# Generation and Partial Characterization of a Regulatory T Cell Phenotype Following Repeated *In Vivo* Treatment with the Staphylococcal Superantigen Toxic Shock Syndrome Toxin-1

by

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## Abstract

Toxic shock syndrome induced by superantigens such as TSST-1 is characterized by the production of lethal proinflammatory cytokines associated with a T helper 1 ( $T_H$ 1) response including IL-2, IFN- $\gamma$ , TNF- $\alpha$  and IL-12. However, repeated exposure to superantigens appears to upregulate production of the anti-inflammatory cytokine IL-10 by CD4<sup>+</sup>  $T_R$ 1 cells.

 $T_R1$  cells have been implicated in suppression of autoimmune diseases, and their mechanism of differentiation and polarization is currently unclear. Therefore, the objective of this study was to determine if the staphylococcal superantigen TSST-1 was capable of inducing IL-10 producing  $T_R1$  cells, and to further characterize these cells based on their cytokine profile, surface marker expression and functional activity both *in vitro* and *in vivo*.

Utilizing a protocol of repeated subcutaneous injections of  $4\mu g$  TSST-1 in BALB/c mice, we observed a significant decrease in serum levels of IL-2 and IFN- $\gamma$ , while IL-10 levels were enhanced. The decrease in serum IL-2 and IFN- $\gamma$  levels observed *in vivo* following repeated TSST-1 stimulation were transferable to naïve mice by adoptive transfer of  $1\times10^7$  CD4<sup>+</sup> T cells intravenously from mice treated repeatedly with TSST-1. The observed *in vivo* suppression of IL-2 was dependent on IL-10 production, as blockade of the IL-10 receptor abrogated IL-2 suppression induced by adoptive CD4<sup>+</sup> T cell transfer.

Repeated *in vivo* treatment with TSST-1 was accompanied by an increase in both IL-10<sup>+</sup>IL-4<sup>+</sup> and IL-10<sup>+</sup>IFN- $\gamma^+$  CD4<sup>+</sup> splenocytes as well as IL-10<sup>+</sup> single-positive cells as revealed by intracellular cytokine staining. Concurrently, there was a decrease in IL-2<sup>+</sup> CD4<sup>+</sup> splenocytes. TSST-1 treatment also induced a significant increase in intracellular CTLA-4 expression, and surface expression of CD25. CD4<sup>+</sup> T cells from mice treated repeatedly with TSST-1 had significantly higher proportion of IL-10<sup>+</sup>CTLA-4<sup>+</sup> double positive and CD4<sup>+</sup>CD25<sup>+</sup>CTLA-4<sup>+</sup> triple positive cells compared to control mice.

 $CD4^+CD25^+$  splenocytes from mice treated repeatedly with TSST-1 were potent in their ability to induce elevated levels of IL-10 and IFN- $\gamma$ , and suppressed IL-2 production when mixed with naïve splenocytes in a ratio as low as 1:20 and restimulated *in vitro* with TSST-1.

Our investigations of regulatory T cells induced by repeated TSST-1 administration have added new insights with potential therapeutic applications in the fields of autoimmune disease, cancer resistance, and infectious disease pathogenesis.

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## List of Abbreviations:

Activation Induced Cell Death
Antigen Presenting Cell
Bovine Serum Albumin
CytoToxic Lymphocyte Antigen-4
Dendritic Cell
Experimental Autoimmune Encephalomyelitis
Enzyme Linked Immunosorbant Assay
Fluorescence Activated Cell Sorting
Forward Scatter
Glucocorticoid Induced TNF Receptor
Graft Versus Host Disease
Horseradish Peroxidase
Inflammatory Bowel Disease
Intra-Cellular Staining
Interferon Gamma
Immunoglobulin
Interleukin 2
Interleukin-4
Interleukin 6
Interleukin 10
Interleukin 12
Interleukin 10 Receptor-Alpha
Intravenous
Lipopolysaccharide
Monoclonal Antibody
Magnetic Activated Cell Sorting
Mean Floresence Intensity
Major Histocompatibility Marker II
Multiple Sclerosis
Phosphate Buffered Saline
Peripheral Blood Mononuclear Cells
Propidium Iodide
T Effector Cell
Cytotoxic T cell
T Cell Receptor
Transforming Growth Factor-Beta
T Helper-0 Cells, Naïve T cells
T Helper-1 Cell

T <sub>H</sub> 2	T Helper-2 Cell
TMB	Tetramethylbenzidine
T <sub>R</sub> 1	T Regulatory-1 Cell
T <sub>REG</sub>	Regulatory T Cell
TSST-1	Toxic Shock Syndrome Toxin-1
sAg	Super Antigens Sodium Dodecvl Sulphate Polyacrylamide Gel
SDS-PAGE	Electrophoresis
SS	Side Scatter
Vβ	V Beta chain of the TCR

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## Chapter I. Introduction: Regulation of helper T Cell Responses to Staphylococcal Superantigens

## Introduction

Staphylococcal exotoxins, including toxic shock syndrome toxin-1 (TSST-1) and the staphylococcal enterotoxins (SEs) A, B, C1-3, D, E, G-K and others (Jarraud, Peyrat et al., 2001), belong to a family of structurally related proteins known as superantigens (sAgs). These sAgs are the causative agents of toxic shock syndrome (Schlievert, Schoettle et al., 1979;Bergdoll, Crass et al., 1981;Bernal, Proft et al., 1999;Schlievert, Jablonski et al., 2000; Zhang, Iandolo et al., 1998; Jarraud, Cozon et al., 1999). They are also implicated in a wide variety of other human diseases including rheumatoid arthritis (Paliard, West et al., 1991; Schwab, Brown et al., 1993), psoriasis (Leung, Travers et al., 1995), multiple sclerosis (Schiffenbauer, Johnson et al., 1993; Soos, Hobeika et al., 1995) (Torres & Johnson, 1998), and the sudden unexpected nocturnal death syndrome (Al Mandani, Gordon et al., 1999) (Table I-1). SAgs bind to the major histocompatibility complex (MHC) class II molecules on the antigenpresenting cells (APCs), and to the T cell receptor- (TCR) bearing specific VB elements (Marrack & Kappler, 1990). This tri-molecular interaction leads to massive proliferation of T cells, and the uncontrolled and systemic release of pro-inflammatory cytokines, including interleukins IL-1, IL-6, interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor (TNF), and others (Kum, Laupland et al., 1993; Jupin, Anderson et al., 1988; Parsonnet, 1989). It is believed that the massive release of these pro-inflammatory cytokines, particularly TNF- $\alpha$ , is the key step leading to life-threatening toxic shock syndrome (Kain, Schulzer et al., 1993; Bohach, Fast et al., 1990; Davies, McGeer et al., 1996; Kotzin, Leung et al., 1993). The effector T cells involved in this hyper-immune response are predominantly of the T helper-1 ( $T_{\rm H}$ ) phenotype. There is also

some evidence that polarization to a T helper-2 ( $T_H2$ ) response with the production of classical anti-inflammatory cytokines (such as IL-4 and IL-6) also occurs. Moreover, the emergence of a novel T regulatory-1 cell ( $T_R1$ ) subset, producing mainly IL-10 but little or no IL-2 and IL-4, has recently been described following repeated sAg stimulation (Sundstedt, Hoiden et al., 1997).

Table I-1: Superantigen-associated human diseases			
Acute	Chronic		
Toxic Shock Syndrome (TSS)	Rheumatoid Arthritis (RH)		
Sudden Unexpected Nocturnal Death	Psoriasis		
Syndrome (SUNDS)	Multiple Sclerosis (MS)		
	Kawasaki Syndrome		

In this review, the current knowledge regarding the regulation of T helper cell subsets in response to staphylococcal sAgs is critically evaluated, and the role of various cytokines which directly influence T cell polarization or differentiation is summarized. Particular emphasis is directed towards pro-inflammatory as well as anti-inflammatory and regulatory effector functions during toxic shock. Although bacterial sAgs from other sources, particularly streptococcal pyrogenic exotoxins A (SPEA) and C (SPEC), have been shown to induce similar immune responses (Muller-Alouf, Alouf et al., 1996;Rink, Nicklas et al., 1997;Rink, Luhm et al., 1996;Fraser, Arcus et al., 2000), they will not be included in this review. Furthermore, for the sake of brevity, the term "sAgs" used in this review refers specifically to staphylococcal sAgs commonly associated with TSS (i.e. TSST-1, SEA, SEB and SEC), unless explicitly indicated otherwise.

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## **Clinical Features and Epidemiology of Staphylococcal TSS**

Staphylococcal TSS is characterized by the abrupt onset of fever, diarrhea, diffuse skin rash followed by desquamation, hyperemia of the mucous membranes, and hypotension or shock. Other organ systems, including hepatic, renal, muscular, cardiopulmonary, hematological, and central nervous systems may also be involved. Published estimates of the incidence of TSS vary (5-15 cases per 100,000) (Hajjeh, Reingold et al., 1999). Although initially described primarily in menstruating women in association with tampon use (Chesney, Davis et al., 1981), non-menstrual cases associated with post-surgical *Staphylococcus aureus* infections are increasingly recognized (Kain, Schulzer et al, 1993). Among 1,069 TSS cases reported in the United States during 1987-1996, approximately 41% were non-menstrual in origin (Hajjeh, Reingold et al, 1999). The overall fatality remains high (4.1%; 3% for menstrual cases and 5% for non-menstrual cases) (Hajjeh, Reingold et al, 1999), and recurrence is frequent (Kain, Schulzer et al, 1984;Andrews, Parent et al., 2001).

## Limitations in Immunobiological Studies of Staphylococcal sAgs

To place this review in proper perspective, it is useful to first consider some limitations in the interpretation of published studies concerning the immunobiological properties of staphylococcal sAgs. Much of our knowledge of sAgs has been derived from *in vitro* studies of human peripheral blood mononuclear cells (PBMC) from healthy donors, perhaps because the mode of action of T cell stimulation seems similar, though not identical, to conventional antigens (Fraser, Arcus et al, 2000). There have been very few studies of human PBMC obtained from patients during acute TSS or convalescence. There are severe limitations in the interpretation of results from such studies. Firstly, it is clear that *in vitro* observations of the biological properties of sAgs

may not parallel *in vivo* results. For example, Vβ-specific T cells that appeared to be anergic *in* vitro following sAg re-stimulation may in fact be functional in vivo (Heeg, Gaus et al., 1995). Secondly, the MHC class II DR allele isotype or the presence of antibodies to various sAgs in the healthy donors are seldom known or reported. It is not clear whether these variables may influence the cytokine profiles or kinetic responses following sAg stimulation. Finally, the source and purity of the sAgs being studied may be an important source of variability. We, and others, previously demonstrated that commercial sources of staphylococcal sAgs may contain impurities which may produce undesirable biological effects that are absent when compared to purified preparations or recombinant counterparts (Kum, Laupland et al, 1993;Fleischer, Necker et al., 1996). The use of Coomassie blue or silver staining after sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) may not be adequate as the sole criterion for determining the purity of sAgs under study (Rosten, Bartlett et al., 1989). More sensitive techniques such as immunoblotting with polyclonal antiserum raised against the crude culture supernatant used for sAg purification may be required to detect minute quantities of co-purified products (Kum, Laupland et al, 1993). Thus, observations obtained from *in vitro* studies must be interpreted with caution.

To investigate toxic shock syndrome *in vivo*, the rabbit model of subcutaneous sAg infusion described by Parsonnet et al. (Parsonnet, Gillis et al., 1987), has been most commonly studied. While this rabbit model accurately reproduces most of the hemodynamic and clinical manifestations of human TSS, the lack of immunological reagents and cytokine gene knock-out constructs precludes a detailed dissection of different T cell subsets and lymphokine responses to sAgs in this animal species. For this reason, the murine model of sAg-mediated lethal shock is commonly used for *in vivo* studies of TSS (Barton, Shortall et al., 1996;Bean, Freiberg et al.,

1993; Matthys, Mitera et al., 1995; Miethke, Wahl et al., 1992; Miethke, Wahl et al., 1993; Miethke, Duschek et al., 1993; Saha, Harlan et al., 1996; Saha, Jaklic et al., 1996; Takahashi, Nakagawa et al., 1995). Since mice have a natural resistance to sAg-induced shock, Dgalactosamine, an amino-sugar isolated from chondroitin sulphate and an inhibitor of transcription in the liver (Decker & Kappler, 1974;Koff & Connelly, 1976), is required to sensitize mice to the lethal effect of bacterial sAgs. While this model system does not perfectly replicate human TSS, it has been an acceptable model to examine some of the in vivo immunobiological effects of sAgs. Furthermore, treatment with D-galactosamine alone does not appear to greatly affect T cell activation or the cytokine profiles either qualitatively or quantitatively (Miethke, Wahl et al, 1993). Occasionally, surgical adrenalectomy or administration of RU-38486, a glucocorticoid receptor antagonist (Gonzalo, Gonzalez-Garcia et al., 1993), has also been used to sensitize mice and counteract the natural resistance to sAgmediated lethal shock. Interestingly, the absence of IL-10, accomplished either by neutralization with anti-IL-10 monoclonal antibodies (Florquin, Amraoui et al., 1994) or by IL-10 gene targeting (Hasko, Virag et al., 1998), also confers enhanced sensitivity to sAg-induced lethality without the need for chemical or surgical sensitization. The basis for this interesting property of IL-10 will be further discussed in this review.

## T Cell Activation and Effector Functions During Toxic Shock

Both MHC class II-expressing APC and T cells bearing V $\beta$ -specific TCR determinants are required for sAg-mediated T cell activation and cytokine secretion, similar to conventional peptide antigens (Kappler, Kotzin et al., 1989). However, sAg presentation by the APC does not require prior processing, and is not MHC-restricted. Furthermore, cell-to-cell contact of the APC and the TCR appears critical for the induction and secretion of sAg-mediated pro-inflammatory cytokines (See, Kum et al., 1992). sAgs bind to a relatively conserved portion of the TCR VB chain (Kappler, Kotzin et al, 1989;Li, Llera et al., 1999), causing "false recognition" of irrelevant antigens displayed on MHC class II molecules (Labrecque, Thibodeau et al., 1993). This results in the activation of both the T cell and the APC, followed by uncontrolled cytokine release, clonal expansion and massive proliferation of activated T cells that constitute 5-20% of the total T cell population. The critical requirement for T cells in sAg-mediated shock is demonstrated by the lack of responsiveness in SCID mice and in cyclosporin A-treated mice, both of which lack a functional T cell signaling pathway. In addition, SCID mice could be made susceptible to toxic shock following immune reconstitution with functional T cells (Miethke, Wahl et al, 1992). In addition to the tri-molecular interaction of cross-bridging by sAgs between MHC class II and V $\beta$ -specific TCR, the strength of co-stimulatory signals also appear pivotal in sAg-mediated T cell activation and cytokine induction. The co-stimulatory molecules implicated include CD80/86 (also known as B7.1/B7.2) on the APC which interacts with CD28 on the T cell (Lane, Haller et al., 1996; Muraille, De Smedt et al., 1995; Kum, Hung et al., 2002), CD40 on the APC which interacts with CD154 (CD40 ligand) on activated T cells (Jabara & Geha, 1996; Mehindate, al Daccak et al., 1996; Kum, Hung et al, 2002), and adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and lymphocyte-function-associated antigen-1 (LFA-1) (See & Chow, 1992; Tessier, Naccache et al., 1998; Ijichi, Yamano et al., 1996).

SAgs induce proliferation and cytokine production in both  $CD4^+$  and  $CD8^+$  T cells *in vitro* and *in vivo* (Fraser, Arcus et al, 2000;Miethke, Wahl et al., 1995). However,  $CD4^+$  or T helper (T<sub>H</sub>) cells are preferentially activated by sAgs due to their high-affinity binding to MHC class II molecules (Labrecque, Thibodeau et al, 1993).  $CD8^+$  cytotoxic T cells also respond to sAg

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stimulation following presentation by MHC class I molecules, resulting in enhanced production of both IFN- $\gamma$  and IL-12 (Mason, Dryden et al., 1998). The polyclonal stimulation of T<sub>H</sub> cells by sAgs results in a large number of effector cells which are responsible for the systemic release of both pro- and anti-inflammatory cytokines (Mosmann, Cherwinski et al., 1986). This exaggerated and systemic T cell response, rather than the more controlled local response to conventional antigens, is the hallmark of sAg-mediated TSS.

## An Introduction to T Cell Polarization During TSS

Helper T lymphocytes have classically been divided into  $T_{\rm H}1$  and  $T_{\rm H}2$  subsets based on their cytokine production profiles (Mosmann, Cherwinski et al, 1986).  $T_{\rm H}$  effector cells typically produce large quantities of IL-2, IFN- $\gamma$ , TNF- $\alpha$  and TNF- $\beta$ . T<sub>H</sub>2 effector cells produce little or no IL-2 (although this cytokine produced from other sources assists their outgrowth) (La Flamme & Pearce, 1999), and large amounts of IL-4, IL-5, IL-6, IL-10 and IL-13 (Moore, O'Garra et al., 1993; Abbas, Murphy et al., 1996). In addition to their cytokine production profiles, T<sub>H</sub> subsets can also be distinguished on the basis of their expression pattern of cytokine and chemokine receptors and other cell surface markers. T<sub>H</sub>1 cells have low cell surface expression of IFN- $\gamma$ R2, whereas the expression of IL-12R $\beta$ 2 and IL-18R is absent on T<sub>H</sub>2 cells. Effector  $T_H$  cell subsets regulate the activity of one another. Thus, the production of IFN- $\gamma$  by  $T_H l$  effector cells inhibits  $T_H 2$  cell differentiation and proliferation, whereas the  $T_H l$  cells themselves have lost responsiveness to IFN-y at the receptor level. The production of cytokines by  $T_{\rm H}$  differentiated cells is inhibited indirectly by the production of IL-10 by  $T_{\rm H}$  effector cells, which acts on the APC compartment (Moore, O'Garra et al, 1993; Abbas, Murphy et al, 1996;O'Garra, Steinman et al., 1997;O'Garra, 1998;O'Garra, Steinman et al, 1997). Antigennaive  $T_H$  cells ( $T_H0$ ) represent an intermediate phenotype capable of producing both  $T_H1$  and  $T_H2$  cytokines in response to antigenic challenge (Abbas, Murphy et al, 1996). Whether polarized  $T_H1$  and  $T_H2$  cells are derived through the differentiation of  $T_H0$  cells that produce both  $T_H1$  and  $T_H2$  cytokines, or whether  $T_H0$  cells represent a separate, stably differentiated T cell population, remains unclear (Abbas, Murphy et al, 1996).

Recently, other types of T cells capable of regulating both  $T_H1$  and  $T_H2$  responses have been identified. These T regulatory ( $T_R$ ) cells function via the production of anti-inflammatory cytokines and/or cell contact mediated suppression. There is considerable evidence for the role of  $T_{REG}$ 's and associated cytokine production, specifically IL-10 and TGF- $\beta$ , following repeated sAg treatment.

A comparison of cytokines implicated in the polarization of various helper and  $T_{REG}$ 's following sAg stimulation is shown in **Table I-2**. There is evidence for the presence and activity of each of these T helper cell subsets during the immune response to sAgs (Sundstedt, Hoiden et al, 1997;Miethke, Wahl et al, 1993;Gonzalo, Gonzalez-Garcia et al, 1993;Florquin, Amraoui et al, 1994;Miller, Ragheb et al., 1999;Hackett & Stevens, 1993;Sriskandan, Evans et al., 1996;Akatsuka, Imanishi et al., 1994;Sriskandan, Evans et al, 1996). The inter-relationship of these T helper cell subsets and their cytokines will be briefly reviewed. While many cytokines are induced by sAgs, this review will focus only on those lymphokines which have been demonstrated to strongly influence T helper cell polarization. Thus, the role of IL-6 and the monokines IL-1 $\beta$ , IL-8 and IL-18 will not be further discussed in this review.

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neiper 1 cells following staphylococcal Superantigen (sAg) stimulation			
	T <sub>H</sub> 1	T <sub>H</sub> 2	T <sub>R</sub>
T cell-derived cytokines	IL-2 IFN-γ TNF-α TNF-β	IL-4 IL-6 IL-10	IL-10* TGF-β
APC-derived cytokines	TNF-α IL-8 IL-12	IL-6	IL-10 (DC derived)

Table I-2: Cytokines involved in the polarization of  $T_H1$ ,  $T_H2$  and  $T_{REG}$  subsets of

\* While APC's (macrophages and dendritic cells) are capable of IL-10 production following sAg treatment, the major source of IL-10, induced by sAg, appears to be T cell-derived.

## *T<sub>H</sub>*1

Based on the profile of cytokine production,  $CD4^+$  T cells rapidly differentiate into effector T<sub>H</sub>1 cells following sAg stimulation (Kappler, Kotzin et al, 1989;Mosmann, Cherwinski et al, 1986), producing high levels of pro-inflammatory cytokines including IL-2, IFN- $\gamma$ , TNF- $\alpha$  and TNF- $\beta$  (Miethke, Wahl et al, 1993;Hackett & Stevens, 1993;Sriskandan, Evans et al, 1996). Concurrently, activated APCs also produce high levels of TNF- $\alpha$  (Miethke, Wahl et al, 1993), IL-1 (Ikejima, Dinarello et al., 1984), IL-8 (Krakauer, 1998), IL-10 (Akdis & Blaser, 1999) and IL-12 (Sriskandan, Evans et al, 1996). TNF- $\alpha$  and TNF- $\beta$  appear to be the key pro-inflammatory cytokines in the pathogenesis of sAg-induced lethality (Miethke, Wahl et al, 1993;Ikejima, Dinarello et al, 1984) (**Figure I-1**). Secondary challenge with sAgs also induces an increase in both IFN- $\gamma$  and TNF- $\alpha$  production, likely due to the induction of IL-12 (Sundstedt, Hoiden et al,

1997;Gaus, Miethke et al., 1994;Koide, Uchijima et al., 1996;Schols, Jones et al., 1995). As a result, the immediate cytokine response to sAgs can be considered to reflect an acute  $T_{\rm H}1$  response.



Figure I-1. Cytokine interactions during a  $T_{\rm H}1$  response to sAg. Activation of the  $T_{\rm H}1$  cell causes the production of IL-2, leading to an autocrine feedback loop which induces T cell proliferation. Activated  $T_{\rm H}1$  cells also produce IFN- $\gamma$ , which increases the expression of MHCII and costimulatory molecules CD80/86 (B7-1/B7-2) on macrophages. Activated macrophages produce IL-12, which enhances IFN- $\gamma$  production by T cells, favoring an enhanced  $T_{\rm H}1$  response. Initial production of TNF $\alpha/\beta$  by T cells, and later production of TNF- $\alpha$  by macrophages also participate in the  $T_{\rm H}1$  response to sAgs.

## Interleukin-2 (IL-2)

SAgs induce the proliferation of a large number of effector T cells via polyclonal activation (Miethke, Wahl et al, 1995). IL-2, a T cell mitogenic cytokine, is consistently produced at high concentrations by all staphylococcal sAgs (Rink, Luhm et al, 1996).  $T_H0$  cells are the chief producers of IL-2, although this cytokine is responsible for the early clonal expansion and proliferation by  $T_H0$ ,  $T_H1$ ,  $T_H2$ , and some subsets of  $T_{REG}$ 's (Mosmann, Cherwinski et al,

1986;La Flamme & Pearce, 1999;Papiernik, de Moraes et al., 1998;Papiernik, 2001). Curiously, whereas primary stimulation by sAgs in vitro (Rink, Nicklas et al, 1997; Rink, Luhm et al, 1996; Akatsuka, Imanishi et al, 1994) and in vivo (Miethke, Wahl et al, 1992; Florquin, Amraoui et al. 1994) leads to massive production of IL-2 by activated T cells, subsequent re-challenge with the same sAgs results in an inhibition of IL-2 production (Sundstedt, Hoiden et al, 1997; Miller, Ragheb et al, 1999; Florquin, Amraoui et al., 1996). This lack of IL-2 induction following repeated sAg stimulation, is accompanied by clonal deletion and programmed cell death (apoptosis) of some but not all activated T cells. Additionally, it is associated with an absence of both T cell proliferation and IL-2 responsiveness (tolerance or anergy) (Sundstedt, Hoiden et al, 1997; Miller, Ragheb et al, 1999; Gonzalo, Moreno, I et al., 1992). The finding that only some activated T cells undergo programmed cell death points to more than one mechanism for the induction of anergy or non-responsiveness by sAgs. In this regard, Florquin et al. (Florquin, Amraoui et al., 1995) showed that some anergic T cells made deficient in IL-2 production by repeated exposure to SEB in vivo, are primed for IL-10 secretion in mice. There is also in vitro evidence that re-stimulation results in the up-regulation of IL-2Ra (Hamel, Eynon et al., 1995), and exogenous IL-2 reversed this sAg-induced anergic state or activation-induced deletion (Belfrage, Dohlsten et al., 1997). Thus, the non-responsiveness of sAg-induced anergic T cells is not absolute. This sAg-induced non-responsiveness is not simply due to sAg presentation in the absence of co-stimulation on non-professional APCs, since presentation of SEB on highly purified dendritic cells during the primary response did not prevent the induction of non-responsiveness (Hamel, Eynon et al, 1995). Furthermore, this sAg-induced nonresponsiveness is not due to the preferential induction of a T<sub>H</sub>1 phenotype, since skewing towards a  $T_{\rm H}2$  phenotype by adding IL-4 and antibodies to IFN- $\gamma$  did not prevent the induction of non-responsiveness by SEB (Hamel, Eynon et al, 1995). It appears that while early stages of IL-

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2 inhibition are due to a synergistic action of TGF- $\beta$  and IL-10, inhibition of IL-2 at later stages arise from more downstream signaling pathways (Miller, Ragheb et al, 1999).

#### Interferon-γ (IFN-γ)

Production of IFN- $\gamma$ , the cytokine most strongly associated with T<sub>H</sub>1 responses (Mosmann, Cherwinski et al, 1986), is well documented in toxic shock syndrome (Sundstedt, Hoiden et al, 1997;Miethke, Wahl et al, 1993;Takahashi, Nakagawa et al, 1995;Bette, Schafer et al., 1993;Nagaki, Muto et al., 1994). *In vivo*, initial production of this pro-inflammatory cytokine is detectable within half an hour of sAg exposure (Miethke, Wahl et al, 1993), and serum concentration is high in murine models of toxic shock (Sundstedt, Hoiden et al, 1997). This is consistent with an early and differentiated T<sub>H</sub>1 effector cell response to sAg stimulation.

IFN- $\gamma$  contributes significantly to the pathogenesis of toxic shock syndrome in the murine model. This is demonstrated by the protective effects of anti-IFN- $\gamma$  monoclonal antibody (mAb) on SEB-induced toxicity in mice (Matthys, Mitera et al, 1995;Billiau, Heremans et al., 1998). This might be expected since TNF release was found to be reduced when IFN- $\gamma$  was neutralized (Sriskandan, Evans et al, 1996), and since direct neutralization of TNF has been shown to reduce lethality in animal models of toxic shock (Miethke, Wahl et al, 1992). It is important to note that studies of IFN- $\gamma$  neutralization have not consistently resulted in a reduction in sAg-induced lethality. For example, when low doses of D-galactosamine were used to sensitize mice to sAgs, IFN- $\gamma$  neutralization did impart a protective effect (Nagaki, Muto et al, 1994). However, when higher doses of D-galactosamine were used for sensitization, IFN- $\gamma$  neutralization only reduced certain systemic manifestations of illness such as weight loss and hypoglycemia, but not

mortality (Matthys, Mitera et al, 1995). These apparently disparate results raised the possibility that D-galactosamine sensitization itself could augment the lethal effects of IFN- $\gamma$  (Matthys, Mitera et al, 1995). Nevertheless, the importance of IFN- $\gamma$  in mediating sAg-induced toxicity is further supported by results in an animal model that does not require D-galactosamine sensitization. When mice were sensitized to toxic shock by prior treatment with specific anti-IL-10 antibodies, in the absence of D-galactosamine, complete protection from SEB-induced lethality was achieved following neutralization of IFN- $\gamma$  (Florquin, Amraoui et al, 1994).

