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Systemic Inflammatory Response Elicited by Superantigen Destabilizes T Regulatory Cells, Rendering Them Ineffective during Toxic Shock Syndrome

Ashenafi Y. Tilahun,* Vaidehi R. Chowdhary,† Chella S. David,* and Govindarajan Rajagopalan*‡

Life-threatening infections caused by *Staphylococcus aureus*, particularly the community-acquired methicillin-resistant strains of *S. aureus*, continue to pose serious problems (1–3). It is becoming increasingly evident that higher prevalence of exotoxins might contribute to greater virulence, increased pathogenicity, and rapid spread of community-acquired methicillin-resistant strains of *S. aureus* strains around the world (4–8). Among the staphylococcal exotoxins, the superantigens (SAg) need a special mention because of their extreme potency and unique biological activities (9). Recent studies from our own group and others have clearly shown that SAg play an important role in the pathogenesis of serious infections caused by *S. aureus* such as pneumonia, infective endocarditis, sepsis, and toxic shock syndrome (TSS) (10–14).

SAg are the most potent biological activators of the immune system (15). Unlike conventional Ags, SAg bind directly to MHC class II molecules outside of the peptide-binding groove. Subsequently, they interact with certain TCR Vβ families expressed on both CD4+ as well as CD8+ T cells and cross-link the TCR. This results in rapid activation of 30–50% of the total T cell pool. Activated T cells carry out their respective effector functions, including production of large quantities of several proinflammatory cytokines and chemokines. This results in a robust systemic inflammatory response syndrome, hypotension, multiple organ failure, and, ultimately, death. Overall, excessive activation of the immune system by SAg appears to be the primary cause for immunopathology and mortality in diseases caused by toxicogenic *S. aureus* (16). Therefore, countering the SAg-mediated immune activation could be beneficial in such diseases. The immune system is endowed with several natural regulatory pathways to control such heightened immune responses and to limit the collateral immune damage to the host. The CD4+CD25+Foxp3+ T regulatory cells (Tregs) are one such extensively characterized system (17).

Tregs, either natural or induced, have been shown to suppress almost any type of adaptive immune response, whether elicited in a physiological state or in a pathological state (18, 19). For example, Tregs have been shown to be protective in several acute systemic inflammatory conditions such as LPS-induced shock (20), zymosan-induced shock (21), graft-versus-host disease (22–24), sepsis caused by Gram-negative bacteria (25), and CD28 superagonist-induced inflammatory response syndrome (26), which are all analogous to SAg-induced TSS. Given these findings, Tregs are attractive candidates for the prevention and/or treatment of acute inflammatory diseases caused by SAg. However, the high morbidity and mortality associated with TSS and other staphylococcal SAg-mediated diseases indicate that the normal numbers of endogenous Tregs are ineffective. Therefore,
increasing the Treg numbers is a possible solution. In the current study, we therefore investigated whether increasing the numbers of endogenous Tregs directly in vivo using IL-2/anti–IL-2 immune complexes (IL2C) (27, 28), or by adoptive transfer of ex vivo expanded Tregs (29, 30), could be protective in TSS using HLA-DR3 transgenic mouse model. Unlike conventional laboratory

**FIGURE 1.** Repeated administration of IL2C increases endogenous Tregs in HLA-DR3 transgenic mice. HLA-DR3 transgenic mice were injected with immune complexes comprising IL-2 and anti–IL-2 Abs on days 0, 1, and 2 (3×IL2C). On day 5, mice were killed, and the distribution of various cell types in the spleens was analyzed by flow cytometry. (A) Representative dot plots and bar charts depicting distribution of CD4+CD25+Foxp3+ Tregs in naive and 3×IL2C-treated mice. (B) Bar chart depicting the distribution of CD4+ and CD8+ T cells expressing indicated TCR Vβ families in naive and 3×IL2C-treated mice and (C) B cells and macrophages in naive and 3×IL2C-treated mice. Each bar represents mean ± SE of data obtained from four to six mice in each group. *p < 0.05 compared with naive mice.
mice expressing endogenous mouse MHC class II molecules, HLA class II transgenic mice respond robustly to staphylococcal enterotoxin B (SEB) and suffer from an acute systemic inflammatory disease mimicking human TSS, without the use of any sensitizing or potentiating agents (31, 32). Hence, the HLA-DR3 transgenic mouse model was chosen.

