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Phospholipase D Signaling in T Cells

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LOYOLA UNIVERSITY CHICAGO

PHOSPHOLIPASE D SIGNALING IN 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
UMA CHANDRASEKARAN
CHICAGO, IL
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<td>AB-MECA</td>
<td>N6-(4-Aminobenzyl)-9-[5-(methylcarbonyl)-b-D-ribofuranosyl]adenine</td>
</tr>
<tr>
<td>Ado</td>
<td>Adenosine</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP ribosylation factor</td>
</tr>
<tr>
<td>CAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation antigen</td>
</tr>
<tr>
<td>CDAD</td>
<td><em>Clostridium Difficile</em> - associated diarrhoea</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
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<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DEX</td>
<td>dexamethasone</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>------------------------------------------------</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated protein kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>Foxp3</td>
<td>Forkhead box protein 3</td>
</tr>
<tr>
<td>FICZ</td>
<td>Formylindolo (3,2-b) carbazole</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-induced TNF receptor family</td>
</tr>
<tr>
<td></td>
<td>related protein</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>IBD</td>
<td>Inflammatory Bowel disease</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>i.p</td>
<td>intraperitoneal</td>
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<tr>
<td>i.v</td>
<td>intravenous</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<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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<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic cell sorting</td>
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<td>MBP</td>
<td>Myelin basic protein</td>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>nTregs</td>
<td>Naturally occurring regulatory T cells</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl Choline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PX</td>
<td>Phox homology domain</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RAG-1</td>
<td>Recombination activating gene-1</td>
</tr>
<tr>
<td>ROR</td>
<td>Retinoid-related orphan receptor</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
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<td>T-bet</td>
<td>T-box transcription factor</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>TcdB</td>
<td>Toxin B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tregs</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>T1D</td>
<td>Type-1 diabetes</td>
</tr>
<tr>
<td>Zap-70</td>
<td>Zeta-chain-associated protein 70</td>
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Antigen stimulation of T lymphocytes induces the activation of phospholipase D (PLD) signaling. Phospholipase D (PLD) is a phosphodiesterase that catalyzes the conversion of phosphatidyl choline (PC) to phosphatidic acid (PA). PA is an important lipid second messenger and is known to mediate a variety of cellular functions. However, the specific role of PA in T lymphocytes has not been established. Previous studies indicated differential requirement for TCR induced PLD signaling in regulatory and non-regulatory T cells. Inhibition of TCR induced PLD signal preferentially suppressed the growth of non-regulatory T cells while allowing the proliferation of regulatory T cells in the presence of exogenous IL-2. Based on this observation, we hypothesized that PLD signaling is a critical factor that balances the population dynamics between regulatory and non-regulatory T cells.

For my dissertation work, I focused on elucidating the functional and molecular regulation of PLD signaling in T cells. In this study, we identified that various exogenous (alcohol and Clostridium difficile toxin) and endogenous factors (adenosine) modulate PLD signaling altering the population dynamics of regulatory and non-regulatory T cells.
PLD has two isoforms namely PLD1 and PLD2 expressed in mammalian cells. These isoforms are 50% identical and have distinct localization in the cell. PLD1 has peri-nuclear localization while PLD2 localizes to the plasma membrane. Molecular analysis of PLD1 and PLD2 using domain deletion suggested that a region unique to PLD1 known as the ‘loop’ confers the distinct peri-nuclear localization of PLD1.

Further, we addressed the specific function of PLD2 in CD4 T cells using PLD2 knock out mice. We found that PLD2 is dispensable for T cell activation and proliferation but might play an important role in effector T cell differentiation. The results from this study delineate some of the functions of PLD signaling in T cells and provide insight into immunological regulation during T cell activation.
INTRODUCTION

The mammalian immune system exists in a state of equilibrium that allows for immune activation (reactivity) against pathogens (foreign antigens) while maintaining tolerance (non-reactivity) towards self-antigens. This balance is achieved primarily through interactions between different subsets of T lymphocytes (Littman and Rudensky, 2010). T cell receptors (TCR) recognize short peptides derived from both self and foreign antigens in the context of major histocompatibility complex (MHC) thus underscoring their role in both host defense and autoimmunity (Sette et al., 1993). The fate of a T cell following antigen recognition is influenced by the environmental cues it receives from the surrounding milieu like the presence of different cytokines. Through the activation of specific transcription factors, various cytokines promote distinct effector T cell lineage commitment (Th1, Th2, Th17) thus conferring protection against a wide variety of pathogens (Bettelli et al., 2007; Delgoffe et al., 2009). To avoid uncontrolled immune activation against foreign and host molecules causing harm to the host, the immune system employs various tolerance mechanisms. Regulatory T cell mediated immune suppression plays a major role in regulating immune response by effector T cells and maintenance of tolerance towards self (Sakaguchi, 2004).
Hence, tightly controlled balance between effector and regulatory T cell populations maintain immune homeostasis. But various ex vivo (alcohol, bacterial toxins) and in vivo factors (anti-inflammatory agents) can shift the balance between these two T cell populations with opposing effects. For example, alcohol and adenosine have long been known to be immunosuppressive (Cook, 1998; Hasko et al., 2008; Nelson and Kolls, 2002). However, the precise molecular mechanism through which these molecules alter the immune balance was largely unknown. Previous reports have suggested that immune balance is sensitive to alterations in TCR signaling pathways (Dragone et al., 2006; Goodnow, 2001). Also, differences in the signaling pathways downstream of TCR activation have been reported in Treg cells versus effector T cells (Gavin et al., 2002). In particular, our lab had previously reported differential requirement for TCR induced phospholipase D (PLD) signaling between effector and regulatory T cells. Inhibition of PLD signaling in effector T cells blocked their proliferation and survival while it had no significant effect on Treg proliferation (Singh et al., 2006). Taken together, these data indicated that PLD signaling in T cells is a critical factor controlling the balance between Treg cells and non-Treg cells.

The goal of my dissertation project was to identify the mechanism of PLD regulation in T cells. Based on previous data, we hypothesized that inhibition of PLD signal in T cells may promote dominance of Treg cells while activation of PLD signaling may promote expansion of effector T cells (Fig 1). We focused on the possibility that external factors (alcohol and bacterial toxin) and an endogenous factor (adenosine) might mediate immune-suppression by
negatively regulating PLD signaling. Also, the specific role of PLD2 isoform in altering the T cell balance was determined using mice with T cell specific deletion of PLD2. Further, we identified the critical region controlling the sub-cellular localization of PLD isoforms.

In the sections to follow, the significance of PLD signaling in T cells and rationale and discussion of the experiments performed to address the hypothesis is described.
Fig 1. A schematic presentation of the hypothesis proposed. Active PLD signaling following TCR stimulation leads to expansion of effector T cells (white circles) accompanied by a modest proliferation of nTreg cells (grey circles). TCR signaling in the presence of various exogenous factors (alcohol, bacterial toxins) and endogenous factors (adenosine) suppresses PLD signaling thus inhibiting the expansion of effector T cells. (Courtesy: Dr.Iwashima)
CHAPTER ONE
LITERATURE REVIEW

THE T LYMPHOCYTE IN IMMUNOLOGY

Introduction

The immune system can be perceived as an army of organs, tissues, cells
and molecules that protect the host against a wide variety of invading pathogens
(Eberl, 2010). The coordinated effort of these various immune components
provides the host with layered defenses of increasing specificity (Alberts, 2002).
At first, the physical barrier like skin prevents bacteria and viruses from entering
the host. In case of entry into the host, the innate immune response provides an
immediate but non-specific immune response. If pathogens escape the innate
immune response, vertebrates are equipped with an adaptive immune system.
The adaptive immune system makes use of variable receptors to target specific
pathogen antigens. Also, adaptive immune responses provide faster and
heightened responses upon re-infection with the pathogen due to maintenance of
immunological memory.

Bone marrow derived (B cells) and Thymus derived lymphocytes (T cells)
are now considered the key mediators of adaptive immune responses. However,
this was not always the case. Until 1950, the only property known about lymphocytes was that they were motile (Miller, 2002). Using tritiated thymidine and cannulation of thoracic duct, James Gowans showed that small lymphocytes continuously re-circulate from the thoracic duct to blood and secondary lymphoid tissue and back to the thoracic duct (Gowans, 1996) (Masopust et al., 2007). Miller et al., in 1961 were the first to demonstrate the existence of two major interacting subsets of lymphocytes, T and B cells. Further research suggested that T cells could be divided into two distinct subsets on the basis of their cell surface markers: CD4 and CD8. In terms of their function, CD4 T cells were thought to help antibody synthesis (thus, helper T cells) while CD8 T cells mediated cytotoxic effect on pathogens. In 1986, Mossman and Coffman showed that CD4 T cells were heterogeneous and based on their pattern of cytokine production can be further divided into Th1 and Th2 cells. Recent investigations have further expanded the helper T cell subsets into Th17, regulatory T cells and follicular helper T cells. Thus, the central role of T cells in orchestrating the immune response against a wide variety of pathogens and maintenance of self-regulatory network has been firmly established.

Despite the tremendous increase in the knowledge of T cell subsets, their specific contribution to disease progression is not well established. Researchers are only now beginning to apply the knowledge gained from scientific experiments to therapeutic interventions.
Establishing the role and function of Thymus

Ancient Greeks noted the presence of a large mass of tissue in the chest above the heart and they concluded that it had to be the seat of the ‘Soul’ (Miller, 2002). The wasting away of the soul after puberty was never addressed (Miller, 2002). As early as 1777, William Hewson noted that the thymus was filled with particles resembling those in lymph and blood. Also, he believed that the thymus exists during the early periods of life only when those particles seem to be most wanted (Miller, 2002). Another hematologist, John Beard also echoed the same views as Hewson and suggested that thymus must be regarded as the parent source of all lymphoid structures of the body and that leukocytes starting from their birth place in the thymus penetrate into all parts of the body and perform functions in the body (Miller, 2002). Although, both Hewson and Beard had the right views they did not have the right tools and technical skills to demonstrate their idea.

In the late 1950s, Gross showed that filtered extracts of leukemic tissues from high leukemic strain mice could induce the disease in a low leukemic strain of mice if the extracts were injected at birth (Gross, 1951; Miller, 1999, 2002). The role of thymus in leukemogenesis was known as it was previously demonstrated that adult thymectomy prevented spontaneous mouse leukemia developing in high leukemic strains of mice as well as leukemia induced by chemical carcinogens. Miller hypothesized that virus given at birth multiplied in the developing thymus and thus led to leukemogenic transformation. In order to test this hypothesis, he neonatally thymectomized mice. If his hypothesis was correct, he expected that neonatal mice lacking a thymus from birth should no
longer be susceptible to virus infection and would not develop leukemia when grafted with thymic tissue later. However, he found that these neonatally thymectomized mice were healthy at first but wasted away after weaning and died irrespective of virus inoculation. On the other hand, adult thymectomized mice survived and never showed any signs of weight loss or any other pathology. This led him to conclude that “the thymus at birth may be essential to life” (Miller, 1961).

Histological examination of the tissues of neonatally thymectomized mice displayed a marked deficiency of lymphocytes in the lymphoid tissues and in the circulation along with the presence of liver lesions suggesting infection by hepatitis virus (Miller, 1999, 2002). Further these mice failed to reject skin grafts derived from foreign mouse strains indicating immune deficiency. Conversely, inoculation of lymph node/spleen cells or implantation of thymic tissue led to the recovery of immunological functions in these mice as efficiently as normal mice (Miller, 2002). In summary, these studies firmly established the crucial role of thymus in normal immune system development and function.

Delineation of two major lymphocyte subsets

Based on the observations of defective cellular immunity and impaired antibody producing capacity in neonatally thymectomized mice, it was accepted that mammalian thymus produced lymphocytes that participated in both cellular and humoral immunity. In 1962, Burnet and colleagues reported division of labor among chicken lymphocytes as they found that early bursectomy was associated
with defects in antibody formation and early thymectomy was associated with
defects in cellular response (failure to reject homografts) (Warner et al., 1962).
Also, Parrott et al., observed that neonatally thymectomized mice had a
deficiency of lymphocytes limited to those areas of lymph node and spleen
associated with histological changes induced by cell mediated immune
responses but not in those areas where antibody producing cells appeared
(Parrott et al., 1966). All these emerging data intrigued Dr. Miller and he set out to
understand the contribution of thymus to the pool of small lymphocytes and to
antibody forming capacity. Using histocompatibility differences as markers, Miller
et al., proposed the existence of two major subsets of lymphocytes. They were
antibody forming cell precursors derived from lymphocytes in bone marrow (B
cells) and thymus-derived cells (T cells) essential to allow the former to respond
to antigen by producing antibody and mediating cellular immunity against foreign

Following the reports of two families of lymphocytes, several physical and
biochemical techniques were developed to distinguish these cell populations on
the basis of their surface markers, response to a number of mitogens etc (Hirst et
al., 1975). By 1970, it was widely accepted that T cells reacted with Thy-1 (θ)
antiseras, thus serving as a specific T cell marker (Masopust et al., 2007) (Raff,
2008).
Sub-division of T lymphocytes: CD4 and CD8

In 1960, Govaerts demonstrated that lymphocytes from immunized animals rather than the serum, destroyed allogeneic targets in vitro (Govaerts, 1960). Interestingly, destruction of T cells with anti-θ antiserum eliminated this capability of lymphocytes to destroy allogeneic targets in vitro (Cerottini et al., 1970). Several researchers corroborated this finding of cytotoxicity mediated by T cells (Golstein et al., 1972).

T lymphocytes were reported to participate in a variety of cell-mediated immune reactions like alloreactivity, cell mediated cytotoxicity and amplification of antibody production by B cells (Cantor and Boyse, 1975). However, it was not known whether T cells acquire these diverse functions following antigenic stimulation or whether functionally distinct T cell subclass were present in non-immune animals. To address this question, Cantor et al., undertook experiments to define cell surface components selectively expressed by T cells mediating these distinct functions. Cantor et al., hypothesized that genes coding for such components would be exclusively expressed in T cells. Previous work had reported that Ly antigens are expressed exclusively on thymus-derived lymphocytes, so Cantor determined if these functionally distinct T cell populations could be classified on the basis of expression of different Ly alloantigens. He reported that helper T cells exclusively expressed Ly-1 while cytotoxic T cells expressed Ly-23. Also, these subclasses of T cells were found in non-immune animals indicating that commitment of T cells to participate in either helper or cytotoxic function is a differentiative process independent of antigen exposure.
Further, Cantor et al., demonstrated using adoptive transfer of Ly-1 and Ly-23 expressing T cells into lethally irradiated mice, the absence of inter/sequential conversion of Ly-1 and Ly-23 expressing T cells in the host mice even after prolonged periods (Huber et al., 1976). Taken together, these studies demonstrated the presence of two functionally different subsets of T-cells, representing distinct lineages of thymus-derived lymphocytes.

Further, researchers extended the surface molecule analysis of T cells to humans and found the human analog of Ly-1 as CD4 and Ly-23 as CD8 (Chess, 2006). Moreover, Rao et al., demonstrated that expression of Ly antigens determined major histocompatibility complex (MHC) specificity of T cells in addition to their function (Rao et al., 1983). Thus, CD4⁺ T cells were found to be MHC class II restricted, whereas CD8⁺ T cells were found to be MHC class I restricted (Chess, 2006). Many years of research culminated in the understanding of the role of CD4 and CD8 as not just surface markers of T cell subsets but rather functionally important molecules involved in the recognition of antigen along with the Ag-specific α,β TCR (Chess, 2006).
HETEROGENEITY OF HELPER T CELLS

Introduction

Following the discovery of B and T lymphocytes, Miller performed additional experiments using neonatally thymectomized mice and suggested possible interactions between B and T cells in antibody responses (Miller, 1999). This idea was further strengthened by work of Claman et al., and led to the proposal of the “two cell” theory of antibody production (Claman and Chaperon, 1969). The discovery of two distinct lymphocyte populations and their synergism in antibody production posed many interesting questions to researchers like the nature of the contact between these two cells, timing, dosage of antigen, method of information transfer etc. The process of answering these questions and much technical advancement led to the identification of heterogeneity among helper T cells (Fig 2).

Identification of Th1 and Th2 cells

By 1970, it was generally accepted that a homogeneous population of thymus derived T cells participated in both cell mediated immunity (delayed–type hypersensitivity reactions) and humoral immunity (helper cells in antibody response (Liew and Parish, 1974). In 1971, Parish et al, found an inverse relationship between humoral and cell mediated immunity and proposed the possibility of different sub-populations of T cells mediating delayed type hypersensitivity and helper functions (Liew, 2002; Liew and Parish, 1974).
Marrack et al further confirmed the existence of different sub-populations of CD4 T cells. They demonstrated the presence of antigen specific and non-specific Th cells (Marrack and Kappler, 1975). Furthermore, Tada et al., demonstrated the presence of two types of helper T cells that act either independently or synergistically to help the B-cell response to an antigen (Tada et al., 1978). He further distinguished these subsets of CD4 T cells on the basis of their adherence to nylon wool column and the expression of Ia antigen. Accordingly, Th1 cells were nylon non-adherent, did not express the Ia antigen and led to modest increase in B cell responses, while Th2 cells were nylon adherent, expressed the Ia antigen and led to maximal stimulation of B cell responses.

All of the above-mentioned experiments were conducted by stimulating whole T cell fractions isolated from spleen with an antigen, thus depicting polyclonal activation of T cells. However, by using the recently established method for deriving alloreactive T cell clones (Fathman and Hengartner, 1978), Mossman and Coffman established a panel of antigen specific helper T cell clones. They categorized these helper T cell clones on the basis of their pattern of cytokine production (Liew, 2002). They tested the effect of these cytokines (derived by stimulating individual Th cell clones with ConA) on the proliferative ability of 3 main cell lines namely HT2, NFS60 and MM3 cell lines (Mosmann et al., 1986). This led to the identification of two distinct types of CD4 T cells. Type I cells produced IL-2, IFN-γ, GM-CSF and IL-3 in response to antigenic stimulation while type II cells produced IL-3, IL-4 and IL-5 (Liew, 2002). In line with previous observations of Tada et al., type I helper T cell clones enhanced
IgG1 production by 10 fold and did not induce Ia expression on B cells while type II T cell clones enhanced IgE production by 100 fold and induced Ia expression on B cells. Hence, Mossman et al., used the same nomenclature proposed earlier and categorized these CD4 T cells as Th1 and Th2 (Liew, 2002).

Following this discovery using T cell lines, attempts were made to define CD4 T cell subsets in mice under physiological conditions. This led to the proposal of four different CD4 T cell subsets: cells producing Th1 cytokines, Th2 cytokines, both Th1 and Th2 cytokines and neither Th1/Th2 cytokine (Hayakawa and Hardy, 1988). Additional results on the same cell lines led to the proposal of a Th0 subset, which produced both Th1 and Th2 cytokines and were thought to be uncommitted precursors of Th1 and Th2 subsets of CD4 T cells (Firestein et al., 1989).

Development and regulation of Th1 and Th2 cells

Confirmation of the existence of Th1/Th2 subsets of CD4 T cells led to further investigations into their development and mechanisms of regulation. Gajewski and Fernandez-Botran et al., reported that the products of Th1 and Th2 cells could act as autocrine growth factors for further expansion of these cells and also reciprocally inhibit the opposite cell type (Liew, 2002). IFN-γ enhanced Th1 cell growth and inhibited the proliferation of Th2 cells while IL-4 promoted Th2 cell expansion and limited the proliferation of Th1 cell (Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988).
These findings posed many important questions: What initiates the development of Th1 versus Th2 cells? Are these cells derived from a common precursor that are instructed to differentiate into various lineages or do they arise from different precursors that are pre-programmed to develop into different lineages? Researchers answered these questions using cells from TCR transgenic mice. Hsieh et al, were the first to demonstrate that naïve T cells from TCR transgenic DO11.10 mice can develop into both Th1 and Th2 phenotypes under appropriate cytokine and stimulation conditions (Hsieh et al., 1992). Similar findings by others established that Th1 and Th2 cells have a common precursor and that the cytokine microenvironment is the primary determining factor of the helper T cell subset fates (Liew, 2002). Over the years, many other factors like antigen dose (Hosken et al., 1995), major histocompatibility complex haplotypes (Constant et al., 1995) have also been shown to influence the differentiation of Th1 and Th2 subsets. Also, studies were conducted to elucidate the mechanism through which cytokines determine the lineage decisions of helper T cells. Evidence so far indicates that IL-12 drives Th1 differentiation through activation of the transcription factor T-bet in T cells through STAT4 signaling (Jacobson et al., 1995) (Szabo et al., 2000). Conversely, IL-4 induces Th2 cell differentiation through activation of another transcription factor, GATA3 via STAT6 signaling (Takeda et al., 1996; Zheng and Flavell, 1997). It is also proposed that the mutually exclusive expression of T-bet and GATA3 might reflect the ability of these factors to antagonize each other (Liew, 2002).
Effector functions and disease implications

One of the earliest evidence for the role of helper T cell lineages in disease pathogenesis was suggested in 1981 based on studies of Leishmania major infections. Howard et al reported that BALB/c mice are highly susceptible to *Leishmania tropica* infection compared to C57BL/6 mice (Howard et al., 1980; Howard et al., 1981). Further using adoptive cell transfer it was shown that the protection of C57BL/6 mice (resistant strain) correlated with an appropriate increase in IFN-γ mediated Th1 response and susceptibility of Balb/c mice correlated with an inappropriate IL-4 mediated Th2 response (Heinzel et al., 1989; Scott et al., 1988). Th1 response and IFN-γ were also found to be critical to protect hosts from a variety of infections mediated by intracellular pathogens, viruses and tumors (Wan and Flavell, 2009). Also, excessive Th1 response has been related to incidence of inflammatory diseases like IBD and autoimmune diseases like rheumatoid arthritis (Wan and Flavell, 2009). On the other hand, Th2 response protects hosts against infections from a variety of extra cellular pathogens like helminthes. Th2 responses also participate in mucosal immunity and in allergic diseases (Wan and Flavell, 2009).

