

Regulatory T Cells Enhance Mast Cell Production of IL-6 via Surface-Bound TGF- β

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Mast cell degranulation is a hallmark of allergic reactions, but mast cells can also produce many cytokines that modulate immunity. Recently, CD25⁺ regulatory T cells (Tregs) have been shown to inhibit mast cell degranulation and anaphylaxis, but their influence on cytokine production remained unknown. In this study, we show that, rather than inhibit, Tregs actually enhance mast cell production of IL-6. We demonstrate that, whereas inhibition of degranulation was OX40/OX40 ligand dependent, enhancement of IL-6 was due to TGF- β . Interestingly, our data demonstrate that the Treg-derived TGF- β was surface-bound, because the interaction was contact dependent, and no TGF- β was detectable in the supernatant. Soluble TGF- β 1 alone was sufficient to enhance mast cell IL-6 production, and these supernatants were sufficient to promote Th17 skewing, but those from Treg–mast cell cultures were not, supporting this being surface-bound TGF- β from the Tregs. Interestingly, the augmentation of IL-6 production occurred basally or in response to innate stimuli (LPS or peptidoglycan), adaptive stimuli (IgE cross-linking by specific Ag), and cytokine activation (IL-33). We demonstrate that TGF- β led to enhanced transcription and de novo synthesis of IL-6 upon activation without affecting IL-6 storage or mRNA stability. In vivo, the adoptive transfer of Tregs inhibited mast cell-dependent anaphylaxis in a model of food allergy but promoted intestinal IL-6 and IL-17 production. Consequently, our findings establish that Tregs can exert divergent influences upon mast cells, inhibiting degranulation via OX40/OX40 ligand interactions while promoting IL-6 via TGF- β . *The Journal of Immunology*, 2012, 188: 594–603.

Best known for their effector functions in allergic and autoimmune inflammation, mast cells have the capacity to produce a plethora of mediators involved in both innate and adaptive immunity (1–3). Of interest to us, mast cells have the capacity to generate a wide variety of cytokines, and the specific profile of cytokines generated by mast cells during an immune response can shape the nature of the ongoing inflammatory response. For example, mast cell-derived IL-10 has been shown to limit skin inflammation during contact dermatitis (4), whereas conversely, mast cell-derived TNF promotes Ag-mediated airway inflammation and enhances T cell activation (5, 6). Recently, IL-6 has been described as an important early cytokine produced by mast cells because mast cell-derived IL-6 was shown to be necessary for bacterial clearance and host survival in a mouse model of *Klebsiella pneumoniae* infection (7). Cytokine production by mast cells can also occur independent of the degranulation re-

sponse (8–10). However, the mechanisms that control the release of selective mediators from mast cells are unknown.

Natural regulatory T cells (Tregs) make up 5–10% of the naive peripheral CD4⁺ T cell population and play critical roles in the maintenance of tolerance and resolution of inflammation (11). Tregs can exert their suppressive effects by a diverse array of mechanisms that include directly engaging other immune cells via a number of cell-surface receptors (e.g., GITR, CTLA-4, and OX40), as well as through the release of cytokines IL-10, IL-35, and TGF- β (12). Although they are best recognized for their ability to mediate Ag-specific suppression of immune responses, recent evidence has shown that Tregs exert influences that are Ag independent, such as infectious tolerance (13). In this study, Tregs cause naive T cells to develop into new Tregs, allowing for an increased suppressive environment and expanded immunological repertoire of tolerance. This function of Tregs is dependent on cell surface-bound TGF- β (14), establishing that TGF- β bound to the surface of Tregs possesses biological activity.

Tregs have recently been shown to be one way that mast cell release of mediators is controlled. Mast cell degranulation upon stimulation by IgE/Ag-mediated activation is suppressed by coculture with Tregs, and in vivo depletion of Tregs enhanced mast cell-dependent anaphylactic responses (15). Although mast cells and Tregs possess a number of potential interaction partners, this suppression of degranulation required OX40/OX40 ligand (OX40L) interactions. Mast cells and Tregs exhibit substantial colocalization in tissues and lymph nodes (15, 16), and Tregs also recruit mast cells into tissues. For example, Tregs promote mast cell progenitor recruitment to the lung during allergic inflammation (17), whereas Treg-derived IL-9 promotes recruitment of mast cells into transplanted allografts, important for maintaining allograft tolerance (18). Therefore, mast cell/Treg interactions are likely to be occurring during homeostasis as well as during the course of an inflammatory response. However, the influence of Tregs on mast cell production of cytokines has not been investigated.

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Abbreviations used in this article: BMDC, bone marrow-derived mast cell; MCPT-1, mast cell protease-1; MHC II, MHC class II; OX40L, OX40 ligand; PDL1, programmed death 1 ligand 1; PGN, peptidoglycan; SEB, staphylococcal enterotoxin B; SEBOVA, OVA plus staphylococcal enterotoxin B adjuvant; Treg, regulatory T cell.

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In this study, we confirm that coculture of Tregs with mast cells suppresses degranulation, but show that this actually enhances the levels of IL-6 being produced from the mast cell. Mechanistically, this is contact dependent and but independent of the OX40/OX40L-dependent inhibitory effects of Tregs on mast cell degranulation. Instead, enhancement of IL-6 is dependent on surface-bound TGF- β and is driven by enhancing the de novo generation of IL-6 upon mast cell stimulation. Using a model of food allergy, we demonstrate that the adoptive transfer of Tregs into sensitized mice prevents mast cell-dependent anaphylaxis upon food challenge. Concomitantly, mice that received Tregs exhibited significantly enhanced IL-6 and IL-17 levels in the intestinal tissue.

Consequently, our data demonstrate that Tregs possess an unappreciated capacity to enhance proinflammatory cytokines from mast cells while simultaneously suppressing their degranulation. Importantly, our findings establish that these events occur via divergent mechanisms.

Materials and Methods

Mice

C57BL/6 and BALB/C mice were purchased from Taconic Farms (Cambridge City, IN). IL-6^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Foxp3-GFP mice were bred and housed under specific pathogen-free conditions at Northwestern University. All experiments were approved by the Northwestern University Animal Care and Use Committee.

Bone marrow-derived mast cell differentiation

Bone marrow was isolated from the femurs of female, age-matched C57BL/6 and IL-6^{-/-} mice and cultured in complete media (RPMI 1640 with 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, and 0.05 mM 2-ME) containing 30 ng/ml rIL-3 (R&D Systems). After 4 wk, bone marrow-derived mast cells (BMMCs) were analyzed for surface expression of Fc ϵ RI and c-Kit by flow cytometry. Cultures of >95% Fc ϵ RI⁺ and c-Kit⁺ BMMCs were used.

Isolation of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells

CD4⁺CD25⁺ T cells were isolated from pooled spleen and lymph nodes of naive C57BL/6, IL-6^{-/-}, or Foxp3-GFP female mice using CD4⁺CD25⁺ T cell isolation kit (Miltenyi Biotec) following the manufacturer's instructions, which involved negative isolation for CD4⁺ cells followed by positive isolation for CD25⁺ cells. CD4⁺CD25⁻ T cells were obtained from the negatively selected fraction of cells after the CD4⁺ enrichment. Purity was determined by flow cytometry by surface CD4, CD25, and intracellular Foxp3 staining using Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. The population of isolated Tregs recovered using this method was >90% double positive for CD4⁺CD25⁺, and, similarly, >90% were also positive for Foxp3⁺.

BMMC and T cell stimulation

Prior to use, BMMCs were sensitized in complete media with 1 μ g/ml DNP-specific IgE (Sigma-Aldrich) overnight. For coculture experiments, isolated T cells (CD25⁺ or CD25⁻) were cultured in a 1:1 ratio with IgE-sensitized BMMCs with 1×10^5 cells. Unless otherwise indicated, T cells were stimulated using anti-CD3/CD28 Dynabeads (Invitrogen). Beads were used at a 1:1 bead to T cell ratio. Where indicated, BMMCs were stimulated by IgE cross-linking using 0.5 μ g/ml DNP-HSA (Sigma-Aldrich), LPS (0127:B8 Sigma L4516; Sigma-Aldrich), peptidoglycan (PGN; 6538 Invivogen #tlrl-pgnsa; Invivogen), or recombinant mouse IL-33 (R&D Systems). For rTGF- β experiments, IgE-sensitized BMMCs were treated with rTGF- β 1 (R&D Systems) at the doses indicated for 24 h followed by activation with 0.5 μ g/ml DNP-HSA for the time periods indicated. For Transwell separation, Transwells (Costar; Corning) with a 0.4- μ m insert were used with BMMCs in the insert and Tregs in the lower chamber. Supernatants were collected from the lower chamber and analyzed.

