STAPHYLOCOCCAL ENTEROTOXIN ENHANCEMENT OF INFLAMMATORY AND REGULATORY CYTOKINE PRODUCTION AND HUMORAL RESPONSES

By

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I dedicate this work to my parents and my brother Tim, for always being there and supporting me in whatever I do; and to Glenn, for always being there for me for anything.

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LIST OF ABBREVIATIONS

Ab:	Antibody
APC:	Antigen presenting cell
BCA:	Bicinchoninic Acid
BSA:	Bovine serum albumin
cDNA:	Complementary deoxyribonucleic acid
ELISA:	Enzyme linked immunosorbent assay
FBS:	Fetal bovine serum
HPBMC:	Human peripheral blood mononuclear cells
HRP:	Horseradish peroxidase
IFN:	Interferon
IL:	Interleukin
NK:	Natural killer cells
PBMC:	Peripheral blood mononuclear cells
PBS:	Phosphate buffered saline
RNA:	Ribonucleic acid
SEA:	Staphylococcal enterotoxin A
SEB:	Staphylococcal enterotoxin B
TGF:	Transforming growth factor
TNF:	Tumor necrosis factor

Abstract of Thesis Presented to the Graduate School
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By

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Superantigens are microbial proteins that are the causative agents of food poisoning and toxic shock syndrome. Furthermore, superantigens can cause deregulation of the immune system and can exacerbate autoimmune diseases such as multiple sclerosis. Superantigens induce a burst of T-cell proliferation and activation. We analyzed *in vivo and in vitro*, superantigen activation of the cells of the immune system in the context of expression of genes associated with various T-cell subsets. Human peripheral blood mononuclear cells (HPBMC) treated with staphylococcal enterotoxin A (SEA) showed increased expression of the cytokines IFN-γ, IL-2, IL-10, TNF-α, TNF-β, TGF-β, and IL-6 as deduced by microarray analysis of cell mRNA. A similar pattern of genes was upregulated in HPBMC treated with staphylococcal enterotoxin B (SEB). Th1, Th2 and T regulatory cells produce these cytokines. Expression for most of these cytokines is

maximal 24 to 48 hours post superantigen treatment. Enzyme Linked Immuno Sorbent Assay (ELISA) tests for IFN-γ, IL-10 and IL-2, showed increased mRNA levels as seen on the microarray correlate with translation, with the exception of TGFβ, which was not detectable by ELISA or Western Blot. In addition, purified CD4+ T-cells treated with SEB show a similar pattern of upregulated cytokines as shown for superantigen treated HPBMC. *In vivo* experiments showed that SEA and SEB increase specific IgG (but not IgM) antibody production in mice immunized against bovine serum albumin (BSA). Thus, superantigens increase both the specific humoral and cellular immune responses against antigens. Based on the cytokine profile elicited by superantigens in vitro and the specific antibody production in vivo, superantigens enhance Th1, Th2 and T regulatory cytokine production. This property of superantigens makes them an ideal candidate to boost specific immunity; and may have prophylactic applications in the prevention of cancer and other immune- mediated diseases.

CHAPTER 1
INTRODUCTION

General Superantigen Information

Superantigens are microbial proteins produced by various bacteria and viruses including (*Staphylococcus* species, *Streptococcus* species, HIV and rabies viruses) and are powerful activators of CD4+ T-cells. (7,10) Common superantigens include the staphylococcal enterotoxins (A-E) and toxic shock syndrome toxin 1 (TSST-1) which are produced by *Staphylococcus aureus* (32). The enterotoxins are common causative agents of toxin-mediated food poisoning and TSST's are implicated in toxic shock syndrome (32). Superantigens are also suspected to play a role in exacerbation of autoimmune diseases such as multiple sclerosis as well as the immunodeficiency associated with HIV infection (8,10,27,36,33). These acute and chronic disease states associated with superantigen are caused by the massive proliferation and activation of T-cells that is induced by superantigens (2).

Interactions of Superantigens with Immune Cells and Peptide Processing

The potency of the superantigens in relation to T-cell activation and proliferation is in part explained by its unique interaction with the T-Cell Receptor (TCR) on T cells and the major histocompatibility complex II (MHC II) on antigen presenting cells (APC) (30). As shown in Figure 1-1, superantigens bind directly to MHC II (16) and this complex interacts with a specific portion of the Vβ region of the TCR. Each superantigen activates different Vβ regions. TSST-1 activates T-cells with Vβ2 , while SEB activates cells with Vβ3, Vβ 12, Vβ 14, Vβ 15, Vβ 17, and Vβ 20 (2,15).

The interaction with the TCR is antigen-independent and requires no further processing as seen with a typical peptide antigen. Typical peptide antigens are processed internally and presented by APC on either MHC I or MHC II molecules on their cell surface. By acting in this manner, superantigens can induce polyclonal activation of up to 20% of the total T-cell population at one time, as compared to typical peptide antigens that only activate up to 0.01 % of the total T-cell population at one time.

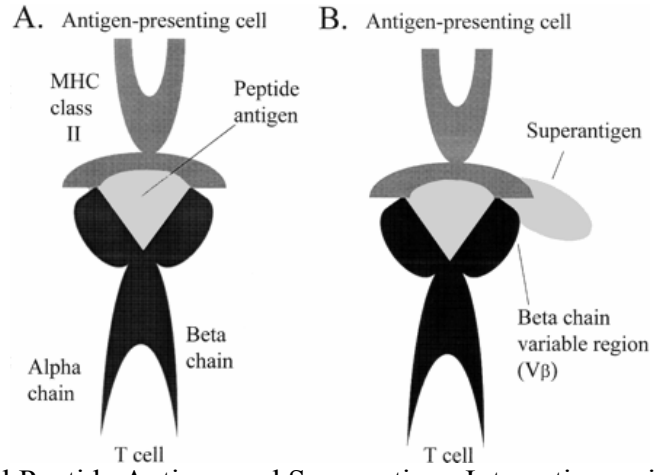


Figure 1-1: Typical Peptide Antigen and Superantigen Interactions with T-cells and APC. (Torres, B.A., S.L. Kominsky, G.Q. Perrin, A.C. Hobeika, and H.M. Johnson. 2001. Superantigens: The good, the bad and the ugly. Exp. Med. Biol. 226:164).

Activation of Cells by Superantigens

Superantigens activate CD4+ T-cells. These T-cells are preferentially activated because of their increased binding affinity for MHC II (12). The CD4+ T cells can be divided into three groups: T helper 1 (Th1), T helper 2 (Th2) and T regulatory cells. Th1 cells are one T-cell group activated by superantigen. Th1 cells are associated with inflammatory responses and produce such cytokines as IL-2, IFN-γ and tumor necrosis factor (21). IL-2 drives further T-cell proliferation and activation. One of the main functions of IFN- γ is macrophage activation through upregulation of MHC I and MHC II. It also has powerful antiviral effects. It enhances CTL, natural killer and macrophage

cell tumoricidal activity (32). Tumor necrosis factor (TNF) induces macrophages to produce NO, as well as activating the vascular endothelium.

The Th2 cells are also activated by superantigens. Th2 cells are associated with humoral responses and anti-inflammatory responses. This group of cells is thought to be the classic adversary to the actions of Th1 cells (3). Th2 cells produce such cytokines as IL-4 and IL-6. Interleukin 4 is an anti-inflammatory cytokine produced by Th2 cells. Interleukin-6 is a cytokine with both anti-inflammatory and inflammatory properties. It can induce acute phase response in the liver and drives B-cell differentiation (3).

A third group of cells called T-regulatory cells also are activated by superantigens, especially cells undergoing repeated treatments with superantigen (17). T regulatory cells are CD 4+ and express CD25. (18) They play important roles in the maintenance of peripheral tolerance. Typical cytokines produced by T-regulatory cytokines include TGF-β and IL-10 and IFN-γ were detected (6,17). Interleukin 10 downregulates MHC II expression and costimulatory molecules like CD 80/86, inducing anergy in CD4+ T cells (22). On the contrary, IL-10 aids in the survival of B-cells and plays a part in their differentiation. It has also been shown in mice knockouts for CD25, that stimulation with superantigens results in uncontrolled release of pro inflammatory cytokines, but that injection with CD4+ CD25 T-cells control this process (20). This indicates that regulatory cells play a role in controlling superantigen activation of T-cells. TGF-β is another type of regulatory cytokine. TGF-β inhibits IL-2 production, and has anti-proliferative effects on T-cells. TGF-β also blocks the differentiation of Th1 and Th2 cells through blocking of transcription factors.

