Cell Biology of Foxp3+ Regulatory T Cells

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LOYOLA UNIVERSITY CHICAGO

CELL BIOLOGY OF FOXP3+ REGULATORY 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
MARIKO TAKAMI

CHICAGO, IL
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<td>AICD</td>
<td>Activation induced cell death</td>
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<tr>
<td>ALPS</td>
<td>Autoimmune lymphoproliferative syndrome</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DISC</td>
<td>Death inducing signaling complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ERK</td>
<td>Extracellular signal regulated protein kinase</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<td>FCS</td>
<td>Fluorescence activated cell sorting</td>
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<td>Foxp3</td>
<td>Forkhead box protein 3</td>
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<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IP3</td>
<td>Ionositol triphosphate</td>
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<td>IPEX</td>
<td>Immune dysregulation polyendocrinopathy, enteropathy, X-linked</td>
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<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<td>iTreg cells</td>
<td>Inducible regulatory T cells</td>
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<td>LAP</td>
<td>Latency associated protein</td>
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<td>LAT</td>
<td>Linker of activated T cell</td>
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<tr>
<td>LCK</td>
<td>Leukocyte-specific protein tyrosine kinase</td>
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<td>LT-α</td>
<td>Lympho toxin-alpha</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility</td>
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<tr>
<td>NFAT</td>
<td>Nuclear translocation of the transcription factor</td>
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<td>NOD</td>
<td>Non obese diabetic</td>
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<tr>
<td>nTreg cells</td>
<td>Naturally arising regulatory T cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PICA</td>
<td>p53-induced and CD28-dependent apoptosis</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13 acetate</td>
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<td>RAG-1</td>
<td>Recombinant activating gene-1</td>
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<td>RasGRP1</td>
<td>Ras guanyl nucleotide releasing proteins 1</td>
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<td>ROR</td>
<td>Retinoid-related orphan receptor</td>
</tr>
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<td>RPMI1640</td>
<td>Roswell park memorial institute 1640</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
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<tr>
<td>SH</td>
<td>Src homology</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SLP76</td>
<td>SH2 domain containing leukocyte protein 76</td>
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<tr>
<td>SOS</td>
<td>Son of seventhless</td>
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<tr>
<td>T-bet</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>ZAP70</td>
<td>Zeta-chain associated protein 70</td>
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The immune system protects us from infection by pathogens such as viruses and bacteria. T cells play a central role in the immune response to fight against pathogens and orchestrate other immune cells by producing cytokines or chemokines. An overreactive immune response can lead to autoimmune diseases, therefore the immune system must possess negative regulation mechanisms. In the periphery, naturally arising regulatory T cells (CD4⁺CD25⁺Foxp3⁺ T cells; nTregs) negatively regulate immune responses and play an important role in maintaining immune homeostasis by suppressing other immune cells. When antigens derived from pathogens are present, conventional CD4⁺ T cells recognize their cognate antigen and proliferate. After pathogen clearance, expanded effector T cells eventually decline in number due to activation induced cell death (AICD) to terminate immune response. However, it is not understood how nTregs respond to antigen stimulation. Since effector T cells are needed for an immune response and nTregs are required for maintaining peripheral tolerance, the responses to antigen stimulation in nTregs might be different from other CD4⁺ T cells.

We previously discovered the phenomenon that nTregs survive and expand when stimulated with plate-bound anti-CD3/anti-CD28 antibodies which mimic antigen stimulation in the presence of IL-2, while conventional T cells undergo massive
apoptosis. This suggests that there is a differential survival mechanism between nTregs and conventional T cells. Further, we found that T cell stimulation under this stimulation was mediated by a novel form of the apoptotic pathway (p53-induced and CD28-dependent apoptosis; PICA) and distinct from classical AICD.

In my dissertation, I hypothesized that this differential survival mechanism between nTreg and conventional CD4+ T cells against PICA might be important in keeping the balance between nTregs and non-Tregs thus maintaining immune homeostasis in a physiological environment. Therefore, I investigated by which mechanisms nTregs survive under PICA inducing stimuli. I found that at least two signaling pathways are altered in nTregs under PICA induced stimulation thus mediating cell survival.

I conducted TCR proximal signaling study and identified that RasGRP1 expression was substantially reduced and its downstream Ras-Erk pathway was impaired in nTregs. nTregs express a transcription factor, Foxp3 which positively or negatively regulates the expression of multiple genes related to nTreg functions. I performed a ChIP assay to determine if rasgrp1 gene expression is negatively regulated by Foxp3 in nTregs, and identified that rasgrp1 gene was indeed a Foxp3 target. Further, loss of RasGRP1 expression rendered conventional CD4+T cells resistant to PICA, suggesting that reduced RasGRP1 expression in Tregs is important for cell survival under PICA inducing stimuli.

Upon TCR activation, T cells produce various cytokines such as IL-2. TGF-b is a pleiotropic cytokine which is also produced by T cells. Tregs, but not other conventional
T cells, express a unique form of membrane bound TGF-b upon TCR activation. I demonstrated that TGF-b signaling is required for Tregs to survive PICA. Conversely, conventional CD4+ T cells became resistant to PICA and underwent robust expansion instead of apoptosis, with the reduction of the proapoptotic protein Bim, when an active form of exogenous TGF-b is present. These data suggest a novel role for TGF-b in nTreg biology and potentially involvement in expansion of effector T cells under PICA inducing stimuli.

Taken together, my work provides insight into how nTregs survive under antigen stimulation while conventional CD4+ T cells undergo apoptosis. My work suggests that reduced RasGRP1 expression and TGF-b signaling mediate the survival of nTregs under PICA inducing stimuli. These altered signals in Tregs are likely regulating the balance of nTreg and conventional T cells during an immune response, thus maintaining immune homeostasis.
CHAPTER ONE
LITERATURE REVIEW

CD4+ T CELL IMMUNITY

Introduction

The immune system protects us from non-self invasions such as viruses or bacterial infections. CD4+ T cells play a major role in orchestrating other immune cells by producing cytokines or chemokines during the adaptive immune response, hence they are also called helper T cells. For example, CD4+ T cells help B cells to make antibodies and also help macrophages to have enhanced activity to fight against pathogens during infections. CD4+ T cells express T cell receptors (TCRs) on their surface and recognize peptides derived from pathogens presented on major histocompatibility class II (MHC class II) that are expressed by antigen presenting cells (APCs). Engagement of TCRs with MHC class II-peptide complex leads to activation of TCR signaling cascades which promote transcription of multiple genes including cytokine genes.

In 1986, Mossman and Coffman reported that CD4+ T cell lines were divided into two distinct populations by cytokine production, IFN-γ producers or IL-4 producers (Mosmann et al., 1986; Mosmann and Coffman, 1989). This was the discovery of Th1
and Th2 subsets. Heinzel et al. reported that BALB/c mice were more susceptible than C57BL/6 mice during *Leishmania major* infections (Heinzel and Maier, 1999). This is because BALB/c mice are biased toward a Th2 response rather than Th1. This promotes a humoral response instead of a cellular immune response against intracellular pathogens. This finding suggests a correct immune response at the right time leads to elimination of pathogens, however dysregulation of differentiation of CD4$^+$ T cells can cause chronic infection.

Today, helper T cell differentiation is known to be much more complex and there are at least 6 subsets reported; Th1, Th2 Th17, iTregs, Th3 and Th9 (Fukaura et al., 1996; Jager et al., 2009; Mosmann and Coffman, 1989; Xing et al., 2011; Zhu and Paul, 2008). Each subset produces its signature cytokines and is responsible for mediating immune responses. Cytokine milieu and antigen stimulations determine the fate of naïve CD4$^+$ T cells.

*Th1 cells*

Th1 cells mediate cellular immune responses against intracellular pathogens by producing IFN-γ, IL-2 and lymphotoxin-α (LTα). IFN-γ is known as type II IFNs and activates macrophages through IFN-γ receptor signaling. This event mediates cellular immune response fighting against mycobacterial infections such as *Leishmania major* infections. LTα is another cytokine produced by Th1 cells which mediates inflammatory responses (Suen et al., 1997). Excess Th1 response can cause tissue damages which lead to autoimmune diseases too. For example, higher levels of LTα is detected in multiple
sclerosis patients, suggesting LTα is involved in the disease progression. Suen et al. has found that LTα deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE), suggesting that LTα mediates EAE progression (Suen et al., 1997).

Th1 cells express a transcription factor, T-bet which is induced by IFN-γ during Th1 differentiation (Szabo et al., 2000). Ectopic expression of T-bet is sufficient to differentiate naïve CD4+ T cells into Th1 cells. It promotes IFN-γ production and inhibits production of IL-4, which is known as Th2 cytokine (Yin et al., 2002). Finotto et al. have shown that T-bet deficient mice develop an asthma like phenotype (Finotto et al., 2005). These data suggest that fine balance of Th1 cells against Th2 cells is critical to maintain immune homeostasis.

Th2 cells

Th2 cells mediate humoral immune responses and are responsible for eliminating extracellular pathogens, such as helminth, by producing IL-4, IL-5, IL-10 and IL-13 (Abbas et al., 1996; Glimcher and Murphy, 2000). Gros et al. and Swain et al. have shown that naïve CD4+ T cells preferentially differentiate into IL-4 producers under anti-CD3 antibody stimulation in the presence of IL-4 in vitro (Gross et al., 1993; Swain et al., 1990). They also show that this culture condition inhibits cells producing IFN-γ and IL-2.

Th2 cells express a transcription factor GATA-3 and expression of GATA-3 is upregulated during Th2 differentiation (Kurata et al., 1999; Zhu et al., 2001). IL-4 mediates IgE class switching in B cells (Shimoda et al., 1996). IgE is known to bind to
FC receptor on basophils and mast cells (Schulman et al., 1988). This event leads to secretion of histamine from these cells, hence excess Th2 responses are often linked to induction of asthma and other allergic diseases (Barnes, 2001; Zhou et al., 2001).

**Th17 cells**

Th17 cells mediate an inflammatory response by producing IL-17 (Ivanov et al., 2006). Naïve CD4+ T cells can differentiate into Th17 in the presence of TGF-β and IL-6 in vitro (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Th17 cells do not produce IL-4 or IFN-γ, so it is considered a distinct subset from Th1 or Th2. IL-23 is another critical cytokine for Th17 (Aggarwal et al., 2003). Recent studies suggest that IL-23 is not required for Th17 differentiation but is important for maintenance and proliferation of Th17 cells (Aggarwal et al., 2003; Bettelli et al., 2006; Veldhoen et al., 2006). Th17 cells express an orphan nuclear transcription factor, RORγt (Ivanov et al., 2006). RORγt directly activates transcription of IL-17 and it is required for Th17 induction (Manel et al., 2008). Further, ectopic expression of RORγt makes CD4+ T cells Th17 like phenotype, suggesting that RORγt is sufficient to induce Th17 (Ivanov et al., 2006; Sofi et al., 2010).

The importance of IL-17 signaling during infections was delineated using mouse models. Ye et al. showed that IL-17 deficient mice became extremely sensitive to intranasal *Klebsiella pneumoniae* compared to wild type mice using a model of *Klebsiella pneumoniae* lung infection (Ye et al., 2001). IL-17 was important to clear the pathogens. On the other hand, accumulating evidence suggests that IL-17 is also involved in the
development of autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis (Gold and Luhder, 2008; Hofstetter et al., 2005; Kotake et al., 1999; Nakae et al., 2003). For example, Komiyama et al. showed that IL-17 knockout mice exhibit a delayed onset of experimental autoimmune encephalomyelitis (EAE) (Komiyama et al., 2006). Blockade of IL-17 signaling using anti-IL-17 neutralizing antibody experiments also showed delayed Type I diabetes in the NOD mouse model (NOD), which produced increased levels of IL-17 (Emamaullee et al., 2009).

In humans, Zao et al. showed that AID patients had higher frequencies of Th17 cells (Zhao et al., 2010). Furthermore, Wong et al reported that increased levels of IL-17 in plasma from SLE patients compared to healthy donors (Wong et al., 2008).

**Th9 cells**

Th9 is the most newly identified helper T cell subset, which produces IL-9. IL-9 is a pleiotropic cytokine which influences multiple cell types including T cells, B cells and Mast cells. For T cells, IL-9 is important for cell growth and can promote Th17 differentiation (Elyaman et al., 2009; Nowak et al., 2009; Schmitt et al., 1994). For Mast cells, IL-9 increases their activity and also works as a growth factor (Wiener et al., 2004). IL-9 producing CD4+ T cells were originally classified as a part of the Th2 subset, however it is now considered as a distinct population from Th2 (Soroosh and Doherty, 2009). Naïve CD4+ T cells differentiate to Th9 in the presence of IL-4 and TGF-β (Schmitt et al., 1994). Recently, PU.1 was characterized as the Th9 hallmark transcription factor (Chang et al., 2009; Chang et al., 2005). PU.1 directly controls IL-9 gene transcription but also represses Th2 cytokine production (Chang et al., 2010).
IL-9 is thought to be involved in proinflammatory responses. Temann et al. showed that overexpression of IL-9 in lungs of transgenic mice caused asthma symptoms such as mucus production and subepithelial fibrosis (Temann et al., 2002). Further, Kearly et al showed that mice treated with IL-9 neutralizing antibody ameliorated allergy inflammation using an acute inflammatory mouse model challenged with house mite allergen (Kearley et al., 2011).

REGULATORY T CELLS FOR TOLERANCE

Introduction

In the early 1970s, the concept of peripheral tolerance attracted attention. Gershon and Kondo found that a T cell subset, termed suppressor T cells, dampened immune responses (Gershon and Kondo, 1970). It was reported that the I-J molecule, which was supposed to be located on MHC gene complex, played a key role in exerting immune suppressive functions. However it turned out to be not true, and it was realized that such I-J molecules did not exist after extensive studies using molecular biology techniques (Kronenberg et al., 1983). In 1995, Sakaguchi et al. drew renewed attention for the concept of suppressor T cells (Sakaguchi et al., 1995). They discovered that CD4+CD25+ T cells have suppressive ability against immune responses. CD25 is an IL-2 receptor α chain and 5-10% of CD4+ T cells express CD25 under the naïve state while all CD4+ T cells upregulate surface expression of CD25 after TCR activation (Levings et al., 2001; Sakaguchi et al., 1995). They performed a cotransfer of CD4+CD25− and CD4+ CD25+ T cells into athymic mice and found that the mice which only received CD4+CD25+ T cells
developed severe autoimmune symptoms including colitis and auto-antibody production (Sakaguchi et al., 1995). This experiment suggests that CD4^+CD25^+ T regulatory (Treg) cells indeed have suppressive functions to control immune responses. Later, accumulated evidence suggested that IL-2 receptor α chain (CD25) was not only a Treg cell surface marker but also critical for development, survival and function of Treg cells (D'Cruz and Klein, 2005; Fontenot et al., 2005).

CD4^+CD25^+ Treg cells develop in the thymus. Itoh et al. found that TCR transgenic mice which also lack Rag expression did not develop Tregs, suggesting that Tregs need TCR specificity for cell development (Itoh et al., 1999). Recent studies suggested that TCR repertoires between CD4^+CD25^+Treg cells and CD4^+CD25^-T cells were comparable but distinct by sequencing the variable region of TCR α chain (Kuczma et al., 2009; Madakamutil et al., 2008; Singh et al., 2010b).

**Foxp3**

Although CD4^+CD25^+ T cells were discovered as a CD4^+ T cell subset which had suppressive functions and termed regulatory T cells by the Sakaguchi group, it was not understood what controlled suppressive function of CD4^+CD25^+Treg cells.

Scurfy mice came about by a spontaneous mutations on the X chromosome. These mice exhibit autoimmune disease symptoms including splenomegaly and lymphadenopathy. In 2001, Benett et al. discovered the scurfy mice had mutations in the foxp3 gene (Bennett et al., 2001). Hori et al. then reported that Foxp3 was specifically expressed in the CD4^+CD25^+ Treg cell population (Hori et al., 2003). Further, they showed that overexpression of Foxp3 rendered non-Treg cells (CD4^+CD25^- T cells) to a
Treg-like phenotype with suppressive functions (Hori et al., 2003). Also, Fontenot et al. showed that ectopic expression of Foxp3 conferred suppressor function on peripheral T cells (Fontenot et al., 2003). In humans, autoimmune syndrome X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy (IPEX) patients have multiple mutations in the foxp3 gene, suggesting there is a link between foxp3 mutations and autoimmune disease (Lopes et al., 2006; Myers et al., 2006). Furthermore, forced expression of Foxp3 using transgenic mice rendered mice more susceptible to virus infection challenges and showed reduced T cell numbers in the spleen and lymph nodes while T cell development occurred normally (Guo et al., 2010). These finding suggested that Foxp3 was a master regulator of Treg cells. Foxp3 is a transcription factor and a member of the forkhead family (Ziegler, 2006). It has a fork head (FKH) domain which binds to core DNA sequences (Coffer and Burgering, 2004; Lin and Peng, 2006). Members of the Foxp3 family function as both transcriptional activators and repressors. Accumulated evidence suggests that Foxp3 also works as transcriptional activator and repressor. For example, the il-2 gene is a known target of Foxp3 and its gene expression is negatively regulated in Treg cells (Fontenot et al., 2003; Hori et al., 2003). Schubert et al. showed that ectopic expression of Foxp3 in Jurkat cells resulted in downregulation of il-2 (Schubert et al., 2001). Recently, mechanisms that make Foxp3 repress the il-2 gene have been proposed. Schubert et al. showed that the il-2 promoter contained a forkhead binding sequence adjacent to the NFAT binding site, therefore they proposed a model in which Foxp3 competes for the binding site with NFAT thus repressing il-2 transcription (Schubert et al., 2001). Betteli et al. showed that NFAT and Foxp3 interact in a cell free system (in cell lysate), therefore they proposed the model where Foxp3 sequestered
NFAT thus inhibiting NFAT binding to the *il-2* promoter (Bettelli et al., 2005). Further, Wu et al. showed that mutations in the NFAT interaction site of Foxp3 upregulated *il-2* gene expression (Wu et al., 2006). This also supports the model that Foxp3 inhibits NFAT binding to the *il-2* promoter by directly interacting with NFAT.