Reagents

Staphylococcal enterotoxin B (SEB; Toxin Technologies) was used under approval by the Northwestern University Office of Research Safety and

under the requirements of the Centers for Disease Control and Prevention and the Department of Agriculture. LPS (0127:B8) and OVA (grade V) were obtained from Sigma-Aldrich.

Neutralizing Abs and recombinant cytokines

Rat anti-mouse OX40L (clone MGP34) was purchased from Alexis Biochemicals. Rat anti-mouse programmed death 1 ligand 1 (PDL1; clone MIH5), rat anti-mouse ICOS ligand (clone HK5.3), and rat anti-mouse MHC class II (MHC II; I-A/I-E) (clone M5/114.15.2) blocking Abs, rat IgG2a, and rat IgG2b controls were purchased from eBioscience. Rat IgG2c control was purchased from BioLegend. Rat anti-mouse GITR (clone DTA-1) was purchased from BD Pharmingen. Recombinant mouse IL-6, recombinant human TGF- β , mouse anti-TGF- β 1, 2, 3 (clone 1D11), normal mouse IgG1, chicken anti-mouse TGF- β 1, and chicken IgY were purchased from R&D Systems. BMMCs or T cells were incubated with blocking Abs for 1 h prior to coculture. Samples were stimulated by IgE cross-linking with DNP-HSA and supernatants collected at 24 h. The blocking Abs remained in the culture for the duration of this whole period. For Th17 generation experiments, anti-IL-4 and anti-IFN- γ were purchased from BioXCell. Soluble anti-CD3 ϵ and anti-CD28 were purchased from BD Biosciences.

In vitro generation of Th17 cells using BMMC supernatants

Naive CD4⁺CD62L⁺ T cells were isolated from pooled spleens of naive C57BL/6 mice using CD4⁺CD62L⁺ isolation kit (Miltenyi Biotec) following the manufacturer's instructions. Twenty-four-well plates were coated with anti-hamster IgG (Jackson ImmunoResearch Laboratories) at 3 μ g/ml. Following incubation, plates were washed with PBS, and naive T cells were plated at 5×10^4 cells/ml. A total of 500 μ l BMMC supernatant from cultures of BMMCs alone, BMMC-TGF- β , and BMMC-Treg, in which BMMCs were activated through IgE/Ag cross-linking, was added to naive T cells with 500 μ l fresh media. Soluble anti-CD3 ϵ and anti-CD28 were added at 0.2 μ g/ml for T cell stimulation. As a positive control for Th17 generation, naive T cells were cultured under the following conditions: 20 ng/ml rIL-6, 2 ng/ml rTGF- β , 5 μ g/ml anti-IL-4, and 5 μ g/ml anti-IFN- γ with anti-CD3 and anti-CD28. Untreated samples received only anti-CD3 ϵ and anti-CD28 stimulation. Cells were cultured for 3 d, and then restimulated with PMA/ionomycin in the presence of monensin (GolgiStop; BD Biosciences) for 4 h. All samples were stained for intracellular IL-17 and analyzed by flow cytometry.

Histamine and cytokine/chemokine analysis

Histamine enzyme immunoassay (BD Biosciences) was used to detect histamine in culture supernatants and was performed following the manufacturer's instructions. IL-6 was detected in culture supernatants and cell lysates by sandwich ELISA (BD Biosciences). Mouse Inflammation CBA kit (BD Biosciences) was used to detect TNF, IFN- γ , IL-2, and MCP-1 in culture supernatants following the manufacturer's instructions.

Real-time RT-PCR and mRNA stability assay

IgE-treated BMMCs were treated with rTGF- β for 24 h. Both untreated and treated BMMCs were stimulated with DNP-HSA for 2 h. For mRNA stability assays, actinomycin D (Sigma-Aldrich) was then added (5 μ g/ml), and cells were collected at the time points indicated (Fig. 6D). Total RNA was isolated using Qiagen RNeasy kits (Qiagen). cDNA was synthesized using a qScript cDNA synthesis kit (Quanta). Gene expression was determined by PCR using an ABI 7500 Thermal Cycler (Applied Biosystems) and specific TaqMan probes (Applied Biosystems) for each gene of interest. Expression of β -actin was used as a housekeeping gene for analysis of changes in the cycle threshold values. The fold induction above BMMCs alone was determined based on changes in the δ cycle threshold values.

Flow cytometry

For staining of cell-surface TGF- β , isolated CD4⁺CD25⁺ T cells were stimulated for 24 h with CD3/CD28 stimulation. Samples were blocked with anti-CD16/CD32 for 15 min at 4°C before being incubated with unconjugated chicken anti-TGF- β or chicken IgY (R&D Systems) at 1 μ g/10⁶ cells for 20 min. After washing, allophycocyanin-conjugated anti-chicken IgY (Jackson ImmunoResearch Laboratories) was added for 20 min and then washed three times before analysis. Samples were gated on CD4⁺ cells and analyzed using FlowJo software (Tree Star). For intracellular IL-17 staining, PE anti-IL-17 (BD Biosciences) or isotype control was used with BD Biosciences intracellular staining buffer set according to the manufacturer's instructions (BD Biosciences).

Experimental food allergy model

Mice (BALB/C, 4–6 wk old, maintained on an OVA-free diet) were sensitized as previously described (19). Briefly, mice received 100 μ g OVA with 20 μ g SEB adjuvant via a ball-ended mouse-feeding needle once per week for 8 wk. Sensitization was confirmed by serum OVA-specific IgE analysis, as previously described (19). At week 9, sensitized mice received 0.5×10^6 Tregs or CD25[−] T cells (isolated from the spleen and mesenteric lymph nodes of naive BALB/C mice) i.v. via retro-orbital injection. Twenty-four hours later, all mice were challenged with an oral administration of 5 mg OVA. Symptom scoring was performed every 15 min for 1 h in a blinded fashion by two independent investigators according to previously described parameters of symptoms for determining IgE-mediated responses in mouse models of food allergy (20). Concurrently, airway obstruction responses, as determined by changes in the enhanced pause (Penh) response, were measured using whole-body plethysmography (BUXCO, Troy, NY). Serum mast cell protease-1 (MCPT-1) levels were assessed by sandwich MCPT-1 ELISA following the manufacturer's instructions (BD Biosciences).

Intestinal cytokine analysis

Intestinal tissue was homogenized in ice-cold PBS containing protease inhibitor mixture (Sigma-Aldrich). Samples were centrifuged at $12,000 \times g$ for 7 min. Supernatants were removed and analyzed for IL-6 and IL-17 by ELISA. Total protein concentration of supernatants was determined by bicinchoninic acid (Pierce).

Statistical analysis

Results are expressed as the means $\pm$ SEM. Data was analyzed using a nonpaired Student *t* test (GraphPad Prism; GraphPad). A *p* value <0.05 was considered significant. The *p* values >0.05 were not considered significant.

Results

Tregs suppress mast cell degranulation but enhance mast cell-derived IL-6 in a cell–cell contact-dependent manner

Initially, we investigated the influence of T cells on Ag-stimulated responses by IgE-sensitized BMMCs. BMMCs pretreated with anti-DNP IgE were cultured with Tregs or CD4⁺CD25[−] T cells and activated by addition of DNP-HSA. In the presence of Tregs, BMMC degranulation was significantly reduced, as previously described (15) (Fig. 1A). We then analyzed cytokine production by ELISA. BMMC-CD4⁺CD25[−] T cell cultures had significantly increased levels of IFN- γ (Fig. 1B) and IL-2 (Fig. 1C) as compared with cultures of BMMC-Tregs or BMMC alone, demonstrating that mast cells alter T cell cytokine production, as previously suggested (6). Of the cytokines we investigated, IL-6 was unique in that it was significantly increased in BMMC-Treg cultures only (Fig. 1D), whereas cultures from BMMC with either T cell population demonstrated increased TNF (Fig. 1E), and MCP-1 was unaltered by coculture (Fig. 1F). Independent experiments using CD4⁺/GFP⁺ cells derived from the Foxp3/GFP knockin that were sorted using a MoFlo high-speed cell sorter (DakoCytomation) demonstrated similar enhancement of IL-6 (data not shown).