Superantigens in Disease

Superantigens are involved in both short- and long- term acute and chronic disease states. Staphylococcal enterotoxins A (SEA) are responsible for the most food poisoning caused by superantigens (9,32). The acute gastrointestinal illness resulting from ingestion of the toxin is short lived. Toxic shock syndrome (TSS) caused by TSST-1 is also an acute disease caused by superantigens (1,24,32). TSS became well studied in the 1980's when it became associated with tampon use in women during menstruation. Clinical manifestations of TSS include rashes, fever, severe hypotension, and possible fatal shock.

While the acute effects of superantigens are somewhat severe, the involvement of superantigens in exacerbation of autoimmune diseases persists over long periods of time.

The involvement of superantigens in autoimmune disease is demonstrated in a murine model of multiple sclerosis (MS), called experimental allergic encephalomyelitis (EAE), which is induced in mice by injection of myelin basic protein (MBP). The T-cell population in mice responsible for EAE has a Vβ8+ specificity (38). Pretreatment of PL/J mice with SEB prevents induction of EAE by MBP (27). This is most likely due to anergy and deletion of VB8+ T-cells by SEB (10). However, SEB has also been shown to reactivate EAE in mice that have been immunized with MBP and recovered from an initial episode of the disease (9). This phenomenon is not limited to SEB, since SEA has also been shown to reactivate EAE (9,26) showing that different Vβ specificities may also be involved in autoimmune disease.

Superantigens are also thought to be involved in some immunodefiency diseases, such as HIV. A regulatory gene product encoded in the 3' LTR of the HIV genome

(called Nef) has been shown to block binding of SEA to Raji cells; and to induce T-cell proliferation, IL-2 production, and IFN-γ production (32,33). Other evidence of superantigen characteristics include selective expansion of particular Vβ sets (Vβ3, 5.3 and 18) (29,34) and need for APC for Nef induced activation of T-cells (37). Another study has shown that blocking Nef with an anti-Nef antibody does not support HIV replication (34) suggesting that Nef is necessary for HIV to maintain itself in a host. HIV requires activated CD4+ T cells for replication (36); and a superantigen-like molecule is one way for the virus to achieve this. These studies together show that Nef has typical characteristics of a superantigen and its action induces a pool of cells for viral replication, which aids in the spread of the virus in a host.

Uses of Superantigens in Vaccines and in the Treatment of Disease

Superantigen effects are generally considered to be negative, especially in the context of food poisoning, TSS, and their involvement in chronic disease states. However, the very characteristics and interactions of superantigens also make them ideal candidates for use in vaccines, prophylactically and as treatment of diseases. In order to achieve this, the proliferation and cytokine production must be controlled and biased to a certain direction. In this way, the effects of superantigens would be used to improve and bolster immune responses as opposed to deregulating immune function.

A mouse model of melanoma has been used to show how superantigens can be prophylactically effective as vaccine against melanoma (14). C57BL/6 mice were injected with irradiated B16F10 melanoma cells, followed by SEA and SEB 6 and 10 days later. Three days after SEA and SEB treatment, mice were challenged with live B16F10 cells. Median survival time was >150 days for those mice injected with

superantigens, as opposed to 14 to 23 days in mice with no treatment or treatment with cells only or superantigen only (14). In addition, surviving mice were rechallenged with live tumor and 75 percent survived. (14) The staphylococcal entertoxins (SE) are helping to boost anti-tumor activity through activation of a large number of T-cells already primed with the tumor antigen.

Another study takes a different approach to the use of staphylococcal enterotoxins in the treatment of melanoma (23). Mice were injected with c215 transfected B16 melanoma cells followed by injection of SEA fused to a C215 tumor reactive antibody, along with IL-2 fused to the same antibody. Prolonged survival was seen in mice receiving both treatments, as opposed to each treatment alone (23). Repeated cycles of treatments also increased survival time (23). This method directs SEA directly to the tumor, with the use of the antibody. The use of IL-2 as well, adds to the proliferation induced alone by SEA.

A third approach uses plasmid encoded SEB and either IL-2 or GMCSF (5). Dogs with melanoma tumors received intratumor injections of lipid complexes with plasmid DNA encoding SEB and either IL-2 or GMCSF. Partial tumor regression, and in some cases, complete regression was seen in dogs receiving this treatment (5). Little toxicity was seen in the dogs given these treatments. The treatment is directed to the tumor through intratumoral injection and SEB is produced in the animal. IL-2 acts as an additional proliferation agent, as in the previous experiment.

All of the above studies show in different ways how superantigens may be used as prophylaxis for cancer as well as ways to treat cancer once tumors have already

established in the body. This is only one approach to the use of superantigens in the treatment and prevention of disease.

In this study, through a combination of molecular biology techniques such as cDNA microarray analysis and traditional immunological procedures, such as ELISA and western blot techniques, specific cytokine production during superantigen stimulation of HPBMC will be assessed in the context of activation of distinct groups of T cells. The studies are designed to gain insight into how superantigens modulate lymphocyte function in disease and immune therapy.

CHAPTER 2
MATERIALS AND METHODS

Human Peripheral Blood Mononuclear cells (HPBMC) Isolation and Cell Culture

Human donor leukocyte packs were obtained from Civitan Regional Blood Center (Gainesville, FL). HPBMC were isolated using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as per the manufacturers instructions. After the removal of the cells from the gradient and 2 washes with RPMI 1640 culture media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 U/mL penicillin, and 200 mg Streptomycin, the cells were resuspended in fresh supplemented culture media and counted using a light microscope. 2 x 10^^{6} cells/well were plated in 24 well plates and incubated at 37°C in a 5% CO2 atmosphere and used immediately for further experimentation

Human CD4+ T Cell Isolation

Human donor leukocyte packs were obtained from Civitan Regional Blood Center (Gainesville, FL). An enrichment cocktail (Rosette Sep)for human CD4+ T cells (Stem Cell Technologies, Vancouver, BC) was used to enrich for CD4+ T cells, followed by isolation of the cells using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as per the manufacturers instructions. After removing cells from the gradient and 2 washes with RPMI 1640 culture media, supplemented with 10% heat inactivated fetal bovine serum (FBS), 200 U/ml penicillin, and 200 mg Streptomycin, the cells were resuspended in fresh supplemented culture media and counted using a light microscope.

2 x 10^6 cells/well were plated in 24 well plates and incubated at 37°C in a 5% CO2 atmosphere and used immediately for further experimentation.

Staphylococcal Enterotoxin Treatment of HPBMC and CD4+ T-cells

Staphylococcal enterotoxin A (SEA) (Toxin Technology, Sarasota, FL) and B (SEB) (Toxin Technology, Sarasota, FL) were cultured with 2 x 10^6 HPBMC per well in a 24 well plate, both at a concentration of 100 ng/ml. CD4+ T-cells (2 x 10^6 cells per well) were treated with 100 ng/ml of SEB. SEB does not require APC for processing (4,30). Media treated cells served as a control. Six wells were used for each treatment at each time point. Cells were incubated as described above and were harvested from the cultures at time points of 8h, 16h, 24h, and 48h. Following centrifugation for 10 minutes at room temperature, cell pellets and supernatant. were separated and used for RNA isolation and ELISA/Western Blot experiments respectively.

Total RNA Isolation

RNA from SEA and SEB or media treated HPBMC as described above was obtained using the following RNA isolation kits: Absolutely RNA (Stratagene, LaJolla, CA), RNAqueous (Ambion, Austin, TX), and TRIZOL Reagent (GibcoBrl, Carlsbad, CA). The protocols were followed as described by the manufacturer. The RNA samples were quantitated by reading absorbance at 260nm on a Gilford Instrument Spectrophotometer 260 (Nova Biotech, El Cajon, CA). The RNA samples were diluted 1:100 in 10mM Tris or DNAse and RNAse free water. The following calculation was used to determine the concentrations of RNA in μg/μl: [A_{260} x 100 x .040 ug/ul]. Following quantitation, RNA samples were stored at -80° C for future use in microarray experiments described below.

Microarray Procedure

Microarray procedures were performed as described by the manufacturer (SuperArray, Frederick, MD). Briefly, total RNA was used to prepare cDNA probes. During this process the cDNA was labeled with biotinylated dUTP's. The probes were denatured and allowed to hybridize overnight at 60°C to the microarray membrane. The nylon microarray membrane is spotted with cDNA from 96 common human cytokines purchased from SuperArray Inc (Frederick, MD). Following a series of washes and further blocking, the membrane was incubated with alkaline phosphatase-streptavidin. CDP Star substrate was then added to the membrane. The gene microarray membranes were exposed to film and developed. This procedure was followed as recommended by the manufacturer (Super Array, Frederick, MD). A schematic of the entire procedure can be found in Appendix B.

Analysis of Microarray Data

The film images of the media and superantigen treated microarray membranes were scanned and converted to a TIFF format. The images were then analyzed using the Image J 1.29 software (NIH, Bethesda, MD) and Scion Image software, to determine the pixel density value for the spot of a desired gene. Each intensity value was normalized to the positive control on its respective membrane, then divided by the corresponding media control values. This value is a fold increase ratio of superantigen treated cells to media treated cells.