Materials and Methods

Mice

The following mice were used. HLA-DR3 transgenic mice expressing HLA-DRA*0101 and HLA-DRB*0301 and IFN-γ–deficient HLA-DR3 transgenic mice have already been described (31–33). These mice do not express any endogenous mouse MHC class II molecules. Mice were bred within the barrier facility of Mayo Clinic Immunogenetics Mouse Colony (Rochester, MN) and moved to a conventional facility after weaning. All the experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Reagents, Abs, and flow cytometry

Endotoxin-reduced, highly purified SEB (Toxin Laboratories, Sarasota, FL) was dissolved in PBS at 1 mg/ml and stored frozen at −80˚C in aliquots. The purity of SEB was verified by SDS-PAGE, followed by Coomassie blue staining, and the absence of certain other staphylococcal SAG was verified using staphylococcal enterotoxin identification visual immunoassay (3M, St. Paul, MN). The following Abs were used for flow cytometry (BD Biosciences, San Jose, CA): CD4 GK1.5, CD8 53-6.7, TCR Vβ6 RR4-7, TCR Vβ8 F23.1, B220 RA3-6B2; Mac-1 M 170, glucocorticoid-induced TNFR family–related receptor (GITR) DTA-1, CD25 7D4, and Foxp3 FJK-16s. Intracellular staining for Foxp3 was done using a kit from eBiosciences (San Diego, CA). In some experiments, in vivo neutralization of IL-17 was achieved by i.p. administration of 100 μg purified rat anti-mouse anti–IL-17 Abs (clone 50104; R&D Systems). Abs were administered 10 min prior to challenge with SEB (50 μg/mouse). Mice were killed at indicated time points; spleens and thymi were collected in PBS and crushed, and the distribution of indicated cell types was determined using flourochrome-labeled Abs using a flow cytometer. Flow data were analyzed using FlowJo software (Version 10; Tree Star, Ashland, OR).

In vivo expansion of Tregs with IL2C

In vivo expansion of Tregs was achieved by administering IL2C, as described by Boyer et al. (27). Briefly, 1 μl murine IL-2 (1 μg/μl; PeproTech, Rocky Hill, NJ) was added to 5 μl anti-murine IL-2 Ab (0.5 mg/ml; JES6-1A12; eBiosciences), mixed well, and incubated for 30 min at 37˚C. Subsequently, 194 μl 1× PBS was added to this mix, and 200 μl mix was injected into each mouse on days 0, 1, and 2. The amount of IL-2 and anti–IL-2 Abs was scaled up depending on the number of animals. Mice were challenged with SEB on day 5 or left untreated. Mice were bled 3 h after SEB challenge, and all animals were killed 3 d later. Injection of IL2C on days 0, 1, and 2 is hereafter referred to as 3×IL2C. In one set of experiments, mice received 3 times the dose of IL2C described above on days 0, 1, and 2 to study whether increasing the concentration of IL2C would confer better Treg-mediated protection.

In some studies, instead of repeated administration of IL2C, as described above, IL2C were delivered continuously using mini-osmotic pumps. For this, 4 μl murine IL-2 (1 μg/μl) was added to 20 μl anti–IL-2 Ab, mixed well, and incubated for 30 min at 37˚C. Subsequently, 76 μl 1× PBS was added to this mix to make the volume to 100 μl, and the entire content was then loaded into a 7-d release Alzet mini-osmotic pump, as per manufacturer’s protocol (Durect, Cupertino, CA). The entire content was then loaded into a 7-d release Alzet mini-osmotic pump, as per manufacturer’s protocol (Durect, Cupertino, CA). The amount of IL-2 during each cycle of expansion, as recommended in the protocol. Tregs were expanded by two rounds of stimulation cycles, each round lasting 6–7 d. The expanded cells were tested by flow cytometry for expression of CD4, CD25, and Foxp3, and >95% of the cells were found to be bona fide Tregs phenotypically. Five million ex vivo expanded Tregs were injected i.v. via the tail vein into unmanipulated recipients. Twenty-four hours later, recipients were challenged with SEB (50 μg/mouse) or left untreated.

SEB-induced in vitro splenocyte proliferation assay

For T cell proliferation, single-cell suspensions of splenocytes from HLA-DR3 transgenic mice were depleted of RBCs by buffered ammonium chloride lysis. Cells were cultured in HEPES-buffered RPMI 1640 containing 5% FCS, serum supplement, streptomycin, and penicillin, at a concentration of 1 × 10^6 cells/well in 100 μl vol in 96-well round-bottom tissue culture plates. SEB was added at indicated concentrations. After 24 h, the cells were pulsed with tritiated thymidine (1 μg/ml). Cells were harvested 18 h later, and the extent of cell proliferation was determined by a standard thymidine incorporation assay.

Serum cytokine quantification and histopathology

For serum cytokine analyses, HLA-DR3 mice were challenged with SEB (50 μg/mouse in 200 μl, i.p.) or with PBS. Three hours later, animals were bled and sera separated. The cytokine concentrations in the sera were determined in duplicates using a multiplex bead assay, per the manufacturer’s protocol, and using their software and hardware (Bio-Plex, Bio-Rad). Tissues collected in buffered formalin were paraffin embedded, cut, and stained with H&E per standard procedure for histopathologic analysis.

Statistical analyses

Unpaired Student t test was applied to determine the statistical significance when comparing two groups. The difference was considered statistically significant when p < 0.05. Statistical analyses and bar charts were generated using GraphPad Prism, version 6.

