

INTRANASAL EXPOSURE TO STAPHYLOCOCCAL ENTEROTOXIN B ELICITS AN ACUTE SYSTEMIC INFLAMMATORY RESPONSE

Govindarajan Rajagopalan,* Moon M. Sen,* Manisha Singh,* Narayana S. Murali,[†] Karl A. Nath,[†] Koji Iijima,[‡] Hirohito Kita,[‡] Alexey A. Leontovich,[§] Unnikrishnan Gopinathan,[§] Robin Patel,^{||} and Chella S. David*

*Department of Immunology; the [†]Division of Nephrology; the [‡]Division of Allergic Diseases Research; the

[§]Genomics Research/Microarray Shared Resource and Cancer Center; and the ^{||}Divisions of Infectious Diseases and Clinical Microbiology, Mayo Clinic College of Medicine, Rochester, MN

Received 27 Oct 2005; first review completed 8 Nov 2005; accepted in final form 10 Jan 2006

ABSTRACT—*Staphylococcus aureus* produces a variety of superantigen exotoxins, including staphylococcal enterotoxin B (SEB). Little is known regarding the pathogenesis of SEB entering through the intranasal route. Intranasal exposure to SEB might occur because of nasal packing following surgical procedure, biologic warfare, or even *S. aureus* colonization. We evaluated the local and systemic effects of intranasally delivered SEB using a series of human leukocyte antigen (HLA) class II transgenic mice as conventional mice expressing endogenous class II molecules mount a poor immune response to SEB. Gene expression profiling using microarrays showed robust up-regulation of genes involved in several proinflammatory pathways as early as 3 h post-intranasal challenge with SEB in HLA class II transgenic mice. This was accompanied by a several hundred-fold increase in serum levels of pro-inflammatory cytokines such as IL-12, IL-6, TNF- α , IFN- γ , as well as MCP-1 in HLA class II transgenic mice but not in C57BL/6 mice; CD4⁺ or CD8⁺ T-cells independently contributed to the systemic cytokine response. Defective IL-12 or IL-4 receptor signaling significantly decreased or increased serum IFN- γ , respectively. Intranasal exposure to SEB resulted in neutrophil influx into bronchoalveolar lavage fluid and caused expansion of both CD4⁺ and CD8⁺ T-cells expressing TCR V β 8 in the spleen. This was accompanied by mononuclear cell infiltration in the liver reminiscent of the systemic inflammatory response syndrome. Thus, we have shown, for the first time, that intranasal administration of SEB can cause systemic immune activation.

KEYWORDS—Rodent, superantigen, inflammation

ABBREVIATIONS—BAL, bronchoalveolar lavage, SEB, staphylococcal enterotoxin B, STAT, signal transducer and activator of transcription

INTRODUCTION

Bacterial superantigens are a family of microbial polypeptide exotoxins that are capable of inducing strong proliferation of T lymphocytes even at extremely low concentrations (1). They are the most potent T-cell activators known to humankind. Mechanistically, unlike conventional antigens that bind to the peptide-binding groove of major histocompatibility complex (MHC) class II molecules after undergoing a series of processing steps, superantigens directly bind to MHC class II molecules outside the peptide-binding groove without undergoing any processing. Whereas exogenous antigens presented by MHC class II molecules activate CD4⁺ T-cells, MHC class II-bound superantigen activates both CD4⁺ and CD8⁺ T-cells. Whereas conventional antigens activate specific T-cells by interacting with both α and β chains of T-cell receptor (TCR) molecules, superantigens activate T-cells by directly binding to the variable region of the TCR β chain (and in rare cases TCR V α families), irrespective of their antigen specificities (1). Superantigens thus activate 30% to

50% of the total T-cell pool, whereas conventional antigens activate approximately 1 in 10⁴ to 10⁶ T-cells. By virtue of their capacity to induce strong immune activation, superantigens can cause a variety of illnesses ranging from acute food poisoning to toxic shock syndrome and have even been implicated in some autoimmune diseases (1).

Bacterial superantigens are produced by *Staphylococcus aureus* and *Streptococcus pyogenes*, which are ubiquitous in nature (2, 3). Humans are natural carriers for these organisms, the nasal passage being the most common site for *S. aureus* colonization (2). Whereas more than 50% of pathogenic isolates of *S. aureus* produce one or more superantigen exotoxins (4), even strains isolated from asymptomatic carriers can produce superantigens (4). Given their ability to elaborate superantigen exotoxins and their anatomic localization, it is likely that the nasal passage is exposed to bacterial superantigens (5). In this context, whereas the pathogenesis of menstrual or nonmenstrual toxic shock associated with superantigens is fairly well understood, little is known regarding the immunopathogenesis of intranasal exposure to staphylococcal enterotoxin (SEB).

Staphylococcal toxic shock is a well-known complication of endonasal surgery and is generally associated with the use of nasal packing, stents, implants, septal splints, etc. (6, 7). It is believed that nasal packing materials, similar to vaginal tampons, facilitate growth of *S. aureus*. Stents and other implants may facilitate biofilm formation by *S. aureus* and enhance exotoxin production. Thus, staphylococcal superantigenic

Reprints: Dr. Chella S. David, Department of Immunology, Mayo Clinic College of Medicine, 200 First Street, SW, Rochester, MN 55905. E-mail: davic4@mayo.edu.

Supported by NIH grant AI14764.

GR is a recipient of Juvenile Diabetes Research Foundation fellowship.

DOI: 10.1097/01.shk.0000209565.92445.7d

Copyright © 2005 by the Shock Society 2005

toxins produced by the organisms growing in the nasal cavity are suspected to cause toxic shock. However, there is no direct evidence to link staphylococcal superantigen exotoxins with toxic shock following endonasal procedures. The nasal passage may also be exposed to bacterial superantigens, especially SEB, in the event of an intentional bioterrorism act or even accidental laboratory exposure.

A significant knowledge gap in the understanding of the immunopathogenesis of superantigen-mediated diseases exists, attributable to the dearth of suitable animal models. Although nonhuman primates serve as a reasonable animal model for studying the pathophysiology of superantigens (8), ethical concerns, exorbitant costs, lack of availability of suitable laboratory reagents, and challenges of performing in vivo manipulations such as immunodepletion and gene knockouts limit the use of nonhuman primates in the study of the immunopathogenesis of bacterial superantigen-mediated diseases. Rabbit models suffer from similar drawbacks. Traditional mouse models are also unsuitable for superantigen studies because bacterial superantigens bind extremely poorly to murine MHC class II molecules (8). As a consequence, they fail to substantially activate murine T-cells. For example, whereas superantigens can activate murine T-cells, about a 1000-fold higher concentration is required to achieve similar activation to that observed with human T-cells (9). Consequently, mice are highly resistant to superantigen-induced toxic shock (8), and the use of sensitizing agents such as bacterial LPS or d-galactosamine (d-gal) is needed to induce superantigen-induced toxic shock in mice (8).

Toxic shock seen in mice after sensitization with LPS or d-gal does not recapitulate superantigen-induced toxic shock in humans. First, LPS is a derivative of Gram-negative bacteria and activates primarily monocyte/macrophages of the innate immune system through toll-like receptor 4, but not T lymphocytes (10). Bacterial superantigens produced by Gram-positive bacteria, on the other hand, primarily activate T lymphocytes. Secondly, d-gal is a hepatocyte-specific transcriptional inhibitor and markedly sensitizes hepatocytes to cytokine-induced apoptosis (11). Therefore, liver damage is the primary cause of mortality in d-gal sensitization models. Transgenic expression of human MHC class II molecules, the high affinity ligands for bacterial superantigens, in mice lacking endogenous class II molecules dramatically increases the magnitude of the murine immune response to bacterial superantigens (12–14). As a result, human leukocyte antigen (HLA) class II transgenic mice are rendered extremely susceptible to toxic shock even in the absence of aforementioned sensitization agents (13–15). Thus, HLA class II transgenic mice are a valuable tool to dissect the immunopathogenesis of diseases caused by bacterial superantigens.