Western Blot for TGFβ3

HPBMC were cultured with or without superantigens described above and the resulting supernatants were saved at -80 C and used for western blot analysis of TGF-β3. The supernatants were concentrated using Amicon centriprep YM-10 filters (Millipore,

Billerica, MA). A Bicinchoninic Acid (BCA) protein assay (Pierce, Rockford, IL) was performed on the supernatants from control (media) or superantigen treated cells. Equal amounts of each sample (26 μg/lane) were loaded on a 15 % reducing SDS-PAGE ready gel (BioRad, Hercules, CA) and run at 100V. Overnight transfer onto nitrocellulose membrane was carried out, after which the membrane was block with 5% non-fat instant milk in Tris-buffered saline (pH 7.5) and .01% Tween-20 for 1 hr. The Immunoblot was incubated with a 1:1000 dilution of rabbit anti-human TGFβ3 (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, a conjugated secondary antibody, goat-anti-rabbit conjugated to horseradish peroxidase (HRP), (Santa Cruz Biotechnology, Santa Cruz, CA) was added at a 1:12,000 dilution and incubated for 1 h. Blots were washed and analyzed through film development.

ELISA for Human Cytokines

HPBMC were cultured with or without superantigens described above and the resulting supernatants were saved at -80 C and used for ELISA assays. The supernatants were concentrated using Amicon centriprep YM-10 filters (Millipore, Billerica, MA). A BCA protein assay (Pierce, Rockford, IL) was performed on the supernatants from control (media) or superantigen treated cells. Equal amounts of each sample were used in the ELISA assays. HPBMC were treated with media or superantigen and supernatants were collected at various time points as described above. IL-2, IL-10, and IFN-γ levels were determined using ELISA kits. The supernatants were tested for IFN-γ using the CytoScreen Immunoassay kit for IFN-γ (Biosource International, Camarillo, CA), IL-2 using the BD-Opt-EIA kit for IL-2 (BD Biosciences), and IL-10 using the BD-OptEIA kit for IL-10 (BD Biosciences). Color development was monitored at 490nm in an ELISA plate reader (Biorad, Richmond, CA) after substrate solution from each respective

cytokine kit was added and reaction stopped with the stopping solution provided in each kit.

Mouse Studies and Detection of Specific Antibodies in Mouse Sera

We used 6 to 8 week old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) in these studies. Mice were bled before injections with the BSA. We injected 50 μg of BSA intraperitoneally (i.p.) into the mice. One week later, mice were injected i.p. with PBS or a combination (25 μg each) of SEA and SEB (Toxin Technology, Sarasota, FL). Mice were bled from the tailvein once a week for one month; and sera were stored at -20°C for further experiments.

Sera from the mice were tested for BSA specific IgG and IgM antibodies using a standard ELISA protocol. Briefly, 50 μl of BSA (25 ng/well) in binding buffer (0.1 M carbonate/bicarbonate, pH 9.6) were placed in wells of 96 well plates and allowed to adhere overnight at room temperature. Plates were washed in wash buffer (150 mM NaCl, 0.05% Tween 20) and free reactive sites were blocked for 2 h with 200 μl/well blocking buffer (PBS (pH 7.2) containing 5% nonfat instant milk). After washing plates, sera were diluted and 50 ul were placed in the wells for 1.5 h. Plates were again washed and alkaline phosphatase- conjugated anti-mouse IgG whole molecule or anti-mouse IgM (50 ul ; Sigma Aldrich, St.Louis, MO) was added to wells. After 45 minutes, plates were washed and 200 ul of substrate (1mg/ml p-nitrophenyl phosphate in binding buffer) was added to the plates. Color was allowed to develop for 30-60 minutes, after which 50 ul of stop solution (2 M H_2SO_4) was added. Absorbance was read at 405 nm using a Model 450 Bio-Rad Microplate reader (BioRad, Hercules, CA).

Proliferation Assay

Human NK-92 cells (ATCC, Mannassas, VA), a natural killer cell line requiring IL-2 for growth, were cultured in alpha minimum essential medium without ribonucleosides and deoxyribonucleosides with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 12.5% horse serum and 12.5% fetal bovine serum at 37 °C. The cells were plated at 4 x 10^4 cells per well in a 96 well plate and treated with either media, IL-2 (Biosource International, Camarillo, CA) (30 U/ml), or IL-2 and TGFβ (25 ng/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) for 48 hours. Cells were then pulsed with 1 μCi per well of ^{3}H-thymidine for 6 hours, after which cells were harvested an cell associated radioactivity was quantified using a β-scintillation counter and activity reported as mean CPM +/- SD. All tests were run in replicates of six.

CHAPTER 3
RESULTS

Superantigens Enhance Specific Antibody Production to Antigens in Mice

It has been shown previously that superantigens enhance the immune cellular response against melanoma cells in vivo in mice (15). Here a determination was made of the ability of SEA and SEB to enhance the humoral antibody response of mice primed with the T dependent antigen bovine serum albumin (BSA). Mice were first injected with BSA alone, BSA followed seven days later by SEA/SEB, SEA/SEB alone, or PBS alone. As can be seen in the ELISA measurements in Figure 3-1A, BSA alone induced an antibody response that was enhanced greater than 2 fold By SEA/SEB at a 1:100 dilution of sera. There was no antibody response to the control antigen gp120 in the same sera. This was evidenced by the low similar ELISA profiles for BSA alone, BSA followed by SEA/SEB, SEA/SEB alone, or PBS alone. Thus, the enhancement of SEA/SEB was specific for the primary antigen BSA. Furthermore, the antibody response to BSA was IgG specific, but not IgM specific. At 14 days following BSA injection there was no evidence of specific IgM antibodies to BSA in sera of mice, as per Figure 3-1B, where the BSA response was compared with that of PBS. Importantly, a comparison of mice injected with BSA and BSA followed by SEA/SEB showed the same profile of IgM response. Thus, SEA/SEB did not enhance the IgM response under the same conditions under which it enhanced the IgG response. Since specific IgG levels were increased against BSA, total IgG levels were compared among the different groups. Mice treated with BSA alone, BSA followed by SEA/SEB, SEA/SEB alone or PBS alone, did not

show any significant differences in total IgG levels, as shown in Figure 3-1C. Thus, an enhancement effect of SEA/SEB on the total IgG levels was not observed. Furthermore, the enhancement of the antibody response to BSA by SEA/SEB could not be attributed to non-specific enhancement of total IgG. Therefore, the SEA/SEB enhancement of the antibody response to BSA was antigen specific and of the IgG isotype.

Superantigens Enhance Cytokine RNA Production in HPBMC

A determination of the ability of SEA and SEB to induce increased cytokine production was first made through microarray analysis of RNA in cells treated with the SAg's. Human PBMC were treated with 100 ng/ml of SEA or SEB, or culture media alone. Cells were harvested from the culture at timepoints of 8, 16, 24, and 48 hours. Total RNA was extracted from the cells and was used to synthesize cDNA. The cDNA was allowed to hybridize to a nitrocellulose membrane that was spotted with various human common cytokine genes. Representative microarray images of media, SEA, and SEB treatments are shown in Figure 3-2. Analysis of the microarray membranes and supernatants of the treated cells revealed that both SEA and SEB induce increased gene expression in HPBMC as compared to those of media control cells. Appendix B contains a list of all the cytokines coded for the on the microarray membrane.

SEA Induces Cytokine Gene Expression in HPBMC

As shown in Figure 3-3, SEA induces upregulation of several genes including IFN-γ, IL-2, CD40L, IL-10, IL-6, TNFβ, TGFβ and IL-1β, with as much as 2-25 fold increase over media in HPBMC. IFNγ expression was similar at all four time points (5-15 fold increase over media), where as expression of IL-2, IL-10 and IL-1β were maximal at 16 hours after SEA incubation. TGF-β, IL-6 and CD40L had maximal expression at 24

hours as compared to media controls. SEA induction of TNFβ gene expression was approximately 2 fold greater than media at 8 hours, where as it was approximately 16 fold greater than media at 16-48 hours. Thus, SEA increased cytokine gene expression in HPBMC as compared to media treated cells.