#### **Tumor Necrosis Factor (TNF)**

High levels of the pro-inflammatory cytokine TNF- $\alpha$  are released systemically during TSS (Miethke, Wahl et al, 1993). *In vivo*, the kinetics of TNF induction is rapid, achieving peak serum levels 1 hour after sAg exposure in rabbits (Miethke, Wahl et al, 1992;Miethke, Wahl et al, 1993;Miethke, Duschek et al, 1993). Neutralization of total TNF (- $\alpha$  and - $\beta$ ) *in vivo* has been shown to completely abrogate sAg-mediated lethality in several animal models (Miethke, Wahl et al, 1992;Miethke, Duschek et al, 1993;Ikejima, Dinarello et al, 1984). Conversely, soluble TNF receptor is effective in neutralizing the deleterious effects of TNF overproduction during toxic shock (Corcoran, Scallon et al., 1998). Therefore, TNF is considered the key effector cytokine in the pathogenesis of toxic shock syndrome (Miethke, Duschek et al, 1993).

#### Interleukin-12 (IL-12)

IL-12 is produced by activated APCs, and is required for the priming of activated T cells to produce IFN- $\gamma$  (McKnight, Zimmer et al., 1994). Neutralization of IL-12 has been shown to inhibit sAg-induced IFN- $\gamma$  by 60%(Sriskandan, Evans et al, 1996). Therefore, it could be

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hypothesized that treatment with IL-12 would exacerbate the immune response to sAgs through up-regulation of IFN- $\gamma$  and subsequent TNF production. Unexpectedly, pre-treatment with IL-12 has been shown to rescue mice from the lethal effects of toxic shock (Takahashi, Nakagawa et al, 1995). This apparent paradox may be explained by the observation that exogenous IL-12 also up-regulates the anti-inflammatory cytokine IL-10, which serves as a negative feedback for IL-12-induced IFN- $\gamma$  production (see further discussion under IL-10) (Segal, Dwyer et al., 1998;Mingari, Maggi et al., 1996;Meyaard, Hovenkamp et al., 1996).

## *T<sub>H</sub>*2

T<sub>H</sub>2 cells are considered to be the classical antagonists to a T<sub>H</sub>1 phenotype. sAg-activated T<sub>H</sub>2 cells induce a humoral immune response and down-regulate the cellular T<sub>H</sub>1 effector function by producing various anti-inflammatory cytokines including IL-4 (Sundstedt, Hoiden et al, 1997;Rink, Luhm et al, 1996;Miethke, Wahl et al, 1993;Florquin, Amraoui et al, 1996), and IL-10 (Sundstedt, Hoiden et al, 1997;Florquin, Amraoui et al, 1994;Hasko, Virag et al, 1998;Miller, Ragheb et al, 1999;Florquin & Goldman, 1996;Koide, Uchijima et al, 1996;Schols, Jones et al, 1995) (**Figure: I-2**). These T<sub>H</sub>2-like cytokines have been shown to be induced by various sAgs both *in vivo* and *in vitro*, although the level of IL-4 expressed has been variable for different sAgs in different studies (Rink, Luhm et al, 1996;Krakauer, 1995b). The T<sub>H</sub>2 response is particularly prominent following chronic sAg stimulation (Florquin, Amraoui et al, 1996). Both IL-4 and IL-10 are capable of suppressing IFN- $\gamma$  through direct and indirect mechanisms, while still stimulating B cell differentiation (Moore, O'Garra et al, 1993;Paludan, 1998). However, exogenous IL-4 is less effective than IL-10 in inhibiting IFN- $\gamma$  and other T<sub>H</sub>1 cytokines induced by SEA and SEB (Krakauer, 1995a). Additionally, there is compelling data indicating that IL-10 production in response to repeated sAg stimulation originates from a unique subset of T helper

cells known as  $T_R1$  cells that are distinct from classical  $T_H2$  cells. Furthermore, as discussed later in this review, IL-10 (along with TGF- $\beta$ ) plays a pivotal role in down-regulating the  $T_H1$ hyperimmune response induced by sAgs and is believed to be critical for the eventual recovery from toxic shock. This regulatory cytokine along with TGF- $\beta$  will be further discussed under the section of T regulatory cells.



Figure I-2: Cytokine interactions during a  $T_H^2$  response to sAg. Production of IL-4 by activated  $T_H^2$  cells contributes to an autorcrine cytokine loop which induces  $T_H^2$  cell proliferation and enhanced  $T_H^2$  responses. IL-4, as well as IL-5, IL-6 and IL-10 all contribute to costimulatory molecule expression, isotype switching and antibody production by B cells.

#### Interleukin-4 (IL-4)

The production of IL-4, an anti-inflammatory cytokine (Mosmann, Cherwinski et al, 1986), can be detected both *in vitro* (Rink, Luhm et al, 1996) and *in vivo* (Sundstedt, Hoiden et al, 1997;Miethke, Wahl et al, 1993;Florquin, Amraoui et al, 1996) following primary exposure to various sAgs, although disparate results have been reported by different authors using different sAg preparations. IL-4 plays a critical role as a promoter of B cell differentiation (Paul & Ohara, 1987) and a suppressor of both IFN-γ as well as TNF-α production (Paludan, 1998) (**Figure I-2**). Suppression of sAg-induced IFN-γ and TNF-α by exogenous IL-4 has been well demonstrated by Krakauer et al. (Krakauer, 1995a). This anti-inflammatory response to exogenous IL-4 may be mediated either by increasing the production of IL-10 or inhibition of IL-12 (Levings & Schrader, 1999). Evidence for an extended IL-4 response following repeated sAg stimulation is lacking, however. While secondary superantigenic challenge may prime for a more enhanced IL-4 response (Sundstedt, Hoiden et al, 1997;Florquin, Amraoui et al, 1996), this is not always observed (Koide, Uchijima et al, 1996). In addition, extended treatment with sAgs seems to abrogate the IL-4 response almost completely (Sundstedt, Hoiden et al, 1997;Miller, Ragheb et al, 1999). This may be due to the production of immunosuppressive cytokines, such as TGF-β (Miller, Ragheb et al, 1999) and IL-10 (Sundstedt, Hoiden et al, 1997)which can down-regulate both IL-4 production (Powrie, Leach et al., 1996) and other T<sub>H</sub>2 responses (Bridoux, Badou et al., 1997) (**Figure I-3**).



**Figure I-3. Classical view of T<sub>H</sub>1 suppression during sAg stimulation.** IFN- $\gamma$  production is inhibited by T<sub>H</sub>1 cells either directly or indirectly by IL-4, produced by T<sub>H</sub>2 cells. While this is an accepted mechanism of IFN- $\gamma$ -mediated suppression in many systems, it should be noted that it represents an immune deviation from a cellular to a humoral response, as opposed to a complete suppression of the immune response. Furthermore, this potential model does not appear to apply to all sAg's, since TSST-1 fails to induce detectable IL-4 *in vivo* or *in vitro*.

In contrast to *in vitro* stimulation, both Florquin et al. (Florquin, Amraoui et al, 1996) and Bourrie et al. (Bourrie, Benoit et al., 1996) were able to detect serum levels of IL-4 in mice following primary or repeated injection with SEB. Sundstedt et al. (Sundstedt, Hoiden et al, 1997) observed that mice challenged repeatedly with SEA at 4-day intervals produced a transient elevation in serum IL-4 levels that subsequently became suppressed following the third SEA injection. This was temporally correlated with a sustained level of serum IL-10. Neutralization with anti-IL-10 antibody in these animals resulted in an enhanced production of IL-4 *in vivo* may be under negative feedback control by IL-10. It has however, been suggested that this cytokine is initially required for IL-10 production (Mendel & Shevach, 2002).

## Regulatory T Cells $(T_R)$

The study of T cells with regulatory function has evolved considerably from early controversies surrounding studies of suppressor T cells (summarized by (Chatenoud, Salomon et al., 2001). Immune tolerance, whereby the immune system is induced to tolerate non-self antigens, has been demonstrated in cattle (Owen, 2003) and mice (Billingham, Brent et al., 1953). Embryonic exposure to allogenic hemopoeitic cells was found to confer tolerance, or immune acceptance, to donor skin transplantation. Observations of tolerance mediated by high doses of sheep red blood cells by Gershon and Kondo strengthened this concept (Gershon & Kondo, 1970). The term infectious tolerance was coined shortly thereafter (Gershon & Kondo, 1971) when it was determined that a adoptive transfer of sheep red blood cell-tolerant T cells to a naïve host can also confer tolerance. Evidence has continued to accumulate in several models of autoimmunity, for a role of T cell-mediated suppression in immune responses to foreign antigens as a mechanisim of tolerance induction (Okumura & Tada, 1971;Baker, Stashak et al., 1974;Baker, Barth et al., 1970;Ha & Waksman, 1973).

The concept of immune tolerance, mediated by T cell suppression, became tainted with various models of complex regulatory cascades (Gershon, Eardley et al., 1981;Green & Gershon, 1984). Furthermore, regulatory function was tenuously associated with a gene locus between I-A and I-E MHC regions (Murphy, 1987). This exciting area of research imploded as a result of several contradictory findings. Sequencing of the locus in question revealed that this region did not contain another MHC gene (Kronenberg, Steinmetz et al., 1983). Additionally, the inability to purify or clone suppressive factors (Webb, Semenuk et al., 1989) and the lack of a specific cell surface marker for T suppressor cells contributed to the discredit of the term suppressor T cell.

From this controversy sprang the pioneering work of Mosmann and Coffman (Mosmann, Cherwinski et al, 1986) that focused instead on immune deviation from  $T_{H1}$  to  $T_{H2}$  responses, and vice versa. For a period of time, immunologists placed suppressive responses into one of these two well accepted categories(Bach & Chatenoud, 2001), avoiding the mention of suppressive T cells. However, observations accumulated that did not fit these categories. Instead, similarities with earlier findings were uncovered, indicating a role for suppressive T cells in the mediation of tolerance (Shevach, McHugh et al., 2001b;Sakaguchi, 2000;Mason & Powrie, 1998;Levings, Bacchetta et al., 2002;Levings & Roncarolo, 2000;Powrie, Correa-Oliveira et al., 1994). Researchers in this field began to adopt a new, less controversial term for T cells with suppressive potential: T regulatory ( $T_R$ ) cells.

There is clear evidence of a role for  $T_{REG}$ 's following sAg treatment (Grundstrom, Cederbom et al., 2003;Pontoux, Banz et al., 2002;Sundstedt, Hoiden et al, 1997;Assenmacher, Lohning et al., 1998;Huang, Huso et al., 2003;Zelenika, Adams et al., 2002). Several CD4<sup>+</sup> T cell subsets capable of inducing tolerance to both self and foreign antigens have been described. These subsets appear to have different mechanisms of inducing suppression, and are characterized by various cell surface markers (**Table I-3**).

Table I-3: Comparison of $T_R1$ , Natural CD4 <sup>+</sup> CD25 <sup>+</sup> $T_{REG}$ , CD45RB <sup>LOW</sup> T Cells, and $T_H3$ Cells				
	T <sub>R</sub> 1 Cells	NaturalCD4 <sup>+</sup> CD25 <sup>+</sup> T <sub>REG</sub> Cells	CD45RB <sup>LOW</sup> T <sub>REG</sub> Cells	T <sub>H</sub> 3 Cells
Developmental Location	Periphery	Thymus	Undetermined	Gut
Cellular Origin	Th0, T <sub>H</sub> 1 or T <sub>H</sub> 2 cells?	CD25 <sup>+</sup> DP, that become CD4 <sup>+</sup> CD8 <sup>-</sup>	Differentiated Memory Cells	Unknown
Site of Localization	Peripheral site of Inflammation	Spleen and Lymph Nodes	Undetermined	MALT Tissue
Antigen Specificity	Peripheral antigens	Self antigens expressed in the thymus	Peripheral antigens	Gut antigens
CD25 <sup>+</sup> Expression	When activated	Constitutive high levels	When activated	Unknown
Central mechanism of suppression	Cytokines	Cell Contact	Cytokines	Cytokines
Factors thought responsible for function	IL-10, TGF <b>-</b> β	CTLA-4, m-TGF-β	IL-10, TGF-β	TGF-β
Putative Surface Markers	CD4, possibly CD25?	CD4, CD25, GITR, CTLA-4, CD5 <sup>HIGH</sup> , CD45RB <sup>LOW</sup> , CD69 <sup>HIGH</sup> , CD62 <sup>LOW</sup>	$\begin{array}{c} \text{CD4,}\\ \text{CD45RB}^{\text{LOW}}, 1/3\\ \text{CD25}^{+}\end{array}$	CD4 <sup>+</sup>

## Interleukin-10 (IL-10)

IL-10 is the regulatory cytokine most consistently identified in human PMBC or murine splenocytes following repeated staphylococcal sAg stimulation (Sundstedt, Hoiden et al, 1997;Florquin, Amraoui et al, 1994;Hasko, Virag et al, 1998;Miller, Ragheb et al, 1999;Florquin, Amraoui et al, 1996;Gaus, Miethke et al, 1994;Koide, Uchijima et al,

1996; Schols, Jones et al, 1995). While IL-10 can be produced by both macrophages and T cells, it is mainly secreted by CD4<sup>+</sup> T cells in toxic shock murine models, as demonstrated using CD4<sup>+</sup>-depleted (Florquin, Amraoui et al, 1994) or CD4<sup>+</sup> knock-out mice, and in macrophagedepleted mice (Sundstedt, Hoiden et al, 1997). SAg activation of CD4<sup>+</sup> T cells results in both upregulation of IL-10 mRNA (Koide, Uchijima et al, 1996) and IL-10 production (Sundstedt, Hoiden et al, 1997; Florquin, Amraoui et al, 1994; Hasko, Virag et al, 1998; Gaus, Miethke et al, 1994:Koide, Uchijima et al, 1996;Schols, Jones et al, 1995), which is detectable in mice 4 hours after a single injection of SEB (Florquin, Amraoui et al, 1994). Scheffold et al. (Scheffold, Assenmacher et al., 2000a) demonstrated by high-intensity immunofluorescence that IL-10 is expressed on the surface of CD4<sup>+</sup> cells following SEB stimulation, and in a conformation that may bind its appropriate receptor on neighboring cells. This implies that this cytokine may play a regulatory role within the immunological synapse, even at a concentration that is undetectable in culture supernatants. The primary exposure to sAgs also appears to consistently prime for more enhanced IL-10 production and evoke a memory-like response during secondary sAg stimulation (Sundstedt, Hoiden et al, 1997; Miller, Ragheb et al, 1999; Florquin, Amraoui et al, 1996; Gaus, Miethke et al, 1994).

IL-10 augments cell survival, perhaps via a Bcl-2-independent pathway (Lelievre, Sarrouilhe et al., 1998). IL-10 has also been shown to activate cell cycle inhibitors (O'Farrell, Parry et al., 2000), and directly inhibit T cell proliferation (Taga & Tosato, 1992) by inducing cell cycle arrest in the G0/G1 phase (Perrin, Johnson et al., 1999). IL-10 also inhibits IFN- $\gamma$ , TNF- $\alpha$  and TNF- $\beta$  production by T cells (de Waal Malefyt, Yessel et al., 1993), and TNF- $\alpha$  production by APCs (Moore, O'Garra et al, 1993;de Waal Malefyt, Abrams et al., 1991). IL-10 further contributes to the control of inflammatory responses by up-regulating the TGF- $\beta$  receptor on

activated T cells(Cottrez & Groux, 2001). Moreover, IL-10 has several inhibitory effects on APC function, including down-regulation of both MHC class II (de Waal Malefyt, Haanen et al., 1991), ICAM-1 (Spittler, Schiller et al., 1995), and CD80/86 (B7.1/B7.2) expression (Flores Villanueva, Reiser et al., 1994; Willems, Marchant et al., 1994). Finally, IL-10 also inhibits the synthesis of IL-12 by APC, a critical factor for the induction of pro-inflammatory and cytolytic T cell responses (Aste-Amezaga, Ma et al., 1998).

The pivotal role of IL-10 in inhibiting the production of pro-inflammatory cytokines and decreasing the severity of toxic shock has been demonstrated in several key studies. Firstly, Bean et al. (Bean, Freiberg et al, 1993) showed that IL-10 treatment, either before or up to 30 min following SEB challenge, protected mice in a dose-dependent manner in the D-galactosaminesensitized mouse model of lethal toxic shock. Secondly, neutralization of endogenous IL-10 with monoclonal antibodies increased SEB-induced lethality, even in the absence of D-galactosamine sensitization (Florquin, Amraoui et al, 1994). The cytokine profile in these mice showed elevated levels of both IL-2 and IFN- $\gamma$ , indicating a strong T<sub>H</sub>1 effector response. The lethal effect induced by IL-10 neutralization was abrogated if these mice concurrently received neutralizing antibodies to IFN-y, again underscoring the important role of IFN-y in mediating toxic shock, and the counteracting role of IL-10 in the suppression of IFN-y (Florquin, Amraoui et al, 1994). In a third study, similar results were obtained with IL-10 knock-out mice in which SEB induced lethal shock even in the absence of D-galactosamine sensitization (Hasko, Virag et al, 1998). Again, the absence of endogenous IL-10 in these knock-out mice following SEB challenge correlated directly with markedly increased levels of several pro-inflammatory cytokines, including IL-2, IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , and IL-12.

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#### Transforming Growth Factor- $\beta$ (TGF- $\beta$ )

TGF- $\beta$  is another potent regulatory cytokine which inhibits IL-2 expression (Brabletz, Pfeuffer et al., 1993) and cell cycle progression (Hocevar & Howe, 1998). The involvement of TGF- $\beta$  in peripheral tolerance following chronic antigen stimulation has been shown in a number of animal models (Miller, Lider et al., 1992). Furthermore, TGF- $\beta$  has been shown to suppress T<sub>H</sub>1mediated autoimmunity in a number of model systems including colitis (Powrie, Leach et al, 1996), non-obese diabetes (Lepault & Gagnerault, 2000), transplant rejection (Zhai & Kupiec-Weglinski, 1999), and multiple sclerosis (Rohowsky-Kochan, Molinaro et al., 2000), as well as T<sub>H</sub>2-mediated autoimmunity (Bridoux, Badou et al, 1997). This cytokine also has a potent ability to alter the direction of antibody responses, suppressing serum antibody levels and directing isotype production towards the IgA subclass(Cazac & Roes, 2000).

Although a number of studies have explored the role of exogenous TGF- $\beta$  in modulating the effector functions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Chen & Wahl, 2003;Heath, Murphy et al., 2000;Bertone, Schiavetti et al., 1999;Crisi, Santambrogio et al., 1995), the induction of TGF- $\beta$  following primary stimulation with sAgs remains to be elucidated. However, Miller et al. (Miller, Ragheb et al, 1999) found that CD4<sup>+</sup> splenocytes obtained from mice which had received repeated injections of SEA, entered into a tolerant state in which little IL-2, IL-4 or IFN- $\gamma$  were produced upon rechallenge with a pigeon cytochrome C peptide. Instead, these cells produced high levels of TGF- $\beta$  as well as IL-10. Furthermore, these CD4<sup>+</sup> cells actively suppressed IL-2 production by naïve splenocytes when stimulated with the peptide. This suppressive activity of SEA-treated T helper cells was only partially reversed with antibodies directed against TGF- $\beta$  and IL-10, suggesting that additional, as yet unidentified, molecules may participate in inducing this profound state sAg-mediated anergy or tolerance.

Groux et al. (Groux, O'Garra et al., 1997) described a unique subset of effector CD4<sup>+</sup> T cells generated after repeated antigenic stimulation of naïve CD4<sup>+</sup> T cells in the presence of IL-10, or a combination of IL-4 and IL-10. These T cell clones, designated T<sub>R</sub>1 cells, displayed low proliferative capacity and a cytokine profile distinct from that of the classical  $T_{H2}$  phenotype in that high levels of IL-10 were produced with low levels of TGF- $\beta$ , while levels of IL-4 or IL-2 were undetectable. Since then, a number of workers have confirmed the existence of this novel subset of regulatory T cells in a variety of model systems (Chen, O'Shaughnessy et al., 2003;Groux, 2003;Kemper, Chan et al., 2003;Wakkach, Fournier et al., 2003;Bacchetta, Sartirana et al., 2002;Barrat, Cua et al., 2002;McGuirk, McCann et al., 2002;McGuirk & Mills, 2002:Levings, Bacchetta et al. 2002;Levings, Sangregorio et al., 2001a:Noel, Florquin et al., 2001; Wakkach, Cottrez et al., 2001; Groux, 2001; Roncarolo, Bacchetta et al., 2001; Roncarolo, Levings et al., 2001;Roncarolo & Levings, 2000;Levings & Roncarolo, 2000). T<sub>R</sub>1 cells grow slowly in vitro and are often characterized as "anergic" due to their inability to produce IL-2 upon antigenic stimulation (Groux, O'Garra et al, 1997). T<sub>R</sub>1 cells are antigen-specific and require TCR engagement by the antigen/MHC complex for activation. Furthermore, the growth and proliferative response of  $T_{R1}$  cells is itself dependent on IL-10.

The origin or cell lineage of  $T_R1$  cells is currently unclear, as it is not yet understood whether they are derived by a further differentiation from Th0,  $T_H2$  or  $T_H1$  cell types, although there is some evidence in support of the latter following SEB stimulation (Assenmacher, Lohning et al, 1998). Using intracellular immunofluorescent staining techniques, Assenmacher et al. (Assenmacher, Lohning et al, 1998) elegantly showed the sequential production of IL-2, IFN- $\gamma$ and IL-10 in T helper cells following primary and repeated stimulation by SEB. As expected from naïve T cells, primary exposure to SEB rarely induced the co-expression of any combination of these cytokines in a given activated T cell. However, following repeated stimulation, IL-2-enriched  $T_H$  cells were seen to sequentially and preferentially express IFN- $\gamma$ , while IFN- $\gamma$ -enriched  $T_H$  cells were seen to sequentially and preferentially express IL-10. In contrast, IL-2 and IL-10 were rarely co-expressed. These data suggest that  $T_R1$  cells may have developed from  $T_H1$  precursors. In other systems,  $T_{REG}$  clones have been shown to express  $T_H2$  gene transcripts (Zelenika, Adams et al, 2002;Mendel & Shevach, 2002).

The induction of  $T_R1$  cells is antigen-specific, while the suppressor function of these  $T_{REG}$ 's, once activated, appears to be antigen-non-specific (Roncarolo, Bacchetta et al, 2001). Such non-specificity is likely due to a "bystander effect" of the paracrine action of the immunosuppressive cytokines induced. This  $T_{REG}$  subset has been implicated in both peripheral tolerance (Kitani, Chua et al., 2000;Groux & Powrie, 1999;Bacchetta, Bigler et al., 1994;Rohowsky-Kochan, Molinaro et al, 2000;Stohlman, Pei et al., 1999;Zhai & Kupiec-Weglinski, 1999), and mucosal tolerance mechanisms (Duchmann, Schmitt et al., 1996;Desvignes, Etchart et al., 2000).

The generation of regulatory T helper cells following repeated sAg stimulation was first reported by Sundstedt et al. (Sundstedt, Hoiden et al, 1997). Following successive subcutaneous injection with high-dose SEA (10  $\mu$ g) in mice, a hyporesponsive state was induced characterized by clonal deletion and anergy of the remaining SEA-reactive T cells. These anergic T helper cells characteristically produced high levels of IL-10, but little or no IL-4, while the production of T<sub>H</sub>1 cytokines including IL-2, IFN- $\gamma$  and TNF, was markedly inhibited. The IL-10 production was sustained with further sAg stimulation in the complete absence of IL-2, IL-4 and IFN- $\gamma$ . Pretreatment of mice with neutralizing anti-IL-10 mAb before the SEA challenges, substantially enhanced IFN- $\gamma$  and TNF serum levels, and led to an increase in IL-4, but not IL-2 production. These data suggest that IL-10 may provide negative feedback to control the accumulation of T<sub>H</sub>1 and perhaps even T<sub>H</sub>2 effector responses. Since the production of IL-10 in these T helper cells did not seem to be linked to IL-4 production, it was proposed that this sAg-induced immunosuppressive effect was not due to the shift to a classical T<sub>H</sub>2 response, but represented a novel T regulatory phenotype. Further work with both SEA and SEB has supported this hypothesis, and demonstrated that sAg generated T<sub>R</sub>1 cells are antigen specific since they have a V $\beta$  specificity that matches the sAg used to generate them (Noel, Florquin et al, 2001).

#### 'Natural' CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T cells (T<sub>REG</sub>)

"Natural" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s migrate from the thymus after the 3<sup>rd</sup> day of life and reach plateau levels around 7-8 weeks of age (Papiernik, de Moraes et al, 1998). These "natural" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s are believed to undergo altered negative selection (Shevach, McHugh et al., 2001a) on the thymic epithelium (Jordan, Boesteanu et al., 2001) in which CD4<sup>+</sup> T cells which recognize self antigen migrate to the periphery rather than undergo thymic deletion. The activation, and proliferation of these cells dependent on exogenous IL-2 or IL-4 (Thornton, Piccirillo et al., 2004;Thornton, Donovan et al., 2004), produced by the very effector T cells that T<sub>REG</sub>'s suppress. These cells are identified by their constitutive high expression of the IL-2 receptor  $\alpha$  chain, CD25, prior to foreign antigen stimulation(Sakaguchi, Sakaguchi et al., 1995). The activated by self or mucosa-associated antigens in the periphery, as chronic antigen stimulation is required for their maintenance (Seddon & Mason, 1999). The lack of these cells *in vivo* invariably results in organ-specific autoimmunity (Sakaguchi, Sakaguchi et al, 1995) including autoimmune gastritis (Suri-Payer, Kehn et al., 1996).

Suppression by "natural" CD4<sup>+</sup>CD25<sup>+</sup> cells is believed to be antigen non-specific (Thornton & Shevach, 2000). This regulatory T cell phenotype is capable of both  $T_H1$  and  $T_H2$  suppression(Jones, Murphy et al., 2003;Mottet, Uhlig et al., 2003;Jiang, Camara et al., 2003;Bellinghausen, Klostermann et al., 2003;Francis, Till et al., 2003;Hadeiba & Locksley, 2003). While such suppression is non-specific *in vitro*, it is believed that these  $T_{REG}$ 's inhibit T effector ( $T_E$ ) cells following active recruitment to the periphery, and stimulate  $T_E$  cells interacting with APC's (Shevach, McHugh et al, 2001a), a model that at least mathematically holds promise (Leon, Perez et al., 2001).  $T_{REG}$ 's have been reported to induce contact-mediated suppression *in vitro*, involving cytotoxic lymphocyte antigen-4 (CTLA-4) (Takahashi, Tagami et al., 2000;Read, Malmstrom et al., 2000;Manzotti, Tipping et al., 2002) and surface expressed TGF- $\beta$  (Chen & Wahl, 2003;Levings, Sangregorio et al., 2002;Nakamura, Kitani et al., 2001, although both the former and later findings are controversial (Shevach, McHugh et al, 2001a;Piccirillo, Letterio et al., 2002;Jonuleit, Schmitt et al., 2002).

#### Antigen-Induced CD45RB<sup>LOW</sup> Regulatory T Cells

Antigen-naïve T cells have high expression levels of the CD45RB marker (CD45RB<sup>HI</sup>), while antigen-specific mature or memory T cells have a decreased expression (CD45RB<sup>LOW</sup>). Removal of this subset, and subsequent transfer of only CD45RB<sup>HIGH</sup> T cells into a rag2 KO mouse results in inflammatory bowel disease (IBD) (Mottet, Uhlig et al, 2003). These cells have been shown to mediate their suppressive effects through both IL-10 (Jump & Levine, 2002;Annacker, Pimenta-Araujo et al., 2001;Asseman, Mauze et al., 1999) and TGF- $\beta$  (Powrie, Leach et al, 1996) in the IBD murine model. While it has been demonstrated that the CD25<sup>+</sup> positive T cell subset within this CD45RB<sup>LOW</sup> population is responsible for this cytokine phenotype (Read, Malmstrom et al, 2000), these cells may in fact be more  $T_R1$ -like than natural  $CD4^+CD25^+$   $T_{REG}$ -like, since their suppressive function is largely cytokine-mediated. The requirement for cytokine-mediated suppression has been suggested to depend on both the strength of the immune response against the antigen, and the type of antigen whether self or foreign (Shevach, McHugh et al, 2001a).