We have previously shown that the systemic immune response to SEB delivered by the intraperitoneal route is far superior in HLA class II transgenic mice when compared with conventional mice strains (e.g., C57BL/6 or BALB/c) (12–14). The systemic immune activation following acute intranasal exposure has not, however, been studied. In the current study, we evaluated whether acute exposure to SEB through the intranasal route can cause systemic immune activation and

dissected the role of T-cell subsets and cytokines in this process using a series of HLA class II transgenic mice.

MATERIALS AND METHODS

Mice

HLA-DR3 transgenic mice expressing functional HLA-DRA1*0101 and HLA-DRB1*0301 transgenes on the complete mouse MHC class II-deficient background (AE^g) (14) were generated and are hereafter referred to as DR3 transgenic mice. HLA-DQ8 transgenic mice expressing HLA-DQA1*0301 and HLA-DQB1*0302 transgenes have been previously described and are hereafter referred to as DQ8 transgenic mice (16). DQ8 mice lacking CD4 and CD8 molecules (16) were also used (DQ8.CD4^{-/-}, DQ8.CD8^{-/-}, respectively). HLA-DQ8 mice were mated to IL-10^{-/-} mice on the B6 background (Jackson Laboratories, Bar Harbor, ME) to generate DQ8.IL-10^{-/-} mice. The offspring were selectively intercrossed to generate a DQ8.IL-10^{-/-} line. Signal transducer and activator of transcription (STAT) 4-deficient HLA-DQ8 transgenic mice and STAT6-deficient HLA-DQ8 mice were generated by Dr. William Stahlman (University of Southern California Keck School of Medicine, Los Angeles, CA). All of the HLA class II transgenic mice lacked endogenous class II molecules, and consequently their T-cell responses are restricted only by the transgenic HLA class II molecules (17). C57BL/6 (B6) mice were from the Jackson Laboratories. Mice were bred within the barrier facility of Mayo Clinic Immunogenetics Mouse Colony (Rochester, MN) and moved to a conventional facility after weaning. All experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee. The experiments were performed in adherence to the National Institutes of Health guidelines on the use of laboratory animals.

Intranasal superantigen challenge

Highly purified endotoxin-reduced (<1 EU/mg) SEB (Toxin Laboratories, Sarasota, FL) was dissolved in sterile endotoxin-free phosphate-buffered saline (PBS) and stored at -80°C. For intranasal challenge, mice were first anesthetized with 2,2,2-tribromoethanol (Avertin, Aldrich Chemical, Milwaukee, WI). The dose of anesthetic agent was adjusted so that the pedal reflexes were totally absent. Subsequently, mice were held in an upright position and 50 μ L of PBS alone or 10 μ g of SEB (unless specifically indicated) dissolved in 50 μ L of PBS was instilled into both the nares. Mice were laid in a supine position until they recovered from anesthesia.

Intraperitoneal superantigen challenge

HLA-DR3 transgenic mice were injected with 10 μ g of SEB dissolved in 200 μ L of PBS or PBS alone. Mice were killed at 6 or 72 h postchallenge, at which time spleen, liver, and lungs were collected for analyses.

Gene expression profiling using microarrays

Gene expression profiling following intranasal SEB exposure was carried out as per standard techniques. Briefly, HLA-DR3 transgenic mice were intranasally challenged with either PBS or 10 μ g of endotoxin-reduced highly purified SEB. Three hours later, mice were killed and total RNA from spleens was extracted using Trizol (Invitrogen Corporation, CA) per the manufacturer's recommendations, followed by purification using Qiagen RNeasy columns (Qiagen Inc., CA). Integrity of RNA was verified by electrophoretic analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples contained 18S and 28S rRNA peaks with no degradation. A minimum of 8 μ g of RNA from each sample was subsequently processed. Synthesis and cleaning of double-stranded cDNA followed by synthesis of biotin-labeled cRNA and fragmentation of biotinylated cRNA into 100- to 150-nt fragments were carried out in the Mayo Clinic Advanced Genomics Technology Center core facility, according to protocols described in the Affymetrix GeneChip Expression Analysis manual (Affymetrix Inc., Santa Clara, CA). For each sample, 15 μ g of fragmented, biotinylated cRNA was hybridized to mouse 430A 2.0 microarray (Affymetrix Inc.), then washed with buffers of varying stringencies, stained with streptavidin-PE and scanned with a Hewlett-Packard GeneArray Scanner (Hewlett-Packard, Santa Clara, CA). The GeneChip Microarray Suite v4.01 software program (Affymetrix Inc.) was then used to generate data for subsequent statistical analysis. The "average difference" in fluorescence between PBS-treated mice (baseline) and SEB-treated mice was determined and tabulated. Microarray data were also analyzed using Ingenuity Pathways Analysis 3.0 (Ingenuity Systems Inc., Redwood City, CA).

Analysis of bronchoalveolar lavage fluid

Experimental animals were injected intraperitoneally with a lethal dose of 2,2,2-tribromoethanol. The trachea was exposed, cannulated with a blunt needle, and the lungs were lavaged twice with 0.5 mL of PBS. Bronchoalveolar lavage

(BAL) fluids were centrifuged and the pelleted cells were counted using a hemocytometer after resuspension in 1 mL of PBS. BAL cell differentials were determined by Wright-Giemsa stain; 200 cells were differentiated using conventional morphologic criteria.

Serum cytokine analysis

PBS- and SEB-challenged mice were bled by tail-bleeding at 4, 8, and 48 h following intranasal challenge and sera separated by centrifugation. Cytokine concentrations in serum were determined using a cytometric bead array, per the manufacturer's protocol (BD Biosciences, San Diego, CA).

T-cell analysis

Mononuclear cells from lymphoid organs were stained with FITC-conjugated anti- $V\beta 6$ or anti- $V\beta 8$, phycoerythrin-conjugated CD4, and PerCP-conjugated CD8. Cells were analyzed by flow cytometry using CellQuest software (BD Biosciences). All antibodies were from BD Pharmingen (San Diego, CA).

Histopathology

After BAL fluid collection, lungs and livers were fixed in 10% formalin and embedded in paraffin. Thin sections were stained with hematoxylin and eosin for histopathologic evaluation.

Statistical analysis

Statistical analysis was performed using GraphPad InStat3 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Gene expression profiling

In the first set of experiments, we studied whether intranasal exposure to SEB can cause systemic immune activation *in vivo*. For this, HLA-DR3 transgenic mice were intranasally challenged with SEB and splenic RNA was analyzed as early as 3 h. Gene expression profiling studies showed that about 1500 genes showed a detectable increase in expression in the SEB-treated over the PBS-treated group. Of these, 850 genes showed more than a 2-fold increase and the remaining 650 genes showed less than a 2-fold increase in the SEB-treated versus the PBS-treated group. Interestingly, genes involved in inflammatory-type immune response such as proinflammatory cytokines, chemokines, chemokine receptors, cytokine/chemokine signaling molecules, as well as genes involved in cell cycle regulation constituted most genes up-regulated in the SEB-treated group. Genes whose expressions were down-regulated at 3 h following intranasal SEB challenge were also analyzed. There were 3 genes whose expression was down-regulated by 20- to 25-fold, 5 genes by 10- to 20-fold, and 19 genes by 5- to 10-fold, with the remainder showing less than 2.5-fold decrease. Only 2 genes of immunologic importance were down-regulated: IL-7 (decreased by 24.4 fold) and IL-16 (decreased by 6.3-fold). Table 1 shows some of the genes of immunologic significance that were up-regulated in SEB-treated in comparison to PBS-treated mice.

