Superantigen-Induced Proliferation of Human CD4 $^+$CD25$^-$ T Cells Is Followed by a Switch to a Functional Regulatory Phenotype

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Superantigen-Induced Proliferation of Human CD4⁺CD25⁻ T Cells Is Followed by a Switch to a Functional Regulatory Phenotype

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Bacterial superantigens are potent T cell activators. In humans they cause toxic shock and Scarlet fever, and they are implicated in Kawasaki’s disease, autoimmunity, atopy, and sepsis. Their function remains unknown, but it may be to impair host immune responses increasing bacterial carriage and transmission. Regulatory (CD25⁺FOXP3⁺) T cells (Tregs) play a role in controlling inflammatory responses to infection. Approximately 2% of circulating T cells are naturally occurring Tregs (nTregs). Conventional Ag stimulation of naive FOXP3⁺ T cells induces Ag-specific Tregs. Polyclonal T cell activation has been shown to produce non–Ag-specific Tregs. Because superantigens are unique among microbial virulence factors in their ability to trigger polyclonal T cell activation, we wanted to determine whether superantigen stimulation of T cells could induce non–Ag-specific Tregs. We assessed the effect of superantigen stimulation of human T cells on activation, regulatory markers, and cytokine production by flow cytometry and T cell suppression assays. Stimulation of PBMCs with staphylococcal exotoxin A and streptococcal pyrogenic exotoxins A and K/L resulted in dose-dependent FOXP3 expression. Characterization of this response for streptococcal pyrogenic exotoxin K/L confirmed its αβ specificity, that CD25⁺FOXP3⁺ cells arose from CD25⁻ T cells and required APCs. These cells had increased CTLA-4 and CD127 expression, typical of the recently described activated converted Treg-like cells, and exhibited functional suppressor activity comparable to nTregs. Superantigen-stimulated CD25⁺FOXP3⁺ T cells expressed IL-10 at lower superantigen concentrations than was required to trigger IFN-γ production. This study provides a mechanism for bacterial evasion of the immune response through the superantigen induction of Tregs. The Journal of Immunology, 2010, 185: 6591–6598.

The superantigen exotoxins of *Staphylococcus aureus* and *Streptococcus pyogenes* are among the most potent T cell mitogens known (1). They are a large family of proteins with high sequence diversity, but a conserved two-domain structure comprising an N-terminal oligonucleotide-oligosaccharide binding-fold and a C-terminal β-grasp domain (1, 2). This structure confers the ability to bind MHC class II and the TCR simultaneously. MHC class II binding occurs at two conserved sites away from Ag-presenting regions of the molecule—a high-affinity interaction with the class II β-chain and a low-affinity interaction with the class II α-chain. Superantigens can be classified into three main groups on the basis of their interactions with MHC class II. One group binds the α-chain with or without contact with antigenic peptide (e.g., streptococcal pyrogenic exotoxin A [SPEA] and staphylococcal enterotoxin B). Another group binds the β-chain (e.g., streptococcal pyrogenic exotoxin C and the toxin we have focused on for this study, streptococcal pyrogenic exotoxin K/L [SPE-K/L]). The third group binds at both sites to cross-link MHC class II (e.g., staphylococcal enterotoxin A [SEA]) (1). TCR binding is, in most cases, through the V region of the β-chain (TCR Vβ region) (3) and in vitro, superantigens trigger Vβ-specific T cell proliferation. Toxins such as SEH that interact with the TCR Vα region trigger Vα-specific T cell proliferation (4). Nonantigen restricted activation of up to 50% of the whole T cell repertoire is considered to be the mechanism by which superantigen cause toxic shock syndrome (TSS).

The biologic relevance of superantigens is best established for TSS (5, 6). This form of sepsis is relatively rare, but fulminant and associated with invasive *S. pyogenes* infections, such as necrotizing fasciitis, and superficial *S. aureus* infections, such as tampon-associated TSS. Superantigens also cause scarlet fever and Kawasaki’s disease, which are childhood illnesses that clinically resemble TSS. They have also been implicated in the autoimmune sequelae of *S. pyogenes* infection such as rheumatic fever, eczema, atopy, and sepsis (1). However, the biologic importance of superantigens goes beyond specific clinical syndromes such as these. It is now established that *S. aureus* and *S. pyogenes* strains typically carry multiple genes encoding superantigens (7, 8). Most healthy people have Ab responses to superantigens, which indicates that exposure must occur in the course of clinically mild disease or asymptomatic colonization (9, 10). It has been proposed that superantigens may function to impair host immune responses and thus prolong carriage in the nose and pharynx and aid transmission of the bacterium (1, 11).