**Th17 cells: new member of effector T cells**

Mossman used the ability of T cell clones to induce footpad swelling as a marker for delayed type hypersensitivity reactions (Mosmann et al., 1986). Based on these experiments, he proposed that DTH was mediated solely by Th1 cells. The lymphocytic infiltrates found in the patients with autoimmune diseases like
rheumatoid arthritis (RA) and multiple sclerosis (MS) bear the hallmarks of DTH (Steinman, 2007). So based on Mossman’s findings it was hypothesized that administration of IFN-$\gamma$ would worsen EAE (animal model of MS) while blocking IFN-$\gamma$ would ameliorate EAE. Further, one would have also predicted that EAE would be attenuated or absent in IFN-$\gamma$ deficient mice.

Contrary to these predictions, the results were just the opposite. For example, paralysis in the EAE model was worsened in IFN-$\gamma$ mice. In 2003, multiple studies demonstrated that IL-23 knock out mice were resistant to the induction of various autoimmune diseases like EAE, IBD and arthritis (Cua et al., 2003) (Murphy et al., 2003). The cytokine IL-23 is a heterodimeric molecule, sharing the p40 subunit with the Th1 cytokine IL-12 but differing from IL-12 because of its p19 subunit (Steinman, 2007). IL-23 does not promote the development of Th1 cells, but instead it is one of the essential factors driving the expansion of CD4 T cells producing IL17, IL17F, IL6 and TNF (Langrish et al., 2005). Using passive transfer studies, authors demonstrated that IL-17 producing T cells induced EAE while IFN-$\gamma$ producing T cells did not (Steinman, 2007). Cua and colleagues showed that EAE, driven by IL-23 and IL-17, was worsened with administration of neutralizing antibody to $\gamma$-IFN suggesting that Th1 and Th17 were reciprocal in terms of their function (Cua et al., 2003).

In an attempt to identify factors that inhibited the TGF-$\beta$ mediated conversion of naïve T cells into Foxp3 expressing T cells, Bettelli et al, discovered that TGF-$\beta$ along with IL-6 led to differentiation of naïve T cells into IL-17 producing T cells (Korn et al., 2009). Not surprisingly, IL-6 knock out mice
are resistant to EAE and anti-IL6 improves disease condition in EAE (Steinman, 2007). IL-6 or IL-21 in the presence of low amounts of TGF-β result in the induction of the steroid nuclear receptor, RORα and RORγt in T cells through STAT3 signaling (Korn et al., 2009). However, the mechanism through which RORγt regulates Th17 cell differentiation is not yet determined.

In addition to inducing tissue inflammation in autoimmune diseases like EAE and RA, Th17 cells also participate in host defense against variety of microbes. Korn et al propose that Th17 cells function in the clearance of specific types of pathogens that require massive inflammatory responses, which cannot be dealt with by Th1 and Th2 immunity (Korn et al., 2009). Pathogens as diverse as *Citrobacter rodentium* and *Candida albicans* can trigger Th17 responses (Huang et al., 2004; Korn et al., 2009). Interestingly, Th17 cells are abundant in the lamina propria (LP) of the small intestine. Ivanov et al, showed that Th17 cells are induced in the LP in response to specific components of the commensal microbiota, underscoring their importance in maintaining intestinal immunity (Ivanov et al., 2008).
**Figure 2. Heterogeneity of CD4 T cells.** Differentiation into different effector CD4⁺ T cell lineages, T helper (Th) 1, Th2, Th17, and T regulatory (T_{reg}) cells is initiated following antigenic stimulation of uncommitted (naïve) CD4⁺ T helper cells (Thp). The effector cell types are characterized by their synthesis of specific cytokines and their immuno-regulatory functions, as indicated on the right. The differentiation along different lineages involves different cytokines and the activation of distinct signaling cascades and transcription factors that result in the induction of additional cyto/chemokines and cyto/chemokine receptors, which may be part of positive and negative feedback loops. Modified from Jetten et al ((Jetten, 2009)
HETEROGENEITY OF REGULATORY T CELLS

Introduction

Immunologists have appreciated the concept of self versus non-self discrimination by the immune system since the late 19th century. Ray Owen was the first to observe the phenomenon of immunological tolerance in vivo when he documented the coexistence of two types of erythrocytes in the blood of dizygotic cattle twins and the lack of immune reactivity against each other (Owen, 1945; Wood et al., 2010). This led Burnet to propose the concept of “Neonatal tolerance” wherein he stated that exposure to an antigen early in life would induce specific tolerance to that antigen. Burnet further postulated in his clonal selection theory that during ontogeny, antibodies against self-antigens would be deleted (Martini and Burgio, 1999; Wood et al., 2010).

In 1970, Gershon and Kondo reported the finding that T cells not only augmented but also dampened immune responses (Gershon and Kondo, 1970; Sakaguchi et al., 2007). This suppressive function was found to be mediated by a subset of T cells called suppressor T cells. It was soon determined that I-J was the key suppressor molecule expressed by this subset of T cells. However, no such gene could be found within MHC that encoded this molecule (Steinman, 2007) (Ishii et al., 1982) (Kronenberg et al., 1983).

In the 80s, using newly developed tools (monoclonal antibodies and TCR transgenic mice) Marrack and von Boehmer’s group reported clonal deletion and anergy as key mechanisms of immunological tolerance (Marrack and Kappler, 1987; Sakaguchi et al., 2007) (Kisielow et al., 1988). Most of these results were
inferred from experiments wherein tolerance was induced towards an exogenous antigen.

The question of tolerance was addressed from a diametrically opposite spectrum by investigators like Sakaguchi and Penhale. They studied the effect of loss of tolerance towards self-antigens and thus the etiology of autoimmune diseases. This led to the identification of a subset of T cell called regulatory T cell (Treg cells) that prevented the onset of autoimmunity and is central to the maintenance of tolerance to self-antigens. An improved understanding of the mechanisms of tolerance has led to new possibilities for the treatment of autoimmune diseases (Martini and Burgio, 1999).

**Identification of regulatory T cell subset**

Ehrlrich postulated “Horror autotoxicus” in 1900 suggesting the unwillingness of the organism to endanger itself by the formation of toxic autoantibodies (Silverstein, 2001). However, Donath and Landsteiner demonstrated that the cause of Paroxysmal cold hemoglobinuria is due to the formation of autoantibodies against patient’s own erythrocytes (Silverstein, 2001). Many other diseases of autoimmune origin were discovered as well but these results faded in the wake of biochemical pursuits by scientists to uncover antibody–antigen interactions.

In the late 1960s many studies reported that neonatal thymectomy of normal mice/rats (day 3 after birth) led to the development of inflammatory tissue damage along with the appearance of tissue-specific autoantibodies in circulation
(Penhale et al., 1973; Sakaguchi et al., 2007). Also, adoptive transfer of CD4 T cells from these mice with autoimmune disease was sufficient to induce disease in T cell deficient recipients (McKeever et al., 1990). Interestingly, adoptive transfer of CD4 T cells from normal syngeneic animals into thymectomized mice inhibited the development of these diseases. Based on these studies, it was inferred that there might exist two types of CD4 T cells in the periphery of normal untreated mice/rats: one capable of mediating autoimmune disease and the other capable of dominantly suppressing them (Sakaguchi et al., 1985).

Following this, attempts were made to separate these two putative CD4+ T cells on the basis of cell surface markers. In 1985, Sakaguchi demonstrated that transfer of the CD5low CD4+ T cell subset into nude mice led to the development of autoimmunity similar to neonatally thymectomized mice while co-transfer with normal untreated CD4+ T cells inhibited autoimmunity (Sakaguchi et al., 1985). Similar findings by Powrie and Morrissey, demonstrated the role of CD45RB<sup>high</sup> CD4+T cells in mediating autoimmune diseases (Powrie et al., 1993) (Morrissey et al., 1993). In 1995, Sakaguchi et al., identified CD25 as a definitive marker of CD4 T cells capable of suppressing autoimmune diseases based on adoptive transfer studies (Sakaguchi et al., 1995). As one would expect based on previous findings, CD25+ T cells were confined in the CD5<sup>high</sup> and CD45RB<sup>low</sup> fraction of CD4 T cells (Sakaguchi et al., 2007). Furthermore, removal of CD25+ T cells not only elicited autoimmune disease but also led to enhanced immune response against non-self antigens (Sakaguchi et al., 1995). Together, all these results collectively led to the identification of thymus derived CD4+ CD25+ T cells
that mediates the maintenance of tolerance to self and controls the magnitude of immune response to non-self. These CD4+CD25+ T cells were termed as naturally occurring regulatory T cells (nTreg cells).

Around the same time as the discovery of nTreg cells, two other reports of CD4 T cells with regulatory properties surfaced. Groux et al., showed that naive T cells from OVA TCR-transgenic mice, repeatedly stimulated with OVA and IL-10, differentiated into T cells with a unique cytokine profile distinct from Th1 or Th2 cells (Groux et al., 1997). They produced IL-10, some IL-5, and IFN-γ, with or without TGF-β, but showed only marginal or no IL-2 and IL-4 production. These cells prevented the development of T cell mediated autoimmune responses. Consequently, these cells were designated as type 1 regulatory T cells (Tr1) (Jonuleit and Schmitt, 2003) (Beissert et al., 2006) (Fig 3).

Also, Chen et al., described a subset of T cells in mice that suppressed the onset of myelin basic protein (MBP)-specific experimental autoimmune encephalitis following induction of oral tolerance to MBP (Jonuleit and Schmitt, 2003). The suppression of these T cells was primarily mediated through TGF (Transforming growth factor)-β and was called T helper type 3 (Th3) cells (Beissert et al., 2006)(Fig 3).

**Phenotypic and functional characteristics of Treg cells**

Naturally arising Treg cells are mostly CD4⁺CD25⁺ and constitute 5–10% of peripheral T cells in normal mice (Fehervari and Sakaguchi, 2004). These cells suppress cytokine production (e.g. IL-2, IL-4, IFN-γ) and proliferation of antigen-
receptor stimulated non-regulatory CD4 and CD8 T cells. Although their mode of suppression is not clearly understood, emerging studies have put forth many interestingly mechanisms that can be broadly classified into three classes: contact dependent, soluble factors and killing of target cells. The contribution of cell contact dependent mechanisms have been suggested based on the inability of Treg cells to suppress effector T cell proliferation when the two populations were separated using a semi-permeable membrane (Thornton and Shevach, 1998). Following cell contact, Treg cells might deliver negative signals to responding T cells through increased cAMP levels (Bopp et al., 2007), which is inhibitory for T cell proliferation and IL-2 production. Also, production of the immunosuppressive nucleoside, adenosine through the action of CD39 and CD73 expressed on the surface of Treg cells has been suggested (Deaglio et al., 2007). Also, Treg cells have been proposed to suppress immune responses through cytokine deprivation induced apoptosis of effector T cells (Pandiyan et al., 2007). IL-2 is a critical growth/survival factor for Treg cells (Fontenot et al., 2005). At the same time, the presence of exogenous IL-2 abrogates the suppressive property of Treg cells.

Although surface phenotype of CD4\(^+\)CD25\(^+\) was originally used to isolate these populations, it is not a definitive marker of natural regulatory T cells since activated effector T cells also express CD25. Many other cell surface markers expressed by Treg cells are CD38 (Martins and Aguas, 1999; Read et al., 1998), CD62L (Ermann et al., 2005; Mudter et al., 2002; You et al., 2004), CD103 (Banz et al., 2003), glucocorticoid-induced tumor necrosis factor (TNF) receptor
(GITR)(Shimizu et al., 2002), or low levels of cell-surface CD45RB (Powrie et al., 1996; Powrie et al., 1994; Read et al., 2000; Taams et al., 2001), though none of these are exclusive to Treg cells. Currently, the most reliable marker that exclusively distinguishes Treg cells from non-Treg cells is a transcriptional repressor Foxp3 (forkhead box P3)(Fontenot et al., 2003; Gambineri et al., 2003; Hori et al., 2003; Khattri et al., 2003; Schubert et al., 2001). Mutations of foxp3 gene result in severe autoimmune disorders both in human and mouse (Bennett et al., 2001; Brunkow et al., 2001; Chatila et al., 2000; Wildin et al., 2001). Foxp3 gene is required for the production and maintenance of regulatory T cells (Fontenot et al., 2003; Kim et al., 2007) and most importantly Foxp3 is not induced upon activation of non-suppressive murine CD4⁺CD25⁻ T cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003).

Accumulating evidence suggests that the function of Treg cells is not limited to suppression of autoimmunity and they play significant roles impairing the immune response to chronic viral infections (Mills, 2004; Mills and McGuirk, 2004; Rouse and Suvas, 2004; Suvas et al., 2004; Suvas et al., 2003; Toka et al., 2004). This was first reported using persistent Friend leukemia virus infection of mice (Iwashiro et al., 2001). Mice infected with this virus have suppressed CD8 T cell-mediated immunity against virus-infected cells. When CD4⁺CD25⁺ T cells are removed from peripheral T cells of infected mice, CD8 T cells markedly reduced viral levels during persistence (Dittmer et al., 2004). Increase of Treg cells was also reported in cases of infection with HIV (Weiss et al., 2004).
Other studies have demonstrated that the frequency of Treg cells increases under tumor bearing conditions (Waldmann, 2006), as well as during pregnancy (Saito et al., 2005; Zenclussen, 2005). Conversely, decrease of Treg cells was reported in cases of autoimmune disease such as multiple sclerosis, type I diabetes, and rheumatoid arthritis (Lan et al., 2005).

As mentioned previously, regulatory T cells (CD4+Foxp3+) can also be induced in the periphery and hence are called “adaptive” or “induced” Treg cells. IL-10 producing Tr1 cells proliferate poorly after polyclonal or Ag-specific activation in vitro and functional studies revealed that these cells have immunosuppressive properties and can prevent the development of T cell-mediated autoimmune responses (Levings et al., 2002) (Jonuleit and Schmitt, 2003). Tr1 can control the activation of naive and memory T cells both in vitro and in vivo and suppress Th1- and Th2-mediated immune responses to pathogens, tumors, and alloantigens (Groux, 2003). Furthermore, supernatants of activated Tr1 cells strongly reduce the capacity of dendritic cells (DC) to induce alloantigen-specific T cell proliferation (Levings et al., 2002) (Roncarolo et al., 2001). The suppressive effects of Tr1 cells are reversed by blocking Abs against IL-10, showing that the inhibitory capacity of Tr1 cells is mainly mediated through production of immunosuppressive IL-10 (Groux et al., 1997; Levings et al., 2002).

Regulatory Th3 cells are a unique T cell subset induced by orally administered Ag in vivo and triggered in an Ag-specific fashion (Jonuleit and Schmitt, 2003). They provide help for IgA production and have suppressive properties for Th1 and Th2 cells (Jonuleit and Schmitt, 2003; Weiner, 2001a).
However, the suppression of immune responses mediated by Th3 cells are Ag nonspecific and can occur as bystander suppression through secretion of TGF-β. Because TGF-β is broadly expressed and influences the functional activity of multiple cell types, TGF-β secreting Th3 cells probably have a major role in many aspects of immune regulation and T cell homeostasis (Weiner, 2001b).
**Fig 3. Different subsets of regulatory T cells.** Naturally occurring regulatory T cells mature and migrate from the thymus and constitute 5–10% of peripheral T cells in normal mice. The inducible populations of regulatory T cells include distinct subtypes of CD4⁺ T cell: Tₐ₁ cells, which secrete high levels of IL-10 and Tₐ₃ cells, which secrete high levels of TGF-β. TGF-β can also induce Foxp3 expression CD25- T cells and thus confer regulatory properties to T cells in the periphery. Modified from Milojevic et al (Milojevic et al., 2008).
The immune system faces the daunting task of protecting the host against a wide variety of pathogens at the same time maintaining peace towards self. CD4 T cells play key roles in orchestrating both the functions of the immune response. As mentioned previously, naïve CD4 T cells specialize to become distinct subsets and produce restricted patterns of cytokines that are tailored to combat various pathogens. The range of infections afflicting HIV infected individuals following decline in their CD4 T cell count underscores the importance of CD4 T cells in host defense (O'Shea and Paul, 2010). On the other hand, heterogenous populations of CD4+ regulatory T cells suppress the immune response to both self and non-self antigens. Adoptive transfer of Treg depleted CD4 T cells leads to development of spontaneous autoimmunity, illustrating the dominant role of Treg cells in mediating tolerance and autoimmunity.

Homeostasis of the immune response depends on the balance of population dynamics and functionality between these effector and regulatory T cell subsets (Mills, 2004). Perturbations in this balance have been reported in many diseases. Tang et al, reported that type I diabetes (T1D) progression in the NOD mice was associated with a progressive loss of functional Treg cells in the inflamed islets (Tang et al., 2008). Conversely, expansion of Treg cells in inflammatory mouse models like EAE has been reported to be associated with disease resolution (Knoechel et al., 2005) (Korn et al., 2007).
Various mechanisms have been proposed to explain the alterations in the balance between Treg cells and effector T cells in vivo. The importance of IL-2 levels in the maintenance of homeostasis is evident from different studies. For instance, Tang et al observed that low dose injections of IL-2 supported the growth and survival of Treg cells over effector T cells (Teff) whereas a high dose of IL-2 led to rapid expansion of effector T cells (Tang et al., 2008). Based on these observations they further proposed that Treg cells versus effector T cells regulate each other through a feedback loop. IL-2 production by activated Teff cells expands and sustains Treg cells, which in turn feeds back to suppress the Teff cell response and maintain normal immune homeostasis. Disruption of this crosstalk can lead to the dysregulation of the Treg cell: Teff balance thus contributing to the development of disease (Tang et al., 2008).

Recent emerging studies suggest that plasticity and interconvertibility among different subset of T cells might also alter the immune balance (Wan and Flavell, 2009). Foxp3 expressing T cells from wild type mice lose Foxp3 expression and exhibit activated-memory phenotype and produce inflammatory cytokines (Zhou et al., 2009). Also, fully differentiated Th17 cells were shown to convert into Th1 cells in the absence of TGF-β (Lee et al., 2009). However, the mechanism and the proportion of T cells undergoing such inter-conversion are not known. This newly defined property of CD4 T cells can have far reaching consequences in the pathogenesis and treatment of variety of diseases ranging from autoimmunity to cancer.
Studies of immune evasion by various pathogens also provide clues to the mechanisms of alteration of immune balance. One of the common immune evasion strategy employed by many pathogens involves increasing the production of various immunosuppressive or anti-inflammatory molecules to skew the immune response towards regulatory phenotype. Many intracellular pathogens and viruses either induce cellular IL-10 or encode their own IL-10 homolog to delay or suppress the host immune response (Redpath et al., 2001).

Various environmental toxins have also been shown to tip the immune balance between the effector and regulatory T cells. Quintana and Veldhoen et al demonstrated that dioxin signaling through aryl hydrocarbon receptor (AHR) increased Treg activity and proliferation while activation of AHR by FICZ led to increased Th17 activity (Quintana et al., 2008) (Veldhoen et al., 2008).