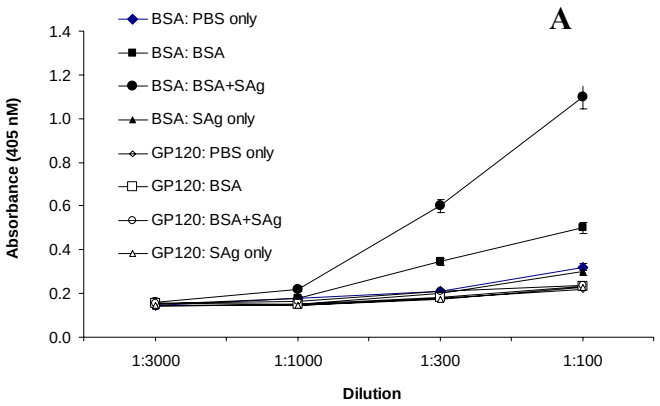
SEB Induces Cytokine Gene Expression in HPBMC

The cytokine gene expression induced by SEB in HPBMC was determined next. As shown in Figure 3-4, SEB increased cytokine expression of several genes as compared to media controls, although expression levels were lower than that was seen with SEA treatment. SEB induction of IFN-γ and IL-1β was maximal at 16 hours, IL-6 and TGFβ at 24 hours, and IL-10, TNFβ, and CD40L at 48 hours as compared to the media control. IL-10 and TGFβ are regulatory cytokines, so the relative delay in their gene expression could indicate a signal to modulate Th1 cell cytokines. Therefore, SEB also upregulates cytokine gene expression in HPBMC.

SEB Induces Cytokine Gene Expression in CD4+ T cells

Previously, it has been shown that superantigens activate CD4+ T cells (37). Here we investigated the profile of cytokine gene expression in CD4+ T cells treated with SEB. SEB does not require antigen presenting cells for processing (4,30), thus CD4+ T cells were purified from HPBMC using the Rosette Sep procedure as described in Materials and Methods, and then treated with 100 ng/ml of SEB for timepoints of 8, 16, 24, and 48 hours. As shown in Figure 3-5, similar cytokine genes were upregulated as those seen above for HPBMC. Expression levels for TGFβ, CD40L, and TNFβ were greater than 50 times than that of media at 24 hours. IFN-γ expression was maximal at 24 hours, IL-2 at 48 hours and IL-6 at 8 hours. Thus, SEB also stimulates cytokine production in CD4+ T-cells.

Figure 3-1: Antigen and isotype specificity of superantigen enhancement of antibody to BSA. Mice were injected with BSA alone, BSA followed by SEA/SEB, SEA/SEB alone or PBS under the same conditions as in Materials and Methods. A) Sera were tested by ELISA for IgG Abs to BSA or gp120 B) Sera were tested by ELISA for IgM Abs to BSA. C) Total IgG levels for the sera of A are presented in C. Students t test: A)BSA vs BSA and SEA/SEB, $P<0.001$; BSA and SEA/SEB, BSA vs gp120, $p<0.001$. No comparisons were significant in B and C. Data are representative of three experiments, each performed in triplicate.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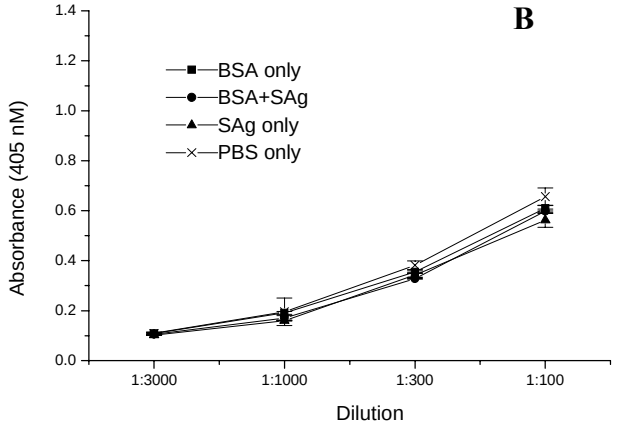
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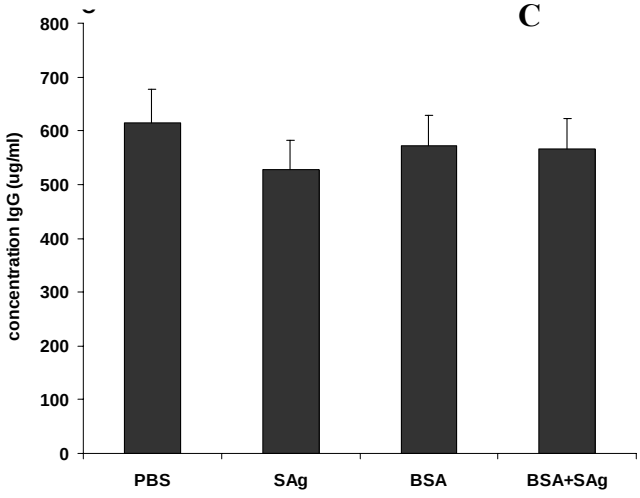
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SAg
BSA
BSA+SAg

MEDIA SEA SEB

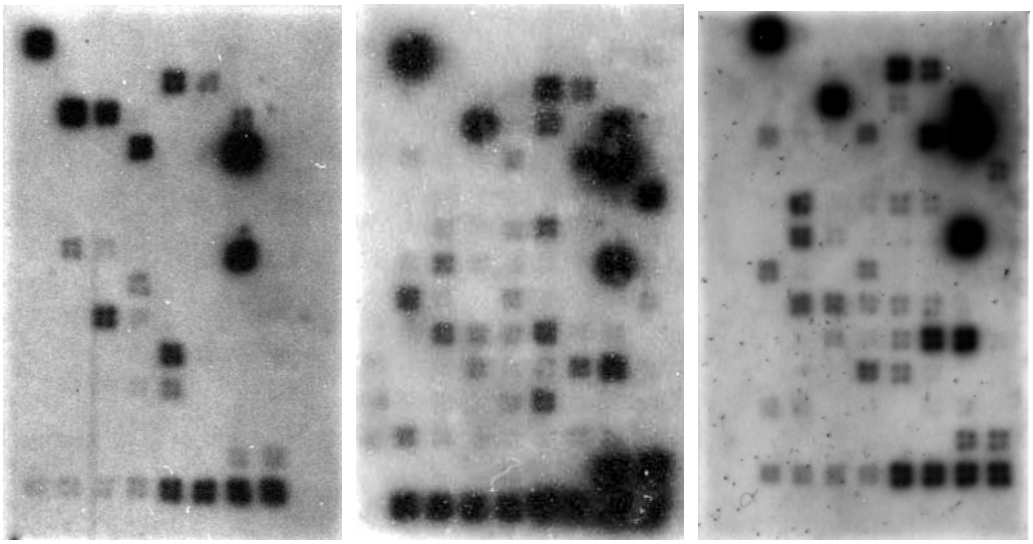


Figure 3-2: Staphylococcal enterotoxin A and staphylococcal enterotoxin B increase expression of various human cytokine genes. Human PBMC were treated with 100 ng/ml of SEA or SEB or media alone and cultured as described in methods. Cells were harvested from the cultures at time points of 8, 16, 24, and 48 hours. Total RNA was extracted from these cells and used to make cDNA. The cDNA was allowed to hybridize overnight with a nylon membrane spotted with cDNA spotted with various common human cytokines. After washes and incubation with AP-streptavidin, and substrate, the membranes were exposed to film and developed. Images seen here represent 24 hours post treatment.