#### T<sub>H</sub>3 cells

 $T_H3$  cells have been identified as an essential CD4<sup>+</sup> T cell subtype that produce high levels of TGF- $\beta$  in response to antigen stimulation, and are involved in gut immunohomeostasis and oral tolerance. The high-level constitutive expression and upregulation of IL-4, IL-10 and TGF- $\beta$  in the gut mucosa (Gonnella, Chen et al., 1998) contributes to the observed polarization towards a T<sub>H</sub>3 response in this organ (Chen, Kuchroo et al., 1994), including the enhancement of IgA isotype switching (Cazac & Roes, 2000). These cells appear distinct from T<sub>H</sub>1 and T<sub>H</sub>2 cells, as it is possible to generate CD4<sup>+</sup> T cells which produce TGF- $\beta$  but not IL-2, IL-4, IL-10 or IFN- $\gamma$ . The relationship of this cell type to other T<sub>REG</sub> subtypes is undetermined, as there are reports that these cells may be similar to both natural T<sub>REG</sub> and T<sub>R</sub>1, since they mediate their suppressive function through both CTLA-4 (Fowler & Powrie, 2002;Samoilova, Horton et al., 1998) and IL-10 (Chen, Kuchroo et al, 1994).

#### Interactions Between Polarized T<sub>H</sub> Subsets Following sAg Stimulation

#### $T_H1$ vs. $T_H2$

Based on our current knowledge, it is clear that the initial outcome of primary sAg stimulation is a polarized  $T_H 1$  response. Whether the eventual outcome of sAg stimulation will shift to a  $T_H 2$ - dominated response may be determined early after sAg exposure, and depends on the net balance between IL-12/IFN-y secretion that favors a T<sub>H</sub>1 response (McKnight, Zimmer et al, 1994), and IL-6/IL-4 production which favors a T<sub>H</sub>2 response (Miethke, Wahl et al. 1993;Mosmann, Cherwinski et al, 1986). However, following repeated sAg exposure, both  $T_{H1}$  and  $T_{H2}$ responses are suppressed (Sundstedt, Hoiden et al, 1997). While T<sub>H</sub>1 and T<sub>H</sub>2 subsets are capable of cross regulation via IFN- $\gamma$  and IL-4 respectively, decreased production of both these polarizing cytokines are seen after repeated sAg exposure (Sundstedt, Hoiden et al, 1997; Miller, Ragheb et al, 1999). This suppression is probably due to the emergence and predominance of a  $T_{REG}$  response accompanied by the secretion of IL-10 with or without TGF- $\beta$ . While the production of IL-10 could support a classical  $T_{H2}$  phenotype, the consistent observation that both IL-4 mRNA (Koide, Uchijima et al, 1996) and IL-4 production (Sundstedt, Hoiden et al, 1997; Miller, Ragheb et al, 1999; Florquin, Amraoui et al, 1996) are markedly inhibited following repeated sAg treatment suggests that a novel regulatory T helper subset such as T<sub>R</sub>1 cells may be activated. The observation that sAg-activated T cells can also induce the secretion of TGF- $\beta$ (Miller, Ragheb et al, 1999) further supports this contention, since this regulatory cytokine has been shown to inhibit IL-4 production, and both T<sub>H</sub>1- and T<sub>H</sub>2-mediated immune responses (Bridoux, Badou et al, 1997;Heath, Murphy et al, 2000).

#### T<sub>H</sub>1 vs. T<sub>R</sub>1

The mechanism for activation of  $T_{REG}$ 's following repeated sAg stimulation is incompletely understood at present. Since IL-10 provokes negative-feedback inhibition of an overwhelming  $T_{H}1$  immune response, one might expect a pro-inflammatory cytokine such as IFN- $\gamma$  to upregulate IL-10 production. However, the link between IFN- $\gamma$  and IL-10 production in the induction of anergy seems to be indirect at best. Neutralization of IFN- $\gamma$ , while inhibiting clonal deletion and apoptosis of reactive lymphocytes, has little effect on preventing sAg-induced anergy or IL-2 non-responsiveness (Kuschnaroff, Valckx et al., 1997). The source of this indirect link between excessive IFN- $\gamma$  production and subsequent induction of IL-10 may be provided by IL-12, an essential IFN- $\gamma$ -inducing cytokine which also up-regulates IL-10 in human CD4<sup>+</sup> T cells (Mingari, Maggi et al, 1996;Meyaard, Hovenkamp et al, 1996). This unexpected ability of IL-12 may explain the curious protective effect of IL-12 when administered prior to sAg exposure, as was observed by Takahashi et al. (Takahashi, Nakagawa et al, 1995). Rather than priming for a T<sub>H</sub>1 response, administration of exogenous IL-12 in this instance may have primed for a greater T<sub>R</sub>1-dependent IL-10 response which counteracted the lethal effects of sAg-induced T<sub>H</sub>1 activation.

Although cytokines contribute to immune suppression following sAg stimulation, cell contact may also play an important role. Such mechanisms are thought to incorporate a triple-cell model, consisting of DC, T effector cell, and  $T_{REG}$  in contact with one another (**Figure I-4**) (Leon, Perez et al, 2001;Shevach, McHugh et al, 2001a), with suppression signals mediated through CTLA-4 (Annacker, Pimenta-Araujo et al, 2001;Fowler & Powrie, 2002;Manzotti, Tipping et al, 2002;Read, Malmstrom et al, 2000;Samoilova, Horton et al, 1998) and other as yet unidentified molecules. This surface regulation may not occur in a single direction, as the glucocorticoid induced TNF receptor (GITR) expressed on  $T_{REG}$ 's can break tolerance when crosslinked (**Figure I-5**), suggesting that it may play a role in turning off  $T_{REG}$ 's (Ronchetti, Nocentini et al., 2002;Shimizu, Yamazaki et al., 2002). Additionally, differences in both the signal strength, and the temporal nature of suppression may affect the eventual outcome between  $T_H l$  and  $T_{REG}$  responses (Barrat, Cua et al, 2002;Baecher-Allan, Viglietta et al., 2002).



Figure I-4. Proposed Triple-Cell Model for  $CD4^+CD25^+$  Mediated Suppression of Effector T cell Responses. A dendritic cell displays organ-specific antigens to both a T effector cell (either  $CD4^+$  or  $CD8^+$ ) and a natural  $CD4^+CD25^+T_{REG}$  cell. Cell-cell contact through as yet unidentified ligands on the  $CD4^+CD25^+T_{REG}$  and T effector cells results in suppression of the immune response directed against the organ-specific antigen (Adapted from Shevach, McHugh et al., 2001a).



**Figure I-5. Regulation of Regulatory T Cells:** Activated T effector cells have been shown to express higher levels of the GITR ligand on their surface. Cross-linking of the GITR receptor has been shown to inhibit the suppressive effects of regulatory T cells (Shimizu, Yamazaki et al., 2002).

#### T<sub>H</sub>2 vs. T<sub>R</sub>1

 $T_R1$  cells can also inhibit  $T_H2$  responses to sAg (Sundstedt, Hoiden et al, 1997). This suppressive activity by  $T_R1$  cells may be mediated by regulatory cytokines. Firstly, although IL-10 was initially characterized as a  $T_H2$  cytokine, it is capable of strongly inhibiting  $T_H2$  cell proliferation under CD28 costimulation (Akdis & Blaser, 2001). This cytokine also suppresses the production of the  $T_H2$  cytokines IL-4 and IL-5(Akdis & Blaser, 1999).  $T_H2$ -specific B cell responses are also affected by IL-10 produced by  $T_R1$  cells, leading to decreased IgE responses to ovalbumin *in vivo* (Cottrez, Hurst et al., 2000). In addition, the activity of both basophils and eosinophils is suppressed by IL-10 (Akdis & Blaser, 2001). Finally, the production of TGF- $\beta$  by activated  $T_R1$ cells further decreases  $T_H2$  responses through the reduction of STAT-6 activation and GATA-3 expression (Heath, Murphy et al, 2000). Through these regulatory mechanisms, strong  $T_R1$  responses induced by chronic sAg treatment are thought to suppress  $T_H2$  responses, as was observed in the repeated SEA exposure murine model (Sundstedt, Hoiden et al, 1997).

#### Summary

Based on this review of T helper cell responses following sAg activation, we believe that the induction of a subset of  $T_{REG}$ 's, particularly  $T_R 1$ , results in the sustained release of the inhibitory cytokines IL-10 and TGF- $\beta$ . Furthermore, this  $T_{REG}$  response plays a pivotal role in reversing the deleterious  $T_H 1$  hyperimmune response to sAgs (Sundstedt, Hoiden et al, 1997;Miller, Ragheb et al, 1999). The kinetics of  $T_{REG}$  subset proliferation and the rapidity and magnitude of IL-10 or TGF- $\beta$  induction may be the critical determinant of the ultimate survival of the host. A schematic representation of the postulated polarization and interactions between the  $T_H 1$ ,  $T_H 2$  and  $T_R 1$  T cell subsets following staphylococcal sAg stimulation is depicted in **Figure I-6**.



Figure I-6. Schematic representation of the proposed interactions between polarized  $T_H1$ ,  $T_H2$ and  $T_R1$  subsets of helper T cells following staphylococcal sAg stimulation. Induction of the  $T_R1$ subset accompanied by the sustained secretion of the regulatory cytokine IL-10 (with or without TGF- $\beta$ ) is believed to play a pivotal role in the down-regulation of  $T_H1$  hyperimmune responses and eventual immunohomeostasis and recovery from toxic shock. IL-10 serves to down-regulate (-) APC function by suppressing both the expression of MHC class II (MHCII) and co-stimulatory molecules CD80/86 (B7.1/7.2), and the secretion of IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$  and IL-12.  $T_H1$  cells are also directly suppressed through the inhibition of IFN- $\gamma$  and TNF- $\beta$  production by TGF- $\beta$ , which also down-regulates the  $T_H2$  responses of T cell proliferation and IL-4 secretion.

#### **Research Questions**

Based on the above literature review, we wish to address whether the staphylococcal sAg TSST-1 may also induce the differentiation of a regulatory T cell phenotype similar to SEB and SEA (Florquin, Amraoui et al, 1996;Noel, Florquin et al, 2001;Sundstedt, Hoiden et al, 1997). Specifically, we ask:

- 1) Does repeated administration of TSST-1 *in vivo* induce a  $T_R$ 1-like serum cytokine profile characterized by high IL-10, low IFN- $\gamma$ , and low IL-2 compared to control animals?
- 2) Is this  $T_R$ 1-like phenotype transferable via CD4<sup>+</sup> T cells *in vivo*?
- Is the suppression effect observed following repeated *in vivo* administration of TSST-1 mediated by IL-10?
- 4) What are the phenotypic characteristics of TSST-1 induced T<sub>R</sub>1-like cells both in terms of cytokine production and cell surface markers?
- 5) What is the role of CD25 as a marker for regulatory T cells in our system?

#### **Chapter II. Materials and Methods**

#### **TSST-1** Purification

Recombinant TSST-1 was purified from culture supernatants of S. aureus RN4220, previously transformed to carry the tst gene. Purification was performed using methods previously developed in our laboratory (Kum, Laupland et al, 1993). Briefly, the Staphylococcus aureus strain which produces recombinant TSST-1 was cultured for 18 h in brain heart infusion broth at 37°C in a shaker. Culture supernatant was centrifuged at 15,000 x g for 30 min at 4°C. Supernatant was then desalted and concentrated 10-fold using the amicon spiral cartridge concentrator, with a 10 kD cutoff. Concentrated supernatant was then fractionated by preparative isoelectric focusing on a Rotofor apparatus (Bio-Rad Laboratories, Hercules, USA). Fractions were tested for TSST-1 by SDS-PAGE followed by silver staining or western blotting with anti-TSST-1 antibodies. Fractions containing TSST-1 were pooled, and dialyzed against water and lyophilized. Lyophilized TSST-1 was then reconstituted in tris-acetate buffer (pH 8.3), and applied to a polybuffer column for chromatofocusing, and fractions were then eluted with Polybuffer 96-acetate (pH 6.0). Eluted fractions were tested for TSST-1 by western blot analysis. Pooled fractions were again dialyzed against water and lyophilized. Following four rounds of chromatofocusing, purified TSST-1 was analyzed by SDS-PAGE and silver stained to determine purity. No traces of contaminants were detectable, as the protein migrated as a single band of 22 kD. Toxin bioactivity was tested by stimulation of human PBMC with various doses of toxin ranging from  $1 \times 10^{-6}$  to  $1 \times 10^{5}$  pM of TSST-1. TNF- $\alpha$  assays on supernatant from purified human monocytes stimulated with TSST-1 alone were used to confirm that such stimulation was independent of potential LPS contamination. TSST-1 will not stimulate TNF-a secretion from monocytes in the absence of T cells, while LPS contamination induces high levels of TNF-α expression. Quantification of protein was achieved by Bradford assay against a BSA standard (Biorad Laboratories, Hercules, USA)

#### Mice Handling and TSST-1 Administration

Female BALB/c mice between 8-12 weeks of age were purchased from Charles River Biotechnology Corporation (Wilmington, MA, USA) or Taconic (German Town, NY, USA) and housed in the UBC Jack Bell Research Centre following established guidelines of the Canadian Council on Animal Care, under protocols A99-0113 and A03-0081 issued to Dr. A.W. Chow. Mice were given 5 days of acclimatization in the Jack Bell facility before any experiments were performed. Animals were housed 5 per cage, using standard feed, water, bedding and enclosures. Prior to the beginning of any experiment, experimental animals were randomly redistributed among cages to negate any cage specific abnormalities that may have been present. Mice received repeated injections of either TSST-1 dissolved in 100  $\mu$ l of PBS, or PBS alone, using a 25 gauge needle at a single location subcutaneously, at 4-day intervals for a total of 3 injections. This dosing interval was based on studies with SEA by Sundstedt el al (Sundstedt, Hoiden et al, 1997). The injection site was not shaved, but was wiped with alcohol as an antiseptic. In preliminary dose-finding experiments, groups containing at least 4 mice each were utilized to determine the most suitable TSST-1 dose that was most likely to induce sustained levels of detectable serum IL-10 following repeated subcutaneous administration in vivo. Four dosing regimens of TSST-1 in addition to PBS alone were evaluated: 40µg/mouse, 4µg/mouse, 0.4µg/mouse and 0.04µg/mouse. Serum was sampled from each mouse 2 hours post TSST-1 injection, in order to determine which cytokines were primed for both production and release in the serum. Based on results from the dose-finding experiments, the 4 µg dose of TSST-1 was chosen for all subsequent experiments to induce a Tr-1 phenotype in our murine model.

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#### Serum Sampling

Following each injection, serum was sampled via the tail vein 2 h post TSST-1 or PBS administration. Whole blood was centrifuged at 800 x g for 5 min at room temperature, and serum was pipetted off and stored at -70°C for subsequent cytokine analysis. Typically 100  $\mu$ l - 250  $\mu$ l of whole blood was taken, yielding between 5-80  $\mu$ l of serum per sample. Mice were euthanized 2 hours after the final treatment via heart puncture with halothane anesthesia. Between 400  $\mu$ l - 1500  $\mu$ l of whole blood was collected using this method, and handled as described for sample storage. Mice were observed daily throughout the duration of the experiment to ensure that the treatment protocol did not induce any complications.

#### Quantitation of Serum Cytokine Levels by ELISA

Serum samples were tested for cytokines utilizing commercial kits from BD Pharmingen (Mississauga, Canada) for IL-2, IL-4, and IL-10. Reagents from BD Pharmingen were purchased for developing the IFN- $\gamma$  ELISA in-house, including capture, detecting and Avidin-HRP (clone R4-6A2 for coating and biotinlylated clone XMG1.2 for detection). All ELISAs were quantified using tetramethylbenzidine (TMB) as a substrate. The reactions were stopped with equal volumes of 1M H<sub>3</sub>PO<sub>4</sub> and read at 450  $\eta$ M, with 570  $\eta$ M subtracted from the detected signal. A list of the reagents used for the cytokine assays is shown in **Table II-1**. A Dynatech MR5000 96 well plate reader and the BioLinx version 2.20 software (Dynatech Laboratories, Inc., Chantilly, VA, USA) were used for the ELISA measurements

Table II-1: List of ELISA Reagents			
Material	Catalogue #	Supplier	
OptEIA <sup>™</sup> Mouse: IL-2 Set	#555148		
OptEIA <sup>TM</sup> Mouse: IL-4 Set	#555232		
OptEIA <sup>™</sup> Mouse: IL-10 Set	#555252	BD Pharmingen,	
Purified Anti-Mouse IFN-γ (Capture)	#551216	Mississauga, ON	
Biotin Rat Anti-Mouse IFN-γ	#554410		
Avidin-HRP	#554058		
TMB Substrate Reagent A&B 300ml	#555214		
Immunolon 1B ELISA Plates	#62402-947	VWR Canlab, Mississauga, ON	
H <sub>3</sub> PO <sub>4</sub>	#A242500	Fisher Scientific, Nepean, ON	

The detection limits of our optimized ELISA assays for various cytokines throughout our experiments are shown in Table II-2.

Table II-2. Lower detection limits of various cytokines measured by ELISA				
Cytokine	Detection Limit Median and range (pg/ml)	Example correlation coefficient (r <sup>2</sup> ) values for standard curves	No. of standard curve replicates	
IL-2	15.6 (1.95-15.63)	0.9785	6	
IL-4	15.6 (15.63-31.25)	0.9911	5	
IL-10	31.2 (15.63-62.50)	0.9916	8	
IFN-γ	31.2 (3.90-125.00)	0.9873	. 7	

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#### Isolation and Purification of Splenocytes

Mice were sacrificed by cervical dislocation. Spleens were removed and homogenized with a 100  $\mu$ M mesh into single cell suspensions, washed and resuspended in 10 ml of RPMI 1640 (Stem cell technology, Vancouver, BC). The cell suspension was then centrifuged at 800x g for 7 min. Red blood cells were lysed with Gey's balanced salt lysis solution containing 0.7% NH<sub>4</sub>Cl for removal of red blood cells, and washed twice in RPMI before being resuspended in complete growth medium.

#### Purification of CD4<sup>+</sup> Splenocytes by Magnetic Separation

 $CD4^+$  T cells were positively isolated from splenocyte single cell suspensions according to the protocol provided by Miltenyi Biotech Inc. (Auburn CA), using MS+ columns, an OctoMACs magnet, and magnetic beads coated anti-L3T4. Following purification, cells were further washed with RPMI 1640 (Stem Cell Technologies, Vancouver, BC), after which they were resuspended in complete growth medium (**Table II-3**). Cells were always cultured at 37°C, with 5% CO<sub>2</sub> in complete growth medium.

Table II-3: List of Reagents for Complete Growth Medium				
Material	Concentration	Catalogue #	Supplier	
RPMI-1640	90% of Volume	36750		
L-Glutamine	2mM	07100		
Penicillin / Streptomycin	80U/ml	07500	Stem Cell Technologies, Vancouver, B.C.	
Sodium Pyruvate	0.8mM	07000		
HEPES buffer	0.24M	07200		
Fetal Calf Serum	10% of volume	SH30071	Hyclone, Logan, UT	
β-mercapthaenthanol	17µl of 1/100 Stock	M-7522	Sigma, Saint Louis, MO	
Polymyxin B	2µg/ml	P4932		

#### Adoptive Transfer of CD4<sup>+</sup> T Cells into Naïve Mice

CD4<sup>+</sup> splenocytes from mice treated repeatedly with TSST-1 or PBS were purified by magnetic separation as described above, and were washed three times with PBS to remove any excess magnetic beads or MACs buffer. Purified cells were resuspended in 300 $\mu$ l PBS at a concentration of 5x10<sup>7</sup>/ml. They were loaded into a 1 cc tuberculin syringe, and 200 $\mu$ l of CD4<sup>+</sup> (~1x10<sup>7</sup>) were injected IV into the tail vein of naïve mice which were first warmed with a heat lamp for 10 min to facilitate the injection. Mice were given 4 days to recuperate before subsequent challenges with repeated subcutaneous administration of TSST-1 or PBS at 4-day intervals as described previously. Sera were collected 2 hours post each injection for quantitation of IL-2, IL-4, IFN- $\gamma$  and IL-10 by ELISA as described above.

#### Treatment with Monoclonal Antibodies

To evaluate the role of IL-10 in mediating the suppression of serum IL-2 and IFN- $\gamma$  following adoptive transfer of CD4<sup>+</sup> T cells from TSST-1 treated donor mice, groups of mice also received either  $\alpha$ IL-10R $\alpha$  mAb or an IgG isotype control during adoptive transfer of CD4<sup>+</sup> T cells. Briefly, CD4<sup>+</sup> T cells for adoptive transfeer were resuspended in 300 µl of either  $\alpha$ IL-10R $\alpha$ mAb (clone 1B1.2) or IgG isotype control (clone GL113) at a concentration of 2.5 mg/ml. Approximately 200µl of the antibody and cell mixture (containing ~1x10<sup>7</sup> CD4<sup>+</sup> T cells and 0.5mg of  $\alpha$ IL-10R $\alpha$  mAb or IgG isotype control) were administered intravenously into each mouse via the tail vein. Mice were given 4 days to recuperate before subsequent challenges with repeated subcutaneous administration of TSST-1 or PBS at 4-day intervals as described previously. A second dose of 0.5 mg  $\alpha$ IL-10R $\alpha$  or IgG isotype control was administered IV 3 days after the first toxin challenge (7 days post T cell transfer).

#### Intracellular Staining for Cytokines and CTLA-4

The technique of intracellular cytokine staining is depicted in **Figure II-1**. Cells restimulated for 5 h under naïve conditions and in the presence of monensin were first stained for various surface markers, and then treated with paraformaldehyde which fixes the cell membrane. The cell membrane is then made permeable by the addition of saponin. Anti-cytokine or anti-CTLA-4 antibodies labeled with fluorochromes are then added, resulting in staining of the golgi apparatus and accumulated cytokines if present. Excess antibody is removed with several washing steps in the presence of saponin and cells are then analyzed by flow cytometry.



Figure II-1. Schema depicting the technique of intracellular cytokine staining.

The use of ICS must be carefully controlled for any non-specific background staining. This is accomplished by the use of fluorochrome-labeled anti-cytokine antibodies in the presence of an excess of unlabeled antibodies of the same specificity to determine the amount of non-specific background staining. This step is essential to control for non-specific staining due to auto-fluorescence, as the targeted population is often small constituting less than 10% of total cells. In order to ensure the results are reproducible, specimens from multiple animals (ranging from 4 to 8 mice per group, depending on the experiment) were collected. In order to detect intracellular cytokines, a Golgi transport inhibitor must be used on *in vitro* stimulated cells. This Golgi transport inhibitor affects the release or display of rapidly produced cytokines as well as other surface molecules. The concentration range in which the Golgi transport inhibitor can be used is relatively small, due to the toxicity of these agents. Therefore, cells must be activated to rapidly

produce cytokines within a narrow 5-hour window.  $CD4^+$  cells, purified from mice treated repeatedly with TSST-1 or PBS, were stimulated polyclonally *in vitro* using  $\alpha$ CD3,  $\alpha$ CD28 and IL-2 in order to induce the strong and rapid production of cytokines.

## *In Vitro Stimulation of Purified CD4<sup>+</sup> Cells Prior to Intracellular Cytokine Staining (ICS)*

96 well flat-bottomed tissue culture plates were coated with 50µl of  $\alpha$ CD3 (10µg/ml; clone 1452C11) in sterile carbonate buffer (pH 9.6) and incubated overnight at 4°C. All reagents were supplied by BD Pharmingen, Mississauga Canada, unless stated otherwise. The next day, plates were washed with RPMI. Subsequently, cells were cultured at 1x10<sup>6</sup> per ml under T<sub>H</sub>0-like conditions in the presence of  $\alpha$ CD28 (2µg/ml; clone 37.51), and IL-2 (10 units/ml or 10ng/ml). Following 4 days of stimulation, cells were washed twice with cold RPMI, resuspended in fresh complete growth medium, and transferred to fresh  $\alpha$ CD3-coated tissue culture plates prepared as described above, with a final volume of 200µl per well. Cells were stimulated under T<sub>H</sub>0-like conditions in the presence of monensin for 5 hours if analysis by ICS was required. Alternatively, cell supernatants were harvested 48 or 96 hours after secondary stimulation in the absence of monensin treatment, and supernatants were collected for subsequent cytokine profile analysis.

Polarized T cells were pelleted in 96-well round-bottomed plates and stained for surface molecules in a 3% FCS-PBS (FACs) buffer. Following this, cells were fixed and permeabilized using an intracellular staining kit (BD Pharmingen, Mississauga Canada) according to the manufacturer's instructions. It is worthwhile to note that fixed, non-permeabilized cells can be stored in FACs buffer at 4°C for at least a week before ICS is performed. Cells were stained for intracellular cytokines using various fluorochrome-labeled anti-cytokine antibodies at the

concentrations listed in **Table II-4**. The need for stringent controls using valid positive gates for ICS is essential, and requires the use of 10 times excess of unlabelled anti-cytokine antibody concurrent with fluorochrome-labeled antibody for a known positive control. These precautionary steps are essential to control for auto-fluorescence seen following activation due to the inevitable increases in forward/side scatter. Thus, following cell fixation and permeabilization, there is an inherent amount of non-specific staining which must be controlled for. Typically, the positive gates are set so that only ~1% of cells are positive for the cytokine of interest in the labeled/unlabeled negative control. This background was subtracted from the experimental readings during analysis. An example of intracellular cytokine staining for IL-10 and IFN-g using labeled/unlabeled anti-cytokine antibodies for setting the positive gate is shown in **Figure II-2**. Cells were resuspended in FACs buffer and then analyzed on a BD FACs Calibur flow cytometer (BD Biosciences, Mississauga, ON). 10,000 events in the live cell gate were counted while dead or apoptotic cells were excluded by Propidium Iodide (PI) staining.

Table II-4: List of antibodies used for intracellular staining					
Reagent	Label	Clone	Staining Dilution	Catalogue #	Supplier
αIL-2	Biotin	JES6-5H4.5	1/50	554426	
alL-2	Unlabled	JES6-5H4.5	1/5	554425	
αIFN-γ	FITC	XMG1.2	1/100	554411	· .
αIFN-γ	Unlabled	XMG1.2	1/10	559065	
aIL-4	PE	11B11	1/50	554435	
aIL4	Unlabled	11B11	1/5	554433	BD Pharmingen
αIL-10	PE	JES5-16E3	1/50	554467	Mississauga, ON
αIL-10	APC	JES5-16E3	1/50	554468	
,αIL-10	Unlabled	JES5-16E3	1/5	559063	
αCTLA-4	PE	UC10-4F10-11	1/100	553720	
Strep-CyC	CyC	N/A	1/100	554062	
Strep-PE	PE	N/A	1/100	13025D	
Strep-PercP	PercP	N/A	1/100	554064	



Facs Analysis Using Propidium Iodide Staining to Establish Live/Dead Cell Gates For Fixed And Permeabilized Splenocytes

The use of PI staining of fixed and permeabilized cells provided a useful tool for setting the live cell inclusion gate for subsequent analysis of intracellular cytokine staining. This method also provided a good positive control for cell permeabilization during the staining process. Dead cells

stained by PI have characteristic changes with low forward scatter due to smaller size, and either high side scatter indicating increased granularity, or low side scatter indicating cell fragments containing nuclei (Dyer, Derby et al., 1983). Briefly, cells that were first fixed and permeabilized (described above) were stained at 2x10<sup>5</sup>cells/well in 100µl of PI stain at a 1/100 dilution, in Perm Wash (BD Pharmingen, Mississauga, ON) for 0.5 h at 4°C in the dark. Following 2 washes with Perm Wash, cells were reconsituted in FACs buffer (comprising of 3% of heat inactivated fetal calf serum in PBS) and placed in FACs tubes. Cells were then gated for PI incorporation, as visualized by a signal on the FL3 channel of the flow cytometer (Elstein & Zucker, 1994;Elstein & Zucker, 1994). Typically, 10,000 events in the live cell gate were counted and retained for each sample. An example of PI staining for setting the dead/live cell gate is shown in **Figure II-3**. The BD FACS Calibur flow cytometer and Cell Quest software package (BD Pharmingen, Mississauga, ON) were used for FACs sample data acquisition, and the EXPO32 ADC version 1.1 software (Applied Cytometry Systems, Inc., Sacramento, CA, USA) was used for flow cytometric analysis.



