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Rapid Regulatory and Effector Immune Responses in Toxic Shock Syndrome

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Abstract

Toxic shock syndrome (TSS) is an acute, potentially fatal condition characterized by high-grade fever, hypotensive shock and systemic inflammation. It is caused by exposure to staphylococcal and streptococcal superantigens (SAgs), which can activate up to 50% of T cells resulting in a hyperinflammatory 'cytokine storm' within hours. This inflammatory cascade progresses to a life-threatening illness with alarming rapidity, and SAg-exposed individuals can develop multi-organ failure within hours of onset of symptoms. However, there are currently no available treatments that efficiently mitigate the cytokine storm, which drives TSS immunopathology. Therefore, identifying and understanding the critical components underlying this process should hold the key to designing effective therapeutics to reduce TSS severity. In this thesis, I have utilized a clinically relevant humanized HLA-DR4 transgenic (DR4tg) mouse model of TSS to reveal the previously unrecognized roles of three rapid host responses in the initiation or control of the cytokine storm. First, genetic and antibody-mediated depletion of invariant natural killer T (*i*NKT) cells in DR4tg mice show that *i*NKT cells are pathogenic in TSS and contribute to the cytokine storm. Targeting *i*NKT cell responses with the T helper type-2 (Th2)-polarizing glycolipid agonist OCH also reduces TSS morbidity and mortality. Second, I found that granulocytic myeloid-derived suppressor cells (MDSCs) are rapidly recruited to the liver of DR4tg mice during TSS. These hepatic MDSCs potently suppress SAg-induced T cell responses and may therefore mitigate tissue injury in TSS. Lastly, I define the rapid production of interleukin-17A (IL-17A) by effector memory T cells as a novel mechanism promoting immunopathology in TSS. Blockade of IL-17A signaling in human blood mononuclear cells reduces the expression of multiple

inflammatory mediators of TSS, suggesting that IL-17A contributes to the cytokine storm. Importantly, the treatment of DR4tg mice with an IL-17A-neutralizing antibody attenuates TSS-induced tissue damage, morbidity and mortality. Collectively, the results presented in this thesis delineate the novel contributions of *i*NKT cells, MDSCs and IL-17A to the early phase of TSS pathogenesis. Furthermore, my findings suggest that therapeutic approaches targeting *i*NKT cells or IL-17A responses may be effective in reducing TSS mortality.

Keywords

Toxic shock syndrome, superantigen, cytokine storm, invariant natural killer T cells, myeloid-derived suppressor cells, interleukin-17A, effector memory T cells, transgenic mice.

Co-Authorship Statement

The investigations presented in this thesis were predominantly carried out by Peter Szabo under the supervision of Dr. S. M. Mansour Haeryfar. Details regarding authorship contributions are listed below:

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Szabo, P. designed and performed experiments, analyzed data, and wrote the first draft of the manuscript. Rudak, P., Choi, J., and Xu, S. aided in performing experiments. Schaub, R. provided the NKT14 reagent. Singh, B., and McCormick, J. provided intellectual and material support. Haeryfar, S.M.M. conceived the project, supervised all aspects of research and wrote the final version of the manuscript text.

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List of Abbreviations

αGC	α-galactosylceramide
act-D	actinomycin-D
APC	antigen-presenting cell
Bcl2	B cell lymphoma 2
CARS	compensatory anti-inflammatory response syndrome
CCAC	Canadian Council on Animal Care
CDR	complementarity determining region
CEBP	CCAAT/enhancer-binding protein
CFSE	carboxyfluorescein succinimidyl ester
COX2	cyclooxygenase 2
D-gal	D-galactosamine
DC	dendritic cell
EGR2	early growth response 2
ERK	extracellular signal-regulated kinase
FMO	fluorescence minus one
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
G-MDSC	granulocytic myeloid-derived suppressor cell
GAS	group A streptococcus
GM-CSF	granulocyte macrophage-colony stimulating factor
H&E	haematoxylin and eosin
HLA	human leukocyte antigen
IEL	intraepithelial lymphocyte

IFN	interferon
IL	interleukin
IMC	immature myeloid cell
*i*NKT	invariant natural killer T
iNOS	inducible nitric oxide synthase
ITAM	immunoreceptor tyrosine-based activation motif
IVIG	intravenous immunoglobulin
i.p.	intraperitoneal
i.v.	intravenous
JAK-STAT	Janus kinase-signal transducers and activators of transcription
JNK	c-Jun N-terminal kinase
mAb	monoclonal antibody
M-CSF	macrophage-colony stimulating factor
M-MDSC	monocytic myeloid-derived suppressor cell
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MDSC	myeloid-derived suppressor cells
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIP-2	macrophage inflammatory protein-2
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	natural killer T cells
NO	nitric oxide

PBS	phosphate-buffered saline
PGE2	prostaglandin E2
PLC	phospholipase C
PLZF	promyelocytic leukaemia zinc finger protein
qPCR	quantitative real-time polymerase chain reaction
R	receptor
ROR	retinoic acid receptor-related orphan receptor
ROS	reactive oxygen species
RPL13a	ribosomal protein L13a
SAg	superantigen
SE	staphylococcal enterotoxin
SEFIR	SEF/IL-17R
SEL	staphylococcal enterotoxin-like
SMEZ	streptococcal mitogenic exotoxin Z
SPE	streptococcal pyrogenic exotoxin
STSS	streptococcal toxic shock syndrome
TBP	TATA-binding protein
TCR	T cell receptor
T_{EM}	T effector memory
Th1/2/17	T helper type-1/2/17
TIM3	T cell immunoglobulin and mucin domain containing-3
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF	tumor necrosis factor

TRAF	tumor necrosis factor receptor associated factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Treg	T regulatory
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin-1
*v*NKT	variant natural killer T
WT	wildtype

Chapter 1

Introduction

Parts of this chapter have been adapted and published in:

Szabo, P. A., Anantha, R. V., Shaler, C. R., McCormick, J. K., and Haeryfar, S. M. 2015. CD1d- and MR1-Restricted T Cells in Sepsis. *Front. Immunol.* **6**: 401.

1.1 Preamble

The immune system is an extraordinary protective entity capable of mounting rapid and effective responses against virtually every type of pathogenic microorganism that we encounter on a daily basis. Through an elaborate network of physical barriers, anti-microbial molecules, and a myriad of cell types (*e.g.*, neutrophils, natural killer cells, monocytes, macrophages, dendritic cells, mast cells, basophils and eosinophils), the innate arm of the immune system provides a non-specific front-line defense against foreign invaders. Innate immune cells also help shape the nature of the highly specific adaptive arm of immunity, where T and B cell responses to antigens from virtually all encountered pathogens work together to limit or outright eliminate infection. This process then gives rise to immunological memory, which promotes an immune response that is both more rapid and greater in magnitude to pathogens that have been encountered previously.

Not to be outdone, pathogenic microbes engage in an evolutionary 'arms race' with the host immune system, developing sophisticated tactics for immune evasion to survive. Through numerous virulence factors, pathogens exploit opportunities to bypass immune defenses and colonize host tissues. The most successful of these pathogenic microbes are typically known to be common causes of devastating disease in humans. The Gram-positive coccus, *Staphylococcus aureus*, is among these pathogens and is currently the most common cause of skin and soft-tissue infections worldwide (1). Interestingly, around 20% of the human population appears to be persistently colonized by *S. aureus* with another 30% being intermittent carriers (2). Most often, *S. aureus* carriage is asymptomatic and considered 'harmless'. However, when afforded the opportunity, *S.*

aureus causes severe, invasive and life-threatening infections including osteomyelitis, endocarditis, pneumonia, enterocolitis, sepsis and toxic shock syndrome (TSS) (3).

One family of virulence factors, the staphylococcal superantigens (SAgs), is known to be the direct causative agent of TSS (4). These potent exotoxins trigger the hyperactivation of the immune system resulting in an avalanche of inflammatory mediators that cause intense fever, hypotensive shock and organ damage. In essence, SAgs 'short-circuit' the immune system, amplifying the immune response to the point of catastrophic collateral damage to the host. Alarmingly, this process can progress to life-threatening disease extremely rapidly, whereby multi-organ failure can occur in as little as 8-12 hours after the onset of symptoms (5) and mortality rates can range as high as one-in-five (6).

Despite the high levels of morbidity and mortality in TSS, there is currently no treatment for the overwhelming inflammation induced by SAgs. Identifying the underlying immunological mechanisms that regulate or drive TSS pathogenesis may hold the key to developing therapeutics to reduce disease severity. Here, I identify and define the role of three previously unrecognized processes mediating TSS immunopathogenesis in its most early phases.

1.2 Toxic Shock Syndrome

A syndrome of disorientation, high fever, and scarlatiniform rash with rapid progression to hypotensive shock and multi-organ dysfunction was first formally associated with *Staphyloccoccus aureus* infection in 1978 (7). It was then that Todd and colleagues coined the term 'toxic shock syndrome'. In the early 1980s, TSS rose to public consciousness following an outbreak in young females that was related to the usage of high absorbency tampons (8-10). Several cases in men and non-menstruating women were also reported during this time. A bacterial toxin from vaginal isolates of *Staphyloccoccus* in TSS patients was identified as the cause of TSS (11, 12). These toxins were later dubbed 'superantigens' due their ability to abnormally stimulate a large fraction of T cells (13). As a result, SAgs trigger a massive inflammatory response leading to the clinical symptoms of TSS (**Table 1.1**).

Cases of TSS are typically defined as either menstrual (14) or nonmenstrual (15) in origin. Menstrual TSS occurs within the first few days before or after the beginning of a woman's menstrual cycle and is strongly associated with tampon usage due, at least in part, to increases in oxygen levels in the normally anaerobic vaginal environment (16, 17). In the vast majority of menstrual TSS cases, vaginal colonization of *S. aureus* can be readily detected (18). Nearly all cases of this form of TSS are caused by *S. aureus* strains that produce the SAg toxic shock syndrome toxin-1 (TSST-1) (11, 12). Although the exact reason for this association is unclear, it has been proposed that it may be a result of the greater ability of TSST-1 to infiltrate mucosal surfaces compared to other SAgs (19, 20). A large proportion of mucosal *S. aureus* strains also more commonly produce TSST-1 relative to other SAgs, which may partly explain the association as well (21).

Clinical Criteria
1. Fever: temperature ≥ 38.9°C (102.0°F)
2. Rash: diffuse macular erythroderma
3. Desquamation: 1-2 weeks after onset of rash
4. Hypotension: systolic blood pressure ≥ 90 mm Hg for adults or less than fifth percentile by age for children aged less than 16 years
5. Multisystem involvement (three or more of the following organ systems):
• Gastrointestinal: vomiting or diarrhea at onset of illness
• Muscular: severe myalgia or creatine phosphokinase level at least twice the upper limit of normal
• Mucous membrane: vaginal, oropharyngeal, or conjunctival hyperemia
• Renal: blood urea nitrogen or creatinine at least twice the upper limit of normal for laboratory or urinary sediment with pyuria (greater than or equal to 5 leukocytes per high-power field) in the absence of urinary tract infection
• Hepatic: total bilirubin, alanine aminotransferase (ALT) enzyme, or aspartate aminotransferase (AST) enzyme levels at least twice the upper limit of normal for laboratory
• Hematologic: platelets less than 100,000/mm^3
• Central nervous system: disorientation or alterations in consciousness without focal neurologic signs when fever and hypotension are absent
Microbiological Laboratory Criteria
Negative results on the following tests, if obtained:
• Blood or cerebrospinal fluid cultures (blood culture may be positive for *Staphylococcus aureus*)
• Negative serology for Rocky Mountain spotted fever, leptospirosis, or measles
Case Classification
Probable: A case which meets the laboratory criteria and in which four of the five clinical criteria described above are present
Confirmed: A case which meets the laboratory criteria and in which all five of the clinical criteria described above are present, including desquamation, unless the patient dies before desquamation occurs

Table 1.1: Clinical criteria for toxic shock syndrome.
Adapted from (22).

In contrast to menstrual TSS, a nonmenstrual form can originate from any infection or colonization of SAg-producing strains of *S. aureus*. Non-menstrual TSS can occur in a variety of clinical settings including skin and soft-tissue infections (15, 23), surgical and post-partum wound infections (24, 25), after influenza infections (26) and burns (27-29). Additional categories also include recalcitrant erythematous desquamating syndrome (30), purpura fulminans (31), extreme pyrexia syndrome (32) and TSS-like staphylococcal enterocolitis (33), which are all associated with *S. aureus* SAgs. Approximately half of nonmenstrual TSS cases are caused by TSST-1, with the other half caused by the staphylococcal enterotoxins (SEs) A, B, and C (23, 34-36).

1.2.1 Epidemiology

At its peak in the early 1980s, the incidence of TSS was estimated to be as high as 13.7 cases per 100,000 (37). With the removal of high-absorbency tampons from the market and increased public awareness, these rates dropped to 0.5 per 100,000 by the late 1980s (24, 38). More recently, a population-based surveillance program between 2000 and 2006 indicated that the annual incidence of TSS remained stable at 0.52 per 100,000 and that approximately half of the reported cases were non-menstrual in origin (39). Notably, the investigators suggested that this might be an underrepresentation of the disease burden, likely due to both the strict CDC case definition (**Table 1.1**) and current advances in supportive therapy that prevent severe manifestations of the disease. Despite its relatively low incidence, TSS has an overall case fatality rate of 2-4% (23, 24, 39). Alarmingly, however, non-menstrual TSS mortality rates can reach as high as 12-22% in some studies (6, 23).

1.2.2 Streptococcal TSS

A significant number of *Streptococcus pyogenes* or invasive group A *Streptococcus* (GAS) infections share clinical similarities to TSS, and were designated as 'toxic shock-like syndrome' or streptococcal TSS (STSS) in the late 1980s (40-42). STSS patients have invasive GAS infections that culminate in shock, multi-organ failure and progressive soft tissue destruction (42). Unlike staphylococcal TSS, which is typically secondary to a localized *S. aureus* infection, STSS is the result of an invasive GAS infection where the majority of patients are bacteremic (42, 43). Streptococcal M types 1 and 3 have been most associated with invasive GAS infections and the SAgs streptococcal pyrogenic exotoxins (Spe) A and C are often detected in streptococcal isolates from STSS patients (40, 44, 45). Annual incidence rates of invasive GAS infections in Europe are 1.5-5.2 per 100,000 and approximately 5-14.4% of these patients developed STSS (46, 47). A study from 11 European countries (Strep-EURO) revealed an overall 7-day case fatality rate of 19% for GAS infections and 44% in patients that progressed to STSS (47).

1.2.3 Therapeutic Strategies

There are currently no specific therapeutics available for the treatment of TSS. The key to successful management of the disease is the rapid identification of the source of infection, appropriate and effective use of antibiotics, removal of tampon in menstrual TSS, and supportive care (48). In the early stages of the disease, similar therapeutic interventions as septic shock are applied, including haemodynamic support, vasopressors and/or inotropes, and intubation, if required (49).

Based on the finding that individuals lacking effective neutralizing antibodies to SAgs may be at increased risk of developing TSS (50-52), intravenous immunoglobulin (IVIG) has been proposed as an adjunctive therapy (48). IVIG is a combination of pooled human antibodies derived from hundreds to thousands of donors, which typically contain antibodies against both staphylococcal and streptococcal SAgs (53). The effectiveness of IVIG in improving clinical outcomes has been demonstrated in a number of studies involving STSS (54-57); however, evidence for its effectiveness in staphylococcal TSS is limited (48). It should also be noted that the mechanism of action for IVIG is unclear. IVIG preparations are able to neutralize both staphylococcal and streptococcal SAg activity *in vitro*, though SAgs from *S. aureus* require much higher doses of IVIG to be effectively inhibited (58). IVIG can also directly opsonize GAS, promoting bacterial clearance by enhancing phagocytosis (59). Lastly, IVIG has a general anti-inflammatory effect mediated by immunoglobulin Fc domains binding to Fc receptors on immune cells (60). While IVIG has been shown to suppress SEB-induced T cell activation, this effect is largely maintained after SEB-specific antibodies are depleted from the IVIG preparations, suggesting that a mechanism independent of toxin neutralization is in effect (61).

Several other strategies to attenuate TSS morbidity have been examined including the inhibition of SAg-receptor interactions, blockade of SAg signal transduction, and active vaccination against staphylococcal/streptococcal cell wall components or the various exotoxins themselves (62, 63). Although promising, these approaches are still in their infancy and are currently undergoing preclinical investigation. Despite the significant advances made towards understanding the immunopathogenesis of TSS, many questions

remain unanswered. A more complete understanding of the cellular interactions and biological effects of SAgs may aid in the quest for a therapeutic invention to reduce TSS severity.

1.2.4 Superantigens

SAgs are a unique group of protein exotoxins secreted by the vast majority of *S. aureus* and GAS strains that are pathogenic to humans (64). *S. aureus* encodes a large number of SAgs including TSST-1 and the SEs A, B, C, D, E, G, H, I, R and T (65). SEs were originally defined by their emetic properties and ability to cause food poisoning when ingested by humans or when administered orally to monkeys (66). A group of SE-like (SEl) toxins that are structurally similar to SEs have also been defined, although these toxins either do not induce, or have not yet been shown to induce emesis (65). Interestingly, almost all staphylococcal SAgs are encoded on mobile genetic elements including pathogenicity islands, plasmids or phages, suggesting an exchange of genetic elements between different strains or even different bacterial species (4). GAS also produces numerous SAgs including SpeA, C, G-M, streptococcal superantigen (SSA) and streptococcal mitogenic exotoxin Z (SMEZ) (64). Similar to staphylococcal SAgs, most streptococcal SAgs are encoded on mobile genetic elements.

It is important to note that SAgs are not specifically limited to *S. aureus* and GAS, nor to bacteria in general. SAgs have also been documented in the human pathogens *Plasmodium falciparum* (67), *Clostridium perfringens* (68), *Toxoplasma gondii* (69), *Yesinia pesudotuberculosis* (70) and *Yesinia enterocolitica* (71) among others.

1.2.5 Superantigen Structure and Binding Properties

SAgs are generated as pro-toxins that require cleavage of a secretion signal to be exported from the cell in a Sec-dependent manner and range in size from 22-29 kDa (65). Crystal structures of SAgs show that they are commonly made of two major protein domains, an N-terminal oligosaccharide/oligonucleotide binding fold with a β barrel motif and a C-terminal β-grasp motif, which are connected together by a central α-helix (72).

SAgs are capable of bypassing conventional mechanisms of antigen presentation and directly cross-link T cell receptors (TCRs) on T cells with major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APCs) in an unprocessed form (73, 74). SAgs interact with MHC class II outside the antigen-binding groove (75) and bind specific TCR β-chain variable domains (Vβ), inducing the activation and clonal expansion of T cells bearing the appropriate Vβ segments (76). Each SAg has a unique pattern of Vβ specificities resulting in a 'Vβ T cell signature' that can be used to identify the SAg(s) a patient is exposed to (77). Several SAgs including TSST-1, SEA and SEB also bind the co-stimulatory molecule CD28 on T cells, which is required for maximal T cell activation (78).

Based on phylogenetic analyses and structural similarities, staphylococcal and streptococcal SAgs can be classified into 5 evolutionary groups (65, 79). Group I consists of TSST-1 as an evolutionarily distinct SAg, which has a unique primary amino acid sequence compared to other SAgs. TSST-1 lacks a cysteine loop associated with emetic activity (80), further distinguishing it from SEs. It also utilizes a low-affinity MHC class II α-chain binding site and forms a TCR-MHC complex that is highly dependent on the

antigenic peptide present within the MHC class II molecule (81, 82). TSST-1 is also specific for Vβ2^{+} T cells in humans, as it recognizes specific amino acids within the complementarity-determining region (CDR) 2 and framework region 3 (83). Group II SAgs include SEB, SEC and SpeA. This group also uses a single low-affinity binding domain to the α-chain of MHC class II; however, this binding appears to be entirely peripheral to the antigenic peptide (84, 85). Notably, group II SAgs bind the TCR Vβ in a manner that is less dependent on specific amino acid side-chains, allowing for a wider range of Vβ specificities (86-88). Group III SAgs include SEA, which, along with group II SAgs, are implicated in the majority of food poisoning cases (89). In addition to the low-affinity binding site on the MHC class II α-chain used by groups I and II, group III SAgs contain a second high-affinity binding site that is zinc-dependent, which engages the MHC class II β-chain and extends over the bound peptide (90-94). It has been suggested that the additional presence of the high-affinity binding site may increase the efficiency of these SAgs to activate T cells and APCs at significantly lower concentrations. Group IV SAgs consist exclusively of streptococcal SAgs, including SPEC and SMEZ, which also bind MHC class II α-chain and β-chain similar to group III SAgs (95, 96). Lastly, group V SAgs include SEI and the SEl toxins, which use similar binding targets as group III and group IV SAgs (97).

1.2.6 Superantigen-mediated T cell activation

The mode of TCR engagement by SAgs is in stark contrast with that of conventional antigens. Typically, T cell activation is initiated by the interaction between the TCR and its cognate antigenic peptide that is presented in the context of self-MHC, called the rule of MHC restriction (98). Given that T cell antigen specificity is the result of the

variability in TCRs generated by the stochastic joining of TCR α- and β-V(D)J segments, at most only 0.01% of naïve T cells can be activated by a specific antigen (99). However, SAgs 'hijack' the immunological synapse, and activate T cells in a TCR Vβ-dependent manner, regardless of T cell antigen specificity. Since there are a limited number of TCR Vβ regions expressed by T cells, approximately 50 in humans, SAgs can activate up to 20-50% of T cells (**Fig. 1.1**) (64, 65). Furthermore, SAgs do this in an exceptionally potent manner, where as low as femtogram (10^{-15} g) quantities can stimulate human T cells *in vitro* (100).

Downstream of the TCR, T cell signaling by SAgs is relatively similar to that of conventional antigens, whereby the three canonical signals required for T cell activation are comparable for both SAgs and conventional antigens. Signal one is induced by SAg engagement of the TCR-CD3 complex, recruiting the *Src* family of protein tyrosine kinases including *Lck* (101). Activated *Lck* phosphorylates the CD3 immunoreceptor tyrosine-based activation motifs (ITAMs), recruits a variety of adaptor molecules, and facilitates a series of phosphorylation events mediated by phospholipase C (PLC)-γ1 that culminate in the activation of protein kinase C and increased intracellular Ca^{2+} (102). Together, these signaling pathways lead to activation and translocation of the nuclear factor of activated T cells (NF-AT) and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to the nucleus, facilitating the process of T cell activation and proliferation. For signal two, T cell co-stimulatory molecules (*e.g.* CD28) can either engage with their receptors (*e.g.* CD80/86) on APCs or be directly stimulated by SAgs (103). Co-stimulation is required for proper T cell activation and ensuing proliferation, cytokine production, and survival (104). The third signal is provided by the cytokine

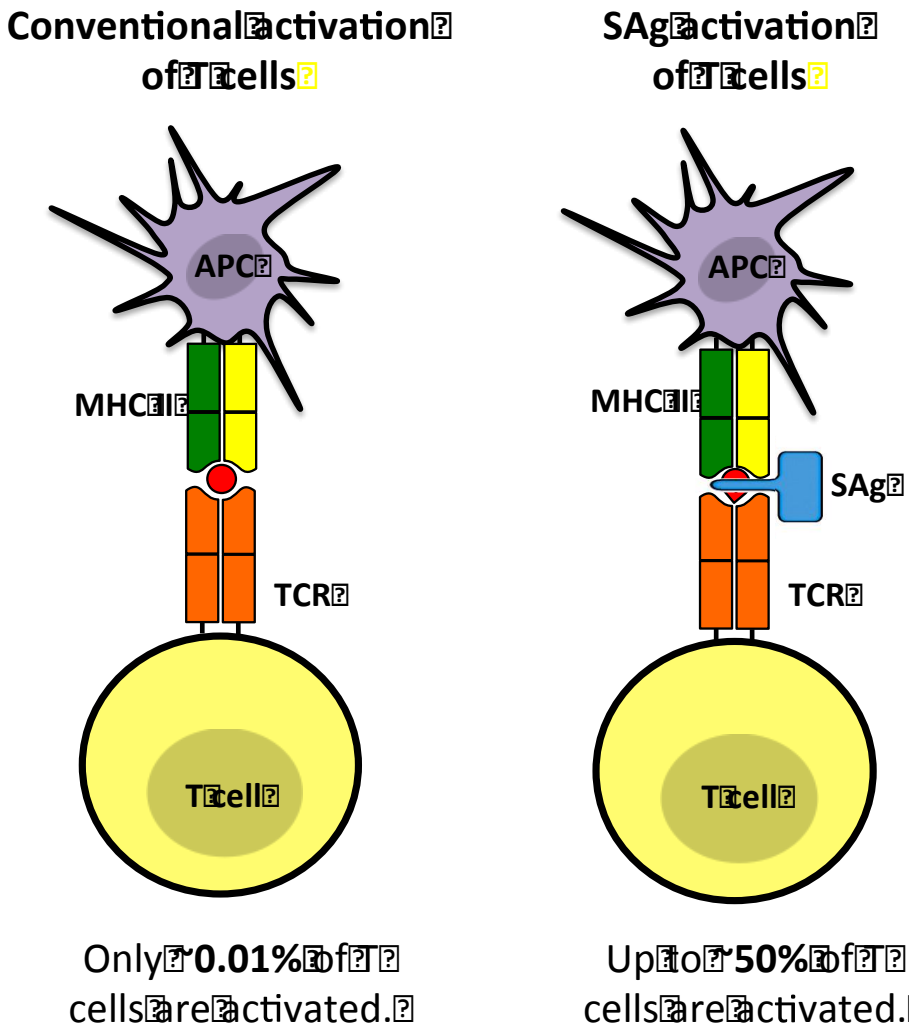

Figure 1.1: SAgs bypass conventional modes of antigen processing and presentation to activate a larger number of T cells.

Conventional protein antigens are taken up, processed into peptide fragments, and presented to T cells in the peptide-binding groove of MHC class II by APCs. In contrast, SAgs bind the lateral surfaces of MHC class II and the Vβ-domain of the TCR in unprocessed form. Whereas conventional antigens activate up to ~0.01% of T cells, SAgs may activate up to 50% of exposed T cells resulting in the overwhelming cytokine storm in TSS. Adapted from (105).

milieu present at the time of T cell activation and is essential for productive T cell responses and polarization of T cell differentiation into distinct effector subtypes (106).

In 2006, Bueno et al. found that SAg-mediated T cell signaling did not strictly require *Lck* activity and that a Gα11/PLC-β-mediated phosphorylation cascade can initiate an *Lck*-independent signaling pathway (107). Interestingly, both *Lck*-dependent and -independent pathways can be utilized by SAgs, where either can lead to the activation of transcription factors that ultimately facilitate T cell activation (108). These findings shed light on how SAgs activate both $CD4^+$ and $CD8^+$ T cells, despite only cross-linking MHC class II molecules. Since one function of the CD4/CD8 co-receptors is to recruit and activate *Lck*, an *Lck*-independent pathway would allow for T cell activation to occur in the absence of CD4/CD8 binding to MHC.

1.2.7 Cytokine Storm

The overwhelming immune activation caused by SAgs triggers a 'cytokine storm' – a rapid and uncontrolled production of cytokines and inflammatory mediators from many cell types including T cells, APCs, epithelial cells and endothelial cells. A variety of inflammatory cytokines are rapidly produced within hours of SAg exposure, including tumor necrosis factor α (TNFα), interleukin (IL)-1, IL-6, and interferon γ (IFNγ) (109, 110).

TNFα is a potent inducer of systemic inflammation and signals a range of cellular responses including apoptosis, differentiation, proliferation, migration and cytokine production (111, 112). TNFα signaling occurs through the TNF receptors (TNFR) 1 and 2, which utilize death domains (*i.e.* TNFR-associated death domain and Fas-associated

death domain) to activate the caspase 8, mitogen-activated protein kinase (MAPK), and NF-κB pathways that mediate its pleiotropic effects. In endothelial cells, TNFα elicits vasodilation, vascular permeability, and expression of pro-coagulant proteins such as tissue factor to promote intravascular coagulation (113). Together with IL-1, TNFα is likely the main mediator of inflammation-driven activation of coagulatory cascades in the vasculature (114). The administration of TNFα alone causes septic shock-like state in animals (115) and induces a systemic inflammatory response syndrome in humans (116-118).

IL-1 can be subdivided into IL-1α and IL-1β that both signal through the IL-1 receptor (IL-1R)-1 and accessory protein (IL-1RAcP) complex (119). IL-1 has similar and often synergistic inflammatory effects with TNFα (120). IL-1β mediates pro-coagulant activity and tissue factor expression in human monocytes when stimulated with SAgs (121). IL-1 and TNFα are often considered the two primary mediators of shock (122), where they act synergistically to induce sustained hypotension, pulmonary edema and hemorrhage (123). These overlapping functions may be due to the fact that both cytokines activate the NF-κB signaling pathway, which plays critical role in the initiation and propagation of the inflammatory cascade in TSS and septic shock (122, 124). This pathway promotes: i) the expression of adhesion molecules and chemokines to recruit and activate neutrophils causing endothelial cell injury, vascular permeability, and tissue damage; ii) expression of tissue factor and other pro-coagulants initiating disseminated intravascular coagulation; iii) induction of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) pathways triggering vasodilator prostaglandins and nitric oxide, respectively, which cause systemic hypotension and vascular hyporeactivity; and iv)

production of inflammatory cytokines (*e.g.* TNFα, IL-1, IL-6 and IFNγ) thus amplifying and propagating the inflammatory response. The extent of NF-κB activation has been shown to correlate with mortality risk in several contexts, including septic shock (125).

IL-6 binds to the IL-6 receptor (IL-6R) and has many different biological effects including the activation of T cells, initiation of the coagulation cascade, and neutrophil activation and trafficking (126). Along with TNFα and IL-1, IL-6 is an endogenous pyrogen, that is, a group of fever-inducing molecules that stimulate prostaglandin E2 (PGE2) synthesis, which acts on the hypothalamus to increase the thermostatic set point (127). IL-6 is also a major mediator of the acute phase response, a systemic reaction to inflammation characterized by the rapid production of acute phase reactants, such as C-reactive protein, from the liver. These proteins trigger complement activation, coagulatory cascades or promote leukocyte chemotaxis (128). Notably, serum IL-6 concentrations are markedly increased in TSS patients (129) and correlate with sepsis severity and mortality (130). In spite of these many pro-inflammatory characteristics, IL-6 can promote anti-inflammatory responses as well. While TNFα and IL-1 synergistically induce IL-6 production, IL-6 can inhibit the release of TNFα and IL-1 and promote the expression of several anti-inflammatory mediators, like IL-10 (131, 132). IL-6 responses also appear to be protective in mouse models of endotoxemia (133) and streptococcal infections (134), likely due to a suppressive effect on TNFα.

IFNγ signals through the interferon gamma receptor (IFNGR), consisting of subunits IFNGR1 and IFNG2, and performs a wide variety of immunoregulatory roles including innate immune cell activation, leukocyte trafficking, augmenting MHC class I and II expression, activation of iNOS, and promoting T helper type 1 (Th1) responses (135).

IFNγ induces the expression of the death receptor Fas, which triggers vascular cell apoptosis (136, 137). Together with TNFα, IFNγ also disrupts ion transport and barrier functions of epithelial cells leading to increased permeability (138). Indeed, IFNγ and TNFα often have synergistic effects, where many IFNγ-induced genes are also induced by TNFα, likely due to multiple avenues of cross-talk between signaling pathways (135).

A variety of chemokines are also rapidly produced in response to SAg stimulation (139, 140). TSST-1 can stimulate the production of chemokines directly from the human vaginal epithelium (141). These chemoattractants facilitate the recruitment of neutrophils, macrophages and T cells to the areas of inflammation and injury. In addition to increasing the accessibility for SAgs to activate T cells, recruited immune cells can also have direct tissue damaging effects. For example, cytokine-activated neutrophils produce cytotoxic molecules and matrix metalloproteases once in peripheral tissues (142). Recruited immune cells can also produce large amounts of inflammatory cytokines upon activation, thus amplifying the cytokine storm. SAgs activate many non-immune cells including fibroblasts, endothelial cells and epithelial cells (141, 143, 144) that produce other inflammatory molecules (*e.g.* adhesion proteins, tissue proteases and reactive oxygen species) in addition to cytokines, resulting in further tissue injury.

Overexpression of inflammatory cytokines in the cytokine storm results in widespread inflammation, fever, disseminated intravascular coagulation, leukocyte migration, vasodilation, capillary leak, shock, and tissue destruction. This process culminates in the dysfunction of multiple organ systems, which, if severe, can lead to death. Two distinct phases of multiple organ dysfunction have been proposed (145). In the early phase, the initial activation of immune cells triggers a wave of inflammatory cytokines that causes

endothelial cell damage, increased vascular permeability, swelling and extravasation. The later phase of organ dysfunction begins as the inflammatory cells reach the interstitial compartments of individual organs. Microvascular coagulopathy, dysregulated apoptosis and tissue hypoxia cause damage to parenchymal cells, thus impairing organ function (146). The reflux of inflammatory cytokines and other mediators back into circulation then creates a positive feedback loop, amplifying this process.

1.2.8 SAg-Induced Immunosuppression

Following the hyperactivation of T cells, SAgs induce a state of immune dysregulation in the body. Almost immediately after activation, surface TCR expression on SAg-reactive T cells is reduced by 50% due to receptor endocytosis (147). The activation and proliferation of SAg-reactive T cells is transient and the majority undergo apoptosis by activation-induced cell death within days (148, 149). This creates a functional 'hole' in the T cell repertoire and is sustained for a period of at least 4 weeks, when a 50% reduction of T cells bearing SAg-reactive Vβ domains can still be observed (150). Neonatal exposure to SAgs in mice also deletes virtually all SAg-reactive mature thymocytes, in addition to the majority of immature thymocytes (73). Activated T cells that escape deletion become anergic, or functionally unresponsive to *in vitro* re-stimulation by SAgs (150, 151). However, whether this translates into anergy *in vivo* is unclear. Multiple studies have demonstrated functional cytokine responses upon SAg re-challenge, though SAg-reactive $CD4^+$ T cells, and $CD8^+$ T cells, to a lesser extent, show impairments in their proliferative capacity (152-154). Exposure to SAgs also generates immunosuppressive T regulatory cells (Tregs) (155-158), which may contribute to the induction of T cell anergy. Whether Tregs play a protective role in TSS is unclear, as

systemic inflammatory responses in TSS destabilize the Treg population, and their expansion or adoptive transfer in mice exposed to SAg fail to mitigate immunopathology (159).

In addition to causing T cell dysfunction, SAgs impair B cell-mediated humoral immunity (160-162). Low or negative antibody titres for TSST-1 are observed in greater than 90% of menstrual TSS patients, with the majority of patients failing to seroconvert within two months (50). Some patients even remain seronegative even after recurrent bouts of the disease. This is in contrast to a healthy population, where 85% of individuals show antibody titres to TSST-1 at a level thought to be at least partially protective (52). The failure to develop effective antibody-mediated immunity is likely due to impairment in the T cell compartment during TSS. T cells anergized by SAgs can no longer provide help for B cells to stimulate antibody production, thus impairing T cell-dependent B cell responses (161, 162). SAgs also induce T cell-dependent B cell apoptosis (163-165). Lastly, SAgs largely promote Th1-type cytokine responses. Therefore, the minimal Th2-type cytokines present may be insufficient to support optimal B cell proliferation and differentiation (124).

1.2.9 Animal Models of TSS

Preclinical animal models that simulate SAg-mediated immunopathology in humans have greatly aided the characterization of molecular and cellular events in TSS. Despite our current understanding, however, there has yet to be an effective therapy translatable to the clinic. The careful and appropriate use of animal models of TSS (listed in **Table 1.2**) remains our most essential tool for the development of novel treatment modalities to reduce TSS severity and, hopefully, save human lives.

Animal	Model	SAgs	Defining Immunopathology	Reference
Mouse	Sensitizing agents (D-gal/act D)	SEA, SEB, TSST-1	TNFα-dependent lethal shock; severe hemorrhagic hepatitis	(166-170)
	SAg + LPS	SEA, SEB, SEC, TSST-1	Rapid cytokine storm; hypothermia; intestinal apoptosis; lethal shock	(169, 171-175)
	HLA-transgenic	SEB SpeA	Rapid cytokine storm; multi-organ inflammation; impaired gut permeability; lethal shock	(170, 176-178)
	Aerosol/ i.n	SEB	Rapid cytokine storm; pulmonary edema, hypothermia; liver neutrophil infiltration and necrosis; lethal shock	(179-184)
Rabbit	Mini-osmotic pump	SEA, SEB, TSST-1 SpeA SpeC	Rapid cytokine storm; fever; conjuctival hyperemia; anorexia, cachexia; lethal shock.	(185-189)
	SAg + LPS	TSST-1, SEH	TNFα production; hyperemia; diarrhea; lethal shock	(190, 191) (192, 193)
	i.v.	SEA, TSST1	IL-2, IFN, TNF production; fever; lethal shock (strain and sex dependent)	(191, 193-195)
Primate	i.v.	SEB	Hypotension; cardiac output decline; decreased blood oxygen content; lethal shock	(196)
	Aerosol	SEB	IL-2, IL-6, IFNγ production; hypotension; fever; diarrhea; vomiting; pulmonary edema; lethal shock	(197-199)

D-gal, D-galactosamine; act D, actinomycin D; LPS, lipopolysaccharide; tg, transgenic; i.n., intranasal; i.v., intravenous.