Regulatory T cells (Tregs), characterized by the expression of the fork-head transcription factor FOXP3 and the IL-2R α-chain CD25, play a central role in maintaining tolerance to self and prevention of an overexuberant inflammatory response to infection (12, 13). Approximately 2% of CD4⁺ T cells in the peripheral blood express FOXP3, are anergic to stimulation by nonspecific T cell mitogens, such as anti-CD3, and are termed naturally
occurring regulatory T cells (nTregs) (14). In contrast, inducible regulatory T cells (iTregs) are generated in the periphery from CD4+CD25+ T cells and acquire FOXP3 expression and suppressor function after TCR stimulation (15, 16).

Early studies of T cell responses to bacterial superantigens described proliferation followed by anergy (17–19). We wished to study the nature and functionality of T cells after superantigen stimulation in more detail. In particular, we wanted to determine whether superantigen-induced T cell proliferation is in fact followed by a switch to a functional regulatory phenotype. If so, it would provide a mechanism for superantigen-induced impairment of immunity. In this study, we show that superantigen-stimulated human T cells express FOXP3 in a dose-dependent, Vβ-specific manner. These cells arise from CD25+FOXP3+ cells rather than through expansion of nTregs. Cell surface marker phenotyping suggests they are activated converted Treg-like cells as suggested recently by Sagakuchi et al. (20). Superantigen-stimulated FOXP3-expressing cells express IL-10 and are functionally suppressive. Our findings indicate that superantigen-induced regulatory-like T cells, restricted only by Vβ specificity, have the potential to suppress T cell-mediated immunity to superantigen-producing organisms.

Materials and Methods

Abs and reagents

Anti-human CD3-Pacific Blue and GITR-PE/Cy7 were purchased from eBioscience (Cambridge, U.K.). Anti-human CD4-AlexaFluor700, CD4-FITC, CD8-PE/Cy5, CD25-allophycocyanin/H7, FOXP3-AlexaFluor 647, IFN-γ-PE/Cy7, and IL-10-PE were purchased from BD Pharmingen (San Diego, CA). Anti-human CD34-EC, TCR Vβ1-PE, and TCR Vβ1-FFTC Abs were purchased from Beckman Coulter (Fullerton, CA). Anti-human CD127-eFluor450 and CTLA-4-PE were purchased from eBioscience (San Diego, CA). Anti-human CD45RA-OD655 and the Aqua Live/Dead fixable dead cell stain were acquired from Invitrogen (Carlsbad, CA). Recombinant SPEA and SEA were purchased from Toxin Technology (Sarasota, FL).

Production of recombinant SPE-K/L

The vector containing spe-K/L was provided by Thomas Proft (University of Auckland, Auckland, New Zealand). Recombinant SPE-K/L was expressed in Escherichia coli as a thioredoxin–SPE-K/L fusion protein, purified on a Nickel affinity column and cleaved from the column using protease 3C as described by Proft et al. (21), with the following exceptions: the fusion protein was expressed in E. coli Origami(B/D3)pLysS (Novagen, Madison, WI) rather than E. coli AD494(DE3) and purified on HisLink Protein Purification Resin (Promega, Madison, WI) instead of Nickel IDA Sepharose.

PBMC in vitro stimulation

Human PBMCs were isolated from heparinized whole blood of healthy volunteers by gradient centrifugation (Ficoll-Plaque PLUS, GE Healthcare, Buckinghamshire, U.K.). The isolated PBMCs were washed twice with PBS and resuspended at 1 × 10^6 cells/ml in RPMI 1640 medium containing 10% heat-inactivated FCS, 2 mM l-glutamine and 1% penicillin-streptomycin. The PBMCs were stimulated with recombinant superantigen or PHA (Sigma-Aldrich, St. Louis) in round-bottom, 96-well microtiter plates at 1 × 10^5 cells per well and incubated in a humidified incubator with 5% CO_2 at 37°C for 3 d.

