams & Rudensky, 2007), which in turn inhibits the growth of conventional T cells and associated IL-2 production (Thornton & Shevach, 1998). T regs, thus, are essential in limiting the effector response and more critically serve to prevent the expansion of autoreactive T effector cells that have escaped thymic deletion (Suri-Payer, et al., 1998). In order to understand the nature of IL-2 signaling in promoting both effector immunity and tolerance, studies evaluating the difference in the activation of the downstream signaling pathways for PI3K and MAPK in both conventional T cells and T regs must be reviewed. After production of IL-2, conventional T cell IL-2 signaling mediates activation of STAT5, PI3K, and MAPK thereby potentiating T cell proliferation. However, unlike conventional T cells, T regs do not produce IL-2, and furthermore SOCS2 and PTEN activation in T regs are thought to prevent the activation of IL-2-induced PI3K and MAPK pathways while still facilitating STAT5-mediated foxp3 induction and suppressive function (Bensinger, et al., 2004; Lali, Crawley, McCulloch, & Foxwell, 2004; Malek, 2008; Sugimoto, et al., 2006; Walsh, et al., 2006). Thus, differences in the signaling pathways induced in conventional T cells and T regs may help to explain the opposing nature of IL-2 in promoting effector function in addition to a role in T reg maintenance and suppressive function. Secondly, recent studies evaluating the downstream effects of IL-2 autocrine signaling on *Il2* gene

expression in effector T cells raise additional questions pertaining to *Il2* gene regulation. Despite original data indicating that IL-2 autocrine signaling promotes DNA replication and proliferation of conventional T cells suggesting positive feedback signaling on *Il2* transcription, Villarino, et al have shown that IL-2 autocrine signaling leads to an auto-inhibitory feedback loop to prevent further IL-2 protein and transcript production in activated T cells(Villarino, et al., 2007). Therefore, despite the proposed proliferative trait of IL-2 on effector cells, the complexity of autocrine and paracrine IL-2 signaling to conventional T cells as well as T regs will require further investigation in order to delineate the role of this cytokine in effector function, tolerance, and contraction of the immune response.

Balance of Il2 Gene Regulation in Autoimmunity

The Idd3 (Insulin-dependent diabetes) locus includes the IL-2, IL-21, TENR, and KIAA1109 genes. Through genetic linkage association studies several single nucleotide polymorphisms (SNPs) upstream of the *Il2* transcriptional start site have been linked to several autoimmune conditions including type I diabetes, celiac disease, rheumatoid arthritis, autoimmune ovarian dysgenesis, and psoriasis in humans as well as type I diabetes in the non-obese diabetic (NOD) mouse model. Thus, the Idd3 locus is considered a generalized autoimmune risk locus (A. C. Anderson, Chandwaskar, Lee, & Kuchroo, 2008; M. S. Anderson & Bluestone, 2005; del Rio, et al., 2008; Glas, et al., 2009; Hunt, et al., 2008; Y. Liu, et al., 2008; Sadlack, et al., 1993; van Heel, et al., 2007; Yamanouchi, et al., 2007; Zhernakova, et al., 2007). Polymorphisms within the *Il2* coding region, which result in the conversion of a serine→proline amino acid in mature IL-2 protein also repeatedly segregate with the Idd3 locus in NOD mice (Denny, et al.,

1997). Santamaria, et al have shown an associated downregulation of IL-2 transcript levels as well as a decrease in the frequency of T regs in NOD mice suggesting a strong association in appropriate regulation of the *Il2* gene and tolerance in type I diabetes (Yamanouchi, et al., 2007). Future studies delineating the genetic alterations within the Idd3 region and more specifically the *Il2* locus that result in aberrant *Il2* expression in autoimmune models such as NOD mice will help to shed light on the pathways of transcriptional regulation that are critical for IL-2 production and downstream maintenance of tolerance.

IL-2-Targeted Therapies

Human trials using IL-2 administration as a therapy for HIV/AIDS and cancer have indicated an expansion of the T reg population further suggesting that IL-2 may preferrentially activate T regs rather than stimulating the effector response *in vivo* (Ahmadzadeh & Rosenberg, 2006; van der Vliet, et al., 2007; Velilla, et al., 2008; Zhang, et al., 2005). This may explain the reduced efficacy of IL-2 as an anti-tumor therapy as increased T regs would presumably interfere with tumor-directed effector T cell populations (Malek, 2008). In contrast to a suggested benefit in HIV/AIDs patients, ongoing human clinical trials evaluating the overall benefit of IL-2 therapy show no substantial benefit in the occurrence of opportunistic infections and death (Levy & Committee, February 8-11, 2009; Losso, Abrams, & Group, February 8-11, 2009). Further studies will be necessary to delineate the value of IL-2 administration as a form of HIV/AIDS and cancer therapy by evaluating the contribution of IL-2 production to the effector response *in vivo* despite clear T reg promoting properties.

Changes in immune cellular components as a result of IL-2 administration may be informative in the design of alternative IL-2-related therapies for autoimmune disease. Antibody therapy targeted to block CD25 (Daclizumab), the alpha subunit of the high affinity IL-2 receptor, has shown promise toward the treatment of autoimmune conditions such as multiple sclerosis. Clinical trials have indicated significant benefit either as a monotherapy or in combination with current mainstream therapies such as IFN-β during active disease. The mechanism of action of daclizumab administration seems to involve a novel and unexpected mechanism of reduced frequency of T regs while showing an increase in the fraction of $CD56^{bright}$ NK cell populations rather than inhibition of conventional T cell expansion. Despite the reported benefits, however, daclizumab therapy in some cases has shown severe side effects calling into question its approval for use in the general population (Bielekova, et al., 2006; Bielekova, et al., 2009; Oh, et al., 2009; Schippling & Martin, 2008). Additional studies as to the mechanism involving changes in NK cell and T reg populations as a result of blocking IL-2 signaling with inhibitory antibodies to CD25 will prove useful in gaining further mechanistic insight into immune imbalance during multiple sclerosis as well as in designing more effective therapies with minimal side effects.

Recent studies have also shown that *in vivo* administration of anti-IL-2/IL2 complexes serve as an IL-2 receptor agonist leading to enhanced T reg development *in vivo* (Boyman, Kovar, Rubinstein, Surh, & Sprent, 2006; Webster, Morley, van Lenthe, & Muller, 2008). The mechanism by which the cytokine:antibody complex treatment leads to enhanced expansion of the regulatory population is currently unknown. However, a clear understanding into a potential mechanism may prove useful in

generation of therapies targeted towards autoimmune conditions involving aggressive autoreactivity.

In conclusion, a greater understanding of the involvement of IL-2 in the effector-tolerance decision will be critical in designing effective therapies for several immunocompromising conditions including cancer and autoimmunity in order to maintain immune homeostasis. Central to this understanding is a greater awareness of the role of *Il2* gene expression in the balance of T cell immunity. Effective IL-2 reporter mouse models will be an essential approach to addressing many biologically relevant questions that will further our understanding of this critical cytokine in T cell homeostasis.

REGULATORY T CELLS SUPPRESS EXPRESSION OF *Il2* BY RESPONDER T CELLS VIA A TRANSCRIPTIONAL MECHANISM

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Abstract

Interleukin-2 (IL-2) production and signaling are integral to maintenance of immune homeostasis. Rapid production of IL-2 upon activation of naïve CD4 T cells has been associated with effector immunity. Conversely, T regulatory cell populations that establish immune tolerance target production of this cytokine in responder CD4 T cells for suppression. Previous reports have suggested a mechanism of suppression of CD4 responder T cells by Tregs involving transcriptional inhibition of IL-2 while others implicate induced cell death through Treg-mediated cytokine starvation. Limitations in conventional staining methods and transgenic reporter mouse models have significantly restrained assessment of IL-2-producing CD4 responder cells in Treg-containing co-cultures. In this study we describe the generation of an *Il2* BAC-In transgenic reporter model (2BiT) in which a Thy1.l reporter is expressed under control of the extended *Il2* locus. Reporter expression is initiated rapidly upon activation and with fidelity to the endogenous *Il2* locus in CD4 T responders. Implementation of our model in Treg suppression co-cultures indicates a model of suppression where Tregs inhibit *Il2* expression resulting in increased CD4 responder T cell death. Our data also are suggestive of a mechanism of Treg suppression involving CD4 responder cell death by inactivation and competition with Tregs for co-stimulation through APCs rather than by cytokine starvation. Taken together, our findings describe the development and implementation of a novel *Il2* BAC transgenic model that serves as an effective tool to study the contribution of *Il2* regulation in CD4 responder T cells to mechanisms of Treg suppression.

Introduction

Interleukin-2 (IL-2) is a type I alpha helical cytokine that is associated with *in vitro* T cell growth and effector responses (1-3). Additional studies have indicated an *in vivo* role for IL-2 signaling in maintaining peripheral T regulatory cell populations (Tregs) (4-6). Tregs are essential in upholding immune tolerance through the inhibition of autoreactive T cells that have circumvented thymic deletion mechanisms (7). While the events involving a mechanism for Treg suppression remain undefined a key hallmark of suppressive activity of Tregs is the inhibition of IL-2 production by CD4 responder T cells (8). However, the mechanism of IL-2 inhibition in responder CD4 T cells remains a point of contention. Previous reports implicate a mechanism of Treg suppression involving transcriptional inhibition of *Il2* expression in CD4 responder T cells (8). However, additional studies suggest Tregs suppress CD4 responder T cells through cytokine starvation and apoptosis (9, 10). A primary limitation preventing clarification of the role of IL-2 inhibition in Treg-mediated suppression resides with constraints in the detection of IL-2-producing cells as a result of short-lived expression and stability of cytokine product and reporter systems.

IL-2 mRNA is rapidly and transiently upregulated upon sufficient TCR and CD28 signaling in antigen-activated naive T cells and is tightly coordinated with protein production (1, 11). Steady state levels of IL-2 mRNA are critically dependent upon changes in gene locus accessibility, initiation of transcription, and stabilization through protein interactions with AU-rich sequence elements within the 3' UT of the existing transcript (12-15). The rapid turnover of IL-2 transcript and protein has presented significant challenges in the detection and analysis of *Il2* gene regulation (14, 16, 17).

Generation of reporter expressing mouse models to track gene regulation as an alternative to monitoring transiently regulated genes presents an attractive alternative to historically limiting staining methods. However, conventional IL-2-reporting transgenic mouse models that incorporate minimal *Il2* promoter sequences have frequently lacked fidelity of reporter expression to endogenous IL-2 regulation patterns due to insufficient *cis*-regulatory sequence or position-dependent integration effects from random insertion into the genome (18-20).

In an effort to generate an *Il2* reporter transgene that reliably and stably reports on *Il2* gene regulation we have constructed an *Il2* BAC-in transgenic mouse model (2BiT) containing an extended regulatory region of endogenous locus driving expression of an SV40 poly adenylation sequence-containing Thy1.1 reporter. Our model shows rapid and robust reporter expression in an *Il2*-specific manner. Using our model we show that Treg-mediated suppression of activated CD4 responder T cells results in an inhibition of *Il2* gene expression during IL-2 peak production and results in an increase in responder cell death during late activation. Suppression of the 2BiT reporter in CD4 T responders was rescued by excess CD28 co-stimulation but not altered by exogenous IL-2. Thus, the 2BiT model is a highly sensitive tool that enables analysis of Treg-mediated suppression to *Il2* expression and activation of CD4 responder T cells.

Methods

Generation of the Il2 BAC-in Thy1.1/SV40pA transgenic mouse model (2BiT)

To generate the IL-2 BAC-in Thy1.1-SV40pA transgenic mouse model (2BiT), a Thy1.1-linked SV40pA sequence was introduced to replace the first exon of the IL-2

gene within the IL-2-containing BAC clone 114D21 (CHORI Oakland, CA) by recombineering techniques as described previously (21). Briefly, frt sequences, used for site-specific recombination, were introduced directly adjacent to a kanamycin resistance cassette. This enabled excision of this drug resistance marker in subsequent BAC engineering stages. The frt-flanked kanamycin resistance cassette was generated by amplifying the kanamycin coding sequence from the Topo pCR 2.1 plasmid (Invitrogen) using the oligonucleotides 5'-CGGGCCGCGGGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTCAGAAGAACTCGTCAAGAAGGCG-3' and 5'-GCGCGAGCTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCCCGGAATTGCCAGCTGGG (Integrated DNA Technologies) containing frt sequences and a unique restriction site and then further sub-cloned into the pBluescript II plasmid. The kanamycin resistance gene allowed for selection of positive recombinants following recombineering. A Thy1.1-linked SV40pA sequence was amplified from the Thy1.1-SV40pA/pcDNA3 plasmid generated previously (22) and cloned upstream of the frt-flanked kanamycin cassette in pBluescript II. Regions of homology**,** both 5' and 3' of the first exon of *Il2*, were PCR amplified using primers 5' CGTTCAAGCTTAGAGCTGCCTATCACCCTTGCTAATCACTCCTCACAGTGACCTCAAGTCCTGCAGGCATGTACCCAGCCATCAGCGTCGCTCT 3' and 5' CGCCACACTTGACCAGTTTGTC 3' for the 5' arm of homology and 5' GCATACCCGGGAGGTTATTAAACACAGCCTTTGGCAAGAAAGCTAAAGGTATTGCCTATAGATGGGATGGCTGTGCTACGACTCACTATAGGGCG 3' and 5' TCGACCAAGCTTATAGCCGAATAGCCTCTCCACC 3' for the 3' arm of homology.

The arms were then digested and sub-cloned upstream of the Thy1.1 coding sequence and downstream of the frt-flanked kanamycin cassette, respectively. The resulting fragment was then recombined into an IL-2 containing BAC DNA clone 114D21 subsequently replacing the first exon of IL-2 and preventing further IL-2 mRNA transcription from the BAC fragment. The chloramphenicol-resistant strain EL250 (Frederick Laboratories), derived from DY380, was used to generate the IL-2 BAC Thy1.1-SV40pA/frt-Kan^r recombinant. The EL250 strain contains a defective lambda prophage encoding the genes exo, bet, and gam, which direct recombination and are regulated through the temperature-sensitive repressor cI857 (6). Prior to recombineering, the 114D21 IL-2-containing BAC clone was introduced into EL250 by electroporation (1.8 kV). 114D21-containing EL250 was subject to transient heat-shock at 42°C, then electroporated with the Thy1.1-SV40pA/frt-Kan^r (120ng) targeting fragment. Electroporated EL250 were then recovered in LB media at 30°C for 1 hour, then plated on LB agar plates containing chloramphenicol (20 μg/ml) and kanamycin (50 μg/ml) and grown at 30°C for 1-2 days. Kanamycin resistant colonies were then screened by PCR and sequencing. EL250 also harbor an arabinose-inducible flp*e* recombinase gene, which can mediate recombination between two identical frt sites (Lee, EC 2001). In order to prevent any adverse effects on transcriptional regulation of the Thy1.1 reporter the kanamycin coding sequence was removed from the BAC construct through transient expression of flp*e* recombinase with the addition of 0.1% L-arabinose to the bacterial cultures. Kanamycin-excised Thy1.1 BAC reporter clones were screened by parallel drug selection with either chloramphenicol (20 μg/ml) or chloramphenicol (20 μg/ml) and kanamycin (50 μg/ml). The final IL-2 Thy1.1/SV40pA BAC reporter was then linearized with NotI digestion and

purified for injection into fertilized embryos of C57BL/6 mice. Three female founder mice were found to be positive for the Thy1.1 reporter by PCR screening, two of which expressed the reporter in 33 ± 3% of $CD4^+$ cells, and the third founder did not express Thy1.1 possibly due to integration site silencing effects. Of the two founder mice that did express the IL-2 Thy1.1/SV40pA reporter only one reproduced offspring. This founder was used to establish the IL-2 BAC-in Thy1.1/SV40pA reporter transgenic mouse line (2BiT) harboring a BAC transgene approximately 200 kb in length encompassing the IL-2 and IL-21 genes.

Mice

C57BL/6 mice (Jackson Labs, Bar Harbor, ME, USA), IL-2 BAC-in Thy1.1 transgenic reporter mice (2BiT), IL2GFP transgenic (3), IL2GFP Knock-in (unpublished), and $Foxp3^{gfp}$ mice were housed in a pathogen-free facility in autoclaved microisolator cages and were used between 6 and 8 weeks of age. $Foxp3^{gfp}$ mice have previously been described (6) and were backcrossed onto C57BL/6.

BAC Copy Number Determination

Genomic DNA purified from 2BiT and C57BL/6 mice were digested with HindIII overnight. Digestion was continued the following day with additional HindIII enzyme then digested DNA was separated on a 0.8% agarose gel at 35 volts. Genomic DNA was then transferred to a nylon membrane and probed with a sequence directed toward the 5' UT of the IL-2 gene. The probe hybridizes to a 6 kb fragment in the BAC transgenic locus and 4.8 kb fragment at the endogenous IL-2 locus. Densitometry analysis showed a transgenic:endogenous stoichiometry of ~1.7 indicating 3 or 4 copies of the transgene present in the genome of the BAC transgenic mice.

BAC Termini Mapping

Integration of the 2BiT transgene was verified by "inside-out" polymerase chain reaction (PCR) using BAC-termini primers 5' GGCCGTCGACATTTAGGTGACACT 3' (Forward primer #1) and 5' TGTGCATGTAAGTGTAGACCCCTG 3' (Reverse primer #2) for the 5' BAC terminus and 5' TCTCCCTATAGTGAGTCGTATTAG 3' (Forward primer #3) and 3' CCTGGCAAAATCGGTTACGG 3' (Reverse primer #4) to the 3' BAC terminus. The absence of amplified product with primers #3 and 4 suggested truncation of the 3' end of the 2BiT reporter. Quantitative PCR analysis with SYBR green (Invitrogen, Carlsbad, CA) using primers 5' GTGCCCAATTCAAACTGAGTGA 3' and 5' TTCCCAGCTTTGCTCAGATG 3' to exon 2 and 5' GTGCCCAATTCAAACTGAGTGA 3' and 5' GCAATAAACCAGCGTGTTGATG 3' to exon 10 of the Tenr gene, which is located in opposing orientation and 3' to IL-2 within the 2BiT BAC transgene, demonstrated a 4-fold difference in the genomic DNA of 2BiT relative to C57BL/6 at exon 10 of the Tenr gene. There was no difference in the genomic content at exon 2 of the Tenr gene. This suggested that the truncation of the BAC transgene occurred between exon 2 and exon 10 of the Tenr gene. Exon 2 and exon 10 are separated by ~30 kb of genomic DNA potentially reducing the original 237 kb BAC transgene to 200 kb.

$CD4^+$ T Cell Isolation And Activation

Single-cell suspensions were generated from spleen and lymph nodes from the indicated mouse strains following red cell lysis. Subsequently, $CD4^+CD25^-CD44^{low}$ and $Thy1.1^-$ (from 2BiT mice) $CD4^+CD25^-CD62L^{high}$ cells were further isolated by flow activated cell sorting. Facs sorting instead of other enrichment methods was found to

yield optimal T cell viability at early activation periods. Negative fractions from splenic $CD4^+$ T cell isolation were irradiated (3000 rads) and used as the source of APCs in co-culture with naïve T cells at a ratio of 8:1 or 5:1, respectively. Cultures were stimulated with 2.5 μg/ml or 5 μg/ml soluble anti-CD3 antibody (clone 145-2C11) in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated FBS, nonessential amino acids (1X), penicillin (100 IU/ml), streptomycin (100 mg/ml), glutamine (2mM), sodium pyruvate (1mM), and β-mercaptoethanol (55μM) ('R10 Medium'). Some cultures were also treated with 1 μg/ml α-CD28 antibody (clone 37.51) (ebioscience).

Proliferation Assays

For proliferation assays, $5x10^4$ $CD4^+CD25^-CD44^{low}$ or $CD4^+CD25^-CD62L^{high}$ naïve T cells were seeded into 96-well polypropylene round bottom plates in triplicate with $4x10^5$ CD4-depleted irradiated splenic feeders (3000 rads) with or without $5x10^4$ live-gated $CD4^+CD25^+$ or $CD4^+Foxp3^{gfp+}$ T regulatory cells. Cultures were incubated with or without 2.5 μg/ml soluble anti-CD3. After 68-72 hours, 1 μCi [^{3}H]thymidine (TdR) was added to each well and incubated for 16-20 hours after which samples were harvested with a cell harvester and then thymidine incorporation determined by a scintillation counter.

Cytokine Analysis

IL-2 protein levels were quantified from the supernatants of unstimulated samples or samples stimulated with soluble anti-CD3 using cytometric bead array flex sets according to manufacturer's recommendations (BD Biosciences). IL-21 protein levels were measured by enzyme-linked immunosorbent assay (ELISA) using α-mouse capture

and biotinylated antibodies to IL-21 (R&D Systems) according to manufacturers recommendations.

Quantitative PCR Analysis

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen; Carlsbad, CA). cDNA synthesis was performed with Superscript III reverse transcriptase and random hexamers (Invitrogen; Carlsbad, CA) according to manufacturer's recommendations. In order to eliminate genomic DNA contamination, RNA was treated with DNA-*free* recombinant DNAse I (0.2 U/μl) (Ambion) for 30 minutes at 37°C before terminating the reaction with stop reagent. Multiplex real time PCR-based analysis of gene expression was performed with a Bio-Rad iCycler Optical System and QPCR Platinum Supermix (Bio-rad Laboratories). The following primer pairs and probes for IL-2, and β 2 microglobulin were used (Integrated DNA Technologies): for IL-2; oligonucleotides 5'-GAAACTCCCCAGGATGCTCA-3' and 5'-GCCGCAGAG GTCCAAGTTC-3' and probe 5'/56-FAM/TTGCCCAAGCAGGCCA CAGAATTG-3'. Cycle threshold values for IL-2 and IL-21 were normalized to β 2-microglobulin values using oligonucleotides 5'-CCTGCAGAGTTAAGCATGCCAG-3' and 5'-TGCTTGATCACATGTCTCGATCC-3' and probe 5'-/5Texrd-XN/TGGCC GAGCCCAAGACCGTCT-3'

mRNA Stability

$CD4^+CD25^-CD44^{low}$ or $CD4^+CD25^-CD62L^{high}$ naïve T cells (3 X 10^5 cells/well) from 6-8 week old C57BL/6 or 2BiT mice were stimulated with or without live-gated $CD4^+Foxp3^{gfp+}$ T regulatory cells (3 X 10^5 cells/well) in the presence of 5 μg/ml soluble anti-CD3 and CD4-depleted irradiated splenic feeders (1.5 X 10^6 cells/well) in 48 well

plates. After 18 hours, actinomycin D (10 μg/ml) was added to each well and samples were homogenized in trizol (Invitrogen) at the indicated time points. Total RNA was prepared followed by cDNA synthesis and analysis of gene expression was carried out by quantitative PCR. Normalized values for individual time points were determined using the difference between IL-2 or Thy1.1 transcript with β 2-microglobulin for Δct. Initial values for Δct for IL-2 or Thy1.1 were then normalized relative to their respective Δct_0 (Δct at time 0) for ΔΔct values at each time point to estimate relative decay. The half-life of transcript was determined by two possible methods; calculated from trendline equations; Tresp [$y=89.576e^{-1.563x}$, $R^2=0.93023$] and Tresp + Treg [$y=84.775e^{-2.81x}$, $R^2=0.96003$], or as previously described where the $t_{1/2}$ = (0.5 hours)/(Δct_{30}-Δct_0) (23).

T Regulatory Cell Polarization

Spleen and lymph nodes were harvested from Foxp3^{gfp} mice. Following red cell lysis with ACK (Quality Biologicals, Inc), $CD4^+$ cells were isolated using $CD4^+$ Dynabead separation according to manufacturer's protocol (Invitrogen). $CD4^+CD25^-$ cells were then further sorted using CD25 Microbead isolation (Miltenyi Biotech). $CD4^+CD25^-$ cells were then cultured with 5 ng/ml $TGF\beta_1$ (R&D Systems), 50 U/ml IL-2 (R&D Systems), 100 nM retinoic acid (Sigma-Aldrich), 10μg/ml anti-IFNγ (XMG 1.2), 10μg/ml anti-IL-4 (11B11) in R-10 complete media. After 4 days in culture, live $CD4^+$ Foxp3^{gfp+} cells were isolated by flow activated cell sorting using CD4-PeCy7, FITC-GFP, and 7AAD staining (BD Bioscience).

Cytokine Capture Assay, surface, and intracellular staining

IL-2 producing cells were detected using the IL-2 cytokine secretion assay according to the manufacturer's recommendations (Miltenyi Biotech). In addition, recombinant

DNAse I (0.1 U/ml) was added during the secretion step to minimize cell clumping (Roche). Following staining with IL-2 APC detection antibody surface staining was performed for using FITC-Vital Dye (Invitrogen Molecular Probes), PE-CD25, PerCP-Thy1.1, PeCy7-CD4 (ebioscience), and Biotin-CD45.1 or Biotin-CD44 (ebioscience) with streptavidin-APCCy7 (BD Pharmingen). Samples were acquired using the LSRII flow cytometer (BD Bioscience) and data were analyzed using FlowJo 8.4.6 (Tree Star, Ashland, OR).

Statistical Analysis

Statistical significance was determined by ANOVA with Tukey post-test or unpaired Student's *t* test using Prism software (Graphpad, San Diego, CA). Values are expressed as either the mean ± s.e.m. or the mean ± s.d. where a p value ≤ 0.05 is considered statistically significant.

Results

Development and Characterization of an Il2 BAC-in reporter mouse model

In an effort to evaluate *Il2* gene expression in CD4 responder T cells during Treg suppression we have engineered a transgenic reporter model containing extended distal regulatory sequence of the *Il2* promoter. Using a bacterial artificial chromosome fragment containing the *Il2* gene as well as 155 kb and 30-60 kb of contiguous 5' and 3' genomic DNA, respectively, a Thy1.1 (CD90.1) coding-SV40 poly adenylation sequence cassette was introduced in-frame into the first exon of the *Il2* gene within the BAC sequence using recombineering methods (Figure 1). A premature stop codon was placed 3' to the Thy1.1 coding sequence to ensure translational termination of the Thy1.1 transcript independently of naïve BAC *Il2* translation signal sequences. The 5' splice site

between exons 1 and 2 of the BAC *Il2* gene was disrupted to abrogate downstream *Il2* expression while still ensuring normal gene regulation within the remainder of the BAC locus. Founder lines on a defined strain background were then generated by injecting the "*Il2* BAC-in transgene" (2BiT) DNA into C57BL/6 single-cell embryos. A single founder line containing ~3-4 copies of the 2BiT transgene (Figure 2) was evaluated for the remainder of our studies. Integration of the 5' terminus of the 2BiT transgene was confirmed while screening for 3' terminus integration indicated a truncation event between exon 2 and exon 10 within the downstream gene TENR estimating our BAC transgene to be approximately ~200 kb in length (Figure 2). Development, reproduction, and survival were normal in 2BiT mice hemizygous for the transgene.