Manipulation of the immune balance serves as an attractive target for therapy for a range of human diseases. The ideal treatment for autoimmune disease would be to decrease the frequency of effector T cells and increase the frequency of Treg cells. Several investigators have devised many therapeutic strategies to tip the Treg: effector T cell balance. For example, administration of monoclonal antibodies to CD3, CD4 or CD40L in the presence of antigen causes selective expansion of Foxp3+ T_{reg} cells, producing the dominancy of T_{reg} cells over effector T cells (Wing and Sakaguchi, 2010). Rapamycin, an inhibitor of the Akt-mTOR pathway, increases the number of Foxp3+ T cells in part by enhancing Foxp3 transcription and by rendering T_{reg} cells resistant to apoptosis (Basu et al., 2008; Strauss et al., 2007c; Wing and Sakaguchi, 2010). Administration of IL-2–
anti-IL-2 complexes selectively expands Foxp3$^+$ T$_{reg}$ cell populations (Webster et al., 2009; Wing and Sakaguchi, 2010). On the other hand, the ideal treatment in the case of tumours would be to increase the frequency of effector T cells and reduce the numbers of Treg cells. Administration of anti-CD25 mAb (PC61) has been reported to cause reduction in the number of CD4$^+$CD25$^+$ cells thus enabling tumour rejection (Onizuka et al., 1999).

Role of TCR mediated signaling pathways in altering the immune balance

The underlying theme in the above mentioned strategies for selective expansion/inhibition of Treg cells/Teff is the manipulation of IL-2 and/or IL-2R signaling in CD4 T cells. The potential caveat in these strategies is that IL-2 is a T cell growth factor for both effector and regulatory T cells and this might lead to high variability in the in vivo effect in a heterogeneous patient population.

An alternative strategy would be to identify specific factors/signaling cascades present exclusively in one subset versus the other and to exploit those molecules for therapeutic applications. Consistent with this notion several studies have reported the role of specific TCR associated signaling molecules in controlling the direction of helper T cell subset differentiation.

Recognition of MHC–antigen peptide complex by T cell receptor triggers a cascade of downstream signaling events. The most proximal biochemical event is the tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR/CD3 complex (Guy and Vignali, 2009; Iwashima, 2003; Nakayama and Yamashita, 2010). Lck, an Src family tyrosine kinase plays
an important role in the tyrosine phosphorylation of ITAMs. Then, the Zap70 Syk family kinase is recruited to the phosphorylated ITAMs through the SH2 domains of ZAP70. The recruited ZAP70 is then phosphorylated and activated by surrounding Lck tyrosine kinase, thus leading to the phosphorylation of LAT molecules (Nakayama and Yamashita, 2010). Following these proximal events, the activation of various distinct signaling pathways is initiated, including (i) the Ras/ERK MAPK cascade, (ii) the Ca/calcineurin/NF-AT pathway, and (iii) the PKC/NF-κB pathway (Nakayama and Yamashita, 2010). Interestingly, the differential requirement for various signaling molecules involved in both proximal and distal events of TCR signaling has been reported in different subsets of CD4 T cells. For instance, Lck activity is required for the generation of Th2 cells as dominant negative (DN) Lck transgenic mice shows impaired Th2 cell generation as against intact Th1 cells (Yamashita et al., 1998). In the same lines, Tan et al., showed that PKC is necessary for IL-17 production in the mice (Tan et al., 2006). Further, inhibition of ERK/MAPK cascade using pharmacological inhibitor or DN Ras results in increased Th1 cell differentiation (Yamashita et al., 1999). Recent studies from our lab group reported the differential requirement for phospholipase D (PLD) signaling between regulatory versus non-regulatory T cells. Suppression of TCR induced increases in PLD activity led to selective inhibition in the proliferation and survival of effector T cells. As a consequence, the frequency of Treg cells increased under these conditions. Thus, manipulation of PLD activity may serve as a tool to alter the immune balance. Hence, identification of
factors/molecules that modulate PLD activity will be of significant therapeutic importance.

BALANCING ACT OF PHOSPHOLIPASE D (PLD) IN T CELLS

PLD: structure, function and expression

PLD belongs to the phosphodiesterase family of proteins, found in bacteria, fungi and animals (Melendez and Allen, 2002). Mammalian PLDs catalyze the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline (Exton, 2002). Phosphatidic acid is an important lipid second messenger, which mediates the downstream effects of PLD signaling. PA is an abundant phospholipid that can be also synthesized from pathways other than PLD signaling (Athenstaedt and Daum, 1999).

A unique characteristic of PLD is that primary (1–) alcohol serves as a more competitive substrate compared to water (> 1000 fold), and the presence of 1–alcohol effectively blocks PLD signaling (Morris et al., 1997) (Fig 4a). In the presence of 1–alcohol (such as ethanol or 1–butanol), PLD prefers transphosphatidylation to hydrolysis and produces phosphatidyl–alcohol, which is poorly metabolized. As a result, the production of PA is significantly reduced. Thus, 1–alcohol is a potent suppressive regulator of PLD signaling.

Two isoforms of PLDs are expressed in mammalian tissues namely PLD1 and PLD2, which share 55% identity (Frohman et al., 1999). The domain structure of PLDs contains phox homology (PX) and pleckstrin homology (PH)
domains at the N-terminal followed by four conserved sequences (I-IV) (Sung et al., 1999) (Fig 4b). PX and PH domains are implicated in phospholipid and protein binding. Results from deletional analysis indicate that PIP$_2$ binds at PH domain and at conserved sequences located between catalytic sites II and III (Hodgkin et al., 1999) (Sciorra et al., 1999). The catalytic sites II and III contain the characteristic ‘HxKxxxxD’ motifs (Frohman et al., 1999). In addition, PLD1 contains an 116 amino acid ‘loop’ region inserted between the II and III catalytic sites. The C-terminal four amino acids of mammalian PLDs are evolutionarily conserved and modification of these residues (mutation/deletion) causes loss of catalytic activity (Xie et al., 2000) (Liu et al., 2001). PLD1 exists as two splice variants, PLD1a and PLD1b that differ in the length of the loop region. This region may function as a negative regulator of PLD1 since deletion of the loop has been shown to cause modest up regulation of basal catalytic activity of PLD1 (Frohman et al., 1999).

Although PLD1 and PLD2 have the same substrate specificity, the activities of PLD1 and PLD2 are regulated differently. PLD1 has low basal activity in vitro and it can be stimulated in vitro using agonists as it is coupled to receptor signaling via the small GTPases, ARF1/6, Rho, CDC42, and RalA. PKC mediated phosphorylation also up regulates PLD1 activity. In contrast, PLD2 is less responsive to small GTPases and may be constitutively active and regulated by inhibition (Colley et al., 1997).
Fig 4. Schematic representation of catalytic reaction of PLD and domain structure of PLD isoforms found in mice. (A) In aqueous solution, PLD catalyzes hydrolysis of phospholipid to yield Phosphatidic acid (PA). But in the presence of small amounts of ethanol, PLD yields Phosphatidyl ethanol (PEt). R1, R2 and R3 are fatty acid side chains. B) Domain structure of PLD isoforms in mice. PX, phox homology; PH, pleckstrin homology domain. Motifs I~IV are conserved regions and II and IV contain HKD sequences. Modified from (Frohman et al., 1999).
PLD1 is mainly found in perinuclear regions, whereas PLD2 localizes predominantly in the plasma membrane (Colley et al., 1997; Sung et al., 1997). Both PLD1 and PLD2 are palmitoylated in the pleckstrin homology (PH) domain and found associated with membrane raft fractions (Sugars et al., 1999; Xie et al., 2001). The site of generation of PA and the availability of effector targets determines its downstream signaling. Hence, the sub–cellular distribution of both the isoforms of PLD is an important factor affecting its function and regulation.

Functions of PA

PA is now recognized as a vital second messenger for many cell types (Andresen et al., 2002; Chen, 2004; Chen and Fang, 2002; Cummings et al., 2002; English, 1996; Ghosh and Bell, 1997; Houslay and Adams, 2003; Rizzo and Romero, 2002). For instance, the direct interaction of PA has been demonstrated for several signaling proteins, namely cRaf-1 (Ghosh et al., 1996; Rizzo et al., 1999; Rizzo et al., 2000; Wang et al., 2002), mTOR (Chen and Fang, 2002; Fang et al., 2003; Fang et al., 2001; Lim et al., 2003), phosphodiesterase 4D3 (El Bawab et al., 1997; Grange et al., 1998; Grange et al., 2000; Savany et al., 1996), PKC \( \zeta \) (Bandyopadhyay et al., 2001; Limatola et al., 1994),SHP-1(Frank et al., 1999; Tomic et al., 1995) and sphingosine kinase-1 (Delon et al., 2004). PA plays a significant role in the regulation of the function of these proteins. The binding of PA to SHP-1, for instance, activates the phosphates in vitro (Zhao et al., 1993). PA also functions in protein recruitment.
Interactions with PA are essential for the recruitment of cRaf-1 to membranes (Rizzo et al., 2000).

PA is also a precursor for a number of alternative signaling molecules. For example, PA can be converted to diacylglycerol (DAG), by phosphatidic acid phosphohydrolases (Balboa et al., 1995; Billah et al., 1989; Lassegue et al., 1993; Mullmann et al., 1990). DAG is an activator of conventional and novel PKC isoforms and other signaling molecules (Cummings et al., 2002; Gudermann et al., 2004; Haeffner, 1993; Kikkawa et al., 1989; Yang and Kazanietz, 2003). DAG is also generated by the hydrolysis of phosphatidylinositol (4,5)-bisphosphate by phospholipase C (Haeffner, 1993; Nishizuka, 1992). The nature of the DAG species generated by the action of phospholipase C differs from that derived from PA through the action of phospholipid phosphohydrolases (Liu et al., 1999; Plo et al., 2000). The consequences of this difference are currently unknown.

Hence, PA mediates a variety of cell functions including cell survival, proliferation, cytoskeletal rearrangement and vesicular trafficking (Fang et al., 2001; Ghosh et al., 1996; Lim et al., 2003; Rizzo et al., 1999). However, the precise role of PA in modulating T cell function and survival is not clear.

Regulation of catalytic activity of PLD

Various in vitro studies have elucidated the role of certain co-factors in the catalytic activation/inactivation of PLD. They can be classified into 3 classes.
i. **Phospholipids**

PIP2 was determined to be essential for catalytic activity of PLDs (Brown et al., 1993). PIP3 is also effective in up regulation of PLD activity. A PIP2 binding site is located between conserved regions II and III of the catalytic domain and mutation in this region abrogates PLD activity without altering their sub cellular localization (Frohman et al., 1999). Another PIP2 binding site has been reported to be present within the PH domain. This binding site affects PLD activity by altering their sub cellular localization (Oude Weernink et al., 2007).

ii. **Small GTPase**

Several small GTPase’s can activate PLD. Activation of PLD1 by ARF is GTP dependent and requires the N-terminal region of PLD. However, the detailed mechanism of PLD activation by ARF is not known (Frohman et al., 1999). Several Rho family proteins, namely RhoA, Rac and CDC42 are also involved in PLD activation. In vitro, GTPγs activated forms of these proteins have been shown to activate PLD (Hammond et al., 1997). Botulinum C3 toxin has been reported to block PLD activation by inactivating Rho (Malcolm et al., 1996; Schmidt et al., 1996). C-terminus of PLD1 is reported to be the interaction site for Rho (Du et al., 2000).

iii **PKC**

Treatment of cells with phorbol esters effectively activated PLD, suggesting that Protein Kinase C (PKC) is upstream of PLD activation (Exton, 2002). Several
phosphorylation dependent and independent modes phosphorylation by PKC has been reported (Hammond et al., 1997).

**TCR signaling and PLD**

Attachment of the T cell receptor complex by its cognate antigen triggers a cascade of downstream signaling events including phosphorylation of ITAM motifs, phosphoinositide hydrolysis and calcium flux. Emerging results indicate that antigen receptors that transduce signals via immuno–receptor tyrosine based activation motifs (ITAMs) are coupled to PLD activation (Melendez and Allen, 2002). Fcγ and ε receptors activate PLD by the antigen-antibody complex (Cockcroft et al., 2002; Gewirtz and Simons, 1997; Gruchalla et al., 1990; Jose Lopez-Andreo et al., 2003; Kusner et al., 1999; Loegering and Lennartz, 2004). TCR stimulation was also shown to upregulate PLD activity by 2~300% (Reid et al., 1997). Inhibition of PLD activity in T cell line caused loss of AP-1 activation, cytokine production and proliferation (Mollinedo et al., 1994). BCR was also shown to activate PLD (Forssell et al., 2000; Hitomi et al., 1999).

Previously our lab group had demonstrated that PLD1 and PLD2 signaling are involved in different stages of TCR signaling (Singh et al., 2006). Over expression of both PLD1 and PLD2 potentiated NF-AT activation by TCR in Jurkat cells. CD2/ZAP-70, a fusion protein between the extracellular and transmembrane domains of CD2 with Zap-70 activates NFAT when expressed in Jurkat cells. When co-expressed with CD2/Zap-70, PLD1 induced marked
upregulation of NFAT activity whereas PLD2 induced only a slight upregulation. Conversely, lipase inactive PLD2 but not PLD1, blocked anti-TCR antibody induced tyrosine phosphorylation of TCR ζ. Together, the data indicated that PLD2 is required for TCR signaling events proximal to the recruitment of Zap-70 to ITAM while PLD1 is involved in events distal to tyrosine phosphorylation of ζ.

It is not yet clear how PLDs are coupled to TCR. Several groups have reported the presence of PLD enzymes in caveolae, which are specialized domains of the plasma membrane, related to sphingolipid and cholesterol-rich micro domains or rafts (Gidwani et al., 2003; Hekman et al., 2002; Zheng et al., 2003). In view of the fact that molecules with a key role in T cell signaling like Lck also localize to these domains, it is tempting to suggest that lipid rafts might provide the perfect platform for PLD and TCR coupling.

In our recent study, we also demonstrated that the increase in PLD activity after TCR stimulation was two fold in CD4+CD25- cells and rather unchanged in CD4+CD25+ T cells. Moreover, PLD1 expression was substantially lower in CD4+CD25+ cells than in CD4+CD25- cells indicating differences in the requirement for PLD signaling between regulatory and non-regulatory T cells. Inhibition of the TCR induced increase in PLD activity in CD4+CD25- T cells substantially affected their activation; proliferation and cytokine secretion while it had no effect on CD4+CD25+ T cells. Consequently, culture of heterogeneous population of naïve CD4 T cells in the presence of TCR stimulation and exogenous IL-2 led to preferential expansion of Foxp3+CD4+ T cells. Moreover, these expanded Treg cells exhibited potent immune suppression in a mouse
model of inflammatory bowel disease (IBD) indicative of their functional capabilities. These data also illustrated the biological significance of PLD signaling in T cells. Investigations to elucidate the anti-proliferative effect of inhibition of PLD signaling on CD4+CD25- T cells suggested that blockade of high affinity IL-2 receptor (CD25) expression may be a factor. However, the precise molecular mechanism of regulation of CD25 expression by PLD is still unknown. Also, the specific function of the PLD isoforms in mediating this differential effect on CD4 T cells is unknown.

*Modulation of PLD activity by extracellular stimuli*

The above mentioned data suggested to us that PLD signal regulation could play a pivotal role in controlling the balance between regulatory and non-regulatory T cells. In vivo suppression of PLD signaling may promote dominance of naturally arising Treg cells under physiological conditions. Conversely, augmenting PLD signaling may promote growth of effector T cells and break the immune suppression by Treg cells. This led us to ask: Is it possible for pathogens/tumours to inhibit PLD activity (directly / indirectly through secretion of factors) to subvert the protective immune responses of the host? Alternatively, does elevated PLD activity contribute to autoimmune responses?

There have been a number of studies that indicate the modulation of PLD activity by various extracellular stimuli. These stimuli in turn mediate their effect on PLD through the help of above-mentioned cofactors of PLD signaling. Alcohol
is an exogenous factor that alters the immune phenotype of the host (Nelson and Kolls, 2002). T cells from individuals who were exposed to alcohol both chronically and acutely showed a higher frequency of T cells expressing CD25 (Cao et al., 1998; Laso et al., 1996a; Laso et al., 1996b; Sacanella et al., 1998; Santos-Perez et al., 1996). One study showed that these T cells were exclusively CD4$^+$ (Laso et al., 1996b). Moreover, the frequency of CD25$^+$ T cell remained high even 9 months after alcohol withdrawal. In another study, clear positive correlation was observed between total estimated life time dose of ethanol consumed and the percentage of PBLs expressing CD25 (Sacanella et al., 1998). Our studies suggested that these effects might be channeled through suppression of PLD signaling and increased frequency of Treg cells.

Pathogens like *Clostridium difficile* might also alter the host immune response through PLD signaling. The major virulence factors produced by *C. difficile* are Toxin A (TcdA) and Toxin B (TcdB) (Genth et al., 2008). Inside the host cell, these toxins mono-glucosylate and thereby inactivate small G proteins of the Rho sub-family (Genth et al., 2008). Since PLD is one of the downstream effector molecules of Rho, these toxins indirectly inhibit PLD activity (Schmidt et al., 1996). We propose that the functional consequence of inhibiting PLD signaling is enrichment of Treg cells. This in turn might facilitate the evasion of host immune response by *C. diff* through elimination of the effector response by non-Treg cells.

The receptor system that has been linked to PLD regulation includes G-protein coupled receptors and tyrosine kinase coupled receptors (Frohman et al.,
G-protein coupled receptors include chemokine receptors (RANTES, IL8) (Bacon et al., 1995; Bacon et al., 1998), adenosine receptors (Selvatici et al., 2006; Thibault et al., 2002), N-formyl peptide receptor (FPR) (Selvatici et al., 2006), P2Z/P2X7 nucleotide receptor (Coutinho-Silva et al., 2003) and prostaglandin (PGE2) receptors (Burelout et al., 2004). Tyrosine kinase coupled receptors include TCR, BCR, FcReRI, and FcRI (Melendez and Allen, 2002) and Insulin receptors (Farese, 1988).

These extracellular stimuli alter PLD activity in both a positive and negative manner. Antigen and chemokine receptors elevate the activity of PLD (Bacon et al., 1995; Bacon et al., 1998). FPR, which recognizes bacterial peptides, also elevates PLD activity (Selvatici et al., 2006). On the other hand, inflammation related receptors, including adenosine receptors (Selvatici et al., 2006; Thibault et al., 2002), lipoxin A (4) receptor (Levy et al., 1999) and PGE2 receptors (Burelout et al., 2004) suppress PLD activation. Interestingly, a recent report indicated that PGE2 induces enrichment of regulatory T cells (Baratelli et al., 2005).

Most of the studies indicate that PLD1 is associated with receptor induced PLD activity and this involves the role of small GTPases or PKCs whereas PLD2 may be constitutively active and is a target of negative regulation. In contrast to the activation process of PLD, the process that inactivates PLD is not well characterized. Adenosine (A2a) receptor signaling in neutrophils (Thibault et al., 2002) and A3 receptor signaling in ischemic heart (Mozzicato et al., 2004) has been shown to modulate PLD activity. PGE2 receptor has also been shown to
inhibit PLD activity. Both A2a and PGE2 stimulate adenylate cyclase and thereby increase the intracellular cAMP levels. These data indicate that downstream targets of cAMP, such as PKA may mediate suppression of PLD activity. However, no clear connection between PKA and PLD has been made at this point. Interestingly, a recent report showed that $\beta\gamma$ subunits of G protein could inhibit PLD1 activity by direct interactions (Preininger et al., 2006). Other negative regulatory factors of PLD include ceramide (Nakamura et al., 1996), presqualene diphosphate (PSDP) (Levy et al., 1999) and munc-18-1 (Lee et al., 2004).

**Adenosine and PLD signaling**

Adenosine (Ado) is a purine nucleoside formed from its precursor ATP by the action of CD39 [nucleoside triphosphate dephosphorylase (NTPD)] and CD73 (5-ectonucleotidase)(Hasko et al., 2008). Adenosine is constitutively present at low concentrations in extracellular space however metabolically stressful conditions (hypoxia, cell death) increase its levels in extracellular space drastically (Fredholm et al., 2001; Linden, 2001). Numerous studies suggest the role of Ado as an immune modulator. First, high concentrations of Ado represent a pre-eminent alarm signal indicating tissue injury to the surrounding cells. Secondly, Ado occupies its cognate receptor present on a variety of immune cells including dendritic cells (Panther et al., 2003; Schnurr et al., 2004), neutrophils (Cronstein et al., 1983) and lymphocytes and mediates immune
suppression (Borsellino et al., 2007; Hasko et al., 2008; Kobie et al., 2006; Zarek et al., 2008). A physiological response of Ado is elicited by engaging one or more of the four G-protein coupled receptors (A1, A2a, A2b and A3) (Jacobson and Gao, 2006).