Cell isolation and stimulation

CD4+ T cells were purified from PBMCs by negative magnetic-bead selection, and CD4+CD25+ and CD4+CD25− cells were separated by positive selection against CD25 using the human CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. The purity of isolated CD4+CD25− T cells and CD4+CD25+ T cells was >90% by flow cytometric analysis (data not shown). The CD4-depleted PBMCs were then irradiated at 50 Gy and used as APCs. In vitro stimulation of CD4+CD25− cells, 5 × 10^4 isolated CD4+CD25+ cells were stimulated with recombinant superantigen or PHA (Sigma-Aldrich) in round-bottom, 96-well microtiter plates with 5 × 10^4 irradiated APCs and incubated in a humidified incubator with 5% CO_2 at 37°C for 3 d.

Ab staining

To characterize proliferative responses to superantigen stimulation, cells were stimulated as described above, washed with FACS buffer (PBS, 0.5% BSA, and 0.1% sodium azide) and stained for 30 min at 4°C with Aqua Live/Dead cell stain, CD4-FITC, CD8-PE/Cy5, CD25-allophycocyanin/H7, CD45RA-Qdot655, CD127-eFluor450, CTLA-4-PE, GITR-PE/Cy5, and TCR Vβ1-FFTC. The human FOXP3 Buffer Set (BD Pharmingen) according to the manufacturer’s instructions. Permeabilized cells were stained with FOXP3-AlexaFluor 647 and CD3-Pacific Blue for 30 min at 4°C. CD3 was included in the intracellular stain to ensure that no stimulated CD3+ cells were excluded.

Phenotypic analysis of the SPE-K/L-stimulated cells was examined by staining with the following cell surface Abs as described above: Aqua Live/Dead cell stain, CD4-EC, CD8-PE/Cy7, CD25-allophycocyanin/H7, CD45RA-Qdot655, CD127-eFluor450, CTLA-4-PE, GITR-PE/Cy5, and TCR Vβ1-FFTC. The human FOXP3 Buffer Set was used to fix and permeabilize the cells before FOXP3-AlexaFluor 647 and CD3-AlexaFluor 700 staining for 30 min at 4°C.

To investigate cytokine production, PBMCs were stimulated as described above but for 5 d. Brefeldin A (Sigma-Aldrich) was added to stimulated cells 14 h before the designated time points. Cells were stained with Aqua Live/Dead cell stain, CD4-EC, CD8-PE/Cy5, CD25-allophycocyanin/H7, and TCR Vβ1-FFTC as described above. Cells were fixed and per-

FIGURE 1.

The frequency of CD25+FOXP3+ cells increases in CD4+ and CD8+ T cells after superantigen stimulation. PBMCs were stimulated with various concentrations of the bacterial superantigens SPE-K/L, SEA, or SPEA for 3 d. Live CD3+CD4+ T cells were analyzed by flow cytometry for expression of CD25 and FOXP3. Data represent the mean ± SEM of three donors with the exception of SPE-K/L, which is the mean ± SEM of six donors. A, SPE-K/L. B, SEA. C, SPEA.
meabilized with the Human FOXP3 Buffer Set and stained with CD3-Pacific Blue, FOXP3-AlexaFluor 647, IFNy-PE/Cy7, and IL10-PE for 30 min at 4°C.

Suppression assays
To determine whether cells expressing CD25*FOX3* after superantigen stimulation are functionally suppressive, we tested their ability to suppress proliferative responses of fresh donor T cells to anti-CD3 measured by dye dilution. PBMCs were stimulated with SPE-K/L (1 ng/ml) for 3 d. After 3 d, the PBMCs were washed twice and the CD4+CD25+ T cells and CD4+CD25-CD4+ T cells were isolated as described above. Fresh CD4+CD25- and CD4+CD25+ T cells were isolated from the same donor. The CD4+CD25- T cells (1 x 10^5) were labeled with 2 x 10^6 M PKH67 Fluorescent Cell Linker (Sigma-Aldrich) according to the manufacturer’s instructions and used as responder cells. Isolated cells (5 x 10^4) were incubated with labeled responder cells (5 x 10^4), irradiated APCs (5 x 10^4), and anti-human CD3 OKT3 (eBioscience) for 4 d at 37°C in 5% CO2. Assays were performed in triplicate in round-bottom 96-well microtiter plates in a final volume of 200 μl. Cells were plated in 50 μl/well as follows: isolated cells, labeled responders, irradiated APCs, and OKT3. Cells were washed with FACS buffer and stained with Aqua Live/Dead cell stain and CD4-FITC for 30 min at 4°C. FACS Lysing Solution (BD Biosciences) and FACS Permeabilizing Solution 2 (BD Biosciences) were used to fix and permeabilize the cells before intracellular staining for CD3-Pacific Blue for 30 min at 4°C.

