Determination of Transforming Growth Factor-β Signaling Targets in Activated T Cells

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LOYOLA UNIVERSITY CHICAGO

DETERMINATION OF TRANSFORMING GROWTH FACTOR-β TYPE I RECEPTOR
(TGF-βRI) SIGNALING TARGETS IN ACTIVATED T CELLS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY
RAMIAH D. JACKS
CHICAGO, ILLINOIS
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ABSTRACT

T lymphocytes are a critical component of the adaptive immune system and their responses in pathogenic and steady state conditions require strict regulation. One mechanism involved in this regulation is Transforming Growth Factor-β (TGF-β) signaling. TGF-β can act on activated T cells to facilitate the differentiation of TGF-β dependent T helper subsets or the suppression of T cell activation and proliferation. However, the molecular mechanism(s) by which TGF-β signaling controls T cell differentiation vs T cell suppression remain poorly understood. TGF-β signaling is propagated by initially binding to the TGF-β type II receptor, which subsequently activates TGF-β type I receptor (TGF-βRI) that phosphorylates the transcription factors Smad2/Smad3. This induces nuclear localization of Smad complexes and transcription of target genes. To understand how TGF-β signaling functions to control T cell fate, we generated TGF-βRI deficient Jurkat T cells using the CRISPR/cas9 genome editing system. Unexpectedly, TGF-βRI deficient Jurkat demonstrate a hyperactivation phenotype and showed highly elevated expression of surface antigens (CD69, CD25), and secreted significantly increased levels of IL-2 and TNF. Analysis of signaling events in TGF-βRI deficient Jurkat reveal a highly elevated and constitutive activity of the critical transcription factor, activator protein-1 (AP-1), constitutive activation of the upstream kinase for AP-1, jun-amino-terminal kinase (JNK), and increased expression of the transcriptional target for c-Jun, JUN. Similarly, in primary T cells, transient expression of a dominant negative mutant of TGF-βRI revealed elevated AP-1 activity,
while a constitutively active TGF-βRI mutant reduced this activity. Further, the upstream kinase for JNK, MKK7, and critical scaffold proteins upstream of JNK activation, CARMA1 and Bcl10, are upregulated in the TGF-βRI deficient Jurkat. Correspondingly, primary T cells stimulated in the presence of TGF-β exhibit reduced levels of CARMA1 and Bcl10 expression.

Taken together, these data describe a novel TGF-β-mediated pathway that functions to suppress the activation of JNK by suppressing the CARMA1/Bcl10 signalosome. Because JNK signaling is involved in T cell activation, differentiation and apoptosis, the suppression of JNK activation by TGF-β signaling could serve as a mechanism used to control T cell functions.
CHAPTER ONE

REVIEW OF LITERATURE

T lymphopoiesis

T cell lymphopoiesis, or the process by which T lymphocytes develop from progenitor cells, occurs in a sequential manner that involves migration of progenitors to the thymus for maturation, selection and export to the periphery. T lymphocyte (or T cell) progenitors arise from bone marrow derived hematopoietic stem cells (HSCs). Although the thymus is the site of T cell development, it has been shown that the thymus does not provide a suitable environment for self-renewal of bone marrow progenitors (Goldschneider et al. 1986). Therefore, to maintain T lymphopoiesis throughout adult life, seeding of the thymus with bone marrow progenitors is required. HSCs are multipotent and therefore have the capacity to become an erythrocyte, megakaryocytic, macrophage, neutrophil, eosinophil, basophil, mast cell, conventional dendritic cell, natural killer cell, B cell or a T cell (Heinzel et al. 2007). HSCs with T cell potential first give rise to multipotent progenitors (MPPs), which give rise to lymphoid primed MPPs (LMPPs) which further give rise to common lymphoid progenitors (CLPs) (Kondo et al. 1997; Adolfsson et al. 2005). LMPPs expressing high levels of the cytokine receptor fms-like tyrosine kinase 3 (Flt3) have been shown to exit the bone marrow and home to the thymus (via the blood) due to a variety of cytokines, adhesion molecules and chemoattractants (Krueger et al. 2010; Schwarz et al. 2007). Once in they thymus, seeded progenitors give rise to early thymic progenitors (ETPs), which are
the earliest known and most efficient intrathymic T cell progenitor. ETPs are known to express high levels of the transmembrane receptor tyrosine kinase, c-Kit, and reside within the Double Negative 1 (DN1) subset in the thymus (Matsuzaki et al. 1993; Porritt et al. 2004). The DN1 subset is the most immature progenitor and is phenotypically defined as CD4⁻ CD8⁻ CD3⁻ CD44⁺ CD25⁻ , with 'DN' corresponding to a lack of expression of the co-receptors CD4 and CD8, and is known to possess multilineage potential (Shimonkevitz et al. 1987; Pearse et al. 1989)(Figure 1). T cell specification of cells within the DN1 subset requires Notch signaling, which not only promotes a T cell lineage, but also limits other alternative fates. Notch signaling accomplishes this by activating several transcription factors such as T cell factor-1 (TCF-1), GATA3 and Hes1. Collectively, these transcription factors function to promote T cell lineage commitment (Pui et al. 1999; Radtke et al. 1999). After this specification to the T cell lineage, cells within the DN1 subset acquire the expression of CD25 (high affinity IL-2 receptor α-chain) and are now classified as DN2 cells which are phenotypically defined as CD3⁻ CD44⁺ CD25⁺ (Figure 1). Pre-committed DN2 cells migrate to the cortex in the thymus and initiate T cell receptor (TCR)-β, TCR-γ and TCR-δ gene rearrangements. Full commitment to the T cell lineage occurs after the DN2 stage to DN3 stage transition. DN3 cells (CD44⁻ CD25⁺) have successfully formed the pre-TCR complex, which is comprised of a rearranged TCR-β-chain in association with an invariant pre-TCRα–chain and CD3 signaling molecules (for TCRαβ T cells), or have successfully rearranged TCR-γ and TCR-δ genes and express the complete TCRγδ receptor (for TCRγδ T cells)(Hoffman et al. 1996; Taghon et al. 2006)(Figure 1). Expression of the complete TCRγδ receptor allows for a burst in proliferation and functional maturation,
without having to progress through any further stages (Prinz et al. 2006). Conversely, expression of the pre-TCR complex rescues cells from apoptosis, enforces tcrb allelic exclusion and increases proliferation, while simultaneously silencing tcrg expression to allow for progression to the αβ lineage (Hager-Theodorides et al. 2007; Falk et al. 2001; Fehling et al. 1995). DN3 cells in the αβ lineage that have successfully expressed a functional TCR β-and pre-TCRα–chain, a process termed β-selection, may proceed to the DN4 stage (CD44 CD25\(^-\)) (Falk et al. 2001; Hager-Theodorides et al. 2007)(Figure1). The DN4 stage to the double positive (DP) stage transition occurs in the subcapsular region of the thymus and involves an upregulation of both CD4 and CD8 and is therefore phenotypically defined as CD4\(^+\) CD8\(^+\) CD3\(^+\) CD44\(^-\) CD25\(^-\) (Figure 1). Cells at the DP stage have successfully rearranged their TCR-α-chain and express the complete TCRαβ.

TCR expression is essential for T cell activation, as T cell activation occurs via antigen recognition in the context of major histocompatibility complex (MHC) by the TCR (Haskins et al. 1983; Reinherz et al. 1982). Additionally, the TCR dictates which antigen(s) will elicit a response by the T cell. These antigens could be derived from foreign pathogens or naturally expressed by the cells in our body (self-antigens). Recognition of foreign pathogens by T cells is advantageous and a critical component of our adaptive immune response. However, recognition of self-antigens by T cells could result in autoimmune diseases. Therefore, cells at the DP stage undergo processes to ensure that the TCR on each T cell can recognize antigens in the context of the MHC molecule while simultaneously eliminating cells that express a TCR that binds too strongly to self-antigens. These processes are termed positive and negative selection,
respectively.

**Figure 1. Steps in T lymphopoiesis.** T cell lymphopoiesis takes place in a series of steps in the thymus. Blood borne T cell precursors give rise to early thymic progenitors (ETPs). Next, a series of sequential double negative (DN) stages arise; with T cell lineage commitment occurring at the DN2 to DN3 transition. Complete acquisition of functional T cell receptor αβ (TCRαβ) and expression of the co-receptors CD4 and CD8 occurs at the DN4 to double positive (DP) transition. Finally, DP cells undergo positive and negative selection before exiting the thymus into the periphery as mature naïve single positive (SP) CD4 or SP CD8 T cells. Modified from (Fayard et al. 2010).

**Positive and Negative selection**

The goal of positive selection is to positively select for thymocytes that express TCRs that are able to bind self-MHC molecules. This is essential for future recognition of antigens from foreign pathogens in the context of self-MHC. Thymocytes that do not interact with self-MHC molecules undergo apoptosis from neglect (Mandal et al. 2008; Aifantis et al. 2006). Positive selection occurs in the cortex of the thymus. In the cortex, cortical thymic epithelial cells (cTECs) mediate positive selection by expressing MHC class I or MHC class II with a self peptide. These self peptides are known to be
relatively weak and induce a low level of TCR signaling that rescues thymocytes from death but does not induce TCR-mediated apoptosis (Hogquist et al. 1994). DP thymocytes that recognize MHC class I in the context of self-peptide differentiate into CD4^+CD8^- Single Positive (SP) T cells. Conversely, DP thymocytes that recognize MHC class II in the context of self-peptide differentiate into CD4^-CD8^+ SP T cells (Konig et al. 1992)(Figure 1).

The goal of negative selection is to remove thymocytes whose TCR strongly recognizes self-MHC in the context of self-peptide. Selecting for thymocytes with a low-affinity TCR for self-MHC and self-peptides is critical for establishing tolerance to self (Kappler et al. 1987). Negative selection takes place in the medulla and is mediated by medullary thymic epithelial cells (mTECs) and medullary dendritic cells (DCs). SP thymocytes migrate to the medulla and encounter mTECs and DCs. These antigen presenting cells (APCs) express a large number of tissue-specific self antigens in the context of MHC class I and MHC class II (Gotter et al. 2004; Derbinski et al. 2001). SP thymocytes with high-affinity receptors to these self-antigens will undergo TCR-mediated apoptosis. However, in the presence of the cytokines transforming growth factor-β (TGF-β) and IL-2, T regulatory cell (Treg) progenitors are prevented from undergoing apoptosis, and instead, can differentiate into Tregs that have suppressive functions (Bautista et al. 2009). This process of thymic Treg (tTreg) differentiation is termed clonal diversion and aids in controlling self-reactive T cells.

After positive and negative selections are complete, SP thymocytes undergo a final functional maturation in the medulla. Functional maturation involves a switch whereby the thymocytes no longer undergo apoptosis when stimulated through their
TCR, but rather proliferate. Functional maturation also involves the upregulation of the cell surface markers CD62L and sphingosine-1-phosphate receptor 1 (S1P₁) and the chemokine receptor CCR7 (Ueno et al. 2004; McCaughtry et al. 2007). It has been shown that the expression of S1P₁, a G-protein coupled receptor, on SP thymocytes facilitates emigration from the thymus into the circulation via lymphatics or blood vessels in the medulla (Matloubian et al. 2004). Once in the bloodstream, these mature naïve T cells can circulate between the blood and peripheral lymphoid tissue until they encounter their cognate antigen and participate in the adaptive immune response.

**T cell Subsets**

CD4⁺ and CD8⁺ T cells that have egressed from the thymus into the periphery are mature naïve T cells. Naïve T cells are T cells that have not seen their cognate antigen. However, upon antigen recognition in the context of MHC, naïve CD4⁺ T cells proliferate and can differentiate into helper and regulatory subsets, while naïve CD8⁺ T cells can differentiate into cytotoxic T cells or Tregs. Each subset has a unique and defining transcription factor and cytokine profile that is discussed in detail in the following sections.

**CD4⁺ T cell Subsets.**

CD4⁺ T cells recognize antigen in the context of MHC class II presented on APCs and this TCR-mediated stimulation is known as the first signal for T cell activation (Bonnefoy-Berard et al. 1992). The second signal for T cell activation is co-stimulation. APCs provide ligation of co-stimulatory molecules expressed on the surface of T cells. The main co-stimulatory molecule expressed on all naïve T cells is CD28 (Burr et al. 2001; June et al. 1987). The third signal for T cell activation is inflammatory cytokine
secretion by APCs (Curtsinger et al. 1999). The cytokine milieu experienced by activated CD4⁺ T cells dictates which helper or regulatory CD4⁺ T cell subset the cell will become. APCs and other local cell’s production of the cytokines interleukin 12 (IL-12) and interferon γ (IFNγ) initiate the signaling cascade to generate T helper 1 (Th1) cells (Hsieh et al. 1993; Smeltz et al. 2002). These cytokines are involved in the expression of the master transcription factor for promoting Th1 differentiation and suppressing the development of other Th subsets, T-box transcription actor (T-bet) (Afkarian et al. 2002)(Figure 2). Th1 cells are characterized by their expression of T-bet and secretion of IFNγ, IL-2 and tumor necrosis factor-α (TNF-α) and function to aid in the elimination of intracellular bacteria and viruses (Szabo et al. 2000). To achieve elimination of intracellular pathogens, Th1 cytokine secretion activates macrophages, natural kill (NK) cells, CD8⁺ T cells and B cells to increase their respective pathogen clearance activities (Suzuki et al. 1988; Murray et al. 1985; Melzer et al. 2008; Smith et al. 2000).

CD4⁺ T cell stimulation in the presence of IL-4 and IL-2 promotes the differentiation of Th2 cells (Kopf et al. 1993; Cote-Sierra et al. 2004). The critical master transcription factor that promotes Th2 differentiation while inhibiting Th1 differentiation is GATA-binding protein (GATA3) (Zheng & Flavell 1997; Zhang et al. 1997)(Figure 2). Th2 cells are characterized by their secretion of IL-4, IL-5, IL-10, and IL-13 and function to mediate the elimination of extracellular pathogens. To achieve this, secretion of IL-4 promotes immunoglobulin E (IgE) class switching in B cells, which can bind to Fcε receptor I (FcεRI) on mast cells to induce degranulation and cytokine secretion to promote parasite clearance (Gurish et al. 2004). Additionally, secretion of IL-5 by Th2
cells recruits eosinophils, which secrete granules that are important for clearing parasitic infections (Coffman et al. 1989). Finally, secretion of IL-13 mediates expulsion of helminthes and promotes resistance to intracellular bacteria (Urban et al. 1998; McKenzie et al. 1998). Of note, it is also known that Th2 responses mediate airway hypersensitivity, which is an important feature of asthma, and other allergic diseases (Woodruff et al. 2009; Dougherty et al. 2010).

The cytokines IL-6, IL-21, IL-23 and TGF-β promote the differentiation of stimulated CD4+ T cells into Th17 cells. Low concentrations of TGF-β in combination with IL-6 induce the critical master regulator for Th17 cells termed retinoid-related orphan receptor gamma-T (RORγt) (Ivanov et al. 2006)(Figure 2). Furthermore, the presence of IL-21 leads to the self-amplification of Th17 cells and the presence of IL-23 allows for the stabilization of Th17 cells (Stritesky et al. 2008; Wei et al. 2007). Th17 cells secrete IL-17a, IL-17f, IL-21 and IL-22 and mediate immune responses against extracellular bacteria and fungi (Mangan et al. 2006b; Liang et al. 2006). IL-17a can induce many inflammatory cytokines and recruit and activate neutrophils to induce their response to pathogens. Additionally, IL-21 acts on CD8+ T cells and NK cells to promote increased cytotoxic activity (Miyamoto et al. 2003; Taylor et al. 2013). Th17 cells have also been greatly appreciated as critical mediators of various autoimmune disorders such as rheumatoid arthritis (RA), psoriasis, juvenile idiopathic arthritis (JIA) and Crohn’s disease (Piper et al. 2014; Rovedatti et al. 2009; Kotake et al. 1999). Their pathogenesis is attributed to the chronic inflammation induced by IL-17a and/or IL-17f and IL-22 in the absence of Treg cells (Furuzawa-Carballeda et al. 2012; Moon et al. 2014).
As discussed above, Tregs play a critical role in maintaining self-tolerance and regulating immune responses. In addition to being generated in the thymus (tTregs), Tregs can also be induced in the periphery by stimulated CD4\(^+\) T cells in the presence of high concentrations of TGF-\(\beta\) and are termed peripheral Tregs (pTregs) (Chen et al. 2003). TGF-\(\beta\) signaling induces the expression of the critical transcription factor Forkhead transcription factor 3 (Foxp3)(Figure 2). Additionally, TGF-\(\beta\) signaling induces the phosphorylation and activation of the transcription factor Smad3, which has been shown to interact with ROR\(\gamma\)t to inhibit Th17 differentiation (Martinez et al. 2009). Tregs express high levels of CD25, as IL-2 signaling is required to enhance Foxp3 expression and promote the differentiation of pTregs over Th17 cells (Laurence et al. 2007). As the name suggests, Tregs possess regulatory and suppressive functions that are mediated via several mechanisms. One mechanism by which Tregs suppress the activation and proliferation of conventional T cells (Tconv) is through the co-inhibitory molecule cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Bachmann et al. 1999; Read et al. 2000). CTLA-4 is constitutively expressed on Tregs and functions to suppress Tconv cells in the presence of APCs by downregulating the expression of co-stimulatory CD80/CD86 molecules on APCs (Oderup et al. 2006). Another mechanism of Treg mediated suppression is through the secretion of immunosuppressive cytokines. These cytokines include TGF-\(\beta\) and IL-10. Tregs produce soluble and membrane-bound TGF-\(\beta\) that functions to suppress Tconv cell proliferation \textit{in vivo} in a colitis model (Read et al. 2000). Furthermore, IL-10 signaling has been shown to prevent T cell-mediated colitis and control IFN-\(\gamma\) production by T cells in the inflamed skin (Sojka & Fowell 2011). Finally, Tregs can also suppress specific CD4\(^+\) T cell effector subsets by expression of
Th1 or Th2 specific transcription factors. It has been demonstrated that a subset of Tregs that are T-bet positive are essential in controlling Th1 mediated responses while interferon regulatory factor 4 (IRF-4) positive Tregs are critical for suppressing Th2 responses (Koch et al. 2009) (Zheng et al. 2009). Therefore, it is well appreciated that Tregs implement a variety of mechanism to regulate immune responses to prevent chronic inflammation and autoimmune diseases.

**Figure 2. Naïve CD4+ T cell Differentiation into Effector or Regulatory Subsets.** Diagram depicting the differentiation of stimulated naïve CD4+ T cells into subsets. Subset specificity is conferred by the cytokine milieu experienced at the time of stimulation. Each subset is driven by a critical transcription factor that regulates acquisition of each subset's effector or regulatory functions. Modified from (Meng et al. 2015).

**CD8+ T cell Subsets.**

CD8+ T cells recognize antigen in the context of MHC class I presented on APCs and after the recognition of all three activation signals described above, differentiate into
cytotoxic T cells (also know as cytotoxic T lymphocytes-CTLs). Generation of effector CTLs requires IL-2 signaling, which stimulates cell proliferation, production of IFN-γ, perforin, and granzymes and promotes trafficking to peripheral tissues (Rollings et al. 2018). CTLs function to eliminate infected and malignant cells by secreting the critical cytolytic mediators, perforin and granzymes (Curtsinger et al. 2003; Thomas & Massague 2005). Because of their potent ability to destroy malignant and transformed cells, CTLs are currently being harnessed and engineered for cancer immunotherapy.

CD8\(^+\) T cell activation in the periphery in the presence of TGF-β leads to the generation of CD8\(^+\) pTregs. CD8\(^+\) Tregs mediate suppression of Tconv using direct cell-to-cell contact, as well as the secretion of immunosuppressive cytokines (Churlaud et al. 2015). CD8\(^+\) Tregs also have the capacity to exert cytotoxic effects against antigen activated CD4\(^+\) Tconv (Varthaman et al. 2011; Tang et al. 2006). Although the study of CD8\(^+\) Tregs is just recently beginning to intensify, it is appreciated that they possess important immunosuppressive functions.

**Perinatal vs Adult T cells**

Upon birth, newborns experience a drastic change from the sterile environment of the womb to the outside world, where a large variety of new antigens exists. It is greatly appreciated that the immune responses in neonates is dampened due to an immunosuppressive environment. This is believed to protect neonates from mounting deleterious immune responses to the onset of harmless antigens encountered at birth. Evidence for the immunosuppressive and tolerogenic nature of the fetal immune system was shown by seminal experiments performed by Billingham, Brent and Medawar. They demonstrated that CBA mice injected in utero with adult tissue from a different inbred
mouse (strain A) accepted a skin graft from strain A when challenged 8 weeks after birth. CBA mice that were not injected with tissue from strain A in utero, or injected with tissue from strain A after birth, rejected skin grafts from strain A upon challenge (BILLINGHAM et al. 1953). These data suggest that the developing fetal immune system has the capacity to tolerate exposure to foreign antigens, as it would self-antigens.

The unique features of the perinatal immune system are conducive to an immunosuppressive environment. For example, Tregs comprise ~15% of the total CD4⁺ T cell population in the human fetus, which is substantially more than the amount found in an infant or adult (Takahata et al. 2004). Further, stimulation of CD25⁺ Treg depleted fetal lymph node and spleen cells from humans in the presence of allogeneic antigen presenting cells exhibited a far greater frequency of Foxp3 positive cells compared to adult CD4⁺ T cells stimulated in the same manner. Further, these fetal Foxp3 positive T cells were functionally suppressive (Mold et al. 2008). Similarly, our lab has discovered that stimulation of white blood cells from human umbilical cord blood with an anti-CD3 antibody in the presence of exogenous IL-2 generates a significantly greater frequency of CD4⁺ and CD8⁺ Tregs, in a TGF-β dependent manner, compared to cells stimulated from peripheral adult blood (Lee et al. 2018). These data suggest that naïve T cells from the fetus and neonates have a greater propensity to become Tregs compared to naïve T cells from adults. This phenomenon is believed to be a mechanism that contributes to the immunosuppressive environment in the fetus.