Additionally, other Treg function related genes such as CD25 and CTLA4 are known to be upregulated by Foxp3 while expression of IL-4, IFN-γ genes is repressed (Ziegler, 2006).

**Suppressive function of Treg cells**

There have been multiple mechanisms proposed for CD4⁺CD25⁺Treg cell suppressive functions. Hori et al. showed that Treg cells required antigenic stimulation via TCR to exert their suppressive function against responder cells (Hori et al., 2003). Takahashi et al. demonstrated that when Treg cells and responder cells (non-Treg cells) were separated using permeable membrane, Treg cells were not able to suppress proliferation of responder cells (Takahashi et al., 2000). Further, Gondek et al. showed that culture supernatant of antigen-stimulated Treg cells failed to suppress responder cells (Gondek et al., 2005). These results indicated that Treg cells suppress responder cells in a cell contact dependent manner.

Treg cells do not produce IL-2, however they express high levels of IL-2 receptor α chain, CD25 on the cell surface. Therefore, Padiyan et al. proposed the model that Treg cells used up IL-2 thus causing deprivation of IL-2 for responder cells leading to apoptosis (Padiyan et al., 2007). They showed that the addition of exogenous IL-2 rescued responder T cells from apoptosis and provoked Treg cell suppressive functions.
The interaction of Treg cells and antigen presenting cells (APCs) has also been proposed to make up a suppressive environment. Oderup et al. found that Treg cells downregulated B7 costimulatory molecules expressed on DCs, suggesting that Treg cells reduced DC function thus preventing activation of responder cells (Oderup et al., 2006). Further, Treg cells activated DCs to upregulate expression of the enzyme indoleamine 2,3-dioxygenase (IDO) (Fallarino et al., 2003; Grohmann et al., 2003). IDO bears off tryptophan by catabolizing the conversion of tryptophan to kyurenine, which is toxic to T cells (Mellor and Munn, 2004). Hence, responder cells lack the essential amino acid tryptophan and are exposed to kyurenin thus eliminating responder cells.

Cytokine secretion is also proposed to be one of Treg cell suppressive mechanism. Nakamura et al. demonstrated that membrane bound TGF-β expressed on Treg cells were essential for Treg cell suppressive function (Nakamura et al., 2001). Further, IL-10 and galectin were proposed to be involved in Treg cell suppressive function (Sugimoto et al., 2006).

Together, these findings suggest that Treg cells suppress the immune response by multiple mechanisms in a direct or indirect manner.

**Inducible Treg (iTreg) cells**

Naïve CD4+ T cells can differentiate to inducible Treg (iTreg) cells when TGF-β is present during antigen stimulation. iTreg cells also express Foxp3 as do Treg cells and exert suppressive function (Knoechel et al., 2005). However, Foxp3 expression in iTreg cells is not stable as nTreg cells. Floess et al. showed that iTreg cells which were generated under TCR stimulation in the presence of TGF-β, reduced Foxp3 expression
and suppressive function when they were restimulated in the absence of TGF-β (Floess et al., 2007). iTreg cells are preferentially generated in gut associated lymphoid organs, since TGF-β level is high in these area (Chen et al., 2003; Selvaraj and Geiger, 2007). Constant antigen exposure such as commensal bacteria or food antigens is also preferred condition for iTreg cell induction (Kretschmer et al., 2005; Mucida et al., 2005).

T CELL RECEPTOR (TCR) PROXIMAL SIGNALING

Introduction

As mentioned above, T cells recognize antigens using its surface expressed TCR, which initiates activation of cells thus leading to cell proliferation and cytokine production. At the molecular level, multiple proteins form signaling cascades and convert input TCR stimulation signals into output such as cytokine production.

Ligation of TCRs to their cognate antigens presented on MHC class II initiates TCR signaling cascade by activating receptor associated src family phosphotyrosine kinases, LCK. LCK then phosphorylates immunoreceptor tyrosine based activation motif (ITAM) on TCRζ chains in the CD3/TCR complex, thus making binding site for the dual src homology 2 (SH2) domain of src family kinase, the tyrosine kinase ζ- associated protein 70 kDa (ZAP-70) (Iwashima et al., 1994; Kane et al., 2000; Straus et al., 1996). This event recruits ZAP-70 to the plasma membrane and activates ZAP-70. Activation of ZAP70 then leads to phosphorylation of multiple side adopter proteins, the linker for activation of T cells (LAT) and induces the formation of protein complex with other
adopter proteins including Grb-2, SHC, SLP76 by recruiting them to its phosphorylated sites (Buday et al., 1994; Gilliland et al., 1992; Zhang et al., 1998).

Son of sevenless (SOS) is a substrate for Grb-2 and is recruited to the plasma membrane following activation of Grb2 in the LAT complex. Activated SOS by phosphorylation then activates a Ras guanyl nucleotide exchange factor thus leading to Erk activation (Ravichandran et al., 1995; Xavier et al., 1998).

Phospholipase C-γ (PLC) is also one of the proteins which make a complex with LAT following LAT activation and is activated by phosphorylation in this protein complex (Nishibe et al., 1990). Activated PLC-γ then produces DAG and IP3 by cleaving phosphatidylinositol-4,5-bisphosphate (PIP₂) (Nishibe et al., 1990). IP3 results in the increase of the intracellular calcium concentration [Ca²⁺] leading to nuclear translocation of the transcription factor, NFAT (Crabtree, 1999; Stankunas et al., 1999). DAG activates another Ras activator, RasGRP1 thus leading to the activation of Ras-Erk pathway (Ebinu et al., 2000).

One of most well studied outputs upon TCR stimulation was IL-2 production. IL-2 was originally identified as a T cell growth factor and essential for T cells to become effector cells and proliferate (Waldmann et al., 2001). Upon TCR activation, il-2 transcription is initiated by transcription factors including NFAT and AP-1 (Jain et al., 1995). NFAT is activated via the calcium signaling pathway and AP-1 is regulated via the Ras-Erk pathway. Erk activation leads to upregulation of c-Fos, which forms the AP-1 complex (Karin et al., 1997). Therefore, both the Ras-Erk signal and calcium signal regulate production of IL-2 in response to TCR stimulation.
RasGRP1

RasGRP1 was originally identified from brain and T cell cDNA library by selecting Ras activation related genes (Ebinu et al., 2000; Tognon et al., 1998). Ebinu et al. showed that the catalytic domain of RasGRP1 promoted activation of H-Ras in vitro using H-Ras overexpressed Rat2 cells (Ebinu et al., 2000). Further, they demonstrated that RasGRP1 translocate to plasma membrane with PMA treatment followed by activation of Erk. They also characterized the catalytic domain by homology search of amino acids and identified that it contains CDC25, named for the prototypic Ras activator from *Saccharomyces cerevisiae*, and Ras exchange motif (REM) domain. Shortly thereafter, Lorenzo et al demonstrated that RasGRP1 possessed C1 domain and bound to DAG and DAG analog, PMA (Lorenzo et al., 2000). Further, they tracked RasGRP1 localization by overexpressing RasGRP1- GFP fusion in Hela cells and showed that RasGRP1 translocated to plasma membrane in response to PMA stimulation. These data suggest that RasGRP1 activated Ras in response to DAG.

RasGRP1 is dominantly expressed in T cells among RasGRP family (RasGRP1, 2, 3, 4) and multiple T cell lines also express RasGRP1 (Ebinu et al., 2000). Especially, Jurkat T cell lines have been used to investigate a role of RasGRP1 in T cells. Ebinu et al. showed that over expression of RasGRP1 in Jurkat cells resulted in enhanced sensitivity to TCR stimulation of Ras-Erk pathway (Ebinu et al., 2000). Further, they showed that overexpression of RasGRP1 led to increased IL-2 production. Roose et al. reported that RasGRP1 deficient Jurkat cell lines had decreased activity of Ras-Erk pathway and decreased expression of CD69, which is early T cell activation marker in response to TCR stimulation (Roose et al., 2005). These data suggest that RasGRP1 links the signal...
between PLC-γ and Ras. Although there is another Ras activator, SOS exists in T cells, these data suggest that RasGRP1 plays essential roles for activating Ras and is not compensated by SOS activity. This model was more clearly supported by using RasGRP1 deficient mice.

Dower et al. engineered RasGRP1 deficient mice and demonstrated that RasGRP1 was essential for thymocyte differentiation (Dower et al., 2000). They showed that RasGRP1 deficient mice exhibited abnormal thymocyte differentiation pattern. The CD4+CD8+ double positive T cell ratio substantially increased while single positive CD4+ or CD8+ T cells decreased compared to littermate control, suggesting that RasGRP1 expression was required for positive selection during thymic development. In contrast, Normant et al. used RasGRP1 transgenic mice which overexpress RasGRP1 under control of the proximal LCK promoter (Norment et al., 2003). T cells which lack expression of Rag1 cannot undergo V, (D), J gene rearrangement, therefore they cannot undergo development process in thymus (Swat et al., 1996). However, RasGRP1 transgenic mice in Rag1 knockout background exhibited single positive CD8+ T cells, suggesting that RasGRP1 affects thymocyte development (Norment et al., 2003).

Dower et al. showed that RasGRP1 knockout mice have splenomegaly and autoimmune lymphoproliferative disorder (Dower et al., 2000). These mice also had antinuclear antibodies. These data suggest that the lack of RasGRP1 expression in T cells leads to expansion of T cells in periphery thus influencing to B cells. Since RasGRP1 deficient mice lack RasGRP1 expression in whole body, Layer et al. further tested loss of RasGRP1 effect specifically on T cells (Layer et al., 2003). They adoptive transferred T cells isolated from lag mice, which has mutations in rasgrp1 gene, into T and B cell
deficient mice (RAG knockout mice). The recipient mice also developed autoimmune lymphoproliferative disease, which suggested that loss of RasGRP1 expression in T cells causes pathogenesis.

IMMUNE HOMEOSTASIS

*TGF-β in immune homeostasis*

TGF-β is a pleiotropic cytokine which mediates multiple physiological functions such as proliferation, apoptosis and differentiation and is produced by many cell types including lymphocyte (Bommireddy and Doetschman, 2004). TGF-β is also known as an essential factor to keep immune homeostasis (Gorelik and Flavell, 2002).

T cells dominantly produce TGF-β 1 among TGF-β family, TGF-β 1, 2 and 3 (Li et al., 2006). The role of TGF-β 1 in immune homeostasis and peripheral tolerance has been demonstrated using TGF-β1 deficient mice or TGF-β receptor deficient mouse models. Kukarni et al. and Shull et al. engineered TGF-β 1 deficient mice and showed that these mice developed an excessive inflammatory response and multifocal inflammatory disease and died at the age of 3 to 5 weeks (Kulkarni et al., 1993; Kulkarni and Karlsson, 1993; Shull et al., 1992). Since T cells have been known to be essential for mediating immune disorders, it was expected that loss of TGF-β 1 caused immune disorders in a T cell dependent manner. Later, Gorelik et al. engineered transgenic mice which expressed dominant negative form of human TGF-β receptor II under control of the CD4 promoter (Gorelik and Flavell, 2000). Hence, TGF-β signaling is blocked specifically in T cells in
these transgenic mice. They showed that these transgenic mice also developed lymphoproliferative disorders, which suggested TGF-β signaling in T cells was required for regulating immune homeostasis. Li et al. further investigated the definitive role of TGF-β signaling using conditional TGF-β receptor knockout mice crossed with CD4 cre mice (Li et al., 2006). These mice lacked expression of TGF-β receptor II in T cells. These mice also exhibited lymphoproliferative disorders and lethal autoimmunity. This result also supports the idea that TGF-β signaling in T cells is required for regulating immune homeostasis.

It is now widely accepted that regulatory T cells are essential for maintaining peripheral tolerance. Li et al. showed that the number of Treg cells in the periphery substantially decreased in the conditional knockout mice, which lacked TGF-β signaling in T cells, while thymic Treg cell numbers were not affected, suggesting that TGF-β signaling affected maintenance of Treg cells (Li et al., 2006). Further, they showed that TGF-β receptor deficient Treg cells proliferated more than wild type Treg cells in response to TCR stimulation, suggesting TGF-β signaling was suppressing Treg cell proliferation. To address whether the loss of peripheral tolerance in these mice is due to a decrease in the number of Treg cells or intrinsic effect of other effector T cells, Li et al. performed adoptive transfer experiment that transferred wild type Treg cells into neonatal TGF-bRII deficient mice. If a reduced number of Treg cells was the cause of autoimmune disorder in TGF-bRII deficient mice, transferred wild type Treg cells into the mice would be expected to rescue these mice from developing autoimmune disease. However, these mice still developed an autoimmune disease and transferred wild type Treg cells did not
suppress T cell activation in the host mice, which suggested that Treg cells are not only mechanisms to keep immune homeostasis, but also that TGF-β signaling in T cells possessed intrinsic effect leading to peripheral tolerance.

In addition to nTreg cells produced in thymus, naïve T cells also can be differentiated to Foxp3+ Treg cells referred to as inducible Treg (iTregs) cells. *In vitro*, naïve CD4+ T cells are differentiated to iTregs with TCR stimulation in the presence of TGF-β (Chen et al., 2003; Wan and Flavell, 2005). Tone et al. showed that TGF-β induces Foxp3 expression through SMAD3 association with Foxp3 promoter (Tone et al., 2008). In an *vivo* mouse model, Peng et al. demonstrated that overexpression of TGF-β 1 in pancreatic islets resulted in expanded CD4+Foxp3+Treg cells and rescued NOD mice from developing diabetes (Peng et al., 2004). These data suggest that TGF-β induces Treg cells thus maintaining peripheral Treg cells not only influencing nTreg cells.

TGF-β can influence naïve CD4+ T cell differentiation into other helper T cell subset besides iTreg cells. Betteli et al. showed that in the presence of IL-6, TGF-β can convert naïve T cells into Th17 and iTreg differentiation was blocked (Bettelli et al., 2006). Th17 produces signature cytokine, IL-17 and leads to proinflammatory response (Bettelli et al., 2006). Further, Gorelik et al. reported that TGF-β inhibited Th1 and Th2 differentiation by downregulating expression of their hallmark transcription factors, T-bet and GATA-3 (Gorelik et al., 2000). This suggests that TGF-β can also contribute to inflammatory responses rather than keep peripheral tolerance.

The ligand, TGF-β 1 is secreted by T cells as homodimer but as an inactive form by associating with homodimer of latency associated protein (LAP) (Annes et al., 2003). To
initiate TGF-β signaling cascade, LAP has to be removed from TGF-β inactive form by integrins or matrix proteins such as Thrombospondin-1 (TSP-1) (Masli et al., 2006; Yehualaeshet et al., 1999).

TGF-β signaling is initiated when active form of TGF-β binds to TGF-β type II receptor and results in phosphorylation of serine/threonine kinase in Type II receptor kinase cytoplasmic domain. This event results in the engagement of tetrameric receptor complex of TGF-β receptor type I and type II, activating type I receptor serine/threonine kinase in the cytoplasmic region. Phosphorylated Type I receptor kinase then activates transcription factors, SMAD proteins thus eliciting multiple cellular functions by activating gene transcriptions (Bommireddy and Doetschman, 2004).

Antigen induced cell death (AICD) in immune homeostasis

Activation induced cell death (AICD) is one of the mechanisms to keep immune homeostasis by decreasing T cell number in the periphery due to apoptosis. Shi et al. originally used the term AICD to describe the phenomenon that the ligation of the TCR on thymocytes leads to apoptotic cell death (Shi et al., 1989). At present, accumulating evidence suggests that mature T cells also can undergo AICD. An in vitro setting of AICD refers to a death caused by restimulation of activated T cells. To stimulate T cells in vitro, antigenic stimulation can be mimicked by using anti-CD3 antibody activating through the TCR and anti-CD28 antibody activating the CD28 costimulatory signal. In an AICD in vitro model, T cells are stimulated with anti-CD3 and anti-CD28 for several days, then restimulated with anti-CD3 or PMA and ionomycin. PMA is a diacylglycerol analog and ionomycin is an ionophore which increases intracellular calcium
concentration. Treating T cells with these chemicals also resembles TCR activation (Krammer, 2000).

In 1991, Lenardo et al. demonstrated that restimulation of T cells induced cell surface expression of Fas ligand leading to apoptosis by using T cell hybridoma (Lenardo, 1991). Fas is a death receptor which belongs to the tumor necrosis factor receptor (TNFR) family and it possesses a death domain in the cytoplasmic tail of the intracellular region (Nagata, 1997). The ligation of Fas and Fas ligand leads to activation of Fas-associated death domain (FADD) followed by the formation of death inducing signaling (DISC) complex (Kischkel et al., 1995). The DISC complex then activates caspase 8 and induces apoptosis in T cells. There are soluble form and membrane bound form of Fas ligand expressed by alternative splicing. LA et al. showed that membrane bound form of Fas ligand was required to cause apoptosis in T cells by using mice which lack either the soluble form or the membrane bound form of Fas ligand (LA et al., 2009). Lenardo et al. showed that Fas mediated AICD was initiated in an IL-2 dependent manner (Lenardo, 1991). As described above, IL-2 is a cytokine produced by T cells and is known to be important factor for cell survival. Further, Refaeli et al. showed that IL-2 signaling upregulates the transcription and cell surface expression of Fas ligand thus inducing apoptosis (Refaeli et al., 1998). They used IL-2 knockout mice and showed that the IL-2 deficient CD4+ T cells did not upregulate cell surface expression of Fas ligand and became resistant to AICD. These data surprisingly suggest IL-2 signaling is not only a cell survival factor but also an important factor to induce AICD.

