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Systemic cytokine and chemokine responses in immunized mice challenged with staphylococcal enterotoxin B

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Abstract

The cytokine storm induced by staphylococcal enterotoxin B (SEB) describes the rapid and dramatic induction of mediators which are likely responsible for the toxin's deleterious effects. However despite the use of numerous animal models for investigating SEB related illness in humans, mechanisms of toxicity and correlates of protection remain unclear. In the present study, we used an LPS-potentiased model of SEB lethality to investigate the toxin-induced cytokine and chemokine responses in untreated and immunized mice. Of 30 separate mediators analyzed, serum levels for 28 or 27 of these cytokines and chemokines were elevated following administration of dosages of 3 or 30 LD₅₀ of native SEB, respectively. Mice immunized with a non-toxic SEB vaccine candidate expressed in either *E. coli* or transgenic soy expression systems were protected from lethality when challenged with potentiased SEB. The majority of SEB-induced cytokines and chemokines (21 of 28 or 23 of 27 following challenge with dosages of 3 or 30 LD₅₀ of native SEB, respectively) were significantly decreased in mice immunized with an SEB vaccine candidate when compared to control animals. Together, these studies provide the most comprehensive evaluation of the cytokine storm induced in this LPS-potentiased model of SEB lethality to date. As with other animal models, the identification of those mediators which are necessary and sufficient for SEB-induced toxicity remains unclear.

Keywords

staphylococcal enterotoxin B; cytokine; chemokine; immunization; mouse model

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1. Introduction

Animal models to evaluate human vaccine candidates have been widely utilized in research and development efforts (Gerdtz et al., 2007) and are required for regulatory agency approvals (Riese et al., 2015). Unfortunately, animal models used for pre-clinical vaccine efficacy studies have some significant limitations (Centlivre and Combadiere, 2015). Rodent models are relatively inexpensive, and there are many reagents available to characterize their immune responses (Riese et al., 2015). The ability to characterize the magnitude of an immunoglobulin or cell mediated response following vaccination of rodents is routine (Schunk and Macallum, 2005). However it has often been difficult to determine whether a particular antibody level, or the expansion of a particular antigen-specific lymphocyte population, represents a correlate of protection in rodents that will translate to vaccine efficacy in humans (Thakur et al., 2012). In an effort to engineer relevant responses in mice, methods such as the xenotransplantation of human immune cells or tissues into immunodeficient mouse strains have been developed (Centlivre and Combadiere, 2015). Non-rodent vaccination models more closely mimic human immune responses, and the relevance of these larger animal models has been touted (Gerdtz et al., 2015). In particular, non-human primate models likely provide the most comparable immunity following vaccination (Rivera-Hernandez et al., 2014). However the expense and limitations, including ethical considerations, for justifying significant numbers of primates for post-immunization challenge studies often restrict their routine use (Rivera-Hernandez et al., 2014).

A variety of animal models have been used to investigate the efficacy of vaccine candidates targeting staphylococcal enterotoxin B (SEB) (Brosnahan, 2016; Fries and Varshney, 2013). The relative insensitivity of normal mice to this toxin has resulted in potentiation of its effects by co-administration of agents such as LPS (Stiles et al., 1993) or D-galactosamine (Miethke et al., 1992) to produce lethality. Alternatively, transgenic mice which express human major histocompatibility class II molecules have been engineered resulting in a more robust response to SEB (Faulkner et al., 2005; Rajagopalan et al., 2009). Larger animal models respond systemically to SEB as a superantigen (Krakauer et al., 2016), including swine (Bost et al., 2016; Hudson et al., 2013) and non-human primates (He et al., 2014; Komisar et al., 2001; Weng et al., 1997). These models have also been used to suggest efficacy of various SEB vaccination formulations (Boles et al., 2003; Hudson et al., 2013; Tseng et al., 1995). Despite such a diversity of studies using a variety of animal models, the immune parameters central for mediating SEB toxicity in humans remain unclear (Fries and Varshney, 2013; Krakauer et al., 2016). An SEB-induced cytokine storm is universally described as being deleterious (Tisoncik et al., 2012). However, those secreted factors which directly initiate this cascade, or the magnitude necessary to mediate SEB's noxious effects, remain poorly defined (Fries and Varshney, 2013; Krakauer et al., 2016).