Another unique feature of the fetal immune system is a bias towards Th2 responses over Th1 responses. This Th2 bias is thought to be more protective than Th1
responses, as Th2 cytokines are anti-inflammatory while Th1 cytokines are pro-inflammatory. In fact, neonatal murine CD4\(^+\) T cells stimulated with anti-CD3 and anti-CD28 antibodies secreted greater levels of IL-4 and IL-13 compared to adult CD4\(^+\) T cells (Rose et al. 2007). Further, neonatal CD4\(^+\) T cells adoptively transferred to adult RAG2 deficient hosts demonstrated enhanced Th2 responses and diminished Th1 responses upon challenge \textit{in vivo} compared to adaptively transferred adult CD4\(^+\) T (Adkins et al. 2002). Collectively, these data suggest that fetal CD4\(^+\) T cells from mice are intrinsically poised to execute a Th2 program. However, studies assessing an intrinsic Th2 bias in perinatal T cells from humans are less conclusive. Indeed, stimulation of T cells from cord blood and 3-month-old infants demonstrated that these T cells do not show an increased propensity for Th2 cytokine production, except for IL-13 (Halonen et al. 2009). Therefore, further studies characterizing perinatal T cells from humans must be performed to determine if the cell intrinsic Th2 bias observed in the mouse perinatal immune system is conserved.

**Transforming Growth Factor-\(\beta\) (TGF-\(\beta\))**

As detailed above, CD4\(^+\) and CD8\(^+\) T cells possess a wide variety of functions that orchestrate immune responses to pathogens, infected cells and tumor cells. Further, if suppressive and regulatory mechanisms are not in place, T cells can mediate chronic inflammation and destructive autoimmune disorders due to aberrant activation. Therefore, to maintain health and homeostasis, the immune system has numerous checkpoints to control T cell activation. One of the most critical regulators of T cell responses is the cytokine transforming growth factor-\(\beta\) (TGF-\(\beta\)). TGF-\(\beta\) is a pleotropic cytokine, meaning that one cytokine can mediate multiple effects. TGF-\(\beta\) received its
name from its first identified function, which is its ability, as a secreted factor, to induce a transformed phenotype in normal fibroblasts in vitro (de Larco & Todaro 1978) (Moses et al. 1981)(Roberts et al. 1981). It was later discovered that TGF-β acts on numerous cell types to regulate development, metastasis, wound healing, fibrosis and immune responses (Sanford et al. 1997) (Maehara et al. 1999) (Sanderson et al. 1995). This dissertation will focus on TGF-β signaling in T cells.

**Processing of TGF-β.**

TGF-β is a highly evolutionary conserved cytokine that has three known mammalian isoforms termed TGF-β1, TGF-β2, and TGF-β3. The TGF-β family of cytokines also includes activins, nodal, bone morphogenetic protein (BMP), and growth differentiation factor (GDF). TGF-β1 is the predominant isoform that is expressed in the immune system (Shull et al. 1992). Before TGF-β can signal to T cells, it must first be proteolytically processed and activated. This process is crucial in controlling TGF-β bioavailability. TGF-β is synthesized as a prepro-TGF-β precursor that contains an amino-terminal pre-region which possesses a signal peptide, a pro-region (also known as the latency-associated peptide (LAP)) and the mature peptide region at the carboxy-terminal (Gray & Mason 1990; Wakefield et al. 1988)(Figure 3).

The first processing event is the cleavage of the pre-region by endopeptidases and this occurs in the Endoplasmic Reticulum (ER). Once cleaved, it forms a dimer that is connected by disulfide bonds and is termed pro-TGF-β (Figure 3 (i)). The second processing event occurs in the Golgi where furin, an endopeptidase that catalyzes the cleavage of precursor proteins, cleaves the pro-peptide, LAP (Dubois et al. 2001; Dubois et al. 1995) (Figure 3 (ii)). After cleavage, LAP non-covalently associates with
the mature TGF-β and holds it in a latent form (Miyazono et al. 1993) (Figure 3 (iii)). The association of the LAP homodimer with the mature TGF-β homodimer is termed the small latency complex (SLC). This formation is required for the correct folding of the mature TGF-β homodimer and successful secretion from cells (Gray & Mason 1990; Miyazono et al. 1991). Additionally, LAP prevents the binding of mature TGF-β to its receptors and renders its inactive. The SLC can be directly secreted from cells or remain in the cell and associate with a large protein, called latent TGF-β binding protein-1 (LTBP-1), through disulfide bonds (Taipale et al. 1994) (Figure 3 (iv)). This complex is termed the large latent complex (LLC) and once formed the inactive/latent TGF-β is secreted from cells. LTBP-1 functions to target and archer latent TGF-β to the extracellular matrix (ECM) to and present it other cells for subsequent activation (Nunes et al. 1997; Olofsson et al. 1995).
Figure 3. The Processing and Secretion of TGF-β. TGF-β is expressed and processed inside of the cell and is subsequently secreted into the extracellular matrix (ECM). (i) Dimerization of the pro-peptide after cleavage by endopeptidases. (ii) Furin mediated cleavage of the latency-associated peptide (LAP). (iii) LAP undergoes a conformational change to hold TGF-β in an inactive form. This is termed the small latency complex (SLC). (iv) The SLC can associate with the latent TGF-β binding protein-1 (LTBP-1) through disulfide bonds. This complex is termed the large latency complex (LLC). LTBP-1 archers the LLC to the ECM to present TGF-β to cells. Modified from (Worthington et al. 2011)

Activation of Latent TGF-β.

Activation of TGF-β is thought to be mediated by a variety of factors including (but not limited to) acidic pH, various proteases, heat, reactive oxygen species and shear stress (Oursler 1994; Schultz-Cherry & Murphy-Ullrich 1993; Sato & Rifkin 1989). However, studies have shown that under physiological conditions the critical activators
of TGF-β are integrins. Integrins are cell adhesion and signaling molecules comprised of a heterodimer of α and β subunits that form a type I transmembrane receptor (Xiong et al. 2001). Four integrins (αvβ3, αvβ5, αvβ6 and αvβ8) have been shown to bind the tripeptide RGD integrin binding motif present on LAP to liberate active TGF-β (Munger et al. 1999). Strong evidence to support the requirement for integrins in the activation of TGF-β was obtained by Yang, Z. et al, who demonstrated that mice that possess a single point mutation in the RGD integrin binding motif (RGD to RGE, Tgfb1RGE/RGE) of LAP only express latent TGF-β and die from vasculogenesis problems during development or from lethal multi-organ inflammation due to a failure of active TGF-β to control the immune system (Z. Yang et al. 2007). Integrins that can activate TGF-β are expressed in many different cell types such as epithelial cells, dendritic cells and CD4+ T cells (Travis et al. 2007).

It is well appreciated that integrins can bind the LCC to generate active TGF-β, but the mechanisms by which active TGF-β is liberated are less clear. Two mechanisms of integrin-mediated activation have been described. One mechanism is termed ‘proteases-independent’ and does not require proteases. Instead, it has been proposed that integrins αvβ5 and αvβ6 bind LAP on the LCC and generate a ‘pulling force’, which is mediated by the actin cytoskeleton connected to the cytoplasmic domain of the integrin. Simultaneously, the LTBP component of the LCC bound to the ECM, specifically fibronectin, creates an opposing ‘holding force’. The combination of these two forces somehow induces a conformational change that permits TGF-β liberation and ability to bind its receptor that is proposed to be on the same cell expressing the αvβ5/αvβ6 integrin (Fontana et al. 2005) (Wipff et al. 2007) (Munger et al. 1999) (Figure
4). However, further work must be performed to understanding the conformational change induced by these forces that facilitates the activation of TGF-β. The second mechanism of integrin-mediated activation requires the activity of proteases or protease-dependent. Integrin αvβ8 has been shown to require the cleavage of LAP by the metalloprotease MT1-MMP to release active TGF-β (Mu et al. 2002). In this mechanism, liberated active TGF-β is believed to diffuse and affect cells distal from the TGF-β activating cell. However, little is known about how this αvβ8-MT1-MMP pathway of TGF-β activation is regulated.

Interestingly, αvβ6 and αvβ8 integrins are critical activators of TGF-β during immune homeostasis, as mice lacking αvβ8 (Itgb8−/−) and treated with anti-β6 neutralizing antibody exhibit extensive multi-organ inflammation, a phenotype that is very similar to that seen in Tgfb1−/− mice (Aluwihare et al. 2009). The integrins αvβ6 and αvβ8 are known to activate TGF-β via different mechanisms; with αvβ6 participating in the protease-independent pathway and αvβ8 participating in the protease-dependent pathway. This suggests that multiple mechanisms of TGF-β activation are key in maintaining immune homeostasis.
Figure 4. The Protease-independent Model of TGF-β Activation. Model depicting the liberation of TGF-β from the large latency complex (LCC). (1) Integrins bind LAP. (2) Actin cytoskeleton connected to the cytoplasmic domain of the integrin generates a ‘pulling force’ on the LCC. (2) Because the LCC is bound to the extracellular matrix (ECM), a simultaneous ‘holding force’ is generated. (3) Active TGF-β is liberated. (4) Liberated TGF-β can bind TGF-β receptors and mediate signaling. Modified from (Hinz 2015).

**TGF-β Signal Propagation**

TGF-β signaling is propagated by active TGF-β binding to the TGF-β type II receptor (TGF-βRII). Further, active TGF-β displays a high affinity for the type II receptors and does not associate with isolated TGF-β type I receptors (TGF-βRI) (Wrana et al. 1992). Both the TGF-βRII and TGF-βRI are transmembrane glycoproteins that contain a TGF-β binding extracellular domain, a single transmembrane region and
a cytoplasmic serine/threonine kinase domain (Lin et al. 1992). However, the TGF-βRI also contains a regulatory segment within its cytoplasmic juxtamembrane region termed the GS region, that is critical for its activation (Wieser et al. 1995). Binding of active TGF-β to the constitutively active TGF-βRII results in autophosphorylation of TGF-βRII and recruitment of TGF-βRI. The association of TGF-βRI to the TGF-βRII and active TGF-β complex occurs in cooperatively. Cooperative binding refers to the sequential ability of both the TGF-βRII and TGF-βRI to bind to active TGF-β, with TGF-βRII binding first (E Zúñiga et al. 2006). Additionally, it has been shown through crystal structures and mutagenesis assays that that direct TGF-βRII-TGF-βRI heterodimer interactions are critical for cooperative binding of active TGF-β (Groppe et al. 2008)(Figure 5).
Figure 5. A Structural Model of the Tetrameric TGF-βRII and TGF-βRI Complex following Sequential Cooperative Binding. In the cooperative bind model of TGF-β signal transduction, TGF-β RI (yellow) binds TGF-β (green and blue strands) first. Next, TGF-βRI (purple) binds TGF-β. This binding of TGF-β facilitates the physical interaction of both receptors to allow for TGF-βRII mediated activation of TGF-βRI. Modified from (Shi & Massague 2003).

Once TGF-βRII and TGF-βRI are in a tetrameric complex, the constitutively active TGF-βRII phosphorylates multiple serine and threonine residues within the TTSGSGSG sequence in the GS region of TGF-βRI (Wrana et al. 1994). Phosphorylation of TGF-βRI switches the GS region from serving as a binding site for the inhibitor FK506-binding protein 1A (FKBP12) to a binding site for regulatory R-Smads (an acronym from the fusion of Caenorhabditis elegans Sma genes and the Drosophila Mad, Mothers against decapentaplegic) substrate (Huse et al. 2001; Huse et al. 1999) (Figure 6). When the GS region is bound by FKBP12, the kinase
domain of TGF-βRI is held in an inactive conformation and remains inactive until phosphorylation by TGF-βRII. Activation of TGF-βRI can facilitate canonical and non-canonical TGF-β signaling, as described below.

**Figure 6. Structure of TGF-βRII and TGF-βRI.** Once bound to ligand the TGF-βRII autophosphorylates and phosphorylates the TGF-βRI within its GS region at the sites indicated. This facilitates the removal of the inhibitory FKBP12 to allow for TGF-βRI-mediated phosphorylation of Smads.

**Canonical TGF-β Signaling.**

Canonical TGF-β signaling is defined by being Smad mediated. Smads contain an amino-terminal MAD homology (MH1) domain, a proline-rich linker and carboxy-terminal MH2 domain (Abdollah et al. 1997). The MH1 domain regulates nuclear import and DNA binding while the MH2 domain mediates recognition by type I receptors and interacts with cytoplasmic adaptors (Lo et al. 1998). The TGF-βRI functions to activate Smad2 and Smad3 by phosphorylating the Ser-X-Ser motif within their respective MH2
domains. To achieve this, Smad anchor for receptor activation (SARA) has been shown to bind Smad2 and Smad3 and present the MH2 domain of the Smad for phosphorylation through direct interaction with TGF-βRI (Qin et al. 2002). Phosphorylation of Smad2 and Smad3 induces the dissociation from SARA and promotes their assembly with co-Smad4 (Tsukazaki et al. 1998). The phosphorylated homodimer of Smad2 or Smad3 associates with Smad4 and this trimeric complex translocates to the nucleus (Correia et al. 2001)(Figure 7). In the nucleus, Smads bind Smad-binding elements (SBEs) and mediate activator or repression of target genes to facilitate TGF-β signaling (Kang et al. 2003).

**Figure 7. Model of Canonical TGF-β Signaling.** TGF-β signaling is propagated by ligand binding to the TGF-β receptor type II (TGF-βRII) first. This recruits the TGF-β receptor type I (TGF-βRI). TGF-βRII phosphorylates and activates TGF-βRI. Once activated TGF-βRI can phosphorylate Smads2 and Smad3. Activated Smad2/3 can complex with co-Smad4 and translocate to the nucleus to mediate downstream TGF-β signaling.
Non-canonical TGF-β Signaling.

TGF-β signaling can also be mediated in a manner that does not involve the phosphorylation of Smads. This is termed 'non-canonical' TGF-β signaling. Non-canonical TGF-β signaling can activate the mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), p38 MAP kinase (Hartsough & Mulder 1995)(Atfi et al. 1997)(Bakin et al. 2002).

Although TGF-βRII and TGF-βRI are serine/threonine kinases, they have also been shown to be phosphorylated on and phosphorylate tyrosine residues (Lawler et al. 1997). After ligand binding and TGF-βRII-TGF-βRI complex formation, TGF-βRI is phosphorylated on a tyrosine residue by a mechanism that is not understood. After tyrosine phosphorylation, TGF-βRI can recruit and directly phosphorylate Src homology domain A (ShcA) on both tyrosine and serine residues (Lee et al. 2007). Phosphorylation of ShcA facilitates its association with growth factor receptor binding protein 2 (Grb2). Grb2 is an adaptor protein that is associated with Son of sevenless (Sos) and upon binding to ShcA gets recruited to TGF-βRI. Once there, Sos activates the membrane-associated GTPase, Ras, by catalyzing the exchange of GDP to GTP (Pronk et al. 1994; Blaikie et al. 1994). Activated Ras can now bind and activate the MAP kinase kinase kinase (MAPKKK) Raf to initiate a signaling cascade that involves the activation of the MAP kinase kinase (MAPKK) MEK and subsequently ERK (Lee et al. 2007). TGF-β signaling has been demonstrated to activate ERK in several of cell types including epithelial cells, breast cancer cells and fibroblasts (Frey & Mulder 1997; Mucsi et al. 1996).
The activation of JNK by TGF-β signaling is mediated by the association of TNF receptor-associated factor 6 (TRAF6) to the TGF-βRII-TGF-βRI complex. TRAF6 is an E3 ubiquitin ligase and its binding to activated TGF-βRII-TGF-βRI complexes induces activation of the RING finger E3 ligase and subsequent K63-linked polyubiquitination of itself (Yamashita et al. 2008). This ubiquitination mediates the interaction of TRAF6 with TGF-β activated kinase 1 (TAK1) and TAK1 polyubiquitination and activation (Sorrentino et al. 2008). Activated TAK1 initiates a signaling cascade that activates MAP kinase kinase kinase 4 (MKK4) and MKK7, which activate JNK.

**TGF-β Signaling in T cells**

In T cells, TGF-β can in induce the differentiation of CD4+ and CD8+ T cell subsets that require TGF-β, while simultaneously inhibiting subsets that do not require TGF-β. Further, TGF-β can mediate the suppression of T cell activation and proliferation. However, the detailed molecular pathways that mediate the distinct functions of TGF-β in T cells are not fully elucidated. Current knowledge regarding TGF-β signaling in T cell differentiation and suppression will be reviewed below.

**TGF-β Signaling in T cell Differentiation.**

As detailed above, the cytokine milieu experienced by activated CD4+ and CD8+ T cells dictates which effector or regulatory subset the cell will become. Naïve CD4+ and CD8+ T cells activated in the presence of antigen, TGF-β, and IL-2 can differentiate into peripheral Tregs (pTregs). Peripheral Tregs have suppressive activity and are critical in maintaining peripheral tolerance. The acquisition of suppressive functions is associated with the expression of the transcription factor Foxp3 (Chen et al. 2003; Zheng et al. 2002). Induction of Foxp3 is meditated by TGF-β signaling via the activation of the
transcription factor Smad3. Smad3 binds to the Foxp3 locus at the conserved non-coding sequence 1 (CNS1) region and, in cooperation with other transcription factors and IL-2 signaling, regulates the transcription of Foxp3 (Tone et al. 2008). Foxp3 plays an essential, non-redundant role in solidifying and stabilizing the Treg lineage and controlling development and function (Gavin et al. 2007; Fontenot et al. 2003). TGF-β signaling is necessary for Foxp3 expression and subsequent Treg generation, as mice deficient TGF-β1 (TGF-β1−/−) develop a lethal lymphoproliferative autoimmune syndrome and succumb to the disease by 20 days after birth (Shull et al. 1992). Further, Foxp3 knockout mice have a similar phenotype to TGF-β1 deficient mice due to a deficiency in Tregs (Fontenot et al. 2003). In addition to the TGF-β requirement for Treg generation, it has also been demonstrated that Tregs express membrane-bound TGF-β1 that suppresses the proliferation of conventional T cells in a contact-dependent manner in vitro, as a neutralizing anti-TGF-β antibody abolishes the suppression (Nakamura et al. 2001). However, in vivo studies of Treg suppression reveal that expression of TGF-β is not the only mechanism which mediates suppression. Indeed, mice with a Treg specific deletion of cytotoxic T lymphocyte antigen-4 (CLTA-4) succumb to a fatal T-cell mediated autoimmune disease (Wing et al. 2008). A more recent study demonstrated that Treg expressed CTLA-4 can actively remove the co-stimulatory molecules CD80/86 on dendritic cells and degrade them in a process termed trans-endocytosis (Qureshi et al. 2011). Therefore, another mechanism of Treg mediated suppression is to reduce the expression of co-stimulator molecules on dendritic cells to inhibit the full activation of Tcov. Further, mechanisms by which Tregs implement to mediate suppression is thought to be dictated by the particular environment in vivo.
Peripheral Tregs share the requirement of TGF-β for differentiation with T helper 17 (Th17) cells. However, pTregs and Th17 cells have opposing functions. While pTregs are suppressive, Th17 cells produce inflammatory cytokines (IL-17, IL-22, and IL-23) and function to recruit neutrophils, promote inflammation at sites of infection and are major contributors to various autoimmune diseases (Veldhoen et al. 2006; Bettelli et al. 2006). Therefore, it becomes clear that TGF-β can direct the differentiation of subsets that play opposing roles in the immune response, but how TGF-β dictates the generation of pTreg vs Th17 is a central focus in the field of T cell biology. Studies have shown that at the initial stage of differentiation, TGF-β functions to induce both the Treg and Th17 program (Manel et al. 2008). TGF-β signaling upregulates the expression of the IL-23 receptor, and signaling through the IL-23 receptor confers full acquisition of Th17’s pathogenic proprieties (Mangan et al. 2006a). Further, IL-6 drives Th17 differentiation in mice by activating the transcription factor STAT3, which induces the expression of Th17 specific genes such as Rorc (encodes for ROR-gamma), and Il17 (encodes for IL-17) (X. O. Yang et al. 2007). STAT3 also functions to inhibit pTreg generation by downregulating TGF-β-mediated expression of Foxp3 (Zhou et al. 2007). Similarly, IL-2 induced STAT5 functions to inhibit Th17 generation by aiding in the induction of Foxp3 (Hori et al. 2003). In addition to the contribution of TGF-β signaling to the generation of both pTregs and Th17 cells, other factors also influence the balance between these two subsets. These factors include other cytokines, such as IL-2 and IL-6/IL-23, costimulatory signaling, metabolic pathways and the microbiota (Gogishvili et al. 2013; Delgoffe et al. 2011; Ivanov et al. 2009).
Not only is TGF-β required for pTreg and Th17 cell differentiation, but TGF-β also inhibits the differentiation of both T helper 1 (Th1) and T helper 2 (Th2) cells. As described above, IFN-γ promotes Th1 differentiation via the induction of critical Th1 associated genes, such as T-bet. However, TGF-β signaling inhibits IFN-γ signaling and T-bet expression (Park et al. 2005). This inhibition is mediated by noncanonical TGF-β-induced MEK/ERK MAP kinase signaling (Park et al. 2007). Further, mice deficient in TGF-β1 succumb to a multifocal inflammatory disorder mediated by Th1 activation and IFN-γ secretion (Gorham et al. 2001). These data suggest that TGF-β functions to inhibit IFN-γ signaling to control Th1 mediated inflammation (Figure 8). Similarly, mice that express a dominant negative TGF-βRII under a T cell-specific promoter, rendering T cells insensitive to TGF-β signaling, develop a large proportion of Th1 and Th2 cells compared to control mice (Gorelik & Flavell 2000). Th2 cell generation requires the critical transcription factor, GATA3. Studies have shown that TGF-β inhibits Th2 differentiation by the inhibition of GATA3 expression (Gorelik et al. 2000; Heath et al. 2000)(Figure 8). Although, the molecular mechanisms by which TGF-β inhibits GATA3 expression are poorly understood.
Figure 8. TGF-β Signaling in CD4+ T cell Differentiation. TGF-β signaling can suppress both the Th1 and Th2 lineages while promoting the differentiation of Th17 cells and Tregs.