Characterization of Il2 BAC-in reporter mice

In order to validate the immunological phenotype of the 2BiT model, lymphoid population distribution and function were analyzed *ex vivo* from hemizygous 2BiT or C57BL/6 mice. Analysis of peripheral lymphoid populations in 2BiT mice revealed normal frequencies of CD4 and CD8 T cells, B cells, and Foxp3^{gfp+} T regulatory cells in lymph nodes and spleen (Figure 3), and there appeared to be no histopathological abnormalities throughout a normal lifespan in these mice. In addition, soluble α-CD3-activated naïve 2BiT CD4 T cells showed similar levels of IL-2 protein production and T cell proliferation at 18 and 72 hours, respectively (Figure 4). The results of the parameters tested suggested that peripheral lymphoid populations in hemizygous 2BiT model showed no overt abnormalities compared to mice of similar background strain. Due to the presence of an additional copy of the IL-21 gene within our BAC transgene we also assessed the level of IL-21 production in activated naïve CD4 T cells from 2BiT

mice compared to mice of the C57BL/6 background strain. Our results indicated that under conditions of high IL-21 production, T helper 17 polarized CD4 T cells from 2BiT mice showed higher levels of IL-21 protein production compared to similarly polarized CD4 cells from C57BL/6 mice. However, under conditions of soluble α-CD3 activation IL-21 transcript levels were lower in CD4 T cells from 2BiT mice compared to CD4 T cells from C57BL/6 mice (Figure 5). In addition, protein production under these conditions during peak IL-2 production showed undetectable levels of IL-21 protein production (data not shown). These results suggested that an additional copy of IL-21 does not lead to an excess of IL-21 transcript or protein production for the conditions tested in this study.

To examine the fidelity of 2BiT transgene expression with endogenous *Il2* regulation, total lymphocyte populations from spleens of 2BiT mice were activated with PMA and Ionomycin and analyzed by flow cytometry. Staining analysis of CD4 and CD8 T cells revealed that populations expressing IL-2 showed coordinate production of the Thy1.1 reporter indicating fidelity of reporter expression to endogenous *Il2* regulation (Figure 6). Similar results were also seen in total lymphocyte populations from mesenteric lymph nodes, and pooled axial, inguinal, and cervical lymph nodes (data not shown). Likewise, Thy1.1^{+} populations showed an association with IL-2 expression demonstrating minimal leakiness of reporter expression (Figure 6). Due to the inherent transient kinetics of *Il2* expression, the presence of Thy1.1^{+}IL-2^{-} populations are likely due to prolonged transcript stability of the Thy1.1 reporter in cells that have ceased endogenous IL-2 production (Figure 7). Furthermore, CD4 T cells are the primary source of IL-2 cytokine production with a contribution to a lesser extent from CD8 T cells (1).

In agreement with lineage-dependent expression patterns of IL-2, activation of $CD4^+$ T cell populations yielded a prominent population of dual-expressing $Thy1.1^+IL\text{-}2^+$ cells where as activated $CD8^+$ T cells expressed both IL-2 and Thy1.1 at a more reduced frequency. Furthermore, activation of $CD4^-CD8^-$ cells with PMA and Ionomycin failed to generate significant levels of Thy1.1 and IL-2 production. These results suggested that the Thy1.1 reporter in our 2BiT model demonstrates lineage-specific activation. In addition, unlike naïve CD4 T cells, $CD4^+$ T regulatory cells are known for their inability to express *Il2* transcript or protein product due to gene silencing upon TCR activation (25). In order to further validate lineage-exclusion of reporter expression in Treg populations from the 2BiT transgenic model, $CD4^+CD25^+Thy1.1^-$ populations were isolated from 2BiT peripheral lymphoid tissues and activated with soluble α-CD3 in the presence of irradiated CD4-depleted splenic APCs followed by analysis for Thy1.1 expression by flow cytometry. In accordance with Tregs being non-IL-2 producers, stimulation of natural Treg populations from 2BiT mice indicated low levels of Thy1.1 surface expression compared to activation of naïve CD4 T cells (Figure 8). These results show that reporter activation adheres to patterns of *Il2* silencing in regulatory T cell populations. In order to evaluate the fidelity of Thy1.1 reporter and endogenous IL-2 expression under conditions of polyclonal activation in $CD4^+$ T cells, naïve $CD4^+CD25^-CD44^{low}Thy1.1^-$ T cells harvested from 2BiT peripheral lymphoid tissues were activated with soluble α-CD3 and CD4-depleted irradiated splenic APCs and analyzed by flow cytometry. Kinetic analysis of reporter expression showed induction of $CD4^+Thy1.1^+$ cells as early as 6 hours with coordinate production of endogenous IL-2. Coordinate regulation of Thy1.1 reporter expression and IL-2 continued to increase to peak

production at 16 hours **(**Figure 9**)**. Reporter expression achieved a higher mean fluorescent intensity and was prolonged after endogenous IL-2 levels declined suggesting IL-2-producing cells are more stably marked in our model (Figure 9). Taken together, in addition to the congruence of Thy1.1 reporter expression with endogenous *Il2* activation, 2BiT reporter activation exhibited lineage-specific expression to the IL-2-producing $CD4^+$ and $CD8^+$ T cell populations, while displaying minimal reporter activation in traditionally IL-2 non-producing Treg populations. Our analysis of reporter expression in response to T cell activation also demonstrates the use of a transgenic reporter that marks *Il2* activation to IL-2-producing lineages.

Thy1.1 reporter expression is suppressed in 2BiT responders by T regulatory cells

Conflicting studies have proposed that *in vitro* Treg suppression of responder CD4 T cells involves inhibition of *Il2* gene expression while other studies have implicated a mechanism of Treg suppression involving cytokine deprivation and cell death (8-10). In previous studies, it has been difficult to definitively assess the effects of Treg suppression on *Il2* gene expression due to technical constraints involved with separating IL-2 production by the Treg and T responder populations, and difficulties in resolving possible differential effects on *Il2* transcription and *Il2* transcript stability (8, 13). Because the 2BiT model permits enhanced detection of assessment of *Il2* expression by transgenic responder T cells, and the 2BiT transcript is stabilized by replacement of the endogenous 3'UTR element with an exogenous 3'UTR element lacking destabilizing AU repeats, we were able to examine the effects of Treg suppression on *Il2* expression in responder T cells independently of effects on mRNA stability. We analyzed the effect of congenically marked Tregs on Thy1.1 reporter expression during peak IL-2 production

periods in 2BiT naïve CD4 T cells activated with soluble α-CD3 and irradiated CD4-depleted splenic APCs. We evaluated conditions containing either naïve CD4 2BiT responders alone or cultures containing naïve 2BiT CD4 responders and an equal number of naïve CD4 C57BL/6 to test if doubling the cell number in our co-culture resulted in suppression of the Thy1.1 reporter. Our results indicated that during peak IL-2 production the presence of Tregs led to a significant reduction in the frequency of Thy1.1 reporter expression (49.3% ± 2.71% reduction) by viable activated naïve 2BiT CD4 responder T cells when co-cultured at a 1:1 ratio with congenically marked Treg cells (Figure 10). However, conditions containing activated naïve 2BiT responder CD4 T cells plus an equal number of naïve C57BL/6 CD4 T cells did not alter the frequency of viable $CD4^{+}Thy1.1^{+}$ 2BiT responder cells (Figure 10). Addition of higher ratios of Tregs to T responders further suppressed Thy1.1 reporter expression (59.7% ± 0.50% reduction) in 2BiT responder CD4 T cells (Figure 2). Furthermore, mean fluorescent intensities (MFI) of Thy1.1 expression in viable responder cell populations also showed a significant reduction, which correlated with the number of T regs. These results suggested a relative inhibition of reporter transcript per cell (Table I). Thus, these results showed that Tregs inhibited *Il2* gene expression in viable responder CD4 T cells *in vitro* during peak IL-2 production and that suppression is due to inherent properties of this regulatory population and not a result of increased total cell number in our co-cultures.

In light of previous reports suggesting that Treg suppression involves a mechanism of cytokine depletion and cell death of responder CD4 T cells we evaluated the effect of Tregs on the viability of 2BiT responder cell populations activated with soluble α-CD3 and irradiated CD4-depleted splenic APCs in parallel with Thy1.1

reporter expression (8-10). We show that during a peak IL-2 production period, Tregs showed no overt effects on CD4 responder T cell viability despite a proportional decline in activated $CD4^+Thy1.1^+CD25^+$ within viable 2BiT responder T cell populations (Figure 11A). These results suggested that Tregs were capable of suppressing *Il2* gene expression in viable responder CD4 T cells during an early activation period without altering overall responder cell viability. However, longer *in vitro* culture times are necessary to assess effects on cell viability. Therefore, we also evaluated the effect of Tregs on 2BiT responder T cell populations during later activation stages. Analysis of 2BiT reporter expression indicated that similar to patterns seen during early activation, Tregs were able to suppress the frequency of $CD4^+Thy1.1^+$ viable 2BiT responder populations. However, unlike early activation, the presence of Tregs diminished 2BiT responder CD4 T cell viability during late activation (Figure 11A, B). Furthermore, inhibition of IL-2 protein production and proliferation of 2BiT responder CD4 T cells occurred in concert with co-cultures containing Tregs during both phases of activation demonstrating suppressive function of regulatory T cell populations (Figure 12). Our results indicated that Tregs inhibited induction of viable CD4 responder T cells capable of expressing Thy1.1 reporter during both early and late activation while also enhancing 2BiT responder cell death during late activation. Together, these results indicated that Treg-mediated suppression of responder CD4 T cells involves early inhibition of *Il2* gene regulation that precedes an overall reduction in cell viability.

Since the presence of Tregs led to a marked decline in the frequency of viable $CD4^+Thy1.1^+CD25^+$ responder T cells we asked if Treg suppression induced cell death of activated Thy1.1-expressing responder CD4 T cells or if Tregs prevented CD4 T

responders from overcoming a threshold of activation. To this end we analyzed total 2BiT CD4 responder cell populations for the frequency of $CD4^+Thy1.1^+$ responder cells in viable and non-viable populations differentiated by vital dye staining. Our results indicated that non-viable populations within 2BiT responder CD4 T cell cultures activated with soluble α-CD3 and irradiated CD4-depleted splenic APCs in the absence of Tregs were predominantly $Thy1.1^-$ where as $Thy1.1^+CD4^+$ T cells were largely among viable responder populations (Figure 13). This trend was seen in both early and later activation periods (Figures 13, 14). The presence of Tregs led to a reduction in viable $CD4^+Thy1.1^+$ responder T cell frequency and an increase in the proportion of non-viable $CD4^+Thy1.1^-$ responder populations. These findings suggested that Treg-mediated suppression prevented naïve CD4 T cells from becoming activated and undergoing *Il2* gene expression rather than rather than targeting cell death of activated and *Il2*-competent responder T cells. In sum, these data suggest that Tregs act to suppress responder CD4 T cells *in vitro* by preventing initial activation and subsequent *Il2* expression necessary, events which mark the potential for *in vitro* cell growth and survival.

Exogenous IL-2 Fails to Rescue Treg-Mediated 2BiT CD4 Responder Suppression

Several studies have suggested that suppression of responder CD4 T cells results from Treg sequestration of available IL-2 by virtue of IL-2 binding to surface CD25 on Tregs (9, 10, 25). Tregs would thus serve to act as an "IL-2 sink" preventing necessary survival signals to the responder population thus leading to premature apoptosis (10). Previous reports have indicated addition of exogenous IL-2 during co-culture of responder cells and Tregs can rescue a Treg-mediated suppressive phenotype *in vitro* (9, 26). To this end, we wanted to determine if excess IL-2 prevented suppression of 2BiT

reporter-positive populations in co-cultures containing Tregs. Our results indicated that addition of exogenous IL-2 in the presence or absence of Tregs to naïve CD4 2BiT responder T cells activated with soluble α-CD3 and irradiated CD4-depleted splenic APCs did not affect the frequency or mean fluorescent intensity of 2BiT reporter expression in 2BiT responder CD4 T cells during early activation (Figures 11A, 15-17, Table I). In addition, IL-2 did not rescue the degree of 2BiT responder cell death after longer periods of activation (Figure 11B). These results suggested that excess IL-2 cytokine during *in vitro* activation of naïve CD4 2BiT T responders did not alter *Il2* gene expression or cell viability in responder CD4 T cells in the presence or absence of Tregs.

Excess CD28 co-stimulation overrides Treg-Mediated 2BiT Reporter Inhibition

Our results thus far have indicated that a primary target of Treg suppression is inhibition of *Il2* expression in activated naïve CD4 T cells. In addition to a threshold level of cognate antigen exposure and subsequent downstream TCR signaling, co-stimulation of the CD28 T cell surface molecule through interactions with APC-derived B7 molecules also contributes to T cell activation and *Il2* gene expression (3, 27). Upon engagement of CD28 with B7 molecules on antigen presenting cells, CD28 signaling can serve to activate TCR-dependent *Il2* gene expression (28). In addition, CD28 can signal stabilization of the IL-2 transcript through a TCR-independent mechanism (29, 30). Thus, co-stimulation through CD28 can act to maintain IL-2 mRNA expression and stability through multiple signaling pathways. Tregs also require CD28 for their development and maintenance in the periphery (31) and may act to limit co-stimulation provided by antigen presenting cells by out-competing for available B7 molecules. Given this possibility we postulated that excess CD28 activation could override the

ability of Tregs to suppress the frequency of 2BiT responder $CD4^+Thy1.1^+$ populations during activation. To this end, we added a CD28 agonist antibody to determine the effect on reporter expression in α-CD3-activated 2BiT responder cells CD4 in the presence or absence of congenically marked Tregs. Activation of naïve 2BiT CD4 T cells with soluble α-CD3 and irradiated CD4-depleted splenic APCs showed a dramatic increase in 2BiT $CD4^+Thy1.1^+$ responder T cells in cultures containing excess agonist CD28 antibody during peak IL-2 production compared to cultures containing α-CD3 alone. In the presence of congenically marked Tregs, 2BiT CD4 responder T cells activated with soluble α-CD3 alone during early activation demonstrated an inhibition of 2BiT $CD4^+Thy1.1^+$ responder populations as previously shown. However, in the presence of CD28 agonist antibody the dramatic induction of 2BiT $CD4^+Thy1.1^+$ T responders remain unaltered in the presence of congenically marked Tregs (Figures 15-17). Taken together, these studies demonstrate that additional co-stimulation induces a more robust activation of *Il2* expression in activated naïve CD4 T cells than induction with soluble α-CD3 antibody alone. Furthermore, our results indicate that excess co-stimulation enhances the frequency of *Il2*-producing CD4 responder T cells and in addition imparts resistance to suppression by Treg populations *in vitro*.

Suppression by T regulatory cells affects IL-2 mRNA stability from responder T cells

Steady-state IL-2 mRNA levels are a result of production from both transcriptional initiation processes as well as fluctuations in post-transcriptional mRNA stabilization important for gene expression. Co-culture assays with responder CD4 cells from 2BiT mice, which demonstrate a highly prolonged half-life, (Figure 7) indicated substantial inhibition of transcript production as a result of inhibition by Tregs. The

inherent design of our 2BiT transgene allows us to eliminate effects by Tregs on post-transcriptional regulation of the Thy1.1 transcript by virtue of the incorporation of a non-native SV40 poly adenylation 3' UTR. In order to determine if Tregs have any effect on post-transcriptional regulation of endogenous IL-2 transcript containing the native 3' UTR sequence elements, CD4 responder T cell-derived endogenous IL-2 transcript stability was assessed in the presence or absence of *in vitro*-induced Tregs (Figure 18A). Suppressive function of *in vitro*-induced Foxp3^{+} Tregs was confirmed by inhibition of activated naïve CD4 T cell proliferation, and IL-2 protein production (Figure 18B). Co-cultures containing 2BiT CD4 responder populations activated with α-CD3 and irradiated CD4-depleted splenic APCs in the presence or absence of *in vitro*-induced Foxp3^{gfp+} Tregs were activated for 18 hours, a period of peak IL-2 transcript production (Figure 19A). Suppression of IL-2 transcript levels in co-cultures containing activated naïve CD4 T cells in the presence of *in vitro*-induced Tregs occurred following an 18 hour period (Figure 19B). These results indicated that Tregs were capable of interfering with IL-2 transcript levels in co-cultures containing activated naïve CD4 T responders. However, IL-2 transcript levels are a measure of transcript degradation as well as production. In order to determine if Tregs have any affect on IL-2 transcript stability we evaluated IL-2 mRNA stability in RNA decay analyses. Co-cultures containing naïve CD4 T cells from C57BL/6 or 2BiT mice, irradiated CD4-depleted splenic APCs in the presence or absence of *in vitro*-induced Tregs were activated for 18 hours then subject to actinomycin D treatment (Figure 19C). After actinomycin D treatment, RNA samples were collected out to 60 or 90 minutes and then subject to analysis by quantitative PCR. Our results indicated that the half-life of endogenous IL-2 transcript produced by

responder CD4 T cells was significantly reduced in the presence of Tregs (Figure 19C, Table II). These findings suggested that the presence of Tregs interferes with signals in CD4 responder T cells necessary for intrinsic IL-2 transcript stabilization. In addition, unlike endogenous IL-2 transcript, Tregs did not alter reporter Thy1.1 mRNA containing a non-native SV40 poly adenylation 3' UTR sequence (Figure 19C). Taken together, our results indicate that in addition to substantial inhibition of *Il2* transcriptional rates, T regulatory cells reduced the mRNA half-life of IL-2 transcripts produced in co-cultures containing responder CD4 T cells and Tregs.

Discussion

In this study we describe the generation and characterization of a novel *Il2* BAC-In transgenic reporter model (2BiT) that follows lineage-specificity and responsiveness of endogenous *Il2* gene expression in activated naïve CD4 T cells to tolerogenic signals imposed by Treg populations. Furthermore, we describe a model for *in vitro* suppression by Tregs where an inhibition of *Il2* gene expression in viable responder CD4 T cells precedes cell death of non-IL-2-producers. Our findings are suggestive of a mechanism of *in vitro* suppression imparted by Tregs that involves limiting the threshold of activation of naïve CD4 T responders and further *Il2* gene expression.

Treg suppression has been shown to involve inhibition of IL-2 protein production as well as IL-2 mRNA levels in responder CD4 T cells (8). These findings suggest a role for Tregs in the inhibition of the *Il2* transcriptional process in effector CD4 T cells. However, additional studies report a mechanism of suppression by which Treg-mediated inhibition of IL-2 production is secondary to active transcriptional events and instead

involves induction of responder cell apoptosis via an "IL-2 sink-induced" cytokine deprivation (9, 10, 26). Among the several potential suppressive mechanisms, the IL-2 "sink" mechanism proposes that T regulatory cells suppress effector cells by binding extracellular IL-2 through CD25 molecules on the cell surface and thus limiting available cytokine for autocrine IL-2 signaling within the responder population (9, 26, 32). Cell death resulting from cytokine-deprivation induced by Treg suppression would thereby limit IL-2 production by virtue of fewer activated responder CD4 T cells. Technical limitations in the detection of IL-2 from responder CD4 T cells in co-cultures containing Tregs have prevented an accurate assessment of the contribution of Treg suppression to responder CD4 T cell-derived *Il2* transcription as opposed to changes in cell viability. Furthermore, technical constraints have limited the evaluation of differences in proximal events in gene expression of *Il2* versus changes in post-transcriptional regulation in responder CD4 T cells as a result of Treg suppression. Due to these inherent caveats in tracking gene expression in suppression co-cultures, the precise effect of Tregs on *Il2* transcription within living responder CD4 T cells requires continued clarification and would provide further insight into understanding mechanisms of immune tolerance.

Our studies show that Tregs suppress Thy1.1 reporter expression in viable responder cells during peak IL-2 production. In addition, inhibition of Thy1.1 reporter expression in viable T responders precedes an increase in cell death of responder CD4 T cells. Furthermore, analysis of viable and non-viable responder CD4 T cells from 2BiT mice revealed that Thy1.1-expressing cells were predominantly viable and suppression by Tregs resulted in an increase in cell death among Thy1.1 non-expressing populations. Collectively, these results suggested that potentiation of early *Il2* expression in the

responder CD4 T cells is a predictor of late-stage viability of this population. We also show that the presence of Tregs limited the frequency of CD25-expressing responder cells suggesting an effect on the activation status as a result of suppression. In addition, co-cultures containing Tregs indicated a decreased frequency of CD25-expressing cells that showed Thy1.1 reporter expression. These observations suggest that the presence of Tregs prevents activation of CD4 responder T cells and further *Il2* expression necessary to overcome a threshold of activation. Activation of naïve CD4 T cells requires both TCR signaling upon recognition of cognate antigen in addition to co-stimulation through CD28 signaling. In assays that provide excess co-stimulation 2BiT responder CD4 T cells show an increased frequency of Thy1.1^{+} viable responders during peak IL-2 production suggesting that CD28 signaling can alter the threshold of activation for T cell activation. Moreover, increased CD4^{+}Thy1.1^{+} viable 2BiT responders in response to excess co-stimulation was not altered in the presence of suppressive Treg populations implicating a role for co-stimulation in immune tolerance mechanisms.

Similar to previous findings, our study finds no evidence for an IL-2-deprivation model during Treg-mediated suppression of responder CD4 T cells (33). Using the 2BiT model we show a model of suppression independent of an "IL-2 sink" as addition of exogenous IL-2 to our co-cultures did not rescue reporter expression in naïve CD4 T responders from 2BiT mice the presence of Tregs. In addition, exogenous IL-2 did not alter 2BiT reporter expression in CD4 responder T cells from these mice suggesting the inability for IL-2 receptor signaling to affect *Il2* gene expression, a significant indicator of Treg-mediated suppression of responder CD4 T cells. However, our results indicated that excess co-stimulation in co-cultures containing 2BiT responder CD4 T cells allowed

for a rescue of reporter suppression imparted by Tregs. The interaction of T cell-derived CD28 with B7 molecules on antigen presenting cells has been well established as the necessary second-signal in the course of T cell activation and prevention of an anergic state (29, 30, 34, 35). Downstream signaling through this costimulatory marker has been shown to enhance both TCR-mediated *Il2* gene activation as well as IL-2 transcript stability independent through a TCR-independent mechanism (28-30). In addition, CD28 signaling has been shown to be critical for Treg development in the thymus and maintenance in the peripheral lymphoid tissues (31). Thus, it remains conceivable that Treg suppression is due to a competition of both naïve responder cells and Tregs for costimulatory signaling. To this effect, addition of CD28 agonist antibody to our co-cultures prevented Treg-mediated suppression of 2BiT reporter expression. This is in contrast to a mechanism of Treg suppression involving insufficient activation and resulting cell death seen from Treg-induced suppression of *Il2* activation. Future evaluation of the role of CD28 signaling in 2BiT reporter expression during Treg suppression will be required to clarify the significance of competition for co-stimulation in responder CD4 T cell activation and *Il2* expression.

A major limitation of previous studies evaluating effects on IL-2 production in CD4 responder T cells by Tregs has been the inability to distinguish the contribution of *Il2* gene expression from viable and non-viable CD4 T responder cells in the presence or absence of Tregs in co-cultures. Attempts to address this caveat by employing an IL-2-GFP "knock-in" transgene (36) poses additional constraints through the disruption of an endogenous allele. IL-2-deficiency generates a lethal autoimmune phenotype (37, 38) due to a deficiency of peripheral Treg populations (39). Perturbing endogenous loci will

reduce the overall production of IL-2 transcript and protein production from cells that normally generate this cytokine (40). Given the innate drawbacks of IL-2 detection, further limiting production of this cytokine as a means to introducing a reporter transgene is not ideal for monitoring changes in locus regulation in CD4 T responders as a result of Treg suppression. Unlike this study, the 2BiT model allows for reporting on *Il2* gene expression without disrupting endogenous *Il2* alleles. Furthermore, the 2BiT model allows us to distinguish effects on gene expression independently of contributions from changes in mRNA stability with the inclusion of a non-native SV40 poly adenylation 3'UTR sequence. In addition, the use of congenic markers enables analysis of effects of Tregs on *Il2* expression specifically within responder CD4 T cells while assessing changes in viability of this population. Taken together, the 2BiT model enables analysis of multiple parameters of responder CD4 T cell viability and *Il2* gene expression within co-cultures that contain additional Treg populations.

A previous attempt to generate a reliable GFP transgenic reporter model containing a 2.7 kb *Il2* promoter-GFP-SV40-hCD2 LCR showed lineage-specific regulation with fidelity to *Il2* expression in naïve T cell responses (3). However, in line with limitations of conventional IL-2-reporting transgenic mouse models, this model was unable to mimic suppression of *Il2* in responder CD4 T cells in co-culture suppression assays with Tregs (Figure 20). The inability of our previous GFP transgenic model to mimic *Il2* endogenous regulation through suppression of reporter expression in the presence of Tregs may be a reflection of the requirement for additional distal cis-regulatory elements from the IL-2 locus. To date, an ICER/CREM regulatory sequence within the proximal *Il2* promoter has been shown to play an integral role in Treg-

mediated suppression of T responder *Il2* transcription (41, 42). However, previous studies using transgenic mice containing *Il2* promoter sequences driving a GFP reporter indicated distal regulatory elements upstream of a 2.0 kb promoter fragment was required for appropriate locus control. Our *Il2* BAC-in model circumvents limitations of insufficient regulatory sequence by including 200 kb of endogenous sequence elements with 155 kb flanking the 5' end of the *Il2* gene and 30-60 kb of flanking 3' sequence. In addition, the large size of the 2BiT transgene limits the degree of copies that integrate randomly into the genome thus ensuring more physiologic expression of the reporter to the endogenous *Il2* gene (43). Random integration of the 2BiT transgene provides the added advantage of reporting on gene regulation from an independent genomic position thus preventing phenotypic changes from altering endogenous sequence or regulatory elements. Due to the rapid turnover of *Il2* transcript and protein, a final benefit to our model is the inclusion of an SV40 poly adenylation sequence, which allows for the stabilization of reporting of *Il2* gene expression.

