

Staphylococcal enterotoxin B inhibits regulatory T cells by inducing glucocorticoid-induced TNF receptor-related protein ligand on monocytes

Ivan D. Cardona, MD,^a Elena Goleva, PhD,^a Liang-Shiou Ou, MD,^b and Donald Y. M. Leung, MD, PhD^a *Denver, Colo, and Taoyuan, Taiwan*

Background: Superantigens inhibit naturally occurring CD4⁺CD25⁺ regulatory T-cell (nTreg) activity, yet the mechanism for this is unknown.

Objective: We tested the hypothesis that staphylococcal enterotoxin B (SEB), a prototypic superantigen, inhibits the ability of nTregs to suppress T-effector cell (Teff) proliferation by an antigen-presenting cell-dependent cell contact mechanism and the induction of glucocorticoid-induced TNF receptor-related protein ligand (GITR-L) on monocytes.

Methods: Cell proliferation assays were performed on immunomagnetic-bead separated CD4⁺CD25⁺, CD4⁺CD25⁻ T cells and CD14⁺ monocytes using anti-CD3, SEB, or a neutralizing anti-GITR-L antibody for stimulation. A cytokine permeable membrane insert separating cells in culture was used to evaluate the role of cell contact. Proliferation was measured by [³H]-thymidine incorporation or a carboxyfluorescein diacetate succinimidyl ester assay. GITR-L expression was determined by RT-PCR and immunostaining. **Results:** Antigen-presenting cell contact was required for proliferation of nTregs and loss of their ability to suppress Teff proliferation as confirmed by carboxyfluorescein diacetate succinimidyl ester. At baseline, monocytes expressed no GITR-L, but after SEB stimulation, GITR-L gene and protein expression increased significantly over a period of 24 hours ($P < .05$). When GITR-L activity was neutralized with blocking antibody, as opposed to isotype control, nTregs suppressed Teff proliferation despite the presence of SEB ($P < .05$). Likewise, loss of GITR-L at 48 hours was associated with restoration of nTreg activity ($P \leq .05$).

Conclusion: Staphylococcal enterotoxin B upregulates GITR-L on monocytes and inhibits nTreg ability to suppress Teff proliferation via a cell contact interaction.

Clinical implications: Prevention of GITR-L induction on monocytes by SEB may be a novel target for control of inflammation in superantigen-driven diseases such as atopic dermatitis. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Inflammation, superantigens, T-regulatory cells, monocytes/macrophages, GITR-L

Staphylococcus aureus and *Streptococcus pyogenes* secrete toxins known as superantigens, which are thought to play a role in various inflammatory and autoimmune diseases, yet the mechanisms involved are poorly understood.¹ Superantigens target the molecules involved in the first step of adaptive immune responses. Specifically, superantigens bind sequentially to the MHC class II molecule on antigen-presenting cells (APCs), and the variable- β chain of the T-cell receptor, without the need for antigen processing. Therefore, superantigens are powerful T-cell mitogens that activate multiple arms of the immune system.²

Naturally occurring CD4⁺CD25⁺ regulatory T cells (nTregs) also have important effects on the adaptive immune system, but in a direction opposing that of superantigens, by suppressing proliferation of autoreactive and T effector cells.³ The suppressive function of nTreg cells occurs via cell-cell contact between nTreg cells and T effector cells; however, the mechanism that triggers this interaction is still unknown.⁴ Within the past 2 years, numerous reports have implicated loss of nTreg cell suppressive function in the pathogenesis of autoimmune and allergic inflammatory conditions.⁵⁻¹³ We recently investigated the role of superantigens as an environmental factor that may abrogate nTreg cell function by microbes. Superantigens were found to cause potent abrogation of nTreg activity, thereby suggesting a novel mechanism by which superantigens could augment T-cell-activated responses in human autoimmune and inflammatory diseases.¹²

Using murine cells, 3 mechanisms have been described for blocking nTreg suppressive activity: LPS-induced production of IL-6 by dendritic cells *in vitro*,¹⁴ triggering of nTreg cells by anti-CD28 *in vitro*,¹⁵ and the engagement of the cell surface receptor glucocorticoid-induced TNF receptor-related protein (GITR; TNFRSF18) with an agonistic anti-GITR antibody *in vitro*¹⁶ as well as its

From ^athe Department of Pediatrics, National Jewish Medical and Research Center, Denver; and ^bthe Department of Pediatrics, Chang Gung Children's Hospital, Taoyuan.

Supported by grants AR41256, HL-36577, HL37260, and 3M01 RR00051 from the National Institutes of Health, University of Colorado Health Sciences Center Cancer Center, the American Academy of Allergy, Asthma and Immunology Fujisawa Skin Disease Award, and the Edelstein Family Chair in Pediatric Allergy-Immunology.

Received for publication June 1, 2005; revised November 22, 2005; accepted for publication November 29, 2005.

Reprint requests: Donald Y. M. Leung, MD, PhD, National Jewish Medical and Research Center, Department of Pediatrics, Room K926i, 1400 Jackson Street, Denver, CO 80206. E-mail: leungd@njc.org.

0091-6749/\$32.00

© 2006 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2005.11.037

Abbreviations used

APC:	Antigen-presenting cell
CFSE:	Carboxyfluorescein diacetate succinimidyl ester
DAPI:	4'-6-Diamidino-2-phenylindole, dihydrochloride
FITC:	Fluorescein isothiocyanate
GITR:	Glucocorticoid-induced TNF receptor-related protein
GITR-L:	Glucocorticoid-induced TNF receptor-related protein ligand
nTreg:	Naturally occurring CD4 ⁺ CD25 ⁺ regulatory T cell
SEB:	Staphylococcal enterotoxin B

injection into a mouse model, leading to the induction of autoimmunity *in vivo*.¹⁷ Similar mechanisms have not been described in human nTreg cells.