TGF-β Signaling in T cell Suppression.

TGF-β signaling is critical in regulating T cell proliferation and homeostasis. Numerous studies have shown that TGF-β reduces T cell proliferation by inhibiting the transcription of the T cell growth factor, IL-2 (Kehrl et al. 1986; Brabletz et al. 1993). IL-2 signaling drives mitosis in activated T cells and has non-redundant roles in T cell expansion (Cantrell & Smith 1983). TGF-β mediates the inhibition of IL-2 transcription through Smad signaling. One mechanism by which Smad signaling inhibits IL-2 transcription is by the upregulation of Foxp3. Foxp3 is known to bind the IL2 promoter and represses the activity of the complex of two other transcription factors, Nuclear Factor of Activated T cells (NF-AT) and Activator Protein 1 (AP-1). Foxp3 competes with AP-1 for co-operative binding with NF-AT at the IL2 promoter to repress IL-2.
transcription (Wu et al. 2006; Chen et al. 2006). Another mechanism of TGF-β-mediated IL-2 transcriptional inhibition describes how Smad2 and Smad3 have redundant roles in recruiting histone 3 lysine 9 (H3K9) methyltransferases and promoting H3K9 trimethylation in the IL2 proximal promoter region to suppress transcription (Wakabayashi et al. 2011). Taken together, these data demonstrate how TGF-β signaling functions to suppress T cell proliferation by controlling the production of IL-2.

To maintain homeostasis, TGF-β has been shown to inhibit naïve T cell proliferation but not activated T cell proliferation, as activated T cells downregulate the expression of TGF-βRII (Cottrez & Groux 2001; Sanjabi et al. 2009). Further, TGF-β promotes the expression of IL-7Rα during thymic T cell development to allow naïve peripheral T cells to receive IL-7 signaling for their survival (Ouyang et al. 2013). These data suggest crosstalk between TGF-β signaling and signals involved in maintaining naïve T cell homeostasis. To allow for priming of naïve T cells upon encounter with APCs, co-stimulation through CD28 has been shown to send signals that inhibit TGF-β’s anti-proliferative effects (Sung et al. 2003). To inhibit proliferation of activated T cells, IL-10 signaling has been shown to upregulate the expression of TGF-βRII to re-establish responsiveness to TGF-β mediated suppression (Cottrez & Groux 2001). Although a few molecular mechanisms have been proposed to describe TGF-β-mediated T cell suppression, fundamental molecular mechanisms concerning both canonical and non-canonical TGF-β signaling in controlling T cells remain poorly understood.
c-Jun Amino-Terminal Kinase (JNK) Signaling in T cells

c-Jun amino terminal kinase (JNK) (also called stress-activated protein kinase [SAPK]) belongs in a group of mitogen-activated protein (MAP) kinases. These proteins are serine/threonine kinases that are activated in the presence of a range of stimuli including cytokines, growth factors, and cellular stress. Other MAP kinases include extracellular signal-regulated kinase (ERK) and p38. The functions of these MAP kinases are to regulate processes such as T cell activation, proliferation, differentiation and death (Dumont et al. 1998; Yang et al. 1998; Adler et al. 2007; Xia et al. 1995) Further, the JNK family includes JNK1, JNK2, and JNK3. Both JNK1 and JNK2 are ubiquitously expressed, while JNK3 is expressed only in the brain, testis and heart (Gupta et al. 1996; Martin et al. 1996) Each JNK gene (JNK1, JNK2 and JNK3) is expressed as a 46 kDa and a 54 kDa isoform as a result of alternative splicing (Gupta et al. 1996). The differential functions of the splice variants remain to be elucidated. However, the JNK1 and JNK2 signaling pathways are becoming increasingly appreciated for controlling immune responses and T cell function and these activities will be reviewed in the following sections.

Upstream Kinases in JNK Activation.

A signaling cascade comprised of MAP kinase proteins function to activate JNK. The first protein to be activated in this cascade is a MAPK kinase kinase (MAPKKK), which phosphorylates and activates the MAPK kinase (MAPKK) that phosphorylates JNK. The specific MAPKKK and MAPKK upstream of JNK are dictated by extracellular stimuli. These stimuli include inflammatory cytokines, such as IL-1 and tumor necrosis
factor-α (TNF-α), osmotic stress and TCR signaling (Clerk et al. 1999; Huangfu et al. 2006; Moriguchi et al. 1997). While the activation of JNK by inflammatory cytokines is well appreciated in innate immune cells, the study of JNK in T cells has focused on JNK activation by TCR signaling. Further, the precise signal transduction pathways that facilitate TCR-mediated induction of JNK activation remain largely unknown.

TCR engagement activates numerous signaling pathways, with CD28 co-stimulation required for full T cell activation (as described above). CD28 co-stimulation enhances JNK activation (Su et al. 1994). This co-stimulation allows for the phosphorylation of the MAPK/ERK kinase kinase (MEKK)(Figure 9). Two isoforms of MEKK exist in mammalian cells, MEKK1 and MEKK2. MEKK1 was the first MEKK thought to be involved in TCR-mediated JNK activation (Kaga et al. 1998). However, further studies revealed that MEKK2, rather than MEKK1, translocates to the immunological synapse during T cell stimulation and specifically activates JNK (Schaefer et al. 1999; Su et al. 2001). MEKKs function to phosphorylate and activate MAPK kinase 4 (MKK4) and MKK7, which directly activate JNK.

Interestingly, another MAPK/ERK kinase kinase that can also phosphorylate and activate MKK4 and MKK7 is transforming growth factor-β-activating kinase 1 (TAK1) (Ninomiya-Tsuji et al. 1999)(Figure 9). As the name suggests, TAK1 is a kinase that mediates TGF-β-induced transcriptional regulation (Yamaguchi et al. 1995). TAK1 is known to be upstream of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), p38 and JNK signaling (Wang et al. 2001). TAK1 is activated by the RING domain ubiquitin ligase TRAF6. TRAF6 catalyzes an activating lysine 63 (K63) polyubiquitination on TAK1 (Ninomiya-Tsuji et al. 1999). Further, TRAF6 is activated by interaction with TGF-
βRI in a kinase-independent manner (Sorrentino et al. 2008). This TGF-β-mediated interaction of TRAF6 and the receptor induces autoubiquitination and activation of TRAF6. Taken together, a TGF-β mediated JNK activation pathway exists through the activation of TRAF6/TAK1/MKK4/7. This pathway has been described in human embryonic kidney cells, and epithelial cells, and neurons (Sorrentino et al. 2008; Liu et al. 2012; Yamashita et al. 2008). However, it remains to be determined if this TGF-β-mediated JNK activation pathway occurs in T cells.

After activation by the MAPKKK, MKK4 and MKK7 directly phosphorylate JNK on conserved threonine and tyrosine residues (Lin et al. 1995; Tournier et al. 1997)(Figure 9). Although, it is clear that there are non-redundant roles for MKK4 and MKK7 in JNK activation. MKK4 phosphorylates JNK on a tyrosine residue (Tyr-185) while MKK7 phosphorylates JNK on a threonine residue (Thr-183) (FLEMING et al. 2000). To determine the requirement of this dual phosphorylation of JNK by MKK4 and MKK7, single and double knockouts were evaluated in mouse embryonic fibroblasts (MEFs). MEFs from dual MKK4 MKK7 deficient mice were severely defective in JNK activation in the presence of proinflammatory cytokines. Further, JNK activation in the presence of proinflammatory cytokines was abolished in MEFs from MKK7 deficient mice but only reduced by 50% in MEFs from MKK4 deficient mice (Tournier et al. 2001). Additionally, in vitro kinase assays have determined that MKK4 can activate both JNK and p38, while MKK7 can only activate JNK (Lin et al. 1995; Moriguchi et al. 1997). These data suggest that mono-phosphorylation of JNK by MKK7 is sufficient to induce JNK activity in the presence of proinflammatory cytokines, while MKK4 might be required for full activation of JNK. Interestingly, MKK4 deficient murine CD4+ T cells are not defective in
JNK activation in the presence of TCR stimulation (Swat et al. 1998). These data further suggest that the requirement for MKK4 or MKK7 in JNK activation may be stimuli and cell-type dependent.

**Figure 9. Kinases upstream of JNK Activation in T cells.** JNK is activated in a sequential process that commences with the activation of a MAPK kinase kinase (MAPKKK) followed by activation of a MAPK kinase (MAPKK). Two MAPKKK upstream of JNK activation in T cells are depicted.

**Adaptor Molecules, CARMA1 and Bcl10, in JNK Activation.**

Adaptor molecules are critical components of signal transduction. Although adaptor molecules don’t have enzymatic activity, they are paramount in providing a key scaffolding function that regulates signal specificity and catalyzes the activation of molecules in the complex. Adaptor molecules recruit specific signaling complexes to help physically assemble multiple signaling components in response to stimuli. In turn, adaptor proteins control activated signaling pathways by their expression levels and phosphorylation status (Therrien et al. 1996; Printen & Sprague 1994). Specifically, for the JNK signaling pathway, caspase recruitment domain-containing membrane-
associated guanylate kinase protein-1 (CARMA1) has been identified as a key scaffolding protein (Blonska et al. 2007).

CARMA1 is comprised of 5 domains connected by linker regions. These domains include an N-terminal caspase activation and recruitment (CARD) domain, a coiled-coil (C-C) domain, a PDZ homology domain, an SH3 domain and a guanylate kinase domain (GUK)(Figure 10). The composition of the PDZ, SH3 and GUK form the membrane-associated guanylate kinase (MAGUK) domain. The MAGUK domain of CARMA1 is required for localization of CARMA1 to the plasma membrane and multimerization of CARMA1 (Tanner et al. 2007). The CARD domain of CARMA1 facilitates interactions with other proteins containing CARD domains. These protein-protein interactions are critical for upstream signaling for both the JNK and NF-κB pathways (Blonska et al. 2007; Pomerantz et al. 2002). Finally, the C-C domain facilitates oligomerization of CARMA1 after activation (Tanner et al. 2007).

CARMA1 is expressed in resting T cells in a closed, inactive form (Figure 10). Studies have identified that the linker region between the MAGUK domain and the C-C domain may facilitate the closed conformation, as deletion of this linker region results in constitutive CARMA1 activity (Sommer et al. 2005). Further, this linker region is required for CARMA1 and protein kinase C-θ (PKC-θ) physical association (Wang et al. 2004). Mutagenesis studies of the serine and threonine residues within this linker region have identified PKC-θ specific phosphorylation sites (Matsumoto et al. 2005). Based on these data, it is proposed that CARMA1 is held in an inactive form, and phosphorylation of the linker region between the MAGUK domain and the C-C domain by PKC-θ facilitates a conformational change, or “opening”, whereby CARMA1 can oligomerize
and associate with other proteins to assemble upstream proteins involved in the activation of both JNK and NF-κB.

Figure 10. Model of CARMA1 Structure in Resting and Activated T cells. In resting T cells, CARMA1 is held in an inactive form. Upon T cell stimulation, protein kinase C-θ (PKC-θ) phosphorylates the linker region between the PDZ and coiled coil (C-C) domains of CAMRA1. PKC-θ phosphorylation mediates activation and opening of CARMA1, thus permitting it to bind Bcl10 through CARD-CARD domain interactions. This complex serves as a scaffold for molecules upstream the activation of JNK and NF-κB. Modified from (Roche et al. 2013).
Upon TCR stimulation, plasma membrane-tethered CARMA1 localizes to the immunological synapse. There, CARMA1 recruits PKC-θ and B cell CLL-lymphoma 10 (Bcl10) to the immunological synapse (Wang et al. 2004). Activated PKC-θ phosphorylates and activates CARMA1. Activated CARMA1 self-associates in multimers bind to Bcl10 through CARD-CARD interactions (Sommer et al. 2005; Matsumoto et al. 2005). This CARMA1/Bcl10 complex then binds to another protein, mucosa-associated lymphoid tissue lymphoma translocation 1 (MALT1), via interactions between MALT1 and the distal end of the CARD domain of Bcl10 (Che et al. 2004). This newly assembled CARMA1/Bcl10/MALT1 (CBM) complex is critical for the activation of NF-κB and JNK in T cells (Figure 11).

In the JNK activation pathway, MALT1, which is a paracaspase with structural similarity to caspase, recruits TRAF6. MALT1 recruitment of TRAF6 is mediated through its two putative c-terminal TRAF6 binding motifs (Sun et al. 2004). Binding of TRAF6 to MALT1 induces oligomerization of TRAF6 and activation of its ligase activity. Next, TRAF6 self-ubiquitinates and can recruit and ubiquitinate TAK1. Ubiquitination of TAK1 by TRAF6 activates its kinase activity and initiates the TAK1/MKK4/7 signaling cascade to activate JNK (Figure 11). Interestingly, in addition to TAK1’s involvement in TGF-β-mediated JNK activation (described above), TAK1 deficient murine effector T cells were impaired in TCR-mediated JNK activation (Wan et al. 2006). These data suggest that TAK1 also integrates TCR-stimulation signaling to activate JNK by recruitment and activation by TRAF6 to this described CBM scaffold.

The CBM/TRAF6/TAK1 JNK activation pathway is the major described signaling pathway that mediates TCR-induced JNK activation in T cells. Indeed, T cells from
CARMA1 deficient mice exhibit defective JNK activation upon anti-CD3 and anti-CD28 antibody stimulation (Hara et al. 2003). Further, JNK activation was inhibited in Jurkat T cells transduced with a dominant negative version of CARMA1 in the presence of TCR-stimulation and CD28 co-stimulation (Gaide et al. 2002). To begin to determine if this CBM/TRAF6/TAK1 pathway selectively activates JNK1 or JNK2, one study found that CARMA1 deficient Jurkat are defective in activation of the p54 isoform of JNK2, but not activation of the p46 isoform of JNK1, in the presence of anti-CD3 and anti-CD28 antibody stimulation (Blonska et al. 2007). They also found that Bcl10 specifically and selectively interacts with the p54 isoform of JNK2 and not the p46 isoform of JNK2. However, the authors noted that their detection of the p54 isoform of JNK1 was low in Jurkat T cells, and further studies are required to determine if the p54 isoform of JNK1 is also regulated by CARMA1. Taken together, these data provide evidence for the importance of the CBM complex in JNK activation in T cells.
Figure 11. The CARMA1/Bcl10/MALT1 Signaling Complex upstream of JNK Activation. T cell activation results in PKC-θ mediated phosphorylation and activation of CARMA1. Active CARMA1 undergoes a conformation change that allows for the assembly of the CARMA1/Bcl10/MALT1 (CBM) scaffold. Once assembled, other molecules upstream of JNK are recruited to facilitate the activation of JNK.

JNK Substrates: The Jun Family of Transcription Factors

JNK serves as the activating kinase for numerous transcription factors. These transcription factors include the Jun family, activating transcription factor 2 (ATF2), c-Myc and NF-AT2 (Westwick et al. 1995; Verheij et al. 1996; Buschmann et al. 2000; Chow et al. 2000). This dissertation will only focus on the Jun family JNK substrates. The Jun family is comprised of c-Jun, JunB, and JunD. All three transcription factors
share a high degree of homology. However, their individual functions in developmental processes, cell growth and apoptosis are different (Jochum et al. 2001) and will be reviewed in the text below.

**c-Jun.**

C-Jun is a transcription factor that was the first identified substrate for JNK, hence how the name c-Jun amino-terminal kinase (JNK) arose (De Rijard et al. 1994). JNK phosphorylates c-Jun by phosphorylating on Serine 63/73 and threonine 91/93 residues. This phosphorylation relieves the repression of c-Jun by an inhibitory complex that is associated with histone deacetylase 3 (HDAC3) and permits its transcriptional activity (Weiss et al. 2003). Specifically, phosphorylation of c-Jun by JNK1 also suppresses c-Jun multi-ubiquitination, reduces its degradation and enhances its stability (Musti et al. 1997). Conversely, JNK2 functions as a negative regulator of c-Jun stability and binds c-Jun in unstimulated cells to target it for degradation (Sabapathy et al. 2004). These data suggest that there are distinct roles for JNK1 and JNK2 in controlling c-Jun stability and function.

Phosphorylated c-Jun interacts with various binding partners to mediate transcription of target genes. Further, c-jun can homodimerize or heterodimerize with another transcription factor in the Fos family to form a complex termed activator protein 1 (AP-1) (Halazonetis et al. 1988). AP-1 aids in the induction of several genes by binding to AP-1 consensus sequences within a gene’s promoter region (Angel et al. 1987). Further, in genes of the immune system AP-1 can also cooperatively bind DNA with the nuclear factor of activated T cells (NF-AT) at NF-AT binding sites. This cooperative binding of DNA functions to increase the stability and lower the dissociation
rate of the AP-1:NF-AT complex for DNA compared to NF-AT alone (Jain et al. 1992; Jain et al. 1993).

The AP-1 complex, formed with c-Jun has a well-appreciated role in promoting proliferation (Figure 12). In fact, c-Jun deficient mice are embryonic lethal. However, fibroblasts derived from c-Jun deficient embryonic murine cells demonstrate a severe proliferation defect and inefficient G1-to-S phase progression (Schreiber et al. 1999). Further, c-Jun can function as an oncogene and is highly overexpressed in many cancers (Jiao et al. 2010; Zhang et al. 2015; Meyer-ter-Vehn et al. 2000).

JunB.

JNK phosphorylates JunB on threonine 102/104 residues to potentiate its transcriptional activity (Adamson et al. 2000). Like c-Jun, JunB can form a homodimer or a heterodimer with a member of the Fos family to compose AP-1. AP-1 complexes containing JunB accumulate in Th2 cells, but not Th1 cells (Rincon & Flavell 1997). Further, stimulation of murine T cells expressing a JunB transgene under Th1 polarizing conditions (IL-2, IL-12 and a neutralizing anti-IL-4 antibody) resulted in the production of both Th1 (IFN-γ) and Th2 cytokines (IL-4 and IL-5) (Li et al. 1999). Similarly, JunB deficient murine CD4^+ T cells stimulated under Th2 polarizing conditions (IL-4 and a neutralizing anti-IL-12 antibody) exhibited aberrant GATA3 expression and significantly reduced expression of IL-4, IL-5 and IL-10 upon TCR stimulation (Hartenstein et al. 2002). Another study demonstrated that murine CARMA1 deficient CD4^+ T cells exhibit reduced JNK activity, reduced levels of JunB and GATA3 and significantly reduced expression of IL-4, IL-5 and IL-10 upon TCR stimulation (Blonska et al. 2012). Taken together, these data
suggest that JNK-activated JunB transcriptional activity is critical in establishing the Th2 lineage and cytokine profile (Figure 12).

**JunD.**

JunD can be phosphorylated by both JNK and ERK1/2 at serine 90/100 and threonine 117 (Gallo et al. 2002). Consistent with the other Jun family proteins, JunD can also form a homodimer or a heterodimer with a member of the Fos family to compose AP-1. As mentioned above, it is well appreciated in fibroblasts that c-Jun enhances cell growth. However, JunD antagonizes c-Jun activity and functions as a negative regulator of cell growth (Weitzman et al. 2000; Pfarr et al. 1994). Further, splenic T cells from JunD deficient mice stimulated in the presence of anti-CD3 and anti-CD28 antibodies exhibited a marked increase in proliferation compared to cells from wild type mice (Meixner et al. 2004). Conversely, more recent data in T cells demonstrated that IL-7 induced JNK activity that increased JunD containing AP-1 complexes, which were shown to upregulate IL-7 induced genes involved in cell survival, metabolism and growth (Ruppert et al. 2012). Because IL-7 is a critical cytokine for T cell growth and homeostasis, these data suggest that IL-7 mediated T cell growth involves JunD/AP-1 signaling (Figure 12).
Figure 12. The Jun Family of Transcription Factors Function in T cells. Jun proteins form homodimers (not shown here) or heterodimers with Fos proteins to create the AP-1 complex. Depicted here are the known functions for c-Jun, JunB and JunD containing AP-1 complexes in T cells.

The Function of JNK1 and JNK2 in T cells

The specific differential or redundant roles of JNK1 vs JNK2 in T cells is still under intense investigation. However, studies have determined that JNK1 and JNK2 contribute to T cell activation, differentiation and apoptosis and will be reviewed below.

JNK1 and JNK2 in T cell Activation.

Upon TCR stimulation, a massive signaling cascade commences that results in the upregulation of many genes. Further, both Jnk1 and Jnk2 expression have been
shown to increase in response to T cell activation in primary murine naïve CD4+ T cells (Weiss et al. 2000). This serves as a mechanism to regulate JNK function, as Jnk1 and Jnk2 levels were undetectable in resting naïve CD4+ T cells. The kinetics of JNK functions from gene expression, subsequent JNK activation and exertion of kinase activity were shown to require 24 hours after anti-CD3 and anti-CD28 antibody stimulation (Rincon et al. 1997; Weiss et al. 2000).

One function of JNK post T cell activation is to stabilize IL-2 mRNA. IL-2 is a critical T cell growth factor produced upon T cell stimulation. In unstimulated T cells, IL-2 mRNA has a relatively short half-life but is prolonged after TCR stimulation (Lindstein et al. 1989). Inhibition of the JNK signaling pathway using a pharmacological JNK inhibitor decreased the half-life of the IL-2 mRNA in a dose-dependent manner in stimulated Jurkat T cells (Chen et al. 1998). Further, inhibition of the ERK or p38 signaling pathways did not affect the half-life of the IL-2 mRNA. These data suggest that JNK signaling plays a non-redundant role in T cell activation induced IL-2 mRNA stabilization.