Recent studies have elucidated an important role for adenosine in mediating the immunosuppressive properties of Treg cells. It was demonstrated that Treg cells express CD39/CD73 and thus hydrolyze extracellular ADP and ATP to adenosine (Deaglio et al., 2007; Kobie et al., 2006). Also, Treg cells isolated from CD39 knockout mice do not suppress the proliferation of CD4+CD25- cells, indicating the role of CD39 in modulating Treg function (Deaglio et al., 2007). Similarly, Treg cells isolated from wild type mice failed to suppress CD4+CD25- cells from A2a receptor knockout mice (Naganuma et al., 2006; Zarek et al., 2008). Numerous studies have established the immunosuppressive function of A2a receptor signaling. Indeed activation of A2a receptor on Treg cells causes upregulation of Foxp3 expression (Zarek et al., 2008). Interestingly, A2a receptor occupancy on neutrophils reduces PLD activity by limiting the recruitment of Arf, RhoA, and PKCα to membranes (Thibault et al., 2000).

The combined effects of activated adenosine A2 and A3 receptors (A2AR, A3AR) on mononuclear cells of the immune system are considered anti-inflammatory (Hasko et al., 2008; Jacobson and Gao, 2006). Activation of A3 receptors on macrophages inhibits the release of tumor necrosis factor alpha (TNF-α) and thus exerts beneficial effect in treating inflammatory disorders like
arthritis (Baharav et al., 2005). Conversely, A3 receptor activation on mast cells triggers histamine release (Salvatore et al., 2000) and thus is an important player in mediating asthma (Hasko et al., 2008). In addition, A3 receptors have been demonstrated to utilize PLD and RhoA in dictating cell functions. However, the precise function of A3 receptor in T cells has not been established.

Based on these studies, we set out to determine the effect of adenosine in modulating PLD signaling in T cells. We also tested if PLD signaling plays any role in mediating the immune suppression of adenosine.

CONCLUSION

The heterogeneity of T cell subsets has led us to appreciate the functional importance of CD4 T cells in fighting pathogens, inducing autoimmunity as well as maintaining tolerance to self. It is widely accepted that the population balance between the various subsets of CD4 T cells is central to maintaining immune homeostasis. Hence, identification of factors controlling this population balance like PLD signaling will provide novel therapeutic strategies for controlling variety of diseases ranging from autoimmunity to tumors.
CHAPTER TWO

MATERIAL AND EXPERIMENTAL METHODS

Mice

All mouse work was done in accordance with the Animal Care and Use Committee guidelines of Medical College of Georgia and Loyola University. Animals were housed in pathogen-free conditions prior to manipulations. C57BL/6 mice and RAG-1 knock out mice were obtained from Jackson laboratory. CD4 CRE mice were obtained from Taconic. P25 TCR transgenic mice was generously gifted by Dr. T. Saito (Japan) (Tamura et al., 2004). D011.10 TCR transgenic mice were housed in pathogen free facility in Medical College of Georgia (Augusta, Georgia, USA).

Generation of PLD2<sup>-/-</sup> mice

PLD2<sup>fl/fl</sup> mice were generated by selecting the PLD<sup>neo</sup> ES cell clone (Mayuko Takezaki). The floxed mouse PLD2 (PLD2<sup>fl/fl</sup>) mice line was bred with FLP transgenic mice to delete the neo transgene. Then PLD2<sup>fl/fl</sup> (minus neo) mice were bred with mice carrying CD4 cre transgene. Mice were routinely screened by genotyping of the tail DNA using PCR. Primers used for genotyping
are listed in Table I. Genomic DNA was isolated from CD4 T cells and analyzed by southern genomic blot for targeted recombination.

**Southern Blot**

Genomic DNA was isolated from CD4 T cells using standard procedures (Blin and Stafford, 1976). Ten micrograms of genomic DNA was digested with the restriction enzyme Bgl II, electrophoresed on a 0.7% agarose gel, transferred to Hybond-N and UV cross-linked according to the manufacturer’s suggestion (Amersham Pharmacia Biotech). Filters containing the immobilized DNA were incubated with 1-5\(\times\)10\(^6\) cpm/ml of radio labeled probe. 25ng cDNA-probe was labeled with \(^{32}\)P using the Prime It\(^\circledR\) II Random Primer Labeling Kit (Stratagene \#300385). The PLD2 targeting probe corresponds to a 340bp Xhol-EcoRI fragment located immediately upstream of the short arm of the targeting construct. Filters were washed at 65 °C in a solution containing 0.1X SSC and 0.1% SDS and analyzed by autoradiography.

**Adoptive transfer**

CFSE labeled (0.5uM) (donor) p25 CD4 T cells (2.5-5\(\times\)10\(^6\)) were injected intravenously into Ly5.1 (recipient) mice on day 0. Recipient mice were injected with 2.5ug of p25 peptide intravenously on day 1. Recipient mice were given intraperitoneal injections of vehicle (DMSO in PBS), AB-MECA (200ug /kg), Ethanol (2.9g/kg body weight in 20% vol/vol) in 200 ul from day 0-6. Mice were sacrificed on day 8 and spleen, mesenteric lymph nodes and all peripheral lymph
nodes (axillary, brachial, cervical, inguinal and popliteal) were harvested and intracellular staining for Foxp3 expression was performed. Proliferation function in flowjo software was used to determine number of cells in each cell division.

**RAG1 -/- mice**

CFSE (0.5uM) labeled CD4 T cells (1-5x10^6) from wild type B6 mice were intravenously transferred into recipient RAG1-/- mice. Mice were injected with ethanol or PBS intraperitoneally as described previously for 5 days. Mice were sacrificed on day 6 and splenocytes were analyzed for Foxp3 expression by flow cytometry.

**Cell isolation and culture**

Splenic CD4 T cells were isolated using either positive or negative selection kit from BD Biosciences or MACS Miltenyi Biotec as per manufacturers protocol. Cell purity was confirmed to be ≥95% by BDFACS Canto. Cells were cultured in vitro in RPMI 1640 medium supplemented with 10% v/v heat-inactivated FBS, 2mM glutamine, 5x10^-5 M 2-ME, and 100 U/mL penicillin and 100 µg/mL streptomycin (all were obtained from Thermo Scientific Hyclone).

**Treg enrichment**

1x10^6 CD4 T cells were pre-incubated with DMSO, AB-MECA (A3R agonist, SIGMA), 0.5 % ethanol (SIGMA, 200 proof) and 2 % ethanol (2ml in 24 well plate). After 16 hrs, we replaced 1.5 ml of culture supernatant with fresh medium containing 0.2ug/ml of anti-CD3 (e-bioscience), γ-irradiated splenocytes (2500 rad) (2-5x10^6 cells/well) and different treatment constituents like DMSO. We
replaced 1 ml of medium for the next 72 hrs with medium containing anti-CD3, 2ng/ml of mouse recombinant IL-2 and respective treatment constituents. We washed the cells on day 4 and cultured them with IL-2 (2ng/ml) for next 3 days. On day 7, cells were harvested, washed and counted. Cells were then stained with different fluorescent antibodies and analyzed by flow cytometry.

**Activation markers**

1×10^6 CD4 T cells were activated with anti-CD3 (0.2ug/ml) and APCs (T cell depleted γ-irradiated splenocytes (2500 rad), (2-5×10^6 cells/well) in the presence of DMSO, AB-MECA, varying concentrations of ethanol and C.difficile culture supernatants. 24 hrs later cells were harvested and analyzed by flow cytometry. In some experiments, CD4 T cells were further sorted into CD4+ CD25+ and CD4+ CD25- T cells using BDFACSria.

**CFSE labeling**

Purified CD4 T cells were labeled with 2.5μM CFSE (Invitrogen) for 10 min at 37°C in PBS. The reaction was quenched by the addition of medium containing 10% FCS and then washed twice in PBS. CFSE labeled cells were cultured for 7 days as mentioned above. In some experiments, cells were labeled with 2.5uM PKH-26 (a red fluorescent cell labeling dye, SIGMA). Cells were harvested on Day 5 after stimulation.

**Cytokine assay**

To analyze cytokine production by ELISA, CD4 T cells were stimulated with plate bound anti-CD3 (2 ug/ml) and soluble CD28 (1 ug/ml) in the presence
of DMSO or AB-MECA. Supernatants were collected at 24, 48 and 72 hrs and enzyme linked immunoassays were performed as described previously.

To detect cytokine production by intracellular staining, CD4 T cells were stimulated with plate bound anti-CD3 (2ug/ml) and soluble CD28 (1ug/ml) for 48-72 hrs. Cells were then rested for 48 hrs in the presence of IL-2. On day 5, cells were washed and 1x10^6 were restimulated with PMA (50ng/ml) and Ionomycin (1ug/ml) for 6 hrs in the presence of Golgistop (Monesin, 2uM). Cells were then harvested and stained with fluorescent antibodies and analyzed by flow cytometry.

**Th1, Th2 and Th17 skewing conditions**

For Th1 skewing conditions, CD4 T cells were stimulated as before and cultured in media supplemented with mIL-12 (10ng/ml) and anti-IL-4 (10ug/ml). After 48 hrs, medium was supplemented with additional IL-2.

For Th2 skewing conditions, CD4 T cells were stimulated as before and cultured in media supplemented with mIL-4 (10ng/ml), anti-IL-12 (10ug/ml) and anti-IFNγ (10ug/ml). After 48 hrs, medium was supplemented with additional IL-4.

Cells skewed to Th17 were supplemented with TGF-β (2ng/ml), mIL-6 (10ng/ml), anti-IL-4 (2ug/ml) and anti-IFNγ (2ug/ml). Th17 skewed cells were harvested on day3 or 4 washed and stimulated with PMA and Ionomycin and analyzed by flow cytometry. Th1 and Th2 skewed cells were harvested on day5 after stimulation and restimulated with PMA and Ionomycin.
**Induction of Treg cells**

To test for induction of Treg cells, CD4+CD25- T cells were stimulated with APCs (whole splenocytes) and anti-CD3 (0.2-0.4ug/ml). Medium was supplemented with AB-MECA or DMSO. TGF-β (5ng/ml) was added to positive control wells. IL-2 (2ng/ml) was added to all wells on day 1 and day 2. Cells were harvested on Day 5 or 6 and analyzed for Foxp3 expression by flow cytometry.

**Flow cytometry**

*Cell surface staining*

Cells in culture were harvested and washed once with FACS buffer (5% FCS and 0.1% azide in 1X PBS). \(1 \times 10^6\) cells were stained with various antibodies for 1hr at 4 degree and cells were washed three times with FACS buffer. Before staining with antibodies, cells were incubated for 5-10 mins with 2.4G2(FcR block). Purified, fluorochrome and biotin-coupled monoclonal antibodies specific for CD4 (RMA 4–4, GK1.5), CD8 (53–6.7), CD25 (PC61, 7D4), CD3 (145-2C11) and FoxP3 (FJK-16s) were purchased from eBiosciences (San Diego, CA). Thy 1.2, Ly 5.2 and \(V\beta11\) were purchased from Biolegend (San Diego, CA). Fluorescent antibodies against IL-2, IL-4, IFN-\(\gamma\) was purchased from e-bioscience and IL-17 was purchased from Biolegend.

*Intracellular cytokine staining*

\(0.5-1 \times 10^6\) cells were surface stained and then fixed for 15 mins with 4% Paraformaldehyde (Sigma) at room temperature. Cells were diluted with FACS
buffer and kept at 4 degrees overnight. The following day, cells were washed and incubated with permeabilization buffer (50mM Nacl, 5mM EDTA, 0.02% Azide, 0.5 % Triton-X pH7.5) for 15 mins followed by blocking (3% BSA in PBS). Cells were then incubated with fluorescent antibodies for 45 mins on ice. Finally, cells were washed three times in FACS buffer and analyzed by flow cytometry.

Intracellular Foxp3 staining

1x10^6 cells were surface stained with anti-CD4 and anti-CD25, fixed overnight with Fix/Perm buffer (eBioscience). After 12-16 hrs, cells were then washed twice with IX permeabilization buffer (eBioscience) and stained with anti-Foxp3 antibody in IX FACS buffer at 4°C for 1hr. Cells were washed thrice with IX FACS buffer and analyzed by flow cytometry.

All the data was acquired using FACSCanto or FACSCanto II (Becton Dickinson) at the LUMC FACS Core Facility, and data was analyzed using FlowJo analysis software (©Tree Star, Inc).

T cell proliferation assay

CD4 T cells (2x10^4 – 3x10^4) were stimulated with anti-CD3 (0.2ug/ml) and (T cell depleted splenocytes, γ-irradiated, 2500 rads) APCs in 96 well plates (200ul). Depending on the experiment, medium was supplemented with DMSO, AB-MECA, and 0.5%-2% ethanol and toxin or no-toxin supernatants from C.difficile culture. 72 hrs after stimulation cells were assayed for proliferation using CellTiter-Glo Luminescent cell viability assay (Promega). The kit uses luciferase as the detection enzyme. Luciferase enzyme requires ATP to generate
light. Since metabolically active cells produce more ATP, the light signal detected is proportional to the amount of ATP present that in turn correlates with the number of viable cells. Luminescence is measured using plate reader (Turner Biosystems) and analyzed using VERITAS software.

**ELISA**

96 well plates were coated with purified mIL-2, mIL-4 or mIFN-γ (capture antibody, 1:500-1:1000) in 0.1M NaHCO₃ buffer overnight at 4 deg. Next day, wells were washed twice in ELISA wash buffer (0.05% Tween in 1xPBS) and blocked with blocking buffer (3% BSA in PBS) at room temperature for 1 hr. Then the wells were washed again and incubated with sample supernatants (serially diluted in 1:3) overnight at 4 deg. The following day wells were washed and incubated with biotin-conjugated antibodies (mIL-2, mIL-4 or mIFN-γ) for 1-2hrs. Further, streptavidin-HRP (1:1000) was added and incubated for 30 mins at room temperature. 100ul of 3,3', 5,5'-Tetramethylbenzidine (TMB) liquid substrate (Sigma, St. Louis, MO) was added to each well after washing the plates five times with wash buffer. Upon color development, the reaction was stopped by adding 50ul of stop buffer (4 water: 1 HCl: 1 H₂SO₄) to the wells. The plates were then read at 450 nm using plate reader (Biotek, EL x 800) and the data was analyzed using KC junior software.
**Phospholipase D Assay**

Lymphocytes were harvested from P25 TCR transgenic mice and incubated overnight with (1-10μg/ml) P25 peptide. The following day cells were harvested, washed with RPMI three times to remove any residual antigen peptide. CD4 T cells were sorted and kept at room temperature until use. Cells were then suspended in 10% RPMI containing 10mM HEPES (1-5x10^6/500ul) and pretreated with DMSO or AB-MECA (50μM) for 30 mins in 37 deg water bath. Following pretreatment, CD4 T cells were stimulated with soluble biotin conjugated anti-CD3 and avidin for different time points (15-30 mins). Cells were centrifuged and resuspended in RIPA lysis buffer (10mM phosphate buffer pH 7.2,150mM NaCl, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS) containing Dnase. Cell lysates were assayed using the commercially available Amplex Red PLD assay kit (Invitrogen (Molecular Probes)). In this assay, PLD activity is monitored indirectly by using 10-acetyl-3, 7-dihydrophenoxasine, called the Amplex Red reagent. PLD cleaves the L-α-phosphatidylcholine (PC) substrate to yield choline and phosphatidic acid. Choline is oxidized by choline oxidase (provided in the kit) (choline oxidase from an Alcaligenes sp.) to betaine and H₂O₂. Finally, H₂O₂ in the presence of horseradish peroxidase reacts with the Amplex Red reagent to generate the highly fluorescent product resorufin (excitation and emission maxima, ~563 and 587 nm, respectively), an H₂O₂ fluorogenic sonde. As per manufacturers manual, this kit is specific for membrane PLD enzymes, with optimal activity at nearly neutral pH (pH 8). Purified PLD from *Streptomyces chromofuscus* and 10 μM H₂O₂ served as
positive controls. The PLD assay kit can detect PLD levels as low as 10 mU/ml. One unit of PLD is defined as the amount of enzyme that liberates 1.0 μmol of choline from PC per minute at pH 8.0 at 30°C. Cell lysates were incubated with the reagents provided in the kit for 1 hr at 37 deg and the fluorescence intensity was read using fluorometer (BMG Labtech).

**cAMP Assay**

CD4 T cells from P25 transgenic mice were sorted and stimulated as previously described in PLD assay. In the case of cAMP assay, CD4 T cells were stimulated for 5 mins. Further, to assess cytosolic cAMP concentrations, T cells were washed three times in ice-cold PBS, lysed in 0.1 N HCl (10^7/ml) for 30 mins. Supernatants were assayed using the acetylated version of cAMP-specific ELISA kit (Correlate EIA Direct Cyclic AMP Enzyme Immunoassay kit; Assay Design).

**Western Blots**

In general, cell pellets were suspended in 1xSDS sample buffer (0.5-1x10^6 cells in 25 ul) and boiled for 5-7 mins with intermediate vortexing. The proteins were then separated on 8% polyacrylamide gels. Proteins were then transferred to nitrocellulose membrane (Millipore) overnight in transfer buffer. The following day membrane was blocked with 5% milk in TBST for 1 hr. Blots were then washed three times in 1X TBST and incubated with the primary antibody overnight at 4 deg. Next day, blots were washed and incubated with secondary antibody for 1 hr at room temperature. ECL substrate (GE healthcare) was
added to the blot for 1 min before developing.

To determine adenosine A3 receptor expression, CD4+CD25+ and CD4+CD25- T cells were sorted using MACS column. Cells were stimulated with plate bound anti-CD3 (2ug/ml) and anti-CD28 (2ug/ml) for 72 hrs. Both unstimulated and stimulated cells were harvested, washed in 1xPBS and pelleted. Cells pellets were processed as described above. Adenosine A3 receptor antibody (Santa Cruz Biotechnology, 1:500) was diluted in antibody dilution buffer (0.2% BSA, 0.1% azide in 1X TBST). Anti-rabbit HRP (Cell Signaling, 1:5000) was used as secondary antibody. The membrane was stripped with buffer containing 2-ME and probed with β-actin (Sigma) antibody.

For phospho PKA analysis, splenocytes from P25 TCR transgenic mice were stimulated as described in PLD assay. Sorted CD4 T cells were incubated with AB-MECA or DMSO for 30 mins before stimulation. T cells were stimulated with biotin anti-CD3 (2C11, 10ug/ml) for 1 min followed by addition of 2.5ug/ml of avidin for 5 mins at 37 deg. Cells were then pelleted and processed as described above. Phospho-PKA substrate antibody (Cell Signaling, 1:1000) was diluted in antibody dilution buffer. Anti-rabbit HRP (1:5000) was used as secondary antibody.

**Generation of PLD YFP fusion constructs**

The plasmid encoding human PLD1 and mouse PLD2 were kindly provided by Dr. Michael Frohman. DNA encoding full length PLD1 and PLD2 were sub-cloned into pMEYFPzeo-C1 vector by Dr. Nagendra Singh. Deletion mutants of
PLD1 and PLD2 in pMEYFPzeo-C1 were constructed using convenient restriction sites or polymerase chain reaction-based strategies and were sequenced at all junctions to ensure that the reading frame was maintained. Plasmids were sequenced to confirm the intended mutation. The mutant constructs of both PLD1 and PLD2 were confirmed to be expressed at similar levels and at the expected sizes (Fig 5 and 6). Primers used for the generation of the constructs are listed in Table 1.