Results

Administration of IL2C increases Treg numbers in HLA-DR3 transgenic mice

Administration of IL2C on days 0, 1, and 2 resulted in a significant (3- to 4-fold) increase in the absolute numbers of CD4^+CD25^+CD122^+cells in SEB-treated mice compared with naive mice.

Purification, expansion, and adoptive transfer in vitro expanded Tregs

CD4^+CD25^+ T cells were isolated from naive HLA-DR3 transgenic mice using magnetic mouse Treg purification kit following the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). The final positive selection step was repeated twice to ensure purity, which was verified by testing a small aliquot by flow cytometry by intracellular staining for Foxp3. Purified Tregs were expanded in vitro by culturing them with anti-CD2, anti-CD28 beads (Dynabeads Mouse T-Activator CD3/CD28; Life Technologies, Grand Island, NY), as per manufacturer’s protocol and published literature (29, 30). Culture medium was supplemented with different amounts of IL-2 during each cycle of expansion, as recommended in the protocol. Tregs were expanded by two rounds of stimulation cycles, each round lasting 6–7 d. The expanded cells were tested by flow cytometry for expression of CD4, CD25, and Foxp3, and >95% of the cells were found to be bona fide Tregs phenotypically. Five million ex vivo expanded Tregs were injected i.v. via the tail vein into unmanipulated recipients. Twenty-four hours later, recipients were challenged with SEB (50 μg/mouse) or left untreated.

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FIGURE 2. Modulatory effect of unfractionated Tregs from HLA-DR3 transgenic mice expanded with IL2C on SEB-induced splenocyte proliferative responses in vitro. Unfractionated splenocytes isolated from naive and 3×IL2C-treated mice were stimulated with indicated concentrations of SEB, and the extent of proliferation was determined by tritiated thymidine incorporation assay. Each bar represents mean ± SE of data obtained from four to six mice in each group. *p < 0.05 compared with naive mice.
Foxp3+ Tregs in the spleens by day 5 (Fig. 1A). In vivo administration of 3×IL2C also resulted in an increase in the total numbers of CD4+ T cells, possibly due to an increase in the Tregs cells. However, there were no significant changes in the numbers of CD8+ T cells, B cells, and macrophages in 3×IL2C-treated mice compared with untreated mice (Fig. 1B, 1C). These findings were consistent with previous studies that used IL2C to expand Tregs in vivo (27).

**FIGURE 3.** SEB-induced systemic cytokine/chemokine surge in naive and IL2C-treated HLA-DR3 transgenic mice. HLA-DR3 transgenic mice were left untreated or injected with immune complexes comprising IL-2 and anti–IL-2 Abs on days 0, 1, and 2 (3×IL2C). On day 5, mice were challenged with SEB (50 μg/mouse) or PBS and bled 3 h later. Concentrations of indicated cytokines/chemokines were determined using multiplex assay. Each bar represents mean ± SE of data obtained from six to eight mice in each group. *p < 0.05 compared with naive mice.
Splenocytes from HLA-DR3 transgenic mice treated with IL2C respond robustly to SEB in vitro

As a measure of Treg suppressor activity relevant to our model, we next determined the extent of SEB-induced proliferation of unfractionated splenocytes isolated from 3×IL2C-treated mice in vitro. This was compared with the responses of splenocytes isolated from naive mice. Splenocytes from 3×IL2C-treated mice consistently had higher thymidine uptake following stimulation with SEB than the splenocytes from untreated mice (Fig. 2). This indicated that even though more Tregs are present in the spleens of 3×IL2C-treated mice, they are unable to suppress the proliferative response elicited by SEB in vitro.

Elevated systemic IFN-γ levels in HLA-DR3 transgenic mice treated with IL2C following SEB challenge

TSS in humans is characterized by a systemic cytokine and chemokine storm, which culminates in multiple organ dysfunction and often death (35). Challenging HLA-DR3 transgenic mice with SEB elicits a similar acute response (31). Therefore, in the next set of experiments, the ability of in vivo expanded Tregs to dampen SEB-induced systemic cytokine and chemokine surge was investigated. For this, naïve and 3×IL2C-treated HLA-DR3 transgenic mice were challenged with SEB and bled 3 h later, and levels of several cytokines and chemokines in the sera were determined. Whereas naïve HLA-DR3 mice had low levels of cytokines and chemokines in their sera, as expected, challenging with SEB resulted in a dramatic elevation in serum levels of all cytokines (Th1, Th2, and Th17) and chemokines tested (Fig. 3), as shown by us earlier (31–33). Sera from control 3×IL2C-treated mice also had low levels of cytokines and chemokines similar to naïve HLA-DR3 mice, suggesting that administration of 3×IL2C does not elicit an inflammatory response nor does it result in elevated basal levels of anti-inflammatory cytokines, such as IL-10. However, contrary to the expectation, the serum cytokine/chemokine levels were still highly elevated in 3×IL2C-treated mice challenged with SEB similar to SEB-challenged HLA-DR3 mice that did not receive 3×IL2C.