Many studies have been performed to determine the role of JNK1 and/or JNK2 in the production of cytokines and proliferation upon T cell activation. One study demonstrated that JNK2 deficient murine peripheral T cells are defective in IL-2, IL-4 and IFN-γ production 36 hours post stimulation with anti-CD3 and anti-CD28 antibodies (Sabapathy et al. 1999). Further, insufficient IL-2 production from JNK2 deficient T cells rendered them defective in proliferation in response to low-levels of TCR and co-stimulation, but not at high levels. This could be explained by a possible partial compensation of JNK2 activity by JNK1, as JNK signaling, as assessed by AP-1
activity, was not completely abolished in JNK2 defective T cells. However, another study showed that murine T cells from JNK1 deficient mice exhibited the same reduction in IL-2 production and proliferation in response to the lower doses of stimuli as JNK2 deficient cells, and that addition of exogenous IL-2 rescued the proliferation defect (Sabapathy et al. 2001). Taken together, these data suggest that JNK1 and JNK2 have similar functions controlling IL-2 production and subsequent proliferation upon T cell stimulation. Although, this conclusion was challenged by another group who demonstrated that CD4+ T cells from mice expressing a dominant negative JNK1 in a JNK2 deficient background (dnJNK1+Jnk2−/−) stimulated with anti-CD3 and anti-CD28 antibodies produced more IL-2 protein and messenger RNA compared to CD4+ T cells from wild type mice (Dong et al. 2000). It is clear that much work remains to be performed to fully elucidate the roles of JNK1 and JNK2 in CD4+ and CD8+ T cell activation.

**JNK1 and JNK2 Signaling in T cell Differentiation.**

JNK signaling contributes to Th1 and Th2 differentiation and effector functions. Indeed, Th1 and Th2 cells contain high levels of both JNK1 and JNK2 (Yang et al. 1998). T cells from JNK1 deficient mice stimulated under neutral conditions demonstrate enhanced production of Th2 cytokines and increased JunB protein compared to T cells from wild type mice (Dong et al. 1998). As stated in the section above, JunB is critical for IL-4 and IL-5 production to establish the Th2 lineage. Further, T cells from JNK1 deficient mice stimulated under Th1 polarizing conditions exhibited no defect in the ability to produce Th1 cytokines, however, these cells also simultaneously made Th2 cytokines (Dong et al. 1998). In line with these data, CD4+ T cells from JNK2
deficient mice stimulated under Th1 or Th2 polarizing conditions revealed that there was no Th2 defect, however, IFN-γ production was significantly reduced and expression of IL-12 receptor β2 (IL-12Rβ2) was impaired compared to wild type Th1 cells (Yang et al. 1998). Taken together, these data suggest that JNK1 functions to repress the Th2 lineage by degrading JunB while JNK2 promotes Th1 differentiation by upregulating IL-12Rβ2 to enhance IFN-γ expression (Figure 13). However, much more work needs to be performed to fully understand how JNK1 and JNK2 contribute to other T cell subsets.

![Diagram](image.png)

**Figure 13. JNK1 and JNK2 in Th1 and Th2 Differentiation.** The model derived from studies that have determined that JNK1 is inhibitory for Th2 differentiation while JNK2 promotes the Th1 lineage. Modified from (Rincón & Pedraza-Alva 2003).

**JNK1 and JNK2 Signaling in T cell Apoptosis**

JNK plays a critical role in mediating apoptotic pathways in a cell type and stimuli dependent manner. This dissertation will focus on TCR and costimulatory signals for
JNK-mediated apoptosis in T cells. To this end, double positive (DP) thymocytes from JNK2 deficient mice administered an anti-CD3 antibody exhibited a partial resistance from apoptosis compared to DP thymocytes from wild type mice (Sabapathy et al. 1999). Similarly, T cells from JNK1 deficient mice had a moderate reduction in activation-induced cell death (AICD) compared to T cells from wild type mice (Dong et al. 1998). Further, T cells from mice deficient in JNK signaling through c-Jun (c-Jun dominant negative) were more resistant to apoptosis at high doses of anti-CD3 antibody stimulation in vitro and in vivo compared to T cells from wild type mice (Dong et al. 1998). Collectively, these data provide evidence for the contribution of both JNK1 and JNK2 to apoptosis in thymocytes and peripheral T cells.

**Purpose of Dissertation**

Previous work in our lab has focused on TGF-β-mediated effects on T cells. We identified that CD14⁺ CD36⁺ monocytes from umbilical cord blood (UCB) induce Foxp3⁺ CD4⁺ and CD8⁺ T cells from UCB in a TGF-β-dependent manner (Lee et al. 2018). These studies further identified that naïve T cells from UCB have a greater propensity to become Foxp3⁺ T cells compared to naïve T cells from adult blood stimulated under the same polarizing conditions. These data suggested that naïve T cells from UCB and adult peripheral blood possess intrinsic differences that influence their capacity to differentiate into Foxp3⁺ cells under these conditions. We sought to further characterize the differences between naïve T cells from UCB and adult peripheral blood. We hypothesized that there are other functional or phenotypic differences between naïve T cells from UCB and adult peripheral blood, based on the differences between the perinatal and adult immune systems (reviewed above). To test this, we characterized
the naïve phenotype, cytokine profile and differentiation capacity between naïve T cells from UCB and adult peripheral blood.

In addition to the role of TGF-β in the differentiation of Foxp3+ Tregs, TGF-β also has pleiotropic roles in T cell functions. As reviewed above, TGF-β signaling is important for the differentiation of some T cell subsets, the inhibition of other T cell subsets, and the inhibition of T cell activation and proliferation. However, the molecular mechanisms by which each function of TGF-β signaling is mediated in T cells remains poorly understood. We sought to identify the direct targets of, or molecules affected by TGF-β signaling in T cells. Identification of molecules within TGF-β-mediated signaling pathways would afford us a more complete understanding of how TGF-β signaling regulates T cell function. To identify novel targets of TGF-β signaling in T cells, we generated a TGF-β type I receptor (TGF-βRI) deficient Jurkat T cell line. We used this cell line as a model system to characterize the phenotypes of T cells that can no longer respond to TGF-β signaling. We speculated that this system would allow us to identify previously un-described molecules and pathways that are regulated by TGF-β signaling in T cells.
CHAPTER TWO
MATERIALS AND METHODS

Cell Preparation and Reagents

Whole umbilical cord blood (UCB) was kindly donated from Gottlieb Memorial Hospital and Loyola University Medical Center from donors that meet our collection criteria (Exclusion criteria: 1. evidence of active malignancies; 2. use of medications that affect the immune system- such as glucocorticoids and immunosuppressants; 3. uncontrolled hyper or hypothyroidism; 4. presence of an autoimmune disease; 5. presence of active infection). Adult peripheral blood was obtained from National Institute of Health. Naïve CD4 T cells were isolated from mononuclear cells enriched from UCB or adult blood via negative selection using an EasySep Human Naïve CD4+ T Cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada). Total T cells were also isolated from mononuclear cells enriched from adult blood using the panning method. Briefly, total monocular cells from adult blood were stained with anti-CD19 antibody (Biolegend, San Deigo, CA), washed and were allowed to adhere to plate bound goat anti-mouse immunoglobulin. Twenty-minutes post incubation at 37°C, non-adhered total T cells were carefully collected. Isolated naïve CD4 T cells from UCB and adult blood were seeded at 0.3 x 10^6 cells per 96 well round bottom in RPMI 1640 (GE Healthcare Hyclone, Chicago, IL) supplemented with 10% fetal calf serum (Gemini Bioproducts LLC, West Sacramento, CA), L-glutamin (Hyclone, Logan, UT), 10% solution of penicillin/streptomycin (Hyclone, Logan, UT), 1mM sodium pyruvate
(Corning, Corning, NY), 0.1 M hepes (Corning, Corning, NY), non-essential amino acids (Gibco, Waltham, MA), essential amino acids (Corning, Corning, NY), and 50 µM 2-ME (Fisher Scientific, Waltham, MA) in the presence of recombinant human IL-7 (20 ng/mL; PeproTech, Rocky Hill, NJ). Media was changed every 2–3 days and IL-7 concentrations were maintained throughout. Cells were maintained in the presence of 5% CO2 at 37°C.

Jurkat T cells were a gift from Dr. Arthur Weiss (UCSF, San Francisco, CA). Jurkat T cells were maintained in RPMI 1640 medium with identical FBS, supplements and antibiotics as above.

C57/BL6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were maintained under specific pathogen-free condition. Splenic total T cells were purified by depletion of non-T cells by the panning method. Briefly, splenocytes were applied and allowed to adhere to plate bound goat anti-mouse immunoglobulin. Twenty-minutes post incubation at 37°C, non-adhered total T cells were carefully collected and maintained in RPMI 1640 medium with identical FBS, supplements and antibiotics as described above.

**Phenotype Analysis and Cytokine Profile of Naïve CD4+ T cells**

On the day of isolation (day 0) and day 7 of maintenance in IL-7, the naïve phenotype was assessed by staining with anti-CD4, anti-CD45RA (BioLegend, San Diego, CA), anti-CD45RO (BD Biosciences, San Jose, CA), anti-CD26, and anti-CD31 (BioLegend, San Diego, CA) antibodies and analyzed on a BD FACSCANTO II Flow Cytometer (BD Biosciences). Additionally, on day 0, 1 x 10^6 cells were stimulated in the presence of fresh media, phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Fisher
Scientific, Hampton, NH) and ionomycin (1 µM; Sigma-Aldrich, St. Louis, MO) for 4 hours. After stimulation, cell supernatants were harvested and analyzed for the expression of IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-13 using the LEGENDplex Human Th Cytokine Panel (BioLegend) on a BD FACSCANTO II Flow Cytometer (BD Biosciences).

**Th1 and Th2 Differentiation Assays**

Isolated naïve CD4+ T cells from UCB and adult blood were maintained in IL-7 for 7 days, then were stimulated with anti-CD3 (OKT3; 5µg/ml; BioLegend) and anti-CD28 (28.2; 5µg/ml; BioLegend) and differentiated in the presence of IL-2 alone (neutral), T helper type 1 (Th1), or T helper type 2 (Th2) inducing reagents using a Human Th1 or Th2 Differentiation Kit (R&D Systems, Minneapolis, MN) according to the manufacture’s protocol. Cells were harvested on day 5 for the Th1 differentiation assay and day 13 for the Th2 differentiation assay and washed once in media. 1 x 10^6 cells from each assay were then stimulated in the presence of fresh media, PMA and ionomycin for 4 hours. After stimulation, cell supernatants were harvested and analyzed for the expression of IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-13 using the LEGENDplex Human Th Cytokine Panel (BioLegend) on a BD FACSCANTO II Flow Cytometer.

**Generation of a TGF-βRI Deficient Jurkat T cell Line using CRISPR/cas9 Genome Editing**

Generation of a TGF-βRI deficient Jurkat T cell line was achieved by co-transfection of three pCRISPR-SG01 vectors encoding CRISPR guide RNA (gRNA) against human TGF-βRI (GeneCopoeia, Rockville, MD). The sequences for the three
corresponding CRISPR gRNAs against TGF-βRI are as follows: 1) 5'-CCTCTAGAGAAGACGTTCG-3', 2) 5'-AATGCTCGACGATGTTCCAT-3', and 3) 5'-CGGCGAGCGGTCTTGCCCATC-3'. Cells were first transfected with a human codon-optimized expression vector for cas9, pCAG-hCas9 (Addgene 51142). The pCAG-hCas9 vector was a gift from Dr. Izuho Hatada (Horii et al. 2013). Transfection was accomplished by electroporation using a Gene Pulser II electroporation system (260V, 800uF) (Bio-Rad Hercules, CA). Forty-eight hours post transfection cells were selected in neomycin and Cas9 expression was confirmed by western blot. Cas9 expressing cells were then co-transfected with the three CRISPR gRNA against TGF-βRI. Forty-eight hours post transfection cells were single cell sorted and seeded in 96 well plates using a FACS Aria Cell Sorter (BD bioscience, San Jose, CA). Cells were cultured in the presence of hygromycin (for gRNA) and neomycin (for pCag-hCas9). After selection, TGF-βRI protein deficiency was assessed by western blot and TGF-βRI loss of function was assessed by flow cytometry.

**Stimulation of the TGF-βRI Deficient Jurkat T cell Line**

For overnight stimulation, the TGF-βRI deficient line and control Jurkat cas9 only were stimulated in the presence of plate bound anti-CD3 antibody (OKT3, 5µg/ml, Biolegend, San Diego, CA) overnight in the presence of 5% CO2 at 37°C. After stimulation, cells were harvested and the supernatant was collected. Where indicated, cells were stimulated overnight in the presence of active TGF-β (10µg/ml, R&D Systems, Minneapolis, MN). The phoshpo-Smad2/3 expression and activation phenotype were assessed by flow cytometry and the production of cytokines was
assessed using the LEGENDplex Human Th Cytokine Panel (BioLegend, San Diego, CA) on a BD FACSCANTO II Flow Cytometer.

For the time-course stimulation, the TGF-βRI deficient line and control Jurkat cas9 only were stimulated in the presence of plate bound anti-CD3 antibody (OKT3, 5µg/ml, Biolegend, San Diego, CA). Two-hours and four hours post stimulation, cells were harvested and lysed for nuclear and cytoplasmic fractionation using 1% Triton X-100 lysis buffer (as described below) and molecules were assessed by western blot.

For the shorter stimulation, the TGF-βRI deficient line and control Jurkat cas9 only were stimulated in the presence of a soluble anti-Jurkat TCR antibody (C305, a kind gift from Dr. A. Weiss) for 5 minutes in a 37°C water bath. After stimulation, cells were harvested and lysates were prepared to assess molecules by western blot.

**Stimulation of the Primary Human 1383I TCR T cell Line**

The 1383I TCR T cell line are tumor infiltrating lymphocytes (TILs) that are HLA-A2 restricted and reactive against the melanoma antigen tyrosinase (368-376). These cells were a kind gift from Dr. Michael Nishimura (Nishimura et al. 1999). The 1383I T cell line was stimulated in the presence of the antigen presenting cell line T2 (ATCC, Manassas, VA). Before co-culture with T cells, the T2s were pulsed with tyrosinase peptide 368-376 (YMDGTMSQV) (Molecular Resources, Fort Collins, CO) by peptide incubation with T2s for two hours in the presence of 5% CO2 at 37°C. T cells to T2s were co-cultured at a 1:1 ratio in the presence or absence of exogenous active TGF-β (10 ng/ml, R&D Systems, Minneapolis, MN). After overnight stimulation, cells were harvested and activation-associated surface markers were assessed by flow cytometry.
Western Blot Analysis

Cells were harvested and whole cell lysates were prepared at 1 x 10^6 cells/50μL in sodium dodecyl sulfate (SDS) lysis buffer (2% SDS, 125 mM DTT, 10% glycerol, 62.5 mM Tris-HCL (pH 6.8). Where indicated, the nuclear and cytoplasmic fractions were extracted by lysing cells in 1% Triton X-100 lysis buffer (10mM phosphate buffer, 150 mM NaCl, 0.1% SDS, protease and phosphatase inhibitors). Proteins were separated using SDS-PAGE gels and transferred onto PVDF membranes. Membranes were probed with the following antibodies: anti-TGF-βRI, anti-phospho JNK, anti-JNK, anti-phospho ERK, anti-ERK, anti-phospho-MKP-1, anti-phospho-MKK7, total MKK7, anti-CARMA1, anti-TAK1, anti-TRAF6, anti-ASK1 (Cell Signaling Technologies, Danvers, MA); anti-Bcl10 (Biolegend San Diego, CA); anti-c-Jun, anti-MKP-1, anti-POSH, anti-JIP, anti-phospho-PKC-θ, anti-PKC-θ (Santa Cruz Biotechnology, Dallas TX) and anti-β-actin (Sigma Aldrich, Milwaukee, WI). Detection of signals was achieved using the ECL system (GE Healthcare, Piscataway, NJ). The relative intensity of each band (indicated under each lane) was normalized to β-actin as the control, or the unphosphorylated form for phosphorylated proteins, and determined by ImageJ software (National Institutes of Health).

Flow Cytometry

Fluorochrome-conjugated antibody specific for phospho-Smad 2/3 was from BD bioscience (San Jose, CA). Anti-CD25, anti-CD69, and anti-PD-1 antibodies were from Biolegend (San Diego, CA). For the surface stain, cells were stained on ice for 30 minutes. Cells were analyzed on a BD FACSCANTO II Flow Cytometer.
Reporter Assays in the TGF-βRI Deficient Jurkat T cell Line and Primary Human T cells

The TGF-βRI Deficient Jurkat T cell line and Jurkat cas9 only control cells were transfected with the transcriptional activity reporter constructs AP-1 Firefly Luciferase (Takara Shiga, Japan) or NF-AT Firefly Luciferase, a gift from Dr. Gerald Crabtree (Sanford University, Sanford, CA) as well as a control Renilla Luciferase construct (Iwashima et al. 2002). Transfection was performed by electroporation using a Gene Pulser II electroporation system (260V, 800uF) (Bio-Rad Hercules, CA). Twenty-four hours post transfection cells were stimulated with plate-bound anti-CD3 antibody (OKT3, 5µg/ml, Biolegend, San Diego, CA) overnight. After stimulation, cells were lysed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) following the manufacture’s instructions. Transfection efficiency was normalized using the activity of the co-transfected Renilla Luciferase in unstimulated samples.

To assess NF-AT activity using the NF-AT-GFP transcriptional activity reporter construct, the TGF-βRI Deficient Jurkat T cell line and Jurkat cas9 only control cells were transfected with NF-AT-GFP, generated by fusing three tandem NF-AT binding sites with enhanced GFP cDNA (Ohtsuka et al. 2004), in the presence of a control mCherry only vector, a kind gift from Dr. Edward Campbell (Loyola University Chicago, Chicago, IL). Twenty-four hours post transfection cells were stimulated with plate-bound anti-CD3 antibody overnight. After stimulation, cells were harvested and NF-AT-GFP activity was assessed by flow cytometry. The analysis was performed by gating on mCherry positive cells as a transfection control first, then assessing NF-AT-GFP expression in mCherry positive cells.
For primary T cell experiments, primary total T cells from adult blood were transfected with a constitutively active TGF-βRI construct pcDNA3-ALK5 T204D, a gift from Aristidis Moustakas (#80877 Addgene, (Moren et al. 2005)), or a dominant negative TGF-βRI construct pCMV5B-TGF-beta receptor I K232R, a gift from Jeff Wrana (#11763 Addgene, (Moren et al. 2005)), or a backbone control vector generated from the pCMV5 TBRI-His, a gift from Joan Massague (#19161 Addgene), by the removal of the wild type TGF-βRI insert. Along with the TGF-βRI constructs, cells were also co-transfected with the AP-1 Firefly Luciferase or NF-AT Firefly Luciferase construct in the presence of the control Renilla Luciferase vector. Transfection was performed using the Human T Cell Nucleofector Kit (Lonza, Walkersville, MD), according to the manufacturer’s protocol. Forty-eight hours post transfection cells were divided and some cells were stimulated with plate-bound anti-CD3 (OKT3, 5µg/ml, Biolegend, San Diego, CA) and anti-CD28 (28.2, 5µg/ml, Biolegend) antibodies overnight. After this stimulation, another set of transfected cells were stimulated with 50ng/ml phorbol 12-myristate 13-acetate (PMA) and 1µM ionomycin for 6 hours. After 6 hours, both set of stimulated cells were lysed and analyzed as described above.

**RNA Sequence Analysis of TGF-βRI Deficient Jurkat T cell Line**

Unstimulated TGF-βRI deficient line and unstimulated Jurkat cas9 only control cells were lysed and RNA was isolated using the RNAAqueous Total RNA Isolation Kit (Thermo Fisher Scientific Waltham, MA) following manufacture’s instructions. RNA sequencing was performed at UT Health in San Antonio Texas by Dr. Zhao Lai using the Illumina HiSeq 3000 platform. Reads were analyzed using the Galaxy bioinformatics software (Cyverse at the Texas Advanced Computing Center).
Activation and Proliferation of Primary Mouse T cells

For the activation assay, total T cells from mice were stimulated with anti-CD3 (OKT3, 5µg/ml, Biolegend) in the absence or presence of active TGF-β (2.5ng/ml, R&D Systems, Minneapolis, MN). On day 3 post stimulation cells were harvested and counted. Cells were then analyzed on a BD FACSCANTO II Flow Cytometer to assess the frequency of CD4+ and CD8+ T cells. Cell count/proliferation for CD4+ and CD8+ T cells was assessed by multiplying the frequency of each subset by the raw cell number.

For western blot analysis, CD8+ T cells were stimulated as described above. Three days post stimulation total SDS western blot lysates were made as described above. CARMA1 and Bcl10 were assessed by western blot.

Statistical Analysis

Statistical significance for experiments comparing two conditions, a (unpaired or paired) Student’s t test was used. For experiments with multiple samples comparisons, p values were calculated using an ANOVA with Tukey’s multiple comparisons test. These analyses were performed using the GraphPad Prism software (San Diego, CA).
CHAPTER THREE

RESULTS

SECTION 1: Comparison of Naïve CD4+ T cells from Umbilical Cord Blood to Naïve CD4+ T cells from Peripheral Adult Blood

CD26 is Highly Expressed by Expression of CD26 by Naïve CD4+ T cells from Umbilical Cord Blood

To determine the cellular and molecular differences between the perinatal and adult immune systems, we sought to characterize the phenotype and functions of naïve CD4+ T cells from umbilical cord blood (UCB) compared to adult peripheral blood. We first determined the surface antigen expression of the naïve CD4+ T cells (as defined by CD45RA+ CD45RO-) from each group. Assessment of surface antigens that are known to be expressed by T cells revealed a significant difference in the expression of CD26. We identified that close to 100% of CD45RA+ naïve CD4+ T cells from UCB express high levels of CD26. Conversely, a fraction of CD45RA+ naïve CD4+ T cells from adult blood express low levels of CD26 while CD45RA- CD45RO+ T cells express high levels of CD26 (Figure 14A and 14B). CD26 is an enzymatic protein with multiple functions, including participating in T cell co-stimulation as well as binding adenosine deaminase (Bailey et al. 2017).