Collectively, our data indicate a model of Treg suppression of responder CD4 T cells *in vitro* that involves prevention of naïve responder CD4 T cells from overcoming a threshold of activation coordinate with an inhibition of *Il2* gene activation and mRNA stability. Responder CD4 T cells receiving suboptimal activation signals in the presence of Tregs would be unable to undergo *Il2* gene expression as well as generate necessary survival signals for maintenance of viability thus resulting in increased cell death among this population in the presence of Tregs. Future studies with the 2BiT model will serve to address questions of *Il2* gene regulation and the role of IL-2-producing cells in effector immunity and tolerance. The 2BiT transgene also serves as a platform from which to

study putative regulatory elements within the entire *Il2* locus *in vivo* without disrupting endogenous regulatory sequence necessary for normal development and function. In addition, the 2BiT model will also be of interest to determine the degree of this reporter to stably mark IL-2 producing cells for the purpose of monitoring the fate of the effector cell and memory responses *in vivo*.

Numerous studies have highlighted the central importance of the IL-2 cytokine in the maintenance of the effector response and immune tolerance. The 2BiT model will serve as a useful tool with which to address many questions regarding the integral nature of *Il2* gene expression and signaling in immune homeostasis. One explanation for this observation is the lack of sufficient distal regulatory elements within our promoter sequence necessary for comprehensive IL-2 control (44).

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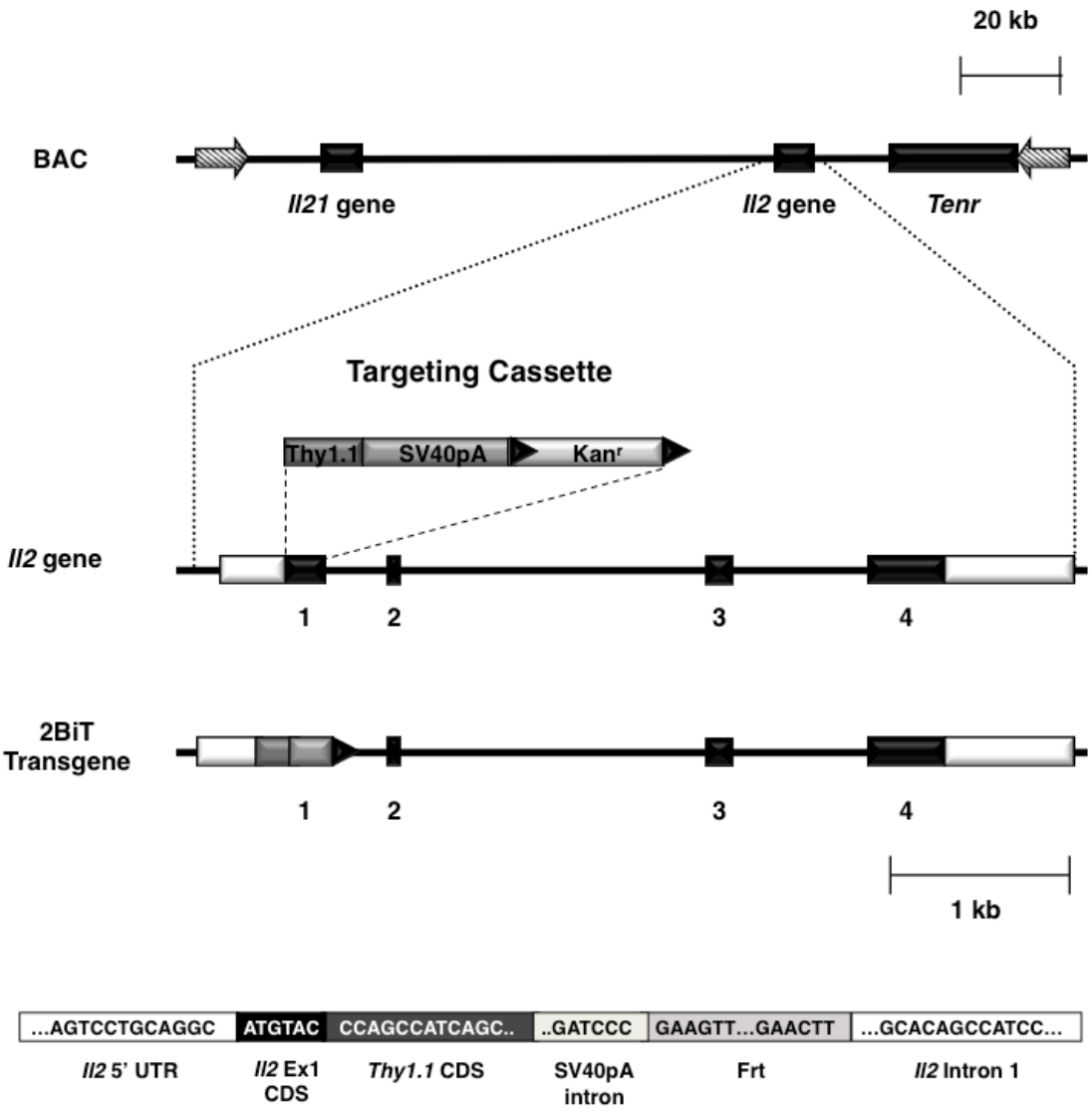


Figure 1. Generation and Characterization of an *Il2* BAC-In Transgenic Mouse Model (2BiT). A Bacterial Artificial Chromosome containing an *Il2* gene was modified to incorporate a Thy1.1-SV40pA-Kanamycin resistance targeting cassette (grey boxes) within the first exon of *Il2* using recombineering techniques. The transgenic *Il2* locus is situated approximately 155kb downstream and 30kb upstream of the 5' and 3' BAC termini, respectively. Positive recombinants for transgene integration were screened based on kanamycin resistance selection, PCR, and sequencing analysis. The kanamycin resistance marker was removed through the introduction of a FLP-recombinase plasmid and then excised recombinants were screened using parallel negative selection by kanamycin, PCR, and sequencing analysis. Transgene product was then linearized with NotI restriction enzyme and column purified for injection into fertilized embryos.

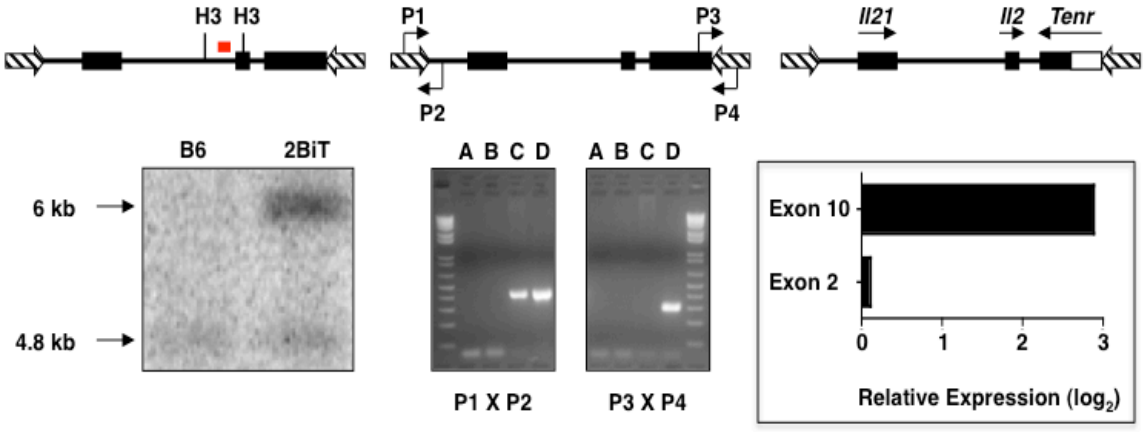


Figure 2. 2BiT BAC Integration Analysis. (Left) Screening for copy number of the 2BiT transgene using Southern Blot analysis. Genomic DNA from 2BiT mice or a C57BL/6 control was digested with HindIII, then blotted to a nylon membrane, followed by hybridization to a sequence fragment to the 5' UTR of the *Il2* locus. The probe binds to both the 4.8kb endogenous band and the 6kb transgenic fragment. Densitometry analysis was used to determine copy number as a measure of intensity of the transgenic band compared to the endogenous band. (Middle) BAC terminus integration was assessed using primer sets specific for the 5' or 3' terminus. (A-H20; B-C57BL/6 genomic DNA; C-2BiT genomic DNA; D-Purified 2BiT BAC DNA). (Right) Determination of 3' terminus truncation using SYBR Green PCR analysis of 2BiT genomic DNA versus C57BL/6 DNA using primers specific to either exon 2 or exon 10 of the TENR gene.

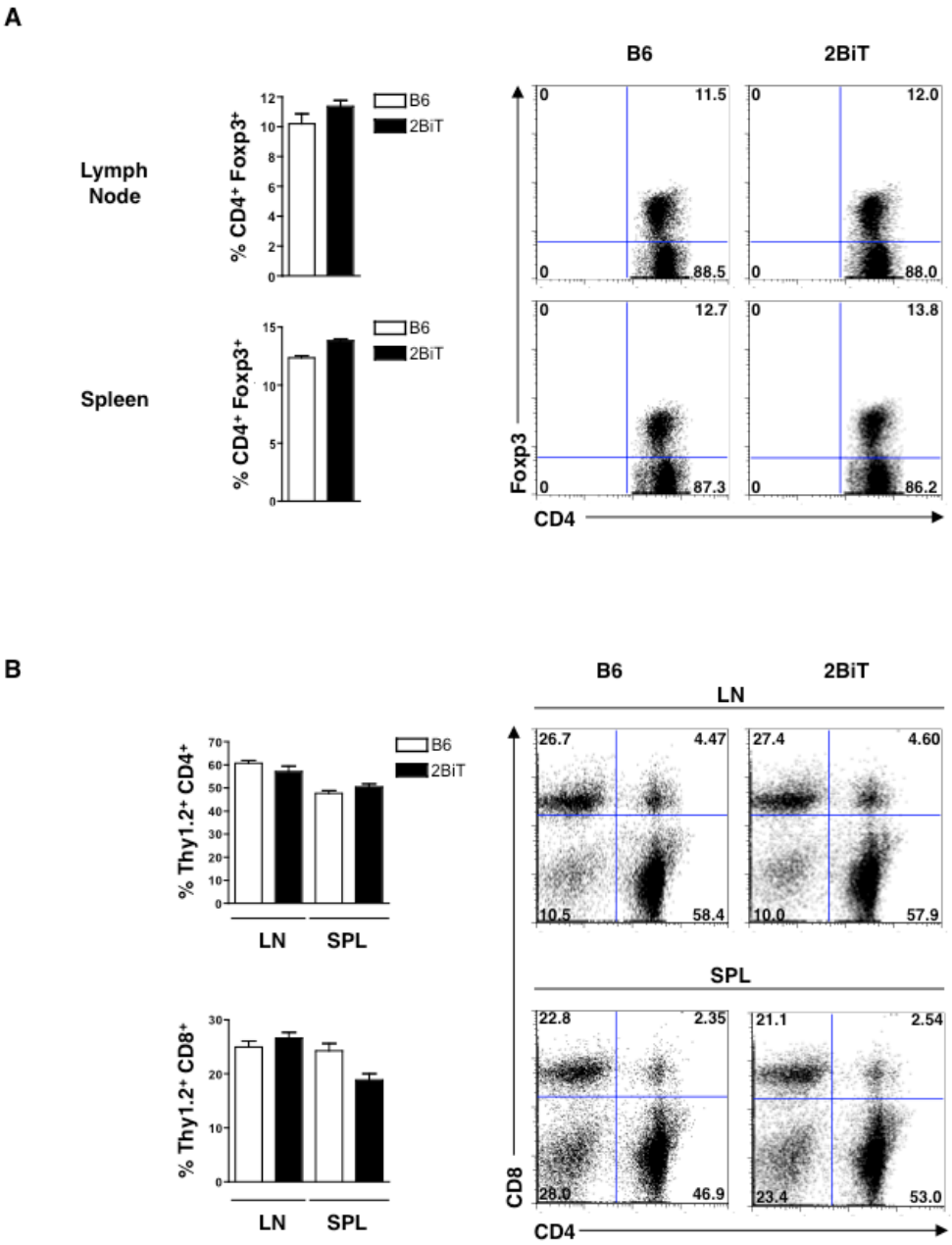
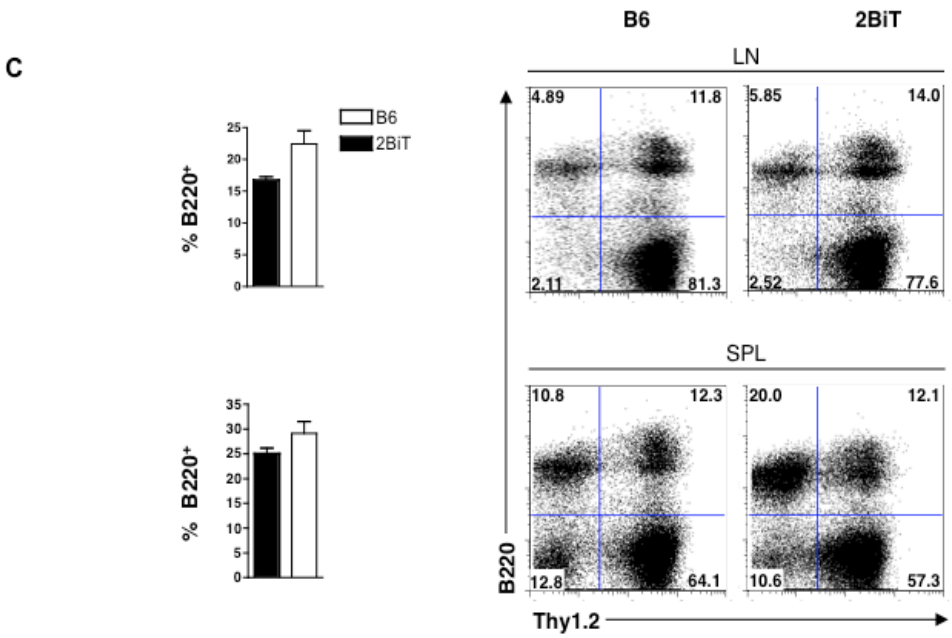


Figure 3. *Ex vivo* Phenotype Analysis of 2BiT Lymphoid Populations. Comparative analysis of peripheral lymphoid populations derived from 2BiT and C57BL/6 mice. Spleen and lymph nodes were harvested and stained with monoclonal antibodies to the indicated cell surface or intracellular proteins. The frequency of (A) $Foxp3^{+}CD4^{+}$ regulatory cells, (B) $Thy1.2^{+}$ $CD4^{+}$ or $CD8^{+}$ populations, (C) $B220^{+}$ B cells. Numbers in quadrants represent frequencies of each population. Bar graphs (left) are represented as the mean percent of the indicated populations ± s.d. Data are representative of 3 2BiT mice and 3 C57BL/6 mice.



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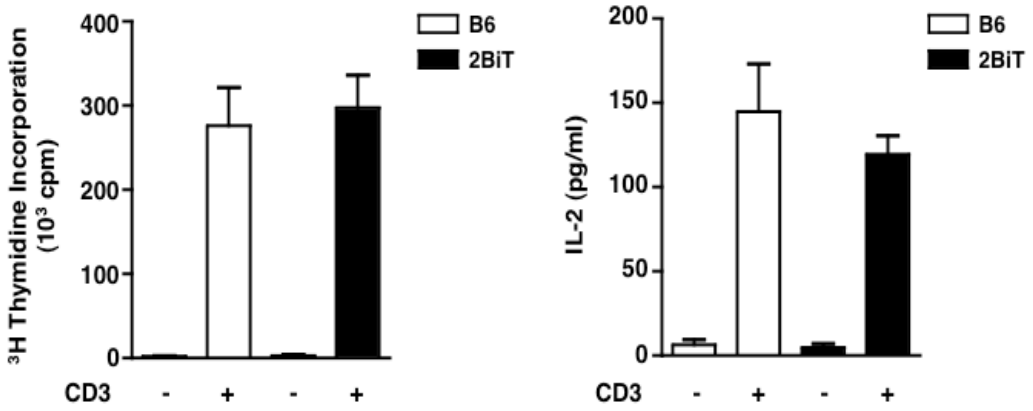

Figure 4. Analysis of Proliferation and IL-2 Production from Activated 2BiT Naïve CD4 T Cells. Analysis of proliferation (left) and IL-2 protein production (right) from CD4 naïve T cells from 6-8 week old 2BiT ($5x10^4$-left; 10^5-right) and C57BL/6 ($5x10^4$-left; 10^5-right) mice activated with previously irradiated CD4-depleted splenic APCs ($4x10^5$-left; $8x10^5$-right) and 2.5 μg/ml soluble α-CD3 for 72 hours and 18 hours, respectively. Proliferation assays were treated with tritated thymidine after 72 hours and then harvested for analysis by scintillation counter. IL-2 protein was analyzed by cytometric bead array. Data are pooled results from three experiments.

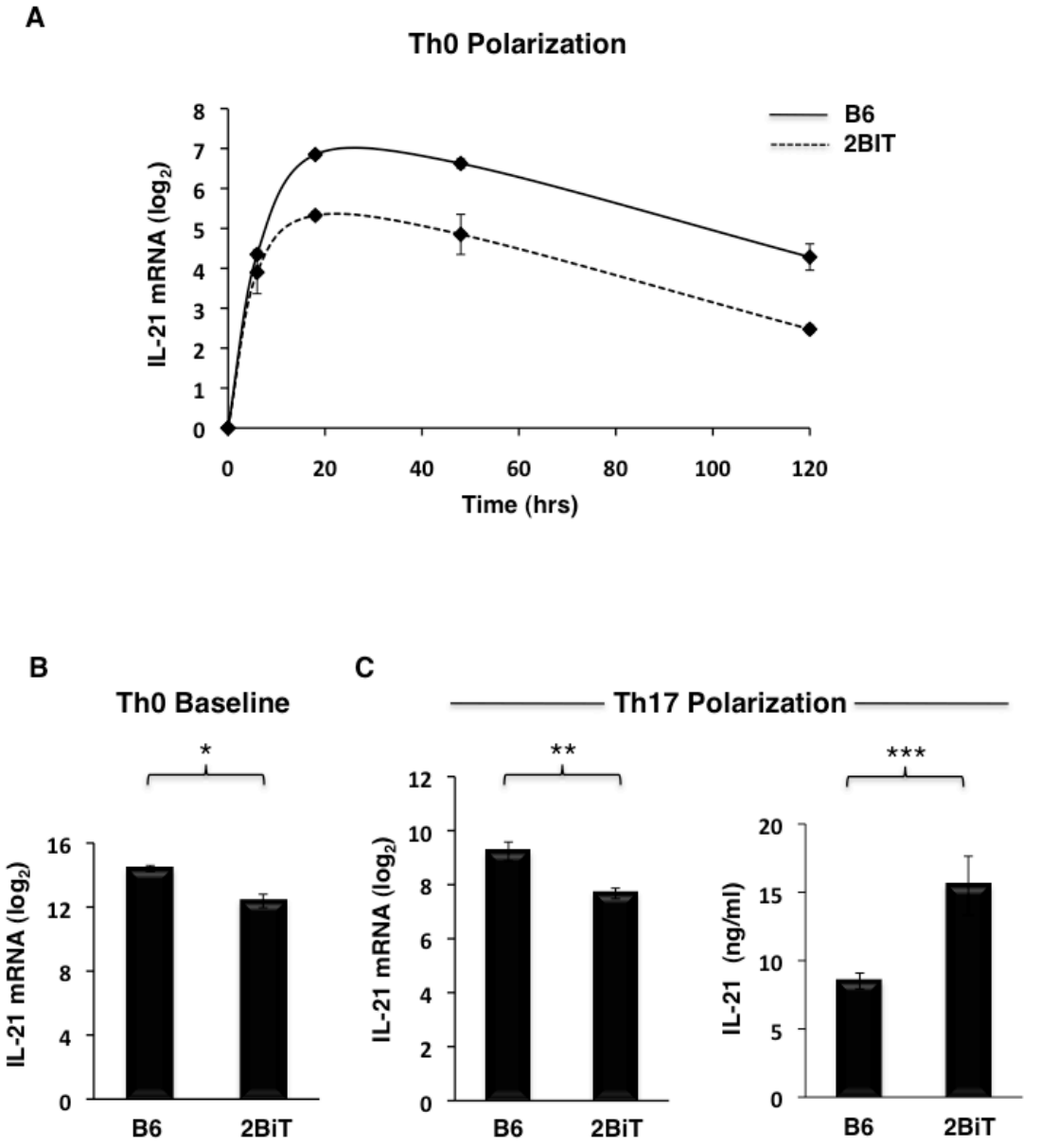

Figure 5. IL-21 Production in Activated 2BiT Naïve CD4 T Cells. (A) IL-21 transcript production in activated naïve CD4 T cells from 6-8 week old C57BL/6 and 2BiT mice. RNA isolated from soluble CD3-activated naïve CD4 T cells ($3x10^5$) from C57BL/6 or 2BiT mice was analyzed by quantitative PCR at 0, 6, 18, 48, and 120 hours (A), at baseline in Th0 conditions (*p = 0.0049). Data are expressed as the mean Δct ± s.e.m. (B), and under Th17 polarization conditions (**p =0.0007) (C) (left). IL-21 protein production was assessed under Th17 polarization conditions by ELISA (C) (right) (***p = 0.0111). RNA analysis was normalized using ΔΔct method with β-2 microglobulin and corresponding Δct values at time zero. Data are expressed as mean ΔΔct values ± s.e.m.

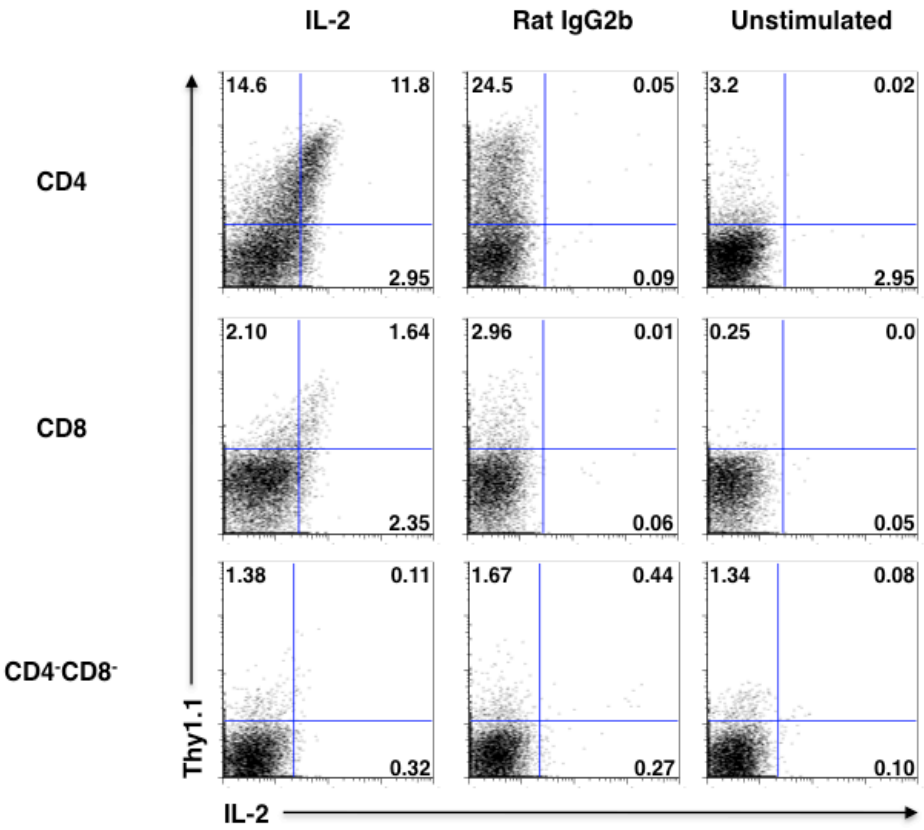

Figure 6 Fidelity of 2BiT Reporter Expression to Endogenous IL-2 Regulation. Analysis of Thy1.1 reporter and IL-2 expression in total lymphocyte populations (10^6) from spleens of 8 week old 2BiT mice activated with Phorbol 12-Myristate 13-Acetate (PMA) (50 ng/ml) and Ionomycin (1μM) and in the presence of golgi plug at the recommended concentration (Pharmingen) for 5 hours. Cells were stained live/dead vital dye (Invitrogen) and monoclonal antibodies to surface markers CD4, CD8, and Thy1.1, and intracellularly for IL-2 then analyzed by flow cytometry. Lymphocytes were gated on viable populations and analyzed for reporter expression in mixed lymphocyte populations. Numbers in quadrants represent frequencies of each population. Data are representative of three experiments.

