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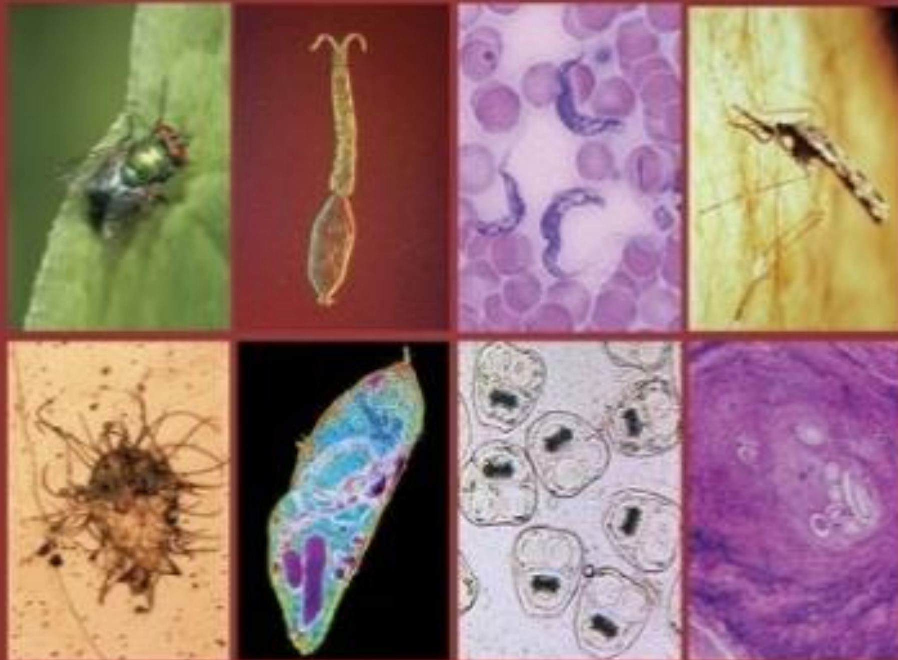

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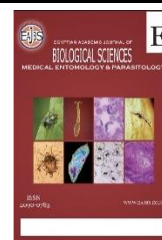

Bacterial Superantigens as An Agent Provocateur to Initiating Chaos in Immune Response

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ABSTRACT

Environmental agents including bacterial superantigens (sAgs) have been shown to contribute to immune response dysregulation, resulting in immunity-related diseases. Staphylococcal enterotoxin B (SEB) is one among the several powerful exotoxins of *Staphylococcus aureus*, causing a range of clinical diseases from mild food poisoning to deadly non-menstrual toxic shock. *Streptococcus pyogenes* infections in the deep tissues can create equally potent sAgs (such as streptococcal pyrogenic exotoxin (SPE) -A) that can cause deadly shock. The innate immune response elicited by sAgs is not against the bacteria themselves; rather sAgs driven T cell and cytokine-based immune response aids bacterial survival.

The current study looked at how healthy people's T cells reacted *in vitro* to common bacterial sAgs such as SEB and SPE-A. Different Th1 and Th2 cytokines were measured to identify the functional activity of T cells. The findings show that the interaction between PBMC and sAgs has a significant impact on the magnitude of sAg-dependent polyclonal T cell ($CD4^+$) elicitation along with cytokine generation *in vitro*, which is governed by IL-4 and IL-10 releasing cells. Infection-related sAg-activity possibly influences the clinical outcome of the infection, implying that sAg-activated T cells and cytokines play a crucial role in initiating immunity-related diseases.

INTRODUCTION

Staphylococcus aureus and *Streptococcus pyogenes* produce superantigens (sAgs) which are very strong immunogens (Fraser and Proft 2008). Among them, Staphylococcal enterotoxin B (SEB) produced by *Staphylococcus aureus* (Dinges *et al.,* 2000) may result in mild to deadly toxic shock (Dinges et al., 2000; Ferry *et al.,* 2008). SEB is one of the most important virulence factors among the staphylococcal infections caused by antibiotic-resistant strains (Fey *et al.,* 2003)(Kashiwada *et al.,* 2012), which is fast becoming a major public health issue. *Streptococcus pyogenes* (group A-hemolytic Streptococcus) infections in the deep tissues can create equally potent sAgs capable of producing fatal shock (Schmid 2008). Streptococcal pyrogenic exotoxins (SPE) -A, -B, and -C, as well as a variety of other pyogenic toxins, are produced by invasive group A streptococcal strains.

