

B CELL MODULATION BY TOXIC SHOCK SYNDROME TOXIN-1 INDUCED CD4⁺
REGULATORY T CELLS GENERATED IN BALB/C MICE

by

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B.Sc. Microbiology and Immunology, University of British Columbia, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Experimental Medicine Program)

THE UNIVERSITY OF BRITISH COLUMBIA

July 2006

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ABSTRACT

Among the most exciting recent advances in the field of immunology is research on the regulatory T cells. Tregs, comprising only 5-10% of the peripheral CD4⁺ T cell population, are known to suppress T cell function. One type of Tregs is the antigen induced Tregs, which can be generated in mice following repeated subcutaneous injections of Toxic Shock Syndrome Toxin-1, a superantigen secreted by the bacteria, *Staphylococcus aureus*. These Tregs have been shown to have the ability to down regulate T cell activity and proliferation via the secretion of cytokines (IL-10 and TGF- β). Recent findings suggest that Tregs cells not only control T cell activity but B cell activity as well. In this study, the ability of these TSST-1 induced Tregs to modulate B cell proliferation, viability and antibody production is explored. It was found that the adoptive transfer of TSST-1 primed CD4⁺ T cells into mice hyperimmunized with TNP-KLH suppressed TNP-specific antibody production while PBS primed CD4⁺ T cells did not. The effect of CD4⁺CD25⁺ versus CD4⁺CD25⁻ T cells was further investigated *in vitro*. Co-culturing B cells with PBS primed CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells in the presence of 1 nM TSST-1 caused B cells to undergo multiple cycles of proliferation. The PBS primed CD25⁺ T cell population induced a T_H1-like response (IgG2a production) while the PBS primed CD25⁻ T cells induced a T_H2-like response (IgG1 production). In contrast, co-culturing B cells with either TSST-1 primed CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells suppressed B cell proliferation and function but through entirely different mechanisms. TSST-1 primed CD4⁺CD25⁺ Tregs prevented B cell proliferation by inducing B cell apoptosis, while TSST-1 primed CD4⁺CD25⁻ Tregs temporally suppressed the onset of a T_H2-like response. B cell suppression appeared to be mediated by both the secretion of IL-10 and TGF- β and by cell-to-cell mediated mechanisms.

These data provide evidence that in addition to modulating T cell activity, TSST-1 induced Tregs also regulate B cell activity both *in vitro* and *in vivo*, thus raising the possibility of using TSST-1 primed Tregs for controlling both T and B cell-mediated autoimmune diseases.

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ABBREVIATIONS

Abbreviations	Definitions
7-AAD	7 Amino-Actinomycin D
APC	Allophycocyanin or Antigen Presenting Cells
CFSE	Carboxyfluorescein succinimidyl ester
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cells
ELISA	Enzyme-linked immuno-sorbent assay
FACS	Fluorescence activated cell sorter
HRP	Horseradish peroxidase
i.p.	Intraperitoneally
i.v.	Intravenously
MHCII	Major histocompatibility complex class II
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SAg	Superantigen
s.c.	Subcutaneous
SEM	Standard error of the mean
TCR/CD3	T cell receptor
TGF- β	Transforming growth factor-beta
Tr1 cells	Type 1 regulatory T cells
Tregs	Regulatory T cells
TMB	Tetramethylbenzidine
TNP-KLH	Trinitrophenol-keyhole limpet haemocyanin
TSST-1	Toxic shock syndrome toxin-1

ACKNOWLEDGEMENTS

The author would like to thank the following people:

Dr. Anthony W Chow – for allowing the author into his laboratory and giving his time, patience, trust, support, and guidance in her project and in life.

Drs. Michael Gold and Cheryl Helgason – for being on the author's supervisory committee despite their busy schedules and for giving the invaluable insight, suggestions and advice into her project.

Dr. Winnie Kum and Haowei Li – for their endless scientific and technical support, guidance, assistance and advice throughout this project.

Shirin Kalyan – for her valuable assistance in interpreting the results.

Ryan Hung – for his help with using the FACS machine and completing the FACS analyses.

Past & present members of the Chow lab – for raising questions and challenging the author to the best of her abilities.

THANKS EVERYONE!!!

1.0 INTRODUCTION

1.1 MAJOR TYPES OF CELLS CONTRIBUTING TO THE ADAPTIVE IMMUNE RESPONSE

1.1.1 MACROPHAGES AND DENDRITIC CELLS

Proinflammatory immune responses begin when the immune system recognizes the presence of a foreign entity in the body. The first cells of the adaptive immunity to respond are the macrophages and dendritic cells (DCs). They are the cells that bridge the gap between the immediate antigen non-specific innate immune response with the antigen specific adaptive response. Macrophages and DCs are termed antigen presenting cells (APCs) for they have the specialized ability to take up soluble antigens, through a process called phagocytosis, from the extracellular environment and present the processed antigen on major histocompatibility complex class II (MHCII) molecules to T cells, leading to T cell activation. Upon phagocytosis, antigens enter the endosomal pathway where antigens are broken down. The resulting peptides are loaded onto MHCII molecules to be presented on the surface of the APC. The peptide bound MHCII molecule binds specifically to corresponding clones of T cells that bear T cell receptors (TCRs) that have specific affinity for the presented peptide and MHCII molecule. This engagement then leads to T cell activation, as discussed below. Of these two types of APCs, DCs play the most pivotal role in dictating and selecting for the type of pro-inflammatory response that would be most appropriate in the clearance of the specific immune challenge.

Besides their antigen presenting activities, these cells also participate in the immune response in other capacities. Macrophages have been shown to secrete various bactericidal molecules, such as nitric oxide and reactive oxygen species, along with pro-inflammatory cytokines, such as IL-12 and TNF- α . There is a further classification of DCs, the plasmacytoid dendritic cells (pDCs), which have been newly described to activate the anti-inflammatory response by inducing regulatory T cells (Moseman *et al.*, 2004), as seen in murine ovarian

cancer, viral infections and transplantation models (Ochando *et al.*, 2006; Wei *et al.*, 2005b; Kawamura *et al.*, 2006).

1.1.2 T CELLS

T cells constitute around 30% of the total splenic population and they play a vital role in controlling and mediating the various types of adaptive immune responses. They are termed "T" cells because they mature in the thymus after migration from the bone marrow. T cells can be divided into $\alpha\beta$ T cells, cells that have antigen specificity and undergo clonal selection and deletion in the thymus, and the $\gamma\delta$ T cells, cells that have broad antigen specificity and do not undergo clonal selection. As a result, $\alpha\beta$ T cells are considered part of the adaptive immune system while $\gamma\delta$ T cells are considered part of the innate immune system. For the purpose of this review, only $\alpha\beta$ T cells will now be discussed. T cells of the adaptive immune response can be further broken down into CD4 or CD8 expressing T cells. However, regardless of whether the T cell is CD4+ or CD8+, the mechanism of T cell activation remains the same, dependent on the type of stimulation transmitted from the APCs to generate Signal 1 and 2 in the T cell. For CD4+ T cells, antigen specific APCs will present processed antigen to T cells via the major histocompatibility class II (MHCII) molecule and those clones that have T cell receptors (TCRs) that react against the presented antigen, will experience Signal 1. The second signal is generated when co-stimulatory molecules, such as CD28 or CD40L, bind their specific ligand on APCs, in this case, CD80/CD86 and CD40, respectively. Having Signal 1 without Signal 2 results in anergy - the state of T cell unresponsiveness even in the face of activation signals. On the other hand, the engagement of co-stimulatory molecules without specific MHCII/TCR interaction would have no effect on T cells.

CD8+ T cells are conventionally known as cytolytic or cytotoxic T lymphocytes (CTLs),

involved in cell-mediated immunity. Their main role is to induce apoptosis of host cells, via the release of granzymes and perforins, that have been virally infected or that have undergone tumour transformation. In addition, several recent reports point to the presence of suppressive CD8⁺ T cells, currently termed cytotoxic regulatory T cells, that modulate the immune system by inducing apoptosis of activated pro-inflammatory cells via granzyme and perforin (Zhao *et al.*, 2006; Kawamura *et al.*, 2006). CD8⁺ T cells recognize antigens presented upon major histocompatibility complex class I (MHCI), a molecule that is present on the majority of cell types in the body. When a cell becomes virally infected, viral peptides will be presented upon the MHCI molecule. This will activate the specific clones of CTLs to all cells that present the same antigen. In essence, CD8⁺ T cells are involved in the clearance of endogenous antigens.

CD4⁺ T cells, on the other hand, are involved in the removal of exogenous antigens and in down-regulation of the immune response. Most of the CD4⁺ T cells are termed "T helper cells" due to their function, not to directly mediate an immune response, but to help induce effector cells, such as B cells and other T cells. CD4⁺ T cells begin life as uncommitted naive T cells. Depending on the type of infection or disease, different chemokines and cytokines generated by innate immune cells and APCs, help drive T cell differentiation after activation to become either a T helper 1 (T_H1) or T helper 2 (T_H2) cell. However, regardless of the type of T helper cells, these T cells, once activated, will upregulate the expression of CD25, the interleukin-2 (IL-2) receptor α -chain, and also the production of IL-2. The autocrine effect of IL-2 will then induce T cell proliferation. T_H1 cells are defined by having an interleukin-2 (IL-2), interleukin-12 (Bastos *et al.*, 2002), interferon- γ (Harris *et al.*, 2005a) and tumour necrosis factor- α (TNF- α) rich profile. This array of cytokines induces T_H1 differentiation and expansion, as resulting T_H1 cells induce further macrophage activation, allowing for a more effective clearance of intracellular pathogens. In short, T_H1 cells are activated in the presence of endogenous antigens. T_H2 cells, on the other hand, help mediate the humoral response, an

immune response that is predicated upon B cell activation and subsequent antibody production to neutralize extracellular soluble antigens. It can therefore be said that T_H2 cells are responsible for the clearance of exogenous antigens such as bacteria and their soluble by-products. These helper cells generate an interleukin-4 (Bober *et al.*, 1994), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-13 (IL-13) dominant cytokine profile. These cytokines will then, in turn, induce B cell activation, differentiation, expansion and isotype switching (details discussed below). Whether the T cell differentiates into a T_H1 or T_H2 cell, both cell types can differentiate into memory T cells. Comparatively speaking, little is known about these cells. What is known is that memory T cells are generated at the end of an infection where they are specialized cells that can help speed up the secondary immune response as these cells require only Signal 1 to be activated. It is hypothesized that these cells are the result of constant stimulations with low levels of the antigen that had not been completely cleared during the primary immune response.

In addition to the T helper cells, there is one more type of CD4⁺ T cells and they are the regulatory T cells (Valmori *et al.*, 2005), first discovered in the early 1970's but have only found prominence in the scientific community within the last 10 years. These cells, comprising of about 5-10% of the total CD4⁺ T cell population, have been shown to have the ability to down-regulate the immune system, particularly the T cells, by way of suppressing T cell proliferation and altering their cytokine profile, shifting from either the T_H1 or T_H2 profile to that of the Tregs - little or no IL-2 and IL-4 production but high IL-10 and transforming growth factor- β (TGF- β) secretion. Many questions remain unanswered with respect to these cells that have much promise in the realm of autoimmune disease and cancer therapy. Thus, these cells will be the focus of this project and the subject of the discussion below. Finally, there is also an unconventional group of T cells, called the natural killer T cells (Cava *et al.*, 2006). These cells are known to be either CD4⁺ or CD4⁻ and CD8⁻. They are similar to $\gamma\delta$ T cells for they have a restricted TCR repertoire but they are also CD8⁺ like for they bind to and are activated by a special class of MHC I

molecules, CD1D, which is known to exclusively present lipids and other hydrophobic molecules. Interestingly, these cells are implicated in the generation of antigen specific regulatory T cells, mediated by the production of IL-4 (Medzhitov and Janeway, Jr., 1998; Jiang *et al.*, 2005). Furthermore, EAE and nonobese diabetic mouse models were observed to have a decreased level of NKT cells and a lack of IL-4 production. The add back of NKT cells was able to reverse the disease (Hammond *et al.*, 1998; Kung *et al.*, 1999). A recent report by Kim *et al.* confirms that NKT cells are indeed implicated in the generation of Tregs, and in addition, are also involved in the process of clonally purging activated T cells, suggesting that not only do NKT cells have the ability to induce regulatory T cells but they, themselves, might have suppressive activity of their own (Kim *et al.*, 2006).

Table 1: Summary of the major types of $\alpha\beta$ T cells

	CTL	T_H1	T_H2	Treg
CD4 or CD8?	CD8	CD4	CD4	CD4
Part of which type of immune response?	Adaptive: Cell-mediated immunity	Adaptive	Adaptive: Humoural Immunity	Suppressive
Targets	Virally infected cells, cancer cells	Macrophages, Other T cells	Soluble antigens (Bacteria, toxins, etc)	T cells and perhaps B cells?
Location of antigen	Intracellular – in the cytosol	Intracellular – in the vesicles	Extracellular	n/a
Mechanism of action	Cell-to-cell contact; apoptosis mediated by release of granzymes and perforins	Via IL-2 and IFN- γ to clonally expand T cells; further activate macrophages	Activate B cells to secrete antibodies	Various mechanisms: cytokine (IL-10 and TGF- β) and/or cell-to-cell contact mediated

1.1.3 B CELLS

Like T cells, B cells are also a complicated class of cells. B cells are termed "B" cells because their generation does not involve the thymus, simply maturing in the bone marrow. Similar to the distinction between $\alpha\beta$ T cells and $\gamma\delta$ T cells, B cells can also be divided into B-1 and B-2 cells where B-1 cells, comprising of about 5% of total B cells, have limited antigen specificity while the B-2 cells are the "conventional" B cells that undergo clonal selection, expansion and deletion. B-1 cells are generally restricted to the mucosal environment and due to the nature of the project, only the characteristics of the B-2 cells will now be described. These B cells lead complex lives as they can function both as a target cells, activated by T_H2 cells to induce differentiation and antibody production, and as APCs. Thus, B cells can play the role of both the upstream activator and the downstream target of T cells. First, as the target cells, B cells respond to cytokines such as IL-4, IL-6 and IL-13 to induce activation, proliferation and differentiation into plasma B cells, terminally differentiated cells where their sole purpose is to produce antibodies. B cells, like T cells, also require specific signals in order to be activated. Signal 1 is provided when B cells bind specific antigens with the B cell receptor (BCR), also known as surface bound IgM (sIgM). Upon binding, sIgM will then be internalized and antigen broken down to be loaded and presented on the B cell MHCII molecule. Antigen peptide loaded MHCII engagement with the appropriate TCR, along with binding between the co-stimulatory molecules, CD40 on B cells with CD40L on T cells, help to activate B cells. At this point, B cells are then induced to differentiate into plasma cells and isotype switching is dictated by the type of cytokines in the surrounding milieu. IgG1 and IgE are produced in the presence of IL-4, part of the T_H2 cytokine profile, while IgG2a is produced in the presence of IFN- γ , part of the T_H1 cytokine profile. Depending on the specific type of antigen, different cytokines will be produced to select for a specific type of antibody isotype production. Antibody coating of antigens induces three effects - neutralization, opsonization and complement activation.

Neutralization of antigens helps to prevent further interaction between the pathogen in the host. Antibody coated antigens also induce macrophages to undergo phagocytosis with increased proficiency in a process called opsonization. Lastly, antibody coated pathogens trigger complement activation, which induces pathogen killing and also strongly facilitates opsonization. Besides plasma B cells, activated B cells can also differentiate into memory B cells. These epitope specific memory B cells are the functional counterpart to memory T cells for B cells, facilitating subsequent immune responses upon encounters with the same antigen.

As effectors of T cells, B cells can furthermore be subdivided into B effector 1 (Be1) or B effector 2 (Be2), both of which work in a similar fashion as T_H1/T_H2 cells for they mediate their effect via cytokine production (Harris *et al.*, 2000; Harris *et al.*, 2005a). Be1 cells act on the immune system by helping to drive CD4+ differentiation into T_H1 cells via the production of IFN- γ while Be2 secrete IL-4 to select for the T_H2 phenotype. Interestingly enough, the generation of Be1 and Be2 cells is dependent on T_H1 and T_H2 cells and the cytokines they produce. In essence, Be1 and T_H1 are co-dependent on each other while the same can be said of Be2 and T_H2 (Harris *et al.*, 2000; Harris *et al.*, 2005b). The question of which cell type is responsible for driving the initial activation remains unanswered.

Having discussed B cells in the light of being T cell targets, it must be pointed out that B cells can also be activated even in absence of T cells by T cell independent (TI) antigens, categorized into two groups – the TI-1 and TI-2 antigens. Both these types of antigens tend to have extensive structural repeats that aid in mediating B cell activation in an epitope non-specific manner. The TI-1 antigens, often termed B cell mitogens, have the ability to activate a large number of B cells via a BCR independent pathway. One example of the TI-1 antigen is the bacterial product, lipopolysaccharide (LPS). When LPS binds to Toll-like receptor-4 (TLR-4), a receptor that is not only expressed on B cells but also on many other immune cells, massive polyclonal activation of B cells results. Similarly, TI-2 antigens also activate a large number of B

cells, however, the mechanism of activation differ from the TI-1 antigens. The TI-2 antigens have the ability to extensively crosslink BCRs, providing an overwhelming Signal 1, thus triggering B cell proliferation. An example of a TI-2 antigen is the polysaccharide capsule that envelopes pyogenic bacteria.

Finally, recent research has shown that B cells can also play the role of a regulatory cell, pointing to the existence of regulatory B cells (Bregs). This topic will be discussed below.

1.2 DOWN-REGULATION OF THE ADAPTIVE IMMUNE RESPONSE: THE CONCEPT OF IMMUNE TOLERANCE

Having seen the complexity of how the adaptive immune response is activated, it would come as no surprise to find that the processes involved in down-regulation would be just as, if not more so, complicated. The ability to down-regulate any immune response is vital to the maintenance of a properly functioning immune system. Firstly, an immune response must be "turned off" once the threat of foreign antigens has been contained, for the mechanisms involved in pathogen clearance also have detrimental effect on host cells. Furthermore, it is not energy effective to the host to allow an immune system to be active indefinitely. Secondly, there are many T and B cell clones which have reactivity against the host's own proteins. Thus, if these cells are allowed to be activated, the host would then experience an autoimmune disease, the destruction of autologous cells by one's own immune system. Since neither of these two scenarios are desired, a mechanism by which the immune response may be controlled is required. This modulation of the immune response is generically termed immune tolerance and can take place in one of three ways: by inducing apoptotic cell death of specific T or B cell clones (Rocha and von Boehmer, 1991; Schwartz, 2003; Marrack and Kappler, 2004), by inducing the state of anergy or immune unresponsiveness even in the presence of specific activating stimuli (Schwartz, 2003; Walker and Abbas, 2002), or by suppression of the function of activated immune cells

(Maloy and Powrie, 2001; Shevach, 2002; Sakaguchi *et al.*, 2001a).

1.2.1 APOPTOSIS AND ANERGY

Apoptosis and anergy induction are two of the most common methods by which the immune system purges cells that are deemed harmful to the body. In the case of controlling self-reactive cells, this is achieved via deletion or inactivation during cell maturation either in the thymus (for T cells) or the bone marrow (for B cells). During the developmental stages for T cells, all the clones come into contact with self epitopes presented by APCs. Those cells that bind tightly to the presented MHCII/peptide complexes are marked for clonal deletion (apoptosis) or anergy. Those that do not bind at all are likewise eliminated, leaving those clones with slight binding affinity - those that can recognize the MHCII molecule but not the presented self peptide - to mature. In addition, as mentioned previously, in the presence of Signal 1 but not Signal 2, T cells will enter into a state of anergy.

The elimination of self-reactive B cells employs similar tactics as that of the T cells. Like T cells, B cells that strongly bind self antigens during the maturation stages in the bone marrow are induced to undergo somatic hypermutation to generate new BCR bearing different epitope specificity (in a process known as receptor editing), and if the resultant receptor still displays strong affinity, those B cells will be purged. B cells that bind with intermediate affinity are induced to become anergic. Only those B cell clones that do not bind self peptides are allowed to mature. In addition, self-reactive B cells that escaped elimination for whatever reason are induced to become anergic since the corresponding self reactive T cells would also be anergic as described. Thus, while only BCR will engage self proteins, these B cells will lack co-stimulatory molecule stimulation. Finally, perhaps as a fail-safe mechanism for self-reactive B cells that manage to escape the previous two elimination strategies, it has been shown that chronic exposure to soluble antigens also induces B cell anergy. This latter concept of chronic

stimulation to induce anergy is hypothesized to be employed by gut commensals to allow for bacterial survival in the gut without eliciting an inflammatory response. The same theory is applied to how many tumours manage to evade immune clearance (Pardoll, 2003). Finally, it has been hypothesized that some types of regulatory T cells might mediate immune modulation via the induction of anergy (Vanasek *et al.*, 2006). However, this remains controversial as the majority of work in the field indicates that Tregs down-regulate the immune system via the suppression of pro-inflammatory cell activity.

While the mechanism of apoptosis induction is relatively clear, the mechanism of anergy has yet to be elucidated. Current opinion suggests that anergy is an intracellular event where changes in calcium signalling and the expression of activator protein-1 alters the levels of calcium-dependent transcription factor, thus preventing activation of T cells (Borde *et al.*, 2006). Furthermore, it has also been found that the deletion of calreticulin, a calcium buffering chaperone, causes severe inflammatory reactions and exaggerated immune response to antigen challenge in mice. This is possibly due to the observed lower threshold of activation in TCR signalling, elevated levels of free calcium in the cytosol of T cells, constant signalling through mitogen-activated protein kinase pathways and accumulation of the calcium-dependent transcription factor in the nucleus (Porcellini *et al.*, 2006). Once again, much work remains to be completed.

1.2.2 SUPPRESSION

While anergy is mediated by a cell intrinsic mechanism, suppression is mediated when other types of cells impose upon the target of suppression. This process has been termed “dominant tolerance” (Graca *et al.*, 2005). The class of T cells that mediate this suppression is known as the regulatory T cells and they remain one of the most controversial cells in the field of immunology to date. According to newly published data, the distinction between these three

mechanisms to mediate tolerance may be restricted to cell type. Self-reactive T cells undergo apoptosis, while the CD25⁺ population is induced to become suppressive regulatory T cells and the CD25⁻ population is induced to enter an anergic state (Knoechel *et al.*, 2006). Regardless, regulatory T cells are the focus of this project and will be the subject of the rest of this introduction.

1.3 REGULATORY CELLS

1.3.1 REGULATORY T CELLS

Since the discovery of regulatory T cells, there has been controversy surrounding their function, mechanism of suppression, surface receptors and cytokine profile. These cells, although only comprising 5-10% of the peripheral CD4⁺ T population, are hypothesized to have control over the host immune response after an infection has been cleared (Sakaguchi *et al.*, 1995). In recent years, researchers have categorized different T regulatory cell subtypes based on surface marker expression and cytokine profile (Sakaguchi, 2003). These subtypes include antigen induced Tregs (Tr1s), natural CD4⁺CD25⁺ regulatory T cells, and T_h3 cells, among others. Regardless of the subtype, however, they are all defined by their ability to produce an anti-inflammatory profile – high IL-10 but low IL-2 and IL-4 – and their functional ability to suppress IL-2 and IFN- γ inflammatory cytokine production. This suppression is usually conferred via cytokines, such as IL-10 or TGF- β , and/or via cell-to-cell contact mediated mechanisms (Sakaguchi, 2003).

1.3.1.1 NATURAL CD4⁺CD25⁺ T_{REGS}

By far the most studied of regulatory T cells are the natural CD4⁺CD25⁺ Tregs (nTregs). These cells have been shown to be responsible for suppressing pro-inflammatory responses towards self antigens by inhibiting autoreactive effector T cells (DiPaolo *et al.*, 2005). It has

been reported that the absence of these cells *in vivo* leads to organ-specific autoimmunity (Sakaguchi *et al.*, 2001b), such as autoimmune gastritis (Laurie *et al.*, 2002; Suri-Payer *et al.*, 1996) and encephalomyelitis (Kohm *et al.*, 2002). It is hypothesized that these cells, along with other CD4⁺ T cells, are located on the thymic epithelium (Jordan *et al.*, 2001) but instead of undergoing negative selection, as would be the fate of regular self-reactive T cells, these natural CD4⁺CD25⁺ Tregs follow an alternate pathway (Shevach *et al.*, 2001). Natural CD4⁺CD25⁺ Tregs are characterized by their ability to constitutively express the IL-2 receptor α -chain, or CD25, without any type of prior antigen activation required (Sakaguchi *et al.*, 2001b). Natural CD4⁺CD25⁺ Tregs are defined by their ability to suppress both T_H1 and T_H2 cells (Bellinghausen *et al.*, 2003; Francis *et al.*, 2003; Hadeiba and Locksley, 2003; Jiang *et al.*, 2003; Jones *et al.*, 2003; Mottet *et al.*, 2003). When natural CD4⁺CD25⁺ T cells are eliminated, an autoimmune disease known as IPEX (Kobayashi *et al.*, 2001) ensues, suggesting the pivotal role of natural Tregs in maintaining self-reactive T cells under control (Wildin *et al.*, 2001). The mechanism of this suppression is via the down-regulation of effector cell CD25 expression, involving cell-to-cell contact between CD80/CD86 and CTLA-4 (Piccirillo *et al.*, 2002; Manzotti *et al.*, 2002; Read *et al.*, 2000; Takahashi *et al.*, 2000). Furthermore, clinical association between specific CTLA-4 polymorphism and the chance of development of an autoimmune disease has been established (Atabani *et al.*, 2005). However, this remains controversial as Kataoka *et al.* have evidence to show that CTLA-4 knockout mice do not have natural Tregs with a stifled suppressive activity nor do CTLA-4 over-expressing mice have increased Treg activity (Kataoka *et al.*, 2005). In addition, surface bound TGF- β and TGF- β receptor have also been shown to be involved (Nakamura *et al.*, 2004; Chen and Wahl, 2003; Levings *et al.*, 2002b; Nakamura *et al.*, 2001). It must be noted that the role of TGF- β (transforming growth factor- β) is more controversial and less well defined as this cytokine is pleiotrophic, playing both stimulatory and inhibitory roles at different times (Cottrez and Groux, 2001). It does, however, appear to adopt

the inhibitory role in the presence of IL-10, suggesting that while IL-10 may not be directly involved in the suppression mechanism, it is required to induce TGF- β to act in an anti-inflammatory way. Recent *in vivo* data has shown that CD4⁺CD25⁺ Tregs are not only antigen specific but also depend on both IL-10 and TGF- β to mediate suppression, as observed in a fetal murine autoimmune pneumonitis model, thus giving credence to this hypothesis of IL-10's indirect involvement (Huang *et al.*, 2005). In addition, the programmed death 1 (PD-1) protein and glucocorticoid-induced tumour-necrosis factor receptor (GITR) are also implicated as possible means of cell-to-cell contact (Shimizu *et al.*, 2002) but the details remain largely unknown (Nishimura *et al.*, 1999; McHugh *et al.*, 2002; Shimizu *et al.*, 2002). With respect to how these cells are generated, it is theorized that nTregs are continuously activated by self antigen and that chronic stimulation is the requirement for their maintenance and survival (Seddon and Mason, 1999).

In addition to mediating tolerance via suppression, natural Tregs have also been shown to induce tolerance via the induction of apoptosis. It was found that Tregs suppress differentiation and activation of T cells by inducing APCs to undergo apoptosis in a Fas/FasL dependent manner which led to decreased effector T cell activation (Zhao *et al.*, 2006). Interestingly, these Tregs have also been shown to mediate suppression by inducing cell death involving granzymes and perforins. However, the details of Treg mediating apoptosis have yet to be understood.

Finally, a third mechanism by which nTregs have been shown to suppress T cells is by interfering with CD4⁺ T cell interaction with dendritic cells, preventing a stable interaction from being established between the two cell types (Tadokoro *et al.*, 2006). Furthermore, the mobility of naïve T cells inside lymph nodes has been shown to become sluggish, effectively decreasing the chances of CD4⁺ T cell interacting with DCs. Thus, in addition to suppressing activated T cells, natural Tregs also have the ability to prevent further activation of T cells.