**Confocal microscopy**

Jurkat T antigen cells were transfected with the PLD YFP fusion constructs by electroporation. Live cells were isolated (16-18 hrs after transfection,) using lymphocyte separation medium (Mediatech, Inc). Approximately 10⁵ cells in 50μl medium were spread on 0.1% lysine coated glass slides. The cells were left to adhere to the glass slide for 5 mins and the excess medium was drained. Cells were fixed with 4% Paraformaldehyde for 10-20 mins and washed with 1x PBS for three times. For nuclear staining, DAPI (Molecular probes) or Propidium iodide was added to cells for 5 mins and washed with 1x PBS three times. One drop of prolong antifade (Molecular probes) was added on top of the cells and mounted with cover slip. Slides were left overnight at room temperature in the dark and analyzed using Zeiss® LSM-510 laser scanning microscope at the LUMC and MCG core imaging facility. LSM image examiner software was used for image acquisition and analysis.
Fig 5. Western blot analysis of PLD1 deletion mutant constructs. Full length and deletion mutant constructs of hPLD1b were transfected into Jurkat T antigen cells by electroporation. The following day live cells were isolated and lysed in 1xSDS sample buffer. A) Proteins were separated on 8% polyacrylamide gel and probed with anti-GFP antibody. B) Proteins were separated on 12% polyacrylamide gel and probed with anti-GFP antibody. Protein markers sizes (kDa) are indicated on the left side of the gel. Protein of expected size was obtained for the different constructs. Vector only: 30 kDa, Full length PLD1: 150 kDa, loop deleted PLD1: 130 kDa, PH+PX+C: 75 kDa, PH + PX only: 62 kDa, N-terminal deletion: 100 kDa, C-terminal only: 40 kDa.
**Fig 6. Western blot analysis of PLD2 deletion mutant constructs.** Full length and deletion mutant constructs of mPLD2 were transfected into Jurkat T antigen cells by electroporation. The following day live cells were isolated and lysed in 1x SDS sample buffer. Proteins were separated on 8% polyacrylamide gel and probed with anti-GFP antibody. Protein markers sizes (kDa) are indicated on the left side of the gel. Protein of expected size was obtained for the different constructs. Full length PLD2: 125 kDa, PH+PX+C: 75 kDa, C-terminal only: 40 kDa.
In contrast to the diffuse cytoplasm and nuclear localization of YFP-vector alone, PLD1YFP and PLD2YFP have peri-nuclear and plasma membrane localization respectively (Fig 7).
Fig 7. Confocal microscopic analysis of YFP fusion constructs. YFP fusion (green) construct of vector alone, full length PLD1 and PLD2 were transfected in Jurkat T antigen cell line. The sub cellular localization of these constructs was determined using confocal microscopy.
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**PLD2 (-/-) mice genotyping primers**

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CHAPTER THREE
EXPERIMENTAL RESULTS

REGULATION OF PHOSPHOLIPASE D SIGNALING BY EXOGENOUS FACTORS

Antigen stimulation of lymphocytes results in increased PLD activity. Primary alcohol is a well-known inhibitor of PLD signaling. Previous studies have documented the inhibition of T cell activation in the presence of primary alcohols in vitro. Singh et al (Singh et al., 2006) analyzed the effect of inhibiting PLD signaling in different subsets of CD4 T cells using 0.3% 1-butanol. Inhibition of PLD signaling selectively blocked the proliferation of CD4+CD25- (effector) T cells and led to the enrichment of Foxp3+ regulatory T cells. This study highlighted the differential requirement for PLD signaling among effector versus regulatory T cell subsets.

We then sought to determine the role of PLD signaling in dictating the immune response of the host. We hypothesized that in vivo suppression of PLD signaling may promote immune suppression through dominance of Treg cells under physiological/pathological conditions. Conversely, enhancement of PLD signaling under certain conditions might promote dominance of effector T cells.
and thus promote immune reactivity. For my studies, I focused on identifying various exogenous and endogenous factors that alter the immune status of the host through modulating PLD signaling.

**Ethanol and CD4 T cell population dynamics**

Based on the effect of 1-butanol, we predicted that another 1-alcohol, ethanol would also inhibit PLD signaling. Ethanol is widely consumed for recreational purposes and is considered as an immunosuppressive agent (Cook, 1998; Nelson and Kolls, 2002; Zhang and Meadows, 2005). Excessive alcohol intake has been associated with increased incidence of infection, cancer and autoimmunity (Song et al., 2002). Previous studies in mice indicate that alcohol administration for short duration causes reduced cell numbers of immune cells like T cell, B cell and NK cells and concomitant reduction in the cellularity of spleen and thymus (Chadha et al., 1991). However, the effect of alcohol on CD4 T cells in specific and the mode of action were not delineated.

To determine if ethanol can enrich for Treg cells, we treated murine CD4 T cells with varying concentrations of ethanol in the presence of anti-CD3 stimulation in vitro for 4 days. After 72 hrs of stimulation, cells were washed and cultured for 3 days in the presence of IL-2. Cells were harvested and stained for regulatory T cell markers, Foxp3 and CTLA4. When T cells were stimulated by anti-CD3 antibody in the presence of low concentrations of alcohol (0.5%), no enrichment of Foxp3+ Treg cells was observed and the frequency of Foxp3+
Figure 8. Effect of ethanol on CD4 T cells in vitro. Total CD4+ T cells were cultured with anti-CD3 plus antigen presenting cells in medium containing increasing concentrations (0.5% - 2%) ethanol for 3 days. After 3 days, cells were placed in medium containing IL-2 (20ng/ml) for 4 days. Seven days after the start of culture, cells were harvested and analyzed by flow cytometry for Foxp3 and CTLA4 expression. Representative data from 3 independent experiments is shown.
cells was similar to medium-treated samples. When higher doses of ethanol were used (1.0%, 2.0%), however, we observed a clear dose dependent increase in Treg frequency (Fig 8) among CD4 T cells and observed 4.9 and 16.5% of CD4 T cells becoming Foxp3+, respectively. Similar to 1-butanol, CD4 T cells expanded in the presence of 2% ethanol contain higher percentages of Foxp3+ cells.

The experiment shown above used stimulation of T cells by anti-CD3 antibodies. To determine if ethanol can enrich for antigen specific Treg cells, we next tested the effect of ethanol on antigen-induced T cell proliferation using CD4 T cells from P25 TCR transgenic mice. The P25 mouse carries a transgene encoding the TCR specific for peptide-25 of Mycobacterium tuberculosis (Tamura et al., 2004). T cells were stimulated with the antigen peptide in the presence of irradiated antigen presenting cells for 3 days followed by culture in the presence of IL-2 for 4 days. Similar to anti-CD3 stimulated cultures, the frequency of Foxp3+ cells in 2% ethanol treated cultures were 22% as against 0.6% in both medium and 0.5% ethanol treated samples (Fig 9).

The increase in percentage of Foxp3+ cells could be due to increased proliferation of Foxp3+ cells in 2% ethanol treated cultures or due to decreased proliferation of Foxp3- T cells. To test for these possibilities, we labeled CD4 T cells with CFSE and measured the dilution of CFSE to monitor proliferation. Compared to 0.5% ethanol, very few Foxp3- T cells proliferated in 2% ethanol treated cultures as seen from the CFSE dilution plots (Fig 10).
Figure 9. Effect of ethanol on CD4 T cells in the presence of antigenic stimulation in vitro. Total CD4+ T cells from P25 TCR transgenic mice were cultured with P25 peptide (0.1μg/ml) plus antigen presenting cells in medium containing different percentages of ethanol for 3 days. After 3 days, cells were placed in medium containing IL-2 (20ng/ml) for 4 days. Seven days after the start of culture, cells were harvested and analyzed by flow cytometry for Foxp3 and CD25 expression. Representative data from 2 independent experiments is shown.
Figure 10. Proliferation kinetics of CFSE labeled CD4 T cells in the presence of ethanol. Total CD4+ T cells were labeled with CFSE (2.5ug/ml) and cultured with anti-CD3 (0.2ug/ml) and APCs in medium containing 0.5% ethanol (solid line) or 2% ethanol (dotted line) for 3 days. Cells were placed in medium containing IL-2 (20ng/ml) for next 4 days. On day 7 after stimulation, cells were harvested and analyzed by flow cytometry for Foxp3 and CFSE expression. A) Line graph depicting number of Foxp3- T cells in different generations B) Line graph depicting number of Foxp3+ T cells in different generations.
As a result, the total Foxp3- T cell numbers recovered from 0.5% ethanol treated cultures were 8 times more than that recovered from 2% ethanol cultures. In contrast, Foxp3+ cells from samples treated with 2.0% ethanol showed no difference in CFSE levels between 0.5% and 2% ethanol treated samples. Together, the data demonstrate that ethanol causes increased frequency of Foxp3+ cells by decreasing the proliferation of Foxp3- T cells.

Epidemiological studies showed that alcohol abusers are predisposed to more frequent and more severe infections (Cook, 1998). In particular, increased incidence of infections and enhanced severity of disease such as pneumonia and tuberculosis were associated with chronic consumption of alcohol (Cortese et al., 1992; Esposito, 1984; Fernandez-Sola et al., 1995; Friedman et al., 1987; Moss et al., 1996; Ruiz et al., 1999; Winterbauer et al., 1969). The incidence of chronic Hepatitis C Virus (HCV) infection is also increased in alcohol abusers (Degos, 1999; Harris et al., 2001; Khan and Yatsuhashi, 2000; Lima et al., 2000; Schiff, 1999). Also, studies on rodents show that animals are more vulnerable to infection after chronic or acute exposure to alcohol. These observations can be interpreted based on our in vitro studies in the following manner: Consistent intake of alcohol might lead to selective elimination of effector T cells and consequent enrichment of regulatory T cells. This in turn might increase the susceptibility of the host to various infections. Also, antigenic exposure in the presence of alcohol might inhibit the proliferation of antigen specific effector T cells thus compromising the overall health of the host.
We tested for these possibilities in vivo in mice using two different experimental set-ups. First, the effect of ethanol on CD4 T cell subsets in mice in the absence of antigen was determined. Wild type C57BL/6 mice were injected with either PBS or 20% ethanol intraperitoneally (i.p) for 7 days. Mice were then sacrificed and their spleen and mesenteric lymph nodes were harvested and stained for regulatory T cell markers. As expected, spleen of mice treated with ethanol contained 23.56% of Foxp3+ cells compared to 17.5% in PBS treated mice (Fig 11a). This increase in frequency of Foxp3+ cells was due to significant decrease in the total Foxp3- CD4 T cell numbers; no significant change in Foxp3+ T cell numbers was observed (Fig 11b). These data confirmed our prediction that ethanol selectively eliminates Foxp3- T cells even in the absence of antigenic proliferation.

Secondly, we determined if ethanol inhibits antigen-induced effector T cell proliferation while allowing Treg cells to proliferate in vivo, as we observed in vitro. To test the effect of ethanol in vivo, we used the protocol that has been previously described by others. Briefly, mice were injected with 2.9g/kg body weight ethanol in 20% vol/vol saline intraperitoneally (i.p) (Meadows et al., 1989; Obradovic and Meadows, 2002; Song et al., 2002). This dose of ethanol injection leads to blood ethanol level of 300mg/dl in 30 minutes. We adoptively transferred CFSE labeled CD4 T cells from P25 TCR transgenic mice into C57.BL/6 CD45.1 recipient mice intravenously. These recipient mice express CD45.1 isoform, which is distinguishable from the CD45.2 isoform expressed by the donor mice.
Figure 11. Flow cytometry analysis and quantification of cell numbers of CD4 T cells in mice injected with ethanol. Wild type mice were injected intraperitoneally with either PBS or 20% ethanol for 7 days. On day 7, mice were sacrificed and their spleen cells were stained for Foxp3, CD4 and CTLA4. A) Representative FACS plots of spleen CD4 T cells from PBS and ethanol treated mice. B) Bar graphs of total Foxp3- T cell numbers in spleen of PBS (white bars) and ethanol (black bars) treated mice. C) Bar graphs total Foxp3+ T cell numbers in spleen of PBS (white bars) and ethanol (black bars) treated mice.
A) 

No antigen | PBS | Ethanol

Foxp3

CFSE

B) 

CONTROL

ETHANOL

Total cell numbers

 GENERATIONS

C) 

CTRL

ETHANOL

Total cell numbers

 GENERATIONS
Figure 12. Effect of ethanol on the proliferation kinetics of Foxp3 positive and Foxp3 negative CD4 T cells in vivo in the presence of antigen. CFSE labeled CD4 T cells from P25 TCR transgenic (Ly5.2) mice were intravenously transferred to Ly5.1 recipient mice. 24 hrs later, the recipients were challenged with 2.5ug of P25 peptide along with either PBS (control) or 20% ethanol (i.p) for a period of 7 days. On day 8, mice were sacrificed and all their peripheral lymph nodes (axial, cervical, inguinal, popliteal) were isolated and pooled for analysis by flow cytometry. A) Representative FACS plots showing CFSE dilution in lymphocytes of control and ethanol treated mice. B) Line graphs depicting the number of foxp3- T cells in each generation of control (dotted lines) and ethanol treated (solid lines) mice. C) Line graphs depicting the number of foxp3+ T cells in each generation of control (dotted lines) and ethanol treated (solid lines) mice. Data is representative of 2 independent experiments.
One day after injection of T cells the recipient mice were injected with the antigen peptide. Immediately after injection of peptide, mice received ethanol injection as described above. Mice were administered ethanol daily for 7 days. On day 8, mice were euthanized and spleen, mesenteric and peripheral lymph nodes (inguinal, brachial, axial, popliteal, cervical) were harvested. The state of proliferation of adoptively transferred T cells was determined by analyzing the CFSE level of CD45.2+ T cells (Fig. 12a). T cells of mice that received no antigen showed no proliferation after 7 days while T cells from mice that received antigen peptide in PBS showed significant level of proliferation in both Foxp3+ and Foxp3- populations. The frequency of Foxp3+ T cells in PBS treated mice was 3.5%. Mice injected with ethanol showed a marked reduction in proliferation of Foxp3- T cells and the frequency of Foxp3+ T cells was 9.75%. The majority of Foxp3- T cells in control mice were in between generation 3 to generation 6 (i.e. most of these cells underwent 3 cell divisions)(Fig 12b). However, there was no clear peak in the cell division of Foxp3- T cells of ethanol treated mice suggesting that majority of cells did not proliferate effectively. In stark contrast, the level of cell division of Foxp3+ cells was comparable between ethanol treated and PBS treated mice (Fig 12c). As a consequence, the total cell numbers that were obtained from ethanol treated and untreated mice were comparable for Foxp3+ T cells but the cell numbers was significantly lower in ethanol treated animals for Foxp3- cells. Together, the data demonstrate that ethanol does block antigen induced Foxp3- effector T cell proliferation while allowing Treg cells to expand in vivo.
Figure 13. Effect of ethanol on homeostatic proliferation of CD4 T cells. CD4 T cells from wild type mice were intravenously transferred into RAG1 KO mice along with intraperitoneal injections of either PBS or 20% ethanol for 5 days. On day 6, mice were sacrificed and their spleen cells were stained for Foxp3 and CD4 T cells. A) Representative FACS plots of PBS and two ethanol treated mice showing CFSE dilution in spleen CD4 T cells. B) Bar graphs of total cell numbers of CD4 T cells in the spleen. C) Bar graphs depicting the number of foxp3- T cells in each of control (white bars) and ethanol treated mice (filled bars). D) Bar graphs depicting the number of foxp3+ T cells in each of control (white bars) and ethanol treated mice (filled bars).
Zhang et al reported that chronic alcohol consumption (2 weeks-6 months) results in T cell lymphopenia, which in turn induces homeostatic proliferation (Zhang and Meadows, 2005). Since we also observed similar reduction in cell numbers in mice injected with ethanol for 1 week (Fig 11b), we investigated whether ethanol affects homeostatic proliferation. We tested the primary effect of ethanol on the proliferation of CD4 T cells under well-established homeostatic condition like RAG1 KO mice. We adoptively transferred CFSE labeled CD4 T cells from wild type mice into RAG-1 knock out mice intravenously followed by ethanol injection for 5 days. Under lymphopenic conditions, T cells are stimulated by self-peptide and self-MHC (Zhang and Meadows, 2005). We monitored proliferation of T cells based on dilution of CFSE. As seen in (Fig 13b), the total CD4 T cell counts in ethanol injected mice were 5 times lower than PBS injected mice. Unlike antigen-induced proliferation of CD4 T cells, we did not observe selective decrease in Foxp3- T cells alone. Both Foxp3- and Foxp3+ T cells counts were reduced by injection of ethanol (Fig 13 c). Thus, the data show that in acute phase of homeostatic expansion, ethanol inhibits proliferation of both Foxp3- and Foxp3+ CD4 T cells. Further experiments could be aimed at looking for specific markers of homeostatic proliferation such as expression of CD44 and lack of CD69.
Effect of alcohol ingestion on the balance between Foxp3+ and Foxp3- CD4 T cells

The data indicate that continuous and high dose exposure of T cells to alcohol may induce a relative increase in the frequency of Foxp3+ T cells. Despite the minute presence of endogenous ethanol, alcohol consumption by humans is the major source of ethanol in the human body (Antoshechkin, 2001). To study the effects of in vivo ethanol exposure on CD4 T cells, we fed mice with liquid diet containing alcohol. DO11.10 TCR (T cell receptor) transgenic mice (receptor specific for ovalbumin (OVA) plus I-Ad) were fed an alcohol-containing nutrient-supplemented liquid diet (Bio-Serv, NJ) (ethanol concentration was gradually increased from 1% to 6.3% w/v every 2 days) or calorie matched control diet for 10 days (sex-matched 12-week-old male mice, 6 mice per group). Mice were euthanized on day 10, and mucosal CD4 T cells were analyzed for expression of Foxp3 by flow cytometry. CD4 T cells from the mesenteric lymph nodes of alcohol-fed mice showed a moderate but clear increase in Foxp3+ CTLA4+ T cells compared to the control group (Fig 14a). Average frequency of Foxp3+ CD4 T cells increased significantly compared to control diet fed animals (Fig 14b). These results show that continuous presence of ethanol in the diet causes relative increase of Foxp3+ T cell in mucosal associated lymphoid organs and suggest that continuous alcohol consumption by humans might lead to selective enrichment of Treg cells and immune suppression leading to heightened susceptibility to opportunistic infections.
Figure 14. Effect of alcohol containing diet on mice CD4 T cells. Mice were fed on liquid diet containing water or varying concentration of ethanol for 10 days. Mice were sacrificed on day 10 and their mesenteric lymph node was stained for Foxp3 and CTLA4. A) Representative FACS plots of mesenteric lymph node of two control and two alcohol fed mice. B) Data from 2 different experiments were pooled and the percentage of Foxp3 positive cells in control versus alcohol fed mice is shown as a graph.
Clostridium difficile toxin: Hijacking the immune system through PLD

Clostridium difficile (C. diff) is a spore forming, anaerobic, gram-positive bacterium (Genth et al., 2008). Its role in the pathogenesis of pseudomembranous colitis is well established. Recently, it has emerged as the leading cause of nosocomial diarrhea in patients (Genth et al., 2008). Bacterial pathogens interact with the mammalian cells through the production of protein toxins (Fiorentini et al., 2003). These toxins serve as virulence factors and aid in establishing their niche in the host. The major virulence factors produced by C. difficile are Toxin A and Toxin B. Following cell entry, these toxins mono-glucosylate and thereby inactivate lower molecular mass GTP binding proteins of the Rho sub family (Genth et al., 2008). As mentioned previously, PLD is one of the downstream effector molecules of Rho. As a consequence, C. difficile toxins are considered inhibitors of PLD signaling.

Thus, we hypothesized that C. difficile might evade the host T cell effector response by inhibiting PLD signaling. If these toxins inhibit PLD signaling in T cells as well, then we expect to see inhibition of effector T cell proliferation in the presence of these toxins. To test the effect of C. difficile toxins on T cell proliferation, PKH-26 (analog of CFSE) labeled CD4 T cells were stimulated with anti-CD3 in the presence of varying amounts of supernatants from toxigenic and non-toxigenic strains of C. difficile (C. difficile supernatants were kindly provided by Michelle Merrigan). As seen in (Fig 15a,b), following TCR stimulation 50% of both Foxp3+ and Foxp3- T cells had undergone one cell division.
Fig 15. Analysis of proliferation kinetics of Foxp3+ and Foxp3- T cells in the presence of *C. difficile* toxin supernatants. CD4 T cells were labeled with 5μM PKH-26 and stimulated with anti-CD3 (0.2μg/ml) and APCs. Varying amounts of supernatants from toxigenic (Tcd) or non-toxigenic (non-Tcd) (0.05%-0.25%) strains of *C. difficile* were added to the culture medium. 72 hrs after stimulation, cell were harvested and stained with anti-Foxp3 antibody. A) Histograms comparing the proliferation of Foxp3- T cells (top row) and Foxp3+ T cells (bottom row) between medium (left side) and 0.15% of *C. difficile* supernatants (right side) treated samples. Left panels: blue line (stimulated sample) and red line (unstimulated sample). Right side: blue line (supernatant from toxigenic C.diff strain) and red line (supernatant from non-toxigenic *C. difficile* strain) B) Graph showing percentage of growth inhibition between samples containing increasing amounts of *C. difficile* culture supernatants. C) Bar graph showing percentage of growth inhibition in Foxp3+ and Foxp3- T cells in the presence of purified TcdB (μg/ml).
In comparison, the Foxp3- T cell proliferation was decreased substantially when the concentration of toxin containing supernatants was increased. Also, we observed some inhibition in the proliferation of Foxp3+ T cells at higher concentrations of the toxin supernatants (Fig 15b). Since we used supernatants from *C. difficile* cultures as the source of toxin, it is possible that components other than toxins might exhibit non-specific effects at high concentrations. Further, to confirm that toxins specifically mediate the effect on T cell proliferation, we performed this experiment with purified Toxin B. Purified toxin B (1ug/ml) inhibited the proliferation of Foxp3- T cells by 60% while inhibition of proliferation of Foxp3+ T cells was 10% (Fig 15c). These results suggest that *C. difficile* toxin B (TcdB) through inhibiting PLD signaling blocks effector T cell proliferation significantly.