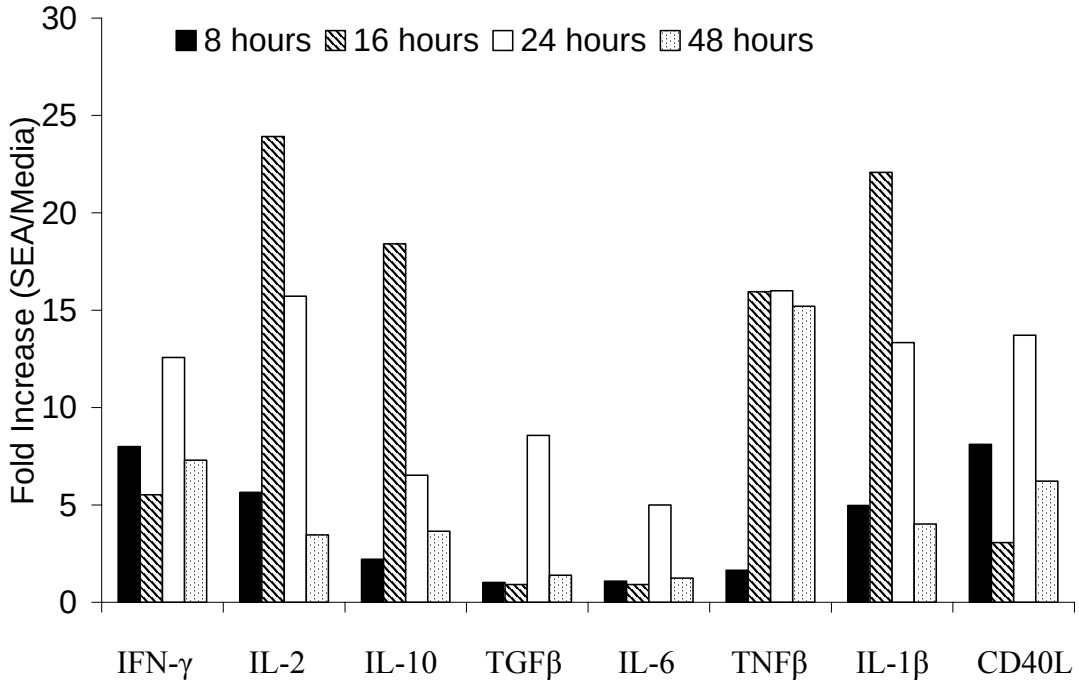


Figure 3-3: Staphylococcal enterotoxin A induces increased expression of various Th1, Th2, and T regulatory cytokine genes. Human PBMC were treated with 100 ng/ml of SEA and cultured as described in methods. Cells were harvested from the cultures at time points of 8, 16, 24, and 48 hours. Total RNA was extracted from these cells and used to make cDNA. The cDNA hybridized overnight to a nylon membrane spotted with cDNA of common human cytokines. The complete list of cytokine genes on the nylon membrane is presented in Appendix 3. After washes and incubation with AP-streptavidin, and substrate, the membranes were exposed to film and developed. Pictures were scanned into TIFF format and the software program ScionImage was used to determine pixel density for each spot. Background values were subtracted from each value, followed by normalization to a positive control on each membrane. This normalized value for SEA treated cells was divided by the same value for media to obtain the fold increase in gene expression of each cytokine.

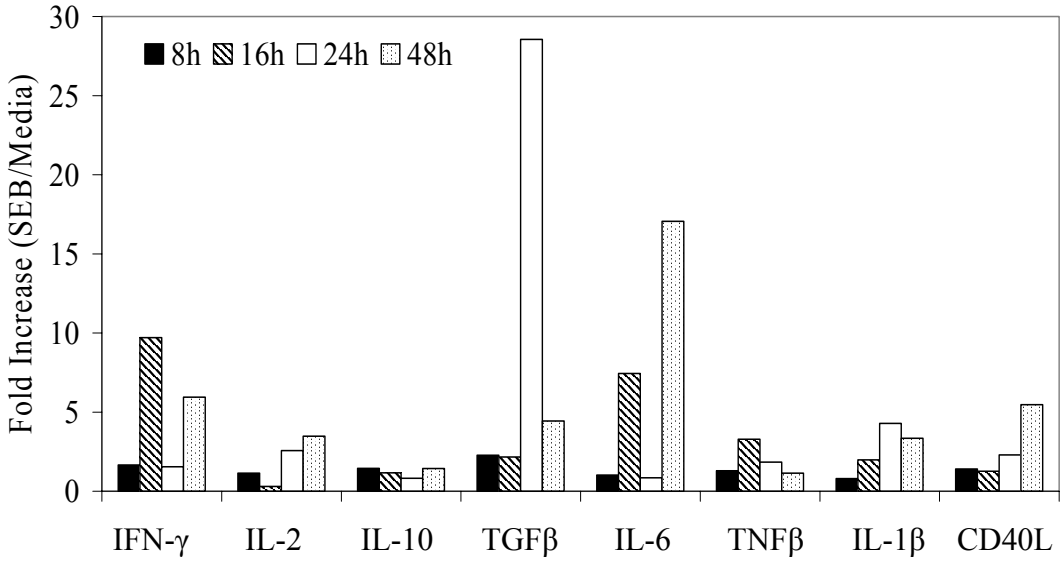
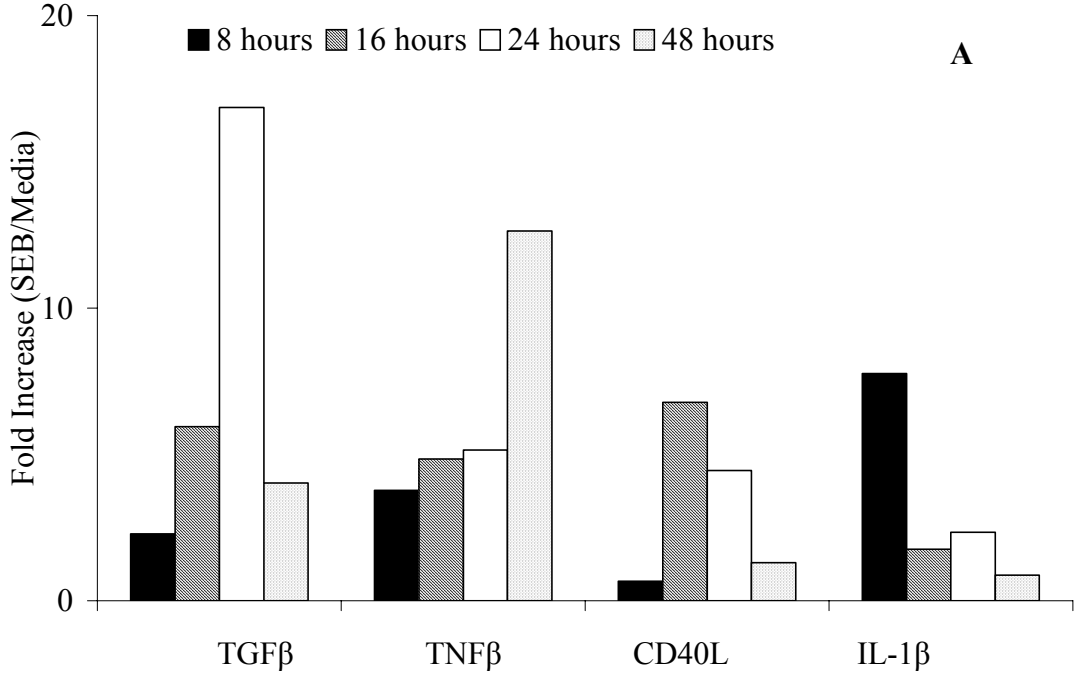
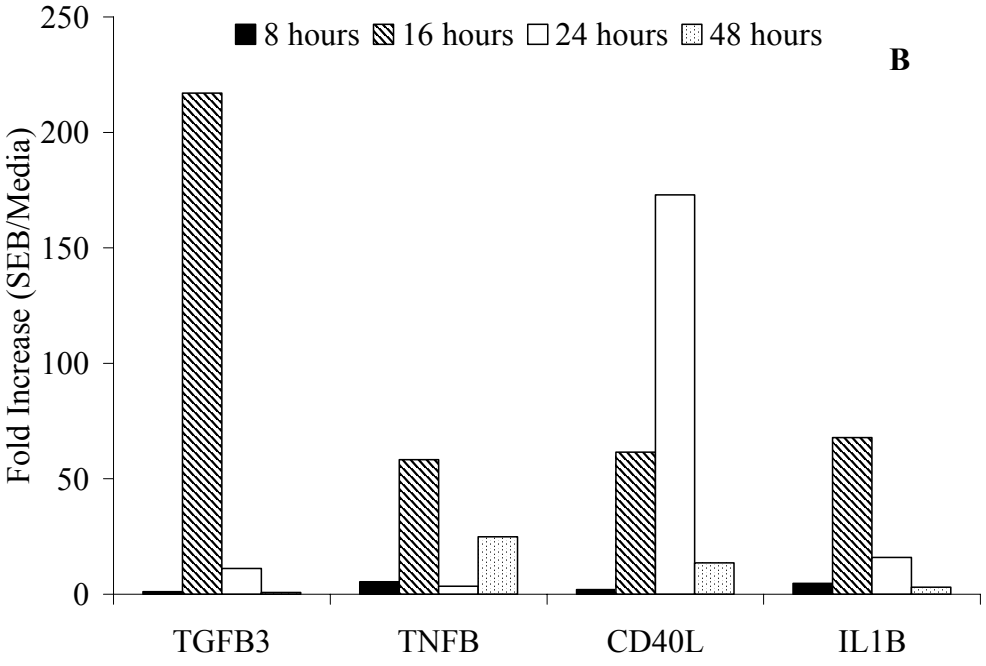


Figure 3-4: Staphylococcal enterotoxin B induces increased expression of various Th1, Th2, and T regulatory Cytokine Genes. Human PBMC were treated with 100 ng/ml of SEB and cultured as described in methods. Cells were harvested from the cultures at time points of 8, 16, 24, and 48 hours. Total RNA was extracted from these cells and used to make cDNA. The cDNA hybridized overnight to a nylon membrane spotted with cDNA of common human cytokines. The complete list of cytokine genes on the nylon membrane is presented in Appendix 3. After washes and incubation with AP-streptavidin, and substrate, the membranes were exposed to film and developed. Pictures were scanned into TIFF format and the software program ScionImage was used to determine pixel density for each spot. Background values were subtracted from each value, followed by normalization to a positive control on each membrane. This normalized value for SEB treated cells was divided by the same value for media to obtain the fold increase in gene expression of each cytokine.