Computer-based pathway analysis showed significant changes in expression of multiple components of a variety of signaling pathways of immunologic importance following SEB treatment (Fig. 1). The y axis in 1A represents ratios between the numbers of functions or pathways analysis genes from the data set file over the number of genes involved in a particular canonical pathway. Larger ratios indicate higher numbers of genes regulated within a given pathway. For example, intranasal administration of SEB caused changes in

the expression of 11 of 18 genes involved in IFN signaling. 1B depicts the statistical significance of changes in selected pathways of immunologic importance in SEB- over PBS-treated mice. The s also indicate that many components of these pathways are up-regulated. Overall, the results indicate that intranasal administration of SEB can cause a profound systemic inflammatory response.

Rapid systemic inflammatory response following intranasal SEB administration in HLA class II transgenic mice

Whereas gene expression analysis gives information about the levels of RNA transcripts specific for various cytokines/chemokines, it does not indicate the levels of actual proteins *in vivo*. Therefore, we next studied the levels of selected proinflammatory cytokines/chemokines in sera by multiplex bead assay. HLA-DR3 mice intranasally challenged with SEB but not PBS had a dramatic elevation in several proinflammatory cytokines (i.e., IL-12, TNF- α , IFN- γ , IL-6) as well as macrophage chemoattractant protein (MCP-1) in the serum. B6 mice challenged with same dose of SEB showed little increase in serum cytokine levels (Fig 2). Levels of cytokines were lower at 8 than at 4 h (data not shown). These data indicate a good correlation between gene expression (mRNA) levels and concentration of actual proteins in sera.

When HLA-DR3 transgenic or B6 mice were intranasally challenged with 50 μ g of SEB, 100% of the HLA-DR3 mice (4/4), but none of the B6 mice (0/4), succumbed to SEB-induced toxic shock, further confirming the ability of intranasally delivered SEB to cause systemic immune activation in HLA class II transgenic mice. In another set of experiments, we challenged HLA-DQ8 and B6 mice with SEB intranasally and serum cytokine levels were measured. HLA-DQ8 mice had significantly elevated levels of all cytokines tested including IL-6 ($P < 0.05$, Student *t* test) when compared with B6 mice (data not shown). Taken together, these results established that intranasal administration of SEB can cause massive systemic immune activation in HLA class II transgenic but not B6 mice.

Role of T-cell subsets in systemic inflammatory response following intranasal SEB challenge

SEB, like other superantigens, has the ability to activate both CD4⁺ and CD8⁺ T-cells. Therefore, to specifically address the roles of these T-cells subsets in the systemic immune activation following intranasal superantigen exposure independent of one another, we challenged HLA-DQ8.CD4^{-/-} and HAL-DQ8.CD8^{-/-} mice with SEB and compared the response generated in these mice with that of wild-type HLA-DQ8 mice with intact CD4⁺ and CD8⁺ T-cells. As there was a strong correlation between gene expression profiling and cytometric bead array, we used the later in subsequent experiments for economical reasons.

As in HLA-DR3 mice, intranasal exposure to SEB caused a dramatic systemic inflammatory response in HLA-DQ8 mice, indicating that this effect could be generated across HLA class II mice (Fig 3). However, CD4-deficient DQ8 mice as well as CD8-deficient DQ8 mice had significantly higher serum IL-12 than wild-type DQ8 mice following intranasal

TABLE 1. Gene expression profiling following intranasal exposure to SEB in spleen

Gene	Common Name	Fold Change*	GenBank	Function
Ptgs2	Prostaglandin endoperoxidase synthase 2	729.7	M94967	Prostaglandin synthesis
Irg1	Immunoresponsive gene 1	373.6	L38281	
Ccl11		345.2	NM_011330	Chemokine
Il2	IL-2	168.4	AF065914	Cytokine
Ifng	IFN- γ	151.8	K00083	Cytokine
Cxcl1		151.7	NM_008176	Chemokine
Ccl2		135.1	AF065933	Chemokine
Ccl24		118.5	AF281075	Chemokine
Il6	IL-6	97.47	NM_031168	Cytokine
Cxcl2		96.54	NM_009140	Chemokine
Il1fb	IL-10-related T-cell-derived inducible factor beta	91.37	AJ249492	Cytokine
Cxcl11		81.53	NM_019494	Chemokine
Cxcl10		68.84	NM_021274	Chemokine
Mmp13	Matrix metalloproteinase 3	62.32	NM_008607	Metalloproteinase
Cxcl5		50.26	NM_009141	Chemokine
Cxcl9		43.45	NM_008599	Chemokine
Ccl12		39.53	U50712	Chemokine
Socs1	Suppressor of cytokine signaling 1	35.96	AB000710	Anti-inflammatory
Slamf1	Signaling lymphocytic activation molecule family member 1	30.6	BB132695	Signaling
Ccl3		26.63	NM_011337	Chemokine
Csf3	Colony stimulating factor 3	23.26	NM_009971	Cytokine
Ccl7		21.83	AF128193	Chemokine
Lif	Leukemia inhibitory factor	20.38	AF065917	Cytokine
Il21	IL-21	17.78	NM_021782	Cytokine
Il4	IL-4	13.72	NM_021283	Cytokine
Ccl17		12.02	NM_011332	Chemokine
Ccl22		11.23	BC012658	Chemokine
Ccl1		10.78	NM_011329	Chemokine
Il2ra	IL-2 receptor, alpha	10.65	AF054581	Cytokine
Il1rn	IL-1 receptor antagonist	10.26	M57525	Cytokine
Ccl2		9.518	AJ318863	Chemokine
Ccl4		6.611	AF128218	Chemokine
Cx3cl1		6.177	AF010586	Chemokine
Tnf	Tumor necrosis factor	6.071	NM_013693	Cytokine
Lta	Lymphotoxin alpha	5.645	NM_010735	Cytokine
Cx3cl1		4.766	AF010586	Chemokine
IL-10	IL-10	4.58	NM_010548	Anti-inflammatory
IL-12b	IL-12b	4.1	MM.239707	Cytokine

*Relative fold increase in mRNA levels in SEB- over PBS-treated mice; n = 4.

administration of SEB, suggesting that CD4⁺ and CD8⁺ T-cells inhibit IL-12 production by one another ($P < 0.01$ for DQ8 vs. DQ8.CD8^{-/-} and DQ8.CD4^{-/-} vs. DQ8.CD8^{-/-}; $P < 0.05$ for DQ8 vs. DQ8.CD4^{-/-}, 1-way ANOVA). Unlike HLA-DR3 mice, levels of IL-12 were similar or slightly increased but not decreased at 8 h in comparison to at 4 h in all 3 strains on the HLA-DQ8 background.