Flow cytometric analysis
Cells were washed with FACS buffer and acquired using an LSR II bench top flow cytometer with three lasers (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one controls were performed to ensure that the upregulation was not due to an Ab effect (data not shown).

Statistical analysis
Data were expressed as mean values ± SEM. Statistical comparisons for the phenotypic analysis and suppression assays were made by two-tailed Mann-Whitney U tests. Relative proliferation for the suppression assays was calculated by conversion of the maximal proliferation frequency of fresh CD4+CD25- T cells to 100% and all further proliferation as a percentage of maximal. Statistical significance was as follows: ***p < 0.001; **p < 0.01; *p < 0.05.

Results
Superantigen stimulation of PBMCs results in dose-dependent expression of CD25 and FOXP3
To investigate whether superantigen stimulation induces a regulatory phenotype in responding T cells, we first examined the effect of a panel of superantigens on the expression of the regulatory T cell marker FOXP3. We selected prototypic superantigens of S. aureus (SEA) and S. pyogenes (SPEA) and the more recently described S. pyogenes superantigen SPE-K/L. PBMCs from healthy donors were incubated with varying concentrations of each toxin for 3 d and then analyzed by flow cytometry for CD25 and FOXP3 expression. For each superantigen, there was a dose-dependent increase the percentage of CD4+ T cells expressing CD25*FOX3* (Fig. 1A-C). The biggest increase in expression (60%) was seen with SEA, which binds both the α- and β-chain of the MHC class II (Fig. 1B). Because the three toxins tested in this study exemplify the three main classes of superantigen based on
MHC class II interaction, it is likely that induction of FOXP3 expression is a feature of stimulation by superantigens generally.

**CD25^+FOXP3^+ T cells induced by SPE-K/L are TCR Vβ1-specific**

To confirm that the CD25^+FOXP3^+ responses observed were truly superantigen responses, we determined what proportion of CD25^+FOXP3^+ cells expressed TCR Vβ1 after stimulation with SPE-K/L. SPE-K/L has been demonstrated to target primarily human TCR Vβ1 expressing T cells (21). SPE-K/L stimulation resulted in a dose-dependent increase in TCR Vβ1 T cell expression of FOXP3 in all six donors examined (Fig. 2A). The CD25^+FOXP3^+ response to SPE-K/L is almost entirely TCR Vβ1 restricted, such that at a SPE-K/L concentration of 0.1 ng/ml, 100% of CD4^+Vβ1^+ T cells were CD25^+FOXP3^+ compared with 11% of non-CD4^+Vβ1^ T cells (Fig. 2B). That all Vβ1 cells are CD25^+ FOXP3^+ does not exclude the possibility that other Vβ types could also be involved.

To examine whether nonspecific stimulation could also result in increased FOXP3 expression, PBMCs were stimulated with the T cell mitogen PHA (1 μg/ml). Stimulation with PHA also resulted in an increase in the proportion of T cells expressing CD25 and FOXP3, but this effect was not TCR Vβ-restricted (Fig. 2A). These results show that PBMCs stimulated with superantigen increase FOXP3 expression in a TCR Vβ-specific manner.