Table 1.2: Common animal models of TSS

Mouse models are most often used to examine the underlying mechanisms of SAg-induced morbidity, largely due to the variety of available immunological tools and reagents, well-defined Vβ signatures, and relatively low cost of maintenance compared to other animals. Problematically, mice are considerably more resistant to SAgs when compared to humans as a result of a much lower binding affinity of SAgs to mouse MHC class II in some strains (200). Therefore, sensitizing compounds such as actinomycin D (167) or D- galacatosamine (D-gal) (166) must be used to amplify the effects of SAgs and induce mortality. The severity of TSS in models using sensitizing agents varies considerably and can skew inflammatory responses or disproportionately impact individual organs. One of the most widely utilized models involves priming mice with D-gal (166, 168, 169, 201, 202) followed by intraperitoneal administration of SAg to induce a cytokine storm and other hallmark features of TSS. D-gal induces transcriptional arrest in hepatocytes leading to extreme sensitivity to TNFα-induced apoptosis (203). When used in combination with SAgs, D-gal induces severe TNFα-dependent hepatotoxicity (204), a pathology not typically seen in human TSS (205). Although TNFα responsible for the lethality in sensitized mice is derived, at least in part, from SAg-mediated T cell activation (166, 206), the dominant event leading to sudden death in this model is likely severe hepatitis. As expected, TNFα plays a key role in this model and anti-TNFα/β neutralizing antibodies confer protection from lethality (166, 207). Conclusions from these models must be drawn with full recognition that sensitizing agents cause cytokine responses that do not necessarily reflect human disease. This problem is illuminated by the notable failure of a TNFR fusion protein to prevent septic shock in humans, including those caused by *S. aureus* (208). Notably, in patients with Gram-positive infections, the

group receiving the highest-dose of the drug exhibited an increase in both disease severity and mortality.

Another common mouse model utilizes bacterial lipopolysaccharide (LPS) as a potentiating agent following SAg exposure (169, 171-174, 209, 210). SAgs are known to substantially enhance LPS lethality due to a synergistic effect on the production of TNFα (192). There is some evidence to suggest this synergy occurs in human TSS, as patients with menstrual TSS show increased colonization of Gram-negative bacteria in the vaginal flora (211) and low levels of LPS can be detected in acute-phase sera of TSS patients (212). In mice, the injection of SAg followed by LPS results in rapid production of IL-1α, IL-6, TNFα and IFNγ within hours, which is not observed in SAg-only controls (171). Lethality in this model can be correlated to increased serum IL-6, TNFα, macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1) by 8 hours and IFNγ and IL-2 by 21 hours after TSS induction (174). However, cytokine responses and TSS mortality are highly influenced by LPS dosage as opposed to SAg. Furthermore, exposure to SAg in human peripheral blood mononuclear cells (PBMCs) strongly upregulates TLR4 expression and enhances production of inflammatory cytokines by LPS independent of T cells (210). Thus, the use of LPS as a potentiating agent may cause unnecessary confounding effects that are more reflective of endotoxicosis than TSS. As the role of LPS in human TSS is undefined, the utility of this mouse model and its relevance to TSS also remains to be determined.

In order to avoid potential pitfalls associated with sensitizing and potentiating agents in mouse models of TSS, mice expressing human MHC class II, known as HLA-transgenic mice, have been used (170, 176-178, 213-215). Due to the higher affinity binding of

SAgs to human leukocyte antigens (HLAs)-DQ and -DR, these transgenic mice respond to much lower doses of SAg (216-219). Accordingly, our lab has previously demonstrated robust cytokine responses in HLA-DR4tg mice injected with 50 μg of SEB (220). Similarly, a single 50 μg dose of SEB is sufficient to induce a rapid inflammatory cytokine response, inflammation in the liver, lungs, kidney, heart and small intestines, and lethal shock in HLA-DR3 transgenic mice (170, 177). Importantly, in comparison to D-gal sensitization where the disease is restricted to the liver, HLA-DR3 mice treated with SAg alone show multi-organ involvement, similar to humans (170). The inhibition of IFNγ in this model attenuates the cytokine storm, prevents severe intestinal pathology, and protects against mortality (177). HLA transgenic mouse models have also been successfully applied to STSS. HLA-DQ8 transgenic mice expressing human CD4 are susceptible to SpeA-induced STSS that is characterized by rapid production of TNFα, IFNγ and IL-6, elevated liver enzymes and lethal shock within 72 hours (178). A potential limitation for using HLA-transgenic mice as disease models is that MHC class II genes are among the most polymorphic genes in the genome and these inbred mouse strains only mimic the responses of one specific haplotype. Furthermore, SAgs preferentially bind some MHC class II molecules over others; SEB and TSST-1 bind HLA-DR more efficiently than HLA-DQ for example (221), and thus care must be taken to select the most appropriate transgenic strain for a given SAg. Nevertheless, HLA-transgenic mice recapitulate many aspects of human TSS and therefore are one of the most useful mouse models of the syndrome.

Several other mouse models have been established to investigate different aspects of TSS pathogenesis by varying the SAg entry point. A model of SAg-induced airway

inflammation was developed involving a single or double dose of SAg introduced intranasally (179-184). TSS in this model culminates in severe lung inflammation, systemic release of cytokines, hypothermia and death within days. However, this may more accurately represent TSS resulting from the inhalation of aerosolized SEB in a bioterrorism setting (222) rather than natural *S. aureus* infection. Intravaginal exposure to SEB in HLA-transgenic mice causes a robust systemic inflammatory response and leukocyte infiltration in the liver and lungs (223). Although almost all human menstrual TSS cases are caused by TSST-1 and not SEB, further investigations in this model can be targeted to understand and/or inhibit the mechanisms by which SAgs can cross mucosal barriers and cause disease.

Historically, rabbits have been the 'gold standard' model for SAg-induced TSS as they mimic almost all symptoms of human TSS after administration of staphylococcal and streptococcal SAgs (185, 186, 191, 192, 194, 224, 225). Rabbits can also be implanted with mini-osmotic pumps that release SAg continuously over the course of 7 days, more accurately mimicking the mode of SAg exposure in a *S. aureus* infection (185-189). Several rabbit models that utilize chambers to localize SAg-producing *S. aureus* have been instrumental in identifying TSST-1 a causative agent of TSS (226-229). The susceptibility to SAg-induced TSS varies among rabbit strains and may require LPS sensitization to induce morbidity (191). Currently, the use of rabbits to model TSS is rare, likely due to the technical and ethical considerations that make larger animals unattractive to study. There are several drawbacks of using rabbits rather than mice, such as increased housing and animal care costs, less availability of immunological reagents and antibodies, and a greater difficulty for genetic manipulation.

In addition to mice and rabbits, several models of TSS utilizing larger animals have been described. Intradermal exposure to SEB in non-human primates elicits skin reactions, cutaneous mast cell degranulation and emesis (230). Non-human primates have also been used to investigate immune responses to aerosolized SEB (197-199) and evaluate the efficacy of vaccine candidates against lethal SEB challenge (231, 232). In the hopes of more closely simulating human TSS, a porcine model was developed where piglets were given SEB intravenously. Intoxicated piglets quickly developed vomiting, diarrhea, temperature spikes, hypotension and multi-organ dysfunction (233). Lastly, goats have been used to study the effects of both SEB and TSST-1-induced TSS, where intravenous injection yields tachycardia, fever and various hematological changes (234). Investigating TSS pathogenesis in larger animals is rare, due to both the high cost of animal maintenance and significant ethical concerns surrounding their use as disease models with high morbidity and mortality.

1.3 Invariant Natural Killer T Cells

Natural killer T (NKT) cells are a specialized group of innate-like T lymphocytes with remarkable immunomodulatory properties. Named for their co-expression of both NK cell markers (e.g. NK1.1 in mice and CD161 in humans) and αβ TCRs, NKT cells possess specific reactivity to glycolipid antigens presented by the MHC class I-like glycoprotein CD1d (235). CD1d is a member of the CD1 family of antigen-presenting molecules that present lipids, rather than peptides, to non-MHC restricted T lymphocytes. In humans, there are five members of CD1 molecules, CD1a-e, while mice only express CD1d (236). CD1d is widely expressed on cells of hematopoietic origin, including typical antigen-presenting cells like DCs, macrophages and B cells (237).

Two individual subsets of NKT cells can be defined based on the structural characteristics of their TCRs in addition to their reactivity to glycolipid antigens. Type I NKT cells, known as *i*nvariant NKT (*i*NKT) cells, express a canonical TCR composed of a unique α-chain (Vα24-Jα18 in humans; Vα14-Jα18 in mice) paired with a limited number of β-chains (Vβ11 in humans; Vβs 2, 7, or 8.2 in mice) (235). These cells exhibit highly conserved reactivity to α-galactosylceramide (αGC) (238) and the invention of αGC-loaded CD1d tetramers has enabled their unambiguous identification by flow cytometry (239, 240). In mice, *i*NKT cells are either $CD4^+CD8^-$ or $CD4^-CD8^-$ (235), and an additional $CD8^+$ population exists in humans (241). *i*NKT cells also constitutively express the activation/memory markers CD25, CD44 and CD69 on their cell surface, consistent with a 'memory-like' phenotype. In contrast, type II NKT cells, known as variant NKT (*v*NKT) cells, express a diverse αβ TCR repertoire and although they are 'CD1d-restricted', they do not respond to αGC (235). A large proportion of *v*NKT cells

can be activated by the self-lipid sulfatide (242). The understanding of *v*NKT cell responses in health and disease remains limited, largely due to a notable lack of robust reagents to specifically identify and characterize *v*NKT cell function.

*i*NKT cells are widely distributed throughout the body and can be found in the thymus, liver, bone marrow, spleen, lymph nodes and intestinal tract (239). They express a range of chemokine receptors that mediate their homing to individual tissues and, unlike conventional T cells, show limited recirculation under homeostatic conditions (243). In mice, *i*NKT cells have been most commonly studied in the liver, where they represent up to 20-30% of lymphocytes (239, 240). However, they are less frequent in all other tissues, where they constitute ≤1% of T cells. In humans, *i*NKT cells are not highly enriched in the liver and are 10-fold less frequent in most locations (244), except the human omentum, where approximately 10% of T cells express the invariant Vα24-Jα18 TCR (245). On average, *i*NKT cells represent ~0.1% of T cells in the peripheral blood of humans, but this fraction can range from virtually undetectable to as high as 1% across individuals (246, 247). The reason for this extreme variation among individuals as well as the differences in abundance and tissue distribution between mice and humans is unknown.

1.3.1 Modes of Activation

*i*NKT cells are activated in response to exposure to a wide range of pathogens, from bacteria to viruses, protozoa and even fungi (248). Many of these microorganisms contain lipid antigens, which can directly stimulate the *i*NKT cell TCR through their presentation by CD1d on APCs. These exogenous antigens are usually glycerol-based lipids (e.g. common bacterial membrane phospholipids) or ceramide-based glycolipids (e.g.

glycolsphingolipids) and can be found in *Spingomonas* spp., *Ehrlichia* spp., *Borrelia burgdorferi*, *Aspergillus fumigatus,* and *Streptococcus pneumoniae* (249-253). Additionally, other *i*NKT cell ligands include mycobacterial phosphatidylinositol mannoside (254), cholesteryl α-glucosides from *Helicobacter pylori* (255), and lipophosphoglycans from the protozoans *Leishmania donovani* (256) and *Entamoeba histolytica* (257).

The prototypical exogenous antigen for *i*NKT cells is the glycosphingolipid αGC. Initially isolated from the extracts of the marine sponge *Agelas mauritanius* (238), αGC is believed to have originated from microbes (possibly *Sphingomonas* spp.) engaged in a symbiotic relationship with the sponge. αGC is composed of a lipid tail that is buried deep in the hydrophobic region of CD1d and galactose head that projects out of the CD1d molecule to be bound by the α-chain of the *i*NKT cell TCR (258). The length of the sphingosine and acyl chains of the lipid tail controls the binding affinity of the TCR-lipid-CD1d complex, which partially determines the strength of *i*NKT cell activation (259). Based on this observation, αGC derivatives with altered sphingosine and/or acyl chains that skew *i*NKT cell responses have been generated. OCH, a sphingosine-truncated analog of αGC promotes the production of Th2-type cytokines, exemplified by IL-4 (260). C20:2, which possesses a shorter acyl chain that contains two sites of unsaturation also potently stimulates the production of Th2-type cytokines (261), perhaps more effectively than OCH in human PBMC cultures (262). Conversely, a C-glycoside analog of αGC is a Th1-skewing *i*NKT agonist that effectively induces IL-12 and IFNγ production (263). In addition to the structure of the lipid agonist, several other factors influence the production of cytokines by *i*NKT cells including the location and kinetics of

glycolipid loading onto CD1d and its association with lipid rafts (264), the type of APC involved (265) and the presence of co-stimulatory molecules on APCs (266).

In the absence of exogenous lipid antigens, *i*NKT cells can be activated by APCs that have been exposed to microbial products or danger signals, which engage pattern recognition receptors such as Toll-like receptors (TLRs) to induce cytokine secretion (267, 268). Interestingly, this mode of activation is dependent on IL-12 in addition to CD1d-lipid signaling, suggesting that the presentation of self-antigens can facilitate *i*NKT cell activation. This is supported by the fact that *i*NKT cells are activated in a variety of settings where exogenous lipids are absent, such as in viral infections, autoimmunity and cancer (269). There has been much debate around the nature of glycolipids involved in this process, in addition to the putative self-lipid responsible for the positive selection of *i*NKT cells in the thymus. Many candidates for self-lipids that mediate these processes have been proposed, including isoglobotrihexosylceramide (270), lysophosphatidylcholine (271), β-glucosylceramide (272), and the plasmogen lysophosphatidylethanolamine (273). A recent report by Teyton and colleagues demonstrates the presence of endogenous α-anomeric glycolipids like αGC in mammalian immune cells (274). However, the biological role of endogenous αGC in humans remains to be confirmed.

As innate T lymphocytes, *i*NKT cells are highly responsive to cytokines for their activation and function. *i*NKT cells constitutively express several cytokine receptors, including the receptors for IL-12 (275), IL-18 (276), IL-23 (277) and IL-25 (278). IL-12 appears to play a critical role in the activation of *i*NKT cells both in the absence or presence of *i*NKT cell ligands. *i*NKT cell-mediated responses to *Salmonella*

typhimurium, which lacks cognate *i*NKT cell antigens, is blocked by IL-12-neutralizing antibodies (267). The activation of *i*NKT cells by the *S. typhimurium* LPS is also IL-12-dependent. Even in microorganisms for which *i*NKT cell lipid antigens have been identified, such as *S. pneumoniae*, effective *i*NKT cell responses also depend on IL-12, suggesting that these cytokine-driven signals may be the dominant pathway for *i*NKT cell activation (279). A recent study using reporter mice that detect TCR signaling showed that while some bacteria that produce *i*NKT cell antigens signaled through the TCR, others thought to require presentation of self-lipids (*e.g.* those derived from *S. typhimurium*) induced robust *i*NKT cell responses in the absence of antigenic stimulation (280). This pathway was likely mediated by IL-12 and IL-18, which are able to induce *i*NKT cell activation entirely independent of TCR stimulation (276). Based on these observations, a general model of *i*NKT cell activation has been proposed where: a) microbes that possess high-affinity cognate *i*NKT cell lipid antigens can activate *i*NKT cells predominantly through the TCR with little or no need for cytokine stimulation; or b) microbes possessing *i*NKT cell antigens or lacking cognate antigens entirely can activate T cells through innate cytokine-driven pathways that may or may not include the presentation of endogenous *i*NKT cell antigens by APCs (281).

Lastly, our group has recently demonstrated that group II bacterial SAgs can activate both mouse and human *i*NKT cells (220). This activation was independent of CD1d but required antigen-presenting cells expressing the human MHC class II molecule HLA-DR.

1.3.2 Effector Functions

As their name suggests, *i*NKT cells are armed with a variety of cytotoxic effector molecules including Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) that

are able to directly kill target cells (282, 283). However, they are best known for their ability to swiftly produce both pro- and/or anti-inflammatory cytokines upon activation. The rapidity of this response may be due, at least in part, to the constitutive expression of cytokine mRNAs (284). The sheer variety of cytokines produced by *i*NKT cells, including IFNγ, TNFα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-21, IL-22 and GM-CSF, further underscores their ability to shape ensuring immune responses downstream of their activation (285-290).

Similar to conventional MHC-restricted $CD4^+$ T cells, *i*NKT cells can be generally categorized into separate functional subsets based on their patterns of cytokine production. The fates of individual *i*NKT cells are therefore determined both by 'master regulator' transcription factors like promyelocytic leukemia zinc finger protein (PLZF) and early growth response 2 (EGR2) as well as 'cytokine-specific' transcriptional regulators like T-bet (Th1-like), GATA3 (Th2-like) and RORγT (Th17-like) (291). Th1-like *i*NKT cells predominantly produce IFNγ in response to αGC or IL-12 stimulation and constitute the majority of *i*NKT cells in the mouse liver (292). They can be $CD4^{+/-}$ and $NK1.1^{+/-}$ but characteristically express the CD122 (IL-15R β chain) and require IL-15 for homeostasis. In contrast, Th2-like *i*NKT cells produce minimal IFNγ and high amounts of IL-4, IL-9, IL-10 and IL-13 (292). Most of these cells express CD4 and IL-25R (IL-17RB), and are enriched in mouse lymph nodes and lungs. Interestingly, Th2-like *i*NKT cells are up to 8-fold more abundant in Balb/c compared to C57BL/6 mice, which may partly explain the predominance of Th2 responses in Balb/c mice (293). Th17-like *i*NKT cells principally produce IL-17A and IL-22 in response to stimulation and are typically $CD4^-$ and $NK1.1^-$ (292). IL-17A-producing *i*NKT cells also express IL-

25R and are enriched in the lungs, skin and peripheral lymph nodes (292, 294). In addition to these three main *i*NKT cell subsets, other functional populations of *i*NKT cells have been defined, most notably NKT10 cells. Characteristically producing the immunosuppressive cytokine IL-10, NKT10 cells are $CD4^+$IL-$25R^+$ and enriched in adipose tissue (292). These cells are also a naturally occurring population in human peripheral blood (61).

Through the expression of such a wide variety of cytokines and surface molecules, *i*NKT cells transactivate many different downstream effector cells and can modulate their function. DCs constitutively express CD1d and are a major APC population responsible for mediating *i*NKT activation *in vivo* (265, 295). During this process, CD40-CD40L interactions trigger the maturation of DCs to secrete IL-12 (275), promote $CD8^+$ T cell cross-priming (296) and enhance conventional $CD4^+$ and $CD8^+$ T cell responses to protein antigens (297, 298). *i*NKT-DC interactions trigger NK cell transactivation, yielding sustained IFNγ production (299). *i*NKT cells can also provide cognate (300) and non-cognate (301) B cell help to promote antibody responses. Furthermore, *i*NKT cells can modulate myeloid-derived suppressor cell (MDSC) responses, though the nature of this interaction is unclear. During influenza infection, the absence of *i*NKT cells results in the expansion of MDSC populations in mice and *i*NKT cell adoptive transfer abrogates the immunosuppressive function of MDSCs (302). In contrast, the activation of *i*NKT cells by αGC induces the expansion and immunosuppressive function of MDSCs in a mouse model of multiple sclerosis (303).

1.3.3 Experimental Models to Study *i*NKT Cell Functions

There are several available mouse models for the studying *i*NKT cells in health and disease. Vα14-Jα18 transgenic mice overexpress the invariant TCRα chain Vα14-Jα18 of *i*NKT cells, significantly biasing T cell populations towards the *i*NKT cell lineage. Numerous mice with this phenotype have been developed to study the role of the invariant TCRα chain on the development of *i*NKT cells or to overcome the paucity of *i*NKT cells in wild-type mice (304, 305). In contrast, $CD1d^{-/-}$ mice are deficient in all NKT cells due to the absence of CD1d, preventing positive selection of all NKT cells in the thymus (306). As such, $CD1d^{-/-}$ mice are devoid of both *i*NKT and *v*NKT cells and therefore any phenotype using these mice cannot be ascribed to one cell subset over the other. $J\alpha18^{-/-}$ mice have a targeted deletion of the Jα18-coding region specific for the *i*NKT cell TCR (307). Since these mice lack only *i*NKT cells many developmental and disease studies utilize both $J\alpha18^{-/-}$ and $CD1d^{-/-}$ mice in combination to decipher the functions of *i*NKT and/or *v*NKT cells individually from one another. However, a recent report revealed that $J\alpha18^{-/-}$ mice demonstrate a significant reduction in TCRα repertoire diversity (308). The authors propose that, as a result of the insertion of a deletion cassette in the TRAJ18 region (coding for Jα18), all gene rearrangements upstream of TRAJ18 have been suppressed. Importantly, novel $J\alpha18^{-/-}$ mice that do not possess this defect have been recently generated (309, 310), but are not yet widely available.

Currently, no commercially available antibody for the selective depletion of *i*NKT cells exists. A recently generated *i*NKT cell-specific antibody called NKT14 was reported to effectively deplete *i*NKT cells *in vivo* for up to 3 weeks (311). Although extensive

characterization of NKT14-mediated *i*NKT cell depletion is lacking, this antibody holds significant future promise once commercially available.

1.4 Myeloid-Derived Suppressor Cells

As the immune system has evolved many different effector mechanisms to combat invading pathogens, it has also developed regulatory pathways to keep runaway inflammatory processes in check. MDSCs have recently emerged as key players in the regulation of immune responses in many different inflammatory diseases. Although the majority of research has focused on MDSC function and manipulation in cancer (312-314), further investigations have highlighted their importance in autoimmunity (315), transplantation (316), bacterial (317), viral (318) and parasitic infections (319) and more. Originally described as 'bone marrow suppressor cells' (320), these cells have the remarkable capacity to potently suppress T cell responses and secrete pro- and anti-inflammatory cytokines to module both innate and adaptive immune responses. Due to these immunoregulatory qualities, MDSCs appear to resolve acute inflammation in some contexts and contribute to immunopathology in others.

1.4.1 Definition

MDSCs are a heterogeneous population of myeloid progenitor cells and immature myeloid cells (IMCs) (*i.e.* neutrophils, monocytes/macrophages and dendritic cells) defined by their ability to suppress T cell responses (312). Rather than a distinct subset of cells, MDSCs are a mix of myeloid populations that have been prevented from differentiating into mature cells and express surface markers indicating various stages of immaturity (321). MDSCs can be uniformly characterized in mice by their expression of the CD11b and Gr-1 surface markers. Gr-1 consists of the antigens Ly-6G, expressed on neutrophils, and Ly-6C, expressed on macrophages. Notably, anti-Gr-1 antibodies simultaneously bind both epitopes. Based on morphological and phenotypic differences,

two major subsets of MDSCs have been defined: granuolocyctic (or polymorphonuclear) MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs) (322). In mice, G-MDSCs express a $CD11b^{+}Gr\text{-}1^{hi}Ly6G^{+}Ly6C^{lo}$ phenotype and possess ‘band-like' nuclei, more typical of neutrophils. M-MDSCs are $CD11b^{+}Gr\text{-}1^{mid}Ly6G^{-}Ly6C^{hi}$ and are mononuclear in appearance, more typical of monocytes. G-MDSCs typically outnumber M-MDSCs in most cases and G-MDSCs appear to expand to a greater extent as well (323, 324). In culture, M-MDSCs largely retain the ability to differentiate in mature myeloid cells, whereas the majority of G-MDSCs remain as immature $CD11b^{+}Gr\text{-}1^{+}$ cells (324). Furthermore, M-MDSCs have been suggested to be more suppressive on a per cell basis (323, 325), although this may also depend on the disease and/or inflammatory context.

In humans, G-MDSCs are defined as $CD11b^{+}CD14^{-}CD15^{-}/CD66b^{+}$ and M-MDSCs as $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}CD15^{-}$ (322). CD33 is often used instead of CD11b as a myeloid marker and can be helpful in separating the two subsets, as G-MDSCs stain as $CD33^{dim}$ and M-MDSCs are $CD33^{hi}$ (326). A third subset of MDSCs that contain a mixed population more indicative of immature progenitors, known as early-stage MDSCs (eMDSCs), are defined in humans, and have no mouse equivalent (322). These cells are Lin^{-} (including CD3, CD14, CD15, CD19, CD56) $HLA\text{-}DR^{-}CD33^{+}$.

Although the surface phenotype of MDSCs is necessary for their identification, it does not distinguish them from their mature myeloid counterparts. Presently, there are no combination of surface markers that can discriminate between G-MDSCs from neutrophils and M-MDSCs from monocytes. Therefore, a suppression assay demonstrating the ability of putative MDSCs to inhibit T cell responses is also required (322). While many different methods to demonstrate T cell suppression by MDSCs have

been employed, most take the form of an *in vitro* T cell co-culture with varying ratios of MDSCs in addition to a T cell stimulus, anti-CD3/CD28 for example. The resulting suppression of T cell activity in the presence of MDSCs is then determined. Typically, ^{3}H-thymidine incorporation or fluorescent carboxyfluorescein succinimidyl ester (CFSE) dye dilution assays are used to evaluate T cell proliferation, while ELISA/ELISPOT can be used to measure T cell-mediated IFNγ responses (322). The presence of surface markers indicative of MDSCs in combination with a demonstrated ability to effectively suppress T cell function remains the 'gold standard' to merit the designation of MDSC (322).

It should be noted that one of the most controversial aspects regarding the definition of MDSCs is why cells with a morphology and surface phenotype resembling neutrophils or monocytes should require separate designations as G-MDSCs or M-MDSCs. Indeed, immunosuppressive neutrophils (327) and monocytes (328) can be found during inflammatory conditions. Koenderman and colleagues go so far as to suggest that G-MDSCs are an alternative functional heterogenic subset of neutrophils (329). During sepsis, the re-programming of monocytes into compensatory anti-inflammatory response (CARS)-monocytes, which have the same function and phenotype of M-MDSCs, can be also thought of as an alternative monocyte differentiation pathway (330). M-MDSCs from breast cancer patients display a more similar gene expression profile to CARS-monocytes from sepsis patients than to monocytes from healthy controls or tuberculosis patients (331). To help resolve this dilemma, a summary of recommendations for MDSC characterization standards was recently established by Bronte et. al (322). In addition to the established requirements to define MDSCs by surface phenotype and

immunosuppressive capability by the assays described above, the investigators propose four distinct molecular and biochemical parameters to distinguish MDSCs from their myeloid counterparts: i) transcription factors and apoptotic regulators (e.g. active STAT3, RORC1, and S100A8/A9 proteins); ii) immunosuppressive genes and molecules (e.g. arginase, iNOS, ROS and PGE2); iii) immunoregulatory cytokine production (e.g. IL-10 and TGFβ); and iv) mediators that contribute to MDSC development and function (e.g. GM-CSF, G-CSF and IL-1). Although these parameters are not exhaustive and may not necessarily define MDSCs in all disease contexts, they constitute an important step forward to guide the growing field of MDSC immunobiology.

1.4.2 Mechanisms of Immunosuppression

MDSCs employ major immunosuppressive pathways to potently inhibit both $CD4^+$ and $CD8^+$ T cell activity. MDSCs produce high levels of arginase-1, which metabolizes L-arginine into L-ornithine, thus depleting L-arginine in the local environment. As a result, T cells lose the expression of the CD3ζ chain, an essential component of the TCR complex responsible for intracellular signal transduction (332). Arginine starvation also causes T cell cycle arrest due to reduced expression of cell cycle regulators cyclin D3 and cyclin-dependent kinases 4 (333). Predictably, T cells activated in the absence of arginine show markedly reduced proliferation and cytokine production, but can be rescued by arginine supplementation *in vitro* (332), or by depletion of arginase-producing MDSCs *in vivo* (334). Both subsets of MDSCs demonstrate arginase-1 activity; however, whether arginase-1 plays a major role as an immunosuppressive factor for either G-MDSCs or M-MDSCs is unclear (323, 325).

MDSCs produce another enzyme that metabolizes L-arginine, iNOS, which catalyzes the synthesis of the potently immunosuppressive molecule, nitric oxide (NO). NO itself impairs T cell responses to IL-2 by directly impairing IL-2R signaling, which is critical for T cell proliferation (335). In a similar manner, NO reduces IL-2 mRNA expression and impairs IL-2 secretion from activated T cells (336). NO also decreases the expression of MHC class II on APCs (337), potentially limiting antigen presentation or SAg binding to reduce T cell activation. Furthermore, iNOS activity in the presence of low cytosolic arginine (depleted by arginase-1) promotes the generation of superoxide (O_2^-) and, consequently peroxynitrite, which is a product of superoxide and NO (338). Peroxynitrites are among the most powerful oxidants in the body and cause nitration or nitrosylation of key amino acids regulating T cell function. The production of peroxynitrite by MDSCs directly induces nitration of tyrosine residues in the TCR and CD8 co-receptor of T cells impairing antigen-specific $CD8^+$ T cell responses (339). Peroxynitrite-mediated nitration in human T cells also triggers apoptosis, due to the inhibition of protein tyrosine phosphorylation in activated T cells (340). The NO pathway is preferentially used by the M-MDSC subset as evidenced by inhibition of iNOS function by L-NG-monomethyl arginine citrate (L-NMMA) in suppression assays, which blocks the activity of M-MDSCs but not that of G-MDSCs (323, 325).

In contrast to M-MDSCs, G-MDSCs generate reactive oxygen species (ROS) as a primary means of immunosuppression (323). Increased ROS production by G-MDSCs is mediated by the up-regulated activity of NADPH oxidase, which generates superoxide (341). In addition to forming peroxynitrites, superoxides also react with protons in water to generate hydrogen peroxide (H_2O_2), which is the main ROS to suppress T cells

responses (342). ROS directly reduce CD3ζ chain expression in T cells, impairing T cell proliferation and cytokine production (343, 344). ROS also promote apoptosis in activated T cells through the downregulation of the anti-apoptotic protein B cell lymphoma 2 (Bcl2) (345). The inhibition of ROS production by the H_2O_2 scavenger catalase reverses G-MDSC mediated T cell suppression in both mice and humans (342, 344).

In addition to the major mechanisms described above, several other pathways of MDSC-mediated T cell suppression have been identified. MDSCs can indirectly promote immunosuppression through the activation of Treg cells by multiple routes. The expression of CD40 (346), CD80 (347) and arginase (348) by MDSCs have been separately shown to induce Treg differentiation and function. MDSC production of the immunoregulatory cytokines IL-10 and tumor growth factor β (TGFβ) also facilitates Treg function (349). Additionally, MDSCs express galactin 9 (350), the ligand for T cell immunoglobulin and mucin-domain containing-3 (TIM-3) expressed on Th1 cells, which triggers apoptosis (351). Lastly, MDSCs can suppress T cells by the local depletion of cysteine, an amino acid essential for T cell activation and survival (352). However, as the vast majority of MDSC function involves the use of murine cancer models, the extent to which these mechanisms are at play in the many different disease contexts that show MDSC involvement has yet to be determined.

1.4.3 Origin, Expansion and Functions

As a heterogeneous population of IMCs, MDSCs originate in the bone marrow under normal conditions of myelopoiesis (330). In this process, hematopoietic stem cells give rise to multi-potent progenitors, which then become common myeloid progenitors and in

turn generate IMCs. Under steady-state conditions, IMCs proceed to differentiate into their respective mature myeloid populations or exit the bone marrow to complete their development in the periphery. In naïve mice, IMCs that express CD11b and Gr-1 can be found at low levels in multiple tissues, including bone marrow (20-30% of myeloid cells), spleen (2-4%), liver (2-5%) and lymph nodes (<1%), and are not immunosuppressive (321). However, during pathological conditions, overwhelming inflammation or cancer for example, IMC populations expand and acquire immunosuppressive qualities to become MDSCs.

The expansion of MDSCs is rooted in 'emergency myelopoiesis', where IMCs are rapidly mobilized from the bone marrow to the blood or sites of inflammation to restore or renew myeloid populations during infection (330). Several growth factors responsible for the regulation and maturation of myeloid cells promote this process, including macrophage colony-stimulating factor (M-CSF), granulocyte-CSF (G-CSF), and granulocyte/macrophage-CSF (GM-CSF) (353). However, a variety of inflammatory mediators also trigger MDSC expansion, including IL-1 (354), hepatic acute phase proteins (355), PGE2 (356) and S100A8/9 (357). These factors commonly converge on the Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3) pathway, a major regulator of MDSC function (312). The activation of STAT3 in MDSCs stimulates their expansion and survival through increasing myelopoiesis and expression of anti-apoptotic proteins. Recently, retinoic-acid-related orphan receptor 1 (RORC1) was identified as another critical transcription factor regulating the accumulation of MDSCs through promoting myelopoiesis and preventing MDSC apoptosis (358). Interestingly, many of the same mediators that stimulate the expansion

of MDSCs also block IMC maturation and differentiation to terminal myeloid cells. S100A9 (359), vascular endothelial growth factor (360) and PGE2 (361) have been shown to mediate accumulation of MDSCs through subverting conventional myeloid differentiation pathways. Both emergency myelopoiesis and defective myeloid cell maturation have been associated with hyperactivation of STAT3, suggesting that these processes may be linked in a positive feedback loop.

In addition to expansion, MDSCs require an 'activation signal' to induce their suppressive activity, many of which are inflammatory mediators produced in abundance during infection. The activation of the NF-κB pathway, through inflammatory signals IL-1, TNFα and TLR ligands, strongly regulates the suppressive capability of MDSCs (362-364). MDSCs deficient in MyD88, a universal adaptor protein used by almost all TLRs and the IL-1R, lose their suppressive abilities and even acquire immunostimulatory functions (365). Notably, the accumulation of MDSCs remains unaffected. TNFα induces the suppressive function of MDSCs through regulation of iNOS activity via NK-κB (366) or through the activity of S100A8/9 proteins (367). However, this appears to primarily affect only M-MDSCs (368), which makes sense as iNOS is often undetectable in G-MDSCs. The inflammatory cytokine IFNγ, linked to the activation of STAT1, is also associated with upregulation of iNOS and arginase-1 expression in M-MDSCs (369). Interestingly, IFNγ signaling in G-MDSCs may actually hinder suppressive ability and cell survival indicating that certain inflammatory signals may have opposite effects on the different subsets (370). The Th2 cytokines IL-4 and IL-13, typically thought of as anti-inflammatory in many cases, stimulate the expression of arginase-1 in MDSCs (371, 372). This signaling pathway mediated by STAT6 is also involved in promoting the

accumulation (373) and survival of MDSCs (374). Lastly, PGE2 augments the suppressive capabilities of MDSCs in addition to its role in MDSC expansion and differentiation blockade. PGE2 upregulates arginase-1 expression in MDSCs (375). It also appears to be involved in a positive feedback loop with the upstream enzyme COX2. PGE2 increases the expression of COX2 in monocytes, blocking their differentiation into DCs converting them to M-MDSCs, and inducing endogenous PGE2 production in addition to other immunosuppressive factors (361).

1.5 Interleukin-17A

IL-17A is a potent inflammatory cytokine that plays a critical role in the host defence against extracellular bacterial, parasitic and fungal infections (376). Impaired IL-17A functions in mice are linked to susceptibility to a wide variety of pathogenic microbes, including *Staphylococcus aureus* (377), *Streptococcus pneumoniae* (378), *Listeria monocytogenes* (379), *Trypanosoma cruzi* (380), and *Candida albicans* (381). In humans, genetic deficiencies in IL-17A signaling also significantly increase susceptibility to chronic mucocutaneous infections caused by *C. albicans* and *S. aureus* (382). However, excessive IL-17A responses are also associated with a variety of chronic inflammatory disorders including psoriasis, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease among others (383). IL-17A exerts its inflammatory effects primarily through induction of downstream inflammatory cytokines, chemokines, effector molecules and antimicrobial proteins. As the IL-17A receptor complex is widely expressed throughout both hematopoietic and non-hematopoietic tissues, IL-17A is a key player in shaping the systemic inflammatory environment.