The current study focused on the potential role of cell-cell contact and GITR ligand (GITR-L) in this process.

METHODS

Subjects

We enrolled 5 normal healthy adult donors with no history of atopic or autoimmune diseases after obtaining informed consent. The Institutional Review Board at National Jewish Medical and Research Center, Denver, Colo, approved this study.

PBMC isolation

Human PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized venous blood from healthy donors as previously described.¹⁸ The PBMCs were resuspended in RPMI 1640 (BioWhittaker, Pittsburgh, Pa) containing 10% heat activated FCS, 40 μ M/L L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 20 mmol/L HEPES buffer solution (GIBCO BRL Life Technologies, Gaithersburg, Md).

CD4⁺CD25⁺ nTreg cell and CD4⁺CD25⁻ T effector cell isolation

Human nTreg cells were isolated from PBMC as previously described.¹² In brief, negative selection of CD4⁺ T cells was performed with immunomagnetic beads (by depletion of CD8⁺, CD11b⁺, CD16⁺, CD19⁺, CD36⁺, and CD56⁺ cells) with the Untouched CD4⁺ T cell Isolation Magnetic Bead Kit (Miltenyi Biotec, Auburn, Calif). CD4⁺ cells were then separated into CD25⁺ (IL-2 receptor α chain) and CD25⁻ T cells by positive selection with a CD4⁺CD25⁺ T cell Isolation Kit (Miltenyi Biotec) or by FACS cell sorting (as described previously¹²) using phycoerythrin-coupled anti-CD25⁺ mAb. Starting with 1×10^8 PBMCs, typically 0.8×10^6 to 1.5×10^6 CD4⁺CD25⁺ T cells were isolated, with a purity range from 96% to 99%. Because CD25 can also be expressed at high levels on activated non-nTreg cells, it has been shown that the best way to select nTreg cells on the basis of cell surface markers is to sort the population that is very high in CD25 expression.¹⁹⁻²¹ Therefore, our isolated CD4⁺CD25⁺ cells were CD25^{hi} (the top 2% of the original CD25⁺ population) as confirmed by flow cytometry. The forkhead transcription factor FoxP3 is a unique molecular marker of nTreg cells and is essential for the development and function of these cells.²² Our CD4⁺CD25⁻ cells were 90% pure and 100% FoxP3-negative.¹² Our purified CD4⁺CD25⁺ T cells displayed phenotypic and functional characteristics of nTreg cells, namely hyporesponsiveness to T cell receptor stimulation, suppression of CD4⁺CD25⁻ T cells, and 100% positive expression for FoxP3 gene and protein.^{12,19-22}

T-cell proliferation and suppression

Functions of nTreg cells (anergy and suppression of T-effector cell proliferation in response to mitogenic stimulation) were assessed by proliferation assays as previously described.¹² To analyze the T-cell proliferative capacity, CD4⁺CD25⁺ T cells were cultured at 5×10^4 cells/well (in a 1:1 ratio) with irradiated (3000 rad) APC (CD4⁻ cell population). We then used 96-well, round-bottom culture plates (Nunc; Nunc Brand, Rochester, NY) with or without cell membrane inserts to physically separate APC from T cells in culture. Cells were stimulated with 0.5 μ g/mL anti-CD3 mAbs (OKT3, Orthoclone; Ortho Biotech Products, LP, St Louis, Mo) or 1 ng/mL staphylococcal enterotoxin B (SEB), a prototypic superantigen (Toxin Technology Inc, Sarasota, Fla). For conditions without APC, plates were coated with 10 μ g/mL anti-CD3 in PBS overnight at 4°C and washed with sterile PBS before the experiment. To analyze suppressive capacity of nTreg cells, CD4⁺CD25⁺ cells were cocultured in a 1:1 ratio with CD4⁺CD25⁻ T effector cells in the presence of irradiated APCs with 1 ng/mL SEB. To analyze the role of GITR-L in superantigen mediated inhibition of nTreg activity, APCs were cultured with 1 μ g/mL anti-GITR-L blocking antibody or mouse IgG₁ isotype control (R&D Systems, Inc, Minneapolis, Minn) for 1 hour before their use in nTreg assays, or the cells were incubated with recombinant human GITR-L (5 ng/mL-5 μ g/mL; Santa Cruz Biotechnology, Santa Cruz, Calif). Anti-GITR-L antibody has been proven to have a neutralizing receptor-ligand interaction. As shown by the manufacturer, approximately 0.02 to 0.06 μ g/mL of this antibody blocks 50% of the binding of 10 ng/mL of recombinant GITR ligand to immobilized recombinant GITR/Fc chimera (0.2 μ g/mL) in a functional ELISA. Complete inhibition was achieved at 0.3 μ g/mL (R&D Systems, Inc). Cells were cultured for 72 hours at 37°C in a humidified, 5% CO₂ containing incubator. Eighteen hours before harvesting, cell cultures were pulsed with 1 μ Ci/well of tritiated thymidine (ICN Biomedicals, Costa Mesa, Calif), and cell incorporated tritium was counted in a liquid scintillation counter. For all proliferation assays, the experimental values reported represent the means of triplicate determinations.