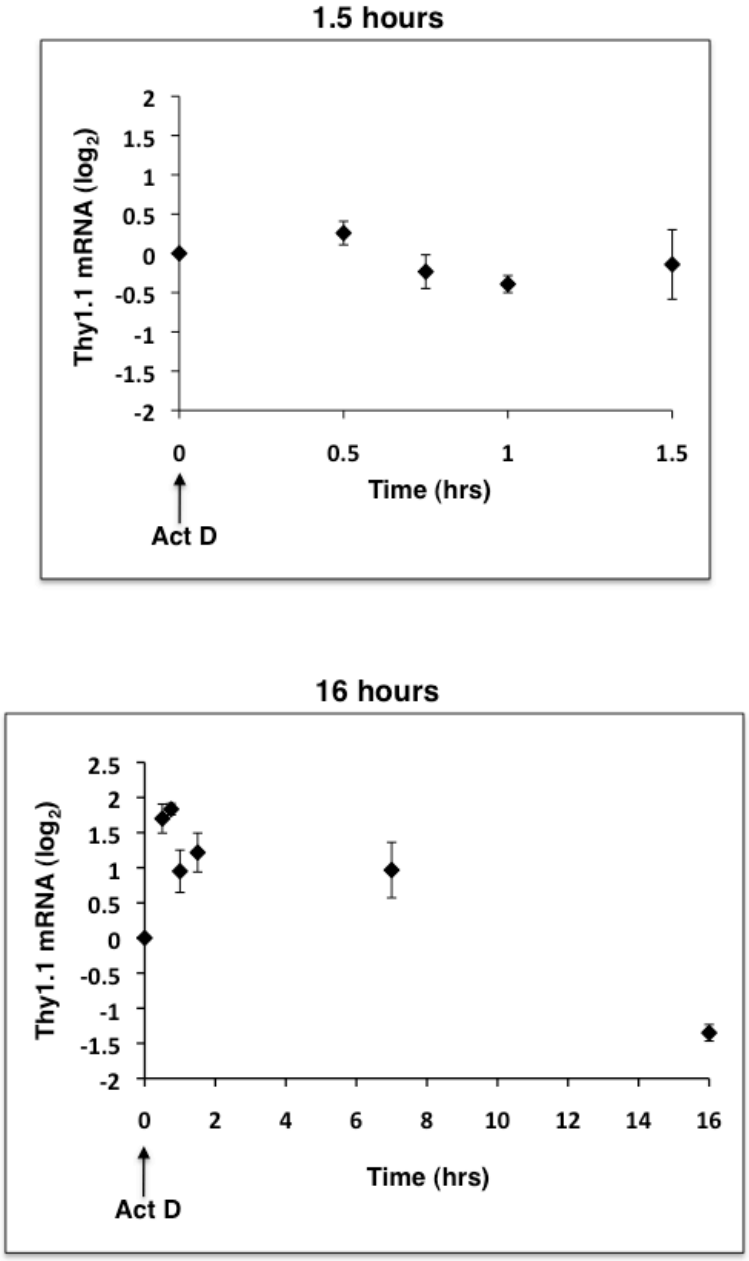

Figure 7. Thy1.1 Transcript Stability in Activated 2BiT CD4 T Responders. RNA decay analysis of Thy1.1 transcript in cultures containing activated naïve CD4 T cells from 2BiT mice. RNA samples were collected at 0, 30, 45, 60, and 90 minutes (top) and 0, 1, 6, 18, 48, 120 hours (bottom) after actinomycin D addition. Isolated RNA was used to synthesize cDNA for quantitative PCR analysis. To calculate the relative change in Thy1.1 transcript levels after addition of actinomycin D, cycle threshold values for Thy1.1 were first normalized to β-2 microglobulin levels (Δct), then 30, 45, 60, and 90 minute time points were normalized to the Δct_0 at time point 0 after actinomycin D treatment (ΔΔct). Data are representative of two experiments and are expressed as the ΔΔct ± s.e.m.

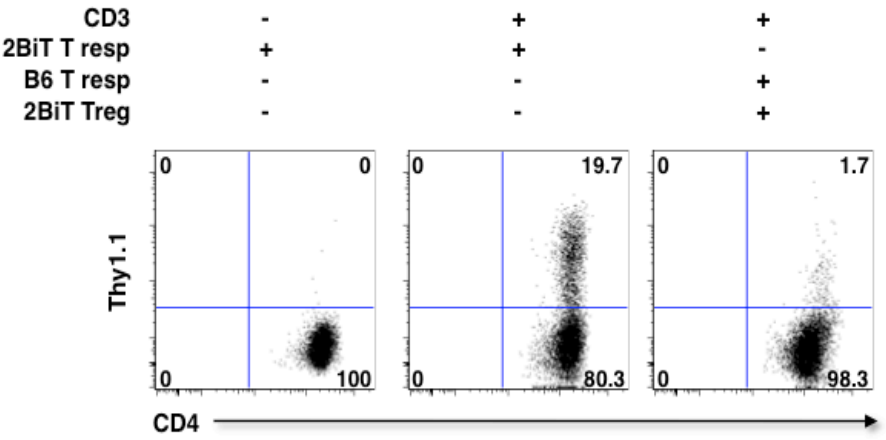

Figure 8. Reporter Expression in $CD4^+CD25^+$ T Cells from 2BiT Mice. Expression of Thy1.1 reporter in $CD4^+CD25^+$ Tregs (10^5) (right) from 2BiT mice activated with 2.5 μg/ml soluble α-CD3 in the presence of irradiated CD4-depleted splenic APCs ($8x10^5$) for 16 hours and stained with live/dead vital dye (Invitrogen) monoclonal antibodies to surface markers for CD4 and Thy1.1. Gated viable $CD4^+CD45.2^+$ lymphocytes in samples containing Tregs were compared to similar cultures containing unstimulated naïve responder T cells (left) or activated naïve CD4 T cells from 2BiT mice (middle). Numbers in quadrants represent frequencies of each population. Data are representative of five experiments.

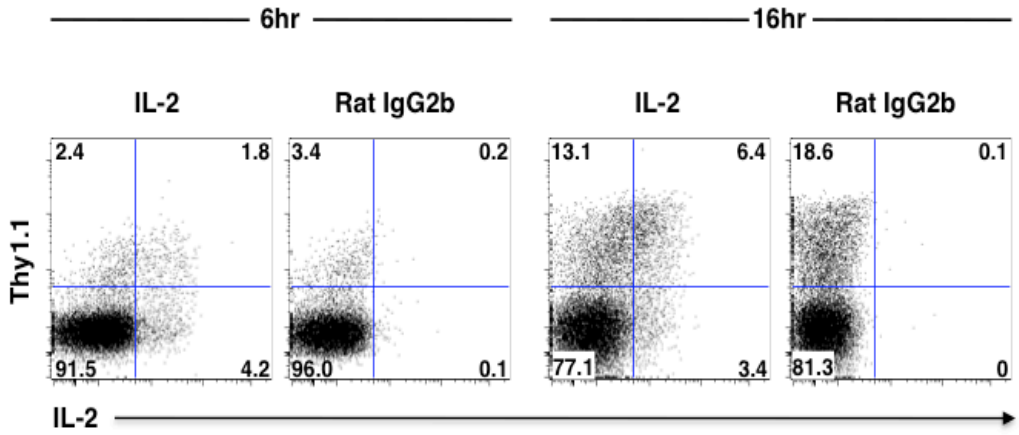

Figure 9. Fidelity of Reporter Expression in 2BiT Naïve CD4 T Cells. Analysis of Thy1.1 reporter and IL-2 expression in naïve CD4 T cells (10^5) from 6-8 week old 2BiT or C57BL/6 mice activated with 2.5 μg/ml soluble α-CD3 in the presence of irradiated CD4-depleted splenic APCs ($8x10^5$) for 6 and 16 hours. Cells were stained with monoclonal antibodies for surface markers CD4, Thy1.1, and IL-2 or a rat IgG isotype control antibody. IL-2-producing cells were detected by cytokine secretion assay (Miltenyi). Lymphocytes were gated on viable $CD4^+$ populations. Numbers in quadrants represent frequencies of each population. Data are representative of three experiments.

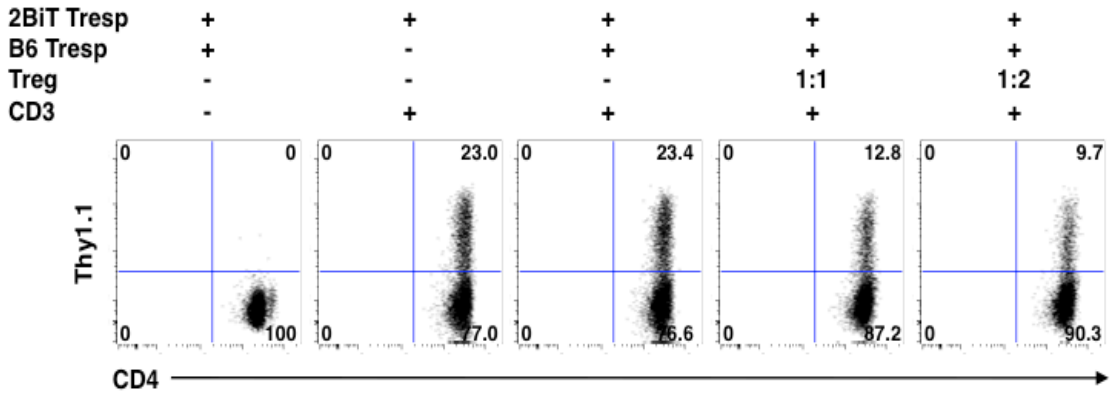

Figure 10. Suppression of IL-2 in 2BiT CD4 T Responder Cells by Tregs. Thy1.1 reporter expression from activated CD4 T cells from 6-8 week old 2BiT mice in the presence or absence of Tregs. Unactivated or activated 2BiT naïve CD4 T cells (10^5) with 2.5 μg/ml soluble α-CD3 in the presence of irradiated CD4-depleted splenic APCs ($8x10^5$) for 15 hours were co-cultured in the presence or absence of $CD45.1^+CD4^+CD25^+$ Tregs (10^5), $CD45.1^+CD4^+Foxp3^{gfp+}$ Tregs, or naïve responder cells from CD45.1 C57BL/6 mice. Cells were stained with live/dead vital dye (Invitrogen), and monoclonal antibodies for CD4, Thy1.1, and CD45.2 and analyzed by flow cytometry. Responder CD4 T cells were gated on viable $CD4^+CD45.2^+$ lymphocyte populations. Numbers in quadrants represent frequencies of each population. Data are representative of six experiments.

2BiT TResp	T Reg	B6 TResp	CD3	IL-2	MFI ± SEM
+	-	-	-	-	1049 ± 211
+	-	-	+	-	4025 ± 137
+	-	+	+	-	3650 ± 71
+	-	-	+	+	3880 ± 106
+	-	+	+	+	3717 ± 77
+	1:1	-	+	-	2945 ± 73
+	2:1	-	+	-	2749 ± 52
+	1:1	-	+	+	2957 ± 84

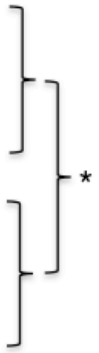

Table I. Mean Fluorescent Intensity of 2BiT CD4$^+$Thy1.1$^+$ T Responder Cells in the Presence of Tregs. The mean fluorescent intensity was determined for 2BiT CD4$^+$Thy1.1$^+$ T responder populations activated from 2BiT naïve CD4 T cells (10^5) stimulated with or without 2.5 μg/ml soluble α-CD3 and CD4-depleted splenic APCs ($8x10^5$) (*p < 0.001 between T responder only groups and T responder + Treg groups; 2BiT + B6 T responder vs Treg 1:1 condition had a $p < 0.01$). Cultures were incubated in the presence or absence of CD45.1$^+$CD4$^+$CD25$^+$ Tregs (10^5), CD45.1$^+$CD4$^+$Foxp3^{gfp+} Tregs (10^5), or naïve responder cells (10^5) from CD45.1 C57BL/6 mice and with or without 50 U/ml IL-2. MFI was determined from CD4$^+$Thy1.1$^+$ populations gated on viable CD4$^+$CD45.2$^+$ lymphocytes. Data are samples pooled from four separate experiments and are represented as the mean ± the standard error of the mean.

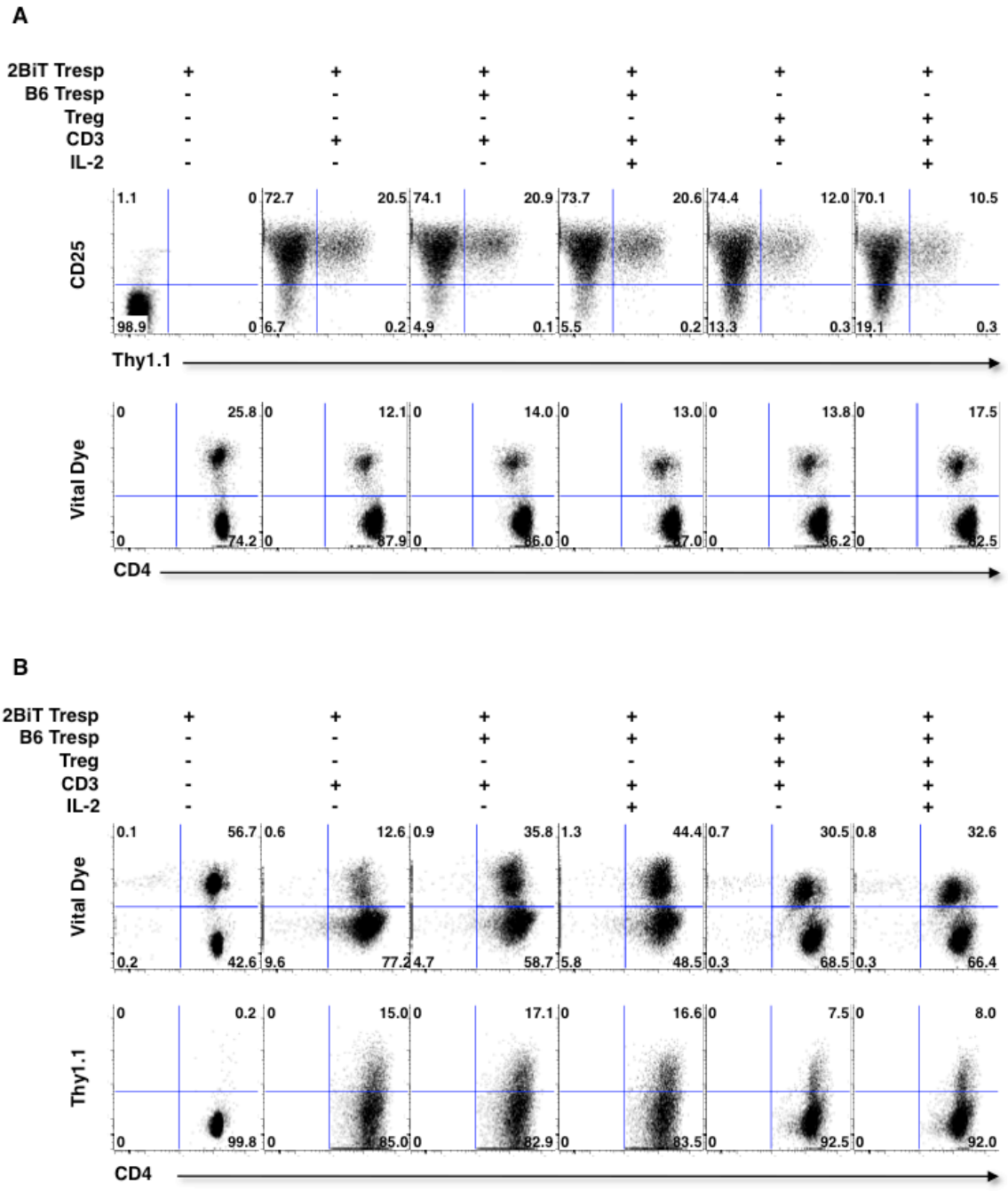

Figure 11. 2BiT CD4 T Responder Reporter Inhibition and Cell Death Induced by Tregs Shows Discordant Regulation. Thy1.1 reporter expression in 2BiT CD4 T cells activated in the presence or absence of Tregs during early (A) (15 hrs) and late (B) (Day 3) activation. Cells from 6-8 week old mice were stained with live/dead vital dye (Invitrogen), and monoclonal antibodies for CD4, Thy1.1, CD25, and CD45.2 and analyzed by flow cytometry. Responder CD4 T cells were gated on either $CD45.2^+$ lymphocytes (A-lower; B-upper) or viable $CD4^+CD45.2^+$ lymphocyte populations (A-upper; B-lower). Numbers in quadrants represent frequencies of each population. Data are representative of four experiments.

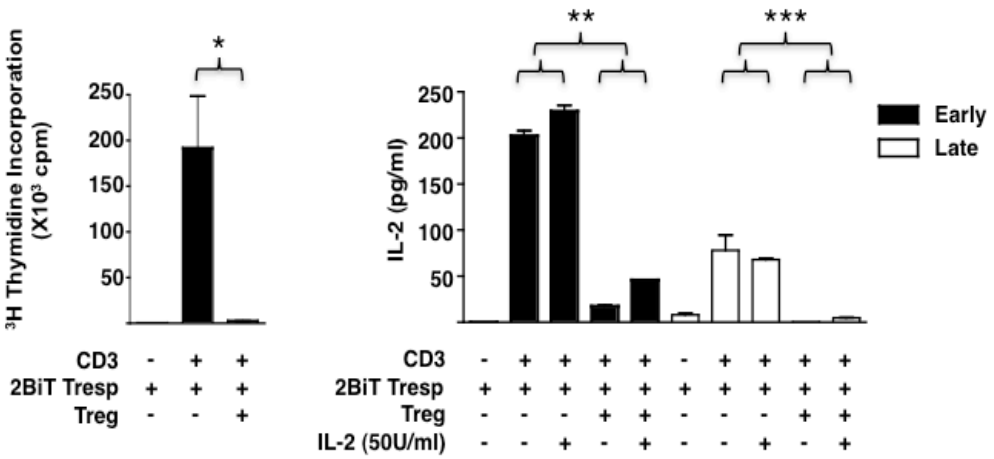

Figure 12. Suppression of 2BiT CD4 T Responder Cell Proliferation and IL-2 Production by Tregs. Proliferation assays (left) of naïve CD4 2BiT T cells ($5x10^4$) activated with or without 2.5 μg/ml soluble α-CD3 in the presence of irradiated CD4-depleted splenic APCs ($4x10^5$) in the presence or absence of Tregs ($5x10^4$) (*$p < 0.001$ as determined by ANOVA). Cultures were stimulated for 68-72 hours in a 96 well round bottom plate and then treated with 1μCi of tritiated thymidine for 16-20 hours then harvested and counted by scintillation counter for tritiated thymdine incorporation. (Right) IL-2 protein production from supernatants (**$p < 0.001$ as determined by ANOVA) of cultures in (Figure 3A) and (Figure 3B). Data are representative of three experiments.

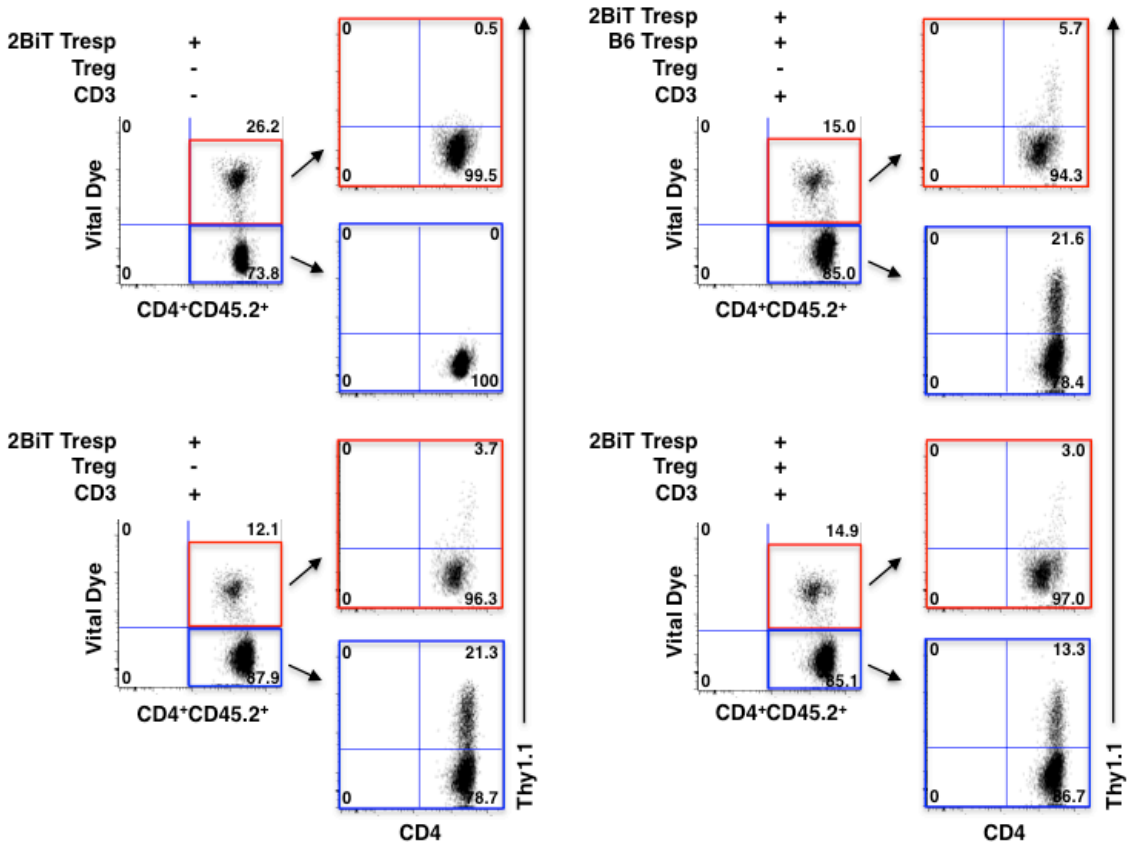

Figure 13. Tregs Suppress Reporter Expression in Viable 2BiT CD4 Responder T Cells. Analysis of viable and non-viable 2BiT responder CD4 T cells for Thy1.1 reporter expression during an early-15 hour activation time point incubated in the presence or absence of Tregs. Naïve CD4 T cells (10^5) from 6-8 week old 2BiT mice were activated with or without 2.5 μg/ml soluble α-CD3 in the presence of irradiated CD4-depleted splenic APCs ($8x10^5$) in the presence or absence of $CD45.1^+CD4^+CD25^+$ Tregs (10^5), $CD45.1^+CD4^+Foxp3^{gfp+}$ Tregs (10^5), or naïve responder cells (10^5) from CD45.1 C57BL/6 mice and cultured with or without 50 U/ml IL-2. Cells were stained with live/dead vital dye (Invitrogen) and monoclonal antibodies to CD4, CD25, and Thy1.1. Lymphocytes were gated on viable $CD4^+$ 2BiT responder T cells to assay cell viability then further subgated on $CD45.2^+$ responders to evaluate Thy1.1 expression in viable and non-viable $CD4^+$ populations. Numbers in quadrants represent frequencies of each population. Data are representative of three experiments.

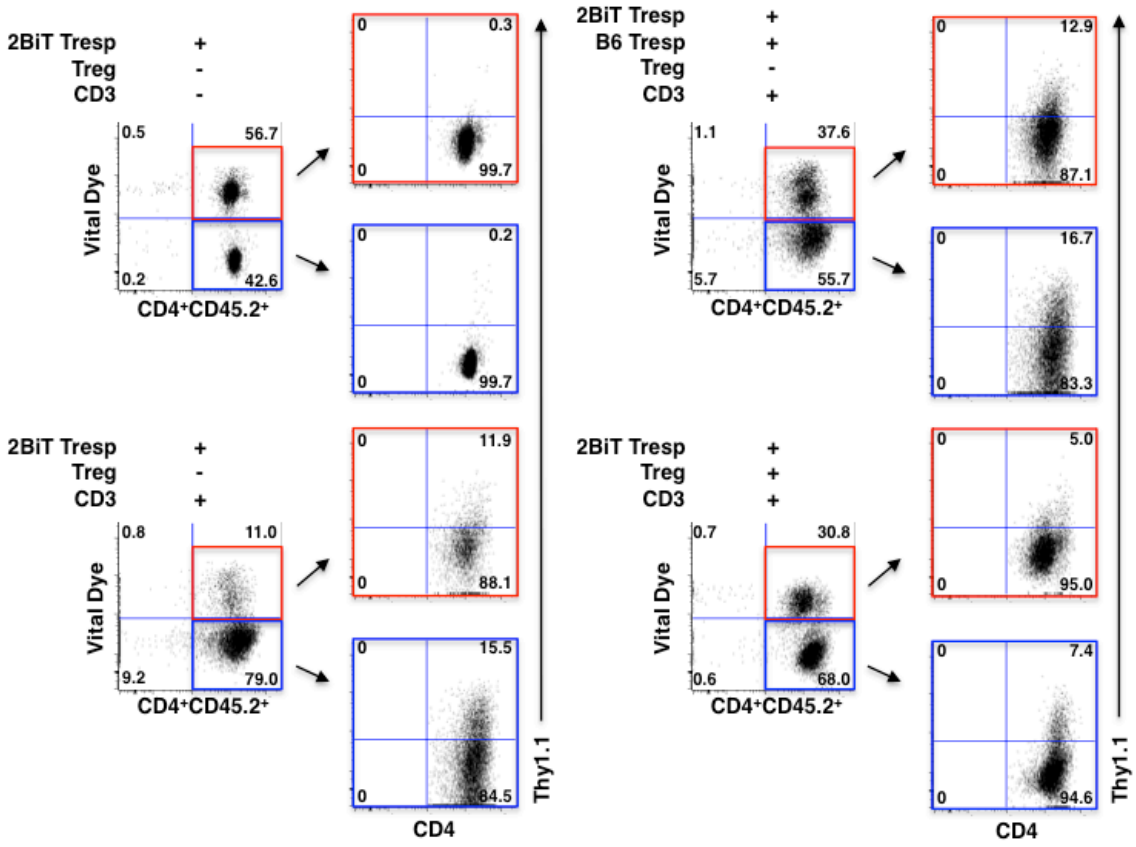

Figure 14. Treg Suppression During Late Activation Leads to an Increase in Non-Viable Responder CD4$^+$Thy1.1$^-$ T Cells. Analysis of viable and non-viable 2BiT responder CD4 T cells for Thy1.1 reporter expression during a late-3 days activation time point incubated in the presence or absence of Tregs. Naïve CD4 T cells (10^5) from 6-8 week old 2BiT mice were activated with or without 2.5 μg/ml soluble α-CD3 in the presence of irradiated CD4-depleted splenic APCs ($8x10^5$) in the presence or absence of CD45.1$^+$CD4$^+$CD25$^+$ Tregs (10^5), CD45.1$^+$CD4$^+$Foxp3^{gfp+} Tregs (10^5), or naïve responder cells (10^5) from CD45.1 C57BL/6 mice and cultured with or without 50 U/ml IL-2. Cells were stained with live/dead vital dye (Invitrogen) and monoclonal antibodies to CD4, CD25, and Thy1.1. Lymphocytes were gated on viable CD4$^+$ 2BiT responder T cells to assay cell viability then further subgated on CD45.2$^+$ responders to evaluate Thy1.1 expression in viable and non-viable CD4$^+$ populations. Numbers in quadrants represent frequencies of each population. Data are representative of three experiments.