1.5.1 The IL-17 Family

There are six members of the IL-17 family: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F (384). IL-17A, the eponymous cytokine of the IL-17 family, was first identified as a product of T cells that induces NF-κB activation and IL-6 secretion from human fibroblasts (385). It is the most completely characterized member of the IL-17 family and is well known to promote systemic inflammatory responses. IL-17F is most similar to IL-17A and shares approximately 50% sequence homology (386). Both cytokines are produced individually as covalent homodimers or as IL-17A/F

heterodimers that share many similar functions (387, 388). Together, IL-17A, IL-17F and IL-17A/F all signal through the same receptor complex composed of IL-17RA and IL-17RC (389, 390). However, the signaling potency of IL-17A is substantially greater than IL-17F, with the IL-17A/F heterodimer exhibiting an intermediate phenotype (388). Interestingly, the individual receptor subunits have differential affinities for IL-17A and IL-17F. IL-17RA binds with high affinity to IL-17A but extremely low affinity to IL-17F, whereas IL-17RC binds IL-17F with greater affinity than IL-17A (389). The expression patterns of IL-17RA and IL-17RC also appear to be reciprocal. In contrast to IL-17RA, which is highly expressed in lymphoid tissues such as the spleen, liver, thymus, lymph nodes, IL-17RC is highly expressed in the intestines and glandular tissue including the adrenal glands, prostate and thyroid (385, 389, 391). Although IL-17A and IL-17F share many overlapping and similar functions, these differences in receptor binding affinity and tissue expression may explain why IL-17A and IL-17F do not always act in a functionally redundant manner. In general, IL-17A appears to have a more central role in promoting autoimmunity and IL-17F contributes to airway hypersensitivity and allergy (392), though both are involved in anti-microbial responses (391).

The other IL-17 family members IL-17B, IL-17C and IL-17D remain poorly characterized. They are classified as inflammatory cytokines based on their ability to stimulate neutrophil responses or induce expression of pro-inflammatory genes (393-396), though their functions in many disease states remain unknown. IL-17B signals through the IL-17RB receptor but the signal transduction pathway has yet to be defined. IL-17C binds to the heterodimeric receptor IL-17RA/IL-17RE and regulates immune responses in epithelial cells in an autocrine fashion (395, 397). IL-17D is the least

characterized member of the IL-17 family and can be found in the brain, heart, lungs, pancreas, muscle and adipose tissue (396). Its receptor has yet to be identified.

The final member of the IL-17 family, IL-17E, is also known as the Th2-promoting cytokine IL-25 (398). IL-25 is expressed in a wide variety of cell types, including both immune and non-immune cells, and signals through IL-17RB in complex with IL-17RA (399). As a strong inducer of Th2 type immune response (e.g. IL-4, IL-5, and IL-13), IL-25 is critical in the defense against parasitic infections but also effectively limits intestinal inflammation (400). Interestingly, IL-25 has the lowest homology to the IL-17 family, and unlike other members, actively limits Th17 cell differentiation by promoting IL-13 production by activated DCs (401).

1.5.2 Sources of IL-17A

IL-17A is the signature cytokine produced by the Th17 subset of $CD4^+$ effector T cells (402, 403). Th17 differentiation from naïve $CD4^+$ T cells is driven by the combination of TGFβ, IL-6 and IL-21 (376), which induce the expression of the 'master regulator' transcription factor RORγT (404). RORγT expression is required for IL-17A production and for upregulation of the IL-23 receptor (IL-23R), a hallmark surface marker for Th17 cells. IL-23 signaling through its receptor is crucial to stabilize the lineage commitment of developing Th17 cells and for their maintenance in the periphery (376). In humans, Th17 cells are long-lived effector memory T (T_{EM}) cells with a high capacity for self-renewal (405). These cells are a terminally differentiated population of memory cells (*i.e.* antigen-experienced) that are capable of rapid effector function upon re-activation. In addition to IL-17A, Th17 cells also secrete copious amounts of IL-17F, IL-21, IL-22, GM-CSF, and TNFα (376). Due to their inflammatory nature, Th17 cells not only play an

important role in the clearance of extracellular microbes, particularly at mucosal surfaces, but also drive inflammatory pathology in autoimmune diseases.

Importantly, IL-17A is also produced by a host of other immune cells given the right inflammatory context. Most prominently, innate-like T lymphocytes like γδ T cells are a rapid source of IL-17A due to their constitutive expression of IL-23R and RORγT (406). They can be activated solely by innate cytokine-driven signals in the absence of TCR engagement and are highly enriched at mucosal surfaces providing first-line defence against invading pathogens. Similarly, Th17-like *i*NKT cells (discussed previously) produce IL-17A when stimulated with their lipid agonists (286). The recently discovered innate lymphoid cell (ILC) lineage also contains a subset of IL-17A producing cells known as group 3 ILCs (407). This group of ILCs is reliant on RORγT for development and consists of lymphoid tissue inducer cells, responsible for the formation of secondary lymphoid organs, and ILC3s, which are innate immune cells that express the NK cell marker NKp46 yet do not have a cytolytic capacity. Lastly, several innate immune myeloid cells have also been reported to produce IL-17A, including neutrophils (408), monocytes (409) and mast cells (410).

1.5.3 Signaling Pathway

Typically, Th1- and Th2-type cytokines signal through JAK-STAT pathways, which promote Th cell differentiation in addition to facilitating effector functions. Instead, IL-17A signaling is more similar to innate immune pathways such as the IL-1 family or TLR ligands. IL-17 family receptors encode conserved cytoplasmic motifs with distinct homology to Toll/IL-1R (TIR) domains, known as SEF/IL-17R (SEFIR) domains (411). Just as TIR domains are essential for the binding of intracellular adaptor MYD88 for IL-

1/TLR signaling, the binding of IL-17A to its receptor complex recruits the signaling adaptor molecule ACT1 via SEFIR domain interactions (412). ACT1 serves both as an adaptor molecule and an E3 ubiquitin ligase, recruiting and ubiqutinating TNFR-associated factors (TRAFs), including TRAF6 (413). TRAF6 is a critical signaling molecule facilitating the activation of NF-κB pathway and several MAPK pathways such as extracellular signal regulated kinase (ERK), p38 and JNK (414). IL-17A signaling also activates the CCAAT/enhancer binding proteins (C/EBPs) C/EBPβ and C/EBPδ, which act in concert with NF-κB to induce IL-17A target genes (415, 416). Furthermore, ACT1 recruits TRAFs 2 and 5 that promote the activation of the RNA-binding protein Hur, which stabilizes the mRNA of both IL-17A and other downstream effector genes (417, 418).

1.5.4 Inflammation

As the direct targets of IL-17A signaling include a host of cytokines, chemokines and inflammatory effector molecules, IL-17A is clearly a potent inducer of systemic inflammatory responses. In fact, the inflammatory cytokine IL-6 is one of the earliest known gene targets of IL-17A (419) and its production still remains the standard bioassay for measuring IL-17A activity. IL-17A signaling triggers a positive feedback loop of IL-6 expression (420) thus amplifying both Th17 cell differentiation and activation of the acute phase response. IL-17A directly stimulates the production of a variety of inflammatory cytokines including TNFα, IFNγ, IL-1β, IL-6, IL-8 and IL-12 from macrophages and DCs (421, 422). Further, IL-17A induces COX-2 and microsomal prostaglandin E-synthase expression that catalyze the production of the inflammatory mediator PGE2 (423, 424).

Many non-hematopoietic cell types such epithelial cells, endothelial cells and fibroblasts express the IL-17RA/C complex and thus are among the main targets of IL-17A. Stimulation of these cells by IL-17A induces the secretion of IL-6, IL-8, G-CSF and PGE2 (419). Endothelial cells also respond to IL-17A by increasing the expression of tissue factor, which promotes coagulopathy (425). Epithelial, endothelial and mesenchymal cells are important sources of chemokines induced by IL-17A, such as the neutrophil chemoattractants CXCL1, CXCL2, CXCL5 and CXCL8/IL-8 (426). Neutrophil responses are also further bolstered by IL-17A through the production of G-CSF, which promotes granulopoiesis and neutrophil accumulation (427). Importantly, IL-17A plays a critical role in triggering granulopoiesis and neutrophil recruitment that is required for host defence against microbial pathogens, such as *Klebsiella pneumoniae* (428). Other chemokine targets of IL-17A include CCL2 and CCL20, which mediate monocyte and dendritic cell migration to inflamed tissue (429, 430), and CXCL9-12, which are potent T cell chemoattractants (431, 432).

A major factor potentiating the inflammatory effects of IL-17A is a strong synergism with other cytokines in regulating its downstream targets. IL-17A induces IL-6 production synergistically with IFNγ, IL-1β, IL-22, and in particular, TNFα (426). The underlying molecular mechanisms for this synergy are not completely understood and likely involve multiple factors. First, IL-17A enhances the mRNA stability of cytokines and chemokines that contain AU-rich elements in their 3’ untranslated region (433, 434). Similarly, IL-17A markedly enhances TNFα-induced IL-6 mRNA expression resulting in significantly augmented protein production (435). IL-17A-mediated stabilization of mRNA has also been demonstrated for G-CSF, GM-CSF, CXCL1, CXCL2, CXCL5, IL-

8 and COX-2 (426). A second potential mechanism of synergy is the convergence of IL-17A and other cytokine signaling pathways at the level of target gene transcription. Both IL-17A and TNFα upregulate the IL-6 promoter through the cooperative activation of the transcription factors C/EBPδ and NF-κB (416). Many IL-17A-target genes have promoters that contain binding sites for C/EBPδ and NF-κB suggesting this may be the case for a variety of IL-17A-induced products (415). Lastly, IL-17A can regulate the expression of cytokine receptors, including TNFR II, to enhance the signaling capacity of inflammatory cytokines (436). In a similar fashion, the synergy between IL-17A and IL-22 in the production of IL-20 subfamily cytokines is due to the upregulation of the IL22R (437). IL17A and IL-22 also synergize in the production of S100A8/9 proteins, though the exact mechanism facilitating this process is unknown (438). It should be noted that although IL-17A itself is a relatively modest inducer of NF-κB *in vitro* (384), due to its synergistic functions with other cytokines it can play a critical role in mediating inflammation or anti-microbial immunity *in vivo*.

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Chapter 2

Rationale and Specific Aims

2.1 Rationale

Toxic shock syndrome (TSS) is a potentially fatal illness characterized by a systemic inflammatory response triggered by exposure to superantigens (Sags) (1). These bacterial toxins directly cross-link T cell receptors (TCRs) on T cells with major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs) to activate an extraordinary number of T cells. This overwhelming activation of T cells causes rapid and massive production of inflammatory mediators known as a 'cytokine storm', beginning hours after SAg exposure. The cytokine storm initiates a cascade of drastic tissue-damaging effects including disseminated intravascular coagulation, vascular leak and tissue hypoxia. If unchecked, this inflammatory process culminates in the dysfunction of multiple organ systems and possibly death. Alarmingly, there currently are no available therapeutic options to attenuate the cytokine storm. Successful treatment of TSS relies almost entirely on supportive therapy and critical care (2). Given the rapidity with which TSS progresses to life-threatening disease, identifying early mediators of the cytokine storm may hold the key to designing effective therapies that mitigate the severity of TSS. Furthermore, much of our understanding regarding TSS immunopathology relies on imperfect animal models that are limited in their ability to reflect human disease. Mouse models of TSS have been hampered by the fact that SAgs bind to certain mouse MHC molecules with very poor affinity resulting in minimal T cell activation and little cytokine production (3). Transgenic mice expressing humanized MHC (*e.g.* HLA-DR) that are exposed to SAgs have been shown to faithfully recapitulate many aspects of human TSS (4-6). Therefore, my objective is to utilize an HLA-DR4 transgenic (DR4tg) mouse model to identify and characterize regulatory and effector

immune responses in the early phase TSS. I hypothesize that rapid effector and suppressor cell responses are critical to TSS pathogenesis and may be targeted to reduce disease severity.

2.2 Specific Aims

2.2.1 Aim 1: To delineate the role of invariant natural killer T cells in TSS immunopathogenesis

Invariant natural killer T (*i*NKT) cells are a subset of T lymphocytes possessing innate-like characteristics and are able to rapidly produce copious amounts of immunomodulatory cytokines upon activation. As a result, they can effectively modulate the function of many downstream regulatory and effector cells types, thus shaping the nature of the ensuing immune response. *i*NKT cells can also be therapeutically targeted by glycolipid agonists to polarize them towards a pro- or anti-inflammatory phenotype. Previous work in our lab utilizing DR4tg mice demonstrated that SAgs activate *i*NKT cells to produce inflammatory cytokines *in vivo* (7). However, whether *i*NKT cells play a regulatory or effector role during TSS was unknown. In light of our previous findings, I hypothesized that *i*NKT cells are pathogenic in TSS and contributed to the cytokine storm. In Chapter 3, I compared TSS morbidity and mortality in DR4tg mice to a newly generated strain of '*i*NKT-deficient' DR4tg mice, dubbed 'DJ' mice. These findings were confirmed using the recently described *i*NKT cell-depleting monoclonal antibody NKT14 (8). To determine whether *i*NKT cells participate in the cytokine storm, I quantified the levels of serum cytokines in DR4tg and DJ mice within the first 24 hours of TSS onset. Finally, I ascertained whether the polarization of *i*NKT cells towards an anti-inflammatory phenotype by the glycolipid agonist OCH (9) could reduce TSS severity. Here, I provided the first report addressing the role of *i*NKT cells in TSS pathogenesis.

2.2.2 Aim 2: To determine the identity and function of a suddenly prominent population of hepatic myeloid cells in a humanized mouse model of TSS

While investigating TSS in a DR4tg mouse model, I detected a suddenly prominent population of cells in the liver within hours of SAg exposure. These cells exhibited a high forward- and side-scatter phenotype by cytofluorimetric analysis, consistent with cells of the myeloid lineage. Given that severe and/or chronic inflammation drives the accumulation of myeloid-derived suppressor cells (MDSCs) in peripheral tissues, such as in sepsis (10) or cancer (11), I hypothesized that these hepatic myeloid cells were MDSCs. Importantly, the presence of MDSCs in TSS was yet to be defined and therefore their role in TSS was unknown. In Chapter 4, I characterized the surface phenotype of putative MDSCs using a panel of fluorochrome-labeled mAbs. I also determined the capacity of these cells to prevent SAg-activated T cell responses via a suppression assay. Using chemical inhibitors of MDSC pathways, I established the major effector molecule responsible for MDSC-mediated T cell suppression. Lastly, I generated human MDSCs *in vitro* to address whether they were able to similarly able to prevent autologous T cells responses to SAg. Taken together, I demonstrated for the first time the accumulation and function of hepatic MDSCs in TSS.

2.2.3 Aim 3: To define the contribution of interleukin-17 to TSS immunopathology

Interleukin-17A (IL-17A) is a potent inducer of systemic inflammation, stimulating the production of a variety of inflammatory mediators including cytokines, chemokines, and transcription factors from many cell types. Importantly, IL-17A both directly triggers and acts synergistically with inflammatory cytokines involved in the TSS cytokine storm, namely tumor necrosis factor α (TNFα), interferon γ (IFNγ), IL-1 and IL-6 (12). It can also be rapidly produced upon activation of conventional $CD4^+$ T helper type 17 (Th17) cells in addition to innate-like T lymphocytes such as γδ T cells and *i*NKT cells (13). However, whether SAgs elicit IL-17A responses that contribute to the cytokine storm and potentiate TSS immunopathogenesis was unexplored. A previous investigation in our lab identified the presence of IL-17A in serum within hours of exposure to SAg in DR4tg mice (7). Thus, I hypothesized that SAgs trigger a rapid IL-17A response that promotes TSS immunopathology. In Chapter 5, I characterized the magnitude, timing and source of IL-17A production in both DR4tg mice and human peripheral blood mononuclear cells (PBMCs) in response to SAgs. Utilizing a mAb to the IL-17A receptor in human PBMCs, I identified the effects of IL-17A signaling on the expression of key downstream inflammatory mediators implicated in TSS-associated cytokine storm. Finally, I investigated the *in vivo* significance of IL-17A function in the DR4tg mouse model of TSS and assessed its impact on disease morbidity, mortality and tissue damage. Collectively, this study reveals for the first time the role of IL-17A in the cytokine storm and TSS-mediated immunopathology.

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Chapter 3

Invariant NKT Cells are Pathogenic in the HLA-DR4-Transgenic Humanized Mouse Model of Toxic Shock Syndrome and Can Be Targeted to Reduce Morbidity

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3.1 Introduction

Staphylococcal enterotoxin B (SEB) and other superantigens (SAgs) secreted by *Staphylococcus aureus* cause human illnesses ranging from mild food poisoning to potentially fatal toxic shock syndrome (TSS) (1). Systemic exposure to SAgs results in polyclonal stimulation of T cells irrespective of their cognate specificity. SAgs are inherently capable of binding to select Vβ regions of the T cell receptor (TCR) and lateral surfaces of MHC class II harbored by antigen-presenting cells. Consequently, rapid and massive release of inflammatory mediators is triggered, which can be followed by fever, shock and organ failure.

Invariant natural killer T (*i*NKT) cells are innate-like T lymphocytes with profound immunoregulatory properties (2). Mouse *i*NKT cells express a canonical TCRα chain with a characteristic Vα14-Jα18 rearrangement, which is paired with one of a limited number of Vβ chains. *i*NKT cells uniquely recognize glycolipid antigens presented by CD1d.

We previously established that mouse and human *i*NKT cells can directly respond to group II bacterial SAgs, including SEB, in a CD1d-independent manner (3). However, whether these cells play a pathogenic or regulatory role during TSS is unknown. This is an important question in light of the facts that: i) *i*NKT cells are strategically located at the host-pathogen interface; ii) they exhibit a ‘pre-activated’ or ‘memory-like’ phenotype (2) and can, as such, be quickly activated; iii) *i*NKT cells possess pre-formed mRNA for certain inflammatory cytokines (4), which enables them to secrete such cytokines amply and speedily; iv) *i*NKT cells exert pleiotropic effects on numerous downstream effector/regulatory cell types including NK, T, B and dendritic cells, macrophages and

myeloid-derived suppressor cells. Therefore, they shape the nature of ensuing immune responses; v) *i*NKT cells are easily 'polarizable' towards a pro- or anti-inflammatory phenotype. Therefore, they can be manipulated by synthetic glycolipids in a disease-tailored fashion (5).

In this work, we have used a humanized mouse model mimicking several aspects of TSS and two distinct experimental strategies, namely genetic deficiency and antibody-mediated depletion of *i*NKT cells, to determine if: i) *i*NKT cells are pathogenic effectors of TSS; ii) deviating *i*NKT cell responses towards a T_H2 phenotype ameliorates the severity of TSS.

3.2 Materials and Methods

3.2.1 Ethics

This study was conducted using a protocol approved by the Western University Animal Use Subcommittee and in full adherence to the Canadian Council on Animal Care guidelines.

3.2.2 Mice

Age- and sex-matched adult mice were used in all experiments. Wild-type C57BL/6 mice were purchased from Charles River Canada. HLA-DR4-transgenic (DR4tg) mice on a C57BL/6 background were bred in our barrier facility. These animals are endogenous MHC class II (IAβ)-deficient but express a chimeric HLA molecule encoded by HLA-DRA-IEα and HLA-DRB1*0401-IEβ transgenes (6).

To develop an *i*NKT cell-deficient mouse strain that is humanized to express HLA II, DR4tg mice were crossed with Jα18$^{-/-}$ mice, on a C57BL/6 background, which lack *i*NKT cells (7). The resultant HLA-DR4tg.Jα18$^{-/-}$ mice, hereafter referred to as DJ mice, were bred to homozygosity and examined for the presence of HLA-DR transgenes, Jα18 and IAβ, or lack thereof, by PCR. Briefly, ear clip genomic DNA was prepared using HotSHOT, and PCR was performed using Platinum Taq DNA polymerase (Thermo Fisher). PCR products were visualized by agarose gel electrophoresis. Primers used for genotyping are listed in **Table 3.1**.

Wild-type C57BL/6, Jα18$^{-/-}$, DR4tg and DJ mice were all housed in the same facility.

Probe/Primer	Sequence (5'-3')/Assay ID	Dye	Source
Vα14-Jα18 Primer (Forward)	TGGGAGATACTCAGCAACTCTGG	N/A	Sigma-Aldrich
Vα14-Jα18 Primer (Reverse)	CAGGTATGACAATCAGCTGAGTCC	N/A	Sigma-Aldrich
Vα14-Jα18 TaqMan Probe	CACCCTGCTGGATGACACTGCCAC	FAM-MGB	Thermo Fisher Scientific
TBP TaqMan Assay	Mm00446973_m1	FAM-MGB	Thermo Fisher Scientific
HLA-DRA-IEα Primer (Forward)	GGGAAGCAGGGGGACTATGAC from Ref (6)	N/A	Sigma-Aldrich
HLA-DRA-IEα Primer (Reverse)	TTAGGGCAATGACTTCGTAGG from Ref (6)	N/A	Sigma-Aldrich
HLA-DRB1*0401-IEβ Primer (Reverse)	TGAAAGCGGTGCGTGCTGTTTAA from Ref (6)	N/A	Sigma-Aldrich
HLA-DRB1*0401-IEβ Primer (Reverse)	CACCCGCTCCGTCCCGTTGAA from Ref (6)	N/A	Sigma-Aldrich
HLA-DRtg Insertion Primer (Forward)	CCATGGACAAGGCAGGGACAAA	N/A	Sigma-Aldrich
HLA-DRtg Insertion Primer (Reverse)	CCGTGACCAAAATGCACATTGAA	N/A	Sigma-Aldrich
IAβ-neo^r Primer (Forward)	GGGAGGAGTACGTGCGCTACGACAG	N/A	Sigma-Aldrich
IAβ-neo^r Primer (Reverse)	GAGAACCTGCGTGCAATCCATCTTG	N/A	Sigma-Aldrich
Jα18 TCR Primer (Forward)	CCCTCTTTCCTCCCTTCCCACCTT	N/A	Sigma-Aldrich
Jα18 TCR Primer (Reverse)	ACAATGCCCTCCACCTTAGTCCTGA	N/A	Sigma-Aldrich
Jα18-neo^r Primer (Forward)	CCCTCTTTCCTCCCTTCCCACCTT [same as Jα18 TCR Primer (Forward)]	N/A	Sigma-Aldrich
Jα18-neo^r Primer (Reverse)	ATCGCCTTCTATCGCCTTCTTGACG	N/A	Sigma-Aldrich

Table 3.1: PCR probes/primers used for genotyping and gene expression

3.2.3 Immunophenotyping

Splenic single cell suspensions were prepared in cold, sterile PBS using a Wheaton Dounce tissue grinder. Non-parenchymal hepatic mononuclear cells were isolated by density gradient centrifugation in 33.75% Percoll PLUS (GE Healthcare). For cytofluorimetric analyses, cells were pre-incubated with 5 μg/mL anti-mouse CD16/CD32 (clone 2.4G2) before they were stained with fluorochrome-conjugated monoclonal antibodies (mAbs) and tetramers listed in **Table 3.2**. A FACSCanto II cytometer and FlowJo software were used to acquire and analyze data, respectively.

3.2.4 *i*NKT cell depletion

DR4tg mice were injected *i.p.* with 200 μg of an *i*NKT cell-depleting mAb, NKT14 (8), which was supplied by NKT Therapeutics Inc. (Waltham, MA). A mouse IgG2a (clone C1.18.4 from BioXCell) was utilized as isotype control. To confirm *i*NKT cell depletion in NKT14-treated mice, splenic and hepatic mononuclear cells were stained with PBS-57-loaded mouse CD1d tetramer or with empty tetramer (staining control), which were both provided by the NIH Tetramer Core Facility. An aliquot of the same cell suspensions were subjected, in parallel, to real-time quantitative PCR (qPCR) assaying for the Vα14-Jα18 TCRα gene rearrangement. In brief, RNA was extracted using a PureLink RNA Mini Kit and converted to cDNA by SuperScript VILO Master Mix (Thermo Fisher). Contaminating genomic DNA was eliminated using on-column PureLink DNase. PCR was performed using a StepOnePlus System, TaqMan Probes listed in **Table 3.1** and TaqMan Fast Advanced Master mix (Thermo Fisher). Gene

Target	Species Reactivity	Host Species	Clone	Isotype	Fluoro-chrome	Source
TCRβ	Mouse	Armenian Hamster	H57-597	IgG	FITC	eBioscience
HLA-DR	Human	Mouse	G46-6	IgG2a, κ	PE	BD Pharmingen
*i*NKT TCR (PBS-57-loaded CD1d tetramer)	Mouse	N/A	N/A	N/A	APC	NIH Tetramer Core Facility
CD4	Mouse	Rat	GK1.5	IgG2b, κ	FITC	eBioscience
CD8	Mouse	Rat	53-6.7	IgG2a, κ	PE	eBioscience
CD11c	Mouse	Armenian Hamster	N418	IgG	APC	eBioscience
CD19	Mouse	Rat	eBio-1D3	IgG2a, κ	FITC	eBioscience
CD45R (B220)	Mouse/ Human	Rat	RA3-6B2	IgG2a, κ	PE	eBioscience
F4/80	Mouse	Rat	BM8	IgG2a, κ	APC	eBioscience
NK1.1	Mouse	Mouse	PK136	IgG2a, κ	PE-Cy 7	eBioscience

Table 3.2: Fluorochrome-conjugated antibodies/tetramers used for *i*NKT cell studies

expression fold-changes were calculated by $\Delta\Delta C_T$ using TATA-binding protein (TBP) as the reference gene.

3.2.5 TSS induction and treatment

Recombinant SEB was cloned from the *Staphylococcus aureus* strain COL, expressed in *E. coli* BL21 (DE3), and purified via nickel column chromatography. Mice were injected *i.p.* with 100 μg SEB and monitored for up to 7 days, the maximum duration allowed under our protocol. Animals were euthanized when moribund or when 20% weight loss was reached.

Separate cohorts of mice were bled 2, 12 and/or 24 hours after they were injected with SEB or with 4 μg of α-galactosylceramide (αGC) (Funakoshi). Serum cytokine analyses were performed by bead-based multiplexing (Eve Technologies), and the matrix2png utility was employed to generate heat maps.

In several experiments, DR4tg mice were treated *i.p.* with the T helper 2 (T_H2)-polarizing *i*NKT cell agonist OCH (NIH Tetramer Facility) at 4 μg/dose every day beginning one day before or 2 hours after SEB injection. Control DR4tg mice received vehicle containing 0.5% Tween-20, 56 mg/mL sucrose and 7.5 mg/mL histidine. Animals were monitored for weight loss and signs of morbidity.

3.2.6 Statistical analysis

Statistical comparisons were made using Student's *t*-test, ANOVA or log-rank test as appropriate. *, **, ***and **** denote $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively.

3.3 Results and Discussion

We previously demonstrated that bacterial SAgs directly activate *i*NKT cells (3). Rieder *et al.* reported that intranasal administration of SEB causes acute lung injury in wild-type mice, but to a lesser degree in Jα18$^{-/-}$ or CD1d$^{-/-}$ mice (9). However, intranasal inoculation of SEB does not provoke a cytokine storm (9) among other features of TSS, and the overall *in vivo* significance of *i*NKT cells in TSS pathogenesis remains unknown – hence the current investigation.

SAgs display poor affinity for MHC II molecules expressed in certain conventional mouse strains including C57BL/6 mice (10), which partially explains why such animals do not provide *bona fide* models in which to study host responses to SAgs. Therefore, we used DR4tg mice that express a humanized MHC II molecule that can establish interactions of sufficient affinity with SEB (11). Using this model also eliminates the need for pre-sensitization of mice with commonly used but notoriously toxic agents such as D-galactosamine (12).

We took two logistically distinct approaches to investigate the role of *i*NKT cells during TSS in the DR4tg model. First, we crossed DR4tg mice with germ-line *i*NKT cell knockout mice (Jα18$^{-/-}$) to generate an *i*NKT cell-deficient, SEB-responsive strain. We confirmed that the resulting "DJ" mice express HLA-DR but are devoid of *i*NKT cells (**Fig. 3.1A**). The frequencies of CD4^{+} and CD8^{+} T cells, B cells, dendritic cells, macrophages and NK cells were comparable in DR4tg and DJ mice (**Fig. 3.2**). When systemically exposed to SEB, DJ mice lost significantly less weight than did *i*NKT-sufficient DR4tg mice (**Fig. 3.1B**). Strikingly, by day 7, 75% of DJ mice had survived, whereas all DR4tg mice succumbed to the SAg challenge (**Fig. 3.1B**).

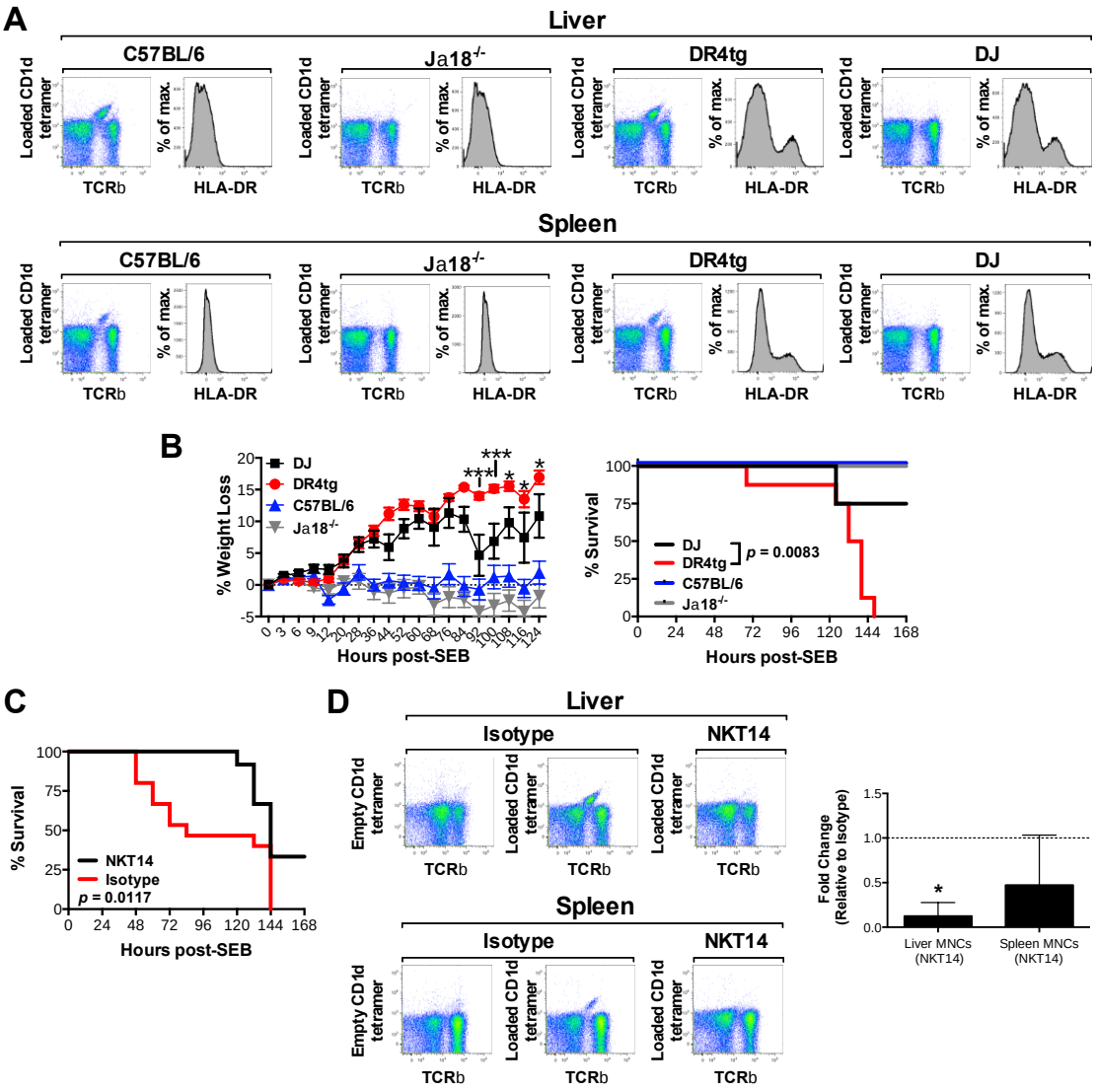
A
Liver
C57BL/6
Ja18-/-
DR4tg
DJ
Loaded CD1d tetramer
% of max.
TCRb
HLA-DR
Spleen
B
DJ
DR4tg
C57BL/6
Ja18-/-
% Weight Loss
Hours post-SEB
% Survival
p = 0.0083
C
NKT14
Isotype
p = 0.0117
D
Liver
Isotype
NKT14
Empty CD1d tetramer
Loaded CD1d tetramer
Spleen
Fold Change (Relative to Isotype)
Liver MNCs (NKT14)
Spleen MNCs (NKT14)

Figure 3.1: Genetic or antibody-mediated depletion of *i*NKT cells reduces morbidity and mortality in SEB-injected DR4tg mice.

(**A**) Splenic and non-parenchymal hepatic mononuclear cells from wild-type C57BL/6, Jα18$^{-/-}$, DR4tg and DJ mice were stained with a FITC-conjugated anti-TCRβ mAb, a PE-conjugated anti-HLA-DR mAb and/or allophycocyanin-conjugated, PBS-57-loaded CD1d tetramer. Dot plots representing samples from 3 mice per group are depicted. (**B**) C57BL/6 (n=6), Jα18$^{-/-}$ (n=6), DR4tg (n=8) and DJ (n=8) mice were injected with 100 μg SEB and monitored for weight loss and survival for up to 7 days. Error bars represent SEM, and * and *** denote $p \leq 0.05$ and $p \leq 0.001$, respectively, for statistical differences (two-way ANOVA; Tukey) between DR4tg and DJ mice at indicated time points. For survival analyses, mice were euthanized when they reached a weight loss of 20% and the log-rank test was used for statistical comparisons. (**C**) DR4tg mice were treated with 200 μg of the *i*NKT cell-depleting mAb NKT14 (n=12) or an isotype control (n=15) 24 hours before they were injected with 100 μg SEB. Mice were monitored for up to 7 days and euthanized when moribund as above. The noted *p* value was calculated using the log-rank test. (**D**) At the end point, hepatic and splenic mononuclear cells were isolated and stained with PBS-57-loaded or empty (control) CD1d tetramers and a FITC-conjugated anti-TCRβ mAb. Representative dot plots are illustrated (n=6/group). In addition, Vα14-Jα18 mRNA levels were quantified by qPCR. Gene expression fold-changes relative to isotype control-treated mice (dotted line) are shown (n=6/group). * denotes a statistical difference with $p \leq 0.05$ (Student's *t*-test; two tailed) between ΔCT values of NKT14- and isotype control-treated DR4tg mice.

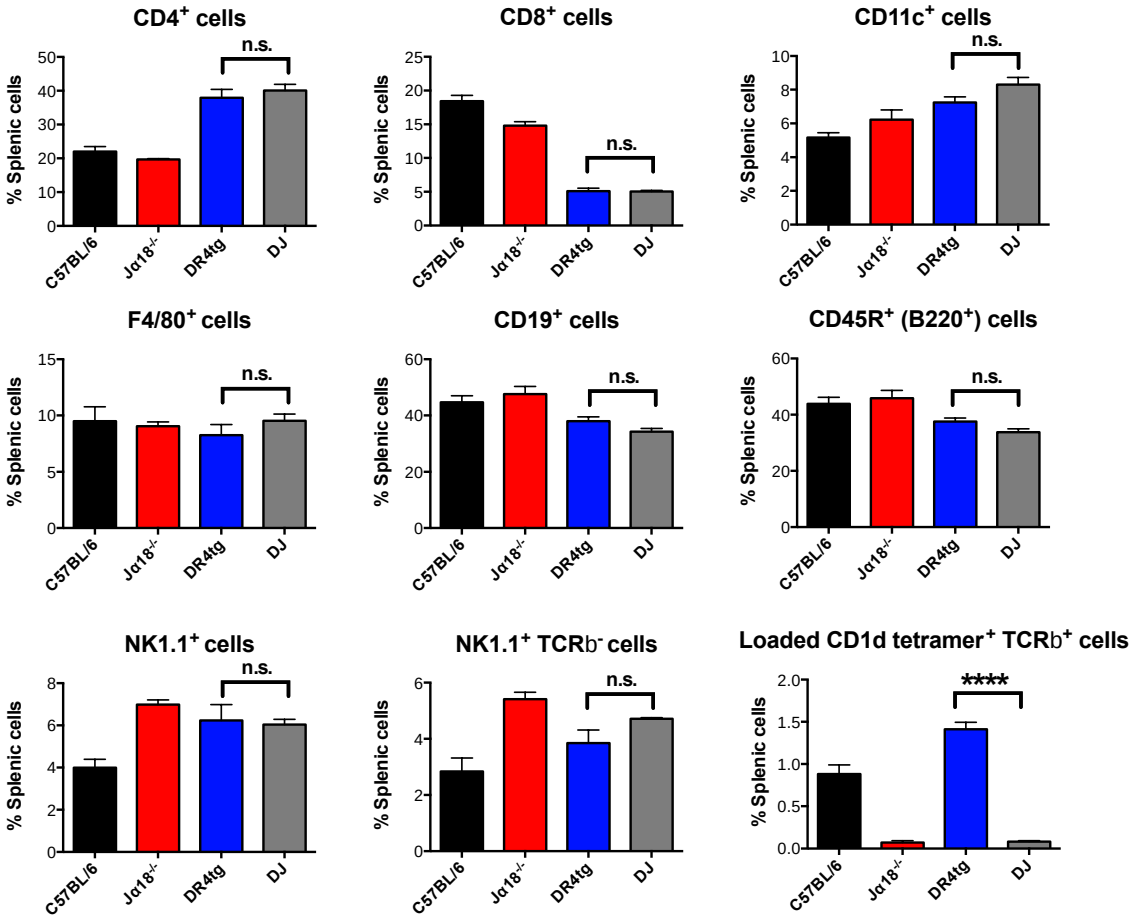

Figure 3.2: DJ mice lack *i*NKT cells but harbor other major immune cell subsets.