CD14⁺ monocyte isolation

From isolated PBMCs, negative selection of CD14⁺ monocytes was performed with immunomagnetic beads by depletion of CD3⁺, CD7⁺, CD16⁺, CD19⁺, CD56⁺, CD123⁺, and glycophorin A using the human Monocyte Isolation Kit II (Miltenyi Biotec). Purified CD14⁺ monocytes were then used in immunostaining and real-time PCR. In addition, purified monocytes from 3 of the donors were used as APC to analyze the role of monocyte-associated GITR-L and nTreg activity after 48 hours of SEB stimulation. Monocytes were stimulated with SEB (1 ng/mL) for 48 hours. Then SEB was washed away, monocytes were irradiated, and purified T effectors were cocultured in a 1:1 ratio (5×10^4 cells/well) with or without nTreg cells from the same donors with 0.5 μ g/mL anti-CD3. As described, cells were then cultured for 72 hours, and for the last 18 hours, they were pulsed with 1 μ Ci/well of tritiated thymidine. Cell incorporated tritium was counted in a liquid scintillation counter. Experimental values reported represent the means of triplicate determinations.

Carboxyfluorescein diacetate succinimidyl ester labeling

To analyze nTreg cells and T-effector cells proliferation in coculture experiments with SEB, nTreg cells (0.5×10^6) or T-effector cells (5×10^6) were labeled with 0.5 μ M/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Vibrant CFDA-SE Cell Tracer kit; Molecular Probes Inc, Eugene, Ore) before stimulation and cocultured with unlabeled T effectors or nTreg cells, respectively, for 72 hours in the presence of irradiated APC. CFSE labeling was

performed on the basis of the manufacturer's recommendations. Division of CFSE labeled cells was analyzed by flow cytometry by gating on the lymphocyte population.

GITR-L protein expression by PBMCs and monocytes

We used the human GITR ligand/TNFSF18 DuoSer ELISA development kit (R&D Systems, Inc) with the appropriate standard to analyze the presence of soluble GITR-L in cell culture supernatants from human PBMCs 24 hours after stimulation with SEB. PBMCs or human CD14⁺ cells purified by negative selection with magnetic microbeads (Miltenyi Biotec) were cultured at 1×10^5 cells/mL on poly-D-lysine coated Lab-Tek II chamber slides (Nalge Nunc International, Naperville, Ill). The cells were stimulated with 1 ng/mL SEB or remained untreated and were fixed at time 0, 6, 24, and 48 hours of incubation. GITR-L expression was examined by immunocytochemistry. In brief, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-GITR-L antibody (eBioscience, San Diego, Calif) and 300 nmol/L 4'-6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Inc) for 1 hour at room temperature, washed, and mounted on slides. To identify cell types that express GITR-L, human PBMCs were stained with anti-CD3 phycoerythrin, anti-CD19 phycoerythrin, or anti-CD14 phycoerythrin antibodies (BD Pharmingen, San Jose, Calif). The specificity of GITR-L staining was controlled by the mouse IgG₁ FITC isotype control (eBioscience). The slides were analyzed by fluorescent microscopy (Leica Microsystems, Bensheim, Germany) with imaging software Slidebook (Intelligent Imaging Innovations, Denver, Colo).

GITR-L gene expression

Using the manufacturer's guidelines (Qiagen, Valencia, Calif), total mRNA was isolated from CD14⁺ monocytes, transcribed into cDNA, and analyzed by RT-PCR using the dual-labeled fluorogenic probe method on an ABI Prism 7000 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, Calif).¹² Primers and probes for GITR-L and 18s RNA were purchased from Applied Biosystems. A standard curve was generated by using the fluorescent data from 2-fold serial dilutions of total RNA of the highest expression sample. Quantities of target gene expression in test samples were normalized to the corresponding 18s RNA transcript.

Statistical analysis

Data were expressed as means $\pm$ SEMs. Cell proliferation and immunofluorescence data were analyzed by using the 2-tailed, paired Student *t* test. A *P* value of less than .05 was considered statistically significant.

RESULTS

Superantigen-mediated inhibition of nTreg activity occurs in an APC-dependent fashion

We initially determined whether the presence of APC contact is required for superantigen-mediated inhibition of nTreg function. With no APCs in cell culture, nTreg cells did not proliferate in the presence of SEB ($P < .05$, compared with nTreg cells with irradiated APCs; Fig 1, A). In addition, these nTreg cells cultured without APCs were able to suppress T-effector cell proliferation ($P < .05$; Fig 1, B). These experiments were performed in the presence of plate-bound anti-CD3 for mitogenic stimulation and are consistent with the anergic state of human nTreg cells to T-cell receptor cross-linking.^{19,20} In contrast,

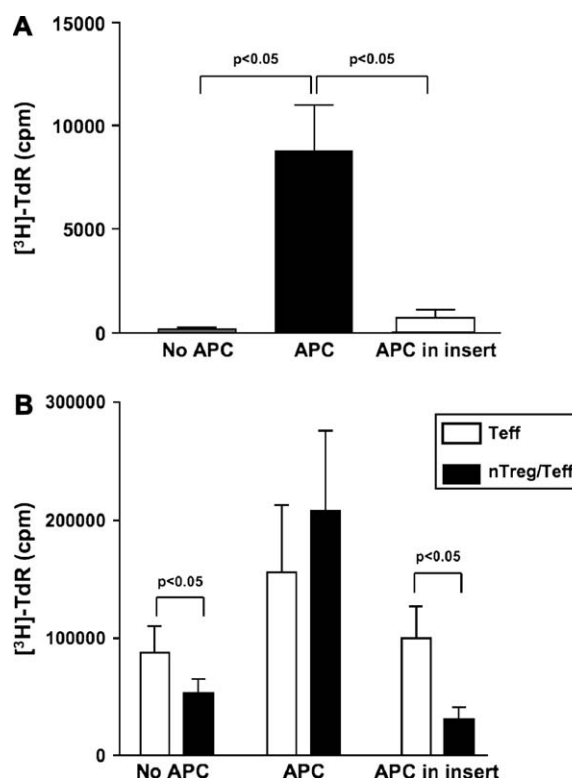


FIG 1. APCs are necessary for superantigen-induced inhibition of nTreg activity. **A**, nTregs stimulated with SEB require APC physical interaction to proliferate ($P < .05$). **B**, T effectors cultured alone or with nTregs and stimulated with SEB under the same conditions as in **A**. nTregs lose ability to suppress T-effector proliferation with APC physical interaction ($P < .05$). Results represent the means $\pm$ SEMs cpm for 5 healthy donors.

when irradiated APCs were cocultured with nTreg cells in the presence of SEB, nTreg cells proliferated (Fig 1, A), and their suppressive function was lost (Fig 1, B).