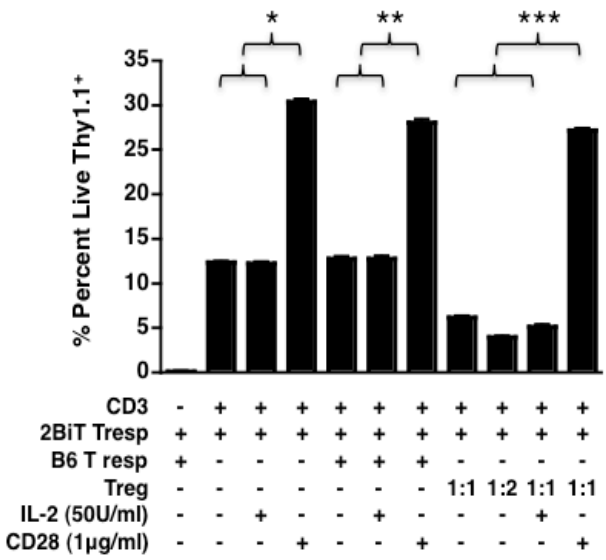

Figure 15. CD28 Signaling, but not IL-2 Signaling, Increases the Proportion of Viable 2BiT CD4$^+$Thy1.1$^+$ T Cells in the Presence of Tregs. The effect of IL-2 or CD28 co-stimulation on the activation of viable CD4$^+$Thy1.1$^+$ 2BiT responder T cells in the presence or absence of Tregs (*, **, *** $p < 0.001$ as determined by ANOVA) during early activation (15 hours). Naïve CD4 T cells from 6-8 week old 2BiT mice were activated with or without 2.5 μg/ml soluble α-CD3 and irradiated CD4-depleted splenic APCs ($8x10^5$) in the presence or absence of CD45.1$^+$CD4$^+$CD25$^+$ Tregs (10^5), or naïve responder cells (10^5) from CD45.1 C57BL/6 mice and cultured with or without 50 U/ml IL-2 or 1 μg/ml CD28 agonist antibody. The mean percentage of viable CD45.2$^+$CD4$^+$Thy1.1$^+$ responders induced. Results are representative of two experiments and are expressed as the mean ± s.d.

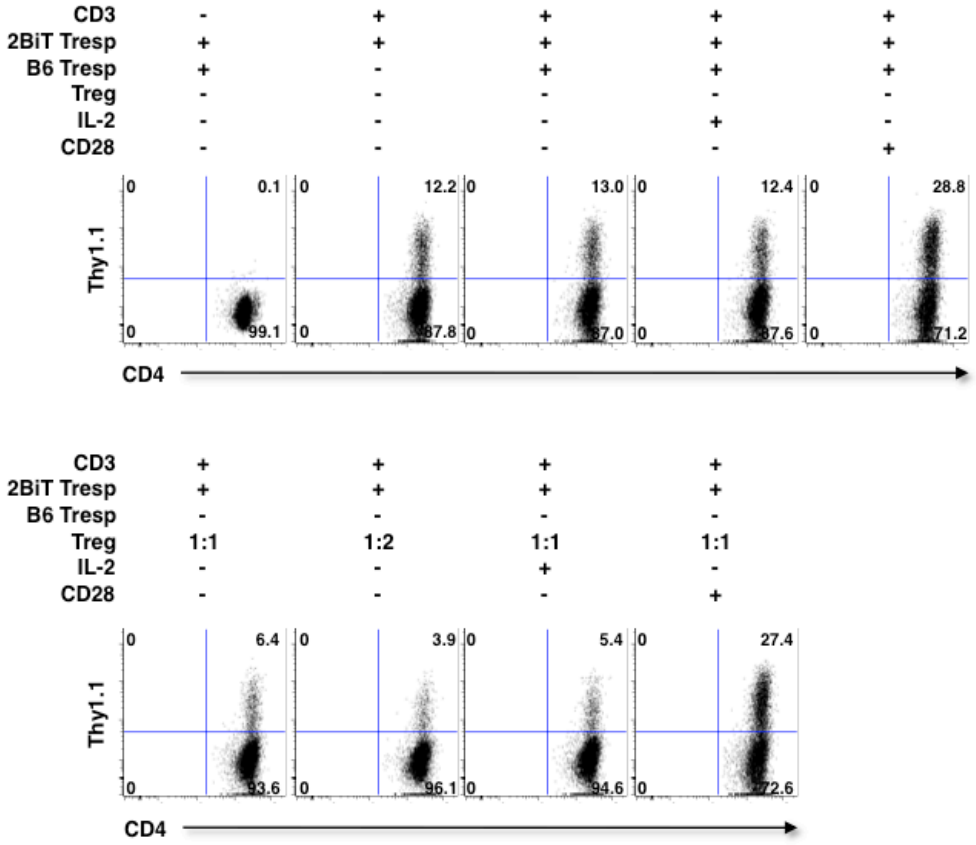

Figure 16. CD28 Signaling, but not IL-2 Signaling, Overrides T Regulatory Cell Suppression of Reporter Expression in Viable CD4 2BiT T Responders. The effect of IL-2 or CD28 co-stimulation on the activation of viable $CD4^{+}Thy1.1^{+}$ 2BiT responder T cells in the presence or absence of Tregs. Naïve CD4 T cells from 6-8 week old 2BiT mice were activated with or without 2.5 μg/ml soluble α-CD3 and irradiated CD4-depleted splenic APCs ($8x10^{5}$) in the presence or absence of $CD45.1^{+}CD4^{+}CD25^{+}$ Tregs (10^{5}), or naïve responder cells (10^{5}) from CD45.1 C57BL/6 mice and cultured with or without 50 U/ml IL-2 or 1 μg/ml CD28 agonist antibody. Flow cytometric analysis of the effect of CD28 agonist antibody and IL-2 on the frequencies of $CD4^{+}Thy1.1^{+}$ populations during early activation (15 hours). Numbers in quadrants represent frequencies of each population. Data are representative of two experiments.

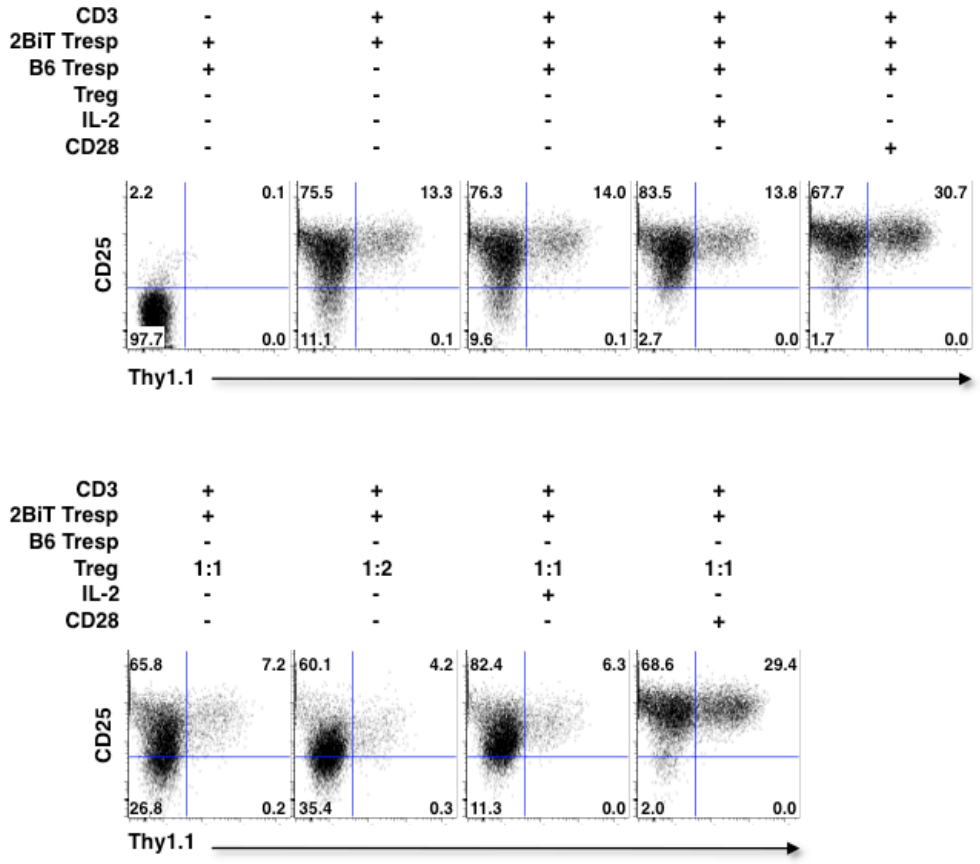

Figure 17. CD28 Signaling, but not IL-2 Signaling, Overrides T Regulatory Cell Suppression of Thy1.1 Reporter Expression in Viable CD25-Expressing 2BiT CD4 T Responders. The effect of IL-2 or CD28 co-stimulation on the activation of viable $CD4^{+}Thy1.1^{+}$ 2BiT responder T cells in the presence or absence of Tregs. Naïve CD4 T cells from 6-8 week old 2BiT mice were activated with or without 2.5 μg/ml soluble α-CD3 and irradiated CD4-depleted splenic APCs ($8x10^{5}$) in the presence or absence of $CD45.1^{+}CD4^{+}CD25^{+}$ Tregs (10^{5}), or naïve responder cells (10^{5}) from CD45.1 C57BL/6 mice and cultured with or without 50 U/ml IL-2 or 1 μg/ml CD28 agonist antibody. Flow cytometric analysis of the effect of CD28 agonist antibody and IL-2 on the frequencies of $CD25^{+}Thy1.1^{+}$ viable 2BiT responder CD4 T cell populations during early activation (15 hours). Cells were stained with live/dead vital dye and monoclonal antibodies to CD4, CD25, CD45.2, and Thy1.1 and analyzed by flow cytometry. Numbers in quadrants represent frequencies of each population. Data are representative of two experiments.

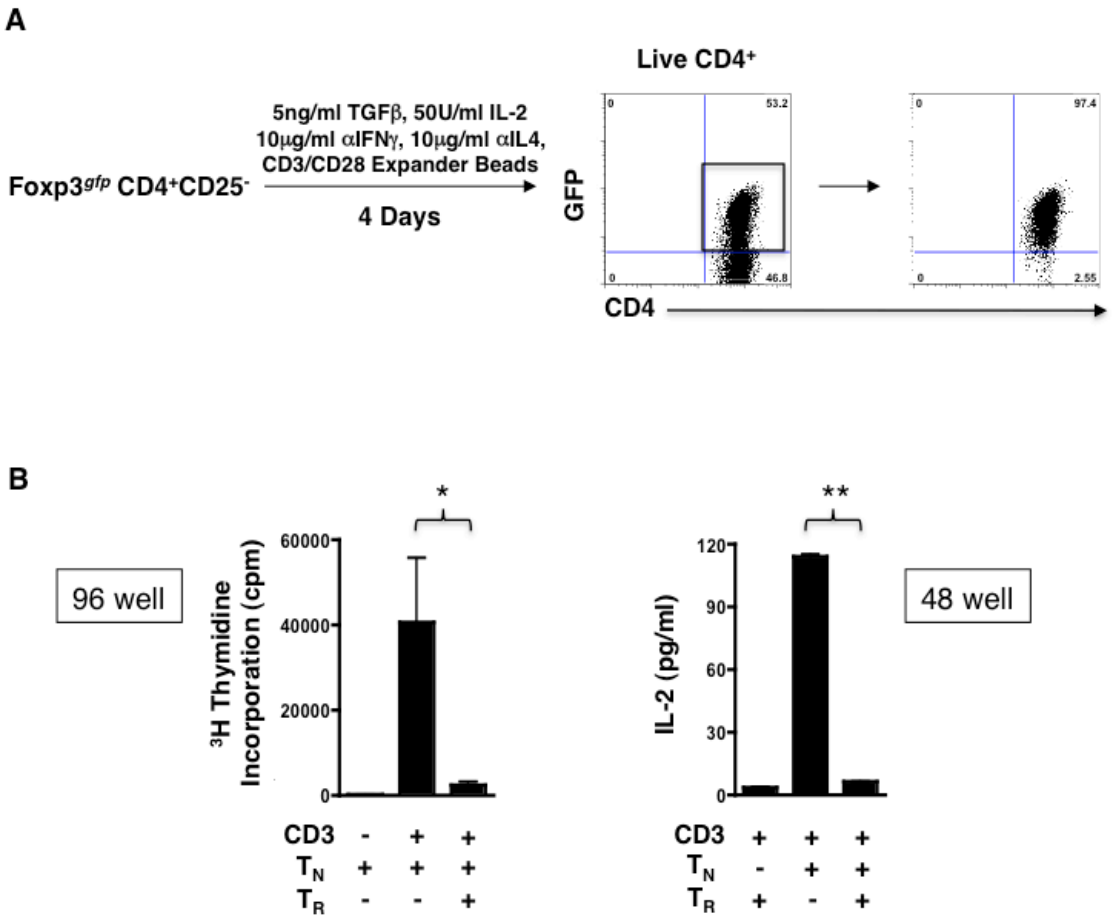

Figure 18. *In Vitro* Treg Polarization. (A) *In vitro* Treg polarization strategy. $CD4^+CD25^-$ T cells from Foxp3GFP mice were cultured with CD3/CD28 expander beads in the presence of TGF-β (5 ng/ml), IL-2 (50 U/ml), α-IFN-λ (10 μg/ml), α-IL-4 (10 μg/ml), and retinoic acid for four days. $CD4^+7AAD^-GFP^+$ Tregs were then sorted by flow cytometric methods and used in RNA decay co-culture analysis (Figure 5). (B) Verification of suppressive ability of *in vitro*-induced Tregs in (left) proliferation assays in a 96 round bottom plate (*$p < 0.05$ as determined by ANOVA) and (right) IL-2 protein analysis of co-cultures containing naïve C57BL/6 CD4 T cells ($5x10^4$ or $3x10^5$) activated with or without 5 μg/ml soluble α-CD3 and irradiated CD4-depleted splenic APCs ($4x10^5$ or $1.5x10^6$) in the presence or absence of *in vitro*-induced $Foxp3^{gfp+}$ Tregs (**$p < 0.0001$ as determined by ANOVA) for 72 or 18 hours, respectively. Proliferation assays were harvested after 72 hours and analyzed by scintillation counter. IL-2 protein was measured by cytometric bead array.

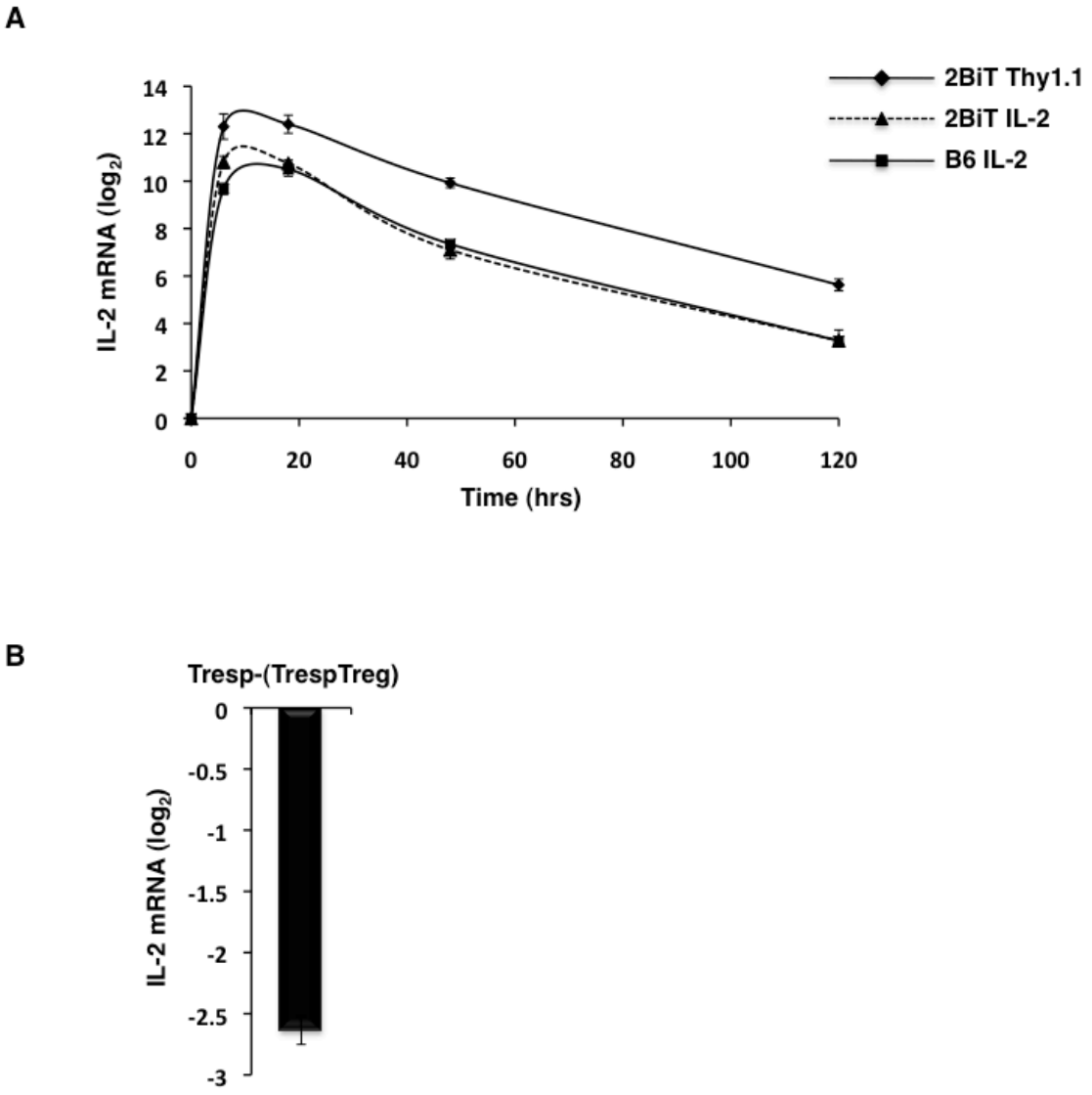

Figure 19. T Regulatory Cells Destabilize IL-2 Transcript in Co-culture Assays Containing CD4 T Responder Cells. The effect of Tregs on IL-2 transcript in co-cultures containing 2BiT or C57BL/6 CD4 T responders activated with irradiated CD4-depleted feeders and soluble α-CD3 in the presence or absence of in vitro-induced $Foxp3^+$ iTregs. Kinetic analysis of IL-2 and Thy1.1 reporter mRNA levels at 0, 6, 18, 48, and 120 hours (A). Suppression of IL-2 transcript in cultures containing CD4 responders and Tregs compared to cultures containing only responder CD4 T cells (B). RNA samples were then isolated and cDNA prepared for quantitative PCR analysis. Cycle threshold values of Treg co-cultures were normalized to β-2 microglobulin and to mean Δct values for conditions containing CD4 responder T cells only. Data are pooled from three separate experiments and are represented as the mean ΔΔct ± s.e.m.

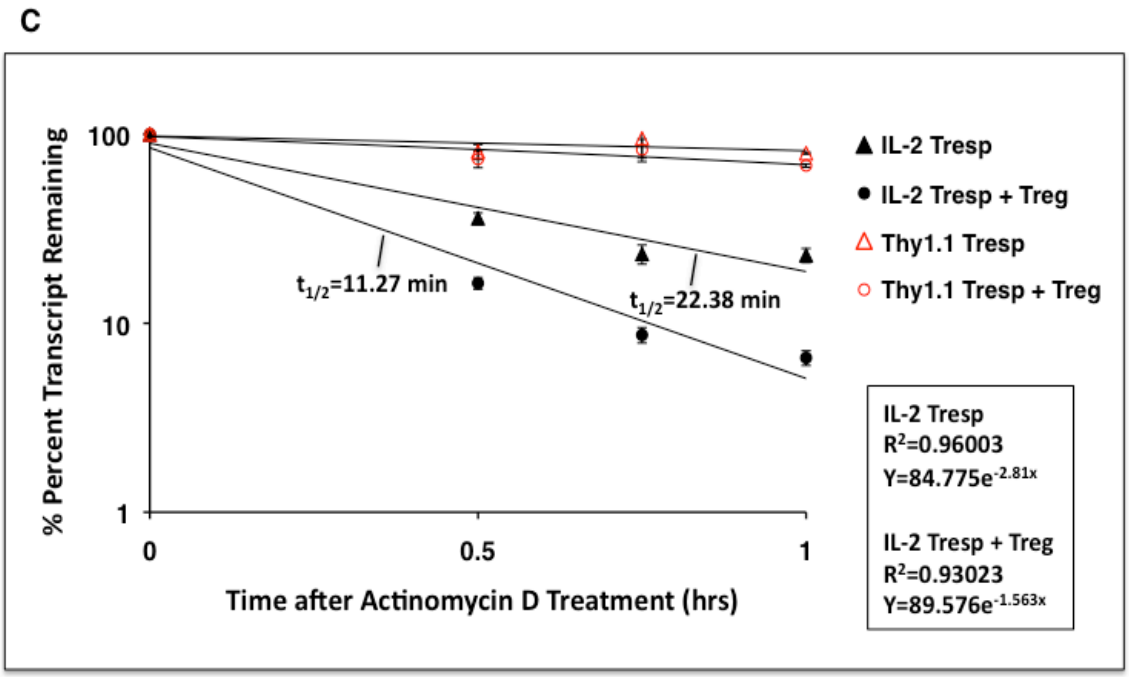

Figure 19. T Regulatory Cells Destabilize IL-2 Transcript in Co-culture Assays Containing CD4 T Responder Cells. The effect of Tregs on IL-2 transcript in co-cultures containing 2BiT or C57BL/6 CD4 T responders activated with irradiated CD4-depleted feeders and soluble α-CD3 in the presence or absence of in vitro-induced Foxp3^{+} iTregs. (C) RNA decay analysis of IL-2 or Thy1.1 transcript in activated co-cultures containing soluble α-CD3 activated naïve CD4 T responders in the presence of Tregs. After 18 hours of activation, cultures were then treated with 10 μg/ml actinomycin D. RNA samples were collected at 0', 30', 45', and 60' after actinomycin D treatment, subject to cDNA synthesis, and used in quantitative PCR analysis. Data are represented as change in cycle threshold for IL-2 relative to β-2 microglobulin levels (Δct), then 30, 45, and 60 minute time points were normalized to the Δct_0 at time point 0 (ΔΔct). The half-life for 50% IL-2 transcript remaining was determined from trendline equations; Tresp [$y=89.576e^{-1.563x}$, $R^2=0.93023$] and Tresp + Treg [$y=84.775e^{-2.81x}$, $R^2=0.96003$]. Data are representative of three experiments.

mRNA half-life	
IL-2 T Resp (0.5 h)	IL-2 T Resp/T Reg (0.5 h)
19.84 ± 1.25 min	13.40 ± 0.86 min
	*p = 0.0006

Table II. Decreased mRNA Half-life of CD4 T Responder-Derived IL-2 in the Presence of Tregs. Calculation of IL-2 mRNA half-life in co-cultures with α-CD3 activated naïve 2BiT T responders with CD4-depleted splenic APCs in the presence or absence of Tregs 30 minutes after actinomycin D treatment (*p = 0.0006 as determined by Student's t test). Half-life values were calculated as $[0.5/(\Delta ct_{30})]$ x 60 minutes. Half-life determination was determined using values from three independent experiments.

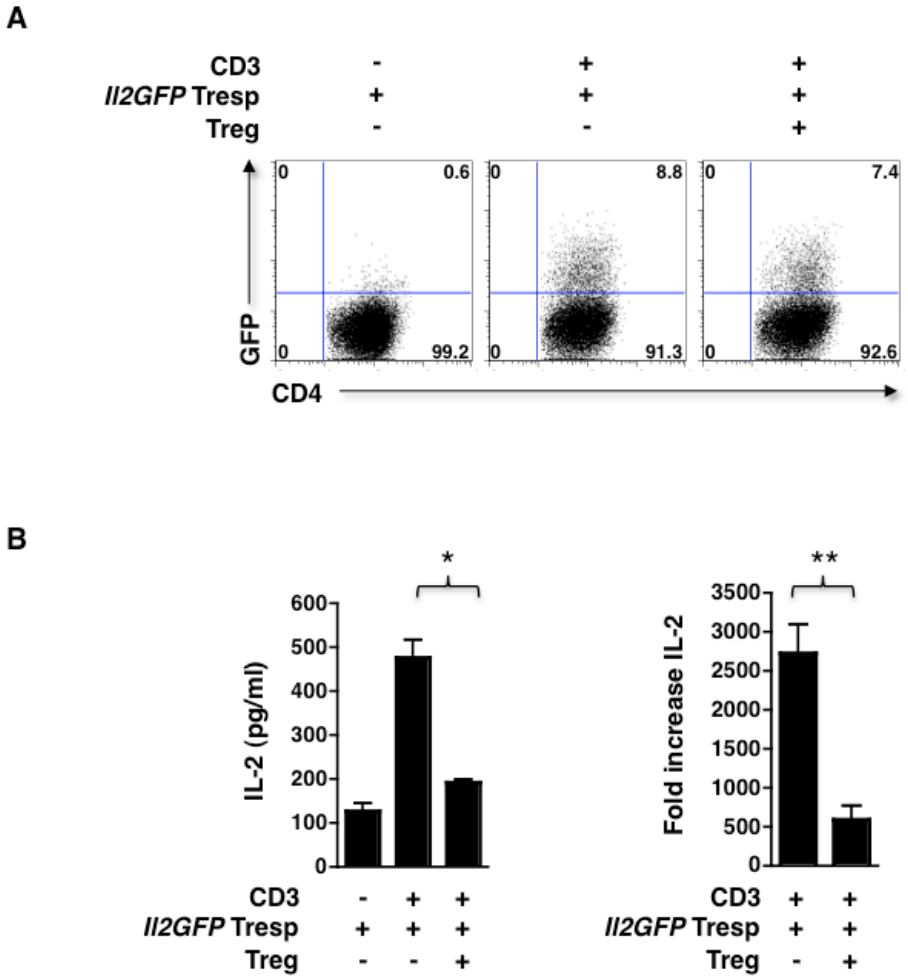

Figure 20. T Regulatory Cells Fail to Suppress Reporter Expression in IL2GFP Responder CD4 T cells. Analysis of endogenous IL-2 and GFP reporter expression in IL2GFP transgenic CD4 responder T cells activated in the presence or absence of Tregs. $CD4^+CD45.2^+CD25^-$ T responders (10^5) from IL2GFP transgenic mice were activated with 2.5 μg/ml soluble α-CD3 and irradiated CD4-depleted splenic APCs ($8x10^5$) in the presence or absence of $CD4^+CD45.1^+CD25^+$ Tregs (10^5) in 96-well round bottom plates for 18 hours. (A) GFP-expressing populations were analyzed by flow cytometry. Cells were stained with monoclonal antibodies to CD4, and CD45.2 then gated on the $CD4^+$ lymphocytes. Numbers in each quadrant represents the percentage of individual cell populations. Data is representative of two independent experiments. (B) (Left) IL-2 protein production in supernatants (*$p < 0.001$ as determined by ANOVA) and IL-2 transcript production (Right) in cultures containing CD4 responders from IL2GFP transgenic mice activated with or without 2.5 μg/ml soluble α-CD3 and irradiated CD4-depleted splenic APCs ($8x10^5$) in the presence or absence of $CD4^+CD25^+$ Tregs (10^5) (**$p = 0.0361$ as determined by Student's t test).