Splenic cells from naïve C57BL/6, Jα18$^{-/-}$, DR4tg, and DJ mice were stained with a panel of fluorochrome-conjugated mAbs (or CD1d tetramer) and immunophenotyped by flow cytometry. Mean values (± SEM) for 3 mice per group are shown. **** denotes $p \leq 0.0001$ (ANOVA). n.s. = not significant

Furthermore, 5 of the 6 surviving DJ mice had either returned to their initial weight or gained weight. SEB-exposed, wild-type C57BL/6 and Jα18$^{-/-}$ mice showed no signs of morbidity, as expected, due to their lack of humanized MHC II (**Fig. 3.1B**).

The observed differences in morbidity and mortality between DR4tg and DJ mice strongly suggested that *i*NKT cells are pathogenic in SEB-induced illness. We are cognizant of the recent report that the cellular deficiency found in the original Jα18$^{-/-}$ mice, which we used to generate DJ mice, is not exclusive to *i*NKT cells (13). Therefore, to unequivocally address the role of *i*NKT cells in TSS, we took advantage of a mAb, clone NKT14, which has become available only recently and which depletes *i*NKT cells selectively (8). Pre-treatment of DR4tg mice with NKT14 before SEB injection effectively reduced TSS mortality in comparison with DR4tg mice receiving an isotype control reagent (**Fig. 3.1C**), thus recapitulating the DJ phenotype. On day 7 post-SEB insult, 3 out of 4 surviving NKT14-treated mice exhibited moderate weight loss, which was not the basis for their euthanization at the experimental endpoint. To verify *i*NKT cell depletion by NKT14, livers and spleens were harvested from NKT14-treated and control mice and examined for the absence or presence of *i*NKT cells. CD1d tetramer staining revealed a near complete absence of splenic and hepatic *i*NKT cell detection in NKT14-treated mice (**Fig. 3.1D**). To rule out the possibility that this was due to *i*NKT cell TCR internalization, as opposed to their depletion, we isolated splenic and hepatic mononuclear cell mRNA from NKT14-treated and control mice and assessed the expression levels of the Vα14-Jα18 TCR gene segment by qPCR. We found the mRNA level corresponding to the Vα14-Jα18 TCRα chain to be dramatically diminished (by ~90%) in the liver of NKT14-treated mice. Interestingly, the mRNA levels in the spleen

showed approximately 50% reduction (**Fig. 3.1D**), suggesting that the efficacy of *i*NKT cell depletion by NKT14 is tissue-specific. This may explain the observed difference in the survival patterns of DJ mice and NKT14-treated DR4tg mice although we find it reassuring that this difference was not statistically significant (**Fig. 3.3**).

TSS is characterized by a rapid and overwhelming cytokine storm. As *i*NKT cells are directly activated by SEB (3) and since they can secrete inflammatory cytokines within hours of activation, we hypothesized that they should participate in the cytokine storm, especially in its early wave. Therefore, DR4tg and DJ mice were injected with SEB or with the prototypic *i*NKT cell superagonist αGC (positive control), and serum cytokine levels were measured 2, 12 and 24 hours later. Consistent with our hypothesis, the majority of cytokines and chemokines induced by SEB in DR4tg mice reached their peak at the earliest time point examined (*i.e.*, at 2 hours) (**Fig. 3.4A**). In fact, several pro-inflammatory cytokines including IL-1α, IL-6, IL-17A and TNFα were only present at the 2-hour time point (**Fig. 3.4A and Fig. 3.5**), suggesting that this early wave of cytokines may initiate or at least contribute to the TSS inflammatory cascade. Importantly, when compared to DR4tg mice, SEB-exposed DJ mice showed reduced levels of several cytokines known to play a direct role in TSS immunopathology, namely IL-1α, IL-2, IL-6, and TNFα (**Fig. 3.4B**). Multiple chemokines (CCL1, CCL4, CCL5, CXCL1 and CXCL10) and growth factors (G-CSF and GM-CSF) were also decreased in DJ mice (**Fig. 3.4B**). Together, these results implicate *i*NKT cells in production of inflammatory mediators early in the course of TSS and suggest that the observed reduction in morbidity and mortality of DJ mice may be owed to an attenuated cytokine storm.

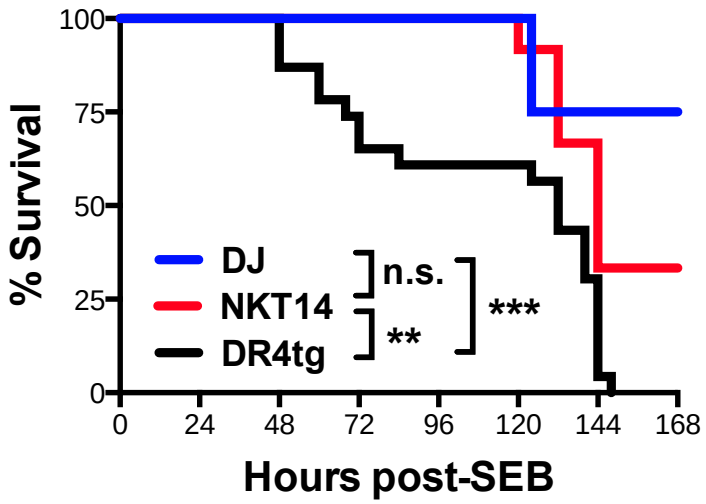

Figure 3.3: TSS mortality is reduced in two separate models of *i*NKT cell deficiency. DJ mice (n=8), NKT14-treated DR4tg (n=12) and naïve or isotype control-treated DR4tg mice (n=23 combined) were injected with 100 μg SEB and monitored for mortality/survival. **, *** denote $p \leq 0.01$ and $p \leq 0.001$, respectively, by the log-rank test. n.s. = not significant

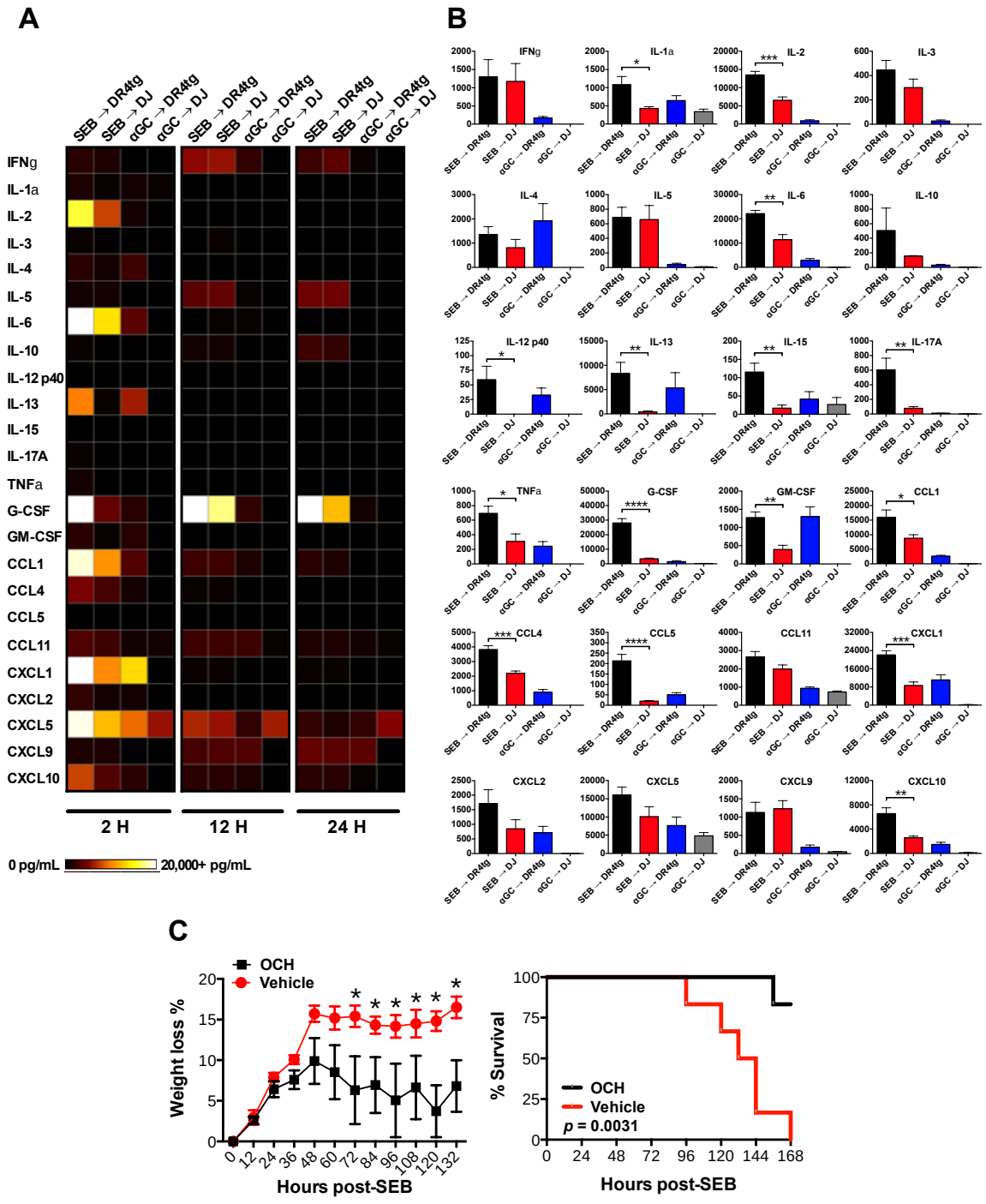
A
SEB → DR4tg
SEB → DJ
αGC → DR4tg
αGC → DJ
IFNg
IL-1a
IL-2
IL-3
IL-4
IL-5
IL-6
IL-10
IL-12 p40
IL-13
IL-15
IL-17A
TNFa
G-CSF
GM-CSF
CCL1
CCL4
CCL5
CCL11
CXCL1
CXCL2
CXCL5
CXCL9
CXCL10
2 H
12 H
24 H
0 pg/mL
20,000+ pg/mL
B
C
OCH
Vehicle
Weight loss %
Hours post-SEB
% Survival
p = 0.0031

Figure 3.4: *i*NKT cells are required for rapid inflammatory mediator production during TSS and their T_H2-polarization reduces TSS severity.

(**A** and **B**) DR4tg and DJ mice were injected with 100 μg SEB or 4 μg αGC (control), and bled 2, 12 and 24 hours later. Serum cytokine concentrations were quantified by bead-based multiplexing for DR4tg mice injected with SEB or αGC (n=6 for each cohort) and for DJ mice injected with SEB (n=6) or αGC (n=3). Mean values for each time point were used to generate a heat map (**A**). In addition, mean ± SEM values (pg/mL) are depicted for the 2-hour time point with *, **, ***, **** denoting $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively (Student's *t*-test; two tailed) (**B**). (**C**) DR4tg mice (n=6 per group) were treated daily with OCH (4 μg/dose) or vehicle beginning one day before SEB injection. The weight loss percentage (± SEM) is shown for up to 132 hours, after which time high mortality was observed in the vehicle-treated group. * denotes $p \leq 0.05$ at indicated individual time points (Student's *t*-test; two-tailed). For survival analysis, mice were sacrificed when moribund (≥20% weight loss) and the *p* value was calculated by the log-rank test.

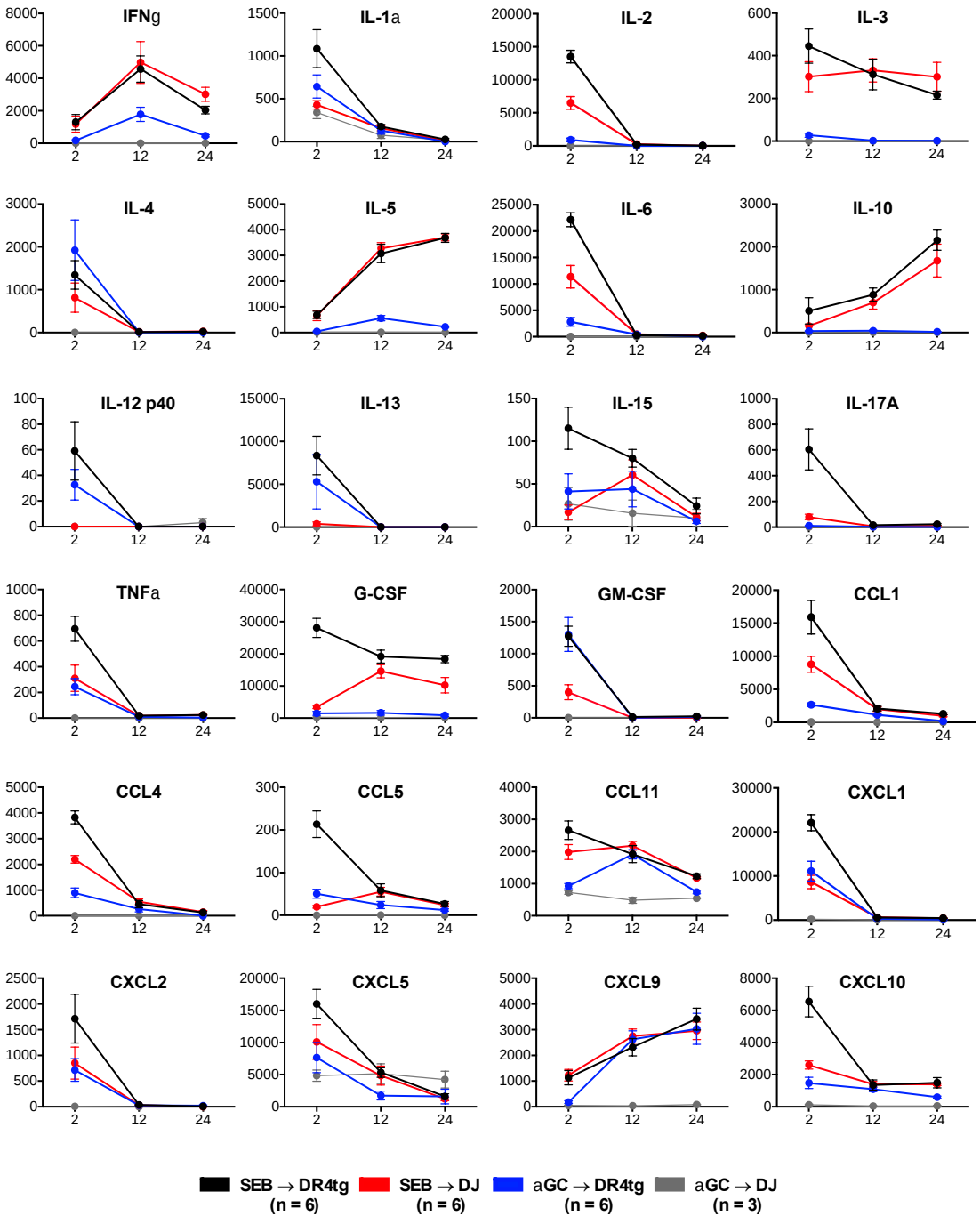

Figure 3.5: SEB-injected DJ mice exhibit a mild cytokine storm.

DR4tg and DJ mice were injected with 100 µg SEB or 4 µg αGC, and serum cytokines levels at 2, 12 and 24 hours were quantified. Mean values (pg/mL) ± SEM are shown.

Since the role of *i*NKT cells in TSS was generally pro-inflammatory, we attempted to skew their responses towards an anti-inflammatory phenotype using a T_H2-polarizing αGC analog called OCH (14). DR4tg mice were administered OCH or vehicle daily before and after receiving SEB. OCH pre-treatment markedly reduced weight loss and rescued ~85% of the animals (**Fig. 3.4C**). Mechanistically, this finding indicates that it is indeed the pro-inflammatory capacity of *i*NKT cells that promotes TSS pathogenesis. To simulate clinical scenarios in which treatments are given after the TSS onset, we started OCH treatment in a separate cohort of DR4tg mice 2 hours after the SEB insult, which coincides with the peak of several inflammatory cytokines (**Fig. 3.4A**). Therapeutic administration of OCH inhibited SEB-induced weight loss and mortality (**Fig. 3.6**).

Our findings are likely translatable to clinical settings because: i) *i*NKT cells' mode of glycolipid antigen surveillance is evolutionarily conserved (15); ii) glycolipid agonists of *i*NKT cells have shown promise in clinical trials for conditions other than TSS (5). Unlike conventional T cells that are restricted by highly polymorphic MHC molecules, *i*NKT cells respond to glycolipid antigens presented by the monomorphic CD1d. Therefore, CD1d-restricted glycolipids can be used in diverse human populations. *i*NKT cells likely represent only one of several early mediators of TSS. Nevertheless, tangible clinical benefits may arise from *i*NKT cell-based therapies, alone or in combination with other modalities, for TSS.

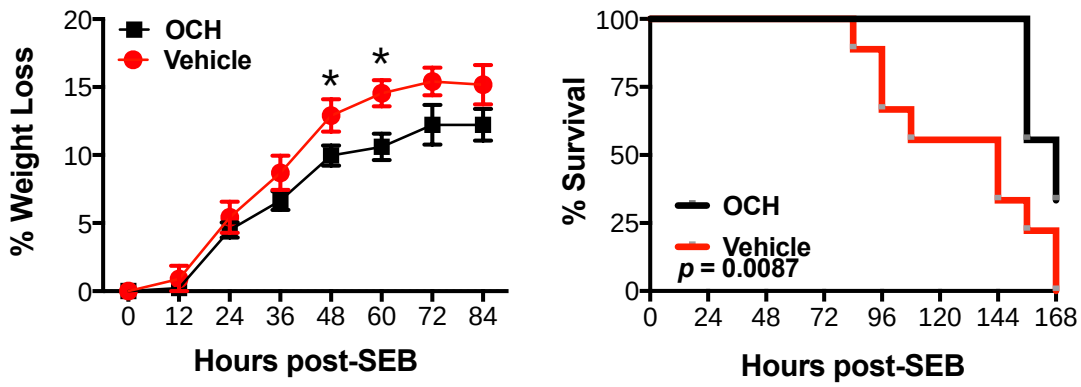

Figure 3.6: Therapeutic administration of OCH ameliorates SEB-inflicted morbidity and mortality in DR4tg mice.

Male DR4tg mice (n=9 per group) were treated daily with OCH (4 mg/dose) or vehicle beginning at 2 hours post-SEB injection. The weight loss percentage (± SEM) is shown for up to 84 hours, after which time high mortality was observed in vehicle-treated mice and to a lesser degree in OCH-treated animals. * denotes $p \leq 0.05$ at indicated time points (Student's *t*-test; two-tailed). For survival analysis, mice were euthanized when they lost 20% of their initial weight and the log-rank test was used to calculate the indicated *p* value.

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Chapter 4

Swift Intrahepatic Accumulation of Granulocytic Myeloid-Derived Suppressor Cells in a Humanized Mouse Model of Toxic Shock Syndrome

This chapter has been previously published:

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4.1 Introduction

Superantigens (SAgs) are exotoxins secreted by select bacterial pathogens including *Staphylococcus aureus* and *Streptococcus pyogenes*. SAgs cause various illnesses ranging from typically self-limiting food poisoning to potentially life-threatening TSS (1). Moreover, if fallen into the wrong hands, SAgs can be weaponized and used against human populations. In fact, staphylococcal enterotoxin B (SEB), a prototype bacterial SAg and a cause of non-menstrual TSS, is considered a "category B priority" bioterrorism agent (2).

SAgs activate a sizeable fraction of the T cell repertoire, up to 50% of exposed T cells, irrespective of their T cell receptor (TCR) specificity for cognate peptide:MHC complexes. This occurs through unconventional contacts SAgs establish, in unprocessed form, with lateral surfaces of MHC class II molecules on antigen-presenting cells and select TCR β-chain variable (Vβ) domains expressed by many T cells (3, 4). The consequent polyclonal T cell activation can lead to a 'cytokine storm', systemic toxicity and hyperinflammation, which is often accompanied or followed by a state of profound immunosuppression. T cells targeted by SAgs in mouse models undergo activation-induced death or become anergic (4, 5). This generates physical or functional 'holes' in the T cell repertoire, thus potentially compromising the host's ability to combat the very bacteria that produce SAgs as well as concomitant or secondary infections. Regulatory/suppressor cells have also been implicated in SAg-mediated immunosuppression. For instance, *in vitro* exposure to SAgs reportedly converts conventional $CD25^-$ T cells to IL-10-producing $CD25^+FoxP3^+$ regulatory T (Treg) cells (6). However, the *in vivo* significance of SAg-induced Treg cells is unclear. The potential

contribution of other suppressor cell types including myeloid-derived suppressor cells (MDSCs) to TSS-associated immunosuppression is ill-defined. Equally important, whether suppressor cell functions may benefit the host by mitigating tissue damage inflicted by unleashed, massive T cell responses remains a matter of debate. We have serendipitously discovered a dramatic, early-onset and tissue-selective influx of MDSCs into the liver of HLA-DR4 transgenic (DR4tg) mice shortly after systemic exposure to SEB. Here, we describe phenotypic and immunosuppressive characteristics of liver-infiltrating MDSCs in a humanized mouse model of TSS. We also extend our findings to an *in vitro* culture system in which human MDSCs modulate the T cell response to SEB.

4.2 Materials and Methods

4.2.1 Ethics

Peripheral blood was drawn from consenting healthy volunteers following a protocol approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects. Animal experiments were conducted in accordance with Canadian Council on Animal Care guidelines.

4.2.2 Animals

DR4tg mice on a B6 background were bred in our barrier facility. These mice lack endogenous MHC class II molecules but express a chimeric HLA encoded by HLA-DRA-IEα and HLA-DRB1*0401-IEβ transgenes (7). Adult wild-type (WT) B6 mice were purchased from Charles River, housed and cared for in the same facility.

4.2.3 Mouse model of TSS

Using an approved biosafety protocol and following the Public Health Agency of Canada regulations, recombinant SEB was cloned from *Staphylococcus aureus* COL, expressed in *Escherichia coli* BL21 (DE3) and purified by nickel column chromatography (8). As a negative control, we generated and used an inactive form of SEB that carries an N→A point mutation at position 23 (9). This mutant, hereafter referred to as SEB_{N23A}, is impaired in binding to mouse TCR Vβ8.2, which is a known target of intact SEB.

Mouse MHC II molecules exhibit poor affinity for SEB (10). Therefore, to simulate staphylococcal TSS, we injected DR4tg mice intraperitoneally (*i.p.*) with 50 μg SEB (8, 9). This model may not mimic all clinical features of TSS (*e.g.*, capillary leakage due to SAgs' toxicity for human endothelial cells), but provides a powerful system in which to

study many inflammatory and immunological aspects of TSS. Control DR4tg animals received PBS or 50 μg SEB_{N23A}. As an additional control, SEB was administered *i.p.* to WT B6 mice.

4.2.3 Cytofluorimetric analyses

Mice were sacrificed by cervical dislocation. Splenic, lymph node and thymic single-cell suspensions were prepared in cold, sterile PBS. Bone marrow cells were flushed, under aseptic conditions, out of femurs and tibias. To obtain non-parenchymal hepatic mononuclear cells, livers were perfused through the central or portal vein with PBS. The tissue was then pressed through a wire mesh, and the resultant homogenate was resuspended in 33.75% Percoll PLUS (GE Healthcare) and spun at room temperature. Erythrocytes among pelleted cells were lysed, and a nylon mesh strainer was used to remove clumps and debris. Cells were incubated on ice with 5 μg/mL anti-mouse CD16/CD32 monoclonal antibody (mAb) (clone 2.4G2) to prevent Fcγ receptor-mediated false positive staining with fluorochrome-labeled mAbs that were subsequently added. These included mAbs to cell surface CD11b, CD11c, CD31, CD244, CCR2, CCR5, Gr-1, Ly-6C, Ly-6G, F4/80 and c-kit. mAbs used in this study are listed in **Table 4.1**. For detection of intracellular Ki-67 after surface staining for indicated markers, cells were fixed and permeabilized using a BD Cytofix/Cytoperm^{TM} kit followed by incubation with an anti-Ki-67 mAb. The 'Fluorescence Minus One' (FMO) control was used to precisely set the gate for Ki-67^{+} events. A FACSCanto II cytometer and FlowJo software were employed for data collection and analysis, respectively.

Marker	Species Reactivity	Clone	Isotype	Source
CD11b	Mouse	M1/70	Rat IgG2b, κ	eBioscience
CD11c	Mouse	N418	Armenian Hamster IgG	eBioscience
CD31	Mouse	390	Rat IgG2a, κ	eBioscience
CD244	Mouse	244F4	Rat IgG2a	eBioscience
CCR2	Mouse	475301	Rat IgG2b	R&D Systems
CCR5	Mouse	HM-CCR5 (7A4)	Armenian Hamster IgG	eBioscience
Ly6G (Gr-1)	Mouse	RB6-8C5	Rat IgG2b, κ	eBioscience
Ly6G	Mouse	1A8	Rat IgG2a, κ	BD Pharmingen
Ly6C	Mouse	HK1.4	Rat IgG2c, κ	BioLegend
F4/80	Mouse	BM8	Rat IgG2a, κ	eBioscience
c-Kit	Mouse	2B8	Rat IgG2b, κ	eBioscience
Ki-67	Mouse	SolA15	Rat IgG2a, κ	eBioscience
CD3ε	Mouse	145-2C11	Armenian Hamster IgG	eBioscience
CD33	Human	WM-53	Mouse IgG1, κ	eBioscience

Table 4.1: Fluorochrome-conjugated antibodies used for MDSC studies

4.2.4 In vitro generation of mouse BMDCs

Bone marrow cells were obtained from femurs and tibias of DR4tg mice, depleted of erythrocytes, filtered and seeded at a density of 1×10^6 cells/mL in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, GlutaMAX™, nonessential amino acids, sodium pyruvate, penicillin and streptomycin, which is referred to as complete medium in this report. Cells were cultured for 6 days in complete medium containing 10 ng/mL recombinant mouse GM-CSF and IL-4 (PeproTech). Every other day, non-adherent cells were discarded and cultures were replenished with fresh medium and cytokines. On day 6, $CD11c^+$ cells were magnetically purified using an EasySep™ Mouse CD11c Positive Selection Kit (STEMCELL Technologies).

4.2.5 In vitro generation of human MDSCs

We used the method of Lechner et al. (11). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by density gradient centrifugation in Ficoll-Paque PLUS medium (GE Healthcare) and cultured for 7 days at 5×10^5 cells/mL complete medium supplemented with 10 ng/mL recombinant human IL-6 and GM-CSF (PeproTech). Adherent cells were then gently harvested using Detachin™ (Genlantis) and stained with an anti-human CD33 mAb before a FACSAria III cytometer was used to sort $CD33^+$ cells.

4.2.6 T cell suppression assays

Hepatic $CD11b^+Gr\text{-}1^{high}$ cells from SEB-treated DR4tg mice were sorted. A small aliquot was stained with Wright-Giemsa for morphological assessments. Splenic T cells from WT B6 mice were passed through nylon wool and magnetically purified using CD90.2

MicroBeads (Miltenyi Biotec). T cells were co-incubated with γ-irradiated BMDCs at a 4:1 (T cell:DC) ratio and stimulated with 100 ng/mL SEB in complete medium for 3 days. $CD11b^{+}Gr\text{-}1^{high}$ cells were absent or present at indicated ratios. Cells were pulsed with tritiated thymidine ([^{3}H]TdR) during the final 18 hours, and [^{3}H]TdR uptake was determined by liquid scintillation counting. T cell proliferation was also judged by carboxyfluorescein succinimidyl ester (CFSE) dye dilution within the $CD3\varepsilon^{+}$ population. In several experiments, N^{G}-monomethy-L-arginine (L-NMMA, Calbiochem), N^{ω}-hydoxy-nor-arginine (nor-NOHA, Calbiochem) or catalase (Sigma-Aldrich) were added to the cultures to inhibit nitric oxide synthase (NOS), arginase or reactive oxygen species (ROS), respectively (12).

For human T cell suppression assays, PBMCs were subjected to two successive rounds of purification using nylon wool columns (Polysciences). T cells were seeded at 8×10^{4} cells/well in a 96-well plate along with 2×10^{4} γ-irradiated, nylon wool-adherent accessory cells, which consist mainly of B cells and monocytes. Autologous $CD33^{+}$ MDSCs were added to the cultures before SEB treatment, and [^{3}H]TdR uptake was quantified.

4.2.7 Statistical analysis

Statistical comparisons were performed using Student's *t*-test, and differences with $p<0.05$ were deemed significant.

4.3 Results and Discussion

While investigating TSS in the DR4tg mouse model (8, 9), we found a rapid and sizable rise in a high forward- and side-scatter population among non-parenchymal hepatic mononuclear cells (**Fig. 4.1A**), but not within the spleen, thymus or lymph nodes (**Fig. 4.2**). Two hours after SEB injection, there was more than a three-fold increase in this population compared with untreated or PBS-treated DR4tg mice (**Fig. 4.1A**). This phenotype was much less pronounced in SEB-injected WT mice, indicating a requirement for higher-affinity human MHC interactions with SEB, which is in agreement with our previous reports (8, 9, 13).

To definitively demonstrate that T cell activation by SEB is a prerequisite for this phenotype, we used SEB_{N23A}, a mutated version of SEB with a partially defective TCR binding capacity (9). As anticipated, injection of DR4tg mice with SEB_{N23A} failed to induce a robust enlargement in the high forward- and side-scatter population within the liver (**Fig. 4.1A**). SEB_{N23A} retains the ability to bind to DR4, and the modest response it elicits is likely contributed by TCR Vβs other than Vβ8.2.

Next, we used a wide panel of mAbs specific for various surface markers, including but not limited to those listed in **Fig. 4.1B**, to characterize the population under our scrutiny. This revealed a $CD11b^{+}Gr\text{-}1^{high}Ly\text{-}6C^{+}$ phenotype for the vast majority of the cells, which fits the granulocytic MDSC (G-MDSC) definition (14). The anti-Gr-1 mAb we used (clone RB6-8C5) may bind to both Ly-6G and Ly-6C. Therefore, in separate experiments, we employed a different mAb (clone 1A8) and confirmed that $CD11b^{+}Gr\text{-}1^{high}$ cells were indeed $Ly\text{-}6G^{+}$ and $Ly\text{-}6C^{+}$ (**Fig. 4.3**). Furthermore, sorted cells stained

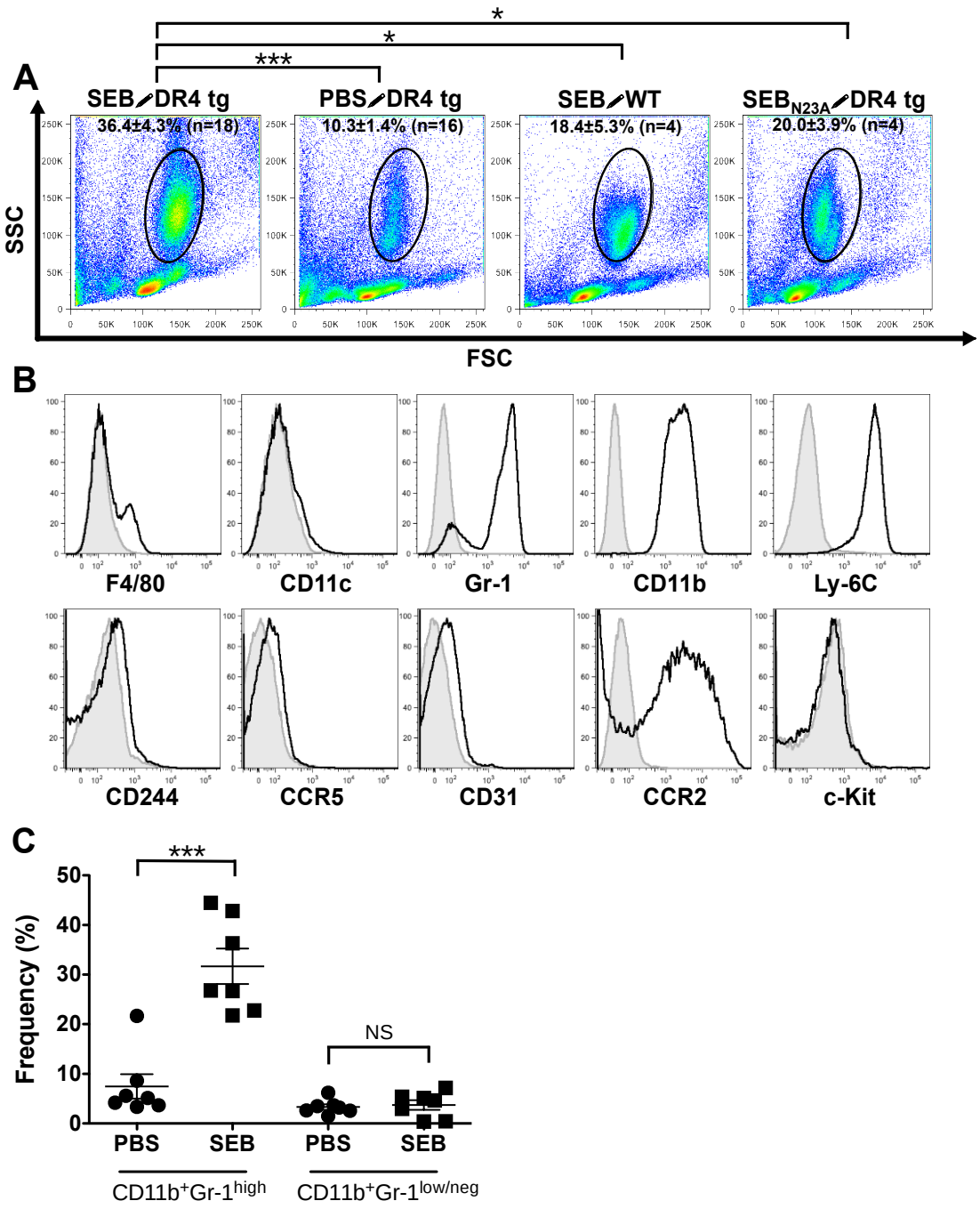
A
SEB/DR4 tg
PBS/DR4 tg
SEB/WT
SEB N23A/DR4 tg
36.4±4.3% (n=18)
10.3±1.4% (n=16)
18.4±5.3% (n=4)
20.0±3.9% (n=4)
*
*
***
SSC
FSC
B
F4/80
CD11c
Gr-1
CD11b
Ly-6C
CD244
CCR5
CD31
CCR2
c-Kit
C
***
NS
Frequency (%)
PBS
SEB
PBS
SEB
CD11b+Gr-1high
CD11b+Gr-1low/neg

Figure 4.1: $CD11b^{+}Gr\text{-}1^{high}Ly\text{-}6C^{+}$ cells amass in the liver of DR4tg mice shortly after SEB injection.

WT or DR4tg mice were injected with SEB, SEB_{N23A} or PBS, and the frequencies of high forward- and side-scatter cells among non-parenchymal hepatic mononuclear cells was determined 2 hours post-SEB exposure. Representative dot plots are illustrated and mean ± SEM values for indicated number of mice per group are shown (**A**). The gated population from SEB-injected DR4tg mice was characterized cytofluorimetrically using mAbs specific for indicated markers (open histograms) and isotype controls (filled histograms) (**B**). The frequencies of $CD11b^{+}Gr\text{-}1^{high}Ly\text{-}6C^{+}$ and $CD11b^{+}Gr\text{-}1^{low/neg}Ly\text{-}6C^{+}$ cells among hepatic mononuclear cells of PBS- and SEB-injected DR4tg mice were determined (**C**). * and *** denote $p<0.05$ and $p<0.001$, respectively.