To prove formally that nTreg cells lose the ability to inhibit proliferation of T effector cells in the presence of SEB, we used the intracellular fluorescent label CFSE to tag proliferating cells and therefore determine the proliferation history of specific cell populations. Anti-CD3 stimulation was used as a control. nTregs or T effectors were labeled with CFSE in coculture experiments and stimulated with either anti-CD3 or SEB for 72 hours in the presence of irradiated APCs. CFSE-labeled cell division was analyzed by flow cytometry by gating on the lymphocyte population (Fig 2). Proliferation of CFSE-labeled T effectors cocultured with nTregs was suppressed in the presence of anti-CD3 stimulation (Fig 2, A) but was not inhibited in the presence of SEB stimulation (Fig 2, D) compared with proliferation of CFSE labeled T effectors only (Fig 2, B and E, for anti-CD3 and SEB induced proliferations, respectively). No difference in proliferation of CFSE-labeled nTregs cocultured with T effector cells was found after anti-CD3 or SEB stimulation (Fig 2, C and F, respectively). We therefore concluded that SEB stimulation renders T-effector cells refractory to suppression by nTreg cells.

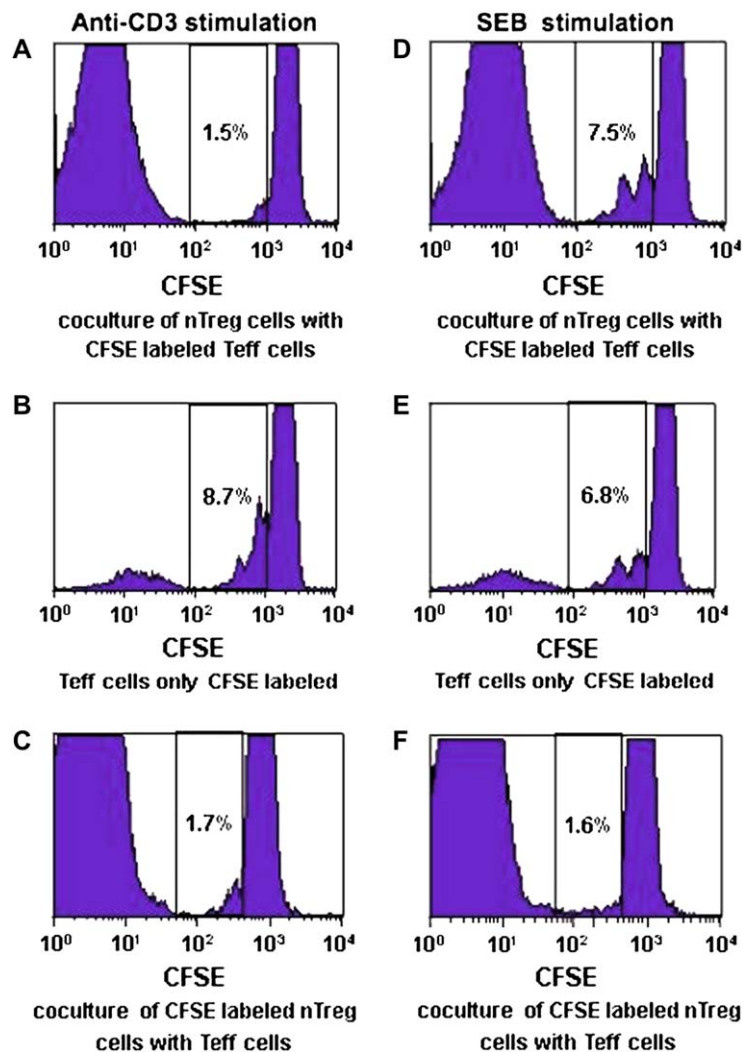


FIG 2. nTregs lose their ability to inhibit proliferation of T effectors in the presence of SEB. nTregs or T effectors were labeled with CFSE in coculture experiments and stimulated with either anti-CD3 (A-C) or SEB (D-F) for 72 hours. CFSE-labeled cell division was analyzed by flow cytometry by gating on the lymphocyte population. Gates represent percent of dividing CFSE-labeled cells. These results are representative of 3 independent experiments. *Teff*, $CD4^+CD25^-$ T-effector cell.

Next, we used membrane inserts to separate nTreg cells from APCs cultured in the presence of SEB. To gain insights into the potential role of cell-cell contact, we used irradiated APCs to inhibit cytokine production (which can contribute to abrogation of nTreg activity).²¹ We found that nTreg cells that were prevented from cell-cell contact with APCs did not proliferate compared with nTreg cells interacting physically with APCs ($P < .05$; Fig 1, A). Likewise, nTreg cells that were prevented from direct cell-cell contact with APCs were also able to suppress T-effector cell proliferation, as opposed to nTreg cells that were not physically separated from APCs ($P < .05$; Fig 1, B). Collectively, these results indicate that superantigen-mediated inhibition of nTreg cell activity requires APC interaction via a cell-cell contact mechanism.