Because IL-6 can be generated by both mast cells and T cells, we used IL-6^{−/−} cells to identify the source. Wild-type (C57BL/6) and IL-6^{−/−} BMMCs were generated and cocultured with Tregs from wild-type or IL-6^{−/−} mice. As shown in Fig. 2A, wild-type BMMCs with IL-6^{−/−} Tregs showed enhanced IL-6 production, whereas no IL-6 was produced in cultures of IL-6^{−/−} BMMCs with wild-type Tregs. This demonstrates that mast cells are the source of IL-6. Furthermore, the enhanced IL-6 production from wild-type BMMCs was observed with either wild-type or IL-6^{−/−} Tregs, suggesting Treg-derived IL-6 is not required.

To determine if this was cell–cell contact or soluble mediator dependent, the cells were cultured using a Transwell system (Costar; Corning), in which cell–cell contact was inhibited but

free exchange of soluble mediators persists. Fig. 2B demonstrates a requirement for cell–cell contact because separation of the Tregs from BMMCs prevented the enhanced IL-6 production.

Tregs enhance mast cell production of IL-6 to various stimuli

During these experiments, we provided both Treg activation using CD3/CD28 beads and mast cell activation via IgE/Ag. We next sought to investigate the requirements for activation on the IL-6 response. IL-6 production was increased in BMMC-Treg cultures either without or with T cell stimulation (CD3/CD28) (Fig. 3A, 3B). Furthermore, whereas IgE-mediated mast cell activation with Ag (DNP-HSA) did enhance the levels of IL-6 (Fig. 3B), Tregs were capable of elevating IL-6 even in the absence of BMMC stimulation (no DNP-HSA) (Fig. 3A). Mast cells have been shown express MHC II and present peptide to Tregs (21), and so, to exclude any potential role of cognate MHC II interactions with TCR, BMMCs were pretreated with a blocking anti-MHC II Ab prior to coculture with Tregs. This had no effect on the increased IL-6 production (Supplemental Fig. 1). Together, these data demonstrate that the ability of Tregs to enhance mast cell production of IL-6 is unlikely to be dependent on TCR/MHC II interactions or mast cell activation and instead is due to an interaction that is independent of Ag-specific requirements.

We next investigated if Tregs could also influence mast cell IL-6 production in response to innate inflammatory stimuli. BMMC-Treg cultures were stimulated with PGN or LPS to trigger TLR2 or TLR4, respectively. As Fig. 3C demonstrates, Tregs significantly enhance production of IL-6 in response to both PGN and LPS. Furthermore, BMMC activation with IL-33, a recently described cytokine that potentially stimulates mast cells (22), results in enhanced IL-6 production in BMMC-Treg cultures. Together, these data suggest that an innate function of Tregs actively primes mast cells for IL-6 production when the mast cell is triggered by either adaptive or innate stimuli.

Tregs suppress degranulation and enhance IL-6 via different mechanisms

As demonstrated in Fig. 2B, Treg priming of mast cells occurred via a cell–cell contact-dependent mechanism. To identify the interaction mediating this response, we began by investigating the OX40/OX40L interaction described to mediate Treg suppression of mast cell degranulation (15). BMMCs were pretreated with a blocking anti-OX40L Ab prior to culture with Tregs. Surprisingly, there was no effect of blocking the OX40/OX40L interaction on the enhanced IL-6 production (Fig. 4A) despite clear abrogation of the Treg suppression of degranulation (Fig. 4B). Blocking Abs to PDL1, ICOS ligand, and GITR were also investigated, with no significant effect on the enhanced IL-6 production from mast cells (Supplemental Fig. 2).

Whereas surface-bound TGF- β has been established as a mechanism through which Tregs can suppress immune responses (14, 23, 24), some investigators have reported being unable to detect TGF- β on the surface of Tregs (25). We were able to detect TGF- β on the surface of our isolated Tregs by flow cytometry (Fig. 4C), whereas soluble TGF- β in the supernatant was undetectable. Addition of anti-TGF- β blocking Ab to the cocultures significantly impaired the enhanced IL-6 production, as compared with isotype control-treated cultures (Fig. 4D). This inhibition was also seen using a different anti-TGF- β Ab clone (Fig. 4E). However, blocking the TGF- β pathway had no effect on degranulation responses, and the Tregs were still able to suppress mast cell histamine release (Fig. 4F). As such, our data suggest that Tregs use two distinct mechanisms to regulate mast cells: degranulation

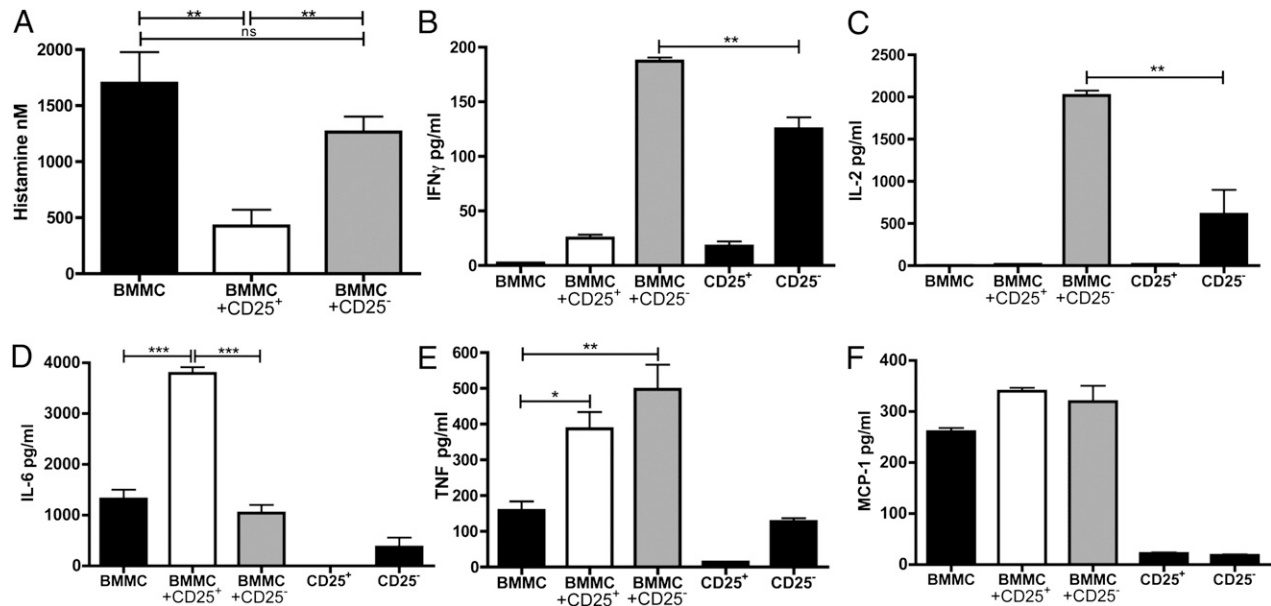


FIGURE 1. In vitro cocultures of BMMCs and Tregs demonstrate Treg suppression of mast cell degranulation and increased IL-6 in supernatant. BMMCs treated with DNP-IgE were cocultured with CD4⁺CD25⁺ Tregs and stimulated by IgE cross-linking using DNP-HSA. Supernatants were collected at 24 h and analyzed for histamine release (A), IFN-γ (B), IL-2 (C), IL-6 (D), TNF-α (E), and MCP-1 (F). Samples were analyzed by ELISA. Representative data are mean ± SEM from two independent experiments ($n = 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

is inhibited by OX40/OX40L interactions, whereas the TGF-β/TGF-βR interaction is necessary for the positive effect on IL-6 production we describe in this study.