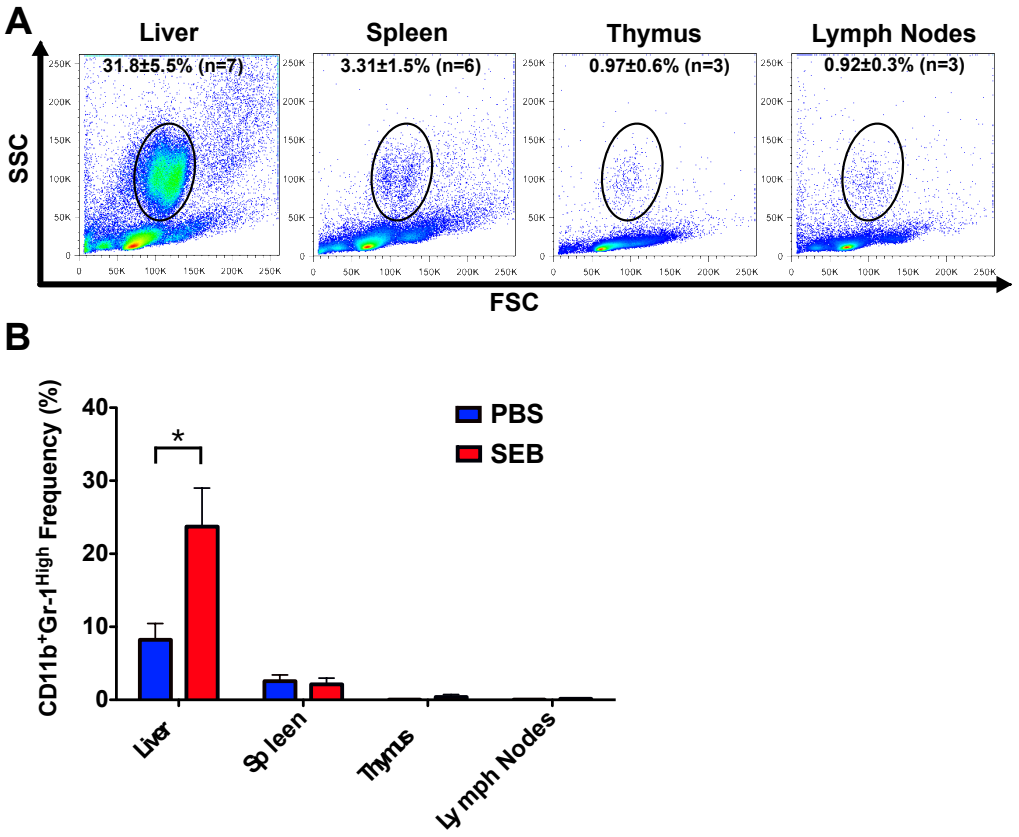

Figure 4.2: G-MDSCs accumulate selectively in the liver following systemic exposure to SEB.

Indicated numbers of DR4tg mice were injected with SEB. Two hours later, non-parenchymal hepatic mononuclear cells as well as splenic, thymic and pooled lymph node cells were isolated and analyzed by a flow cytometer. Representative forward- and side-scatter dot plots are shown, and the mean frequency [± standard error of the means (SEM)] of the gated population is stated for each group (**A**). The percentages of $CD11b^{+}Gr\text{-}1^{high}$ cells were determined in the same cohorts and compared with values corresponding to PBS-injected DR4tg mice (**B**). Error bars represent SEM, and * denotes $p<0.05$.

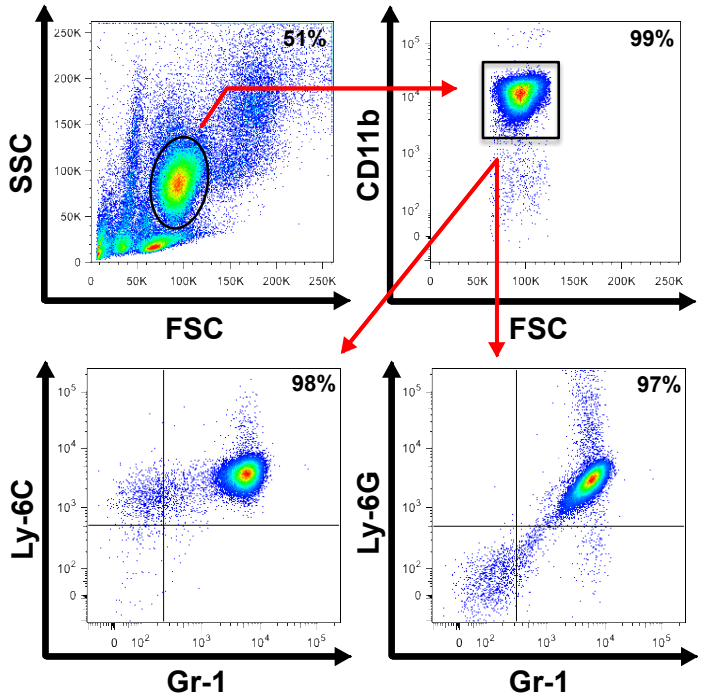

Figure 4.3: Systemic exposure to SEB results in intrahepatic accumulation of CD11b$^+$ cells that concomitantly express Gr-1, Ly-6G and Ly-6C.

A DR4tg mouse was injected *i.p.* with SEB. Two hours later, non-parenchymal hepatic mononuclear cells were isolated and co-stained with fluorochrome-labeled mAbs to CD11b (clone M1/70), Gr-1 (clone RB6-8C5), Ly-6G (clone 1A8) and Ly-6C (clone HK1.4) before they were analyzed by a flow cytometer.

with Wright-Giemsa mostly exhibited a ring-shaped nucleus (**Fig. 4.4**), which is again consistent with the G-MDSC morphology in mice (14). It is noteworthy that a minority fraction of the high forward- and side- scatter cells were $CD11b^{+}Gr\text{-}1^{low/neg}Ly\text{-}6C^{+}$, stained positive for F4/80, and had a monocytic morphology. However, their frequencies in SEB- and PBS-treated DR4tg mice were similar (**Fig. 4.1C**).

Some of the markers expressed by MDSCs can be found on other cell types (*e.g.*, neutrophils). It was therefore crucial to verify the immunosuppressive nature of $CD11b^{+}Gr\text{-}1^{high}$ cells before we could define them as MDSCs. To this end, we demonstrated that $CD11b^{+}Gr\text{-}1^{high}$ cells sorted from the livers of SEB-treated DR4tg mice significantly attenuated *ex vivo* T cell proliferation in response to SEB (**Fig. 4.5A**) or anti-CD3ε mAb (data not shown). We next investigated the mechanism by which MDSCs suppressed T cell proliferation using the pharmacological inhibitors L-NMMA, nor-NOHA or catalase, which inhibit NOS, arginase and ROS, respectively. MDSC-mediated suppression was abrogated in the presence of catalase, but not L-NMMA or nor-NOH, indicating that the suppressive effect of MDSCs was mediated by ROS (**Fig. 4.5B**).

MDSCs are known to amass in certain inflamed or tumor-bearing tissues, albeit at much later time points. The rapidity with which G-MDSCs accumulate in the liver of TSS-afflicted mice suggests their recruitment to the liver as opposed to their *in situ* proliferation. This notion is supported by two lines of evidence. First, the expression of the proliferation marker Ki-67 by $CD11b^{+}Gr\text{-}1^{high}$ cells was lower in SEB-treated mice in comparison with controls (**Fig. 4.6A**). Second, $CD11b^{+}Gr\text{-}1^{high}$ cell accumulation in the liver was accompanied by a simultaneous decrease in the bone marrow (**Fig. 4.6B**), suggesting a bone marrow-to-liver trafficking route. The exact cellular and/or soluble

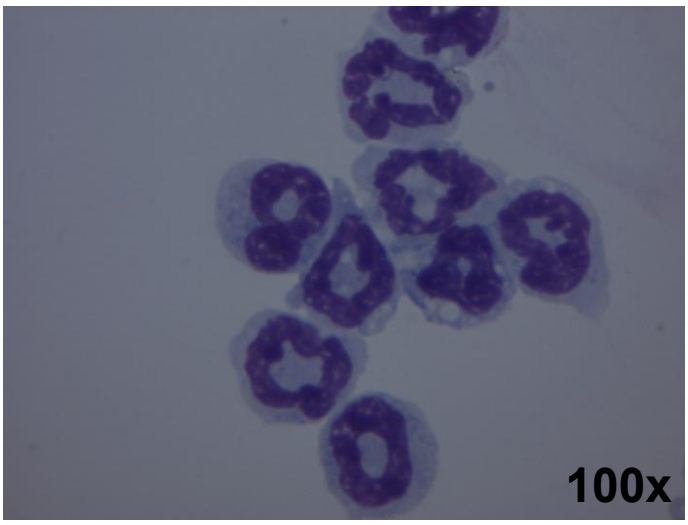

Figure 4.4: Hepatic CD11b^{+}Gr-1^{high} cells from SEB-treated DR4tg mice exhibit a ring-shaped nucleus.

DR4tg mice were injected i.p. with SEB. Two hours later, non-parenchymal hepatic mononuclear cells were isolated and stained for CD11b and Gr-1. CD11b+Gr-1high cells were sorted cytofluorimetrically, stained with Wright-Giemsa, and evaluated morphologically under a light microscope.

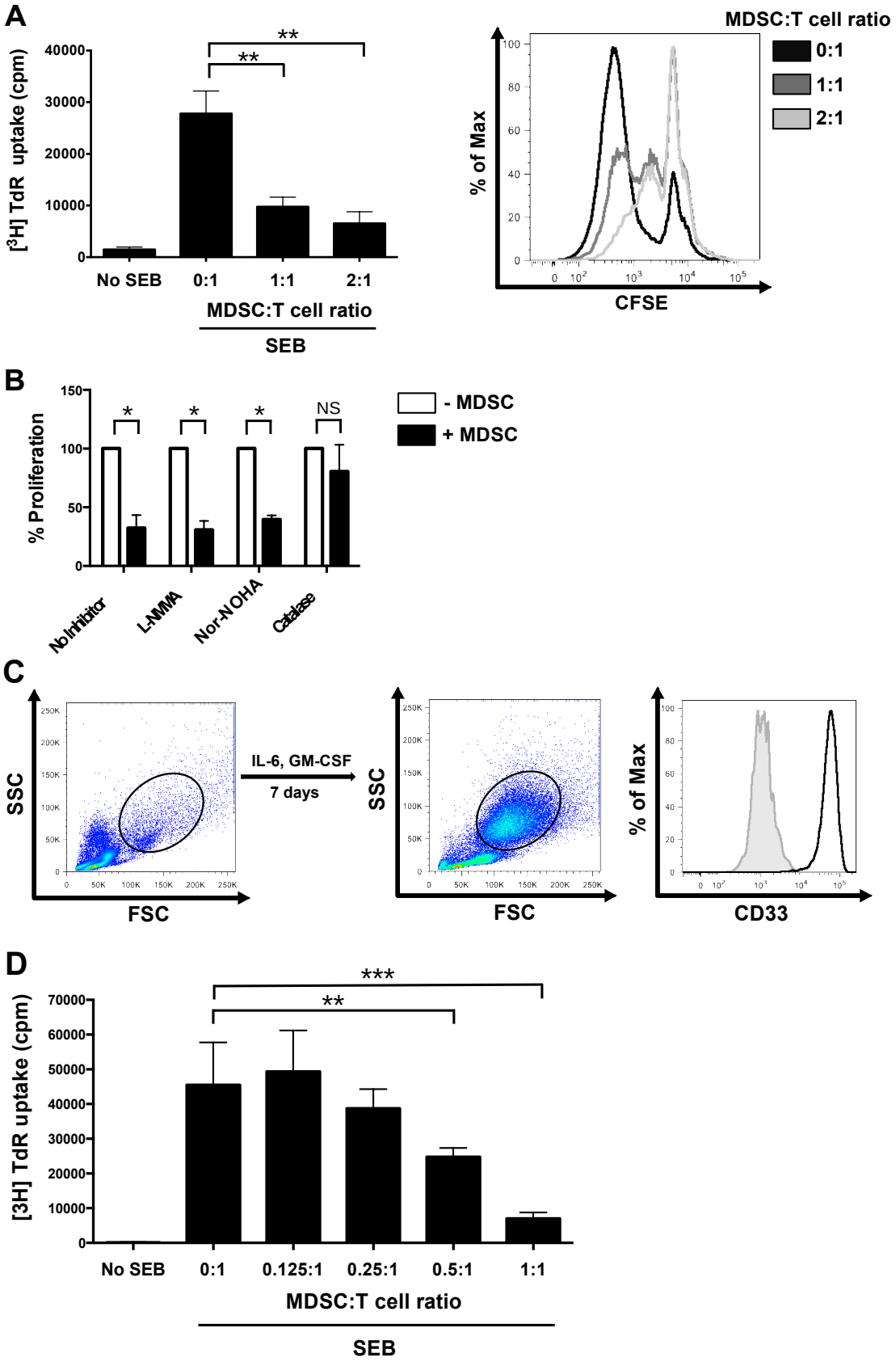
A
[3H] TdR uptake (cpm)
40000
30000
20000
10000
0
**
**
No SEB
0:1
1:1
2:1
MDSC:T cell ratio
SEB
MDSC:T cell ratio
0:1
1:1
2:1
% of Max
CFSE
B
% Proliferation
150
100
50
0
*
*
*
NS
- MDSC
+ MDSC
No Inhibitor
L-NMMA
Nor-NOHA
Catalase
C
SSC
FSC
IL-6, GM-CSF
7 days
SSC
FSC
% of Max
CD33
D
[3H] TdR uptake (cpm)
70000
60000
50000
40000
30000
20000
10000
0
***
**
No SEB
0:1
0.125:1
0.25:1
0.5:1
1:1
MDSC:T cell ratio
SEB

Figure 4.5: Mouse and human MDSCs suppress SEB-induced T cell proliferation. $CD11b^{+}Gr\text{-}1^{high}$ cells were sorted from the livers of DR4tg mice 2 hours after injection with SEB. They were then co-cultured with naïve mouse T cells and *in vitro*-generated DR4tg BMDCs in the presence or absence of SEB for 3 days. T cell proliferation was assayed by [^{3}H] TdR incorporation or by CFSE dye dilution. Representative data from two independent experiments are illustrated. Error bars correspond to standard deviation (SD), and ** denotes $p<0.01$ (**A**). In separate experiments, L-NMMA (0.5 mM), nor-NOHA (0.5 mM) or catalase (1000 U/mL) were added to the cultures and T cell proliferation was measured by [^{3}H] TdR incorporation. Three independent experiments yielded similar results, which were pooled. Error bars represent SEM, and * denotes $p<0.05$ (**B**). Human MDSCs were generated in a 7-day culture system in the presence of recombinant human GM-CSF and IL-6 (10 ng/mL each). This method led to a dramatic expansion of $CD33^{+}$ cells among PBMCS isolated from all the 3 donors recruited to this study (**C**). Sorted $CD33^{+}$ cells were co-incubated for 3 days with autologous T cells and accessory cells and tested for their ability to suppress SEB-triggered T cell proliferation as described in Methods. Similar results were obtained in 3 independent experiments, each of which utilized PBMCs from one of the 3 donors recruited to this study (**D**). Error bars represent SD, and ** and *** denote $p<0.01$, $p< 0.001$, respectively.

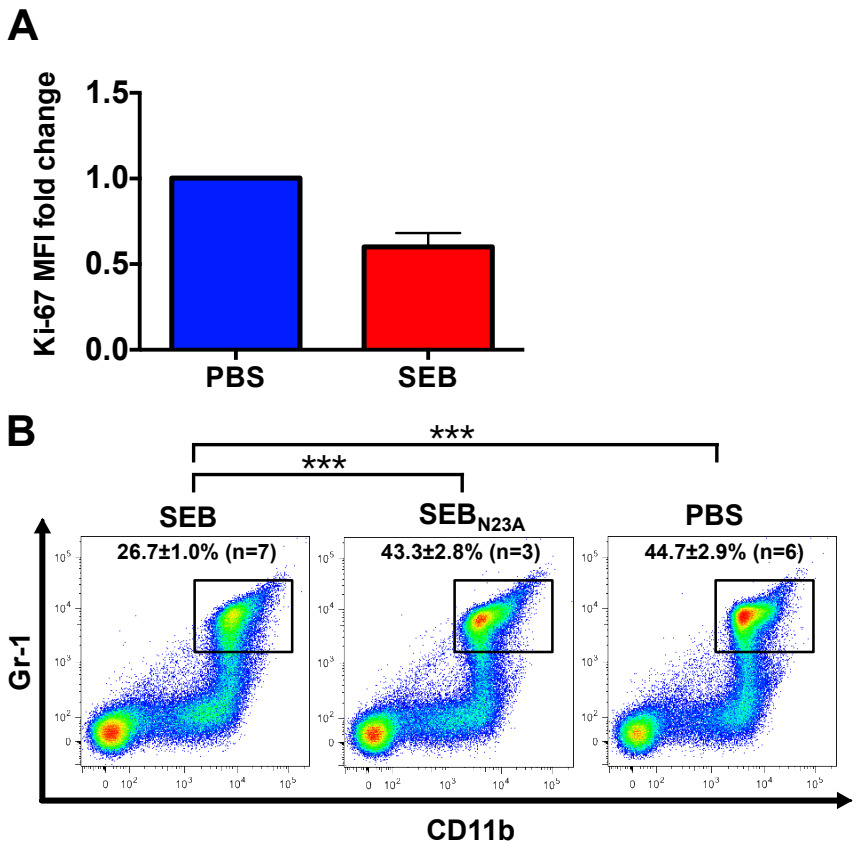

Figure 4.6: SEB administration does not elevate Ki-67 expression by CD11b^{+}Gr-1^{high} cells in the liver but lowers their frequency in the bone marrow.

DR4tg mice (n=3/group) were given SEB or PBS *i.p.* Two hours later, the mean fluorescence intensity (MFI) of Ki-67 on hepatic CD11b^{+}Gr-1^{high} cells was determined and normalized to PBS controls (**A**). The frequencies of CD11b^{+}Gr-1^{high} cells were determined by flow cytometry in the bone marrow of indicated numbers of DR4tg mice 2 hours after they were injected with SEB, SEB$_{N23A}$ or PBS (**B**). Representative dot plots and mean ± SEM values are demonstrated. *** denotes $p<0.001$.

factors that mediate $CD11b^{+}Gr\text{-}1^{high}$ cells' recruitment to the liver during TSS remain unknown. Invariant natural killer T (*i*NKT) cells, which we demonstrated are directly responsive to SEB (9), comprise up to 40% of lymphocytes in the mouse liver. However, genetic deficiency or Ab-mediated depletion of *i*NKT cells did not affect the G-MDSC buildup in the liver nor did systemic depletion of $CD4^{+}$ or $CD8^{+}$ cells (unpublished data). Regardless, once present in the liver, G-MDSCs appear to be retained in this organ as evidenced by their elevated frequencies even at 24 hours post-SEB exposure (data not shown).

The liver is uniquely tolerogenic among immunological sites. This is dictated in part by anti-inflammatory properties of liver-resident cells and by expression of co-inhibitory receptors that dampen T cell responses to self and foreign antigens (15). We have now defined a novel mode of immunosuppression during TSS, which may benefit the host by diminishing hepatic tissue damage. A substantial proportion of SAg-mediated illnesses are non-menstrual. Staphylococcal enterotoxins released in the gastrointestinal tract, due for instance to food poisoning, may cross the perturbed epithelium and access the liver through the portal vein. Therefore, it is fitting that MDSCs can swiftly and selectively home to the liver (**Fig. 4.2A-B**) to help protect this vital organ from SAgs' deleterious effects with inflammatory and metabolic repercussions.

Finally, *ex vivo*-generated human MDSCs were able to inhibit autologous T cell responses to SEB (**Fig. 4.5C-D**), which recapitulates our findings on immunosuppressive function of sorted mouse MDSCs. Whether human liver attracts MDSCs in the early phase of TSS and/or other SAg-mediated illnesses warrants further investigation.

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Chapter 5

Rapid and Rigorous IL-17A Production by a Distinct Subpopulation of Effector Memory T Lymphocytes Constitutes a Novel Mechanism of Toxic Shock Syndrome Immunopathology

This chapter has been previously published:

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5.1 Introduction

Toxic shock syndrome (TSS) is a life-threatening illness characterized by high-grade fever, diffuse erythematous rash formation, desquamation, severe hypotension and multi-organ dysfunction (1). It is caused by systemic exposure to bacterial toxins known as superantigens (SAgs), which are secreted by *Staphylococcus aureus* and *Streptococcus pyogenes*. TSS can be of menstrual (2) or non-menstrual (3) origin. The vast majority of menstrual TSS cases, which are linked to high-absorbency tampon usage (4), are caused by *S. aureus* strains expressing the powerful SAg toxic shock syndrome toxin-1 (TSST-1) (5). In contrast, non-menstrual TSS can occur with virtually any *S. aureus* infection and is primarily associated with TSST-1 and staphylococcal enterotoxin B (SEB) (6). The expression of streptococcal pyrogenic exotoxin A (SpeA) is also strongly correlated with streptococcal TSS (7).

SAgs are a unique family of exotoxins that activate a large proportion of T cells irrespective of their TCR specificity. Cognate peptide antigens presented in the context of self MHC by APCs typically activate 1 in every 10,000 T cells. In contrast, SAgs simultaneously bind MHC class II molecules on APCs outside their antigen-binding groove (8) and select TCR Vβ domains on T cells (9). By doing so, SAgs circumvent conventional modes of antigen processing and presentation to induce the activation and proliferation of up to 50% of all exposed T cells (10). The overwhelming activation of T cells by SAgs results in excessive production of inflammatory mediators, which is commonly referred to as ‘cytokine storm’. SAgs directly stimulate the secretion of IL-2, IFNγ and lymphotoxin-α from T cells, as well as TNFα, IL-1β and IL-6 from APCs (10, 11). Additionally, SAgs initiate secondary inflammatory cytokine and chemokine

responses from various non-hematopoietic cell types such as epithelial cells, endothelial cells and fibroblasts (12). The massive and uncontrolled release of these inflammatory mediators has drastic tissue damaging effects through the activation of the coagulatory cascade, vasodilation, edema, and vascular leakage (13-16). SAgs also promote the production of chemokines CXCL8, CCL2, CCL3, and CCL4 (17, 18), resulting in further recruitment of leukocytes to areas of tissue injury. The net effect of the cytokine storm is a systemic inflammatory response syndrome that may culminate in fatal multi-organ failure.

IL-17A is a potent inducer of systemic inflammation, potentiating the production or activation of inflammatory cytokines (*e.g.*, TNFα, IL-1β, IL-6), chemokines, matrix metalloproteases and transcription factors in both hematopoietic and non-hematopoietic cell types (19). IL-17A also acts synergistically with other inflammatory cytokines, including TNFα, IL-1β and IFNγ, to stabilize mRNA transcripts or activate promoter regions of other inflammatory mediators (20). Although IL-17A is the archetypal cytokine of the $CD4^+$ Th17 cell lineage (19), it can also be quickly produced by innate-like T lymphocytes such as γδ T cells, invariant natural killer T (*i*NKT) cells and mucosa-associated invariant T (MAIT) cells (21, 22). IL-17A is often associated with early and protective host responses to bacteria, and little is known about its pathogenic potentials during infection. For example, whether exposure to SAgs elicits an early IL-17A response that may contribute to the cytokine storm is essentially unexplored.

Identification of early inflammatory mediators of the cytokine storm and their cellular sources is of utmost importance due to the rapidity with which TSS may progress to life-threatening disease. The clinical status of a SAg-exposed individual can deteriorate to

multi-organ failure as early as 8-12 hours after the onset of the symptoms (23), and estimates of TSS mortality range from 4% to 22% (24-26). Also alarmingly, there are currently no available therapeutics to attenuate the cytokine storm, which drives the immunopathology of TSS. Therefore, understanding the critical components of this process may hold the key to designing effective therapies that reduce TSS-associated morbidity and mortality.

In this investigation, using humanized mice and human PBMCs, we found and characterized a vigorous IL-17A response that was detectable within the first few hours of SAg exposure. The kinetics of this response initially suggested the involvement of innate-like T cells (21), which we we were able to rule out. Instead, we identified effector memory T (T_{EM}) cells as the major source of mouse and human IL-17A, which were surprisingly unable to co-produce IFNγ. IL-17A receptor blockade diminished the expression of several pro-inflammatory mediators by SAg-stimulated human PBMCs and also prevented the secondary upregulation of the transcription factor C/EBP delta (CEBPD) in human dermal fibroblasts. These findings suggested a pathogenic role for IL-17A in TSS, which we confirmed using a humanized mouse model of the syndrome. Therefore, this work defines a previously unrecognized mechanism of TSS immunopathology that is dependent upon rapid IL-17A production by a distinct subpopulation of T_{EM} cells.

5.2 Materials and Methods

5.2.1 Mice

Adult 6-to-12-week-old mice closely matched for age and sex were used in all experiments. Wild-type C57BL/6 mice were purchased from Charles River Canada Inc. (St. Constant, Quebec). IL-17A-GFP mice that express enhanced GFP as a marker of IL-17A expression were obtained from The Jackson Laboratory (Bar Harbor, Maine) and bred in a barrier facility at Western University. HLA-DR4tg (DR4tg) mice, which are devoid of endogenous MHC class II and instead express HLA-DRA-IEα and HLA-DRB1*0401-IEβ on a C57BL/6 background (27), were bred in the same facility.

To develop a SAg-sensitive IL-17A reporter mouse strain, DR4tg and IL-17A-GFP mice were crossed. The resultant "D17 mice" were bred to homozygosity and confirmed to express HLA-DR transgenes and the IL-17A-GFP reporter construct but not IAβ. In brief, genomic DNA was extracted from mouse ear clips using Hot Sodium Hydroxide and Tris (HotSHOT). PCR-amplified products were generated using Platinum Taq DNA polymerase (Thermo Fisher Scientific, Ottawa, Ontario) and primer sets that are listed in **Table 5.1**. Agarose gel electrophoresis was performed to visualize PCR products.

Wild-type C57BL/6, IL-17A-GFP, DR4tg and D17 mice were cared for in the same facility. All animal experiments were conducted in accordance with an approved institutional animal use protocol (AUP# 2010-241) and in compliance with the Canadian Council on Animal Care (CCAC) guidelines.

Target (reference if applicable)	Species	Sequence (5'-3') or Accession #	Source
HLA-DRA-IEα Forward (27)	Human	GGGAAGCAGGGGGACTATGAC	Sigma-Aldrich
HLA-DRA-IEα Reverse (27)	Human	TTAGGGCAATGACTTCGTAGG	Sigma-Aldrich
HLA-DRB1*0401-IEβ Forward (27)	Human	TGAAAGCGGTGCGTGCTGTTTAA	Sigma-Aldrich
HLA-DRB1*0401-IEβ Reverse (27)	Human	CACCCGCTCCGTCCCGTTGAA	Sigma-Aldrich
HLA-DR Transgene Insertion Site Forward*	Mouse	CCATGGACAAGGCAGGGACAAA	Sigma-Aldrich
HLA-DR Transgene Insertion Site Reverse*	Mouse	CCGTGACCAAAATGCACATTGAA	Sigma-Aldrich
IL-17A-GFP Common Forward (Jackson Primer #15240)	Mouse	AAGCTGGACCACCACATGA	Sigma-Aldrich
IL-17A-GFP Wildtype Reverse (Jackson Primer #15241)	Mouse	TGAATCCACATTCCTTGCTG	Sigma-Aldrich
IL-17A-GFP Mutant Reverse (Jackson Primer #11188)	Mouse	GACATTCAACAGACCTTGCATTC	Sigma-Aldrich
IAβ-neo^r Forward	Mouse	GGGAGGAGTACGTGCGCTACGACAG	Sigma-Aldrich
IAβ-neo^r Reverse	Mouse	GAGAACCTGCGTGCAATCCATCTTG	Sigma-Aldrich
IAβ Forward	Mouse	GGCATTTCGTGTACCAGTTCATGG	Sigma-Aldrich
IAβ Reverse	Mouse	GTCTCCGGCCCCTCGTAGTTGTGT	Sigma-Aldrich
CCL11	Human	Hs00237013_m1	Thermo Fisher
CCL2	Human	Hs00234140_m1	Thermo Fisher
CCL20	Human	Hs00355476_m1	Thermo Fisher
CCL5	Human	Hs00982282_m1	Thermo Fisher
CCL7	Human	Hs00171147_m1	Thermo Fisher
CEBPB	Human	Hs00270923_s1	Thermo Fisher
CEBPD	Human	Hs00270931_s1	Thermo Fisher
CSF2	Human	Hs00929873_m1	Thermo Fisher
CSF3	Human	Hs00738432_g1	Thermo Fisher
CXCL1	Human	Hs00605382_gH	Thermo Fisher
CXCL10	Human	Hs01124252_g1	Thermo Fisher
CXCL11	Human	Hs04187682_g1	Thermo Fisher
CXCL12	Human	Hs03676656_mH	Thermo Fisher
CXCL2	Human	Hs00601975_m1	Thermo Fisher
CXCL5	Human	Hs01099660_g1	Thermo Fisher
CXCL6	Human	Hs00605742_g1	Thermo Fisher
CXCL9	Human	Hs00171065_m1	Thermo Fisher
GATA3	Human	Hs00231122_m1	Thermo Fisher
IFNG	Human	Hs00989291_m1	Thermo Fisher
IL10	Human	Hs00961622_m1	Thermo Fisher
IL12A	Human	Hs01073447_m1	Thermo Fisher
IL12B	Human	Hs01011518_m1	Thermo Fisher
IL13	Human	Hs00174379_m1	Thermo Fisher
IL17A	Human	Hs00174383_m1	Thermo Fisher
IL17F	Human	Hs00369400_m1	Thermo Fisher
IL18	Human	Hs01038788_m1	Thermo Fisher
IL19	Human	Hs00604657_m1	Thermo Fisher
IL1B	Human	Hs00174097_m1	Thermo Fisher
IL2	Human	Hs00174114_m1	Thermo Fisher
IL22	Human	Hs01574154_m1	Thermo Fisher
IL23A	Human	Hs00900828_g1	Thermo Fisher
IL4	Human	Hs00174122_m1	Thermo Fisher
IL6	Human	Hs00985639_m1	Thermo Fisher
IL8	Human	Hs00174103_m1	Thermo Fisher
LTA	Human	Hs04188773_g1	Thermo Fisher
MYD88	Human	Hs01573837_g1	Thermo Fisher
NFKB1	Human	Hs00765730_m1	Thermo Fisher
NFKBIZ	Human	Hs00230071_m1	Thermo Fisher
NOS2	Human	Hs01075529_m1	Thermo Fisher
PTGS2	Human	Hs00153133_m1	Thermo Fisher
RORC	Human	Hs01076112_m1	Thermo Fisher
RPL13A	Human	Hs04194366_g1	Thermo Fisher
TBP	Human	Hs00427620_m1	Thermo Fisher
TBX21	Human	Hs00203436_m1	Thermo Fisher
TGFB1	Human	Hs00998133_m1	Thermo Fisher
TNFA	Human	Hs01113624_g1	Thermo Fisher
ZBTB16	Human	Hs00957433_m1	Thermo Fisher

Table 5.1: PCR primers/probes used in IL-17A studies. *Primer was designed to span the insertion site of the HLA-DR4 transgenes in the mouse genome, thus enabling discrimination between homozygous and heterozygous transgenic mice.

5.2.2 Generation of bone marrow chimeras

To obtain donor cells, IL-17A-GFP mice were euthanized by cervical dislocation, and bone marrow was flushed out of femurs and tibias with ice-cold PBS. Erythrocytes were lysed by addition of ACK lysis buffer for 2 minutes, and resulting cells were filtered through a 70-μm nylon mesh strainer. Twenty million donor cells were injected *i.v.* into recipient DR4tg mice, which were lethally irradiated at 900 cGy using a ^{137}Cs γ-irradiator before adoptive transfer. Recipient mice were maintained on drinking water supplemented with 2 mg/mL neomycin sulfate (Teknova Inc., Hollister, California) for 2 weeks after irradiation to prevent infection.

5.2.3 Bacterial toxins

Recombinant staphylococcal and streptococcal SAgs were generated using an approved institutional biosafety protocol that follows established Public Health Agency of Canada regulations as described previously (28). Briefly, SAgs were expressed in BL21 (DE3) *E. coli* and purified by nickel column chromatography. As an additional control, an attenuated mutant of SEB that is impaired in binding to mouse TCR Vβ8.2 (29) was generated by site-directed mutagenesis (30). The mutant SEB carries an N→A point mutation at position 23 and is referred to as SEB_{N23A}. LPS was purchased from Sigma-Aldrich (Oakville, Ontario).

5.2.4 TSS mouse model and *in vivo* IL-17A neutralization

Animals were injected *i.p.* with indicated amounts of SEB or the equivalent volume of PBS as a control. They were bled via the saphenous vein, and serum samples were obtained following centrifugation of blood at 17,000 × g for 40 minutes and analyzed for

their cytokine content using ELISA kits from eBioscience (San Diego, California). For *in vivo* IL-17A neutralization, DR4tg mice were treated with 200-µg *i.p.* doses of an anti-IL-17A mAb (clone 17F3, BioXCell, West Lebanon, New Hampshire) or a mouse IgG1 isotype control (clone MOPC-21) 3 hours before and 1 hour after they were injected with 100 µg of SEB. Mice were monitored every 3 hours for the initial 12 hours and every 8 hours thereafter for up to 7 days. Weight loss was assessed at indicated time points, and mice were sacrificed when 20% weight loss was reached following institutional protocols and CCAC guidelines. For histological analyses, mice were sacrificed at 48 hours post-SEB stimulation. Liver and small intestine were extracted, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned and stained with H&E. Sections were scored by a board-certified veterinary pathologist who was blinded to the experimental conditions.

5.2.5 Human PBMC isolation and stimulation with SAgs

Peripheral blood was collected in heparinized vacutainers from healthy volunteers after their written consent was secured and following a protocol approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB #5545). PBMCs were isolated by density centrifugation using 50-mL SepMate tubes from Stemcell Technologies Inc. (Vancouver, British Columbia) and low-endotoxin (<0.12 EU/mL) Ficoll-Paque Plus (GE Healthcare Life Sciences) according to manufacturer's instructions. PBMCs were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 0.1 mM MEM non-essential amino acids, 2 mM GlutaMAX-I™, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin and 100 µg/mL streptomycin. PBMCs were stimulated with 100 ng/mL of indicated SAgs for

up to 24 hours at 37°C in polystyrene U-bottom plates at a density of 2×10^6 per well. To block IL-17 receptor A (IL-17RA) prior to SEB stimulation, PBMCs were incubated with 10 μg/mL of an anti-IL-17RA mAb (clone #133617) or mouse IgG1 isotype control (clone #11711) from R&D Systems (Minneapolis, Minnesota) for 30 minutes.

For cytokine assays, culture supernatants were harvested at indicated time points and analyzed by a bead-based multiplex assay (Eve Technologies, Calgary, Alberta). Heat maps were generated using the matrix2png utility (31). For RNA studies, PBMCs were isolated in RNAlater (Thermo Fisher Scientific) and RNA was extracted using the Purelink RNA Mini Kit (Thermo Fisher Scientific). To eliminate contaminating genomic DNA, on-column Purelink DNase (Invitrogen Canada, Burlington, Ontario) was used as per manufacturer's instructions. RNA quantity and purity was measured by a NanoDrop ND-1000 spectrophotometer. RNA was converted to cDNA via SuperScript VILO Master Mix (Invitrogen Canada). Quantitative real-time PCR (qPCR) was performed on a StepOnePlus Real-Time PCR System using TaqMan Gene Expression Assays (**Table 5.1**) and TaqMan Fast Advanced Master Mix, all from Thermo Fisher Scientific. qPCR reactions and cycling conditions were set up according to manufacturer's instructions. Fold changes between conditions were calculated by the $\Delta\Delta C_T$ method. For low-expressing gene targets that consistently amplified with C_T values >37, C_T values were set to 37 for analysis.

As reliable comparisons of gene expression by qPCR require normalization to stably expressed reference genes, we first tested several commonly used reference genes for changes in SEB-stimulated PBMCs when compared to unstimulated controls. The mean fold changes of TATA-binding protein (TBP) and ribosomal protein L13a (RPL13A)

were no more or less than 20% (data not shown). TBP and/or RPL13A were thus used as reference genes in our qPCR assays.

To analyze RNA stability/degradation, PBMCs were stimulated with SEB for 4 hours followed by treatment with 5 μg/mL of the transcriptional inhibitor actinomycin D (act-D). Cells were harvested at indicated time points, RNA was extracted and converted to cDNA, and qPCR for target genes was performed as described above using 18S RNA as the reference gene.

5.2.6 Human PBMC-dermal fibroblast co-culture

Using an approved ethics protocol (HSREB #104888), primary human fibroblasts were derived from surgically resected normal skin samples as previously described (32). Fibroblast cultures were maintained for a maximum of six passages in DMEM medium containing 10% heat-inactivated FCS, 2 mM GlutaMAX-I™ and 1X Antibiotic-Antimycotic (Thermo Fisher Scientific). Fibroblasts were seeded at a density of 5×10^5/well in a tissue culture-treated polystyrene flat-bottom plate. Four million freshly isolated PBMCs were plated into a Nunc™ Polycarbonate Membrane Insert (pore size: 0.4 μm), which was placed inside the fibroblast culture well. Co-cultures were then stimulated with 100 ng/mL of SEB. Eight hours later, the insert was removed and fibroblasts were harvested and examined by qPCR for their CEBPD expression. In several experiments, anti-IL-17RA mAb or isotype control was added to co-cultures at 10 μg/mL 30 minutes before SEB stimulation.