Superantigens induce monocytes (APCs) to express GITR-L

Recently, it has been reported that stimulation of murine nTreg cells through GITR blocks their suppressor function.²³ The soluble form of GITR-L triggers proliferation of murine nTreg cells and enhances proliferation and IL-2 secretion by activated $CD4^+$ cells. By using an ELISA, we were able to detect purified GITR-L on the standard curve from 8000 pg/mL to 125 pg/mL but were unable to find soluble GITR-L in culture supernatants of superantigen-stimulated PBMCs. Thus, we hypothesized that superantigens might induce GITR-L cell surface expression on APCs, which leads to the loss of nTreg cell ability to suppress T-effector cell proliferation via direct cell-cell contact.

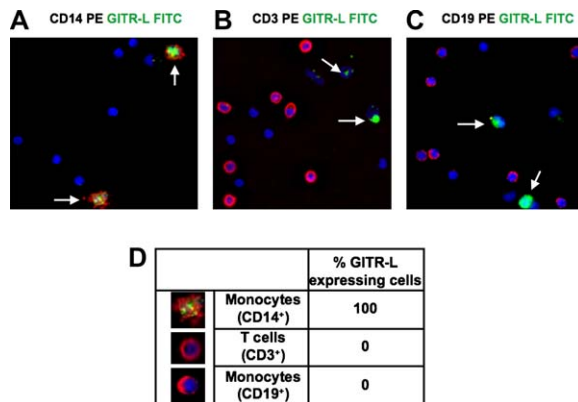


FIG 3. GITR-L expression in PBMCs after SEB stimulation. PBMCs stimulated with SEB for 24 hours and stained with anti-GITR-L antibody (FITC, green). PBMCs were also stained with anti-CD14 (A), anti-CD3 (B), and anti-CD19 (C) (phycoerythrin [PE], red) antibodies. Cell nuclei were counterstained with DAPI (blue; magnification $\times 400$). Arrows indicate GITR-L-expressing cells. D, Percent expression of GITR-L by different cells after SEB stimulation. Results represent 3 independent experiments.

Therefore, by using immunostaining techniques, we set out to determine which cell type in PBMCs could be induced to express GITR-L on its cell surface. A FITC-fluorochrome directly conjugated antibody (represented by the green stain in Fig 3, A-D) was used to stain for cell surface GITR-L protein expression. Comparing the percent of cells expressing GITR-L, monocytes were the only cell type upregulating GITR-L after 24 hours of SEB stimulation, compared with B cells and T cells (Fig 3, A-D). By using real-time PCR, we then analyzed the kinetics of GITR-L gene expression in monocytes and found that GITR-L gene expression peaks at 6 hours post-SEB stimulation (Fig 4, A). The time course for GITR-L protein expression on monocytes after SEB stimulation was also performed for as long as 48 hours using immunostaining techniques. We found that GITR-L protein expression was on the surface of monocytes as early as 6 hours after SEB stimulation and increased significantly compared with baseline by 24 hours of culture (480.7 ± 45.5 , $P = .0001$, compared with media-treated cells; Fig 4, B). By 48 hours, GITR-L protein expression was no longer present (Fig 4, B). No GITR-L expression by monocytes was detected at the baseline or after 24 hours of culture in media alone (Fig 4, B). These results demonstrate that superantigen can directly induce GITR-L gene and protein expression on the surface of monocytes.

Superantigen-induced inhibition of nTreg cell suppression of T-effector proliferation requires upregulation of GITR-L

Next, we examined whether GITR-L was involved in superantigen-induced inhibition of nTreg cell function. To determine whether GITR-L was playing a critical role in SEB-induced inhibition of nTreg cell function, a

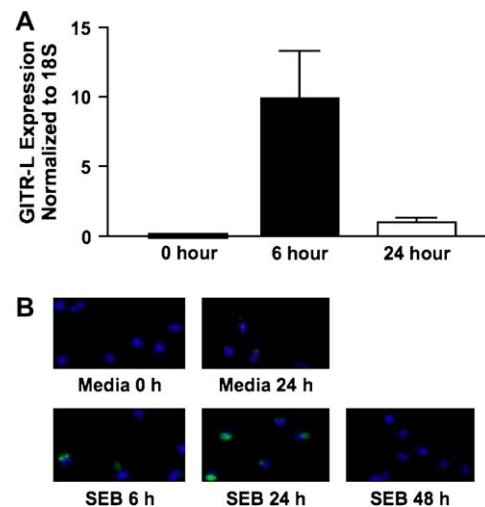


FIG 4. SEB transiently upregulates GITR-L expression by monocytes. A, Expression levels of GITR-L mRNA in purified monocytes after SEB stimulation over time ($n = 5$). B, Immunostaining of GITR-L expression in monocytes after media or SEB stimulation over time (GITR-L-FITC, green; cell nuclei-DAPI, blue; magnification $\times 400$). Results represent 3 independent experiments.

neutralizing anti-GITR-L antibody was added to irradiated SEB-treated APCs. We found that when the neutralizing antibody (and not the isotype control) was added to irradiated APCs, cultured with nTreg cells, and SEB, nTreg cells proliferated less and were able to suppress T-effector cell proliferation ($P < .01$, Fig 5, A; and $P < .05$, Fig 5, B). In contrast, nTreg cells proliferated in the presence of SEB and were not able to suppress T-effector cell proliferation when cocultured with APCs in media or in presence of the isotype control antibody. Thus, our data support the hypothesis that superantigen-induced inhibition of nTreg cell function requires upregulation of GITR-L, because the presence of the neutralizing anti-GITR-L antibody and not the isotype control antibody restored nTreg cell suppressive function on T-effector cell proliferation.