**SPE-K/L induced CD25^+FOXP3^+ T cells originate from CD25^− T cells**

Because nTregs (FOXP3^+CD25^+) comprise ~2% of unstimulated peripheral blood CD4^+ T cells, we sought to determine whether the increase in CD25^+FOXP3^+ cells observed after superantigen stimulation originates from CD4^+CD25^− T cells or expansion of nTregs. Initial experiments with isolated nTregs showed no capacity for proliferation after exposure to superantigen (data not shown). Therefore, to examine whether the FOXP3^+ cells originated from naive T cells, CD4^+CD25^− T cells were isolated from PBMCs and stimulated with and without irradiated APCs and SPE-K/L. To ensure that no CD4^+CD25^− cells remained in the preparation, the removal of CD25^+ cells was performed twice and determined to be <0.1% in all three donors. SPE-K/L stimulation of CD4^+CD25^− T cells in the presence of irradiated APCs resulted in expansion of Vβ1-specific T cells (Fig. 3A). Furthermore, expression of CD25 and FOXP3 in response to SPE-K/L stimulation was markedly less when APCs were not present (Fig. 3B). In the absence of APCs, only 6% of CD4^+ T cells were CD25^+FOXP3^+ at 1 ng/ml SPE-K/L, compared with 26% when APCs were present. Superantigen presentation by activation-induced T cell expression of MHC class II is the most likely explanation for the minor increase in frequency of T cells seen in the absence of APCs. PHA was also able to induce CD25^+FOXP3^+ cells from naive CD4^+CD25^− T cells, although again no TCR Vβ preference was seen (data not shown). These results indicate that the superantigens induce naive T cells to express CD25 and FOXP3 in an APC-dependent manner.

**Phenotypic analysis of CD4^+Vβ1^+ T cells**

Expression of cell surface markers associated with activation and regulatory function was assessed for five healthy donors on day 3 following stimulation with 1 ng/ml SPE-K/L (Fig. 4). TCR Vβ1^+
cells displayed increased CD4 expression on stimulated cells compared with TCR Vβ1− cells. TCR Vβ1+ T cells also showed increased expression of CTLA-4 and CD127. CTLA-4 is a negative regulator of T cell function and CD127, the receptor for IL-7, and it is known to be upregulated in association with FOXP3 following T cell activation (22). Four of the five donors showed an increase in GITR on TCR Vβ1+ T cells. GITR has been shown to be associated with nTregs, but not activated effector T cells (22). After stimulation with SPE-K/L, expression of the naive T cell marker CD45RA was altered on TCR Vβ1+ T cells such that it was intermediate between the CD45RA− and CD45RA+ populations present in Vβ− cells. The bimodal distribution of CD45RA expression in unstimulated cells means that this effect cannot be expressed as a change in mean fluorescence intensity ratio (Fig. 4A). However, this effect was seen in all five donors tested. These results indicate that, for the five donors tested, superantigen-stimulated T cells have a phenotype in keeping with the activated converted Treg-like cells recently proposed by Sakaguchi et al. (20).

**CD4+ T cells stimulated with low concentrations of SPE-K/L produce IL10**

To determine whether the superantigen-induced changes resulted in functional changes, we first analyzed cytokine production. The expression of IFN-γ and IL-10 was examined, because it is well documented that PBMCs stimulated with superantigen produce IFN-γ, and IL-10 is an anti-inflammatory cytokine. To ensure that cytokine responses were T cell mediated, intracellular cytokine staining of CD4+ T cells was used. We noted that expression of TCR after superantigen stimulation was markedly reduced by brefeldin A in these experiments, probably as a result of impaired TCR trafficking (data not shown). For this reason, responses were assessed as a percentage of total CD4+ T cells, rather than TCR Vβ1+ T cells, expressing cytokine at daily intervals. IL-10 production was observed at SPE-K/L concentrations down to 0.01 pg/ml and appears to occur later with a greater proportion of cells producing IL-10 at day 5 than at day 3 (Fig. 5A). Maximal IFN-γ production was observed at days 3–4 and at SPE-K/L concentrations >1 pg/ml (Fig. 5B). As shown in Fig. 5C, almost all responding cells produced either IL-10 alone or both IL-10 and IFN-γ. Only at SPE-K/L concentrations >1 pg/ml were any cells observed to produce IFN-γ in the absence of IL-10. This finding suggests a dose-dependent superantigen effect on T cell cytokine production.