TGF-β is sufficient to enhance mast cell-derived IL-6

Soluble TGF-β has been previously shown to inhibit mast cell growth and activation (26–28). However, the effects on mast cell-derived cytokines have not been reported. We therefore investigated if TGF-β (rTGF-β) would be sufficient to enhance IL-6. IgE-sensitized BMMCs were treated with rTGF-β for 24 h. Treated BMMCs were then stimulated with Ag (DNP-HSA). rTGF-β dose dependently increased mast cell production of IL-6 but only upon IgE/Ag stimulation (Fig. 5A). Furthermore, treatment with rTGF-β did not affect IgE/Ag-triggered degranulation, because there was no change in histamine release between untreated and rTGF-β-treated BMMCs (Fig. 5B). These findings further support the concept of independent mechanisms that enhance mast cell IL-6 production versus degranulation.

TGF-β promotes increased IL-6 gene expression in mast cells

We investigated the mechanism by which IL-6 production is enhanced in mast cells. Mast cell granules are capable of storing a variety of mediators, including cytokines (29), and so we initially explored if TGF-β might increase the storage of IL-6 protein. Cell lysates from rTGF-β-treated BMMCs showed no difference in total IL-6 levels in either unstimulated or stimulated BMMCs (Fig. 6A), suggesting no influence on IL-6 storage. Treatment with cycloheximide to block de novo protein synthesis prior to stimulation also demonstrated a requirement for stimulation-induced synthesis (Fig. 6B). Therefore, we next ascertained the impact on IL-6 gene expression. Treatment with rTGF-β significantly increased IL-6 gene expression as compared with untreated BMMCs (Fig. 6C). rTGF-β-treated BMMCs also exhibited increased *mcp1* (mast cell chymase) and decreased *fcεRIα* gene expression, as has been previously reported (Supplemental Fig. 3) (26, 30). This increase in IL-6 gene expression was not due to increased mRNA stability because IL-6 mRNA decayed at the same rate in both rTGF-β-treated and untreated samples (Fig. 6D) and supports the elevated IL-6 being due to increased gene transcription. We next tested these findings in the mast cell-Treg coculture system. Wild-type BMMCs were cultured with T cells derived from IL-6^{-/-} mice, which do not generate IL-6 mRNA. Gene expression analysis

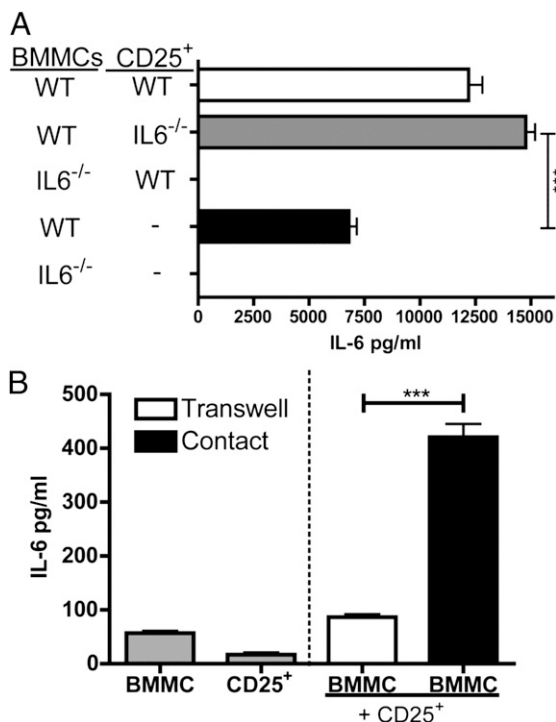


FIGURE 2. Tregs enhance BMMC derived-IL-6 in a cell-cell contact-dependent manner. A, IL-6^{-/-} or wild-type (WT) BMMCs were cocultured with WT or IL-6^{-/-} Tregs to determine the source of the enhanced IL-6. WT BMMCs cocultured with WT Tregs served as the positive control (white bar). Cultures were stimulated with DNP-HSA and supernatants collected at 24 h and analyzed for IL-6 by ELISA. B, BMMC-Treg cocultures were conducted using a Transwell system (CoStar; Corning) in which the cells are separated from contact (white bar). As a positive control, cultures were set up with cells in contact (black bar). Representative data are mean ± SEM from two independent experiments ($n = 6$). ** $p < 0.01$, *** $p < 0.001$.

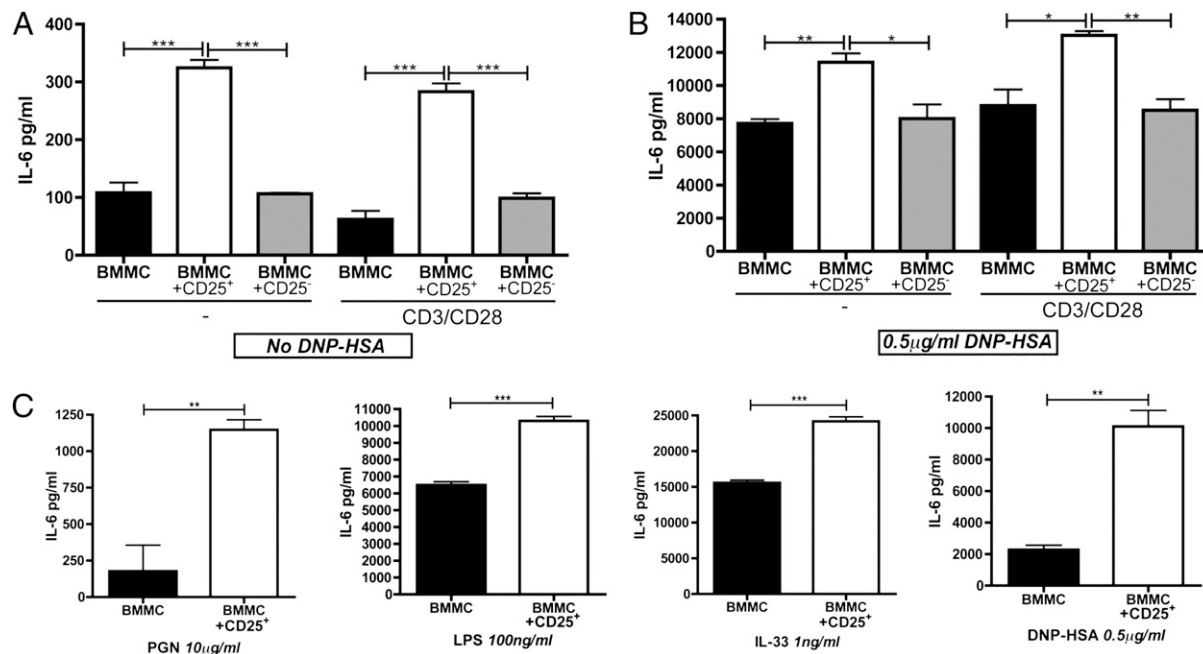


FIGURE 3. Tregs enhance BMMC production of IL-6 to various stimuli. BMMCs and Tregs were cocultured in the absence of anti-CD3/CD28 or in the presence of anti-CD3/CD28 stimulation either without (A) or with DNP-HSA (B) for BMMC stimulation. C, BMMC-Treg cocultures were treated with PGN, LPS, or IL-33 at the doses indicated. In all cultures, supernatants were analyzed at 24 h. Representative data are mean $\pm$ SEM from two independent experiments ($n = 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

demonstrated significantly increased IL-6 gene expression in BMMCs cocultured with Tregs (Fig. 6E) versus CD25⁺ T cells or BMMCs alone. These studies demonstrate that TGF- β and Tregs exert their influence by increasing IL-6 gene expression in mast cells that promotes elevated de novo cytokine synthesis and release of IL-6.