Why does inhibiting PLD signaling selectively affect the proliferation of Foxp3- (effector) T cells? In our studies with 1-butanol, we showed that PLD signal inhibition abrogates activation induced expression of CD25 on Foxp3- (effector) T cells. In contrast, Foxp3+ Treg cells express CD25 constitutively. CD25 is IL-2 receptor alpha chain, up regulated on effector T cells following their activation. IL-2 is an important survival/proliferation cytokine for activated T cells. To test the effect of *C. difficile* toxin on the expression of CD25, CD4 T cells were stimulated with anti-CD3 both in the presence and absence of toxin containing supernatants. CD25 expression on these samples was determined 24 hrs after stimulation. Stimulation of T cells resulted in significant up regulation of CD25 and CD69 in medium or non-toxigenic supernatant containing samples (Fig 16).
Figure 16. Flow cytometric analysis of activation markers expressed by CD4 T cells following antigenic stimulation in the presence of C. difficile supernatants. CD4 T cells were stimulated with anti-CD3 and APCs. Equal amounts (0.15%) of culture supernatants from either toxigenic or non-toxigenic C. difficile strains were added. 24 hrs after stimulation, cells were harvested, stained for activation markers, CD25 and CD69 and analyzed by flow cytometry.
In contrast, the expression of CD25 was not increased in samples treated with toxin containing supernatants. This result is in agreement with our previous finding that inhibition of PLD signal blocks CD25 up regulation. Future experiments can be performed to determine if _C. difficile_ toxin B specifically inhibits CD25 expression on Foxp3- T cells.

Based on our data on alcohol diet fed mice, we predicted that mice exposed to _C. difficile_ toxin/spores would have increased frequency of Treg cells. To test this possibility, we analyzed the frequency of Foxp3+ T cells in mice infected with _C. difficile_ spores. Treatment with antibiotics disrupts the normal commensal microbiota in the host intestine and is often associated with increased incidence of _Clostridium Difficile_- associated diarrhoea (CDAD) (Giel et al., 2010; Vollaard and Clasener, 1994). Based on these studies, mice were treated with various antibiotics prior to introduction of _C. difficile_ spores to induce CDAD. Mice were treated with antibiotics and infected with _C. difficile_ spores by Pehga Mohseni. Compared to wild type mice, mesenteric lymph nodes of mice colonized with _C. difficile_ had higher percentages of Foxp3+ T cells (Fig 17 a). As expected, this increase in Foxp3+ percentage was due to concomitant decrease in Foxp3- T cell numbers (Fig 17b). However, a potential caveat of this experiment is the absence of mice injected with spores derived from non-toxigenic strain of _C. difficile_ as a negative control. It is possible that the stringent treatment with antibiotics might have contributed to the increased frequency of Treg cells. Future experiments could be aimed at comparing mice colonized with spores from both toxigenic and non-toxigenic strains.
Fig 17. Flow cytometric analysis of Foxp3+ T cell percentage in mice infected with *C. difficile* spores. C57BL/6 mice were treated with broad-spectrum antibiotics for 72 hrs. For the next 48 hrs, these mice were fed normal drinking water and injected intraperitonealy with clindamycin. 24 hrs later, these mice were given (50,000 CFU) *C. difficile* spores by oral gavage. 72 hrs later these mice were sacrificed and their mesenteric lymph nodes were harvested and stained using anti-CD4 and Foxp3 antibodies. A) FACS plots from wild type
mice (WT) and two different mice infected with *C. difficile* spores showing the percentage of CD4 T cells expressing Foxp3. B) Bar graph of Foxp3- T cell numbers in wild type and *C. difficile* spores-infected mice. C) Bar graph of Foxp3+ T cell numbers in wild type and *C. difficile* spores-infected mice.
Nevertheless, based on evidence from other models of PLD signal inhibition, it is tempting to speculate that exposure to *C. difficile* toxins might lead to preferential enrichment of Treg cells *in vivo*.

**REGULATION OF PHOSPHOLIPASE D SIGNALING BY NATURALLY PRESENT ENDOGENOUS FACTORS**

Adenosine (Ado) is an endogenous molecule that mediates a wide spectrum of biologic effects by engaging 4 kinds of cell surface receptors: A1, A2a, A2b and A3. Ado is found in very high concentrations under hypoxic conditions like tissues surrounding inflammatory sites and tumors. Notably, these Ado rich environments have an immune suppressive phenotype. For example, increased frequency of Treg cells has been reported in most human solid tumors (Beyer and Schultze, 2006). Several reports highlight the immune modulatory effects of Ado acting through A2a receptor on T cells. However, there is little experimental evidence for a role of A3 receptors mediating effects of Ado in T cells. Interestingly, both A2a and A3 receptors have been previously reported to modulate cell functions through regulating PLD activity. We hypothesized that one mechanism by which adenosine can decrease immune responsiveness is through inhibiting PLD signaling.

TCR stimulation has been shown to modulate the expression of adenosine receptors in mice (Lappas et al., 2005) and humans (Gessi et al., 2004b). To understand the role of adenosine A3 receptor signaling on T cell activation, we investigated the kinetics of A3 receptor expression on CD4+CD25+ regulatory T
cells (Treg cells) and CD4+CD25+ T cells (non-Treg cells) under resting and activated conditions. We purified CD4+CD25- and CD4+CD25+ T cells from C57.BL/6 mouse splenocytes and stimulated them with anti-CD3 and anti-CD28 antibodies. Total cell lysates from each group of T cells were separated by SDS-PAGE and analyzed by Western blot for expression of the A3 receptor. Under resting conditions, freshly isolated CD4+CD25- T cells did not express detectable level of adenosine A3 receptor while CD4+CD25+ T cells expressed barely detectable levels of the receptor. However, following stimulation both CD4+CD25+ and CD4+CD25- T cells express comparable levels of adenosine A3 receptor (Fig 18).

We then examined the functional relevance of A3 receptor expression on CD4 T cells using adenosine A3 receptor selective agonist, AB-MECA. We stimulated splenic CD4 T cells with anti-CD3 antibody plus irradiated T-depleted splenocytes (APCs) in the presence or absence of AB-MECA. We first examined the effect of AB-MECA on the expression of early activation markers on T cells namely CD69 and CD25 (IL-2 receptor α chain) following 24 hrs of stimulation. Under these conditions, 65% of stimulated T cells expressed both CD25 and CD69 whereas CD69 and CD25 alone were expressed by 5% and 13% respectively (Fig 19a). In contrast, in cultures treated with AB-MECA, the frequency of CD69+CD25+ T cells decreased to 40% and CD25 alone increased to 21% and we observed little effect on CD25+CD69- T cells as the frequency remained at 5%. Since naturally occurring regulatory T cells (nTreg cells) constitutively express CD25 and non-Treg (CD4+CD425-) T cells express CD25
Fig 18. Western blot analysis of adenosine A3 receptor expression on CD4 T cells. Treg cells (CD4+CD25+) and non-Treg (CD4+CD25-) T cells were sorted from wild type mice by flow cytometry. Lysates of unstimulated versus stimulated T cells were separated on 8% polyacrylamide gel, transferred onto nitrocellulose membrane and blotted with antibody against A3 receptor (66kDa)(Top blot). Molecular weights are indicated on the left side of the blot. The membrane was stripped and probed with antibody against β-actin as loading control (bottom row).
Fig 19. Analysis of activation markers on T cells following antigenic stimulation in the presence of A3 receptor signaling. CD4 T cells or CD4+CD25- T cells were stimulated with anti-CD3 (0.2ug/ml) and T cell depleted splenocytes (APCs) for 24 hrs in the presence of DMSO or AB-MECA. T cells were stained with anti-CD25, anti-CD69 and analyzed by flow cytometry. A) Dot plots of CD4 T cells expressing CD25 and CD69. B) Histograms of CD4+ CD25- T cells expressing CD25 after TCR stimulation. Shaded peaks represent unstimulated controls and solid black lines denote activation induced up-regulation of CD25.
only upon activation, we sorted Treg cells and non-Treg cells and determined the expression of CD25. 70% of non-Treg cells in control cultures were CD25 positive in comparison to 45% in AB-MECA treated cultures (Fig 19 b). These data collectively indicate that AB-MECA inhibits the expression of CD25 on non-Treg cells and has no effect on Treg cells as they constitutively express CD25.

Next, we examined the effect of AB-MECA on CD4 T cell proliferation at 72 hrs post stimulation (Fig 20a). In the presence of AB-MECA, there was approximately 3-fold reduction in the proliferation of CD4 T cells. A similar inhibitory effect of AB-MECA was observed even in the presence of exogenous IL-2 consistent with the reduced expression of CD25 (IL-2 receptor α chain) (Fig 20b). Also, we measured cytokine secretion by CD4 T cells both in the presence and absence of AB-MECA. For this experiment, purified CD4 T cells were stimulated with plate-bound anti-CD3 antibody plus soluble anti-CD28 antibody. After 24~72 hours of stimulation, culture supernatant was collected and analyzed by ELISA (Fig 20c). Stimulation of CD4 T cells under these conditions induced substantial amount of IL-2, IFN-γ, and IL-4 and the level of cytokines in the supernatant continued to increase for 72 hours. When AB-MECA was present, the initial 24 hours of cytokine production was not significantly affected. However, the level of cytokine production in AB-MECA treated samples showed profound reduction compared to the control samples at later time points (48 and 72 hrs). Among the cytokines examined, IFNγ and IL-4 production were severely reduced, potentially due to inhibition of proliferation of activated T cells.
A) (10^3)

- **DMSO**
- **AB-MECA**

\( p = 0.02265 \)

\( p = 0.04 \)

B) IL-2

- **DMSO**
- **AB-MECA**

IFN-\( \gamma \)

O.D vs HRS

0 24 48 72 HRS
Fig 20. Comparison of proliferation and cytokine secretion profile of DMSO and AB-MECA treated CD4 T cells. A) CD4 T cells were stimulated anti-CD3 (0.2ug/ml) and APCs in the presence of DMSO or AB-MECA (50uM) for 72 hrs. Cells were harvested and proliferation was measured using cell titer glo kit (Molecular probes). (A) in the absence of IL-2 (B) in the presence of IL-2 (20ng/ml) C) CD4 T cells were stimulated with plate bound anti-CD3 (2ug/ml) and anti-CD28 (1ug/ml). Supernatants were collected at different time points and analyzed using ELISA.
Adenosine and CD4 T cell population dynamics

In addition to determining the effect of A3 receptor signaling on the effector functions of CD4 T cells, we investigated the effect of A3 receptor signaling on CD4 T population dynamics. Based on the previous observation of inhibition of CD25 expression on non-Treg cells by AB-MECA, we predicted that culture of CD4 T cells with exogenous IL-2 and AB-MECA would cause preferential enrichment of Treg cells. To test this hypothesis, CD4 T cells were stimulated with anti-CD3 in the presence of DMSO or AB-MECA for 3 days followed by expansion in IL-2 containing medium for 3 days. The culture medium was supplemented with fresh AB-MECA and anti-CD3 every 24 hrs for the first 3 days. As predicted, flow cytometry showed 22% of CD4 T cells were Foxp3 and CTLA4+ in AB-MECA treated samples in contrast to <2% in DMSO treated samples (Fig 21 a).

The increased percentage of Foxp3+ CD4 T cells in AB-MECA treated cultures could result from either expansion of Foxp3+ T cells or from induction of Foxp3 expression in CD4+CD25- T cells. To test for the possibility of increased expansion of Foxp3+ cells, we tested the effect of AB-MECA on the proliferation of CD4+CD25- and CD4+CD25+ T cells separately. AB-MECA substantially reduced the proliferation of CD4+CD25- T cells (50% reduction) and had no significant effect on the proliferation of CD4+CD25+ T cells (Fig 21b). This result of inhibition of CD4+CD25- T cell proliferation was confirmed using CFSE proliferation assay as well.
Fig 21. Effect of adenosine A3 receptor signaling on CD4 T cell population dynamics. A) Total CD4 T cells were cultured with anti-CD3 antibody plus APCs in medium containing DMSO or adenosine A3 receptor specific agonist (AB-MECA, 50uM) for 72 hrs. After 3 days, cells were placed in medium containing IL-2 (20ng/ml) for 4 days and analyzed for Foxp3 and CTLA4 expression. B) CD4+CD25- and CD4+CD25+ T cells were sorted from mice splenocytes and cultured with anti-CD3 and APCs along with DMSO or AB-MECA for 72 hrs. Proliferation was assessed using cell titer glo kit (Molecular probes).
Relative increase in Foxp3+ T cells with AB-MECA could also be caused by *de novo* induction of Foxp3+ cells from naïve T cells (Chen et al., 2003). To address this possibility, CD4+CD25− T cells were stimulated with anti-CD3 in the presence or absence of AB-MECA. TGF-beta has been shown previously to induce Foxp3 expression in CD4+ CD25- T cells and thus served as positive control for this experiment. However, similar treatment of naïve T cells with AB-MECA did not lead to induction of Foxp3+ expression in CD4 T cells (Fig 22, top row). Interestingly, co-culture of AB-MECA with TGF-β led to significant reduction in the percentages of Foxp3+ CD4 T cells (8%) compared to 20% of TGF-β alone treated cultures (Fig 22, bottom row). This result could mean that A3 receptor signaling antagonizes TGF-β signaling in naïve T cells. Taken together, these data rule out the possibility of Foxp3 induction by AB-MECA and rather strongly suggest preferential enrichment of Foxp3+ T cells.

Next, we asked whether adenosine A3 receptor signaling could inhibit effector T cell proliferation *in vivo* in mice. To address this question, CFSE labeled transgenic mouse derived CD4 T cells were intravenously transferred into congenic Ly5.1 mice and challenged in vivo with the cognate antigenic peptide with or without AB-MECA. As the source of CD4 T cells, we used p25 TCR transgenic mouse T cells, specific to *Mycobacterium Tuberculosis* antigen p25 peptide (Tamura et al., 2004). The antigen challenged mice also received AB-MECA or vehicle control through intraperitoneal (i.p) injection daily for 1 week. Expression levels of Foxp3 and CFSE were measured on day 8 after antigen administration.
Fig 22. Flow cytometric analysis of Foxp3 induction in CD4+CD25- T cells following adenosine A3 receptor signaling. CD4+CD25- T cells were cultured with anti-CD3 stimulation and APCs in medium containing DMSO or adenosine A3 receptor agonist (AB-MECA) for 72 hrs. After day 3, cells were placed in medium containing IL-2 (20ng/ml) for 4 days and analyzed for Foxp3 and CTLA4 expression (Top row). Exogenous TGF-β (5ng/ml) was added to the medium (bottom row).
Intravenous injection of 2.5μg antigen peptide in the recipient mice caused effective proliferation of CD4 T cells as seen from the progressive dilution of CFSE in dividing cells (Fig 23a). As expected from in vitro data, CFSE labeled CD4 T cells proliferated considerably less in AB-MECA treated mice compared vehicle treated control mice. Also, we observed increased frequency of Foxp3+ CD4 T cells in AB-MECA treated mice. Further, we compared the total cell numbers in each cell division of the Foxp3- and Foxp3+ T cell fraction (Fig 23b). Consistent with the in vitro data, we found reduced cell numbers in Foxp3- T cell fraction alone of AB-MECA treated mice. This result led us to suggest that A3 receptor (A3R) signaling selectively inhibits effector T cell proliferation in vivo as well. Thus, under adenosine rich conditions (eg: excessive inflammation, hypoxia induced cell death in solid tumors), A3R activation might inhibit effector T cell proliferation with a concomitant increase in the frequency of Treg cells and change the balance towards immune suppression.

Cross talk between Adenosine receptor and Phospholipase D signaling

Ado receptors, A2a and A3 have been reported to utilize PLD in mediating cell functions (Thibault et al., 2000) (Mozzicato et al., 2004). In light of our findings with A3 receptor signaling in CD4 T cells, we next determined the effect of A3R signaling on PLD activity in CD4 T cells. TCR induced PLD activity was measured in AB-MECA treated CD4 T cells using a commercially available PLD assay kit (Molecular Probes). First, whole splenocytes from P25 TCR transgenic mice were stimulated with the cognate P25 peptide overnight to induce the
Figure 23. Effect of A3 receptor signaling on the proliferation kinetics of Foxp3 positive and Foxp3 negative CD4 T cells in vivo. CFSE labeled CD4 T cells from P25 TCR transgenic (Ly5.2) mice were intravenously transferred to Ly5.1 recipient mice. 24 hrs later, the recipients were challenged with 2.5ug of P25 peptide along with either DMSO (control) or AB-MECA (200ug/Kg) (i.p) for a period of 7 days. On Day 8, mice were sacrificed and all their peripheral lymph nodes (axial, cervical, inguinal, popliteal) were isolated and pooled for analysis by flow cytometry. A) Representative FACS plots showing CFSE dilution of adoptively transferred T cells in DMSO and AB-MECA treated mice. B) Line graphs depicting the number of foxp3- T cells in each generation of DMSO (dotted lines) and AB-MECA treated (solid lines) mice. C) Line graphs depicting the number of foxp3+ T cells in each generation of DMSO (dotted lines) and ethanol treated (solid lines) mice. Data is representative of 2 independent experiments.
Fig 24. Assay of PLD activity in AB-MECA treated CD4 T cells. Total splenocytes from P25 TCR transgenic mice were incubated overnight with the P25 peptide (1ug/ml). Following day, CD4 T cells were sorted and pre-treated with DMSO or AB-MECA for 30 mins. Cells were stimulated with soluble biotin conjugated anti-CD3 (10ug/ml) and avidin for 30 mins. Lysates were then probed for PLD activity as per manufacturers instruction. Filled bars are DMSO treated samples and open bars are AB-MECA treated samples.
expression of A3 receptor. The following day CD4 T cells were sorted and pretreated with AB-MECA for 30 min before restimulation with soluble anti-CD3 for 30 min. The total cell lysate from these samples were then used for PLD assay as per manufacturers instruction. As reported previously, we observed an increase in PLD activity in CD4 T cells following stimulation and this increase is not seen in samples treated with AB-MECA (Fig 24). Thus, A3 receptor signaling in CD4 T cells blocks TCR induced PLD activity. This indicates that the selective inhibition of Foxp3- T cell proliferation in AB-MECA treated cells is caused in part by blockade of PLD signaling.

**Adenosine A3 receptor signaling and cAMP**

A3 receptor activation is linked to G\textsubscript{i}-mediated inhibition of adenylyl cyclase (Gessi et al., 2008). Recent reports indicate that A3 receptors dictate cellular responses such as mast cell degranulation in part by decreasing intracellular cAMP concentration (Hasko et al., 2008). To determine the effect of A3 receptor activation on cAMP levels in CD4 T cells, splenocytes from P25 TCR transgenic mice were stimulated with P25 peptide overnight to induce expression of A3 receptor. On the following day, CD4 T cells were sorted by MACS column and incubated with DMSO or AB-MECA for 30 min. CD4 T cells were then stimulated with biotin conjugated anti-CD3 and avidin for 5 mins. Cells were then lysed in 0.1N HCL and the supernatants were assayed using cAMP-specific ELISA (Fig 25a).
A) 

B) 

- DMSO Unstimulated
- DMSO Stimulated
- AB-MECA Unstimulated
- AB-MECA Stimulated

A) 

B) 

- cAMP (1uM) 
- cAMP (10uM) 

- cAMP (100uM) 
- cAMP (1mM) 

**FOXP3**

**CTLA4**
Fig 25. Assay for cAMP levels and activity in CD4 T cells. A) Total splenocytes from P25 TCR transgenic mice were incubated overnight with the P25 peptide (1μg/ml). On the following day, CD4 T cell were sorted and pretreated with DMSO or AB-MECA for 30 mins. Cells were stimulated with soluble biotin conjugated anti-CD3 (10μg/ml) and avidin for 5 mins and lysed in 0.1N HCL. A) The supernatants were assayed for cAMP levels using cAMP specific ELISA. (* p<0.07, # p= 0.065) B) CD4 T cells were cultured with anti-CD3 and APCs along with varying doses of cAMP (1μM to 1mM) for 3 days. Cells were further cultured in IL-2 containing medium for 4 days and analyzed by flow cytometry. C) Total Foxp3- T cell numbers (left) and Foxp3+ T cell numbers (right) in exogenous cAMP treated cultures on day 7.
We did not observe any increase in cAMP levels in either stimulated or non-stimulated cells of control (DMSO) treated samples. In contrast, we detected high concentration of cAMP in both unstimulated and stimulated samples treated with AB-MECA. This observation is in stark contrast to what has been reported in other immune cells regarding A3 receptors decreasing intracellular cAMP concentrations (Jin et al., 1997).