5.2.7 Isolation and cytofluorimetric analyses of mouse cells

PBS- and SAg-primed mice were sacrificed at indicated time points to harvest spleen, pooled lymph nodes (cervical, axillary, brachial, mesenteric, inguinal and popliteal nodes), liver, lungs and small intestine. Spleen and lymph nodes were homogenized using a Wheaton Dounce tissue grinder. To obtain hepatic and lung non-parenchymal mononuclear cells (MNCs), tissue specimens were pressed through a wire mesh, and the extracted cells were washed, resuspended in 33.75% low-endotoxin Percoll Plus (GE Healthcare Life Sciences, Mississauga, Ontario) and centrifuged at 700 × *g* for 12 minutes at room temperature. Pelleted cells were treated with ACK lysis buffer, washed, filtered and resuspended in cold sterile PBS.

Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated from the small intestine as described by Sheridan and Lefrançois (33). Briefly, the small intestine was aseptically removed, flushed with HBSS containing 5% FCS and 10 mM HEPES, digested at 37°C with 1 mM dithioerythritol and filtered through a cell strainer (pore size: 70 μm) to generate an IEL-containing homogenate. To prepare LPLs, the remaining intestinal pieces were additionally digested, first in 1.3 mM EDTA and then in a solution containing 100 U/mL collagenase, and subsequently filtered. IEL- and LPL-containing homogenates were washed, resuspended in 44% Percoll Plus (GE Healthcare Life Sciences), underlaid with 67% Percoll Plus and spun at 600 × *g* for 20 minutes. IELs and LPLs were harvested by a Pasteur pipette from the buffy coat formed at the gradient interface, washed and resuspended in cold PBS.

Freshly isolated mouse cells were incubated with 5 μg/mL of an anti-mouse CD16/CD32 mAb (clone 2.4G2) to block Fcγ receptors and stained at 4°C with fluorochrome-conjugated mAbs to cell surface molecules listed in **Table 5.2**. Corresponding isotype controls were used in parallel for appropriate gating. A FACSCanto II cytometer (BD Biosciences, Mississauga, Ontario) was used for data collection. Data were analyzed by FlowJo software (Tree Star, Ashland, Oregon).

5.2.8 Surface and intracellular staining of human cells and RNA flow cytometry

Freshly isolated human PBMCs were stimulated with SAgs for 4 hours, unless otherwise stated, in the presence of 1 μM brefeldin A (Sigma-Aldrich). Cells were thoroughly washed and stained at 4°C with fluorochrome-conjugated mAbs to cell surface molecules (**Table 5.2**). For intracellular detection of cytokines, the Intracellular Fixation and Permeabilization Buffer Set from eBioscience was used. We also employed a Mouse IL-17A Secretion Assay kit from Miltenyi Biotec (Auburn, California) to confirm the secretion of this cytokine. For detection of transcription factors, the FoxP3 Staining Buffer Set (eBioscience) was used. For RNA staining, the Prime FlowRNA Assay (eBioscience) was used with probes designed for the detection of IL-17A, IFNγ and RPL13A signals. Cells were treated and stained for surface and intracellular molecules (**Table 5.2**) following the manufacturer's instructions. Isotype and/or fluorescence-minus-one (FMO) controls were used in parallel for proper gating. Samples were run on a BD FACSCanto II or a BD LSR II (when using violet fluorochrome-conjugated mAbs) and analyzed by FlowJo software (Tree Star).

Target	React.	Host Species	Clone/ Accession #	Isotype	Fluorochrome	Source
Surface Molecules						
TCRβ	Mouse	Arm. Hamster	H57-597	IgG	PE-Cyanine7	eBioscience
CD4	Mouse	Rat	GK1.5	IgG2b, κ	Alexa Fluor® 700	eBioscience
CD44	Human/ Mouse	Rat	IM7	IgG2b, κ	PerCP-Cyanine5.5	eBioscience
CD62L	Mouse	Rat	MEL-14	IgG2a, κ	APC-eFluor® 780	eBioscience
CCR7	Mouse	Rat	4B12	IgG2a, κ	APC	eBioscience
CD127	Mouse	Rat	A7R34	IgG2a, κ	PE	eBioscience
*i*NKT TCR (detected by CD1d loaded tetramer)	Mouse	Mouse	N/A	N/A	APC	NIH Tetramer Core Facility
CD3ε	Human	Mouse	OKT3 UCHT1	IgG2a, κ IgG1, κ	FITC eFluor® 450	eBioscience eBioscience
CD4	Human	Mouse	RPA-T4 SK3	IgG1, κ IgG1, κ	Alexa Fluor® 700 Brilliant Violet™ 510	eBioscience BD Biosciences
CD8	Human	Mouse	RPA-T8	IgG1, κ	Alexa Fluor® 700	eBioscience
CD45RA	Human	Mouse	HI100	IgG2b, κ	PE PE-Cyanine7	eBioscience eBioscience
CD45RO	Human	Mouse	UCHL1	IgG2a, κ	APC Brilliant Violet™ 785	eBioscience BD Biosciences
CCR6	Human	Mouse	G034E3	IgG2b, κ	PE	BioLegend
CCR7	Human	Rat	3D12	IgG2a, κ	PerCP-eFluor® 710 PE-eFluor® 610	eBioscience eBioscience
CD161	Human	Mouse	HP-3G10	IgG1	APC PerCP-Cyanine5.5	eBioscience eBioscience
Vα7.2 TCR	Human	Mouse	3C10	IgG1, κ	PE PerCP-Cyanine5.5	BioLegend BioLegend
*i*NKT TCR (detected by CD1d loaded tetramer)	Human	Human	N/A	N/A	APC	NIH Tetramer Core Facility
γδ TCR	Human	Mouse	B1 B1.1	IgG1, κ IgG1, κ	PE PerCP-eFluor® 710	BD Biosciences eBioscience
Cytokines						
IL-17A	Human	Mouse	eBio64-DEC17	IgG1, κ	PE PE-Cyanine7	eBioscience eBioscience
IFNγ	Human	Mouse	4S.B3	IgG1, κ	PE PE-Cyanine7	eBioscience eBioscience
Transcription Factors						
RORγT	Human	Mouse	Q21-559	IgG2a, κ	Alexa Fluor® 647	BD Biosciences
FoxP3	Human	Mouse	236A/E7	IgG1, κ	APC	eBioscience
Prime-Flow RNA						
IL-17A	Human		NM_002190		Type 1 Alexa Fluor® 647	Affymetrix
IFNγ	Human		NM_000619		Type 4 Alexa Fluor® 488	Affymetrix
RPL13A	Human		NM_012423		Type 6 Alexa Fluor® 750	Affymetrix

Table 5.2: Antibodies/tetramers used in IL-17A studies

5.2.9 Statistical analyses

Analyses were carried out using GraphPad Prism 7 software (La Jolla, California). The Shapiro-Wilk test was performed to verify the normal distribution of our data sets. Statistical comparisons were made using parametric Student's *t*-tests, ANOVA with Holm-Sidak post-hoc analysis, or log-rank test, as appropriate and as indicated in figure legends. Differences with $p \leq 0.05$ were deemed statistically significant.

5.3 Results

5.3.1 IL-17A is rapidly produced by $CD4^+$ T_{EM} cells in a humanized mouse model of TSS

SAgs have notoriously poor binding affinity for MHC class II molecules expressed in certain conventional mouse strains such as C57BL/6 mice (34). Thus, we investigated the pathogenesis of TSS in humanized DR4tg mice that allow for high-affinity interactions with SAgs and consequently recapitulate many aspects of TSS-mediated immunopathology (28, 29, 35-39). Using this model, we serendipitously found a dramatic serum IL-17A spike at 2 hours post-SEB exposure (**Fig. 5.1A**). Given that the role of IL-17A in TSS is unknown and that IL-17A induces and synergizes with many inflammatory mediators implicated in the cytokine storm, we sought to fully characterize this phenomenon. IL-17A was present in the serum at 2 hours but not detectable at 12 or 24 hours after *i.p.* injection of SEB (**Fig. 5.1A**). In contrast, IL-17A was not detectable in SEB-injected wild-type C57BL/6 mice (**Fig. 5.1A**) neither were several other cytokines tested (*e.g.*, IFNγ and IL-4). This highlights the requirement for human MHC class II molecules in the optimal activation of T cells by SAgs. The injection of SEB_{N23A}, a mutant version of SEB with impaired binding to Vβ8.2 TCR (29), into DR4tg mice also resulted in no detectable IL-17A in the serum (**Fig. 5.1A**), indicating that SEB-induced IL-17A production is highly T cell-dependent.

We next tested a panel of bacterial SAgs other than SEB for their ability to induce IL-17A production in DR4tg mice. These included TSST-1, staphylococcal enterotoxin A (SEA), SpeA, streptococcal pyrogenic exotoxin I (SpeI) and streptococcal mitogenic exotoxin Z (SmeZ). TSST-1 administration did not result in IL-17A appearance in the

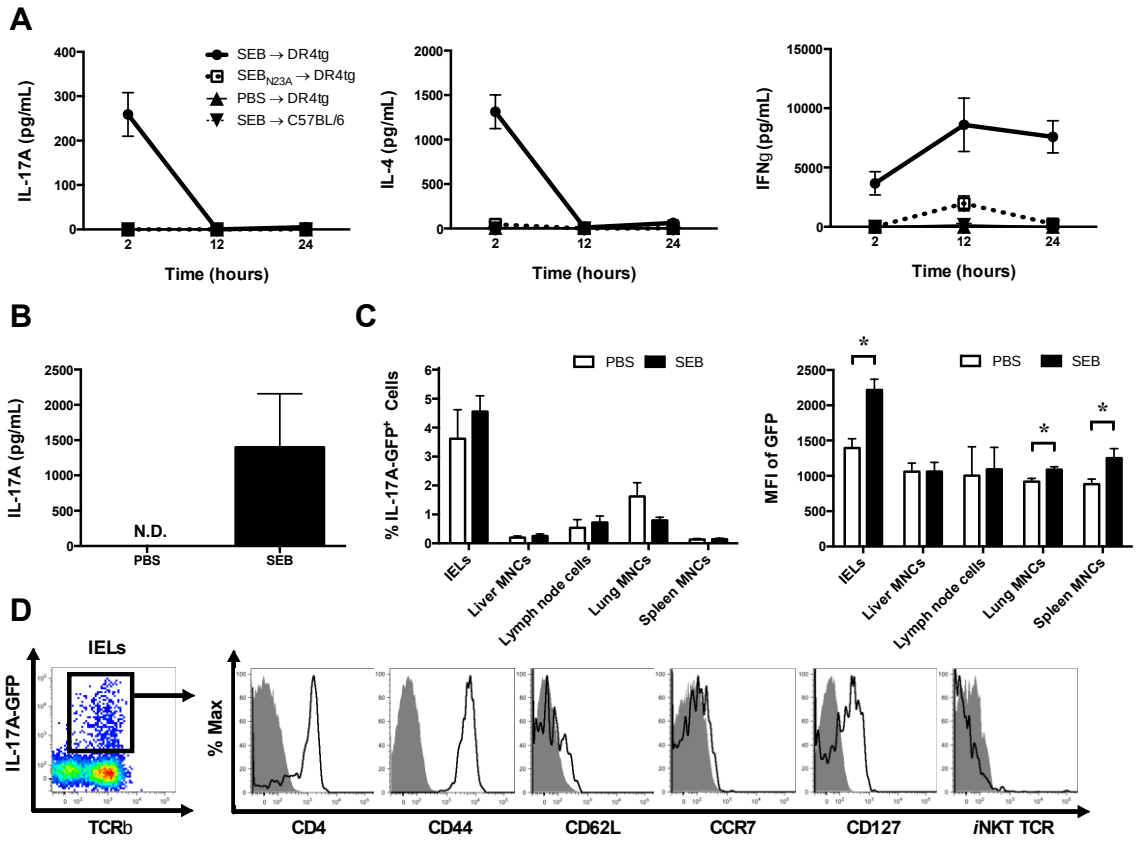
A
IL-17A (pg/mL)
SEB → DR4tg
SEB$_{N23A}$ → DR4tg
PBS → DR4tg
SEB → C57BL/6
Time (hours)
IL-4 (pg/mL)
IFNg (pg/mL)
B
N.D.
PBS
SEB
C
% IL-17A-GFP$^+$ Cells
MFI of GFP
IELs
Liver MNCs
Lymph node cells
Lung MNCs
Spleen MNCs
D
IELs
IL-17A-GFP
TCRb
% Max
CD4
CD44
CD62L
CCR7
CD127
iNKT TCR

Figure 5.1: Rapid IL-17A production by CD4$^+$ T$_{EM}$ cells in a humanized mouse model of TSS.

(**A**) DR4tg mice were injected *i.p.* with 50 μg SEB, SEB$_{N23A}$ or PBS, and bled at indicated time points for serum cytokine measurements. Mean ± SEM values from 2 independent experiments are shown (n = 4). (**B-D**) IL-17A-GFP/DR4tg chimeric mice were injected *i.p.* with PBS or 50 μg SEB and sacrificed after 2 hours. (**B**) Serum IL-17A levels in SEB- and PBS-treated IL-17A-GFP/DR4tg bone marrow chimeric mice were determined by ELISA. N.D.= not detectable. Mean values ± SEM from 3 independent experiments are shown (n = 4-5 mice per group). (**C**) The frequencies of IL-17A-GFP$^+$ cells and the geometric MFI of IL-17 were determined by flow cytometry among non-parenchymal mononuclear cells obtained from indicated organs of SEB- and PBS-treated mice. Mean values ± SEM from 3 independent experiments are shown (n = 6-7 per group). * denotes $p \leq 0.05$ (Student's *t*-test; two-tailed). (**D**) The phenotype of IL-17A-GFP$^+$ cells among IELs from SEB-treated mice was determined using fluorochrome-conjugated antibodies and tetramers for indicated markers (open black histograms) and isotype controls (filled, grey histograms). Representative dot plot and histograms from 3 independent experiments are shown (n = 4-6).

serum neither did SpeI or SmeZ (data not shown). As such, DR4tg mice do not appear to provide a suitable model in which to study the role of IL-17A in the context of TSST-1-mediated TSS or SpeI/SmeZ-triggered host responses. In contrast, animals injected with SEA and SpeA had detectable IL-17A levels in their serum (55 pg/mL and 286 pg/mL, respectively). Therefore, the early IL-17A production may not be limited to staphylococcal TSS, and may also play a role in streptococcal infections. Of note, LPS, a potent stimulus that activates immune cells through a different mechanism than SAgs, failed to trigger an IL-17A response in DR4tg mice (data not shown).

To identify the cellular source of IL-17A, we generated IL-17A-GFP/DR4tg bone marrow chimeric mice. This system allows for quantification of IL-17A responses as measured by GFP fluorescence in SAg-sensitive, human MHC II-expressing mice. Three weeks after the adoptive transfer of bone marrow cells, chimeric mice were injected with 50 μg SEB and sacrificed 2 hours later to obtain blood, spleen, lymph nodes (cervical, axillary, brachial, mesenteric, inguinal and popliteal), liver, lungs and small intestine. When compared to PBS controls, which showed no detectable IL-17A in serum, SEB-injected chimeric mice produced substantial amounts of IL-17A after 2 hours (**Fig. 5.1B**). The greatest frequency of IL-17A-expressing cells was found among IELs of the small intestine (**Fig. 5.1C**). Notably, the frequencies of IL-17A-expressing cells did not significantly differ between SEB and PBS treatment groups (**Fig. 5.1C**). However, the mean fluorescence intensity (MFI) of GFP, an indicator of IL-17A production on a per cell basis, increased upon *in vivo* SEB exposure in IELs and to a lesser degree among lung and splenic non-parenchymal MNCs (**Fig. 5.1C**).

Having determined that IL-17A expression was highly upregulated and most abundant in the IEL compartment after SEB exposure, we next sought to immunophenotype these rapidly IL-17A-producing cells. The majority of IL-17A$^+$ cells were defined as CD4$^+$ T_{EM} cells due to their TCRβ^+CD4$^+$CD44$^+$CCR7$^-$CD62L$^-$CD127$^+$ surface phenotype (**Fig. 5.1D**) (40). Similarly, CD4$^+$ T_{EM} cells constituted the majority of IL-17A$^+$ cells among IELs of PBS-treated mice (data not shown).

We previously reported that *i*NKT cells can be activated by group II bacterial SAgs, including SEB, leading to a swift cytokine response (29). In addition, a subset of *i*NKT cells is capable of producing IL-17A in response to glycolipid antigens (41). Therefore, it was important to rule in or rule out the participation of *i*NKT-17 cells in our model. We found that *in vivo* stimulation with SEB gives rise to IL-17A-expressing cells that do not possess the canonical TCR of *i*NKT cells as judged by their lack of staining with α-galactosylceramide-loaded CD1d tetramer (**Fig. 5.1D**).

As a confirmatory approach, we generated D17 mice by crossing DR4tg mice with IL-17A-GFP reporter animals. We found a pattern similar to that observed in the bone marrow chimeras, namely increased IL-17A MFI among IELs of SEB-primed D17 mice (**Fig. 5.2A-C**). IL-17-producing cells in D17 mice also similarly exhibited a T_{EM} phenotype. Having an adequate supply of D17 animals enabled us to additionally investigate the lamina propria, a site in which IL-17-secreting T cells are known to be abundant (42). These experiments revealed that systemic exposure to SEB raises both the frequency IL-17$^+$CD4$^+$ T_{EM} cells in the lamina propria and the intensity of IL-17A production by these cells. In fact, the IL-17A response was stronger in the LPL compartment in comparison with IELs (**Fig. 5.2B-C**).

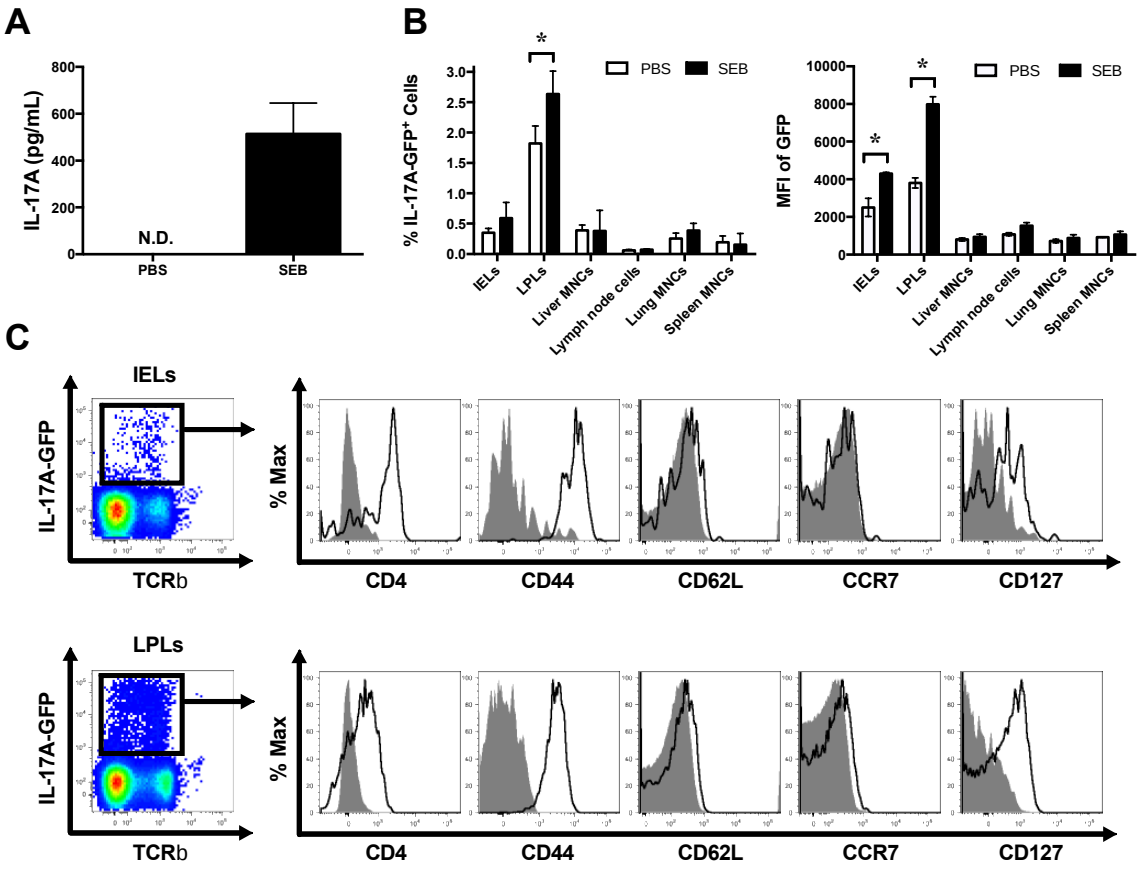
A
IL-17A (pg/mL)
800
600
400
200
0
N.D.
PBS
SEB
B
% IL-17A-GFP+ Cells
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PBS
SEB
*
IELs
LPLs
Liver MNCs
Lymph node cells
Lung MNCs
Spleen MNCs
MFI of GFP
10000
8000
6000
4000
2000
0
C
IELs
IL-17A-GFP
TCRb
% Max
CD4
CD44
CD62L
CCR7
CD127
LPLs

Figure 5.2: SEB-primed D17 mice launch a rapid IL-17A response and harbor an enriched population of IL-17A-producing CD4+ T_{EM} cells in their small intestine.

D17 mice, generated by crossing DR4tg and IL-17A-GFP reporter mice, were injected *i.p.* with 50 μg SEB or with PBS. (**A**) Two hours later, mice were euthanized and the IL-17A content of serum samples was measured. N.D.: not detectable. Error bars represent SEM (n=3/group). (**B**) The frequencies of GFP^+ cells and the MFI of GFP were determined in non-parenchymal mononuclear cells (MNCs) from indicated tissues and among intestinal intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs). * denotes p ″ 0.05 by two-tailed Student's *t*-test. (**C**) GFP^+ IELs and LPLs from SEB-injected mice were immunophenotyped using fluorochrome-labeled mAbs to indicated markers (open histograms) and isotype controls (filled histograms). Depicted data represent findings from 3 mice used in independent experiments.

Together, the above findings show that select SAgs induce rapid IL-17A production by $CD4^+$ T_{EM} cells in a clinically relevant mouse model of TSS. The IL-17A response by mouse $CD4^+$ T_{EM} cells requires high-affinity interactions between SAgs and human MHC, and is most pronounced among intestinal IELs and LPLs.

5.3.2 SAgs induce a rapid IL-17A response by human PBMCs *in vitro*

After we established that SAgs were able to stimulate early IL-17A production in DR4tg mice, we assessed whether SAgs also elicit IL-17A responses in human PBMCs. First, PBMCs from healthy donors were stimulated with SEB or TSST-1 and analyzed for changes in cytokine mRNA levels by qPCR. Samples from all donors showed massive IL-17A mRNA upregulation after stimulation by SEB and, to a slightly lesser extent, by TSST-1 (**Fig. 5.3A**). IL-17A mRNA was highly upregulated at 2 hours, reached a maximum at 4 hours, and gradually decreased until 24 hours post-stimulation (**Fig. 5.3A**). Interestingly, the magnitude and the kinetics of the IL-17A mRNA response differed considerably when compared with IFNγ (**Fig. 5.3A-B**), a prototypic pro-inflammatory cytokine that is induced by SAgs in humans (43) and that is considered pathogenic in mouse models of TSS (44). IL-17A mRNA upregulation was far greater in magnitude at early time points and began to decline after 4 hours whereas IFNγ mRNA levels steadily increased until 24 hours (**Fig. 5.3A-B**).

To ascertain whether human IL-17A was actively secreted among other rapidly produced inflammatory mediators (43), culture supernatants from SAg-stimulated PBMCs were assayed for cytokines by bead-based multiplexing. Both SEB and TSST-1 provoked rapid IL-17A and IFNγ responses, which were detectable as early as 2 hours and continued to

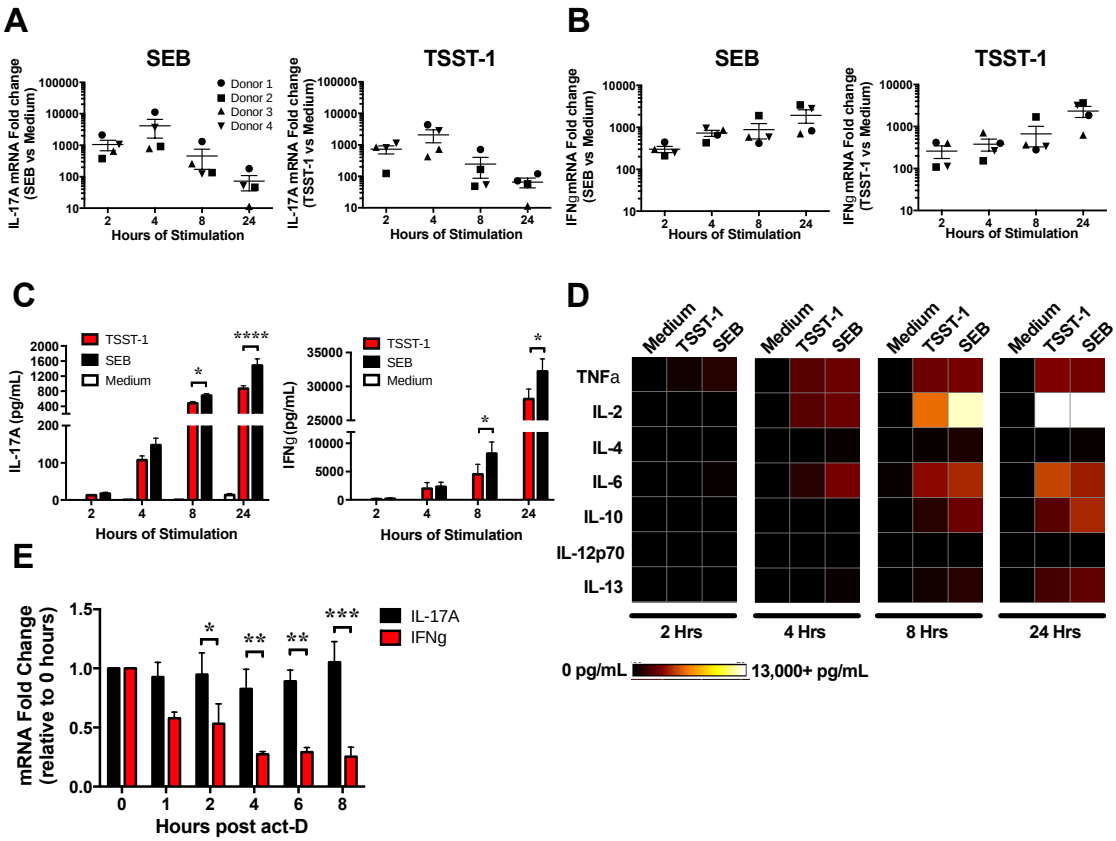
A
SEB
TSST-1
IL-17A mRNA Fold change (SEB vs Medium)
IL-17A mRNA Fold change (TSST-1 vs Medium)
Donor 1
Donor 2
Donor 3
Donor 4
Hours of Stimulation
B
IFNg mRNA Fold change (SEB vs Medium)
IFNg mRNA Fold change (TSST-1 vs Medium)
C
TSST-1
SEB
Medium
IL-17A (pg/mL)
IFNg (pg/mL)
D
Medium
TSST-1
SEB
TNFa
IL-2
IL-4
IL-6
IL-10
IL-12p70
IL-13
2 Hrs
4 Hrs
8 Hrs
24 Hrs
0 pg/mL
13,000+ pg/mL
E
mRNA Fold Change (relative to 0 hours)
IL-17A
IFNg
Hours post act-D

Figure 5.3: SAgs induce rapid IL-17A synthesis by human PBMCs.

Human PBMCs were left untreated or stimulated with 100 ng/mL of SEB or TSST-1 for indicated durations. (**A**) IL-17A or (**B**) IFNγ mRNA upregulation in SAg-stimulated PBMCs was determined by qPCR. Data are shown as fold change relative to unstimulated medium control at each time point. (**C** and **D**) Cytokine concentrations in the supernatant of SAg-stimulated PBMCs at indicated time points were determined by bead-based cytokine multiplexing. Mean values ± SEM from 4 individual donors are shown. Statistical comparisons were made by two-way ANOVA ($p \leq 0.0001$) with Holm-Sidak post-hoc analysis (* and **** denote $p \leq 0.05$ and $p \leq 0.0001$, respectively). (**E**) Human PBMCs were stimulated with SEB for 4 hours prior to act-D treatment. IL-17A and IFNγ mRNA fold changes normalized to time zero (*i.e.*, addition of act-D) were determined by qPCR. Data pooled from 3 individual donors are shown. Statistical comparisons were made by two-way ANOVA ($p = 0.0072$) with Holm-Sidak post-hoc analysis (*, ** and *** denote $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively).

accumulate by 24 hours (**Fig. 5.3C**). The inflammatory cytokines TNFα and IL-6 as well as the growth-promoting cytokine IL-2 were also produced within hours of SAg stimulation (**Fig. 5.3D**). While the kinetics of cytokine expression was consistent for both SEB and TSST-1 stimulation, SEB elicited a more robust response for most cytokines tested when compared to TSST-1 (**Fig. 5.3C-D**).

Thus far, our findings revealed an unexpected discordance vis-à-vis the kinetics of IL-17A expression at mRNA and protein levels – that is while IL-17A mRNA was rapidly upregulated and gradually declined after 4 hours, IL-17A protein continued to accumulate in the supernatant up to 24 hours (**Fig. 5.3A-C**). This discordance was especially apparent when compared to the IFNγ expression kinetics that followed a similar pattern for both cytokine mRNA and protein (**Fig. 5.3B-C**). We hypothesized that disparities in mRNA stability between IL-17A and IFNγ may account for the noted differences as more stable mRNA may not require consistent upregulation for translation into increasing amounts of protein. Since the degree of IL-17A mRNA stability in primary human T cells was unknown, we tested our hypothesis by comparing the half-lives of IL-17A and IFNγ mRNA in SEB-stimulated PBMC cultures using the transcriptional inhibitor act-D (45). PBMCs were challenged with SEB for 4 hours consistent with the IL-17A mRNA response peak (**Fig. 5.3A**), treated with act-D, and analyzed for cytokine mRNA expression in the subsequent 8 hours after transcriptional arrest. Intriguingly, while IFNγ mRNA was quickly degraded within the first few hours, IL-17A mRNA expression remained remarkably stable after the addition of act-D (**Fig. 5.3E**).

These results indicate that SAgs potently activate human PBMCs to rapidly upregulate IL-17A mRNA and secrete IL-17A protein within hours. Interestingly, the initial IL-17A

mRNA upregulation is greater in magnitude and yields more stable mRNA in comparison with the canonical inflammatory cytokine IFNγ although both cytokines are quickly secreted in culture.

5.3.3 SAg-exposed human $CD4^+$ T_{EM} cells are rapid and potent producers of IL-17A

As this work provides the initial report of IL-17A secretion in human PBMCs within the first few hours of SAg exposure, we next aimed to identify its cellular source. Exposure to either SEB or TSST-1 resulted in substantial intracellular IL-17A production by $CD3^+$ T cells within 4 hours (**Fig. 5.4A**). SEB was significantly more potent than TSST-1 and caused IL-17A production by 0.182% of all PBMCs (**Fig. 5.4A**). The MFI of IL-17A staining was equivalent though, indicating that SEB- and TSST-1-stimulated cells synthesized similar amounts of IL-17A on a per cell basis (**Fig. 5.4A**). We also observed that IL-$17A^+$ cells among TSST-1-stimulated PBMCs showed reduced CD3 expression suggesting partial TCR downregulation. Lastly, we confirmed that only $CD3^+$ T cells were actively secreting IL-17A using a cytokine secretion assay in which IL-17A that is about to be secreted binds to the cell surface via a bivalent antibody molecule specific for both CD45 and IL-17A (data not shown).

To immunophenotype IL-17A-producing cells, PBMCs were challenged with SEB for 4 hours and analyzed for a variety of surface markers and transcription factors by flow cytometry. The vast majority of IL-$17A^+$ cells expressed high levels of CD4 and CD45RO, and were $CCR7^-$ (**Fig. 5.4B**), indicating a surface phenotype characteristic of $CD4^+$ T_{EM} cells (46). In line with previous studies (47-49), IL-$17A^+$ cells also expressed CCR6 and the transcription factor RORγT (**Fig. 5.4B**), which are hallmarks of the Th17

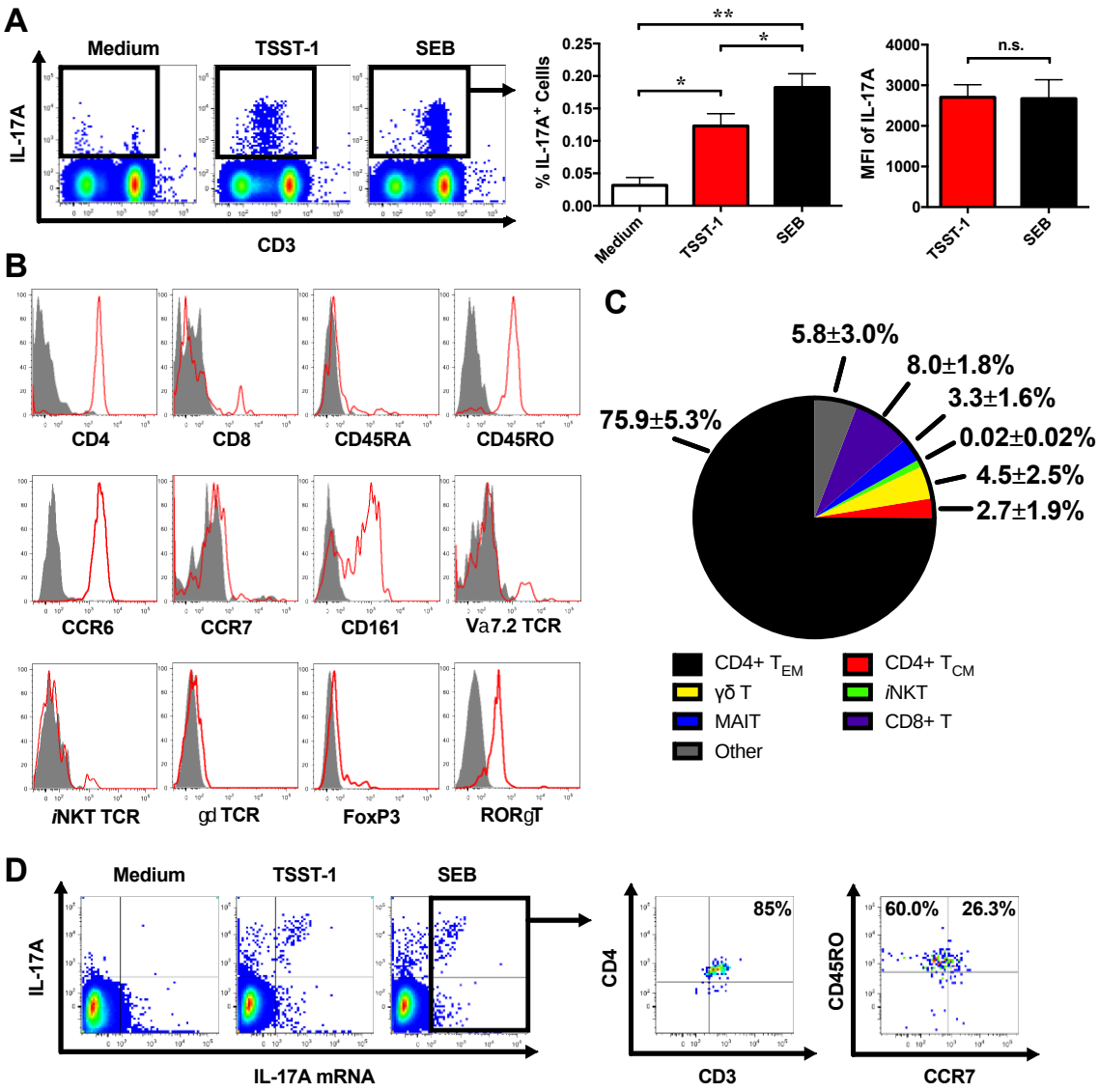
A
Medium
TSST-1
SEB
IL-17A
CD3
% IL-17A+ Cells
MFI of IL-17A
n.s.
B
CD4
CD8
CD45RA
CD45RO
CCR6
CCR7
CD161
Va7.2 TCR
iNKT TCR
gd TCR
FoxP3
RORgT
C
5.8±3.0%
8.0±1.8%
3.3±1.6%
0.02±0.02%
4.5±2.5%
2.7±1.9%
75.9±5.3%
CD4+ TEM
CD4+ TCM
γδ T
iNKT
MAIT
CD8+ T
Other
D
IL-17A mRNA
CD4
CD3
85%
CD45RO
CCR7
60.0%
26.3%

Figure 5.4: $CD4^+$ T_{EM} cells are the major source of IL-17A in SAg-stimulated human PBMCs.