SPE-A is linked to streptococcal toxic shock syndrome in particular (Silversides *et al.,* 2010). Both staphylococcal and streptococcal sAgs have been shown to behave similarly.

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To combat the toxicity and fatality of bacterial sAgs, many preventive and therapeutic interventions are explored in preclinical research. Passive injection of neutralizing antibodies (Hamad *et al.,* 1994), toxoid vaccinations (Boles *et al.,* 2003), recombinant sAg mutants with reduced immunotoxicity (Coffman *et al.,* 2002; Stiles *et al.,* 2001), and designed highly effective inhibitors (Buonpane *et al.,* 2007; Yang *et al.,* 2008) are some of these options. However, no effective therapies or vaccinations against sAgs have yet been licensed for human use.

Bacterial surface antigens have been shown to contribute to immune response dysregulation, resulting in autoimmunity or immunodeficiency (Schiffenbauer *et al.,* 1998). They are strong $CD4^+$ T cell activators, capable of activating a substantial proportion of circulating T cells independent of a specific antigen, resulting in considerable cytokine release into the circulation. sAgs are thought to alter the course of autoimmune illnesses by either beginning the immunological response or provoking a relapse in someone who is in clinical remission. The stimulation of autoreactive T/B cells or the activation of antigen-presenting cells is among the two putative ways that these sAgs initiate autoimmune processes. The sAgs can trigger autoreactive T cells, causing autoimmune damage via cytokine release or cytotoxic processes (Swedo 2001). Autoreactive B cells can be activated by MHC-II sites on B cells or by sAg-mediated macrophage activation, which releases cytokines and causes inflammation. The sAg triggers an immune response through a non-specific T cell and cytokine-mediated immune response which contribute to the bacterial survival rather than clearing it from the system. Among many cytokines secreted, the excessive production of three T cell cytokines - IL-2, IFN-γ, and TNF-α is implicated in acute injury (Bette *et al.,* 1993; Miethke *et al.,* 1993).

Autoimmune reactions and tissue damage are caused by molecular mimicry between sAgs and particular proteins in the body, including pro-inflammatory cytokines and reduced IL-4 secretion (Fae *et al.,* 2005; Guilherme *et al.,* 2007; Guilherme and Kalil 2010).

Invading bacteria and their hosts can resemble one other's immune determinants or epitopes, resulting in an immune response that recognizes both the microbial determinant and the shared host self-antigen. There is evidence that many autoimmune illnesses are linked to bacterial infections that stimulate the creation of antibodies and immune cells targeted against bacterial proteins that are very similar to host self-proteins, resulting in cross-reactivity with healthy tissues. As a result, molecular mimicry is a plausible theory for studying the origin, pathophysiology, therapy, and prevention of immune-related illnesses. Furthermore, persistent exposure to bacterial sAg stimuli can cause clonal energy, which manifests as a reduced response to recall antigen stimulation, such as *Mycobacterium tuberculosis* pure protein derivative (PPD).

To better understand the relationship between sAg-producing bacteria and immunity-related disorders, this study hypothesized that assessing an individual's immunologic potential by estimating changes in immune cells and their cytokine profile after challenging PBMC with bacterial sAg (SEB and SPE-A) stimuli would provide key indicators in monitoring the process of associated immune dysregulation.

MATERIALS AND METHODS

Blood Samples:

The study included 20 healthy people who had not taken any medication in the previous six months. Everyone's peripheral venous blood was taken aseptically and processed for PBMC isolation in EDTA vacutainer tubes. Before being enrolled in the study, each participant signed a written informed consent form.

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Purification of PBMC from Blood:

Blood samples diluted 1:1 with Hank's Balanced Salt Solution (HBSS) were layered over an equivalent volume of HiSep LSM (Hi-Media Laboratories) at 45° angle to allow spin on its side, 500 x g for 30 minutes at room temperature. The hazy layer at the interface was gently collected and washed with HBSS three times and RPMI 1640 once (Hi-Media Laboratories). During the washing procedure, 250 x g centrifugation at room temperature for 10 minutes was used. Above 95% cell viability assessed by trypan blue dye exclusion was chosen as the standard.