**Figure II-3. PI staining for setting dead/live cell gates. (A)** .Representative histogram plot of fixed and permeabilized CD4<sup>+</sup> splenocytes stained with propidium idodie. **(B)** FACs dot plots demonstrating live cells gates established by excluding dead or apoptotic cells (shown in red) identified by PI staining.

#### Staining for Cell Surface Markers

Cells were stained for various surface markers using monoclonal antibodies conjugated to various fluorochromes (**Table II-4**). Briefly, cells were either single stained (for single channel controls), or stained in combination with other markers using reagents from BD Pharmingen (Mississauga, ON) as depicted in **Figure: II-4**. Unless otherwise stated, FACs buffer comprising of 3% heat-inactivated fetal calf serum in PBS was used for all cell surface stains and washes.

Table II-5: List of antibodies used for cell surface staining					
Reagent	Label	Clone	Staining Dilution	Catalogue #	Supplier
αCD4	PE	GK1.5	1/100	09425A	
αCD4	FITC	H129.19	1/100	553650	
αCD4	CyC	H129.19	1/100	09008A	
αCD25	Biotin	7D4	1/100	553069	
aCD25	APC	7D4	1/100	557192	
αCTLA-4	PE	UC10-4F10-11	1/100	553720	Mississauga, ON
αVβ4	Biotin	KT4	1/100	553364	
aCD19	PE	1D3	1/100	557399	
Strep-CyC	CyC		1/100	554062	
Strep-PE	PE		1/100	13025D	
Strep-PercP	PercP		1/100	554064	
αGITR	Biotin	Polyclonal	1/50	BAF524	R&D Systems



Figure II-4. Experimental protocol for cell surface marker characterization of CD4<sup>+</sup> CD25<sup>+</sup> T cells from mice treated repeatedly with TSST-1 or PBS in vivo. Splenocytes from mice treated with either TSST-1 or PBS were isolated and surface stained with aCD4-FITC, aGITRbio-Strep-PerCp, and aCD25-APC. In some instances, cells were also stained intracellularly with aCTLA-4-PE, and analyzed by FACs.

## Co-Culture of TSST-1-Primed Splenocytes with PBS-Primed Naïve Splenocytes and In Vitro Stimulation with TSST-1

BALB/c mice were administered repeated subcutaneous injections of 4  $\mu$ g TSST-1 or PBS for 3 doses at 4-day intervals as described earlier. Two hours after the last injection, mice were euthanized and their spleens were harvested. Various ratios of splenocytes isolated from mice treated repeatedly with TSST-1 were mixed with naïve splenocytes isolated from mice treated repeatedly with PBS, and were stimulated with 1 nM of TSST-1 *in vitro* for 48 h. Following this, the production of various cytokines in the supernatants, including IL-2, IFN- $\gamma$ , IL-4 and IL-10 was assessed by ELISA as described earlier.

#### FACs Purification of CD4<sup>+</sup>CD25<sup>+</sup> And CD4<sup>+</sup>CD25<sup>-</sup> Murine Splenocytes

BALB/c mice were administered repeated subcutaneous injections of 4  $\mu$ g TSST-1 or PBS for 3 doses at 4-day intervals as described earlier. Two hours after the last injection, mice were euthanized and their spleens were harvested. Isolated splenocytes (8 x 10<sup>6</sup> per sample) were stained at a concentration of 6 x 10<sup>6</sup> cells/ml for CD4-PE and CD25-biotin and strepavadin-CyC at a 1/100 dilution. Cells were then sorted into CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> fractions (>90% purity) using a FACS sorter (BD FACS Vantage SE Turbo Sort Cell Sorter, BD Pharmingen, Mississauga, ON) at the UBC Biomedical Research Centre.

# Co-culture of FACs Purified TSST-1-Primed CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25 Murine Splenocytes with Naïve T Cells and In Vitro Stimulation with TSST-1

TSST-1 or PBS primed  $CD4^+CD25^+$  or  $CD4^+CD25^-$  splenocytes were isolated as described above, and added to naïve splenocytes from PBS treated mice at a ratio of 1:20 (5%) and cocultured in 96 well flat-bottom plates at  $2x10^5$  total cells per well. Cells were then stimulated *in vitro* with 1ηM TSST-1 at 37<sup>0</sup>C for 48 h, and the supernatants were stored for subsequent cytokine analysis by ELISA as described earlier.

#### Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 3.02 (GraphPad Software Incorporated, San Diego, CA, USA). The tests used to determine statistical significance depended on the number of groups and variables present. If two experimental groups were being compared with a single variable, for example, levels of IFN- $\gamma$  production after second *in vivo* treatment with PBS or TSST-1, an unpaired Student T test was used. A two-tailed test was used

if there was not a supporting hypothesis for change in a particular direction; a one-tailed test was used if the change was expected to be only in one direction. In the displayed graphs, standard error of the mean (SEM) was utilized for all error bars, and the level of statistical significance for differences between groups were depicted by symbols as shown in Table II-6.

Table II-6: Graphical symbols for p values			
p Value	Symbols used in figures to indicate the level of statistical significance		
>0.05	NS (not significant)		
0.01 to 0.05	*		
0.001 to 0.01	**		
< 0.001	***		

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### Chapter III. Murine Serum Cytokine Profiles Following Repeated TSST-1 Administration in BALB/c Mice Display a T<sub>R</sub>1-like Cytokine Phenotype

#### Introduction

There is considerable evidence that T cells with regulatory function develop after chronic, repeated stimulation with the bacterial sAgs SEA (Grundstrom, Cederbom et al, 2003;Sundstedt, Hoiden et al, 1997;Miller, Ragheb et al, 1999;Grundstrom, Cederbom et al, 2003) and SEB (Noel, Florquin et al, 2001;Feunou, Poulin et al., 2003) in murine models. Regulatory T cell function in these systems is demonstrated by the ability of sAg-specific CD4<sup>+</sup> T cells to suppress further *in vivo* IL-2 and IFN- $\gamma$  responses. This regulatory T cell response induced by sAgs is mediated by the immunosuppressive cytokine IL-10. IL-10 can be detected in serum at high concentrations very early following secondary and subsequent sAg administration (Grundstrom, Cederbom et al, 2003;Sundstedt, Hoiden et al, 1997;Miller, Ragheb et al, 1999;Noel, Florquin et al, 2001).

Whether or not TSST-1, the key sAg involved in staphylococcal toxic shock syndrome, is capable of inducing a similar IL-10-mediated regulatory T cell response is unknown. TSST-1 exhibits immunologic responses that appear different from the other sAgs previously studied in "repeated stimulation" murine models. For example, TSST-1 does not mediate activation induced cell death (AICD) in T cells as the other sAgs (Ryan Hung, and Anthony W. Chow, unpublished data). Also, TSST-1 fails to induce elevated levels of the  $T_H2$  cytokine IL-4 in human PBMC (Kum, Cameron et al., 2001). In addition, differences in the TCR-V $\beta$  repertoire among different sAg (Kappler, Kotzin et al, 1989;Marrack & Kappler, 1990) may make some sAgs better suited for the development of regulatory responses *in vivo* than others. In this

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chapter, the serum cytokine profile induced in BALB/c mice after repeated administration of TSST-1 was investigated. In particular, the possibility of a serum cytokine response consistent with the induction of  $T_R1$  cells was examined.

#### Experimental Approach

We wished to determine whether repeated administration of the staphylococcal sAg, TSST-1 in BALB/c mice may induce a dominant IL-10 cytokine response in vivo. Various doses of sAgs have been used in previous in vitro and in vivo studies with different results (Noel, Florquin et al, 2001; Miethke, Wahl et al, 1992; Miethke, Duschek et al, 1993; Grundstrom, Cederbom et al, 2003; Sundstedt, Hoiden et al, 1997; Florquin, Amraoui et al, 1996; Feunou, Poulin et al, 2003; Grundstrom, Cederborn et al, 2003). Accordingly, we first wished to determine an optimal dose for inducing a strong and sustained serum IL-10 cytokine response in BALB/c mice. A subcutaneous route of TSST-1 administration was chosen because this approach brings together several components of the immune system thought to be important for the generation of regulatory T cells, including the activation of tissue-localized and immature dendritic cells (Bhardwaj, Friedman et al., 1992;Roncarolo, Levings et al, 2001;Wakkach, Fournier et al, 2003;Xia & Kao, 2003;Morel, Feili-Hariri et al., 2003). Similar to previous studies with SEA and SEB, we chose a model of repeated TSST-1 stimulation in BALB/c mice, a strain that has a well characterized in vitro and in vivo response to various sAgs (Florquin, Amraoui et al, 1996;Florquin, Amraoui et al, 1995;Noel, Florquin et al, 2001;Miethke, Wahl et al, 1992; Miethke, Duschek et al, 1993). In order to determine the nature of the serum cytokine response, several key proinflammatory and anti-inflammatory cytokines were examined. IL-2 and IFN- $\gamma$  were measured as markers of a predominant T<sub>H</sub>1 response, while IL-4 was measured as a marker of T<sub>H</sub>2 differentiation. IL-10 levels were quantified to assess possible T regulatory

cell differentiation and function. The 4  $\mu$ g dose of TSST-1 was chosen for repeated administration based on earlier dose optimization pilot studies (for details, see Materials and Methods in Chapter II). These pilot dose optimization experiments were performed to determine which dose of TSST-1 was capable of inducing maximum IL-10 production following three treatments. The repeated treatment protocol of 3 doses spaced 4 days apart was based on that of Sundstedt et al (Sundstedt, Hoiden et al, 1997). This model was utilized to study the characteristics of IL-10 producing T cells, which were generated after three stimulations with superantigens. Since this protocol induced significant IL-10 production for our studies, further stimulations with TSST-1 were not characterized. Twenty mice per group were administered either TSST-1 or PBS subcutaneously at 4-day intervals for a total of 3 injections. Serum was sampled via the tail vein 2 h post TSST-1 or PBS administration for cytokine determinations. In addition, CD4<sup>+</sup> T cells were obtained from the spleens of these mice for adoptive transfer experiments as described in Chapter IV, and for characterizing intracellular cytokine production profiles as described in Chapter VI. An outline of the experimental protocol is shown in Figure III-1 below. The materials and methods for TSST-1 purification, mice handling, TSST-1 or PBS administration, serum sampling, and quantitation of serum cytokine levels by ELISA are summarized in Chapter II.

## Results: Repeated administration of TSST-1 in BALB/c mice led to suppression of proinflammatory serum cytokine levels

The serum IL-10, IL-2 and IFN- $\gamma$  levels in mice following repeated administration of 4 µg TSST-1 or PBS are shown in **Figures III-2**, **III-3 and III-4**, respectively. These results represent pooled data from 5 independently performed experiments. With this large sample size, several significant trends became apparent. Firstly, serum IL-10 levels were significantly increased from the second to the third injection with TSST-1 (4,584.0±613.7 pg/ml vs. 7,154.9±946.7 pg/ml; p<0.01, one-tailed T test) (**Figure III-2**). IL-2 production, while high



Figure III-1: Experimental schematic for the generation of  $T_R1$  phenotype in vivo. Groups of 8-12 week old BALB/c mice were administered subcutaneous TSST-1 or PBS at repeated 4-day intervals for 3 doses. Sera were sampled from the mice two hours after each injection for determination of cytokine levels by ELISA.

after the second injection with TSST-1, significantly decreased following the third injection (p<0.001, T test) (**Figure III-3**). Similarly, serum IFN- $\gamma$  levels also decreased significantly from the second to the third TSST-1 injection ( $8,690\pm1328$  pg/ml vs.  $5,874\pm1316$  pg/ml; p<0.05, T test) (**Figure III-4**). Consistent with our earlier dose optimization experiment results, IL-4 was not detected above background levels (**Figure III-5**).



Figure III-2: Serum IL-10 following repeated injection with a 4  $\mu$ g dose of TSST-1. BALB/c mice were treated 3 times, at 4 day intervals with 4 $\mu$ g TSST-1 injected subcutaneously. Serum was sampled two hours following TSST-1 treatment, and assayed for cytokines by ELISA. While IL-10 was not detectable after the first injection, it significantly increased in concentration following each subsequent treatment with TSST-1 (\*\*p<0.01, paired one-tailed T test).



Figure III-3: Serum IL-2 following repeated injection with a 4  $\mu$ g dose of TSST-1. BALB/c mice were treated 3 times, at 4 day intervals with 4 $\mu$ g TSST-1 injected subcutaneously. Serum was sampled two hours following TSST-1 treatment, and assayed for cytokines by ELISA. Serum IL-2 levels peaked after the second injection of TSST-1, only to significantly diminish after the third injection (\*\*\* p<0.001, paired one-tailed T test).



Figure III-4: Serum IFN- $\gamma$  following repeated injection with a 4 µg dose of TSST-1. BALB/c mice were treated 3 times, at 4 day intervals with 4µg TSST-1 injected subcutaneously. Serum was sampled two hours following TSST-1 treatment, and assayed for cytokines by ELISA. Similar to IL-2, serum IFN- $\gamma$  levels peaked after the second injection of TSST-1, only to significantly decrease after the third injection (\*p<0.05, paired one-tailed T test).



Figure III-5: Serum IL-4 following repeated injection with a 4  $\mu$ g dose of TSST-1. BALB/c mice were treated 3 times, at 4 day intervals with 4 $\mu$ g TSST-1 injected subcutaneously. Serum was sampled two hours following TSST-1 treatment, and assayed for cytokines by ELISA No significant difference in IL-4 production was seen following repeated treatment with TSST-1 compared to PBS treated control animals.
#### Discussion

A large sample size of 20 mice per group was used for the studies with optimized dosing of TSST-1. There were several reasons for this. Firstly, a smaller sample size might have produced unreliable results due to unexpected variations or confounding variables during *in vivo* experimentation with mice. Secondly, we wished to obtain purified CD4<sup>+</sup> splenocytes from these mice in order to determine if this phenotype was transferable via CD4<sup>+</sup> T cells, and to characterize the intracellular cytokine production profile of CD4<sup>+</sup> T cells following repeated TSST-1 administration (described in detail in Chapters IV and VI, respectively). The serum cytokine profiles obtained from these mice clearly demonstrated a sustained production of IL-10 accompanied by an apparent suppression of IL-2 and IFN- $\gamma$  production after the third dose of TSST-1.

During toxic shock, there is an overwhelming bias towards an initial  $T_H1$  response. This can be seen in the production of high levels of IL-2, IFN- $\gamma$ , and TNF- $\alpha$  (Miethke, Duschek et al, 1993;Miethke, Wahl et al, 1993;Miethke, Wahl et al, 1992). The acute  $T_H1$  cytokine response following the primary dose of TSST-1 is strong. Not surprisingly, this primary cytokine response is slow to develop, as is evidenced by our observation that serum IL-2, IFN- $\gamma$  and IL-10 were undetectable when measured 2 hours after the first dose of TSST-1. However, following repeated sAg administration, T cells are primed for memory-like cytokine responses, and high serum levels of IL-2, IL-10 and IFN- $\gamma$  could be detected even at 2 hours after the second injection of TSST-1 (Sundstedt, Hoiden et al, 1997;Noel, Florquin et al, 2001;Florquin, Amraoui et al, 1996;Miller, Ragheb et al, 1999;Grundstrom, Cederbom et al, 2003). This priming for a rapid cytokine response indicates both the development of memory and the potential for highly biased immune responses. If the initial priming conditions are strongly polarized, it is likely that later responses will also be strongly polarized.

Several groups (Florquin, Amraoui et al, 1996;Noel, Florquin et al, 2001;Sundstedt, Hoiden et al, 1997;Grundstrom, Cederbom et al, 2003;Miller, Ragheb et al, 1999) have thus far investigated the early cytokine responses following repeated sAg stimulation. In each case, the primary and secondary responses seemed to prime for polarization towards a strong  $T_{H1}$  response. However, high levels of serum IL-10 could be detected within 2 hours following a second and third stimulation with TSST-1. This late response to sAgs has been characterized as  $T_{H2}$ -like by some investigators (Florquin, Amraoui et al, 1996), since IL-4 production is also detected following repeated stimulation with either SEA, or SEB (Sundstedt, Hoiden et al, 1997;Florquin, Amraoui et al, 1996). Interestingly, IL-4 was not induced above background levels following repeated TSST-1 stimulation in our studies. This is consistent with our previous experience of the lack of IL-4 detection in culture supernatants of human PBMC following TSST-1 stimulation (Kum, Cameron et al, 2001). It remains possible, however, that these findings with TSST-1 may be related to the dose of TSST-1 used in our studies (Brandt, van der et al., 2002).

The studies undertaken thus far have several important limitations. The quantitation of serum cytokine levels following repeated TSST-1 administration does not take into account the local immune response at the site of antigenic challenge, or the immune environment of the draining lymph nodes, where much of the superantigenic stimulation of both APCs and T cells occur. It has been demonstrated that  $T_R1$  cells migrate preferentially to sites of inflammation (Cottrez & Groux, 2004), and we would expect higher levels of both pro- and anti-inflamatory cytokines to be present at the site of subcutaneous TSST-1 injection.

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Several key cytokines were chosen as indicators of immune polarization in our studies, but this was somewhat limited in scope due to resource considerations and the small volumes of sera available from the mice. Inclusion of other T cell polarizing cytokines could possibly provide additional useful information in our studies. These include the T<sub>H</sub>1 cytokines TNF- $\alpha/\beta$  and IL-12, the T<sub>H</sub>2 cytokine IL-5, and the T regulatory cytokine TGF-β. Furthermore, a more complete characterization of the kinetics of cytokine production has not been performed in the murine system. Our choice of a two-hour post-injection sampling time was intended to measure rapid, memory-like cytokine responses. Failure to detect increases in certain serum cytokines such as IL-4 in this system may only indicate that this cytokine is not produced rapidly in response to TSST-1 administration in these mice. The use of only three repeated injections was based on the cytokine profile and resulted in a IL-10 dominant response. Since we were interested in cytokine responses that are characterized by high levels of IL-10, this protocol was sufficient to induce T cells with characteristics were wished to study. This is by no means a 'natural' model system, but rather a method to induce T cells that produce high levels of IL-10, thus the endpoint that would be achieved by continual treatements with TSST-1 was not the goal of our studies. It is possible further treatment of mice with superantigen may modify the cytokine response that was observed. However, using SEA, it has been shown that this strong IL-10 production with decreased IL-2 production phenotype persists following (Sundstedt, Hoiden et al, 1997) a 5<sup>th</sup> challenge, and following a span of 21 days between the  $2^{nd}$  and  $3^{rd}$  challenge with superantigen.

Serum IL-10 levels following the third injection of TSST-1 in our study (mean of ~7.15 ng/ml) was considerably lower than levels detected by others following repeated injection of either SEA (~45 ng/ml) (Sundstedt, Hoiden et al, 1997), or SEB (~25 ng/ml) (Florquin, Amraoui et al, 1996). Similarly, TSST-1 induced an order of magnitude less IL-2 compared to SEA (2.5ng/ml vs. 28ng/ml, respectively). Following 3 injections, TSST-1 induced similar levels of IFN- $\gamma$ 

compared with SEA (~5.9ng/ml vs. ~7ng/ml, respectively), while SEB induced less IFN- $\gamma$  (~0.1ng/ml). It is unclear whether the differences in these cytokine levels were unique to the specific sAgs studied, or due to differences in the experimental protocols used. Higher doses of SEA and SEB were used for repeated *in vivo* stimulation by these other investigators. Additionally, Florquin et al. (Florquin, Amraoui et al, 1996) performed experiments with six repeated sAg injections and measured IL-2 at 90 minutes post-injection, while IL-4, IL-10 and IFN- $\gamma$  were measured at 4 h post injection. Despite these differences in the experimental protocols used by ourselves and other investigators, serum IL-10 levels in BALB/c mice detected after repeated injection of various sAgs were quite comparable, and only varied within an order of magnitude among the different studies.

The serum cytokine profile observed after repeated TSST-1 administration supports the possibility of the induction of  $T_R1$  cells by TSST-1 in our animal model. This possibility is further addressed by determining if this response can be adoptively transferred via CD4<sup>+</sup> T cells in the next chapter.

## Chapter IV. The Suppression of Serum IL-2 and IFN-γ Following Repeated TSST-1 Administration Can Be Adoptively Transferred by CD4<sup>+</sup> T Cells from TSST-1 Treated Donor Mice

#### Introduction

Repeated subcutaneous injections of TSST-1 in BALB/c mice resulted in decreased serum cytokine levels of IL-2 and IFN- $\gamma$ , and increased levels of IL-10. Whether this suppressive effect was mediated by the induction of CD4<sup>+</sup> T cells with T<sub>R</sub>1 characteristics following repeated TSST-1 administration is not clear. One way to address this would be to determine whether adoptive transfer of CD4<sup>+</sup> T cells from the mice that were repeatedly injected with TSST-1 into naïve mice would result in further suppression of serum IL-2 and IFN- $\gamma$ , and higher IL-10 response to TSST-1 in the recipient mice following repeated TSST-1 administration. Since there are no clear markers for T<sub>R</sub>1 cells except for their cytokine production profile and their ability to suppress proinflammatory cytokines by other cells, careful characterization of serum cytokine levels in recipient mice before and after adoptive transfer of T cells, and with or without repeated TSST-1 administration, would be critically important. Such experiments were performed and are described in this Chapter.

#### Experimental Approach

 $CD4^+$  splenocytes from mice that received repeated injections of TSST-1 or PBS (as described in Chapter III) were purified and transferred intravenously into naïve mice. For the sake of simplicity,  $CD4^+$  T cells from TSST-1 treated mice are referred to in this Chapter as "T<sub>R</sub>1" cells, and  $CD4^+$  T cells from PBS treated mice as "naïve" cells. These assertions will be further supported with data presented in later chapters. Various control groups were used in these experiments in order to clarify the effects of T cell transfer and TSST-1 challenge (**Table IV-1**). Mice receiving  $CD4^+$  splenocytes from TSST-1 treated ("T<sub>R</sub>1" cells) or PBS treated ("Naïve" T cells) donor mice were administered repeated subcutaneous injections of either 4 µg TSST-1 or PBS at 4 day intervals for 3 injections, as described in Chapter III. Sera were again collected 2 hours post each injection for quantitation of IL-2, IL-4, IFN- $\gamma$  and IL-10 by ELISA as described in Chapter II. If the suppressive effect of "T<sub>R</sub>1" cells could be adoptively transferred, then there would be further suppression of serum IL-2 and IFN- $\gamma$  levels after the second TSST-1 injection compared to control mice that received "naïve" T cells and repeatedly challenged with TSST-1.

Table IV-1. Evaluation of the effects of adoptive transfer of CD4 <sup>+</sup> T cells from TSST-1
treated donor mice ("T <sub>R</sub> 1" cells) vs. PBS-treated donor mice ("naïve" T cells) on serum
IL-2, IFN-y and IL-10 levels in BALB/c mice following repeated TSST-1 administration

Group #	Experimental and Control Groups	Treatment of Donor Mice	Treatment of Recipient Mice	No. mice per group
1	"Naïve" T cell transfer control	PBS	PBS	5
2	"T <sub>R</sub> 1" cell transfer control	TSST-1	PBS	5
3	"Naïve" T cell transfer control followed by repeated TSST-1 administration	PBS	TSST-1	5
4	" $T_R$ 1" cell transfer followed by repeated TSST-1 administration	TSST-1	TSST-1	5

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## Results: Adoptive transfer of CD4<sup>+</sup> splenocytes from TSST-1 treated mice further suppressed serum cytokine levels of IL-2 and IFN- $\gamma$ in vivo following repeated TSST-1 administration

Serum cytokine levels of IL-2, IFN- $\gamma$  and IL-10 were undetectable from either the Naïve T cell transfer control mice (**Table IV-1**, Group 1) or from the T<sub>R</sub>1 cell transfer control mice (Group 2) that received repeated subcutaneous injection of PBS (data not shown). Serum IL-4 was again not above background levels in any of the experimental or control groups (data not shown).

Serum IL-2 levels following the second TSST-1 injection were significantly suppressed in mice with adoptive transfer of CD4<sup>+</sup> T cells from TSST-1 treated donor mice-(Group 4), compared to mice with adoptive transfer of CD4<sup>+</sup> T cells from PBS treated donor mice (Group 3) (4,448 pg/ml  $\pm$ 360 vs. 6,591 pg/ml  $\pm$  1125; p<0.05) (Figure IV-1). This difference remained significant after the third TSST-1 injection, despite the fact that serum IL-2 levels after the third injection were lower than that after the second injection in both groups.

Similarly, serum IFN- $\gamma$  levels following the second TSST-1 injection were significantly reduced in mice with adoptive transfer of TSST-1 induced "T<sub>R</sub>1" cells (Group 4), compared to mice treated with adoptive transfer of "naïve" T cells (Group 3) (7,568 pg/ml ± 920 vs. 20,111 pg/ml ±3964; p<0.05) (**Figure IV-2**).



Figure IV-1. Serum IL-2 levels following adoptive transfer of CD4<sup>+</sup> T cells and subsequent repeated TSST-1 administration. BALB/c mice were treated 3 times, at 4 day intervals with either  $4\mu$ g TSST-1 injected subcutaneously or PBS as a control. Following the 3<sup>rd</sup> injection, mice were sacrificed, splenocytes removed, and CD4<sup>+</sup> T cells were purified using magnetic columns.  $1\times10^7$  CD4<sup>+</sup> T cells were then adoptively transferred intravenously to mice that were subsequently challenged with either PBS or TSST-1 at 4 day intervals. Serum was sampled two hours after each treatment and assayed for cytokines by ELISA. Similar to previous results (Figure III-3), serum IL-2 levels peaked after the second injection, and decreased by the third TSST-1 stimulation in both T cell transfer groups treated with TSST-1. However, IL-2 levels in mice with adoptive transfer of TSST-1 induced "T<sub>R</sub>1" cells were significantly suppressed compared to mice with adoptive transfer of "naïve" T cells following both the second and third TSST-1 injection (\*p<0.05, one-tailed T test).



Figure IV-2. Serum IFN-γ levels following adoptive transfer of CD4<sup>+</sup> T cells and subsequent repeated TSST-1 administration. BALB/c mice were treated 3 times, at 4 day intervals with either 4µg TSST-1 injected subcutaneously or PBS as a control. Following the 3<sup>rd</sup> injection, mice were sacrificed, splenocytes removed, and CD4<sup>+</sup> T cells were purified using magnetic columns.  $1\times10^7$  CD4<sup>+</sup> T cells were then adoptively transferred intravenously to mice that were subsequently challenged with either PBS or TSST-1 at 4 day intervals. Serum was sampled two hours after each treatment and assayed for cytokines by ELISA. Similar to previous results (Figure III-4), serum IFN-γ levels peaked after the second injection, and decreased by the third TSST-1 stimulation in both T cell transfer groups treated with TSST-1. However, IFN-γ levels in mice with adoptive transfer of TSST-1 induced "T<sub>R</sub>1" cells were significantly suppressed compared to mice with adoptive transfer of "naïve" T cells following the second TSST-1 injection (\*p<0.05, one-tailed T test).

Serum IL-10 levels following adoptive transfer of TSST-1 induced " $T_R1$ " cells were again markedly elevated after the second TSST-1 injection and these levels were maintained after the third injection. Surprisingly, serum IL-10 levels in mice with adoptive transfer of TSST-1 induced " $T_R1$ " cells (Group 4) were not significantly higher than those from mice after adoptive transfer of "naïve" T cells (Group 3) 2 hours after either the second or third TSST-1 injection (**Figure IV-3**).