To understand the high expression of CD26 in CD45RA+ naïve CD4+ T cells from UCB, we reasoned that these cells could be Recent Thymic Emigrants (RTEs). It is known that CD26 expression is upregulated as thymocytes mature, with the highest
expression observed in mature CD4 or CD8 single positive T cells in the thymus (Ruiz et al. 1998). Further, expression of the cell surface marker CD31 on CD45RA+ naïve CD4+ T cells is a marker for RTEs (Kimmig et al. 2002). Therefore, if CD45RA+ naïve CD4+ T cells from UCB express high levels of CD26 because they are RTEs, then we expect that CD26hi CD45RA+ naïve T cells co-express CD31 in cells from both UCB and adult blood. We identified that CD31 is expressed on CD45RA+ cells from both UCB and adult blood, unlike the expression of CD26 (Figure 14C). Interestingly, CD26hi expressing T cells from UCB co-express CD31, while T cells from adult blood do not co-express CD26 and CD31 (Figure 14D an 14E). These data suggest that CD26 expression is not strictly associated with RTEs.

It is appreciated that CD26 participates in T cell co-stimulation and is upregulated after antigen stimulation (Ohnuma et al. 2008). Therefore, we hypothesize that anti-TCR stimulation will increase CD26 expression. To test this, we stimulated total CD4+ T cells with plate-bound anti-CD3 and anti-CD28 antibodies (Figure 14F). We observed that naïve CD4+ T cells from adult blood exhibited an increase in CD26 expression upon stimulation, while CD26 expression from naïve CD4+ T cells from UCB did not significantly change upon stimulation. Taken together, these data suggest that CD26 serves as a unique surface antigen expressed by naïve CD4+ T cells from UCB and is differentially regulated in naïve CD4+ T cells from UCB compared to naïve CD4+ T cells from adult blood.
Figure 14. Expression of CD26 and CD31 in CD4⁺ T cells from UCB and Adult Blood. CD4⁺ T cells were isolated from umbilical cord blood (UCB) and adult peripheral blood and surface markers were assessed by flow cytometry. **A**, CD26 expression was assessed on CD45RA positive and CD45RO positive T cells from UCB and adult blood. **B**, Quantification of data obtained in **A**. Each dot represents data from an independent experiment. Data were analyzed using a Student's t-test. *P<0.05. **P<0.005. **C**, CD31 expression was assessed on CD45RA positive T cells from UCB and adult blood. **D**, CD31 expression was assessed on CD26 high T cells from UCB and adult blood. **E**, Quantification of data obtained in **D**. Each dot represents data from an independent experiment. Data were analyzed using a Student's t-test. *P<0.05. **P<0.005. **F**, CD4⁺ T cells from UCB and adult blood were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies overnight and expression of CD26 on CD45RA positive cells was assessed.
**IL-7 Induced Expansion of Naïve CD4⁺ T cells from UCB**

We next sought to determine if there are intrinsic differences between naïve CD4⁺ T cells from UCB and adult blood in their ability to respond to IL-7. IL-7 is an anti-apoptosis and survival cytokine for naïve T cells and is known to play a critical role in the homeostatic expansion of T cells (Fry & Mackall 2001). Further, IL-7 is required to *ex vivo* expansion of naïve and memory T cells (Tan et al. 2001). Previous studies have also demonstrated that CD31⁺ human naïve T cells proliferate in the presence of IL-7 (Azevedo et al. 2009). We hypothesized that because naïve CD4⁺ T cells from UCB are mostly CD31⁺, they will respond differently to IL-7 when compared to naïve CD4⁺ T cells from adult blood, where only a fraction of cells are CD31⁺. To test this, we purified naïve CD4⁺ T cells from UCB and adult blood and maintained them in the presence of IL-7 *in vitro*. After 7 days of culture, we harvested and analyzed the T cells for their surface antigen expression and cellularity (Figure 15 2A and 2B). We observed that naïve CD4⁺ T cells from both UCB and adult blood maintained their CD45RA/CD45RO expression after 7 days in culture in the presence of IL-7. Further, naïve CD4⁺ T cells from adult blood did not proliferate in the presence of IL-7 but maintained their cellularity. Conversely, naïve CD4⁺ T cells from UCB expanded approximately 2-fold in 1 week in the presence of IL-7. These data confirm that naïve CD4⁺ T cells from UCB respond differently to IL-7 when compared to naïve CD4⁺ T cells from adult blood, which could be due to their short durance after thymic egress.
Figure 15. Phenotype and Cellularity of Naïve CD4$^+$ T cells from UCB and Adult Blood Maintained in IL-7. Naïve CD4$^+$ T cells were isolated from umbilical cord blood (UCB) and adult peripheral blood and maintained in the presence of IL-7 for seven days. 

A. On the day of isolation (day 0) and after 7 days in the presence of IL-7, cells were harvested and expression of CD45RA and CD45RO was assessed by flow cytometry.

B. Percentage of CD45RA positive and CD45RO positive cells from UCB and adult blood were quantified.

C. Naïve CD4$^+$ T cells were counted and seeded on day 0. On day 7, cells were counted again and the percent difference from day 0 was assessed. Each dot represents data from an independent experiment. Data were analyzed using a Student's t-test. **P<0.005.
Cytokine Profile and Differentiation Ability of Naïve CD4$^+$ T cells from UCB

We tested if naïve CD4$^+$ T cells from UCB are intrinsically different than CD4$^+$ T cells from adult blood by assessing the type of cytokines they produce. Previous studies demonstrated that perinatal T cells from mice have an elevated propensity to express Th2 type cytokines, such as IL-4, IL-5, and IL-10 (Opiela et al. 2008; Chen et al. 1995). Further, one study reported that CD26$^+$ T cells produce IL-17 (Bengsch et al. 2012). Therefore, to investigate the cytokine profile of naïve T cells from UCB, we stimulated purified naïve CD4$^+$ T cells from UCB and adult blood with PMA and ionomycin for 4 hours and assessed cytokine profiles. We observed that naïve T cells from adult blood produce IL-2, TNF, and IFN-$\gamma$ (Figure 16). Naïve T cells from UCB also produce IL-2 and TNF, but significantly lower levels of IFN-$\gamma$ (Figure 16). Further, naïve CD4$^+$ T cells from UCB did not produce detectable levels of IL-4, IL-5 or IL-13 (not shown). In addition, we did not observe detectable levels of IL-17 production by naïve CD4$^+$ T cells from UCB or adult blood. These data suggest that naïve CD4$^+$ T cells from UCB do not produce Th1 or Th2 cytokines at a comparable level to naïve CD4$^+$ T cells from adult blood and are not pre-programmed to produce Th2 cytokines.
Figure 16. Cytokine Profile of Naïve CD4\(^+\) T cells from UCB and Adult Blood. Naïve CD4\(^+\) T cells were isolated from umbilical cord blood (UCB) and adult peripheral blood and stimulated with PMA and ionomycin for 4 hours. After stimulation, the supernatant was collected and cytokine production was assessed by a Th1/Th2/Th17 cytometric bead array. Each dot represents data from an independent experiment. Data were analyzed using a 1-way ANOVA with Tukey’s multiple comparisons test. *P<0.05.

We next sought to determine if naïve CD4\(^+\) T cells from UCB have an elevated propensity to become Th2 type T cells compared to naïve CD4\(^+\) T cells from adult blood. To investigate this, we stimulated in vitro maintained naïve CD4\(^+\) T cells from UCB and adult blood under neutral (Th0), Th1 or Th2 inducing conditions. Cells were then harvested and re-stimulated using PMA and ionomycin. After the 4-hour stimulation, the supernatant was collected and the cytokine profile was assessed (Figure 17A – C). We observed no significant difference in the cytokine profiles between naïve CD4\(^+\) T cells from UCB and adult blood under Th0 conditions. However, the Th1 polarizing conditions induced much greater levels of IFN-\(\gamma\) production in CD4\(^+\) T cells from UCB compared to CD4\(^+\) T cells from adult blood (Figure 17B). In addition, Th1 polarizing conditions induced comparable levels of TNF production from CD4\(^+\) T cells from UCB and adult blood. However, Th2 polarizing conditions induced lower levels of IL-4 production by CD4\(^+\) T cells from UCB compared to adult blood (Figure 17C). Further, CD4\(^+\) T cells from UCB produced higher levels of IL-5 and IL-13 compared to adult blood,
demonstrating a dichotomy among the Th2 cytokines. Therefore, these data suggest that naïve CD4 T cells from cord blood differ from adult naïve CD4 T cells in their ability to produce Th1 vs Th2 type cytokines. While cord blood naïve T cells are less effective in producing cytokines in the early stage of antigen stimulation, they can acquire a higher capacity than adult T cells to produce both Th1 (IFN-γ) and Th2 (IL-5, IL-13) type cytokines when exposed to Th1 or Th2 polarizing conditions.
Figure 17. Cytokine Profile of Naïve CD4\(^+\) T cells from UCB and Adult Blood under Th1 and Th2 inducing Conditions. Naïve CD4\(^+\) T cells were isolated from umbilical cord blood (UCB) and adult peripheral blood and maintained in the presence of IL-7 for 7 days. Cells were then stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies under A) neutral (Th0), B) Th1 polarizing conditions or C) Th2 polarizing conditions. Cells were then re-stimulated with PMA/ionomycin for 4 hours. The supernatant was collected and cytokine production was assessed by a Th1/Th2/Th17 cytometric bead array. Data were analyzed using a 1-way ANOVA with Tukey’s multiple comparisons test. *P<0.05. **P<0.005
SECTION 2: Identification of TGF-β Signaling Targets in Activated T cells

Generation of TGF-βRI Deficient Jurkat T cells

It is well appreciated that TGF-β signaling has pleiotropic effects in T cells, as discussed in Chapter 1, however, the molecular mechanisms by which TGF-β mediates the differentiation of T cell subsets that require TGF-β, the inhibition of other T cell subset lineages, and the suppression of T cell activation and proliferation are not well understood. Therefore, we sought to identify molecules downstream of TGF-β signaling to determine the precise molecules in the signaling pathways elicited by TGF-β signaling that mediate differentiation or suppression. To this end, we used the CRISPR/cas9 genome editing system to target the TGF-β type I receptor (TGF-βRI) in Jurkat T cells. We chose the TGF-βRI because this receptor is responsible for phosphorylating Smads and it participates in non-canonical TGF-β signaling (Zhang 2017). To generate TGF-βRI deficient cells, we transfected Jurkat with a construct encoding a human codon-optimized Cas9 endonuclease. Forty-eight hours post-transfection cells were selected in neomycin and Cas9 expression by neomycin-resistant cells was confirmed by western blot (Figure 18A). Next, Cas9 positive cells were co-transfected with three constructs containing three different CRISPR guide RNA against the cytoplasmic region of TGF-βRI (Figure 18B). Forty-eight hours post-transfection cells were selected in neomycin (for Cas9 expression) and hygromycin (for CRISPR guide RNA expression). Finally, selected cells were single cell sorted and clones were assessed for the loss of TGF-βRI expression. Western blot analysis revealed that the expression of TGF-βRI is significantly reduced in one CRISPR/cas9 applied Jurkat T cell clone compared to control Jurkat that were only transfected with
Cas9 (Figure 18C). We termed this clone the ‘TGF-βRI deficient line’. We used the term ‘deficient’ because we acknowledge that there is a faint but detectable band corresponding to the expression of TGF-βRI protein in this line (Figure 18C). We reasoned that this could be the result of an incomplete knockout, whereby only one allele in this line was cleaved by the cas9 endonuclease, which is a known limitation of the CRISPR/cas9 editing system. We next sought to determine the ability of the TGF-βRI deficient line to respond to TGF-β signaling. To test this, we stimulated cells overnight with plate-bound anti-CD3 antibody in the presence of exogenous active TGF-β. After stimulation we assessed phospho-Smad2/3 expression with the expectation that the TGF-βRI deficient line would have reduced phospho-SMAD2/3 expression compared to control. We observed that in the presence of active TGF-β the TGF-βRI deficient line exhibited a decreased level of phospho-SMAD2/3 (7%) compared to control Jurkat (~26%) (Figure 18D). Together, these data support the successful generation of a TGF-βRI deficient Jurkat T cell line.
Figure 18. Establishment of a TGF-βRI Deficient Jurkat T cell Line. A. Jurkat T cells were transfected with a human codon-optimized Cas9 construct and selected in neomycin. Cas9 expression was assessed in neomycin-resistant cells by western blot. B. The protein amino acid sequence for TGF-βRI (NP_004603.1). Domains and location of each CRISPR guide RNA are indicated. C. Cas9 expressing Jurkat T cells were transfected with three constructs containing three CRISPR guide RNAs against TGF-βRI. The expression of TGF-βRI was assessed by western blot analysis. The relative intensity of each band is shown below. D. The TGF-βRI deficient Jurkat T cell line was stimulated overnight with plate-bound anti-CD3 antibody in the presence of PBS (dashed line) or exogenous active TGF-β (solid line). Cells were stained for PSMAD2/3 expression and analyzed by flow cytometry.

The TGF-βRI Deficient Jurkat T cell Line Exhibits a Hyper-Activation Phenotype

After generating a tool to study how the absence of TGF-βRI signaling affects T cell functions, we determined the activation phenotype of the TGF-βRI deficient line. Because TGF-β signaling is known to suppress T cell activation and proliferation (Kehrl
et al. 1986; Marie et al. 2006), we expected that the TGF-βRI deficient line would have an increased activation phenotype. To test this, we stimulated the TGF-βRI deficient line with plate-bound anti-CD3 antibody overnight. Cells were then harvested and assessed for T cell activation surface markers and production of cytokines. Among the surface markers that are known to be expressed by activated T cells, the TGF-βRI deficient line expressed significantly higher levels of CD25 and CD69 upon stimulation compared to control cells (Figure 19A and B). Interestingly, upon stimulation the TGF-βRI deficient line exhibited a population of PD-1 low expressing cells and a population of PD-1 high expressing cells, and this PD-1 low expression population is not observed in control cells upon stimulation (Figure 19A). The cytokine profile upon stimulation in the TGF-βRI deficient cells revealed a significant increase in the production of IL-2 and TNF compared to control (Figure 19C). Other Th1, Th2, and Th17 type cytokines were not detected (data not shown). Taken together, these data suggest that upon stimulation, the TGF-βRI deficient line has a hyper activation phenotype compared to control cells.
Figure 19. Activation Phenotype and Cytokine Profile of the TGF-βRI Deficient Jurkat T cell Line. The TGF-βRI deficient Jurkat T cell line was stimulated overnight with the plate-bound anti-CD3 antibody. Cells were then harvested and activation-induced surface molecules were assessed by flow cytometry. A. Representative flow cytometry histograms showing expression of CD25, CD69 and PD-1. The dashed line (unstimulated) and solid line (stimulated). B. Percent increase in expression of each marker upon stimulation. Each dot represents data from an independent experiment. Data were analyzed using a paired Student’s t-test. *P<0.05. **P<0.005. C. The supernatant was collected one day after stimulation and IL-2 and TNF concentrations were determined by human Th1/Th2/Th17 cytometric bead array. Error bars represent SD. Data analyzed using a 2-way ANOVA with Tukey’s multiple comparisons test. ****P<0.0001.

Primary Human T cells Exhibit Low CD25 and High PD-1 Expression upon Activation in the Presence of TGF-β

Jurkat T cells are an immortal line derived from a patient with acute lymphoblastic leukemia (Schneider et al. 1977), and we sought to confirm the activation phenotype of the TGF-βRI deficient line using primary human T cells to ensure that the phenotypes observed are not specific to transformed cancer cells. To test this, we
obtained a human total T cell line (containing both CD4+ and CD8+ T cells) that was transduced with a T cell receptor (TCR) (termed 1383I) that recognizes the 368-376 epitope from tyrosinase in the context of (Histocompatibility Leukocyte Antigen) HLA-A2. We chose this line to mimic physiologically-relevant antigen-based stimulation instead of the more artificial anti-CD3 and anti-CD28 antibody stimulation. To this end, we stimulated the 1383I TCR T cells in the presence of a tyrosinase peptide-pulsed antigen presenting cell line, T2, in the presence or absence of exogenous active TGF-β. After overnight stimulation, cells were harvested and assessed for T cell activation surface markers. Based on activation data obtained from the TGF-βRII deficient line, we expected that the 1383I TCR T cells stimulated in the presence of active TGF-β would exhibit decreased expression of CD25 and CD69 with increased expression of PD-1 compared to cells stimulated in the absence of TGF-β. Indeed, we observed that upon stimulation in the presence of TGF-β, there was a significant decrease in CD25 expression and an increase in PD-1 expression in both CD4+ and CD8+ cells compared to control (Figure 20A and B). However, there was no difference in the expression of CD69 in the presence or absence of TGF-β in CD4+ or CD8+ cells (Figure 20A and B). These data suggest that in primary human T cells TGF-β signaling functions to suppress CD25 expression and enhance PD-1 expression. These findings coincide with data obtained in the TGF-βRII deficient line whereby, in the absence of TGF-β signaling, CD25 expression was enhanced and PD-1 expression was reduced. Further, these data are consistent with data showing that TGF-β signaling decreases CD25 expression on antigen-stimulated T cells to control proliferation by limiting IL-2 signaling (Tiemessen et al. 2004). These data are also consistent with recent reports demonstrating that TGF-β
signaling increases PD-1 expression on T cells (Park et al., 2016; Stephen et al., 2017). Also, the cytokine data obtained in the TGF-βRI deficient line are consistent with literature stating that TGF-β inhibits IL-2 and TNF-α production (Kehrl et al., 1986; Ahmadzadeh and Rosenberg, 2005). However, the link between TGF-β signaling and the expression of CD69 has yet to be fully elucidated in T cells.
Figure 20. Activation Phenotype in Primary T cells in the Presence of TGF-β.
A. A primary human 1383I TCR total T cell line was stimulated with tyrosinase pulsed T2s in the presence or absence of exogenous active TGF-β. After overnight stimulation, cells were harvested and the expression of CD25, CD69 and PD-1 was assessed on CD4+ or CD8+ cells by flow cytometry. Numbers on the histogram represent the percent difference (either increase or decrease) between unstimulated (dashed line) and stimulated (solid line) cells. B. Frequency of CD25, CD69 and PD-1 expression on CD4+ and CD8+ cells stimulated as in A. Each dot represents data from an independent experiment. Data were analyzed using a 2-way ANOVA with Tukey’s multiple comparisons test.*P<0.05. **P<0.005. Experiment performed by Aleksandra Vuchkovska.
Constitutively High AP-1 and High NF-AT Activity in the TGF-βRI Deficient Line

To understand the hyper activation phenotype observed in the TGF-βRI deficient line, we investigated two critical transcription factors that are upregulated upon T cell stimulation and drive activation phenotypes. Activator protein 1 (AP-1) and nuclear factor of activated T cells (NF-AT) are transcription factors that are activated upon T cell stimulation and function to mediate T cell differentiation and effector functions (Rincón & Flavell 1994; Northrop et al. 1994). We hypothesized that the hyper activation phenotype exhibited by the TGF-βRI deficient line is caused by increased activity of AP-1 and/or NF-AT. To test this, we transfected the TGF-βRI deficient line with AP-1 and NF-AT transcriptional activity luciferase reporter constructs and stimulated the cells with plate-bound anti-CD3 antibody overnight. After stimulation we harvested the cells and assessed AP-1 and NF-AT activity. Strikingly, we observed significantly high AP-1 activity in both the absence and presence of stimulation in the TGF-βRI deficient line (Figure 21A and B). Further, upon stimulation, there is significantly high NF-AT activity in the TGF-βRI deficient line (Figure 21A and B). To explain the constitutive activity of only AP-1, but not NF-AT, we considered that 1) NF-AT activation is mediated by kinases and phosphatases that are calcium dependent. An influx of this required calcium is only initiated upon T cell activation (Trebak & Kinet 2019) and 2) it is well established that NF-AT participates in co-operative binding of NF-AT binding sites with AP-1 to mediate its transcriptional activity (Jain et al. 1992; Feske et al. 2001). Therefore, T cell stimulation initiates calcium signaling that activates NF-AT transcriptional activity which co-operates with the already high activity of AP-1 resulting
in much greater NF-AT activity levels in the TGF-βRI deficient line compared to control under stimulated conditions. We further assessed NF-AT activity in the TGF-βRI deficient line using an NF-AT-GFP transcriptional activity reporter construct. To this end, we co-transfected the TGF-βRI deficient line with this NF-AT-GFP reporter construct with a mCherry only control construct. Cells were then stimulated overnight in the presence of anti-CD3 antibody. After stimulation, the cells were harvested, and NF-AT-GFP activity was assessed in mCherry positive cells. Based on the NF-AT luciferase reporter data, we expected there to be more NF-AT-GFP activity in the TGF-βRI deficient line compared to control. Indeed, we observed greater NF-AT activity as assessed by NF-AT-GFP in the TGF-βRI deficient line compared to cas9 only Jurkat (Figure 21 C and D). These data suggest that the hyper-activation phenotype of the TGF-βRI deficient line could be caused by the constitutive AP-1 activity and high NF-AT activity upon stimulation observed.
Figure 21. AP-1 and NF-AT Transcriptional Activity in the TGF-βRI Deficient Jurkat T cell line. A. The TGF-βRI deficient Jurkat T cell line was transiently transfected with NF-AT or AP-1 transcriptional activity reporter luciferase constructs in the presence of a renilla control plasmid. Cells were stimulated overnight in the presence of plate-bound anti-CD3 antibody. Cells were harvested and AP-1 and NF-AT activity were assessed by dual luciferase assay. A. Representative data for AP-1 and NF-AT activity. The transfection efficiency was adjusted by the expression of Renilla luciferase in the unstimulated condition. Data analyzed using a 2-way ANOVA with Bonferroni’s multiple comparisons test. *P<0.05. ****P<0.0001. B. Fold increase in AP-1 and NF-AT activity compared to control normalized to the J.cas9 unstimulated condition. Each dot represents data from an independent experiment. Error bars represent SD. Analyzed using a 1-way ANOVA with Bonferroni’s multiple comparisons test. **P<0.005. ****P<0.00005. C. The TGF-βRI deficient Jurkat T cell line was transiently transfected with an NF-AT-GFP activity reporter construct in the presence of a control mCherry only vector. Cells were stimulated as in A. Cells were harvested and NF-AT GFP was assessed by flow cytometry. Representative data of cells gated on mCherry (for transfection efficiency) for NF-AT-GFP expression. The dashed line (unstimulated) and solid line (stimulated). D. Fold increase in NF-AT-GFP expression in mCherry positive cells upon stimulation. Each dot represents data from an independent experiment. Data analyzed using a paired Student’s t-test.
High AP-1 and NF-AT Activity in Primary Human T cells in the Absence of TGF-βRI Signaling