In the present study, we utilized an LPS-potentiated model of SEB lethality to investigate the toxin-induced cytokine and chemokine responses in mice. While previous studies using this model have focused on systemic production of a few mediators (Stiles et al., 1993; Stiles et al., 2001; Ulrich et al., 1998), we were able to provide a more comprehensive characterization using a fluorescent multiplex assay. In addition, mice immunized with a SEB vaccine candidate produced using two different recombinant protein expression

platforms (Hudson et al., 2013; Hudson et al., 2014) were challenged with increasing doses of native toxin to investigate the ability of vaccination to reduce the lethal, SEB-induced cytokine storm. For those cytokines and chemokines which were induced by native SEB challenge, vaccinated mice demonstrated significantly reduced serum levels in most of these toxin-induced mediators. The relative magnitude of this reduction was variable depending on the particular endogenous mediator being quantified. Unfortunately, the large number of SEB-induced cytokines and chemokines, and their magnitude of expression, provided little insight as to which factors were required or were necessary for the initiation or lethality of this toxin-induced cascade.

2. Methods

2.1 Expression and purification of a non-toxic SEB vaccine candidate in *E. coli*

A non-toxic form of SEB containing the three amino acid mutations, L45R, Y89A, Y94A, was expressed in *E. coli* (Hudson et al., 2013; Hudson et al., 2014). This vaccine candidate, designated *E. coli*-mSEB, was purified as previously described (Hudson et al., 2013; Hudson et al., 2014) and used in these studies. Briefly, the mSEB open reading frame containing the amino acid changes, L45R, Y89A, Y94A, was cloned into a pET expression vector and transformed into *E. coli*. Cultures containing the expression plasmid were grown to stationary phase in terrific broth at 37°C for 16 hours, and cells were harvested by centrifugation. The cell paste was resuspended in 50 mM phosphate buffer (pH 6.3) and sonicated on ice. Insoluble material was removed by centrifugation, and nickel resin was used to bind protein from the soluble fraction. Recombinant protein was eluted with 50 mM phosphate buffer containing 400 mM imidazole. Eluted *E. coli*-mSEB was quantified using the Bradford reagent (with BSA as a standard) and purity was determined by Coomassie-blue staining of SDS-PAGE gels.

2.2 Expression and purification of a non-toxic SEB vaccine candidate in transgenic soybean seeds

A non-toxic form of SEB containing the three amino acid mutations, L45R, Y89A, Y94A, was expressed in transgenic soybean seed as previously described and was designated soy-mSEB (Hudson et al., 2013; Hudson et al., 2014). Briefly, *agrobacterium*-mediated soybean transformations were performed and transgenic events were taken to maturity. T₁ seeds were collected and genomic and protein assays were conducted to confirm DNA integration and protein expression. Based on results from these assays, select seeds were germinated and propagated over several generations.

Soy-mSEB was isolated as previously described (Hudson et al., 2013; Hudson et al., 2014). Briefly, transgenic seeds were ground to a fine powder, and protein extracted by sonication in 50 mM Tris-HCl (pH 7.8). The sonicated protein mixture was clarified by centrifugation, and the pH was lowered to 4.5 to precipitate acidic proteins. Insoluble material was removed by centrifugation, and the soluble extract was sequentially passed over DEAE cellulose, and then CM cellulose, columns to purify soy-mSEB. Purified mSEB was quantified using the Bradford reagent (with BSA as a standard) and purity was determined by Coomassie-blue staining of SDS-PAGE gels.

2.3 Immunization of BALB/c mice with *E. coli*-mSEB or soy-mSEB

These procedures for immunizing mice were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Charlotte. Groups of 4 week old BALB/c mice (N = 7) were immunized and boosted intramuscularly with 50 ug of purified *E. coli*-mSEB or soy-mSEB emulsified in Freund's adjuvant on days 0, 15, and 29, respectively. Groups of control animals (N = 6) received intramuscular injections of 50 ug of an irrelevant antigen (keyhole limpet hemocyanin, KLH) emulsified in Freund's adjuvant on the same days. Blood was taken from individual animals in each group via the saphenous vein prior to each immunization (days 0, 15, and 29), and also at day 58. Sera was isolated by centrifugation and stored at -80°C until used for anti-SEB antibody quantification.