Mast cell-derived IL-6 is sufficient to promote conversion to Th17

Despite previous reports suggesting that mast cells might promote naive T cell conversion to Th17 (16, 31), the cellular sources of

IL-6 for Th17 differentiation have remained unclear. We asked if Treg/TGF- β -induced mast cell-derived IL-6 was sufficient to promote Th17 differentiation in vitro. Supernatants from cultures of BMMC alone, BMMC-TGF- β , and BMMC-Treg, in which BMMCs were activated with IgE/Ag, were used to condition naive CD4⁺CD62L⁺ T cells. Both BMMC-TGF- β and BMMC-Treg culture supernatants were analyzed for IL-6 production, which were significantly increased as compared with BMMC alone (data not shown). Untreated naive T cells were negative for IL-17 production, whereas cells conditioned under Th17-skewing con-

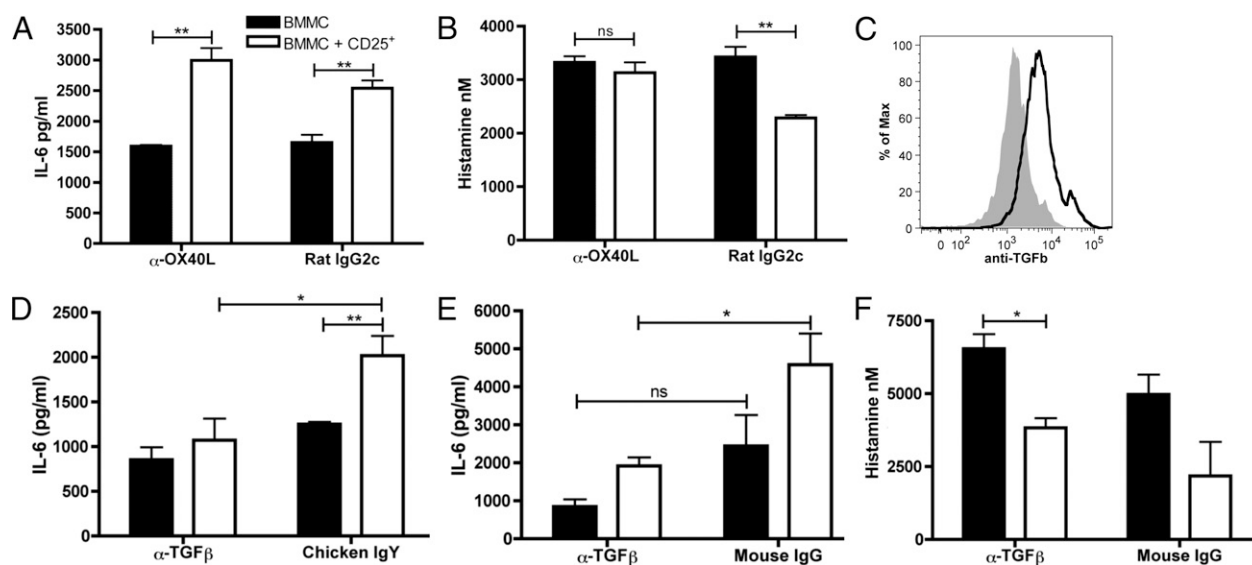


FIGURE 4. Tregs suppress degranulation and enhance IL-6 via different mechanisms. BMMCs were pretreated with anti-OX40L or isotype controls prior to coculture with Tregs. Cultures were stimulated with DNP-HSA and supernatants analyzed at 24 h for IL-6 (A) and histamine (B). C, CD4⁺CD25⁺ Tregs were stained for surface-bound TGF- β (shaded peak, isotype control; solid line, anti-TGF- β). D, Tregs were pretreated with 100 μ g/ml blocking chicken anti-TGF- β or isotype control chicken IgY prior to culture with BMMCs in the presence of DNP-HSA. Supernatants were analyzed at 24 h. E, Tregs were pretreated with mouse anti-TGF- β prior to culture with BMMC in the presence of DNP-HSA. F, Supernatants from E were analyzed for histamine release by histamine ELISA. Representative data are mean $\pm$ SEM from two to three independent experiments ($n = 4-6$). * $p < 0.05$, ** $p < 0.01$.

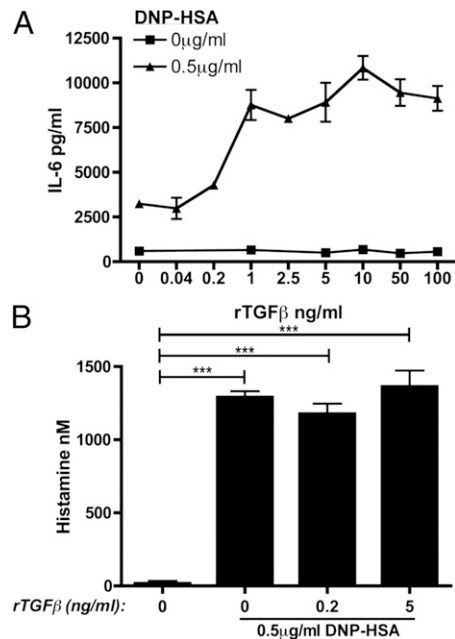


FIGURE 5. TGF- β enhances BMMC-derived IL-6 under stimulation and does not influence histamine release. BMCMs were treated with a dose response of rTGF- β for 24 h. Samples were then stimulated with DNP-HSA or left unstimulated. Supernatants were analyzed at 24 h for IL-6 production (A) and histamine release (B) by ELISA. Representative data are mean $\pm$ SEM from two independent experiments ($n = 6$). *** $p < 0.001$.

ditions (IL-6 + TGF- β) were IL-17 positive (Fig. 7). Interestingly, naive T cells conditioned with supernatants from BMMC-TGF- β cultures had significantly increased proportions of IL-17-positive

cells, despite no exogenous IL-6 being added. In contrast, BMMC-Treg supernatants, in which there was no detectable soluble TGF- β (data not shown), did not promote Th17 development. Thus, our data demonstrate that the IL-6 promoted by TGF- β from mast cells is functional and sufficient to promote Th17 skewing in vitro but that soluble TGF- β is required. This does not occur when the IL-6 is driven by contact with Tregs in vitro.

Adoptive transfer of Tregs into food-allergic mice prevents anaphylaxis and promotes intestinal IL-6 production

Using a mouse model of food allergy we described, whereby mice exposed to OVA plus SEB adjuvant (SEBOVA) exhibit intestinal mastocytosis, elevated Ag-specific IgE, and anaphylaxis upon oral challenge (19), we had previously demonstrated a reduced expression of both TGF- β and Foxp3 in the intestine of mice as compared with controls. Therefore, we examined the expression of IL-6 and IL-17 in this model also. As shown in Fig. 8A and 8B, gene expression for both IL-6 and IL-17 were significantly reduced in anaphylactic SEBOVA mice.

We next examined whether the adoptive transfer of Tregs into sensitized mice would alter either of these responses. Using a similar approach to Gri et al. (15), who demonstrated inhibition of passive anaphylaxis by Treg transfer, 0.5×10^6 Tregs or CD25⁺ T cells were isolated from naive BALB/C mice and i.v. transferred into fully sensitized SEBOVA mice 24 h prior to oral challenge. As depicted in Fig. 8C, all SEBOVA mice exhibited significantly elevated serum levels of OVA-specific IgE, demonstrating that the T cell transfer had no influence on sensitization. However, upon oral challenge with OVA, the anaphylactic response observed in SEBOVA mice was absent in five of the eight SEBOVA mice that received Tregs but persisted in those that re-