Antigen Stimulation of PBMC:

PBMC ($1x10^6$ cells/ml) in RPMI 1640 were seeded into anti-human CD3 antibody precoated wells in a 12-well cell culture plate (Nalgene Nunc, Rochester, NY) and were treated with different sAgs concentrations (SEB and SPE-A; Toxin Technologies, Inc., USA) and PPD tuberculin. All the experiments and treatments were repeated thrice at 37°C for 72 hours in humidified air with 5% CO_2 incubation conditions.

Staining of PBMC:

Cell-free culture supernatant was achieved by centrifuging PBMC at 500 x g for 5 minutes and stored at -80°C for cytokine measurement. Harvested PBMC were labeled with a cocktail of monoclonal antibody conjugates (mAbs) (BD Biosciences) after washing thrice with buffer containing 0.5 percent BSA and 0.1 percent NaN3 in 1X PBS [pH 7.4]. The cells were stained in the dark on ice for 30-60 minutes, then washed three times with wash buffer, fixed with 300 ml ice-cold 2% paraformaldehyde in 1X PBS, and stored at 4^oC.

Fluorescence-Activated Cell Sorting (FACS):

Flow cytometry was performed within 18 hours of sample processing using the BD FACS CaliburTM device. At least 20,000- 30,000 occurrences per condition were recorded before the data evaluation using WinMDI 2.9. Isotype-matched control antibodies were used in every experiment to ascertain the specificity of antibody binding on an equally gated population of cells.

Cytokine Estimation by ELISA:

ELISA kits from BD Biosciences were used to measure cytokines such as IL-2, IL-4, IL-10, and IFN-γ. The amounts of the cytokines were measured in the culture supernatants of PBMC that had been preserved previously, according to the kit guidelines. The absorbance was measured at 450nm 30 minutes after the operation ended. The concentrations of IL-2, IL-4, IL-10, and IFN-γ in samples were determined by plotting the standard curves with concentration versus absorbance. The dilution factor was multiplied by the concentration of diluted samples. The detection limit for all of the kits was ≥ 4 pg/ml.

Data Analysis:

An independent t-test is used to calculate the difference between the observed means of T cell subpopulations and secreted cytokine levels. The difference is given a significance value (*p*-value). A *p*-value of <0.05 was considered statistically significant and was calculated using SPSS 16.0. (SPSS Inc).

RESULTS

Samples:

PBMC were sourced from the healthy volunteers (n=20, mean age 38 years) who were unrelated to each other, were not on any medications, and had no clinical history of any disease.

T-cell Alterations with sAg Stimulation:

The appropriate antigen doses for PBMC stimulation were determined in preliminary trials. Flow cytometry was used to undertake a comprehensive T-cell phenotypic study after antigen stimulation of PBMC. The data were reported as a percentage mean with standard deviation.

A substantial difference in $CD4^+$ T lymphocyte levels between unstimulated and sAg stimulated cells was found, with the percentage of $CD4^+$ T lymphocytes increasing after SEB and SPE-A

stimulation. $CD8^+$ T cells, on the other hand, either did not respond well to sAg activation or the increase in these cell numbers failed to reach statistical significance. Similarly, there was no statistically significant change in the percentages of the other T cell subpopulations identified. When sAg stimulated, cells were compared to unstimulated cells, there was a nominal increase (albeit insignificant) in the number of $CD4^+CD25^+$ T lymphocytes (Table 1).

When PBMC was activated with PPD, the number of $CD8^+CD45RA^+$ T cells increased significantly compared to unstimulated cells. However, there was no discernible alteration in any other T cell subset. (Table 1)

Table 1: Alterations in T cells from healthy subjects after antigen stimulation of PBMC *in-vitro*.

T cells / Stimulant	**No stimuli**	**SEB**	*p*-value	**SPE-A**	*p*-value	**PPD**	*p*-value
$CD3^+CD4^+$	41.16±3.74	45.45±2.96	0.0003*	45.20±3.16	0.0007*	41.84±2.53	0.5047
$CD4^+CD45RA^+$	24.10±3.30	24.60±2.70	0.6030	23.43±1.91	0.4368	25.77±2.09	0.0634
$CD4^+CD45RO^+$	16.26±3.65	17.14±2.55	0.3823	16.16±2.17	0.9167	16.40±1.42	0.8738
$CD3^+CD8^+$	37.43±5.98	37.73±4.61	0.8599	39.51±4.16	0.2048	37.81±3.26	0.8021
$CD8^+CD45RA^+$	18.55±2.20	19.24±3.08	0.4200	18.17±2.20	0.5881	21.61±1.70	<0.0001*
$CD8^+CD45RO^+$	12.36±1.21	12.70±1.17	0.3720	12.45±1.40	0.8290	13.49±2.59	0.0851
$CD4^+CD25^+$	10.94±3.15	12.72±3.08	0.0787	12.41±3.12	0.1464	11.05±3.20	0.9133