While CD25 has been traditionally the marker for Tregs, its expression is transient and therefore challenging to clearly separate out a population of cells that are CD25+. Clearly, a better way of sorting out Tregs is required. GITR, Glucocorticoid induced TNF receptor, (Shimizu *et al.*, 2002) has been shown to be the surface ligand responsible for modulating regulatory T cell suppression (Caton *et al.*, 2004; Ji *et al.*, 2004). It has been shown that the upregulation of the GITR ligand allows for GITR/GITR-L binding, abrogating the ability of Tregs to suppress target cells (Caton *et al.*, 2004; Cardona *et al.*, 2006). GITR neutralization and knockout in mice suffering from a form of inflammatory bowel disease has been shown to ameliorate disease pathology (Santucci *et al.*, 2006). The same is seen in murine allergic asthma and arthritis models (Patel *et al.*, 2005). In a pivotal paper, Valzasina *et al.* showed, that the neutralization of either GITR or OX40, another member of the TNF receptor family, has the ability to reverse Treg mediated tolerance in graft versus host disease (GvHD) (Valzasina *et al.*, 2005). Theoretically, the proposition that there are molecules that can suppress suppression is very attractive and logical. However, many questions remain unanswered regarding GITR and OX40 and thus relatively few groups have used GITR as a marker thus far. Currently, the most popular marker used to define Tregs is Foxp3. Foxp3, belonging to the family of forkhead/winged-helix transcriptional factors, has been found to be expressed in natural CD4+CD25+ Tregs but not present or only expressed at a low level in other T cell types (Fontenot *et al.*, 2003; Hori *et al.*, 2003). Empirically, patients suffering from IPEX – patients without any natural CD4+CD25+ Tregs – are found to have a mutation in the Foxp3 gene (Kobayashi *et al.*, 2001; Bacchetta *et al.*, 2006). Indeed, the bulk of the work in this field has employed Foxp3 as a defining marker for natural Tregs. However, as it turns out, the assumption that Foxp3 identifies Tregs might prove to be erroneous as many groups have found evidence to suggest this transcription factor is not specific for Tregs but is simply upregulated in all activated cells.

As evidence showing the unreliability of Foxp3 as a marker for Tregs piles up, the search is on to find a better marker that may be used to define Tregs. One exciting lead is that of CD127, the IL-7 receptor α -chain, a molecule that promotes T cell proliferation (Seddon and Zamoyska, 2002). Unlike the other markers, negative selection is desired with CD127 to identify the Treg population (Liu *et al.*, 2006). Cells having low expression levels of CD127 despite having been activated reflect the anergic property of Tregs. Cells that are CD4+CD127^{low} have been shown to be CD25+ and Foxp3+. Another possible marker of Tregs is the HLA-DR+ cells. HLA-DR is a type of MHCII molecule, the expression of which, is generally restricted to APCs. However, it is known that CD4+ T cells may also express MHCII molecules, which when engaged with other T cells, mediate antigen unresponsiveness (LaSalle *et al.*, 1992; Lamb and Fledmann, 1982; Ko *et al.*, 1979). Separation based on HLA-DR+ was able to select out a population of cells that is CD25+ and Foxp3+ and mediates suppression on effector T cells by way of cell-to-cell contact dependent mechanisms (Baecher-Allan *et al.*, 2006). In short, HLA-DR based purification appears to have the ability to isolate natural Treg like cells.

Much of the attention has now shifted into the cellular level as many groups are trying to determine how Tregs alter target cell signalling. The latest findings suggest that natural Tregs inhibit T cell proliferation by uncoupling IL-2 signalling from proliferation, blocking the normal pathway of IL-2 stimulation of division (Duthoit *et al.*, 2005).

1.3.1.2 INDUCIBLE T REGULATORY 1 CELLS (T_R1s)

Antigen specificity is one of the most important distinctions between antigen induced Tregs (Tr1s) and other classes of regulatory T cells, such as the natural CD4+CD25+ Tregs (Mills and McGuirk, 2004). The presence of Tr1s was first reportedly generated only after repeated antigenic stimulation in the presence of IL-10, having the ability to prevent colitis (Groux *et al.*, 1997). IL-10 is an important cytokine in immune regulation because it has been

shown to not only have the ability to alter T_H1/T_H2 responses, but also dampen innate cell-mediated immune responses (Fiorentino *et al.*, 1991; O'Farrell *et al.*, 1998). Tr1 cells are known to proliferate slowly *in vitro* and are described as “anergic” as a result of their insensitivity to TCR activation and therefore inability to produce IL-2 even upon antigenic stimulation (Groux *et al.*, 1997). Because Tr1s are antigen-specific, antigen activation is required, at which point CD25 expression and cytokine production is induced. These Tregs are characterized to secrete high levels of IL-10, possibly alongside TGF- β and IL-5, with little or no IL-2 and IL-4 (Mills and McGuirk, 2004). As might be implied, antigen induced Tregs can further be divided into different types of antigen specific Tr1s (Mills and McGuirk, 2004). Tr1s have been shown to be important in mediating suppression towards self antigens (Arif *et al.*, 2004; Kitani *et al.*, 2000; Veldman *et al.*, 2006; Yudoh *et al.*, 2000; Hall *et al.*, 2002), and externally derived antigens such as nickel (Cavani *et al.*, 2000), insect venoms (Saloga *et al.*, 1999), and cat allergens (Reefer *et al.*, 2004). But while Tr1 activation is antigen specific, Tr1 mediated suppression is antigen non-specific, influencing other immune cells via the bystander effect (Thornton and Shevach, 2000). The bystander effect refers to the ability of cytokines that are produced to exert an effect on all cells in the vicinity in a paracrine manner. It has been shown that IL-10 is the main cytokine involved in inducing Tr1 proliferation and suppression of effector cells as both activities are observed to be abrogated upon the addition of neutralizing IL-10 antibodies (Bacchetta *et al.*, 2002). Interestingly, recent investigations have linked Foxp3 expression to the maintenance of Tr1s where the inhibition of this transcription factor converts Tr1 cells into T_H2 -like cells, indicating that Foxp3 might play an integral role in the decision to become Tr1 cells (Veldman *et al.*, 2006). How Foxp3 differentiates between different types of Tregs, since Foxp3 has been shown to be implicated in both natural CD4⁺CD25⁺ Treg and Tr1 cell differentiation, has yet to be elucidated.

1.3.1.3 T_H3 AND OTHER TYPES OF T_{REGS}

Like $Tr1s$, T_H3 cells are antigen induced or via some other method where the TCR is activated (possibly using anti-CD3 antibodies), leading to the characteristically high expression levels of TGF- β , alongside IL-4 and IL-10 (Akdis and Blaser, 2001; Chen *et al.*, 1994). Functionally, these cells have been shown to select for IgA production from mucosal B cells (Cazac and Roes, 2000). It has been shown that both TGF- β and IL-10 are required for the mediation of suppression, as the T_H3 cells are antigen non-specific, exerting bystander suppression on neighboring cells (Chen *et al.*, 1994; Weiner, 2001). Furthermore, CTLA-4 has been likewise implicated in suppression (Samoilova *et al.*, 1998; Fowler and Powrie, 2002). Hitherto this point, T_H3 has only been found associated with the mucosal environment.

Even less is known about the CD45RA and the CD45RO regulatory T cells (Seddiki *et al.*, 2006). These two markers are two splice variants of the same T cell surface marker, CD45. T cells in the thymus express CD45RO (Fukuhara *et al.*, 2002), which is converted into CD45RA once the cell migrate out of the thymus (Vanhecke *et al.*, 1995). Upon antigen activation, CD45RA reverts back to the CD45RO form (Merkenschlager *et al.*, 1988). CD45RO Tregs have high CD25 expression while CD45RA Tregs have a slightly lower level of CD25. Both types of Tregs have been shown to express high levels of Foxp3 and have suppressive activities on other T cells in functional assays (Seddiki *et al.*, 2006). Recent work by Valmori *et al.* indicate that these CD45RA expressing cells might be the precursors of natural Tregs, termed the natural naïve Tregs (Valmori *et al.*, 2005). Unlike natural Tregs, these cells have been shown to proliferate dramatically upon stimulation with autologous MHCII molecules, thus indicating that the TCR specificity of these cells is directed against the self. Clinically, CD4+CD25+CD45RA+ Tregs have shown to be significantly increased in patients with multiple myeloma (Beyer *et al.*, 2006). Furthermore, these Tregs have been shown to play a vital role in mediating clinical ulcerative colitis as total colectomy was able to restore the previously low levels of Tregs to that

observed in healthy patients (Furihata *et al.*, 2006). Combined, these results suggest that CD45RA⁺ Tregs are a true and distinct type of regulatory T cells that when abnormally increased or decreased, leads to immune dys-regulation.

Finally, there are also other types of regulatory T cells that are non CD4⁺. CD8⁺ Tregs have been theorized to exist (Weiner, 1997) having the ability to control the proliferation of CD4⁺ T cells in a HLA-E dependent fashion (Hu *et al.*, 2004). Furthermore, CD8⁺CD28⁻FOXP3⁺ cells have been implicated in being able to induce inhibitory receptors and dampening the expression of co-stimulatory molecules and adhesins (Manavalan *et al.*, 2004).

Table 2: Comparison of characteristics of the major types of Tregs

	T_R1 Cells	Natural CD4⁺CD25⁺ T_R Cells	T_H3 Cells
Antigen Specificity	Peripheral exogenous antigens	Self antigens in thymus	Gut antigens
CD25⁺ Expression	When activated	Constitutive high levels	Unknown
Mechanism of suppression	Cytokines (via IL-10, TGF- β) + Cell Contact?	Cell Contact (CTLA-4, m-TGF- β)	Cytokines (mainly TGF- β)
Surface Markers	CD4, possibly CD25?	CD4, CD25, GITR, CTLA-4	CD4 ⁺

1.3.2 REGULATORY B CELLS

Due to the B cell ability to influence T_H1/T_H2 responses, it is natural to suggest that perhaps there are specialized cells in the B cell population which mediates suppression. The first hint that certain B cells may have suppressive activity came in the model of autoimmune encephalomyelitis (Wolf *et al.*, 1996) as a small subset of B cells, mediated by IL-10 and TGF- β , facilitated recovery from EAE (Fillatreau *et al.*, 2002). Further evidence can be gleaned in other disease models. Lundy *et al.* found that B-1 cells, have regulatory activity which can modulate T_H2 response by decreasing cytokine production, CD4+ T viability and pulmonary inflammation in the cockroach allergen induced murine allergic asthma model (Lundy *et al.*, 2005). Similar hints of B cell influence over T_H1/T_H2 responses have been observed in many autoimmune models such as inflammatory bowel disease (Wei *et al.*, 2005a; Ostanin *et al.*, 2006), rheumatoid arthritis (Korganow *et al.*, 1999; Mauri *et al.*, 2003) and systemic lupus erythematosus (Lenert *et al.*, 2005; Brummel and Lenert, 2005), and in various infection models (Gillan *et al.*, 2005; Mangan *et al.*, 2004). Thus, the postulate that Bregs, B cells with regulatory function, might exist was made in early 2006 (Mizoguchi and Bhan, 2006). Bregs are theorized to balance T_H1/T_H2 response, down-regulate innate immune response and DC activity. Because B cells have been shown to generate both IL-10 and TGF- β (Harris *et al.*, 2000; Tian *et al.*, 2001), it is hypothesized that Breg activity is predominantly cytokine mediated but there are other mechanisms of suppression involving Breg production of antibodies or Breg prevention of CD40/CD40L co-stimulatory molecule ligation. At this point, however, a cautionary note should be made since the suppression of the T_H2 response may simply be the result of T_H1 response induction, and vice versa. Thus, one must keep in mind the distinction between suppressive activity that modulates the entire immune system by down-regulating both T_H1 and T_H2 responses, or tipping the balance of the T_H1/T_H2 response to preferentially select for one response over the other, as might be the case with Bregs.

1.3.3 MYELOID SUPPRESSOR CELLS

While it is clear that the concept of Bregs remains in its infancy, the possible existence of these cells suggests that each type of immune cells may have its own set of regulatory cells. From work done on macrophages, it is known that macrophages may be induced to be in either the pro-inflammatory/tumourcidal (Sinha *et al.*, 2005) (Bastos *et al.*, 2002; Mantovani *et al.*, 2002; Sinha *et al.*, 2005) or the anti-inflammatory/tumour supporting (M2) states (Mantovani *et al.*, 2002; Bastos *et al.*, 2002). It has been shown both *in vivo* and *in vitro* that M2 macrophages have the ability to confer T cell apoptosis by providing Signal 1 but not Signal 2 by down-regulating the expression of the CD80/CD86 co-stimulatory molecules (Orlikowsky *et al.*, 1999). In addition, many groups showed that in many cancer patients, there exist IL-1 β induced myeloid suppressor cells (MSC), which are the result of chronic inflammation. They function to assist tumour survival by inhibiting both CD4+ and CD8+ T cell activity (Kusmartsev and Gabrilovich, 2002; Serafini *et al.*, 2004; Bunt *et al.*, 2006). Clearly, there exists many types of regulatory or suppressor cells beyond those of regulatory T cells.

1.4 STAPHYLOCOCCUS AUREUS AND THE TOXIC SHOCK SYNDROME

TOXIN-1

Staphylococcus aureus is a Gram-positive spherical bacteria that reside commonly in human nasal passages. Most people carry this opportunistic organism and it is only when the host is immuno-compromised will this bacteria be of concern. The reason why *S. aureus* presents with such complications for the host is because of the wide range of virulence factors the bacteria possess, one of which is the ability to produce and release exotoxins. There are two different families of exotoxins that are produced by these bacteria – the pyrogenic toxin superantigen and the hemolysins (Dinges *et al.*, 2000). Within the family of the pyrogenic toxin superantigens

(PTSAGs) are such exotoxins as SEA, SEB and TSST-1, all of which are classified by their ability to cause fever and their superantigenic property. This superantigenic characteristic refers to the ability to non-specifically activate lymphocytes. Conventional antigens specifically activate T cells with epitopes that recognize that antigen, "turning on" about 0.01% of the total T cell population. Activation with a superantigen, on the other hand, is non T cell epitope specific as this toxin has the ability to bind the V β region on the T cell receptor (Marrack P and Kappler J., 1990; Kappler J et al., 1989b; Li H et al., 1999) and cross-linking it with the major histocompatibility complex class II (Labrecque *et al.*, 1993) molecule expressed on an antigen presenting cell (Labrecque *et al.*, 1993). SAg presentation does not require APC processing nor is it MHC-restricted (Kappler J et al., 1989a). In addition, co-stimulatory molecules play a pivotal role in sAg activation of T cells. CD80/86 on APCs interacting with CD28 on T cells provide one form of co-stimulatory signal (Kum *et al.*, 2002; Lane *et al.*, 1996; Muraille *et al.*, 1995) while CD40 on APCs with CD154 on T cells also provide the same signal (Kum *et al.*, 2002; Mehindate *et al.*, 1996; Jabara and Geha, 1996). Furthermore, various adhesion molecules, such as intercellular adhesion molecule-1 (Tessier *et al.*, 1998) and lymphocyte-function-associated antigen (LFA-1), have also shown to be involved (Ijichi *et al.*, 1996; See *et al.*, 1992; Tessier *et al.*, 1998). The result of this direct cell-to-cell interaction is the non-specific activation of 5-25% of all T cells where these cells are simultaneously induced to undergo proliferation, generating a massive amount of T_H1 pro-inflammatory cytokines such as IL-1, IL-2, IL-12, TNF- α and IFN- γ (Labrecque *et al.*, 1993; See and Chow, 1992), regardless of the peptide that is presented on the MHCII molecule (Jupin *et al.*, 1988). Of the PTSAGs, TSST-1 is the one exotoxin that has the ability to traverse the vaginal mucosal barrier and enter the blood stream. Once this sAg enters the circulatory system, the toxin becomes systemic, traveling unregulated throughout the body. It then will induce a massive pro-inflammatory response, elevating production of inflammatory mediators, such as cytokines, chemokines, and chemoattractants.

Soon, blood vessels all over the body will vasodilate as a result of the activation of the immune system, and allow immune cells to migrate from the blood stream into the surrounding tissues (Bohach GA *et al.*, 1990; Kotzin BL *et al.*, 1993). However, as the vessels become “leaky”, plasma, or the liquid portion of blood, also diffuses out into the tissues, leading to edema and, more importantly, a marked drop in blood pressure. This sudden drop in blood pressure hinders circulation, leading to oxygen and nutrient deprivation at the organs as the heart cannot compensate for this drastic change in pressure. As a result, the patient experiences what is termed, toxic shock syndrome, leading ultimately to multiple organ failure and finally, death.

1.5 TSST-1 PRIMED T_{REGULATORY} CELLS

In the arena of antigen induced Tregs, there are many groups that have used a multitude of different proteins, everything ranging from bacterial to plant antigens. However, the significance of using sAg to induce the production of Tregs is that any T cells with the same TCR-V β region will be activated. Essentially, there will be a large number of polyclonal Tregs generated (Bohach GA *et al.*, 1990). While a single dose of Staphylococcal superantigen induces a strong T_H1 response and a second dose generates a memory like response (Florquin and Goldman, 1996; Sundstedt *et al.*, 1997; Miller *et al.*, 1999), three doses of superantigens have been shown to be effective in generating T cells that have suppressive properties, marked by a drastic increase in IL-10 production (Florquin and Goldman, 1996; Sundstedt *et al.*, 1997; Florquin *et al.*, 1994; Miller *et al.*, 1999). Some papers have reported that *Staphylococcus aureus* SEA sAg can induce Tregs that are anergic and hypoproliferative (Sundstedt *et al.*, 1997; Grundstrom *et al.*, 2003; Miller *et al.*, 1999). Others have reported the same using SEB, which binds to a different V β than that of SEA in the murine model used (Noel *et al.*, 2001; Feunou *et al.*, 2003). These cells displayed the expected anti-inflammatory cytokine profile, such as high IL-10, little or no IL-4 etc (Florquin *et al.*, 1994; Hasko *et al.*, 1998; Miller *et al.*, 1999). Tr1

cells secrete IL-10 and TGF- β (Akdis and Blaser, 2001; Jutel *et al.*, 2003; Akdis and Blaser, 1999), the neutralization of which enhances T_H2 IL-4 and IL-5 production. When Tr1 suppression is blocked, or when the ratio of T_H2 to Tr1 cells in co-culture increases, a T_H2 response is markedly increased (Florquin *et al.*, 1994; Akdis *et al.*, 2004). TGF- β also appears to play a role in decreasing T_H2 responses. Furthermore, these sAg induced Tregs have the ability to down-regulate IFN- γ (Huang *et al.*, 2005), TNF- α and IL-2 production by T_H1 cells (Sundstedt *et al.*, 1997). This suggests that while Tr1 cells generate an immunosuppressive cytokine profile, the IL-10 and TGF- β cytokines made are involved in the mediation of suppression (Maloy *et al.*, 2003; Levings *et al.*, 2002a). In addition, surface CTLA-4 (Read *et al.*, 2000) and PD-1 (Nishimura *et al.*, 1999; Carter *et al.*, 2002; Raimondi *et al.*, 2006) both have been implicated as ways cell-to-cell contact can be made. Like SEA and SEB, TSST-1 is also capable of inducing Tregs (Cameron and Chow, 2005; Li H and Chow, 2006). However, much less is known about the role of TSST-1 than that of SEA/SEB generated regulatory T cells.

Past experiments by other members from the Chow Lab showed that TSST-1 generated CD4⁺ T cells down-regulate the proliferation and function of other effector T cells (Cameron and Chow, 2005; Li H and Chow, 2006). What is known about these cells is that they, in the murine *in vitro* setting, suppress the production of pro-inflammatory cytokines by T_H1 and T_H2 cells while the production of IL-10 was markedly increased. These antigen induced T cells have also been shown to have the ability to suppress target T cell activation and proliferation. The mechanism through which suppression is mediated involves IL-10 as the neutralization of IL-10 *in vivo* reverses the suppression of effector T cell pro-inflammatory cytokine production. The contribution of TGF- β to suppression is less clear as only one group has shown that the suppressive activity of SEA induced Tregs is partially reversed when neutralizing TGF- β antibodies are added (Miller *et al.*, 1999). Thus, much work still needs to be done to fully

elucidate the involvement, if any, of TGF- β to TSST-1 primed CD4⁺ T cell suppression. Furthermore, whether or not other cytokines or cell-to-cell contact are involved remains to be seen. Certainly, all the possible interactions discussed previously regarding natural CD4⁺CD25⁺ Tregs – PD-1, CTLA-1, GITR – are also possible means of mediating suppression for the TSST-1 primed CD4⁺ T cells. To summarize, TSST-1 induced CD4⁺ T cells may contain antigen induced Tregs, which are characterized to be suppressive cells that mediate their activity mainly via IL-10, thus exerting a regulatory role on other T cells.

Perhaps then, it is strange for *S. aureus* to employ such a deadly toxin in an attempt to generate regulatory T cells. However, it appears that TSST-1 exerts a dose dependent effect on lymphocytes. At high doses, TSST-1 is found to induce apoptosis while at low doses, it does not (Hung and Chow, 2005). In addition, a single challenge of low dose TSST-1 leads to a T_H1 response while repeated low doses of TSST-1 generates a suppressive response. Furthermore, TSST-1 induced CD4⁺ T cells have the ability to be hyperproliferative, unlike other regulatory T cells discovered to this point, and to be antigen non-specific, or at least, has a wide range of antigen specificity due to its superantigenic property. In short, while there are so many different types of Tregs and even more ways to activate them, TSST-1 primed CD4⁺ T cells were chosen to be the topic of study because these cells are polyclonal, hyperproliferative (which makes them easy to propagate and handle in culture) and are easily generated both *in vivo* (Cameron and Chow, 2005; Li H and Chow, 2006) and *in vitro* (Kum and Chow, 2005). These qualities make the TSST-1 superantigen a prime candidate to be used to induce Tregs that have broad and easily inducible suppressive functions. While the suppressive effect of Tregs, whatever the type may be, on CD4⁺ T cells has been studied for many years, scientists have only recently looked into the effect TSST-1 generated Tregs have on B cells.

1.6 T_{REGS} AND B CELLS

Hitherto this point, the bulk of the work in the field of regulatory T cell research has focused on the interaction between Tregs and their ability to down-regulate other T cells. It is only within the last few years that a few publications investigating the interaction between Tregs and B cells have appeared. Certainly, there are several empirical observations that make this investigation worthwhile. In the clinical setting, it has long been observed that a large number of patients (85%) who suffered from *S. aureus* induced toxic shock make very little or no TSST-1 specific antibodies, resulting in a propensity for the recurrence of toxic shock syndrome (Schlievert, 1993; Bohach GA *et al.*, 1990). Patients with toxic shock also demonstrate a precipitous drop in live B cell count, suggesting that TSST-1 specific B cell viability is altered by TSST-1 or its product, leading ultimately to the attenuation of B cell function. Furthermore, TSST-1 is a difficult antigen to raise antibodies against *in vivo* in small animal models, such as rabbits, which likewise suggests that TSST-1 has some hand in altering B cell function. From a theoretical standpoint, B cells do have the ability to interact with Tregs since B cells have many receptors which would bind cytokines or other mediators produced by Tregs. In addition, there are many surface ligand/receptor interactions that can take place between the two types of lymphocytes. It is thus hypothesized that these TSST-1 primed CD4⁺ T cells can suppress B cell activation and activity.

Within the available body of literature, there have been five different reports studying Treg interaction with B cells. In 2003, Janssens *et al.* showed that cytolytic natural CD4⁺CD25⁺ Tregs lysed antigen presenting B cells by Fas-Fas ligand interaction in an epitope-specific manner (Janssens *et al.*, 2003). This study was momentous for it linked, for the first time, regulatory T cells and B cells together directly, where the suppression is specifically manifested through cell-to-cell dependent contact to induce apoptosis of certain B cell clones. However, a possibly conflicting report surfaced three years later as Zhao *et al.* showed that activated

CD4+CD25+ Tregs selectively suppress the proliferation of B cells and kill them via perforins and granzymes and not via the Fas-Fas ligand pathway in murine cells (Zhao *et al.*, 2006). At this point, it must be pointed out that while both groups focused on CD4+CD25+ Tregs, these Tregs were activated via different protocols, which likely explains the apparent conflict in B cell response. The role that Foxp3 plays in generating Tregs was taken into consideration by Lim *et al.* as they showed that natural CD4+CD25+ Foxp3+ Tregs exert a direct suppressive effect on B cell antibody production and class switching in human tonsil cells (Lim *et al.*, 2005). For the first time, this paper indicated that B cells are affected in their function where antibody production is hindered by Tregs in addition to the reported effect on B cell proliferation and viability. Likewise in human cells, Satoguina *et al.* showed that T regulatory-1 cells induced the production of IgG4 (a human only T_H2-type antibody) induced via an IL-10 dependent mechanism (Satoguina *et al.*, 2005). This paper found that Tr1s select for a T_H2 B cell response mediated via IL-10. Fields *et al.* also investigated the same interaction but in an *in vivo* murine model where they showed that natural CD4+CD25+ Tregs inhibit the maturation, but not the initiation, of an antibody response (Fields *et al.*, 2005). Here, they found that natural Tregs have the ability to down-regulate auto-IgM-antibody production. Kitani *et al.* generated self-MHC-reactive regulatory T cells that suppressed B cells via TGF- β production (Kitani *et al.*, 2000). In short, all these studies point to the importance of the type of regulatory T cells and suggest that there are many different types of these cells which exert different effects on B cells via different mechanisms. The unifying theme remains the same - that Tregs exert a suppressive effect on B cell activation, viability and function in both *in vitro* and *in vivo* settings.

The work that has been done with natural Tregs and Tr1 cells allow a perfect platform from which TSST-1 primed CD4+ T cells may be examined in their interaction with B cells, since previous work with these different types of Tregs gives some ideas of how B cells might respond to TSST-1 primed CD4+ T cells. It is hypothesized that TSST-1 primed CD4+ T cells

exert a suppressive effect on B cell proliferation, viability and antibody production via both cytokine and cell-to-cell contact mediated mechanisms. In this project both *in vivo* and *in vitro* models were used to study the effect of TSST-1 induced Tregs on B cells. Trinitrophenol-keyhole limpet haemocyanin (TNP-KLH), containing a carrier protein (KLH) conjugated to a hapten (TNP), was used to hyperimmunize mice to generate a T_H2 response (Marrack and Kappler, 1975). TSST-1 primed CD4⁺ T cells were generated in another mouse and introduced adoptively into the TNP-KLH immunized mice to observe the effect of these T cells on B cell TNP-specific antibody response. The TSST-1 primed CD4⁺ T cell interaction with B cells was further studied *in vitro* so that the contribution of cytokines, specifically IL-10 and TGF- β , and cell-to-cell contact to the suppression could be assessed. Whether TSST-1 primed CD4⁺ T cells interact directly with B cells or indirectly via interaction with T_H2 cells was also examined. Finally, an attempt was made to separate out the CD25⁺ and CD25⁻ group to assess the effect of TSST-1 primed CD4⁺ T cells on B cells. In particular, IgG1, IgG2a, IgM and IgE will be focused on because IgG1 and IgE production is the result of T_H2 cell activation of B cells while IgG2a results from T_H1 cell activation.

The fight against autoimmune diseases, both mild and severe, can depend largely on the ability to control rampant B cells. One such example are allergies, since allergic responses are the result of IgE overproduction by B cells. Many groups have hitherto looked at the application of allergen induced Regulatory T cells in allergen-specific immunotherapy (Akdis and Blaser, 2001; Akdis *et al.*, 2006; Akdis *et al.*, 2005; Jutel *et al.*, 2003). Some have found that Tregs are able to prevent the development of T_H2 based allergic responses (Cottrez *et al.*, 2000; Akdis *et al.*, 2004), while others on T_H1 based allergic responses (Taylor *et al.*, 2006). Elucidating the mechanism of B cell suppression could potentially lead to new techniques in treating allergies by suppressing IgE production. Similarly, an improved understanding of B cells can lead to answers to the question of B cell memory (McHeyzer-Williams and Ahmed, 1999). Many studies have

shown that memory is conferred when a pathogen is allowed to persist at a low concentration after an infection (Belkaid *et al.*, 2002). Thus, insight into how B cells are down-regulated can lead to the production of better vaccines allowing for longer lasting immunity.