Serum IL-10 following adoptive transfer of CD4<sup>+</sup> T cells and repeated TSST-1 injections (n=5)

Figure IV-3. Serum IL-10 levels following adoptive transfer of CD4<sup>+</sup> T cell and subsequent repeated TSST-1 administration. BALB/c mice were treated 3 times, at 4 day intervals with either 4µg TSST-1 injected subcutaneously or PBS as a control. Following the 3<sup>rd</sup> injection, mice were sacrificed, splenocytes removed, and CD4<sup>+</sup> T cells were purified using magnetic columns.  $1x10^7$  CD4<sup>+</sup> T cells were then adoptively transferred intravenously to mice that were subsequently challenged with either PBS or TSST-1 at 4 day intervals. Serum was sampled two hours after each treatment and assayed for cytokines by ELISA. IL-10 levels in mice following the second and third TSST-1 injection were not significantly different between mice with adoptive transfer of TSST-1 induced "T<sub>R</sub>1" cells and mice with adoptive transfer of "naïve" T cells.

#### Discussion

Adoptive transfer of CD4<sup>+</sup> T cells from PBS treated "naïve"donor mice followed by repeated TSST-1 administration resulted in higher peak serum levels of IL-2, IFN- $\gamma$  as well as IL-10 compared to treated mice without adoptive transfer (see Chapter III). This could be attributed to stress in the recipient mice caused by the adoptive transfer of T cells prior to repeated TSST-1 administration. Regardless of the cause, adoptive transfer of CD4<sup>+</sup> T cells from TSST-1 treated donor mice resulted in significantly lower serum levels of both IL-2 and IFN- $\gamma$  in the recipient

mice following repeated TSST-1 administration, compared to recipient mice with adoptive transfer of  $CD4^+$  T cells from PBS treated "naïve" donor mice (Figure IV-1 and Figure IV-2, respectively). These data are similar to the results obtained by Noel et al. (Noel, Florquin et al, 2001) in a murine model of adoptive transfer of splenocytes following repeated SEB stimulations. Taken together with our earlier results in mice following repeated TSST-1 administration without adoptive transfer described in Chapter III, our data strongly suggest that the TSST-1 induced suppression of serum IL-2 and IFN- $\gamma$  levels was mediated by CD4<sup>+</sup> T cells, possibly through a regulatory T cell subset.

Serum IL-10 levels were elevated after both the second and third TSST-1 injections following adoptive transfer of CD4<sup>+</sup> T cells from TSST-1 treated recipient mice. However these levels were not significantly different from controls. This was expected, as IL-10 production is already maximally stimulated at this point, reducing the observable role of transferred IL-10 producing cells. In addition, we expected a rapid memory like production of IL-10 after the first stimulation with TSST-1 due to the presence of transferred cells capable of rapid IL-10 production. Furthermore the observed decrease in IL-2 and IFN- $\gamma$  observed was thought to be due to the suppressive effects of IL-10. We expected that following CD4<sup>+</sup> T cell transfer, we would see a rapid memory like production of IL-10 after the first stimulation with TSST-1. Suprisingly, we did not observe rapid IL-10 production following the first TSST-1 stimulation. However, in preliminary experiments, when cells were adoptively transferred IP rather than IV, adoptive hosts did produce detectable levels of IL-10 after the first stimulation with TSST-1. Therefore, in the next chapter we sought to determine if IL-10 plays a roll in the suppression of IL-2 and IFN- $\gamma$  that is observed following adoptive transfer of CD4<sup>+</sup> T cells.

## Chapter V. The Role of IL-10 in Mediating Serum IL-2 and IFN- $\gamma$ Suppression Following Adoptive Transfer of CD4<sup>+</sup> T cells from TSST-1 Treated Donor Mice

#### Introduction

Adoptive transfer of CD4<sup>+</sup> T cells from mice treated repeatedly with TSST-1 caused suppression of serum IL-2 and IFN- $\gamma$  levels in response to further TSST-1 stimulation. We hypothesized that CD4<sup>+</sup> regulatory T cells that produce abundant IL-10 (i.e. T<sub>R</sub>1 cells) were induced by repeated TSST-1 administration, and that the suppressive effect was mediated by IL-10, a potent antiinflammatory cytokine. However, serum IL-10 levels following repeated TSST-1 stimulation in mice receiving adoptive transfer of TSST-1 induced "T<sub>R</sub>1" cells were no different from mice receiving adoptive transfer of PBS-treated "naïve" T cells. To further investigate the role of IL-10 in mediating the suppression of serum IL-2 and IFN- $\gamma$  in our murine model, we examined the effect of blocking IL-10 activity during adoptive transfer of "T<sub>R</sub>1" cells with a neutralizing mAb to the IL-10 receptor.

#### Experimental Approach

Five groups of mice, including four different control groups, were used to examine the effect of IL-10 on the suppression of serum IL-2 and IFN-g after adoptive transfer of " $T_R1$ " cells from TSST-1 treated donor mice following repeated TSST-1 administration (**Table V-1**). A neutralizing mAb to the IL-10 receptor  $\alpha$ -chain ( $\alpha$ IL-10R $\alpha$ ) and an isotype-matched IgG control antibody were used in these experiments. Treatment with the  $\alpha$ IL-10R $\alpha$  mAb has previously been shown to inhibit IL-10 mediated signaling events (O'Farrell, Liu et al., 1998;O'Farrell,

Parry et al, 2000;Pontoux, Banz et al, 2002). The experimental protocol is depicted in Figure V-

1.

Table V-1. Evaluation of the effects of  $\alpha$ IL-10R $\alpha$  mAb vs. IgG isotype control on sereum IL-2 and IFN- $\gamma$  levels in BALB/c mice after adoptive transfer of T<sub>R</sub>1 cells from TSST-1 treated donor mice and repeated TSST-1 administration

Group #	Experimental Groups	Treatment of Donor Mice	Treatment of Recipient Mice	mAb or IgG Treatment	No. Mice per Group
1	Effect of $\alpha$ IL-10R $\alpha$ on adoptive transfer of "T <sub>R</sub> 1" cells after repeated TSST-1 administration	TSST-1	TSST-1	aIL-10Ra	8
2	IgG Isotype control for αIL- 10Rα after repeated TSST-1 administration	TSST-1	TSST-1	IgG Isotype Control	5
3	IgG Isotype control on adoptive transfer of "naive" T cells after repeated TSST-1 administration	PBS	TSST-1	IgG Isotype Control	5
4	αIL-10ra and PBS treatment control	-	PBS	aIL-10Ra	3
5	IgG Isotype and PBS treatment control	-	PBS	IgG Isotype Control	3



Figure V-1. Experimental protocol for adoptive transfer of CD4<sup>+</sup> splenocytes from TSST-1 or PBS treated mice and treatment with  $\alpha$ IL-10R $\alpha$  mAb or IgG isotype control. Following adoptive IV transfer of CD4<sup>+</sup> T cells, mice were given 4 days to recover and were then repeatedly challenged with TSST-1 or PBS by subcutaneous injection. The dose of  $\alpha$ IL-10R $\alpha$  or IgG isotype control was repeated 7 days after the first dose. Serum samples were collected 2 h post TSST-1 or PBS challenge for cytokine analysis by ELISA.

## Results: Suppression of serum IL-2 following adoptive transfer of " $T_R$ 1" cells and repeated TSST-1 administration is mediated by IL-10

Serum IL-2 levels in mice which received adoptive transfer of TSST-1 induced "T<sub>R</sub>1" cells and  $\alpha$ IL-10r $\alpha$  neutralizing antibody (Group 1) were significantly higher than those in mice with an identical adoptive transfer in the presence of an isotype-matched IgG control antibody (Group 2) (2,846±563 pg/ml vs. 1,155±408 pg/ml; p<0.05), or in mice with adoptive transfer of "naïve" T cells receiving the isotype-matched IgG control antibody (Group 3) (1,842±399 pg/ml) (**Figure V-2**). This supports our hypothesis that the suppression of serum IL-2 was mediated by 1L-10, since treatment with a neutralizing mAb against the IL-10R $\alpha$  abrogated this suppression of IL-2.



#### Serum IL-2 Levels Following Adoptive T Cell Transfer and mAb Treatment

#### In Vivo Treatment

Figure V-2: Serum IL-2 Levels after the second TSST-1 injection in mice with adoptive transfer of TSST-1 induced  $T_R1$  or naïve T cells in the presence of  $\alpha$ IL-10R $\alpha$  or control mAbs.  $1\times10^7$  CD4<sup>+</sup> T cells from mice treated 3 times with either TSST-1 or PBS were adoptively transferred intravenously to mice along with either neutralizing antibodies to the  $\alpha$  subunit of the IL-10 receptor, or isotype controls (this antibody treatment was repeated on day 7 post transfer). Mice were given 4 days to recover before subsequent challenge with TSST-1 at 4 day intervals. Serum was sampled two hours after the second treatment and assayed for cytokines by ELISA. The controls displayed represent mice treated with antibody alone.  $\emptyset$  indicates no cytokines were detectable. Blocking of the IL-10 receptor by  $\alpha$ IL-10-r $\alpha$  mAb treatment resulted in a significant increase in serum IL-2 levels (\* p<0.05, one way ANOVA).

Serum IFN- $\gamma$  levels were also affected by  $\alpha$ IL-10R $\alpha$  mAb treatment *in vivo* following repeated TSST-1 administration. However, in this instance, serum IFN- $\gamma$  levels were significantly lower compared to mice treated with the isotype-matched control mAb (583±180 pg/ml vs. 1,228±119 pg/ml; p<0.05), or mice with adoptive transfer of "naïve" T cells and treatment with the isotype matched control mAb (1,250±186pg/ml; p<0.05) (**Figure V-3**).



Serum IFN- $\gamma$  Levels Following Adoptive CD4<sup>+</sup> T Cell

Figure V-3. Serum IFN- $\gamma$  levels after the second TSST-1 injection in mice with adoptive transfer of TSST-1 induced T<sub>R</sub>1 or naïve T cells, in the presence of  $\alpha$ IL-10R $\alpha$  or control mAbs.  $1x10^7$  CD4<sup>+</sup> T cells from mice treated 3 times with either TSST-1 or PBS were adoptively transferred intravenously to mice along with either neutralizing antibodies to the  $\alpha$  subunit of the IL-10 receptor, or isotype controls (this antibody treatment was repeated on day 7 post transfer). Mice were given 4 days to recover before subsequent challenge with TSST-1 at 4 day intervals. Serum was sampled two hours after the second treatment and assayed for cytokines by ELISA. The controls displayed represent mice treated with antibody alone.  $\emptyset$  indicates no cytokines were detectable. Blocking of the IL-10 receptor by  $\alpha$ IL-10-R $\alpha$  mAb treatment resulted in a significant decrease in serum IFN- $\gamma$  levels (\* p<0.05, one way ANOVA).

#### Discussion

In order to further clarify the role of IL-10 in mediating the suppression of serum IL-2 and IFN- $\gamma$  following adoptive transfer of CD4<sup>+</sup> regulatory T cells induced by TSST-1, we repeated the adoptive transfer experiments in the presence of a neutralizing mAb to the IL-10R $\alpha$  subunit. This mAb has been shown by others to inhibit the activity of IL-10 both *in vitro* and *in vivo* (O'Farrell, Liu et al, 1998;O'Farrell, Parry et al, 2000;Pontoux, Banz et al, 2002). Our results demonstrated that whereas adoptive transfer of TSST-1 induced "T<sub>R</sub>1" cells resulted in lower serum IL-2 levels after repeated TSST-1 administration compared to mice with adoptive transfer

of "naïve" T cells, the addition of neutralizing  $\alpha$ IL-10R $\alpha$  antibody abrogated this suppressive effect and resulted in a significant increase in serum IL-2 levels. Serum IL-2 levels in the presence of  $\alpha$ IL-10R $\alpha$  were also higher than those in mice with adoptive transfer of "naïve" T cells and treated repeatedly with TSST-1 in the presence of an isotype-matched control antibody. These data provided further evidence in support of the role of IL-10 in mediating the suppression of IL-2 *in vivo* in our murine model. These results are also consistent with similar findings by others following repeated administration of SEA and SEB (Noel, Florquin et al, 2001;Florquin, Amraoui et al, 1994;Sundstedt, Hoiden et al, 1997). This IL-10 – IL-2 negative feedback loop has been considered one of the key markers for T<sub>R</sub>1 function (Bacchetta, Sartirana et al, 2002;Levings & Roncarolo, 2000;Levings, Sangregorio et al., 2001b;Roncarolo, Bacchetta et al, 2001;Groux, 2003).

Our results concerning the role of IL-10 in the suppression of serum IFN- $\gamma$  are more difficult to explain at first sight. Serum levels of this cytokine were significantly suppressed following repeated TSST-1 administration (Chapter III), a phenotype that was transferable by CD4<sup>+</sup> T cells (Chapter IV). IL-10 is known to suppress IFN- $\gamma$  production in several systems (Fiorentino, Zlotnik et al., 1991;Groux & Cottrez, 2003;Krakauer, 1995b). The observation that serum IL-10 levels were elevated following the third treatment with TSST-1 *in vivo*, led us to anticipate that an increase in serum IFN- $\gamma$  levels would accompany the neutralization of IL-10 signaling via treatment with  $\alpha$ IL-10R $\alpha$  mAb in the mice adoptively transferred with "T<sub>R</sub>1" cells. To our surprise, serum IFN- $\gamma$  levels significantly decreased compared to control mice receiving the IgG isotype control antibody. Interestingly, other investigators have reported that neutralization of IL-10 in mice following repeated SEA or SEB administration in BALB/c mice (in the absence of T cell transfer) also resulted in decreased serum IFN- $\gamma$  levels (Noel, Florquin et al, 2001;Florquin, Amraoui et al, 1994;Sundstedt, Hoiden et al, 1997). These results may be due to several factors. Firstly, it has been suggested that  $T_R1$  cells may in fact produce IFN- $\gamma$  in addition to IL-10 under some circumstances (Assenmacher, Lohning et al, 1998). Since IL-10 is known to be a growth factor for  $T_R1$  cells (Asseman & Powrie, 1998;Groux, 2003;Levings & Roncarolo, 2000;Groux, O'Garra et al, 1997), blockade of the IL-10 receptor may have prevented the outgrowth of these dual IL-10<sup>+</sup>IFN- $\gamma^+$   $T_R1$  cells, leaving only  $T_H1$  cells to produce IFN- $\gamma$ . The net effect would therefore be a decrease in the total serum IFN- $\gamma$  level detected by ELISA. In addition, other cytokines such as TGF- $\beta$  and IL-12 may be involved in the regulation of IL-10 and IFN- $\gamma$ , respectively. It is clear that further experiments to specifically address the relationship between IL-10 and IFN- $\gamma$  production by TSST-1 induced CD4<sup>+</sup> regulatory T cells will be required. In the next chapter, the intracellular cytokine profiles and associated marker expression of these regulatory T cells induced by repeated TSST-1 administration are further studied using flow cytometry.

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### Chapter VI. Murine Splenocyte Intracellular Cytokine Profiles and Surface Marker Expression Following Repeated TSST-1 Administration

#### Introduction

Our previous studies indicated that following repeated subcutaneous administration of  $4\mu g$  TSST-1, a strong serum IL-10 cytokine response was induced. This IL-10 response was associated with decreased serum levels of IL-2 and IFN- $\gamma$  and absence of concurrent induction of IL-4. This cytokine response could be adoptively transferred with CD4<sup>+</sup> T cells, and the observed decrease in IL-2 was mediated by IL-10. Consequently, we suspected that this unique cytokine profile might be induced by a T<sub>R</sub>1 subtype of CD4<sup>+</sup> regulatory T cells following repeated TSST-1 administration. To characterize this phenotype further, we first determined the intracellular cytokine production of CD4<sup>+</sup> splenocytes in BALB/c mice following repeated TSST-1 administration, particularly IL-2, IL-4, IL-10 and IFN- $\gamma$ . We also determined the cell surface expression of these cells for several markers that have been linked to T<sub>REG</sub>'s, including CTLA-4, GITR, CD45RB and CD25. Finally, we wished to determine if there was a correlation between the intracellular production of IL-10 and specific cell surface markers.

#### **Experimental Approach**

Although the production of specific cytokines *in vivo* following repeated TSST-1 administration can be readily assayed by ELISA, this approach does not provide any information on the cellular origin for these cytokines. In contrast, the intracellular production of specific cytokines can be assessed on a cell by cell basis using the technique of IntraCellular Staining (ICS) (Sander, Andersson et al., 1991) in combination with flow cytometry. The production of several cytokines

can be assayed simultaneously in each cell with the use of different fluorochrome markers. This can be performed in combination with cell surface marker determination to identify the phenotype of the cytokine producing cells. We therefore adopted an experimental protocol for the intracellular staining of these cytokines (**Figure VI-1**).



Figure VI-1. Experimental schema for detecting intracellular cytokine production in murine splenocytes following repeated TSST-1 stimulation *in vivo*.

Following repeated subcutaneous injection with either  $4\mu g$  TSST-1 or PBS alone at 4-day intervals for 3 doses as described in Chapter II, murine splenocytes were harvested and CD4<sup>+</sup> cells were isolated from single cell suspensions using magnetic separation by positive selection. Isolated CD4<sup>+</sup> T cells were re-stimulated *in vitro* for 4 days with  $\alpha$ CD3,  $\alpha$ CD28 and IL-2, in the presence of the golgi transport inhibitor monensin, to induce rapid and strong cytokine production. Intracellular cytokine staining (ICS) was performed on these CD4<sup>+</sup> T cells 5 hours after the secondary restimulation in the presence of monensin. Following this, cells were harvested and fixed for intracellular staining. The materials and methods related to purification of  $CD4^+$  splenocytes by magnetic separation, *in vitro* cell stimulation, intracellular staining for cytokines and CTLA-4, staining for cell surface markers, FACs analysis using live cell inclusion gates for fixed and permeabilized splenocytes, and positive gates of intracellular cytokine staining are described in Chapter II. Utilizing this experimental approach, we wished to determine any differences in the intracellular cytokine profile as well as cell surface markers of  $CD4^+$  splenocytes harvested from mice following repeated subcutaneous administration of 4 µg of TSST-1 or PBS alone.

#### Results

#### A. Intracellular cytokine staining revealed significantly higher IL-10producing CD4<sup>+</sup> splenocytes from mice following repeated TSST-1 adminstration compared to mice receiving PBS alone.

Triple staining for IFN- $\gamma$ , IL-4, and IL-10 was performed in one group of experiments while double staining for IL-2 and IL-10 was performed in separate experiments. The distributions of single cytokine positive cells (IFN- $\gamma$ , IL-4, or IL-10) from mice treated with TSST-1 or PBS alone are shown in **Figures VI-2**, **VI-3**, **and VI-4**, respectively. The distributions of double cytokine positive cells from mice treated with TSST-1 or PBS alone are shown in **Figures VI-5**, **VI-6**, **VI-7**, **and VI-8**. These studies revealed significantly higher IL-10 producing CD4<sup>+</sup> splenocytes following TSST-1 treatment compared to PBS alone, either as single positive cells (IL-10<sup>+</sup>/IL-4<sup>-</sup>/IFN- $\gamma$ <sup>-</sup>), or as dual positive with IL-4 (IL-10<sup>+</sup>/IL-4<sup>+</sup>/IFN- $\gamma$ <sup>-</sup>) or with IFN- $\gamma$  (IL-10<sup>+</sup>/IL-4<sup>-</sup>/IFN- $\gamma$ <sup>+</sup>). These findings are summarized in **Table VI-1**.



**Figure VI-2:** (A) Repeated TSST-1 stimulation *in vivo* did not result in statistically significant differences in the distribution of  $T_{H1}$  cells (defined as cells with the IFN- $\gamma^+$ , IL-4<sup>-</sup>, IL-10<sup>-</sup> phenotype) compared to mice treated with PBS alone (p>0.05; two-tailed T test). BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at 1x10<sup>6</sup> cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were fixed, and later permiabilized for intracellular staining. Representative histogram plots of CD4<sup>+</sup> T splenocytes that were IFN- $\gamma^+$  but IL-4<sup>-</sup> and IL-10<sup>-</sup> are shown from a mouse treated repeatedly with PBS (B) or TSST-1 (C). For representative dot plots, see the lower right quadrant of Figure VI-5 (B) and (C).



**Figure VI-3:** (A) Repeated TSST-1 stimulation *in vivo* did not result in statistically significant differences in the distribution of  $T_{H2}$  cells (defined as cells with the IFN- $\gamma^{-}$ , IL-4<sup>+</sup>, IL-10<sup>-</sup> phenotype) compared to mice treated with PBS alone (p>0.05; two-tailed T test). BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at 1x10<sup>6</sup> cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were fixed, and later permiabilized for intracellular staining. Representative histogram plots of CD4<sup>+</sup> splenocytes that were IL-4<sup>+</sup> but IL-10<sup>-</sup> and IFN- $\gamma^{-}$  are shown from a mouse treated repeatedly with PBS (B) or TSST-1 (C). For representative dot plots, see the lower right quadrant of Figure VI-6 (B) and (C).



**Figure VI-4:** (A) Repeated TSST-1 stimulation *in vivo* induced a three-fold higher number of  $T_R1$  cells (defined as cells with the IFN- $\gamma^-$ , IL- $4^-$ , IL- $10^+$  phenotype) compared to mice treated with PBS alone (\*\*\*p<0.001, one-tailed T test). BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at 1x10<sup>6</sup> cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were fixed, and later permiabilized for intracellular staining. Representative histogram plots of CD4<sup>+</sup> splenocytes that were IL-10<sup>+</sup> but IL-4<sup>-</sup> and IFN- $\gamma^-$  are shown from a mouse treated repeatedly with PBS (B) or TSST-1 (C). For representative dot plots, see the upper left quadrant of Figure VI-6 (B) and (C).

There were no significant differences in the distribution of IFN- $\gamma$  single positive cells ('classical' T<sub>H</sub>1) between mice treated with TSST-1 (0.4% ± 0.3%) or PBS (0.8% ± 0.4%) (**Figure VI-2**). IL-4 single positive cells ('classical' T<sub>H</sub>2) showed a similar pattern, with no significant differences between mice treated with TSST-1 (4.9% ± 0.8%) or. PBS (5.0%± 1.5%) (**Figure VI-3**). However, the distribution of single IL-10 positive cells was significantly higher following repeated TSST-1 administration compared with PBS treatment (3.5% ± 0.5% vs. 1.2% ± 0.5%; <sup>\*\*\*</sup>p<0.001) (**Figure VI-4**). This intracellular cytokine profile is highly suggestive of the T<sub>R</sub>1 pheonotype (Groux, O'Garra et al, 1997;Groux, 2003;Levings & Roncarolo, 2000;Roncarolo, Bacchetta et al, 2001).

The distribution of double cytokine positive staining cells was also analyzed. Dual IFN- $\gamma^+$  and IL-10<sup>+</sup> positive cells were increased significantly after repeated TSST-1 treatment *in vivo*, compared to control mice receiving PBS alone (1.7% ± 0.3% vs. 0.3% ± 0.1%, respectively; \*\*\*p<0.001; **Figure VI-5**). A similar pattern was observed in the distribution of IL-4<sup>+</sup> IL-10<sup>+</sup> double positive cells in TSST-1 and PBS-treated mice (4.8% ± 1.0% vs. 1.3% ± 0.5%, respectively; \*\*p<0.01; **Figure VI-6**). The frequency of IFN- $\gamma^+$  and IL-4<sup>+</sup> double positive CD4<sup>+</sup> splenocytes was extremely low in both TSST-1 and PBS treated mice (<0.2%) (**Figure VI-7**), indicating that, as expected, these two cytokines were seldom co-expressed in a given CD4<sup>+</sup> splenocyte. Collectively, these results demonstrate a three-fold increase in IL-10 single positive cells, and at least a three-fold increase in the total number of IL-10-producing CD4<sup>+</sup> splenocytes following repeated TSST-1 administration. This increase in IL-10-producing splenocytes correlated with the high and sustained serum IL-10 levels detected after repeated TSST-1 administration, and strongly supports our contention that repeated administration with TSST-1 induces a population of CD4<sup>+</sup> regulatory T cells that are T<sub>R</sub>1-like.



Figure VI-5: (A) Repeated TSST-1 stimulation *in vivo* induced a six-fold higher number of dual positive IL-10<sup>+</sup> IFN- $\gamma^+$  cells compared to PBS treatment alone (\*\*\*p<0.001; one-tailed T test). BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at 1x10<sup>6</sup> cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were fixed, and later permiabilized for intracellular staining. Representative dot plots of IFN- $\gamma$ -FITC vs IL-10-APC staining of CD4<sup>+</sup> splenocytes are shown from a mouse treated repeatedly with PBS (B) or TSST-1 (C).



**Figure VI-6.** (A) Repeated TSST-1 stimulation *in vivo* induced a three-fold higher number of dual positive IL-10<sup>+</sup> IL-4<sup>+</sup> cells compared to PBS treatment alone (\*\*p<0.01, one-tailed T test). BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at  $1\times10^6$  cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were fixed, and later permiabilized for intracellular staining. Representative dot plots of IL-4-PE vs IL-10-APC staining of CD4<sup>+</sup> splenocytes are shown from a mouse treated repeatedly with PBS (**B**) or TSST-1 (**C**).



Figure VI-7: Repeated TSST-1 stimulation *in vivo* induced no difference in the number of dual IFN- $\gamma^+$  IL-4<sup>+</sup> cells (defined as cells with the IFN- $\gamma^+$ , IL-4<sup>+</sup>, IL-10<sup>-</sup> phenotype) compared to mice treated with PBS alone. Less than 0.2% cells were counted in this quadrant for all samples. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at 1x10<sup>6</sup> cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were fixed, and later permiabilized for intracellular staining. Representative histogram plots of CD4<sup>+</sup> splenocytes that were IL-10<sup>-</sup> but IL-4<sup>+</sup> and IFN- $\gamma^+$  are shown from a mouse treated repeatedly with PBS (A) or TSST-1 (B).

Table VI-1 Statistical significance in the distribution of IL-10 <sup>+</sup> single or double							
cytokine	positive	murine	CD4 <sup>+</sup>	splenocytes	following	repeated	TSST-1
administration compared to mice receiving PBS alone (% gated respectively).							

Cytokine	Single Positive	Double positive			
		IFN- $\gamma^+$	IL-4 <sup>+</sup>	IL-10 <sup>+</sup>	
IFN- $\gamma^+$	NS (0.4%:0.8%)		N/A	*** (1.7%:0.3%)	
IL-4 <sup>+</sup>	NS (4.9%:5.0%)		-	** (4.8%:1.3%)	
IL-10 <sup>+</sup>	*** (3.5%:1.2%)	-	-		

Legend:

NS= non significant; N/A= too few cells to count (<0.2%); \*\* p<0.01; \*\*\*p<0.001

We also examined the relationship between IL-2 and IL-10 production in CD4<sup>+</sup> splenocytes from mice following repeated treatment with TSST-1 or PBS (**Figure VI-8**). The vast majority (>70%) of CD4<sup>+</sup> splenocytes from both groups of mice were negative for both IL-2 and IL-10. Only a small proportion were IL-2<sup>+</sup> IL-10<sup>-</sup> (<20%), while very few were either IL-2<sup>-</sup> IL-10<sup>+</sup> or IL-2<sup>+</sup> IL-10<sup>+</sup> double positive (<5% for each category). This pattern supports the notion that these two cytokines are seldom co-expressed in the same CD4<sup>+</sup> T cell. Following repeated TSST-1 administration, a significantly higher proportion of IL-2<sup>-</sup>IL-10<sup>+</sup> cells were observed compared to controls following PBS treatment (5.6±0.8% vs.  $1.5\pm0.2\%$ , respectively; ••p<0.01) (**Figure VI-8**). Conversely, the proportion of IL-2<sup>+</sup>IL-10<sup>-</sup> CD4<sup>+</sup> splenocytes was significantly lower following repeated TSST-1 administration compared to PBS treatment (8.9%±1.6% vs. 21.4±5.9%, respectively; \*p<0.05). These observations further support our contention that repeated TSST-1 administration in BALB/c mice induced a T<sub>R</sub>1-like phenotype, since these CD4<sup>+</sup> regulatory T cells have been reported to be IL-10<sup>+</sup> but IL-2<sup>-</sup> by a number of other investigators (Levings & Roncarolo, 2000;Roncarolo, Bacchetta et al, 2001).