To determine whether loss of GITR-L at 48 hours is associated with restoration of nTreg ability to inhibit T-effector proliferation, monocytes were purified and stimulated with SEB for 48 hours. We then washed away the SEB, irradiated the monocytes, and added from the same respective donors, purified T effectors with or without nTregs in coculture with anti-CD3. Cell culture and proliferation was performed as described. We found that the ability of nTregs to inhibit T-effector proliferation was restored after 48 hours of SEB stimulation (Fig 6; $P \leq .05$) as opposed to nTreg cell ability after 24 hours of SEB, shown in Fig 1, B. Results of 3 independent experiments are shown in Fig 6.

DISCUSSION

In the current study, we found that superantigen mediated inhibition of nTreg cell activity occurs because of

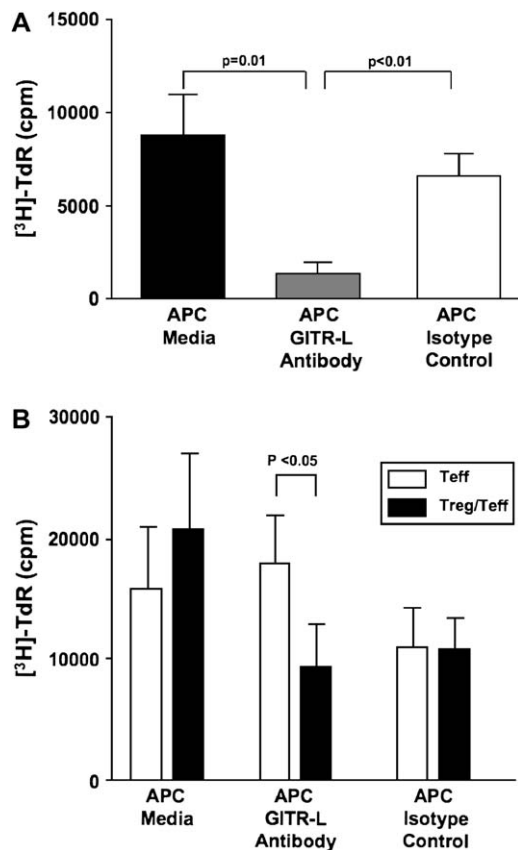


FIG 5. nTreg cells regain their function after blocking GITR-L. **A**, nTregs stimulated with SEB under 3 conditions. **B**, T effectors cultured alone or with nTreg and stimulated with SEB under the same conditions. Of note, data for nTregs cultured alone or with T effectors, in the presence of APCs, were shared between Figs 1 and 5. Results are cumulative mean $\pm$ SEM cpm for 5 healthy donors.

induction of GITR-L expression on human monocytes. This leads to nTreg cell proliferation as well as loss of nTreg cell ability to suppress proliferation of T-effector cells. The presence of a neutralizing anti-GITR-L antibody, but not the isotype control, prevented GITR-GITR-L interaction and restored nTreg cell functions despite the presence of superantigen. Our data clearly demonstrate that inhibition of the nTreg proliferation status by superantigen occurs because of direct cell-cell contact with APC engaging GITR-L-GITR.

Although our understanding of the factors that regulate nTreg cell suppression is advancing,²⁴ the specific mechanisms that control suppressor activity of nTreg cells are still not well understood. CD4⁺CD25⁺ nTreg cells exert their suppressor activity by inhibiting IL-2 production by T-effector cells and promoting cell cycle arrest.²¹ Evidence provided by different research groups suggests that signaling through several receptors appears to be critical for the functional activity of nTreg cells. GITR is highly expressed by nTreg cells.¹⁷ In a mouse model, triggering of GITR both *in vitro* and *in vivo* abrogated nTreg cell-mediated suppression.^{16,17} Several other TNF

receptor superfamily members (4-1BB and OX40) have also been shown to modulate the suppressor function of nTreg cells.^{25,26} In addition, ligation of CD28²⁷ or blockade of CTLA-4 on nTreg cells can abrogate suppression.²⁸

Glucocorticoid-induced TNF receptor-related protein is a constitutively expressed marker for naturally occurring nTreg cells and is also upregulated on conventional activated CD4⁺ T cells.²⁹ The high level of constitutive GITR on nTreg cells suggests an important role for this molecule on this subset of T cells.²¹ For instance, when an antimouse GITR mAb is used to block nTreg cell function *in vitro*,^{16,17} its injection into mice leads to the induction of autoimmunity *in vivo*.¹⁶ Although it was originally presumed that triggering of GITR on the surface of nTreg cells transmits a suppression-blocking signal to these cells,¹⁶ recent data, using GITR deficient mice, suggest that ligation of GITR on activated T-effector cells, not nTreg cells, renders them resistant to suppression.³⁰ Our experiments looking at superantigens effect on nTreg cell suppressive function are consistent with this mechanism, because nTreg suppressive function was lost when APC contact was allowed between nTreg and T-effector cells. It is likely that superantigen-induced expression of GITR-L on APCs interacted in a cell-cell contact mechanism with GITR on T-effector cells, making these cells resistant to nTreg suppression. When APCs were physically separated from T cells by the membrane insert, nTreg suppressive function was maintained, presumably because APC-GITR-L could not engage GITR on T-effector cells. Because our coculture experiment was dealing with a mixed cell population (ie, nTregs with T effectors), we wanted to prove formally that nTregs lose the ability to inhibit T-effector proliferation in the presence of SEB. Therefore, by using a CFSE assay to label different cell populations (nTregs or T effectors), we confirmed that proliferation of CFSE-labeled T effectors cocultured with nTregs was suppressed in the presence of anti-CD3 stimulation, but was not inhibited in the presence of SEB stimulation.