2.0 WORKING HYPOTHESIS & SPECIFIC AIMS

2.1 HYPOTHESIS

TSST-1 induced CD4⁺ T cells will exert a suppressive effect on B cell proliferation, viability and function while PBS induced CD4⁺ T cells will not.

2.2 SPECIFIC AIMS

- 1) To examine the effect of TSST-1 induced CD4⁺ T cells on TNP-KLH specific T_H2 antibody response by B cells *in vivo*.
- 2) To examine if TSST-1 induced CD4⁺ T cells have a suppressive effect on B cells *in vitro*:
 - 2.1) B cell proliferation
 - 2.2) B cell viability
 - 2.3) B cell antibody production
- 3) To examine the contribution of the CD25⁺ vs. CD25⁻ subpopulations of TSST-1 induced CD4⁺ T cells to the suppressive effect on B cells
- 4) To examine the mechanism by which this suppression is mediated:
 - 4.1) Via cytokine production – IL-10 and TGF- β
 - 4.2) Via cell-to-cell dependent interaction
 - 4.3) Via bystander effect

2.3 RATIONALE

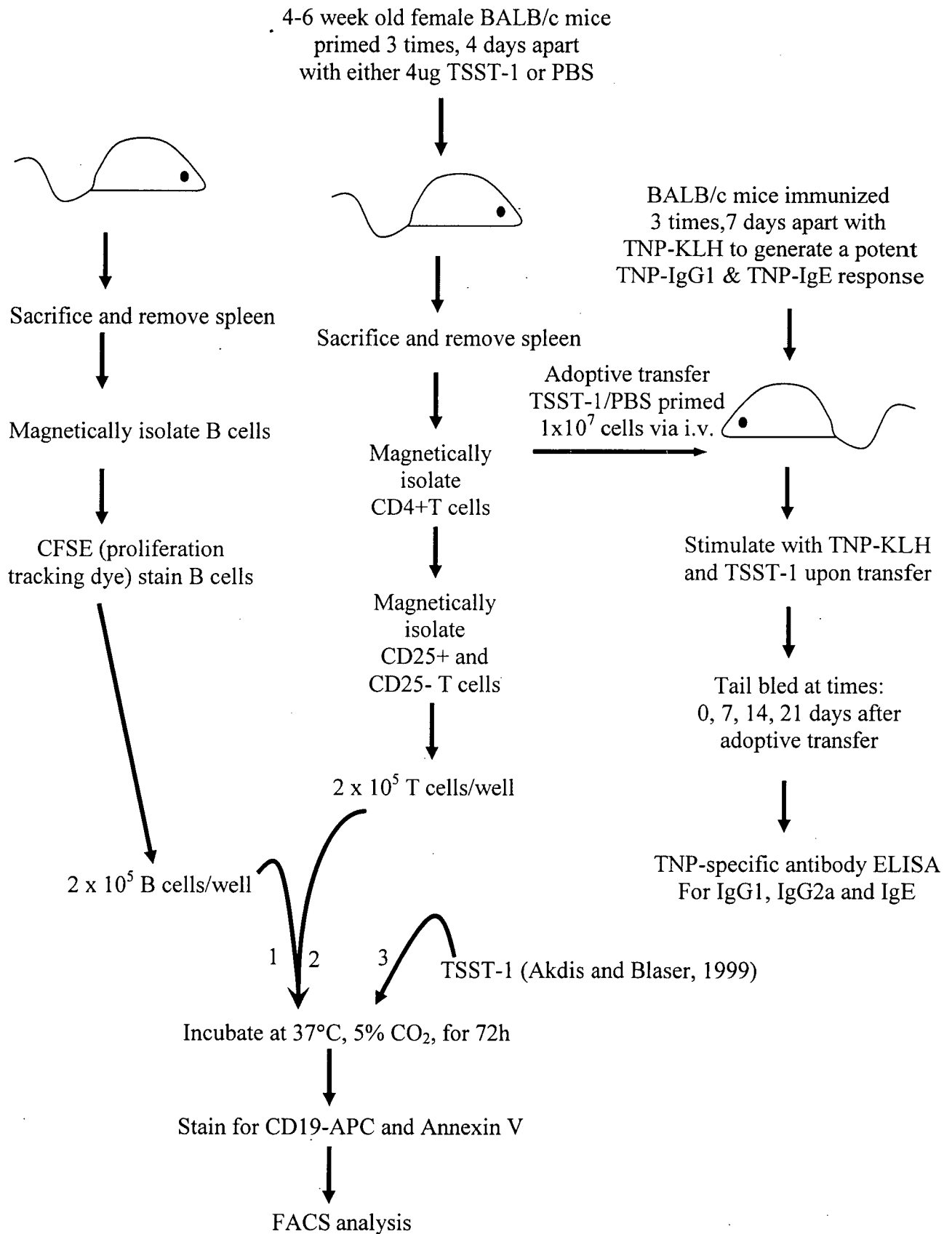
B cell over-activity is the root of many diseases, ranging from allergic asthma to multiple sclerosis. Recently, it has been shown that repeated low dose challenge with the staphylococcal superantigen, TSST-1, induces CD4⁺ T cells that exert suppressive function on T cells. In light of clinical observations that patients suffering from TSST-1 induced toxic shock exhibit low

viable B cell count along with a relative inability to generate TSST-1 specific antibodies, the question is asked whether TSST-1 induced CD4⁺ T cells have any suppressive effect on B cells, and if so, what might be the mechanism that mediates this effect.

2.4 OVERALL EXPERIMENTAL APPROACH

Described in a flow chart in Figure 1, repeated low dose injections of TSST-1 were made in BALB/c mice and CD4⁺ T cells were obtained from the spleen. Here, this project was approached from two directions. First, BALB/c mice were hyperimmunized with TNP-KLH, a T_H2 response biased compound, and TSST-1 primed CD4⁺ T cells were adoptively transferred to examine if TSST-1 primed CD4⁺ T cells have any effect on altering TNP-specific antibody responses. Blood was collected at day 0, 7, 14 and 21 after adoptive transfer and TNP-specific antibodies were assessed using ELISA. The second approach was to set up a co-culture system *in vitro* to examine how TSST-1 primed CD4⁺ T cells affect B cell proliferation, viability and antibody production. CD4⁺ T cells were also separated into their respective CD25⁺/⁻ constituents to observe how each subset influences B cells. Finally, the mechanism of how this suppressive effect is mediated was investigated by asking what the contribution of cytokines, cell-to-cell contact and the bystander effect make on the interaction between the two types of lymphocytes.

Figure 1: Flow chart to describe the general experimental approach taken in this project



3.0 EXPERIMENTAL MATERIALS AND METHODS

3.1 ANIMALS

Female 8-12 week old BALB/c mice were obtained from Charles River Biotechnology Corporation (Wilmington, MA, USA). Upon arrival, all mice were given 5-7 days to acclimate before experiments were performed. All mice were housed in common mouse pathogen free conditions at the UBC Jack Bell animal facility, following guidelines provided by the Canadian Council on Animal Care (protocol A03-0081). Five animals were housed per cage using standard sterile feed, water, bedding and plastic enclosure with a hepa filter to remove pathogens from the air.

3.2 TNP-KLH PREPARATION AND INJECTIONS

TNP-KLH (Biosearch Technologies, CA, USA) was precipitated on alum beads and diluted using PBS so that 50 µg of TNP-KLH was injected per mouse. Likewise, 50 µg of alum, diluted in PBS was also prepared for each 200 µl injection per mouse. A 27 gauge needle (BD Biosciences, San Diego, CA) was used to inject TNP-KLH-alum or alum alone intraperitoneally at 7-day intervals for a total of 3 rounds of injections.

3.3 TSST-1 PREPARATION AND INJECTIONS

Recombinant TSST-1 was isolated and purified in-house from *Staphylococcus aureus* cell free supernatant as previously described (Kum *et al.*, 1996) and subsequently stored at -70°C until use. 4 µg of TSST-1, diluted with PBS up to a volume of 100 µl, was injected per mouse. A 31 gauge needle was used to inject TSST-1 or PBS subcutaneously at 4-day intervals for a total

of 3 rounds of injections. The same volume of PBS was injected per mouse in control experiments.

3.4 ADOPTIVE TRANSFER OF CD4⁺ T CELLS AND SERUM COLLECTION

Mice immunized with TSST-1 or PBS were sacrificed and spleens purified for CD4⁺ T cells (method described below in the cell purification section). TSST-1 or PBS CD4⁺ T cells were then diluted to a concentration of 1×10^7 cells/200 μ l with RPMI and 200 μ l of cell suspension was then adoptively transferred via the tail vein into either TNP-KLH-alum or alum hyperimmunized mice. Tails of recipient mice were bled prior to injection. Following transfer, mice were then injected with 200 μ l of 50 μ g TNP-KLH i.p. and either 100 μ l of 4 μ g TSST-1 or 100 μ l of PBS was i.v. injected. Blood was taken from tail bleeds 7, 14 and 21 days after adoptive transfer.

3.5 ISOLATION AND PURIFICATION OF SPLENOCYTES BY MAGNETIC SEPARATION

Mice were sacrificed using cervical dislocation. Spleens were removed and homogenized through a 100 μ m mesh using a plunger. The single cell suspensions were subsequently washed, centrifuged at 800 g for 7 min and resuspended in RPMI 1640 (StemCell Technologies Inc., Vancouver, BC). Gey's balanced salt lysis solution was used to lyse red blood cells. CD4⁺ T cells were then isolated from splenocytes using CD4⁺ negative magnetic cell selection while CD19⁺ B cells were separated by negative selection. Separations were carried out according to the manufacturer's instructions (StemCell). CD25⁺ T cell positive selection was also carried out

by magnetic cell separation using LD columns, an OctoMACs magnet, BioAb cocktail against CD25 and α -biotin beads (Miltenyi Biotech Inc., Auburn CA). Effluent cells were resuspended in complete growth media. Complete growth media was made using RPMI 1640 (StemCell) supplemented with 10% FBS (Hyclone, Logan, UT), 10 mM HEPES (StemCell), 2 μ g/mL polymyxin B (Sigma, St Louis, MO), 1 mM pyruvate (StemCells), 100 U/mL penicillin/100 mg/mL streptomycin (StemCell), 2 mM L-glutamate (StemCell) and 50 mM β -mercaptoethanol (Sigma).

3.6 CELL CULTURE AND CFSE STAINING

B cells were stained using CFSE proliferation tracking dye (Molecular Probes Inc, Eugene OR) at a concentration of 1 mM per 1×10^6 - 1×10^8 cells for 10 min at room temperature. Dye uptake was stopped using an equal volume of FCS and washed using RPMI. Resultant cells were resuspended with complete growth media, plated in 96 multiwell gas permeable U-shaped plates (VWR) at a density of 2×10^5 cells/well. Purified CD4+, CD25+ or CD25- T cells were also plated into the respective wells at 2×10^5 cells/well. Finally, wells were stimulated with 1 nM of TSST-1 or RPMI control and cultured at 37°C, 5% CO₂ for 24, 48 or 72 h.

3.7 NEUTRALIZATION ASSAY

CD4+ T cells (2×10^5 cells/well) were co-cultured with equal numbers of B cells with or without 1 nM TSST-1 stimulation in 96 well plates. 2 μ g/ml of α IL-10 receptor mAb (IBI.2 raised in-house in rabbits) and/or 2 μ g/ml of α TGF- β mAb (R&D Systems, Minneapolis, MN) were added to specific wells. 1 μ g/ml of GL113 (raised in-house in rabbits) was used as the α IL-

10 receptor isotype control while equal volume of rabbit serum was used as the α TGF- β isotype control. Growth medium was added to make the final volume up to 200 μ l/well.

3.8 TRANSWELL STUDY

1×10^6 B cells stained with CFSE and 1×10^6 naïve CD4⁺ T cells were first plated in 24 multiwell plates (VWR). A semi-permeable filter with 0.4 μ M pore size was then placed into the wells (VWR). Finally, 1×10^6 cells/well of either TSST-1 or PBS primed CD4⁺ T cells were added on top of the filter. 1 nM of TSST-1 was then added to the wells. The final volume of every well amounted to 1 ml.

3.9 BYSTANDER SUPPRESSION ASSAY

TSST-1 or PBS primed CD4⁺ T cells were cultured *in vitro* with or without the stimulation of 1 nM TSST-1 for 72 h. Supernatants were collected and stored at -70°C. Naïve mice were sacrificed and B cells purified and stained with CFSE. 2×10^5 B cells/well were seeded into 96 multiwell plates in the presence of 50 μ l of supernatant from the respective CD4⁺ T cell cultures above. Finally 1 μ g/ml of the B cell mitogen, lipopolysaccharide (LPS) isolated from *Escherichia coli* 055:B5 (Sigma), was added to stimulate B cell proliferation.

3.10 IL-2, IL-4 AND IL-10 CYTOKINE ELISAS

IL-2, IL-4 and IL-10 cytokine assays were performed using commercially available ELISA kits (BD Pharmingen, San Diego, CA). Each well of the Immunolon 1B polystyrene 96

well ELISA plates (VWR) were coated with 50 μ l of α -murine cytokine antibodies (IL-2 at 8 μ l/ml, IL-4 at 4 μ l/ml, and IL-10 at 8 μ l/ml), diluted in carbonate buffer, pH 9.6 and incubated overnight at 4°C in a humidified environment. PBS with 0.05% Tween (Sigma) was used to wash plates three times before blocking with 10% FBS in PBS for one hour at room temperature. Standards were prepared using two-fold serial dilutions at a starting concentration of 1000 ng/ml and incubated overnight at 4°C. Samples were diluted 1 in 2. Detection antibodies (IL-2 at 1 μ l/ml, IL-4 at 4 μ l/ml, and IL-10 at 4 μ l/ml) were diluted in blocking solution and left at room temperature for 1 h. Streptavidin-HRP (BD Pharmingen) was then added, after three washes, at a dilution of 1:1000, and samples were incubated in the dark at room temperature for 30 min. Plates were developed using the tetramethylbenzidine (TMB) substrate reagent A&B system (BD Pharmingen) and the reaction was stopped using 50 μ l of 1 M H_3PO_4 per well. All plates were read at OD₄₅₀ with correction at OD₅₇₀ using a Dynatech MR 5000 ELISA plate reader (Dynatech Laboratories Inc., Chantilly, VA). The MJS BioLynx software (Brockville, ON) was used for the readout.

3.11 QUANTIFICATION OF TOTAL SUPERNATANT AND SERUM TNP-SPECIFIC ANTIBODIES BY ELISAS

Total supernatant antibody ELISAs were performed using 30 μ g/ml of α -mouse-IgG1, IgG2a, IgM or IgE (BD Pharmingen) to coat 96 multiwell ELISA plates (Immunolon 1B, VWR). Standards were prepared using mouse IgG1, IgG2a, IgM or IgE, serially diluted 7 times starting at the first dilution concentration of 1000 pg/ml (BD Pharmingen). Samples were not diluted. 50 μ l of detection antibody was added to the wells at a 1:10,000 dilution for biotinylated α -murine-IgG1, 1:1,000 for biotinylated α -murine-IgG2a, 1:10,000 for biotinylated α -murine-IgM and

1:1,000 for biotinylated α -murine-IgE antibodies (BD Pharmingen). For detecting TNP specific antibodies, 96 well ELISA plates were coated with 30 μ g/ml of either α -mouse-IgG1 or α -mouse-IgE. 100 μ g/ml of TNP-KLH was coated for the IgG2a ELISA using PBS, pH 7.2 as the coating buffer (Biosearch). The concentration standard used for the total antibody ELISA was used here as well. Serum samples were diluted 1 in 1000. For IgG2a, 1:1000 dilution of α -mouse-IgG2a antibody was used. For IgG1, 5 μ g/ml and 25 μ g/ml concentrations of TNP -biotin were used (Biosearch).

3.12 VIABILITY ASSAY, SURFACE STAINING AND FLOW CYTOMETRY

After incubation, cells were washed with 1% FBS in PBS and centrifuged for 7 min at 1,500 RPM. 1:100 dilution of α CD4-PE, α CD19-APC, α IgM-PE-Cyc7 or α CD25-biotin with Strep-Cyc (all from BD Pharmingen except for α IgM-PE-Cyc7, which was from eBiosciences, San Diego, CA) were added to stain the cells for various surface markers prior to assessment using flow cytometry. Cell viability, as well as early and late apoptosis, were assessed using Annexin V following manufacture's instructions (BD Pharmingen) and 7-AAD following manufacture's instructions (EMD Biosciences, San Diego, CA). The BD FACS Calibur flow cytometer and Cell Quest software package (Becton Dickinson, San Jose, CA) were used for FACS sample data acquisition and the WinMDI version 2.8 software (Scripps Research Institute) was used for flow cytometric data analysis.

3.13 STATISTICAL ANALYSIS

All graphs were generated using the Graph Pad Prism 4.0 software and are displayed with means and standard error of the means (SEMs). Two-tailed Student's t tests were performed using the same software unless otherwise stated. P-values < 0.05 are deemed significant and denoted with *. P-values < 0.01 are denoted with ** and p-values < 0.001 are denoted with ***.

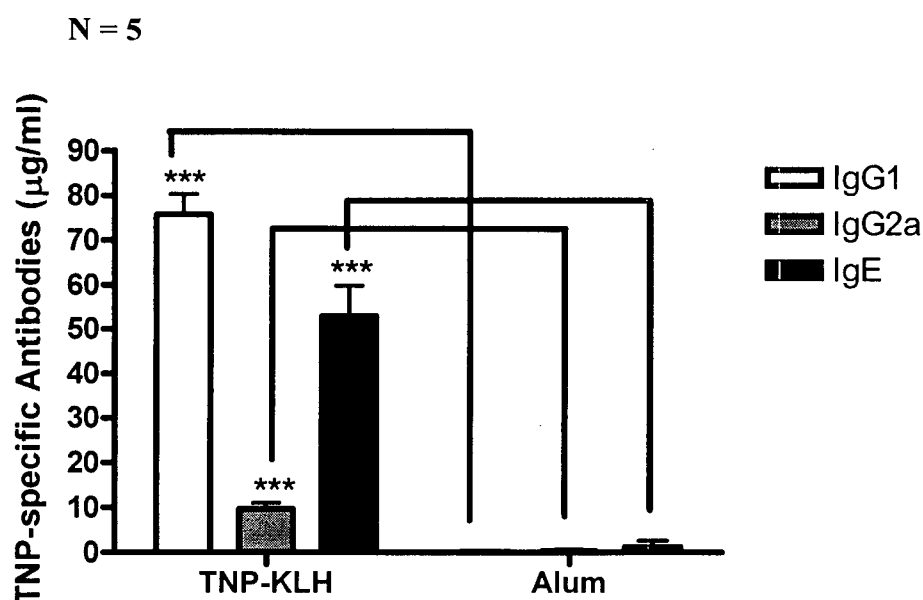
4.0 RESULTS

4.1 EFFECT OF ADOPTIVELY TRANSFERRING TSST-1 VS. PBS PRIMED CD4+ T CELLS ON TNP-KLH SPECIFIC ANTIBODY RESPONSES *IN VIVO*

In this study, the effect of adoptively transferred TSST-1 primed CD4+ T cells on BALB/c mice producing a strong TNP specific antibody response was assessed *in vivo*. At 7, 14 and 21 days following transfer, the production of TNP-specific IgG1, IgG2a and IgE antibodies were quantified. IgG2a is associated with a T_H1 response while IgG1 and IgE are associated with a T_H2 response.

TNP-KLH was chosen as the antigen with which to hyperimmunize mice because this compound is well established in being able to generate a potent T_H2 response, characterized by elevated production of IgG1 and IgE (Kohm *et al.*, 2000; Rizzo *et al.*, 1995; van den Eertwegh *et al.*, 1994). In essence, TNP-KLH hyperimmunization allows for the generation of an allergy-like model to study the possible suppressive effect of TSST-1 primed CD4+ T cells on animals with a strong T_H2 mediated allergic response. The first step in this study was to generate a TNP-KLH hypersensitivity model. In Figure 2, the data confirms that repeated i.p. injections of 50 µg TNP-KLH-alum in BALB/c mice can generate a potent T_H2 response 7 days post-immunization, culminating in high levels of TNP-specific IgG1 and IgE (IgG1: 90 µg/ml vs. 0.2 µg/ml, $p < 0.0001$; IgE: 53 µg/ml vs. 1.3 µg/ml, $p < 0.0001$) while IgG2a is elevated to a lesser degree (9 µg/ml vs. 0.4 µg/ml, $p = 0.0002$). Hyperimmunization with alum did not generate any detectable amounts of IgG1, IgG2a or IgE TNP-specific antibodies.

Figure 2: Effect of repeated TNP-KLH i.p. injections, 3 times, 7 days apart on the generation of TNP-specific antibodies. Repeated i.p. injections of TNP-KLH generated high levels of TNP-specific IgG1 and IgE antibodies and, to a lesser degree, TNP-specific IgG2a antibodies. TNP-KLH was precipitated on alum beads and 50 μ g of TNP-KLH-alum diluted in PBS was injected into 5 mice while an equal volume of alum beads, also diluted in PBS, was injected into another 5 mice. 7 days after the final injection, all mice were bled via the tail vein and serum TNP-specific IgG1, IgG2a and IgE antibodies were measured by ELISA. The graph was generated using the mean and the SEM while all statistical analyses were done using two-tailed Student's t tests.



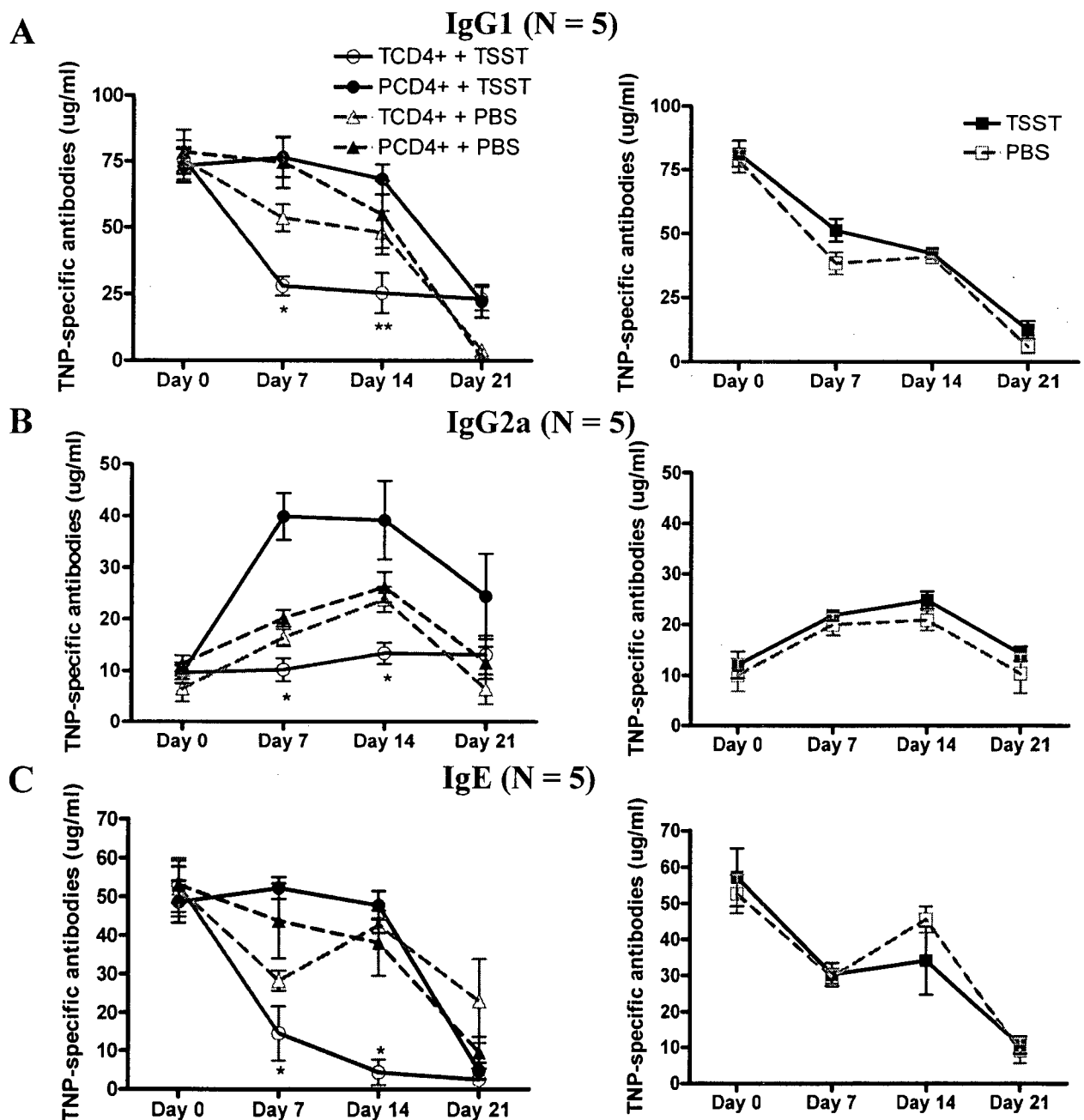
The next step was to generate TSST-1 primed CD4⁺ T cells which were then adoptively transferred into TNP-KLH-alum immunized mice. Previous work conducted in the Chow lab demonstrated that repeated s.c. injections with 4 µg of TSST-1 in BALB/c mice generated CD4⁺ T cells which display a type 1 regulatory T cell (Tr1) phenotype that suppresses cytokine and proliferative responses from naive effector CD4⁺ T cells (Cameron and Chow, 2005). Here, the same method was utilized to generate TSST-1 and PBS primed CD4⁺ T cells. The effect that TSST-1 primed CD4⁺ T cells may have on B cells was thus able to be observed in this model by tracking the changes that occur in antibody production following the adoptive transfer of these *in vivo* generated suppressive T cells. TSST-1 or PBS primed CD4⁺ T cells were then adoptively transferred into TNP-KLH hyperimmunized mice along with a booster dose of TNP-KLH and an activating dose of TSST-1. Mice were tail bled at times 0, 7, 14, and 21 days following adoptive transfer and sera were analyzed for TNP-specific IgG1, IgG2a and IgE by ELISA.

First comparing mice that received the TSST-1 vs. PBS s.c. injection indicates that no significant alteration was made to TNP-specific antibody production when TSST-1 was introduced (Fig.3., right panels). Both groups of mice show a natural decline of TNP-KLH antibodies to nearly undetectable levels 21 days after the last injection of TNP-KLH. Compared to the TSST-1 group, the mice that received TSST-1 primed CD4⁺ T cells along with the activating dose of TSST-1 showed a quicker and more dramatic drop in all antibody isotype levels tested, suggesting that the adoptive transfer of TSST-1 primed CD4⁺ T cells has the ability to suppress B cell function. This effect is TSST-1 specific since mice receiving TSST-1 primed CD4⁺ T cells with or without the activating dose of TSST-1 are significantly different. Further comparison between TSST-1 induced CD4⁺ T cells with PBS primed CD4⁺ T cells shows that TSST-1 priming specifically was able to generate CD4⁺ T cells that have the ability to suppress TNP-specific IgG1 (Day 7: $p < 0.05$; 14: $p < 0.05$, Fig.3.A.), IgG2a (Day 7: $p < 0.05$; 14: $p < 0.05$, Fig.3.B.) and IgE (Day 7: $p < 0.05$; 14: $p < 0.05$, Fig.3.C.) antibody production

significantly for up to 14 days. Thus, the injection of these TSST-1 primed CD4⁺ T cells, when activated with TSST-1, were able to exert a suppressive effect on B cell TNP-specific IgG1, IgG2a and IgE production. Because IgG1 and IgE isotypes are associated with a T_H2 response and IgG2a is associated with the T_H1 response, these results suggest that TSST-1 primed CD4⁺ T cells, and not PBS primed CD4⁺ T cells are able to “turn off”, or at least tone down, both T_H1 and T_H2 type immune responses. Evidently, these TSST-1 primed CD4⁺ T cells appear to have acquired, through repeated low dose priming, the unique property of being able to not only dampen T cell but also B cell function, something that the PBS primed CD4⁺ T cells do not do. In fact, PBS priming seems to generate CD4⁺ T cells that induce an IgG2a dominant B cell response. Again, this effect is TSST-1 specific since mice that were injected with PBS primed CD4⁺ T cells without an activating dose of TSST-1 did not show an increase in IgG2a production. Certainly, this finding is consistent with the fact that TSST-1 stimulation induces a T_H1 response.