Interestingly, SEB-challenged, 3×IL2C-treated mice had significantly higher levels of IFN-γ (6642±1840 pg/ml, n=6) compared with 3×IL2C-untreated SEB-challenged HLA-DR3 mice (1836±932.4 pg/ml, n=8, p=0.02) (Fig. 3). Even though 3×IL2C-treated mice challenged with SEB had higher mean serum levels of IL-4, IL-5, and IL-10 than 3×IL2C-untreated SEB-challenged HLA-DR3 mice, these differences were not statistically significant (Fig. 3). With respect to chemokines, 3×IL2C-treated mice challenged with SEB had significantly higher serum levels of MIP-1α compared with SEB-challenged HLA-DR3 mice not treated with 3×IL2C (2306±307.2 pg/ml versus 934.3±120.9 pg/ml, p=0.0006) (Fig. 3).

We next tested using a small cohort of HLA-DR3 mice whether increasing the concentration of IL2C by 3-fold would suppress SEB-induced systemic cytokine/chemokine storm. HLA-DR3 transgenic mice received thrice the normal dose of IL2C on days 0, 1, and 2. On day 3, mice were challenged with SEB, and sera were collected 3 h later and assayed. Whereas we did not enumerate the Treg numbers in these mice, the serum levels of most of the cytokines/chemokines were much higher than in SEB-challenged HLA-DR3 mice treated with regular dose of IL2C. For example, the serum level of IFN-γ was 13,720±1,149 pg/ml, IL-17 was 8,093±643 pg/ml, and IL-6 was 16,693±520 pg/ml (n=4, mean ± SE) in this group (additional data not shown). Overall, these results showed that, even though IL2C treatment...
increased the number of Tregs in vivo, the SEB-induced systemic inflammatory response was largely unaffected. On the contrary, some cytokines, such as IFN-γ, were significantly elevated in the IL2C-treated group.

Expansion of peripheral T cells bearing SEB-reactive TCR is unaffected in mice treated with IL2C

In addition to the systemic cytokine/chemokine storm, expansion of mature, peripheral CD4+ and CD8+ T cells bearing certain TCR Vβ families (which peaks by day 3) is the hallmark of SAg-mediated T cell activation in vivo. Because Tregs are known to suppress T cell activation and expansion either directly or indirectly (36), we next determined the numbers of splenic CD4+ and CD8+ T cells bearing SEB-reactive, TCR Vβ8, and the SEB-nonreactive, TCR Vβ6 as controls. As shown in Fig. 4A, administration of SEB resulted in a significant increase in CD4+ and CD8+ T cells bearing TCR Vβ8, but not TCR Vβ6, compared with naive mice. However, CD4+ and CD8+ T cells bearing TCR Vβ8 were also significantly elevated even in 3×IL2C-treated HLA-DR3 mice challenged with SEB consistent with the serum cytokine/chemokine data.

Superantigen-induced deletion of double-positive thymocytes is more pronounced in IL2C-treated mice

Although the mature peripheral T cells expressing certain TCR Vβ families undergo robust expansion following administration of SEB, the immature CD4CD8 double-positive thymocytes undergo massive apoptosis in a TCR- and cytokine-dependent manner (37, 38). Therefore, we next examined whether Tregs can protect CD4CD8 double-positive (DP) thymocytes from SEB-induced apoptosis. Challenging HLA-DR3 transgenic mice with SEB resulted in a massive reduction in the CD4CD8 DP thymocytes, causing a profound reduction in the total thymocyte numbers as well (Fig. 4B). However, in vivo expansion of Tregs with 3×IL2C conferred little protection from SEB-induced CD4CD8 DP thymocyte deletion (Fig. 4B).

Course of SEB-induced TSS in IL2C-treated HLA-DR3 transgenic mice

We have previously shown that SEB-induced TSS in HLA-DR3 transgenic mice is characterized by extensive inflammatory changes in various vital organs, which culminates in death, similar to human TSS (31). Therefore, we next studied the extent of inflammation in lung, liver, and the small intestines, the organs that are primarily affected during TSS. As shown in Fig. 5, lung, liver, and small intestines from naive as well as HLA-DR3 mice treated with 3×IL2C showed no signs of inflammation. However, mice challenged with SEB had extensive inflammation in the lungs, liver, and the small intestines irrespective of whether they received 3×IL2C or not, indicating that in vivo administration of 3×IL2C had little protective effect from organ pathology.

We also studied the effect of 3×IL2C treatment on overall protection from TSS in HLA-DR3 transgenic mice. Throughout the study, 40% (4 of 10 mice) of the 3×IL2C-untreated HLA-DR3 transgenic mice succumbed to SEB-induced TSS. However, 50% (6 of 12 mice) of 3×IL2C-treated HLA-DR3 mice died of SEB-induced TSS. There was no statistically significant difference in the survival between these two groups (additional data not shown). Taken together, these results suggested that, even though administration of 3×IL2C resulted in an increase in the total number of Tregs in vivo, neither SEB-induced systemic inflammatory response, multiple organ pathology, nor mortality was reduced.