THE ROLE OF IL-21 IN THE GENERATION OF IL-2-PRODUCING CD4 T CELLS

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Abstract

Maintenance of immune homeostasis requires a balance of T cell activation and self-regulating signals. The type I cytokine family members, IL-2 and IL-21, have been shown to play a critical role in the reciprocal regulation of effector and tolerogenic responses. IL-2 and IL-21 are members of the type I cytokine family and are encoded within the *Idd3* autoimmune susceptibility locus. Polymorphisms within this region have been associated with a wide variety of autoimmune conditions establishing the *Idd3* interval as a general autoimmune risk locus. Previous studies have indicated an association of aberrant IL-21 and IL-2 transcript levels in a murine model of autoimmunity suggesting tight regulation of these two cytokines may be a critical factor for immune homeostasis. In addition, IL-21 has been associated with an increased proportion of IL-2-producing cells and effector-associated immunity suggesting de-regulated IL-21 levels in cases of autoimmunity may alter IL-2-driven responses. Herein we describe the generation of a novel *Il2* GFP knock-in reporter mouse model which we have utilized to address the role of IL-21 in the activation of IL-2-producing CD4 T cells. To date, our results indicate IL-21 enhances the frequency of CD4 T cells that produce IL-2. These findings suggest a role for IL-21 in promoting immunity associated with *Il2* expression in CD4 T cells.

Introduction

Antigen activation leads to rapid production of the IL-2 cytokine in CD4 T cells that achieve a threshold level of activation (Saparov, et al., 1999; Sojka, et al., 2004). Differentiation of T helper lineages from these IL-2-producing precursors is associated

with enhanced effector and recall responses (Saparov, et al., 1999). IL-21, similar to IL-2, is a member of the type I family of cytokines and both *Il2* and *Il21* have gene loci that directly flanking each other on chromosome 3 in mice and chromosome 4 in human (Bird, et al., 2005; Malek, 2008). The *Il2-Il21* "looped domain" is included within the *Idd3* autoimmune susceptibility locus (M. S. Anderson & Bluestone, 2005; Parrish-Novak, et al., 2000). Due to the *Idd3* locus association with type I diabetes, rheumatoid arthritis, celiac disease, inflammatory bowel disease, and psoriasis, the *Idd3* locus has been designated as a general autoimmune risk locus (Adamovic, et al., 2008; M. S. Anderson & Bluestone, 2005; Glas, et al., 2009; Hunt, et al., 2008; King, Ilic, Koelsch, & Sarvetnick, 2004; Y. Liu, et al., 2008; van Heel, et al., 2007; Yamanouchi, et al., 2007; Zhernakova, et al., 2007). Additional studies have suggested a possible discord in *Il2* and *Il21* expression associated with type I diabetes in NOD mice as these mice show a decrease in *Il2* production associated with decreased Treg population as well as an increase in IL-21 production and inflammation (King, et al., 2004; Yamanouchi, et al., 2007). Growing evidence indicates a reciprocal relationship in immune components critical for effector responses and immune tolerance (Bettelli, et al., 2006; Gavin, Rasmussen, Fontenot, Vasta, Manganiello, Beavo, et al., 2007; Laurence, et al., 2007; Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006). IL-2 was previously shown to be required for the maintenance of Treg populations in peripheral lymphoid tissues and associated immune tolerance (Fontenot, et al., 2005), while IL-21 has been implicated in T helper 17 differentiation (R. Nurieva, et al., 2007). In a reciprocal manner, IL-2 has been shown to inhibit T helper 17 differentiation (Laurence, et al., 2007). Likewise, IL-21 diminishes Treg suppressive potential and is associated with expansion of $Foxp3^-$

populations after *in vitro*-polarizing Treg conditions (Fantini, et al., 2007; Parrish-Novak, et al., 2000; Peluso, et al., 2007). These studies implicate IL-21 as a co-stimulator of effector T cell expansion. IL-21 production has also recently been shown in follicular T cell populations (Tfh) and is critical for germinal center formation and IgG1 antibody production in response to antigenic stimuli (Ozaki, et al., 2002). Several studies show an association of IL-21 exposure and signaling to an increased fraction of IL-2-producing cells (Kim-Schulze, Kim, Fan, Kim, & Kaufman, 2009; Yi, Du, & Zajac, 2009; D. Yu, et al., 2009). As IL-2 production is associated with effector cell development, these findings further support a role for IL-21 in promoting effector CD4 T cell development. In order to evaluate the role of IL-21 in effector CD4 T cell responses we have generated a novel *Il2* GFP knock-in mouse model that enables reporting on *Il2* expression with fidelity utilizing total endogenous loci regulatory sequence. To date, we have confirmed the previous findings that IL-21 acts as a co-stimulatory of α-CD3 induced T cell activation and reduces the Treg-suppressive capacity. In addition, we show that IL-21 increases the frequency of IL-2-producing CD4 T cell populations *in vitro*. Additional studies will be necessary to delineate the role of IL-21 in modifying the effector T cell response *in vitro* and the context of disease *in vivo*.

Methods

Mice

C57BL/6 mice (Jackson Labs, Bar Harbor, ME, USA), *Il2* GFP Knock-in, and Foxp3^{gfp} mice were housed in a pathogen-free facility in autoclaved microisolator cages

and were used between 6 and 8 weeks of age. Foxp3^{gfp} mice have previously been described (Fontenot, et al., 2005) and were backcrossed onto a C57BL/6 background.

Il2 GFP Knock-in Generation

The BAC clone 114D21 containing the *Il2* locus was obtained from CHORI. A minigene vector containing proximal regions of homology to *Il2* flanking a ECMV IRES-NEO^r cassette was targeted to BAC clone 114D21 using recombineering methods (P. Liu, Jenkins, & Copeland, 2003). The ECMV IRES-eGFP cassette was inserted downstream of the stop codon within the last exon of *Il2* and upstream of native 3'UTR sequence elements. The final targeting vector was retrieved from the minigene recombinant 114D21 BAC clone through gap repair recombination using a gap repair vector containing distal regions of homology to *Il2* flanking an HSV-TK coding sequence. The retrieved plasmid was then linearized with BamH1 restriction endonuclease and the final *Il2* eGFP targeting vector was used to electroporate Bruce4 mouse embryonic stem cells as described (Harrington, Janowski, Oliver, Zajac, & Weaver, 2008). Homologous recombined ES cell clones resistant to neomycin were selected for by Southern Blot analysis followed by microinjection into albino C57BL/6-blastocysts at the UAB Transgenic Mouse Facility. Chimeric mice were used to establish founder lines as described (Harrington, et al., 2008). Founders were subsequently crossed to the EIIA Cre-recombinase-expressing mice for excision of the neomycin resistance cassette and screened by Polymerase Chain Reaction for neo^r-deleted founders.

PCR Genotyping

Mice positive for eGFP were screened using forward primer 5' TGGTGGACTTTCTGAGGAGATGG 3' and reverse primer 5'

TGTTGAATACGCTTGAGGAGAGC 3' and for the neomycin resistance cassette with forward primer 5' CTGAATGAACTGCAGGACGA 3' and reverse primer 5' ATACTTTCTCGGCAGGAGCA 3' using the following PCR conditions; 94°C 3 minutes for one cycle; 94°C 30 seconds, 55°C for 30 seconds, 72°C 30 seconds for 35 cycles; 72°C 7 minutes for one cycle. Standard Taq polymerase reagents and protocols were used for eGFP and neomycin analysis (Roche). Screening for homozygosity of the *Il2* GFP targeted allele was assayed using forward primer 5' TGGTGGACTTTCTGAGGAGATGG 3' and reverse primer 5' GGTGTAGATTCCAAACCAGCAACC 3' with the following PCR conditions; 94°C 2 minutes for one cycle; 94°C 15 seconds, 60°C for 30 seconds, 72°C 45 seconds plus a 5 second extension time per cycle for 10 cycles; 94°C 15 seconds, 60°C for 30 seconds, 72°C 45 seconds plus a 5 second extension time per cycle for 20 cycles; 72°C 7 minutes for one cycle. High fidelity Taq polymerase (Roche) was used according to manufacturer protocals for homozygosity screening.

IL-21 Titration Studies

For proliferation assays and flow cytometric analysis, IL-21 was added at 10ng/ml (R&D Systems) unless otherwise indicated. For flow cytometric studies, $CD4^+CD25^-CD62L^{high}$ or $CD4^+CD25^-CD44^{low}$ T cells were harvested from spleen and lymph nodes from *Il2* GFP mice. Following red cell lysis with ACK lysis buffer (Quality Biologicals, Inc), $CD4^+$ cells were isolated using $CD4^+$ Dynabead separation according to manufacturer's protocol (Invitrogen). *In vitro* activation was set up in 96-well polypropylene round bottom plates seeded with $1X10^5$ naïve T cells from *Il2* GFP or C57BL/6 mice and $8X10^5$ irradiated CD4-depleted splenic feeders (3000 rads). Cultures

were incubated with or without 2.5 μg/ml soluble anti-CD3 for 18 hours unless otherwise indicated. Samples were then subject to surface stain with CD4-PeCy7. IL-2-APC cytokine capture assay detection was used for kinetic analysis of GFP expression. Samples were acquired using the LSRII flow cytometer (BD Bioscience) and data were analyzed using FlowJo 8.4.6 (Tree Star, Ashland, OR).

Proliferation Assays

For proliferation assays, $5x10^4$ $CD4^+CD25^-CD44^{low}$ or $CD4^+CD25^-CD62L^{high}$ naïve T cells from C57BL/6 mice were seeded into 96-well polypropylene round bottom plates in triplicate with $4x10^5$ CD4-depleted irradiated splenic feeders (3000 rads) with or without $5x10^4$ live-gated $CD4^+Foxp3^{gfp}$ T regulatory cells. Cultures were incubated with or without 2.5 μg/ml soluble anti-CD3. After 68-72 hours, 1 μCi [^{3}H]thymidine (TdR) was added to each well and incubated for 16-20 hours after which samples were harvested with a cell harvester and then thymidine incorporation determined by a scintillation counter.

T Regulatory Cell Polarization

Spleen and lymph nodes were harvested from $Foxp3^{gfp}$ mice. Following red cell lysis with ACK (Quality Biologicals, Inc), $CD4^+$ cells were isolated using $CD4^+$ Dynabead separation according to manufacturer's protocol (Invitrogen). $CD4^+CD25^-$ cells were then further sorted using CD25 Microbead isolation (Miltenyi Biotech). $CD4^+CD25^-$ cells were then cultured with 5 ng/ml $TGF\beta_1$ (R&D Systems), 50 U/ml IL-2 (R&D Systems), 100 nM retinoic acid (Sigma-Aldrich), 10μg/ml anti-IFNγ (XMG 1.2), 10μg/ml anti-IL-4 (11B11) in R-10 complete media. After 4 days in culture, live $CD4^+$

Foxp3^{gfp} cells were isolated by flow activated cell sorting using CD4-PeCy7, FITC-GFP, and 7AAD staining (BD Bioscience).

Statistical Analysis

Statistical significance was determined by ANOVA with Tukey post-test using Prism software (Graphpad, San Diego, CA). All values are expressed at the mean ± s.d. where a p value ≤ 0.05 is considered statistically significant.

Results

Naïve T Cell Proliferation is Responsive to IL-21

In order to test the effect of IL-21 on CD4 effector T cell expansion we measured tritiated thymidine incorporation of activated naïve CD4 T cells in the presence or absence of exogenous IL-21 at several doses. Our results indicate that addition of exogenous IL-21 at a minimal dose of 10ng/ml led to a dramatic enhancement of CD4 T cell proliferation (Figure 1). Doses above 10ng/ml generated similarly higher levels of proliferation as the 10ng/ml dose indicating saturation of the co-stimulatory properties of this cytokine above the effective minimal dose. Thus, we show that in our *in vitro* culture system that proliferative capacity of α-CD3-activated naïve CD4 T cells derived from peripheral lymphoid tissues is responsive to exogenous IL-21.

IL-21 Reduces the Suppressive Capacity of T Regulatory Cell In Vitro-Induced Populations

Previous studies have indicated increased proliferative capacity of Foxp3^{-} fractions from *in vitro*-induced Treg polarization cultures activated in the presence of exogenous IL-21. These results suggested that IL-21 signaling counteracted the Treg-

inducing properties of TGFβ and led to expansion of existing naïve T cells thereby shifting the Treg:Effector ratio within these cultures and associated suppressive capacity (Fantini, et al., 2007).

In order to further evaluate this observation we *in vitro* activated $CD4^+CD25^-$ T cells under Treg-polarizing conditions in the presence or absence of exogenous IL-21 and then assayed associated proliferative potential of these populations during secondary culture. Similar to previous reports, we found that the presence of IL-21 in primary Treg-polarization cultures (1° iTreg) reduced the suppressive capacity of this population in secondary proliferation assays (2° iTreg) containing newly isolate naïve CD4 T responders (Figure 2A). Flow cytometric analysis of *in vitro*-induced Treg populations showed a trend toward a decrease in the percent $Foxp3^+$ in the presence of IL-21 (Figure 2B). Likewise, the presence of IL-21 in 1° iTreg cultures showed a more pronounced blasting population compared to conventional Treg-polarizing conditions. This observation suggested an increase in the ability of the founding naïve T cell population to undergo proliferation in the presence of enhanced IL-21 signaling, a trend that is coordinate with a decreased suppressive capacity of this IL-21-1° iTreg population in 2° cultures (Figure 2B). These results suggest that excess IL-21 signaling antagonizes the Treg-polarizing affect of TGFβ and IL-2 thereby enabling expansion of naïve CD4 T cells. In addition to preventing Treg suppression, exogenous IL-21 appeared to also have proliferation-enhancing properties due to the marked increase in proliferation as compared to cultures with α-CD3 alone. We next analyzed the suppressive ability of $Foxp3^+$ Tregs sorted from the 1° iTreg cultures for intrinsic affects from IL-21 signaling. Sorted 1° iTregs were re-cultured in 2° cultures with freshly isolated naïve CD4 T cells

and assessed for their ability to suppress proliferation. Our results indicate that sorted Foxp3$^+$ 1° iTreg in the absence of IL-21 suppressed proliferation of activated naïve T cells. However, Foxp3$^+$ 1° iTreg differentiated in the presence of exogenous IL-21 showed a marked decline in suppressive ability (Figure 2A). These results suggest that in addition to shifting the effector:Treg ratios within *in vitro*-induced Treg cultures, IL-21 signaling also leads to a decrease in the intrinsic ability of Tregs to suppress proliferation of T responders, while also enhancing their expansion.

Blockade of IL-2 Inhibits IL-21-Mediated Effects on Foxp3$^+$ iTreg

IL-2 has been shown to be necessary for the maintenance of Tregs in peripheral lymphoid tissues *in vivo* (Malek, 2008). In addition, IL-2 has been shown to have T cell growth factor properties *in vitro*. Several studies have indicated that IL-2 autocrine signaling facilitates a G1 to S cell cycle phase transition necessary for T cell proliferation (Cantrell & Smith, 1983, 1984). Due to the enhanced proliferative response of naïve CD4 T cells in the presence of exogenous IL-21 we investigated the role of IL-2 in IL-21-mediated Treg inhibition. Addition of blocking antibody to IL-2 in 1° iTreg cultures led to a marked decline in the frequency of Foxp3$^+$ Tregs and likewise a decrease in ability of this iTreg population to suppress CD4 T responders in 2° cultures. These results agree with the precept that IL-2 is necessary for the maintenance of Treg populations *in vivo*, as well as, *in vitro*-induced polarization of Tregs. In the presence of IL-21 and blocking antibody to IL-2, similar to cultures with IL-21 alone, 1° iTreg cultures showed significant proliferation. However, sorted Foxp3$^+$ Tregs from the IL-21αIL-2 1° iTreg culture condition were able to induce suppression of activated CD4 T responders as compared to IL-21 1° iTreg in secondary cultures (Figure 2A). In relation to Foxp3$^+$

Tregs from normal *in vitro*-Treg polarization conditions, the degree of IL-21αIL-2 1° iTreg suppression in secondary cultures was still compromised. The findings that blockade of IL-2 does not prevent the marked proliferation seen in IL-21αIL-2 iTreg suggests that IL-2 does not mediate IL-21-enhanced proliferation. However, due to the dramatic increase proliferation in these 1° iTreg cultures, subsequent 2° culture may have such a high Teff:Treg ratio that addition of IL-2 blocking antibody would have little impact. Subsequent isolation of $Foxp3^+$ Tregs from the IL-21αIL-2 1° iTreg did show a partial rescue of suppressive capacity of these Tregs compared to IL-21-induced $Foxp3^+$ Tregs. This suggested that inhibition of IL-2 during 1° iTreg culture could, in part, counteract the Treg suppression-inhibitory and T effector expansion properties of IL-21 from 1° iTreg cultures.

Generation of an Il2 GFP Knock-in Reporter Mouse Model

The existing models that report on *Il2* gene expression have several limitations. First, previous transgenic models are limited in their ability to report on *Il2* expression without the use of non-native regulatory sequence as many rely on artificial 3'UTR sequences that over-amplify reporter transcript stability (Brombacher, et al., 1994; Crabtree & Clipstone, 1994; Reed, et al., 1997; Saparov, et al., 1999; Yui, et al., 2001). In addition, many of these reporters are transgenes that rely on promoter sequences that do not contain sufficient regulatory sequence to confer lineage-specific expression with fidelity to *Il2* regulation. Some transgenic models attempt to establish this lineage-specific control with the addition of superficially placed locus control regions or extended cis-regulatory sequence (Saparov, et al., 1999; Yui, et al., 2001). However, a genuine reporter system for *Il2* that relies entirely on endogenous regulatory mechanisms for

reporter expression while maintaining lineage-specific regulation has not been generated to date. It also remains a possibility that insertion of reporter coding sequences in-frame with endogenous gene sequences may lead to production of a gene-reporter protein fusion product that may affect normal translation efficiency as well as native protein function and detection due to masking of critical binding sites and/or epitopes for antibody recognition. A previous attempt to generate a knock-in model targeting the GFP coding sequence to one of the endogenous *Il2* alleles relied on a non-native SV40 poly adenylation sequence to regulate post-transcriptional control of the GFP transcript. In addition, this reporter disrupted native *Il2* transcription from one of the endogenous alleles (Naramura, et al., 1998). Thus, this and other models are limited in their ability to utilize the natural sequence elements necessary to control reporter expression without disrupting native *Il2* expression from endogenous loci or generating altered protein products that may impede normal translation and protein detection. This prompted us to generate a reliable knock-in *Il2* GFP reporter model where GFP is targeted to the endogenous *Il2* locus without disrupting normal function of existing regulatory sequence and is regulated through both native promoter and 3'UTR regulatory sequence elements. To this end we inserted the *GFP-IRES-Neo*^{r} cassette downstream of the stop codon within exon 4 of the endogenous *Il2* locus (Figure 3A, 3B). Thus, our *Il2* GFP knock-in reporter is placed into the endogenous *Il2* locus in a position that does not disrupt native regulatory sequence and is translated independently of IL-2 protein production. Genotyping analyses were developed to detect mice for the presence of the targeted allele. A positive screen for GFP uses primers specific for the enhanced GFP coding sequence and exon 4 of the *Il2* locus (500 bp) (Figure 3C). A negative screen was

developed to determine if mice were homozygous for *Il2* GFP containing two targeted alleles. Mice that were determined to be positive for GFP were then rescreened for the absence of wild-type endogenous product (500 bp). *In vitro* polyclonal activation of naïve CD4 T cells from *Il2* GFP knock-in mice shows reporter expression with fidelity to endogenous *Il2* gene regulation during a kinetic analysis of GFP reporter activation with respect to endogenous IL-2 production (Figure 4A). As early as six hours, the frequency of early IL-2-producers begins in a coordinate manner to the proportion of GFP reporter expressors (Figure 4A). Similarly, analysis of populations for both IL-2 and GFP expression shows that cells expressing endogenous IL-2 are also expressing the GFP reporter indicating a direct relationship of GFP regulation with the endogenous locus (Figure 4B). Furthermore, with additional co-stimulation using α-CD28 agonist antibody shows a greater proportion of IL-2-producers that also correlates well with GFP reporter expression (Figure 4A, 4B). Taken together, single-cell expression analysis indicates that our *Il2* GFP knock-in model serves as a reliable reporter to monitor changes in IL-2 production in CD4 T cells.

IL-21 Increases the Frequency of IL-2-Producing Cells

Previous studies have implicated an association with enhanced IL-21 signaling and an increased frequency of IL-2-producing cells (R. I. Nurieva, et al., 2008; Vogelzang, et al., 2008; D. Yu, et al., 2009). Our previous results showing a strong co-stimulatory effect on T cell proliferation led to further investigate the role of IL-21 on the generation of IL-2-producing cells. In order to evaluate the effect of IL-21 signaling on CD4 T responders we activated naïve CD4 T cells in the presence or absence of IL-21. In the presence of IL-21 at several IL-21 concentrations our results show that addition of

IL-21 enhances the frequency of IL-2-producing cells in a dose-dependent manner (Figure 5). These results are in agreement with IL-21 acting as a co-stimulator of α-CD3 activated expansion of T cells.

Discussion

The major findings from this study confirm previous reports showing that IL-21 acts to both markedly enhance CD4 T cell proliferation upon polyclonal antigen activation as well as diminishing Treg-mediated suppressive properties in suppression assays (Fantini, et al., 2007; Parrish-Novak, et al., 2000; Peluso, et al., 2007). We also find that with the use of a novel *Il2* GFP knock-in reporter mouse model that exogenous IL-21 enhanced the frequency of IL-2-producing cells as measured by GFP expression in activated naïve CD4 T cells. This is in support of several reports that show a significant correlation of IL-21 addition to an increased frequency of IL-2-producing populations (R. I. Nurieva, et al., 2008; Vogelzang, et al., 2008; D. Yu, et al., 2009).

The IL-2 cytokine is produced early and shows rapid and transient kinetics after antigen activation of effector CD4 T cells (Sojka, et al., 2004). *Il2* gene expression during a primary immune response has been linked to enhanced IL-7 receptor production during recall responses. This observation suggested a mechanism by which *Il2* gene expression and protein production promoted memory T cell responses (Dooms, et al., 2007; Z., et al., 2000). The use of a novel *Il2* GFP reporter indicates that IL-21 promotes the generation of IL-2-producing CD4 T cell populations in a dose-dependent manner. Our observations in addition to previous findings suggest a role for IL-21 in promoting

CD4 T cells responses through its enhancing effects on IL-2-producing populations. Further studies will be required to validate this possibility.

Despite a putative role as a T cell growth factor *in vitro*, IL-2 has been shown to be dispensable for T cell proliferation *in vivo* (Cantrell & Smith, 1983, 1984; Sadlack, et al., 1995; Sadlack, et al., 1993; Schorle, et al., 1991; Smith, 1988b; Willerford, et al., 1995). IL-2 has, however, been shown to play a role in specific T helper cell lineages. IL-2 is a product of T helper 1 cells and also promotes T helper 2 generation. Conversely, studies have shown that IL-2 suppresses the generation of T helper 17 cells (Laurence, et al., 2007). Analysis of effector CD4 T cell fate of IL-2-producing populations *in vivo* using our *Il2* GFP mouse model will give further clarification into the role of these defined cell populations in T helper cell lineage commitment. Furthermore, Th17 cells and follicular helper T cells (Tfh) are known to be significant producers of IL-21. In light of our findings that IL-21 enhances IL-2-generating CD4 T cell populations, the role of these lineages in T cell homeostasis through triggering further enhancement of IL-2-positive T cells remains to be determined. Previous studies using the NOD mouse model suggest a possible discord in IL-21 and IL-2 gene regulation in murine type I diabetes (King, et al., 2004; Yamanouchi, et al., 2007). If validated through additional studies, this possibility raises key questions as to the importance of balancing IL-21 and IL-2 production in the context of immune tolerance. In addition, Tfh cells play a significant role in germinal center formation and antibody production (R. I. Nurieva, et al., 2008; Vogelzang, et al., 2008). Does IL-21 production from Tfh cells alter the frequency of IL-2-producing cells that play a role in germinal center responses and how does this population influence immune homeostasis?

Finally, previous reports suggest intrinsic differences within a clonal CD4 T cell population that enable individual cells to overcome a threshold of activation (Saparov, et al., 1999; V., et al., 2003). Additional analyses of activation-dependent alterations in chromatin architecture have indicated stable changes in accessibility of the *Il2* locus with activation state (McKarns & Schwartz, 2008; Rao, et al., 2003; Rao, et al., 2001). Given the increase in the fraction of CD4 T cells that produce IL-2 in the presence of exogenous IL-21, does IL-21 increase the frequency of IL-2-producing CD4 T cells by lowering the threshold of activation? Future studies will be required to validate this possibility and possible mechanism. In addition, future studies will be required to verify the immunological outcome of IL-21 on CD4 primary effector responses as well as memory T cell generation and maintenance. Use of our *Il2* GFP knock-in mouse model provides a novel reporting tool to follow endogenous *Il2* expression with fidelity. Furthermore, this model allows for ease of tracking and separating IL-2-producing populations that will enable us to address many questions of IL-2-driven immunology.