Interestingly, the mice receiving PBS primed CD4⁺ T cells with an activating dose of TSST-1, when compared to mice receiving TSST-1 alone, showed a great difference in that the adoptive transfer of PBS primed CD4⁺ T cells resulted in higher TNP-specific antibodies. This observation suggests one of two possibilities – that repeated PBS injections into mice effect CD4⁺ T cells in some way, enhancing their ability to induce B cell antibody production, or that the process of adoptive transfer itself has an effect on antibody production. Regardless, the comparison between TSST-1 and PBS primed CD4⁺ T cells would control for these experimental factors and since there is a significant difference between the two, it would appear that TSST-1 primed CD4⁺ T cells exert a suppressive effect on B cell function *in vivo*.

Figure 3: Effect of adoptive transfer of TSST-1 vs. PBS primed CD4⁺ T cells on antibody production *in vivo*. Two groups of mice were either primed with 4 μ g of TSST-1 or PBS s.c., 3 times, 4 days apart. Two hours post final injection, TSST-1 or PBS primed mice were sacrificed and CD4⁺ T cells were purified from the spleen. Cells were diluted with RPMI and i.v. injected into the TNP-KLH hyperimmunized mice, immediately followed by boosting with 50 μ g of TNP-KLH and activation with either 4 μ g of TSST-1 or an equal volume of PBS. These mice were bled via their tails prior to adoptive transfer and 7, 14 and 21 days after transfer (Day 0 indicates time immediately before transfer). This experiment was repeated in 5 different animals for each condition. TNP-specific IgG1 (Fig.3.A), IgG2a (Fig.3.B) and IgE (Fig.3.C) antibodies were measured by ELISA. One-way ANOVA analyses with repeated means were used to compare the antibody levels among the different groups. TCD4⁺ refers to TSST-1 primed CD4⁺ T cells while PCD4⁺ refers to PBS primed CD4⁺ T cells. * indicates $p < 0.05$ and ** indicates $p < 0.01$.

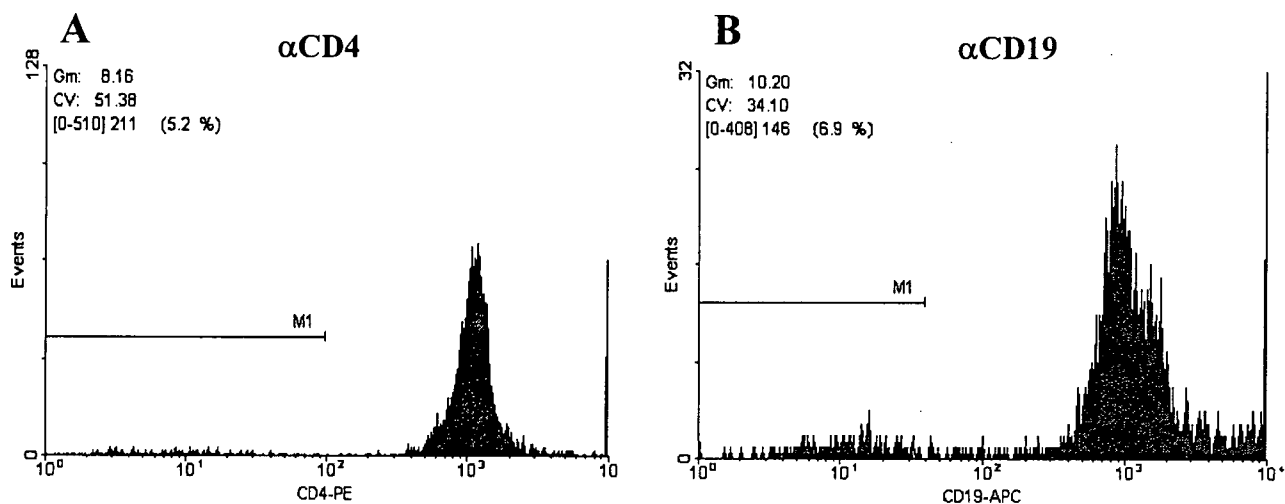


4.2 EFFECT OF TSST-1 VS. PBS PRIMED CD4+ T CELLS ON B CELLS

4.2.1 B CELL PROLIFERATION

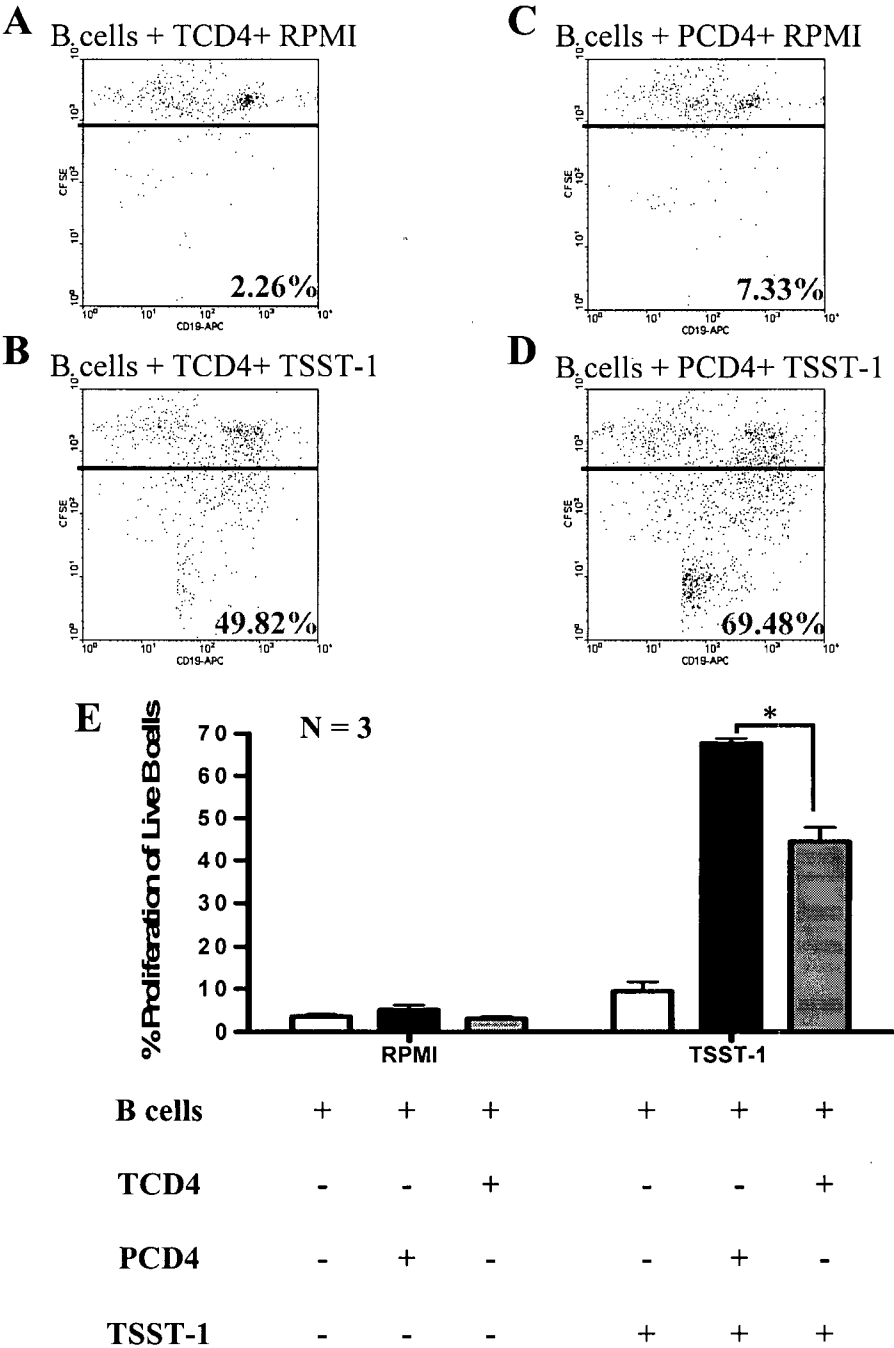
In order to examine the mechanism of TSST-1 primed CD4+ T cell suppression on B cells, a co-culture system that brings together TSST-1 primed CD4+ T and B cells was chosen for further studies. Thus, it is imperative that relatively pure populations of CD4+ T cells and CD19+ B cells would be obtained. As a result, the effectiveness of the respective separation techniques was assessed. Negative selection of CD4+ T cells and CD19+ B cells was performed following manufacturers' instructions. Upon purification, cells were analyzed using flow cytometry. From Figure 4, it appears that both separation techniques are highly efficient at isolating relatively pure populations for both CD4+ T cells (94.8%) and CD19+ B cells (93.1%).

Figure 4: Purity of CD4+ T cells and CD19+ B cells obtained by negative magnetic separation techniques. Upon purification, cells were stained with either α CD4-PE antibodies (Fig.4.A) or α CD19-APC antibodies (Fig.4.B) and analyzed by flow cytometry. Histograms are gated with forward scatter and side scatter for live cells.



The effect of TSST-1 primed CD4⁺ T cells on B cells can then be examined in one of three ways – proliferation, viability and antibody production as indicators of B cell activation and function. Either TSST-1 or PBS primed CD4⁺ T cells were co-cultured with naïve B cells, with or without 1 nM TSST-1 stimulation, and incubated *in vitro* for 72 h. B cells were stained with CFSE, a proliferation tracking dye that diminishes in fluorescence as cells divide. First looking at Figure 5.A and Figure 5.B, it appears that TSST-1 stimulation activates TSST-1 primed CD4⁺ T cells to induce B cell proliferation. Similarly, TSST-1 stimulation activates PBS primed CD4⁺ T cells to induce B cell proliferation (Fig.5.C vs. Fig.5.D). However, comparing Figure 5.B with Figure 5.D, it is clear that TSST-1 induced CD4⁺ T cells do not induce as much B cell proliferation as the PBS induced CD4⁺ T cells. The rate of B cell proliferation is reproducible in three separate experiments (45% vs. 67%, $p < 0.05$, Fig.5). This data suggests that there is some suppressive property in the TSST-1 primed CD4⁺ T cells to reduce B cell proliferation when compared to the PBS primed CD4⁺ T cells co-cultured with B cells. This difference can have several explanations; the most likely two possibilities are that TSST-1 primed CD4⁺ T cells either do not induce as many B cells to proliferate (or induces fewer divisions in the same number of B cells) or that TSST-1 primed CD4⁺ T cells induce B cell apoptosis.

Figure 5: Effect of TSST-1 or PBS primed CD4+ T cells on B cell proliferation. Either TSST-1 or PBS primed CD4+ T cells were co-cultured at a 1:1 ratio with naïve CFSE stained B cells. Samples were assessed via FACS analysis after 72 h of co-culture with or without TSST-1 stimulation. Plots were gated for live B cells. The bar indicates cells that have proliferated (having low CFSE fluorescence). Fig.5.A. is TSST-1 primed CD4+ with B cells without TSST-1 stimulation. Fig.5.B. is the same with TSST-1 stimulation. Fig.5.C. is PBS primed CD4+ with B cells without TSST-1 stimulation. Fig.5.D. is the same with TSST-1 stimulation. Fig.5.E shows the percentage of live B cells that exhibited lower CFSE over the total number of live B cells for 3 experiments using the mean and SEM. TCD4+ refers to TSST-1 primed CD4+ T cells while PCD4+ refers to PBS primed CD4+ T cells. * $p < 0.05$ using a two-tailed Student's t test.

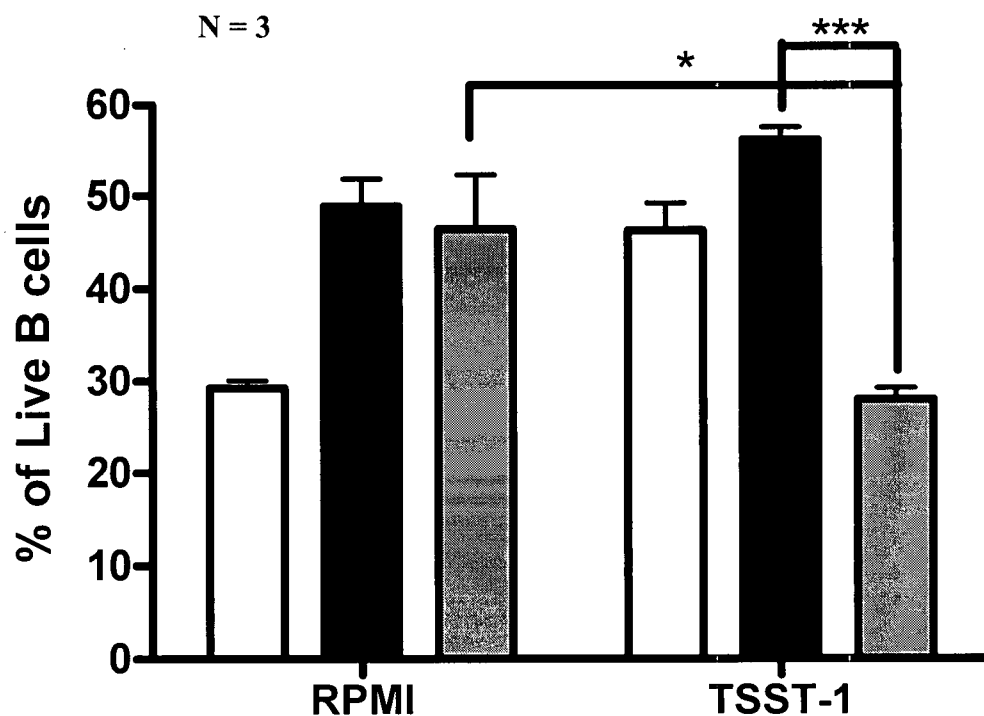


One of the limitations of employing CFSE as a means of tracking proliferation is that when cells undergo apoptosis, the CFSE that was taken up would then be released. It is therefore important to assess the ability of initially unstained T cells to pick up the CFSE dye while in culture. Surface α IgM, which is also known as the B cell receptor (BCR), was employed as a marker to assess the percentage of non-B cells in the CFSE stained population. It appears that roughly 4.5% of the CFSE stained population are T cells (data not shown). Thus, while some T cells inadvertently picked up free CFSE released into the medium by apoptotic B cells, the percentage of these accidentally stained cells were relatively minor and therefore would not significantly alter the interpretation of experiments using CFSE. Furthermore, since the purity assessment of the CD19 separation is at 94%, there is 6% of non-B cells that were also stained alongside B cells which could also contribute to this CD19-CFSE+ population.

4.2.2 B CELL VIABILITY

While it is clear that the TSST-1 induced CD4⁺ T cells exert a markedly different effect on B cells than the PBS induced CD4⁺ T cells with respect to proliferation, the reason behind the hinderance of B cell proliferation by TSST-1 primed CD4⁺ T cells is unclear. It might be attributed to a delay in cell cycle signalling, inhibition of proliferation, or simply killing of B cells via the induction of apoptosis. To examine these possibilities, B cell viability was assessed using Annexin V, a marker of early apoptosis (Fig 6), and 7-AAD, a marker of late apoptosis (data not shown). Both methods yielded similar results. The precedence for B cells undergoing apoptosis as a result of interaction with T cells with downregulatory function has been set as several groups have observed B cell apoptosis as a means of down-regulation (Zhao *et al.*, 2006; Janssens *et al.*, 2003). According to Figure 6, TSST-1 primed CD4⁺ T cells are the only group that causes a decrease in B cell viability, indicating that TSST-1 primed CD4⁺ T cells, and not PBS primed CD4⁺ T cells, induce B cell apoptosis (52% vs. 30%, $p < 0.05$). Thus, the suppression of proliferation seen previously by TSST-1 primed CD4⁺ T cells is likely the result of the induction of B cell apoptosis. Interestingly, B cells alone in culture mostly die after 72 h but co-culture with T cells, regardless of how they were primed, and even without further TSST-1 stimulation, protects B cells from dying to the same degree as stimulation of B cells with TSST-1 alone. Thus, it should be noted that both TSST-1 and T cells can protect B cells from undergoing apoptosis in culture. A possible explanation for this observation might be that both TSST-1 and T cells are able to provide pro-survival signals through activating B cells or cytokine production.

Figure 6: The effect of TSST-1 primed CD4+ T cells on B cell viability. TSST-1 or PBS primed CD4+ T cells were co-cultured with B cells for 72 h with or without 1 nM TSST-1 stimulation. Cells were then washed and stained using Annexin V following the directions of the manufacturer. Stained cells were then analyzed by FACS. Results (mean and SEM) were compiled from three independent experiments. * $p < 0.05$ using a two-tailed Student's t test.



B cells	+	+	+	+	+	+
PCD4	-	+	-	-	+	-
TCD4	-	-	+	-	-	+
TSST-1	-	-	-	+	+	+

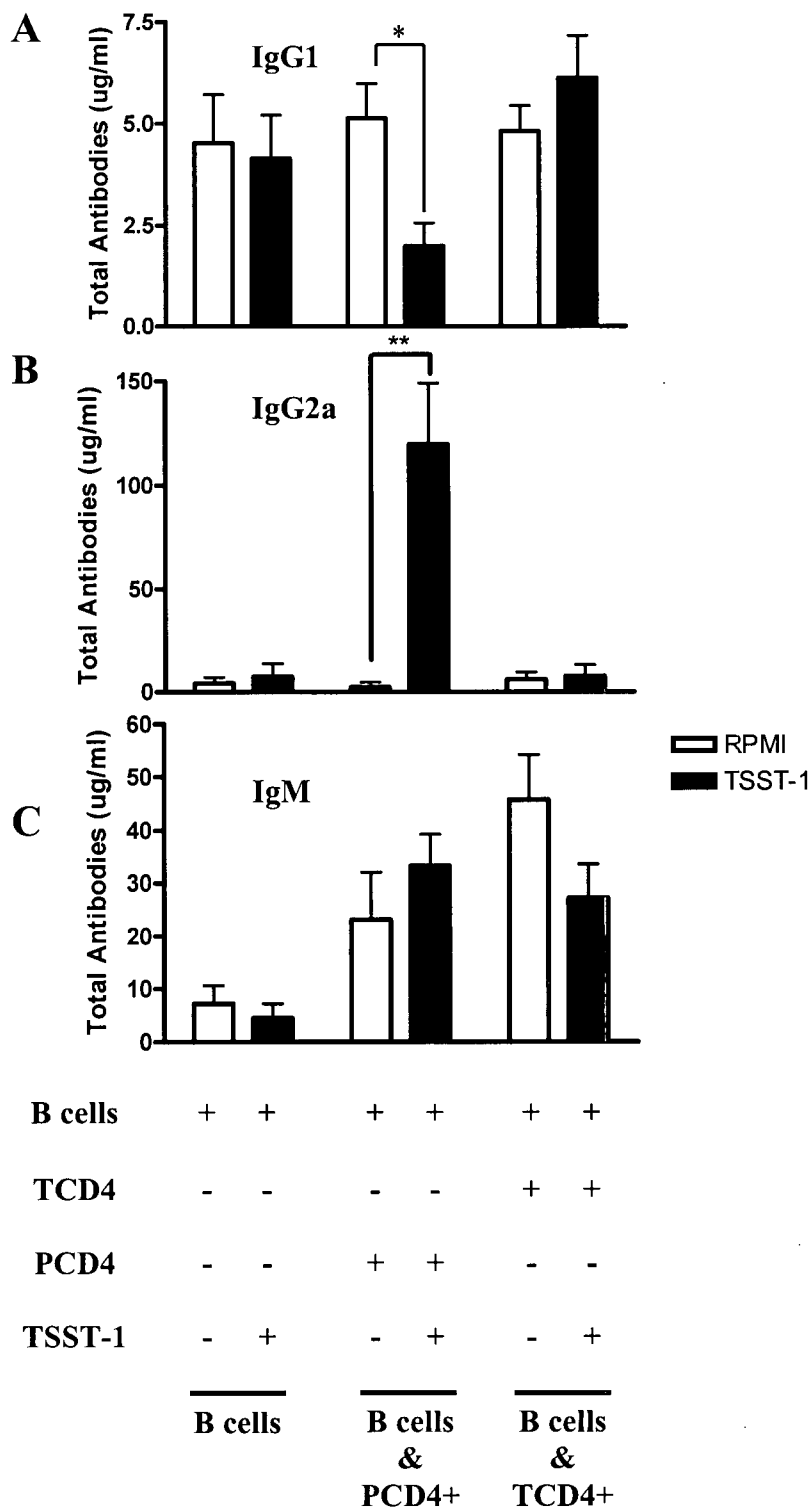
4.2.3 TOTAL ANTIBODY PRODUCTION

Total antibody levels were assessed from the co-culture supernatant in an attempt to assess B cell function. TSST-1 or PBS primed CD4⁺ T cells are co-cultured with B cells with or without 1 nM TSST-1 stimulation for 72 h. From the supernatant, IgG1 was assessed as indicative of the T_H2 response, while IgG2a was assessed as indicative of the T_H1 response. IgM levels were also assayed. From Figure 7, B cells cultured alone did not increase production of any of the tested isotypes of antibodies. It is always possible that B cells were producing antibodies of a different isotype than those tested but it is not likely that there would be an increase in other isotypes either. Likewise, while TSST-1 primed CD4⁺ T cells co-cultured with B cells induced greater antibody production than B cells alone, the addition of TSST-1 to activate the T cell population did not significantly increase antibody production. This suggests that the addition of T cells to a B cell culture stimulates B cells to produce more IgM but not other isotypes. On the other hand, the co-culture of PBS primed CD4⁺ T cells with B cells, when activated with TSST-1, induced an isotype switch to IgG2a by decreasing the production of IgG1 (5 µg/ml vs. 2 µg/ml, $p < 0.05$) and statistically significantly increasing IgG2a production level (125 µg/ml vs. 3 µg/ml, $p < 0.005$). Thus, TSST-1 primed CD4⁺ T cells appear to either have no effect on B cell antibody production or have a suppressive effect by preventing significant secretion of antibodies, while PBS primed CD4⁺ T cells appear to induce B cells to respond with T_H1 type antibody production.

However, in light of the hypothesis that the suppression of B cell proliferation by TSST-1 primed CD4⁺ T cells is mediated by the induction of B cell apoptosis, it would appear that TSST-1 primed CD4⁺ T cells not only exert an effect on B cell function, they are likely also exerting a suppressive effect on antibody secretion, regardless of the isotype. These results, taken together, duplicate the effect observed *in vivo* and further suggest that the observed TSST-1

primed CD4⁺ T cell suppression of B cells may be mediated by the induction of B cells to undergo activation induced cell death (AICD) (where activation is not followed by antibody production but rather with apoptosis).

Figure 7: The effect of TSST-1 primed CD4+ T cells on B cell antibody production. TSST-1/PBS primed CD4+ T cells were co-cultured with B cells for 72 h with or without 1 nM TSST-1 stimulation. Supernatant was collected after the incubation period and antibody levels were assessed. Fig.7.A is the IgG1 antibody levels in the supernatant of B cells alone, TSST-1 induced CD4+ T cell (TCD4+) with B cell or PBS induced CD4+ T cell (PCD4+) with B cell co-culture. Fig.7.B is the IgG2a levels while Fig.7.C is the IgM levels. Results (mean and SEM) were compiled from five independent experiments. * $p < 0.05$ and ** $p < 0.005$, analyzed using Student's t tests.



N = 5

4.2.4 CYTOKINE PRODUCTION

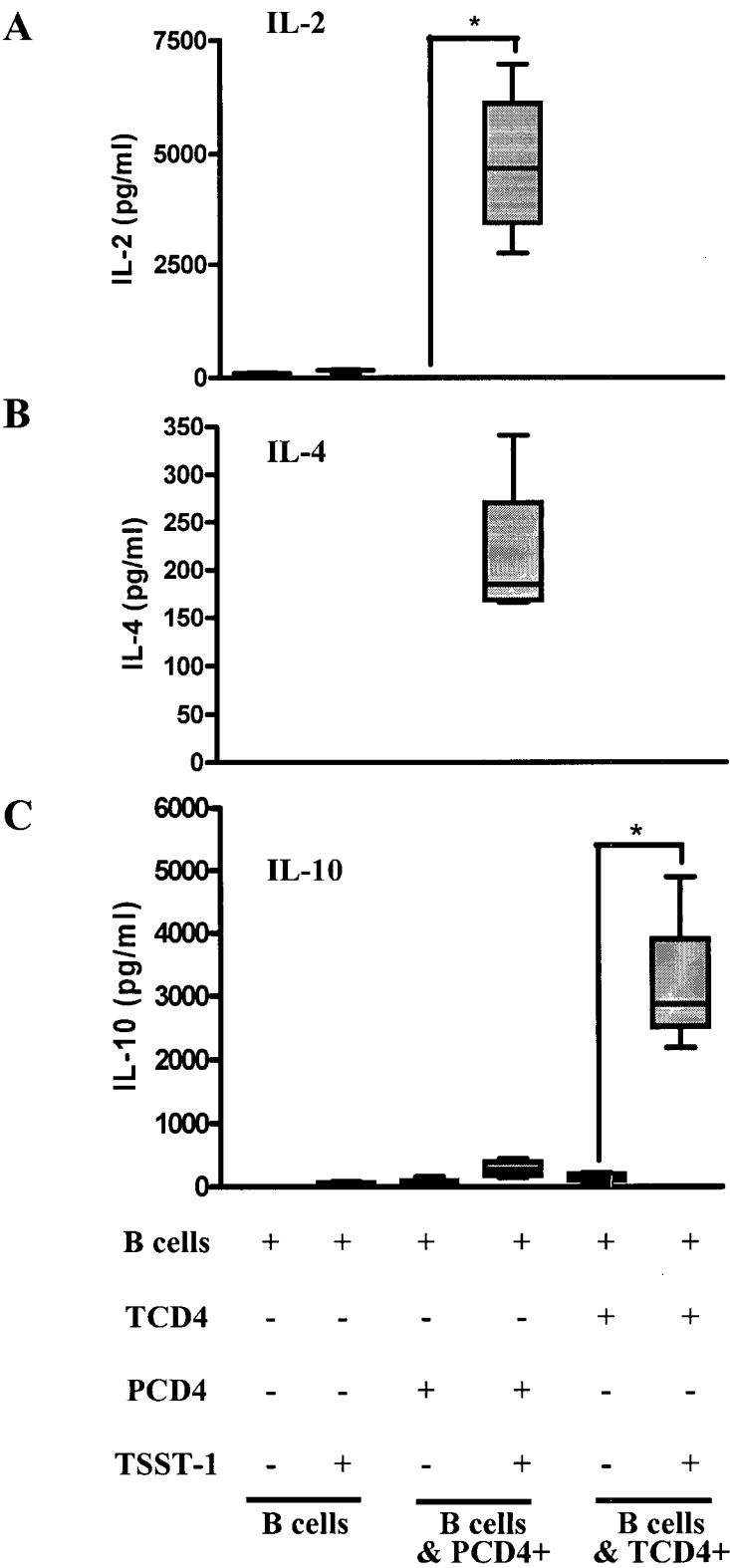
The cytokine profile was examined concurrently to give an indication of the activity of CD4⁺ T cells during the co-culture period. IL-2 levels were measured because it is the hallmark of a T_H1 response. IL-4 was assessed because it is indicative of a T_H2 response while IL-10 was examined because it is the key cytokine produced by regulatory T cells.

It must be noted, however, that because the level of cytokine in the supernatant is assessed, there are several issues with this approach. First, besides T cells, B cells are also known to make cytokines (O'Garra *et al.*, 1990). Thus, measuring the total level of cytokines in the supernatant is not an exact way to measure cytokine production by the CD4⁺ T cells. In an attempt to take this limitation into consideration, cytokine production by B cells alone in culture was assessed (Fig.8). However, even this only takes into account the cytokine production by B cells when stimulated by TSST-1 and not the cytokine production level by B cells when stimulated by TSST-1 while co-cultured with TSST-1 or PBS primed T cells. Thus, it is assumed that the majority of cytokine production is generated by the T cells. The second issue here is that only the net level of cytokines is known. In other words, the measured level of cytokines is the resultant level between production and consumption of a particular cytokine. The use of intracellular cytokine staining would be one way to circumvent this problem.