With respect to TNF- α , at 4 h, only CD4-deficient DQ8 mice had significantly higher levels than wild-type DQ8 mice

($P < 0.05$, 1-way ANOVA). There were no significant differences in TNF- α between the other groups at 4 h. The levels of TNF- α at 8 h were lower than the levels at 4 h and were comparable in all 3 groups. With respect to IFN- γ , only CD4-deficient DQ8 mice had significantly higher levels than other 2 groups at 4 h ($P < 0.05$, 1-way ANOVA). At 8 h, IFN- γ levels were elevated by nearly 4-fold in CD4-deficient DQ8 mice; IFN- γ levels were elevated in the other 2 groups but not to the extent seen HLA-DQ8.CD4^{-/-} mice. Although the levels of

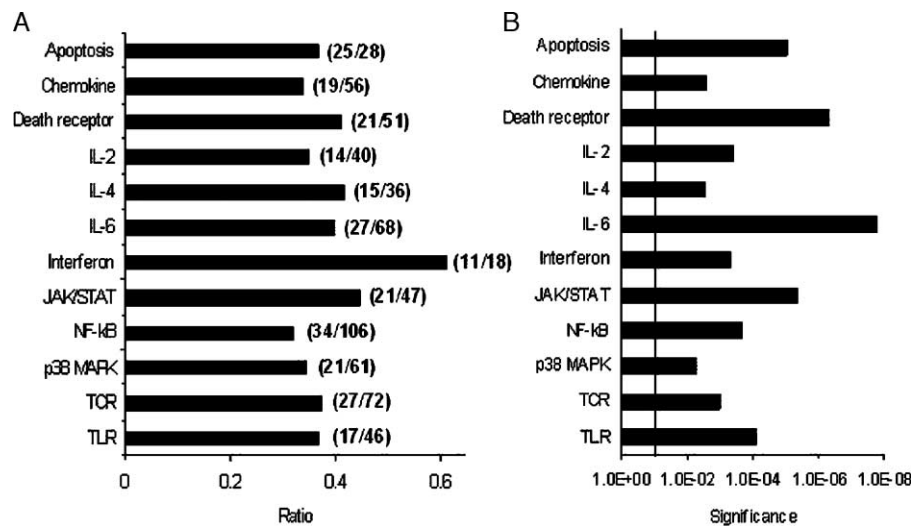


FIG. 1. **Gene expression profiling following intranasal SEB exposure.** Age- and sex-matched HLA-DR3 mice were intranasally challenged with 10 μ g of SEB. Three hours later, splenic RNA was extracted and biotinylated cRNA was hybridized to mouse 430A 2.0 microarray as described in detail in the Materials and methods section. The GeneChip Microarray Suite v4.01 software program (Affymetrix Inc.) was then used to generate data for subsequent statistical analysis. The "average difference" in fluorescence between PBS-treated mice (baseline) and SEB-treated mice was determined and tabulated. Microarray data were also analyzed using Ingenuity Pathways Analysis 3.0 (Ingenuity Systems Inc.). A, x axis represents a ratio between the number of functions/pathways analysis genes from the data set file over the number genes involved in a particular canonical pathway. B, x axis represents *P* values for each pathways in SEB- over PBS-treated mice.

MCP-1 were comparable between the 3 lines at 4 h ($P > 0.05$), they remained elevated in HLA-DQ8.CD4^{-/-} mice at 8 h whereas they fell dramatically in the other 2 lines. With respect to IL-6, CD4-deficient DQ8 mice had significantly higher levels at 4 h ($P < 0.05$, 1-way ANOVA), whereas at 8 h IL-6 levels fell significantly. Together, these data suggest that CD4⁺ and CD8⁺ T-cells can independently contribute to superantigen-induced cytokine production and that these 2 subsets regulate cytokine production by one another in a complex manner. Overall, although the levels of TNF- α tended to decrease with time, IFN- γ levels increased with time.

Role of IL-4 and IL-12 signaling in superantigen-induced cytokine production

Superantigen-induced toxic shock is generally considered as a T_H1-type response, and TNF- α is believed to be the key

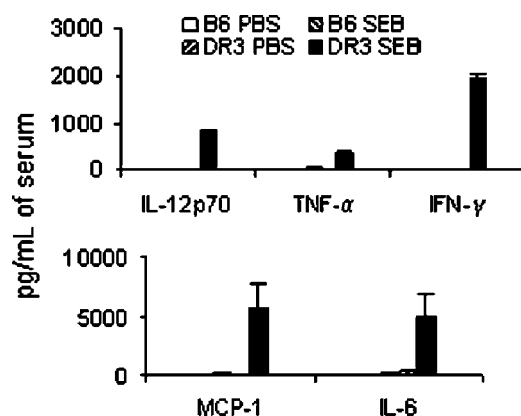


FIG. 2. **Systemic inflammatory response following intranasal exposure to SEB.** C57BL/6 and HLA-DR3 mice were intranasally challenged with SEB (10 μ g). Serum cytokines levels at 4 h postchallenge were measured by cytometric bead assay. PBS-challenged mice had less than 20 pg/mL of IL-12, TNF- α , and IFN- γ . Bars represent mean $\pm$ SEM from 3 (PBS group) to 4 (SEB) mice/group.

cytokine involved in its pathogenesis (8). To identify the contribution of IL-12 and IL-4 signaling components to superantigen-induced systemic cytokine release, DQ8 transgenic mice as well as DQ8 mice deficient in STAT4 (i.e., defective IL-12 signaling) or STAT6 (i.e., defective IL-4 signaling) were challenged with 10 μ g of SEB intranasally and serum cytokines measured 4 and 8 h postchallenge. As depicted in Fig 4, DQ8, STAT4-deficient DQ8, as well as STAT6-deficient DQ8 mice had comparable levels of all cytokines tested except IFN- γ at 4 h. STAT4-deficient mice had the lowest levels of IFN- γ , whereas STAT6-deficient mice had the

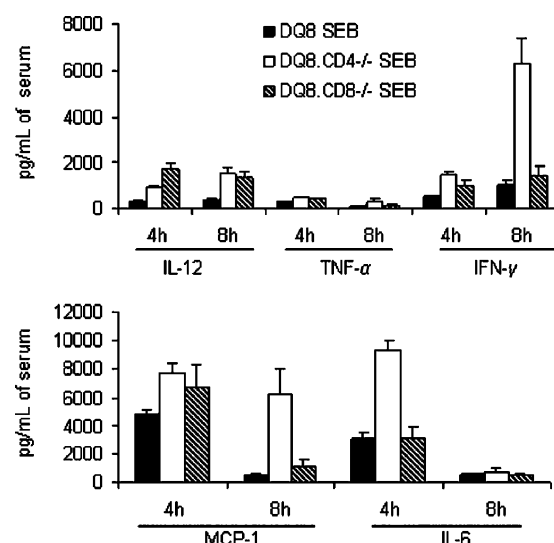


FIG. 3. **Role of CD4⁺ and CD8⁺ T-cells in systemic inflammatory response following intranasal exposure to SEB.** HLA-DQ8 transgenic mice and CD4^{-/-} and CD8^{-/-} DQ8 mice were challenged with intranasal SEB. Serum cytokines levels at 4 and 8 h postchallenge were measured by cytometric bead assay. Bars represent mean $\pm$ SEM from at least 3 mice/group.

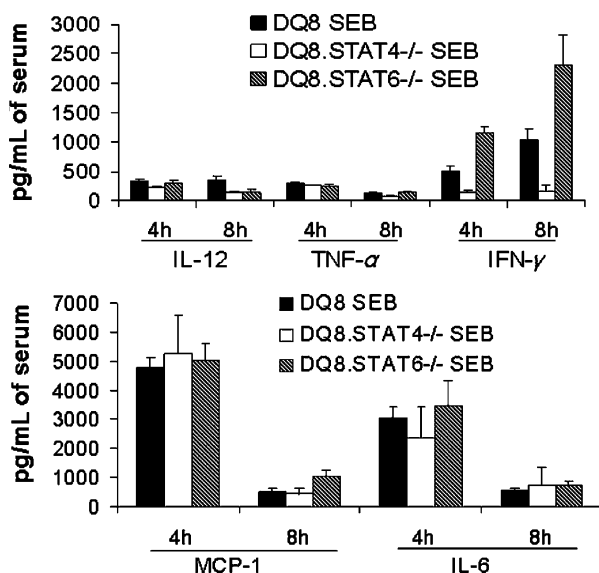


FIG. 4. Role of STAT4 and STAT6 in systemic inflammatory response following intranasal exposure to SEB. HLA-DQ8 transgenic mice and STAT4- and STAT6-deficient DQ8 mice were intranasally challenged with SEB. Serum cytokine levels at 4 and 8 h postchallenge were measured by cytometric bead assay. Bars represent mean $\pm$ SEM from at least 3 mice/group.