**Figure VI-8. Distribution of IL-2<sup>+</sup> and IL-10<sup>+</sup> CD4<sup>+</sup> splenocytes.** BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at  $1\times10^{6}$  cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were fixed, and later permiabilized for intracellular staining. (A) The proportion of IL-2<sup>-</sup>IL-10<sup>+</sup>CD4<sup>+</sup> splenocyte is significantly higher following repeated TSST-1 administration compared to PBS treatment (5.6±0.8% vs. 1.5±0.2%, respectively;  $^{\bullet}$  p<0.01, one-tailed T test). Conversely, IL-2<sup>+</sup>IL-10<sup>-</sup> cells were significantly lower following repeated TSST-1 administration compared to PBS treatment (8.9±1.6% vs. 21.4±5.9%, respectively; \*p<0.05). Representative dot plots of IL-2-PE vs IL-10-APC staining of CD4<sup>+</sup> splenocytes are shown from a mouse treated repeatedly with PBS (B) or TSST-1 (C). Both dot plots demonstrate very few CD4<sup>+</sup> splenocytes that were IL-2<sup>+</sup>IL-10<sup>+</sup> double positive.

#### B. CD4<sup>+</sup>CD25<sup>+</sup> splenocytes were more abundant and intracellular expression of CTLA-4 was more frequent following repeated TSST-1 administration compared to PBS treatment.

The expression of several cell markers putatively linked to different regulatory T cell, including CD25, CTLA-4, GITR and CD45RB (see Table I-3), were investigated. The proportions of  $CD4^+CD25^+$  splenocytes were significantly higher (4.9± 0.2% vs. 3.6±0.2%, respectively; p<0.01, one-tailed T test), while that of CD4<sup>+</sup>CD25<sup>-</sup> splenocytes were significantly lower (22.8±1.2% vs. 28.5±1.1%, respectively; p<0.01) following repeated TSST-1 administration compared to PBS treatment (Figure VI-9). The expression of CTLA-4 and GITR was also examined. There was a small but significant increase in the proportion of CTLA-4<sup>+</sup>GITR<sup>-</sup> total splenocytes following repeated TSST-1 administration compared to PBS treatment (2.3±0.12% vs. 1.7±0.11%, \*p<0.05,two-tailed T test) (Figure VI-10). In contrast, the proportion of CTLA-4 GITR<sup>+</sup> T cells was not significantly different between TSST-1 and PBS treatment groups. However, the proportions of total splenocytes that were either single-positive or dual-positive for CTLA-4 or GITR were generally quite small in both treatment groups (<2.5%). Furthermore, there was no significant difference in the expression of these markers in CD4<sup>+</sup>CD25<sup>+</sup> (Figure VI-11A) or CD4<sup>+</sup>CD25<sup>-</sup> (Figure VI-11B) splenocytes derived from either mice treated repeated with TSST-1 or PBS. Nevertheless, it should be noted that the expression of CTLA-4 and GITR were greater than ten-fold in frequency among CD4<sup>+</sup>CD25<sup>+</sup> splenocytes compared to CD4<sup>+</sup>CD25<sup>-</sup> splenocytes regardless of TSST-1 or PBS treatment (Figure: VI-11). Thus, the higher rate of CTLA-4 expression in total splenocytes from TSST-1 treated mice compared to PBS controls could be partially attributed to the small but significantly higher proportion of triple positive CD4<sup>+</sup>CD25<sup>+</sup>CTLA-4<sup>+</sup> cells in TSST-1 treated mice compared to control mice  $(0.97\% \pm 0.08 \text{ vs. } 0.65\% \pm 0.05; **p<0.01, \text{ one-tailed T test})$  (Figure VI-12).



Figure VI-9. (A) Distribution of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> splenocytes following repeated treatment with TSST-1 or PBS. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested, and stained for surface markers for analysis by flow cytometry. The differences in the proportion of these subpopulations in the two treatment groups were significant (\*\*p<0.01 and \*\*p<0.01, respectively, two-tailed T test). Representative dot plots of CD4<sup>+</sup>FITC vs. CD25-APC staining of splenocytes are shown from a mouse following repeated treatment with PBS (B) or TSST-1 (C).



Figure VI-10. Expression of GITR and CTLA-4 on total splenocytes following repeated treatment with TSST-1 or PBS. BALB/c mice were treated 3 times with either  $4\mu g$  TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested, and stained for surface markers for analysis by flow cytometry. The proportion of CTLA-4<sup>+</sup>/GITR<sup>-</sup> splenocytes was significantly higher following TSST-1 treatment compared to mice receiving PBS alone (\*p<0.05 two-tailed T test).





Figure VI-11. Expression of CTLA-4 and GITR on (A)  $CD4^+CD25^+$  and (B) $CD4^+CD25^-$  splenocytes from mice treated *in vivo* with TSST-1 or PBS. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested, and stained for surface markers for analysis by flow cytometry. There were no statistically significant differences in marker expression between TSST-1 or PBS treated mice (two-tailed T test).



Figure VI-12: The proportion of triple positive  $CD4^+CD25^+CTLA-4^+T$  cells in total splenocytes. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested, and stained for surface markers for analysis by flow cytometry. This proportion of these triple positive cells was significantly higher in mice following repeated TSST-1 administration compared to mice receiving PBS alone (\*p<0.05, one-tailed T test).

# C. Enhanced IL-10 production in CD4<sup>+</sup> splenocytes following repeated TSST-1 administration was positively correlated with both CD25<sup>+</sup> and CD25<sup>-</sup> T cells and the co-expression of CTLA-4.

Our observations of significantly enhanced IL-10 production among CD4<sup>+</sup> splenocytes, as well as increased expression of some markers such as CD25 and CTLA-4 following repeated TSST-1 administration compared to PBS-treatment alone, led us to investigate the relationship between IL-10 production and these other markers. There was a strong correlation between intracellular IL-10 production and CTLA-4 expression (**Figure VI-13**). The majority of TSST-1 derived IL- $10^+$  cells were also CTLA-4<sup>+</sup> rather than IL-10<sup>+</sup>CTLA-4<sup>-</sup> (17.974±1.0% vs. 1.5±0.3%, respectively; \*\*\*p<0.001), suggesting that IL-10 production is linked to CTLA-4 expression following repeated TSST-1 administration. However, the converse was not observed, since CTLA-4 expression was not necessarily associated with IL-10 production. In fact, the majority of TSST-1 derived CD4<sup>+</sup> splenocytes were CTLA-4<sup>+</sup>IL-10<sup>-</sup> (48.6±4.8%), rather than CTLA-4<sup>+</sup>IL-10<sup>+</sup> (18.0±1.0%). Furthermore, there were very few IL-10<sup>+</sup>CTLA-4<sup>-</sup> CD4<sup>+</sup> splenocytes from either TSST-1 or PBS-treated mice ( $1.5\pm0.3\%$  and  $1.7\pm0.6\%$ , respectively). The proportion of CTLA-4<sup>+</sup>IL-10<sup>-</sup> splenocytes was significantly higher following repeated TSST-1 administration compared to PBS treatment ( $48.6\pm4.8\%$  vs.  $30.5\pm3.0\%$ , respectively; p<0.01, one-tailed T test). The proportion of IL-10<sup>+</sup>CTLA-4<sup>+</sup> double positive splenocytes was similarly higher following TSST-1 treatment ( $18.0\pm1.0\%$  vs.  $3.9\pm1.2\%$ , respectively; p<0.001).

Surprisingly, surface expression of CD25 and IL-10 production were not strongly correlated (**Figure VI-14**). In CD4<sup>+</sup> splenocytes from TSST-1 treated mice, the majority of CD25<sup>+</sup> cells were IL-10<sup>-</sup>, whereas the majority of IL-10<sup>+</sup> cells were CD25<sup>-</sup>. There were no discernable differences between TSST-1 and PBS treatment groups in single positive CD25<sup>+</sup>IL-10<sup>-</sup> cells. Thus, the previously observed increase in CD25<sup>+</sup> cells from TSST-1 treated mice (**Figure VI-9**) was primarily due to an increase in dual positive IL-10<sup>+</sup>CD25<sup>+</sup> splenocytes ( $3.6\pm0.5\%$  vs.  $1.4\pm0.2\%$ , respectively; p<0.01). Of note, the proportion of IL-10<sup>+</sup>CD25<sup>-</sup> cells was also significantly higher in TSST-1 treated mice than PBS controls ( $11.4\pm0.7\%$  vs.  $2.9\pm0.6\%$ , respectively; p<0.001).

The relationship between IL-10 production and GITR expression was also examined (Figure VI-15). There was no correlation between IL-10 production and GITR expression, since the majority of GITR<sup>+</sup> cells are IL-10<sup>-</sup> rather than IL-10<sup>+</sup> from both TSST-1 and PBS-treated mice, and IL-10<sup>+</sup> splenocytes were equally likely to be GITR<sup>+</sup> or GITR<sup>-</sup>. Consistent with our earlier results depicted in Figure VI-9, there were no significant differences in GITR+ single positive cells between TSST-1 and PBS-treated mice. TSST-1 treatment, however, did induce a 5-fold increase in IL-10<sup>+</sup>GITR<sup>-</sup> cells (6.4±1.2% vs. 1.1±0.5%, respectively; p<0.01, one-tailed T test), and
a 3-fold increase in IL- $10^+$ GITR<sup>+</sup> cells (9.0±1.0% vs. 2.4±0.8%, respectively; p<0.001) compared to PBS treated controls.

The relationship between IL-10 production and surface expression of the CD45RB marker was next examined. IL-10 producing cells demonstrated a wide range of CD45RB expression as measured by mean fluorescence intensity (**MFI**) (**Figure VI-16**). There was no significant difference in CD45RB MFI in CD4<sup>+</sup> splenocytes following repeated TSST-1 administration compared to PBS treatment (data not shown). However, it can be seen that the MFI for CD45RB was significantly lower in IL-10<sup>+</sup> cells than in IL-10<sup>-</sup> cells for both TSST-1 and PBS-treated mice (p<0.001 and p<0.05, respectively).

The statistically significant differences in IL-10 production and and its association with other markers in CD4<sup>+</sup> splenocytes TSST-1 or PBS-treated mice are summarized in **Table VI-2**.

Table VI-2. Statistical significance in the distribution of single or double marker positive CD4 <sup>+</sup> T cells from mice following repeated TSST-1 administration or PBS alone (% gated respectively)									
Cell Marker	Single Positive	Double Positive							
		IL-10 <sup>+</sup>	IL-10 <sup>-</sup>	<b>CD25</b> <sup>+</sup>	CD25	CTLA-4 <sup>+</sup>	CTLA-4	<b>GITR</b> <sup>+</sup>	GITR <sup>.</sup>
IL-10 <sup>+</sup>	*** (5.6:1.5%)		-	** (3.6:1.4 %)	*** (11.4:2 .9%)	-	N/S (1.5:1.7%)	-	** (6.4:1.1%)
<b>CD25</b> <sup>+</sup>	** (4.9:3.6%)	-	NS	-	-	-	NS		NS
CTLA-4 <sup>+</sup>	* (3.1:2.6%)	*** (18.0:3.9 %)	** (48.6%:30 .5%)	** (1.0:0.6 %)	NS	-	-	NS	* (2.3:1.7%)
GITR⁺	NS	*** (9.0:2.4 %)	NS	NS	NS	NS	NS	-	and the second s

NS= not significant; N/A= not applicable (too few cells to count; <0.2%); \* p<0.05;\*\* p<0.01; \*\*\*p<0.001

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Figure VI-13. Distribution of CD4<sup>+</sup> splenocytes expressing both IL-10 and CTLA-4. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at  $1\times10^6$  cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were stained for surface molecules, fixed, and later permiabilized for intracellular staining. (A) The proportion of CD4<sup>+</sup> splenocytes expressing CTLA-4 with or without co-expression of IL-10 was significantly higher following repeated TSST-1 administration compared to PBS treatment (18.0±1.0% vs. 3.9±1.2% for CTLA-4<sup>+</sup>/IL-10<sup>-</sup> cells,  $\bullet \bullet \bullet$ p<0.001, one-tailed T test; and 48.6±4.8% vs. 30.5±3.0% for CTLA-4<sup>+</sup>/IL-10<sup>-</sup> cells, \*\*p<0.01, respectively). Representative dot plots of CTLA-4-PE vs. IL-10-APC staining of CD4<sup>+</sup> T cells are shown from a mouse treated repeatedly with PBS (B) or TSST-1 (C).



**Figure VI-14. Distribution of CD4<sup>+</sup> splenocytes expressing both IL-10<sup>+</sup> and CD25<sup>+</sup>.** BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at  $1\times10^6$  cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were stained for surface molecules, fixed, and later permiabilized for intracellular staining. (A) Repeated TSST-1 treatment resulted in a significant increase in IL-10<sup>+</sup>CD25<sup>+</sup> cells compared to PBS controls (3.6±0.5% vs. 1.4±0.2%, respectively;  $\bullet p$ <0.01, one-tailed T test). Of note, the proportion of IL-10<sup>+</sup>CD25<sup>-</sup> cells was also significantly higher in TSST-1 treated mice than PBS controls (11.4±0.7% vs. 2.9±0.6%, respectively; \*\*\*p<0.001, one-tailed T test). (B) Representative dot plot of CD25-PercP vs. IL-10-APC staining of splenocytes from a mouse treated repeatedly with PBS or (C) TSST-1 *in vivo*.



Figure VI-15. Distribution of CD4<sup>+</sup> splenocytes expressing both IL-10 and GITR. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at  $1\times10^6$  cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were stained for surface molecules, fixed, and later permiabilized for intracellular staining. (A) The proportion of IL-10<sup>+</sup> GITR<sup>-</sup>CD4<sup>+</sup> splenocytes was 5-fold higher following repeated TSST-1 administration compared to PBS treatment (\*\*p<0.01, one-tailed T test). Similarly, the proportion of IL-10<sup>+</sup>GITR<sup>+</sup> cells was 3-fold higher following TSST-1 treatment (\*\* $\phi$ +p<0.001). (B) Representative dot plots of GITR-PercP vs. IL-10-APC staining of CD4<sup>+</sup> splenocytes are shown from a mouse treated repeatedly with PBS or (C) TSST-1 *in vivo*.



Figure VI-16: Mean Florence Intensity (MFI) for CD45RB in IL-10<sup>+</sup> and. IL-10<sup>-</sup> CD4<sup>+</sup> splenocytes following repeated TSST-1 or PBS treatment. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control, after which CD4<sup>+</sup> T cells were purified via magnetic columns, and cultured at  $1\times10^6$  cells/ml. Cells were cultured on plates coated with  $\alpha$ CD3, in the presence of IL-2 and  $\alpha$ CD28 for 4 days, after which they were washed. They were then transferred to fresh plates and stimulated under identical conditions for 5 hours in the presence of monensin. Following this cells were stained for surface molecules, fixed, and later permiabilized for intracellular staining. (A) In both TSST-1 and PBS derived CD4<sup>+</sup> splenocytes, MFI for CD45RB was significantly lower in IL-10<sup>+</sup> cells compared to IL-10<sup>-</sup> ( $\performed producing cells and CD45RB MFI are shown from a mouse treated repeatedly with PBS or (C) TSST-1.$ 

#### Discussion

The main finding by intracellular cytokine staining summarized in this Chapter is that the CD4<sup>+</sup> splenocytes induced in BALB/c mice after repeated TSST-1 administration exhibit enhanced production of IL-10 with little or no co-expression of IL-2. This intracellular cytokine phenotype, accompanied by their ability to inhibit TSST-1 induced proinflammatory cytokine responses in naïve mice by adoptive transfer, strongly suggests that they are regulatory T cells of the classical  $T_R1$  subclass as described by Groux et al. (Groux, O'Garra et al, 1997), Bacchetta et al. (Bacchetta, Sartirana et al, 2002) and others (see review in Chapter I). The induction of a  $T_R$ 1-like phenotype by repeated administration of TSST-1 is similar to what has been described with other staphylococcal superantigens such as SEA (Sundstedt, Hoiden et al, 1997; Grundstrom, Dohlsten et al., 2000) and SEB (Noel, Florquin et al, 2001). Our observation that the number of  $IL-2^+/IL-10^-CD4^+$  splenocytes were significantly decreased while that of IL-10<sup>+</sup>/IL-2 splenocytes significantly increased following repeated TSST-1 administration compared to PBS-treated control mice also raises the possibility that increased IL-10 production may have led to suppression of IL-2, a phenomenon which has been well documented in other murine models of sAg-induced regulatory T cells (Noel, Florquin et al, 2001;Sundstedt, Hoiden et al, 1997).

Our finding that the co-expression of IFN- $\gamma$  and IL-10 by CD4<sup>+</sup> splenocytes (Figure VI-5), although uncommon (<2%), was significantly higher following repeated TSST-1 administration compared to PBS treatment is intriguing. Whether T<sub>R</sub>1 cells also produce IFN- $\gamma$  has been somewhat controversial in the published literature, and appears to depend on the model system studied. In the human system, the co-production of IL-10 and IFN- $\gamma$  by T<sub>R</sub>1 cells have been reported (Levings, Sangregorio et al, 2001a). However, in the murine system, some T<sub>R</sub>1 cells

induced by immunosuppressive drugs appear to produce little or no IFN- $\gamma$  (Barrat, Cua et al, 2002). There is also circumstantial evidence that individual T<sub>R</sub>1 cells induced by SEB may have evolved by the sequential and preferential expression of IL-10 from IFN- $\gamma^+$  cells (Assenmacher, Lohning et al, 1998). It is unlikely that dual expression of IL-10 and IFN- $\gamma$  following TSST-1 treatment observed in our study is due to non-specific stimulation by  $\alpha$ CD3,  $\alpha$ CD28 and IL-2 in our ICS protocol, since the occurrence of double positive IL-10<sup>+</sup>/IFN- $\gamma^+$  or IL-10<sup>+</sup>/IL-4<sup>+</sup> CD4<sup>+</sup> splenocytes in PBS-treated control mice was uncommon. Furthermore, although we saw a doubling in the number of dual positive IL-10<sup>+</sup>/IL-4<sup>+</sup> and IL-10<sup>+</sup>/IFN- $\gamma^+$  cells following TSST-1 administration, single positive IL-4<sup>+</sup> or IFN- $\gamma^+$  CD4<sup>+</sup> splenocytes were not enhanced following repeated TSST-1 administration. The existence of an enhanced subpopulation of IL-10<sup>+</sup>/IFN- $\gamma^+$  T<sub>R</sub>1 cells following repeated TSST-1 administration may also explain the apparent paradoxical effect of anti-IL-10 treatment which resulted in decreased, rather than increased, serum IFN- $\gamma$  levels observed in Chapter V.

Another important finding is that the proportion of IL-10<sup>+</sup>/CD25<sup>+</sup> and IL-10<sup>+</sup>/CD25<sup>-</sup> CD4<sup>+</sup> splenocytes were both significantly higher following repeated TSST-1 administration compared to PBS-treated control mice. In fact, the majority of IL-10 producing CD4<sup>+</sup> splenocytes were CD25<sup>-</sup> rather than CD25<sup>+</sup> (Figure VI-15). This finding, together with our observation that the inhibitory activity induced by repeated TSST-1 administration is mediated by IL-10 (as demonstrated by the effect of a neutralizing  $\alpha$ IL-10R $\alpha$  mAb in Chapter V) clearly establishes that these TSST-1 induced regulatory T cells are T<sub>R</sub>1 rather than "natural" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s.

The final major finding in this Chapter is that intracellular expression of CTLA-4 in CD4<sup>+</sup> splenocytes is significantly increased following repeated TSST-1 administration compared to PBS-treated control mice, and that this cell marker was positively correlated with IL-10

production (Figure VI-13). In contrast, neither the surface marker GITR nor CD45RB<sup>LOW</sup> were upregulated in CD4<sup>+</sup> splenocytes following repeated TSST-1 administration compared to PBS-treated control mice.

In summary, the inhibitory function of  $T_R1$  cells induced by TSST-1 appears to be dependent on IL-10 production, but the causal relationship and functional significance between IL-10<sup>+</sup> CD4<sup>+</sup> splenocytes and their intracellular CTLA-4 production and various surface marker expression are far from clear. Whether these  $T_R1$  cells induced *in vivo* following repeated TSST-1 administration in BALB/c mice can be shown to mediate suppression of TSST-1 induced proinflammatory cytokine response of naïve splenocytes *in vitro* will be addressed in the next chapter.

### Chapter VII. Co-culture of Naïve Splenocytes with TSST-1 Primed CD4<sup>+</sup> Cells Suppressed IL-2 But Enhanced IL-10 And IFN-γ Production Following TSST-1 Stimulation *In Vitro*

#### Introduction

Our results thus far demonstrated that serum levels of IL-2 and IFN-y significantly decreased while IL-10 levels significantly increased in BALB/c mice following repeated administration of TSST-1 in vivo. Intracellular cytokine staining of splenocytes from TSST-1 treated mice revealed an increased proportion of IL-10 producing cells that were associated with various markers, including CD25 and CTLA-4, compared to PBS-treated control mice. TSST-1 primed CD4<sup>+</sup> splenocytes adoptively transferred to naïve mice resulted in inhibition of TSST-1 induced serum IL-2 and IFN-y in response to TSST-1 administration. Finally, the inhibitory effect of adoptive transfer could be abrogated by a neutralizing  $\alpha$ IL-10 $\alpha$  mAb, indicating that this inhibitory activity was mediated by IL-10. These data strongly suggest that repeated TSST-1 treatment results in the differentiation of a  $CD4^+$  T<sub>R</sub>1 phenotype. Since T<sub>R</sub>1 cells are reported to suppress immune responses, we wished to determine if T cells from mice treated repeatedly with TSST-1 were capable of suppressing the production of T<sub>H</sub>1 cytokines while enhancing the production of IL-10 by naïve T cells following TSST-1 stimulation in vitro. Additionally, we wished to clarify whether the observed T<sub>R</sub>1-like activity of TSST-1 primed splenocytes was mediated by CD4<sup>+</sup>CD25<sup>+</sup> vs. CD4<sup>+</sup>CD25<sup>-</sup> T cells in vitro to determine the CD25<sup>+</sup> expression phenotype of our cells.

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#### Experimental Approach

To further examine the dose-dependent inhibitory activity of TSST-1 induced  $T_R1$ -like T cells generated in BALB/c mice, we co-cultured various ratios of TSST-1 primed splenocytes with PBS-primed naïve splenocytes and stimulated the mixed populations *in vitro* with 1 nM TSST-1 for 48 h. The concentration of various cytokines in the supernatants, including IL-2, IL-4, IL-10 and IFN- $\gamma$ , were determined by ELISA as described under Materials and Methods (Chapter II). The total number of TSST-1 and PBS primed splenocytes in the co-culture were kept constant, but the ratio of TSST-1 primed to naïve splenocytes varied from 0% to 100% in order to assess the potency and dose-dependent effect of TSST-1 induced  $T_R1$  cells. A fixed number of total cells in the co-cultures were necessary to control for potential confounding variables that might affect cytoktine production, such as cell density and viability, and increased uptake of cytokines by cytokine receptors expressed on the surface of both donor and recipient cells. The experimental protocol is depicted in **Figure VII-1**.



Figure VII-1. Experimental protocol for examining the dose-dependent effect of TSST-1 primed splenocytes on cytokine production by naïve splenocytes.

Additionally, since our ICS studies revealed that the proportion of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>C25<sup>-</sup> IL-10 producing splenocytes were both significantly higher following repeated TSST-1 administration compared to PBS-treated control mice, we wished to determine which of these fractions could mediate a suppressive effect on the production of proinflammatory cytokines in the co-culures following TSST-1 stimulation *in vitro*. TSST-1 primed splenocytes were separated into CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> fractions by FACS sorting as described under Materials and Methods. These TSST-1 primed and fractionated T cells were co-cultured in a ratio of 1:20 (5%) with PBS-primed naïve splenocytes, and stimulated with 1 nM TSST-1 *in vitro* for 48 h. A ratio of 5% of TSST-1 primed splenocytes was chosen for these co-culture experiments since we previously determined that CD4<sup>+</sup>CD25<sup>+</sup> T cells comprised approximately

3-5% of total splenocytes in both TSST-1 and PBS treated mice (Figure VI-8). The experimental protocol for this aspect of the study is depicted in Figure VII-2.



**Figure VII-2.** Experimental protocol for examining the effect of TSST-1 primed CD4<sup>+</sup>CD25<sup>+</sup> vs. CD4<sup>+</sup>CD25<sup>-</sup> splenocytes on cytokine production by naïve splenocytes following TSST-1 stimulation *in vitro*.

#### Results

A. TSST-1 primed splenocytes in co-cutlure with naïve splenocytes suppressed the production of IL-2 but enhanced both IL-10 and IFN-γ in a dose-dependent manner following TSST-1 stimulation in *vitro*.

Naïve splenocytes from PBS-treated mice in the absence of TSST-1 primed T cells produced high levels of IL-2 (5,922±1,421 pg/ml) at 48 h after TSST-1 stimulation *in vitro* (**Figure VII-3**). In contrast, IL-2 levels induced by TSST-1-primed splenocytes in the absence of PBS-primed splenocytes were undetectable following TSST-1 restimulation *in vitro*, suggesting that these cells have become tolerisized to further TSST-1 stimulation. The addition of 5% TSST-1 primed

(T<sub>R</sub>1) splenocytes to PBS-treated naïve splenocytes significantly suppressed the production of IL-2 (4,035±1,469 vs. 5,922±1,421 pg/ml; p<0.05, one-tailed paired T test). The suppressive effect of TSST-1-primed splenocytes was clearly dose-dependent, with complete suppression of IL-2 in cell cultures containing ~66% TSST-1-primed splenocytes. This suppressive effect was most likely due to the generation of T<sub>R</sub>1 cells following three repeated injections of TSST-1 *in vivo*, since splenocytes from mice receiving a single injection of TSST-1 *in vivo* only had an intermediate suppressive effect on IL-2 production when restimulated with TSST-1 *in vitro* (1,032±256 vs 0 pg/ml; p=0.02, one-tailed T test).



Figure VII-3: Effect of various ratios of TSST-1-primed and PBS-primed naïve splenocytes on IL-2 production following TSST-1 stimulation *in vitro*. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested. Splenocytes were mixed at various ratios, stimulated with TSST-1 and cultured for 48 hours to determine the effect that splenocytes from mice primed with TSST-1 had on cytokine production by naïve splenocytes. TSST-1-primed splenocytes from TSST-1 treated mice significantly suppressed IL-2 production by naïve splenocytes (\*p<0.05, one-tailed paired T test). Splenocytes from mice that received only a single injection of TSST-1 *in vivo* produced significantly higher IL-2 levels when restimulated with TSST-1 *in vivo* (p=0.02; one-tailed T test).

As expected, naïve splenocytes from PBS-treated mice in the absence of TSST-1 primed T cells induced barely detectable IL-10 levels ( $3.54\pm3.54$  pg/ml) following TSST-1 stimulation *in vitro* (**Figure VII-4**). In contrast, TSST-1 primed splenocytes in the absence of PBS treated T cells induced maximal IL-10 levels following TSST-1 restimulation *in vitro* ( $5,553\pm1,202$  pg/ml), likely due to the generation of T<sub>R</sub>1 cells. The addition of 10% TSST-1 primed splenocytes to PBS-treated naïve splenocytes significantly enhanced the production of IL-10 ( $482.4\pm224.7$  vs.  $3.54\pm3.54$  pg/ml; p<0.05, one-tailed paired T test). The enhancing effect of TSST-1-primed splenocytes was clearly dose-dependent, reaching a plateau with the addition of ~50% TSST-1primed splenocytes. This enhancing effect was likely due to the generation of T<sub>R</sub>1 cells following three repeated injections of TSST-1 *in vivo*, since splenocytes from mice treated with a single injection of TSST-1 *in vitro* (745.1±253.8 vs 5,553±1,202 pg/ml; p<0.005, one-tailed T test). Of interest, no IL-4 (a key T<sub>H</sub>2 cytokine) could be detected for any ratio of TSST-1 and PBS primed splenocytes (data not shown). This suggests that the IL-10 response was independent of IL-4.