Values are presented in terms of mean (±SD, standard deviation); *The mean difference in comparison to 'no stimuli' is significant at the indicated *p*-value.

Secretion of Cytokines by Stimulated PBMC:

SEB and SPE-A stimulation significantly boosted IL-2 levels relative to unstimulated cells' baseline levels, but PPD stimulation resulted in a substantial decrease in IL-2 secretion. The level of IFN-γ increased in unstimulated cells, but it dropped dramatically after exposure to all of the antigens. With PPD, the decrease is greater than with sAgs (Fig. 1).

When compared to baseline levels in unstimulated PBMC, IL-4 increased significantly with sAgs but not with PPD. IL-10 levels, on the other hand, increased significantly in response to sAgs and PPD, with the increase being even greater when PBMC was stimulated with SEB and SPE-A. (Fig. 1).

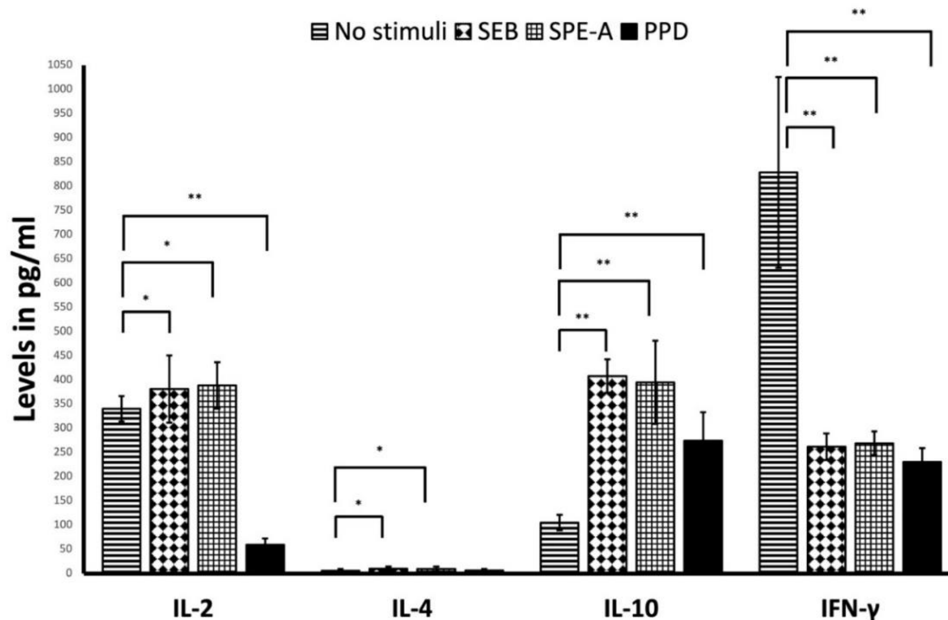

Fig. 1: Cytokine IL-2, IL-4, IL-10, and IFN-γ levels (pg/ml) produced by stimulated PBMC from healthy subjects *in-vitro*. Values are presented in terms of mean (±SD, standard deviation); *$p<0.05$, **$p<0.0001$ in comparison to 'no stimuli'.

DISCUSSION

The most frequently stated environmental elements suggested as causes of different human diseases are infectious pathogens. They have the potential to cause the breakdown of immune tolerance and the emergence of immunity-related disorders. However, the complete understanding of this aspect remains a mystery. This investigation was motivated by a lack of knowledge about the risk of certain bacterial stimulants functioning as triggers for immune-related disorders. This study's analysis of immunologic changes in response to bacterial sAgs provides important indicators for tracking the immune-related disease process and increasing overall living quality.