2.4 ELISAs to quantify the developing anti-SEB serum antibody response and endpoint IgG anti-SEB titers

To quantify the anti-SEB serum antibody response following immunizations, ELISA plates (Corning, Corning, NY) were coated with 100 ng/well of native SEB (Toxin Technologies, Inc., Sarasota, FL) in 100 µL of carbonate buffer overnight at 4°C. Wells were washed, and blocked with 3% BSA in PBS for one hour at room temperature. A 1:3,000 dilution of sera from each mouse was added to wells and incubated overnight at 4°C. Wells were then washed and a horseradish peroxidase conjugated anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL) added at a dilution of 1:2500 in 1% BSA for 1 hour at room temperature. After washing, TMB substrate (BioFX, Owings Mills, MD) was added. Enzymatic reactions were stopped by addition of 1M sulfuric acid and absorbance at 450 nm recorded.

To determine anti-SEB antibody endpoint titers (day 58), a similar ELISA procedure was used. For this ELISA, serum from each mouse was serially diluted as indicated before performing the analysis.

2.5 In vivo challenge with native SEB potentiated by LPS

These SEB challenge studies were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Charlotte. On day 58 post immunization, groups of *E. coli*-mSEB, soy-mSEB, and KLH immunized mice were injected with 1 ug or 10 ug of native SEB (Toxin Technologies, lot number 103010B, < 40 endotoxin units per mg using a limulus amebocyte lysate assay) at time 0, and 4 hours later injected intraperitoneally with 75 ug of LPS (*E. coli* LPS, Sigma Chem. Co.). In previous studies, it was determined that these particular commercially available lots of SEB and LPS, in combination, represented dosages of approximately 3 and 30 LD₅₀ for BALB/c mice, respectively. Mice were also monitored for deleterious clinical effects resulting from toxin administration. When mice became clearly moribund (e.g. hunched posture, decreased mobility, and/or trembling), they were euthanized.

2.6 Quantification of serum cytokine and chemokine levels in immunized mice challenged with native SEB potentiated by LPS

Groups of mice were immunized with *E. coli*-mSEB, soy-mSEB, or KLH as described above. On day 58 post immunization, mice were injected intramuscularly with 1 ug or 10 ug

of native SEB (Toxin Technologies, lot number 103010B, < 40 endotoxin units per mg using a limulus amebocyte lysate assay) at time 0, and 4 hours later injected intraperitoneally with 75 ug of LPS (*E. coli* LPS, Sigma Chem. Co, St. Louis, MO). In previous studies, it was determined that these particular commercially available lots of SEB and LPS, in combination, represented dosages of approximately 3 and 30 LD₅₀ for BALB/c mice, respectively. At 6 hours and 12 hours following the administration of native SEB, blood was taken from the saphenous vein of each mouse. Blood was diluted four fold in PBS and centrifuged to isolate sera. Immune sera was then stored at -80°C until used for cytokine and chemokine quantification. For comparison, sera from naïve mice that received no treatments were also isolated, diluted, and stored at -80°C.

Thirty different cytokines and chemokines were quantified simultaneously using a Discovery Assay called the Mouse Cytokine/Chemokine Array 32-plex (Eve Technologies Corp, Calgary, AB, Canada). The multiplex assay was performed at Eve Technologies using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and a Milliplex Mouse Cytokine/Chemokine kit (Millipore, St. Charles, MO, USA) according to the manufacturer's protocol. For data analysis purposes, cytokine and chemokine experimental values were extrapolated from standard curves. The assay sensitivities for these 30 different mediators range from 0.1 to 33.3 pg/ml. Individual analyte values, assay coefficient of variations, and percent recoveries are available at the websites for Millipore, Inc. or Eve Technologies Corp.

2.7 Statistical analysis

For analysis of immune responses in mice, data obtained from Eve Technologies was transformed using $Y = \log_{10}(Y)$, then one-way ANOVA was performed followed by the post hoc Tukey-Kramer test using GraphPad Prism 7 software (La Jolla, CA). Statistical significance was determined at $P < 0.05$ and data are presented as the log₁₀ value of means +/- standard error.