![FIGURE 5. Organ pathology during SEB-induced TSS in HLA-DR3 transgenic mice and its modulation by IL2C. HLA-DR3 transgenic mice were left untreated or injected with IL2C on days 0, 1, and 2 (3×IL2C) to expand endogenous Tregs. On day 5, mice were challenged with SEB (50 μg/mouse) or PBS and killed 3 d later. Organs were collected in buffered formalin, paraffin embedded, and processed. H&E-stained sections were evaluated by light microscopy. Representative low and high magnification images are shown. Scale bar, 100 μM.](http://www.jimmunol.org/)
Rather, some of the key inflammatory mediators, such as IFN-γ and MIP-1α, were increased in 3×IL2C-treated mice.

**Tregs expanded in vivo with IL2C delivered via mini-osmotic pump also fail to attenuate SEB-induced inflammatory responses**

Considering the mechanisms by which IL2C cause expansion of Tregs (39, 40), we hypothesized that continuous delivery of IL2C using mini-osmotic pumps would cause a more robust expansion of Tregs, which could be more effective in mitigating the systemic inflammatory response seen during TSS. As we hypothesized, pump-IL2C mice harbored more Tregs than all other groups (mean ± SE of CD25+Foxp3+ T cells within CD4+ gated cells in the spleens were as follows: naive = 0.95 ± 0.13; IL-2 = 2.43 ± 0.19; anti-IL-2 = 1.59 ± 0.14; and pump-IL2C = 4.3 ± 0.35 millions; naive versus IL-2, naive versus pump-IL2C, and IL-2 versus pump-IL2C, p < 0.05). However, chronic delivery of IL2C using mini-osmotic pump did not significantly increase the Treg numbers beyond to that seen in mice treated with 3×IL2C. This might suggest the presence of homeostatic threshold for total number of Tregs. We next tested the efficacy of Tregs expanded with pump-IL2C in attenuating SEB-induced systemic cytokine/chemokine surge. SEB-unchallenged naive and pump-IL2C-treated mice had comparable low levels of cytokines and chemokines tested (Supplemental Fig. 1). Interestingly, serum levels of IFN-γ in pump-IL2C mice challenged with SEB were significantly higher than control SEB-challenged mice, a phenomenon that was observed even with 3×IL2C-treated mice (Supplemental Fig. 1). Serum IL-5 was also elevated in SEB-challenged pump-IL2C-treated mice. Whereas the serum levels of IL12p40, IL-17, and KC were statistically lower in pump-IL2C mice challenged with SEB compared with control SEB-challenged mice, these mediators were still very high compared with naive SEB-unchallenged pump-IL2C mice (Supplemental Fig. 1). Neither the expansion of mature peripheral T cells expressing certain TCR Vβ family nor deletion of CD4CD8 DP thymocytes was blocked in SEB-challenged pump-IL2C-treated mice (data not shown). As would be expected from the above findings, lungs, livers, kidneys, and intestines from SEB-challenged pump-IL2C-treated mice displayed similar pathology to that of SEB-challenged HLA-DR3 mice without pump-IL2C treatment (data not shown).

**Adoptive transfer of ex vivo expanded Tregs**

Because in vivo expansion of endogenous Tregs using IL2C did not present any appreciable benefits, we next investigated whether adoptive transfer of ex vivo expanded Tregs would be protective in TSS. Naïve Tregs isolated from HLA-DR3 transgenic mice were expanded using anti-CD3/anti-CD28 beads. Phenotypic analysis of the expanded cells prior to adoptive transfer confirmed that >95% of the cells were CD4+CD25+Foxp3+. One day after adoptive transfer of 5 million Tregs, the recipients were challenged with SEB; serum cytokine and chemokine levels were quantified 3 h later. As shown in Supplemental Fig. 1, HLA-DR3 mice challenged with SEB had significantly elevated levels of all the cytokines and chemokines tested compared with naive mice concordant with previous results. Although adoptive transfer of Tregs per se did not cause any significant elevation in cytokines or chemokines (Supplemental Fig. 2), SEB-challenged HLA-DR3 mice that had received Tregs still had significantly elevated levels of these mediators. However, certain chemokines (such as eotaxin, MCP-1, and KC) as well as IL-6 were significantly lower (p < 0.05) in SEB-challenged Treg-treated HLA-DR3 mice compared with SEB-challenged HLA-DR3 mice that did not receive any Tregs. Interestingly, IL-5 was significantly elevated in the sera of SEB-challenged HLA-DR3 mice that received Tregs compared with those that did not receive Tregs (4236 ± 229 pg/ml versus 1410 ± 147 pg/ml, p < 0.05, respectively; Supplemental Fig. 2). This indicated that, whereas adoptive transfer of Tregs suppressed some chemokines, most of the potentially pathogenic cytokines/chemokines, such as IL-1, IL-12, IL-17, IFN-γ, MIP, MCP, and RANTES, were still elevated. Moreover, SEB-induced expansion of peripheral T cells and deletion of CD4CD8 DP thymocytes were not attenuated in Treg-treated, SEB-challenged HLA-DR3 mice (data not shown). Notably, organ pathology in these mice was also similar to SEB-challenged HLA-DR3 mice that did not receive any Tregs (data not shown). Overall, these results indicated that increasing the Treg numbers, either by expansion of endogenous Tregs or by adoptive transfer, does not confer protection from SEB-induced TSS.