The results indicate that B cells alone in culture secrete IL-2 and IL-10 when stimulated with TSST-1 but these concentrations were much lower when compared to the cytokine levels in a B and T cell co-culture (Fig.8). TSST-1 primed CD4⁺ T cells produced predominantly high levels of IL-10 (3200 pg/ml vs. 150 pg/ml, $p < 0.05$), consistent with the regulatory T cell cytokine profile, while PBS primed CD4⁺ T cells produced mainly elevated levels of IL-2 (4800 pg/ml vs. 0.40 pg/ml, $p = 0.05$) along with lower levels of IL-4 and IL-10, consistent with the T_H1 T cell response in the presence of TSST-1.

Taken together with the observations that TSST-1 primed CD4⁺ T cells suppress B cells possibly by causing B cell AICD, it seems that TSST-1 primed CD4⁺ T cells, which contribute to a supernatant milieu that exhibits predominantly IL-10, may contain regulatory cells, which, through the secretion of immunosuppressive cytokines, prevent B cell antibody production. PBS primed CD4⁺ T cells, on the other hand, which contribute to a supernatant milieu that exhibits predominantly IL-2 with some IL-4, contain T_H1 cells that mediate an IgG2a or a T_H1 response from naïve B cells via the activity of IL-2.

Figure 8: Cytokine levels in various subset co-cultures. TSST-1/PBS primed CD4+ T cells were co-cultured with B cells for 72 h with or without 1 nM TSST-1 stimulation. Supernatant was collected after the incubation period and the cytokine levels were assessed. Results (mean and SEM) were compiled from four independent experiments. * $p < 0.05$ analyzed using Mann-Whitney test.



N = 4

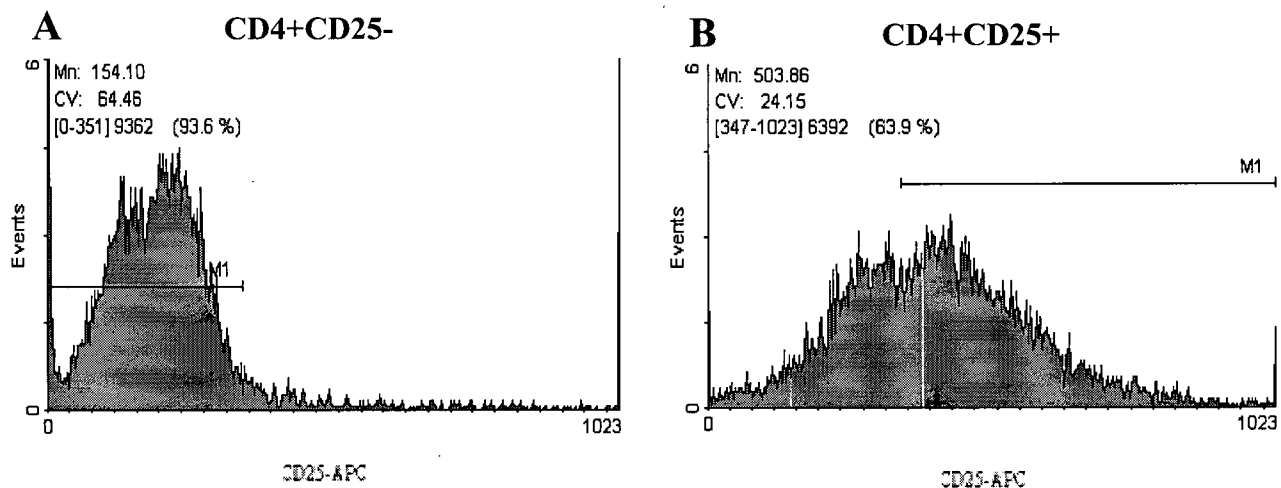
4.3 EFFECT OF TSST-1 OR PBS PRIMED CD4+CD25+ VS. CD4+CD25- T CELLS ON B CELLS *IN VITRO*

The *in vitro* study confirmed that TSST-1 primed CD4+ T cells exert some sort of suppressive effect on B cells, the details of which remain unclear. Within the purified CD4+ T cell population, there are still many types of T cells present, such as T helper 1 and 2 cells, natural CD4+CD25+ Tregs and possibly TSST-1 induced CD4+ Tregs. Thus, in order to further dissect the population that is inducing the observed suppressive effect, it was attempted to further purify the CD4+ population into the CD25+ and CD25- subsets. CD25, or the IL-2 receptor α -chain, is an important marker for natural regulatory T cells. CD25 is expressed on T cells when they become activated. It was found that natural regulatory T cells constitutively express CD25 even without activation, thus garnering the name of natural CD4+CD25+ regulatory T cells. Since then, CD25 has been used as a marker to identify regulatory T cells. Here, in order to further characterize the suppressive effect of TSST-1 primed CD4+ T cells on B cells, the CD4+ population was further purified into the CD25+ and the CD25- cells. Each subset was individually co-cultured with B cells to identify the subpopulation that is responsible for mediating the suppression.

One potential problem is the dynamic nature of CD25 expression. Furthermore, the process of purifying CD25+ populations by positive magnetic separation may itself induce CD25 expression. Cells not expressing CD25 may be stimulated to express CD25 for a brief period during the separation process. Thus, while the CD25- population is relatively pure (93.6%) (Fig.9.A), the CD25+ population is highly impure (63.9%) (Fig.9.B). In short, separation based on CD25 is artificial at best and this must be kept in mind for future CD25 separation based studies. Furthermore, while CD25 is expressed constitutively on natural CD4+CD25+ Tregs, the nature of CD25 expression on antigen induced Tregs remains unclear. Thus, separation based on

CD25 may not only result in populations with mixed CD25 expression but also populations with mixed subtypes of regulatory T cells.

Figure 9: Purity of CD25⁻ T cell negative selection (Fig.9.A) and CD25⁺ T cell separated by positive selection (Fig.9.B) techniques. Cells were separated following manufacturer's instructions. Upon purification, cells were stained with α CD25-APC antibodies to examine the proportion of CD25⁺/CD25⁻ T cells via flow cytometry. Histograms are gated with forward scatter and side scatter for live cells.



4.3 EFFECT OF TSST-1 OR PBS PRIMED CD4+CD25+ VS. CD4+CD25- T CELLS ON B CELLS *IN VITRO*

4.3.1 B CELL PROLIFERATION

TSST-1 or PBS primed CD4+ T cells were separated into CD25+ and CD25- subpopulations and separately co-cultured with CFSE stained B cells in the presence of 1 nM TSST-1 for 72 h. The results indicate that both TSST-1 primed CD25+ and CD25- T cells induced B cell proliferation upon TSST-1 stimulation similar to either PBS primed CD25+ or CD25- T cells (Fig.10 and Fig.11, respectively). Similar to the CD4+ T cells, the TSST-1 primed CD4+CD25+ T cells (Fig.10.B) were unable to induce as much B cell proliferation as the PBS primed CD4+CD25+ T cells (Fig.10.D) (22.2% vs. 64.6%; $p < 0.0001$, Fig.12). Likewise, the TSST-1 primed CD4+CD25- T cells (Fig.11.B) were unable to induce as much B cell proliferation as the PBS primed CD4+CD25- T cells (Fig.11.D) (43.5% vs. 73.8%; $p < 0.05$, Fig.12). In addition to these observations, it is also clear that each subset, be it TSST-1 or PBS primed CD25+ or CD25- T cells, exerted a different effect on B cells. TSST-1 primed CD25+ T cells (Fig.10.B) induced a lower percentage of B cells to undergo proliferation when compared to the TSST-1 primed CD25- T cells (Fig.11.B) (22.2% vs. 43.5%; $p < 0.01$, Fig.12). Furthermore, most B cells that proliferated in the TSST-1 primed CD25- T cell co-culture were mainly in the first and last division cycle while the same cannot be said of the TSST-1 primed CD25+ T cell co-culture. This suggests that B cells may be suppressed by both the TSST-1 primed CD25+ and CD25- T cell populations and that the interaction between B cells and TSST-1 primed CD25+ T cells is a unique one, different from the effect that TSST-1 primed CD25- T cells have on B cells. Likewise, PBS primed CD25+ T cells exert a different effect than PBS primed CD25- T cells on the progression of B cell proliferation as these two populations appear to have induced B cells into different degrees and stages of proliferation.

All these observations in combination hint at two possibilities - that TSST-1 primed CD4+CD25+ T cells may be interacting with B cells at a stage of proliferation different than that of the TSST-1 primed CD4+CD25- T cells, or that the fundamental mechanism of suppression exerted by the CD25+ T cell subset is different from that of the CD25- T cell subset. These results are consistent and statistically significant as indicated in the graph of percentage proliferation (Fig.12).

Figure 10: The effect of TSST-1 primed CD25+ T cells on B cell proliferation. Either TSST-1 or PBS primed CD25+ T cells were co-cultured at a 1:1 ratio with naïve CFSE stained B cells. Samples were assessed via FACS analysis after 72 h of co-culture. Plots were gated for live CD19+ B cells. The bar indicates cells that have proliferated (having low CFSE fluorescence). Fig.10.A. is TSST-1 primed CD25+ with B cells without TSST-1 stimulation. Fig.10.B. is the same with TSST-1 stimulation. Fig.10.C. is PBS primed CD25+ with B cells without TSST-1 stimulation. Fig.10.D. is the same with TSST-1 stimulation. Data are representative of 3 separate experiments.

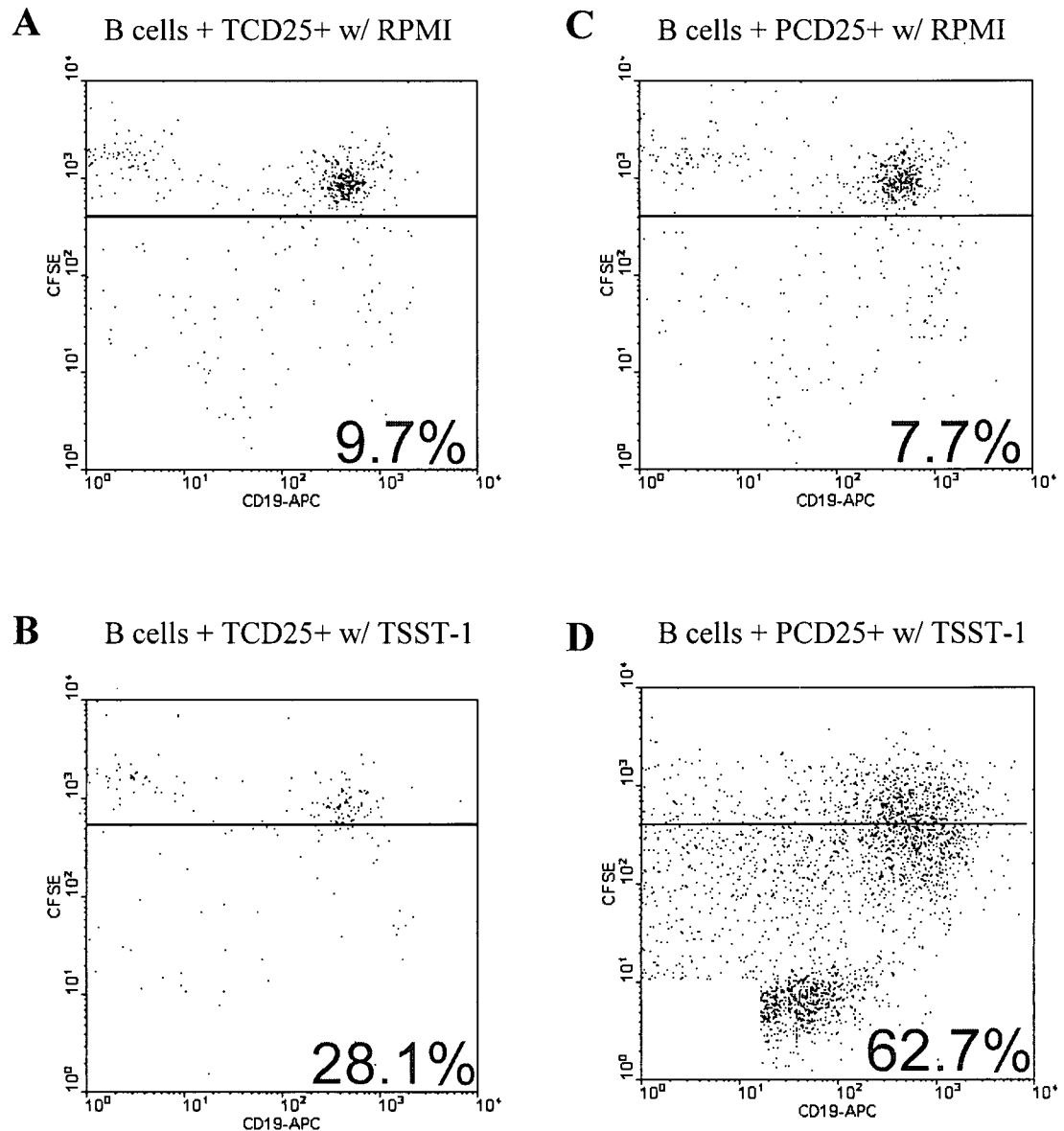


Figure 11: The effect of TSST-1 primed CD25- T cells on B cell proliferation. Either TSST-1 or PBS primed CD25- T cells were co-cultured at a 1:1 ratio with naïve CFSE stained B cells. Samples were assessed via FACS analysis after 72 h of co-culture. Plots were gated for live CD19+ B cells. The bar indicates cells that have proliferated (having low CFSE fluorescence). Fig.11.A. is TSST-1 primed CD25- with B cells without TSST-1 stimulation. Fig.11.B. is the same with TSST-1 stimulation. Fig.11.C. is PBS primed CD25- with B cells without TSST-1 stimulation. Fig.11.D. is the same with TSST-1 stimulation. Data are representative of 3 separate experiments.

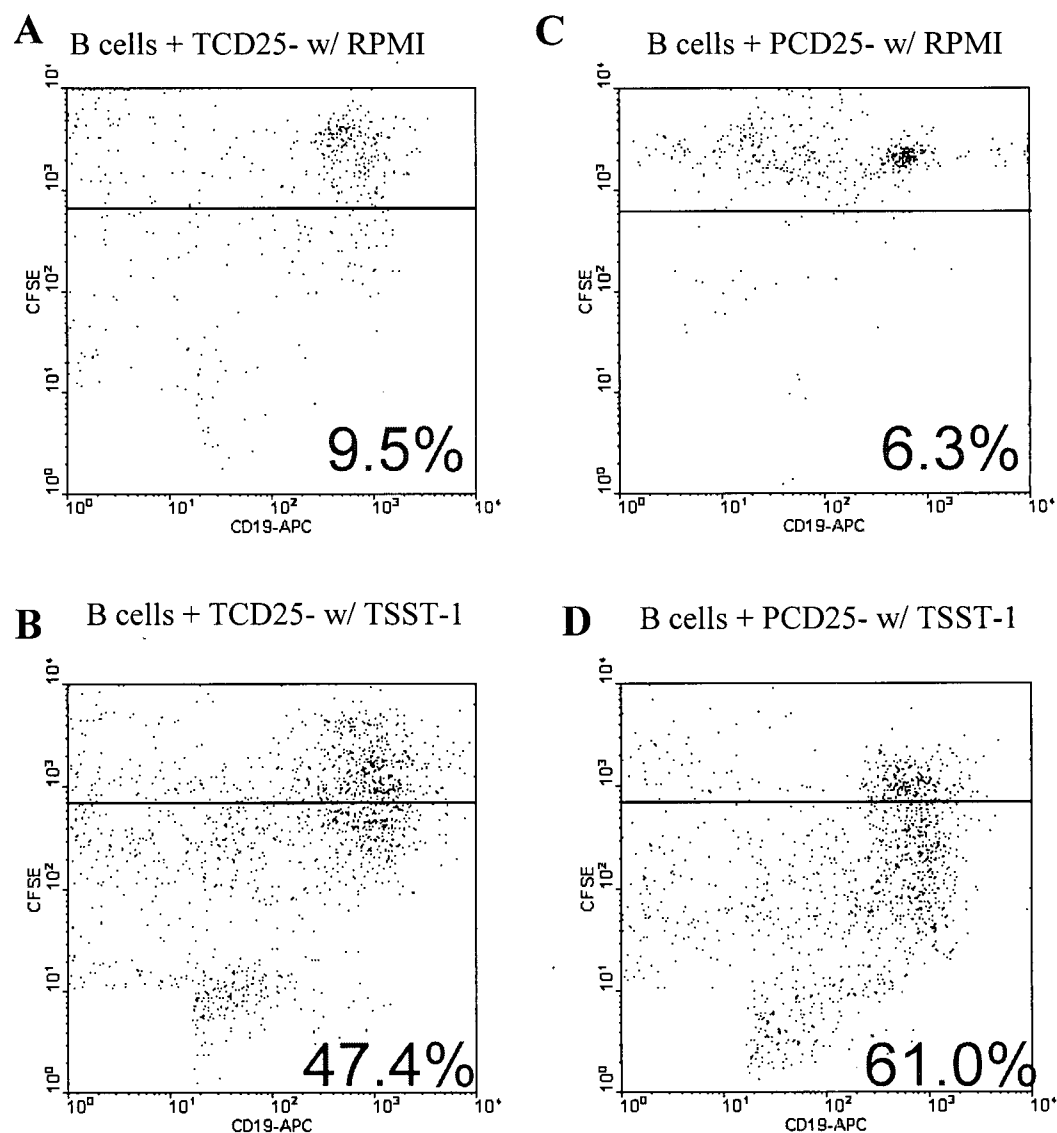
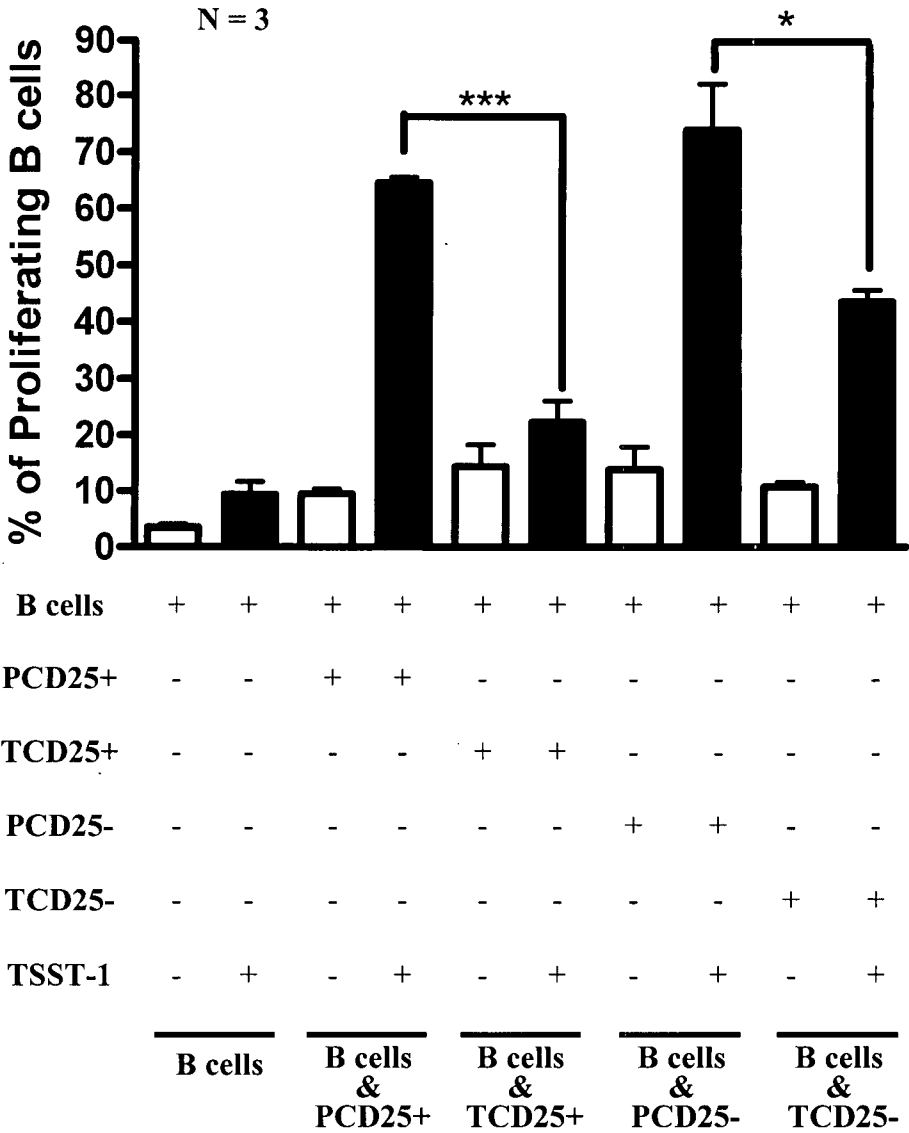


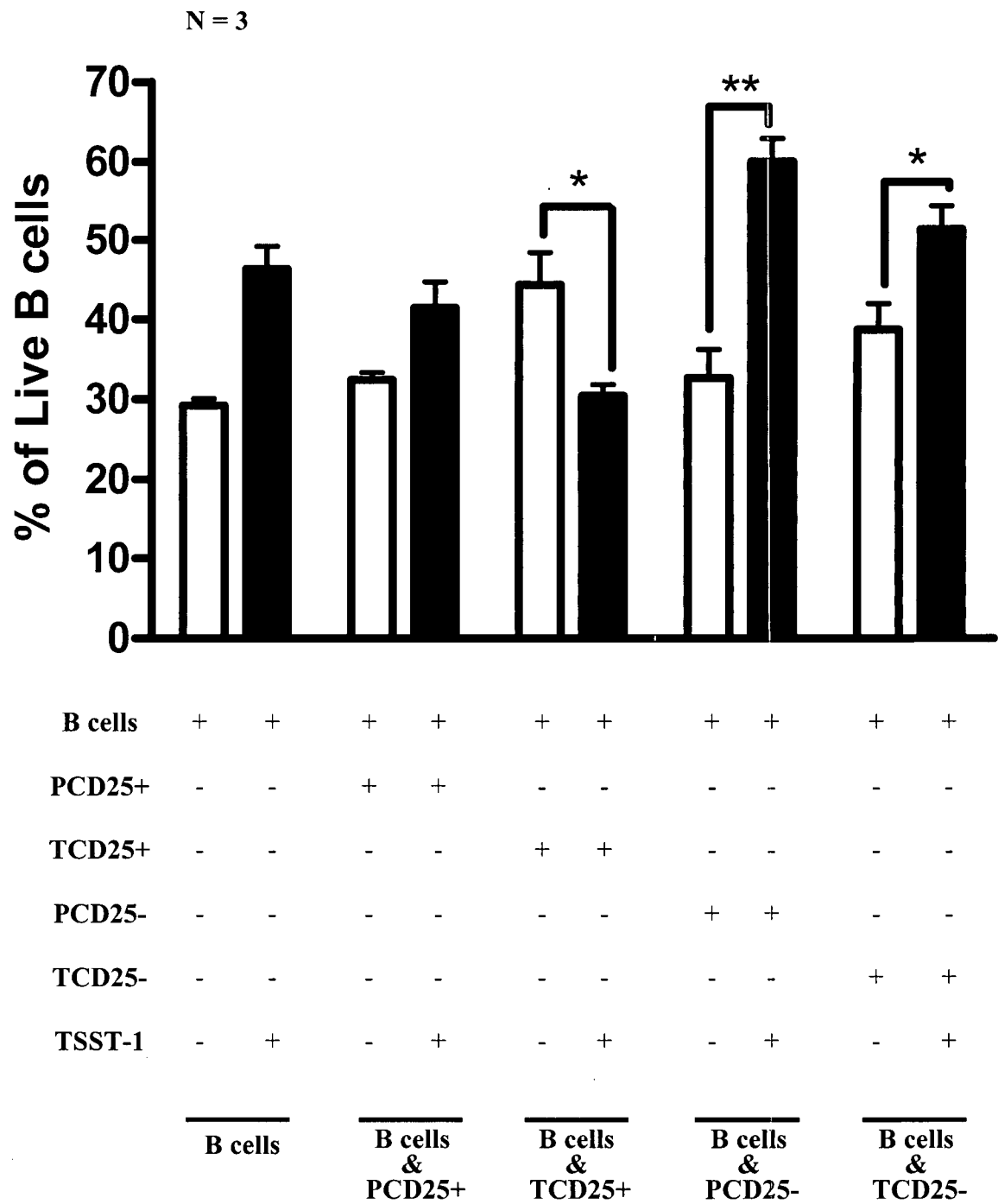
Figure 12: The effect of TSST-1 primed CD25+ or CD25- T cells on B cell proliferation as expressed by percentage proliferation. The same protocol as described in Fig.10 and Fig.11 was used. Data (mean and SEM) were from three independent experiments. * $p < 0.05$ and *** $p < 0.0001$, analyzed using Student's t tests.



4.3.2 B CELL VIABILITY

As before, the cause of the difference seen in the progression and percentage of proliferation is possibly due to the induction of cells undergoing apoptosis. To test this, cells were analyzed with Annexin V and 7-AAD. Figure 13 indicates that only TSST-1 primed CD4+CD25+ T cells exerted a statistically significant apoptotic effect on B cells (44% vs. 30%; $p < 0.05$) while all other subsets, even the TSST-1 primed CD4+CD25- T cells, promoted an increase in B cell survival. While both CD25- subsets helped prevent B cell apoptosis upon TSST-1 stimulation, the degree of survival is much greater for the PBS primed CD25- T cell subset (33% vs. 60%; $p < 0.005$) than the TSST-1 primed CD25- T cell subset (39% vs 51%; $p < 0.05$). These results harken back to the earlier proliferation data (Figure 10-12) in that T cell subsets that induced more B cell proliferation also induced a greater increase in B cell survival. Furthermore, it appears that the suppression of proliferation by TSST-1 primed CD4+CD25+ T cell subset correlates with the induction of B cell apoptosis while the suppression exerted by TSST-1 primed CD4+CD25- T cells is not mediated predominantly by the induction of B cell death. Interestingly, the effects of co-culturing different subsets with B cells even without TSST-1 stimulation differ. B cells alone and the two PBS primed subsets displayed less viability than either of the two situations in which B cells were cultured with TSST-1 primed T cell subsets.

Figure 13: The effect of TSST-1 primed CD25+ or CD25- T cells on B cell viability. TSST-1 or PBS primed CD25+ or CD25- T cells were co-cultured with B cells for 72 h with or without 1 nM TSST-1 stimulation. Cells were then washed and stained using Annexin V following the directions of the manufacturer. Stained cells were then analyzed by FACS. Results (mean and SEM) were compiled from three independent experiments. * $p < 0.05$ and ** $p < 0.005$, analyzed using Student's t tests.



4.3.3 TOTAL ANTIBODY PRODUCTION

The influence upon B cell function, with respect to total IgG1, IgG2a and IgM production, was likewise examined for all T cell subsets. However, this time, cells were cultured over a range of time points – 0, 24, 48 and 72 h following TSST-1 stimulation. The purpose of following antibody production over time is so that the time point at which the down-regulatory effect of TSST-1 induced CD4⁺ T cells on B cells may be identified. In addition, following the course of antibody production over time allows for the analysis of how TSST-1 primed CD4⁺ T cells affect isotype switching. Figure 14 indicates that TSST-1 primed CD25⁺ T cells induced a slight but statistically significant decrease (4.2 µg/ml vs. 5.6 µg/ml, $p < 0.05$) in IgG1 (Fig.14.B). No IgG2a was detected (Fig.14.A). PBS primed CD25⁺ T cells had a different effect on B cells as these cells induced a decrease in IgM (44 µg/ml vs. 11.5 µg/ml, $p < 0.01$), no observable difference in IgG1 production, but a marked increase in IgG2a levels (11.5 µg/ml vs. 52 µg/ml, $p = 0.0001$). TSST-1 primed CD25⁻ T cells exerted yet a different effect on B cells as they induced an increase in IgG1 (5.1 µg/ml vs. 10.9 µg/ml, $p < 0.05$) and a decrease in IgM production (26 µg/ml vs. 58 µg/ml, $p < 0.005$). No IgG2a was detected. Finally, PBS primed CD25⁻ T cells induced an increase in IgG1 levels (9 µg/ml vs. 4.8 µg/ml, $p < 0.005$). Once again, no IgG2a production was detected.