We next sought to determine if we could observe similar AP-1 and NF-AT activity phenotypes in primary T cells where TGF-βRI signaling is ablated. To do this, we obtained a TGF-βRI dominant negative construct. This construct has a point mutation in the kinase domain (lysine to arginine at position 232) such that the TGF-βRI no longer possess kinase activity and can not mediate TGF-β signaling (Moren et al. 2005; Wieser et al. 1995). We also obtained a TGF-βRI constitutively active construct that has a point mutation in the GS region (threonine to aspartic acid at position 204) that renders the receptor in a perpetually active conformation and always able to mediate TGF-β signaling (Wieser et al. 1995). We hypothesized that T cells transfected with the dominant negative TGF-βRI construct will phenocopy the TGF-βRI deficient Jurkat T cell line, and would exhibit constitutively high AP-1 and high NF-AT activity compared to T cells transfected with the constitutively active TGF-βRI construct. To test this, we transfected primary human T cells purified from adult peripheral blood with the constitutively active TGF-βRI construct or the dominant negative TGF-βRI construct in the presence of AP-1 and NF-AT activity luciferase reporter constructs. After resting the T cells for 24 hours, we stimulated them in the presence of plate bound anti-CD3 and anti-CD28 antibodies or with PMA/ionomycin for 6 hours. After stimulation, cells were harvested and AP-1 and NF-AT activities were assessed. We observed that in the presence of the dominant negative TGF-βRI construct there are significantly greater levels of AP-1 and NF-AT activity compared to in the presence of the constitutively active TGF-βRI construct and backbone control (Figure 22A and B). However, we do
not observe constitutively high AP-1 activity in the presence of the dominant negative TGF-βRI construct. This could be due to the transient nature of the assay, whereby the dominant negative TGF-βRI is not stably expressed and is only present in the cells for a total of 48 hours and therefore may not manifest the same phenotype in the unstimulated condition as the stable TGF-βRI deficient Jurkat T cells line. We also observe that in the presence of the constitutively active TGF-βRI construct the levels of AP-1 and NF-AT activity are less than backbone control (Figure 22A and B). These data confirm that the AP-1 and NF-AT activity observed in the TGF-βRI deficient line is also found in human primary T cells and suggests that TGF-βRI signaling reduces AP-1 and NF-AT activity.
Figure 22. AP-1 and NF-AT Transcriptional Activity in the Absence of TGF-βRI Signaling in Primary T cells. Total human T cells were transiently transfected with either backbone control, constitutively active or dominant negative TGF-βRI constructs in the presence of AP-1 or NF-AT luciferase constructs in the presence of a Renilla control plasmid. One-day post transfection cells were simulated with plate bound anti-CD3 and anti-CD28 antibodies or PMA/ionomycin for 6 hours. Cells were harvested and AP-1 and NF-AT activity was assessed by dual luciferase assay. A. Representative data for AP-1 and NF-AT activity. The transfection efficiency was adjusted by the expression of Renilla luciferase in the unstimulated condition. Analyzed using a 1-way ANOVA with Tukey’s multiple comparisons test. *P<0.05. B. Fold increase in AP-1 and NF-AT activity compared to backbone control. Each dot represents data from an independent experiment. Analyzed using a 1-way ANOVA with Tukey’s multiple comparisons test. **P<0.005.
Constitutive JNK Activation and Activity in the TGF-βRI Deficient Line

To explain the constitutive AP-1 transcriptional activity in the TGF-βRI deficient line, we wanted to access the activation status of the molecules upstream of AP-1 activation. The kinases responsible for the activation of the AP-1 complex are c-Jun amino terminal kinase (JNK) and extracellular signaling-regulated kinase (ERK). JNK functions to phosphorylate Jun family transcription factor proteins, such as c-Jun. ERK functions to phosphorylate Fos family transcription factor proteins, such as c-Fos (Figure 23). Phosphorylation of c-Jun and c-Fos potentiates their transcriptional activity (Hess et al. 2004). The AP-1 complex is comprised of a heterodimer of c-Jun and c-Fos or a homodimer of c-Jun proteins (Karin 1995)(Figure 23). We hypothesized that the activation and activity of JNK and/or ERK would be increased in the TGF-βRI deficient line compared to control. To test this, we stimulated the TGF-βRI deficient in the presence of an anti-Jurkat TCR antibody for 5 minutes. Cells were then harvested and phospho-JNK and phospho-ERK were assessed. Interestingly, we detected phospho-JNK in both the absence and presence of stimulation in the TGF-βRI deficient line (Figure 24A). Conversely, phospho-ERK was only detected in the presence of stimulation and at comparable levels to control (Figure 24A). These data suggest that there is constitutively active JNK in the TGF-βRI deficient line.
Figure 23. Model of AP-1 Regulation. The model of AP-1 regulation and complex formation is depicted. The JNK and ERK kinases function to phosphorylate their substrates c-Jun and c-Fos, respectively. AP-1 can be formed from a heterodimer of the transcription factors c-Jun (purple) and c-Fos (blue) or a homodimer of c-Jun.

We next asked if the kinase activity of JNK was also increased in the TGF-βRI deficient line. To test this, we stimulated the TGF-βRI deficient line in the presence of plate-bound anti-CD3 and harvested cells at 2 and 4 hours post stimulation. Cells were then lysed and both the nuclear and cytoplasmic fractions were extracted. Expression of the phosphorylated form and total protein of the JNK substrate, c-Jun, were assessed in both fractions. We expected that if JNK kinase activity was constitutively increased in the TGF-βRI deficient line, then we would observe more phospho-c-Jun and total c-Jun in the nuclear fraction in the deficient line compared to control. This is because the nucleus is the site by which active c-Jun exerts its transcriptional activities. We were unable to visualize phospho-c-Jun (data not shown), however, total c-Jun protein levels in the nuclear fraction are 2-fold higher at the 0 hour time-point (unstimulated) in the
TGF-βRI deficient line compared to control (Figure 24B). Further, there is a greater increase in the levels of total c-Jun protein in the nuclear fraction at the 2 and 4-hour time-points in the deficient cells compared to control. In addition, there are higher levels of total c-Jun protein in the cytoplasmic fraction at the 2 and 4-hour time-points in the deficient line compared to control (Figure 24B). These data suggest that there are greater levels of c-Jun protein in the nucleus of the TGF-βRI deficient line in the absence of stimulation and this level further increases upon stimulation.

Our next question was whether the high levels of c-Jun protein in the nucleus of the TGF-βRI deficient line is due to transcriptionally active c-Jun, since we were unable to visualize phospho-c-Jun. Evidence supporting high levels of transcriptionally active c-Jun in the TGF-βRI deficient line was observed upon RNA sequence analysis of the TGF-βRI deficient line. This analysis was performed using RNA isolated from unstimulated TGF-βRI deficient cells and control cells. RNA sequence analysis revealed a significant increase in the expression of c-Jun (JUN) in the TGF-βRI deficient line. (Figure 24C). This is consistent with literature that states that JUN expression is positively up-regulated by the transcriptional activity of activated c-Jun (Angel et al. 1988). These data suggest that the increased levels of total c-Jun protein in the nuclear fraction of the TGF-βRI deficient line at time-point 0 are transcriptionally active, as evidenced by greater auto-regulation of the c-Jun transcriptional target, JUN, in the unstimulated condition. Taken together, these data reveal that the constitutive AP-1 activity observed in the TGF-βRI deficient line can be explained by the constitutive activation and activity of JNK.
Figure 24. Phospho-JNK Activation and Activity in the TGF-βRI Deficient Jurkat T cell Line. A. The TGF-βRI deficient Jurkat T cell line was stimulated in the presence of soluble C305 (anti-Jurkat TCR) antibody for 5 minutes. Cells were then harvested, lysed and expression of phospho-JNK, total JNK, phospho-ERK1/2, and total ERK1/2 were assessed by western blot. B. The TGF-βRI deficient Jurkat T cell line was stimulated in the presence of plate-bound anti-CD3 antibody for 0 hours, 2 hours or 4 hours. Cells were then harvested, lysed and nuclear and cytoplasmic fractions were prepared. Expression of c-Jun was assessed by western blot. The relative intensity of each band is shown below. C. RNA sequence analysis was performed on unstimulated TGF-βRI deficient Jurkat T cells and the fold change in JUN expression is depicted. Data were analyzed using a paired Student's t test. *P<0.05.

Decreased Expression of Phosphorylated MKP-1 and Increased Expression of Active MKK7 in the TGF-βRI Deficient Line

We next sought to determine the molecular mechanism to explain the constitutively active JNK observed in the TGF-βRI deficient line. To this end, we assessed the expression of molecules that are known to regulate JNK. JNK activity is
downregulated by the phosphatase mitogen-activated protein kinase 1 (MKP-1). MKP-1 is a JNK and p38 specific phosphatase, unlike other MKPs, and functions to deactivate JNK activity by dephosphorylating JNK. Further, phosphorylation of MKP-1 can increase its stability by preventing degradation (Wancket et al. 2012).

We hypothesize that JNK activity is constitutively high in the TGF-βRI deficient line due to a decreased expression of MKP-1. To test this, we stimulated the TGF-βRI deficient Jurkat line in the presence of an anti-Jurkat TCR antibody for 5 minutes. Cells were then harvested and phospho-MKP-1 and total MKP-1 were assessed. Strikingly, there is a drastic reduction in phosphorylated MKP-1, at the expected molecular weight, in the TGF-βRI deficient line in the absence and presence of stimulation (Figure 26A). However, there is an intense expression of a lower molecular weight band that is detected by the anti-phospho-MKP-1 antibody in the TGF-βRI deficient line that is not present in control (Figure 26A). Further, the band at this lower molecular weight is not detected by the anti-total MKP-1 antibody in the TGF-βRI deficient line or control. Finally, the expression of total-MKP-1 is comparable between the TGF-βRI deficient line and control. These data suggest that the phosphorylated form of MKP-1 in the TGF-βRI deficient line is either strongly reduced or modified such that it appears at a lower molecular weight. However, the total-MKP-1 data demonstrates that there are comparable levels of total MKP-1 detected at the same molecular weight between the TGF-βRI deficient line and control. Further tests to determine the identity of the bands detected by the anti-phospho-MKP-1 antibody in the TGF-βRI deficient line remain to be performed.
**Figure 25. Model of JNK Regulation.** The model of JNK regulation is depicted. JNK is deactivated by the phosphatase MKP-1. JNK is activated by the MAP kinase-signaling cascade that commences with activation of the kinases TAK1 or ASK1, followed by the activation of the kinases MKK4 or MKK7 that directly activate JNK.

We also assessed molecules that are involved in positively regulating JNK. The JNK activation signaling cascade commences with the activation of a MAP kinase kinase kinase (MAPKKK). MAPKKKs are activated by various stimuli to initiate the JNK activation pathway. The MAPKKK, TGF-β activated kinase 1 (TAK1), can integrate TGF-β signaling (in non-T cells) or TCR stimulation to initiate the JNK activation pathway (Kim et al. 2009; Liu et al. 2006)(Figure 25). Conversely, in fibroblasts another MAPKKK, apoptosis signal-regulating kinase 1 (ASK1), is activated in response to stress signals such as TNF and reactive oxygen species, to initiate the JNK signaling cascade (Tobiume et al. 2001)(Figure 25). The active MAPKKK activates the next molecule in the pathway, the MAPKK. MAP kinase kinase 4 and MAP kinase kinase 7 (MKK4/7) are the MAPKK targets for both TAK1 and ASK1 and function to directly
phosphorylate JNK (Davis 2000)(Figure 25). To explain the hyper active JNK phenotype observed, we hypothesized that the active form of TAK1 or ASK1 and MKK4 or MKK7 were constitutively increased in the TGF-βRI deficient line. To test this, we stimulated the TGF-βRI deficient Jurkat line in the presence of an anti-Jurkat TCR antibody for 5 minutes. Cells were then harvested and expression of phoshpho-TAK1, ASK1, phospho-MKK4 and phospho-MKK7 was assessed. We observed no change in the expression of ASK1 in the TGF-βRI deficient line compared to control (Figure 26B). While we were unable to visualize phospho-TAK1 (data not shown), total-TAK1 expression is comparable in the deficient line compared to control (Figure 26B). We were also unable to visualize phospho-MKK4 or total MKK4 (data not shown), however, we observed a 2-fold increase in the expression of phospho-MKK7 in the TGF-βRI deficient line in the absence and presence of stimulation compared to control (Figure 26B). Because we were unable to visualize phospho-TAK1, these data do not provide us with direct evidence for which MAPKKK is involved in this hyper JNK activation pathway. However, these data do suggest that the constitutively active MKK7 could contribute to the constitutively active JNK phenotype observed in the TGF-βRI deficient line.
Figure 26. Expression of a JNK phosphatase and JNK Kinases in the TGF-βRI Deficient Jurkat T cell Line. The TGF-βRI deficient Jurkat T cell line was stimulated in the presence of soluble C305 (anti-Jurkat TCR) antibody for 5 minutes. Cells were then harvested, lysed and protein expression assessed by western blot. A. Expression of the JNK phosphatase phosphorylated MKP-1 and total MKP-1. B. Expression of JNK kinases ASK1, TAK1 and phosphorylated MKK7 and total MKK7. The relative intensity of each band is shown below. The relative intensity of phosphorylated proteins was normalized to the unphosphorylated form.

Increased CARMA1, Bcl10 and TRAF6 Expression in the TGF-βRI Deficient Line

To continue to elucidate the molecular pathway upstream of the hyper JNK activation observed in the TGF-βRI deficient line, we examined the expression of various scaffold proteins in the JNK activation pathway. It is well appreciated that scaffold proteins play a critical role in facilitating the assembly and physical interaction between the MAPKKK, MAPKK, and JNK. While scaffold proteins have no catalytic function, they do contain specific docking sites for a single MAPKKK and therefore mediate selectively for specific signaling molecules upstream of JNK activation in
response to stimuli (Johnson & Nakamura 2007). One scaffold protein for the JNK activation pathway is termed JNK Interacting Protein 1 (JIP1). JIP1 specifically binds to MAPKKK of the mixed lineage kinase (MLK) group, which are activated by cellular stress, and functions to phosphorylate MKK7 to mediate JNK activation (Gallo & Johnson 2002). Another scaffold protein is plenty of SH3s (POSH). POSH also selectively recruits MLKs in response to cellular stress and phosphorylates both MKK4 and MKK7 to activate JNK (Xu et al. 2003). Finally, another scaffold protein is caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1). CARMA1 is activated by protein kinase C-θ (PKC-θ) upon T cell stimulation (Thome 2004). Active CARMA1 undergoes a conformation change to allow for the binding of various signaling molecules involved in both the JNK and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation pathways (Roche et al. 2013). The first molecule that is recruited to CARMA1 is the adaptor protein B cell lymphoma 10 (Bcl10). Bcl10 binding to CARMA1 induces Bcl10 polyubiquitination, oligomerization, and recruitment of the protease mucosa-associated lymphoid tissue lymphoma translocation 1 (MALT1) (Qiao et al. 2013). This CARMA1/Bcl10/MALT1 (CBM) complex then recruits the ubiquitin ligase TNF receptor-associated factor 6 (TRAF6), the MAPKKK TAK1, the MAPKK MKK7 and JNK to facilitate JNK activation (Blonska et al. 2007; David et al. 2018). We hypothesized that the constitutive activation of JNK is due to increased expression of one of the JNK scaffold protein complexes in the TGF-βRI deficient line. To test this, we stimulated the TGF-βRI deficient line in the presence of an anti-Jurkat TCR antibody for 5 minutes and then assessed the expression of JIP, POSH, CARMA1, and Bcl10. While
we observed no difference in the expression of JIP or POSH in the TGF-βRI deficient line compared to control, we did observe that there is an increase in both CARMA1 and Bcl10 expression in the absence and presence of stimulation in the TGF-βRI deficient line compared to control (Figure 28A). As described above, Bcl10 undergoes extensive oligomerization upon association with CARMA1. Therefore, we speculate that the heavier upper band (indicated by the arrow) is representing more Bcl10 oligomerization in the TGF-βRI deficient line (Figure 28A). These data suggest that the constitutive JNK activation observed in the TGF-βRI deficient line could be mediated by the constitutive increase in CARMA1 and Bcl10 scaffold molecules upstream of JNK activation.

**Figure 27. Model of Scaffold Proteins involved in JNK Regulation.** The model of the scaffold proteins upstream of JNK activation is depicted. JIP and POSH initiate JNK activation by specifically recruiting MLKs as the MAPKKK while the CARMA1/Bcl10/MALT1 (CBM) complex recruits TAK1 as the MAPKKK upstream of JNK activation.

To further assess the usage of the CARMA1 signaling scaffold as the mechanism that facilitates the hyper JNK activation observed in the TGF-βRI deficient line, we
assessed the expression of another molecule that is recruited to the CBM complex, TRAF6. TRAF6 functions to activate TAK1, the MAPKKK in the CBM JNK activation pathway, by lysine 63-linked polyubiquitination (Wang et al. 2001)(Figure 27). Further, another ubiquitin ligase in the same family, TRAF2, activates the MAPKKK ASK1 and does not utilize the CBM scaffold for JNK activation (Nishitoh et al. 1998). We expected that if the hyper JNK activation observed in the TGF-βRI deficient line is mediated by the CBM scaffold pathway, then we would see increased expression of TRAF6, and not TRAF2, in the deficient cells compared to control. Indeed, we observed an increase in the expression of TRAF6 in the TGF-βRI deficient line in the absence and presence of stimulation compared to control (Figure 28B). Taken together, these data suggest that CARMA1/Bcl10/TRAF6 pathway is mediating hyper JNK activation in the TGF-βRI deficient line.

Finally, we sought to determine if the kinase of CARMA1 is hyper active in the TGF-βRI deficient line, which could explain the hyper JNK activation. CARMA1 is activated by phosphorylation by PKC-θ (Sommer et al. 2005). We hypothesized that there would be increased levels of active PKC-θ would be found in the TGF-βRI deficient line compared to control. However, we observed comparable levels of phospho-PKC-θ in the absence and presence of stimulation in the TGF-βRI deficient line compared to control (Figure 28C). These data suggest that increased active PKC-θ expression is not the mechanism responsible for enhancement of the CBM JNK activation pathway in the TGF-βRI deficient line.
Figure 28. Expression of JNK Scaffold Proteins and the CARMA1 Activator, PKC-θ in the TGF-βRI Deficient Line. The TGF-βRI deficient Jurkat T cells were stimulated in the presence of soluble C305 (anti-Jurkat TCR) antibody for 5 minutes. Cells were then harvested, lysed, and protein expression assessed by western blot. A. Expression of the JIP1, CARMA1, and Bcl10. Arrow in Bcl10 blot indicates possible Bcl10 oligomerization. B. Expression of TRAF6 and TRAF2. C. Expression of the CARMA1 activator PKC-θ. The relative intensity of each band is shown below. The relative intensity of phosphorylated proteins was normalized to the unphosphorylated form.