highest levels of IFN- γ at 4 h ($P < 0.001$ for DQ8.STAT4^{-/-} vs. DQ8.STAT6^{-/-} and DQ8 vs. DQ8.STAT6^{-/-} groups; $P < 0.01$ for DQ8.STAT4^{-/-} vs. DQ8, 1-way ANOVA). At 8 h, the levels of TNF- α , MCP-1, and IL-6 were lower than at 4 h in all the 3 lines. However, the levels of IFN- γ were significantly higher in both HLA-DQ8 and DQ8.STAT6^{-/-}, being highest in the latter. These data suggest that SEB-induced IFN- γ production is dependent on a functional IL-12 signaling cascade. Moreover, when the IL-4 signaling cascade is defective, elevated IFN- γ levels were observed indicating an inhibitory effect of IL-4 on IFN- γ production. STAT6-deficient DQ8 and wild-type DQ8 but not STAT4-deficient DQ8 mice seemed sick following intranasal SEB challenge as

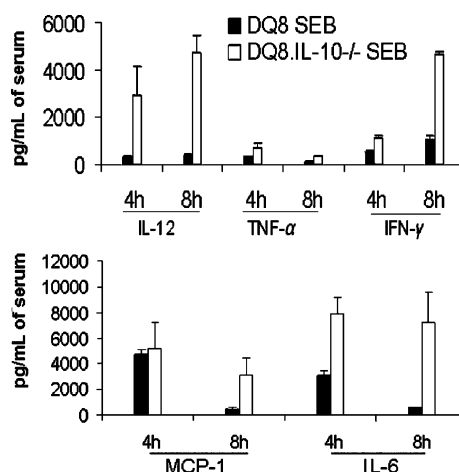


FIG. 5. Role of IL-10 in systemic inflammatory response following intranasal exposure to SEB. HLA-DQ8 transgenic mice and IL-10-deficient HLA-DQ8 mice were intranasally challenged with SEB. Serum cytokines levels at 4 and 8 h postchallenge were measured by cytometric bead assay. Bars represent mean $\pm$ SEM from at least 3 mice/group.

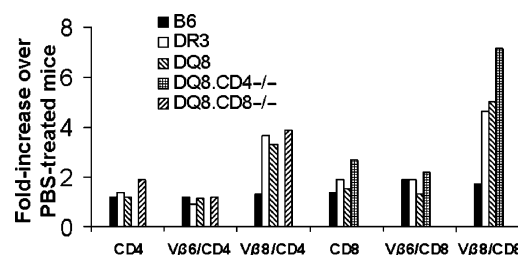


FIG. 6. Splenic T-cell expansion following intranasal SEB exposure. C57BL/6 and indicated HLA class II transgenic mice were challenged with SEB. Mice were killed 48 h later, and the distribution of CD4⁺ as well as CD8⁺ T-cells expressing indicated TCR V β or V β 8 was analyzed by flow cytometry. Bars represent Mean $\pm$ SD from 3 to 5 mice/group.

evidenced by ruffled fur, hunched backs, and listlessness (data not shown).

Role of IL-10 in systemic inflammatory response induced by SEB delivered through the intranasal route

IL-10 is a potent anti-inflammatory cytokine and a strong inhibitor of inflammatory cytokines (18). Targeted inactivation of the *IL-10* gene in mice generally exaggerates the inflammatory status (18). Therefore, we studied the role of IL-10 in the systemic immune response induced by intranasal SEB. IL-10-deficient HLA-DQ8 mice seemed to be sick following intranasal SEB challenge as evidenced by ruffled fur, hunched backs, and listlessness. As shown in Fig. 5, the levels of IL-12, IFN- γ , and IL-6 were significantly elevated at both 4 and 8 h ($P < 0.05$, Student *t* test) in IL-10-deficient HLA-DQ8 in comparison to wild-type HLA-DQ8 mice. Whereas IL-10-deficient HLA-DQ8 showed a trend toward increased TNF- α production ($P = 0.06$), serum levels of MCP-1 were comparable between both groups at 4 h. Overall, these results indicate that in the IL-10-deficient state cytokine levels, with the exception of TNF- α , remained significantly elevated. When wild-type or IL-10-deficient HLA-DQ8 mice were challenged with 50 μ g of SEB, all 4 IL-10-deficient and none of the 4 wild-type HLA-DQ8 mice tested succumbed to SEB-induced toxic shock.

Peripheral T-cell expansion following intranasal SEB challenge

Forty-eight hours after intranasal challenge with SEB, spleens were harvested and the distribution of TCR V β 8⁺ (SEB-reactive) and TCR V β 6⁺ (SEB-nonreactive) T-cells was

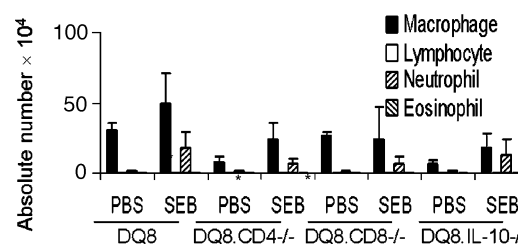


FIG. 7. Analysis of BAL fluid following intranasal SEB exposure. PBS- or SEB-challenged mice were killed 48 h later and BAL fluid collected. Giemsa-stained cytospin preparations from BAL fluid were microscopically evaluated for the distribution of different leukocyte subsets. Bars represent mean $\pm$ SD from 3 to 5 mice/group. * $P < 0.05$ when compared with respective PBS-treated groups.

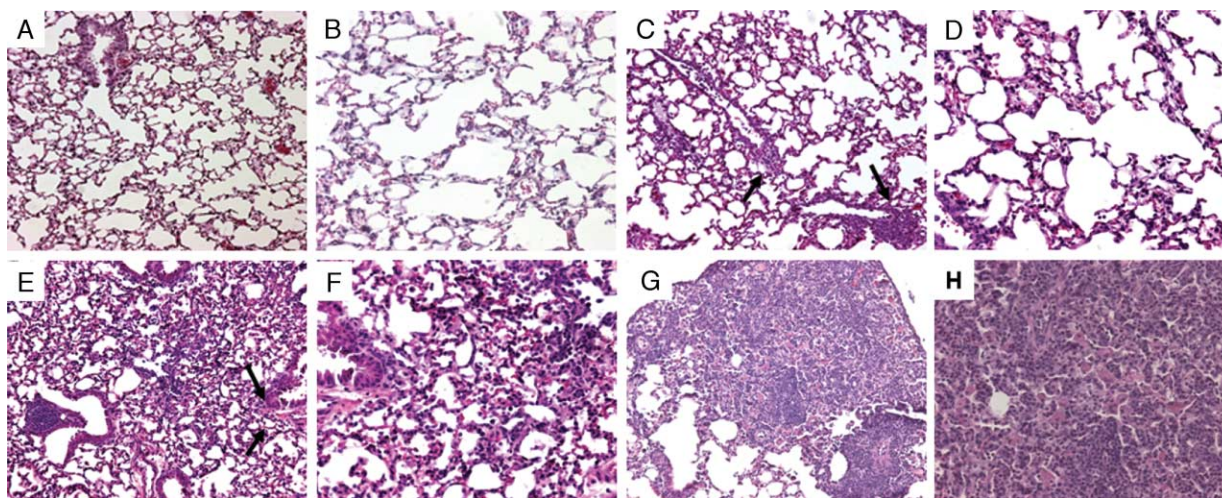


FIG. 8. **Lung inflammation following intranasal SEB challenge.** PBS- or SEB-challenged mice were killed 48 h later and lungs were collected in buffered formalin after harvesting BAL fluid. Representative hematoxylin-and-eosin-stained sections from different groups of mice are given. A and B, PBS-challenged HLA-DQ8 mice; C and D, SEB-challenged B6 mice; E and H, SEB-challenged DQ8 mice. Left panels, lower magnification; right panels, higher magnification.

analyzed by flow cytometry. As expected, there was less than 2-fold expansion in $CD4^+$ and $CD8^+$ T-cells bearing TCR $V\beta 8$ in spleens of SEB-treated B6 mice (Fig 6). All the HLA class II transgenic mice challenged with SEB demonstrated a 4- to 7-fold increase in TCR $V\beta 8^+$ in both $CD4^+$ as well as $CD8^+$ subsets, whereas levels of SEB-nonreactive TCR $V\beta 6^+$ T-cells were comparable between all the groups. These results further confirm that exposure to SEB through the intranasal route can induce strong systemic immune activation.