Observing antibody production over time, it is clear that TSST-1 stimulated TSST-1 primed CD25⁺ T cells prevented B cell antibody production over 24, 48 and 72 hours *in vitro* (Fig.15) while the PBS primed CD25⁺ T cells induced antibody isotype switch to IgG2a. TSST-1 primed CD25⁻ T cells caused an increase in IgG1 along with a decrease in IgM but only at the later time point of 72 h while PBS primed CD25⁻ T cell induction of IgG1 production could be seen as early as 24 h post co-culture, suggesting that TSST-1 primed CD25⁻ T cells induce a

delayed B cell response when compared to the effect of PBS primed CD25- T cells on B cell antibody secretion. Certainly, recalling the proliferation results, B cells were mostly found in the first cell cycle when co-cultured with TSST-1 primed CD25- T cells after 72 h while most of the B cells in the PBS primed CD25- T cell co-culture were evenly distributed in all cycles of cell division (18 h per cell division).

Combining the results from the proliferation, viability and antibody production studies, it appears that both TSST-1 primed CD25+ and CD25- T cell subsets are suppressing B cells but via completely different mechanisms. TSST-1 primed CD25+ T cells prevent B cell proliferation and antibody production (for all isotypes) by inducing B cell death. TSST-1 primed CD25- T cells suppress B cells by delaying activation of a T_H2 -type IgG1 antibody response, consistent with slowing the rate of proliferation. PBS primed CD25+ T cells activate the T_H1 -type IgG2a response by B cells, consistent with the response of naïve T helper cells when challenged with TSST-1. Finally, the PBS primed CD25- T cell subset likewise activates B cells but, instead, selects for a T_H2 -type IgG1 response. These results suggest that each subset of T cells exert a markedly different effect on B cells and that a particular B cell response may be selected for by altering the type of T cells present in the co-culture.

Figure 14: The effect of TSST-1 primed CD25+ or CD25- T cells on B cell IgG1, IgG2a and IgM antibody production. TSST-1 or PBS primed CD25+ or CD25- T cells were co-cultured with B cells for 72 h with or without 1 nM TSST-1. Supernatant was collected and antibody levels were assessed. Results (mean and SEM) were from five separate experiments. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$, analyzed using Student's t tests.

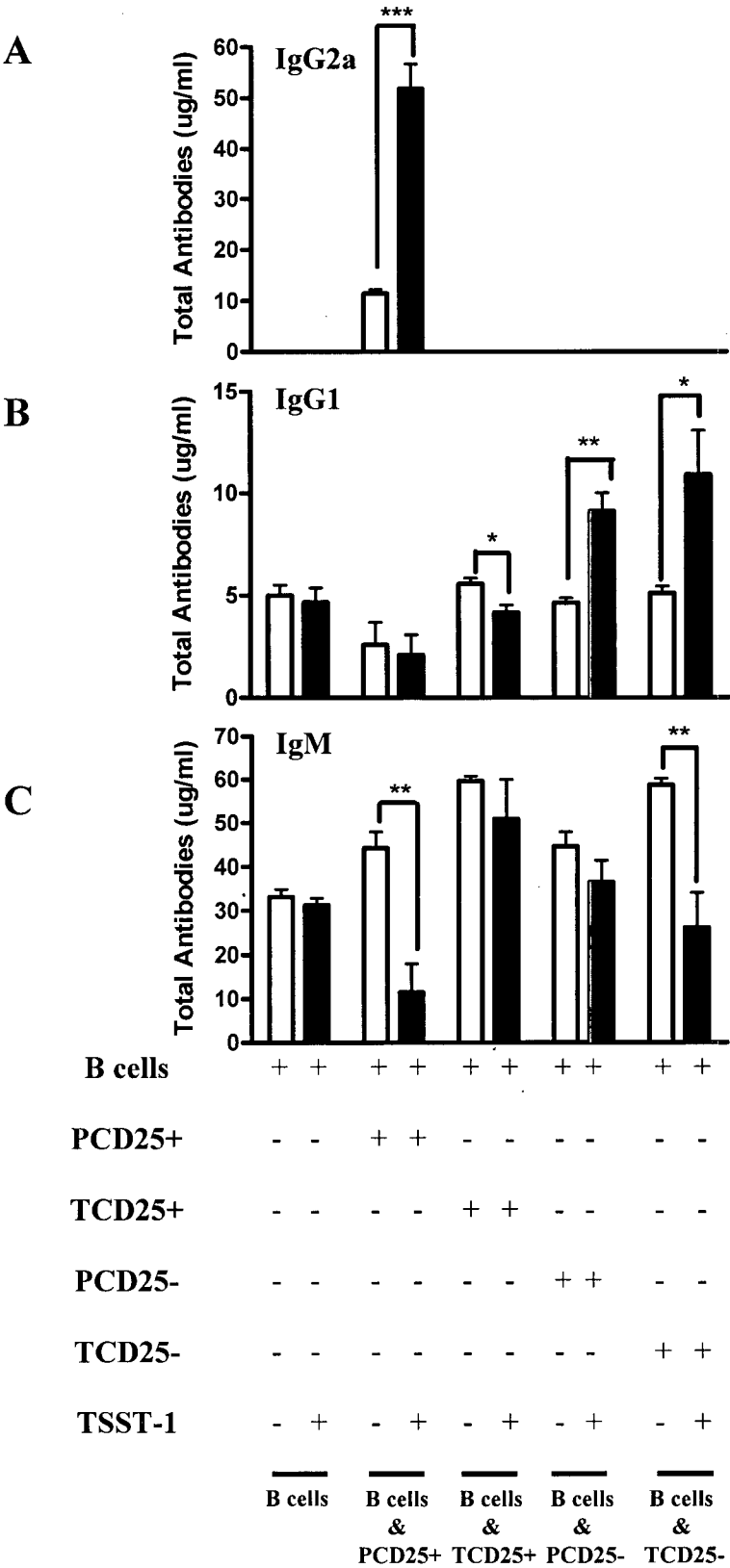
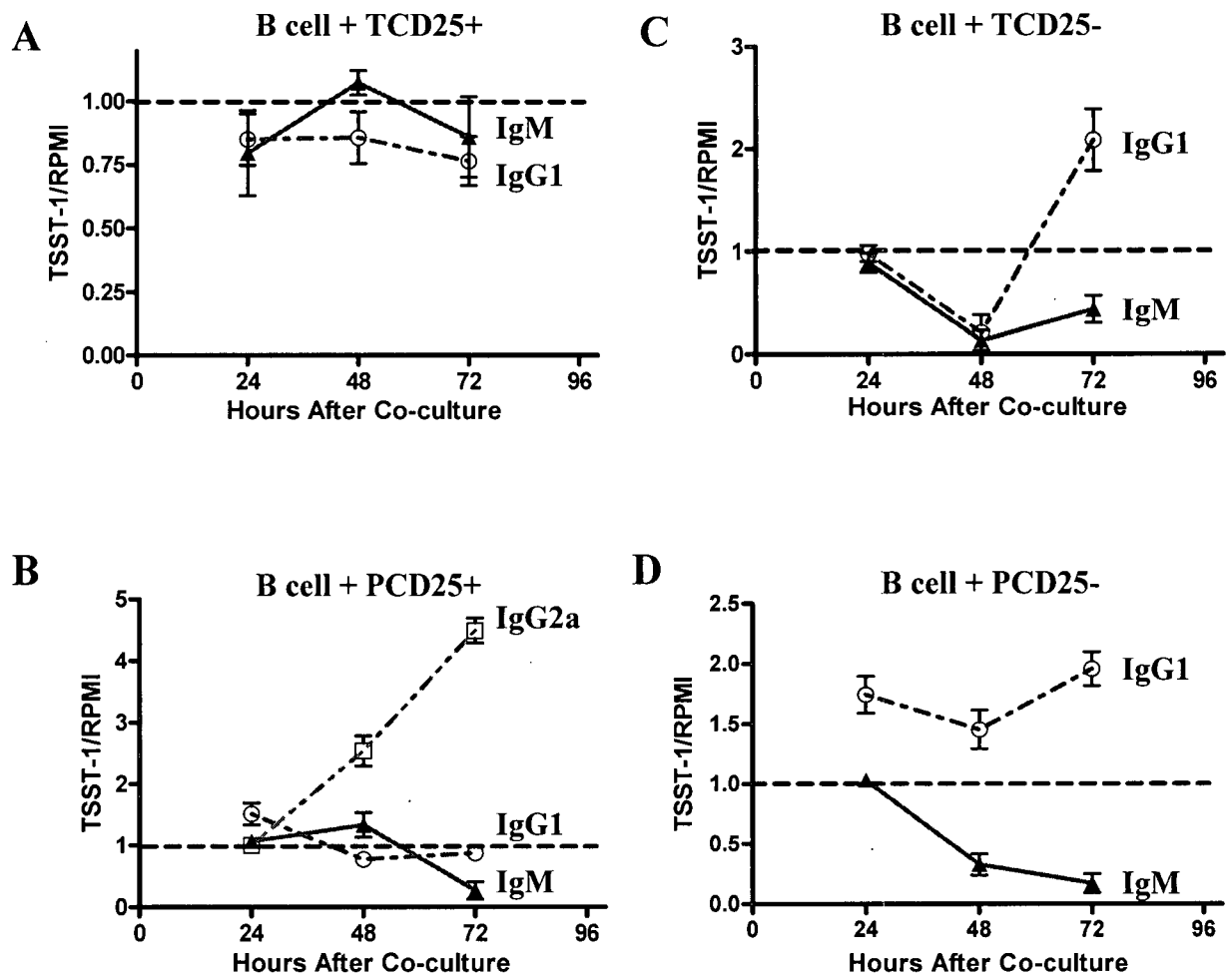


Figure 15: The effect of TSST-1 primed CD25+ or CD25- T cells on B cell antibody production over time. Cells were co-cultured for 24, 48 and 72 h with or without 1 nM TSST-1 stimulation. Supernatant was collected after the incubation period and IgG1, IgG2a and IgM antibody levels were assessed by ELISA. Results were compiled and expressed by dividing the level of antibody production under *in vitro* TSST-1 stimulated levels by the corresponding RPMI (TSST-1 unstimulated) levels. Fig. 15.A displays TSST-1 primed CD4+CD25+ co-cultured with B cells where only IgG1 and IgM were detected. Fig.15.B. displays PBS primed CD4+CD25+ co-cultured with B cells. Fig.15.C. displays TSST-1 primed CD4+CD25- co-cultured with B cells where only IgG1 and IgM were detected. Fig.15.D. displays PBS primed CD4+CD25- co-cultured with B cells where IgG1 and IgM were detected. Results (mean and SEM) were compiled from five independent experiments.

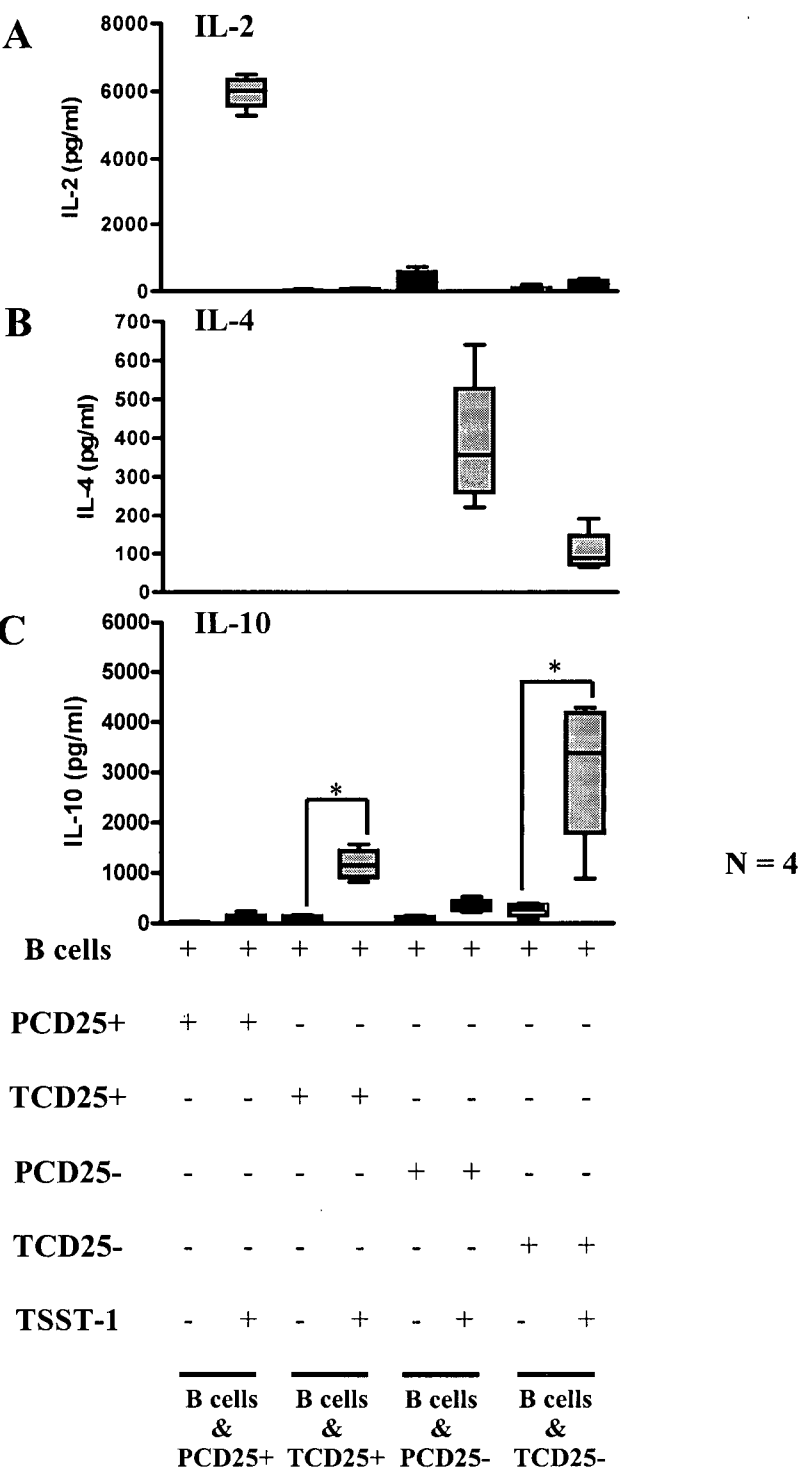


4.3.4 CYTOKINE PRODUCTION

Once again, cytokine production was analyzed as an indication of the activity of each T cell subtype. In Figure 16, TSST-1 primed CD25+ T cells, when activated with TSST-1, generated an IL-10 predominant cytokine profile (1170 pg/ml vs. 85 pg/ml, $p < 0.05$). There were high levels of IL-2 detected in the PBS primed CD25+ T cell co-culture. TSST-1 primed CD25- T cells generated IL-4 and IL-10 (3000 pg/ml vs. 260 pg/ml, $p < 0.05$) while the PBS primed CD25- T cells co-culture displayed a IL-4 rich cytokine profile.

Combining Figure 15 and 16, these results indicate that each of the different subsets induced a different effect on B cell antibody production and isotype switching. TSST-1 activated TSST-1 primed CD25+ T cells produced IL-10 and suppressed antibody production. TSST-1 activated PBS primed CD25+ T cells produced IL-2 and induced a T_H1 associated IgG2a antibody response. TSST-1 activated TSST-1 primed CD25- T cells produced IL-4 and IL-10 and induced a delayed T_H2 type IgG1 response. Finally, TSST-1 activated PBS primed CD25- T cells produced predominantly IL-4 and induced a IgG1 response but to a greater degree than the TSST-1 primed CD25- T cells.

Figure 16: Cytokine levels in the various subset co-cultures. TSST-1 or PBS primed CD25+ or CD25- T cells were co-cultured with B cells for 72 h with or without 1 nM TSST-1 stimulation. Supernatant was collected after the incubation period and cytokine levels were assessed by ELISA. Results were compiled from four independent experiments. * $p < 0.05$, analyzed using Mann-Whitney tests.



4.4 MECHANISM OF TSST-1 PRIMED CD4⁺ T CELL SUPPRESSION ON B CELLS

It was suspected, at this point in the project, that the suppressive effect exerted upon B cell activation and function by the TSST-1 primed CD4⁺ T cells might be attributed to the possible generation of TSST-1 specific Tr1 cells. Thus, in an attempt to examine whether regulatory T cells might be the reason for B cell suppression, the mechanism of suppression was scrutinized in order to understand if regulatory T cells are at work, and if so, which type of Tregs is involved. Within the field of regulatory T cells, the two most studied mechanisms to explain the suppressive effect exerted by Tregs on T cells is either cytokine based (IL-10 and TGF- β dependent) or cell-cell contact based (via possibly Fas/FasL or CTLA-4/CD80 or CD86 interactions). To determine how TSST-1 induced CD4⁺ T cells suppress B cells, both these major mechanisms was studied to see which, or if both, mechanisms are involved in mediating suppression. Finally, the question of whether the presence of cognate T_H cells is required for TSST-1 primed CD4⁺ T cells to exert their suppressive effect on B cells was addressed.

4.4.1 CYTOKINE DEPENDENT MECHANISMS

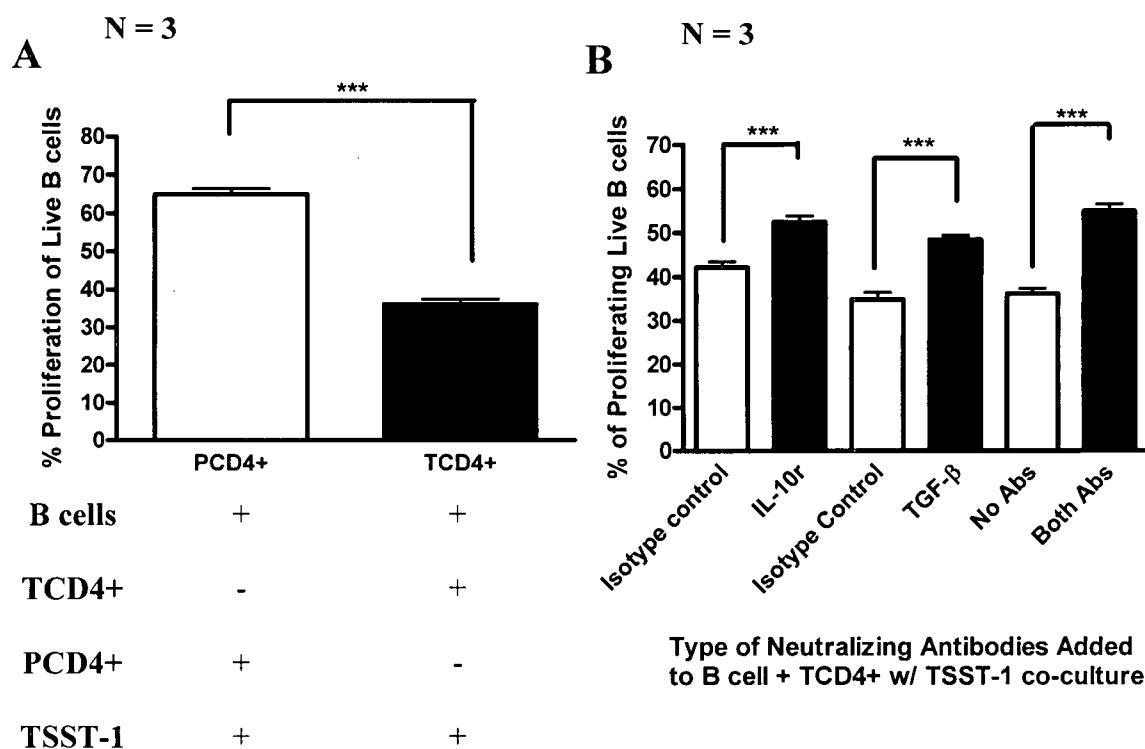
To assess the contribution, if any, of cytokines to the suppression of B cells by TSST-1 induced CD4⁺ T cells, a neutralization assay was conducted. B cells and TSST-1 or PBS primed CD4⁺ T cells were co-cultured with or without the presence of 1 nM TSST-1 for 72 h, as before. However, prior to incubation, α IL-10 receptor and/or α TGF- β neutralizing antibodies were added to different co-cultures to effectively prevent cytokine binding. Figure 17 indicates that both IL-10 and TGF- β were involved in mediating TSST-1 induced CD4⁺ T cell suppression on B cells as the respective neutralization antibodies were able to reverse, at least in part, the

suppression exerted on B cells. While the addition of α IL-10 receptor antibodies increased the percentage of proliferation to 53%, α TGF- β antibodies restored the percentage of proliferation to roughly 50% (Fig.17.B). In contrast, the percentage proliferative B cells in the presence of PBS primed CD4+ T cells was around 65% (Fig.17.A). The addition of both antibodies restored more proliferation (55% vs. 36%, $p < 0.0001$). In short, IL-10 and TGF- β were found to mediate TSST-1 primed CD4+ T cell suppression on B cell proliferation, but they were not the only components in mediating this suppression since the proliferation rate was not restored to the control levels of the PBS primed CD4+ T cell co-culture.

It is surprising to find that the combination of these neutralizing antibodies did not have an additive or a synergistic effect on restoring proliferation given the fact that IL-10 has the ability to induce TGF- β receptor expression in T cells (Yamashita *et al.*, 2006). This is not likely due to experimental error since the same results were duplicated by other members of the same lab (unpublished data). There are several possible explanations for this unexpected observation. First, perhaps the two antibodies, when used together have some non-specific interaction with each other where one antibody is binding to the other antibody instead of its intended epitope. Second, it is foreseeable that there is some cross-reactivity for the antibodies where α -IL-10 receptor antibody neutralizes the TGF- β receptor as well and vice versa. The third possibility, and perhaps the most probable, is that IL-10 and TGF- β are involved in mediating suppression by acting at different steps in the suppressive pathway. In effect, it is possible that IL-10 acts on TSST-1 primed CD4+ T cells to induce the production of TGF- β whereby TGF- β then acts upon B cells to induce apoptosis. Thus, the neutralization of either cytokines leads to the same abolition of suppression on B cells but the combination of neutralizing both cytokines would have no additional effect on reversing the suppression since either antibody would have already been sufficient to disrupt the pathway.

Regardless of the lack of additive or synergistic effect when the two neutralization antibodies were combined, it remains clear that cytokines alone are not enough to explain the entire suppressive effect seen on B cells.

Figure 17: The effect of neutralizing α IL-10 receptor and/or TGF- β on the suppression of TSST-1 primed CD4+ T cells on B cells. Fig.17.A. B cells were co-cultured with either TSST-1 or PBS primed CD4+ T cells in the presence of 1 nM TSST-1 stimulation ($p < 0.0001$). Fig.17.B. B cells were cultured with TSST-1 primed CD4+ T cells with the addition of TSST-1. In addition, either 2 μ g/ml of α IL-10 receptor mAb (IBI.2) and/or 2 μ g/ml of α TGF- β mAb were added to various wells. 1 μ g/ml of GL113 was used as the α IL-10 receptor isotype control while equal volume of rabbit serum was used as the α TGF- β isotype control. Results were compiled from five independent experiments (α IL-10 receptor: $p = 0.0005$; α TGF- β : $p < 0.0001$; both antibodies: $p < 0.0001$). *** $p \leq 0.0005$, analyzed using Student's t tests.

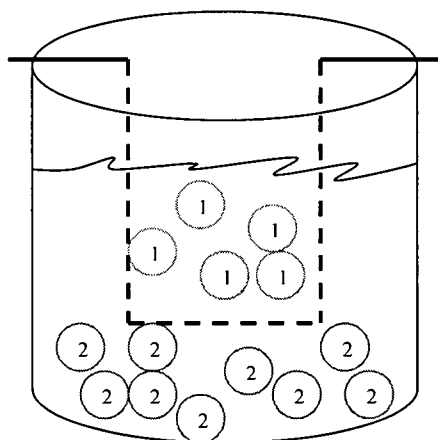


4.4.2 CELL-TO-CELL CONTACT DEPENDENT MECHANISMS

From the neutralization study, it was apparent that there must be an additional mechanism to fully account for how the TSST-1 primed CD4⁺ T cells suppress B cell function. Along with cytokines, cell-to-cell contact has often been cited as vital for regulatory T cells to mediate suppression. With respect to B cells, there are various surface expressed ligands that can potentially bind with receptors present upon regulatory T cells. Thus, an experiment which focuses on cell-to-cell dependent interactions between TSST-1 or PBS primed CD4⁺ T cells and B cells was performed. A transwell system was employed in this study so that, through the use of a cell impermeable filter, the contribution of direct T/B cell contact may be eliminated. However, there is one critical problem with this experimental setup – because TSST-1 is not a B cell mitogen, in order to induce B cell proliferation, direct T_H2 and B cell contact is required. Thus, using this transwell system, the co-culture of PBS primed CD4⁺ T cells and B cells, which is normally the positive control, would not be able to induce B cell proliferation since cell-to-cell contact would have been abolished. With this in mind, the transwell insert system was modified to allow for B cell proliferation by co-culturing naïve CD4⁺ T cells with B cells underneath the filter while TSST-1 or PBS primed CD4⁺ T cells were added into the upper compartment, thus allowing for the presence of T cell help for B cell proliferation while only allowing medium and cytokines to flow through from the TSST-1 or PBS primed CD4⁺ T cells (strategy is described in Figure 18). Figure 19 indicates that in the presence of the insert (Fig.19.A), B cells underwent more divisions than when the insert was absent to separate the B cells from the TSST-1 primed CD4⁺ T cells (50% vs. 40%, $p < 0.05$). However, the percentage of proliferation did not reach the same level as that of the B cell and PBS primed CD4⁺ T cell co-culture (50% vs. 60%, $p = 0.005$, Fig.19.B.), indicating that the suppression of TSST-1 primed CD4⁺ T cells was only

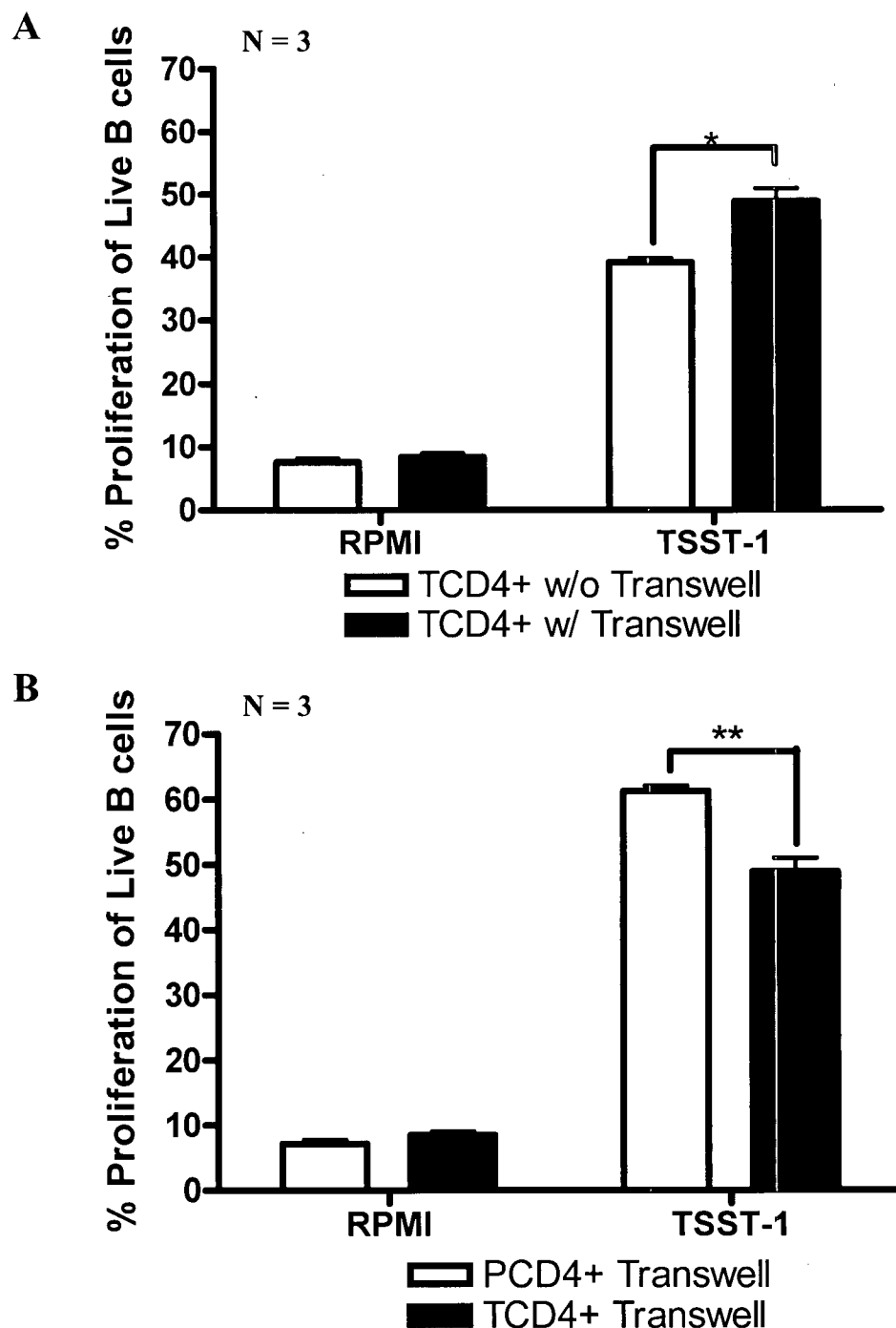
partially reversed. These results suggest that cell-to-cell mediated mechanisms alone cannot fully account for TSST-1 primed CD4⁺ T cell suppression of B cells. In conjunction with the neutralization study, it would appear that TSST-1 primed CD4⁺ T cells suppress B cells via both cytokine and cell-to-cell dependent mechanisms.