Decreased CARMA1 and Bcl10 Expression in the Presence of TGF-β in Primary T cells

Based on data obtained in the TGF-βRI deficient Jurkat T cell line, we next investigated if our findings are applicable in physiologically relevant conditions in primary mouse T cells. We reasoned that since previous work in our lab has shown that human T cells produce their own TGF-β (Takami et al. 2015), and effector T cells from
mice do not produce their own TGF-β, mouse T cells will allow us to determine the phenotype of cells with and without TGF-β present. We first determined the conditions to assess the TGF-β mediated inhibition of proliferation on both CD4+ and CD8+ T cells from mice, as this is a well-appreciated role for TGF-β signaling in T cells. To do this, we purified total mouse T cells and then stimulated in the presence of plate-bound anti-CD3 antibody in the presence or absence of exogenous active TGF-β. Three days post stimulation the cells were harvested and counted to assess cell number. Cell were also assessed by flow cytometry to determine the frequency of CD4+ and CD8+ T cells. Cell count/proliferation of each subset was determined using the frequency obtained by flow cytometry and the raw total cell count. We expected that cells stimulated in the absence of TGF-β would proliferate more compared to cells stimulated in the presence of TGF-β. Interestingly, CD4+ T cells proliferated comparably in the absence and presence of TGF-β (Figure 29A). Conversely, CD8+ T cells proliferated about 8 fold in the absence of TGF-β, however, in the presence of TGF-β there is a significant decrease in proliferation (Figure 29A). These data demonstrate that CD8+ T cells are more sensitive to TGF-β mediated inhibition of proliferation compared to CD4+ T cells. Based on this, we further assessed TGF-β signaling in CD8+ T cells at this time-point that we observed the inhibition of proliferation. We hypothesized that in the presence of TGF-β signaling there will be decreased phospho-JNK, CARMA1 and Bcl10 expression compared to in the absence of TGF-β signaling. To test this, we purified mouse CD8+ T cells and stimulated them in the presence of plate-bound anti-CD3 antibody in the presence or absence of exogenous active TGF-β. Three days post stimulation cells were harvested and the expression of phospho-JNK, CARMA1 and Bcl10 was assessed. While we were
unable to visualize phospho-JNK (data not shown), we did observe that in the absence of TGF-β signaling the expression of CARMA1 and Bcl10 was comparable to day 0 (Figure 29B). Conversely, in the presence of TGF-β signaling there is substantially less CARMA1 and Bcl10 expression (Figure 29B). Of note, there are three bands detected by the anti-Bcl10 antibody and we reasoned that the upper two bands are ubiquitinated Bcl10 (Figure 29B). These data demonstrate that the presence of TGF-β signaling strongly inhibits CARMA1 and Bcl10 expression after stimulation and this reduction correlates with the time point by which T cell proliferation is suppressed by TGF-β signaling in CD8+ T cells. These data also oppose the TGF-βRI deficient Jurkat T cell data whereby CARMA1 and Bcl10 and phospho-JNK are increased in the absence of TGF-β signaling.
Figure 29. Proliferation and Expression of CARMA1 and Bcl10 Expression in the Presence and Absence of TGF-β in Primary T cells. A. Primary mouse CD4^+ and CD8^+ T cells were purified, seeded at 1x10^6 and stimulated with plate-bound anti-CD3 antibody in the presence or absence of exogenous active TGF-β. Three days post stimulation cells were harvested, counted and frequency of each subset was assessed by flow cytometry to determine the proliferation of each subset. Error bars represent standard deviation with three independent experiments. *P < 0.05. B. Primary mouse CD8^+ T cells were purified and stimulated as in (A). Three days post stimulation cells were harvested and expression of CARMA1 and Bcl10 was assessed. The relative intensity of each band is shown below. This experiment was performed by Shena Geisinger.
CHAPTER FOUR
DISCUSSION

Summary of Data

We first sought to compare the phenotype and function of naïve CD4$^+$ T cells from umbilical cord blood (UCB) to that of naïve CD4$^+$ T cells from adult blood. Data obtained previously in our lab demonstrated that naïve CD4$^+$ T cells from UCB have a higher propensity to become regulatory T cells (Tregs) compared to naïve CD4$^+$ T cells from adult blood, in a TGF-β dependent manner (Lee et al. 2018). We examined if naïve CD4$^+$ T cells from UCB have other intrinsic differences between their counterparts in adult blood. We determined that naïve CD4$^+$ T cells from UCB uniquely express high levels of the enzymatic protein CD26 (Figure 14). We also determined that while naïve CD4$^+$ T cells from UCB do not produce cytokines at comparable levels to that of naïve CD4$^+$ T cells from adult blood at a basal level (Figure 16), they do acquire the ability to produce elevated levels of both Th1 (IFN-γ) and Th2 (IL-5 and IL-13) type cytokines when stimulated under Th1 and Th2 polarizing conditions, respectively (Figure 17A, B and C). Taken together, these data identify that CD26 is a unique surface marker for naïve CD4$^+$ T cells from UCB and demonstrate there is no Th2 bias in naïve CD4$^+$ T cells from UCB, rather, they have the capacity to produce high levels of both Th1 and Th2 type cytokines upon differentiation.

We next sought to determine the molecular mechanism(s) by which TGF-β elicits its pleiotropic effects in T cells. To determine the possible target molecules in one or
more of these pathways, we generated a TGF-βRI deficient Jurkat T cell line to characterize the phenotypes of T cells in the absence of TGF-β signaling (Figure 18). We observed that upon activation, the TGF-βRI deficient line exhibited significantly increased expression of the early activation markers CD25 and CD69 and a subpopulation of cells exhibited a substantial decrease in the checkpoint molecule PD-1 (Figure 19A). Further, we observed a significant increase in the expression of the T cell growth factor IL-2, as well as TNF, in the TGF-βRI deficient line upon stimulation (Figure 19B). These data collectively demonstrate a hyper activation phenotype upon stimulation in the TGF-βRI deficient line.

We next sought to confirm our findings obtained in Jurkat T cells, as these are transformed cancer cells and might not reflect the phenotypes observed primary T cells. To do this, we assessed the activation phenotype of the primary human tyrosinase reactive 1383I TCR T cell line in the presence of TGF-β signaling. We observed a significant decrease in the expression of CD25 and an increase in the expression of PD-1 upon stimulation in the presence of TGF-β in CD4+ and CD8+ T cells (Figure 20). Unexpectedly, we did not observed a decrease in CD69 expression in the presence of TGF-β, instead, CD69 expression levels in the presence of TGF-β were comparable to no treatment (Figure 20). Taken together, these data support the findings obtained in the TGF-βRI deficient line, where TGF-β signaling is absent, and demonstrate in primary human T cells that the presence of TGF-β signaling decreases CD25 expression and increases PD-1 expression. However, TGF-β signaling does not effect CD69 expression in primary human T cells.
To explain the hyper activation phenotype observed in the TGF-βRI deficient line, we assessed the activity of two critical transcription factors that are activated upon T cell stimulation and mediate the T cell activation phenotype, AP-1 and NF-AT. We observed that the TGF-βRI deficient line possessed constitutive AP-1 activity and highly elevated NF-AT activity upon stimulation (Figure 21). These data suggest that the hyper activation phenotype observed in the TGF-βRI deficient line could be attributed to the constitutively high AP-1 and high NF-AT activity upon stimulation.

Based on the constitutive AP-1 and high NF-AT activity in the TGF-βRI deficient line, we asked if we could recapitulate these findings in primary human T cells using a dominant negative TGF-βRI construct to inhibit TGF-β signaling in these cells. We observed that primary human T cells transfected in the presence of the dominant negative TGF-βRI construct exhibited significantly higher AP-1 and NF-AT activity upon stimulation compared to T cells transfected with a backbone control construct (Figure 22). Of note, we did not observe constitutive AP-1 activity in the presence of the dominant negative TGF-βRI construct (Figure 22). We reasoned that this is because these assays were performed in cells that were transiently transfected with the dominant negative TGF-βRI construct and this condition might not completely reflect the conditions in the stable TGF-βRI deficient cell line. However, we did observe that in T cells transfected with a constitutively active TGF-βRI construct, where TGF-β signaling is always on, both AP-1 and NF-AT activity were less than T cells transfected with a backbone control construct (Figure 22). Together, these data confirm in primary T cells that the absence of TGF-β signaling enhances AP-1 and NF-AT activity upon stimulation and that the presence of TGF-β signaling has the opposite effect.
Next, we sought to understand why we observed the constitutive activity of AP-1 in the TGF-βRI deficient line. To do this, we examined the expression of the kinases upstream of AP-1 activation, JNK and ERK. Our results demonstrated that there is constitutive phospho-JNK expression in the TGF-βRI deficient line compared to control (Figure 24A). Importantly, the expression of phospho-ERK in the TGF-βRI deficient line is only detected upon stimulation as is at comparable levels to control (Figure 24A). Further, the expression of the JNK substrate, c-Jun, is 2-fold higher in the nuclear fraction in the unstimulated TGF-βRI deficient cells compared to control cells (Figure 24B). Additionally, there is a 2-fold increase in the expression of the c-Jun transcriptional target, JUN, in unstimulated conditions in the TGF-βRI deficient line compared to control (Figure 24C). Collectively, these data demonstrate constitutive activation of JNK and constitutive activity of the JNK substrate, c-Jun, in the absence of TGF-β signaling in the TGF-βRI deficient line. Importantly, these data are the first to identify that the absence of TGF-β signaling enhances JNK activation in T cells.

To determine the molecular pathway that is diminished or enhanced in the TGF-βRI deficient line to mediate the constitutive activation of JNK, we assessed the expression of molecules known to regulate JNK activation. We determined that there is a significant reduction in phosho-MKP-1, the phosphatase responsible for deactivating JNK, at the expected molecular weight in the TGF-βRI deficient line compared to control (Figure 26A). However, in the deficient line, there is an emergence of a lower molecular weight band detected by the anti-phospho-MKP-1 antibody that is not detected by the total anti-MKP-1 antibody in deficient or sufficient cells (Figure 26A). In addition, there are comparable levels of total MKP-1 expression between the TGF-βRI deficient line
compared to control (Figure 26A). Further, we observed a 2-fold increase in the expression of the active form of the JNK kinase, phospho-MKK7, in deficient cells compared to control (Figure 26B). These data indicate that the constitutive activation of JNK in the TGF-βRI deficient line could be the result of the decreased expression of phospho-MKP-1 and/or the increased expression of phospho-MKK7.

To determine the specific JNK activation pathway that is enhanced in the TGF-βRI deficient line, we assessed the expression of the critical scaffold proteins that mediate the association of kinases upstream of JNK activation. We observed a striking increase in the expression of CARMA1, about a 2-fold increase in the expression of Bcl10 and a 2-fold increase in the expression of TRAF6 in the absence and presence of stimulation (Figure 28A and B). Further, although the protein expression of CARMA1 is enhanced, we observed no difference in the expression of the active form of the kinase of CARMA1, phospho-PKC-θ, between deficient and sufficient cells (Figure 28C). These data are the first to identify that the absence of TGF-β signaling results in an increased expression of CARMA1, Bcl10 and TRAF6.

Finally, we also determined that in primary mouse CD8⁺ T cells are more sensitive to TGF-β-mediated inhibition of proliferation (Figure 29A). Further, CD8⁺ T cells stimulated in the presence of TGF-β signaling demonstrated strikingly low expression CARMA1 and Bcl10 compared to cells stimulated in the absence of TGF-β (Figure 29B). These data confirm, in primary T cells, that TGF-β signaling suppresses CARMA1 and Bcl10 expression.
Early studies seeking to characterize naïve CD4$^+$ T cells from UCB vs. adult blood showed that CD45RA expression correlated positively with CD26 expression in T cells from cord blood, thus identifying immature T cells (Amlot et al. 1996). However, more recent studies demonstrated that memory T cells, and not naïve T cells, express CD26 (Waumans et al. 2015). Our data show that antigen receptor stimulation of naïve CD4$^+$ T cells from adult blood induces expression of CD26 (Figure 14). Conversely, stimulated naïve CD4$^+$ T cells from UCB to maintain or slightly increase their expression of CD26. Importantly, the majority of CD26 expression in adult blood are on T cells that are CD45RA$^-$ CD45RO$^+$ while CD26 expression in UCB is on T cells that are CD45RA$^+$ CD45RO$^-$. Therefore, our data demonstrate that expression of CD26 by naïve T cells from UCB fundamentally differs from naïve CD4 T cells from adult blood.

CD26 is serine exopeptidase that cleaves X-proline dipeptides found on polypeptides such as chemokines (Matteucci & Giampietro 2009). Further, CD26 is known to regulate glucose metabolism and inactivate a group of insulinotropic proteins, such as glucagon-like peptide 1 (GLP1) (Mentlein et al. 1993). In addition, an inhibitor of CD26 has been used clinically to treat insulin-resistant diabetic patients (Holst & Deacon 2004). CD26 also functions to bind adenosine deaminase (ADA), which an essential factor for immune competence of neonates, as a metabolic deficiency of ADA results in severe lymphopenia and impaired cellular and humoral immunity (Whitmore & Gaspar 2016). However, the functions of CD26 in T cells specifically are not fully elucidated. Some studies indicate that CD26 plays an inhibitory role in T cell activation. Evidence in support of this include studies from CD26 knockout mice which
demonstrate an increase in the severity of Experimental Autoimmune Encephalomyelitis (EAE) (Preller et al. 2007). In addition, CD26 deficient mouse T cells show a significantly elevated response to antigen stimulation, as assessed by their proliferation and cytokine production (IFN-γ and TNF). Conversely, studies using human T cells demonstrate that CD26 can deliver a robust co-stimulatory signal and contribute to activation of CD4⁺ helper and memory T cells (Morimoto & Schlossman 1998). Further, it has been demonstrated that CD26 is more efficient in responding to stimulation and promoting activation of CD45RO⁺ effector/memory T cells, compared to CD45RA⁺ naïve T cells (Salgado et al. 2003). More evidence for CD26 serving as a co-stimulatory molecule in human T cells comes from a clinical analysis that demonstrated that a CD4⁺ CD45RO⁺ CD26⁺hi T cell subset is correlated with Multiple Sclerosis (MS) disease severity, and this subset was found to be enriched for Th1 effector functions (Krakauer et al. 2006). However, previous work in mice demonstrates that CD26 on T cells is not directly linked to T cell activation (Cordero et al. 2007). Taken together, it is clear that there are species-specific differences for the function of CD26 in T cells. Our data indicate that CD26 may function to enhance CD45RO⁺ T cell effector functions while inhibiting T cell activation in CD45RA⁺ T cells to control naïve vs. memory functions. This idea is supported by the observation that cytokine production, such as IFN-γ, is decreased in freshly isolated naïve CD4⁺ T cells from UCB (Figure 16) and we speculate that this could be due to the highly elevated CD26 expression. While CD26 expression might contribute to the capacity for cytokine production in activated CD45RO⁺ T cells. However, the mechanism(s) by which CD26 control T cell activation and/or cytokine production remains to be elucidated.
Enhanced Effector Cytokine Production by Naïve CD4⁺ T cells from UCB

An unexpected outcome of these studies was the observation of enhanced effector cytokine production by naïve CD4⁺ T cells from UCB when differentiated ex vivo under Th1 or Th2 polarizing conditions (Figure 17). This was unexpected because previous reports in mice suggest that neonates have a Th2 bias in neonates featuring a reduced capacity of neonatal mouse T cells to produce IFN-γ and TNF-α (Chen et al. 1995; Rose et al. 2007). However, in humans, an epidemiological study involving over 400 children demonstrated that in freshly isolated T cells from peripheral blood mononuclear cells (PBMCs) there was no significant bias towards Th2 cytokine production, except for IL-13 (Halonen et al. 2009). Our data correlate with these data obtained in T cells from humans, as we also found no bias in the production of Th2 cytokines by freshly stimulated naïve CD4⁺ T cells from UCB (Figure 16). Further, naïve CD4⁺ T cells from UCB produced significantly high levels of both IFN-γ and IL-5, greater than cells from adult blood, when stimulated under the Th1 or Th2 polarizing conditions, respectively (Figure 17). Of note, naïve CD4⁺ T cells from UCB did not produce detectable levels of IL-4, which functions to promote the differentiation into Th2 cells. Instead, the production of IL-5 functions to promote eosinophils and B cells differentiation and growth (Takatsu 2011). These data support the epidemiological study’s results that demonstrate that the human perinatal immune system is not skewed toward canonical Th2 responses, as it is in mice. Further, our work shows that naïve CD4⁺ T cells from UCB are able to produce high levels of both Th1 and Th2 cytokines, with the exception of IL-4. Collectively, these data provide the field with evidence that the presence of environmental factors (such as cytokines produced by innate immune
cells) could contribute to the overall outcomes of the perinatal immune response upon antigen exposure.

**Implications of these Data: TGF-β Signaling and the Suppression of T cell Activation**

It is well appreciated that one of the pleiotropic roles of TGF-β signaling is to suppress T cell activation and proliferation (Gorelik & Flavell 2000; Letterio & Roberts 1998). Previous studies have identified that TGF-β signaling reduces the expression of CD25 and the production of cytokines (such as IL-2 and TNF-α) and enhances the expression of PD-1 in T cells (Tiemessen et al. 2004; Kehrl et al. 1986; Ahmadzadeh & Rosenberg 2005; Park et al. 2016; Stephen et al. 2017). Our data correlate with these data and demonstrate that upon stimulation in the absence of TGF-β signaling, in the TGF-βRI deficient line, there is significantly higher expression of CD25, CD69, IL-2, TNF and a population of cells that exhibit a lower expression of PD-1 (Figure 19). It is important to note that no exogenous active TGF-β was added to the Jurkat cells in any experiments. Therefore, these activation data in the TGF-βRI deficient line also suggest that there must be tonic TGF-β signaling happening at a steady state and removal of this tonic signaling in the TGF-βRI deficient line results in these hyper activation phenotypes observed. This tonic signaling may be caused by the presence of a low level of active TGF-β or by a low but constant activation and signaling of TGF-β receptors. Interestingly, in a primary human 1383I TCR T cell line stimulated in the presence of exogenously added active TGF-β, we did not observe a decrease in CD69 expression, as would be expected from the data obtained in the TGF-βRI deficient line (Figure 20). Previous studies examining the function of CD69 in T cells yielded
conflicting results. Some studies demonstrated that ligation of CD69 in T cells results in increased proliferation and increased production of proinflammatory cytokines \textit{in vitro} (Cebrián et al. 1988; Sancho et al. 2000). However, other studies showed that ligation of CD69 in T cells \textit{in vivo} results in the production of the anti-inflammatory cytokine, TGF-β (Brandes et al. 1991; Sancho et al. 2003). Further, one study showed that CD69 expression increased in the presence of TGF-β in primary human T cells (Tiemessen et al. 2003). Therefore, the link between TGF-β signaling and the expression of CD69 merits further investigation to determine how TGF-β signaling can control CD69 expression to regulate T cell functions.

It has been shown that TGF-β signaling functions to suppress IL-2 production via canonical Smad signaling, specifically Smad3, and this suppression of IL-2 reduces the IL-2 signaling dependent expression of the IL-2 high affinity alpha chain, CD25 (McKarns et al. 2004; Shatrova et al. 2016; Sereti et al. 2000). This could explain the highly elevated levels of CD25 expression in the TGF-βRI deficient line, as we also observed significant production of IL-2 in the deficient cells. Further, PD-1 expression has recently been shown to be upregulated by TGF-β signaling in T cells in a Smad3 dependent, Smad 2 independent manner (Park et al. 2016). The dramatic decrease in the phosphorylation of Smads in the TGF-βRI deficient line (Figure 18D) could explain the population of cells that exhibit a sharp decrease in PD-1 expression upon stimulation (Figure 19A and B). Collectively, our work is consistent with previous reports on the function of TGF-β signaling in T cell activation.
Novel TGF-β mediated Suppression of JNK Activation in T Cells

TGF-β signaling has been previously shown to activate JNK in a variety of cell types. This TGF-β mediated JNK activation pathway commences with activation of the TGF-βRII and TGF-βRI complex in the presence of the TGF-β ligand. Once active, TRAF6 binds to the TGF-βRI and this binding induces autoubiquitination of TRAF6. TRAF6 now activates TAK1 through lysine 63-linked polyubiquitination. Activated TAK1 can now activate MKK4 or MKK7 to activate JNK (Sorrentino et al. 2008). This TGF-β mediated JNK activation pathway has been shown to be critical for epithelial-mesenchymal transition, promoting fibrosis in kidney disease and promoting apoptosis in neurons (Wang et al. 2018; Choi et al. 2012; Gui et al. 2012). However, it is appreciated that the activation of JNK, as well as the consequence of JNK activity are cell-type and stimuli-specific (Hochedlinger et al. 2002). Indeed, our data indicate that there is constitutive activation of JNK in the TGF-βRI deficient line (Figure 24A). Further, JNK activity is constitutively increased, as evidenced by constitutive AP-1 activity (Figure 21A and B), and increased protein expression and activity of the JNK substrate, c-Jun (Figure 24B and C). Correlative evidence for TGF-β mediated suppression of JNK activation and activity is observed in primary human T cells transfected with a constitutively active TGF-βRI construct that exhibit a substantial decrease in AP-1 activity upon stimulation compared to control cells (Figure 22A and B). Therefore, contrary to what is reported for other cell types, our data identified that TGF-β signaling suppresses JNK activation in T cells.
Implications of these Data: TGF-β mediated Suppression of JNK on T cell Functions

Interestingly, there is a constitutive increase in both the 46 kDa and 54 kDa isoforms of JNK in the TGF-βRI deficient line (Figure 24A). Because JNK1 and JNK2 are known to express both splicing forms (Davis 2000), it is difficult to determine if one or both JNKs are constitutively activated in our deficient line. However, studies have demonstrated that JNK2 functions to bind c-Jun and targets it for degradation in unstimulated cells while JNK1 functions to activate c-Jun’s transcription activity and enhances its stability upon stimulation (Musti et al. 1997; Sabapathy et al. 2004). Because we observed a 2-fold increase in c-Jun protein in the nuclear fraction of the TGF-βRI deficient line in unstimulated conditions (Figure 24B), we hypothesize that both JNK1 and JNK2 are constitutively active in the absence of TGF-β signaling such that activation of JNK2 releases its negative regulation of c-Jun and activation of JNK1 allows for the activation and stabilization of c-Jun. Therefore, these data presented here suggest that the pathway(s) which TGF-β signaling suppresses in our model is responsible for the activation of both JNK1 and JNK2.

Our data also indicate that constitutive JNK activation and activity permits a hyper-activation phenotype upon stimulation (Figure 19). It is well appreciated that AP-1 is a transcription factor formed from a homodimer of JNK substrates, c-Jun or JunB, or a heterodimer of c-Jun/JunB and c-Fos and participates in mediating the transcription of IL-2, Cd25 and TNF (with other transcription factors) upon T cell stimulation (Hermann-Kleiter & Baier 2010). Therefore, our data provide evidence that TGF-β signaling could inhibit T cell activation by the suppression of JNK activation to control the expression of
T cell surface markers and cytokines associated with T cell activation. Of note, further work to assess the expression and activity of other JNK substrates, such as JunB and JunD, in our model to determine if they contribute to the phenotypes observed must be performed.

JNK1 and JNK2 also have distinct roles in T cell differentiation. Previous studies have demonstrated the importance of JNK2 for promoting Th1 differentiation and IFN-γ production (Yang et al. 1998). Further JNK1 functions to suppress the Th2 lineage by degrading JunB, which is critical for IL-4 and IL-5 production, as well as establishing the Th2 lineage (Dong et al. 1998). However, because Jurkat T cells are an effector T cell line and are not naïve T cells, we could not assess T cell differentiation using this model. Therefore, more studies need to be performed to determine the functions of JNK in T cell differentiation and how TGF-β signaling can control/influence T cell differentiation by the suppression of JNK.