Mutant mice which have mutations in fas or fas ligand genes have been used to study the effect of Fas/ Fas ligand pathway. Lpr mice have mutations in fas gene locus.
and do not produce functional Fas. *Gld* mice have mutations in fas ligand gene locus and do not produce functional Fas ligand (Cohen and Eisenberg, 1991). These mice develop lymphoadenopathy with accumulated T cells and produce autoreactive antibodies. In human, Aspinall et al. reported that autoimmune lymphoproliferative syndrome (ALPS) patients had mutations in the *fas* gene (Aspinall et al., 1999). This evidence suggests that Fas/ Fas ligand signaling plays a critical role in maintaining immune homeostasis in the periphery.

Recent studies have shown that a mechanism other than Fas/ Fas ligand interaction is also involved in AICD in T cells. Hildeman et al. showed that *lpr* and *gld* mice were sensitive to T cell death induced by staphylococcal enterotoxin B (SEB), suggesting T cell death with SEB activation was induced in a Fas/FasL independent manner (Hildeman et al., 2002). Further, they found that Bcl-2 interacting mediator of cell death (Bim) is responsible for this apoptosis induced by SEB activation using T cells isolated from Bim knockout mice. Snow et al. reported that Bim also played an important role in inducing AICD. They knocked down Bim expression in primary human T cells isolated from healthy donors and showed that T cells, which lacked Bim expression were rescued from AICD in vitro (Snow et al., 2008). They further found that T cells derived from ALPS patients expressed lower level of Bim and the cells were more resistant to AICD in vitro.

Bim is a member of BCL-2 family and BH-3 only protein, which has only one of Bcl-2 homology region, BH-3. BCL-2 family mediates intrinsic apoptosis associated with mitochondrial membranes and at least 20 family members have been identified (Gross et al., 1999). Some of them are constitutively expressed on mitochondrial membrane or translocate to mitochondria upon stimulation. They are separated into two groups by their
function, pro-apoptotic and anti-apoptotic. Anti-apoptotic proteins include BCL-2, BCL-X and BCL-W. Pro-apoptotic proteins include Bim, BAK, BAD and BAX. Bim antagonizes BCL-2 which inhibits BAK and BAX pro-apoptotic function by associating with them (O'Connor et al., 1998). Hence, Bim releases Bak and BAX and leads to activation of caspase 9 and apoptosis in cells.

Bim expression is regulated at both the transcriptional and the post-transcriptional level. Bim has three isoforms, Bim EL, Bim L, and Bim S (O'Connor et al., 1998). These isoforms are produced by alternative splicing and all of them can induce apoptosis. In T cells, Bim is upregulated upon TCR stimulation through protein kinase C and in a calcineurin dependent manner (Sandalova et al., 2004).

Bim deficient mice exhibit lymphadenopathy and contain increased number of T cells and B cells compared to littermate controls (Bouillet et al., 1999). These mice also develop severe autoimmune disease phenotypes as seen in Fas, Fas ligand mutant mice (lpr, gld mice), suggesting that Bim also plays a critical role in mediating immune homeostasis.

Recent studies suggest that Bim and Fas have a complementary effect. Hutcheson et al. generated mice which lacked expression of both Bim and Fas by crossing lpr and Bim knockout mice and showed that the mice exhibited much more severe autoimmune disease phenotype depicted by lymphoadenopahy compared to Bim or Fas single deficient mice (Hutcheson et al., 2008). These mice accumulated a large number of T cells and B cells in lymphoid organs and it expanded to half of the animal’s body mass. These data suggest Bim and Fas pathway has synergistic effect on developing autoimmune disease.
PROPOSED MODEL AND P53 DEPENDENT APOPTOSIS (PICA)

We previously discovered the phenomenon that non-Treg cells undergo apoptosis when CD4$^+$ and CD8$^+$ T cells are stimulated with plate-bound anti-CD3/anti-CD28 antibodies coated on plastic plate in the presence of IL-2 over 4 days (Singh et al., 2010a). Activation induced cell death (AICD) is a well known mechanism that causes T cell apoptosis. Boehme et al. showed that T cell blasts isolated from p53 deficient mice were sensitive to anti-CD3 antibody induced cell death, suggesting that AICD did not require p53 expression (Boehme and Lenardo, 1996). p53 is a transcription factor which is activated upon cellular stresses, including UV damage and inappropriate cell proliferative signals. p53 regulates cell cycle and other cellular functions (Levine, 1997). We showed that p53 deficient CD4$^+$ T cells became resistant to apoptosis induced by plate-bound anti-CD3/anti-CD28 antibody stimulation, suggesting the apoptosis induced by this stimulation was in a p53 dependent manner and distinguishable from classical AICD. Hence, we named this novel form of apoptosis caused by plate-bound anti-CD3/anti-CD28 antibody stimulation “p53-induced CD28-dependent apoptosis” (PICA) to distinguish it from classical AICD (Singh et al., 2010a). Interestingly, CD4$^+$CD25$^+$ Treg cells were resistant to PICA and selectively expanded under PICA-inducing conditions while non-Treg cells (CD4$^+$CD25$^-$ T cells) underwent massive apoptosis. The Treg cell number increased 7,000 fold in 7 days under plate-bound anti-CD3/anti-CD28 antibody stimulation.
We further showed that PICA was induced in non-Treg cells in a Bim and Fas/FasL dependent manner due to upregulation of each molecule upon plate-bound anti-CD3/anti-CD28 antibody stimulation by using Bim deficient and lpr (Fas mutant) mice. In contrast, Treg cells suppressed Bim and FasL expression under PICA inducing stimuli.

The p53 deficient mice were also equally susceptible to AICD compared to wild type mice. On the other hand, accumulated evidence suggests that the lack of p53 expression in mice leads to earlier onset or exacerbated autoimmune diseases in various mouse models. Simelyte et al. showed that experimental induced arthritis mice, which lack p53 expression exhibited exacerbated disease symptoms compared to wild type mice (Simelyte et al., 2005). Okuda et al. used an experimental autoimmune encephalomyelitis (EAE) model, which showed p53 deficient mice injected with myelin oligodendrocyte glycoprotein developed more severe disease than the wild type control (Okuda et al., 2003). Zheng et al. reported that p53 deficient mice are more susceptible to streptozocin-induced diabetes which was used as a model for type I diabetes (Zheng et al., 2005). In humans, Maas et al. reported that peripheral blood mononuclear cells isolated from rheumatoid arthritis patients expressed lower level of p53 at the mRNA level compared to healthy donors in response to gamma-radiation (Maas et al., 2005). These observations suggest that p53 dependent cell death rather than AICD might be important to maintain immune homeostasis. However, it is not understood what causes dysregulation in immune homeostasis and exacerbated autoimmune disease phenotype in p53 deficient mouse models.

Our in vitro data using plate-bound anti-CD3/anti-CD28 antibody stimulation showed that p53 dependent apoptosis (PICA) caused apoptosis in non-Treg cells but
expand Treg cells, so it was predicted that PICA inducing condition would increase the ratio of CD4+CD25+ Treg cells to non-Treg cells (CD4+CD25- T cells). Hence, we propose the model that the differential survival mechanism between Treg cells and non-Treg cells against PICA is important to balance Treg cells and non-Treg cells thus maintaining immune homeostasis. Therefore, we herein will determine the differential survival mechanisms of Treg cells and non-Treg cells using plate-bound anti-CD3/anti-CD28 antibody stimulation.
CHAPTER TWO
MATERIALS AND METHODS

Mice

BALB/c, C57BL/6 and CD4dnTgfbr2 were purchased from Jackson Laboratory (Bar Harbor, ME). RasGRP1 knockout mice were a gift from Dr. James C Stone (University of Alberta, Canada). All mice were maintained under specific pathogen-free conditions. All procedures were approved and monitored by the Institutional Animal Care and Use Committee of Loyola University Chicago.

Flow cytometry

Fluorochrome-conjugated antibodies specific for Foxp3 (clone FJK-16s) and IL-17A (clone ebio17B7) (eBioscience, San Diego, CA), anti-CD4 (clone GK1.5) and anti-IL-9 (clone RM9A4) (Biolegend, San Diego, CA), annexin V, 7-aminoactinomycin D (7AAD), anti-CD25 (7D4), anti-Fas (Jo2) and anti-FasL (MFL3) (BD Biosciences, San Jose, CA) were used in the experiments. Cells were pelleted by centrifugation at 1,500 rpm for 5 min at 4°C. 10 µl of culture supernatant from 24G2 hybridoma (FC receptor blocker) was added and tubes were kept at room temperature for 5min. Cells were then washed with FACS buffer (1% FCS, 0.1% sodium azide in 1× PBS). Cell surface staining was performed using
50 µl of antibodies (1:100 to 1:500 dilution in FACS buffer) for 30 min on ice. After staining, cells were washed with FACS buffer. For Foxp3 staining, cells were fixed and permeabilized using FOXP3 Staining Buffer Set (eBiosciences, San Diego, CA) as described in the manufacture’s protocol. Briefly, cells were washed twice with 1× PBS after cell surface staining followed by fixation with 300µl of 1× FixPerm buffer overnight at 4°C. The next day, cells were washed twice with FACS buffer and incubated with 1× permeabilization buffer for 10 min on ice. Cells were then stained with anti-Foxp3 antibody (diluted as 1:200) for 1 hour on ice followed by washing with FACS buffer. For annexin V and 7AAD staining, cells were stained in annexin binding buffer (10 mM HEPES pH7.4, 140 mM NaCl, 2.5 mM CaCl₂) with 7AAD (diluted as 1:50) and annexin V (diluted as 1:50) for 15min at room temperature in the dark. Cells were kept on ice after staining and data was collected within 30min. Data was collected on a flow cytometer (FACS Canto II, BD Biosciences, San Jose, CA or an Accuri’s C6 flow cytometer, Accuri Cytometers, Ann Arbor, MI) and analyzed using FlowJo software (TreeStar, Ashland, OR).

Cytokine staining

For intracellular cytokine staining, cells were harvested and restimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Fisher scientific, Pittsburgh, PA) and 1 µM ionomycin (Sigma-Aldrich, St. Louis, MO) in the presence of 2 µM monencin for 4 hours. Cells were then washed twice with 1× PBS followed by fixation using 4% Paraformaldehyde for 10 min at room temperature. Tubes were then filled with FACS buffer and kept overnight at 4 °C. The next day, cells were washed twice with FACS buffer
and permeabilized with 250 µl of permeabilization buffer (containing 50 mM NaCl, 0.02% NaN₃, 5 mM EDTA, 0.5% TritonX, pH7.5) on ice for 10 min. Cells were then washed twice with FACS buffer and incubated with 250 µl of 3% bovine serum albumin (BSA) for blocking. Intracellular staining with anti-IL-17 (diluted as 1:400) or anti-IL-9 (diluted as 1:200) antibodies was performed on ice for 1 hour followed by washing with FACS buffer. Data was collected on a flow cytometer (FACS Canto II, BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

**Preparation of CD4⁺CD25⁺ Treg cells and CD4⁺CD25⁻ T cells from the mouse spleen**

Splenic CD4⁺ T cells were purified by depletion of non-CD4⁺ T cells by the panning method. Spleens were mashed using the frosted side of glass slides to release cells. Splenocytes were treated with ACK lysis buffer (Gibco, Grand Island, NY) for 1 min to remove red blood cells by hypotonic shock. Cells were washed with washing media (containing 1% FCS in RPMI1640) followed by an incubation with 20% of anti-CD8 (using 3-155 hybridoma culture supernatant) on ice for 30 min. Cells were then washed twice with washing media. Washed cells were plated on goat anti-mouse immunoglobulin pre-coated plates and allowed to adhere to plate-bound anti-mouse immunoglobulin for 30 min. Non-adherent cells were collected and this crude fraction of CD4⁺ T cells was then resuspended in sorting media (containing 2% FCS, 10 mM Hepes, 4.2 mM Sodium bicarbonate in Hanks balanced salt solution, 1× Hank’s buffer) at a concentration of 50×10⁶ cells/ml and then labeled with fluorochrome-conjugated anti-CD4 and anti-CD25 antibodies on ice for 45 min. Cells were then washed with sorting medium. CD4⁺CD25⁺ T
cells /CD4⁺CD25⁺ T cells were then sorted using a cell sorter (FACS ARIA, BD Biosciences, San Jose, CA or MoFlo cell sorter, Beckman Coulter, Brea, CA). Sorted cells were kept overnight at 4°C in culture media and then used for experiments.

Culture media

RPMI 1640 media supplemented with 10% FCS (Atlanta Biologicals, Lawrenceville, GA), β-mercaptoethanol (50 µM), glutamine, Hepes (10 mM), sodium pyruvate (1 mM), and non-essential amino acids (Invitrogen, Grand Island, NY) were used in the experiments.

Treg cell expansion

Sorted CD4⁺CD25⁺ Treg cells resuspended into 5 mls of culture media with 10ng/ml of mouse IL-2 (PeproTech, Rocky Hill, NJ) and were plated in 60 mm petri dishes (USA scientific, Orlando, FL) pre-coated overnight at room temperature with 2 ml of anti-CD3 (clone 2C11) and anti-CD28 (clone 37-51) (Biolegend, San Diego, CA) antibodies (5µg/ml each) diluted in 0.1M Borate buffer (pH 8.5). After 4-5 days of culturing, cells were scraped from dishes by pipetting and split into newly anti-CD3/ anti-CD-28 pre-coated dishes (~2.5× 10⁶ cells per plate). Cells were harvested by pipetting after 2-3 days of culture.

Non-Treg cell (CD4⁺CD25 T cells) expansion

The anti-CD3/anti-CD28 coated beads were used for stimulation and expansion of
CD4⁺CD25⁻ T cells. 100 μl of 4.5 μmeter polystyrene beads (Polysciences, Inc, Warrington, PA) (containing ~2.5× 10⁷ beads) were resuspended in 1ml borate buffer (0.1M Boric acid, pH 8.5) containing anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml) antibodies and kept at room temperature overnight with gentle rocking. The next day, anti-CD3 and anti-CD28 coated beads were washed three times with borate buffer to remove unbound antibodies followed by blocking with 10% FCS for 30 min. The anti-CD3/ anti-CD28 antibodies coated beads were stored in 10% FCS culture medium. Sorted CD4⁺CD25⁻ T cells (1× 10⁶ cells) were pelleted and resuspended in 50 μl culture medium containing 4× 10⁶ pre-coated beads. Cells were then kept in a 37 °C water bath for 45 min to promote cell-bead contact. After 45 min of cell-beads incubation, cells bound to beads were then carefully transferred to 2 mls of culture media containing 10 ng/ml of IL-2.

_T cell receptor stimulation using biotin-conjugated anti-CD3 antibody cross-linked by avidin_

CD4⁺CD25⁻ T cells or CD4⁺CD25⁺ Treg cells were sorted on FACS Aria and expanded _in vitro_ for 7 days as described above. After expansion, cells were washed three times with wash media (RPMI1640 containing 1% FCS) to remove stimulation and then cultured in 10% FCS complete media in the presence of 1ng/ml IL-2 for overnight. The next day, cells were resuspended in 10% FCS culture media. 5× 10⁶ cells resuspended in 1ml of 10% FCS culture media were allowed to sit in a 1.5 ml microcentrifuge tube and warmed for 10min in a 37°C water bath. Cells were then stimulated with 5 μg/ml functional grade biotin-conjugated anti-CD3 antibody (2C11) (eBioscience, Sandiego, CA) cross-
linked by 5 μg/ml avidin egg (calbiochem, EMD Chemicals, Rockland, MA).

**T cell stimulation by plate-bound anti-CD3 and anti-CD-28 antibodies**

Sorted CD4+CD25+ or CD4+CD25- T cells were placed into 60 mm dishes pre-coated overnight at room temperature (with 2 ml of anti-CD3, clone 2C11 and anti-CD28, clone 37-51, Biolegend at concentration of 5 μg/ml each in 0.1M borate buffer pH 8.5) containing 5 mls of RPMI culture media in the presence of recombinant IL-2 (10ng/ml). TGF-β signaling in cell culture was blocked using 5 μg/ml anti-TGF-β 1, 2, 3 antibody (clone 1D11) (R&D Systems, Minneapolis, MN) or 10 μM SB431542 (Sigma-Aldrich). 2.5 nM of recombinant human TGF-β (R&D Systems, Minneapolis, MN) was used as an active form of TGF-β. IL-4 signaling was blocked in cell cultures using 10% 11B11 hybridoma culture supernatant (which contains anti-IL-4).

**Western blot**

Equal number of cells was lysed in SDS sample buffer (2% SDS, 125mM DTT, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8). Cell lysates were then boiled for 10 min and loaded onto 8-15% SDS PAGE gels. After gel electrophoresis, separated proteins were transferred overnight onto PVDF membranes (Millipore, Birellica, MA) at 30 V at 4°C. Blotted membranes were then blocked with 5% skim milk in 1× TBST buffer (containing 20 mM Tris-Hcl pH6.8, 137 mM NaCl, 0.1% Tween 20) for one hour and probed with antibodies specific for phospho-ERK (clone E10), phospho-Mek, phospho-cRaf, Bim, Mek (Cell signaling technology, Danvers, MA), Erk (Millipore, Billerica, MA), Raf 1(clone C-20),
RasGRP (clone 199), Fas ligand (kay-10), Sos1 (clone C-23) (Santa Cruz biotechnology, Santa Cruz, CA), Ras (clone 18/Ras) (BD Biosciences, San Jose, CA), or β-actin (clone AC15) (Sigma-Aldrich, St. Louis, MO). All antibodies were diluted in dilution buffer (containing 0.2-5% BSA, 0.1% sodium azide in TBST) for probing. The membranes were further probed with anti-rabbit or anti-mouse HRP conjugated secondary antibodies (Cell Signaling Technology). Signals were detected by enhanced chemiluminescence (ECL) plus western blotting detection reagents (GE Healthcare, Piscataway, NJ).