Previous work by McHugh et al¹⁷ demonstrated that GITR ligation by anti-GITR antibodies led to T-cell receptor-independent proliferation of nTreg cells in the presence of IL-2. In addition, exogenous IL-2 has been shown to block regulatory T cell suppressive activity.^{14,28} In our experiments, APCs were taken from a CD4⁺ cell population, which allows the possibility for contaminating T-effector cells to be introduced to the system and produce IL-2 on stimulation, thus influencing nTreg cell proliferation. However, this is not the case, because irradiated APCs were physically separated from nTreg cells by the cytokine permeable membrane insert, and still nTreg cells did not proliferate and were able to suppress T-effector cell proliferation efficiently in response to SEB. The data presented support the idea that abrogation of nTreg activity, specifically loss of anergy and suppressive function induced by superantigens, is dependent on GITR-L on APCs.

The natural ligand for GITR has been cloned recently.³¹ It was found that the soluble recombinant form of mouse

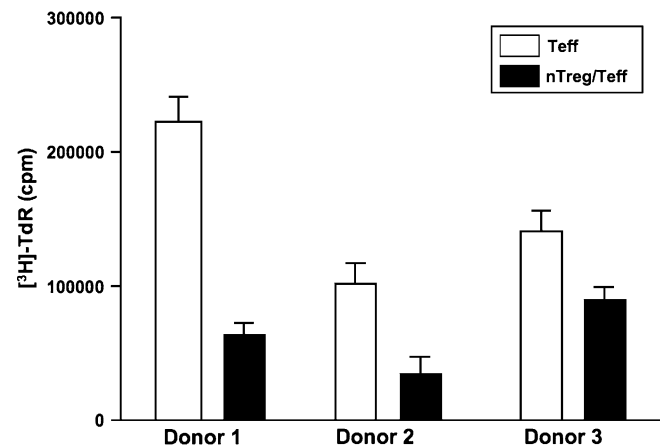


FIG 6. nTreg ability to inhibit proliferation of T effectors is restored 48 hours after SEB. Monocytes were stimulated with SEB for 48 hours. Purified nTregs and/or T effectors were then isolated from the same donors and cocultured with 0.5 μ g/mL anti-CD3. nTreg cell ability to inhibit T-effector proliferation was restored after 48 hours of SEB ($P \leq .05$). Results of 3 independent experiments are shown.

GITR-L induces GITR-dependent nuclear factor- κ B activation and blocks *in vitro* suppression mediated by nTreg cells.²³ GITR-L stimulation upregulates IL-2 secretion by activated T-effector cells and in the presence of IL-2 breaks the anergic state of nTreg cells. Of note, Ji et al²³ reported that soluble GITR-L is able to block nTreg-mediated suppression, but its functional capability was substantially enhanced in the presence of an anti-Flag antibody (Flag tag was artificially added to the recombinant soluble GITR-L), most likely by cross-linking of GITR–GITR-L complexes. This suggested that natural scaffolding of GITR-L on the cell surface is a plausible mechanism for sufficient engagement of GITR. This may explain why we were not able to reproduce these experiments in our human model system by using just soluble recombinant human GITR-L in combination with anti-CD3 stimulation. Furthermore, we did not find soluble GITR-L in the cell culture supernatants from SEB-stimulated human PBMCs after 24 hours of culture.

A recent study reported LPS-induced blockade of murine nTreg cell-mediated suppression.¹⁴ In an attempt to identify factors responsible for this blockade of nTreg cell-mediated suppression, conditioned media from dendritic cells treated with LPS were tested with a variety of blocking antibodies and soluble fusion constructs, including a GITR fusion construct that prevented GITR–GITR-L interaction. The investigators found that IL-6 was a critical cytokine that is induced by LPS and rendered T-effector cells refractory to the suppressor function of nTreg cells. However, it was also found that recombinant IL-6 alone or together with MyD88^{-/-} dendritic cell-conditioned media, despite LPS stimulation, did not lead to blockade of suppression, and nTreg cell-mediated suppression was efficient. These data suggested that IL-6 has to act synergistically with another Toll-like receptor-induced signal for efficient blockade of nTreg cell function. Another group has reported transient upregulation of GITR-L by LPS in murine macrophages³² that, together with the

previous work, could suggest that GITR-L was the missing factor needed to complement IL-6-mediated suppression of nTreg cell function. However, preliminary work in our group suggested that in contrast with murine nTreg cells, human nTreg cells stimulated with superantigen and exogenous IL-6 *in vitro* do not abrogate nTreg function. Specifically, nTreg cells did not proliferate (cpm 264 ± 113) and efficiently suppressed T-effector cell proliferation (cpm 7273 ± 1426 and 1466 ± 451 for T-effector cells and T-effector cells cocultured with nTreg cells, respectively; $n = 3$; $P = .03$). Moreover, anti-IL-6 did not restore the human nTreg anergy and suppressive function. These data highlight an important difference between the murine and human model of GITR–GITR-L interactions, suggesting that cell-cell contact is an important early step in the mechanism of nTreg suppression.