BAL and lung histopathology analysis following intranasal SEB challenge

Mice were challenged with SEB, and 48 h later BAL samples were collected for analysis. Lung tissues were also collected for histopathologic analysis. Intranasal administration of SEB caused a significant increase in neutrophils in BAL fluid ($P < 0.05$) in all the mice tested when compared with respective PBS-treated groups, whereas PBS-challenged mice had only macrophages (Fig. 7). Other inflammatory cells were undetectable in the BAL fluid of SEB-challenged mice; there were no significant differences in macrophage or neutrophil counts between different lines of mice tested.

Histopathologic analysis of lung tissue showed normal lung architecture in PBS-challenged mice (Fig 8A and B). Whereas B6 mice challenged with SEB showed minimal perivascular leukocytic infiltration as indicated by arrows (Fig. 8, C and D), HLA class II transgenic mice challenged with SEB had marked perivascular infiltration and interstitial inflammation (Fig. 8, E and F). Prominent lymphoid accumulation near the bronchi was also evident in lung sections from SEB-treated but not PBS-treated mice indicative of proliferation of lymphocytes within the bronchus-associated lymphoid tissue (indicated by arrows) following SEB administration (Fig. 8E). Interestingly, closer examination of the lung sections from SEB challenged HLA transgenic mice revealed areas of heavy inflammation interspersed with areas with minimal inflammatory changes (Fig. 8, G and F). We did not find striking differences in the extent of inflammation between the differ-

ent knockout mice tested. We also examined the livers from mice intranasally treated with SEB. Whereas livers from PBS-treated mice seemed normal (Fig. 9A), marked perivascular leukocytic infiltration was present in the liver of SEB-treated HLA class II transgenic mice (Fig. 9B).

As evident from the above results, intranasal delivery of SEB caused lung inflammation as well as systemic immune activation. We next wanted to study whether systemic administration of SEB could also cause lung inflammation. For this, HLA-DR3 mice were challenged intraperitoneally with SEB and killed at 6 or 72 h later. As depicted in 10, DR3 mice challenged systemically with SEB had marked inflammation of the lung both at 6 h (Fig 10, A and B) as well as 72 h (Fig 10, C and D).

DISCUSSION

Staphylococcal superantigens are potent mitogens, especially to human T-cells, and have been implicated in a spectrum of diseases ranging from food poisoning to toxic shock (1). The enterotoxigenic staphylococcal superantigens including SEB are absorbed through gastrointestinal mucosa and are associated with food poisoning (8). Toxic shock syndrome toxin-1 is the only staphylococcal superantigen exotoxin that has been shown to be absorbed through nonenteric (i.e., vaginal) mucosa causing menstrual toxic shock (8). Irrespective of the route of exposure, superantigen-induced toxic shock shares similar etiopathogenesis, namely, a profound systemic inflammatory response and multiorgan failure (8).

The ability of SEB to cause systemic immune activation following exposure through nonenteric mucous membranes, especially through the nasal tract, has not been previously demonstrated. This has immense clinical significance, as the nasal passage is the most common site of staphylococcal colonization. Since *S. aureus* in the "carrier state" or in the "disease state" can elaborate a variety of superantigens (4, 19), there is a potential possibility that the nasal passage may be exposed to SEB. Moreover, packing materials, stents,

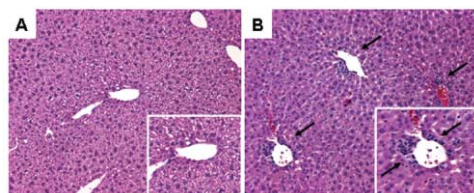


FIG. 9. **Liver involvement following intranasal SEB challenge.** PBS- or SEB-challenged mice were killed 48 h later and liver sections were collected in buffered formalin. Representative hematoxylin-and-eosin-stained sections from different groups of mice are shown. A, PBS-challenged HLA-DQ8 mice; B, SEB-challenged HLA-DQ8 mice. Inserts show higher magnification.

and splints that are widely used in endonasal surgical procedures can also support the growth of *S. aureus* capable of elaborating SEB. Interestingly, toxic shock complicating endonasal surgery involving the use of these abovementioned materials is widely reported (6, 7). There are also clinical observations suggesting that staphylococcal toxic shock syndrome may complicate upper or lower respiratory tract infection (20, 21). Nevertheless, there is no direct evidence to show that intranasal exposure to staphylococcal superantigenic exotoxins can cause toxic shock. To our knowledge, this is the first study to report that intranasal exposure to SEB elicits robust systemic immune activation characterized by a systemic cytokine storm and splenic T-cell expansion.

HLA class II transgenic mice but not C57BL/6 mice exposed to SEB through the intranasal route mounted a robust systemic immune response. Intraperitoneal as well as intravenous routes of superantigen administration also elicit a robust immune response in HLA class II transgenic mice (13, 14). However, the current work focuses on the outcome following intranasal SEB administration. Among the HLA class II transgenic mice tested, the cytokine response was more pronounced in HLA-DR3 than in HLA-DQ8 transgenic mice. Differential interaction of SEB with HLA-DR and HLA-DQ might explain this observation. It is accepted in the superantigen field that superantigens differentially interact with different HLA class II molecules. It is established that HLA-DR is more efficient than HLA-DQ at presenting SEB (22). On the other hand, some super-

antigens, such as streptococcus pyrogenic exotoxin A, are presented very efficiently by HLA-DQ but not HLA-DR molecules (23). Therefore, heightened inflammatory response to SEB in HLA-DR transgenic mice is not unexpected. This might also explain why the levels of IL-12 and IFN- γ in HLA-DR3 mice were lower at 8 h than at 4 h, whereas in HLA-DQ8 mice the reverse was observed. Since HLA-DR3 is more efficient than HLA-DQ8 at presenting SEB, T-cells in HLA-DR3 transgenic mice could have attained maximal stimulation earlier resulting in higher levels of cytokines at earlier time points.

Gene expression profiling showed a significant increase in transcripts encoding several pro-inflammatory cytokines and chemokines in SEB-treated mice as early as 3 h following intranasal SEB exposure. This was accompanied by a dramatic elevation of the actual cytokines in the sera. This effect was observed in HLA class II transgenic, but not conventional mice (C57BL/6). Pathway analysis using a Web-based tool developed by Ingenuity Systems Inc. showed that not only were the transcripts encoding for proinflammatory cytokines (such as IL-2, IL-6, IFN- γ) and several chemokines up-regulated, but transcripts for their receptors, proteins involved in their signal transduction, nuclear factors, proteins involved in cell cycle regulation etc., were all also regulated (Supplementary figures). At the same time, transcripts encoding various anti-inflammatory factors such as IL-4, IL-10, and their receptors, suppressor of cytokine signaling, were also up-regulated during this systemic inflammatory response. This highlights the natural response to counteract the ongoing inflammatory response with these anti-inflammatory proteins and pathways. Heightened susceptibility of IL-10-deficient HLA-DQ8 transgenic mice to SEB-induced toxic shock underscores the importance of endogenous anti-inflammatory pathways.