Figure 3-5: Staphylococcal enterotoxin B induces increased expression of various Th1, Th2, and T regulatory cytokine genes. Human CD4+ T cells were treated with 100 ng/ml of SEB and cultured as described in methods. Cells were harvested from the cultures at time points of 8, 16, 24, and 48 hours. Total RNA was extracted from these cells and used to make cDNA. The cDNA hybridized overnight to a nylon membrane spotted with cDNA of common human cytokines. The complete list of cytokine genes on the nylon membrane is presented in Appendix B. After washes and incubation with AP-streptavidin, and substrate, the membranes were exposed to film and developed. Pictures were scanned into TIFF format and the software program ScionImage was used to determine pixel density for each spot. Background values were subtracted from each value, followed by normalization to a positive control on each membrane. This normalized value for SEB treated cells was divided by the same value for media to obtain the fold increase in gene expression of each cytokine. A) Fold increase of IFN-γ, IL-2, IL-10 and IL-6 in CD4+ T cells treated with SEB. B) Fold increase of TGFβ, TNFβ, CD40L, and IL-1β in CD4+ T cells treated with SEB.


20
10
0
8 hours
16 hours
24 hours
48 hours
A
Fold Increase (SEB/Media)
TGFβ
TNFβ
CD40L
IL-1β


250
200
150
100
50
0
8 hours
16 hours
24 hours
48 hours
B
Fold Increase (SEB/Media)
TGFB3
TNFB
CD40L
IL1B

Superantigens Enhance Cytokine Protein Production in HPBMC

Since superantigens enhance the expression of various cytokine genes, we next determined cytokine protein levels in supernatants of human PBMC treated with SEA or SEB. ELISA and Western Blots were performed on the supernatants of HPBMC treated with 100 ng/ml of either SEA, SEB or media. IL-2, IFN-γ, IL-10 and TGFβ levels were determined. As shown in Figure 3-6, IL-2 was present in cultures treated with SEA and SEB, but not in media. Concentrations of IL-2 were greater than 600 ng/ml in both SEA and SEB treated groups at all four timepoints. Media treated cells supernatants had IL-2 levels of less than 50 ng/ml at all timepoints. This was in contrast to the IL-2 mRNA levels, which decreased after 16 hours. Similarly, IFN-γ and IL-10 protein levels were higher in supernatants from SEA and SEB treated cells than the media treated cells.

IFN-γ levels in cell supernatants were higher at 16 hours than at 24 hours, but were maximal at 48 hours (Figure 3-7). Media treated cell supernatants had low IFN-γ protein (<0.05 OD). IL-10 levels were highest at 48 hours (Figure 3-8) (>1500 ng/ml). Media treated cells had no detectable IL-10 at any of the timepoints. These protein levels were fairly consistent with the mRNA levels seen on the microarray. Higher concentrations of IL-10 at the later time points may be acting to down regulate the action of IL-2 (See discussion). Although TGFβ gene expression was increased as shown on the microarray, there was no detectable protein in the supernatants as determined by ELISA (data not shown) and Western Blots (Figure 3-9). Although TGFβ may be expressed at a later timepoint than 48 hours, cells begin to die after 48 hours incubation with superantigen and generally undergo apoptosis or anergy. Therefore, cytokine measurements may be inaccurate after 48 hours. Thus, superantigens also induce cytokine protein production in conjunction with increased cytokine gene expression in HPBMC in culture.

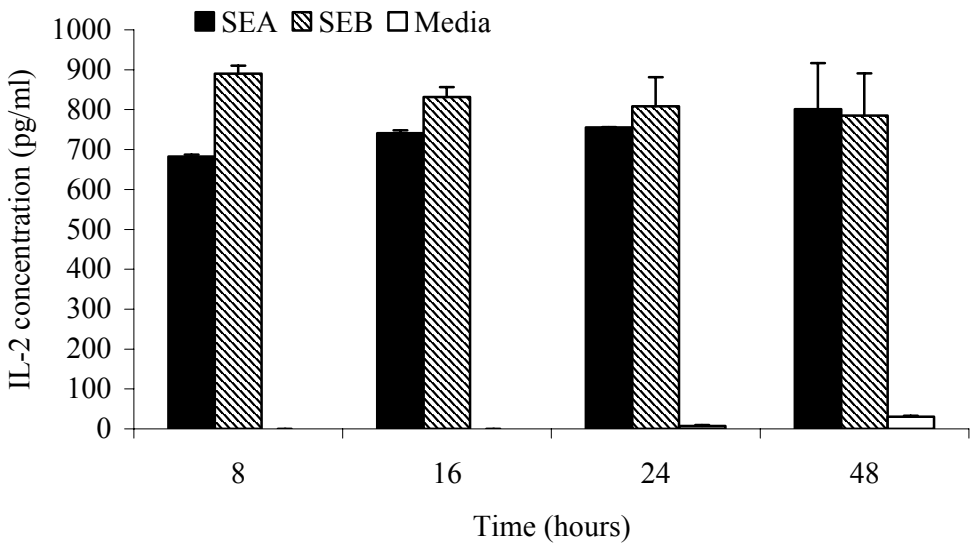


Figure 3-6: Staphylococcal enterotoxin A and staphylococcal enterotoxin B increase IL-2 production in human PBMC. HPBMC were treated with 100 ng/ml of either SEA or SEB, or culture medium alone. Cells were cultured as described in methods. Supernatants were harvested from the cultures at timepoints of 8, 16, 24, and 48 hours. An ELISA was performed on the supernatant to determine levels of IL-2. Data shown here represent mean and SD of duplicate experiments.

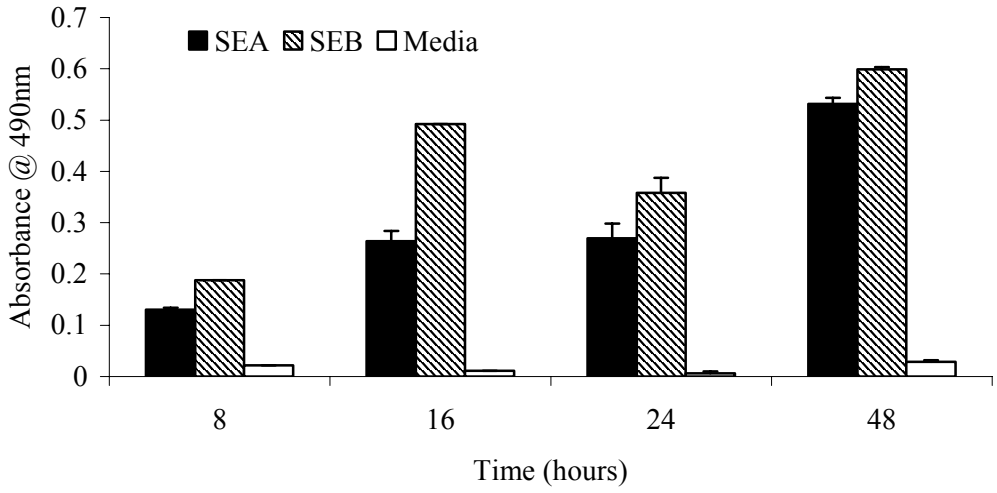


Figure 3-7: Staphylococcal enterotoxin A and staphylococcal enterotoxin B increase IFN-γ production in human PBMC. HPBMC were treated with 100 ng/ml of either SEA or SEB, or culture medium alone. Cells were cultured as described in methods. Supernatants were harvested from the cultures at timepoints of 8, 16, 24, and 48 hours. An ELISA was performed on the supernatant to determine levels of IFN-γ. Data shown here represent mean and SD of duplicate experiments.