Recently, Stephens et al³⁰ suggested that GITR-L is constitutively expressed by murine APCs (dendritic cells, macrophages, and B cells), but is downregulated 48 to 60 hours after activation through TLRs. GITR-L expression was also observed on the surface of T cells 48 hours after anti-CD3 stimulation. In contrast, the data reported by Tone et al³² showed that GITR-L expression can be transiently upregulated on the surface of murine macrophages and bone marrow dendritic cells by LPS stimulation, and no GITR-L mRNA expression was observed in resting or anti-CD3-stimulated T cells. These authors observed constitutive GITR-L expression by only a portion of murine splenic B cells, macrophages, peritoneal macrophages, and bone marrow dendritic cells. In human beings, constitutive GITR-L cell surface expression has been reported only in umbilical vein endothelial cells, and most recently, in retinal pigment epithelium and photoreceptors.^{31,33} Our data indicate that unlike mouse mononuclear cells, human peripheral blood monocytes do not constitutively express GITR-L but rather upregulate GITR-L transiently, after SEB stimulation. By 48 hours, the ability of these cells to abrogate nTreg function *in vitro* is lost. These data

suggest that relative distribution patterns of GITR on activated responder T cells and nTreg cells and of GITR-L on APCs represent a complex dynamic interaction.

In conclusion, our current study demonstrates for the first time that superantigens upregulate human GITR-L expression on human monocytes. This results in proliferation of nTreg cells and abrogation of their immunosuppressive activity via a cell-cell contact interaction. Prevention of GITR-L induction by superantigens may be a novel approach for control of inflammatory responses mediated by superantigens.

We thank Maureen Sandoval for her assistance in the preparation of this manuscript.

REFERENCES

- Kotzin BL, Leung DY, Kappler J, Marrack P. Superantigens and their potential role in human disease. *Adv Immunol* 1993;54:99-166.
- Proft T, Fraser JD. Bacterial superantigens. *Clin Exp Immunol* 2003;133:299-306.
- Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;155:1151-64.
- Baecher-Allan C, Viglietta V, Hafler DA. Human CD4+CD25+ regulatory T cells. *Semin Immunol* 2004;16:89-98.
- Chatila TA. Role of regulatory T cells in human diseases. *J Allergy Clin Immunol* 2005;116:949-59.
- Seroogy CM, Gern JE. The role of T regulatory cells in asthma. *J Allergy Clin Immunol* 2005;116:996-9.
- Sicherer SH, Leung DY. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects. *J Allergy Clin Immunol* 2005;116:153-63.
- Chinen J, Shearer WT. Advances in Asthma, Allergy and Immunology Series 2004: basic and clinical immunology. *J Allergy Clin Immunol* 2004;114:398-405.
- Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. *Clin Exp Allergy* 2004;34:1364-72.
- Ehrenstein MR, Evans JG, Singh A, Moore S, Wames G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med* 2004;200:277-85.
- Kriegel MA, Lohmann T, Gabler C, Blank N, Kalden JR, Lorenz HM. Defective suppressor function of human CD4+ CD25+ regulatory T cells in autoimmune polyglandular syndrome type II. *J Exp Med* 2004;199:1285-91.
- Ou LS, Goleva E, Hall C, Leung DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. *J Allergy Clin Immunol* 2004;113:756-63.
- Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 2004;199:971-9.
- Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 2003;299:1033-6.
- Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 2003;171:3348-52.
- Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002;3:135-42.
- McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, et al. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002;16:311-23.
- Hauk PJ, Hamid QA, Chrousos GP, Leung DY. Induction of corticosteroid insensitivity in human PBMCs by microbial superantigens. *J Allergy Clin Immunol* 2000;105:782-7.
- Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001;193:1285-94.
- Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25 high regulatory cells in human peripheral blood. *J Immunol* 2001;167:1245-53.
- Fehervari Z, Sakaguchi S. CD4+ Tregs and immune control. *J Clin Invest* 2004;114:1209-17.
- Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003;4:330-6.
- Ji HB, Liao G, Faubion WA, Abadia-Molina AC, Cozzo C, Laroux FS, et al. Cutting edge: the natural ligand for glucocorticoid-induced TNF receptor-related protein abrogates regulatory T cell suppression. *J Immunol* 2004;172:5823-7.
- Goleva E, Cardona ID, Ou LS, Leung DYM. Factors that regulate naturally occurring T regulatory cell-mediated suppression. *J Allergy Clin Immunol* 2005;116:1094-100.
- Choi BK, Bae JS, Choi EM, Kang WJ, Sakaguchi S, Vinay DS, et al. 4-1BB-dependent inhibition of immunosuppression by activated CD4+CD25+ T cells. *J Leukoc Biol* 2004;75:785-91.
- Valzasina B, Guiducci C, Dislich H, Killeen N, Weinberg AD, Colombo MP. Triggering of OX40 (CD134) on CD4+CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. *Blood* 2005;105:2845-51.
- Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 2000;12:431-40.
- Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 2000;192:303-10.
- Kanamaru F, Youngnak P, Hashiguchi M, Nishioka T, Takahashi T, Sakaguchi S, et al. Costimulation via glucocorticoid-induced TNF receptor in both conventional and CD25+ regulatory CD4+ T cells. *J Immunol* 2004;172:7306-14.
- Stephens GL, McHugh RS, Whitters MJ, Young DA, Luxenberg D, Carreno BM, et al. Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells. *J Immunol* 2004;173:5008-20.
- Kwon B, Yu KY, Ni J, Yu GL, Jang IK, Kim YJ, et al. Identification of a novel activation-inducible protein of the tumor necrosis factor receptor superfamily and its ligand. *J Biol Chem* 1999;274:6056-61.
- Tone M, Tone Y, Adams E, Yates SF, Frewin MR, Cobbold SP, et al. Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. *Proc Natl Acad Sci U S A* 2003;100:15059-64.
- Kim BJ, Li Z, Fariss RN, Shen de F, Mahesh SP, Egwuagu C, et al. Constitutive and cytokine-induced GITR ligand expression on human retinal pigment epithelium and photoreceptors. *Invest Ophthalmol Vis Sci* 2004;45:3170-6.