**SPE-K/L-induced CD25+FOXP3+ T cells function as Tregs**

To determine whether the superantigen-induced CD25+FOXP3+ cells were functionally suppressive, we examined whether superantigen-induced CD25+ T cells were capable of suppressing CD25+ T cell responses to anti-CD3 OKT3. We isolated CD4+ CD25+ T cells from PBMCs that had been stimulated with SPE-K/L for 3 d, using magnetic beads. These cells were then co-incubated with fresh CD4+CD25− responder cells from the same donor, with OKT3 and irradiated APCs. Because of the ability of SPE-K/L to induce proliferation of the CD25+ (SPE-K/L) T cells being examined for suppressive function, the traditional thymidine incorporation assay could not be used. Therefore, we labeled the CD25− responder cells with the membrane dye PKH67 to distinguish proliferation of the responder cells from the superantigen-stimulated cells. The addition of superantigen-induced CD25+ (CD25+ [SPE-K/L]) led to significant inhibition of the proliferation of responder cells to OKT3 (Fig. 6A). The suppressive activity of

![Figure 5](http://www.jimmunol.org)
superantigen-induced CD25+ T cells was comparable to that of fresh nTregs (Fig. 6A). To ensure that the suppressive effect was due to the CD25+ cells, we decreased the ratio of CD25+ to responder cells in one donor. Both superantigen-induced CD25+ FOXP3+ T cell and nTreg suppression was dependent on the ratio of suppressor cells to responder cells (Fig. 6B).

Discussion

The bacterial superantigens of *S. pyogenes* and *S. aureus* are among the most potent bacterial toxins known, triggering T cell proliferation in vitro at concentrations <1 pg/ml. Their medical importance is most clearly established in the staphylococcal or streptococcal TSS, streptococcal scarlet fever, and for those streptococcal superantigens that act as enterotoxins (e.g., SEA) in the context of staphylococcal food poisoning. It is not clear what evolutionary advantages are conferred by superantigens for the bacteria. However, it is now well established that, rather than some specific strains being toxigenic, clinical isolates of *S. aureus* and *S. pyogenes* almost invariably carry multiple superantigen genes (7, 8). Furthermore, healthy adults commonly have good Ab responses to bacterial superantigens, indicating that superantigen exposure must occur during either trivial infections or asymptomatic colonization (9, 10). These observations indicate that, in addition to playing a role in the etiology of specific disease manifestations, such as TSS and food poisoning, superantigens must confer a major biologic advantage on *S. pyogenes* and *S. aureus*. This observation most likely relates to local activation of T cells at early stages of infection in the throat and nose, allowing persistence at these sites (1). Our findings provide evidence for a specific mechanism by which superantigens might do this; induction of a regulatory T cell phenotype restricted only by the Vβ specificity of the toxin or toxins produced.

Regulatory T cells expressing CD25 and FOXP3 are one of the main mechanisms by which the immune system regulates Ag-specific T cell responses (20); they prevent both autoimmunity and excessive inflammatory responses to infection. Thymically derived, nTregs typically compose ~2% of circulating CD4+ T cells (14); they are characterized by the expression of CD25 and FOXP3, but also lack of CD127 expression. In humans, CD4+FOXP3+ T cells are heterogeneous. Naive CD45RA+FOXP3low cells are highly proliferative in vitro and differentiate into CD45RO+FOXP3high effector Tregs, which require further TCR stimulation to exert their suppressive effect (20).

In mice, several adaptively derived Treg subsets expressing CD25 and FOXP3 have been described. These subsets have been termed iTregs and arise after low-dose antigenic stimulation (23). Other cells with regulatory function include Tr1 (IL-10 producing) and Th3 (TGF-β producing) cells that exhibit cytokine-mediated suppression of effector T cell responses. The situation in humans is less clear. Although in vitro stimulation of CD25+ T cells induces FOXP3, some studies have demonstrated suppressive function whereas others have not (15, 16, 24).

We have demonstrated that superantigen stimulation of human PBMCs is followed by Vβ-specific expression of CD25 and FOXP3 for three superantigens, one staphylococcal (SEA) and two streptococcal (SPEA and SPE-K/L), selected because they exemplify the three main superantigen families classified by mode of interaction with MHC class II (1). We focused on SPE-K/L for subsequent experiments for the practical reason that we express this toxin in our laboratory. SPE-K/L was first identified as SPE-K/L in the genome sequence of an M3 *S. pyogenes* strain from the United States in 2003 and subsequently found to be identical to SPE-L identified in invasive M3 strains from Japan and rheumatogenic M89 strains from New Zealand (21, 25, 26). Proft et al. (21) characterized the toxin as a Vβ1-specific, MHC class II β-chain binding superantigen of the same family as streptococcal pyrogenic exotoxin C. The gene for SPE-K/L has since been identified in a wide range of different M types in studies from Australia and Europe (8, 27).