Figure VII-4: Effect of various ratios of TSST-1 primed and PBS-primed naïve splenocytes on IL-10 production following TSST-1 stimulation in vitro. BALB/c mice were treated 3 times with either  $4\mu$ g TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested. Splenocytes were mixed at various ratios, stimulated with TSST-1 and cultured for 48 hours to determine the effect that splenocytes from mice primed with TSST-1 had on cytokine production by naïve splenocytes. Addition of only 10% splenocytes from TSST-1 treated mice to naïve splenocytes from PBS treated mice induced a significant increase in the production of IL-10 following TSST-1 stimulation *in vitro* (\*p<0.05, one-tailed paired T test). Splenocytes from mice after a single dose of TSST-1 *in vivo* resulted in an intermediate phenotype that produced significantly lower levels of IL-10 compared to splenocytes from mice after repeated treatment with three doses of TSST-1 *in vivo*.

Naïve splenocytes from PBS-treated mice in the absence of TSST-1 primed T cells produced low levels of IFN- $\gamma$  (79.9±62.4 pg/ml) at 48 h after TSST-1 stimulation *in vitro* (**Figure VII-5**). In contrast, IFN- $\gamma$  levels induced by TSST-1-primed splenocytes in the absence of PBS-treated splenocytes were significantly higher following TSST-1 restimulation *in vitro* (1920±419 pg/ml; p<0.005, one-tailed T test), suggesting that memory T cells were activated following TSST-1 restimulation. The addition of 5% TSST-1 primed splenocytes to PBS-treated naïve splenocytes significantly enhanced the production of IFN- $\gamma$  (464.9±215.1 vs. 79.9±62.4 pg/ml; p<0.05, one-tailed T test). The enhancing effect of TSST-1-primed splenocytes was clearly dose-

dependent, reaching a plateau with the addition of ~33% TSST-1-primed splenocytes. This enhancing effect was likely due to the generation of memory T cells following three injections of TSST-1 *in vivo*, since splenocytes from mice treated with a single dose of TSST-1 *in vivo* only had an intermediate boosting effect when restimulated with TSST-1 *in vitro* (589.3±227.4 vs  $1,920\pm419$  pg/ml; p<0.05, one-tailed T test).



IFN-γ Production at Different Cell Ratios (n=5)

Figure VII-5: Effect of various ratios of TSST-1-primed and PBS-primed naïve splenocytes on IFN- $\gamma$  production following TSST-1 stimulation *in vitro*. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested. Splenocytes were mixed at various ratios, stimulated with TSST-1 and cultured for 48 hours to determine the effect that splenocytes from mice primed with TSST-1 had on cytokine production by naïve splenocytes. Addition of only 5% splenocytes from TSST-1 primed mice significantly enhanced the production of IFN- $\gamma$  following TSST-1 stimulation *in vitro* (\*p<0.05, one-tailed paired T test). Splenocytes from mice that received only a single injection of TSST-1 *in vivo* produced significantly lower IFN- $\gamma$  levels when restimulated with TSST-1 *in vivo* (p<0.05; one-tailed T test).

#### B. TSST-1 primed CD4<sup>+</sup>CD25<sup>+</sup>, but not CD4<sup>+</sup>CD25<sup>-</sup>, splenocytes significantly suppressed IL-2 and enhanced IL-10 production by naive splenocytes following TSST-1 stimulation *in vitro*.

PBS-treated naïve splenocytes produced significant levels of IL-2 following stimulation with 1 nM TSST-1 for 48 h *in vitro*, while TSST-1 primed splenocytes produced no detectable IL-2 in culture supernatants ( $5,923\pm1,421$  vs. 0 pg/ml) (Figure VII-6).. However, co-culture of naïve splenocytes with 5% CD4<sup>+</sup>CD25<sup>+</sup> splenocytes derived from TSST-1 treated mice followed by stimulation with 1 nM TSST-1 for 48 h *in vitro* significantly suppressed the production of IL-2 by naïve splenocytes ( $1,126\pm160$  vs.  $5,923\pm1,421$  pg/ml; p=0.005, one-tailed T test). In contrast, co-culture of naïve splenocytes with 5% CD4<sup>+</sup>CD25<sup>-</sup> splenocytes derived from TSST-1 treated mice had no significant effect on the production of IL-2 by naïve splenocytes ( $3,653\pm613$  vs.  $5,923\pm1,421$  pg/ml; p>0.05, one-tailed T test). Similarly, the addition of either CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> T cells from PBS-treated mice had no effect on IL-2 production by naïve splenocytes following TSST-1 stimulation *in vitro* (data not shown).

![](_page_125_Figure_0.jpeg)

Figure VII-6: IL-2 production by naïve splenocytes in the presence of 5% CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> splenocytes derived from TSST-1 treated mice. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested. Flow cytometry based sorting was utilized to separate out CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells. These cells were then added back to naïve splenocytes at a 5%:95% ratio *in vitro*, and stimulated for 48 hours with TSST-1. Supernatants were then harvested for cytokine ELISA's. The addition of 5% CD4<sup>+</sup>CD25<sup>+</sup> but not CD4<sup>+</sup>CD25<sup>-</sup> splenocytes from TSST-1 treated mice significantly suppressed IL-2 production by naïve splenocytes (\*p<0.05, one-tailed T test) following TSST-1 stimulation *in vitro*.

The production of IL-10 by PBS-treated naïve splenocytes following stimulation with 1 nM TSST-1 for 48 h *in vitro* was negligible ( $3.54\pm3.54$  pg/ml) (**Figure VII-7**). In contrast, TSST-1 primed splenocytes from TSST-1 treated mice produced significant IL-10 levels following TSST-1 stimulation *in vitro* (5,553 pg/ml  $\pm$  1,202). Again, the addition of 5% CD4<sup>+</sup>CD25<sup>+</sup> but not CD4<sup>+</sup>CD25<sup>-</sup> T cells from TSST-1 treated mice significantly enhanced the production of IL-10 by naïve splenocytes ( $1,730 \pm 835$  pg/ml vs.  $3.54\pm3.54$  pg/ml; p<0.05, one-tailed T test).

![](_page_126_Figure_0.jpeg)

Figure VII-7. IL-10 production by naïve splenocytes in the presence of 5% CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> splenocytes derived from TSST-1 treated mice. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested. Flow cytometry based sorting was utilized to separate out  $CD4^+CD25^+$  and  $CD4^+CD25^-$  cells. These cells were then added back to naïve splenocytes at a 5%:95% ratio *in vitro*, and stimulated for 48 hours with TSST-1. Supernatants were then harvested for cytokine ELISA's. Naïve splenocytes produced little IL-10 compared to splenocytes from TSST-1 treated mice. The addition of 5% CD4<sup>+</sup>CD25<sup>+</sup> but not CD4<sup>+</sup>CD25<sup>-</sup> T cells significantly enhanced IL-10 production by naïve splenocytes (\*p<0.05, one-tailed T test) following TSST-1 stimulation *in vivo*.

TSST-1 primed CD4<sup>+</sup>CD25<sup>-</sup>

5%

#### C. TSST-1 primed CD4<sup>+</sup>CD25<sup>+</sup>, as well as CD4<sup>+</sup>CD25<sup>-</sup>, splenocytes significantly enhanced IFN-γ production by naive splenocytes following TSST-1 stimulation *in vitro*.

PBS-treated naïve splenocytes produced little IFN-y (79.9.0±62.4 pg/ml) compared to TSST-1

primed splenocytes when stimulated with TSST-1 in vitro (1,920±418 pg/ml; p<0.005, one-

tailed T test) (Figure VII-8). The addition of either 5% CD4<sup>+</sup>CD25<sup>+</sup> or 5% CD4<sup>+</sup>CD25<sup>-</sup> TSST-1

primed splenocytes both significantly enhanced the production of IFN- $\gamma$  by naïve splenocytes (4,386 ±1,573 pg/ml and 1,274 ±118 pg/ml, respectively; p<0.025).

![](_page_127_Figure_1.jpeg)

Figure VII-8. IFN- $\gamma$  production by naïve splenocytes in the presence of 5% CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> splenocytes derived from TSST-1 treated mice. BALB/c mice were treated 3 times with either 4µg TSST-1 or PBS control. Two hours after the final treatment, splenocytes were harvested. Flow cytometry based sorting was utilized to separate out CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells. These cells were then added back to naïve splenocytes at a 5%:95% ratio *in vitro*, and stimulated for 48 hours with TSST-1. Supernatants were then harvested for cytokine ELISA's. The addition of 5% CD4<sup>+</sup>CD25<sup>+</sup> or 5% CD4<sup>+</sup>CD25<sup>-</sup> splenocytes to naïve splenocytes both significantly enhanced IFN- $\gamma$  production compared to naïve splenocytes alone (\*p<0.05, one-tailed T test) following TSST-1 stimulation *in vitro*.

#### Discussion

The experiments described in this chapter further demonstrate the T<sub>R</sub>1-like functional capacity of

splenocytes derived from mice treated repeatedly with TSST-1. The production of IL-2 by naïve

splenocytes was markedly suppressed while that of IL-10 was enhanced when naïve splenocytes were co-cultured with TSST-1 primed splenocytes and re-stimulated with TSST-1 *in vitro*. The effect was dose-dependent, with a demonstrable effect after the addition of only 5-10% of TSST-1 primed splenocytes to naïve splenocytes. Thus, TSST-1-induced  $T_R$ 1-like splenocytes exhibited potent activity on naïve splenocytes in response to TSST-1 stimulation *in vitro*. Furthermore, the  $T_R$ 1-like function was dependent on the presence of the CD4<sup>+</sup>CD25<sup>+</sup> subpopulation, but not CD4<sup>+</sup>CD25<sup>-</sup> subpopulation, from TSST-1 treated mice. These results provide further evidence based on our earlier intracellular cytokine staining data (**Figure VI-8**), confirming that a decrease in intracellular IL-2 production and an increase in intracellular IL-10 resulted from repeated TSST-1 administration *in vivo*.

# Chapter VIII. General Discussion, Significance, Future Directions and Conclusions

#### TSST-1 Induced Regulatory T Cells

The main findings from this research are that repeated administration of TSST-1 in vivo in BALB/c mice generated  $CD4^{+}CD25^{+}$  regulatory T cells that down-regulated T<sub>H</sub>1 cytokine responses and appear to be of the T<sub>R</sub>1 phenotype. To our knowledge, this is the first report that TSST-1 can also induce regulatory T cells similar to other staphylococcal sAgs such as SEA and SEB (Noel, Florquin et al, 2001; Grundstrom, Cederborn et al, 2003; Feunou, Poulin et al, 2003). Utilizing a protocol of repeated subcutaneous injection of 4µg TSST-1 in BALB/c mice, we observed a significant decrease in serum levels of IL-2 and IFN-y, while serum IL-10 levels were enhanced (Chapter III). This suppressive activity could be adoptively transferred to naïve mice by the infusion of  $1 \times 10^7$  CD4<sup>+</sup> T cells intravenously from mice treated repeatedly with TSST-1 (Chapter IV). In vivo, the observed suppression of IL-2 was dependent on IL-10 production, as blockade of the IL-10 receptor with neutralizing antibodies abrogated IL-2 suppression induced by adoptive CD4<sup>+</sup> T cell transfer (Chapter V). Repeated TSST-1 stimulation in vivo was accompanied by an increase in single positive  $IL-10^+$  T cells, double-positive  $IL-4^+/IL-10^+$  T cells, as well as IFN- $\gamma^+$ /IL-10<sup>+</sup> T cells as revealed by intracellular cytokine staining (Chapter VI). Repeated TSST-1 treatment also resulted in a decrease of  $IL-2^+$  CD4<sup>+</sup> splenocytes. Furthermore, IL-10 producing CD4<sup>+</sup> T cells rarely co-produced IL-2. TSST-1 treatment *in vivo* also induced a significant increase in intracellular CTLA-4 expression, as well as surface expression of CD25. Mice treated repeatedly with TSST-1 had a significantly higher proportion of IL-10<sup>+</sup>CTLA-4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> CTLA-4<sup>+</sup> splenocytes compared to control mice treated

with PBS. Splenocytes and purified  $CD4^+CD25^+$  T cells from TSST-1-treated mice were potent in their ability to enhance IL-10 and suppress IL-2 production when mixed with naïve splenocytes in a ratio as low as 1:20 and re-stimulated with TSST-1 *in vitro* (Chapter VII). Unexpectedly, serum IFN- $\gamma$  levels decreased following IL-10 blockade with  $\alpha$ IL-10R $\alpha$  antibody treatment in naïve mice with adoptive transfer of TSST-1 primed CD4<sup>+</sup> splenocytes and repeated TSST-1 stimulation *in vivo*. Co-culture of naïve splenocytes with either TSST-1 primed total splenocytes, or fractionated CD4<sup>+</sup>CD25<sup>+</sup> as well as CD4<sup>+</sup>CD25<sup>-</sup> T cells also enhanced the production of IFN- $\gamma$  following TSST-1 stimulation *in vitro*.

These findings lead us to conclude that repeated administration of TSST-1 *in vivo* in BALB/c mice induced regulatory T cells of the  $T_R1$  phenotye. The biologic basis of these intriguing observations, and the significance of TSST-1 induced regulatory T cells, as well as potential therapeutic applications and future directions of this research are discussed below.

#### Suppression of T<sub>H</sub>1 Cytokine by TSST-1 *In Vivo*

Following the first injection of TSST-1 in BALB/c mice, there is typically a prominent  $T_{H1}$  response within the first 48 hours, as demonstrated by the production of high levels of IL-2, IFN- $\gamma$  and TNF- $\alpha$  by a number of other investigators (Miethke, Duschek et al, 1993;Miethke, Wahl et al, 1993;Miethke, Wahl et al, 1992). In our studies with repeated subcutaneous injection of 4 µg TSST-1 in BALB/c mice, serum IL-2 and IFN- $\gamma$  levels were undetectable at 2 h after the first injection. This was expected since transcriptional activation of proinflammatory cytokine genes do not occur until 4-6 h after TSST-1 stimulation (Miethke, Duschek et al, 1993). Our two hour post-injection sampling timepoint was chosen in order to select for primed, memory like T cell responses and has been utilized in other superantigen model systems to study the response to repeated stimulation (Sundstedt, Hoiden et al, 1997;Florquin, Amraoui et al, 1996). Using a 2 hour sample time point we found after the second injection, there was a rapid and strong, memory-like, proinflammatory cytokine response similar to that following repeated SEA and SEB administration (Sundstedt, Hoiden et al, 1997;Noel, Florquin et al, 2001;Florquin, Amraoui et al, 1996;Miller, Ragheb et al, 1999;Grundstrom, Cederbom et al, 2003). Following the third TSST-1 injection, the levels of IL-2 and IFN- $\gamma$  decreased markedly. Although a downregulatory T<sub>H</sub>2 response could be responsible for suppressing the potent primary T<sub>H</sub>1 response, the lack of concomitant augmentation of serum IL-4 levels, a key T<sub>H</sub>2 cytokine, made this unlikely. On the other hand, the significant increase in serum IL-10 levels following the third TSST-1 injection led us to suspect that a memory-like regulatory T cell response may be responsible for this serum cytokine profile *in vivo*. This was strengthened by our finding that this T<sub>H</sub>1 suppressive response following repeated TSST-1 administration could be adoptively transferred to naïve mice with CD4<sup>+</sup> splenocytes from TSST-1-treated mice. The suppression of serum IL-2 and IFN- $\gamma$  to TSST-1 stimulation *in vivo* following adoptive transfer of CD4<sup>+</sup> splenocytes from TSST-1 treated mice into naïve mice is similar to the findings in a murine model of chronic SEB administration (Noel, Florquin et al, 2001).

This cytokine phenotype of high serum IL-10, with decreased IL-2 and IFN-γ production following repeated superantigen stimulation, although artificial, was utilized by us as a model system to study the charactersitics of the T cells induced under these conditions. Only three treatements with TSST-1 were required to induce an IL-10 dominant cytokine response. This model was not utilized to determine an endpoint at which this cytokine response may change, and for this reason the results of further stimulations with TSST-1 were not characterized. Additional repeated stimulation with TSST-1 may result further cytokine polarization, as seen with SEA (Sundstedt, Hoiden et al, 1997).

High levels of IL-10 production were observed following repeated TSST-1 stimulation in vivo. Since IL-10 has been demonstrated to be a potent suppressor of IL-2 and IFN-y production (Joss. Akdis et al., 2000; Taga & Tosato, 1992), we expected that repeated TSST-1 administration following adoptive transfer of TSST-1-primed CD4<sup>+</sup> splenocytes would lead to more rapid induction and higher serum IL-10 levels compared to adoptive transfer of PBS-treated splenocytes. However, although serum IL-10 levels continued to rise in both groups of mice following repeated TSST-1 stimulation, they were not higher in mice with adoptive transfer of  $T_{R}$  cells compared to mice with adoptive transfer of naïve T cells (Figure IV-3). There are two possible explanations for this observation. Following adoptive transfer by the intravenous route,  $T_R l$  cells may be recruited to a site of immune activation where they exert their suppressive effect locally on the production of cytokines (Iellem, Colantonio et al., 2003;Cottrez & Groux, 2004). Thus, any increased IL-10 production by the adoptive transfer of  $T_R1$  cells would be localized in tissues and may not be detected systemically in the serum. Alternatively, it is conceivable that IL-10 was not responsible for the changes observed in serum IL-2 and IFN-y levels. However, our results utilizing adoptive transfer of CD4<sup>+</sup> T cells from TSST-1 treated mice in the presence of a neutralizing antibody shown to inhibit the IL-10 receptor (O'Farrell, Liu et al, 1998;O'Farrell, Parry et al, 2000;Pontoux, Banz et al, 2002) demonstrate that IL-10 is responsible for the decrease observed in serum IL-2 levels following adoptive transfer of TSST-1 treated CD4<sup>+</sup> T cells. These results are also consistent with similar findings in murine models following repeated administration of SEA and SEB (Noel, Florquin et al, 2001;Florquin, Amraoui et al, 1994;Sundstedt, Hoiden et al, 1997). Furthermore, our intracellular cytokine staining data supports this finding, as expression of IL-10 and IL-2 was mutually exclusive in CD4<sup>+</sup> T cells. This IL-10 – IL-2 negative feedback loop has been considered one of the key

markers for  $T_R1$  function (Bacchetta, Sartirana et al, 2002;Levings & Roncarolo, 2000;Levings, Sangregorio et al, 2001b;Roncarolo, Bacchetta et al, 2001;Groux, 2003).

#### Intracellular Cytokine Profiles and Surface Marker Expression in TSST-1 Induced Splenocytes

The intracellular cytokine profile and surface marker expression of TSST-1 induced splenocytes were determined in order to further characterize the nature of these regulatory T cells. Intracellular cytokine staining of CD4<sup>+</sup> T cells from mice treated repeatedly with TSST-1 showed a striking increase in the number of IL-10 positive cells. There was a 3-fold increase in IL-10<sup>+</sup>IL-4<sup>-</sup>IFN- $\gamma$ <sup>+</sup> cells, and a similar increase in IL-10<sup>+</sup>IL-4<sup>+</sup> and IL-10<sup>+</sup>IFN- $\gamma$ <sup>+</sup> double positive cells.

The role of IL-10<sup>+</sup>IL-4<sup>+</sup> and IL-10<sup>+</sup>IFN- $\gamma^+$  double positive cells in our murine model is somewhat obscure. In the human system, concurrent production of both IL-10 and IFN- $\gamma$  has been observed in T<sub>R</sub>1 like cells (Levings, Sangregorio et al, 2001a). However, in the murine system, some regulatory T cells induced by immunosuppressive drugs appear to produce little or no IFN- $\gamma$  (Barrat, Cua et al, 2002). There is also circumstantial evidence that individual T<sub>R</sub>1 cells induced by SEB may have evolved by the sequential and preferential expression of IL-10 from IFN- $\gamma^+$  cells (Assenmacher, Lohning et al, 1998). Furthermore, although we saw a tripling in the number of dual positive IL-10<sup>+</sup>/IL-4<sup>+</sup> and IL-10<sup>+</sup>/IFN- $\gamma^+$  cells, the number of single positive IL-4<sup>+</sup> or IFN- $\gamma^+$  cells did not increase as a result of repeated TSST-1 treatment *in vivo*. Despite this uncertainty surrounding the role of IL-10<sup>+</sup>IL-4<sup>+</sup> or IL-10<sup>+</sup>IFN- $\gamma^+$  double positive cells, we surmised that the significant increase in the proportion of IL-10<sup>+</sup> single positive (IL-10<sup>+</sup>, IL-4<sup>+</sup>, IFN- $\gamma^-$ ) cells is the clearest indication of a T<sub>R</sub>1-like phenotype following repeated TSST-1 administration *in vivo*. The relationship between IL-10 production and co-expression of several putative markers for regulatory T cells in TSST-1 primed CD4<sup>+</sup> splenocytes was further explored. Although the association of IL-10 production with co-expression of GITR, low-level expression of CD45RB, or CD25 was tentative, intracellular co-expression of IL-10 and CTLA-4 was strongly correlated. These descriptive data did not examine the functional significance of the markers but relied exclusively on IL-10 production as a characteristic of T cells with regulatory function.

IL-10 expression in our system was strongly correlated with CTLA-4 coexpression, since 92% of IL-10<sup>+</sup> cells from TSST-1 treated mice were also positive for CTLA-4 (Figure VI-13). CTLA-4 (or CD152) is a homologue of the T cell co-receptor CD28, but engages its ligands (CD80/86 or B7-1/2) with higher affinity (Bluestone, 1997; Alegre, Frauwirth et al., 2001). It functions as a negative regulator of activation (Bluestone, 1997;Allison, Chambers et al., 1998;Alegre, Frauwirth et al, 2001). This molecule is highly expressed on CD4<sup>+</sup>CD25<sup>+</sup> T cells (Zelenika, Adams et al, 2002;Read, Malmstrom et al, 2000;Takahashi, Tagami et al, 2000), a characteristic thought to ensure that these T regulatory cells do not get optimal activation via CD28 in the event of strong stimulation (Waldmann, 2002). It has been suggested that CTLA-4 expression in "natural" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s is the result of the chronic activation state of these cells and not required for their regulatory function, while CTLA-4 signaling may be required to induce IL-10 producing  $T_R1$  cells (Thornton, Piccirillo et al, 2004). Our finding of a significant correlation between CTLA-4 expression and IL-10 production in T<sub>R</sub>1 cells induced by repeated TSST-1 administration is consistent with this view. Expression of CTLA-4 has been associated with the function of regulatory cells in other systems, as antibody blockade of CTLA-4 results in an ablation of suppressive function (Takahashi, Tagami et al, 2000; Read, Malmstrom et al, 2000). Measurements of intracellular CTLA-4 indicate that cells expressing IL-10 are at least primed for CTLA-4 expression. This result is supported by data obtained in a model of repeated SEA

exposure in TCR transgenic animals (Grundstrom, Cederbom et al, 2003), although it is contrary to the findings of Feunoe et al, in which a correlation between  $T_{REG}$  function and CTLA-4 expression was not observed among SEB specific  $T_{REG}$ 's (Feunou, Poulin et al, 2003). This disparity may either be due to the methodologies of the studies in question, or the differential responses to various sAgs used. Further investigation of the kinetics of CTLA-4 surface expression and the functional role of this coreceptor in TSST-1 induced regulatory T cells is clearly waranted.

GITR has been implicated in T cell homeostasis (Ronchetti, Nocentini et al. 2002), and was identified by gene chip technology to be associated with 'natural' CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s (McHugh, Whitters et al., 2002; Shimizu, Yamazaki et al. 2002). Crosslinking of this receptor with activating antibodies has been reported to transiently block suppression mediated by regulatory T cells (Shimizu, Yamazaki et al, 2002), suggesting that expression of the GITR ligand may be a negative regulator of T<sub>REG</sub> function. However, treatment with aGITR antibodies has been hypothesized to block interaction with its ligand rather than induce GITR mediated activation of T<sub>REG</sub>'s (Suri, Shimizu et al., 2004). In our studies, the surface expression of GITR following repeated TSST-1 administration was not significantly different from PBS-treated control mice, and GITR expression also did not correlate with IL-10 production. Our results are in contrast to recent findings indicating that GITR expression correlated with CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> function. Uraushihara et al. (Uraushihara, Kanai et al., 2003) recently demonstrated that transfer of GITR<sup>+</sup> cells, either CD25<sup>+</sup> or CD25<sup>-</sup>, conferred protection in a model of IBD induced by transfer of CD45RB<sup>HIGH</sup> T cell. GITR expressing cells in this system were shown to produce more IL-10 than their GITR<sup>-</sup> counterparts. Differences between these findings and our own could represent a fundamental distinction in T<sub>REG</sub> subtypes induced by TSST-1 vs. those induced by GITR<sup>+</sup> cells

in the IBD model, or could reflect a difference in the activation state of the cells purified via their GITR receptor.

We did not identify any significant difference in the mean fluorescence intensity of CD45RB between CD4<sup>+</sup> splenocytes from TSST-1 treated vs. control.mice. Furthermore, IL-10 producing splenocytes from both TSST-1-treated and control mice demonstrated a wide range of CD45RB expression. However, we did observe that the CD45RB MFI was significantly lower in IL-10<sup>+</sup> than IL-10<sup>-</sup> CD4<sup>+</sup> splenocytes from both TSST-1 and PBS-treated mice (Figure VI-16).

#### T<sub>H</sub>1 Cytokine Suppression *In Vitro*: Role of CD4<sup>+</sup>CD25<sup>+</sup> T Cells

Our *in vitro* studies revealed that TSST-1 primed CD4<sup>+</sup>CD25<sup>+</sup>, but not CD4<sup>+</sup>CD25<sup>-</sup>, splenocytes significantly suppressed IL-2 and enhanced IL-10 production by naive splenocytes following TSST-1 stimulation. Furthermore, the activity was potent, since addition of only 5% of TSST-1 primed CD4<sup>+</sup>CD25<sup>+</sup> splenocytes was sufficient to demonstrate a significant effect on naïve splenocytes in response to TSST-1 stimulation *in vitro*. The finding that only CD4<sup>+</sup>CD25<sup>+</sup> but not CD4<sup>+</sup>CD25<sup>-</sup> splenocytes from TSST-1 treated mice enhanced the production of IL-10 *in vitro* appears to contradicts our intracellular cytokine staining data which indicated that CD25 expression did not completely correlate with IL-10 production in CD4<sup>+</sup> T cells from TSST-1 treated mice, as ~75% of the IL-10<sup>+</sup> cells were CD25<sup>-</sup>. This discrepancy may be due to our restimulation protocol that is required for intracellular cytokine staining: the induction of polyclonal T cell activation through CD3, CD28 and in particular, IL-2 stimulation may have altered expression of CD25 (the  $\alpha$  component of the IL-2 receptor).

#### The Causative Role of IL-10

The enhanced production of IL-10 by naïve splenocytes following TSST-1 stimulation in vitro was observed only in the presence of 5% CD4<sup>+</sup>CD25<sup>+</sup> but not CD4<sup>+</sup>CD25<sup>-</sup> splenocytes derived from TSST-1 treated mice. The observation that CD4<sup>+</sup>CD25<sup>+</sup> T cells are capable of suppressing IL-2 production while producing high levels of IL-10 has been well documented by others (Annacker, Pimenta-Araujo et al, 2001;Pontoux, Banz et al, 2002;Francis, Till et al, 2003). We suspect that rather than being thymus-differentiated "natural" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s, TSST-1 primed CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s are likely derived from effector T cells in the periphery, as has been suggested by Assenmacher et al. and others (Huang, Huso et al, 2003;Zelenika, Adams et al, 2002;Assenmacher, Lohning et al, 1998). These CD4<sup>+</sup> T cells are likely TSST-1 activated T<sub>R</sub>1 cells which have upregulated their CD25 receptor. This does not exclude the possibility of the presence and contribution of "natural" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s in our system, as these cells have also been suggested to induce IL-10 producing T<sub>R</sub>1 cells through a cell-contact-dependent but cytokine-independent mechanism (Dieckmann, Bruett et al., 2002). This interplay between "natural"  $CD4^+CD25^+$  T<sub>REG</sub>'s and T<sub>R</sub>1 cells has also been demonstrated by the chronic administration of other sAgs including SEA and SEB (Grundstrom, Cederbom et al, 2003; Feunou, Poulin et al, 2003; Pontoux, Banz et al, 2002). This leads us to believe that the addition of 5% TSST-1 primed CD4<sup>+</sup>CD25<sup>+</sup> T cells to naïve splenocytes in our studies may have helped to further differentiate TSST-1 specific T<sub>R</sub>1 cells which suppressed IL-2 production via the induction of IL-10.