Human PBMCs were left untreated in medium or stimulated with 100 ng/mL of SEB or TSST-1 for 4 hours. (**A**) IL-17A-producing cells were identified by intracellular cytokine staining and further analyzed by flow cytometry. Representative dot plots of SAg-stimulated PBMCs are depicted. The percentage of IL-17A-expressing cells and geometric MFI of IL-17A are shown as mean values ± SEM for 6 individual donors. One-way ANOVA ($p = 0.0007$) with Holm-Sidak post-hoc analysis was used (* and ** denote $p \leq 0.05$ and $p \leq 0.01$, respectively); n.s. = not significant by two-tailed Student's *t*-test. (**B**) $IL\text{-}17A^+$ cells were immunophenotyped based on their expression of indicated cell surface and intracellular markers (open red histograms) relative to isotype control (filled, gray histograms). Illustrated data represent 4 individual donors displaying similar results. (**C**) Values represent mean percentages ± SEM of 4 individual donors. Subtypes were defined as: $CD4^+$ T_{EM} ($CD4^+CD3^+CD45RO^+CCR7^-$), $CD4^+$ T_{CM} ($CD4^+CD3^+CD45RO^+CCR7^+$), γδ T ($\gamma\delta$ TCR^+), *i*NKT ($CD3^+$CD1d $tetramer^+$), MAIT ($CD3^+V\alpha7.2$ TCR^+CD161^+), $CD8^+$ T ($CD3^+$ $CD4^-$ $CD8^+$). (**D**) IL-17A protein and mRNA co-expressing cells were identified using the PrimeFlow RNA technology and analyzed by flow cytometry. Dot plots are representative of 4 individual donors.

lineage. Notably, the majority of IL-17A$^+$ cells expressed the lectin receptor CD161 (**Fig. 5.4B**). CD161 is highly expressed on innate-like T cells including γδ T, MAIT and *i*NKT cells but only moderately expressed on Th17 cells (50). Thus, we investigated γδ TCR, Vα7.2 TCR and *i*NKT TCR expression to rule in/out γδ T, MAIT and *i*NKT cells, respectively (**Fig. 5.4B**). These innate-like T cell subsets, in addition to a small population of CD8$^+$ T cells, constituted only minor fractions of the overall IL-17A$^+$ cell population (**Fig. 5.4C**). In contrast, CD4$^+$ T_{EM} cells were the dominant IL-17A-expressing population, which comprised greater than 75% of IL-17A$^+$ cells (**Fig. 5.4C**).

Strikingly, our results revealed thus far that only a small fraction of PBMCs produced IL-17A protein in response to SAgs (**Fig. 5.4A**) despite the massive upregulation of IL-17A mRNA observed in bulk PBMCs (**Fig. 5.3A**). We initially postulated that other cells types in addition to CD4$^+$ T_{EM} cells contributed to the substantial IL-17A mRNA response, but were not detectable by intracellular IL-17A staining. To address this, we utilized the PrimeFlow RNA assay, a new technology that utilizes fluorescent *in situ* hybridization to enable simultaneous detection of cells expressing IL-17A mRNA and intracellular IL-17A protein by flow cytometry. As expected, SEB stimulation enhanced the expression of IL-17A mRNA (**Fig. 5.4D**). Moreover, CD4$^+$ T_{EM} cells (CD3$^+$CD45RO$^+$CCR7$^-$) comprised the majority of IL-17A mRNA$^+$ cells (**Fig. 5.4D**), consistent with our intracellular IL-17A protein findings (**Fig. 5.4C**). Notably, a small proportion of IL-17A mRNA-producing CD4$^+$ T cells stained positively for CCR7 (**Fig. 5.4D**), indicating that CD4$^+$ central memory (T_{CM}) cells may be a source of IL-17A protein at later time points. These results indicate that CD4$^+$ T_{EM} cells are primarily

responsible for rapid IL-17A secretion among SAg-stimulated PBMCs despite their relative rarity.

As multi-cytokine-producing T cells are reportedly induced by SEB stimulation at relatively late time points (51), we explored whether early IL-17A-producers would also co-express IFNγ. Surprisingly, only a minute subset of IL-17A/IFNγ co-expressors was detectable following SAg stimulation over a 4-hour period, and the overwhelming majority of cytokine-producing cells exclusively expressed either IL-17A or IFNγ (**Fig. 5.5A**). A similar trend for IL-17A and IFNγ expression was also evident at 24 hours post-SAg stimulation (**Fig. 5.5B**). Interestingly, at 4 hours post-SEB, $CD4^+$ T_{EM} cells constituted a large proportion of $IFN\gamma^+$ cells (**Fig. 5.5C**), similar to $IL\text{-}17A^+$ cells observed above. This finding prompted us to examine whether IL-17A-producing $CD4^+$ T_{EM} cells had the potential to produce IFNγ mRNA, but were not synthesizing appreciable amounts of IFNγ protein. We found that only a small fraction of $IL\text{-}17A^+$ cells showed detectable IFNγ mRNA transcripts (**Fig. 5.5D**).

Collectively, the above results indicate that SAg-based stimulation of human PBMCs induces a rapid and potent IL-17A response that is chiefly mediated by $CD4^+$ T_{EM} cells. Although $CD4^+$ T_{EM} cells are responsible for substantial early IFNγ production as well, the IL-17A- and IFNγ-producing subsets were two independent populations.

5.3.4 IL-17RA blockade reduces inflammatory gene expression in SEB-exposed human PBMCs and secondarily activated dermal fibroblasts

Many downstream targets of IL-17A signaling are inflammatory cytokines and chemokines (52), which likely augment or perpetuate the overwhelming inflammatory

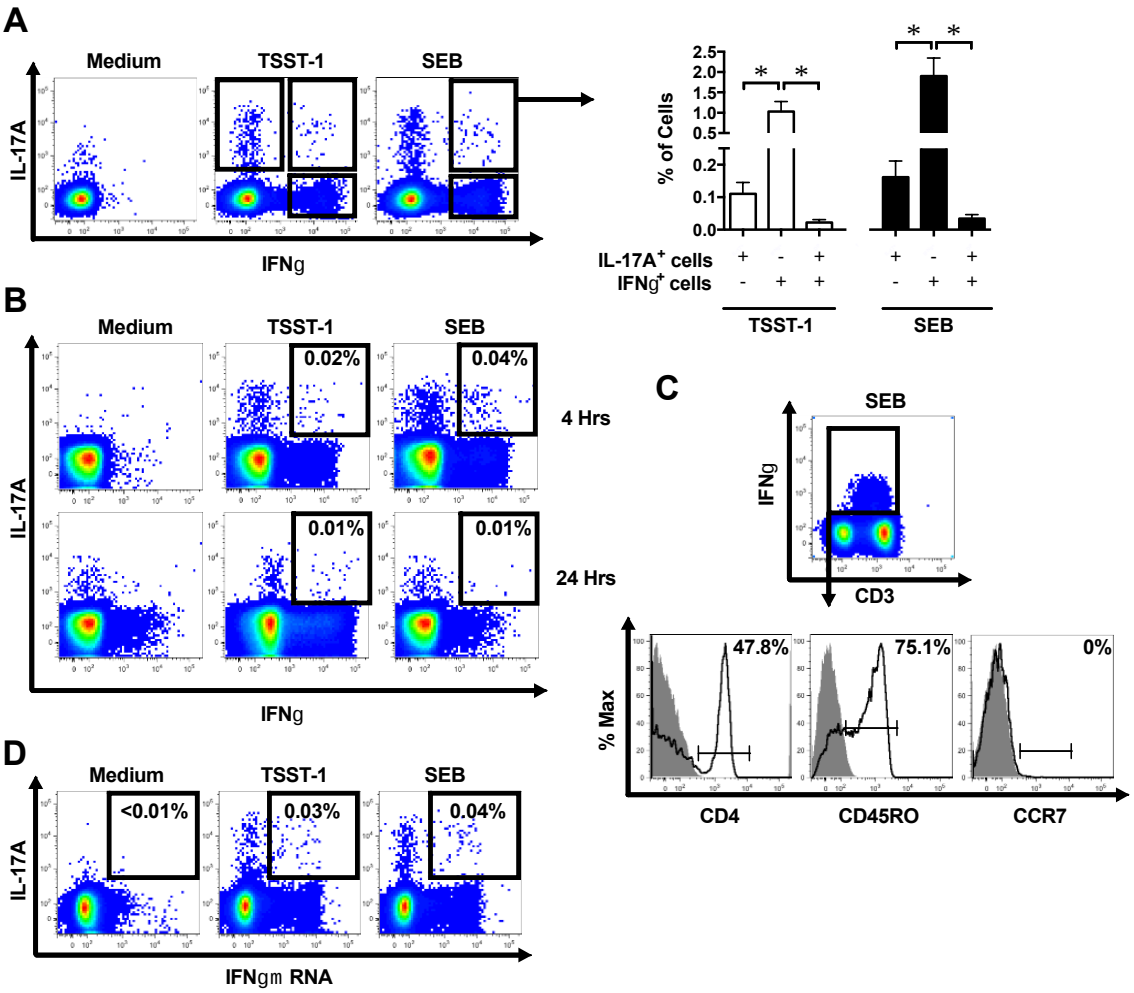
A
Medium
TSST-1
SEB
IL-17A
IFNg
% of Cells
IL-17A+ cells
IFNg+ cells
TSST-1
SEB
B
0.02%
0.04%
0.01%
0.01%
4 Hrs
24 Hrs
C
SEB
IFNg
CD3
47.8%
75.1%
0%
% Max
CD4
CD45RO
CCR7
D
<0.01%
0.03%
0.04%
IFNgm RNA

Figure 5.5: SAg-stimulated IL-17A- and IFNγ-producing cells are two distinct populations.

Human PBMCs from healthy volunteers were left untreated or stimulated with 100 ng/mL of SEB or TSST-1 for 4 hours (**A**) or 24 hours (**B**). IL-17A- and IFNγ-producing cells were identified by intracellular cytokine staining and quantified by flow cytometry. Representative dot plots of SAg-stimulated PBMCs and mean percentages ± SEM from 2-4 individual donors are shown. Statistical comparisons were made by one-way ANOVA (p = 0.0255 and p = 0.0224 for SEB and TSST-1 treatment conditions, respectively) with Holm-Sidak post-hoc analysis (* denotes $p \leq 0.05$). (**C**) Gated IFNγ-producing cells were immunophenotyped by their expression of surface markers (open black histograms) relative to isotype control (filled, gray histograms). Data are representative of 4 individual donors. (**D**) IL-17A and IFNγ mRNA co-expressing cells were identified using the PrimeFlow RNA technology and analyzed by flow cytometry. Dot plots are representative of 4 individual donors.

cascade encountered in TSS. Accordingly, stimulation of human PBMCs with SEB for 8 hours resulted in upregulation of many different pro- and anti-inflammatory genes known to be modulated by IL-17A (**Table 5.3**). Also highly upregulated was the related inflammatory cytokine IL-17F, which signals through the same IL-17RA/IL-17RC complex (53). Thus, we sought to evaluate the contribution of IL-17RA signaling to induction of inflammatory genes in the initiation of the cytokine storm.

PBMCs were pre-incubated with a blocking IL-17RA mAb or isotype control, stimulated with SEB for 8 hours and evaluated for changes in gene expression by qPCR. IL-17RA blockade resulted in diminished expression of several key inflammatory cytokines involved in TSS, including TNFα, IFNγ and IL-6 (**Fig. 5.6A**). Additionally, the expression of many chemokines such as CCL5, CCL7, CXCL1 and CXCL6 were highly downregulated. Interestingly, the blockade of IL-17RA signaling also resulted in upregulation of notable anti-inflammatory cytokines IL-4, TGFβ1 and IL-10 (**Fig. 5.6A**). It is noteworthy that in these experiments, we chose to work with a mAb dose (*i.e.*, 10 µg/mL) that was previously reported to efficiently block IL-17RA (54, 55). In a limited number of experiments, we reconfirmed that this dose could significantly inhibit the upregulation of CEBPD, a transcription factor linked to acute inflammatory conditions (56), in SEB-exposed PBMCs. In contrast, a 0.1 µg/mL-dose of anti-IL-17RA was completely ineffective, and a 1 µg/mL-dose inhibited CEBPD upregulation only marginally (data not shown).

In the next series of experiments, we utilized a human PBMC-dermal fibroblast co-culture system to mimic the secondary effect of IL-17A signaling in non-hematopoietic cells. To this end, PBMCs and fibroblasts were cultured in physically separate

Target	Fold Change (SEB relative to medium control)		
	Donor 1	Donor 2	Donor 3
IL22	1059.25	355.71	2198.29
IL17F	737.24	137.67	780.40
IFNG	304.81	661.94	445.69
IL17A	253.73	96.47	651.44
CSF3	583.76	20.88	296.10
IL6	111.03	6.77	140.41
IL19	70.28	50.68	115.96
IL13	45.96	24.00	84.64
IL2	32.45	58.60	19.38
IL12B	32.24	2.08	71.23
CCL20	40.69	2.49	45.23
CSF2	17.49	26.89	21.14
IL10	28.65	5.74	29.33
CXCL9	2.59	37.62	4.11
CXCL1	10.29	0.74	31.46
PTGS2	14.72	1.86	12.92
LTA	5.37	13.70	10.01
CXCL11	0.51	14.43	1.33
IL1B	6.39	1.20	5.01
TNFA	2.26	3.47	6.43
CXCL5	3.93	0.19	7.15
CXCL2	4.29	0.56	5.42
TBX21	3.48	3.80	0.95
IL8	3.98	0.82	3.03
IL12A	2.12	3.13	1.63
IL4	3.13	1.22	2.53
IL23A	2.13	2.12	2.20
NFKB1	2.43	1.53	2.02
CXCL6	2.75	0.08	3.14
NFKBIZ	2.11	1.29	1.74
CCL2	1.86	0.39	2.68
RORC	1.87	2.01	1.00
CXCL10	0.33	3.50	0.61
C/EBPB	1.11	0.89	0.90
ZBTB16	0.93	1.05	0.73
TGFB1	0.70	1.06	0.66
GATA3	0.50	0.66	0.82
CCL7	0.55	0.26	1.00
CCL5	0.68	0.60	0.34
MYD88	0.58	0.63	0.32
NOS2	0.58	0.12	0.36
C/EBPD	0.23	0.33	0.32
CXCL12	0.14	0.27	0.15
IL18	0.12	0.16	0.06

Table 5.3: Fold changes in gene expression of SEB-stimulated human PBMCs relative to medium control.

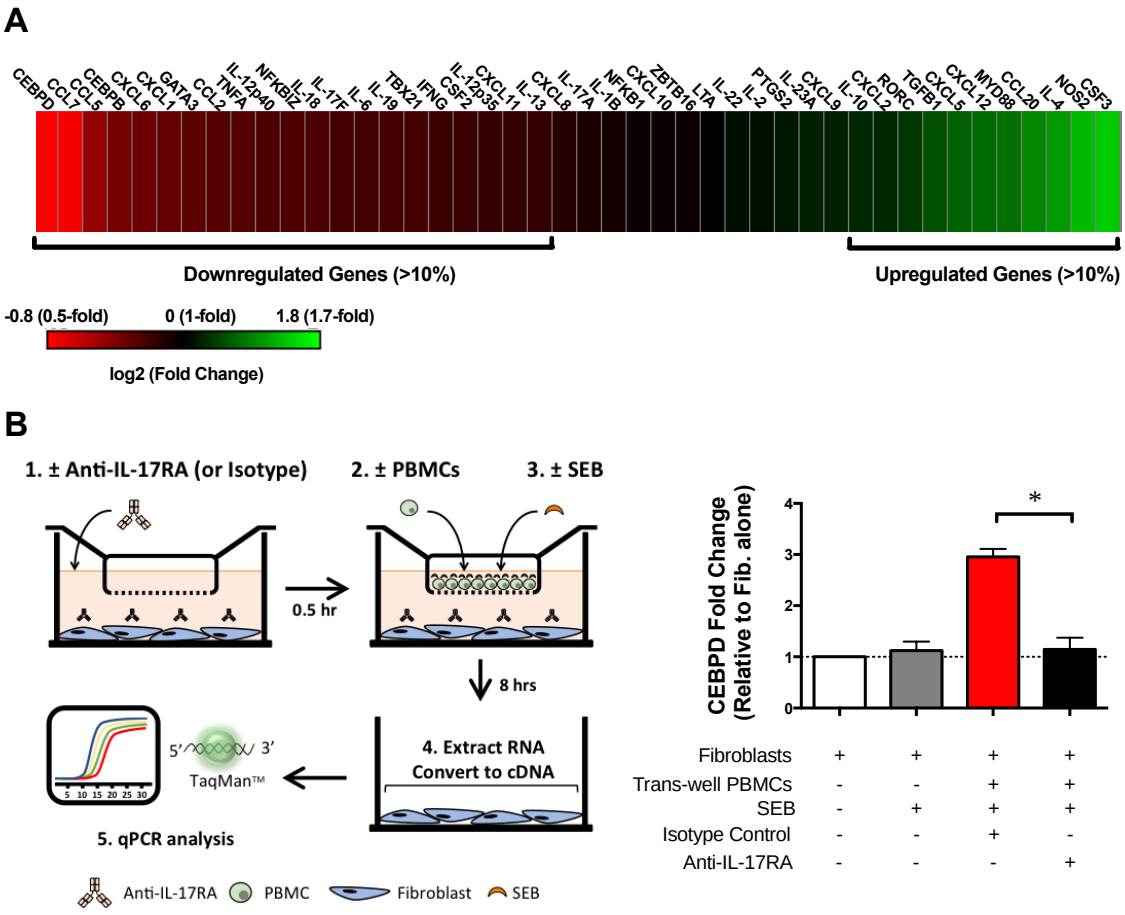
A
CEBPD
CCL7
CCL5
CEBPB
CXCL6
CXCL1
GATA3
CCL2
TNFA
IL-12p40
NFKBIZ
IL-18
IL-17F
IL-6
IL-19
TBX21
IFNG
CSF2
IL-12p35
CXCL11
IL-13
CXCL8
IL-17A
IL-1B
NFKB1
CXCL10
ZBTB16
LTA
IL-22
IL-2
PTGS2
IL-23A
CXCL9
IL-10
CXCL2
RORC
TGFB1
CXCL5
CXCL12
MYD88
CCL20
IL-4
NOS2
CSF3
Downregulated Genes (>10%)
Upregulated Genes (>10%)
-0.8 (0.5-fold)
0 (1-fold)
1.8 (1.7-fold)
log2 (Fold Change)
B
1. ± Anti-IL-17RA (or Isotype)
2. ± PBMCs
3. ± SEB
0.5 hr
8 hrs
4. Extract RNA
Convert to cDNA
5′
3′
TaqMan™
5. qPCR analysis
Anti-IL-17RA
PBMC
Fibroblast
SEB
CEBPD Fold Change
(Relative to Fib. alone)
*
Fibroblasts + + + +
Trans-well PBMCs - - + +
SEB - + + +
Isotype Control - - + -
Anti-IL-17RA - - - +

Figure 5.6: Blockade of IL-17RA signaling attenuates SEB-induced inflammatory gene expression in human PBMCs and in downstream non-hematopoietic cells.

(**A**) Human PBMCs were pre-incubated with 10 μg/mL of an anti-IL-17RA blocking antibody or isotype control and subsequently stimulated with 100 ng/mL of SEB for 8 hours. Changes in gene expression were determined by qPCR and represented as log2 (fold-change) of the anti-IL-17RA group normalized to isotype control. Differentially regulated genes were defined as >10% fold-change difference between treatment groups. Fold-changes in gene expression were pooled from 3 individual donors and are represented as mean values. (**B**) Human dermal fibroblasts were grown in a flat-bottom plate and co-cultured with human PBMCs that were seeded into a cell culture insert with a permeable membrane. Fibroblasts and PBMCs were in two physically separate compartments. Co-cultures were left untreated or stimulated with 100 ng/mL of SEB. Eight hours later, the insert was removed and fibroblasts were harvested and examined by qPCR for their CEBPD expression. Anti-IL-17RA or isotype control (10 μg/mL each) was added 30 minutes before SEB stimulation as indicated. Fold changes in CEBPD gene expression relative to the 'fibroblast-only' condition was calculated for 3 PBMC samples prepared from 3 individual donors. Data are shown as mean ± SEM, and * denotes $p \leq 0.05$ (Student's *t*-test; two-tailed).

compartments prior to SEB stimulation. We found a ~3-fold increase in the expression level of CEBPD by dermal fibroblasts that were co-cultured with SEB-primed PBMCs (**Fig. 5.6B**). Importantly, this response was abrogated in the presence of 10 µg/mL of anti-IL-17RA mAb. Also as expected, SEB did not change the expression of CEBPD in fibroblasts alone, a condition that was used as a control (**Fig. 5.6B**).

Taken together, the above findings strongly suggest that IL-17RA signaling plays an important pro-inflammatory role in TSS by increasing the expression of inflammatory mediators that are expected to contribute to TSS pathogenesis.

5.3.5 IL-17A is pathogenic in an *in vivo* model of TSS

Since the cytokine storm drives many symptoms of TSS, attenuation of rapidly produced cytokines that further perpetuate the inflammatory cascade, such as IL-17A, may be of prime importance to reduce TSS severity. Furthermore, IL-17A functions through induction of inflammatory mediators in both hematopoietic and non-hematopoietic compartments (19). Thus, we utilized the DR4tg mouse model, which provides a powerful *in vivo* system in which to delineate the role of early IL-17A responses in TSS. We treated DR4tg mice with an anti-IL-17A mAb 3 hours prior to and 1 hour after the administration of SEB and monitored disease progression. Neutralizing IL-17A at the TSS onset resulted in significantly attenuated weight loss as early as 20 hours after SEB injection (**Fig. 5.7A**). Importantly, by 7 days after SEB injection, 67% of mice that were treated with anti-IL-17A, compared to only 27% of mice receiving isotype control, had survived (**Fig. 5.7B**).

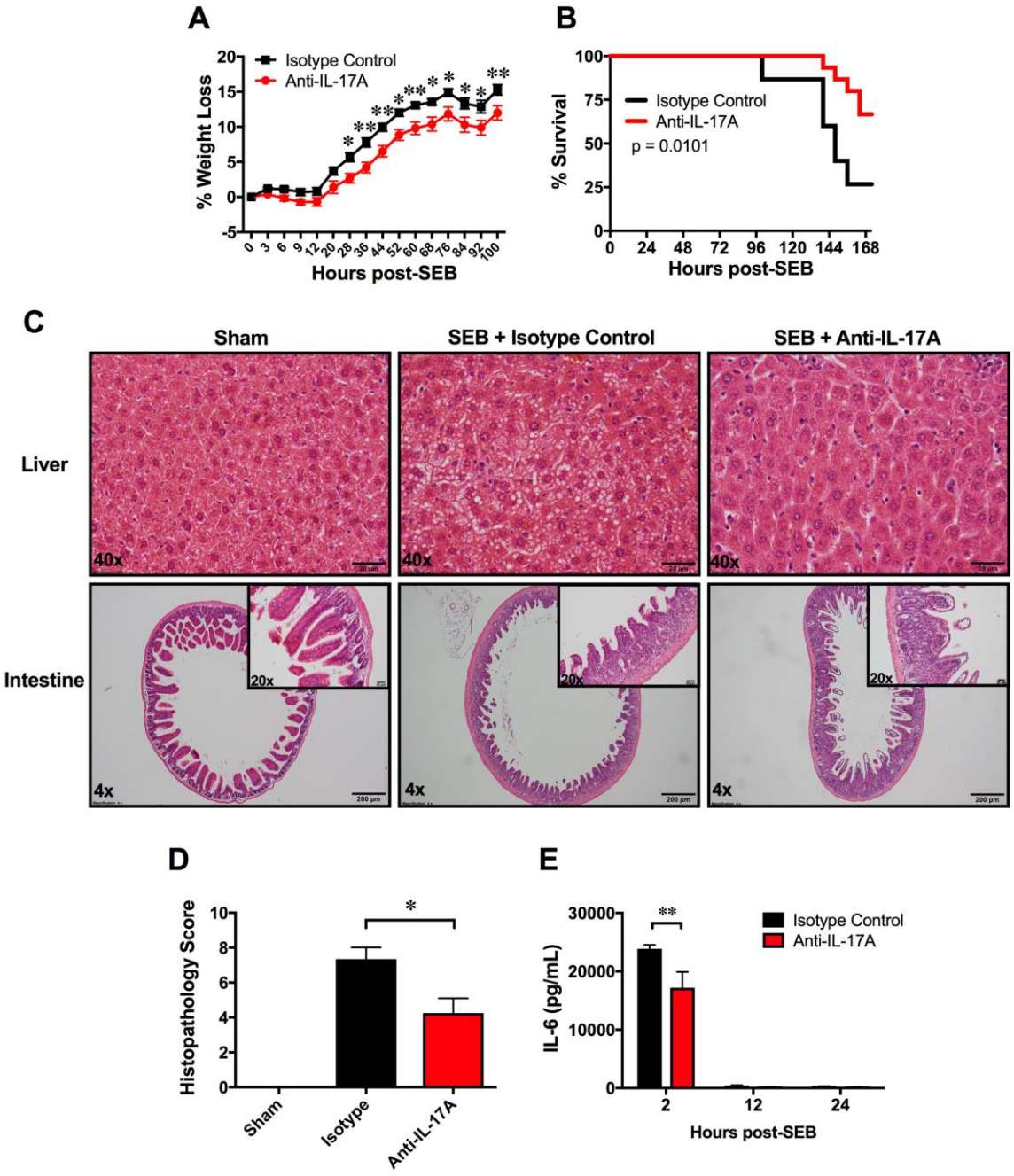
A
Isotype Control
Anti-IL-17A
% Weight Loss
Hours post-SEB
B
Isotype Control
Anti-IL-17A
p = 0.0101
% Survival
Hours post-SEB
C
Sham
SEB + Isotype Control
SEB + Anti-IL-17A
Liver
Intestine
40x
20x
4x
D
Histopathology Score
Sham
Isotype
Anti-IL-17A
E
Isotype Control
Anti-IL-17A
IL-6 (pg/mL)
Hours post-SEB

Figure 5.7: IL-17A neutralization in a humanized mouse model of TSS reduces morbidity, mortality and tissue damage.

DR4tg mice were injected *i.p.* with 200 μg of an anti-IL-17A neutralizing antibody or an isotype control 3 hours before and 1 hour after receiving 100 μg of SEB. (**A**) Weight loss was monitored every 3 hours for the first 12 hours and every 8 hours thereafter (n = 15 per group). Statistical comparisons were made using two-way ANOVA ($p = 0.0012$) with Holm-Sidak post-hoc analysis (* and ** denote $p \leq 0.05$ and $p \leq 0.01$, respectively). (**B**) Survival was monitored up to 7 days after SEB (n = 15 per group). Mice were sacrificed when moribund (≥20% weight loss). $p = 0.0101$ (log-rank test). (**C** and **D**) At 48 hours after SEB injection, mice were sacrificed for their organs and H&E staining was performed as per standard protocols. Sections were scored by a veterinary pathologist blinded to the experimental conditions. Histopathology scores represent sum of changes in the liver (periportal inflammation + microvesicular steatosis) and small intestine [inflammatory cell infiltration + mucosal score (epithelial cell damage + villi blunting)] observed in each section. Parameters received scores from 0-3, with 0 = normal, 1 = mild, 2 = moderate and 3 = severe. * denotes $p \leq 0.05$ (Student's *t*-test; two-tailed). Stained sections are representative of sham (n = 2), SEB + isotype control (n = 3) and SEB + anti-IL-17A (n = 4) groups. (**E**) Two hours after SEB injection, anti-IL-17A- and isotype control-treated mice (n=4/group) were bled and the IL-6 content of serum samples was quantified by ELISA. Mean ± SEM values are shown. Two-way ANOVA was used ($p = 0.05$) with Holm-Sidak post-hoc analysis (** denotes $p \leq 0.01$).

Since TSS is characterized by multi-organ dysfunction, we investigated whether IL-17A neutralization affects SAg-induced liver and intestinal damage. DR4tg mice receiving SEB and either an anti-IL17A mAb or an isotype control were sacrificed at 48 hours post-SEB, and liver and the small intestine were collected for histopathological analyses. SEB induced moderate periportal inflammation in the livers of both anti-IL-17A- and isotype control-treated mice (**Fig. 5.7C**). However, hepatic steatosis, an early indication of hepatotoxicity (57), was greatly reduced in anti-IL-17A-treated mice compared to those receiving the isotype control (**Fig. 5.7C**). Furthermore, the small intestines of mice receiving anti-IL-17A showed less inflammation, epithelial cell damage and villar blunting compared to the control cohort (**Fig. 5.7C**). As scored by a veterinary pathologist blinded to the cohorts, the overall TSS pathology for mice receiving anti-IL-17A was significantly reduced (**Fig. 5.7D**). Finally, we found a statistically significant decrease in the serum concentration of IL-6, a downstream target of IL-17A signaling, in anti-IL-17A-treated mice 2 hours after SEB administration (**Fig. 5.7E**).

Collectively, these results provide the first report that IL-17A is pathogenic in TSS and that neutralizing IL-17A ameliorates morbidity, mortality and tissue damage in a humanized mouse model of TSS.

5.4 Discussion

The inflammatory response mediated by IL-17A is the result of the induction and activation of a large network of immune effector molecules including cytokines, chemokines, acute phase reactants, tissue remodeling enzymes, antimicrobial peptides and transcription factors (19). In this way, IL-17A plays a critical role in the immune response against many different pathogens including *S. aureus* (58) and *S. pyogenes* (59). However, unbalanced or dysregulated IL-17A responses may also lead to excessive inflammation and can therefore be detrimental in several diseased states (52). In this study, we report a pathogenic role for IL-17A during infection.

Much of the existing knowledge of TSS pathogenesis has relied on conventional mouse models (12). However, SAgs exhibit poor affinity for certain mouse MHC class II molecules (34), and as a result, many strains of laboratory mice are resistant to TSS. Sensitizing agents such as D-galactosamine (D-galN) (60) or LPS (61) have been employed to amplify the effects of SAgs and induce lethality, but the extent to which these models accurately reflect human TSS is questionable. For example, D-galN pretreatment leads to extreme sensitivity to TNFα-induced apoptosis in hepatocytes (62), which causes rapid mortality solely due to fulminant hepatitis, which does not represent human TSS (63). Here, we investigated SAg-induced cytokine responses utilizing DR4tg mice that transgenically express the human MHC class II molecule HLA-DR4. This allows for high-affinity SAgs-MHC class II interactions resulting in robust activation of T cells and a cytokine storm that, if left untreated, simulates the potentially catastrophic human TSS (35-37).

We found that exposure of DR4tg mice to SEB resulted in swift production of several cytokines in serum, including the inflammatory cytokine IL-17A. Cytokines secreted in the early phase of the cytokine storm are especially important as they trigger endothelial cell damage that compromises vascular homeostasis and precipitates multi-organ dysfunction (64). Furthermore, rapid secretion of IL-17A not only has inflammatory effects on a wide variety of cell types throughout the body, it can directly induce and also synergize with other cytokines that are produced in the same timeframe. Other studies have found mouse IL-17A secretion in response to SAgs (36, 44, 65). IL-17 is also reportedly detectable in SEB-stimulated human PBMC cultures, albeit at relatively late time points (66, 67). Here, we demonstrate that very early IL-17A is of $CD4^+$ T_{EM} cell origin in mice and in human PBMC cultures. Furthermore, we show that the rapid induction of IL-17A, in addition to other cytokines, is dependent on T cell stimulation since the injection of DR4tg mice with a TCR-binding mutant form of SEB (SEB_{N23A}) resulted in minimal cytokine secretion. We believe the observed presence of IFNγ, at moderate levels, after SEB_{N23A} exposure is likely the consequence of SEB engaging T cells that bear Vβ domains other than Vβ8.2 (29). Lastly, we extended our investigation to other SAgs and found that SpeA and SEA could also induce early IL-17A secretion. Therefore, we propose that rapid IL-17A secretion plays a pathogenic role in both staphylococcal and streptococcal TSS.

To identify the location of IL-17A production in DR4tg mice, we generated IL-17A-GFP/DR4tg bone marrow chimeric mice as well as D17 mice in which IL-17A expression can be quantified by GFP fluorescence. Using these models, we found an increase in the frequency of $IL\text{-}17A^+$ cells and/or the signal intensity of IL-17A in several

tissues, but chiefly among IELs and LPLs from the small intestine, upon exposure to SEB. Interestingly, although IL-17A was consistently absent from the serum of PBS-injected mice, IL-17A-producing cells were readily detectable in these animals, suggesting that IL-17A is expressed at basal levels in multiple organs. This observation is in agreement with those of previous investigations utilizing alternatively generated IL-17A-GFP mice (68) or IL-17A fate-mapping reporter mice (69) that showed resting levels of IL-17A among cells of the small intestine. We expand on these findings to implicate $CD4^+$ T_{EM} cells as the main source of constitutively expressed IL-17A in naive mice. Nevertheless, it stands to reason that additional sources/locations of IL-17A may also exist.

Memory T cells are highly enriched in the intestinal mucosa and their propensity for immediate effector function upon re-activation (70) puts them in a unique position to be rapidly activated by SAgs. It has also been suggested that SAgs produced in the intestinal tract by food-borne pathogens may cross the gut mucosa directly (71), placing memory T cells among the first responders to SAgs. In support of this hypothesis, IL-17A was preferentially induced in the intestinal epithelial layer by $CD4^+$ T_{EM} cells when mice were exposed to SEB. Furthermore, a study by Tilahun *et al.* demonstrated that most NF-κB expression in SEB-injected HLA-DR3 mice was induced in the intestines (36).

The IL-17A response to SEB in mice appears to be translatable to humans, as PBMCs from healthy human volunteers stimulated with SAgs *in vitro* also exhibited rapid IL-17A production. IL-17A was secreted within hours of SAg exposure along with other inflammatory cytokines such as IFNγ, TNFα and IL-6. This inflammatory profile was most pronounced within the first few hours of SAg stimulation, with anti-inflammatory

cytokines IL-10 and IL-13 only reaching appreciable levels towards later time points. This pattern of expression provides additional evidence suggesting that potential therapies aimed at curbing the overwhelming cytokine storm in TSS may be most effective early after exposure to SAgs. A study by Krakauer *et al.* supports this line of reasoning, where mice surviving TSS showed reduced inflammatory cytokine and chemokine levels, though not including IL-17A, at 8 hours post-SEB administration (72). Furthermore, in a TSS model utilizing HLA-DR1 mice, lethality was prevented by anti-TNFα treatment, which dampened the early but not later phase of cytokine release (73). Therefore, it is likely that the early phase of the cytokine storm is responsible for TSS lethality and that attenuating this response may be a fruitful strategy to prevent mortality.

Unexpectedly, we observed apparently paradoxical differences between the kinetics of mRNA upregulation and resulting protein expression between IL-17A and IFNγ responses in SAg-stimulated PBMCs. We propose that the variation of kinetics between the two cytokines may be due, at least in part, to differences in mRNA stability. However, the mechanisms of post-transcriptional regulation of IL-17A remain ill-defined. In mice, the RNA-binding protein Hu antigen R positively regulates IL-17A expression and mRNA stability (74). Conversely, another RNA-binding protein, tristetraprolin, binds directly to the 3' untranslated region of IL-17A mRNA to mediate its decay (75). Lastly, the positive correlation between microRNAs and IL-17A expression in many diseases other than TSS also suggest that microRNAs may play a critical role in regulating IL-17A responses (76). Whether RNA-binding proteins or microRNAs post-transcriptionally regulate human IL-17A in TSS remains to be elucidated.

Our findings indicate that $CD4^+$ T_{EM} cells predominate in the early production of both IL-17A and IFNγ in SAg-stimulated human PBMCs. We also observed that IL-17A and IFNγ responses by $CD4^+$ T_{EM} cells were mutually exclusive, with only a small number of cells capable of producing both cytokines simultaneously. Thus, we propose that SAgs stimulate pre-existing and independent lineage-committed $CD4^+$ T_{EM} cell subsets to rapidly produce IL-17A (T_{EM}-17 cells) and IFNγ (T_{EM}-γ cells) in the initial phases of the cytokine storm. Our results may extend the traditional paradigm of Th1-, Th2- and Th17-lineages of activated effector $CD4^+$ T cells, which proceed to generate the body's long-lived pool of T_{EM} cells (77). This notion is supported by recent reports that human Th17 cells are a terminally differentiated T_{EM} cell population with a high capacity of self-renewal (78, 79).