3. Results

3.1 Antibody responses against native SEB in *E. coli*-mSEB and soy-mSEB immunized mice

To demonstrate immunogenicity of *E. coli*-mSEB and soy-mSEB, groups of mice were immunized, and the developing anti-SEB antibody response followed over time. Fig. 1A shows an increasing antibody response against native SEB in both immunized groups (blue and red bars) over the 58 day experiment. Additional studies were performed to determine the end point anti-SEB titers at day 58. Fig. 1B shows that groups of mice immunized with *E. coli*-mSEB (red bars) or soy-mSEB (blue bars) had higher reactivity at sera dilutions of 1:2,430,000 when compared to mice immunized with the irrelevant antigen, KLH (green bars). We concluded from these studies that immunization with *E. coli*-mSEB or soy-mSEB using this prime and 2 boost regimen resulted in high levels of serum antibody which could bind native SEB in an ELISA.

3.2 In vivo challenge of immunized mice with native SEB potentiated with LPS

To demonstrate the protective nature of the antibody response mounted in mice immunized with *E. coli*-mSEB or soy-mSEB, challenge studies were performed. Fig. 2 shows survival responses for groups of mice given dosages of 3 (Fig. 2A) or 30 (Fig. 2B) LD₅₀ of native SEB potentiated by LPS, respectively. Before 12 hours post-SEB injection, all mice immunized with KLH (green triangles) became moribund. This result was in contrast to mice immunized with *E. coli*-mSEB (red squares) or soy-mSEB (blue circles) which remained active and alive until the experiment was terminated 72 hours later. Based on survival data, we concluded that immunizations with *E. coli*-mSEB or soy-mSEB were equally effective in limiting the lethal effects of these high doses of native SEB potentiated by LPS.

3.3. Quantification of cytokine and chemokine levels following in vivo challenge of immunized mice with native SEB potentiated with LPS

While survival following toxin challenge is the customary endpoint for protection by vaccine candidates, we questioned whether reductions in specific SEB-induced cytokines or chemokines might also serve as specific correlates for such protection. In addition, the possible identification of cytokines or chemokines, which might, or might not, mediate SEB's noxious effects, was an objective.

For these studies, groups of mice were immunized as before (Figs. 1 and 2) with *E. coli*-mSEB, soy-mSEB, or KLH. At day 58 following immunization, mice were challenged 3 (Fig. 3) or 30 (Fig. 4) LD₅₀ dosages of native SEB potentiated with LPS. At 6 and 12 hours post-SEB administration, serum was taken from each mouse and 30 different cytokines or chemokines were quantified using a Discovery Assay called the Mouse Cytokine/Chemokine Array 32-plex. Surprisingly, with the exception of IL-7 and GM-CSF for mice challenged with 3 LD₅₀ (Fig. 3), and IL-7, IL-15, and GM-CSF for mice challenged with 30 LD₅₀ (Fig. 4) of native SEB, all of the other cytokine or chemokine serum levels were significantly elevated at 6 and/or 12 hours post SEB administration when comparing values for untreated mice that received no toxin challenge (gray bars, Figs. 3 and 4) with those of the KLH immunized mice (black or blue bars, Figs. 3 and 4). Based on these studies, we concluded that IL-7 and GM-CSF were unlikely contributors to SEB's noxious effects in this LPS-potentiated mouse model. However due to the fact that the remaining 28 (Fig. 3) or 27 (Fig. 4) cytokines or chemokines were significantly elevated, it was difficult to make any definitive conclusions about each of these mediators' relative importance in this model of SEB-induced lethality.

Since mSEB immunized mice survived when challenged with native SEB (Fig. 2), it was logical to assume that a reduction in the lethal, toxin-induced cytokine storm occurred in these animals. We therefore questioned whether it was possible to identify specific SEB-induced cytokines or chemokines which were reduced in these vaccinated mice since this result would suggest those mediators that correlated with protection. Of the 28 mediators (Fig. 3) induced by 3 LD₅₀ of SEB potentiated with LPS, 21 of these showed statistically significant reductions when comparing *E. coli*-mSEB (green or yellow bars, Fig. 3) or soy-mSEB (orange or red bars, Fig. 3) immunized mice with the higher levels detected in KLH