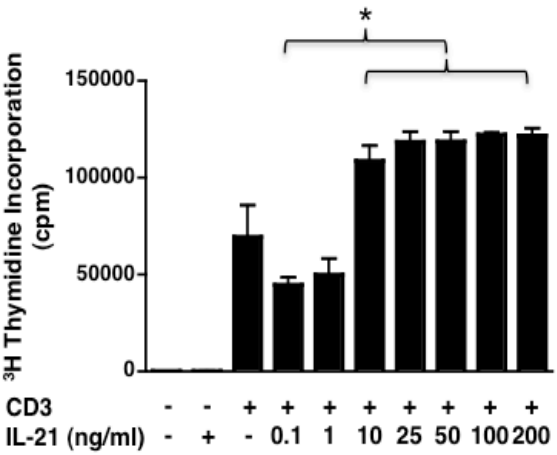

Figure 1. Proliferation of Naïve CD4 T Cells is Responsive to Exogenous IL-21. Naïve CD4 T cells were activated with or without soluble α-CD3 in the presence or absence of IL-21 at the indicated concentrations for 72 hours with irradiated CD4-depleted feeders. Cells were then treated with tritiated thymidine then further incubated for an addition 16-20 hours. Samples were then harvested for tritiated thymidine incorporation and counted on a scintillation counter. (*$p < 0.05$). Data are representative of three independent experiments.

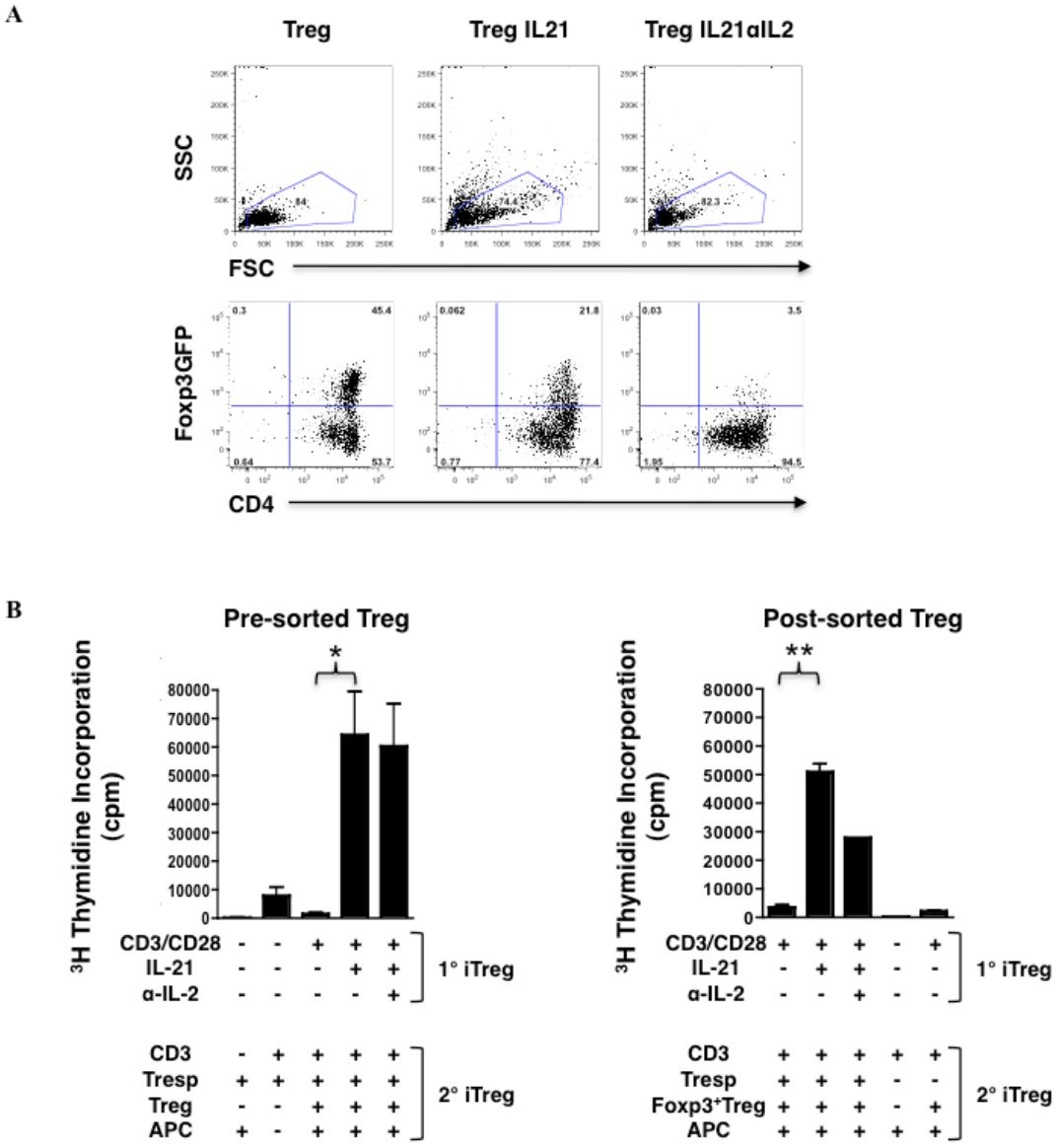

Figure 2. IL-21 Diminishes Treg Suppression and Co-stimulates Proliferation of CD4 T Cells. $CD4^+CD25^-$ T cells from *Foxp3* GFP mice were activated under in vitro Treg-polarizing conditions for four days in the presence or absence of IL-21 and α-IL-2 monoclonal antibody then subject to (A) Flow cytometric analysis for blasting populations (top) and Foxp3 expression (bottom). (B) Pre-sorted primary iTreg cultures (left) or post-sorted $Foxp3^+$ Tregs (right) were then used as a Treg population in proliferation assays with naïve CD4 T responder cells (left). Samples were activated for 72 hours then subject to tritiated thymidine treatment for 16-20 hours. Cells were then harvested for tritiated thymidine incorporation and counted on a scintillation counter. (*$p < 0.001$) Data are representative of one experiment.

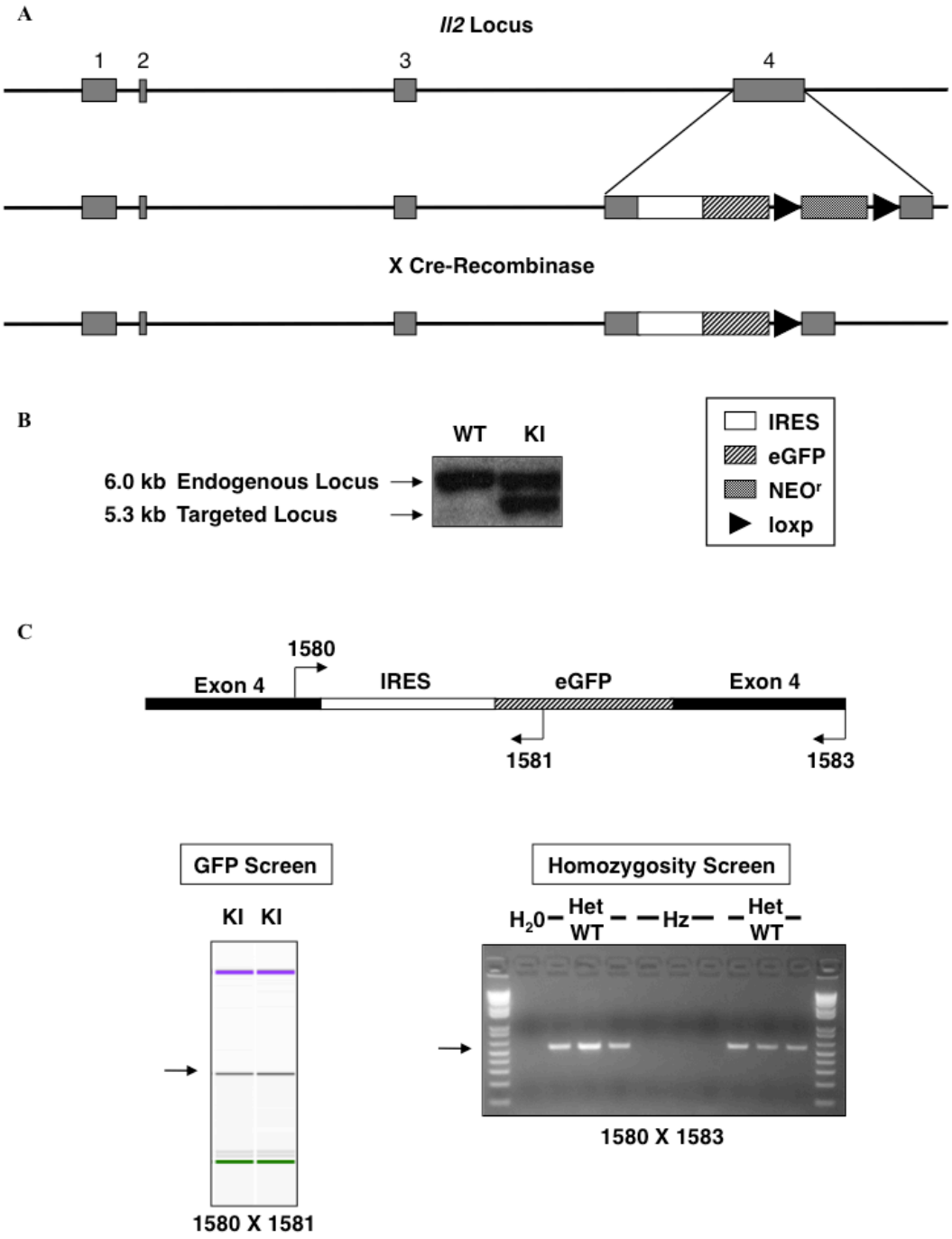

Figure 3. Gene-targeted Insertion of an Enhanced Green Fluorescent Protein Reporter Sequence into the *Il2* Endogenous Locus. (A) Schematic of the gene-targeting insertion of the ECMV IRES-eGFP cassette into the *Il2* locus of embryonic stem (ES) cells. ES cells that underwent homologous recombination were screened by Southern Blot analysis (B). Founders were then screened (C) for the presence of the eGFP reporter (left), then screened for homozygosity (right).

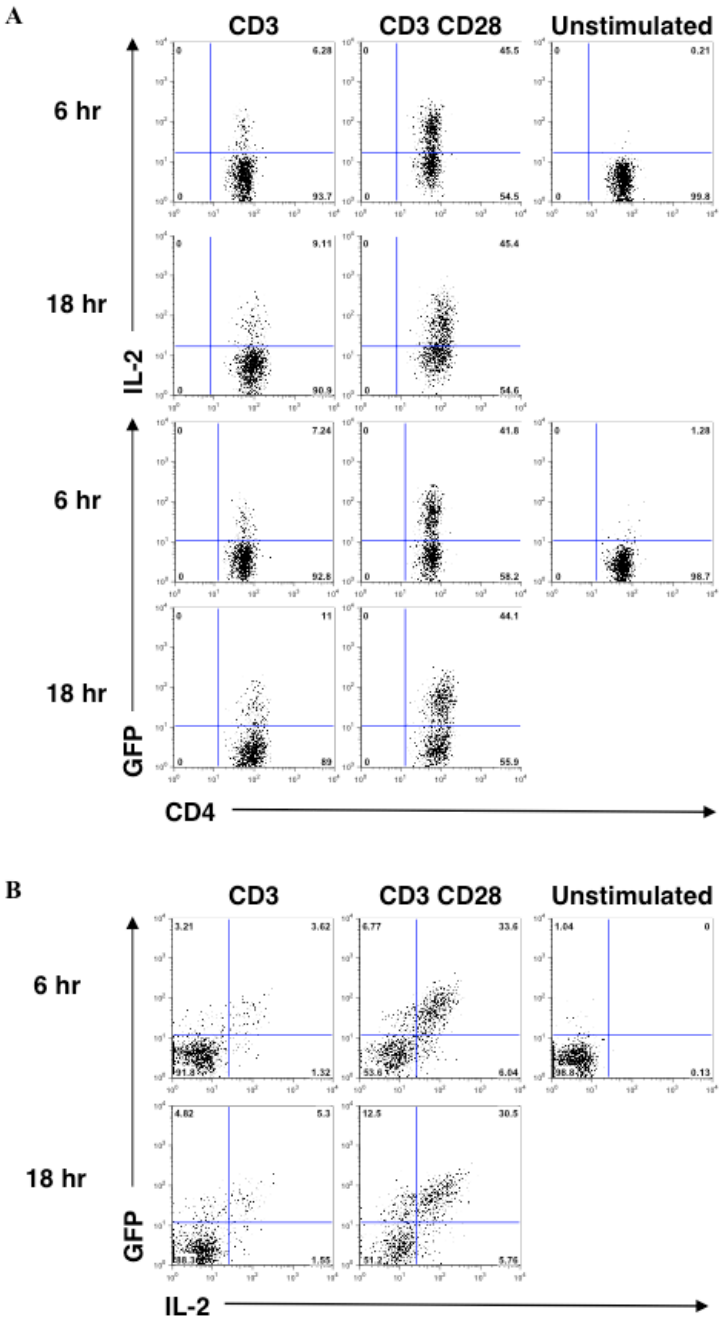

Figure 4. Fidelity of *Il2* GFP Reporter Expression to Endogenous *Il2* Gene Regulation. Naïve CD4 T cells from either *Il2* GFP mice or C57BL/6 mice were activated with or without soluble α-CD3 for 6 or 18 hours in the presence of irradiated CD4-depleted feeders. Cells were then stained for IL-2-producing cells using the IL-2 cytokine capture assay (Miltenyi) followed by surface staining using antibodies targeted for the CD4 surface marker. The lymphocyte population was gated on CD4-positive populations and evaluated for GFP and IL-2 expression. Data are representative of three independent experiments.

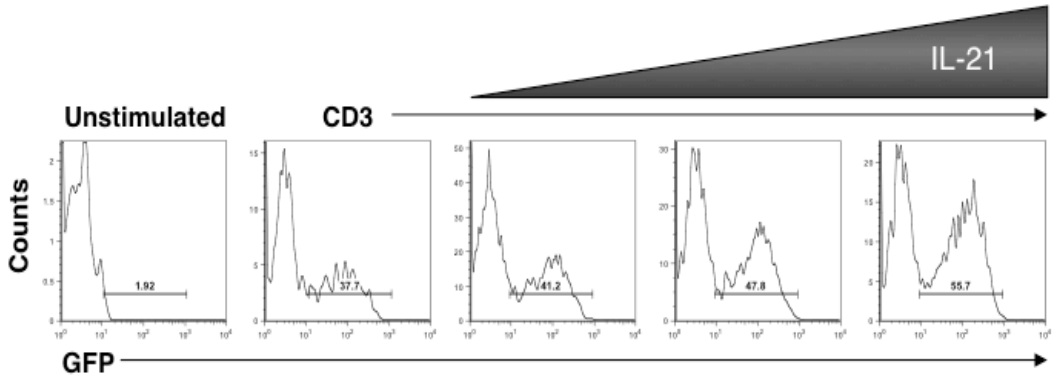

Figure 5. IL-21 Enhances the Generation of IL2-Producing CD4 T Cells. Naïve CD4 T cells were activated with or without soluble α-CD3 in the presence or absence of IL-21 at 0, 0.5, 5, and 50 ng/ml for 18 hours with irradiated CD4-depleted feeders. Populations are gated on the lymphocyte gate followed by $CD45.2^{+}CD4^{+}$ cells and represented as a histogram of GFP^{+} cells. Data are representative of two independent experiments.

DISCUSSION

Major Findings

Monitoring the expression of transcript and protein products that undergo rapid turnover presents a significant challenge when addressing questions that examine the role of these gene products in biological processes. Like many other cytokines, proto-oncogenes, and early response genes, IL-2 undergoes rapid mRNA degradation (Caput, et al., 1986; C. Y. Chen & Shyu, 1995; Yang, et al., 2003) in addition to rapid protein turnover through ligand-mediated internalization of the IL-2 receptor:cytokine complex followed by lysosomal degradation (Donohue & Rosenberg, 1983; Letourneau, et al., 2009; Levy & Committee, February 8-11, 2009; Muhlradt & Opitz, 1982). Existing techniques that aim to detect IL-2-producing cells are intrinsically limited by the relative instability of *Il2* gene product. IL-2 production and signaling plays a central role in bridging both the effector response and tolerogenic cellular components. Many questions regarding the function of this soluble mediator in immune homeostasis remain to be adequately addressed. Our findings describe the generation of two novel reporter systems for monitoring the expression of *Il2* with fidelity and sensitivity of detection to endogenous locus expression. We show that in the context of *in vitro* polyclonal T cell activation that both an extended *Il2* transgenic model (2BiT) and an *Il2* GFP knock-in

model with minimal disruption to endogenous elements display inducible reporter expression in a lineage-specific manner. Reporter expression in both models shows robust activation and show fidelity to endogenous *Il2* gene expression patterns. The 2BiT model is distinguished from its *Il2* GFP counterpart in that Thy1.1 reporter expression follows *Il2* gene activation while maintaining transcript stability to more stably mark IL-2-producing cells. Furthermore, we show using the 2BiT transgenic model that activated conventional naïve CD4 T cells show Treg-mediated suppression of reporter expression. In addition, 2BiT reporter inhibition by Tregs was not rescued by addition of exogenous IL-2 while excess co-stimulation with the addition of exogenous agonist α-CD28 antibody prevented suppression. Thus, this demonstrates the responsiveness of our 2BiT model to external stimuli. These data also imply that Tregs compete with responder CD4 T cells for co-stimulatory signals on antigen presenting cells *in vitro* as excess co-stimulatory activity enables escape of reporter activation from suppression. However, further experiments are required to substantiate this possibility. In analyzing the kinetic parameters of responder CD4 T cell viability in conjunction with reporter suppression in the presence of Treg-associated suppression, we observed inhibition of reporter expression in responder T cells during peak IL-2 production time points while enhancement of cell death occurred at later stages following T cell proliferation. Due to the longer half-life of our reporter it is conceivable that Tregs prevented initial reporter activation in the responder CD4 T cell population rather than suppression of existing reporter expression in activated cells. These findings suggested a Treg mechanism that involves preventing activation of responder T cells to produce the Thy1.1 reporter followed by an association of enhanced cell death. Additional experiments will be

required to validate whether responders that fail to produce the Thy1.1 reporter during early activation stages are the source of cell death during late activation. Studies using the *Il2* GFP knock-in model indicate inducible reporter expression that follows a pattern more closely with endogenous kinetic parameters. In addition, reporter expression is responsive to exogenous IL-21 affecting the proportion of reporter-positive CD4 T cell populations. In agreement with previous studies indicating that IL-21 is a co-stimulator of α-CD3 activated T cells, addition of exogenous IL-21 enhanced the frequency of IL-2-producing cells (Chapter 2-Figure 5) (Parrish-Novak, et al., 2000). Taken together, our data show that 2BiT and *Il2* GFP knock-in reporter activation is inducible in under conditions of T cell activation and is responsive to specific exogenous signals *in vitro*. Future studies will be required to validate these observations in an *in vivo* setting.

Significance of High Fidelity Reporter Models to Immunology

Many of the current mouse models that incorporate *Il2* promoter regulatory elements driving the expression of an easily identifiable marker fail to report gene expression with fidelity to endogenous gene regulation due to insufficient cis-regulatory sequence (Brombacher, et al., 1994; Crabtree & Clipstone, 1994; Reed, et al., 1997). In addition, previous transgenic knock-in targeting attempts that disrupt one endogenous *Il2* allele pose a risk to normal gene dosage effects and biallelic expression patterns under conditions of strong activation signals (Hollander, 1999; Naramura, et al., 1998). Furthermore, despite transgenic approaches that show lineage-specificity of reporter expression, these models still remain limited in the extent of long-range distal regulatory

elements that may be necessary for appropriate *Il2* locus control as well as balancing appropriate regulation of the *Il2* gene with the neighboring *Il21* locus (Yui, et al., 2001). In an effort to generate a reporter that maintains the precision of *Il2* locus activation with minimal perturbation of endogenous regulatory sequence our *Il2* reporter models represent a novel and innovative platform from which to study many aspects of *Il2* locus regulation and T cell immunity.

Reciprocal Relationship of IL-2 and IL-21

Generation of the 2BiT transgenic and *Il2* GFP knock-in reporter models presents a unique opportunity to address several important biological questions regarding *Il2* gene expression and signaling. IL-2 is a member of the type I cytokine family and is encoded on chromosome 4 in humans and chromosome 3 in mice (Malek, 2008). Several studies have begun to establish a unique relationship between IL-2 and other type I cytokine family members such as IL-21. Immune homeostasis depends on a reciprocal balance of pathogen-induced effector responses and contraction of immune regulators upon clearance of pathogen while maintaining tolerance to self-antigen. Several studies have defined a reciprocal relationship between the pro-inflammatory T helper 17 lineage and Treg development and suppressive function (Bettelli, et al., 2006; Gavin, Rasmussen, Fontenot, Vasta, Manganiello, Beavo, et al., 2007; Laurence, et al., 2007; Veldhoen, et al., 2006). The IL-2 and IL-21 cytokines have been shown to play counter-regulatory roles in these effector-tolerance decisions (Fantini, et al., 2007; Fontenot, et al., 2005; Peluso, et al., 2007; Piao, et al., 2008). IL-2 has been shown to promote the development

and maintenance of Tregs while having a suppressive effect on the generation of T helper 17 populations (Fontenot, et al., 2005; Laurence, et al., 2007). However, IL-21 has been shown to have an opposing role by promoting the pro-inflammatory T helper 17 lineage and inhibiting Treg suppressive function (Fantini, et al., 2007; Korn, et al., 2007; R. Nurieva, et al., 2007; Peluso, et al., 2007; Piao, et al., 2008). Many of these reciprocal effects by IL-2 and IL-21 responses may reside in their differential ability to signal STAT5 and STAT3 activation and downstream transcriptional targets (Zeng, et al., 2007). Future studies addressing the context and cell-specific activation of the *Il2* and *Il21* gene loci and the consequences of downstream signaling from the IL-2 and IL-21 gene products will help to shed new light on the balance of effector immunity and the maintenance of tolerance. Conversely, several studies have implicated a role for IL-21 enhancement of IL-2-expressing T cell populations. $CD4^+$ T follicular helper cells (Tfh) have been shown to express high levels of IL-21 and function to bridge pathogen-induced effector T cell responses and humoral immunity (R. I. Nurieva, et al., 2008; Vogelzang, et al., 2008). In addition to expressing CXCR5, PD-1, BTLA, ICOS, and Bcl-6, recent findings indicate that Tfh cells are enriched in $CD4^+CXCR5^{hi}$ cells expressing both IL-2 and IL-21 (D. Yu, et al., 2009). This raises the possibility that *Il21* and *Il2* expression are coordinately regulated within this population. Alternatively, IL-21 signaling may stimulate proliferation of IL-2-producing cells (D. Yu, et al., 2009). The association of IL-21 production and an increased frequency of IL-2-producing cells have been documented in tumor animal models that evaluate the induction of CD8 responses as a result of viral infection. Injection of a B16 cell line engineered to produce IL-21 into a model of murine melanoma was performed to analyze the anti-tumor activity of this

cytokine. In addition to a regression in tumor size and a decrease in accumulation of Foxp3$^+$ Treg populations, increased local levels of IL-21 cytokine also enhanced tumor antigen-specific T cell responses and increased the percentage of IL-2-producing CD4 and CD8 T cells (Kim-Schulze, et al., 2009). A recent study also showed in a model of LCMV infection that IL-21-deficiency was associated with a decline in the frequency of IL-2-producing antigen-specific CD8 T cells. These results further solidified a role for CD4-generated IL-21 in CD8 cytotoxic T cell-mediated antiviral activity (Yi, et al., 2009). Moreover, our studies using the *Il2*GFP knock-in model indicate that addition of exogenous IL-21 enhances the activation of reporter expression and proliferation of naïve CD4$^+$ T cells during *in vitro* activation (Chapter 2-Figures 1,5). Taken together, these findings suggest a synergistic relationship between the presence of IL-21 and the proportion of IL-2-expressing cells both in *in vitro* and *in vivo* models of infection and disease. Future studies evaluating the role of IL-21 signaling on expansion of IL-2-producing T cells will be essential for understanding the role of these cytokines in the primary effector response and development of memory T cell populations. Our *Il2* reporter models provide us with new tools to differentiate IL-2-producing populations from non-producers after primary activation *in vitro* or *in vivo*. Assaying the long-term effect of IL-21 signaling on IL-2$^+$ effector T cells will permit analysis of subsequent effects on survival and long-term fate of effector populations as well as production of memory T cell responses. Since *Il2* expression during the primary effector response has been shown to be critical for memory T cell responses through upregulation of the IL-7 receptor, isolation and tracking the fate of IL-2-producers will bring new insights into the role of IL-7 receptor on memory T cell development(Dooms, et al., 2007; Z., et al.,

2000). Thus, with the use of our novel *Il2* reporter systems, we can potentially answer many biological relevant questions regarding effector immunity and memory T cell responses.