*Enzyme-linked immunosorbent assay (ELISA)*

IL-17, IL-4, IFN-γ and IL-9 production was measured by enzyme-linked immunosorbent assay (ELISA). Purified or biotin conjugated antibodies specific for IL-4 (clone 11B11) (Biolegend, San Diego, CA), IL-4-biotin (clone 13VD6-24G2), IFN-γ (clone R4-6A2) (eBioscience, San Diego, CA), IFN-γ-biotin (clone XMG1.2) (BD Biosciences, San Jose, CA) were used in these experiments. Mouse IL-17A ELISA MAX™ Standard Sets and mouse IL-9 ELISA MAX™ Set Deluxe (Biolegend, San Diego, CA) were used for detection of IL-17 and IL-9. ELISA for IL-4 and IFN-γ were performed in 96 well flat bottom ELISA microplates (BD Bioscience) coated with 50 µl of polyclonal anti-IL-4 (1:500) or anti-IFN-γ antibodies (1:500) diluted in bicarbonate buffer (pH8.0) and incubated overnight at 4°C. Plates were washed the next day using washing buffer (containing 0.05% TritonX in 1× PBS) to remove unbound antibodies and then incubated with 50 µl of cell culture supernatants overnight at 4°C. Cell culture supernatant was removed by washing followed by incubation with biotin-conjugated anti-IL-4 and anti-
IFN-γ antibodies for 1 hour at room temperature. Excess antibodies were washed and plates were incubated with HRP conjugated avidin for 30 min at room temperature followed by washing wash buffer. Plates were then incubated with 50 µl of 3,3’,5,5’-tetramethylbenzidine (TMB substrate) (Sigma Aldrich, St. Louis, MO) for a few minutes and then the enzyme reaction was stopped by adding 50 µl of stopping solution (containing 2M H₂SO₄). Data was collected on microplate reader, KCjunior (Bio-Tek instruments, Winooski, VT) using 450 nm wavelength. Serial dilutions of standards were used to generate a standard curve which was then used to calculate cytokine concentration.

**Chromatin Immunoprecipitation (CHIP) Assay**

A ChIP assay was performed using a ChIP assay kit (Usb, Cleveland, Ohio) as described in the manufacture’s protocol with some modifications. CD4⁺CD25⁺ Treg cells were sorted on a FACS Aria and then expanded *in vitro* for 7 days as described above. 1× 10⁷ Treg cells were used for each set of experiments. Harvested cells were centrifuged at 1,500 rpm for 5 min at 4°C followed by washing the cell pellet with 10 ml of 1× PBS (containing 2% FCS). Cells were then resuspended in freshly made 1% formaldehyde in 1× PBS (containing 2% FCS). Cells were incubated for 15 min at room temperature with gentle rocking thoroughly on rotating shaker platform to promote crosslinking. This was followed by the addition of glycine to the cells for a final concentration of 0.125M for 5 min at room temperature to halt crosslinking. Cells were then washed twice with 10 ml of chilled 1× PBS (containing 2% FCS). Cells were pelleted and frozen in liquid nitrogen quickly and stored at -80°C for future use or lysed immediately in 1 ml of cell lysis buffer. Cell lysate
was sonicated for chromatin fragmentation using Sonifier250 (BRANSON, Fisher scientific, Pittsburgh, PA). The condition of sonication was optimized to make the chromatin DNA fragment size between 200 and 800bp. The optimized condition is Duty cycle 80%, Level 2.5 for 15 sec for 4 sets and Duty cycle 80%, Level 3.0 for 10sec once. Samples were chilled on ice for 2 min between each cycle. Samples were then centrifuged at 13,000 rpm at 4°C for 10 min and supernatants were transferred to a new 1.5 ml microcentrifege tubes. 100 µl of cell lysate (1× 10^6 cells) was diluted in 500 µl lysis buffer and pre-cleared by using 50 µl of pre-blocked beads with gentle rocking for 1 hour at 4°C. Beads were then centrifuged at 3,000 rpm for 5 min at 4°C and the supernatant was carefully transferred to new 1.5 ml microcentrifuge tube followed by incubation with 4 µg of rabbit polyclonal anti-Foxp3 antibody (Novus biologicals, Littleton, CO) or polyclonal rabbit IgG (Cell signaling technology, Danvers, MA) as a negative control overnight at 4°C with gentle agitation. The next day, samples were centrifuged at 3,000 rpm for 5 min at 4°C and were washed with 1 ml of lysis buffer for 10 min with gentle rocking followed by washing with 1 ml of high salt buffer, 1 ml of lithium salt buffer and 1 ml of TE buffer. 120 µl of elution buffer was then added and the samples were incubated for 20 min on a rotisserie shaker at room temperature. Beads were pelleted by centrifuge at 3,000 rpm for 5 min at room temperature and supernatants were transferred to a new 1.5 ml microcentrifuge tube. Beads pellets were again resuspended in 120 µl of elution buffer and incubated for 20 min on rotisserie shaker at room temperature. Beads were centrifuged at 3,000 rpm for 5 min at room temperature and supernatants were combined with 1st collection followed by incubation with 9 µl of 5 M NaCl overnight at 65°C to reverse crosslink. The next day,
DNA was purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA) and eluted using 40 µl of 10 mM Tris-HCl, pH8.5. PCR was performed using TaKaRa ExTaq (Fisher Scientific) and primer sets (Table 1).

**Quantitative RT-PCR**

Total RNA was isolated as described in the manufacture’s protocol (Ambion, Applied Biosystems, Carlsbad, CA). Cells were centrifuged at 3,000 rpm for 5 min at 4°C and lysed in 500 µl of TRI reagent (Ambion) by pipetting followed by an incubation for 5 min at room temperature. 200 µl of chroloform was added and the samples were incubated for 10min at room temperature followed by centrifugation at 13,000 rpm for 10 min at 4°C. The top aqueous phase (~400 µl) was transferred to new tube and mixed with 400 µl of isopropanol followed by an incubation for 5 min at room temperature. Samples were then centrifuged at 13,000 rpm for 10 min at 4°C and washed with 500 µl of 70% EtOH. Total RNA was resuspended in 15-30 µl of RNase free water.

cDNA was synthesized from total RNA using OligodT primers and Superscript III cDNA synthesis kit (Invitrogen, Grand Island, NY). ~1 µg of total RNA was incubated with 1 µl of 50 µM oligo (dT) primers and 1 µl of 10 mM dNTP for 5 min at 65°C. Samples were then chilled on ice for 2 min and incubated with cDNA synsthesis mix (1× RT buffer, 5 mM MgCl₂, 10 mM DTT, 40 U RNaseOUT and 200 U SuperScriptIII RT) for 50 min at 50°C. The reaction was then terminated by incubation at 85°C for 5 min and chilled on ice for 5 min. Remaining RNA was digested by incubation with 1 µl of RNaseH for 20 min at 37°C. cDNA synthesis reaction was stored at -20°C.
Realtime PCR was performed using Maxima™ SYBR Green qPCR master mix (Fermentus, Fisher scientific, Pittsburgh, PA) and primer sets (Table 1).

**Measurement of intracellular free calcium**

CD4^+^CD25^-^ T cells or CD4^+^CD25^+^ Treg cells were sorted on FACS Aria and expanded for 7 days as described above. After expansion, cells were washed three times with wash media (RPMI1640 containing 1% FCS) to stop stimulation and cultured in 10% FCS culture media in the presence of 1 ng/ml IL-2 overnight. The next day, cells were resuspended in 10% FCS culture media and loaded with 6 µl of 1 mM Fura-2 AM (at a final concentration of 6 µM) (Molecular probe, Invitrogen, Grand Island, NY) for 15 min at 37°C in dark. Cells were then washed with 1× Hanks buffer (without calcium) (Gibco, Invitrogen, Grand Island, NY) to quench excess dye and then resuspended in 1 ml of 1× Hanks buffer (without calcium). Intracellular free calcium was measured by transferring cells into a cuvette of a spectrofluorophotometer (RH500, Shimadzu, Columbia, MD) and the data were calculated at 340 nm/380 nm excitation. First, the spectrofluorophotometer was paused after monitoring the basal level of [Ca^{2+}] for 60 sec. The spectrofluorophotometer was then resumed and cells were stimulated with 5 µg/ml functional grade biotin-conjugated anti-CD3 antibody (2C11, eBioscience, San Diego, CA) crosslinked by 5 µg/ml avidin egg (calbiochem, EMD chemicals, Philadelphia, PA). Data were collected for 10 min after stimulation.

**Statistical analysis**
Statistical significance was determined by 2-tailed Student T tests.
Table 1. PCR primers

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CHAPTER THREE
EXPERIMENTAL RESULTS

Proximal T cell receptor (TCR) signaling in CD4⁺CD25⁺Treg cells and CD4⁺CD25⁻T cells

Since CD4⁺CD25⁺ Treg cells but not non-Treg cells (CD4⁺CD25⁻ T cells) survive PICA inducing conditions, it is expected that CD4⁺CD25⁺ Treg cells respond to plate-bound anti-CD3/anti-CD28 antibody stimulation differently compared to non-Treg cells. In plate-bound anti-CD3/anti-CD28 antibody stimulation, immobilized (plate-bound) anti-CD3 antibody activates TCR signaling and immobilized anti-CD28 antibody activates CD28 costimulatory signaling. Although CD28 costimulatory signaling is required for full activation of T cells, the main T cell activation signaling is mediated through TCR. Therefore, we hypothesized that proximal TCR signaling might be altered in CD4⁺CD25⁺ Treg cells, thus becoming resistant to PICA. To study TCR signaling in CD4⁺CD25⁺ Treg cells, we decided to take a biochemical approach. It has been challenging to obtain enough Treg cells to perform signaling studies for two reasons. First, CD4⁺CD25⁺ Treg cells are madeup only 5-10% of CD4⁺ T cells in humans and mice. Second, commonly used bead-bound anti-CD3/anti-CD28 antibody stimulation or irradiated antigen presenting cells (APC) with anti-CD3 stimulation have difficulties expanding pure populations of CD4⁺CD25⁺ Treg cells, because a small number of contaminated non-Treg cells can expand
Fig. 1 Western blot analysis of Foxp3 expression in *ex vivo* expanded CD4*⁺*CD25*⁺* Treg cells and non-Treg cells. CD4*⁺*CD25*⁺* Treg cells or CD4*⁺*CD25*⁻* T cells were isolated from the spleen of BALB/c mice. CD4*⁺*CD25*⁺* Treg cells were stimulated and expanded with plate-bound anti-CD3/anti-CD28 antibodies in the presence of IL-2. CD4*⁺*CD25*⁻* T cells were stimulated with bead-bound anti-CD3/anti-CD28 antibodies in the presence of IL-2. Cells were harvested at day 7 and rested for overnight without anti-CD3/anti-CD28 antibody stimulation. Cells were then directly lysed into SDS sample buffer and subjected for western blot analysis using anti-Foxp3 and anti-β actin (for loading control) antibodies.
Fig. 2 Western blot analysis of proximal TCR signaling molecules in in *ex vivo* expanded CD4⁺CD25⁺ Tregs and non-Tregs (CD4⁺CD25⁻ T cells) upon TCR activation. *Ex vivo* expanded CD4⁺CD25⁺ Tregs or non-Tregs (CD4⁺CD25⁻ T cells) isolated from the spleen of BALB/c mice were stimulated with biotin-conjugated anti-CD3 crosslinked with avidin for 15, 30 min. Cells were then directly lysed into SDS sample buffer and subjected for western blot analysis using anti-phospho-LCK (tyrosine 394), anti-phospho-LCK (tyrosine 505), anti-LCK, anti-TCRζ (A), and phospho-tyrosine (B) antibodies. Phosphotyrosine levels of some proteins indicated with arrows were downregulated in Treg cells compared to non-Treg cells. In contrast, bands indicated with asterisks were detected at higher levels in Treg cells.
faster than Treg cells and take over the culture. Hence, until now, proximal TCR signaling in Treg cells has not been well understood. We discovered the phenomenon that Treg cells can expand more than 7,000 fold under plate-bound anti-CD3/anti-CD28 antibody stimulation while non-Treg cells undergo massive apoptosis. By taking advantage of this plate-bound anti-CD3/anti-CD28 antibody stimulation system, we expanded electronically sorted CD4⁺CD25⁺ Treg cells for 7 days and examined TCR signaling. Since non-Treg cells (CD4⁺CD25⁻ T cells) undergo apoptosis under plate-bound anti-CD3/anti-CD28 antibody stimulation, we expanded non-Treg cells with bead-bound anti-CD3/anti-CD28 antibody stimulation for 7 days as a control. After 7 days, both Treg cells and non-Treg cells were removed from stimulations and rested overnight to examine short term TCR stimulation effect in Treg cells. First, we examined Foxp3 expression levels in ex vivo expanded Treg cells by western blot. Expanded Treg cells expressed high levels of Foxp3 while non-Treg cells had very low levels of Foxp3, suggesting that Foxp3⁺ Treg cells were successfully expanded (Fig. 1). Although expanded CD4⁺CD25⁺ T cells also showed a faint Foxp3 band, this could be a small contamination of Foxp3⁺ Treg cells. Previously, we reported that the amount of Foxp3⁺ Treg cells was over 95% after 7 days of plate-bound anti-CD3/anti-CD28 antibody stimulation using flow cytometry analysis (Singh et al., 2010).

To determine if proximal TCR signaling is altered in Treg cells compared to non-Treg cells, ex vivo expanded CD4⁺CD25⁺ Treg cells or non-Treg cells (CD4⁺CD25⁻ T cells) were stimulated with biotinylated anti-CD3 antibody cross-linked with avidin. Cells were then harvested for western blot after 15 or 30 min of stimulation. The signaling events following
TCR activation are depicted by the activation of kinases and phosphorylation of tyrosine residues. First, TCR activation leads to phosphorylation of the Src family tyrosine kinase, LCK, followed by phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the TCRζ chain. ZAP70 then binds to phosphorylated ITAMs on the TCRζ chain leading to the activation of downstream signaling (Iwashima et al., 1994; Straus et al., 1996). Therefore, we determined activation and expression levels of these molecules in Treg cells compared to non-Treg cells. Treg cells expressed comparable amounts of LCK compared to non-Treg cells and phosphorylation level of LCK residue 394, 505 stayed at basal levels even after stimulation in both Treg cells and non-Treg cells (Fig. 2A). In contrast, TCRζ expression was substantially reduced in Treg cells compared to non-Treg cells (Fig. 2A). Total phosphotyrosine levels were also altered in Treg cells (Fig. 2B). Phosphotyrosine levels of some proteins indicated with arrows were downregulated in Treg cells compared to non-Treg cells. In contrast, bands indicated with asterisks were detected at higher levels in Treg cells compared to non-Treg cells. These data suggest that proximal TCR signaling in Treg cells is mediated differently compared to non-Tregs.

Next, we next focused on the downstream signaling cascade of Zap70 and assessed activation of the major signaling pathways downstream of TCR signaling, the Ras-Erk pathway. Surprisingly phosphorylation of Erk was significantly reduced in Tregs and barely detectable while non-Treg cells showed strong activation of Erk after 15 or 30 min after TCR stimulation (Fig. 3A). Further, upstream of the Erk signaling cascade, Mek and cRaf, also showed substantially reduced levels of phosphorylation while total protein
Fig. 3 Western blot analysis of proximal TCR signaling, Ras-Erk pathway in *ex vivo* expanded CD4⁺CD25⁺ Treg cells and non-Treg cells (CD4⁺CD25⁻ T cells) upon TCR activation. *Ex vivo* expanded CD4⁺CD25⁺Treg cells or non-Treg cells (CD4⁺CD25⁻ T cells) isolated from the spleen of BALB/c mice were stimulated with biotin-conjugated anti-CD3 crosslinked with avidin for 15, 30 min. Cells were then directly lysed into SDS sample buffer and subjected for western blot analysis using anti-phospho-Erk, anti-Erk, anti-phospho-Mek, anti-Mek, anti-phospho-cRaf, anti-cRaf (A), anti-Ras, anti-RasGRP1 and SOS (B) antibodies.
expression was comparable in Treg cells compared to non-Treg cells (Fig. 3A). These data suggest Ras-Erk signaling activity in response to TCR stimulation is substantially blocked in Treg cells compared to non-Treg cells though Ras expression was equal between Treg cells and non-Treg cells.

In T cells, Ras activity is controlled by two Ras activators, RasGRP and SOS. Since Ras-Erk pathway is significantly blocked in Treg cells, expression of Ras activators might be altered in Treg cells. We then sought to determine the expression of RasGRP1 and SOS in Treg cells compared to non-Treg cells. As we expected, RasGRP1 expression was substantially reduced in Treg cells compared to non-Treg cells (Fig 3B). SOS expression was also slightly reduced in Treg cells. These data suggest that the blockade of Erk signaling might be due to reduced expression of RasGRP1 and SOS.