(black or blue bars, Fig. 3) immunized mice at either 6 hours and/or 12 hours post toxin administration. Of the 27 mediators (Fig. 4) induced by 30 LD₅₀ of SEB potentiated with LPS, 23 of these showed statistically significant reductions when comparing *E. coli*-mSEB (green or yellow bars, Fig. 4) or soy-mSEB (orange or red bars, Fig. 4) immunized mice with the higher levels detected in KLH (black or blue bars, Fig. 4) immunized mice at either 6 hours and/or 12 hours post toxin administration. We concluded from these studies that vaccination of mice with *E. coli*-mSEB or soy-mSEB could limit production of the majority of cytokines and chemokines which were induced in this model of SEB potentiated lethality. Unfortunately, due to the large number of mediators that were reduced in the mSEB immunized mice, it was not altogether clear which cytokine or chemokine responses might represent specific correlates of protection following vaccination.

Conversely, of the 28 mediators (Fig. 3) induced by administration of 3 LD₅₀ of native SEB potentiated with LPS, 7 (IL-1 α , IL-9, IL-13, IL-15, CCL5, G-CSF, and M-CSF) were not significantly reduced by immunization with *E. coli*-mSEB or soy-mSEB. Of the 27 mediators (Fig. 4) induced by administration of 30 LD₅₀ of native SEB potentiated with LPS, 4 (IL-6, CCL5, CXCL1, and G-CSF) were not significantly reduced by immunization with *E. coli*-mSEB or soy-mSEB. Since all *E. coli*-mSEB or soy-mSEB immunized mice survived a challenge with 3 LD₅₀ (Fig. 2A) or 30 LD₅₀ (Fig. 2B) of native SEB, the lack of a vaccination-induced reduction in these mediators suggested that their contribution to SEB-induced lethality is minimal in this model.

4. Discussion

For SEB, its ability to function as a superantigen results in the release of high levels of numerous cytokines, chemokines, and growth factors (Fries and Varshney, 2013; Krakauer et al., 2016). This activity can easily be demonstrated in cell cultures of human leukocytes (Krakauer, 1999; Sperber et al., 1995), and has been inferred from food contamination (Marrack and Kappler, 1990) and a limited number of cases of human exposure (Dembek, 2007; Rusnak et al., 2004; Ulrich et al., 1997). Because of this remarkable ability to induce a cytokine storm (Tisoncik et al., 2012), it is likely that this superantigen activity explains some clinical symptomology (Dembek, 2007; Rusnak et al., 2004; Ulrich et al., 1997). Unfortunately, it is not clear which particular mediators are necessary and sufficient for initiating and/or propagating SEB-induced toxicity (Fries and Varshney, 2013; Krakauer et al., 2016).

Animal models of SEB-induced toxicity have provided some insight. Non-human primate responses to SEB exposure can include lethality (Tseng et al., 1995), as well as elevated serum cytokine levels (Boles et al., 2003; He et al., 2014; Krakauer et al., 1997; Weng et al., 1997). Swine models of SEB-induced superantigen activity can also include lethality (Bi et al., 2009), with the magnitude and composition of toxin-induced cytokines varying significantly depending upon the report (Bi et al., 2009; Hudson et al., 2013). Non-human primates and swine respond to SEB as a superantigen, and therefore likely represent the most relevant models. It is unfortunate that studies using these models have not provided a clearer understanding of the specific relationship between the superantigen-induced cytokine storm and the subsequent pathophysiology.