Figure 18: Depiction of the protocol followed in the experiment done in Figure 19.B. Figure 18 illustrates the transwell system with the addition of either TSST-1 or PBS primed CD4+ T cells inside the insert.



① = TCD4+ or PCD4+ T cells ② = B cells and naïve T cells

Figure 19: The involvement of cell-to-cell contact in mediating the TSST-1 induced CD4+ T cell effect on B cells. Fig.19.A. PBS primed CD4+ T cells were co-cultured with B cells on one side of the 0.4 μ m transwell membrane filter insert while TSST-1 primed CD4+ T cells were added on the other side with or without 1 nM TSST-1 stimulation. This is compared to B cells co-cultured with equal numbers of TSST-1 primed CD4+ T cells without insert. Fig.19.B PBS primed CD4+ T cells were co-cultured with B cells on one side of the insert while either TSST-1 or PBS primed CD4+ T cells were added on the other side with or without 1 nM TSST-1 stimulation. Cells were cultured for 72 h and B proliferation was analysed using CFSE and flow cytometry. Results (mean and SEM) were compiled from three independent experiments. * $p < 0.05$ and ** $p < 0.005$, analyzed using Student's t tests.



4.4.3 BYSTANDER EFFECT

As mentioned, B cells play many roles, commonly functioning as antigen presenting cells as well as effector antibody producing cell. Because of this duality, the study of B cells presents a unique challenge to separate which role the B cell is playing at any one time. In the case of TSST-1 induced CD4⁺ T and B cell interaction, it is unclear if B cells are directly suppressed by TSST-1 primed CD4⁺ T cells or indirectly via suppression of cognate T_H2 CD4⁺ T cells, which, having been suppressed, will be unable to activate B cells. To examine this question, an add back experiment was performed. Firstly, B cells were activated by the common B cell mitogen, lipopolysaccharide (LPS), and the supernatant generated from the *in vitro* culture of TSST-1 or PBS primed CD4⁺ T cells with or without TSST-1 stimulation was added to the B cell culture to investigate if the effect of TSST-1 induced CD4⁺ T cells is exerted even in absence of T cell involvement (strategy is described in Figure 20). Furthermore, this experiment is also a confirmatory experiment for the antibody neutralization experiment which could indicate if cytokines are involved in mediating the suppression. According to Figure 21, the addition of supernatant from an *in vitro* culture of TSST-1 activated TSST-1 primed CD4⁺ T cells has the ability to suppress B cell proliferation when compared to the supernatant free control (25% vs. 18%, $p < 0.05$). On the other hand, TSST-1 activated PBS primed CD4⁺ T cell supernatant exerted no statistically significant effect on B cell proliferation when compared to the supernatant free control. The addition of supernatant from TSST-1 activated TSST-1 primed CD4⁺ T cells also induced a significant decrease on B cell proliferation when compared to supernatant from un-activated TSST-1 primed CD4⁺ T cells (27% vs. 18%, $p < 0.005$). Thus, TSST-1 primed CD4⁺ T cells are able to suppress B cell proliferation even in the absence of cognate T_H2 cells, suggesting that TSST-1 primed CD4⁺ T cells can interact directly with B cells. These results also confirm observations made during the neutralization study that cytokines are

at least partially accountable for B cell suppression by TSST-1 primed CD4⁺ T cells as the supernatant from a TSST-1 activated TSST-1 primed CD4⁺ T cell culture caused a decrease in LPS induced B cell proliferation.

Figure 20: Flow chart to illustrate the procedure performed to demonstrate a bystander effect of TSST-1 primed CD4⁺ T cells on B cell proliferation.

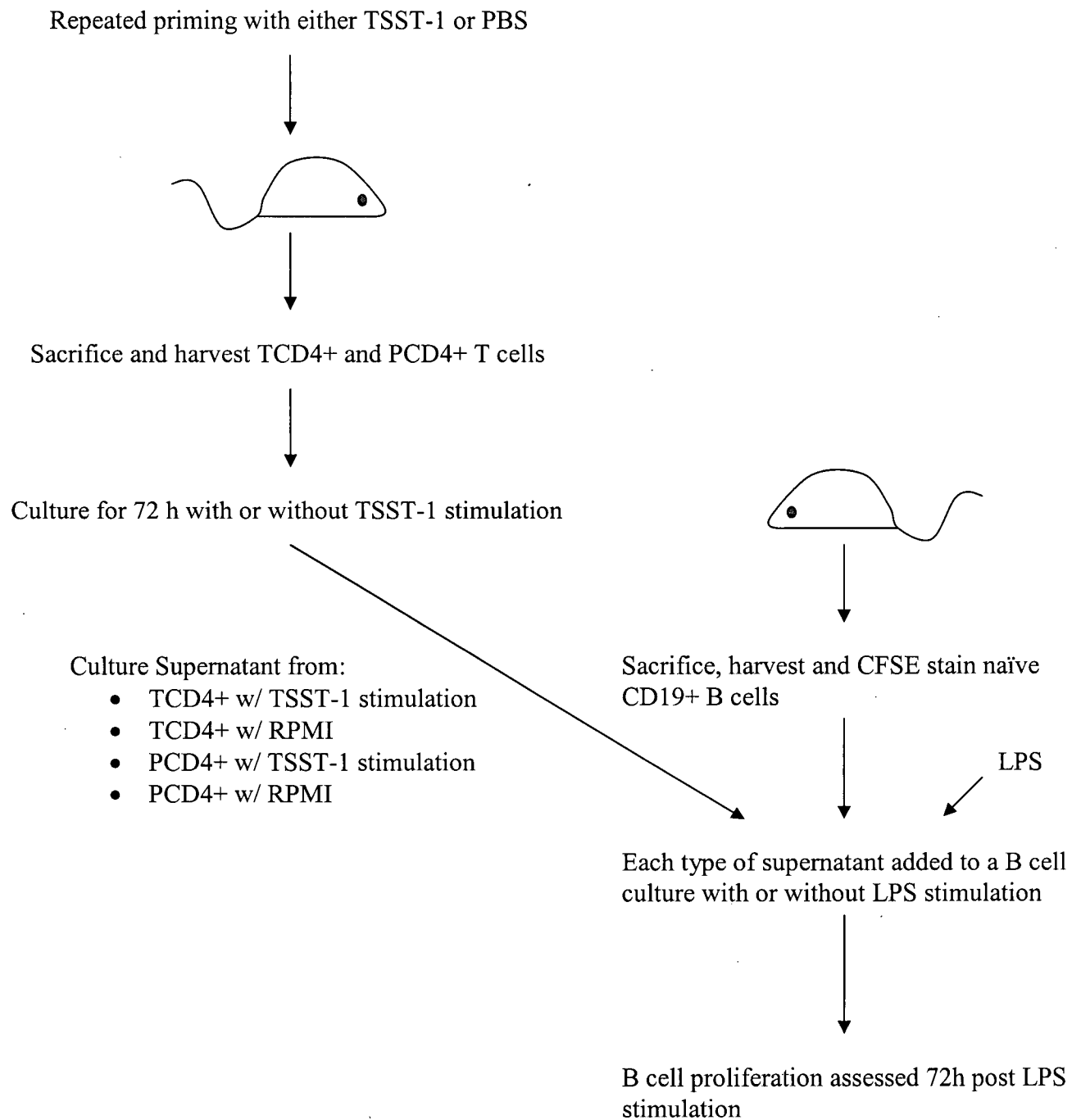
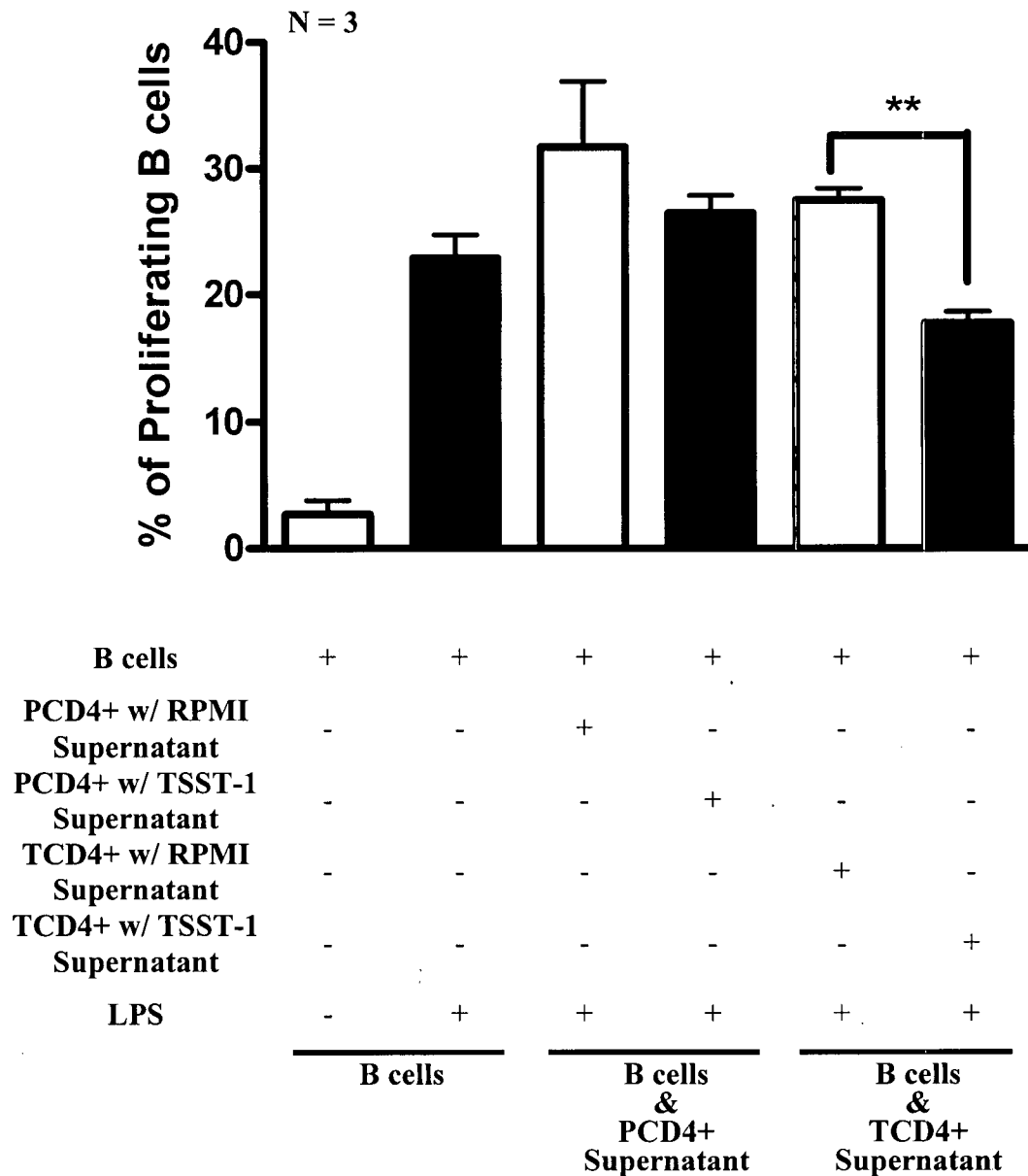


Figure 21: The effect of TSST-1 primed CD4+ T cell supernatant alone on LPS-induced B cell proliferation rate. Splenic TSST-1 or PBS primed CD4+ T cells were isolated and cultured with or without 1 nM TSST-1 stimulation *in vitro* for 48h, at which point the supernatant was collected. Naïve mice were then sacrificed and splenic B cells purified. B cells were then cultured with 1 µg/ml LPS stimulation with or without the addition of supernatant from the TSST-1 or PBS primed CD4+ T cell culture that had been activated with or without TSST-1 stimulation. Results (mean and SEM) were compiled from three independent experiments. ** p < 0.005, analyzed using Student's t tests.



5.0 DISCUSSION

5.1 ROLE OF TSST-1 INDUCED T_{REGS} IN THE TSST-1 AND NOT PBS PRIMED CD4+ T CELL POPULATION WHICH LEADS TO B CELL SUPPRESSION

To this point, the TSST-1 primed CD4+ T cell population in this thesis has not been called “Tregs” by name. However, all the data collected suggest that the TSST-1 induced CD4+ T cells were able to exert a suppressive effect on B cells likely because TSST-1 primed Tregs were generated within that CD4+ T cell population. Regulatory T cells are defined by their immunosuppressive IL-10 dominant cytokine profile and their ability to down-regulate other cells of the immune system, namely the T cells, in an IL-10 dependent manner. In conjunction with the work done by others in the lab investigating the interaction between TSST-1 primed CD4+ T cells and helper T cells (Cameron and Chow, 2005; Li H and Chow, 2006), the work reported here suggests that functionally, this population of TSST-1 induced T cells has the ability to suppress B cell function as well. With respect to the cytokine profile, TSST-1 primed CD4+ T cells certainly exhibit the classical immunosuppressive profile of Tregs – high IL-10 and no IL-2 or IL-4 – while the PBS primed CD4+ T cells do not have such a profile, generating high IL-2, IL-4 and no IL-10 upon TSST-1 stimulation. Thus, this cytokine profile is priming specific. Furthermore, not only is IL-10 made, it is also shown to be vital in the suppressive mechanism of the TSST-1 primed CD4+ T cells. The neutralization and supernatant add back studies indicate that IL-10 is present (from the cytokine ELISA results) and is involved in mediating suppression. Thus, it would appear that there likely contains, in the TSST-1 primed CD4+ T cell population, putative TSST-1 primed Tregs, since the TSST-1 primed CD4+ T cell population fits the two criteria that defines Tr1 cells. However, because steps could not be taken to eliminate other Tregs, such as natural CD4+CD25+ Tregs, from the models of study without exerting an influence on the generation of antigen induced Tr1 cells, it is unclear which population in the TSST-1 primed CD4+ T cell population is responsible for B cell suppression. Furthermore, the

characterization of the mechanism of suppression – being based on IL-10 and TGF- β , also applies to that of the T_H3 cells, making distinction between Tr1 and T_H3 cells impossible. Thus, while there is no doubt that Tregs are at work, the type of Treg, however, is unclear, although TSST-1 induced Tr1s are the prime candidate. Thus, a more generic label of simply “Tregs”, T cells that exhibit a suppressive cytokine profile and can exert a suppressive effect on other lymphocytes, is chosen to describe the suppressive cells within the TSST-1 primed CD4⁺ T cell population.

5.2 SUPPRESSION OF B CELLS BY TSST-1 PRIMED CD4⁺ T CELLS IS LIKELY TO BE MAINLY ATTRIBUTED TO THE TSST-1 PRIMED CD4⁺CD25⁺ T CELL SUBSET

While the separation of CD4⁺ T cells into CD25⁺ and CD25⁻ subsets is problematic and artificial, nevertheless, CD25 remains one of the most commonly used marker to identify regulatory T cells. From these results, it would appear that TSST-1 primed CD4⁺CD25⁺ T cells is the subset that most closely resembles the TSST-1 primed CD4⁺ T cells functionally as the B cell response in co-cultures with these two types of T cells are highly similar with respect to the suppression of proliferation, drop in cell viability and suppression of antibody production. This implies that while the CD25⁺ T cell population may only make up approximately 10%-20% of the CD4⁺ T cell population, they are nevertheless the dominant cell type that dictates the action of the entire CD4⁺ T cell population. Based on the current knowledge in the literature, it is certainly expected to find regulatory T cells residing in the CD25⁺ T cell population. The surprise, therefore, was to find that the CD25⁻ T cell population also exerts a down-regulatory effect on B cells. As mentioned, CD25 based separation techniques are far from perfect and thus, it is plausible for there to be contaminating CD25⁺ T cells in the CD25⁻ T cell population. Given how powerful these TSST-1 primed CD25⁺ T cells are, it is entirely possible that these cells still

are able to exert a suppressive effect on B cells. However, due to the low numbers, they can only exert an attenuated form of suppression, having only the ability to delay B cell responses instead of abolishing them entirely through the possible mechanism of AICD.

Of course, the other possibility is that there might actually be two different types of regulatory T cell populations, one in the CD25⁺ T cell subset and one in the CD25⁻ T cell subset. Certainly, from the studies looking at the mechanism of suppression, there is currently no known classification of Tregs that mediates suppression via both cytokines and cell-to-cell mediated contact. Thus, if the classification criteria are correct, then this would suggest that the suppression of B cells is mediated by at least two different Treg populations. It is known that natural CD4⁺CD25⁺ T cells reside in the CD25⁺ T cell fraction. CD25 expression on antigen induced Tr1s, however, remain in doubt. Thus, it might be that the putative TSST-1 primed Tr1s are CD25⁻, mediating their suppression of B cell antibody production via cytokine production, while the CD25⁺ fraction, containing nTregs, induces B cell apoptosis via cell-to-cell mediated contact. Natural Tregs, however, may not be the culprit as repeated TSST-1 stimulations may generate two populations of regulatory T cells – one residing in the CD25⁺ T cell population, and one in the CD25⁻ T cell population (discussed further in Section 5.5; Fig.23). Since the neutralization and transwell studies were done using whole CD4⁺ T cell populations, it is therefore not known if the two mechanisms of suppression are attributed to different populations of T cells or both to the same one.

At this point, it must be pointed out that there is a danger in concluding that TSST-1 primed CD4⁺CD25⁺ T cells are the cells exerting the dominant response. This is because the *in vitro* study is highly artificial and not at all representative of the human immune system. Cells are co-cultured at a 1:1 ratio (50% of T cells and B cells) while the spleen has a much different percentage breakdown with 35% of the total population as B cells and 30% as CD4⁺ T cells, as 5-10% of this population are Tregs while only 20% of the CD4⁺ T cell population are CD25⁺

after repeated TSST-1 stimulation. Of course, there are other types of T cells, such as CD8+ T cells, to further complicate the situation. Thus, whatever the effect is observed *in vitro*, it must be kept in mind that the results might not be representative of conditions *in vivo*. Nevertheless, the *in vitro* model affords researchers a closed environment to study cellular interactions between cells.

5.3 TSST-1 INDUCED T_{REGS} SUPPRESSION IS MEDIATED BY BOTH CYTOKINE BYSTANDER EFFECT AS WELL AS DIRECT CELL-TO-CELL MECHANISMS ON B CELLS

While there are no currently described category of Tregs whose suppression is mediated by both cytokine and cell-to-cell dependent mechanisms, many of the definitions for different subtypes of Tregs are not absolute. Indeed, little is known about the suppression mechanism of Tr1 cells other than that it is IL-10 dependent. Here, the argument is made for a new type of regulatory T cells that require both cytokine and cell-to-cell contact dependent mechanisms to fully mediate suppression.

The suppression of TSST-1 primed CD4+ T cells was seen to be mediated by both cytokine and cell-to-cell dependent mechanisms as cytokine neutralization restores about 83% of B cell proliferation (B cell proliferation in presence of TSST-1 primed CD4+ T cells and α IL-10r and α TGF- β neutralizing antibodies vs. B cell proliferation in presence of PBS primed CD4+ T cells; 54%/65%; Fig.17) while transwell system restores 77% of B cell proliferation (B cell proliferation in the presence of TSST-1 primed CD4+ T cells in a transwell membrane vs. B cell proliferation in the presence of PBS primed CD4+ T cells; 50%/65%; Fig.19). These two combined appeared to have the ability to restore B cell proliferation to the level observed in the co-culture with PBS primed CD4+ T cells (from 40% of B cells undergoing proliferation as seen in the TSST-1 primed CD4+ T cell co-culture). Of course, this hypothesis must be tested. One

way to do so is to conduct an experiment that combines both the neutralization and the transwell studies together – that by adding neutralization antibodies to the transwell system, B cells can then be tracked to see if proliferation rate can then be restored. Nevertheless, it would appear that TSST-1 primed Tregs are unlike all other described Tregs since both mechanisms are required for full suppression. Since it does not appear that the effects are synergistic, this suggests that the two suppression mechanisms may be interdependent on the same pathway, which together, works holistically to mediate suppression. Given that several groups have reported cell-to-cell contact between Tregs and B cells resulting in B cell apoptosis, it is therefore proposed that direct cellular contact between TSST-1 primed Tr1s and B cells lead to B cell apoptosis (Janssens *et al.*, 2003; Zhao *et al.*, 2006). On the other hand, B cells in the periphery not engaging in direct contact with TSST-1 primed Tr1s will be functionally suppressed (and not killed) by the immunosuppressive cytokines, as has been reported here and elsewhere (Satoguina *et al.*, 2005). This proposed model is further discussed below in section 5.5.

The type of ligand/receptor interaction that might occur between TSST-1 primed CD4⁺ T cells and B cells is currently unknown but based on the available data in the field, there are several interesting possibilities. Fas/FasL interactions might be one such interaction. Fas/FasL is known to mediate apoptosis and consequently, the involvement of Fas would explain the drop in viability. Certainly, one group has already found that Tregs mediate B cell apoptosis via Fas/FasL engagement (Janssens *et al.*, 2003). While T and B cells might interact through some ligand/receptor system that leads to suppression, interaction between the two types of lymphocytes might also involve a surface ligand that acts as a competitor for the activating ligand, blocking ligand/receptor binding that promotes B cell activation. One example is CD28 binding with CD80/CD86 versus CTLA-4 interaction with CD80/CD86. CD80/CD86 are co-stimulatory molecules on APCs which, when bound to CD28 on T_H2 cells, help to induce B cell proliferation. CTLA-4 is a surface ligand found on many types of regulatory T cells and this

ligand is known to bind CD80/CD86, as discussed before. Thus, it is hypothesized that CTLA-4 on regulatory T cells competes with CD28 for CD80/CD86 sites to prevent the induction of an activating signal. Mechanistically, Tregs compete for CD80/86 possibly via one of two ways. First, it is possible that the TSST-1 primed CD4⁺ T cells upregulate the expression of CTLA-4 so that while there may be a much lower number of these cells than helper T cells, the availability and affinity of the ligand is high. Second, and likely the more probable mechanism, is that TSST-1 primed CD4⁺ T cells are not only exerting a suppressive effect directly on B cells, as seen in the supernatant add back study, but is also down-regulating the T_H2 cells simultaneously, inducing the internalization of surface CD28 and CD40L expression. In essence, TSST-1 primed CD4⁺ T cells might exert a suppressive effect on B cells by suppressing B cell proliferation and by “turning off” the mechanism by which B cells are activated.

Furthermore, the observations made that there is a direct interaction between TSST-1 primed Tregs and B cells suggest that B cells might take up immunosuppressive cytokines, such as IL-10 and TGF- β , leading possibly to the modulation of B cell activation and function. B cells are known to take up IL-10 and TGF- β but the effect of these cytokines on B cells is controversial (Parekh *et al.*, 2003; Bober *et al.*, 1994). IL-10 is traditionally thought of as a B cell inducer, increasing B cell proliferation, activation and differentiation (Go *et al.*, 1990). Certainly, T_H2 cells make IL-10 which, along with other cytokines, induces IgG1 or IgE production. This situation, therefore, presents an apparent contradiction of the duality of the role of IL-10 – why would TSST-1 primed CD4⁺ T cells make IL-10 which possibly contributes to B cell suppression while IL-10 made by T_H2 causes the diametrically opposite response? Perhaps one of the most probable explanations for this conundrum would be that of concentration. T_H2 production of IL-10 is relatively low, generating around the order of 100 pg/ml. TSST-1 primed CD4⁺ T cells, on the other hand, generates IL-10 on the order of 3,000 pg/ml. Thus, it is hypothesized that at a low concentration of IL-10, B cells are activated while at a high

concentration, B cells are suppressed. One way to address this speculation would be to observe the dosage affect of IL-10 in a culture of B cells undergoing LPS induced proliferation. Any changes to the rate of proliferation would give clues as to how valid this IL-10 dosage theory might be.

5.4 TSST-1 INDUCED T_{REGS} EXERT ANTIGEN NON-SPECIFIC SUPPRESSION ON B CELL FUNCTION *IN VIVO* AND *IN VITRO*

One of the unique characteristics of antigen induced Tr1 cells is that these regulatory cells are antigen specific in their activation but antigen non-specific in their suppressive function. With respect to these TSST-1 induced CD4⁺ T cells which contain putative TSST-1 primed Tregs, it would appear that they certainly are antigen specific in their activation while the effect is antigen non-specific (suppression of both TNP and LPS specific B cell responses). However, this assertion has yet to be proven because there are two possible explanations for why TNP and LPS specific responses were both suppressed. What is hypothesized to happen during this method of repeated TSST-1 priming is that TSST-1 is able to cross react between T cells that bear the V β region to the MHCII molecule displayed on APCs, leading to the activation of those T cells. Over repeated TSST-1 challenges, the interaction between V β +T cells and MHCII is hypothesized to change from inducing T_H1 cells, as would occur after one challenge, to the generation of immunosuppressive Tregs. First, it is possible that the TNP-specific T cell clones also contain the appropriate V β region, which then upon repeated TSST-1 stimulations, activate, among others, TNP-specific TSST-1 induced CD4⁺ T cells simply by virtue of clonal expansion of the V β specific CD4⁺ T cells. Because TSST-1, being a superantigen, activates about 5-20% of the total T cell population polyclonally, it is hypothesized that these cells are not epitope non-specific in the way they exert their suppressive effect, but rather, simply has a wide range of epitope specificity (since a conventional antigen activates specific T cells clones, which

comprises about 0.01% of the total population). Second, TSST-1 primed T cells are antigen non-specific in their suppression where TNP-specific or LPS activated B cells may simply be down-regulated as the result of the bystander effect. The neutralization and bystander studies certainly support the notion that IL-10 and TGF- β , at least in part, mediate B cell suppression, thus implying non-specificity as any cells with IL-10 or TGF- β receptors may bind these cytokines. Between these two possibilities, it is suspected that TSST-1 primed CD4⁺ T cells are antigen non-specific in their activity since the clinical observation has been that patients who suffered from TSST-1 induced toxic shock have low B cell count even after recovery, implying non-specific induction of B cell apoptosis and suppression. Presently, however, the only assertion that can be made is that TSST-1 primed CD4⁺ T cells have a broad range of epitope specificity.

5.5 TWO PROPOSED MODELS TO DESCRIBE THE DELICATE BALANCE BETWEEN ACTIVATION AND SUPPRESSION

The immune system operates upon an exquisite balance between activation and suppression, the loss of either one, would lead to disease outcomes, such as autoimmune diseases, various forms of cancers or infections. Thus, the immune response may be skewed one way or another simply by tipping the balance. Through evolution, many bacteria have discovered different ways to alter the balance so that the immune system would encounter difficulty when attempting to clear the pathogen. *Staphylococcus aureus* is one such organism seemingly capable of immune modulation, specifically by the secretion of bacterial superantigens, such as TSST-1. The work done in this project expands upon the conventional role of TSST-1 as that of a superantigen, a potent immune activator, and two new models of how TSST-1 interacts with immune cells is proposed.