It seems most likely that the expansion of CD25+FOXP3+ cells in this study arises from CD25+FOXP3+ cells, because removal of CD25+ cells prior to stimulation was greater than 99.9%. It is not impossible that proliferation of any FOXP3low naive natural regulatory T cells that remained after CD25 depletion could explain our findings, at least in part. Our findings are in keeping with early reports of the T cell response to bacterial superantigens in which proliferation was found to be followed by anergy and increased CD25 expression (17–19).

Our observations are not surprising because similar changes have been reported previously after CD25+ T cell stimulation using anti-CD3 and peptide–MHC complexes (15, 16, 24). In keeping with the findings of Walker et al. (15, 16), we find that superantigen-induced FOXP3 expressing CD4+ T cells are functional suppressor cells. We have demonstrated IL-10 production by these cells as one marker of a suppressor phenotype. We con-

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**FIGURE 6.** SPE-K/L CD4+CD25- T cells functionally suppress effector T cells. CD4+CD25- T cells were isolated from PBMCs stimulated with SPE-K/L (1 ng/ml) for 3 d and incubated with fresh PKH67-labeled CD4+CD25- T cells from the same donor. Cells were stimulated with irradiated APCs from the same donor and anti-CD3 for 4 d before flow cytometric analysis. A, CD4+CD25+ T cells isolated from SPE-K/L-stimulated PBMCs suppress CD4+CD25- T cells. Proliferation of CD4+ T cells was analyzed by the percentage of cells with decreased PKH67 expression. Data represent mean ± SEM of three donors. B, Suppression by CD4+CD25+,CD25-SPE-K/L+ T cells is dependent on the number of CD4+CD25+ T cells present. Proliferation relative to maximal proliferation frequency is displayed. Data represent mean of triplicates from one donor ± SEM.
clude that this IL-10 production is truly a T cell response to superantigen stimulation rather than an artifact of any endotoxin contamination, because activated T cells do not mount cytokine response to endotoxin although they may express TLR4 (28). Although we have not explored the mechanism of their suppressive activity, the predominant immunosuppressive ability of IL-10 is the inhibition of monocytes and macrophage function (29). The use of irradiated APCs in this research would suggest that the suppression we have observed is not IL-10 mediated. A particularly striking observation is the identification of FOXP3 and IL-10 production at lower concentrations than required to detect pro-inflammatory IFN-γ production. It is likely that such low superantigen concentrations are more biologically relevant to the situation of early and local mucosal infection or colonization than TSS. In the former, very low quantities of superantigen may be present in patients with atopic dermatitis who are colonized by superantigen-producing strains of *S. aureus*. Our findings support those of Ou et al. (31) who identified increased CD25+FOXP3+T cells expressing the skin-homing marker cutaneous-lymphocyte associated Ag in patients with atopic dermatitis.

The systemic inflammatory response triggered by infection in sepsis is accompanied by defective immune responses, including impaired phagocytic function, and increases in immunosuppressive cytokines (e.g., IL-10) and CD25+FOXP3+T cells (32–34). Apoptosis of lymphoid and myeloid cells is considered to be primarily responsible for these changes (35). Our findings suggest an additional mechanism when sepsis is caused by superantigen-producing organisms in which direct superantigen-stimulation of CD25+FOXP3+T cells results in more T cells with regulatory function. In light of our findings, further study of this effect in vivo is warranted. This study should involve superantigen-sensitive, HLA class II transgenic, and FOXP3 knockout mouse models and the study of patients with different forms of sepsis.

Although bacterial superantigens are notorious for their role in TSS, the ecologic niche of *S. aureus* and *S. pyogenes* is the human nasopharynx. Consequently, it is likely that the role of superantigens in infection is exerted locally at this site and probably is to corrupt the host immune response to superantigen-producing bacteria, thus prolonging carriage and aiding transmission (1, 11). In this study, we provide a potential mechanism for such a role. Just as induction of Tregs appropriately restricts Ag-specific and localized immune responses to infection, superantigen-induced regulatory-like T cells restricted only by Vβ specificity have the potential to suppress T cell-mediated immunity to superantigen-producing bacteria.

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**Disclosures**

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