Next, we next monitored the mobilization of intracellular calcium in Treg cells. Calcium signaling is another major proximal TCR signaling pathway. LAT, which is a substrate of ZAP70, recruits and activates phospholipaseCγ (PLCγ) (Nishibe et al., 1990). PLCγ then leads to the production of IP3 leading to the mobilization of intracellular calcium (Crabtree, 1999). In non-Treg cells (CD4+CD25− T cells), phosphorylation of PLCγ increased in response to TCR stimulation. In contrast, Tregs showed higher basal level of PLCγ activation compared to non-Treg cells. Interestingly, the level of PLCγ phosphorylation decreased after TCR stimulation in Treg cells. The total expression of PLCγ was comparable between Treg cells and non-Treg cells (Fig 4A).

We then assessed calcium levels in response to TCR stimulation in Treg cells compared to non-Treg cells. As described above, Treg cells and non-Treg cells were
A

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B

![Graph showing [Ca^{2+}] (μM) over time (sec) for CD4+CD25- and CD4+CD25+ samples.](image)
Fig. 4 Calcium signaling in in *ex vivo* expanded CD4⁺CD25⁺ Treg cells and non-Treg cells (CD4⁺CD25⁻ T cells) upon TCR activation. (A) *Ex vivo* expanded CD4⁺CD25⁺Treg cells or non-Treg cells (CD4⁺CD25⁻ T cells) isolated from the spleen of BALB/c mice were stimulated with biotin-conjugated anti-CD3 cross-linked with avidin for 15, 30 min. Cells were then directly lysed into SDS sample buffer and subjected for western blot analysis using anti-phospho-PLC γ and anti-PLC γ antibodies. (B) *Ex vivo* expanded CD4⁺CD25⁺ Treg cells or non-Treg cells (CD4⁺CD25⁻ T cells) isolated from the spleen of C57BL/6 mice were loaded with Fura2-AM and stimulated with biotin-conjugated anti-CD3 cross-linked with avidin. Intracellular calcium level was determined by the ratio between emission fluorescence intensities at 340nm and 380nm.
expanded and rested overnight. Cells loaded Fura-2-AM were stimulated with avidin crosslinked anti-CD3 and calcium mobilization in these cells was assessed. In response to TCR stimulation, the elevation of intracellular calcium concentration, \([\text{Ca}^{2+}]\) was observed in both Treg cells and non-Treg cells. However, the \([\text{Ca}^{2+}]\) in Treg cells reached to a much lower peak compared to non-Treg cells and declined to basal level. These data suggest that calcium signaling is also altered in Treg cells. Altogether, data above indicate that proximal TCR signaling is highly impaired in Treg cells compared to non-Treg cells.

*Lack of RasGRP1 expression rescues non-Treg cells from PICA*

We have demonstrated that proximal TCR signaling is drastically altered in CD4\(^+\)CD25\(^+\) Treg cells compared to non-Treg cells (CD4\(^+\) CD25\(^-\) T cells). This difference may be the key that renders Treg cells resistant to PICA and the reason why the same plate-bound anti-CD3/CD28 antibody stimulation leads to different outcomes for CD4\(^+\)CD25\(^+\) Treg cells and non-Treg cells (CD4\(^+\)CD25\(^-\) T cells), either survival or death. The most drastic differences in CD4\(^+\)CD25\(^+\)Treg cells were downregulation of RasGRP1 and the blockade of its down stream Ras-Erk activity in response to TCR stimulation. RasGRP1 transmits the TCR activation signal to Ras by converting the Ras-GDP bound form to the active Ras-GTP binding form. Whereas there are two Ras activators in T cells, SOS and RasGRP1, Roose et al. demonstrated that RasGRP1 dominantly controls Erk activation in response to TCR stimulation (Roose et al., 2005). Further, Dower et al. showed that TCR dependent Erk activation is impaired in RasGRP1 deficient thymocytes (Dower et al., 2000). Hence, it is expected that the Ras-Erk pathway is unresponsive
during TCR activation in Treg cells because of the downregulation of RasGRP1. Ras-Erk signaling is highly conserved among vertebrates and plays crucial roles in determining cellular physiology such as cell survival, proliferation and differentiation (Ramos, 2008). In the case of T cells, Erk signaling plays an important role in thymic positive and negative selection (Bommhardt et al., 2000; Daniels et al., 2006; Mariathasan et al., 2001; Werlen et al., 2003). Whereas it is well studied that Erk activation is important for cell survival, Martin et al. reported that sustained Erk signaling promotes cell death using multiple cell types including the human lymphoblastoid Bolet cell line (Martin et al., 2006). Therefore, we focused on RasGRP1 expression in CD4⁺CD25⁺ Treg cells and non-Treg cells (CD4⁺CD25⁻ T cells) and its down stream Ras/Erk pathway. We hypothesized that the downregulation of RasGRP1 expression in CD4⁺CD25⁺ Treg cells might be important for cell survival under PICA inducing condition, plate-bound anti-CD3/anti-CD28 antibody stimulation. In other words, CD4⁺CD25⁻ T cells might undergo apoptosis in a RasGRP1/ Ras/ Erk signaling dependent manner under PICA inducing conditions. To test our hypothesis, we used RasGRP1 knockout mice, which exhibit abnormal T cell development in the thymus due to impaired positive selection (Dower et al., 2000). These mice develop autoimmune disorders. These mice exhibit splenomegaly and enlarged spleens contain higher number of CD4⁺ T cells whereas fewer numbers of CD4⁺CD25⁺ Treg cells in the periphery (Chen et al., 2008). The rationale of this experiment is if the downregulation of RasGRP1 is important for CD4⁺CD25⁺ Treg cell survival under plate-bound anti-CD3/anti-CD28 antibody stimulation, then RasGRP1 deficient CD4⁺CD25⁻ T cells would also become
Fig. 5 The lack of RasGRP1 expression rescues non-Tregs from PICA. CD4⁺CD25⁻ T cells isolated from the spleen of RasGRP1 knockout mice (RasGRP−/−) or littermate control (Wt) were stimulated with anti-CD3/anti-CD28 antibodies. Cells were harvested at day 4. (A) Total number of live cells was determined. (B) Cells were stained with annexin V and analyzed by flow cytometry.
Fig. 6 Effect of RasGRP1 expression on Erk activity by CD4^{+}CD25^{-} T cells under plate-bound anti-CD3/anti-CD28 antibody stimulation. CD4^{+}CD25^{-} T cells isolated from the spleen of RasGRP1 knockout mice (KO) or littermate control (WT) were stimulated with plate-bound anti-CD3/anti-CD28 antibodies supplemented with IL-2. Cells were harvested at day 3. Cells were lysed into SDS sample buffer and subjected for western blot analysis using anti-phospho-Erk, anti-Erk and anti-β actin (for loading control) antibodies.
resistant to PICA as wild type CD4⁺CD25⁺ Tregs do. To address this question, we cultured electronically sorted CD4⁺CD25⁻ T cells isolated from the spleen of RasGRP1 knockout mice or littermate control with plate-bound anti-CD3/anti-CD28 antibody stimulation. After 4 days of stimulation, cells were harvested and cell viability was assessed by flow cytometry analysis. As expected, cells isolated from wild type littermates underwent massive apoptosis and over 80% of the cells became annexinV⁺ (Fig. 5A). In contrast, RasGRP1 deficient cells isolated from the spleen of RasGRP1 knockout mice showed a substantial decrease in the percentage of annexinV⁺ cells (37%) (Fig. 5A). The total live cell number of wild type CD4⁺CD25⁻ T cells decreased 30 fold from day 0 (Fig. 5B). In contrast, the total live cell number of RasGRP1 deficient CD4⁺CD25⁻ T cells increased 4 fold from day 0. These data suggest that non-Treg cells (CD4⁺CD25⁻ T cells), which lack RasGRP1 expression avoid apoptosis and expand under PICA inducing condition with plate-bound anti-CD3/anti-CD28 antibodies. We then determined whether Erk activation is impaired in RasGRP1 deficient CD4⁺CD25⁻ T cells under PICA inducing condition. To address this question, we cultured electronically sorted CD4⁺CD25⁻ T cells isolated from the spleen of RasGRP1 knockout mice or littermate control mice with plate-bound anti-CD3/anti-CD28 antibody stimulation. After 3 days of culturing, cells were harvested and lysed in SDS sample buffer. Erk activation, determined by levels of phosphorylated Erk, was assessed by western blot analysis. Phosphorylation of Erk was observed from wild type cells, but Erk activation was barely detectable from RasGRP1 deficient cells, suggesting that RasGRP1 deficient cells
exhibited impaired Erk activity in response to plate-bound anti-CD3/anti-CD28 antibody stimulation (Fig. 6).

*Lack of RasGRP1 expression downregulates Bim expression*

It is now clear that the lack of RasGRP1 expression renders CD4\(^+\) T cells resistant to apoptosis and its downstream Erk activity is impaired in the absence of RasGRP1 under PICA inducing condition. We next sought to determine how lack of RasGRP1 expression changes the fate of non-Tregs from death to survival under PICA inducing stimuli. T cell apoptosis is mainly mediated by two different pathways, Fas and Bim (Hutcheson et al., 2008). Fas belongs to the death receptor, Tumor necrosis factor (TNF) receptor family. The Fas/Fas ligand interaction induces activation of caspase 8 through the formation of the death inducing signaling complex (Kischkel et al., 1995). Cell surface expression of Fas is highly upregulated upon TCR stimulation. Bim belongs to the Bcl-2 family and initiates apoptosis via the intrinsic pathway by activating caspase 9. Both Fas and Bim are crucial for maintaining cell homeostasis. We previously reported that CD4\(^+\)CD25\(^-\) T cells isolated from Bim knockout mice or lpr mice which lack functional Fas ligand are resistant to PICA and are able to survive and expand under plate-bound anti-CD3/anti-CD28 antibody stimulation (Singh et al., 2010). These data indicate that PICA is induced in a both Bim and Fas/FasL dependent manner. Further, the cell surface expression of FasL/Fas and Bim were substantially upregulated when non-Treg cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies. In contrast, Treg cells did not exhibit increased expression of those proapoptotic proteins and kept basal level of Bim and Fas ligand cell surface
**Fig. 7** Effect of RasGRP1 expression on apoptotic protein expression by CD4<sup>+</sup>CD25<sup>-</sup> T cells under plate-bound anti-CD3/anti-CD28 antibody stimulation. CD4<sup>+</sup>CD25<sup>-</sup> T cells isolated from the spleen of RasGRP1 knockout mice (KO) or littermate control (WT) were stimulated with plate-bound anti-CD3/anti-CD28 antibodies. Cells were lysed in SDS sample buffer at day 3 and subjected for western blot analysis using anti-Bim, anti-Fas ligand and anti-β actin (for loading control) antibodies. EL and L forms of Bim are indicated with arrowheads. Membrane-bound and soluble forms of Fas ligand are indicated with arrowheads.
expression under plate-bound anti-CD3/anti-CD28 antibody stimulation. To determine if the lack of RasGRP1 expression in CD4⁺CD25⁻ T cells blocks upregulation of Bim and Fas/ FasL, we cultured electronically sorted CD4⁺CD25⁻ T cells isolated from the spleen of RasGRP1 knockout mice or littermate control under plate-bound anti-CD3/anti-CD28 antibody stimulation. After 3 days, cells were harvested and lysed in SDS sample buffer. Bim, Fas and Fas ligand expression were assessed by western blot analysis. At day 3, wild type cells exhibited increased Bim expression compared to unstimulated cells. In contrast, RasGRP1 deficient cells expressed basal levels of Bim after stimulation (Fig. 7A). Unstimulated cells also showed lower Bim expression compared to wild type cells. Fas ligand expression was increased in both RasGRP1 knockout cells and wild type cells, suggesting there is no effect of RasGRP1 on FasL expression (Fig. 7B). These data suggest that upregulation of Bim under plate-bound anti-CD3/anti-CD28 antibody stimulation is in a RasGRP1 expression dependent manner.

*Regulation of RasGRP1 expression in CD4⁺ T cells*

The data above indicate that the level of RasGRP1 expression correlates with CD4⁺ T cell fate of death or survival. As described in Fig. 3B, we demonstrated that Treg cells substantially downregulate RasGRP1 expression compared to non-Treg cells. This experiment was done using *ex vivo*-expanded cells to obtain enough CD4⁺CD25⁺ Treg cells for western blot analysis. Since it is not understood how the *rasgrp1* gene is regulated in response to TCR stimulation, we next compared the RasGRP1 expression levels in freshly isolated cells and activated state of CD4⁺CD25⁺ Treg cells/ CD4⁺CD25⁻ T cells.
Fig. 8 Western blot analysis of RasGRP1 expression on CD4^+^CD25^+^ Treg cells and CD4^+^CD25^-^ T cells. CD4^+^CD25^+^ Treg cells or CD4^+^CD25^-^ T cells were isolated from the spleen of BALB/c mice. CD4^+^CD25^+^ Treg cells were stimulated and expanded with plate-bound anti-CD3/anti-CD28 antibodies in the presence of IL-2. CD4^+^CD25^-^ T cells were stimulated with bead-bound anti-CD3/anti-CD28 antibodies in the presence of IL-2. Cells were harvested at day 0 or day 7. Cells were then lysed into SDS sample buffer and subjected for western blot analysis using anti-RasGRP1 and anti-β actin (for loading control) antibodies.
**Fig. 9** The *rasgrp1* gene expression in CD4⁺CD25⁺ Treg cells and CD4⁺CD25⁻ T cells.

CD4⁺CD25⁺ Treg cells or CD4⁺CD25⁻ T cells were isolated from the spleen of BALB/c mice. CD4⁺CD25⁺ Treg cells were stimulated and expanded with plate-bound anti-CD3/anti-CD28 antibodies in the presence of IL-2. CD4⁺CD25⁻ T cells were stimulated with bead-bound anti-CD3/anti-CD28 antibodies in the presence of IL-2. Cells were harvested at day 7 and total RNA was isolated for real time PCR. * p<0.05
Electronically sorted CD4^+CD25^+ Treg cells were stimulated with plate-bound anti-CD3/CD28 antibodies. FACS sorted CD4^+CD25^- T cells were stimulated with bead-bound anti-CD3/anti-CD28 antibodies. Cells were harvested at day 0 and day 7 and lysed in SDS sample buffer. RasGRP1 expression was assessed by western blot analysis. If RasGRP1 expression changes in response to TCR stimulation, then we would expect to see different expression patterns from freshly isolated cells compared to activated cells. Surprisingly, both CD4^+CD25^+ Treg cells and non-Treg cells (CD4^+CD25^- T cells) expressed comparable levels of RasGRP1 at day 0 (unstimulated) (Fig. 8A). After 7 days of in vitro stimulation, non-Treg cells showed a substantial increase of RasGRP1 expression from basal level, while CD4^+CD25^+ Treg cells slightly downregulated RasGRP1 expression. These data indicate that large differences of RasGRP1 expression previously observed between CD4^+CD25^+ Treg cells and non-Treg cells was due to upregulation of RasGRP1 in non-Treg cells in response to TCR stimulation, since freshly isolated cells (unstimulated cells) showed basal levels of RasGRP1 expression in both Treg cells and non-Treg cells. To determine if reduced expression of RasGRP1 in Treg cells is regulated at the transcriptional level, we isolated RNA from in vitro expanded CD4^+CD25^+ Treg cells or CD4^+CD25^- T cells and performed quantitative PCR (Fig. 9). At day 7, non-Treg cells expressed higher amount of rasgrp1 compared to Treg cells, suggesting that reduced RasGRP1 expression in Treg cells is mediated by transcriptional regulation.

CD4^+CD25^- T cells can be differentiated into Foxp3^+ Treg cells (inducible Treg cells) in the periphery when TGF-β is present (Chen et al., 2003; Wan and Flavell, 2005). Next, we determined if inducible Tregs also downregulate RasGRP1 expression under TCR
Fig. 10 RasGRP1 expression by CD4^+CD25^- T cells in iTreg cell polarizing condition. CD4^+CD25^- T cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies. Cells were harvested at day 0, 1, 3, 4 and 7. Cells were lysed into SDS sample buffer and subjected for western blot analysis using anti-RasGRP1, anti-Foxp3 and anti-β actin antibodies.
stimulation. To address this question, electronically sorted CD4^+CD25^- T cells were
stimulated with plate-bound anti-CD3 plus soluble anti-CD28 antibodies in the presence or
absence of TGF-β. Stimulation with plate-bound anti-CD3 plus soluble anti-CD28 in the
presence of TGF-β has previously been reported as iTreg cell polarizing conditions
(Fantini et al., 2007). After 1, 3, 5 or 7 days of culturing, cells were lysed in SDS sample
buffer. RasGRP1 expression and Foxp3 expression were assessed by western blot analysis.
In the absence of TGF-β, no Foxp3 induction occurred while RasGRP1 expression
gradually increased with the highest peak observed at day 7 (Fig. 10). In contrast, cells
stimulated in the presence of TGF-β started to express Foxp3 after one day of stimulation
and gradually increased its expression level with longer activation, suggesting that cells
differentiated into iTreg cells. Although increased expression of Foxp3 expression is either
due to an increase of Foxp3 expression per cell or an increase in the proportion of Foxp3^+ T
cells, RasGRP1 expression was inversely correlated to Foxp3 expression and was reduced
from basal level. As Foxp3 expression increased, RasGRP1 expression reduced in the
presence of TGF-β suggesting that iTreg cells also downregulated expression of RasGRP1.