IL-2 and IL-21 signaling are integral to the reciprocal regulation of pro-inflammatory responses and immune tolerance through Treg maintenance. Development of our *Il2* reporter models allows us the unique opportunity to address the relationship of IL-2 expression and signaling in the context of T helper 17 and Treg reciprocal regulation *in vivo*. A representative model of this reciprocal relationship in the Th17 and Treg lineages can be seen in models of cancer. Increased frequencies of Treg populations are present in tumor microenvironments and contribute to the inability of tumor antigen-specific T effector cells to facilitate tumor regression (Curiel, et al., 2004; Sakaguchi, et al., 2001; Spiotto & Schreiber, 2005; Turk, et al., 2004; Zou, 2005, 2006). One way to address the role of IL-2 and IL-21 production in the counter-balance of Th17 and Treg populations would be to evaluate the IL-21-induced synergy of IL-2-producing populations in cancer models. This would also provide a more in-depth mechanistic understanding as to the benefits of combined IL-21 and IL-2 therapy seen in murine melanoma models (He, et al., 2006). The use of *in vivo* cancer models such as the B16 melanoma murine model provides a platform with which to evaluate the effect of *Il2* reporter positive cells and the effect of IL-2 signaling resulting from their IL-2 production on T cell lineage commitment in the context of tumor regression or exacerbation (Kryczek, et al., 2007). Transferring previously activated *Il2*-positive cells into a tumor microenvironment allows us to analyze the effects of IL-2 production on tumor reduction by monitoring changes in the ratio of CD8:CD4 Tregs to favor CD8

cytotoxic T cell help (Spiotto & Schreiber, 2005). Alternately, IL-2 produced from these transferred cells may result in further Treg maintenance and differentiation thereby exacerbating tumor progression. Similar experiments could be performed in models of autoimmunity where pro-inflammatory signals prevail among diminished Treg responses (Rennick & Fort, 2000). Since IL-2 has been shown to suppress Th17 differentiation (Laurence, et al., 2007), we would predict that transfer of IL-2 producing cells into a model of autoimmunity might shift a predominantly pro-inflammatory biased population toward a propensity of Treg development and function thereby regaining immune homeostasis in these systems. Thus, in addition to addressing questions of IL-2-producers and effector cell fate in primary and recall responses, our new *Il2* reporter models enable us to evaluate the role of IL-2 in the balance of effector immunity and tolerance.

The *Il2* and *Il21* genes are encoded on chromosome 4 in humans and chromosome 3 in mice and have been termed a "looped domain" possibly arising from a gene duplication event (Brandt, Singh, Bulfone-Paus, & Ruckert, 2007; Parrish-Novak, et al., 2000). The *Il2* gene is positioned downstream and directly flanking the *Il21* gene (Bird, et al., 2005). Since *Il2* and *Il21* share intergenic regulatory sequence this raises the possibility that specific intervening sequences may jointly regulate both gene loci. The presence of T cell populations that show dual production of both IL-2 and IL-21 suggest coordinate regulation in these populations (D. Yu, et al., 2009). Alternatively, several studies indicate that a lack of synchrony in expression of the two cytokines may result in a general autoimmune risk (Zhernakova, et al., 2007). The *Il2* and *Il21* gene loci are included within the *Idd3* locus on human 4q27 in addition to *KIAA1109* and the *Tenr*

genes, a susceptibility locus genetically linked to several autoimmune conditions (M. S. Anderson & Bluestone, 2005). Studies using the NOD mouse model, a model of type I diabetes (TID), showed an increased production in IL-21 and an increased frequency of IL-21 receptor expression on T lymphocyte populations (King, et al., 2004). Additional studies using the same NOD mouse model showed a decreased proportion of Treg populations in association with autoimmunity as well as decreased IL-2 and increased IL-21 transcript levels(Yamanouchi, et al., 2007). Collectively, this suggests a discord the in the regulation of expression from the *Il2* and *Il21* loci during a disruption in immune tolerance. In addition to Type I diabetes, additional supporting evidence shows a link between the *Idd3* locus and celiac disease, psoriasis, rheumatoid arthritis, and ulcerative colitis (UC) (Adamovic, et al., 2008; M. S. Anderson & Bluestone, 2005; Glas, et al., 2009; Hunt, et al., 2008; King, et al., 2004; Y. Liu, et al., 2008; van Heel, et al., 2007; Yamanouchi, et al., 2007; Zhernakova, et al., 2007). Furthermore, genome-wide analysis shows the presence of highly relevant SNPs within the *Il2/Il21* intergenic sequence associated with celiac disease (van Heel, et al., 2007). Thus, genome-wide association studies that find linkage of the 4q27 region to several autoimmune conditions strongly suggest integrity of critical intergenic regulatory sequences between the *Il2* and *Il21* loci are necessary to maintain immune tolerance. In addition, a discord in the regulation of these two genes in a murine model of type I diabetes that also shows linkage to Idd3 locus SNPs implicates the presence of cis-regulatory sequences common or unique to both *Il2* and *Il21* that maintain joint regulation of this locus in a lineage-specific manner during immune homeostasis. Both the 2BiT and *Il2*GFP knock-in models maintain the potential for re-targeting of intergenic regulatory elements that may regulate expression

patterns specific to the conjoined locus in a T cell context-dependent manner. Due to the ease of manipulation, the 2BiT transgene may also serve as a platform to not only re-target putative intergenic regulatory sequences but also can used to generate an *Il2/Il21* dual transgenic model due to the presence of the *Il21* gene in its entirety. Dual reporting of this transgene would allow for detection of conditions and lineages that express *Il2* and *Il21* in a coordinate manner and those that show more of a discord. It would also enable us to detect aberrant regulation of the joint loci under conditions of autoimmunity. In light of existing data, discordant regulation of the *Il2* and *Il21* gene may result in a tilting of the immune response toward pro-inflammatory and autoreactive potential. A loss of *Il2* expression may generate a decreased frequency of Treg populations some of which could be defined as ex-Tregs derived from $Foxp3^+$ Tregs that have lost Treg supportive signals and have decreased Foxp3 expression. Given the preponderance of the Treg population for self-antigen specificity it remains feasible that ex-Tregs, given the appropriate antigen stimulus and cytokine environment, may become autoreactive (Zhou, et al., 2009). Tfh cells present an ideal population with which to study coordinate regulation of *Il2* and *Il21* due to the presence of cells that express both IL-21 and IL-2 (D. Yu, et al., 2009). In addition, this population functions at a critical intermediary position linking signals from effector T cells that have undergone antigen activation and B cell responses that induce isotype antibody switching. This may poise the Tfh population to play a crucial role in autoantibody production and activation of autoreactive T cells. The use of a dual reporter will allow us to monitor the contribution of aberrant *Il2* and *Il21* gene expression in these autoimmune-prone conditions. Collectively, the 2BiT and *Il2* GFP knock-in reporter models are valuable tools that would enable identification of

uncharacterized transcription factor binding sites involved in the regulation of *Il2* and *Il21* gene expression.

Importance of *Il2* Gene Expression and Locus Accessibility

In addition to surveying intergenic sequences for regulatory potential our reporter platform affords the ability to evaluate the contribution of regulatory elements contained within the *Il2* locus alone. Several transcription factor binding sites have been classified within a minimal *Il2* promoter, which are critical for transcriptional activation (Jain, et al., 1995). However, additional studies using transgenic mouse models with either a 2.0 kb or 8.4 kb *Il2* promoter sequence driving transcriptional regulation of a GFP reporter indicate that distal regulatory elements apart from the previously characterized proximal promoter are necessary for lineage-specific *Il2* gene expression *in vivo* (Yui, et al., 2001; Yui, et al., 2004). This emphasizes that distal sequences within the *Il2* locus may have critical locus control activity. Furthermore, using sequencing alignment tools, analysis of sequence conservation of the *Il2* locus across several species indicates the presence of several candidate conserved non-coding sequences that may represent important transcription factor binding sequences necessary for locus regulation. Conserved non-coding sequences are at least 100 bp in length and show 70% sequence conservation in cross-species sequence comparisons (Dermitzakis, Reymond, & Antonarakis, 2005). Their high degree of conservation throughout evolution implicates potential importance of these sequence regions in locus regulation. It is important to consider that conservation of non-coding elements does not, however, guarantee regulatory or

transcription factor binding potential. Yet, *in vitro* transcription factor binding assays in addition to promoter-cassette-reporter *in vitro* expression studies utilizing transfection of reporter plasmids containing *Il2* promoter sequence fused with potential regulatory sequence cassettes can quickly provide informative results that would allow for *in vivo* targeting of these sites within the context of a transgenic or knock-in model (Hatton, et al., 2006). Thus, gene expression studies on candidate regulatory sequence within the extended *Il2* locus will provide necessary insight into critical locus control regions and their partner transcription factors that ensure appropriate locus control.

Prior to antigen exposure, the *Il2* locus within naïve T cells displays limited accessibility as determined by studies evaluating chromatin architecture (Acuto, Mise-Omata, Mangino, & Michel, 2003; Fehervari, Yamaguchi, & Sakaguchi, 2006; Malek & Bayer, 2004; Nelson, 2004; Schimpl, et al., 2002). Changes in chromatin accessibility of the locus precede transcriptional initiation. These modifications in the chromatin state of the *Il2* gene locus are necessary for accessibility of inducible transcription factors as well as general transcription factor machinery (Barton & Crowe, 2001). Several studies indicate that antigen exposure of naïve CD4 T cells induces rapid accessibility of the *Il2* proximal promoter in regions where several transcription factor binding site have been well documented (Rao, et al., 2003; Rao, et al., 2001; Rothenberg & Ward, 1996; Schimpl, et al., 2002; Siebenlist, et al., 1986; Ward, et al., 1998). Chromatin remodeling within this *Il2* promoter region has also been shown to require binding of c-rel, a NF-κB transcription factor family member, to the CD28 response element within the proximal promoter region (Rao, et al., 2003). The state of local chromatin architecture can have a profound influence on accessibility of transcription factor machinery and gene expression

(Barton & Crowe, 2001). Locus control regions and boundary sequence elements within gene loci are critical for maintaining lineage-specific control of gene expression and enabling gene transcription independent of position variegation effects from neighboring chromatin influences, respectively (Maston, Evans, & Green, 2006). Furthermore, appropriate activation signals that interact with these sites can facilitate changes in chromatin accessibility (Maston, et al., 2006). The implication of distal regulatory sequences in lineage control of *Il2* gene expression raises the possibility that key sequences within the extended *Il2* locus may act as vital locus control regions and boundary elements (Yui, et al., 2001; Yui, et al., 2004). A more in-depth understanding as to the identity and function of these sequence elements also has significant implications in defining the locus changes that accompany memory T cell development. Memory CD4 T cells have been shown to undergo a more robust activation of *Il2* gene expression which is associated with an "opening" of chromatin domains including release of a nucleosome normally positioned within the proximal promoter (McKarns & Schwartz, 2008). Furthermore, this study shows differences in the activation requirements for *Il2* expression in naïve CD4 T cells as opposed to memory CD4 T cells. These results are in support of previous observations that show a progression toward a lower threshold of activation and lack of co-stimulation requirement for *Il2* expression in memory CD4 T cells as compared to naïve T cells (Dutton, Bradley, & Swain, 1998). The mechanism by which the *Il2* locus undergoes evolution of chromatin landscape resulting in greater competency for gene expression during peripheral lymphoid maturation still remains unknown. It is likely that several additional transcription factors and co-activators facilitate this locus transition. Thus the question remains as to the

identity of these additional factors and the mechanism by which they establish initiation and maintenance of chromatin remodeling of the *Il2* locus. In addition, chromatin remodeling events thus far have only been characterized within the proximal region of the *Il2* locus. Chromatin remodeling events that occur within other regions of the *Il2* locus that are critical for gene expression in a lineage-specific manner await investigation. Taken together, future studies delineating the regulatory sequences critical for transcription factor binding and chromatin remodeling will ultimately provide significantly greater insight into the mechanism of *Il2* locus control.

Intrinsic T Cell Differences In *Il2* Expression

Locus regulation of *Il2* accessibility may also be implicated in the control of the frequency with which antigen specific T cells undergo complete activation into an effector-like state. Activation of *Il2* GFP antigen-specific CD4 T cells shows that a large proportion of the cells express activation markers, however a minority of the cells initiate *Il2* gene transcription (Saparov, et al., 1999). These results suggested that either inherent T cell differences were responsible for overcoming a threshold of activation or that variations within the antigen presenting cell population attributed to the under representation of IL-2 producing cells upon antigen exposure. Further studies distinguishing these possibilities showed that differences in *Il2* expression from a clonal CD4 T cell population were not attributed to variations in antigen presenting cell populations (V., et al., 2003). These findings suggested that even within a clonal population of T cells there exists an intrinsic variation in *Il2* expression potential. A

recent study evaluating changes in chromatin architecture of the *Il2* locus in memory T cells indicated that stable chromatin remodeling alterations in this CD4 T cell population associated with more rapid production of IL-2 compared to naïve populations (McKarns & Schwartz, 2008). This and other studies showing tight regulation of *Il2* mRNA and protein production together suggest that collective changes in chromatin structure affect the propensity for *Il2* expression and subsequent protein secretion (Bruniquel & Schwartz, 2003). Antigen activation involves signaling through the TCR, which induces rapid transcriptional upregulation of *Il2* gene expression. During T cell activation, TCR signaling events are accompanied by co-stimulatory signals through the CD28 surface molecule from contacts made with B7 molecules on antigen presenting cells. Combined TCR signaling with CD28 co-stimulation signals leads to initial activation of effector T cells (Acuto & Michel, 2003). However, a threshold of activation must be overcome in order to achieve *Il2* production and effector status (Iezzi, Karjalainen, & Lanzavecchia, 1998; Saparov, et al., 1999). These studies together with the required changes in chromatin remodeling of the *Il2* locus suggests that a threshold of antigen activation corresponds to sufficient changes within the *Il2* locus to allow for accessibility of promoter elements and gene expression necessary for T cell expansion. Variable capability of T cells within a clonal population to express *Il2* under conditions of sufficient antigen exposure may result from differences in prior antigen exposure of individual T cells (V., et al., 2003). T cells are exposed to antigen during thymic education as well as during routine trafficking through the peripheral lymphoid tissues. Cumulative TCR signals from these antigen encounters may result in incremental changes chromatin structure and locus accessibility. These gradual changes may

ultimately reduce the threshold of activation for a particular T cell clone resulting in a more competent gene locus for future antigen exposure. A simple assay to perform to address the question of intrinsic T cell variation resulting from differences in *Il2* locus accessibility would be to compare clonal populations of CD4 T cells that have experienced varying degrees of antigen exposure and assay their ability to induce reporter expression upon antigen exposure. Both the 2BiT and the *Il2* GFP knock-in reporter models would be useful tools in addressing this question. In addition to comparisons of reporter expression between populations of differential antigen exposure, general locus accessibility of resting and activated populations within these groups could reveal important insights into the role of *Il2* locus accessibility in responsiveness to T cell effector immunity and recall responses. Taken together, intrinsic T cell differences determine the ability of a clonal T cell to overcome the threshold of activation. Studies determining the role of differences in chromatin architecture at the *Il2* locus may provide mechanistic insight to the nature of this population variability. Furthermore, these inherent differences in *Il2* competence may be an essential feature to population control of T cell responses and immune homeostasis ensuring that a subset of the total population can generate an effector response to a threshold level of antigen at any given time but that there is always a reservoir of naïve T cells "on deck" being incrementally triggered by small doses of antigen to achieve "readiness" for future pathogenic insults. Studies evaluating the contribution of intrinsic differences to this population phenomenon will be informative for a greater understanding into the role of gene locus accessibility to T cell education and maturation and ultimately to the immune response. Furthermore these studies could demonstrate how the immune response is orchestrated from a pool of naïve

T cells and the importance of immune surveillance in facilitating *Il2* locus maturation and T cell responsiveness.

Future Implications For Studying IL-2 Production In Innate Immune Populations

It is well established that antigen-activated CD4 and CD8 T cells are the primary contributors to IL-2 production. However, additional cellular components of the immune response have been shown to express IL-2 such as DCs, NK cells, and NKT cells (Malek, 2008). Specifically, gene array analysis of bacterial-activated dendritic cells indicated IL-2 transcript induction. Furthermore, IL-2 produced from activated DCs was able to induce T cell activation as shown by induction of CD4 and CD8 T cell proliferation (Granucci, et al., 2001). Thus, this study indicated an ability of DCs in the activation of acquired immunity through IL-2 production. In addition, similar to activated T cells, LPS activated DCs showed an upregulation of CD25, the IL-2 receptor α subunit, on their surface. Moreover, inhibition of DC CD25 molecules with blocking antibody showed a disruption in the ability of DCs to mediate T cell proliferative responses (Mnasria, Lagaraine, Velge-Roussel, Lebranchu, & Baron, 2009). This suggested that IL-2 autocrine signaling in DC populations plays a role in DC-mediated T cell proliferation. Moreover, these studies emphasize the role of DC-mediated IL-2 production in bridging activation of adaptive cellular components with pathogen-induced innate mediators. In addition to cell mediators of the adaptive response, bacteria-primed DC production of IL-2 also mediated NK activation (Granucci 2004). Collectively, these studies suggest a role for IL-2 production by DCs in regulating both innate and acquired immunity. A key

question raised by these studies is the contribution to DC-derived IL-2 production to the initiation of effector and memory responses to bacterial infection. In addition, what role does IL-2 generated from innate immune components have on *Il2* locus priming in CD4 T cells in the context of infection? Interestingly, DC-induced IL-2 production was generated only in the presence of microbial products. Addition of inflammatory cytokines failed to lead to IL-2 production indicating DCs retain the capacity to differentiate between active bacterial infection and local inflammatory signals (Granucci, Feau, Angeli, Trottein, & Ricciardi-Castagnoli, 2003). This raises the question as to whether increased local concentrations of IL-2 as a result of bacterial-mediated DC production allows neighboring T cells to respond more readily by lowering the threshold of activation (Granucci, et al., 2003). Furthermore, NK cells have been shown to produce IL-2 (Malek, 2008). We could also address the role of IL-2 production from NK cells as well as additional innate immune components in signaling adaptive cell mediators. Determining the whether NK-mediated production of IL-2 is strictly for mediating viral infection and activation of acquired immunity would provide further insights into the functional compartmentalization of innate cells and pathogen-specific responses. The biological importance of these questions in the future holds significant value to our understanding of how innate immunity prompts acquired immune responses in the context of infection.

The Role of IL-2 In Activation Induced Cell Death (AICD)

Finally, the role of IL-2 signaling in activation induced cell death (AICD) still remains elusive. T cells that have undergone extensive TCR signaling have been shown to facilitate AICD in the presence of IL-2 signaling through upregulation of FasL (Lenardo, 1991; Malek, et al., 2001). We could address the question of IL-2 contribution to AICD in a target population of T cells by comparing the degree of AICD in co-cultures containing targets T cells with congenically marked activated IL-2-producers or activated IL-2-non-producers. Thus, if IL-2 signaling plays a role in AICD of activated T cells we would predict that the presence of IL-2-producers would induce more cell death in an antigen-activated target T cell population undergoing excessive TCR stimulation. The findings that IL-2 signaling can cooperate with TCR activation to induce cell death suggests a mechanism for T cell contraction in the presence of activated T cell populations beyond the requirement for antigen clearance. AICD studies would allow us to gain further insight as to the mechanisms of antigen activation and T cell homeostasis. Thus, the generation and characterization of the 2BiT and *Il2* GFP knock-in mouse models provides novel and reliable tools of detection of from which to study the role of *Il2* expression and signaling on the balance of effector responses and T cell tolerance.

SUMMARY

Overview

In order to maintain immune homeostasis, both effector immunity and tolerance mechanisms must uphold appropriate counter-regulatory measures. A compromise in the balance of these key arms of immunity can give rise to debilitating conditions and in many cases prove to be life threatening such as is the case with autoimmunity and cancer. A clear understanding of the mechanisms and mediators crucial for driving antigen-specific effector events sufficiently while retaining the capability to bring equilibrium to inflammatory responses are essential for developing effective life-saving and life-altering therapeutic strategies to individuals inflicted with diseases of immune dysregulation. The cytokine IL-2 is a soluble factor, produced primarily by activated T cells, shown to play an integral role in mediating effector-tolerance decisions. Originally characterized based on its T cell growth-promoting properties *in vitro*, gene disruption studies have since shown that *Il2* expression is strongly implicated in the maintenance of immune tolerance by regulating the generation of vital T regulatory cell populations *in vivo*. In addition, *Il2* expression during the primary effector phase of T cell activation contributes to memory responses critical for immune clearance during subsequent rounds of antigen exposure. Thus, the ability to effectively monitor *Il2* gene expression during several states of immune activation is ultimately necessary to gain a further understanding of the role of this vital mediator in immune tolerance and effector immunity.

Primary Question

Il2 gene expression and protein production are initiated rapidly in activated T cells and display rapid turnover and short-lived kinetics following antigen exposure. This feature of *Il2* expression imposes severe limitations on the detection and effector fate tracking of IL-2-producing cells during an immune response using conventional methodology. Thus, generation of reporting tools capable of simulating *Il2* expression patterns have been necessary to more effectively address questions of the importance of IL-2 production and the immune cells from which it is produced to immune regulation. Prior to this work several murine reporter models were generated in an effort to track changes in *Il2* gene expression *in vivo*. However, there are several limitations inherent to the design and reporter product expression of the existing models that prevent lineage-specific expression of reporter product with fidelity to endogenous *Il2* regulation. In addition, many of these models contain limited endogenous regulatory sequence and thus are insufficient to serve as a platform from which to modify extended regulatory elements for questions relating to gene expression and locus control. Furthermore, attempts to generate reporters by targeting endogenous loci not only incorporate non-native regulatory elements but also result in deletion of a single *Il2* allele preventing bi-allelic expression. Therefore, there remains a pressing need for reliable reporter systems that maintain locus control through the use of extended endogenous regulatory sequence and minimal perturbation to the native locus.

Major Findings

In order to address the question of generating reliable mouse *Il2* reporter tools in this work we describe the generation and characterization of two novel murine systems to

monitor *Il2* expression. We first describe the generation of an *Il2* BAC transenic (2BiT) model that contains 200 kb BAC transgene of endogenous regulatory sequence driving the expression of a Thy1.1-SV40pA reporter. This study shows that the 2BiT model reports with stability and fidelity to endogenous *Il2* regulation during conventional T cell activation. In addition, this model indicates a mechanism of Treg suppression involving suppression of CD4 responder T cell activation and *Il2* expression, which results in an increase in non-IL-2-producing responder CD4 T cell death. Furthermore, under the conditions of the existing study, analysis with the 2BiT model implicates a mechanism of competition for co-stimulation between CD4 T responder cells and Tregs rather than IL-2 cytokine-deprivation suggesting Tregs limit access of CD4 responder T cells to optimal activation signals. Future studies will be necessary to delineate the impact of Tregs on limiting a threshold of activation of naïve CD4 responder T cells. Alternatively, this model is an ideal platform from which to generate further modifications to evaluate locus regulation due to the presence of an extended endogenous regulatory sequence contained within the transgene. The design of the 2BiT transgene leaves the endogenous *Il2* locus unperturbed thereby avoiding potential phenotypic changes due to disruption of endogenous regulatory sequence. Further modifications of regulatory sequence elements of interest within this additional *Il2* locus will provide an innovative tool for addressing questions of *Il2* gene regulation during the immune response. Taken together, this study describes the generation of a reliable transgenic reporting tool, which enables the opportunity to evaluate many questions regarding the role of *Il2* gene expression in both effector immunity and tolerogenic responses within the context of an extended locus region. An *Il2* GFP knock-in was developed in a second study in order to generate a

knock-in reporter model with minimal disturbance to the endogenous sequence while utilizing all native regulatory regions for control of reporter expression. Our findings from this study indicate coordinate regulation of the GFP reporter to endogenous *Il2* gene regulation. In addition, our *Il2* GFP model is responsive to cytokine signaling from IL-21 showing an increased frequency of IL-2-producers demonstrating the effector-enhancing potential of this cytokine on T cell immunity. Thus, from this study we describe the generation of the first *Il2* knock-in model that shows reporter expression with fidelity to endogenous gene patterns using all *Il2* native regulatory elements and with minimal perturbation to the locus. Furthermore, we show that the GFP reporter is capable of responding to additional cytokine signals differentially from T cell receptor activation.

Potential Applications

Collectively, we describe the generation of two highly reliable *Il2* reporting tools that will enable an expanding list of future studies to evaluate the integral role of IL-2 immune homeostasis. An understanding of the mechanisms that dictate effector-to-memory differentiation will be vital for answering questions related to host immunity and response to infection. The 2BiT and *Il2* GFP reporter models provide powerful tools to study T cell effector fate determination and function arising from IL-2-producing populations. In addition, the 2BiT transgene stably marks IL-2-producing cells making it a highly sensitive model to evaluate infrequent populations that generate IL-2 such as memory T cells. Deciphering the inherent differences within a clonal T cell population that give rise to only a subset of IL-2-producers that overcome a threshold of activation will be essential to understanding the decisions and mechanisms involved in T cell population dynamics and commitment to an effector fate during antigen activation

necessary for immune homeostasis. Our reporter systems will allow us to address these questions readily by analyzing T cell populations that show variable potentials for IL-2-production based on the degree of antigen experience. In addition, differences in *Il2* locus accessibility due to the degree of antigen exposure have been predicted to account for variations in IL-2-responsiveness. T cell populations defined as producers or non-producers of IL-2 could be evaluated for differences in accessibility due to antigen exposure in addition. These studies and others would also allow for a further understanding into the mechanisms that control *Il2* locus regulation. Several recent findings implicate the intergenic region between *Il2* and *Il21* within the Idd3 locus as a general autoimmune risk locus. In addition, recent findings also implicate dysregulation of the *Il2* and *Il21* genes in models of autoimmunity. Utilization of the 2BiT and *Il2* GFP reporter models will enable the analysis of differences within the *Il2*-*Il21* "looped domain" for sequence elements that may co-regulate both gene loci. Finally, IL-2 suppression in conventional T cells is a hallmark measure of Treg suppression *in vitro.* The use of our transgenic and knock-in reporters to develop useful tolerogenic models *in vivo* will contribute to understanding immune tolerance mechanisms.

Taken together, the function of IL-2 is central to balancing the necessity to undergo active inflammation while preventing responsiveness to self-antigen. Further examination of the counter-regulatory mechanisms required to stabilize these opposing arms of immunity will be essential in the discovery of new therapeutic targets and strategies of several debilitating diseases. The application of novel and reliable *Il2*-reporter systems will undoubtedly provide valuable tools necessary to confidently address

many of these questions and gain a further understanding of the vital role of IL-2 in immune regulation.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: September 17, 2008

TO: Casey T. Weaver, M.D.
BBRB-870 2170
FAX: 975-8310

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Post-Thymic T Cell Functional Heterogencity
Sponsor: NIH
Animal Project Number: 080907829

On September 17, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	250
Mice	A	1000

Animal use is scheduled for review one year from September 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080907829 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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