In an attempt to mimic SEB's superantigen activity in mice, transgenic strains have been developed which express human HLA-DR molecules and result in animals that are more sensitive to the toxin (Faulkner et al., 2005; Rajagopalan et al., 2009). Using these mice, one study suggested that SEB toxicity is not a simple TH1 cytokine storm, but is dependent on an early TNF- α response for lethality (Faulkner et al., 2005). A more recent study using an HLA-DR3 transgenic mouse model provided a more comprehensive analysis of the cytokine and chemokine serum responses at a single time point of 3 hours following administration of a lethal dose of SEB (Rajagopalan et al., 2009). Surprisingly every cytokine, chemokine, or factor investigated was significantly, and sometimes dramatically, increased. The authors noted that TNF- α levels were only modestly increased relative to many of the other mediators investigated (Rajagopalan et al., 2009). Chemokines as a group were among the most dramatically increased, which the authors interpreted as an implication for their importance in toxicity (Rajagopalan et al., 2009). If the magnitude of expression of a particular mediator equates to its importance, then there are a large number of candidates contributing to SEB-induced toxicity in these mouse models (Faulkner et al., 2005; Rajagopalan et al., 2009). However, we know from human clinical trial data that the magnitude of deleterious side effects resulting from recombinant cytokine therapies are not only concentration dependent, but are also dependent on the mediator itself. Some exogenously delivered cytokines appear to be quite toxic (Cohen, 1995; Creaven et al., 1987; Zaidi and Merlino, 2011), while others given at similar dosages are relatively well tolerated (Asadullah et al., 2003). Both the mediator and its concentration over time must be taken into account when considering relative in vivo toxicities whether endogenously produced or exogenously administered. Further, there is reason to suggest that there may be synergy in their protective or deleterious effects when particular cytokines are present at the same time (Cosgrove et al., 2010; Talmadge, 1992). Therefore, while mouse models have demonstrated the lethality of SEB and its ability to stimulate systemic cytokine and chemokine production, the focus on a few pro-inflammatory cytokines (Faulkner et al., 2005) or a single timing of mediator expression (Rajagopalan et al., 2009) limit these characterization studies.

In performing the studies presented here, we were hopeful that a more comprehensive quantification of the cytokine storm at two very different time points (i.e. early and late) during SEB-induced lethality would help clarify those mediators of importance and eliminate those which were less critical. While these results (Figs. 3 and 4) succeeded in broadening the characterization of an SEB potentiated cytokine storm in mice (Stiles et al., 1993; Stiles et al., 2001; Ulrich et al., 1998), whether they provided compelling insight as to the mediators that are necessary and sufficient for initiating and/or propagating SEB-induced toxicity is not clear. Surprisingly 28 (Fig. 3) or 27 (Fig. 4) of the 30 cytokines or chemokines quantified had significant elevations when compared to naïve mice. Therefore, merely considering the increase of a particular mediator does not provide much clarity regarding its relative importance in this toxin's lethality. Further data analyses ranking the fold increases, or absolute magnitude of increase, for each cytokine or chemokine over time also provided little insight when attempting to prioritize their importance. For this reason, such data analyses were not included here. Our expansion of the number of cytokines and chemokines quantified does allow us to confirm some previously reported results (Rajagopalan et al., 2009), while also adding to the list of potential mediators that might require additional

consideration (i.e. IL-9, IL-15, CCL3, CXCL2, CXCL9, CXCL10, and LIF), or might need to be excluded (i.e. IL-7 and GM-CSF), as contributors to SEB lethality in this mouse model.

Perhaps the most unexpected observation was the general magnitude and persistence of each of the 9 chemokines which were quantified (Figs. 3 and 4). One previous study using an HLA-DR3 transgenic mouse model demonstrated increased mRNA expression for numerous chemokine and chemokine receptors, and reported quantification of 5 of these chemokines in sera (Rajagopalan et al., 2009). However, the contributions made by such high levels of circulating chemokines to mechanisms of SEB-induced lethality in mouse models remain unclear and largely unexplored.

Assessing the relevance of SEB-induced chemokine and cytokine production in potentiated (Miethke et al., 1992; Stiles et al., 1993; Stiles et al., 2001; Ulrich et al., 1998) and HLA-DR transgenic (Faulkner et al., 2005; Rajagopalan et al., 2009) mouse models with swine (Bi et al., 2009; Hudson et al., 2013), and non-human primate (Komisar et al., 2001; Weng et al., 1997) studies is challenging. In vitro stimulation of human (Krakauer, 1999; Sperber et al., 1995) and non-human primate (Kenway-Lynch et al., 2014) leukocytes by SEB induces an array of cytokine and/or chemokine release, as in vivo exposure of non-human primates to this toxin does (Komisar et al., 2001; Weng et al., 1997). Studies using piglet models of SEB toxicity have also shown the rapid and dramatic induction of numerous cytokines (Hudson et al., 2013). Unfortunately, these human, non-human primate, or swine studies have not provided any clear indication of the importance of particular cytokines or chemokines in human SEB-induced toxicities. With such a lack of understanding of the mechanisms involved in human disease, it is difficult to validate animal models since relevant correlates of protection or damage remain ill defined.