*Foxp3 directly associate with rasgrp1 gene*

We next determined by which mechanism CD4^+CD25^- Treg cells downregulate
RasGRP1 expression. As we described in Fig. 9, downregulation of *rasgrp1* is mediated at
the transcriptional level. Further, we showed that RasGRP1 expression was reciprocally
correlated with Foxp3 expression in the iTreg cell induction model (Fig. 10). Foxp3 is a
transcription factor recognized as the most descriptive marker of Tregs and expressed by
A

\[ \text{Forkhead consensus: } T_A TT G_T \]

B

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Fig. 11 Foxp3 interaction with rasgrp1 gene in CD4^+CD25^+ Treg cells. (A) Forkhead consensus sequences were found in rasgrp1 promoter region indicated with red ellipses. CR1, 2, 3, 4 and 5 are regions amplified by PCR. (B) Expanded CD4^+CD25^+ Treg cells isolated from the spleen of C57BL/6 mice were lysed and cross-linked for ChIP assay. DNA was extracted from precipitated samples for PCR primer sets which amplify forkhead consensus regions from rasgrp1 promoter.
both nTreg cells and iTreg cells in mice. Foxp3 negatively or positively controls many
genes which are related to Treg functions. Overexpression of Foxp3 is sufficient to make T
cells suppressive function (Hori et al., 2003). Therefore, we hypothesized that Foxp3 might
downregulate rasgrp1 expression by directly associating with the rasgrp1 transcription
control region. To address this question, we determined if Foxp3 associates with the
transcription control region of rasgrp1. Foxp3 belongs to the fork head family that binds to
fork head consensus TRTTKW as previously reported (Overdier et al., 1994). Therefore,
we first searched if there are any forkhead consensus sequences upstream of rasgrp1 from
the transcription starting site. We found that there are 4 potential Foxp3 binding sites up to
4 kb upstream of the rasgrp1 gene (Fig. 11A). To determine if Foxp3 binds to these
forkhead consensuses which exist upstream of the rasgrp1 transcription starting site, we
designed the primers to amplify these regions and performed a ChIP assay. Expanded Treg
cells were lysed and cross-linked. Cell lysates were precipitated using anti-Foxp3 antibody
or an isotype control antibody. If Foxp3 binds to upstream of the rasgrp1 transcription
starting site, we would expect to see that DNA fragment precipitated with Foxp3 and
amplified by PCR reaction. Among the Foxp3 antibody precipitated samples, only the CR1
and CR2 primer sets amplified clearly detectable bands (Fig. 11B). Since no bands were
detected from isotype precipitated samples, these data suggest that Foxp3 directly
associates with the rasgrp1 gene and possibly downregulates rasgrp1 transcription in Treg
cells.
TGF-β signaling in CD4⁺ T cells and PICA

The data above focused on the difference of proximal TCR signaling between CD4⁺CD25⁺ Treg cells and other non-Treg cells (CD4⁺CD25⁻ T cells) and found that Ras guanyl nucleotide exchange protein 1 (RasGRP1) is downregulated in CD4⁺CD25⁺ Treg cells compared to other CD4⁺ T cells. Further, we showed RasGRP1 expression in non-Treg cells (CD4⁺CD25⁻ T cells) is critical to promote apoptosis under PICA inducing condition stimulated with plate-bound anti-CD3/anti-CD28 antibody. Altogether, these data indicate that downregulation of RasGRP1 in CD4⁺CD25⁺ Treg cells might be one of the survival mechanisms against apoptosis under plate-bound anti-CD3/anti-CD28 antibody stimulation.

Is downregulation of RasGRP1 in CD4⁺CD25⁺ Treg cells the only mechanism to control cell survival? Next, we focused on the downstream events of proximal TCR signaling, which includes cytokine production. CD4⁺ T cells produce a variety of cytokines such as IL-2, IL-4 and IFN-γ in response to TCR stimulation along with CD28 co-stimulation. TGF-β is a pleiotropic cytokine, which is also produced by CD4⁺ T cells and influences cell proliferation, apoptosis and differentiation (Chang et al., 2003; Conery et al., 2004; Jung et al., 2004; Murillo et al., 2005; Sillett et al., 2001). TGF-β is secreted in an inactive form by noncovalently attaching to latency associated peptide (LAP) (Li and Flavell, 2008). Nakamura et al. reported that CD4⁺CD25⁺ Treg cells produce more TGF-β compared to other CD4⁺ effector T cells (Nakamura et al., 2001). Furthermore, CD4⁺CD25⁺ Treg cells but not other CD4⁺ effector T cells (CD4⁺CD25⁻ T cells) express TGF-β on their surface by interacting with LAP and anchor protein, GARP
CD4\(^+\)CD25\(^+\) Treg cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence or absence of SB431542 (TGF-\(\beta\) super-family type I receptor kinase inhibitor) or anti-TGF-\(\beta\) antibody in media supplemented with IL-2. (A) Cells were harvested at day 3 and total number of live cells was determined. (B) At day 3, cells were stained with annexin V and analyzed by flow cytometry. ** p< 0.01
(Glycoprotein A repetitions predominant) (Tran et al., 2009). Surface bound TGF-β on CD4+CD25+ Treg cells exerts a suppressive function toward effector T cells (Nakamura et al., 2001). Although it is not well understood if there are any other specific roles of surface bound TGF-β, there are clear differences about TGF-β production and its expression style between CD4+CD25+ Treg cells and other CD4+ effector T cells. Hence we hypothesized that there is more active TGF-β signaling in Treg cells and that might be important for CD4+CD25+ Treg cell survival under PICA inducing condition, plate-bound anti-CD3/anti-CD28 antibody stimulation. To test our hypothesis, we first determined if TGF-β signaling is required for the survival of Treg cells. To inhibit TGF-β signaling in Treg cells, we used a TGF-β super-family type I receptor kinase inhibitor (SB431542) or a TGF-β neutralizing antibody. SB431542 is a small molecule inhibitor that blocks TGF-β type I receptor serine/threonine kinase activity by competing with the ATP binding site of its kinase. TGF-β neutralizing antibody binds to TGF-β thus blocking TGF-β association with the TGF-β receptor. Purified CD4+CD25+ Treg cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence of TGF-β signaling inhibitor (SB431542) or anti-TGF-β neutralizing antibody (anti-TGF-β-1, 2, 3 antibody). If TGF-β signaling is important for CD4+CD25+ Treg cell survival, we would expect to see that CD4+CD25+ Treg cells inhibited TGF-β signaling would undergo apoptosis under plate-bound anti-CD3/anti-CD28 antibody stimulation. After three days of stimulation, cells were harvested and analyzed by flow cytometry. CD4+CD25+ Treg cells expanded ~2 fold compared to the starting cell number (Fig. 12A). When TGF-β super-family type
Fig. 13 Effect of TGF-β signaling blockade on PICA of CD4⁺CD25⁺ Treg cells.

CD4⁺CD25⁺ Treg cells isolated from the spleen of dnTgfbr2 mice or wildtype littermate control were stimulated with plate-bound anti-CD3/anti-CD28 antibodies. (A) Cells were harvested at day 3 and total number of live cells was determined. (B) At day 3, cells were stained with annexin V and analyzed by flow cytometry. **p < 0.01
I receptor kinase inhibitor (SB431542) was added, cell growth was substantially blocked and the cell number decreased approximately 5 fold. Similarly, when CD4^+CD25^+ Treg cells were treated with anti-TGF-β antibody, live cell number decreased substantially compared to the starting number (Fig. 12A). Flow cytometric analysis showed that this decrease in cell numbers corresponds to an increase in annexin V^+ apoptotic/dead cell frequency (Fig. 12B). These data suggest that TGF-β signaling in CD4^+CD25^+ Tregs is critical for cell survival under PICA inducing condition, stimulation with plate-bound anti-CD3/ anti-CD28 antibodies.

To further confirm these results, we examined PICA resistance by CD4^+CD25^+ Treg cells isolated from transgenic mice expressing a dominant-negative form of TGF-β receptor type II under the control of mouse CD4 promoter (CD4dnTgfbr2). These mice have a normal level of Foxp3^+CD4^+CD25^+ Treg cells (~8 weeks old) although TGF-β receptor signaling is substantially blocked in T cells. We isolated splenic CD4^+CD25^+ Treg cells from CD4dnTgfbr2 mice or their wild type littermate control and stimulated them with plate-bound anti-CD3/anti-CD28 antibodies in the presence of IL-2. After 3 days of culture, we harvested cells and assessed their survival (Fig. 13A). While the number of wild type littermate Treg cells increased from day 0, the number of CD4^dnTgfbr2 Treg cells was less than 10% of the control and decreased compared to the starting cell number. As observed with the chemical inhibitor of TGF-β signaling and TGF-β neutralizing antibody, the frequency of annexinV^+ cells were about 2 fold higher in CD4^dnTgfbr2 T cell culture compared to that of the littermate control (Fig. 13B). Since we did not add exogenous TGF-β to the culture, the data strongly suggest that CD4^+CD25^+ Treg cells provide TGF-
Fig. 14 Effect of TGF-β on PICA by CD4+CD25− T cells. CD4+CD25− T cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence or absence of recombinant TGF-β in media supplemented with IL-2. (A) Cells were harvested at day 3 and stained with annexin V for flow cytometry analysis. (B) Total number of live cells was determined at day 3. **p< 0.01
β in an autocrine manner to maintain CD4⁺CD25⁺ Treg cell resistance against PICA.

**Exogenous TGF-β renders CD4⁺CD25⁻ T cells resistant to PICA**

Since TGF-β signaling is important for the survival of CD4⁺CD25⁺ Treg cells in an autocrine manner, it is possible that non-Tregs (CD4⁺CD25⁻ T cells) undergo apoptosis under plate-bound anti-CD3/anti-CD28 antibody stimulation due to less or lack of TGF-β signaling. Ouyang et al. reported that CD4⁺CD25⁻ T cells express comparable level of TGF-β receptor although it is slightly lower than Treg cells (Ouyang et al., 2010). Since the TGF-β receptor is expressed in non-Tregs (CD4⁺CD25⁻ T cells), the addition of the active form of TGF-β might render a survival signal to non-Tregs (CD4⁺CD25⁻ T cells) from PICA under plate-bound anti-CD3/anti-CD28 antibody stimulation. We cultured FACS sorted CD4⁺CD25⁻ T cells under PICA-inducing conditions, stimulation with anti-CD3/anti-CD28 antibodies, in the presence or absence of exogenous TGF-β. After 3 days of culturing, we harvested cells and assessed their survival. As observed previously, cells that were stimulated by plate-bound anti-CD3/anti-CD28 antibodies underwent apoptotic cell death detected by an increase of annexin V⁺ cells (Fig 14A). When exogenous TGF-β was added to the culture, as we predicted, the frequency of apoptotic/dead cells decreased substantially. This change with the addition of TGF-β was due to expansion of the number of live cells and not due to a decrease of annexin V⁺ cell numbers (Fig. 14B). When CD4⁺CD25⁻ T cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies, the final live cell number after 3 days of stimulation was about the same as the starting sample (1.2 fold increase). In contrast, the annexin V⁺ cell number increased by 2.8 fold when
CD4+CD25− T cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence of TGF-β. These data show that TGF-β renders CD4+CD25− T cells resistant to PICA and allows them to expand.

**TGF-β signaling downregulates Bim expression**

We have demonstrated that CD4+CD25+ Treg cells resist PICA in an autocrine TGF-β signaling dependent manner and non-Treg cells (CD4+CD25− T cells) are rescued from apoptosis in the presence of TGF-β, suggesting TGF-β signaling is a key to controlling CD4+ T cell fate (survival or apoptosis) under PICA inducing conditions. We then sought to determine by which mechanisms TGF-β signaling rescues CD4+ T cells from PICA under plate-bound anti-CD3/anti-CD28 antibody stimulation. As we described above, T cell apoptosis is mainly mediated by two different pathways, Fas and Bim. We previously reported that CD4+CD25− T cells isolated from Bim knockout mice or lpr mice which lack functional Fas ligand are resistant to PICA and are able to survive and expand under plate-bound anti-CD3/anti-CD28 antibody stimulation (Singh et al., 2010). These data indicate that PICA is induced in both a Bim and Fas/FasL dependent manner. Further, those proapoptotic molecules, Bim and Fas ligand (cell surface) expression were substantially upregulated when non-Treg cells were stimulated with plate-bound anti-CD3/anti-CD28 antibody stimulation. In contrast, Treg cells did not exhibit increased expression of those proapoptotic proteins and kept basal level of Bim and Fas ligand in the same stimulation condition. We therefore hypothesized that TGF-β signaling in CD4+ T cells might downregulate these apoptotic proteins. Since TGF-β rescued CD4+CD25− T cells from
Fig. 15 Effect of TGF-β on Bim expression by CD4+CD25− T cells. CD4+CD25− T cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence or absence of recombinant TGF-β in media supplemented with IL-2. Cells were harvested at day 3. Cells were lysed into SDS sample buffer and subjected for western blot analysis using anti-Bim and anti-β actin (for loading control) antibodies. EL and L forms are indicated with arrows.
Fig. 16 Effect on TGF-β signaling on cell surface expression of Fas and FasL by CD4^+CD25^- T cells. CD4^+CD25^- T cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence or absence of recombinant TGF-β in media supplemented with IL-2. (A) Cells harvested at day 3 were stained for Fas ligand (FasL) expression and analyzed by flow cytometry. (B) Cells harvested at day 3 were stained for Fas expression and analyzed by flow cytometry.
**Fig. 17** Effect of TGF-β on *bim* transcript by CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ T cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence or absence of recombinant TGF-β in media supplemented with IL-2. Cells were harvested at day 3 and total RNA was isolated for real time PCR. *Bim* gene expression was normalized to *hprt* transcript.
PICA under plate-bound anti-CD3/anti-CD28 antibody stimulation, we first determined whether addition of exogenous TGF-β reduces expression of Bim and/or Fas ligand by CD4⁺CD25⁻ T cells. If our hypothesis is correct, we would expect to observe that CD4⁺CD25⁻ T cells cultured in the presence of TGF-β decreases the expression of Bim or Fas/Fas ligand. Unstimulated CD4⁺CD25⁻ T cells expressed two forms (L and EL isoforms) of Bim at a basal level. When stimulated with plate-bound anti-CD3/anti-CD28 antibodies, CD4⁺CD25⁻ T cells expressed both forms of Bim at a level clearly higher than that seen in unstimulated T cells (Fig 15). Stimulated CD4⁺CD25⁻ T cells in the presence of TGF-β, on the other hand, showed a markedly reduced level of Bim protein expression, even lower than that in unstimulated T cells. In contrast to Bim expression, TGF-β treatment caused a mild reduction in expression of FasL in CD4⁺CD25⁻ T cells (Fig. 16A), while expression of Fas did not differ between TGF-β treated or untreated samples (Fig. 16B). Together, the data clearly show that TGF-β suppresses the expression of molecules required for apoptosis, particularly Bim, by CD4⁺CD25⁻ T cells stimulated by PICA-inducing conditions. Bim is regulated at both transcriptional and posttranscriptional regulations (Ewings et al., 2007). We then determined if TGF-β reduced the expression of Bim in CD4⁺CD25⁻ T cells at transcriptional level by performing real time PCR. Bim gene expression level in TGF-β treated cells were about 3 fold lower compared to untreated cells under plate-bound anti-CD3/anti-CD28 antibody stimulation, suggesting that TGF-β negatively regulates the transcription of bim (Fig 17).

We next determined if TGF-β signaling in CD4⁺CD25⁺Treg cells plays the same role as in conventional T cells. If TGF-β signaling in CD4⁺CD25⁺ Treg cells keeps cells
resistant to PICA, it would be expected that CD4^+CD25^+ Treg cells treated with TGF-β signaling inhibitor (SB431542) would express higher levels of Bim. To test this, CD4^+CD25^+ Treg cells were purified and stimulated with plate-bound anti-CD3/anti-CD28 antibodies for 3 days with or without TGF-β signaling inhibitor (SB431542). Three days later, cells were harvested and tested for the expression of Bim, Fas and FasL (cell surface) (Fig. 18 and Fig. 19). Stimulated Treg cells expressed a comparable level of Bim protein to unstimulated cells and showed a stark contrast to Bim expression by CD4^+CD25^- T cells as we reported previously. In contrast, Treg cells that were stimulated in the presence of TGF-β signaling inhibitor showed a substantial upregulation of Bim expression (Fig. 18). The increase was more evident for the EL form of Bim than for the L form. The EL form is considered to play a major role in apoptosis by inducing the release of apoptotic proteins Bax and Bak. Unlike Bim, Fas and FasL expression by stimulated CD4^+CD25^+ Treg cells did not increase with TGF-β treatment (Fig. 19A, B). We then determined if TGF-β signaling in Treg cells negatively regulate Bim expression at transcriptional level as CD4^+CD25^- T cells. If TGF-β signaling in Treg cells negatively regulates Bim expression at transcriptional level, then we would expect to see upregulation of bim transcription in TGF-β signaling inhibitor treated Treg cells under plate-bound anti-CD3/anti-CD28 antibody stimulation. After 2 days of plate-bound anti-CD3/anti-CD28 antibody stimulation in the presence or absence of TGF-β signaling inhibitor, Tregs were harvested and total RNA was isolated to perform quantitative PCR. As we expected, bim gene expression was 3 fold higher in TGF-β signaling inhibitor (SB431542) treated Treg cells compared to DMSO treated control, suggesting that TGF-β signaling in Treg cells also
CD4⁺CD25⁺ Treg cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3/antiCD28 antibodies in the presence or absence of SB431542 (TGF-β super-family type I receptor kinase inhibitor) in media supplemented with IL-2 for 2 days. Cells were harvested and lysed in SDS sample buffer and subjected for western blot analysis using anti-Bim or anti-β actin (for loading control) antibodies.
Fig. 19 Effect of TGF-β signaling on cell surface expression of Fas and FasL by CD4^+CD25^+ Treg cells. CD4^+CD25^+ Treg cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3/antiCD28 antibodies in the presence or absence of SB431542 (TGF-β super-family type I receptor kinase inhibitor) in media supplemented with IL-2. (A) Cells harvested at day 2 were stained for Fas ligand (FasL) expression and analyzed by flow cytometry. (B) Cells harvested at day 2 were stained for Fas expression and analyzed by flow cytometry.
Fig. 20 Effect of TGF-β signaling on bim transcript by CD4⁺CD25⁺ Treg cells.