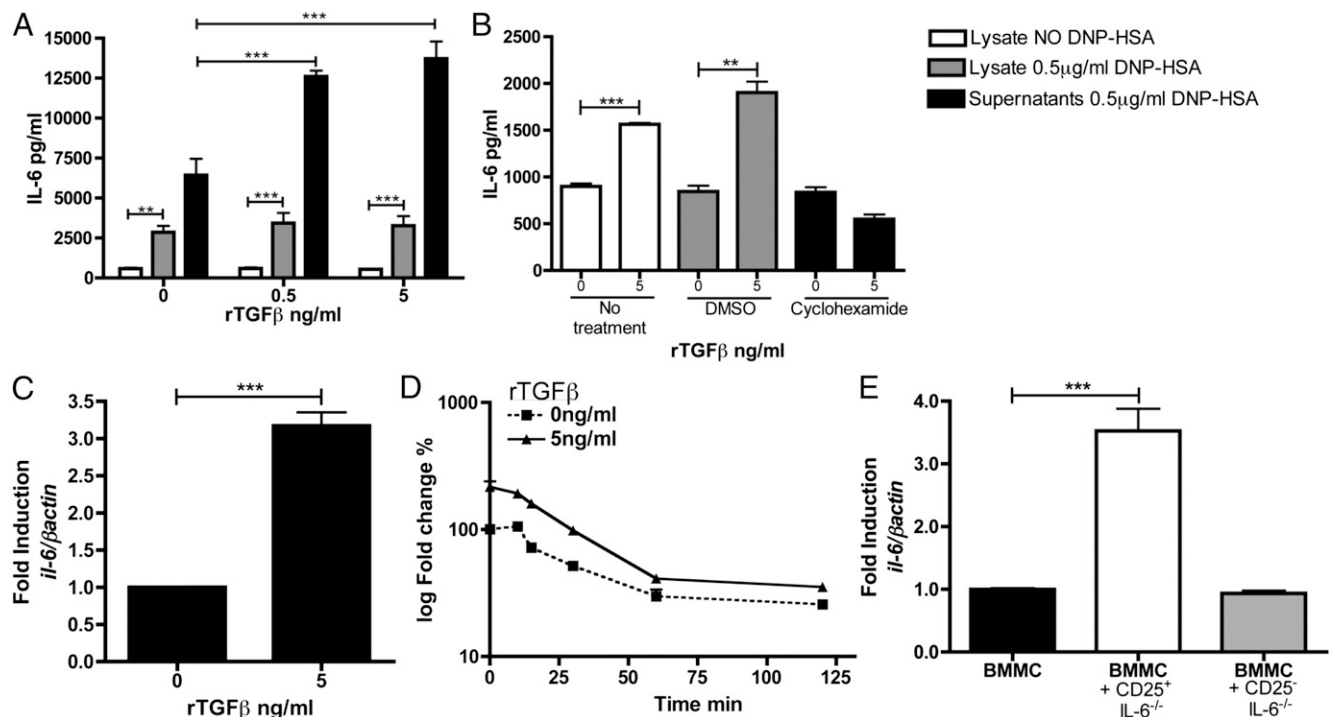


FIGURE 6. TGF- β promotes increased IL-6 gene expression in BMCMs without enhancing IL-6 protein storage or mRNA stability. BMCMs were treated with rTGF- β for 24 h. A, Cell lysates from unstimulated BMCMs (white bars) and stimulated BMCMs (gray bars) as well as supernatants from stimulated BMCMs (black bars) were analyzed for IL-6. B, rTGF- β -treated BMCMs subjected to cycloheximide demonstrate a requirement for de novo IL-6 protein synthesis. C, BMCMs were treated with 5 ng/ml of rTGF- β for 24 h, and then stimulated for 2 h. Samples were collected for RNA processing and analyzed for IL-6 gene expression. Data depicted as fold induction in comparison with untreated BMCMs. D, Untreated and rTGF- β -treated BMCMs were stimulated for 2 h with DNP-HSA. Actinomycin D was added to cultures and samples collected for gene expression analysis at time points indicated. E, IL-6^{-/-} Tregs or CD25⁺ T cells were cultured with WT BMCMs for 2 h, and samples were collected for gene expression analysis. Representative data are mean $\pm$ SEM from two to three independent experiments ($n = 3-6$). ** $p < 0.01$, *** $p < 0.001$.

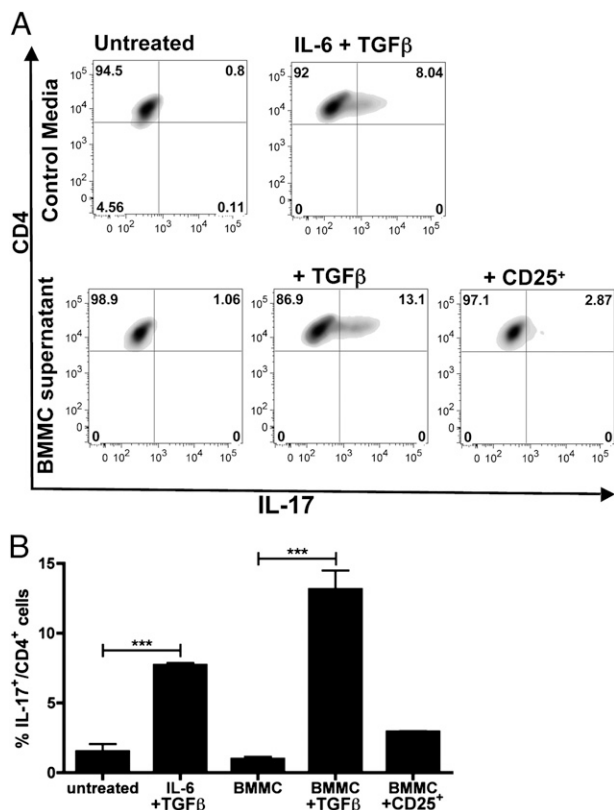


FIGURE 7. Mast cell-derived IL-6 is sufficient to skew naive T cells to Th17. Naive T cells (CD4⁺CD62L⁺) were placed under untreated or Th17-skewing conditions (IL-6 + TGF-β). BMMCs, BMMC + rTGF-β, or BMMC + CD25⁺ cultures were stimulated with DNP-HSA and supernatants collected. Supernatants were then used to treat naive T cells. Cells were collected at day 3 and analyzed for IL-17 production by intracellular flow cytometry. *A*, Representative flow plots depicted. *B*, Quantification of flow cytometry plots ($n = 5$). *** $p < 0.001$.

ceived CD25⁻ cells (Fig. 8D). Nonsubjective measurements of this response, including respiratory distress (Fig. 8E) and the mast cell protease MCPT-1 (Fig. 8F), were also significantly reduced, supporting the conclusion that Tregs directly impair mast cell degranulation in vivo. Using Foxp3-GFP Tregs, we detected Tregs within the mesenteric lymph nodes and lamina propria of the small intestine but not the Peyer's patches (Supplemental Fig. 4), suggesting that the transferred Tregs were capable of trafficking to the intestine. However, whereas SEBOVA mice actually had reduced intestinal IL-6 protein levels as compared with control mice that received OVA alone (Fig. 8G), the adoptive transfer of Tregs but not CD25⁻ T cells into SEBOVA mice restored the IL-6 levels in the intestine. Interestingly, we observed a concomitant increase in intestinal IL-17 production in SEBOVA mice that received Tregs (Fig. 8H).

Discussion

In response to IgE-mediated activation, mast cells are well characterized for the ability to degranulate and release preformed mediators, such as histamine, that elicit immediate hypersensitivity responses. However, they also generate an incredibly diverse range of cytokine mediators and possess a variety of activation pathways, which includes innate as well as adaptive stimuli. Recent work has shown that contact with Tregs suppresses mast cell degranulation (15). In this study, we sought to determine the effects of Tregs on the production of cytokines from mast cells. Our data demonstrate

that cytokine production is also affected but rather than suppress, Tregs actually promote mast cell production of IL-6.

Foxp3⁺ Tregs possess an arsenal of methods through which they have been shown to inhibit inflammation and immunity, including release of soluble factors, such as IL-10, IL-35, and TGF-β, as well as contact-dependent mechanisms, such as OX40 interacting with OX40L (12). This OX40/OX40L mechanism was previously shown to mediate Treg inhibition of in vitro mast cell degranulation, whereas depletion of Tregs was sufficient to enhance anaphylaxis in vivo (15), suggesting that Tregs constitutively inhibit mast cell degranulation. Our data suggest that this is only one mechanism through which mast cells and Tregs interact and that surface TGF-β on Tregs can alter mast cell cytokine production.

In addition to its secretion, recent studies have shown TGF-β can be expressed on the cell surface of activated Tregs in a complex with latency-associated peptide and GARP (32). Functionally, disruption of this complex reduces the capacity of Tregs to suppress T cell proliferation or induce differentiation of new Tregs during the process of infectious tolerance (14, 32). As a result, the functions of surface-bound TGF-β have been generally seen as inhibitory in nature. Although previous studies have established that soluble TGF-β can alter the expression of mast cell proteases and FcεRI (26), we were unable to detect soluble TGF-β in supernatants from our mast cell-Treg coculture experiments and so we examined the Tregs used for their surface expression. Our data show cell-surface expression of TGF-β and that Ab blockade was sufficient to prevent the enhancing effects of Tregs on mast cell production of IL-6 in our cocultures. Therefore, we conclude that Tregs mediate their influence on mast cells via a contact-dependent, surface-bound TGF-β mechanism and that this is a mechanism that Treg possess to exert positive, stimulatory influences over other cells.