The timing and the intensity of SAg stimulation in human PBMCs also apparently affect the phenotype and cytokine profiles of activated cells. We found that at 4 hours post-SEB stimulation, IL-17A-producing $CD4^+$ T_{EM} cells exclusively expressed the Th17-lineage transcription factor RORγT but did not co-produce IFNγ. In contrast, a recent study by Björkander *et al.* demonstrated that 24-hour stimulation of human PBMCs with SAgs induces IL-17A in $CD4^+$ T cells that express the regulatory T cell transcription factor FoxP3 (80). As FoxP3 has been previously shown to be upregulated in SAg-stimulated $CD4^+$ T cells over days of SAg exposure (81), it remains to be seen whether early IL-17A-producing $CD4^+$ T_{EM} cells may acquire FoxP3 expression after longer periods of SAg stimulation. In a separate study, overnight stimulation of human PBMCs with 10 μg/mL of SEB (a 100-fold greater concentration of SAg than the dose used in this study) induced multi-functional $CD4^+$ T_{EM} cells that often co-expressed IL-17A and IFNγ (51).

Both at 4 and 24 hours after SAg stimulation, we detected only a minute subpopulation of co-producing $CD4^+$ T_{EM} cells. It is possible that the high concentration of SAg used by McArthur *et al.* may have been responsible for the co-producer phenotype. Circulating levels of SAgs in patients' serum samples range from ~40 pg/mL in intensive care units (82) to 50-100 ng/mL in adult burn patients (83), and as high as 1,000 ng/mL in streptococcal TSS patients (84). As such, our findings utilizing a more conservative concentration of SAg may represent a more physiologically relevant outcome for SAg stimulation of human PBMCs.

We found that the blockade of IL-17RA signaling in SEB-stimulated PBMCs attenuated the expression of multiple inflammatory genes including IL-6, TNFα, and IFNγ, which mediate many toxic effects of TSS (12). Concomitantly, the expression of anti-inflammatory cytokine genes (IL-10, TGFβ1 and IL-4) was upregulated by IL-17RA blockade. To our surprise, CCL20, a chemokine that binds CCR6 to mobilize lymphocytes and dendritic cells, was also upregulated. Nevertheless, the overall picture emerging from our IL-17RA blockade experiments strongly suggests a pro-inflammatory role for IL-17RA signaling in the context of human PBMC responses to SAgs. Equally important, IL-17RA blockade in dermal fibroblasts prevented their secondary response to SEB-exposed PBMCs. Therefore, this approach may work at multiple levels and may have therapeutic potentials for TSS. When patients with severe plaque psoriasis were treated with brodalumab, a mAb against IL-17RA, reduced expression of inflammatory cytokine transcripts was noted (85). Our results are also supported by the findings of Crowe *et al.* that IL-17RA knockout mice show greatly attenuated cytokine storm when

infected with influenza virus (86). In this model, the absence of IL-17RA signaling decreased lung inflammation, weight loss and morbidity.

Remarkably, *in vivo* neutralization of IL-17A in DR4tg mice at the onset of TSS led to a significant reduction in weight loss and a striking increase in survival. We also observed drastic pathological changes in both the liver and the epithelium of the small intestine in DR4tg mice exposed to SEB. These mice developed hepatic microvesicular steatosis, an early toxic change in hepatocytes that signals serious disease. Characterized by the accumulation of small lipid vesicles in hepatocytes, microvesicular steatosis is indicative of impaired mitochondrial β-oxidation of fatty acids and can lead to multi-organ dysfunction (57). Importantly, microvesicular steatosis occurs in both human TSS (87, 88) and sepsis (89). To our knowledge, our work is the first description of its reversal in an animal model of TSS. We also observed considerable epithelial damage and villi blunting in the small intestine of mice injected with SEB. These pathological changes likely impair intestinal function and contribute to the weight loss observed in SAg-exposed mice. Importantly, *in vivo* neutralization of IL-17A in this model markedly attenuated both hepatic steatosis and damage to the intestinal epithelium. As almost all human TSS patients show multi-organ dysfunction (90), our results suggest that IL-17A may be an attractive clinical target to reduce TSS severity.

The key to successful treatment of TSS remains the rapid identification of the source of infection, appropriate and effective use of antibiotics, drainage of wounds (or removal of tampon in menstrual TSS) and supportive care (91). Intravenous immunoglobulin has also been shown to improve clinical outcomes in TSS caused by group A *Streptococcus* (92). However, there is currently no cure for the cytokine storm induced by SAgs. Here,

we define the rapid production of IL-17A by $CD4^+$ T_{EM} cells as a novel mechanism underlying TSS immunopathology. Our findings suggest that neutralizing IL-17A function may serve to lessen systemic inflammatory cytokine levels and decrease tissue damage in TSS. Thus, a therapeutic approach targeting the early induction of multiple inflammatory mediators that are involved in the cytokine storm, including IL-17A, may be effective in averting TSS mortality.

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Chapter 6

General Discussion and Conclusions

6.1 Discussion

Over the last few decades of intensive research, much has been learned about the biological and molecular determinants of TSS pathogenesis. It is clear that bacterial SAgs cause TSS through the abnormal activation of large numbers of T cells, resulting in a massive release of systemic inflammatory mediators in a matter of hours (1, 2). This 'cytokine storm' defines many, if not most, signs and symptoms of clinical TSS, which can progress to multi-organ failure as early as 8-12 hours after the onset of symptoms (3). Despite our current understanding of the factors that trigger TSS pathology, there currently are no available therapeutic options that decrease the severity of the cytokine storm. Successful clinical management of TSS still relies almost wholly on supportive care (4). Together, these issues highlight the need to identify the key players of the early stages of the cytokine storm, where clinical intervention is most likely to be effective. Towards this goal, this thesis aimed to utilize clinically relevant humanized mouse models and human cells to characterize rapid immune responses in TSS.

6.1.1 *i*NKT cells are pathogenic in TSS

In Chapter 3, I defined the role of *i*NKT cells in TSS pathogenesis using a humanized DR4tg mouse model. These mice express the human MHC class II molecule HLA-DR4, which, unlike some mouse MHC class II, allows for potent T cell activation by SAgs similar to human TSS. To generate a mouse strain that is *i*NKT cell-deficient yet retains is ability to potently respond to SAgs, I crossed DR4tg mice with germ-line *i*NKT cell knockout mice ($J\alpha18^{-/-}$). When challenged with SAg, these novel *i*NKT cell-deficient 'DJ' mice showed significantly attenuated weight loss and mortality compared to the *i*NKT cell-sufficient DR4tg mice (**Fig. 3.1B**), strongly suggesting that *i*NKT cells were

acting in a pathogenic manner. However, a relatively recent report highlights that Jα18$^{-/-}$ mice may have additional cellular deficiencies (5). Therefore, I confirmed my findings utilizing an *i*NKT cell-depleting antibody (6) that selectively eliminated *i*NKT cells from the liver and, to a somewhat lesser extent, from the spleen (**Fig. 3.1C-D**). Both experimental approaches demonstrated that *i*NKT cells are pathogenic in TSS.

*i*NKT cells are known for their rapid and copious production of both pro- and anti-inflammatory cytokines, due to the constitutive expression of cytokine mRNAs (7). To evaluate the contribution of *i*NKT cells to the TSS cytokine storm, I quantified the levels of serum cytokines of DR4tg and DJ mice after exposure to SEB. Strikingly, when compared to DR4tg mice, DJ mice showed reduced quantities of many inflammatory cytokines and chemokines consistent with an attenuated cytokine storm (**Fig. 3.4A**). Importantly, several inflammatory cytokines known to play a direct role in TSS immunopathology were present only at the 2-hour time point after SEB injection and were markedly decreased in DJ mice (**Fig. 3.4B**). These results indicate that *i*NKT cells are major contributors to the early cytokine storm in TSS. The finding that TSS morbidity/mortality was reduced when *i*NKT cells were polarized towards Th2-type, anti-inflammatory cytokine responses by OCH (**Fig. 3.4C and 3.6**) further strengthens this notion.

In light of the results presented in this chapter, further investigations dissecting the mechanistic contribution of *i*NKT cells to TSS immunopathology are clearly warranted. However, this study, like the vast majority of *i*NKT cell research utilizing germ-line knockout mouse models, is hindered by the lack of 'clean' *i*NKT cell-deficient mice. CD1d$^{-/-}$ mice lack NKT cells in general (8) and therefore the contribution of either *i*NKT

and *v*NKT cells cannot be disentangled. As noted previously, Jα18$^{-/-}$ mice show reduced TCR α-chain diversity, which does not seem to impact the frequency of major immune cell subsets (**Fig. 3.2**) yet may have unknown ramifications to immune responses in these mice. Additional studies with Jα18$^{-/-}$ mice should use newly generated strains that do not possess TCRα repertoire defects, once they become available.

The *i*NKT cell-specific mAb NKT14 shows considerable promise in the depletion of *i*NKT cells in mouse models. Scheuplein et. al. demonstrated the efficient and selective depletion of *i*NKT cells in NKT14-injected mice by flow cytometry (6). However, the authors did not rule out whether the absence of *i*NKT cells is due to TCR downregulation, a common characteristic of activated *i*NKT cells (9). To address this issue, I evaluated gene expression levels of the *i*NKT cell TCR α-chain (Vα14-Jα18) in NKT14- and isotype control-treated mice. Although Vα14-Jα18 expression in mice injected with NKT14 was greatly reduced in the liver (by ~90%), the mRNA levels in the spleen showed only a 50% reduction compared to controls (**Fig. 3.1D**). This finding suggests that the efficacy of *i*NKT cell depletion by NKT14 is tissue specific and may be incomplete. The presence of *i*NKT cells that have downregulated their TCR is potentially problematic because *i*NKT cells can be activated independently of TCR signals, such as through IL-12 and IL-18 for example (10). Thus, they may still participate in the cytokine storm. Although, DJ mice and NKT14-injected mice showed similar reductions in TSS mortality compared to *i*NKT-sufficient controls (**Fig 3.3**), my findings highlight the need for a comprehensive evaluation of the NKT14 effectiveness throughout individual mouse tissues.

6.1.2 MDSCs rapidly accumulate in the liver during TSS

In Chapter 4, I discovered a dramatic influx of myeloid cells into the liver of DR4tg mice shortly after SEB exposure (**Fig. 4.1A**). These cells resembled G-MDSCs in both cell surface phenotype ($CD11b^{+}Gr\text{-}1^{high}Ly\text{-}6C^{+}$) and morphology (**Figs. 4.1B, 4.3 and 4.4**). I classified these cells as *bona fide* MDSCs based on their ability to potently suppress SEB-induced T cell proliferation (**Fig. 4.5A**). Using several pharmacological inhibitors, I also showed that MDSC-mediated T cell suppression was mediated by ROS, as opposed to arginase or nitric oxide, confirming the identity of these cells as G-MDSCs (**Fig. 4.5B**). Collectively, my results demonstrated that SAg exposure in DR4tg mice rapidly triggers the accumulation of G-MDSCs in the liver.

During severe inflammation, MDSCs are known to amass in peripheral lymphoid organs, typically after several days (11). Contrastingly, I demonstrated the hepatic accumulation of MDSCs in just 2 hours after TSS onset. The rapidity of this response and a lack of Ki67 upregulation in the liver, in conjunction with decreased MDSC frequency in bone marrow (**Fig. 4.6**), is highly suggestive of direct MDSC recruitment from the bone marrow to the liver. The exact cellular and chemotactic factors that mediate this process are unknown. In mouse models of hepatocellular carcinoma, tumor derived GM-CSF and CXCL1 facilitate the recruitment of MDSCs to the liver (12). In addition, IL-17A derived from γδ T cells indirectly triggers MDSC migration to liver via inducing the secretion of CXCL5 by tumor cells (13). Importantly, I found that GM-CSF, CXCL1, IL-17A and CXCL5 were all rapidly produced within 2 hours of SAg exposure in DR4tg mice, as detailed in Chapter 3 (**Fig. 3.4B**). Interestingly, when injected with SEB, DJ mice did not show a reduction in hepatic MDSC accumulation (data not shown), despite the absence of

*i*NKT cells and a marked decrease in many inflammatory mediators in serum (**Fig. 3.4B**). The dramatic reduction of GM-CSF, CXCL1 and IL-17A in DJ relative to DR4tg mice suggests these factors may not play a dominant role in this response. Although CXCL5 levels were also reduced in DJ mice, the difference was minor and deemed not statistically significant, which leaves open the possibility that CXCL5 may promote this process. Nevertheless, further inquiry into the mechanisms behind MDSC recruitment to the liver in TSS is required.

Using a suppression assay, I established that MDSCs were potent suppressors of SAg-mediated T cell proliferation *in vitro*. However, the capacity of MDSCs to mitigate hepatic tissue damage *in vivo* during TSS needs to be explored. To this end, I attempted to deplete MDSCs in DR4tg mice using an anti-Gr-1 mAb and the chemotherapy agent gemcitabine. Anti-Gr-1 antibodies are commonly used to deplete MDSCs in tumor bearing mice, although they are not necessarily selective in doing so (14). Previous studies also show that anti-Gr-1 antibodies have difficulty depleting MDSCs in the liver (15). Accordingly, mice treated with anti-Gr-1 antibody did not reduce MDSC recruitment to the liver in TSS (data not shown). Alternatively, the chemotherapy drug gemcitabine has also been shown to be effective in selectively depleting MDSCs in mouse models of cancer (16, 17). Treatment with gemcitabine prior to SAg exposure failed to prevent MDSCs recruitment to the liver (data not shown). Several other alternatives to gemcitabine can be used to selectively deplete MDSCs, such as 5-fluorouracil (18), which may be effective in our mouse model of TSS and warrant further study. Qin et al. recently demonstrated a promising approach where the depletion of intratumoral MDSCs was achieved by a therapeutic ‘peptibody’ generated by fusing

MDSC-binding peptides with the Fc portion of a mouse IgG2b (19). It remains to be seen whether this method proves to be useful in depleting MDSCs in peripheral tissues or other disease contexts. An efficient and selective method for MDSC depletion *in vivo* would allow for a thorough investigation into the role of recruited MDSCs during TSS.

In this chapter, I showed that *ex-vivo* generated human MDSCs were able to potently inhibit autologous T cell responses to SEB (**Fig. 4.5D**), indicating that human MDSCs possess the same SAg-suppressive capacity as in mice. However, MDSCs generated in this manner resemble M-MDSCs in both surface phenotype and morphology (20), as opposed to the G-MDSCs I detected in mice. Therefore, whether G-MDSCs expand and home to the liver in human TSS remains an unanswered but important question. A recent study in human sepsis found that as early as 12 hours after diagnosis, both M-MDSCs and G-MDSCs were present in high quantities in patients' peripheral blood (21). Like TSS, the septic immune response begins with a cytokine storm (22), and thus the presence of early MDSCs in septic blood suggests that a similar process may occur in TSS patients as well.

6.1.3 IL-17A is pathogenic in TSS

In Chapter 5, I characterized the cellular source of rapid IL-17A production in both DR4tg mice and human PBMCs. Using IL-17A-GFP/DR4tg bone marrow chimeric mice, I identified $CD4^+$ T_{EM} cells in the intestinal epithelium as a major source of the rapid IL-17A response to SEB (**Fig. 5.1C-D**). This phenotype was confirmed using D17 mice, which were generated by crossing DR4tg and IL-17A-GFP mice to homozygosity (**Fig. 5.2A-C**). Furthermore, PBMCs from healthy human volunteers stimulated with SAgs also exhibited IL-17A production within hours, suggesting that rapid IL-17A responses in

TSS are translatable to humans (**Fig. 5.3C**). Similar to DR4tg mice, $CD4^+$ T_{EM} cells constituted the vast majority of IL-17A-producing cells among human PBMCs (**Fig. 5.4B-C**). Notably, $CD4^+$ T_{EM} cells were also responsible for a large proportion of early IFNγ as well (**Fig. 5.5C**). Thus, my findings implicate $CD4^+$ T_{EM} cells as major players in the rapid production of inflammatory cytokines during TSS in both mice and humans.

Importantly, I defined the role of rapidly produced IL-17A in response to SAgs as pathogenic in TSS. Since many downstream targets of IL-17A include cytokines known to participate in cytokine storm, I evaluated changes in inflammatory gene expression in SEB-stimulated human PBMCs after IL-17A signaling blockade. Several key inflammatory mediators of TSS including TNFα, IFNγ and IL-6 were downregulated as a result of incubation with anti-IL-17RA antibody (**Fig. 5.6A**), suggesting that IL-17A contributes to the cytokine storm and is therefore pathogenic in TSS. Accordingly, IL-17A receptor blockade also abrogated the secondary effect of SAg-stimulated PBMCs on human dermal fibroblasts as judged by C/EBP delta (CEBPD) expression (**Fig. 5.6B**). In the DR4tg mouse model of TSS, *in vivo* neutralization of IL-17A significantly attenuated SEB-induced morbidity and mortality (**Fig. 5.7A-B**). Remarkably, mice receiving anti-IL-17A antibody also demonstrated a stark reduction in intestinal and hepatic tissue damage compared to non-neutralized controls (**Fig. 5.7C-D**). Collectively, my findings reveal the previously unrecognized contribution of IL-17A to the initiation of the cytokine storm and the development of TSS immunopathology.

The gene expression of many different cytokines, chemokines and inflammatory mediators in addition to those implicated in TSS pathogenesis were highly upregulated in human PBMCs 8 hours after SAg exposure. Strikingly, IL-22 was most highly

upregulated amongst all genes examined including IL-17A, IFNγ and IL-6 (**Table 5.3**), suggesting it may be produced in abundance after SAg stimulation. Similar to IL-17A, IL-22 is essential in immunity to several Gram-negative extracellular pathogens, yet in excess can be pathogenic in inflammatory disorders such as psoriasis and rheumatoid arthritis (23). Importantly, IL-22 acts synergistically with IL-17A to induce inflammation (24, 25) and may therefore be involved in the TSS cytokine storm. Future studies addressing the role of IL-22 and other highly upregulated genes in SAg-stimulated PBMCs may uncover novel mechanisms that drive or inhibit TSS pathogenesis.

In this chapter, I provided the first report that rapid IL-17A responses induced by SEB exposure in mice are translatable to human PMBCs. However, the fundamental question is whether human patients show high levels of IL-17A during the cytokine storm in TSS. Although this question remains unanswered, there is accumulating evidence for the rapid production of IL-17A in the cytokine storm during sepsis. A recent study demonstrated that human neonates with sepsis exhibit IL-17A receptor signaling (26). In fact, IL-17RA emerged as a critical effector pathway upon gene expression analysis of whole blood from septic infants when compared to healthy controls. More conclusive, yet preliminary data was recently presented at the American Thoracic Society 2016 International Conference by Mikacenic et al., who measured IL-17A levels in septic patients within 24 hours of diagnosis (27). A doubling of IL-17A concentration was associated with increased severity score, progression to septic shock and 28-day mortality. These studies demonstrate that IL-17A is rapidly produced during cytokine storm and is pathogenic in sepsis. Whether this is also the case with TSS requires further investigation.

6.2 Conclusions and Implications For Future Studies

A recurrent theme throughout this thesis is that early immune responses to SAgs are critical for the development of TSS immunopathology. This is because the cytokine storm, which defines many symptoms of TSS, begins within hours of exposure to SAgs. Accordingly, I found that both DR4tg mice and human PBMCs rapidly produce a variety of inflammatory mediators in as little as 2 hours after stimulation with SAgs. The initial wave of the cytokine storm in both mice and humans was dominated by inflammatory cytokines such as TNFα, IFNγ, IL-2, IL-6 and IL-17A. Contrastingly, anti-inflammatory cytokines, like IL-10, appeared well after the inflammatory cytokine cascade reached excessive levels. These observations suggest that the early phase of the cytokine storm may be a critical period of systemic inflammation that culminates in tissue damage and eventually multi-organ failure. In support of this hypothesis, neutralizing IL-17A in DR4tg mice at the beginning of TSS onset attenuated hepatic and intestinal tissue damage in addition to mortality. Similarly, in a DR1tg mouse model of TSS, SAg-induced lethality was prevented only by pre-treatment of anti-TNFα antibody, whereas a 4-hour delay in antibody treatment after the onset of TSS was ineffective (28). Therefore, early immune responses to SAgs are likely responsible for initiating the inflammatory cascade that leads to organ damage and lethality in TSS. Interestingly, the rapid recruitment of MDSCs to the liver in DR4tg mice shortly after SEB exposure may represent an attempt by the host to mitigate hepatic pathology. Thus, future studies targeting this early timeframe should be fruitful in identifying novel mechanisms driving and/or attenuating TSS pathogenesis, or aid in the generation of therapies to reduce TSS severity.

Given that SAgs rapidly trigger the hyperactivation of the immune cells to cause disease, identifying the cellular processes that promote or inhibit the initial phases of the cytokine storm is critical to the understanding of TSS pathogenesis. In Chapter 3, I showed that *i*NKT cells were major contributors to the production of inflammatory mediators in TSS using DR4tg mice. In Chapter 4, I discovered a rapid influx of MDSCs into the liver in SAg-exposed DR4tg mice, which represents a novel mode of immunosuppression that may limit tissue damage in TSS. In Chapter 5, I demonstrated that $CD4^+$ T_{EM} cells in both DR4tg mice and human PBMCs were the main source of early IL-17A cytokine responses. Thus, my findings uncovered the previously unrecognized role of *i*NKT cells, MDSCs and T_{EM} cells in the early stages of TSS. Future investigations targeting these cells and/or their downstream effector responses for potential therapeutics may prove useful in preventing TSS mortality.

As the early cytokine storm is paramount to the pathogenesis of TSS, prospective therapies aimed at reducing disease severity are likely to be most effective early after exposure to SAgs. My findings suggest two novel approaches that target the early cytokine storm to reduce TSS severity. First, I demonstrated that skewing *i*NKT cell responses towards an anti-inflammatory phenotype using Th2-polarizing agonist OCH significantly attenuated both morbidity and mortality in a mouse model of TSS. Therefore, glycolipid agonists like OCH may be considered as potential therapeutics in TSS. Previous studies from this lab have similarly shown success with OCH in the prevention/treatment of inflammatory disorders in mouse models such as autoimmune arthritis (29), cardiac allograft rejection (30) and sepsis (31). Importantly, *i*NKT cell agonists exhibit significant promise in pre-clinical and clinical trials of cancer and viral

diseases (32). However, *i*NKT cell populations in humans are less abundant than in mice, at least in circulation, and whether their therapeutic manipulation has the same beneficial effect in this context needs to be confirmed. Another approach to the treatment of TSS would be to directly target inflammatory mediators in the cytokine storm. To this end, I demonstrated that neutralizing early IL-17A reduced the expression of many inflammatory mediators in SEB-stimulated human PBMCs and decreased tissue damage, morbidity and mortality in a mouse model of TSS. Therefore, targeting the multiple early inflammatory mediators of TSS, including IL-17A, may be effective to attenuate the inflammatory cascade and prevent tissue damage. However, a potential limitation to therapies aimed at mitigating rapid mediators of TSS is the need to identify patients early in the cytokine storm. As this thesis identifies novel contributors to TSS pathology, investigating the downstream mechanisms of these pathways is an important avenue for future research.

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Appendices

Appendix A: Copyright approval

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March 28, 2017

Peter Szabo
University of Western Ontario
Schulich Medicine & Dentistry
Department of Microbiology and Immunology
[redacted]
Canada
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Dear Dr. Szabo,

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Appendix B: Human Ethics Approval

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice

Date: October 03, 2016
Principal Investigator: Dr. Mansour Haeryfar
Department & Institution: Schulich School of Medicine and Dentistry\Microbiology & Immunology,Western University

Review Type: Expedited
HSREB File Number: 5545
Study Title: Regulation of Immune Responses by Natural Killer T (NKT) and Natural Killer (NK) cells - 15439E
Sponsor: Canadian Institutes of Health Research

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2017/10/31
Expiry Date -2017/10/30

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

[redacted]
Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer: Erika Basile ✓ Katelyn Harris___ Nicole Kaniki___ Grace Kelly ___ Vikki Tran ___ Karen Gopaul ___

Appendix C: Animal Ethics Approval

2010-241::5:

AUP Number: 2010-241
AUP Title: Cell-mediated Immune Responses
Yearly Renewal Date: 11/01/2015

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-241 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

PETER ANTHONY SZABO

Department of Microbiology & Immunology, Schulich School of Medicine & Dentistry,
Western University, London, Ontario, Canada.

EDUCATION

PhD in Microbiology & Immunology, Western University, London, Canada 2011-2017
Supervisor: S.M. Mansour Haeryfar
Thesis: Rapid Regulatory and Effector Immune Responses in Toxic Shock Syndrome

Honours B.Sc. in Life Science, McMaster University, Hamilton, Canada 2006-2011

PUBLICATIONS

* denotes co-primary authorship

1. **Szabo PA,** Goswami A, Mazzuca DM, Kim K, O'Gorman DB, Hess DA, Welch ID, Young HA, Singh B, McCormick JK, Haeryfar SMM. Rapid and Rigorous IL-17A Production by a Distinct Subpopulation of Effector Memory T Lymphocytes Constitutes a Novel Mechanism of Toxic Shock Syndrome Immunopathology. ***J Immunol*** (2017). doi:10.4049/jimmunol.1601366

2. **Szabo PA**, Rudak PT, Choi J, Xu SX, Schaub R, Singh B, McCormick JK, Haeryfar SMM. Invariant NKT Cells are Pathogenic in the HLA-DR4-Transgenic Humanized Mouse Model of Toxic Shock Syndrome and Can Be Targeted To Reduce Morbidity. ***J Infect Dis*** (2016). doi:10.1093/infdis/jiw646

3. **Szabo PA***, Goswami A*, Memarnejadian A, Mallett CL, Foster PJ, McCormick JK, Haeryfar SMM. Swift Intrahepatic Accumulation of Granulocytic Myeloid-Derived Suppressor Cells in a Humanized Mouse Model of Toxic Shock Syndrome. ***J Infect Dis*** (2016). doi:10.1093/infdis/jiw050

4. **Szabo PA***, Anantha RV*, Shaler CR, McCormick JK, Haeryfar SMM. CD1d- and MR1-restricted T Cells in Sepsis. ***Front Immunol*** (2015). doi:10.3389/fimmu.2015.0040

5. Xu SX, Gilmore KJ, **Szabo PA**, Zeppa JJ, Baroja ML, Haeryfar SMM, McCormick JK. Superantigens Subvert the Neutrophil Response To Promote Abscess Formation and Enhance *Staphylococcus aureus* Survival In Vivo. ***Infect Immun*** (2014). doi:10.1128/IAI.02110-14

6. Woods MW, Tong JG, Tom SK, **Szabo PA**, Cavanagh PC, Dikeakos JD, Haeryfar SMM, Barr SD. Interferon-induced HERC5 is evolving under positive selection and inhibits HIV-1 particle production by a novel mechanism targeting Rev/RRE-dependent RNA nuclear export. ***Retrovirology*** (2014). doi:10.1186/1742-4690-11-27

7. Rytelewski M*, Meilleur CE*, Atef Yekta M, **Szabo PA**, Garg N, Schell TD, Jevnikar AM, Sharif S, Singh B, Haeryfar SMM. Suppression of Immunodominant Antitumor and Antiviral $CD8^+$ T Cell Responses by Indoleamine 2,3-Dioxygenase. ***PLoS ONE*** (2014) doi:10.1371/journal.pone.0090439

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SCHOLARSHIPS AND AWARDS

Canadian Institutes of Health Research (CIHR) National Student Research Poster Competition Honourable Mention (Top 10% of Poster Presenters)	June 2016
CIHR Travel Award ($1000) to attend the National Student Research Poster Competition and Canadian Student Health Research Forum	June 2016
Invitation to CIHR National Student Research Poster Competition (Top 5% of PhD students in Canada)	June 2016
Canadian Society for Immunology (CSI) 2016 Honourable Mention, One-Minute One-Slide Presentation	April 2016
F.W. Luney Travel Award ($1000)	April 2016
London Health Research Day First Place Feature Platform Presentation ($700)	March 2016
Ontario Graduate Scholarship ($15000)	May 2015
CSI 2015 Travel Award ($500)	May 2015
American Association of Immunologists (AAI) 2015 Travel Award ($500)	May 2015
F.W. Luney Travel Award ($1000)	May 2015
F.W. Luney Travel Award ($1000)	March 2014
PhD Candidacy Exam: Pass with Distinction	March 2013
Centre for Human Immunology Travel Award ($500)	June 2012
Federation of Clinical Immunology Societies (FOCIS) 2012 Travel Award ($500)	June 2012
F.W. Luney Travel Award ($1000)	May 2012
Western Graduate Research Scholarship (~$6000/year)	Sept 2011-16
McMaster University Senate Scholarship ($800)	May 2010
McMaster University Dean’s Honour List	Sept 2008-11
McMaster University Honour Award ($500)	Sept 2006

ORAL PRESENTATIONS

1. **Szabo PA**, Goswami A, Welch, I, McCormick JK, Haeryfar SMM. Staphylococcal superantigens trigger a rapid IL-17A response in memory T cells that is pathogenic in toxic shock syndrome. London Health Research Day. London, ON, Canada. March 2016.
   −**Selected as one of 32 platform podium presentations out of 424 accepted abstracts**
   −**Awarded First Place Feature Platform Presentation Award**
2. **Szabo PA**, Goswami A, Welch, I, McCormick JK, Haeryfar SMM. IL-17A is pathogenic in toxic shock syndrome. Lawson Health Research Institute Seminar Series. London, ON, Canada. January 2016.
3. **Szabo PA**, Goswami A, Welch, I, McCormick JK, Haeryfar SMM. IL-17A is rapidly triggered by staphylococcal superantigens and is pathogenic in toxic shock syndrome. Infection and Immunity Research Forum. London, ON, Canada. December 2015.
   −**Selected as one of 10 podium presentations out of 77 accepted abstracts**
4. **Szabo PA**, Goswami A, McCormick JK, Haeryfar SMM. Staphylococcal superantigens trigger rapid human IL-17A production by a novel population of memory T cells. Canadian Society for Immunology. Winnipeg, MA, Canada. June 2015.
   −**Selected as one of 8 presentations in the Immune Mediated Diseases workshop**
5. **Szabo PA**, Goswami A, McCormick JK, Haeryfar SMM. Staphylococcal superantigens trigger rapid human IL-17A production by a novel population of memory T cells. American Association of Immunologists. New Orleans, LA, USA. May 2015.
   −**Selected as one of 8 presentations in the Cell Subsets, Cytokines and Inflammation block symposium.**
6. **Szabo PA**, McCormick JK, Haeryfar SMM. *i*NKT17 cells in toxic shock syndrome. Canadian Society for Immunology. Quebec City, QC, Canada. March 2014.
   −**Selected as one of 8 presentations in the Immune Mediated Diseases workshop**

PUBLISHED ABSTRACTS

1. **Szabo PA**, Goswami A, Welch, I, McCormick JK, Haeryfar SMM. Rapid IL-17A production by memory T cells is critical for toxic shock syndrome pathogenesis. Canadian Student Health Research Forum: CIHR Nation Student Research Poster Competition. Winnipeg, MA, Canada. June 2016.
2. Cottrell J, **Szabo PA,** Zebian N, Lester J, Siroen D, Haeryfar SMM, Adams P, Cruezenet C. Variability in Dendritic Cell Immunological Responses to the Secreted *Helicobacter pylori* Protein HcpE. Canadian National Medical Student Research Symposium. Winnipeg, MA, Canada. June 2016.
3. **Szabo PA**, Goswami A, Welch, I, McCormick JK, Haeryfar SMM. Rapid IL-17A production by memory T cells is critical for toxic shock syndrome pathogenesis. Canadian Society for Immunology. Ottawa, ON, Canada. April 2016.

4. Cottrell J, **Szabo PA,** Zebian N, Lester J, Siroen D, Haeryfar SMM., Adams P, Cruezenet C. Variability in Dendritic Cell Immunological Responses to the Secreted *Helicobacter pylori* Protein HcpE. London Health Research Day. London, ON, Canada. March 2016.
5. **Szabo PA**, Goswami A, McCormick JK, Haeryfar SMM. Staphylococcal superantigens trigger rapid human IL-17A production by a novel population of memory T cells. Canadian Society for Immunology. Winnipeg, MA, Canada. June 2015.
6. **Szabo PA**, Goswami A, McCormick JK, Haeryfar SMM. Staphylococcal superantigens trigger rapid human IL-17A production by a novel population of memory T cells. Joint Microbiology/Immunology & Infectious Disease Retreat. Grand Bend, ON, Canada. May 2015.
7. **Szabo PA**, Goswami A, McCormick JK, Haeryfar SMM. Staphylococcal superantigens trigger rapid human IL-17A production by a novel population of memory T cells. American Association of Immunologists. New Orleans, LA, USA. May 2015.
8. Cottrell J, Lester J, Zebian N, **Szabo PA,** Siroen D, Haeryfar SMM, Adams P, Cruezenet C. Effect of HcpE on maturation of dendritic cells and their cytokine production. London Health Research Day. London, ON, Canada. March 2015.
9. Cottrell J, Lester J, Zebian N, **Szabo PA,** Siroen D, Haeryfar SMM, Adams P, Cruezenet C. Effect of immuno-modulatory proteins from *Helicobacter pylori* on dendritic cells. Immunity and Infection Research Forum. London, ON, Canada. November 2014.
10. Woods M, Tong J, Tom S, **Szabo PA,** Cavanagh P, Dikeakos J, Haeryfar SMM, Barr S. Interferon-induced HERC5 protein inhibits HIV-1 replication by two novel mechanisms and is evolving under positive selection. The 20th International AIDS Conference. Melbourne, Australia. July 2014.
11. **Szabo PA**, McCormick JK, Haeryfar SMM. *i*NKT17 cells in toxic shock syndrome. Canadian Society of Immunology. Quebec City, QC. March 2014.
12. Goswami A, **Szabo PA,** Haeryfar SMM. Intrahepatic accumulation of immunosuppressive myeloid cells in response to superantigen administration. Canadian Society of Immunology. Quebec City, QC. March 2014.
13. **Szabo PA,** McCormick JK, Haeryfar SMM. *i*NKT17 cells in toxic shock syndrome. London Health Research Day. London, ON. March 2014.
14. Goswami A, **Szabo PA,** Haeryfar SMM. Intrahepatic accumulation of immunosuppressive myeloid cells in response to superantigen administration. London Health Research Day. London, ON. March 2014.
15. Goswami A, **Szabo PA,** Haeryfar SMM. Intrahepatic accumulation of immunosuppressive myeloid cells in response to superantigen administration. Infection and Immunity Research Forum. London, ON. November 2013.

16. Goswami A, **Szabo PA,** Haeryfar SMM. A staphylococcal superantigen mediates $CD11b^{+}$ $Ly6C^{+}$ $Ly6G^{+/-}$ myeloid cell accumulation in the liver. Ontario Quebec Undergraduate Immunology Conference. Toronto, ON. May 2013.
17. **Szabo PA**, McCormick JK, Haeryfar SMM. Exploring the role of iNKT17 cells in toxic shock syndrome. Infection and Immunity Research Forum. London, ON. November 2012.
18. Goswami A, **Szabo PA**, Haeryfar SMM. Hepatocytes bearing suppressor cell profiles accumulate following superantigen exposure. Infection and Immunity Research Forum. London, ON. November 2012.
19. **Szabo PA**, McCormick JK, Haeryfar SMM. Exploring the role of iNKT17 cells in toxic shock syndrome. Canadian Society of Immunology, St. John's, NL. June 2012.
20. **Szabo PA**, McCormick JK, Haeryfar SMM. Identification of iNKT17 cells in toxic shock syndrome. Federation of Clinical Immunology Society Conference Trainee Satellite Symposium, Vancouver, BC. June 2012.

TEACHING AND MENTORING

- Future Professor Workshop Series (15 hours instruction), Teaching Support Centre, Western University, Jan 2017
- Guest Lecturer (1 hr), MICROIMM 3820A: Microbiology and Immunology for Nursing Students, Western University, Oct 2016.
- Teaching Mentorship Program Graduate, Teaching Support Centre, Western University, May 2016
- Advanced Teaching Program Certificate (17.5 hours instruction), Teaching Support Centre, Western University, May 2016.
- Guest Lecturer (1 hr), MICROIMM 3820A: Microbiology and Immunology for Nursing Students, Western University, Sept 2015.
- Teaching Assistant, MICROIMM 3820A: Microbiology and Immunology for Nursing Students, Western University, Sept-Dec 2015.
- Teaching Assistant, Medicine 5116 Infection & Immunity, Schulich School of Medicine, Oct-Dec 2012-2014.
- 4^{th} Year Microbiology and Immunology Undergraduate Thesis Mentor to Ankur Goswami, Haeryfar Laboratory, Sept 2012-Apr 2013.

ADMINISTRATION

Microbiology & Immunology Graduate Student Representative on the Appointments Committee, Western University, Sept 2013-2016.