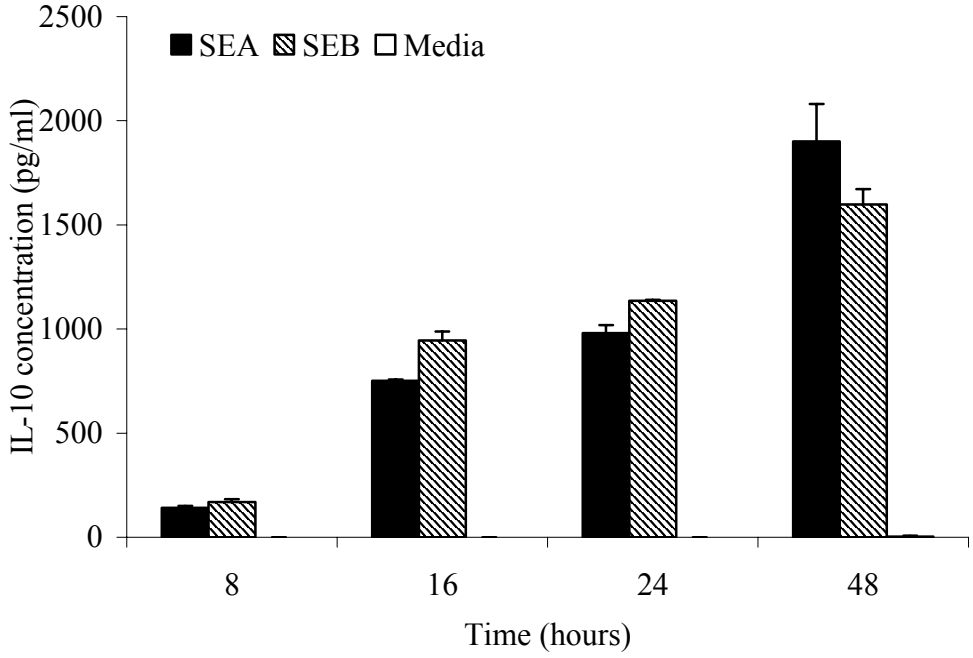


Figure 3-8: Staphylococcal enterotoxin A and staphylococcal enterotoxin B increase IL-10 Production in Human PBMC. HPBMC were treated with 100 ng/ml of either SEA or SEB, or culture medium alone. Cells were cultured as described in methods. Supernatants were harvested from the cultures at timepoints of 8, 16, 24, and 48 hours. An ELISA was performed on the supernatant to determine levels of IL-10. Data shown here represent mean and SD of duplicate experiments.

Figure 3-9: Staphylococcal enterotoxin A and staphylococcal enterotoxin B do not increase TGFβ production in human PBMC. HPBMC were treated with 100 ng/ml of either SEA or SEB, or culture medium alone. Cells were cultured as described in methods. Supernatants were harvested from the cultures at timepoints of 8, 16, 24, and 48 hours. A Western Blot was performed on the supernatants to determine levels of TGFβ. Data shown here represent 24 and 48 hour timepoints. A: TGFβ MW marker (25 kDa), B: blank, C: blank, D: 24h SEA, E: 24h SEB, F: 24h media, G: 48h SEA, H: 48h SEB, I: 48h media, J: media control K: Molecular weight ladder

TGFβ Suppresses IL-2 Induced Growth in NK92 Cells *in vitro*

NK92 cells, an IL-2 dependent cell line, were treated with either media, IL-2 (30 U/ml), or IL-2 and TGFβ (25 ng/ml) for 48 hours. Cells were then pulsed with ^{3}H-Thymidine for 6 hours and cell associated radioactivity was counted on a β-scintillation counter. As shown in Figure 3-10, cells cultured with IL-2 and TGFβ had almost 50% less proliferation than those cells cultured with IL-2 alone. Cells cultured with media alone, had low proliferation, showing that IL-2 is necessary for significant proliferation. Therefore, TGFβ can suppress IL-2 induced proliferation.

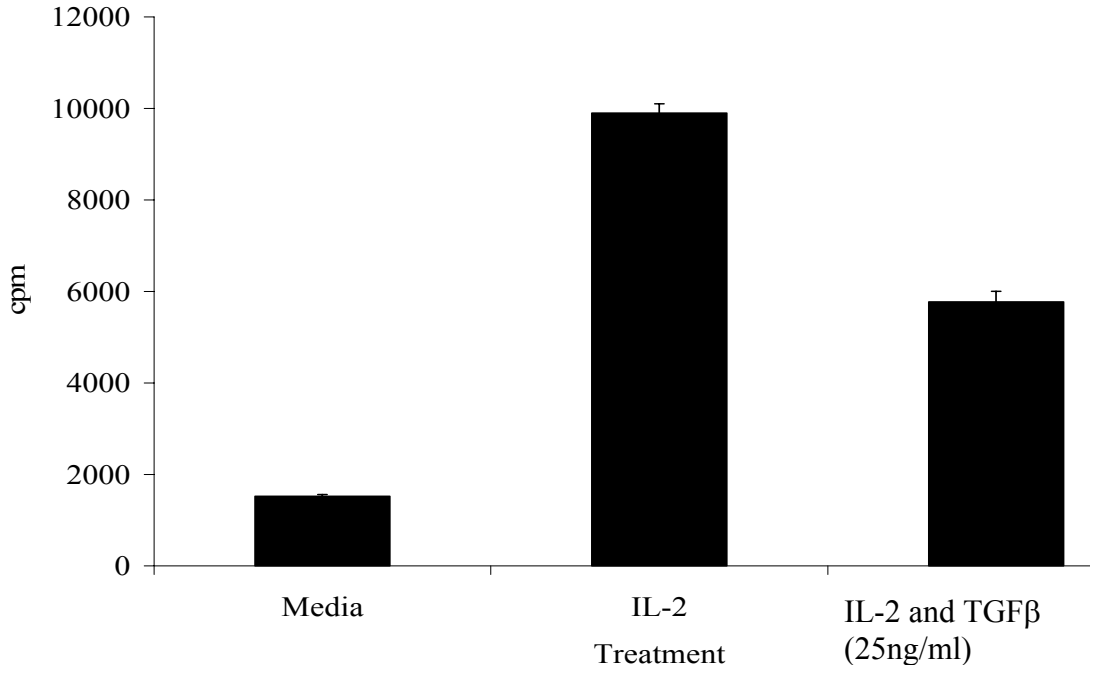


Figure 3-10: Transforming growth factor beta suppresses IL-2 induced proliferation of NK-92 cells. Human NK92 cells were plated at 4 x 104 cells per well in a 96 well plate and treated with either media, IL-2 (30 U/ml), or IL-2 and TGFβ (25 ng/ml) for 48 hours. Cells were then pulsed with 1 μCi per well of 3H-thymidine for 6 hours, after which cells were harvested and cell associated radioactivity was quantified using a β-scintillation counter and activity reported in CPM. All experiments were performed in replicates of six.

CHAPTER 4
DISCUSSION

In the first part of this study the immunoenhancing effects of superantigens on the humoral arm of the immune response were studied. Immunization of mice with the prototype T-dependent Ag BSA followed by SEA/SEB resulted in increased IgG antibody response to BSA. Thus, enhancement effects of superantigens were specific for the primary antigen BSA. The results presented here, combined with those of previous findings on superantigen enhancement of tumor-specific immunity to mouse melanoma (14), are evidence that superantigens such as SEA and SEB can significantly boost Ag-specific immune responses.

Microarray experiments of human PBMC treated with SEA, SEB or media alone showed increases in mRNA levels in various cytokines. These cytokines include but are not limited to IFN-γ, IL-2, TNF-β, IL-6, IL-10 and TGF-β. The presence of IL-2 mRNA is seen as early as 8 hours, peaking at 16 hours, followed by a decline after that through 48 hours (Figure 3-3). IFN-γ mRNA expression levels peak at 24 hours, followed by a decline (Figure 3-3, Figure 3-4, Figure 3-5). TNF-β mRNA expression increases at 16 h and is maintained through 48 hours (Figure 3-3). These three cytokines are indicative of a typical Th1 inflammatory type response. In the case of Th2 cytokine expression, IL-6 mRNA expression levels are maximal at 24 hours followed by a rapid decline

(Figure 3-3). TGF-β and IL-10, which are produced by T regulatory CD4+ T cells are maximal at 16 and 24 hours respectively. The studies on the cytokine profile of

superantigen activated cells and the enhancement of the humoral response against BSA presented here and the studies on enhancement of cellular response against melanoma cells suggest distinct T cell populations are being activated by superantigens (14). Thus, superantigens activate Th1, Th2, and T regulatory cells *in vivo* and *in vitro.*

In conjunction with the microarray studies, ELISA and Western blot studies were run to determine if the increases in mRNA levels were associated with translation of the message. ELISA (Figure 3-7) and Western Blots for IFN-γ (data not shown) showed increases in IFN-γ over time, with very little production of the IFNγ in media treated cells. Furthermore, IL-2 levels increased as early as 8 hours and remained constant through 48 hours (Figure 3-6). This is in contrast to the mRNA levels of IL-2, which started to decrease at the same time, possibly due to action of T regulatory cells. This may be due to a lag period between RNA message decline and seeing an actual decline in the protein produced. Carrying out the experiment for a much longer period of time may show the actual decrease in protein levels. However, this may prove to be difficult, as cells exposed to these high a concentrations of SEA or SEB begin to die after 48 hours. The fact that IL-10 protein expression in superantigen treated cells increased over time and is maximal at 48 hours (Figure 3-8) indicates how the T regulatory cells control the Th1 and Th2 cell types and plays an important role in helping the immune system to recover from encounters with superantigen. TGFβ expression increases after superantigen stimulation of cells, but is not detected at the protein level one to two days after superantigen stimulation (Figure 3-9). Others have shown that IL-10 enhances the expression of TGFβ (4,13,25). Thus, TGFβ protein production may occur beyond the 48 hour time point measured here but may be difficult to detect for the same reason

discussed above for IL-2. It has been previously shown in mice lacking CD4 25+ T-cells, which is the phenotype of T regulatory cells, have sustained production of inflammatory cytokines and that this production can be corrected by injections with CD4 25+ T-cells (20). As per Figure 3-9, TGFβ suppresses IL-2 induced proliferation by almost 50% in NK92 cells, an IL-2 dependent cell line. This suggests how the regulatory cytokines produced at later time points during superantigen stimulation, may act to down regulate the effects of Th1 cytokines produced earlier. It also identifies a potential target to block regulatory cytokines or a particular cell type, to bias and sustain an inflammatory or humoral response for a longer period of time. Thus, similar to an increase in RNA expression, IL-10, IL-2 and IFN-γ protein expression increase in culture supernatants taken from superantigen treated cells.