CD4⁺CD25⁺ Treg cells isolated from the spleen of C57BL/6 mice were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence or absence of recombinant TGF-β in media supplemented with IL-2. Cells were harvested at day 2 and total RNA was isolated for real time PCR. Bim gene expression was normalized to hprt transcript.
downregulates Bim at transcriptional regulation (Fig. 20). Taken together with the data from studies with CD4⁺CD25⁻ T cells, the data demonstrate that TGF-β signaling plays a pivotal role in suppression of the Bim under PICA inducing conditions.

**Effect of TGF-β for CD4⁺ T cell differentiation under PICA inducing conditions**

As mentioned above, TGF-β signaling also promotes T cell differentiation. It is well established that TGF-β can induce differentiation of naïve CD4⁺ T cells into Foxp3⁺ inducible Treg (iTreg) cells. Since CD4⁺CD25⁺ naturally arising Treg cells are resistant to PICA, we then hypothesized that the survival of CD4⁺CD25⁻ T cells observed with exogenous TGF-β may have been due to the conversion of CD4⁺CD25⁻ T cells to Foxp3⁺ iTreg cells. To test this possibility, we stimulated FACS sorted CD4⁺CD25⁻ T cells with plate-bound anti-CD3 plus either soluble or plate-bound anti-CD28 antibodies in the presence of TGF-β (including IL-2). Stimulation with plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of TGF-β was used for inducible Treg cell polarizing conditions as previously reported (Fantini et al., 2007). After 3 days of stimulation, expression of Foxp3 was examined by flow cytometry. When stimulated by plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of TGF-β (inducible Treg cells polarizing condition), a significant proportion of cells (37.3%) expressed Foxp3 (Fig. 21). However, only 5.3% of cells expanded with both the anti-CD3 and anti-CD28 antibodies being plate-bound expressed Foxp3. The percentage of Foxp3⁺ T cells from plate-bound anti-CD28 antibody stimulated cells was comparable to those stimulated without TGF-β,
Fig. 21 Effect of TGF-β on Foxp3 expression by CD4⁺CD25⁻ T cells under PICA inducing stimuli. CD4⁺CD25⁻ T cells isolated from the spleen of C57BL/6 mice were stimulated with either plate-bound anti-CD3/anti-CD28 antibodies or plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of absence of TGF-β in media supplemented with IL-2. Cells were harvested at day 3 were stained for CD4 and Foxp3, and analyzed by flow cytometry.
**Fig. 22** Effect of TGF-β and IL-6 on PICA by CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ T cells isolated from the spleen of C57BL/6 mice were stimulated with either plate-bound anti-CD3/anti-CD28 antibodies or plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of absence of TGF-β, IL-6 in media supplemented with IL-2. (A) Cells were harvested at day 3 were re-stimulated with PMA and Ionomycin for 4 hours in the presence of monensin and stained for IL-9 and IL-17 expression, then analyzed by flow cytometry. (B) Total number of IL-17⁺ cells and IL-9⁺ cells were determined based on the data obtained in (A).
suggesting inducible Treg induction did not occur with PICA inducing condition, plate-bound anti-CD3/ anti-CD28 antibody stimulation. Together, the data show that CD4⁺CD25⁻ T cells treated with TGF-β become resistant to PICA not because CD4⁺CD25⁻ T cells differentiated into inducible Treg cells.

_TGF-β promotes differentiation of Th9 cells under PICA-inducing condition_

TGF-β is not only involved in iTreg cell differentiation but also for other helper T cell subset differentiations, such as Th9 or Th17 (Soroosh and Doherty, 2009; Yang et al., 2010). Since TGF-β rescued CD4⁺CD25⁻ T cells from PICA without inducing Foxp3⁺ Tregs, we then determined whether cells survived PICA in the presence of TGF-β differentiated into other effector T cell subsets. To address this question, we stimulated purified CD4⁺CD25⁻ T cells with plate-bound anti-CD3 plus either soluble or plate-bound anti-CD28 antibodies in the presence or absence of TGF-β. After 3 days of stimulation, cells expressing IL-9 or IL-17 were assessed by intracellular cytokine staining. CD4⁺CD25⁻ T cells stimulated by plate-bound anti-CD3 plus anti-CD28 without TGF-β did not express IL-9, but a significant portion of the cells stimulated by the same manner in the presence of TGF-β expressed IL-9 (14%) (Fig. 22A). The actual number of cells producing IL-9 also increased significantly with TGF-β (Fig. 22B), showing that TGF-β induced differentiation of a group of CD4⁺CD25⁻ T cells into Th9 cells under PICA-inducing conditions. In contrast, CD4⁺CD25⁻ T cells stimulated by plate-bound anti-CD3 plus soluble anti-CD28 antibodies did not express IL-9 either with or without TGF-β. No increase in Th17 cells was observed under either of these conditions (Fig. 22B). These data suggest that PICA
Fig. 23 Effect of TGF-β and IL-4 on PICA by CD4+CD25− T cells. CD4+CD25− T cells isolated from the spleen of C57BL/6 mice were stimulated with either plate-bound anti-CD3/anti-CD28 antibodies or plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of absence of TGF-β, anti-IL-4 antibody in media supplemented with IL-2. (A) Cells harvested at day 3 were re-stimulated with PMA and Ionomycin for 4 hours in the presence of monensin and stained for IL-9 and IL-17 expression, then analyzed by flow cytometry. (B) Culture supernatant was collected at day 3. IL-4 production was determined by ELISA. ** p< 0.01
inducing conditions, stimulation with plate-bound anti-CD3/anti-CD28 antibody, promotes differentiation of CD4+CD25− T cells to Th9 subset.

Dardalhon et al. reported that Th9 is induced in an IL-4 dependent manner (Dardalhon et al., 2008). We then determined if Th9 cells were induced in an IL-4 dependent manner under plate-bound anti-CD3/anti-CD28 antibody stimulation. To address this question, we cultured electronically sorted CD4+CD25− T cells stimulated with plate-bound anti-CD3 antibody plus either plate-bound anti-CD28 or soluble anti-CD28 antibodies in the presence or absence of anti-IL-4 (2C11 hybridoma culture supernatant). After 3 days of culture, we assessed IL-9 producing cells by cytokine staining. Indeed, addition of anti-IL-4 antibody abrogated induction of Th9 cells by TGF-β and plate-bound anti-CD3/anti-CD28 antibodies (Fig. 23). Whereas IL-4 producing cells were not detectable by cytokine staining after three days of stimulation (data not shown), culture supernatants from cells stimulated with plate-bound anti-CD3/anti-CD28 antibodies contained a clearly detectable level of IL-4 either in the presence or absence of TGF-β (Fig. 23B). TGF-β abrogated IL-4 production in cells stimulated with plate-bound anti-CD3 and soluble anti-CD28 while no decrease of IL-4 was observed for cells stimulated with plate-bound anti-CD3/anti-CD28 antibodies (Fig. 23B). In contrast to IL-4, the level of IFN-γ in the cytoplasm and culture supernatants from cells stimulated by plate-bound anti-CD3/antiCD28 antibody was moderately higher than that of cells stimulated by plate-bound anti-CD3 and soluble anti-CD28 antibodies (Fig. 24). The data suggest that T cells stimulated with plate-bound anti-CD3/anti-CD28 antibodies resist suppression of IL-4 production by TGF-β and differentiate into Th9 in part due to the presence of autocrine IL-4.
Fig. 24 Effect of TGF-β and IL-6 on PICA by CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ T cells isolated from the spleen of C57BL/6 mice were stimulated with either plate-bound anti-CD3/anti-CD28 antibodies or plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of absence of TGF-β, IL-6 in media supplemented with IL-2. (A) Cells harvested at day 3 were re-stimulated with PMA and ionomycin for 4 hours in the presence of monensin and stained for IFN-γ expression, then analyzed by flow cytometry. Total number of IFN-γ⁺ cells was determined based on the data obtained in flow cytometry analysis. (B) Culture supernatant was collected at day 3. IFN-γ production was determined by ELISA. ** p< 0.01, * p< 0.05
Fig. 25 Effect of TGF-β and IL-6 on PICA by CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ T cells isolated from the spleen of C57BL/6 mice were stimulated with either plate-bound anti-CD3/anti-CD28 antibodies or plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence or absence of TGF-β, IL-6 in media supplemented with IL-2. Culture supernatant was collected at day 3. IFN-γ production was determined by ELISA. ** p<0.01
IL-6 plays a critical role in regulating the balance between Th17 and Treg cells and induces Th17 along with TGF-β (Kimura and Kishimoto, 2010). Since IL-6 is mainly produced by non-T cell populations such as antigen presenting cells or non-hematopoietic cells including keratinocytes and fibroblasts, we next tested if exogenous IL-6 plus TGF-β changes the fate of CD4+CD25− T cells under PICA-inducing conditions. When CD4+CD25− T cells were stimulated in the presence of TGF-β and IL-6, the frequency of IL-17+ cells showed a modest increase over the TGF-β only control groups (Fig. 22A). The increase was higher for plate-bound anti-CD28 antibody stimulation than soluble anti-CD28 stimulation (3.7% over 1.4%). In addition, we observed a substantial increase in the amount of IL-17 detected in the culture supernatant for cells stimulated with plate-bound anti-CD28 antibody than with soluble anti-CD28 controls (Fig. 25). This was not merely due to an increase in total live cell numbers in the presence of exogenous IL-6 (Fig. 22B). Rather, there was a marked increase in the cell number of IL-17+ cells, suggesting that plate-bound anti-CD3/anti-CD28 antibody stimulation promotes differentiation and/or expansion of Th17 cells.
CHAPTER FOUR
DISCUSSION

Introduction

CD4+CD25+ Treg cells comprise about 5-10% of CD4+ T cells in the periphery of mice and humans. Since Tregs suppress immune responses, the balance between Treg cells and effector T cells is critical in determining an immunogenic or tolerogenic environment in the body. During an infection, the immunogenic environment is needed to fight against pathogens, thus effector CD4+ T cells undergo clonal expansion by recognizing their cognate antigen via TCR and orchestrate other immune cells by producing a variety of cytokines. On the other hand, excess immunity leads to tissue damage and can cause autoimmune disorders. Therefore, expanded effector T cells undergo apoptosis following an immune response. This event is known as activation induced cell death (AICD) (Shi et al., 1989). However, it has not been addressed how CD4+CD25+ Treg cells respond to antigen stimulation compared to effector CD4+ T cells. Since effector T cells are needed for an immune response and Treg cells are required for maintaining peripheral tolerance, the responses to antigen stimulation in Treg cells might be different from other CD4+ T cells.

Our laboratory recently discovered the phenomenon that CD4+CD25- T cells undergo apoptosis when stimulated with plate-bound anti-CD3/anti-CD28 antibodies.
over 4 days in the presence of IL-2, while Treg cells survive and expand (Singh et al., 2010). Classical AICD occurs in a p53 independent manner, however we found that the cell death due to plate-bound anti-CD3/CD28 antibody stimulation is mediated by a novel form of the apoptosis pathway (p53-induced and CD28-dependent apoptosis: PICA) and is distinct from classical AICD. Other groups have reported that p53 deficient mice develop exacerbated experimental autoimmune arthritis, experimental autoimmune encephalomyelitis and streptozotocin-induced diabetes even though p53 deficient T cells are sensitive to AICD (Okuda et al., 2003; Simelyte et al., 2005; Zheng et al., 2005). These observations led us to hypothesize that PICA is important to control the balance of Treg cells versus non-Treg cells in the periphery. Understanding the differential survival mechanisms between Treg cells and non-Treg cells against PICA might give us a better understanding of peripheral tolerance and help us to identify therapeutic targets to treat immune dysregulation such as autoimmune diseases, GVHD, and cancer.

In this study, we focused on two different aspects that mediate differential survival mechanisms of Treg cells compared to non-Treg cells under PICA inducing condition, plate-bound anti-CD3/anti-CD28 antibody stimulation *in vitro*. First, from a TCR proximal signaling study, we found that the Ras activator, Ras guanyl nucleotide releasing proteins (RasGRP1) expression was substantially reduced in Treg cells compared to effector T cells (non-Treg cells) and its downstream Ras-Erk pathway was significantly impaired. Further, non-Treg cells which lack RasGRP1 expression were resistant to PICA and they expanded under PICA inducing stimuli. Second, we found that TGF-β signaling rendered CD4⁺CD25⁺ T cells resistant to PICA and was required for
survival and expansion by CD4⁺CD25⁺ Treg cells when stimulated with plate-bound anti-CD3/anti-CD28 antibodies. These data suggest that both TGF-β and RasGRP1 signaling play crucial roles in mediating PICA thus balancing the proportion of Treg cell and non-Treg cell subsets.

*RasGRP1 expression in CD4⁺ T cells and PICA*

Our data demonstrates that RasGRP1 expression is substantially reduced in Treg cells compared to non-Treg cells (CD4⁺CD25⁻ T cells) after TCR activation. To determine if reduced RasGRP1 expression in Treg cells is one of the survival mechanisms under PICA inducing conditions, we used RasGRP1 deficient non-Treg cells (CD4⁺CD25⁻ T cells). Our data show that RasGRP1 deficient non-Treg cells are resistant to PICA under plate-bound anti-CD3/CD28 antibody stimulation, suggesting that RasGRP1 signaling is required for inducing PICA in non-Treg cells.

RasGRPs are activators for Ras by converting the Ras-GDP bound form to the active Ras-GTP form (Dower et al., 2000). TCR signaling leads to activation of Phospholipase C γ (PLCγ) which produces diacylglycerol (DAG). The DAG recruits RasGRP1 to the plasma membrane allowing RasGRP1 to interact with Ras thus activating Ras-Erk signaling. T cells dominantly express RasGRP1 among RasGRP family members (RasGRP1, 2, 3, 4) and RasGRP1 plays a critical role in T cell immunity have been demonstrated using RasGRP1 deficient mice or lag mice which have mutations in the *rasgrp1* gene. Layer et al. showed that lag mice develop lymphoadenopathy at 3 to 4 month old (Layer et al., 2003). These mice possess enlarged spleens containing a larger
number of CD4+ T cells compared to wild type littermate controls and developed an autoimmune disorder which is depicted by high levels of antinuclear antibodies in the serum. Transferring the T cells isolated from lag mice (RasGRP1 mutant mice) into T and B cell deficient mice (Rag knockout mice) causes the host to develop an autoimmune disorder, suggesting RasGRP1 expression in CD4+ T cells is required for maintaining immune homeostasis. Priatel et al. reported that adoptive transfer of CD4+ T cells isolated from RasGRP1 knockout mice into congenic host mice also develop exacerbated autoimmune disorder phenotype compared to wild type CD4+ T cells transferred into the host (Priatel et al., 2007). These data also support the idea that RasGRP1 expression in CD4+ T cells is required for maintaining immune homeostasis. RasGRP1 is known to be required for T cell development in the thymus (Dower et al., 2000). Positive selection is impaired in RasGRP1 deficient mice but negative selection is normal in these mice, suggesting central tolerance is kept intact. Why do RasGRP1 deficient mice develop an autoimmune disorder? One of the mechanisms might be dysregulation of peripheral tolerance in RasGRP1 deficient mice. Our data demonstrate that RasGRP1 expression is substantially upregulated in non-Treg cells in response to TCR stimulation. Further, CD4+CD25+ T cells isolated from RasGRP1 deficient mice become resistant to PICA under stimulation with plate-bound anti-CD3/anti-CD28 antibodies over 4 days while wild type non-Tregs undergo massive apoptosis. Altogether, we propose the model that effector CD4+ T cells which lack RasGRP1 expression cannot undergo apoptosis and survive, hence the increased ratio of effector CD4+ T cells (non-Treg cells) against Treg cells break a tolerance thus causing an autoimmune syndrome.
Recently, Yasuda et al. reported systemic lupus erythematosus (SLE) patients express lower levels of RasGRP1 compared to healthy donors (Yasuda et al., 2007). They also showed that SLE patients had a higher frequency of alternating splicing of rasgrp1 which produces a dysfunctional protein. Wen et al. also reported that RasGRP1 expression is lower in SLE patients compared to healthy donors (Pan et al., 2010). Their data suggest that microRNA-21 is highly expressed in SLE patient and further microRNA-21 directly the downregulates RasGRP1 expression. These data also support our model that PICA is balancing Treg cells and non-Treg cells in an RasGRP1 dependent manner.

Our previous report shows that RasGRP1 expression is downregulated in Treg cells compared to non-Treg cells. Since lowered RasGRP1 expression seems to induce dysregulation of effector T cells, reduced expression of RasGRP1 in Treg cells may be critical to maintain immune homeostasis.

We have previously shown that PICA is induced in a Bim and Fas/Fas ligand interaction dependent manner (Singh et al., 2010). Our data demonstrate that the proapoptotic protein, Bim, is substantially reduced in RasGRP1 deficient non-Treg cells (CD4^+CD25^- T cells) under plate-bound anti-CD3/anti-CD28 antibody stimulation compared to littermate control. This suggests that RasGRP1 signaling mediates upregulation of Bim under PICA inducing conditions. Further, reduced expression of Bim in Treg cells under plate-bound anti-CD3/anti-CD28 antibody stimulation might also be due to downregulated RasGRP1 expression. Recently, Stang et al. reported that activating RasGRP1 signaling by the DAG analogue, breostatin, leads to apoptotic cell
death in the Tredo non-Hodgkin’s B cell lymphoma line (Stang et al., 2009). They also demonstrated that apoptotic cell death is mediated by phosphorylation of the proapoptotic protein, Bim, through the activation of the RasGRP-Ras-Erk pathway. We have not examined if PICA is induced by ERK activity. We can address this question by treating non-Treg cells (CD4⁺CD25⁻ T cells) with a Mek inhibitor (PD98059 or UO1). If PICA is induced in an Erk acrivity dependent manner, we would expect to observe that non-Treg cells treated with the Mek inhibitor avoid apoptosis and survive under plate-bound anti-CD3/CD28 antibody stimulation.