Finally, JNK1 and JNK2 both contribute to apoptosis in thymocytes and peripheral T cells (Dong et al., 1998; Sabapathy et al., 1999). While we did not directly assess apoptosis in the TGF-βRI deficient line, we did observe that these cells had a substantial growth disadvantage compared to control cells (data not shown). Further tests need to be performed to determine if there is enhanced apoptosis occurring the in TGF-βRI deficient line due to the constitutive activation and activity of JNK.

**Identification of links between TGF-β Signaling and JNK Regulation Pathways**

We observed that numerous molecules in the pathway upstream of JNK activation were constitutively altered in the TGF-βRI deficient line. While there were significantly reduced levels of the negative regulator of JNK, phospho-MKP-1, at the
expected molecular weight, there was a new, lower molecular weight band detected by the anti-phospho-MKP-1 antibody in the TGF-βRI deficient line (Figure 26A). It is known that phosphorylation of MKP-1 does not function to enhance its phosphatase capacities, rather, functions to stabilize the molecule by preventing degradation (Sabapathy et al. 1999; Dong et al. 1998). However, we observed no decrease in total-MKP-1 expression at the expected molecular weight in the TGF-βRI deficient line compared to control. Therefore, further studies must be conducted to determine the identity of the unique phospho-MKP-1 band and to determine the MKP-1 phosphatase activity in the TGF-βRI deficient line to assess if a downregulation in this pathway contributes to the constitutive activation of JNK observed.

In addition, we also observed constitutive expression of molecules that positively regulate JNK activation, including a 2-fold increase in the active form of the JNK kinase MKK7, a dramatic increase in the scaffold protein CARMA1, and a 2-fold increase in the adaptor molecule Bcl10 and the ubiquitin ligase TRAF6 (Figures 26B and 28A and B). Of note, while the expression of total CARMA1 protein was increased, there was no corresponding increase in the activating kinase of CARMA1, PKC-θ (Figure 28C). Therefore, further experiments to determine the activation/phosphorylation status of CARMA1 in the TGF-βRI deficient line are required to determine if/how TGF-β signaling functions to inhibit this pathway.

When confirming our findings in primary mouse T cells, we observed that upon stimulation in the absence of TGF-β signaling the expression of CARMA1 and Bcl10 gradually increases over the 3-days assessed. However, in the presence of TGF-β, we observed that while CARMA1 expression was only mildly suppressed by day 3-post
stimulation, Bcl10 expression was suppressed day 2 and day 3-post stimulation (Figure 29). Interestingly, this day 3 time-point post stimulation is also the time-point whereby we observed the greatest TGF-β mediated inhibition of T cell proliferation (data not shown). Therefore, the reduction of Bcl10 expression day 2 and day 3-post stimulation in the presence of TGF-β correlates with TGF-β’s anti-proliferative functions. We speculate from data obtained in the TGF-βRI deficient line that this decrease in Bcl10 expression in the presence of TGF-β signaling results in the suppression of JNK activation, which would function to suppress T cell activation and proliferation. Therefore, this could serve as a novel mechanism by which TGF-β signaling suppresses T cell activation and proliferation. However, more work is required to determine the suppression of JNK activation in the presence of TGF-β signaling and if/how this is linked to T cell proliferation in primary T cells. We are currently assessing the expression of phospho-JNK in the presence of active TGF-β in primary T cells expecting to see a reduction in the expression of phospho-JNK in the presence of TGF-β compared to in the absence. We also plan to directly inhibit JNK using commercially available pharmacological inhibitors as well as a dominant negative JNK expression construct to assess if direct inhibition of JNK reduces T cell proliferation.

To explain why we did not observe a more striking decrease in CARMA1 expression in the primary mouse T cells in the presence of TGF-β signaling, as expected from the TGF-βRI deficient line data, we reasoned that perhaps a more prolonged time course is required to see more significant differences. Another possibility is that because the CARD11 (gene that encodes for CARMA1) promoter region is predicated to contain AP-1, c-Jun and NF-κB (Fishtleivich et al. 2017), there might be a
positive feedback loop occurring whereby the known constitutive activity of AP-1 and perhaps a hypothesized constitutive activity of NF-κB function to keep the expression of CARMA1 high in the TGF-βRII deficient line. While our data are the first to establish a link between TGF-β signaling and the expression of CARMA1, more studies must be performed to understand this difference seen between Jurkat T cells and the primary mouse T cells.

**Novel TGF-β mediated Regulation of a Signalosome in T cells**

CARMA1 serves as a nucleation site for Bcl10, which undergoes extensive oligomerization that is a critical step to provide the scaffold for other molecules upstream of JNK activation, such as MALT1, TRAF1, TAK1, MKK7 and JNK (Wancket et al. 2012; Qiao et al. 2013)(Figure 27C). Here, we are the first to demonstrate that TGF-β signaling suppresses the expression of CARMA1 and Bcl10 in primary CD8+ T cells. While studies in mice deficient in the Tgfβ1 or mice with a T cell-specific deletion of the TGF-βRII have shown that the T cells are hyper-activated, hyperproliferative and mediate a lethal inflammatory disorder, the specific molecular pathways which mediated TGF-β suppression of T cell functions remained poorly understood (Marie et al. 2006; Christ et al. 1994). Using our model, we were able to identify a pathway that TGF-β signaling suppresses to alter T cell functions. To this end, we propose that the suppression of Bcl10 in the presence of TGF-β signaling could reduce the CARMA1/Bcl10 scaffold complex, which would reduce the activation of JNK. Reduction in the expression of Bcl10 could affect the entire CBM scaffold complex because it is known that optimal signal propagation mediated by scaffold proteins is dependent on the concentration of both the scaffold proteins and the kinases (Levchenko et al. 2000).
Therefore, data presented here represent a novel finding in that TGF-β signaling does not merely mediate the suppression of one molecule, instead, the downregulation of Bcl10 suppresses the multiple components of the TCR-mediated JNK activation pathway in T cells. Further, these data provide evidence that TGF-β signaling suppresses a signalosome or a protein complex involved in the regulation of a protein (JNK). In addition, we acknowledge that we may not have identified all of the molecules in the signalosome that are affected by this observed TGF-β-mediated suppression of Bcl10. In fact, the CARMA1/Bcl10 scaffold is also a critical upstream pathway for the activation of NF-κB in T cells (Marie et al. 2006; Shull et al. 1992). Assessment of NF-κB activation and activity, as well as its upstream kinases, in the TGF-βRI deficient line must be performed to determine if the NF-κB pathway is also constitutively active in the absence of TGF-β signaling.

**TGF-β Signaling in CD4⁺ T cells vs. CD8⁺ T cells**

While determining the conditions to study TGF-β’s effect on primary T cells, we demonstrated that CD4⁺ T cells stimulated with an anti-CD3 antibody proliferated comparably in the absence and presence of TGF-β (Figure 29A). Conversely, CD8⁺ T cells stimulated using the same conditions showed a significant reduction in proliferation in the presence of TGF-β compared to in the absence (Figure 29A). Of note, these cells were not stimulated in the presence of an anti-CD28 antibody to provide CD28 co-stimulation signals. CD28 signaling is important for T cell activation, proliferation and specifically enhances the production of IL-2 through the activation of the NF-κB pathway (Boomer & Green 2010). However, in the absence of TGF-β CD4⁺ and CD8⁺ T cells were able to proliferate even without CD28 co-stimulation, with CD8⁺ T cells
proliferating about 2 fold more than CD4\(^+\) T cells (Figure 29A). Therefore, we sought to understand why TGF-\(\beta\) signaling robustly inhibits proliferation in CD8\(^+\) T cells but not CD4\(^+\) T cells. A previous report demonstrated that upon antigen exposure or anti-CD3 antibody stimulation CD4\(^+\) T cells proliferate in a limited manner and undergo proliferation arrest in early subsequent divisions while CD8\(^+\) T cells proliferate robustly (Foulds et al. 2002). These data are in line with our findings and suggest that there are intrinsic differences in the proliferation response between CD4\(^+\) and CD8\(^+\) T cells. Indeed, it is known that CD4\(^+\) T cells and CD8\(^+\) T cells have different requirements for activation and expansion (Crispe et al. 1985). These differences could contribute to the sensitively to the presence of TGF-\(\beta\) signaling. Previous reports have identified that CD4\(^+\) T cells are negatively regulated by cell surface inhibitory receptors, such as CTLA-4, that control cell cycle progression (Liu et al. 2001; Doyle et al. 2001). We reasoned that perhaps because CD4\(^+\) T cells CD8\(^+\) T cells have intrinsically different proliferation responses they are not regulated in the same manner. While CD4\(^+\) T cells may rely more on cell surface inhibitory receptors to inhibit proliferation, TGF-\(\beta\) signaling may be the prominent signal to control proliferation in CD8\(^+\) T cells in the conditions that we assessed.

**Potential TGF-\(\beta\) Signaling and CD28 co-stimulation Crosstalk in CD8\(^+\) T cells**

Based on our findings, we reasoned that TGF-\(\beta\) signaling suppresses CD8\(^+\) T cell proliferation by suppressing the activation of JNK. Interestingly, CD28 co-stimulation signaling enhances the activation of JNK in T cells (Rivas et al. 2001). The precise molecular pathway has not been fully elucidated. However, CD28 co-stimulation enhances the membrane translocation of PKC-\(\theta\), which is the first step in the activation
of the NF-κB pathway (Coudronniere et al. 2000). As described above, PKC-θ is the kinase that phosphorylates and activates CARMA1, and CAMRA1 and its associated complex of proteins is upstream of both the NF-κB and JNK activation pathways. We hypothesized that because CD28 co-stimulation enhances JNK activation, that the presence of CD28 signaling could antagonize TGF-β-mediated suppression of proliferation by activating the precise pathways that TGF-β signaling functions to suppress. Indeed, one report demonstrated that CD28 co-stimulation overcomes TGF-β-mediated suppression of proliferation in antigen-specific transduced human CD4+ and CD8+ T cells, but a mechanism was not proposed (Koehler et al. 2007). We are currently assessing proliferation and the expression of CARMA1 and Bcl10 in the presence and absence of both TGF-β and CD28 stimulation in CD8+ T cells to determine if an antagonistic crosstalk exists between these two pathways.

**Potential Mechanism(s) for TGF-β-mediated Regulation of CARMA1 and Bcl10**

At this point, we do not know the mechanism by which the suppression of CARMA1 or Bcl10 protein is mediated by TGF-β. The two possibilities of TGF-β mediated suppression include post-translation or translation regulation. CARMA1 protein levels are regulated by lysine-48 mediated polyubiquitination and proteasome-dependent degradation (Moreno-García et al. 2010) Bcl10 protein levels can be regulated by polyubiquitination mediated degradation through the autophagy pathway (Roche et al. 2013). To determine if TGF-β signaling suppresses CARMA1 and Bcl10 by influencing protein degradation, future work seeks to assess the ubiquitination status
in the presence and absence of TGF-β and protein expression in the presence of a pharmacological proteasome inhibitor.

To explore the possibility of translation regulation of CARMA1 and Bcl10 by TGF-β, we assessed what is known about the CARMA1 and Bcl10 genes. While there are no known Smad2 or Smad3 binding sites on the CARD11 or BCL10 promoter in humans, there is a predicted Smad4 binding site on the BCL10 promoter (Fishilevich et al. 2017). TGF-β signaling is known to suppress the transcription of target genes via Smad binding to the TGF-β inhibitory element (TIE) (Paul et al. 2012). Therefore, it remains to be elucidated if TGF-β signaling suppresses Bcl10 in this manner. One way to test this is to determine the expression of Bcl10 and CARMA1 in the presence and absence of TGF-β in Smad4 deficient cells, expecting that we would not observe a reduction in the expression of Bcl10 and CARMA1 in the presence of TGF-β but in the absence of Smad4.

It is also possible that TGF-β signaling could function to induce the expression of an inhibitory molecule that functions to suppress CARMA1 and Bcl10 expression. Indeed, previous reports have identified that expression of the microRNA, miR-155, is induced by TGF-B signaling in epithelial cells (White et al. 2000). MicroRNAs are short, non-coding RNAs that inhibit protein production by translational silencing (Kong et al. 2008). Further, miR-155 has been shown to target Bcl10 cells of the central nervous system (CNS) and in lung infiltrating mononuclear cells in mice (Cannell et al. 2008). Finally, a recent study demonstrated that CD4 T cells from mice deficient in miR-155 exhibit enhanced phospho-JNK expression in the absence and presence of stimulation compared to wild type mice, while total JNK levels where comparable between deficient
and sufficient mice (Rao et al. 2015; Tili et al. 2018). Further experiments must be performed to determine if microRNAs that target Bcl10, such as miR-155, could be a mechanism by which TGF-β signaling suppresses Bcl10 in T cells. Further, TGF-β signaling could function to induce microRNAs against other proteins in the CBM complex, such as CARMA1, or induce the expression of other target genes that function to reduce the activation of JNK in T cells.

Taken together, data presented here are the first to identify the activation of JNK as a target of TGF-β signaling in T cells. Further, these data are also the first to identify the CARMA1/Bcl10 signalosome as a potential target to facilitate TGF-β mediated suppression of JNK activation. Based on our findings, we hypothesize that TGF-β signaling could function to suppress Bcl10 expression to downregulate the activity of the CARMA1/Bcl10 signalosome and suppress the activation of JNK (Figure 30). We speculate that this suppression of Bcl10 could be directly facilitated by Smad signaling to the Bcl10 promoter to mediate transcriptional repression or through the induction of an inhibitory molecule (like a microRNA) that mediates translational repression (Figure 31). Importantly, our data suggest that the suppression of JNK activation by TGF-β signaling could be a crucial target pathway to control T cell activation. Much work is required to fully elucidate the precise molecular pathway of TGF-β-mediated signaling suppression of the activation of JNK through the CARMA1/Bcl10 signalosome to understand how TGF-β signaling modulates T cell functions. These experiments include determining how TGF-β signaling suppresses the expression of CARMA1/Bcl10 and assessing the posttranslational modifications, such as ubiquitination and phosphorylation, of CARMA1 and Bcl10 in the presence of TGF-β signaling.
Figure 30. Model of TGF-β mediated Suppression of JNK Activation in T cells. The JNK activation pathway is activated upon T cell stimulation. T cell stimulation in the presence of TGF-β signaling suppresses the activation of JNK by suppressing the expression of the adaptor molecules CARMA1 and Bcl10. The CARMA1/Bcl10 complex is crucial for the association and activation of kinases upstream of JNK activation and we propose that the reduction in Bcl10 expression by TGF-β suppresses the JNK activation signalosome to inhibit T cell activation in primary T cells.
Figure 31. Model for how TGF-β signaling could Suppress Bcl10 Expression. 1) TGF-β signaling could directly function to repress Bcl10 expression at the transcriptional level. 2) TGF-β signaling could induce the expression of an inhibitory molecule, like a microRNA, that functions to suppress Bcl10 expression at the translational level.


Aifantis, I. et al., 2006. Regulation of T-cell progenitor survival and cell-cycle entry by the pre-T-cell receptor. *Immunological reviews*, 209, pp.159–169.


Bautista, J.L. et al., 2009. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. , 10(6), pp.610–617.


Che, T. et al., 2004. MALT1/paracaspase is a signaling component downstream of CARMA1 and mediates T cell receptor-induced NF-kappaB activation. *The Journal of biological chemistry, 279*(16), pp.15870–15876.


E Zúñiga, J. et al., 2006. Assembly of TβRⅠ:TβRⅡ:TGFβ Ternary Complex in vitro with Receptor Extracellular Domains is Cooperative and Isoform-dependent,


Gaide, O. et al., 2002. CARMA1 is a critical lipid raft–associated regulator of TCR-induced NF-kB activation. *Nature Immunology*, 3(9), pp.836–843. Available at: https://doi.org/10.1038/ni830.

Gallo, A. et al., 2002. Menin uncouples Elk-1, JunD and c-Jun phosphorylation from


Ivanov, I.I. et al., 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell, 126(6), pp.1121–1133.


Jain, J. et al., 1993. The T-cell transcription factor NFATp is a substrate for calcineurin


Koehler, H. et al., 2007. CD28 Costimulation Overcomes Transforming Growth Factor-


Krueger, A. et al., 2010. CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. *Blood*, 115(10), pp.1906–1912.


affect the levels of mitogen-activated protein kinase signaling and reduce its
threshold properties. *Proceedings of the National Academy of Sciences*, 97(11),

Li, B. et al., 1999. Regulation of IL-4 expression by the transcription factor JunB during
T helper cell differentiation. *The EMBO journal*, 18(2), pp.420–432. Available at:

Liang, S.C. et al., 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and
cooperatively enhance expression of antimicrobial peptides. *The Journal of
experimental medicine*, 203(10), pp.2271–2279.

Lin, A. et al., 1995. Identification of a dual specificity kinase that activates the Jun
kinases and p38-Mpk2. *Science*, 268(5208), p.286 LP-290. Available at:
http://science.sciencemag.org/content/268/5208/286.abstract.

TGF-beta type II receptor, a functional transmembrane serine/threonine kinase.
*Cell*, 70(6), p.1069.

Lindstein, T. et al., 1989. Regulation of lymphokine messenger RNA stability by a
Available at: http://science.sciencemag.org/content/244/4902/339.abstract.

Liu, H.-H. et al., 2006. Essential role of TAK1 in thymocyte development and activation.
Available at: http://www.pnas.org/content/103/31/11677.abstract.

Liu, J. et al., 2001. Enhanced CD4+ T cell proliferation and Th2 cytokine production in

Liu, Q. et al., 2012. A Crosstalk between the Smad and JNK Signaling in the TGF-β-
Induced Epithelial-Mesenchymal Transition in Rat Peritoneal Mesothelial Cells.
*PLOS ONE*, 7(2), p.e32009. Available at: https://doi.org/10.1371/journal.pone.0032009.

Lo, R.S. et al., 1998. The L3 loop: a structural motif determining specific interactions
between SMAD proteins and TGF-beta receptors. *The EMBO journal*, 17(4),
pp.996–1005.

Maehara, Y. et al., 1999. Role of transforming growth factor-beta 1 in invasion and
metastasis in gastric carcinoma. *JOURNAL OF CLINICAL ONCOLOGY*, 17(2),

Mandal, M. et al., 2008. Regulation of lymphocyte progenitor survival by the
proapoptotic activities of Bim and Bid. *Proceedings of the National Academy of
Sciences*, 105(52), p.20840 LP-20845. Available at:
http://www.pnas.org/content/105/52/20840.abstract.

cells requires transforming growth factor-beta and induction of the nuclear receptor

Mangan, P.R. et al., 2006b. Transforming growth factor-β induces development of the TH17 lineage. *Nature*, 441(7090), pp.231–234. Available at: https://doi.org/10.1038/nature04754.


Matloubian, M. et al., 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*, 427(6972), pp.355–360. Available at: https://doi.org/10.1038/nature02284.


Mu, D. et al., 2002. The integrin alpha(v)beta8 mediates epithelial homeostasis through


Ninomiya-Tsuji, J. et al., 1999. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature*, 398(6724), pp.252–256.


Prinz, I. et al., 2006. Visualization of the earliest steps of γδ T cell development in the adult thymus. *Nature Immunology*, 7, p.995. Available at: https://doi.org/10.1038/ni1371.


Rollings, C.M. et al., 2018. Interleukin-2 shapes the cytotoxic T cell proteome and immune environment – sensing programs.


Sommer, K. et al., 2005. Phosphorylation of the CARMA1 linker controls NF-kappaB activation. Immunity, 23(6), pp.561–574.


Tanner, M.J. et al., 2007. CARMA1 coiled-coil domain is involved in the oligomerization and subcellular localization of CARMA1 and is required for T cell receptor-induced NF-kappaB activation. *The Journal of biological chemistry*, 282(23), pp.17141–17147.

Taylor, P.R. et al., 2013. Activation of neutrophils by autocrine IL-17A–IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORγt and dectin-2. *Nature Immunology*, 15, p.143. Available at: https://doi.org/10.1038/ni.2797.


Tournier, C. et al., 2001. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes &
development, 15(11), pp.1419–1426.


Tsukazaki, T. et al., 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell, 95(6), pp.779–791.


Wakabayashi, Y. et al., 2011. Histone 3 lysine 9 (H3K9) methyltransferase recruitment to the interleukin-2 (IL-2) promoter is a mechanism of suppression of IL-2 transcription by the transforming growth factor-beta-Smad pathway. The Journal of biological chemistry, 286(41), pp.35456–35465.


Wan, Y.Y. et al., 2006. The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nature Immunology, 7(8), pp.851–858. Available at: https://doi.org/10.1038/ni1355.


Wang, D. et al., 2004. CD3/CD28 costimulation-induced NF-kappaB activation is mediated by recruitment of protein kinase C-theta, Bcl10, and IkappaB kinase beta to the immunological synapse through CARMA1. Molecular and cellular biology,


Wu, Y. et al., 2006. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell, 126(2), pp.375–387.


VITA

Ramiah Jacks was born in Indianapolis, Indiana to Greg and Regina Jacks on December 14th, 1990. She attended DePaul University in Chicago, Illinois, where she earned her Bachelor’s of Science, cum laude, in Biology, with a concentration in Pre-Health, in 2013. While at DePaul University, Ramiah received her first research experience in the laboratory of Dr. Catherine Southern. In Dr. Southern’s lab, Ramiah studied the conformations of the fragment crystallizable (Fc) region of human IgG antibody using single molecule Förster resonance energy transfer (FRET).

Ramiah matriculated into the Loyola University Chicago Interdisciplinary Program in Biomedical Sciences in 2013. While at Loyola, Ramiah joined the Department of Microbiology and Immunology and performed her doctoral work in the laboratory of Dr. Makio Iwashima. Ramiah’s doctoral work focused on understanding the molecular mechanisms by which TGF-β signaling facilitates the regulation of T cell functions and fate. This work was supported by the T32 Immunology Training Grant awarded to Dr. Katherine Knight and the Predoctoral Ruth L. Kirchstein National Research Service F31 Diversity Award from the National Institute of General Medical Sciences (NIGMS).

Upon completion of her graduate studies, Ramiah will continue her research endeavors as an Institutional Research and Academic Career Development Award
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