Superantigens enhance specific humoral and cellular responses against antigens, such as BSA in vivo and this immune enhancement is due to the action of superantigen on CD4+ T cells that produce inflammatory, helper and regulatory cytokines in a time dependent manner. Inflammatory cytokines produced by Th1 cells are induced initially by superantigen after which regulatory and suppressor cytokine, produced by Th2 and T regulatory cells, levels increase. There is an inherent characteristic of superantigen effects on naive vs Ag-primed T cells that is a plus for their immunoenhancing properties. Naive T cells initially undergo cell division when treated with superantigens, followed shortly by anergy and/or deletion. Ag-primed T cells also expand when treated with superantigen but, in contrast, do not undergo the anergy/deletion characteristic of naive T cells (11). Thus, the Vβ-specific polyclonal expansion associated with superantigens is tilted toward

primed Ag-specific T cell. This effect may be of beneficial use in vaccinations to boost immunity to a particular pathogen or disease.

APPENDIX A
MICROARRAY PROTOCOL

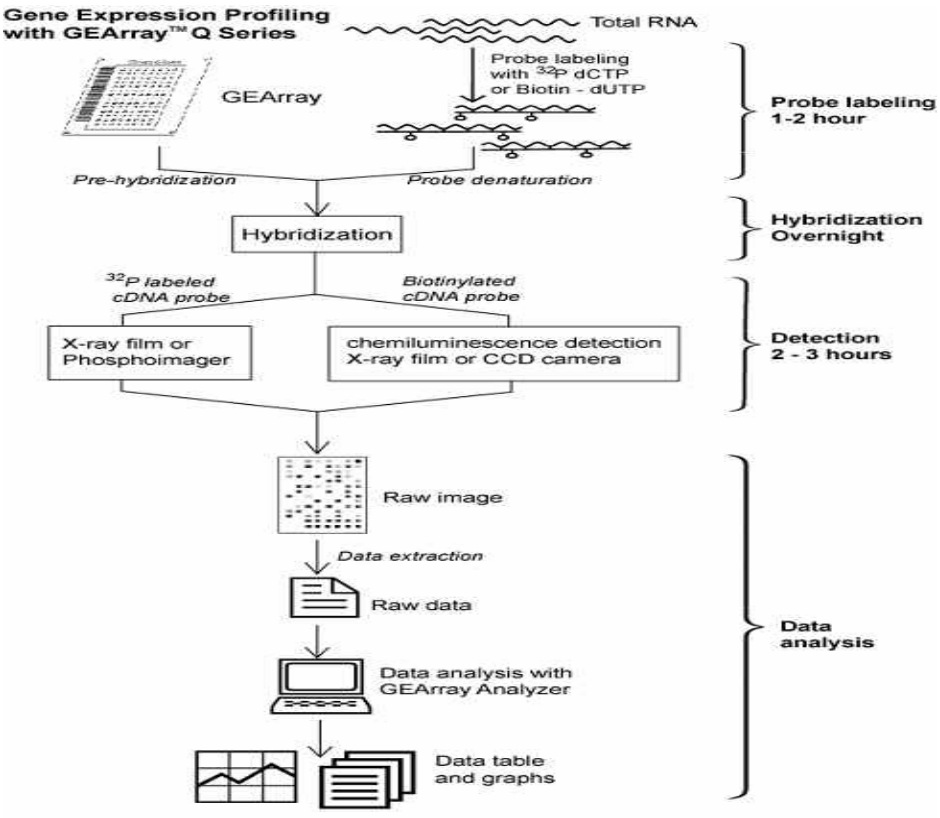


Figure A-1: Microarray Protocol (GEArray Q series protocol from Superarray, Frederick, MD)

APPENDIX B
MICROARRAY GENE LIST

Allograft inflammatory factor 1	c-fos induced growth factor	Interleukin 4
Bone morphogenetic protein 1	Hepatocyte growth factor	Interleukin 5
bone morphogenetic protein 2	interferon, alpha 1	Interleukin 6
Growth differentiation factor 10	Interferon, alpha 2	Interleukin 7
Bone morphogenetic protein 4	Interferon, alpha 4	Interleukin 8
Bone morphogenetic protein 6	Interferon, alpha 5	Interleukin 9
bone morphogenetic protein 8	Interferon, alpha 6	Leptin
Colony stimulating factor 1	Interferon, alpha 7	Lymphotoxin-alpha
Colony stimulating factor 2	Interferon, beta 1, fibroblast	Lymphotoxine-beta
Colony stimulating factor 3	Interferon, gamma	Platelet-derived growth factor-BB
Homo sapiens erythropoietin	Interferon, omega 1	Platelet-derived growth factor alpha polypeptide
Fibroblast growth factor 1	Insulin like growth factor IA	Pleiotrophin
Fibroblast growth factor 10	Insulin-like growth factor 2	Transforming growth factor, alpha
Fibroblast growth factor 11	Interleukin 10	Transforming growth factor, beta 1
Fibroblast growth factor 12	Interleukin 11	Transforming growth factor, beta 2
Fibroblast growth factor 12B	Interleukin 12A, p35	Transforming growth factor, beta 3
Fibroblast growth factor 14	Interleukin 12B,p40	Thrombopoietin
Fibroblast growth factor 16	Interleukin 13	Tumor necrosis factor
Fibroblast growth factor 17	Interleukin 14	Tumor necrosis factor (ligand) superfamily, member 10
Fibroblast growth factor 19	Interleukin 15	Tumor necrosis factor (ligand) superfamily, member 11
Fibroblast growth factor 2	Interleukin 16	Homo sapiens TNF (ligand) superfamily, member 4
Fibroblast growth factor 20	Interleukin 17	CD40 ligand
Fibroblast growth factor 21	Interleukin 18	Ligand for Fas
Fibroblast growth factor 23	Interleukin 19	CD27 ligand/CD70 antigen
Fibroblast growth factor 3	Interleukin 1, alpha	CD30 ligand
Fibroblast growth factor 4	Interleukin 1, beta	Tumor necrosis factor (ligand) superfamily, member 9
Fibroblast growth factor 5	Interleukin 2	Vascular endothelial growth factor
Fibroblast growth factor 6	Interleukin 20	Vascular endothelial growth factor B
Fibroblast growth factor 7	Interleukin 22	Vascular endothelial growth factor C
Fibroblast growth factor 9	Interleukin 3	PUC18 Plasmid DNA (negative control)
Glyceraldehyde-3-phosphate dehydrogenase (positive control	Homosapiens peptidylprolyl isomerase A	Ribosomal protein L13a (positive control)
		Beta Actin (positive control)

Table B-1: Microarray gene list. (GEArray Q series protocol from Superarray, Frederick, MD)

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BIOGRAPHICAL SKETCH

Amy Kristin Anderson was born in New Jersey on May 2, 1977. Her family got larger, when her brother Tim was born in March of 1980. Her family lived in NJ until she was 10 years old, when they moved to Tinmouth, Vermont in December of 1987. Amy enjoyed living in the rural town of about 400 people, having a horse and attending smaller sized elementary and high schools. After graduation from Mill River Union High School in 1995, Amy began attending the University of New Hampshire, on a partial academic scholarship, where she pursued a Bachelor of Science in Medical Laboratory Science. Amy graduated magna cum laude from UNH in 1999 and got a job at Duke Medical Center in Durham, NC, in the immunohematology lab as a Medical Technologist. While living in NC, Amy met Glenn, who was working on his Master of Science at North Carolina State University. Glenn's job search after graduation in 2000, took him all the way to Florida and Amy decided to look at graduate school there. Amy began attending the University of Florida in the fall of 2001 to pursue a Master of Science degree, studying in the department of Microbiology and Cell science. In November of 2001, Amy decided to do her master's research work in the laboratory of Dr. Howard M. Johnson. Amy and Glenn were married on June 28, 2003 in Vermont. After graduation, Amy plans to pursue a career in the biotechnology industry, hopefully doing research.