IL-7 and IL-16 showed reduction in expression levels. IL-7 is mainly required for thymocytes and naive T-cells survival. The current understanding is that IL-7 plays an important role in thymic T-cell lymphopoiesis and peripheral T-cell homeostasis (24). Some reports have suggested that IL-7 can also prevent apoptosis of activated T-cells. Because the robust superantigen-induced activation of mature T-cells in the periphery is always followed by massive apoptosis (25) (as also indicated by pathway analysis), it is conceivable that IL-7, as an antiapoptotic factor, may be down-regulated. Interestingly, massive thymocyte apoptosis is also a hallmark of superantigen-induced immune activation (13, 14). Although we did not measure thymic IL-7 levels, it is possible that thymic levels of IL-7 are also reduced in such instances contributing to thymic apoptosis.

IL-16 is a chemokine for CD4⁺ T lymphocytes believed to play a role in allergic diseases such as asthma and atopic dermatitis (26). Expression and secretion of IL-16 in the context of systemic inflammatory response has not been previously reported. Further studies are needed to address its relevance in intranasal SEB-induced systemic immune activation.

Gene expression profiling showed that prostaglandin endoperoxide synthase (PTGS) 2 [also called as cyclooxygenase

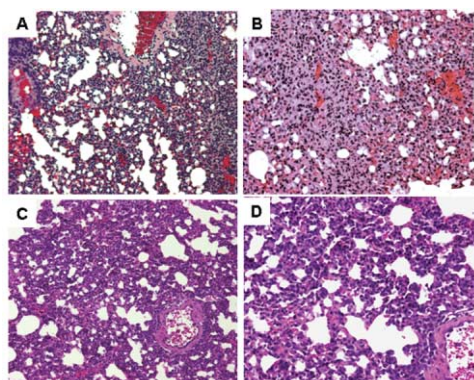


FIG. 10. **Lung inflammation following systemic SEB challenge.** SEB-challenged HLA-DR3 mice were killed 6 or 72 h later and lungs were collected in buffered formalin. Representative hematoxylin-and-eosin-stained sections are given. A and B, 6 h postchallenge; C and D, 72 h postchallenge. Left panels, lower magnification; right panels, higher magnification.

(cox)-2] was up-regulated by nearly 750-fold in SEB-treated mice in comparison to PBS-treated mice. PTGS2 is involved in prostaglandin biosynthesis, which is a very potent mediator of inflammation. Various proinflammatory mediators such as CCL2, CXCL1, CXCL2, CXCL10, IL-2, IL-6, IFN- γ , and TNF- α that are up-regulated in mice exposed to SEB can induce PTGS2 expression. The role of PTGS2 in endotoxic shock and sepsis-related shock is being realized (27, 28). Our study suggests that PTGS2 might also play a role in superantigen-induced toxic shock and that drugs that specifically block the PTGS2 pathway could play a beneficial role in systemic inflammatory response induced by intranasal SEB. We are currently evaluating the therapeutic role of cox-2 inhibitors in superantigen-induced toxic shock using the HLA class II transgenic mouse models.

Although superantigens do not bind directly to toll-like receptors (TLR) unlike other microbial derivatives such as bacterial double stranded DNA containing CpG motifs, lipopolysaccharide, etc., recent studies have shown that bacterial superantigens can up-regulate the expression of TLR on human monocytes (29). Gene expression profiling indicated that transcripts coding for certain TLR as well as signaling molecules involved in TLR pathway were up-regulated following intranasal SEB exposure (Supplementary Fig. 5). Although the significance of this process is currently not known, bacterial superantigens could potentially sensitize the host to other microbial derivatives by this process.

Superantigens bind to MHC class II molecules and activate both CD4⁺ and CD8⁺ T-cells. However, the outcome of activation of these 2 subsets would be expected to be different as they perform distinct functions in vivo. The consensus is that CD4⁺ T-cells are "helper" cells where as CD8⁺ T-cells are "cytotoxic" cells. The cytokine profile of these 2 subsets is also different (30). The independent roles played by these 2 subsets in superantigen-induced immune activation, especially following intranasal exposure, have not been studied. We have shown that CD4⁺ and CD8⁺ T-cells can independently contribute to the systemic inflammatory response following intranasal exposure to SEB.

CD4⁺ T helper cells are functionally divided at least in to 2 types, T_H1 and T_H2, based on their cytokine production. IL-12 is a key cytokine that drives the production of T_H1 cytokines such as IFN- γ by T_H1 cells. T_H2 cells primarily produce IL-4. Both IL-12 and IL-4 reciprocally inhibit the synthesis and function of each other (31). Since superantigens activate T-cells directly by interacting with their TCR, superantigens should theoretically be able to activate both T_H1 as well as T_H2 cell types if these cell types express appropriate TCR V β elements. Because IL-12 and IFN- γ levels are significantly elevated during superantigen-induced toxic shock, it is considered to be a T_H1-mediated disease (1, 32). Nonetheless, only TNF- α is considered a key cytokine in the pathogenesis of toxic shock and the roles of IL-12 and IL-4 signaling pathways in the pathogenesis of superantigen-induced systemic inflammatory response have not been previously reported.

We have shown that mice with defective IL-4 signaling (i.e., STAT6^{-/-} mice) have significantly elevated serum IFN- γ

while, whereas mice with defective IL-12 signaling (i.e., STAT4^{-/-} mice) have lower IFN- γ levels when compared with wild-type HLA class II transgenic mice following intranasal exposure to SEB. Similar results have been reported in a murine model of plague (33). Interestingly, levels of TNF- α were comparable between STAT4- and STAT6-knockout mice in our studies. We have observed in other experiments that repeated intranasal challenge with lower concentrations of SEB results in higher mortality in wild-type as well as STAT6-deficient HLA-DQ8 mice, whereas a significantly lower mortality is observed in STAT4-deficient HLA-DQ8 mice under same conditions (Rajagopalan et al., unpublished data). Other studies have shown that STAT4-deficient mice are protected from endotoxin-induced shock (34) as well as from systemic inflammatory response following IL-12 injection (35).

Considering these factors along with the finding that TNF- α levels were comparable between the 3 strains studied and that IFN- γ was increased and decreased in STAT6 and STAT4-deficient DQ8 mice, respectively, we suggest that IFN- γ might play a major role in the pathogenesis of superantigen-induced toxic shock. The other consistent observation was that levels of TNF- α tended to decrease with time whereas levels of IFN- γ continued to rise between 4 and 8 h, except in HLA-DR3 mice. This cytokine pattern further underscores our hypothesis that IFN- γ but not TNF- α may play a detrimental role in toxic shock caused by SEB. Gene expression profiling also strongly supports our hypothesis. Whereas IFN- γ transcripts were increased by 150-fold, TNF- α transcripts were increased only by 6-fold. This indicates that therapeutic use of soluble TNF- α receptors may be ineffective in clinical settings of superantigen-induced toxic shock. Therapeutic interventions designed to block production or functioning of IL-12 and/or IFN- γ may be beneficial after acute airway exposure to SEB or other systemic response to superantigens.