Furthermore, different animal models do not always provide similar responses, and this is especially true for mouse models. For example in our study using a potentiated SEB model no significant IL-1 β , IL-12p40, or GM-CSF serum increases were observed at 6 hours post native SEB administration when compared to naïve mice (Figs. 3 and 4). However in a previous work using an HLA-DR transgenic mouse model, these mediators were highly inducible in sera at 3 hours post administration (Rajagopalan et al., 2009). Whether these differences can be attributed to our use of an LPS-potential model and/or to the kinetics of the response (i.e. 3 versus 6 hours post administration) is not clear. More recently, a lethal mouse model using a double hit of low dose SEB given intranasally to C3H/HeJ mice resulted in widespread upregulation of type I and type II interferon-associated mRNA expression (Ferreira et al., 2014). It is difficult to reconcile these results (Ferreira et al., 2014) with the widespread upregulation of SEB-induced mRNAs for cytokines, chemokines, and their receptors observed previously (Rajagopalan et al., 2009). Such differences may be attributed to differences in toxin dose, method of administration, tissues analyzed, or the particular mouse strain utilized. However such possible explanations do little to clarify which mouse model is most relevant to human disease or which pathways are necessary and sufficient for SEB-induced toxicities.

The usefulness of an animal model for evaluating the potential efficacy of a human vaccine candidate requires defined correlates of protection. For toxins that are quite noxious, like SEB, it is difficult to define what such correlates might be useful to follow since human clinical trials cannot be carried out. In the present study, we used immunized groups of mice in an attempt to suggest some possible correlates of protection. As expected (Hudson et al., 2013), mice immunized with *E. coli*-mSEB or soy-mSEB (Fig. 1) were protected from lethality following SEB administration (Fig. 2). These immunized mice allowed us to observe which SEB-induced cytokine and chemokine responses were most affected in these protected animals. Logically, reduction of particular SEB-induced mediators in *E. coli*-mSEB (green or yellow bars, Figs. 3 and 4) or soy-mSEB (orange or red bars, Figs. 3 and 4) immunized mice when compared with the higher levels detected in KLH immunized mice (black or blue bars, Figs. 3 and 4) might indicate those most important for lethality. Given that 21 (Fig. 3) or 23 (Fig. 4) mediators at 6 and/or 12 hours post challenge with LPS-potentiated SEB, respectively, were significantly reduced in immunized mice, it was not clear which of these cytokines or chemokines might be most important for toxin-induced lethality. Considering the absolute magnitude of reduction alone, IL-2, IL-12p70, IL-17, CCL3, CCL4, CXCL2, LIF, IFN- γ , and TNF- α were notable in this respect at 6 hours and/or 12 hours post challenge (Figs. 3 and 4). Perhaps this list of mediators includes those most likely to affect survival. However the analysis becomes even more complicated when other variables are examined. If one considers only 6 hours post toxin challenge, there are a number of cytokines and chemokines which were not significantly reduced in vaccinated animals, including IL-1 β , IL-6, IL-7, IL-10, IL-12p40, IL-13, IL-15, CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, G-CSF, GM-CSF, and M-CSF. One must consider the possibility that during a toxin-induced cytokine storm the kinetics of a particular mediator's increase may be as important as the magnitude of its expression.

In summary, the present work expands the characterization of the cytokine storm induced in this LPS potentiated mouse model of SEB lethality. While a goal of this study was to identify mediators of toxicity and eliminate those unlikely to be involved, we were surprised by the breadth and kinetics of the systemic response. As with all other characterization studies that have attempted to define the cytokine storm, regardless of the animal model, the mediators responsible for SEB-induced toxicity remain ill-defined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

mSEB	a recombinant form of staphylococcal enterotoxin B (SEB) containing the three amino acid mutations, L45R, Y89A, Y94A
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KLH keyhole limpet hemocyanin

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Most comprehensive chemokine and cytokine profile in a mouse model of staphylococcal enterotoxin B toxicity to date.

Immunizing with a vaccine candidate protects mice and reduces selected toxin induced chemokine and cytokine production.

Despite this comprehensive profiling effort, identifying mediators necessary and sufficient for toxicity remains unclear.

Fig. 1.