In considering the mechanisms through which IL-6 may be enhanced, we define that the enhancing influence of TGF-β was at the level of enhanced gene transcription, rather than mRNA stability or increased protein storage. TGF-β receptor activation can engage both Smad-dependent and -independent signaling pathways (33). In human bronchial epithelial cells and prostate cancer cells, TGF-β activation has been shown to promote IL-6 gene expression through Smad-dependent pathways (34, 35). In this study, Smad proteins can couple with other IL-6-associated transcription factors (e.g., c-Jun/AP-1 and NF-κB) to drive IL-6 gene transcription. Smad3 was shown to directly act with c-Jun/c-Fos components to mediate TGF-β-stimulated transcription (36), suggesting this might be a pathway through which TGF-β could influence IL-6 transcription. Preliminary studies aimed at investigating the requirement for Smad3 in our responses have yielded contradictory results, with pharmacological inhibition using a Smad3 inhibitor reducing the IL-6 levels but no apparent difference in BMMC derived from Smad3^{-/-} mice (data not shown). We are continuing to investigate this further. However, TNFR-associated factor 6, which is a Smad-independent TGF-β-activated factor (37), has also been shown to regulate IL-6 production in mast cells (38) and B cells (39). Consequently, the specific mechanisms through which TGF-β, via Treg presentation, might promote IL-6 production are an exciting topic for future studies but may theoretically occur via either of the described major pathways from the TGF-β receptors.

One intriguing component of our study is that, despite both cells having critical roles in adaptive immunity, mast cells and Tregs clearly cooperate via mechanisms that are not Ag specific. Mast cells and Tregs have been demonstrated to reside in close proximity to one another in secondary lymphoid tissues as well as in mucosal tissues during homeostasis (15, 40), suggesting that these inter-

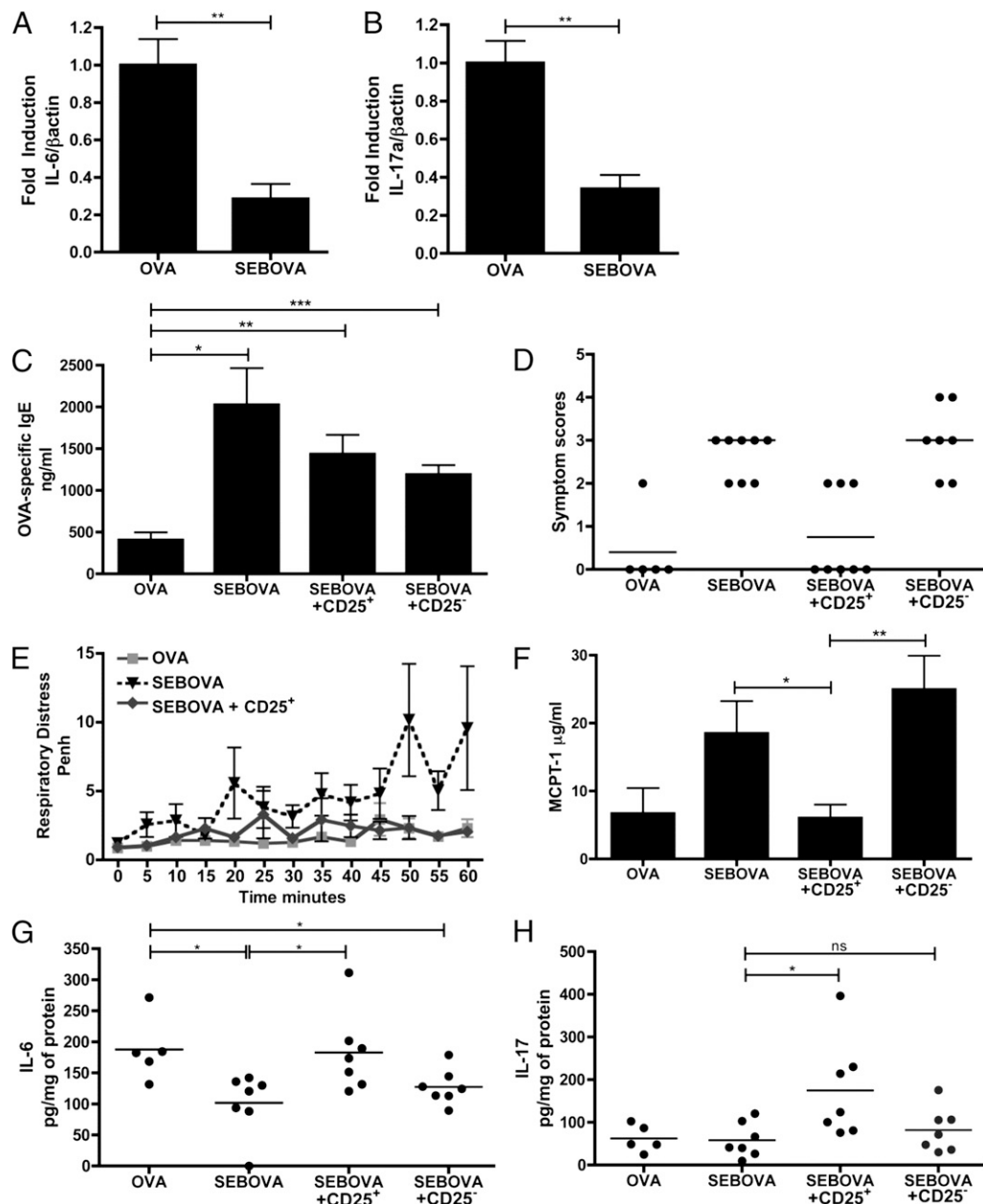


FIGURE 8. Adoptive transfer of Tregs inhibits anaphylaxis and promotes intestinal IL-6 production. Mice were treated with OVA alone or SEBOVA. Twenty-four hours after challenge, the expression of IL-6 (A) and IL-17 (B) from cells in the mesenteric lymph nodes was determined. Following the same sensitization protocol, control-treated or mice sensitized to OVA (SEBOVA) received Tregs (CD25⁺) or CD25⁻ T cells 24 h before oral Ag challenge. C, Serum levels of OVA-specific IgE were measured to determine effective sensitization. D, Forty-five minutes postchallenge, anaphylactic symptom scores were assigned. E, Airway obstruction responses were measured using whole-body plethysmography. Data represent mean Penh value during each 5-min period. F, Serum MCPT-1 levels were measured by ELISA. IL-6 (G) and IL-17 (H) protein levels were determined by ELISA 24 h postchallenge in supernatants from intestinal tissue homogenates. Data are mean $\pm$ SEM from two independent experiments ($n = 5-8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

actions likely occur in the absence of inflammation and Ag-specific requirements. Mast cell-derived IL-6 has been shown to be important in a diverse range of immune contexts, including driving B cell activation and IgA secretion (41), limiting tumor growth (42) and providing protection against *Klebsiella pneumoniae* (7). We anticipate that Tregs and TGF- β would also likely regulate such responses.

Functionally, our data demonstrate that mast cells are a potential source of IL-6 for Th17 differentiation and that this is regulated by Tregs. This supports a recently published study showing mast cells can promote Treg and naive T cell differentiation to Th17 in vitro (16) but contrasts with their conclusions that proposed mast cells

functioned to promote IL-6 production from the naive T cells. Our coculture experiments using mast cells or T cells from IL-6^{-/-} mice conclusively demonstrated that the mast cell is the critical source of IL-6 production in our studies. Our data would also suggest that Tregs or mast cells alone are not capable of supplying sufficient soluble TGF- β to drive Th17 differentiation because activated BMMC supernatants or BMMC-Treg culture supernatants were not able to induce IL-17 production. However, it remains to be determined if Tregs might use surface-bound TGF- β to both drive IL-6 production from mast cells and also provide TGF- β -dependent signals to naive T cells concurrently or whether an alternative source of TGF- β would be required. Furthermore,

Tada et al. (43) previously demonstrated that rTGF- β inhibited IL-6 production from macrophages but enhanced production from Langerhans cells upon activation, raising the possibility of different cell types possessing differential potential for IL-6 production via this mechanism. Also, in contrast to our findings in this paper, coculture of islet cells for 2 d with Tregs was shown to reduce IL-6 mRNA levels (44). Consequently, it is intriguing to postulate a scenario whereby Tregs might inhibit IL-6 from cells in one tissue compartment but actively enhance those in another.