The second messenger cAMP is shown to be a negative regulator of T cell activation and proliferation (Bopp et al., 2007; Ramstad et al., 2000). A previous study showed that cAMP-dependent signal transduction interferes with CD25 expression and IL-2 production (Ramstad et al., 2000). To test if A3 receptor signaling blocks effector T cell activation/proliferation via cAMP, CD4 T cell proliferation was determined following addition of exogenous cAMP (dibutyryl-cAMP, Db-cAMP, a cell permeable analog of cAMP, Calbiochem). Total CD4 T cells were stimulated with anti-CD3 antibody and APCs in the presence of increasing concentrations of Db-cAMP for 3 days followed by culture in IL-2 containing medium for 4 days. Flow cytometric analysis of Foxp3 and CTLA4 expression show that low concentrations (1uM-10uM) of cAMP have no effect of CD4 T cells population dynamics, whereas high concentration (100-1000μM) cause increased frequency of Treg cells (Fig 25 b, c). Hence, increase in cAMP level selectively causes inhibition of Foxp3- T cell proliferation similar to those observed with AB-MECA. Taken together, these findings indicate that cAMP is involved in mediating the effects of adenosine A3 receptor on CD4 T cells.
Various studies suggest that the effect of cAMP in T cells is mediated by the cytosolic cAMP-dependent protein kinase A (PKA) type I (Ramstad et al., 2000). It is reported that cAMP binds to the two regulatory subunits in the inactive PKA tetrameric holoenzyme to release two active catalytic subunits. In turn, these catalytic subunits phosphorylate diverse proteins to regulate their activity (Yao et al., 2002). Based on these observations, it was hypothesized that the increase in cAMP levels in A3R agonist treated samples would lead to activation of PKA followed by increased phosphorylation of its downstream effector molecules. To test this experimentally, western blot analysis was performed on CD4 T cells stimulated with anti-CD3 in the presence of AB-MECA. The lysates were assayed for phosphorylation patterns by western blot analysis using anti-phospho-PKA substrate antibody. In comparison to both unstimulated and stimulated control (DMSO) treated samples, stimulated AB-MECA treated samples showed increased phosphorylation of PKA substrates (Fig 26a). cAMP assay showed increased cAMP levels in both unstimulated and stimulated samples treated with AB-MECA. We further determined the effect of A3 receptor signaling on PKA activity in both stimulated and unstimulated T cells. As predicted from the data on cAMP, AB-MECA increased PKA substrate phosphorylation in unstimulated AB-MECA treated CD4 T cells (Fig 26b). A mild increase in PKA substrate phosphorylation was observed in AB-MECA treated CD4 T cells stimulated with anti-CD3.
Fig 26. Western blot analysis of phosphorylation patterns of PKA substrates in AB-MECA treated CD4 T cells. Total splenocytes from P25 TCR transgenic mice were incubated overnight with the P25 peptide (1ug/ml). The following day, CD4 T cell were sorted and pre-treated with DMSO or AB-MECA for 30 mins. Cells were stimulated with soluble biotin conjugated anti-CD3 (10ug/ml) and avidin for 5 mins and the lysate were separated on 8% polyacrylamide gels, transferred on to nitrocellulose membrane and blotted with antibody against phospho-PKA substrates. A and B) (Top blot) phosphoPKA blot, (bottom) beta actin, loading control. US = Unstimulated sample, S = Stimulated sample.
In conclusion, these data indicate that adenosine signaling through A3 receptor blocks T cell proliferation via an increase in cAMP and decrease in PLD signaling.

**PLD signaling: synergy between ethanol and adenosine**

Since both ethanol and adenosine A3 receptor agonist inhibit PLD activity and result in enrichment of Treg cells, we examined if there is synergy between the A3R agonist (AB-MECA) and ethanol. CD4 T cells from mouse spleen were stimulated with anti-CD3 antibody both in the presence and absence of ethanol and AB-MECA. Lower concentrations of ethanol (0.5%) alone did not cause increased frequency of Treg cells. However, when 0.5% ethanol was combined with 50μM AB-MECA, substantial increase in the percentage of Foxp3+ T cells was observed (Fig 27 a). Also, the comparison of Foxp3+ and Foxp3-T cell numbers indicate decreased proliferation of Foxp3- T cells in cultures treated with either AB-MECA alone or AB-MECA and 0.5% ethanol (Fig 27 b) indicating enrichment of Treg cells in these culture conditions. The synergy between these two factors may be due to increased suppression of PLD signaling and/or inhibition of multiple signaling pathways that collectively block non-Treg activation.

Previously, it was reported that ethanol increases adenosine receptor stimulated cAMP levels in human lymphocytes (Hynie et al., 1980) (Gordon et al., 1986). Increased cAMP levels in the presence of both ethanol and adenosine could contribute to the observed synergy in Treg enrichment. However, we
A)  DMSO  AB-MECA (50μM)

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<tr>
<td>0.5% ethanol</td>
<td>0.78</td>
<td>4.74</td>
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Foxp3 vs. CTLA4
**Fig 27. Combinatorial effect of ethanol and adenosine on CD4 T cell population dynamics.** Total CD4 T cells were cultured with anti-CD3 and APCs along with ethanol alone or in combination with AB-MECA(50uM) for 3 days. Cells were further cultured in IL-2 containing medium for 4 days and analyzed by flow cytometry. A) Flow cytometric plots depicting Foxp3 in the x-axis and CTLA4 in the y-axis. B) Total Foxp3- T cell (left) and Foxp3+ (right) T cell numbers in 0.5% ethanol, AB-MECA and in AB-MECA + 0.5% ethanol treated cultures.
Fig 28. Western blot analysis of phosphorylation patterns of PKA substrates in CD4 T cells treated with a combination of ethanol and AB-MECA. Total splenocytes from P25 TCR transgenic mice were incubated overnight with the P25 peptide (1μg/ml). Following day, CD4 T cell were sorted and pre-treated with ethanol (0.5%) or AB-MECA or both for 30 mins. Cells were stimulated with soluble biotin conjugated anti-CD3 (10μg/ml) and avidin for 5 mins and the lysate were separated on 8% polyacrylamide gels, transferred on to nitrocellulose membrane and blotted with antibody against phospho-PKA substrates. A) (Top blot) phospho PKA blot, (bottom) beta actin, loading control.
observed very little, if any, increase in phosphorylation of PKA substrates in samples treated with the combination of AB-MECA (50μM) and 0.5% ethanol compared to 0.5% ethanol alone (Fig 28). Since there are many potential bands that are not separated by 1D gel analysis, further analysis is needed to determine whether ethanol and AB-MECA have synergistic effect on PKA activity.

**STRUCTURE-FUNCTION ANALYSIS OF PHOSPHOLIPASE D**

The data presented above show that various exogenous and endogenous factors influences the immune outcome by regulating PLD signaling. However, the contribution of PLD to T cell signaling is not clearly understood. As described previously, the catalytic action of PLD on phosphatidyl choline (PC) results in the formation of phosphatidic acid (PA) and soluble choline. The formation of PA constitutes the crucial contribution of PLD to cellular signaling and function. PA is an important lipid second messenger that can also be synthesized by several other routes and thus the contribution of PLD to the overall mass of PA in cells is quite miniscule (Ktistakis et al., 2003). Therefore, PLD is thought to only influence the local levels of PA. Thus, delineation of the spatial regulation of PLD isoforms in T cells will help us understand its function in T cells.

As mentioned previously, the two isoforms of PLD localize at different locations in the cell. Over expression and epitope tag studies reveal that PLD1 is found mainly in punctate structures around perinuclear regions while PLD2 is primarily found in the plasma membrane. To determine the region that controls the localization of PLD1 and PLD2, a series of mutants of both PLD1 and PLD2
were generated. In order to assess the localization of these mutants in the cell, these mutants were tagged with YFP in the N-terminal and transfected in Jurkat T antigen cells, which express SV40 large T antigen to allow high level of expression of transfected genes (Northrop et al., 1993). After transfection (24 hrs), live cells were separated using Ficoll gradient, mounted on glass slides coated with lysine, fixed and covered with cover slip. These slides were then analyzed using confocal microscopy.

**Determinants of PLD1 perinuclear localization**

At first, we addressed the region responsible for the differential localization of PLD1 and PLD2. The primary sequence of PLD1a revealed the presence of 116 amino acid ‘loop region’ inserted between catalytic sites II and III (Frohman et al., 1999). Previous studies in which the loop region of PLD1 was deleted have reported modest increase in the basal activity of PLD1 (Sung et al., 1999). Based on this observation, we hypothesized that deletion of the loop region redistributes PLD1 similar to PLD2 and this results in increased catalytic activity of PLD1. To test this idea, mutant human PLD1b with loop (aa. 504-585) deletion was constructed and its localization was assessed using confocal microscopy. The loop deletion mutant of PLD1 lost its ability to localize in punctate structures surrounding the nucleus and localized to the plasma membrane, similar to PLD2 (Fig 29). These data suggested that the loop region is a required component for PLD1 localization in vesicular structures.
Fig 29. Confocal microscopic analysis of the sub-cellular localization of PLD1 loop deletion mutant. PLD1 and PLD2 full length and loop deletion mutant are tagged with YFP. Green indicates the localization of the respective construct and red in propidium iodide staining of the nucleus. These constructs were transfected in jurkat T antigen cells. Live cells were isolated 24 hours later by ficoll gradient and plated on lysine coated glass slides. Cells were then imaged using the confocal microscope and images were processed using LSM image examiner software.
Fig 30. Confocal microscopic analysis of the sub-cellular localization of PLD1 loop region alone or loop region and full length PLD1 combinations. PLD1 full length is tagged with YFP. Green indicates the localization of the full-length PLD1 construct. Loop alone construct was tagged with FLAG. Blue is the DAPI staining of the nucleus. Loop alone construct was detected using anti-FLAG conjugated with fluorochrome. PLD1 and loop construct were transfected in jurkat T antigen cells either alone or together. Live cells were isolated 24 hours later by ficoll gradient and plated on to lysine coated glass slides. Cells were then imaged using the confocal microscope and the images were processed using LSM image examiner software.
Next, we determined if the loop region is sufficient to determine the localization of PLD1 into vesicular structures. Furthermore, if loop interacts with unknown target molecules in the cell that dictate the localization of PLD1 then expression of the loop alone construct could interfere with PLD1-target molecule interaction. Therefore, flag tagged ‘loop alone’ construct was co-transfected with YFP fusion full length PLD1. Full length PLD1 localized in punctate structures and when co-transfected with the ‘loop alone” construct, there were no observable differences in its localization (Fig 30). The loop alone construct was mainly detected in the plasma membrane. The data suggest that the loop region is required for PLD1 sub-cellular localization in the vesicular structures but loop alone (aa 504-585) is not sufficient to create the structure of PLD1 that interacts with the cellular target that determines the localization of PLD1.

Also, we determined the contribution of PX, PH and C-terminal regions to the peri-nuclear localization of PLD1 by deletion of the corresponding regions from full length PLD1. These constructs were transfected into Jurkat T antigen cells and their localization was determined by confocal microscopy. The construct lacking PX and PH domain (a.a 1-333 deletion) localized entirely in the cytoplasm (Fig 31). The mutant with only PX and PH domain (a.a 1-342) had diffuse localization in both nucleus and cytoplasm, similar to vector alone. Interestingly, the construct with only the C-terminal (aa. 933-1036) localized entirely in the nucleus. This suggests that the C-terminal (aa-933-1036) has nuclear import property. Indeed, PLD1b localization in the nucleus has been reported before (Freyberg et al., 2001).
Full length PLD1b

N-terminal deletion of PLD1

PX+ PH only

C-terminal only

Full length PLD1b

N-terminal deletion

PX+PH only

C-terminal only

Merged

YFP

DAPI
Fig 31. Schematic representation of the mutant PLD1 constructs and confocal microscopy of their sub-cellular localization. All the constructs are tagged with YFP. Green indicates the localization of these constructs. Blue is the DAPI staining of the nucleus. These constructs were transfected in jurkat T antigen cells. Live cells were isolated 24 hours later by ficoll gradient and plated on lysine coated glass slides. Cells were then imaged using the confocal microscope and images were processed using LSM image examiner software.
Together, these data suggest that the interaction of PX, PH domain with the rest of PLD1 domains determines the perinuclear localization of PLD1. It is evident that interactions between different domains rather than a single domain are key to the sub cellular localization of PLD1.

**Determinants of PLD2 plasma membrane localization**

PLD2 has strikingly restricted distribution to the plasma membrane. Thus, YFP fusion constructs of PLD2 deletion mutants were made to determine the domain necessary for its plasma membrane localization. The construct containing PX+PH+ C-terminal localized to the cytoplasm (Fig 32). Similar to PLD1, 'C-terminal only’ construct of PLD2 localized to the nucleus. This observation is in agreement with previous studies indicating highly homologous C-termini of both PLD isoforms (Liu et al., 2001). Together, these observations suggest that the interaction of PX, PH and C-terminal with the catalytic region determines the plasma membrane localization of PLD2.

Future studies can be aimed at assaying the catalytic activities of both PLD1 and PLD2 mutant constructs and its effect of TCR signaling to pinpoint with certainty the contribution of PLDs to the functioning of the immune system.
**Fig 32. Schematic representation of the mutant PLD2 constructs and confocal microscopy of their sub-cellular localization.** All the constructs are tagged with YFP. Green indicates the localization of these constructs. Red is the propidium iodide staining of the nucleus. These constructs were transfected in jurkat T antigen cells. Live cells were isolated 24 hours later by ficoll gradient and plated on lysine coated glass slides. Cells were then imaged using the confocal microscope and the images were processed using LSM image examiner software.
EFFECT OF PLD2 GENE DELETION ON T CELL RESPONSES

Delineating the isoform specific contribution of PLD1 and PLD2 would be critical to address their role in regulatory T cell enrichment. In order to delineate the potential role of PLD2 in regulating the balance between effector and regulatory T cell subsets, we generated mice deficient in PLD2 specifically in T cells. Mice carrying floxed PLD2 genes were crossed with CD4 CRE mice in order to delete the gene in double positive (CD4+CD8+) and CD4 and CD8 single positive T lymphocytes. PLD2 gene was successfully deleted in CD4 T cells as detected by the differential size of DNA segments (WT mice = 6 kb, PLD2 +/- = 6 and 5.2kb, PLD2 -/- = 5.2Kb) on southern blots (Fig 33a).

At first, we characterized PLD2^flox/flox^CD4cre mice to determine the effect of deletion of PLD2 on thymocyte differentiation and peripheral T cell activation. The absence of PLD2 had minimal effect on T cell development as determined by flow cytometric analysis of thymocytes from WT and PLD2 (+/- and -/-) mice (Fig 33B). Also, CD4:CD8 ratios in the spleen were similar between PLD2 (+/-) and PLD2 (-/-) mice (Fig 33 C). Based on our previous in vitro findings, we expected increased frequency of regulatory T cells in PLD2 knock out mice. Surprisingly, deletion of PLD2 did not alter the development of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells (Fig 33D). These observations suggest that deleting PLD2 at the double positive stage of T cell development does not affect the development of CD4, CD8 and regulatory T cells.
Fig 33. Phenotypic analysis of conditional PLD2 deficient mice. A) Southern blot analysis of CD4 T cells from WT or PLD2 (+/-), PLD2 (-/-) mice. B) Flow cytometric analysis of thymocytes from PLD2 deficient mice. X-axis corresponds to CD4 T cells and y-axis corresponds to CD8 T cells. C and D) Flow cytometric analysis of splenocytes from PLD2 deficient mice. Foxp3 versus CD25 is gated on CD4+ T cells.
Next, we determined the functional consequences of TCR induced signaling in PLD2 deficient T cells. Sorted CD4 T cells were stimulated with anti-CD3 and irradiated APCs overnight. Expression of CD69 (very early activation antigen) and CD25 (IL-2Rα) was determined by flow cytometry. Both WT and PLD2 (-/-) mice upregulated activation markers to the same extent (Fig 34A). Concomitant with activation status, both WT and PLD2 (-/-) mice had similar proliferation kinetics 72 hrs after activation (Fig 34B). Although, our previous in vitro experiments suggested that inhibition of PLD signaling using 1-alcohol/adenosine blocks proliferation of effector CD4 T cells, proliferation of CD4 T cells from PLD2 knock out mice did not exhibit any significant differences. This result indicates that the ability of PLD2 (-/-) CD4 T cells to initially become activated is not affected by their lack of PLD2.

In addition, we determined the cytokine secretion by CD4 T cells of PLD2 knock out mice. Sorted CD4 T cells were added to tissue culture plates coated with anti-CD3 along with soluble anti-CD28 for 72 hrs. After 72 hrs of stimulation, cells were rested for 48 hrs and then re-stimulated with PMA and ionomycin. Intracellular cytokine staining on these cells indicate that in PLD2 (-/-) mice, the CD4 T cells have 24% IL-2 positive cells compared to 5% (WT) and 12% (PLD2+/-) mice (Fig 35). In contrast, the percentage of IL-4 secreting cells was lower in PLD2 (-/-) mice (6%) compared to WT (18%) and PLD2 (+/-) mice (12%). We did not observe any significant differences in the cytokine levels of IL-17 and TNF-α between WT and PLD2 (-/-) mice. Thus, the data indicate that
Fig 34. Characterization of CD4 T cell function in PLD2 deficient T cells. A) Activation markers, CD69 (y-axis) and CD25 (x-axis) following stimulation. Cells are gated on CD4 T cells after 24 hrs of stimulation with anti-CD3. B) Proliferation analysis of PLD2 deficient CD4 T cells after 72 hrs of stimulation with different concentrations of anti-CD3. WT, PLD2 (+/-) and PLD2 (-/-) mice are littermates.
**Fig 35. Cytokine profiles of PLD2 deficient CD4 T cells.** A) CD4 T cells from WT and PLD2 KO mice were stimulated with plate bound anti-CD3 and soluble CD28 for 72 hrs, following which cells were rested for 2 days. Cells were re-stimulated with PMA and ionomycin and intracellular FACS staining for IL-2, IFN-γ, IL-4, IL-17 and TNF-α was performed.
PLD2 deficient CD4 T cells remain as IL-2 producers after three days of stimulation and have impairment in differentiation into Th2 type T cells.

To test if PLD2 is necessary for effector T cell differentiation into different subsets namely Th1, Th2 or Th17, CD4 T cells from WT and PLD2 knock out mice were activated under Th1 (in the presence of IFN-γ, IL-12 and anti-IL-4), Th2 (in the presence of IL-4 and anti-IFN-γ) or Th17 (in the presence of TGF-β and IL-6) skewing conditions and FACS staining was performed to detect intracellular cytokine expression. Under Th1 induction conditions, 78% of WT CD4 T cells were IFN-γ positive and 11% were IL-2 positive while in PLD2 (-/-) mice 21% were IL-2 positive and 66% were IFN-γ positive (Fig 36, top row) indicating slight resistance to differentiate into Th1 phenotype. On the other hand, under Th2 induction conditions 1.8% of WT CD4 T cells were IL-4 positive in comparison to 0.8% of PLD2 (-/-) T cells (Fig 36, middle row). Under Th17 skewing conditions, ~8% of CD4 T cells in PLD2 (-/-) mice were IL-17 positive compared to 6% of WT T cells (Fig 36, bottom row). Together, the data suggest that PLD2 (-/-) mice have slight impairment in differentiation into Th1/Th2 cell types and remain at IL-2 secreting stage. We can further assess effector T cell differentiation in PLD2 (-/-) mice in vivo (eg: by using P25 peptide, a strong inducer of Th1 response).