We have demonstrated that non-Treg cells which lack RasGRP1 expression are resistant to PICA. Hence, it is expected that the downregulation of RasGPR1 expression in Treg cells is important for cell survival under PICA inducing stimuli. However, we haven’t addressed if the downregulation in Treg cells is required for Treg cell survival under plate-bound anti-CD3/anti-CD28 antibody stimulation. It would be interesting to address if overexpression of RasGRP1 renders Treg cells sensitive to PICA. Due to technical difficulties with the use of a retrovirus or lentivirus transduction system on murine Treg cells to overexpress RasGRP1, we were not successful in addressing this question. In future studies, an alternative approach such as co-overexpressing Foxp3 and RasGRP1 in non-Treg cells can be tested. If downregulation of RasGRP1 expression is important for Treg survival under plate-bound anti-CD3/anti-CD28 antibody stimulation, we would expect to see CD4⁺ T cells co-overexpressed Foxp3 and RasGRP1 undergo apoptosis under PICA inducing stimuli.
Regulation of RasGRP1 expression in T cells

As described above, previous reports show that RasGRP1 deficient mice exhibit substantially reduced single positive T cells in the thymus. Since they have normal numbers of double positive T cells, RasGRP1 is required for positive selection but not for negative selection. RasGRP1 is expressed in all stages of thymocyte development as well as peripheral T cells. Han et al. showed that EL4 lymphoma cells derived from murine thymoma also expresses RasGRP1 and EL4 cells downregulate RasGRP1 expression in response to PMA stimulation (Han et al., 2007). PMA, a DAG analogue, renders RasGRP1 to translocate into the plasma membrane by directly binding to C1 thus allowing RasGRP1 to associate with Ras. Since DAG is produced upon TCR stimulation, this data led us to think that RasGRP1 expression might be downregulated in response to TCR stimulation. Contrary to what was expected from previous study, our data demonstrate that RasGRP1 expression is substantially increased after 7 days of \textit{ex vivo} stimulation in non-Treg cells (CD4\(^+\)CD25\(^-\)T cells). Interestingly, Treg cells continuously expressed basal levels of RasGRP1. Presently, it is not known if upregulation of RasGRP1 expression in non-Treg cells (CD4\(^+\)CD25\(^-\)T cells) has any specific roles in effector CD4\(^+\) T cells. Ebinu et al. reported overexpression of RasGRP1 in human Jurkat T cell lines leads to a higher production of IL-2 (Ebinu et al., 2000). Perhaps, this upregulation of RasGRP1 expression might be required for non-Treg cells to possess effector functions such as cytokine production. Future study is needed to address this question. On the other hand, Treg cells retained basal levels of RasGRP1 expression after \textit{ex vivo} stimulation for 7 days. We found that the difference of RasGRP1 expression
in \textit{ex vivo} expanded Treg cells and non-Treg cells is controlled at the transcriptional
level. The \textit{rasgrp1} mRNA level was about 15 fold lower in expanded Treg cells
compared to non-Treg cells.

Foxp3 is a transcription factor which belongs to the forkhead family and is
specifically expressed in Treg cells. Since Foxp3 positively or negatively control many
genes related to Treg cell functions, we hypothesized that Foxp3 controls \textit{rasgrp1}
transcription. To address this question, we performed a ChIP assay on expanded Treg
cells using anti-Foxp3 antibody. We found 4 sites that a contain forkhead consensus
sequence up to 4kb upstream of the \textit{rasgrp1} transcription starting site. Primers designed
to detect two of the sites amplified a strong band from the Foxp3 antibody precipitated
sample but not from the isotype IgG control antibody precipitated sample, suggesting that
Foxp3 indeed binds to the \textit{rasgrp1} gene transcription regulation region. Recently, Marson
et al. performed that ChIp assay combined with gene chip microarray using murine T cell
hybridoma that overexpressed Foxp3. They reported that Foxp3 controls about 1,100
genes including well known Foxp3 target genes such as IL-2 and CTLA4 (Marson et al.,
2007). Zheng et al. also performed a similar approach as Mason et al. and reported that
Foxp3 controls about 1,200 genes using CD4⁺CD25⁺Treg cells isolated form C57BL/6
mice (Zheng et al., 2007). Neither of them identified the \textit{rasgrp1} gene as a Foxp3 target.

Our study is the first to report Foxp3 targets the \textit{rasgrp1} gene. There are a few
possibilities why the \textit{rasgrp1} gene was not identified as a Foxp3 target. A microarray
based approach might select a target gene with stringent conditions, thus \textit{rasgrp1} was not
picked up. Another possibility is that Foxp3 might bind to the \textit{rasgrp1} gene only after
cells are activated. Since we used *ex vivo* expanded Treg cells stimulated for 7 days with plate-bound anti-CD3/anti-CD28 antibodies for the ChIP assay, Treg cells were in an activated state. We also observed that freshly isolated Treg cells and non-Treg cells expressed comparable level of RasGRP1. This also supports the idea that Foxp3 binds to the *rasgrp1* gene after TCR activation. Future studies are needed to determine if Foxp3 binds to *rasgrp1* before or after TCR stimulation.

We have demonstrated that Foxp3 targets the *rasgrp1* gene, but we have not addressed if Foxp3 binding leads to the downregulation of *rasgrp1* transcription in Treg cells. One mechanism by which Foxp3 negatively regulates gene expression is competition of the binding site with the transcription factor, nuclear factor of activated T cells (NFAT) (Schubert et al., 2001). NFAT mediates immune functions such as cytokine production and the activity of NFAT is regulated by calcium signaling. We found that the NFAT binding consensus sequence exists adjacent to one of the potential Foxp3 binding sites, 3kb upstream of the *rasgrp1* transcription starting site. In future studies, it will be interesting to address if downregulation of *rasgrp1* transcription is due to Foxp3 competing with NFAT for its binding site. This question can be examined by performing a ChIP assay using NFAT antibody. We can compare NFAT binding to the *rasgrp1* gene in Foxp3 overexpressed CD4^+^CD25^−^ T cells using a retroviral transduction system. We can also test if Foxp3 negatively regulates RasGRP1 by transiently overexpressing Foxp3 in non-Treg cells using retrovirus transduction and assess *rasgrp1* transcription levels by real time PCR. If Foxp3 downregulates RasGRP1 expression, we would expect to see
decreased \textit{rasgrp1} gene expression in Foxp3 transduced non-Treg cells compared to control cells.

A recent report shows that microRNA-21 directly targets RasGRP1 expression and downregulates expression levels (Pan et al., 2010). Other groups have reported that microRNA-21 is highly expressed in Treg cells compared to non-Treg cells (Rouas et al., 2009). Taken together, there might be a RasGRP1 downregulation mechanism by microRNA-21 along with transcripitional regulation of \textit{rasgrp1} by Foxp3. Understanding the role of microRNA-21 in Treg cells might give us another regulation mechanism of RasGR1P1 in Treg cells. In the future, it would be interesting to examine the role of microRNA-21 under PICA inducing condition.

\textit{TGF-β signaling decides the fate of non-Treg cells and Treg cells under PICA inducing conditions}

Our data demonstrate that TGF-β signaling is required for Treg cell survival under plate-bound anti-CD3/anti-CD28 antibody stimulation. Nakamura et al. reported that TGF-β production in response to TCR stimulation was higher in Treg cells compared to non-Treg cells by measuring total TGF-β from culture supernatants (Nakamura et al., 2001). However, this assay does not tell how much active TGF-β, which can be associated with TGF-β receptor is present in culture supernatant of Treg cells or non-Treg cells. Our data show that Treg cells undergo apoptosis under plate-bound anti-CD3/antiCD28 antibody stimulation when TGF-β signaling is blocked by a chemical inhibitor (SB431542) or an anti-TGF-β neutralizing antibody. In contrast, non-Treg cells
became resistant to PICA in the presence of exogenous active TGF-β. Taken together, it is expected that Treg cells might possess specific mechanisms to release the active form of TGF-β. To convert latent TGF-β to the active form, latent associated protein (LAP) needs to be cleaved from TGF-β by TGF-β activators such as integrins or thrombospondin-1 (TSP-1) (Masli et al., 2006). Currently, it is not understood if Treg cells express these molecules. It has been demonstrated that Treg cells but not other T cells express a membrane bound form of TGF-β. There might be specific mechanisms to convert latent TGF-β into the active form of the membrane bound TGF-β, thus Tregs but not non-Tregs are able to survive under PICA inducing conditions. Recently, Oida et al. reported that murine CD4⁺CD25⁻ T cells also express the membrane bound of TGF-β without inducing Foxp3⁺ inducible Treg cells (iTreg) when cells were stimulated in the presence of exogenous TGF-β (Oida and Weiner, 2010). This observation supports the idea that membrane bound TGF-β might be important for Treg and non-Treg cell survival under PICA inducing conditions. In future studies, we can address the production of the active-form of TGF-β by Treg cells or non-Treg cells under plate-bound anti-CD3/anti-CD28 antibody stimulation. Further, it would be interesting to determine the role of membrane bound TGF-β in Treg cell survival under PICA inducing conditions. To address this question, we can knockdown GARP which anchors TGF-β on the cell surface. If membrane bound TGF-β has a specific role for Treg cell survival under plate-bound antibody stimulation, GARP knockdowned Treg cells might become sensitive to PICA.
Our data show that TGF-β signaling reduces expression of Bim. Recent reports showed that TGF-β regulates expression of Bim in non-lymphoid cells and mitogen- and stress-activated protein kinase-1 (MSK-1) plays a critical role in the anti-apoptotic functions of TGF-β (Hoshino et al., 2011; van der Heide et al., 2011). Currently, it is not known if MSK plays any role in T cell activation or death. In the future, it will be interesting to determine by which mechanisms TGF-β signaling downregulates Bim expression. Complex and intricate regulation of Bim by TGF-β potentially reflects what has been reported on the role of microRNA-25 (Petrocca et al., 2008). In CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells, Bim protein levels are negatively regulated by TGF-β. Notably, recent reports show that microRNA-25, which regulates Bim protein synthesis and promotes anti-apoptotic responses, was much reduced in Treg cells from patients with multiple sclerosis (De Santis et al., 2010; Li et al., 2009). Loss of this microRNA could lead to an increase in Bim protein expression by Treg cells and their death, hence less effective maintenance of self-tolerance. In the future, we can determine the role of microRNA-25 in Bim expression in Treg cells under PICA inducing conditions.

Recently, Ouyang demonstrated that TGF-β promotes nTreg cells cell survival during negative selection, where Bim plays a critical role (Ouyang et al., 2010). Though thymic selection does not require p53, the data suggest that TGF-β signaling can be anti-apoptotic under certain conditions in connection to Bim expression. Though PICA is an *ex vivo* event established by use of anti-receptor antibodies, our previous work shows that PICA can be induced by extended stimulation from allogenic dendritic cells *in vitro*.
Therefore, it will be interesting to see if TGF-β rescues effector T cells from PICA \textit{in vivo}.

\textit{PICA inducing conditions skews differentiation of CD4$^+$ T cells}

We have demonstrated that TGF-β signaling promotes the differentiation of CD4$^+$CD25$^-$ T cells that receive PICA inducing stimuli. Our data show that non-Tregs (CD4$^+$CD25$^-$ T cells) become resistant to PICA in the presence of exogenous TGF-β under plate-bound anti-CD3/anti-CD28 antibody stimulation. Since TGF-β is known to promote inducible Treg cell differentiation, we expected that Foxp3$^+$ T cells were induced under PICA inducing stimuli, thus they became resistant to PICA. However, this phenomenon is not due to induction of Foxp3$^+$ inducible Treg cells. Our data suggest that non-Treg cells preferentially differentiated into Th9 (IL-9 producing cells) in an IL-4 dependent manner instead of becoming iTreg cells in the presence of TGF-β under PICA inducing stimuli. Further, IL-17 production is also substantially increased under PICA inducing stimuli compared to plate-bound anti-CD3/ soluble anti-CD28 antibody stimulation. Currently, the molecular mechanisms that underlie this phenomenon are unknown. TGF-β may be simply providing a signal required for survival of T cells and IL-4 provides differentiation signaling for Th9. Similarly, TGF-β might allow T cells to survive PICA so that exogenous IL-6 can induce differentiation of surviving cells into Th17 cells. Alternatively, TGF-β could also provide the signaling required for initiation/establishment of differentiation. In either case, the plasticity of CD4$^+$ T cell
Fig. 26 Schematic model of CD4+ T cells under PICA inducing stimuli. Conventional CD4+ T cells undergo PICA when stimulated with plate-bound anti-CD3/CD28 antibodies, whereas nTreg cells expand robustly under same conditions. TGF-β signaling and reduced RasGRP1 expression mediate survival of nTreg cells under PICA inducing stimuli. Conversely, when an active form of exogenous TGF-β is present, non-Treg cells become resistant to PICA and undergo robust expansion instead of apoptosis, with reduction of the proapoptotic protein Bim. Substantial fraction of PICA-resistant T cells expressed IL-9. Moreover, the presence of IL-6 along with TGF-β led to the generation of Th17.
differentiation provided by TGF-β and PICA stimulation might play significant roles in determining the outcomes in vivo (Fig. 26).

Accumulated evidence suggests that TGF-β promotes iTreg cell differentiation with antigen stimulation. However, we have discovered that PICA inducing stimuli does not induce iTreg cell differentiation even in the presence of TGF-β. Conversely, non-Treg cells differentiated into Th9 cells instead of becoming iTreg cells under PICA inducing stimuli when TGF-β was present. Furthermore, in Th17 polarizing conditions, PICA inducing stimuli induced more IL-17 producing cells compared to soluble anti-CD28 stimulation. Since Th9 and Th17 cells mediate effector immune responses by producing IL-9 and IL-17, these data indicate that the condition of PICA inducing stimuli might be able to shift physiological conditions from a tolerogenic to an immunogenic environment even in the presence of TGF-β, which possesses immune suppressive effects. In our study, we have used plate-bound anti-CD3/anti-CD28 antibody stimulation as PICA inducing stimuli compared to plate-bound anti-CD3 plus soluble anti-CD28 antibody stimulation. At present, it is not clear how the difference caused by plate-bound anti-CD28 antibody compared to soluble anti-CD28 antibody leads to a different outcome on the survival of non-Treg cells in the presence of TGF-β. It would be interesting to address the mechanism by which plate-bound anti-CD28 antibody stimulation skews the difference of CD4⁺ T cells compared to soluble anti-CD28 antibody stimulation. Since the difference of these stimulations is only the type of anti-CD28 antibody stimulation, we propose to determine the downstream CD28 signaling pathway by co-immunoprecipitating CD28 associating molecules using anti-CD28 antibody under plate-
bound anti-CD28 stimulation. We expect that CD28 interacting protein such as AKT might be differentially regulated between plate-bound anti-CD28 and soluble anti-CD28 antibody stimulated cells. Although it still remains unclear and needs to be addressed, the molecular mechanisms behind skewing CD4⁺ T cell differentiation under PICA inducing stimuli has potential applications in clinical settings as an immunotherapy tool for treating disease or immune disorders.

We propose the potential applications of PICA inducing stimuli as cancer vaccines for the following reasons. First, PICA inducing stimuli blocks iTreg cell differentiation in the presence of TGF-β. TGF-β plays an important role in tumor development. Many types of tumor cells produce large amounts of TGF-β in the tumor microenvironment and TGF-β production leads to conversion of CD4⁺ T cells into iTreg cells. For example, Lu et al. demonstrated that gastric cancer cells directly convert CD4⁺ T cells into iTreg cells in a TGF-β dependent manner (Lu et al., 2011). Liu et al. demonstrated that non-Treg cells converted into iTreg cells at the tumor site in a TGF-β dependent manner using a mouse model (Liu et al., 2007). This is known to be one of the tumor evasion mechanisms to escape immune surveillance (Curiel, 2007; Nishikawa and Sakaguchi, 2010). If we can mimic PICA inducing stimuli at the tumor site, we would expect to see the blockade of iTreg cell induction at the tumor site thus gaining a more efficient immune response to fight against cancer.

Secondly, PICA inducing stimuli made non-Treg to differentiate into Th9 cells in the presence of TGF-β. Further, they differentiated into Th17 and produced large amounts of IL-17 when TGF-β and IL-6 were present. If we can match PICA inducing stimuli at the
tumor sites, Th17 and Th9 cells might trigger an effective immune response by producing IL-17 and IL-9.

Therefore, we would like to further address how we can duplicate PICA inducing stimuli at the tumor site. Since CD28 signaling is thought to play a key role, we will examine modulation of CD28 signaling for T cells at the tumor site by administration of anti-CD28 antibody or overexpression of B7, which is the ligand for CD28 on APCs.

Conclusion remarks

My work provides insight into how nTreg cells survive under antigen stimulation while conventional CD4+ T cells undergo apoptosis. My work suggests that reduced RasGRP1 expression and TGF-β signaling mediate the survival of nTreg cells under PICA inducing stimuli. These altered signals in Treg cells are likely regulating the balance of nTreg cells and conventional T cells during immune response thus maintaining immune homeostasis. These could be new therapeutic targets to treat immune dysregulations.
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