**Homeostasis of Tregs in IL2C-treated mice challenged with SEB**

As most of the SAg-driven immunopathological processes were not attenuated by Tregs either expanded in vivo or transferred adoptively after two rounds of ex vivo expansion, we next investigated the fate of Tregs in mice undergoing TSS. Surprisingly, the numbers of Tregs still remained elevated in mice treated with IL2C even on day 8 (i.e., 6 d after cessation of treatment of IL2C) when not challenged with SEB. However, the numbers of Tregs in these mice fell drastically when they were challenged with SEB (Fig. 6). Similar results were observed with pump-IL2C–treated mice and mice with adoptively transferred Tregs. In both these groups, the Tregs remained elevated in recipients that were not challenged with SEB. However, in recipients that were challenged with SEB, the Treg numbers were significantly reduced. In pump-IL2C mice,
the Treg numbers fell to the level seen in naive mice, and, in mice that received ex vivo expanded Tregs, the Treg numbers were significantly below that seen in naive mice (data not shown). Overall, a reduction in the Treg numbers in mice challenged with SEB could partly explain why SAg-induced inflammatory responses were unabated (quantitative defect).

Another possibility is that the SAg-induced inflammatory milieu somehow compromised the immunoregulatory capabilities of Tregs (qualitative defect), at the same time rendering the effector T cells less amenable to Treg-mediated suppression. GITR is one such pathway that can accomplish both these consequences (41). GITR is a classical cell surface marker that is constitutively expressed on Tregs. On the contrary, naive conventional T (Tconv) cells express very low levels of GITR. However, GITR expression is upregulated on Tconv cells upon activation and functions as a costimulatory molecule (42). Engagement of GITR on Tregs by GITR-L can negatively affect their suppressor activity, whereas ligation of GITR expressed on activated Tconv cells could render them less susceptible to Treg-mediated suppression (43). Given its overall anti-immunosuppressive property, we next investigated the expression profile of GITR on Tregs as well as on Tconv cells during TSS in HLA-DR3 mice.

As shown in Fig. 7, in naive HLA-DR3 mice, close to 80% of the Foxp3+CD4+ T cells (Tregs) were GITR+, as expected. Even the median fluorescent intensity (MFI) of GITR was also high on naive Foxp3+CD4+ T cells. Whereas IL2C-treated mice had slightly higher percentage of GITR+ Tregs as well as higher MFI of GITR at day 6, they reduced to naive levels by day 7. Interestingly, in mice challenged with SEB, almost 100% of the Foxp3+CD4+ T cells expressed GITR, and the MFI of GITR on Tregs also increased. SEB-induced upregulation of GITR expression on Tregs could also be appreciated in 3×IL2C-treated mice (Fig. 7). These results suggested that, in the systemic inflammatory milieu, the expression of GITR on Tregs increases significantly.

In the Tconv cell (Foxp3−) compartment, only 10% of the CD4+ T cells expressed GITR in naive mice, and even the MFI was also very low (Fig. 7B, 7C), consistent with previous results (42). However, challenging with SEB resulted in a time-dependent increase in percentage of GITR+ CD4+ Tconv cells. At 24 h, close to 60% of the CD4+ Tconv cells were GITR+, whereas, by 48 h, nearly 80% of them were GITR+. Even the MFI of expression of GITR was also high on Tconv cells (Fig. 7, Supplemental Fig. 3). A similar pattern was seen in IL2C-treated HLA-DR3 mice.

**FIGURE 7.** Expression profile of GITR on Tregs and Tconv cells in HLA-DR3 transgenic mice. HLA-DR3 transgenic mice were left untreated or injected with IL2C on days 0, 1, and 2 to expand Tregs. On day 5, mice were challenged with SEB (50 μg/mouse) or PBS. Mice were killed 24 (day 6) or 48 h (day 7) later, and splenocytes were isolated and stained with indicated Abs. (A) Representative dot plots depicting distribution of CD4+Foxp3+ and CD4+Foxp3− T cells in different groups of mice. (B) Histogram overlays depicting expression of GITR on the cells gated as above. (C) Bar chart depicting percentages of GITR+ cells within the indicated gates, and (D) bar chart depicting MFI of GITR expression on cells within the indicated gates. Each bar represents mean ± SE of data obtained from three mice in each group. *p < 0.05 compared with naive mice.
challenged with SEB (Fig. 7, Supplemental Fig. 3). We also analyzed the expression of GITR on CD4+ and CD8+ T cells expressing SEB-reactive TCR Vβ8 and SEB-nonreactive TCR Vβ6 during TSS. As shown in Supplemental Fig. 4, a low percentage of TCR Vβ6 as well as Vβ8+ CD4+ and CD8+ T cells from naive HLA-DR3 mice expressed GITR. Upon challenge with SEB, almost 100% of SEB-reactive Vβ8+ CD4+ and CD8+ T cells expressed GITR, as expected. Interestingly, even though TCR Vβ6 does not bind to SEB (and hence, TCR Vβ8+ CD4+ and CD8+ T cells are not directly activated by SEB), >75% of TCR Vβ6+ CD4+ and CD8+ T cells from SEB-challenged mice expressed GITR. Thus, challenging with SEB resulted in upregulation of GITR, an important anti-immunoregulatory molecule, on the Tregs and to a much higher degree in the Tconv cells.