Environmental factors, such as bacterial surface antigens, have been shown to contribute to immune response dysregulation, resulting in autoimmunity or immunodeficiency (Schiffenbauer et al., 1998). Gram-positive cocci's surface antigens, such as those found in *Staphylococcus aureus* and *Streptococcus pyogenes*, are among the most potent T cell activators known (Commons *et al.,* 2014; Fraser and Proft 2008). Because they work to direct a non-specific T cell and cytokine-mediated immune response that aids in the survival of the bacteria that produce them, these sAgs are becoming a growing health problem. Despite having a fairly similar protein structure, each sAg has developed its own method of binding to MHC and TCR, and this extraordinary binding variety obviously provides *Staphylococcus aureus* and *Streptococcus pyogenes* with a significant survival advantage.

Both SEB and SPE-A are powerful activators of $CD4^+$ T cells and cytokine release, according to this study. Without going through any intracellular processing, sAgs bind straight to the α- or β chain of cell surface MHC class II molecules (outside of the peptide-binding groove). They then activate T cells by engaging directly with the variable region of the β-chain (or α-chain in rare circumstances) of the T cell receptor (TCR), regardless of antigen specificity (Li *et al.,* 1999). sAgs do not require CD4 or CD8 co-receptors to activate T cells. As a result, MHC class II-bound sAgs can activate $CD4^+$ and $CD8^+$ T lymphocytes in a TCR-dependent but cognate antigen-independent way. As a result, sAgs can activate a high number of T

cells, whereas typical peptide antigens only activate a small number of T cells. Bacterial sAgs have also been shown to activate $CD8^+$ T cells with the necessary TCR Vβ elements (Li et al., 1999), suggesting that $CD8^+$ T cells may be contributing to the cytokine storm triggered by these antigens. Although no significant changes in $CD8^+$ T cells were seen in this investigation, some reports have suggested that $CD4^+$ T helper cells can also cause cytotoxicity, resulting in the death of target cells (Collison and Workman 2008).

Both $CD4^+$ and $CD8^+$ T lymphocytes are activated by bacterial sAgs (Seo *et al.,* 2007). Based on their cytokine secretion characteristics, $CD4^+$ T cells could be classed as distinct T helper cells (Bettelli *et al.,* 2007). This research shows that sAgs cause acute cell damage, resulting in high IL-2 and IL-10 levels, as well as moderate IL-4 levels and low IFN-γ response. T helper subset differentiation takes days in a traditional immune response, whereas sAgs appear to trigger a rapid and robust differentiation of naive cells into these T helper subsets. Among many cytokines released in response to sAg, the varied response of IL-2, IFN-γ, and TNF-α is responsible for acute damage (Arad *et al.,* 2011; Miethke *et al.,* 1993). Although the amount of TNF-α produced in this study was not calculated, the high activation of $CD4^+$ T cells could imply that its expression is altering. In rheumatic heart disease patients, molecular mimicry, including proinflammatory cytokines and reduced secretion of IL-4, results in the progression of autoimmune response and cardiac tissue damage (Fae *et al.,* 2005; Guilherme *et al.,* 2007).

Bacterial sAgs can suppress the host immune system by affecting immune cells causing an eventual diseased state (Broeker *et al.,* 2014; Kotb 2008). sAgs forming a trimolecular complex with both MHC class II molecules and the TCR (vβ element) is the basis of their superantigenic activity that non-specifically stimulate a substantial fraction of T cells (almost 10% of resting T cells) (Fernández *et al.,* 2006; Marrack and Kappler 1990; Ono *et al.,* 2008). Unlike conventional antigens, APC fails to process sAgs internally and in turn, these antigens are not presented as peptide antigens in association with MHC class II molecule. Because bacterial sAgs are known to trigger a number of cytokines, the dysregulation or imbalance between regulatory and inflammatory cytokines, as well as the number of cytokines, may influence the course of the disease. Thus, the cells that produce immunosuppressive (IL-4 and IL-10) cytokines appear to dominate the cytokine response to sAg activation. This suggests that host variables determine the amount of sAgs induction of cytokine production as well as the clinical outcome of the infection.

Conclusion

SEB and SPE-A used as prototype sAgs show that the interaction between PBMC and sAgs has a significant impact on the magnitude of resultant polyclonal T cell response and cytokine production *in vitro*. Following lymphocyte activation by sAgs, incongruent cytokine production alters cell proliferation, differentiation, and functioning, resulting in immune response dysregulation and the triggering of immunity-related diseases.

Conflict of interest There is no conflict of interest declared by the author.

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