Histopathologic studies did not reveal significant changes in lung pathology. However, it should be noted that histology was performed at only 1 time point, 48 h. A detailed time course study is required to address histopathologic changes in the context of cytokine levels. We are also currently performing studies to measure the functional status of major organs such as lungs, livers, and kidneys to identify the events that lead to multiple organ failure and death, the hallmarks of human toxic shock syndrome.

In conclusion, we have shown that exposure to SEB through nasal route can result in massive systemic immune activation characterized by production of several proinflammatory mediators.

ACKNOWLEDGMENTS

The authors thank Julie Hanson and her crew for excellent mice husbandry and Michelle Smart for characterizing the transgenic mice.

REFERENCES

1. Proft T, Fraser JD: Bacterial superantigens. *Clin Exp Immunol* 133: 299–306, 2003.
2. Chambers HF: The changing epidemiology of *Staphylococcus aureus*? *Emerg Infect Dis* 7:178–182, 2001.

3. Bisno AL, Brito MO, Collins CM: Molecular basis of group A streptococcal virulence. *Lancet Infect Dis* 3:191–200, 2003.
4. Becker K, Friedrich AW, Lubritz G, Weiler M, Peters G, von Eiff C: Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J Clin Microbiol* 41:1434–1439, 2003.
5. Bachert C, Gevaert P, van Cauwenberge P: *Staphylococcus aureus* enterotoxins: a key in airway disease? *Allergy* 57:480–487, 2002.
6. Wagner R, Toback JM: Toxic shock syndrome following septoplasty using plastic septal splints. *Laryngoscope* 96:609–610, 1986.
7. Abram AC, Bellian KT, Giles WJ, Gross CW: Toxic shock syndrome after functional endonasal sinus surgery: an all or none phenomenon? *Laryngoscope* 104:927–931, 1994.
8. McCormick JK, Yarwood JM, Schlievert PM: Toxic shock syndrome and bacterial superantigens: an update. *Annu Rev Microbiol* 55:77–104, 2001.
9. Dohlsten M, Björklund M, Sundstedt A, Hedlund G, Samson D, Kalland T: Immunopharmacology of the superantigen staphylococcal enterotoxin A in T-cell receptor V beta 3 transgenic mice. *Immunology* 79:520–527, 1993.
10. Dalpke AH, Heeg K: Synergistic and antagonistic interactions between LPS and superantigens. *J Endotoxin Res* 9:51–54, 2003.
11. Silverstein R: D-Galactosamine lethality model: scope and limitations. *J Endotoxin Res* 10:147–162, 2004.
12. Yeung RS, Penninger JM, Kundig T, Khoo W, Ohashi PS, Kroemer G, Mak TW: Human CD4 and human major histocompatibility complex class II (DQ6) transgenic mice: supersensitivity to superantigen-induced septic shock. *Eur J Immunol* 26:1074–1082, 1996.
13. Rajagopalan G, Sen M, David CS: In vitro and in vivo evaluation of staphylococcal superantigen peptide antagonists. *Infect Immun* 72:6733–6737, 2004.
14. Rajagopalan G, Singh M, Sen M, Murali N, Nath KA, David CS: Endogenous superantigens shape response to exogenous superantigens. *Clin Diagn Lab Immunol* 12:1119–1122, 2005.
15. Welcher BC, Carra JH, DaL Silva J, Hanson CS, David MJ, Aman S: Lethal shock induced by streptococcal pyrogenic exotoxin A in mice transgenic for human leukocyte antigen-DQ8 and human CD4 receptors: implications for development of vaccines and therapeutics. *J Infect Dis* 186:501–510, 2002.
16. Taneja V, Taneja N, Paisansinsup T, Behrens M, Griffiths M, Luthra H, David CS: CD4 and CD8 T cells in susceptibility/protection to collagen-induced arthritis in HLA-DQ8-transgenic mice: implications for rheumatoid arthritis. *J Immunol* 168:5867–5875, 2002.
17. Taneja V, David CS: HLA class II transgenic mice as models of human diseases. *Immunol Rev* 169:67–79, 1999.
18. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A: Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683–765, 2001.
19. Kluytmans J, van Belkum A, Verbrugh H: Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* 10:505–520, 1997.
20. MacDonald KL, Osterholm MT, Hedberg CW, Schrock CG, Peterson GF, Jentzen JM, Leonard SA, Schlievert PM: Toxic shock syndrome. A newly recognized complication of influenza and influenzalike illness. *JAMA* 257:1053–1058, 1987.
21. Micek ST, Dunne M, Kollef MH: Pleuropulmonary complications of Pantone-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus*: importance of treatment with antimicrobials inhibiting exotoxin production. *Chest* 128:2732–2738, 2005.
22. Jardetzky TS, Brown JH, Gorga JC, Stern LJ, Urban RG, Chi YI, Stauffacher C, Strominger JL, Wiley DC: Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature* 368:711–718, 1994.
23. Norrby-Teglund A, Nepom GT, Kotb M: Differential presentation of group A streptococcal superantigens by HLA class II DQ and DR alleles. *Eur J Immunol* 32:2570–2577, 2002.
24. Fry TJ, Mackall CL: The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J Immunol* 174:6571–6576, 2005.
25. Krakauer T: Immune response to staphylococcal superantigens. *Immunol Res* 20:163–173, 1999.
26. Conti P, Kempuraj D, Kandere K, Di Gioacchino M, Reale M, Barbacane RC, Castellani ML, Mortari U, Boucher W, Letourneau R: Interleukin-16 network in inflammation and allergy. *Allergy Asthma Proc* 23:103–108, 2002.
27. Suliburk JW, Helmer KS, Gonzalez EA, Robinson EK, Mercer DW: Ketamine attenuates liver injury attributed to endotoxemia: role of cyclooxygenase-2. *Surgery* 138:134–140, 2005.
28. Viridis A, Colucci R, Fomai M, Blandizzi C, Duranti E, Pinto S, Bernardini N, Segnani C, Antonioli L, Taddei S: Cyclooxygenase-2 inhibition improves vascular endothelial dysfunction in a rat model of endotoxic shock: role of inducible nitric-oxide synthase and oxidative stress. *J Pharmacol Exp Ther* 312:945–953, 2005.
29. Hopkins PA, Fraser JD, Pridmore AC, Russell HH, Read RC, Sriskandan S: Superantigen recognition by HLA class II on monocytes up-regulates toll-like receptor 4 and enhances proinflammatory responses to endotoxin. *Blood* 105:3655–3662, 2005.
30. Woodland DL, Dutton RW: Heterogeneity of CD4(+) and CD8(+) T cells. *Curr Opin Immunol* 15:336–342, 2003.
31. Constant SL, Bottomly K: Induction of T_H1 and T_H2 CD4 + T cell responses: the alternative approaches. *Annu Rev Immunol* 15:297–322, 1997.
32. Florquin S, Goldman M: Immunoregulatory mechanisms of T-cell-dependent shock induced by a bacterial superantigen in mice. *Infect Immun* 64:3443–3445, 1996.
33. Elvin SJ, Williamson ED: Stat 4 but not Stat 6 mediated immune mechanisms are essential in protection against plague. *Microb Pathog* 37:177–184, 2004.
34. Kamezaki K, Shimoda K, Numata A, Matsuda T, Nakayama K-I, Harada M: The role of Tyk2, Stat1 and Stat4 in LPS-induced endotoxin signals. *Int Immunol* 16:1173–1179, 2004.
35. Biber JL, Jabbour S, Parihar R, Dierksheide J, Hu Y, Baumann H, Bouchard P, Caligiuri MA, Carson W: Administration of 2 macrophage-derived interferon-[gamma]-inducing factors (IL-12 and IL-15) induces a lethal systemic inflammatory response in mice that is dependent on natural killer cells but does not require interferon-[gamma]. *Cell Immunol* 216:31–42, 2002.