**IFN-γ and IL-17 destabilize Tregs during SEB-induced systemic inflammatory response syndrome**

Recent studies have suggested that the phenotype of Tregs is not stable and that, under certain inflammatory conditions, particularly in the presence of IFN-γ and IL-17, the Tregs may lose their Foxp3 expression, convert to proinflammatory type, and may actively produce proinflammatory cytokines such as IFN-γ and IL-17 (44–46). Because TSS is characterized by a profound elevation in systemic levels of several inflammatory mediators, particularly IFN-γ and IL-17, we hypothesized that this inflammatory milieu may not be conducive for the maintenance of Tregs. Therefore, we next investigated the roles of IFN-γ and IL-17 in Treg homeostasis during TSS using IFN-γ-deficient HLA-DR3 transgenic mice and neutralizing anti–IL-17 Abs.

First of all, the number of Tregs in the spleens of HLA-DR3 mice challenged with SEB and treated with isotype control Abs was significantly reduced compared with naive animals, consistent with the previous results (Fig. 8). Interestingly, neutralization of IL-17 in vivo with Abs restored the numbers of Tregs recovered from HLA-DR3 transgenic mice undergoing TSS to that seen in naive mice, suggesting that IL-17 plays some role in Treg homeostasis. With respect to IFN-γ, interestingly, even the naive HLA-DR3-IFN-γ−/− mice had significantly higher numbers of Tregs compared with HLA-DR3-IFN-γ+/+ mice. Surprisingly, HLA-DR3-IFN-γ−/− mice challenged with SEB and treated with isotype control Abs yielded more Tregs, whereas maximum Tregs were present in HLA-DR3-IFN-γ−/− mice treated with anti–IL-17 Abs. These results suggested that IFN-γ and IL-17 act together to reduce the number of Tregs, whereas blocking IFN-γ and IL-17 led to an increase in Tregs during TSS.

**Modulation of GITR expression by IFN-γ during TSS**

In the next set of experiments, we investigated the effect of IFN-γ on expression of GITR on Tconv cells and Tregs during TSS. For this, HLA-DR3-IFN-γ−/− and HLA-DR3-IFN-γ+/+ mice were challenged with 10 μg SEB and killed 48 and 72 h later, and the expression of profile of GITR was analyzed by flow cytometry. As shown in Fig. 9A, both CD4+ as well as CD8+ TCR Vβ8+ T cells from naive HLA-DR3-IFN-γ−/− mice expressed reduced levels of GITR compared with naive HLA-DR3-IFN-γ+/+ mice. Even though the CD4+ as well as CD8+ TCR Vβ8+ T cells from HLA-DR3-IFN-γ−/− mice upregulated GITR expression following SEB challenge at 48 h, their GITR levels were lower when compared with cells from HLA-DR3-IFN-γ−/− mice. The overall expression of GITR fell by 72 h and was comparable between HLA-DR3-IFN-γ−/− and HLA-DR3-IFN-γ+/+ groups. Similar pattern was seen with CD4+ as well as CD8+ TCR Vβ6+ T cells and CD4+ Foxp3+ T cells (summarized in Fig. 9B).

**Discussion**

*S. aureus* is still one of the leading causes of lethal infections in humans (47). Recent reports have shown that, at least in the United States, *S. aureus* causes more deaths than HIV/AIDS (48, 49). Higher prevalence of various exotoxins, including SAg, could be one of the contributing factors for the greater virulence of certain *S. aureus* strains (4–8). As SAg are the most potent biological activators of the immune system, it is believed that SAg that are produced in vivo following an infection with toxigenic *S. aureus* causes a rapid immune activation, a robust systemic inflammatory response syndrome, multiple organ failure, and, ultimately, death (10–13). Therefore, attenuating the magnitude of immune activation using Tregs could prove to be beneficial in diseases involving SAg, such as TSS. Observations that such an approach is effective in certain infectious disease models (50) and in other models of acute systemic inflammation that are analogous to TSS (20–26) lend support to this hypothesis. Nonetheless, high morbidity and mortality associated with TSS suggest that normal numbers of endogenous Tregs are ineffective in countering SAg-mediated immune activation. Therefore, we investigated whether increasing the Tregs above the endogenous levels could be beneficial in TSS. Our study revealed that increasing the Treg numbers, either by in vivo expansion of endogenous Tregs using IL2C or adoptive transfer of Tregs expanded ex vivo using anti-CD3/anti-CD28 beads, was ineffective. Although the precise reasons as to why Tregs were ineffective in our model could not be identified, we put forth the following hypotheses.