Using in vitro approaches and a passive anaphylaxis model, Tregs have previously been demonstrated to inhibit mast cell degranulation (15). In this study, we demonstrate that the Tregs also inhibit active anaphylaxis, because SEBOVA mice that received Tregs exhibited reduced symptom scores, an absence of airway obstruction responses, and significantly reduced serum MCPT-1 levels in a model of food allergy. This demonstrates that Tregs possess the potential to prevent anaphylactic responses despite the presence of Ag-specific IgE and dominant Th2-skewed immunity. Clinically, it is well described that the majority of individuals who possess food Ag-specific IgE show no clinical reactivity (45). This nonresponder tolerance might be due to efficient control over mast cell degranulation by Treg cells, as we achieved in our mice.

Although Th17 cells exert pathogenic functions in many diseases, their role in the intestine seems to include an important role in homeostasis. Th17 cells reside in the intestinal compartment and have been shown to be critically important for controlling responses to the commensal microflora (46, 47). We found reduced expression of both IL-6 and IL-17 in the intestinal tissues of SEBOVA mice. Furthermore, the adoptive transfer of Tregs appeared to restore these back to control levels. We are continuing to explore the mechanisms behind this reduction of IL-6 and IL-17 in our allergy model, but it is interesting to note that TNF, a preformed mediator released during mast cell degranulation, has been shown to decrease intestinal IL-6 levels in the jejunum (48). Although the precise role of Th17 cells in the pathogenesis of food allergy remains unknown at this time, our data demonstrate a decrease in this arm of immunity in our food allergy model and suggest that Tregs participate in sustaining this response. Whether this is mechanistically functioning via TGF- β -dependent interactions with mast cells remains to be determined. Furthermore, although we were able to detect Treg movement into the intestine, the requirement for this interaction to occur within the local tissue remains to be determined. It is interesting that i.v. transfer of Tregs has been shown to influence inflammatory responses at a diverse range of target tissues, including those in experimental autoimmune encephalomyelitis (49–52), colitis (53, 54), and asthma models (55, 56). One possibility is that some Treg-mediated influences, including those on mast cells, might actually occur centrally, outside of specific tissues.

In conclusion, we have defined a novel interaction between Tregs and mast cells that is initiated by surface-bound TGF- β . This serves to prime mast cells for production of IL-6 upon their activation to both innate and adaptive signals. Functionally, we demonstrate that mast cell-derived IL-6 is sufficient to promote T cell differentiation to Th17 in the presence of TGF- β . As such, we believe that Tregs serve to limit the degranulation role of mast cells to establish an altered inflammatory environment by actively promoting the release of mast cell-derived cytokines.

Disclosures

The authors have no financial conflicts of interest.

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SUPPLEMENTAL DATA:

Figure S1: *Tregs enhance BMMC derived IL-6 in a Class II MHC independent manner.*

BMMCs were pre-incubated with blocking anti-Class II MHC antibody prior to co-culture with Tregs. Cultures were stimulated with 0.5µg/ml DNP-HSA and supernatants were collected at 24hrs for IL-6 analysis. Representative data are mean $\pm$ SEM from 2 independent experiments.

Figure S2: *PD1/PD-L1, ICOS/ICOSL, GITR/GITRL interactions do not appear to mediate Treg priming of mast cell IL-6.*

(A) BMMCs were pre-incubated with blocking antibodies to PDL1, ICOSL or Rat IgG2c isotype control for 1-hr prior to co-culture with Tregs. (B) Tregs were pre-incubated with anti-GITR for 1-hr prior to co-culture with BMMCs. All cultures were stimulated with DNP-HSA and supernatants analysed for IL-6. Representative data are mean $\pm$ SEM from 2 independent experiments, **p<0.01, ***p<0.001.

Figure S3: *rTGFβ-treated mast cells exhibit increased *mcpt1* and decreased *fcεRI* gene expression.*

rTGFβ treated BMMCs were stimulated with DNP-HSA for 2hrs and analysed for changes in gene expression for (A) *mcpt1* and (B) *fcεRI*. Representative data are mean $\pm$ SEM from 2 independent experiments, **p<0.01, ***p<0.001.

Figure S4: *Transferred Tregs traffic to intestinal compartments.*

0.5x10⁶ FoxP3-GFP Tregs were intravenously transferred into naïve Balb/C mice. 24 post-transfer, mesenteric lymph nodes (mLN), Peyers Patch (PP) and Lamina Propria (LP) were investigated for the presence of FoxP3-GFP positive cells. Samples were gated on CD4⁺ cells. Representative flow plots of data from 6 individual animals.

Figure S1

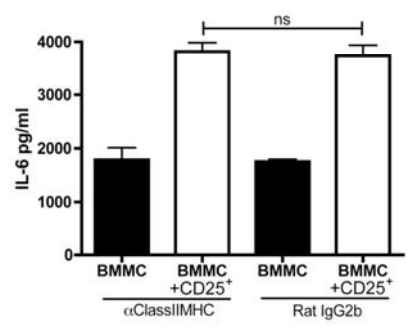


Figure S2

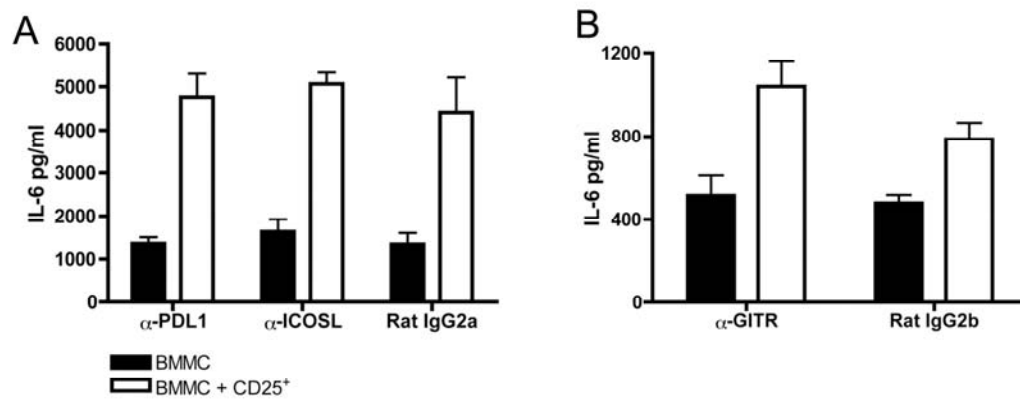


Figure S3

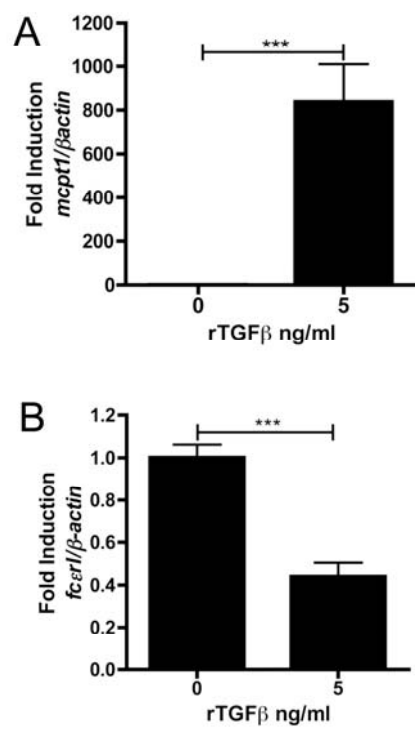


Figure S4