Normal interaction involving a conventional antigen takes place when APCs present processed antigen peptides via the MHCII molecule. The loaded MHCII molecule interacts with

TCR on specific T cell clones and induces clonal T cell activation and expansion (Fig.22.A). With a superantigen such as TSST-1, it has the ability to cross-link and mediate non-specific APC and T cell interaction, resulting in polyclonal activation and expansion of all T cell clones bearing the appropriate V β segments (V β 2 in humans and V β 15 in mice) (Fig.22.B). Finally, with chronic TSST-1 exposure, TSST-1 induced Tr1s may be generated, which help to suppress the immune response, inhibiting the host's ability to clear *S. aureus*, allowing for immune evasion and bacterial survival (Fig.22.C).

Based on the observations made in this project, there are two possible models to explain the influence of TSST-1 primed Tr1s on B cells. First, it is possible that repeated TSST-1 priming generates two populations of Tr1s, one residing in the CD25+ T cell population, one in the CD25- T cell population (Fig.23). TSST-1 primed CD25+ Tr1 cells suppress B cell clones that are specific for TSST-1 by inducing apoptosis, mediated via direct cell-to-cell contact (Fig.23.A). Likewise, TSST-1 primed CD25- Tr1 cells also suppress B cells. However, these Tr1 cells suppress B cells that are not TSST-1 specific via a bystander effect, mediated by the production of immunosuppressive cytokines (Fig.23.B). These two populations of Tr1 cells combined help to maintain B cell tolerance towards *Staphylococcus aureus*, the bacteria that produces TSST-1. The second model proposes that repeated TSST-1 priming generates one population of TSST-1 primed Tr1 cells that suppress B cells in several ways. The model for B cell suppression by TSST-1 primed Tr1 cells requires, like B cell activation, two signals. The putative Signal 1 that is required for B cell suppression is the same as for B cell activation, generated when BCR engages specific antigens in the extracellular space. The second signal is mediated when TSST-1 primed Tr1 cells are cross-linked to B cells via TSST-1 along with the binding of other co-stimulatory molecules such as Fas/FasL or CTLA-4/CD80/CD86. These two signals combined to induce B cell apoptosis, leading to the depletion of B cells in an antigen specific manner (Fig.24.A). In the absence of Signal 1, cross-linking MHCII on B cells with

TCR on TSST-1 primed Tr1s would not induce an apoptotic effect since these B cells are not TSST-1 specific and hence would not have been activated (Fig.24.B). However, these non-TSST-1 recognizing B cells would still be suppressed by TSST-1 primed Tr1 cells but via cytokine production (Fig.24.C). As a result, there is a global antigen non-specific suppression exerted upon the B cell population by TSST-1 primed Tr1 cells but specifically, B cells that are reactive against TSST-1 will be purged from the system. From the bacteria point of view, this would also achieve the goal of immune evasion.

Figure 22: The induction of various types of T cells by conventional antigens, TSST-1 superantigens or chronic TSST-1 stimulations. Fig.22.A, specific interaction of MHCII molecules presenting conventional antigen peptides to T cells having the TCRs with appropriate epitope specificity. This leads to the activation of specific T cell clones. Fig.22.B, the TSST-1 superantigen non-specifically cross-links APCs with T cells, leading to polyclonal activation and T_H1 differentiation. Fig.22.C, upon chronic TSST-1 stimulations, the interaction between APCs and T cells changes in an as-yet unknown way whereby T cells are induced to become TSST-1 primed Tr1 cells.

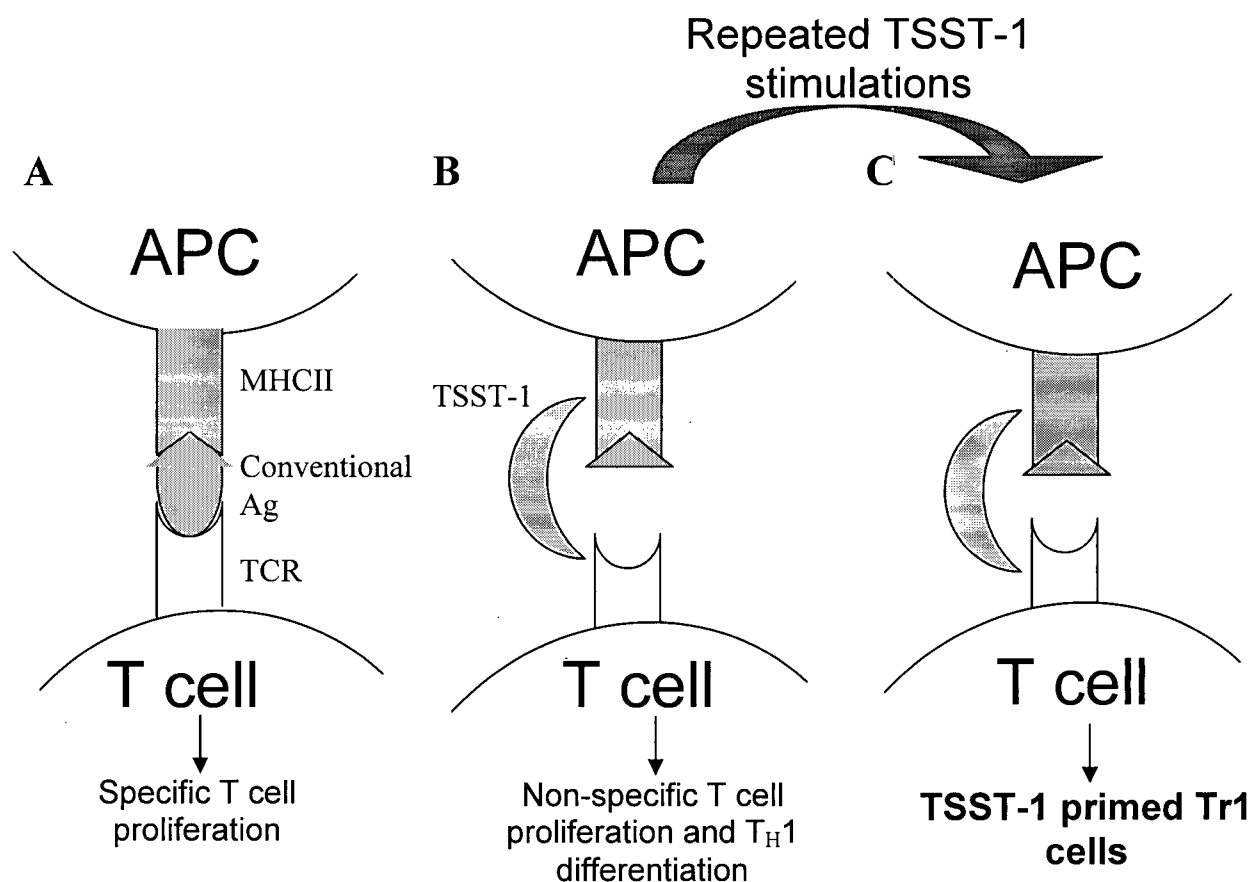


Figure 23: First proposed model of TSST-1 primed Tr1 cell suppression of B cells. Fig.23.A, TSST-1 primed CD25+ Tr1 cells induce TSST-1 specific B cells to undergo apoptosis via cell-to-cell contact. Fig.23.B, TSST-1 primed CD25- Tr1 cells suppress non-TSST-1 specific B cell function via cytokine production.

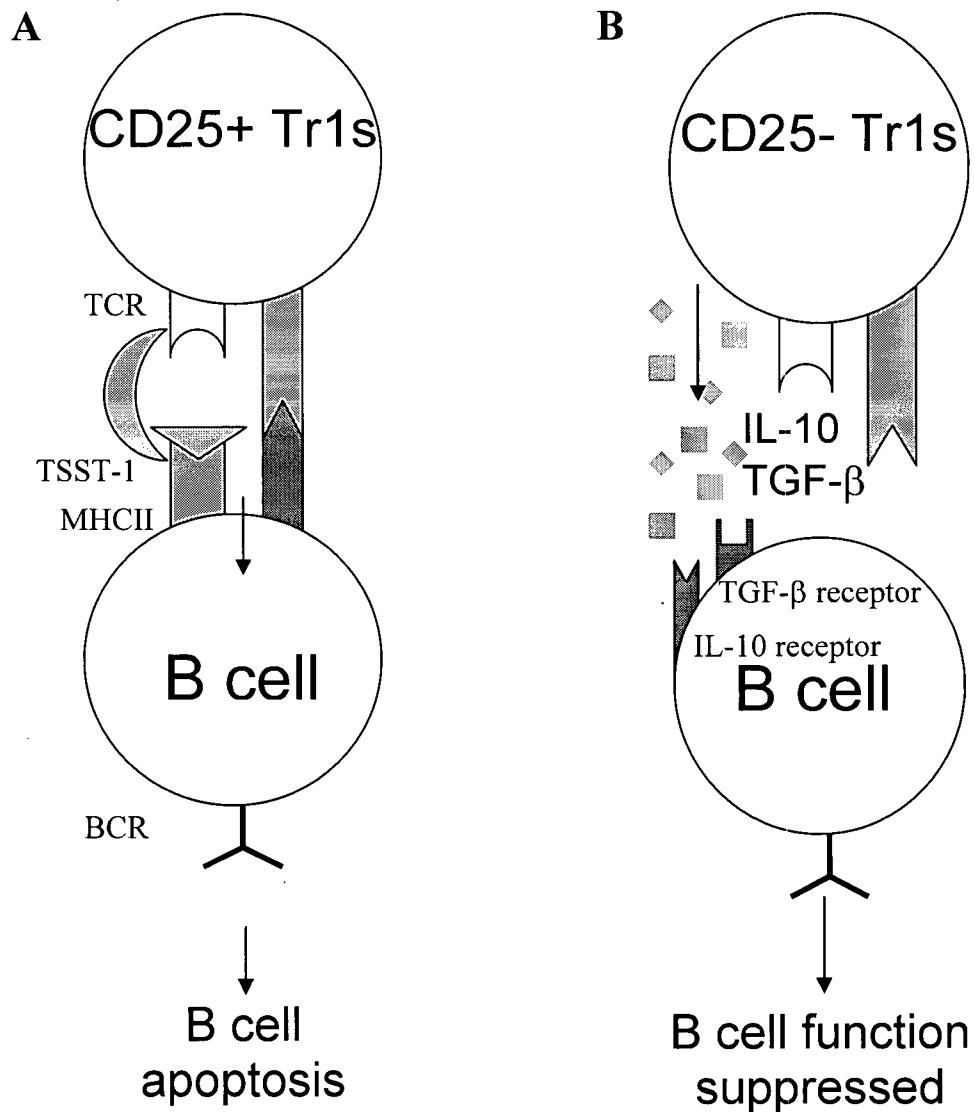
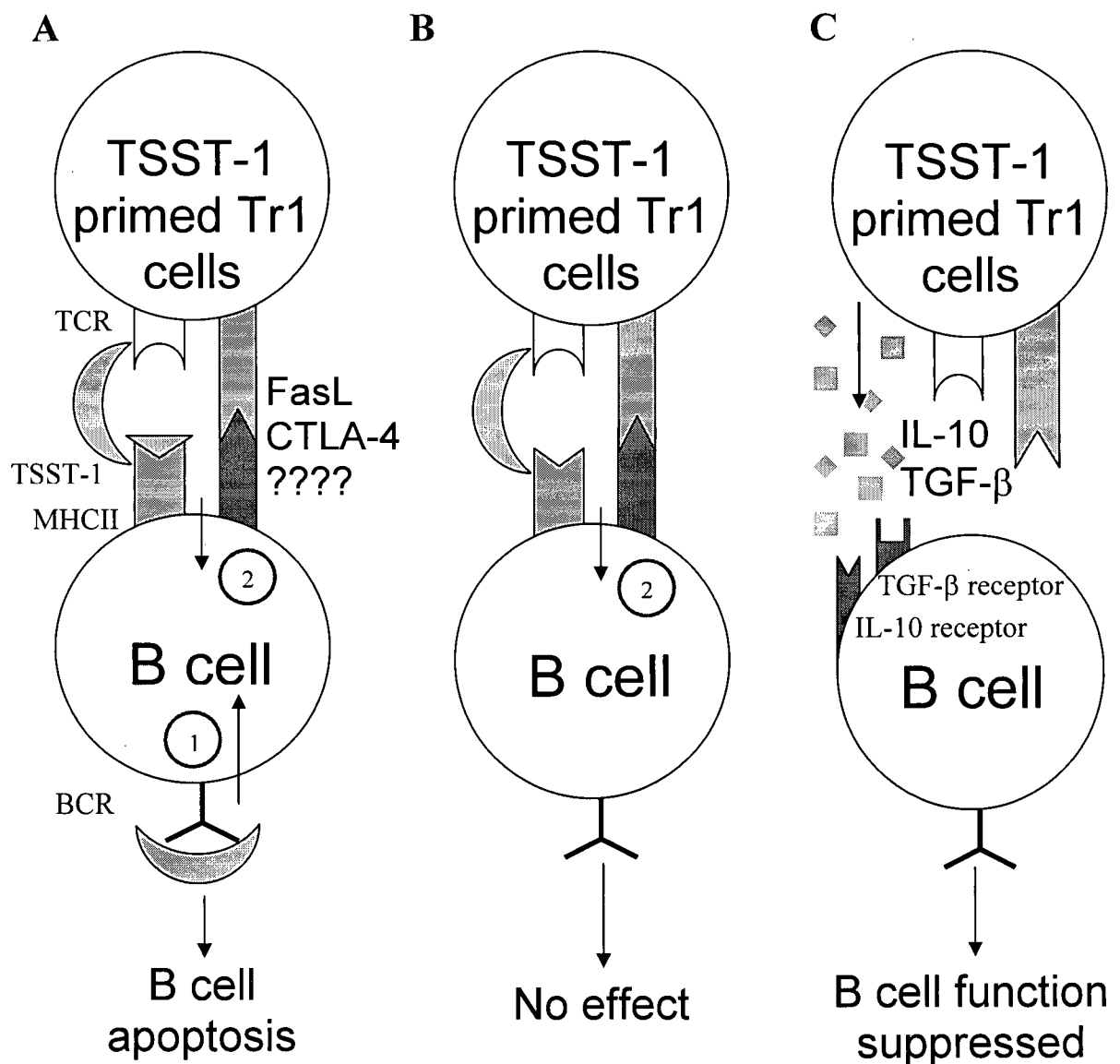


Figure 24: Second proposed model of TSST-1 primed Tr1 cell suppression of B cells. Fig.24.A, TSST-1 specific B cells bind to TSST-1 through the BCR, which provides Signal 1, while cross-linking to the TCR on TSST-1 primed Tr1 cells via TSST-1, which along with the binding of co-stimulatory molecules, generates Signal 2. Together, Signal 1 and 2 induce apoptosis in TSST-1 specific B cells. Fig.24.B, Signal 2 in absence of Signal 1 has no effect on B cells. Fig.24.C, the function of surrounding TSST-1 non-specific B cells is suppressed by cytokines - IL-10 and TGF- β - that are produced by TSST-1 primed Tr1 cells.



5.6 POTENTIAL APPLICATIONS OF TSST-1 PRIMED Tregs IN B CELL MEDIATED AUTOIMMUNE DISEASES

The possible generation of TSST-1 induced Tregs that have the ability to control T and B cells certainly brings about an exciting promise to the field of regulatory T cells from a therapeutic point of view for people suffering from various autoimmune diseases. If a normal immune response is the net result of all pro-inflammatory and anti-inflammatory signals from all immune cells, then autoimmune diseases might be thought of as the result of immune dysregulation – where either the signals for pro-inflammation are overwhelming that of the anti-inflammation signals from immune cells or that the anti-inflammatory responses are somehow prevented from exerting their influence. Thus, if suppressive regulatory cells may be generated and deployed under tight control, these cells have the potential to be used therapeutically to bring balance back to the immune system. Within the literature, there have already been multiple attempts to employ Tregs as immune modulating therapy. Because a deficiency in Tregs has been observed in rheumatoid arthritis (Ehrenstein *et al.*, 2004), type I diabetes (Lindley *et al.*, 2005) and multiple sclerosis (Viglietta *et al.*, 2004; Hafler *et al.*, 2005), it is logical to suspect that all of these autoimmune diseases may be the result of the lack of functional Tregs in the body and hence, these diseases are prime candidates for Treg adoptive transfer and “add back” immune therapy. Attempts to study the *in vivo* effectiveness of Tregs in controlling various autoimmune diseases have already begun. Myelin basic protein reactive Tregs introduced into the autoimmune encephalomyelitis murine model (Francis *et al.*, 2003) helped to ameliorate disease symptoms and reduce morbidity (Mekala *et al.*, 2005; Mekala and Geiger, 2005). In NOD mice, the type I diabetes model, several groups have already shown the effectiveness of Tregs as immunotherapy (Battaglia *et al.*, 2006b). Furthermore, Jaeckle *et al.* has also shown that Foxp3 transduced T cells may control established type 1 diabetes in mice. Furthermore, in transplantation, suppression of B cell reactivity towards allografts may benefit from this therapy.

Battaglia *et al.* have used rapamycin and IL-10 to induce the generation and expansion of antigen-specific Tr1 cells which have been able to prevent graft versus host transplantation rejection diseases (Battaglia *et al.*, 2006a).

While many groups have found varying success in their particular murine disease models, the limitations of human clinical application of Treg therapy remain. Due to the anergic state that natural Tregs are in, they pose a problem when one is trying to expand these cells to collect large enough numbers for *in vivo* therapy (Bluestone, 2005). Several groups have found distinct ways to bypass this problem by generating Tregs using α CD3/ α CD28 coated beads, mitogens, peptide-MHCII multimer complexes or specific antigen pulsed DCs. However, these methods, while successful, also pose another potential problem – that of possible uncontrollable expansion *in vivo*. Finally, there is the concern of antigen specificity where transferred Tregs lack antigen specificity thus conferring suppression on even unrelated inflammatory responses, rendering the patient severely immuno-compromised. In effect, this is the concern of Tregs mediating their suppression via cytokines for this will result in the bystander effect.

With TSST-1 primed Tregs, some of these problems are likely avoided. Due to their ability to undergo hyperproliferation, there will be little difficulty in generating enough TSST-1 induced Tregs. As for being able to control Tregs, it has been shown in this project, and in others, that TSST-1 primed Tregs will only be activated in the presence of TSST-1. Presumably, introduction of a TSST-1 neutralizing agents would then be able to “turn off” TSST-1 primed Treg suppression. Also, it is predicted that the activity of TSST-1 primed Tregs is only observed for a short period, on the order of several weeks. However, these assertions have yet to be substantiated. As for the question of antigen specificity, TSST-1 primed Tregs would indeed have a wide antigen specificity. Since cell-to-cell mediated contact is required for TSST-1 induced Tregs suppression as well as cytokine production, bystander suppression may not be a great concern. Furthermore, there are several additional advantages to using TSST-1 primed

Tregs. First, because of the range of antigen specificity that TSST-1 induced Tregs have, there is no need to define the specific antigen that triggers an autoimmune disease. Certainly, in many instances, there is more than one antigen that triggers autoimmunity. Second, the availability of a “non-toxic” TSST-1 mutant (S14N) that is non-lethal to both human PBMCs and murine splenocytes while retaining the ability to generate Tr1 cells (Kum *et al.*, 2001; Kum *et al.*, 2004) allows for possible clinical usage while minimizing the potential of triggering toxic shock syndrome in the patient.

Based on the current knowledge of different autoimmune diseases, the candidate that would most likely receive benefit from TSST-1 induced Treg therapy would be allergy and allergic asthma patients since B cell over-reactivity is the sole or main cause of pathology (Tournoy *et al.*, 2006). Currently, the only treatment of allergies is allergen specific immunotherapy (SIT). Combined with the knowledge of inducible Tregs, it appears that SIT works by inducing host tolerance to allergens possibly by way of inducing Treg responses (Taylor *et al.*, 2006; Ling *et al.*, 2004). Instead of SIT, however, TSST-1 induced Tr1s may be directly administered instead. Patients’ blood may be drawn and CD4+ T cells isolated so that TSST-1 induced Tregs may be generated *in vitro*, adhering to the protocol described by another member of the Chow Lab (Kum and Chow, 2005a). These Tregs can then be purified into the specific subsets so that whichever response, be it regulatory or even T_H1 or T_H2 based, may be selected. The purified cells can then be reintroduced back into the patient along with a low dose of non-toxic TSST-1 mutant, such as S14N, to activate the transferred cells. If the *in vivo* mouse model is any indication, this effect would then exert a suppressive effect on the immune system which would last for up to 14 days – long enough to “quiet down” the immune system but likely not strong enough to damage the immune system. While this assertion has yet to be substantiated, it is not expected that there will be any major side effects or complications because these are autologous cells, taken out from the same patient and therefore would have no

complications involving rejection, and this therapeutic method selects for a natural response, and therefore little chance for therapy resistance. In essence, this method of generating TSST-1 induced Tregs is simply taking a normal response in the immune system and heightening it when that response, for whatever reason, has been prevented from being activated in cases of allergic asthma or any other types of autoimmune diseases.

5.7 FUTURE WORK

As touched upon in the discussion, there is a great deal of work that remains to be done in this promising field. However, only the most pressing and immediate future directives shall be discussed here. First, one of the biggest needs of this project is to shed some light upon the question of epitope specificity. Are the TSST-1 primed CD4⁺ T cells exerting suppression upon all B cells (non-specific suppression via the bystander effect) or is there some sort of discrimination based on epitope specificity (which would then involve specific cell-to-cell interaction)? With respect to TSST-1, epitope specificity would refer to all the clones that bear the V β region and thus all those B cells that bear the same epitopes would be suppressed. One way to answer this question is to isolate T cell clones that are specific for TNP and assess if they have the V β reactive TSST-1 binding region. Thus, if these clones do not have the V β region then this would indicate that TSST-1 primed CD4⁺ T cell exerts a non-specific immunosuppressive response. Perhaps another way of approaching this problem is to use V β knockout mice and to hyperimmunize them using an antigen that does not require the TSST-1 specific V β segment on T cells for activation. TSST-1 primed CD4⁺ T cells will once again be generated in normal BALB/c mice and following adoptive transfer if antibody levels decrease, then this would indicate that TSST-1 primed CD4⁺ T cells have an antigen non-specific suppressive response. It is hypothesized that TSST-1 primed CD4⁺ T cells have an epitope non-specific suppressive effect.

What is not known is the ability of B cells to mount an immune response after 21 days and thus it will be interesting to follow up on this model and ask the question whether the mice that received the injection of TSST-1 induced CD4⁺ T cells would then have trouble mounting future immune responses to TNP-KLH and other antigens. Thus, another possible direction that this project could take is that the *in vivo* studies should be revisited so that the ability of TSST-1 primed CD4⁺ T cell recipients to respond to further challenges by TNP-KLH, TSST-1 or an

unrelated antigen may be observed. The reason for this approach is to find out the long term effects of the adoptively transferred TSST-1 primed CD4⁺ T cells, giving some ideas of how potent these cells are in controlling the immune system. Therapeutically, it would not be advantageous for these cells to be suppressing the immune system indefinitely. While there has not been any work by any members in the field of regulatory T cells to look at the long term effects of T regulatory cell suppression (ie; interaction between Tregs and memory T or B cells), it is predicted that if this *in vivo* model were to receive an additional challenge with TNP-KLH 21 days after the initial adoptive transfer there would not be a sustained immunosuppressive effect on the recipients of TSST-1 induced CD4⁺ T cells. This is hypothesized because the transferred cells are not likely to have the ability to survive indefinitely in the recipient and thus, the observed suppressive effect is only going to last for as long as these TSST-1 induced CD4⁺ T cells are alive and activated. Nevertheless, the ability of the native immune system to recover and respond to future challenges is of paramount importance in the possible application of this novel therapeutic modality. This study would give some ideas about the limitations and side effects of this technique applied clinically *in vivo*.

The third direction to take with this project is to find more relevant markers so that the TSST-1 primed CD4⁺ T cells may be properly purified to isolate the putative TSST-1 primed Tregs. As discussed above, CD25 is a poor marker for Tregs due to its inconsistent nature. Much of the difficulty and confusion in the field of regulatory T cells may simply be due to this very problem of the lack of a reliable marker to use to differentiate between different types of Tregs as natural CD4⁺CD25⁺ Tregs, antigen induced Tregs and even CD8⁺ Tregs all have overlapping surface markers (Scotto *et al.*, 2004). With the recent focus on FoxP3, perhaps TSST-1 primed CD4⁺ T cells could be separated based on FoxP3 expression to isolate the putative antigen induced Tregs as was recently done (Veldman *et al.*, 2006). Another possible marker might be the CTLA-4 as it is known that one of the surface ligands that natural CD4⁺CD25⁺ Tregs use to

mediate suppression is CTLA-4. Beyond these interactions, there are many more ligand/receptor interactions that have yet to be discovered and studied. Thus, it is proposed that efforts should be spent in trying to identify what type of specific interactions is involved in cell-to-cell contact mediation of suppression.

One of the biggest question that arose from this project is whether the suppression observed is mediated by one type of Treg or two types of Tregs, each with a different mechanism of suppression, since no one type of regulatory T cell is currently described to have both cytokine and cell-to-cell contact dependent suppression mechanisms. Thus, one possible future direction would be to closely examine if the two suppression mechanisms may be attributed to the same T cell population or different populations. To do so, the neutralization and transwell studies can be repeated with purified CD25⁺ vs. CD25⁻ T cell populations to observe the mechanism of suppression mediated by each population. It is hypothesized that both suppression mechanisms will be found to be attributed to the same TSST-1 primed CD25⁺ T cell population.

Another immediate aim would be to conduct experiments designed to test the proposed model of B cell suppression by TSST-1 primed Tr1 cells. One of the main ideas of the model is that cytokine mediated suppression results in functional down-regulation while cell-to-cell suppression leads to apoptosis. This hypothesis can be addressed by assessing viability in the neutralization and transwell experiments. If cell-to-cell suppression mediates apoptosis, then the abrogation of cell-to-cell contact should increase B cell viability. Furthermore, if IL-10 and TGF- β do not stimulate cell death then the neutralization of these cytokines should have no effect on B cell viability. Another postulate of the model proposes that TSST-1 specific B cells receive Signal 1, and then along with Signal 2, leads to apoptosis. To test this, TNP-specific B cell clones can be isolated and co-cultured with TSST-1 primed CD4⁺ T cells with or without the addition of TNP-KLH and viability can also be assessed. If viability drops, then that would confirm that BCR engagement with antigen specific epitopes is required to induce apoptosis.

Finally, because of the different types of T cells in each population besides the Tregs in the CD4⁺ population, it is likely that TSST-1 induced T cells exert one effect on B cells but another cell type in the population are exerting another effect on B cells. Thus, another future focus would be to set up a competition between TSST-1 primed CD4⁺ T cells and PBS primed CD4⁺ T cells in their respective abilities to influence B cells – essentially to ask if B cells would be preferentially activated or suppressed in the presence of the two types of T cells. Thus, the net effect of the two differing responses will dictate how B cells will respond. For this experiment, equal numbers of each cell type should be plated. However, it is highly likely that if the ratio is changed to heavily favour the PBS primed CD4⁺ T cells in cell numbers then the suppressive effect exerted by TSST-1 primed CD4⁺ T cells would then diminish. It would be of interest to set up a real competition assay between TSST-1 primed CD4⁺ T cells and PBS primed CD4⁺ T cells, and to perform a dosage assay to observe the changes in B cell proliferation and antibody production as the ratio of TSST-1 primed CD4⁺ T cells to PBS primed CD4⁺ T cells is varied. This experiment would also ask the question of what is the lowest number of TSST-1 primed CD4⁺ T cells required for B cell suppression in an environment where naïve and TSST-1 primed T cells are both competing for B cell attention in an attempt to simulate the *in vivo* situation.

These are just some potential leads this project could take as there is still a great deal of work that remains to be done before the ultimate goal of therapeutic applications in various human autoimmune diseases can be realized. Nevertheless, the promise for something novel and exciting is offered by these TSST-1 primed CD4⁺ T cells that could perhaps one day change the way allergies or autoimmune diseases are viewed and treated.

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