Instability of the Tregs in an inflammatory milieu could be one of the mechanisms. Even though higher numbers of Tregs persisted for a long period (even at day 8) in mice that were treated with IL2C or adoptively transferred with Tregs, challenging with SEB led to
a rapid decline in the numbers of Tregs. This could be due to deletion of Tregs and/or conversion of Tregs to effector T cells. Recent studies have demonstrated that an inflammatory environment, such as in TSS, not only leads to loss of Treg suppressor functions, but can also promote conversion of Tregs to proinflammatory T effector cells, thereby accentuating the inflammation, rather than suppressing inflammation (44–46, 51–56). Elevated serum IFN-\(\gamma\) levels in SEB-challenged IL-2C–treated mice (Fig. 3, Supplemental Fig. 1) support this Treg to T effector cell conversion theory. Notably, IFN-\(\gamma\)–producing Tregs have also been demonstrated in humans under inflammatory conditions (57, 58). Increased recovery of Tregs from SEB-challenged mice, in which IL-17 and IFN-\(\gamma\) were blocked (Fig. 8), further supports the emerging theme that excessive amounts of proinflammatory cytokines, such as IL-17 and IFN-\(\gamma\), destabilize Tregs, rendering them ineffective as suppressor cells, and covert them to inflammatory cells (59–61). Given the pathogenic role of IFN-\(\gamma\) in TSS, conversion of Treg to IFN-\(\gamma\)-producing type may be detrimental during TSS (33). It is also possible that administration of IL-2C led to a small, but appreciable increase in Tconv cells in vivo, which resulted in elevated cytokine levels.

In addition to the quantitative defect in Tregs, as discussed above, there could be qualitative defects in Tregs in mice undergoing TSS. Increased resistance of effector T cells to Treg-mediated immune regulation could be another reason for the ineffectiveness of Tregs in our model. Multiple factors could compromise Treg function, at the same time helping the effector T cells overcome Treg-mediated immunosuppression. These include the TLR pathway (62, 63) and the GITR pathway (41, 64). The TLR pathway might disrupt Treg functions during infection with toxigenic S. aureus in which staphylococcal TLR agonists (such as lipoteichoic acid, CpG DNA motifs, cell wall peptidoglycan, etc.) could contribute to overriding Treg-mediated suppression (65). However, in the SAg-induced TSS model, we believe that the GITR pathway plays a major role.

GITR is a member of the TNFR superfamily. GITR is constitutively expressed on Tregs, whereas it is rapidly upregulated on activated T cells, where it functions as a costimulatory molecule (42). Several studies have demonstrated that ligation of GITR on Tregs can detrimentally affect their suppressor activity, although ligation of GITR on effector T cells makes them more resistant to Treg-mediated suppression (41, 43, 66). In our study, the SAg-induced systemic inflammatory milieu not only increased the expression of GITR on Tregs, it caused a rapid upregulation of GITR Tconv cells such that 80–90% of the CD4\(^+\) as well as CD8\(^+\) Tconv cells were GITR\(^+\). As GITRL is constitutively expressed on several cell types, including DC, B cells, and macrophages (43, 67), ligation of GITR on Tregs could abrogate their suppressor activity, whereas in T effector cells, GITR could protect them from Treg-mediated immune regulation.

**FIGURE 9.** Role of IFN-\(\gamma\) on upregulation of GITR on Tconv cells and Tregs during TSS. IFN-\(\gamma\)-sufficient and IFN-\(\gamma\)-deficient HLA-DR3 transgenic mice were challenged with SEB (10 \(\mu\)g/mouse). Mice were killed 48 or 72 h later, and splenocytes were isolated and stained with indicated Abs. (A) Representative histogram overlays depicting the expression profile of GITR on indicated gated population. (B) Bar charts depicting MFI of GITR expression (mean \(\pm\) SE) on cells within the indicated gates. *\(p < 0.05\) between IFN-\(\gamma\)-sufficient and IFN-\(\gamma\)-deficient group.
Further studies using HLA-DR3.3FN-γ−/− and HLA-DR3.3FN-γ−/− mice suggested that upregulation of GITR on Tconv cells and Tregs during TSS could be partly mediated through IFN-γ. Nonetheless, GITR was still upregulated on T cells from HLA-DR3.3FN-γ−/− mice, suggesting that other pathways are also involved. Overall, contradictory to the widespread notion that Tregs could be beneficial in several inflammatory conditions, our study showed that, at least in SAG-induced TSS, Tregs are ineffective due to several reasons. On the contrary, Tregs may even promote immunopathology by converting into an inflammatory phenotype.

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References