In light of our previous findings, we next determined if ethanol/adenosine inhibits effector T cell proliferation through inhibiting PLD2. To test this, we treated WT, PLD2 (+/-) and PLD2 (-/-) mice with DMSO, A3 agonist or A3 agonist plus 0.5% ethanol. We predicted that if A3 agonist inhibited PLD2 signaling, then
Fig 36. Differentiation of PLD2 CD4 T cells into Th1, Th2 and Th17 Effector cells. Spleen CD4 T cells were stimulated with anti-CD3 in vitro and rested 5 days in the presence of Th1, Th2 or Th17 skewing conditions. T cells were re-stimulated with PMA and Ionomycin and intracellular cytokine staining was performed.
Fig 37. Effect of A3 receptor agonist on PLD2 deficient CD4 T cells. CD4 T cells isolated from WT and PLD2 KO mice spleen were stimulated with APC and anti-CD3 either in presence of A3 agonist (AB-MECA) or carrier control (DMSO) for 3 days. On day 4, cells were washed and placed in medium containing IL-2 for 4 days. On day 7, cells were harvested and stained for detection of Foxp3 expression and analyzed using flow cytometry.
Fig 38. Effect of ethanol on PLD2 deficient CD4 T cells. CD4 T cells isolated from WT and PLD2 KO mice spleen were stimulated with APC and anti-CD3 in the presence of varying concentrations of ethanol for 3 days. On day 4, cells were washed and placed in medium containing IL-2 for 4 days. On day 7, cells were harvested and stained for detection of Foxp3 expression and analyzed using flow cytometry.
treatment of PLD2 (+/-) with A3 agonist and ethanol would lead to increased frequency of Foxp3+ cells. However, we found no differences in the frequency of Foxp3+ cells between WT and PLD2 knock out mice under these treatment conditions (Fig 37 and 38). This suggests that A3 agonist/ethanol mediated enrichment of Treg cells might involve additional signaling pathways other than PLD2. Future experiments can be conducted to determine the level of PLD activity in PLD2 (-/-) T cells. Based on the experiments of Treg enrichment using AB-MECA/ethanol it is very likely that PLD1 compensates for the lack of PLD2 in PLD2 (-/-) mice thus exhibiting no difference in PLD activity levels.

Future studies in PLD2 (-/-) mice can be conducted to determine its role in T cell signaling and function. Previous data from the lab indicated that PLD2 is involved in proximal TCR signaling events. We can determine if PLD2 (-/-) mice exhibit altered TCR signaling pathway compared to WT mice.
CHAPTER FOUR
DISCUSSION

Introduction

The response to pathogenic attack and maintenance of immune homeostasis requires precise coordination between different cell types of helper T cell subsets. Population balance between different types of cells is key to sustaining a balanced immune system. Many studies have reported altered balance between effector versus regulatory T cell subsets as the prime cause of disease pathogenesis. For instance, increased frequency and suppressor function of Treg cells have been reported and proposed as potential mechanism of immune suppression in patients with tumor and HIV-infection (Strauss et al., 2007a) (Strauss et al., 2007b) (Sempere et al., 2007). On the other hand, reduced number/function of Treg cells and increased effector T cell function have been reported in patients with allergic/autoimmune diseases (Dejaco et al., 2006) (Bacchetta et al., 2006).

Hence, ideal treatment for conditions of excessive immune suppression would be to enhance the frequency/function of effector T cells and vice-versa for autoimmune diseases. Therapeutic potential of this strategy has spurred the
interests of researchers leading to the discovery of many mechanisms for specific enrichment of either Treg cells or effector T cells.

Previously, our lab reported the differential requirement for PLD signaling in Treg cells versus effector T cells. As a consequence, inhibition of PLD signaling led to preferential enrichment of Treg cells by inhibiting effector T cell proliferation. Interestingly, other reports suggest increased PLD expression and signaling in autoimmune myocarditis and chronic inflammation (Ahn et al., 2004). For my dissertation work, I focused on identification of mechanisms regulating PLD signaling in T cells. The data demonstrated that various exogenous (ethanol, bacterial toxins) and endogenous (adenosine) factors alter the immune balance through regulating PLD signaling. PLD2 deficient mice showed that the effect of these molecules on the Teff: Treg balance is not dependent on PLD2 alone. Further the structure that controls the sub cellular localization of PLD1 and PLD2 was determined using mutant construct analysis.

**IMMUNE BALANCE: EFFECT OF EXOGENOUS FACTORS**

The human body is exposed to myriads of pathogens, pollutants, and environmental and bacterial toxins in day-to-day life. The immune system has evolved in many ways to combat these intruders and to maintain peace with self through mechanisms like plasticity of helper T cell subsets. But some pathogens and various exogenous molecules selectively manipulate the immune balance to their advantage by shifting the equilibrium between these activities one way or
the other. For instance, environmental toxin like dioxin has been shown to cause immune suppression by increasing Treg frequency (Quintana et al., 2008). Our data demonstrate that by inhibiting PLD signaling, exogenous factors like ethanol and \textit{C. difficile} toxin tip the balance towards immune suppression.

Our results suggest that these external factors by means of inhibiting PLD signaling, block the up regulation of activation induced CD25 expression on naïve CD4 T cells. IL-2 is an important T cell growth factor and CD25 is required for the proper function/signaling of IL-2. However, Treg cells constitutively express CD25 and thus expand in the presence of exogenous IL-2. The functional significance of ethanol/\textit{C. difficile} toxin mediated inhibition of PLD signaling is suggested by the decreased proliferation of antigen specific effector T cells and increased frequency of antigen specific Treg cells in vivo.

There is extensive evidence that ethanol consumption leads to immune suppression. Recently, both acute and chronic alcohol intake have been shown to result in specific defects in innate and adaptive immunity (Nelson and Kolls, 2002). It is suggested that ethanol results in loss of splenic and circulating T and B cells through apoptosis (Shao et al., 1995) (Sibley and Jerrells, 2000). Our experiments using ethanol provide evidence to suggest that this loss of T cells might be due to lack of PLD signaling and its effect on naïve T cells. In addition to mediating its effect through CD4 T cells, alcohol has been shown to modulate the functioning of other immune cells like macrophages, neutrophils, dendritic cells with antigen presenting capabilities (Szabo et al., 1993) (Nelson and Kolls,
The lack of activation induced marker expression on CD4 T cells might also be mediated through lack of antigen presentation through these APCs. Thus, in our experimental set up we cannot rule out the effect of ethanol on these APCs and the consequent reduction in TCR activation and PLD signaling.

In contrast to mice injected with ethanol intraperitoneally, mice fed on alcohol diet showed increased frequency of Treg cells only locally (mesenteric lymph nodes). This result can be explained based on previous studies suggesting that oral administration of ethanol results in increased availability and metabolism of ethanol in the gut resulting in less entry of ethanol into the bloodstream while intraperitoneal injections result in rapid appearance of ethanol in the bloodstream (Livy et al., 2003). Thus oral exposure of alcohol reflects the localized effect of ethanol on T cells while intraperitoneal injection reflects systemic effect of ethanol on T cells. This phenomenon of local immune suppression could in principle be exploited for treatment of gut associated inflammatory diseases like IBD and colitis.

Our studies with *C. difficile* toxin demonstrate a new facet of immune evasion strategy through PLD signaling. *C. difficile* toxins have long been known to induce reorganization of actin filaments due to modification of Rho proteins (Ottlinger and Lin, 1988). Since PLD is an effector molecule of Rho, *C. difficile* was also reported to inhibit PLD signaling. However, functional relevance for the lack of PLD signaling was not previously established. On the basis of our experimental results, we propose that by inhibiting PLD signaling in naïve T cells
through toxin B, *C. difficile* eliminates effector T cell functions. The consequent increase in the frequency of Treg cells aids the pathogen’s success in promoting illness and furthering its own survival. Although we report the effect of Toxin B on altering the immune balance, we have not tested the effect of blocking Toxin B using anti-Toxin B antibodies. Based on our results, we predict that blocking of Toxin B would mitigate the effects of *C. difficile* on the immune system and ameliorate the disease progression by promoting effector and memory T cell expansion.

**IMMUNE BALANCE: EFFECT OF ENDOGENOUS FACTORS**

One of the drawbacks of a powerful immune response is uncontrolled immune response against pathogens and associated collateral damage to self. The restraint of uncontrolled immune response is mediated through negative feedback mechanisms. Adenosine (Ado) is an endogenous molecule well known for its role in mediating the negative feedback mechanisms of immune responses. Previous studies have reported inhibition of inflammation following activation of adenosine receptors. Adenosine receptors are expressed by all immune cells and A2a receptor signaling on macrophages, dendritic cells and T cells have been shown to inhibit effector function (Zarek et al., 2008). Based on many studies, A2a receptors are considered the dominant receptor dictating lymphocyte responses in T cells.
Here we demonstrate the role of A3 receptor in inhibiting effector T cell responses. The data indicate that stimulation of T cells in the presence of A3 receptor agonist inhibits TCR induced activation of PLD signaling. This inhibition of PLD signaling abrogates CD25 expression, effector T cell proliferation, and cytokine secretion. Although, signaling through A3 receptor in other immune cells results in low cAMP concentrations, we found that signaling through A3 receptor in CD4 T cells results in elevated cAMP levels and increased PKA activity.

Previous studies on A3 receptors report both pro and anti-inflammatory effects depending on the system investigated (mast cells, macrophages) and species examined (rat, humans) etc (Gessi et al., 2008). However, the specific effect of activating A3 receptors on CD4 T cells has never been studied. In the heart, A3 receptors mediate cardioprotection through modulating PLD signaling (Mozzicato et al., 2004). This led us to determine the effect of A3 receptor signaling on CD4 T cells. We found that A3 receptor signaling blocked TCR induced PLD activation. At present, we do not know the exact mechanism of A3 receptor signaling leading to PLD signal inhibition. Inhibition of PLD by A2a receptors is mediated by interference with the translocation of small GTPases, Arf and Rho (Thibault et al., 2000). In future, we can test if A3 receptor inhibits PLD through translocation of small GTPases as well. One recent study also demonstrated inhibition of PLD1 activity directly by G\beta\gamma subunits (Preininger et al., 2006).
Since adenosine receptors are coupled to G-protein, signaling is thought to occur either through the inhibition or stimulation of adenylyl cyclase resulting in a decrease or increase of intracellular cAMP concentration respectively. The classical signaling pathway associated with A3 receptor suggests Gi mediated inhibition of adenylyl cyclase with a concomitant decrease in cAMP concentrations. We experimentally determined the effect of A3 receptor activation on cAMP concentrations. Contrary to previous reports, we found increased cAMP levels in A3 receptor agonist treated CD4 T cells. This increased cAMP levels also resulted in increased PKA activity. The effect of A3 receptor activation on inhibition of effector T cell proliferation and concomitant increase in Treg frequency was indeed reproducible by the cell permeable analog of cAMP, db-cAMP. Future experiments can be done to determine if the effect of A3 receptor is solely mediated by cAMP and PKA by co-culturing CD4 T cells with A3R agonist and PKA inhibitor H-89.

A3 receptor is coupled to trimeric GTP binding proteins, Gαi and/or Gαo and Gβγ dimers. The αi subunits directly inhibit adenylyl cyclase. However βγ dimers from Gi/o are known to activate adenylyl cyclase (AC) isozymes II and IV (Yao et al., 2002). We speculate that the increase in cAMP levels caused by A3R activation is mediated through βγ dimers. We can test if this is indeed the case by inhibiting βγ signaling. One way to inhibit βγ signaling is through over expression of the carboxyl terminal of βARK1 retrovirally in CD4 T cells. Previous evidence
suggests that expression of carboxyl terminus of βARK1 binds free βγ dimers and thus inhibits βγ signaling (Yao et al., 2002) (Koch et al., 1994).

Depending on the cell type studied, cAMP has been reported to inhibit (Le Stunff et al., 2000; Thibault et al., 2002) or activate (Mamoon et al., 1999) PLD activity through PKA signaling. In our experiments, we detected increased cAMP levels in CD4 T cells treated with A3R agonist 5 mins following T cell stimulation and observed a decrease in the PLD activity 15-30 mins after TCR stimulation. Based on these results, it is very likely that increased cAMP levels caused by A3 receptor signaling results in inhibition of PLD signaling. The experiment using inhibitor of PKA (H-89) and/or constitutively active form of PKA could also be used to determine PLD activity following PKA signaling.

Based on our various observations, we propose the following model depicting A3 receptor signaling in CD4 T cells (Fig 39). Two different pathways can mediate the effect of A3 receptor signaling in T cells. A central role for Gi/o βγ is proposed. A3 receptor activation can lead to increased cAMP levels and PKA signaling. PKA has been demonstrated to inhibit PLD activation. Another possibility is βγ dimers can directly inhibit PLD activity (Preininger et al., 2006).

The ability of A3 receptor agonist to promote regulatory T cell enrichment in vivo suggests that it could be used for treatment of autoimmune diseases. Studies in mice and rats demonstrate that the anti-inflammatory effects of methotrexate are in part mediated via A3 receptors (Montesinos et al., 2003).
Perhaps, methotrexate acting through A3 receptor on CD4 T cells blocks PLD signaling and leads to Treg enrichment.

Interestingly, it has been demonstrated that A3 receptor is over expressed in cancer tissues in comparison to normal tissues (Gessi et al., 2004a). It should be noted that the concentration of adenosine in solid tumor reaches as high as 100μM due to high levels of hypoxia (Hasko and Cronstein, 2004). Thus, it is highly likely that T cells activated by MHC class II positive APCs in these tissues are selectively blocked via A3 receptor signaling. Moreover, since A3 receptor agonists inhibit IFN-γ secretion, A3 receptor antagonists could be effective in tumor immunotherapy.
Fig 39. Schematic representation of A3 receptor signaling in CD4 T cells. A central role for Gi/o βγ subunits is proposed. A3 receptor activation leads to increased cAMP levels and activates PKA. PKA has been demonstrated to inhibit PLD activation. Another possibility is βγ dimers directly inhibit PLD activity (Preininger et al., 2006).
AB-MECA AND ETHANOL: POTENTIAL USE AS A TOLEROGENIC ADJUVANT

Our data show that concurrent presence of PLD suppressive factors such as adenosine and ethanol impose substantial suppression of immune response. We observed that 0.5% ethanol had no effect of inhibition of effector T cell proliferation or expansion of Treg cells. However, combination of 0.5% ethanol with A3 receptor agonist led to substantial decrease in effector T cell proliferation. This suggests that there exists a threshold for PLD activity in T cells and the combination of ethanol and AB-MECA pushes PLD activity well below this threshold. Under these conditions, we see a sizable effect of PLD inhibition on effector T cell proliferation and subsequent enrichment of Treg cells.

A very high concentration of ethanol leads to cell death due to the toxic effects of by products of ethanol (Lieber, 1997). However, we can avoid these toxic effects by using a combination of low doses of ethanol and AB-MECA.

Induction/expansion of antigen-specific Treg cells is of great therapeutic interest for the treatment of autoimmunity, allergy and organ transplantation. Our studies using AB-MECA/ethanol show expansion of antigen specific Treg cells in vivo. Therefore, AB-MECA alone or combination of AB-MECA and low dose ethanol may be used in immunotherapy of various diseases.

A recent study proposed the use of immunosuppressants like dexamethasone (DEX) as tolerogenic adjuvants (Kang et al., 2008). They demonstrated that antigenic immunization combined with DEX led to block of
dendritic cell (DC) maturation and preferential expansion of antigen specific Foxp3+ cells. Based on the similar effect observed with AB-MECA /ethanol, we propose that agents inhibiting PLD signaling can be used as tolerogenic adjuvants. However, we do not know the effect of AB-MECA and ethanol on the status of DC maturation. Future experiments can be designed to evaluate the effect of PLD signaling in antigen presenting cells like DC.

**PLD LOCALIZATION: INTERACTION BETWEEN MULTIPLE STRUCTURES**

PLD1 and PLD2 are 50% identical and display similar domain structures. However, they localize to different regions in the cell. It is suggested that localization determines intermolecular interaction with downstream target molecules thus serving different functions in signal transduction. A previous report shows that deletion of the loop region in PLD1 led to an increase in its basal activity levels but the localization of the loop deletion mutant was not determined. We over expressed loop deletion mutant in the Jurkat T cell line and found that it had plasma membrane localization similar to PLD2. Since PLD2 is characterized by high basal activity it is very likely that the increased activity of the loop deletion mutant is in part due to its plasma membrane localization and potential modification by membrane attached enzymes (eg: phosphorylation).

A puzzling observation was that the isolated loop domain had plasma membrane localization as well. Since deletion of loop from PLD1 abrogates its distribution from vesicular structures we hypothesize that the coordinated
interaction of loop within PLD1 and with unknown structure in the cell sequesters it to vesicular structures. One way to test this hypothesis is to insert the isolated loop domain into full length PLD2. If the interaction of loop with the other domains is the factor governing its vesicular localization, then PLD2 will localize similar to PLD1.

A previous report suggests that PH, PX and palmitoylation sites on the N-terminal region of PLD1 serve as potential membrane binding determinants (Sugars et al., 2002). In agreement with previous studies, in a deletion mutant encompassing all 3 potential sites (N-terminal deletion mutant), the membrane binding determinants redistributed to the cytoplasm. We also tested the cellular distribution of the isolated ‘PH+PX’ domain of PLD1. The localization of this mutant did not resemble that of full length PLD1. Rather it was found to have a diffuse localization in both cytoplasm and nucleus similar to vector construct. Based on this result, we suggest that ‘PH+PX’ domain cannot be studied in isolation. Other studies suggest that an intact C-terminus of PLD1 is required for its function (Liu et al., 2001). We made a deletion mutant containing the C-terminus fused with YFP. Interestingly, this mutant localized only in the nucleus of the cell. Taken together, we propose that a single domain does not determine the localization of PLD1. Rather interactions between different domains of PLD1 and with other cellular components regulate localization of PLD1 in sub cellular structures. The presence of C-terminal region alone determines its nuclear localization while interaction of the C-terminal region with catalytic domains and
the loop region redistributes PLD1 to the cytoplasm. Further, the interaction of N-terminal domains (PX+PH) with the rest of PLD1 determines its localization in membranous vesicles like golgi.

We undertook experiments to determine the domains necessary for the plasma membrane localization of PLD2. As proposed for PLD1, similar interaction between domains determines its plasma membrane localization. Isolated C-terminus mutant of PLD2 had nuclear localization while the mutant with the presence of PH+PX along with the C-terminus redistributed to the cytoplasm. Further, the interaction of the region containing the catalytic domains with the rest of PLD2 results in its plasma membrane localization. Our studies provide evidence for the role of different domains of PLD1 and PLD2 in determining their respective localizations under basal condition. Future experiments can be conducted to determine the cellular binding target of PLD proteins particularly the loop and N-terminal half. It is possible that various modifications of typical residues mediate the hierarchy of localization signals. This can be addressed using a site directed mutagenesis approach. Also, we have not examined the functional effect of these mutations. Functional studies in future will provide a better understanding of the role of localization in determining specific functions.

**PLD2 AND T CELL FUNCTIONING**

As a result of the critical role of PLD in development, PLD null mutants are embryonic lethal (LaLonde et al., 2006). In PLD2\(^{\text{flox/flox}}\) CD4 cre mice, PLD2 is
deleted relatively late (double positive stage). Our data demonstrate that PLD2 is dispensable for T cell development after the double positive stage of thymocyte differentiation. Our data does not address the role of PLD2 in earlier aspects of T cell development.

Also, deficiency of PLD2 did not affect CD4 T cell activation and proliferation. On the other hand, we did observe differences in cytokine secretion profiles of PLD2 deficient T cells. PLD2 deficient T cell cultures had more IL-2 positive cells than wild type T cells and showed resistance to differentiate in to Th1 and Th2 lineage in vitro. This effect on effector T cell differentiation might be caused by blockade of transcription factors like T-bet and GATA3. These possibilities can be investigated in the future.

Surprisingly, PLD2 deletion had very little effect, if any, on the expansion of regulatory T cells. One possible explanation is that PLD1 compensates for the lack of PLD2. We can test for this possibility by inducibly deleting PLD1 alone in PLD2 CD4 cre knock out mice. Another possibility is that PLD2 deficient T cells are inherently different due to their thymocyte development and differentiation in the absence of PLD2 signals. The other exciting possibility is perhaps that PLD2 is more important under conditions of physiological stress. This possibility can be addressed by inducing autoimmunity or by observing the immunological response to pathogens in PLD2 deficient mice in vivo. Nonetheless, these experiments in PLD2 knock out mice have brought to light previously unknown questions and answers.
CONCLUDING REMARKS

PLD signaling in T cells was discovered in 1991. However, until now the specific role of PA and PLD in T cell functioning has not been clear. Our lab group was the first to highlight the differential requirement of PLD signaling in effector versus regulatory T cells. This led us to investigate the role of PLD signaling in T cells. My dissertation work has focused on identifying the regulation of PLD signaling in T cells. I found that physiological molecules like adenosine maintain immune homeostasis by modulating PLD signaling. In addition, I discovered that pathogens/ exogenous molecules could alter the host immune response by virtue of their PLD inhibiting properties. Further, I determined the structure that controls the sub-cellular localization of PLD isoforms and their specific role in T cell function. These studies highlight the complex array of functions mediated by PLD in T cells. In the process of studying PLD, we have also discovered the role of adenosine A3 receptor signaling in T cells. This study highlights the role of crosstalk between different signaling pathways in human health and disease.
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