## The Identity of TSST-1 Induced Regulatory T Cells: Why they are likely $T_R1$ cells and not "natural" CD4<sup>+</sup>CD25<sup>+</sup> $T_{REG}$ 's

The purification and definitive identification of  $T_R1$  cells has been hampered by the lack of suitable markers for these cells. Since there are currently no definitive markers for  $T_R1$  cells, the identification of  $T_R1$  cells by their cyptokine expression profile and  $T_H1$  suppressive function *in vitro* and *in vivo* is critical. In our murine model, we defined  $T_R1$  cells as CD4<sup>+</sup> T cells that are IL-10<sup>+</sup> and IL-4<sup>-</sup> (making them distinct from  $T_H2$  cells), and capable of suppressing IL-2 by a IL-10 and/or TGF- $\beta$  dependent mechanism (Asseman & Powrie, 1998;Bacchetta, Sartirana et al, 2002;Barrat, Cua et al, 2002;Cottrez, Hurst et al, 2000;Groux, 2001;Groux, O'Garra et al, 1997;Groux, 2003;Levings & Roncarolo, 2000;Roncarolo, Bacchetta et al, 2001). In addition to intracellular cytokine staining for IL-10, several other cell surface markers have been suggested to be helpful for the detection of  $T_{REG}$ 's. In the murine system, CTLA-4 (Read, Malmstrom et al, 2000), GITR (Shimizu, Yamazaki et al, 2002), CD45RB (Powrie, Correa-Oliveira et al, 1994), and CD25 (Shevach, McHugh et al, 2001a) expression have all been suggested as potentially useful markers for regulatory T cells. The lack of more definitive surface markers for regulatory T cell subsets has unfortunately led many investigators to resort to designating CD25<sup>+</sup> expression in CD4<sup>+</sup> T cells as a surrogate marker for this cell type.

The cytokine phenotype of  $CD4^+$  T cells generated by repeated TSST-1 administration in our murine system fits a T<sub>R</sub>1 profile (Groux, O'Garra et al, 1997;Roncarolo, Bacchetta et al, 2001;Levings & Roncarolo, 2000), as there are significant increases in single and dual positive IL-10<sup>+</sup> cells, and the *in vivo* suppression of IL-2 production in response to TSST-1 was mediated by IL-10. Since IL-10 has been shown to induce  $CD25^+$  expression (Cohen, Katsikis et al., 1994), the increased numbers of  $CD4^+$   $CD25^+$  IL-10<sup>+</sup> cells in response to repeated TSST-1

stimulation *in vivo* lead us to believe that CD25 expression in these cells may have been transiently induced by IL-10. This suppressive effect was not due to "natural"  $CD4^+CD25^+$  regulatory T cells, since IL-10 is not produced by "natural"  $CD4^+CD25^+$  regulatory T cells but was found to be at least partially responsible for the suppressive effect of TSST-1 induced regulatory T cells. Based on the above, we believe that these regulatory T cells induced by repeated TSST-1 administration are indeed  $T_R1$  rather than 'natural'  $CD4^+CD25^+$   $T_{REG}$ 's, although whether the latter also participate in the inhibitory function induced by TSST-1 will require further study.

 $T_R1$  and "natural" CD4<sup>+</sup>CD25<sup>+</sup>  $T_{REG}$ 's differ in several respects, including their origin and mechanism of action (**Table 1-3**). Thus, 'natural' CD4<sup>+</sup>CD25<sup>+</sup> cells have been shown to mediate their inhibitory function *in vitro* by direct cell-cell contact with effector T cells independent of IL-10 (Baecher-Allan, Brown et al., 2001;Dieckmann, Bruett et al, 2002;Levings, Sangregorio et al, 2001b;Levings, Bacchetta et al, 2002;Nakamura, Kitani et al, 2001;Shevach, McHugh et al, 2001a). In contrast, the inhibitory effect of  $T_R1$  cells is at least partially IL-10-dependent (Asseman & Powrie, 1998;Bacchetta, Sartirana et al, 2002;Boussiotis, Tsai et al., 2000;Groux, 2001;Groux, 2003;Levings, Bacchetta et al, 2002;Roncarolo, Bacchetta et al, 2001). CD25 (the high affinity  $\alpha$  subunit of the IL-2 receptor) is strongly expressed on unstimulated "natural"  $T_{REG}$ 's (Shevach, McHugh et al, 2001a), while resting  $T_R1$  cells are CD25<sup>-</sup>. However, these two subsets are indistinguishable based on CD25<sup>+</sup> expression following stimulation, since CD25 upregulation is a common feature of T cell activation. As a result, many researchers inadvertently lump these two cell types together as CD4<sup>+</sup>CD25<sup>+</sup> "natural"  $T_{REG}$ 's despite their heterogeneity following stimulation. Confusion as to the role played by either subset of  $T_{REG}$ 's is increased by the suggestion that the chief function of "natural" CD4<sup>+</sup>CD25<sup>+</sup> T cells is to induce  $T_R1$  cells which then serve to suppress the immune response in an IL-10 dependent fashion (Jonuleit, Schmitt et al, 2002;Dieckmann, Bruett et al, 2002).

#### Molecular Mechanisms of T<sub>R</sub> Control

The work presented here makes a strong case for the role of IL-10 in the suppression of TSST-1 mediated responses. Several molecular mechanisms have been implicated in the suppressive effects mediated by IL-10 induced regulatory T cells (**Figure VIII-1**). IL-10 has been shown to directly suppress the effects of APC's as well as T cells. It inhibits APC's by suppressing TNF- $\alpha$  production (de Waal Malefyt, Abrams et al, 1991;Moore, O'Garra et al, 1993), and down regulating MHCII (de Waal Malefyt, Haanen et al, 1991) as well as the costimulatory molecules ICAM-1 (Spittler, Schiller et al, 1995) and CD80/86 (B7.1/B7.2) (Flores Villanueva, Reiser et al, 1994;Willems, Marchant et al, 1994). IL-10 has also been shown to inhibit T cell proliferation (Taga & Tosato, 1992) and the production of IL-2 (Taga & Tosato, 1992), IFN- $\gamma$ , TNF- $\alpha$ , and TNF- $\beta$  (de Waal Malefyt, Yessel et al, 1993) by T cells. Other immune supressive cytokines, such as TGF- $\beta$  may also play a role in T<sub>R</sub> induced immunosuppression (Powrie, Leach et al, 1996).

While cytokines play a role in the suppression of T cell responses, there is considerable data that other molecules, such as CTLA-4 may inhibit T cell responses. CTLA-4 has been shown to induce the expression of indoleamine 2,3-dioxygenase (IDO) when interacting with dendritic cell expressed CD80/86 (Grohmann, Orabona et al., 2002;Munn, Sharma et al., 2004). This enzyme catalyzes the breakdown of tryptophan to kynurenine and other metabolities which serve to suppress local immune responses (Fallarino, Grohmann et al., 2003;Grohmann, Fallarino et al., 2003). CTLA-4 may also further inhibit APC costimulation of effector T cells by competing for

CD80/86 with higher affinity than CD28 (Alegre, Frauwirth et al, 2001;Allison, Chambers et al, 1998). In addition,  $T_R$  cells may inhibit T cell activation via their surface expression of CD25, the high affinity alpha portion of the IL-2 receptor, by mopping up this cytokine and making it unavailable to nearby T cells clones (de la, Rutz et al., 2004). Other as yet unidentified surface molecules may also play a role in inducing immune suppression.

Immune suppression induced by each of these modalities may occur concurrently during a  $T_R$  response, as these mechanisms are not mutually exclusive. Immunosuppression may result either by the activation of  $T_R$ , or possibly by the differentiation of a reactive T cell clone into a  $T_R$  phenotype, as has been suggested from some models of superantigen-induced peripheral tolerance (Grundstrom, Cederbom et al, 2003).

![](_page_142_Figure_0.jpeg)

**Figure VIII-1: Molecular Mechanisms of T**<sub>R</sub> **Suppression.** T<sub>R</sub> cells likely supress T helper cell responses via multiple mechanims. CTLA-4 stimulation of APC's by T<sub>R</sub>s has been suggested to induce the synthesis of indoleamine 2,3-dioxygenase (IDO), and the subsequent production of suppressive metabolities such as kynurenine which may inhibit T cell activation. T<sub>R</sub> cells may modify costimulation of T<sub>H</sub> cells by APCs through stimulation with CTLA-4 and cytokines such as IL-10 and TGF- $\beta$ . T<sub>R</sub> cells might induce direct proximal suppression of effector T cells through direct CTLA-4 stimulation, IL-2 absorption or through some as yet unidentified mebrane ligand. T<sub>R</sub> cells may also induce cytokine dependent suppression, through the actions of IL-10 and TGF- $\beta$ . The resulting effect on the effector T cell may be either a suppressed response, or the conversion of the effector T cell into a T<sub>R</sub> cell that expresses the transcription factor Foxp3. These mechanisms are not necissarily mutually exclusive and may operate concurrently (Fehervari & Sakaguchi, 2004).

#### The Special Case of IFN- $\gamma$

Since IL-10 is known to suppress IFN-y production (Fiorentino, Zlotnik et al, 1991;Macatonia, Doherty et al., 1993) we anticipated an increase in serum IFN-y levels in mice following adoptive transfer of TSST-1 primed splenocytes and repeated TSST-1 administration in the presence of  $\alpha$ IL-10R $\alpha$ . To our surprise, IFN- $\gamma$  instead significantly decreased following blockade of IL-10R $\alpha$  (Figure V-3). However, other investigators have also reported that neutralization of IL-10 in BALB/c mice following repeated SEA or SEB administration (in the absence of T cell transfer) resulted in decreased serum IFN-y levels (Noel, Florquin et al, 2001; Florquin, Amraoui et al, 1994;Sundstedt, Hoiden et al, 1997). This unexpected effect of aIL-10Ra blockade on IFN- $\gamma$  may be due to several factors. Firstly, it has been suggested that T<sub>R</sub>1 cells may produce IFN- $\gamma$ under some circumstances (Assenmacher, Lohning et al, 1998). This possibility is supported by our intracellular cytokine staining studies, since a significantly higher proportion of CD4<sup>+</sup> T cells in mice following repeated TSST-1 administration were IFN- $\gamma^+$ IL-10<sup>+</sup> double positive cells compared to PBS treated control mice (Figure VI-5). Since IL-10 is known to be a growth factor for T<sub>R</sub>1 cells (Asseman & Powrie, 1998;Groux, 2003;Levings & Roncarolo, 2000;Groux, O'Garra et al, 1997), blockade of the IL-10 receptor may have prevented the outgrowth of these dual positive IL-10<sup>+</sup>IFN- $\gamma^+$  T<sub>R</sub>1 cells, leaving only T<sub>H</sub>1 cells to produce IFN- $\gamma$ . The net effect would therefore be a decrease in the total level of serum IFN-y detected by ELISA. In addition, other cytokines such as TGF- $\beta$  and IL-12 may be involved in the regulation of IL-10 and IFN- $\gamma$ . respectively. Also unexpectedly, IFN- $\gamma$  levels produced by naïve splenocytes were enhanced by the addition of 5% CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> splenocytes from TSST-1 treated mice. It is possible that the  $T_R1$  cells within the CD4<sup>+</sup>CD25<sup>+</sup> population may be primed for IFN- $\gamma$ production as has been reported previously (Roncarolo, Bacchetta et al, 2001;Groux, 2003).
Alternatively, the CD4<sup>+</sup>CD25<sup>+</sup> subpopulation is likely heterogeneous, containing both 'natural'  $CD4^+CD25^+$  T<sub>REG</sub>'s as well as TSST-1 induced T<sub>R</sub>1 and T<sub>H</sub>1 cells. Therefore, the observed enhancement of IFN- $\gamma$  production may be mediated by the IFN- $\gamma$  producing T<sub>H</sub>1 subset within both the CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> TSST-1 primed T<sub>H</sub>1 cells. This latter hypothesis is further supported by the heterogenicity of cytokine production observed in CD4<sup>+</sup> CD25<sup>+</sup> human T cell clones (Levings, Sangregorio et al, 2002), and by data in a murine model of repeated SEB administration (Feunou, Poulin et al, 2003). It is clear that further experiments to specifically address the relationship between IL-10 and IFN- $\gamma$  production by TSST-1 induced T<sub>R</sub>1 cells will be required.

### Significance of Our Research

Our research on the induction of regulatory T cells by TSST-1 has a number of important applications, particularly for the study and treatment of autoimmune diseases.

### Clarification of T<sub>R</sub>1 Cells in Host Immunity and Immunopathology

Regulatory T cells have enormous implications in a number of disease, particularly Graft Versus Host Disease (GVHD) (Jones, Murphy et al, 2003), Experimental Autoimmune Encephalomyelitis (EAE) (Chen, Kuchroo et al, 1994;Hori, Haury et al., 2002), and Inflammatory Bowel Disease (IBD) (Duchmann, Schmitt et al, 1996;Groux & Powrie, 1999). Aside from autoimmunity,  $T_R1$  cells are thought to play a role in inducing immune deviation resulting in decreased immune responses of the host to cancer and chronic infections, such as tuberculosis, leishmaniasis and malaria, in which a deleterious role for dysfunctional regulatory T cells has been suggested (Brady, Eckels et al., 1999;McGuirk, McCann et al, 2002;Belkaid, Piccirillo et al., 2002;Sharma, Stolina et al., 1999;Antony & Restifo, 2002;Boussiotis, Tsai et al, 2000;Plebanski, Flanagan et al., 1999;McGuirk & Mills, 2002;Sakaguchi, 2003;Satoguina, Mempel et al., 2002;Zuany-Amorim, Sawicka et al., 2002). We are particularly interested in the role of sAgs in staphylococcal infections. While sAgs are capable of inducing acute toxic shock syndrome and subsequent death, we believe the selective advantage of this toxin is in its potent ability to induce  $T_R1$  cells following chronic exposure, resulting in immune deviation. Since  $T_R1$  cells can induce bystander suppression in nearby activated T cells through IL-10 signaling, we believe they may cause suppressed responses to other staphylococcus specific antigens. This may allow for chronic infection or colonization by *S. aureus*. Thus, we are currently developing an infection model to examine the role  $T_R1$  cells play in the pathogenesis as well as host responses to chronic staphylococcus infections.

### Potential Applications for TSST-1 Mediated Immunotherapy

Our work has provided exciting results that raise the possibility of using TSST-1 and perhaps other staphylococcal superantigen induced T<sub>R</sub>1 cells to suppress autoimmunity. During a deleterious autoimmune response, several activation markers are expressed on T cells activated by self-antigens. It should be possible to isolate and purify these activated autoreactive cells using FACs or MACs sorting. It may be possible to generate self-antigen specific  $T_R1$  cells by repeated stimulation of the identified self-reactive cells in vitro with a panel of different sAgs in the presence of self APC's such as immature dendritic cells. We expect that re-infusion of sAggenerated and self-antigen-specific T<sub>R</sub>1 cells would localize to the site of immune activation and suppress the deleterious autoimmune responses by producing IL-10 or other anti-inflammatory cytokines locally rather than systemically. This form of "targeted" treatment may minimize toxicities that invariably severe accompany conventional therapy with systemic immunosuppressive agents. Additionally, it may also result in better control of the autoimmune process rather than relying on life-long immunosuppressive chemotherapy. Although both T<sub>R</sub>1

and "natural" CD4<sup>+</sup>CD25<sup>-</sup> T<sub>REG</sub> subsets seem capable of mediating bystander suppression (Barrat, Cua et al, 2002;Kemper, Chan et al, 2003;Thornton & Shevach, 2000;Groux, O'Garra et al, 1997), the antigen-specific and inducible nature of the T<sub>R</sub>1 subset has the most therapeutic potential for the treatment of autoimmunity and transplantation. These T<sub>R</sub>1 cells could be targeted against autoantigens or graft antigens of interest, since they can be derived from antigen-specific T cells clones (Bacchetta, Sartirana et al, 2002;Groux, O'Garra et al, 1997). In contrast, "natural" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s appear to be preselected for self antigen specificity (Shevach, McHugh et al, 2001a) and are only specific to antigens expressed in the thymus (Cottrez & Groux, 2004), making their therapeutic potential for suppressing immunopathological responses to foreign antigens questionable.

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In order to demonstrate proof of concept, it will be necessary to develop a suitable murine model for autoimmunity in order to examine the potential therapeutic value of sAg-generated  $T_R1$  cells for immunotherapy. EAE, a model for multiple sclerosis, is an excellent candidate for such studies. The immunological response in EAE is antigen-specific and directed at unique epitopes within myelin basic protein. Thus, this murine model may provide an excellent opportunity to evaluate whether TSST-1 or other sAg-generated  $T_R1$  cells may have therapeutic potential *in vivo* for the treatment of an autoimmune disease with T cells that can be tested for their antigenspecificity *in vitro*. Establishment of this model system for use with our TSST-1 generated  $T_R1$ cells is currently under consideration.

In addition to autoimmune diseases, TSST-1 or other sAg-generated  $T_R1$  cells may also prove to be beneficial in the treatment of diseases affected by the immune response such as graft rejection, cancer and certain chronic infections. Our investigations of the development and characterization of regulatory T cells induced by repeated TSST-1 administration have added new insights with potential therapeutic applications to this important and exciting field of research.

### Advantages for Using TSST-1 to Generate T<sub>R</sub>1 Cells

Research into therapeutic applications of T<sub>R</sub>1 cells has been hampered by difficulties both in differentiating  $T_{R}$  cells, and getting them to proliferate. In addition, many of the model systems utilized to study this T cell subset rely on stimulation of the T cells with specific antigens presented on irradiated splenocytes in the presence of IL-10. This limits the generation of  $T_{R1}$ cells to those T cell clones which react with known antigens. Many of the therapeutic applications for  $T_R1$  cells rely on developing cells specific for human self antigens. Identification of such autoreactive antigens in order to stimulate these regulatory T cells in an antigen-specific manner is an enormous undertaking due to the polymorphic nature of the autoantigens, as well as MHC alleles, and TCR genes within the human population. For example, an antigen that stimulates cells specific for multiple sclerosis in one individual, may not work for another. Although enrichment of viable  $T_{R1}$  cells based on IL-10 production is feasible in both the human and murine systems (Scheffold, Assenmacher et al., 2000b;Siewert, Herber et al., 2001;Becker, Pohla et al., 2001;Assenmacher, Lohning et al, 1998), this procedure is both time-consuming, expensive, and technically difficult. There is also the possibility of contamination by a population of false positive T cells due to cross capture of IL-10 on the cell surface.

The system we have developed has a number of advantages over other techniques to generate regulatory T cells. The polyclonal, V $\beta$ -specific nature of sAg stimulation may eliminate the need to identify and activate antigen-specific autoreactive human cells of interest. Rather, a panel of sAgs with varied V $\beta$  specificity may be used to ensure activation of the cells of interest

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once the autoreactive cell clones have been isolated and identified from PBMC on the basis of their unique activation profile and cell surface marker expression. Our choice of TSST-1 has an additional advantage over other sAgs. Unlike SEA and SEB, purified TSST-1 does not appear to induce any detectable increase in the production of IL-4 in murine or human cells, either *in vitro* or *in vivo*. Interestingly, some of the differences in cytokine responses seen with different sAgs may be related to their V $\beta$  specificity and the V $\beta$  repertoire of the host. Although TSST-1 appears to be a useful tool for generating  $T_R1$  cells in our murine system, further research to examine the response to these other sAgs will be beneficial for broadening the potential therapeutic applications of sAg-generated  $T_R1$  cells. This is important since it is likely that a panel of different sAgs with wide ranging V $\beta$  specificities for autoreactive T cell clones of interest will be required for antigen-specific immunosuppression. Finally, our system overcomes difficulties encountered in generating a large number of these cells in a timely fashion, requiring only 3 *in vivo* treatments over the course of 8 days to induce functional  $T_R1$  cells.

### Future Directions

There are a number of questions that need to be addressed in future studies of TSST-1 induced regulatory T cells which are summarized below.

### Is There a Requirement for CD4<sup>+</sup>CD25<sup>+</sup> Cells in Our System?

Although we believe that the regulatory T cells induced by repeated TSST treatment are  $T_R1$  cells, studies have suggested a link between the "natural" CD4<sup>+</sup>CD25<sup>+</sup> subset of  $T_{REG}$ 's and  $T_R1$  cells. There are suggestions that the former  $T_{REG}$  subtype may be responsible in part for the differentiation of the latter (Dieckmann, Bruett et al, 2002;Grundstrom, Cederbom et al, 2003;Feunou, Poulin et al, 2003). Since only CD4<sup>+</sup>CD25<sup>+</sup> but not CD4<sup>+</sup>CD25<sup>-</sup> splenocytes from TSST-1 treated mice demonstrated suppression of IL-2 and enhanced production of IL-10 *in* 

*vitro* (Figures VII-6 and VII-8), it is possible that our  $T_R1$  cells may have derived from CD4<sup>+</sup>CD25<sup>-</sup> cell that became CD25<sup>+</sup> following TSST-1 activation. Work by Grundstrom et al. (Grundstrom, Cederbom et al, 2003), utilizing repeated SEA treatment in a TCR transgenic mouse backcrossed against a rag2<sup>-/-</sup> background, reported that potent suppression of  $T_H1$  responses can develop in the absence of "natural" CD4<sup>+</sup>CD25<sup>+</sup> T cells. However, such suppression has a different phenotype than that seen when "natural" CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub>'s are present. The requirement for CD4<sup>+</sup>CD25<sup>+</sup> cells in our system could be addressed by reconstituting rag2<sup>-/-</sup> T cell knockout mice by adoptive transfer of naïve T cells that are either CD25<sup>+</sup> or CD25<sup>-</sup>, and determining if CD25<sup>+</sup> cells are required for the development of a T<sub>R</sub>1 like serum cytokine profile after repeated TSST-1 treatment. Additionally, since the forkhead transcription factor Foxp3 is specifically expressed in "natural" CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells and is required for their development (Fontenot, Gavin et al., 2003), it would be important to determine if this transcription factor is upregulated in TSST-1 induced regulatory T cells.

# Does Suppression of $T_H1$ Cytokines by TSST-1 Induced $T_R1$ Cells Require Cell Contact?

In vivo, we showed that IL-10 played a key role in TSST-1 induced suppression of serum IL-2, since this effect was abrogated by treatment with an  $\alpha$ -IL-10 mAb. *In vitro*, we demonstrated by intracellular cytokine staining that TSST-1 induced CD4<sup>+</sup>CD25<sup>+</sup> splenocytes produced high levels of IL-10. This is consistent with the development of a T<sub>R</sub>1-like phenotype following repeated TSST-1 treatment in BALB/c mice. However, it remains unclear whether cell contact is also necessary for the suppressive function on the IL-2 response, and whether other cytokines besides IL-10, such as TGF- $\beta$ , may play a contributory role. This could be addressed by the use of a transwell system in concert with appropriate TGF- $\beta$  neutralizing antibodies to determine if cell contact and TGF- $\beta$  signalling are required for the IL-2 suppressive effect of TSST-1.

Furthermore, any results from *in vitro* observations must be validated by *in vivo* studies, since *in vitro* and *in vivo* results do not necessarily agree with each other (Grundstrom, Cederbom et al, 2003).

### Do TSST Induced T<sub>R</sub>1 Cells Also Affect a Classical T<sub>H</sub>2 Response?

The effects of  $T_RI$  regulatory T cells have, to date, chiefly focused on down-regulation of deleterious  $T_HI$  mediated immunological responses, including GVHD (Jones, Murphy et al, 2003), EAE (Chen, Kuchroo et al, 1994;Hori, Haury et al, 2002), and IBD (Duchmann, Schmitt et al, 1996;Groux & Powrie, 1999). The effects of this  $T_{REG}$  subtype in  $T_H2$  mediated immune responses has also been studied to some extent (Cottrez, Hurst et al, 2000), but is undetermined in our model system. Our *in vivo* data points to IL-10 being a key regulator of cytokine suppression. IL-10 was originally characterized as a  $T_H2$  like cytokine, since it is capable of inducing B cell blast proliferation, and can mediate isotype switching (Moore, O'Garra et al, 1993;Wakkach, Cottrez et al., 2000). Thus, it is likely that repeated treatment with TSST-1 *in vivo* may also affect  $T_H2$ -mediated immunological responses. We suspect that total antibody isotype production, as well as antigen specific antibody production will be altered by an ongoing  $T_R1$  response. Preliminary work to determine the effect of TSST-1 generated  $T_R1$  cells on a TNP-KLH specific B cell response has been promising, and further work with this model system is ongoing.

### What is the Developmental Origin of TSST-1 Induced T<sub>R</sub>1 Cells?

Whether the  $T_R1$  like phenotype observed after repeated TSST-1 administration *in vivo* in our study was derived from  $T_H0$ ,  $T_H1$  or  $T_H2$  cells is unclear. It has been suggested that  $T_R1$  cells might derive either from predetermined precursors, or they may have differentiated from regular effector cells (Huang, Huso et al, 2003;Zelenika, Adams et al, 2002;Assenmacher, Lohning et al,

1998). If the latter scenario is true, then our IL-10<sup>+</sup>IL-4<sup>+</sup> or IL-10<sup>+</sup>IFN- $\gamma^+$  dual positive cells may in fact represent pre-T<sub>R</sub>1 cells in an intermediate stage of differentiation. Data from T<sub>R</sub>1-like cells generated by chronic stimulation with the related superantigen SEB appear to support this possibility, since there was a sequential and preferential expression of IL-2, IFN- $\gamma$  and finally IL-10 in CD4<sup>+</sup> T cells that were apparently derived from T<sub>H</sub>1 cells following repeated SEB stimulation (Assenmacher, Lohning et al, 1998). On the other hand, there is also some evidence that T<sub>R</sub>1 cells may have been derived from T<sub>H</sub>2 cells (Zelenika, Adams et al, 2002;Mendel & Shevach, 2002). This question could be addressed by utilizing a rag27 T cell knockout system with adoptive transfer of unpolarized T<sub>H</sub>0 cells, or polarized T<sub>H</sub>1 or T<sub>H</sub>2 cells to determine which phenotype further develops a T<sub>R</sub>1 serum cytokine response after repeated stimulation with TSST-1. It is also evident that immature dendritic cells may play a role in the differentiation of T<sub>R</sub>1 cells (Morel, Feili-Hariri et al, 2003;Roncarolo, Levings et al, 2001;Wakkach, Fournier et al, 2003). Further work should also determine if T<sub>R</sub>1 functions via a three-cell model of simultaneous interactions with both the APC and the effector T cell, as has been suggested (Leon, Perez et al, 2001;Shevach, McHugh et al, 2001a).

#### Can TSST-1 Induced T<sub>R</sub>1 Cells be Generated In Vitro?

While our murine system shows promise in the generation of  $T_R1$  cells *in vivo*, it would be important to determine if  $T_R1$  cells can also be generated by TSST-1 *in vitro*. This would be particularly important for work with human cells, for which *in vivo* techniques are not practical. *In vitro* generation of  $T_R1$  cells is also essential for their potential therapeutic use in human diseases. Additionally, the generation of a TSST-1 specific T cell clone for use in generating  $T_R1$ cells *in vitro* will simplify our current model system, facilitating the study of gene expression and signal transduction by these cells. For this reason, our lab is actively working on the development of both murine and human  $T_R1$  clones for *in vitro* studies.

## Conclusion

Repeated TSST-1 administration *in vivo* induces a functional  $T_R1$  phenotype based on the data presented.

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