Antibody responses against native SEB in immunized mice. Groups of mice were immunized with 50 μ g of purified *E. coli*-mSEB (red bars, N = 7), soy-mSEB (blue bars, N = 7), or KLH (green bars, N = 6). At the indicated times post immunization, serum was taken from each mouse and analyzed for anti-SEB antibody responses using an ELISA. Results are presented as mean absorbance values ($\pm$ S.E.) for each immunization group. Asterisks represent statistically significant differences ($p < 0.05$) when compared to KLH immunized animals. (A) A 1:3,000 dilution of sera was used to determine the kinetics of the

developing anti-SEB response. Primary immunization was on day 0, followed by two booster immunizations on days 15 and 29. (B) Endpoints titers were determined at day 58 post immunization. Sera were serially diluted as indicated, and reactivity against native SEB determined. This entire study was performed two separate times with similar results.

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Fig. 2.

Survival following *in vivo* challenge of immunized mice with native SEB potentiated with LPS. Groups of mice were immunized with 50 ug of purified *E. coli*-mSEB (red squares, N = 7), soy-mSEB (blue circles, N = 7), or KLH (green triangles, N = 6) as described in Fig. 1. On day 58 post-immunization, mice in each group were injected intraperitoneally with 1 ug (2A) or 10 ug (2B) of native SEB at time 0, and 4 hours later injected intraperitoneally with 75 ug of LPS. This combination represented dosages of approximately 3 LD₅₀ (panel A) or 30 LD₅₀ (panel B), respectively. Mice were monitored for deleterious clinical effects resulting from toxin administration. When mice became clearly moribund, they were euthanized.

Fig. 3.

Quantification of cytokine and chemokine levels following *in vivo* challenge of immunized mice with an LPS-potentiated SEB dosage of approximately 3 LD₅₀. Groups of mice were immunized with *E. coli*-mSEB, soy-mSEB, or KLH as described in Fig. 1. On day 58 post-immunization, mice in each group were injected intraperitoneally with 1 ug of native SEB at time 0, and 4 hours later injected intraperitoneally with 75 ug of LPS. This combination represented a dosage of approximately 3 LD₅₀. At 6 hours and 12 hours following the administration of native SEB, sera was isolated and stored at -80°C. For comparison, sera

from naïve mice that received no treatments were also isolated and stored at -80°C . Quantification of cytokine and chemokine serum levels were performed using a Discovery Assay called the Mouse Cytokine/Chemokine Array 32-plex. Results are presented as mean pg/ml ($\pm$ S.E.) for the indicated cytokines or chemokines for each immunization group. Statistically different increases between naïve mice (gray bars) and KLH-immunized mice (black and blue bars) are indicated with “a”. Statistically different decreases between KLH-immunized mice (black and blue bars) and either *E. coli*-mSEB-immunized mice (green and yellow bars) or soy-mSEB-immunized mice (orange and red bars) are indicated with “b”. The designation “#” indicates mean serum concentrations <1 pg/ml.

Fig. 4.

Quantification of cytokine and chemokine levels following *in vivo* challenge of immunized mice with an LPS-potentiated SEB dosage of approximately 30 LD₅₀. Groups of mice were immunized with *E. coli*-mSEB, soy-mSEB, or KLH as described in Fig. 1. On day 58 post-immunization, mice in each group were injected intraperitoneally with 10 ug of native SEB at time 0, and 4 hours later injected intraperitoneally with 75 ug of LPS. This combination represented a dosage of approximately 30 LD₅₀. At 6 hours and 12 hours following the administration of native SEB, sera was isolated and stored at -80°C. For comparison, sera

from naïve mice that received no treatments were also isolated and stored at -80°C . Quantification of cytokine and chemokine serum levels were performed using a Discovery Assay called the Mouse Cytokine/Chemokine Array 32-plex. Results are presented as mean pg/ml ($\pm$ S.E.) for the indicated cytokines or chemokines for each immunization group. Statistically different increases between naïve mice (gray bars) and KLH-immunized mice (black and blue bars) are indicated with “a”. Statistically different decreases between KLH-immunized mice (black and blue bars) and either *E. coli*-mSEB-immunized mice (green and yellow bars) or soy-mSEB-immunized mice (orange and red bars) are indicated with “b”. The designation “#” indicates mean serum concentrations <1 pg/ml.