Immunity
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$CD4^+CD25^+$ Regulatory T Cells Suppress Mast Cell Degranulation and Allergic Responses through OX40-OX40L Interaction

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DOI 10.1016/j.immuni.2008.08.018

SUMMARY

T regulatory (Treg) cells play a role in the suppression of immune responses, thus serving to induce tolerance and control autoimmunity. Here, we explored whether Treg cells influence the immediate hypersensitivity response of mast cells (MCs). Treg cells directly inhibited the FcεRI-dependent MC degranulation through cell-cell contact involving OX40-OX40L interactions between Treg cells and MCs, respectively. When activated in the presence of Treg cells, MCs showed increased cyclic adenosine monophosphate (cAMP) concentrations and reduced Ca^{2+} influx, independently of phospholipase C (PLC)-γ2 or Ca^{2+} release from intracellular stores. Antagonism of cAMP in MCs reversed the inhibitory effects of Treg cells, restoring normal Ca^{2+} responses and degranulation. Importantly, the in vivo depletion or inactivation of Treg cells caused enhancement of the anaphylactic response. The demonstrated cross-talk between Treg cells and MCs defines a previously unrecognized mechanism controlling MC degranulation. Loss of this interaction may contribute to the severity of allergic responses.

INTRODUCTION

Allergies are increasingly prevalent in the population of western countries (Ring et al., 2001). Allergic hypersensitivity is associated with both immunoglobulin E (IgE) and T helper 2 (Th2) cell responses to environmental allergens. In allergic individuals, priming of allergen-specific $CD4^+$ Th2 cells by antigen-presenting cells (APCs) results in the production of Th2 cell cytokines, which are responsible for initiating B cell production of allergen-specific IgE. IgE binds to the high-affinity receptor for IgE (FcεRI) on mast cells (MCs) and basophils. Allergen crosslinking of cell-surface-bound allergen-specific IgE leads to the release of preformed and granule-stored allergic mediators like histamine, as well as the secretion of de novo-synthesized prostaglandins, cysteinyl leukotrienes, cytokines, and chemokines. Granule-stored mediators are key to the immediate (acute) allergic reactions, such as the wheal and flare response in the skin (Williams and Galli, 2000), whereas de novo-synthesized mediators are more important in the late (chronic) phase of the allergic response.

The homeostatic mechanisms regulating MC number and function in peripheral tissues are largely dependent on Th2 cell cytokines, such as interleukin-3 (IL-3), IL-4, IL-5, IL-9, and IL-13 (Shelburne and Ryan, 2001). Some of these cytokines are key in enhancing MC survival (IL-3) or recruitment (IL-9) to effector sites, but in general, Th2 cell cytokines establish a positive feedback loop that maintains the Th2 cell response (Lorentz et al., 2005). Environmental factors, such as exposure to allergens, infections, and air pollution, interact with genetic factors to influence the progression of the immune response toward a Th2 cell phenotype, resulting in allergen-specific IgE production and subsequent allergen-mediated activation of MCs promoting allergic disease (Umetsu et al., 2002). However, the immunological mechanisms that control in vivo Th2 cell-driven inflammation or that dampen MC-mediated allergic response are not fully understood.

Regulatory T cells are crucial in preventing the development of autoimmune diseases, maintaining self-tolerance, and regulating the development and the intensity of the immune response to foreign antigens, including allergens (Lohr et al., 2006). In recent years, the naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells and an inducible population of allergen-specific IL-10-secreting type 1 Treg (Tr1) cells have been implicated in promoting or suppressing allergic diseases (Akdis, 2006; Wing and Sakaguchi, 2006). Allergen-specific Treg and Tr1 cells are thought to control allergy by secreting IL-10 and TGF-β, suppressing IgE production by B cells, and decreasing Th2 cell

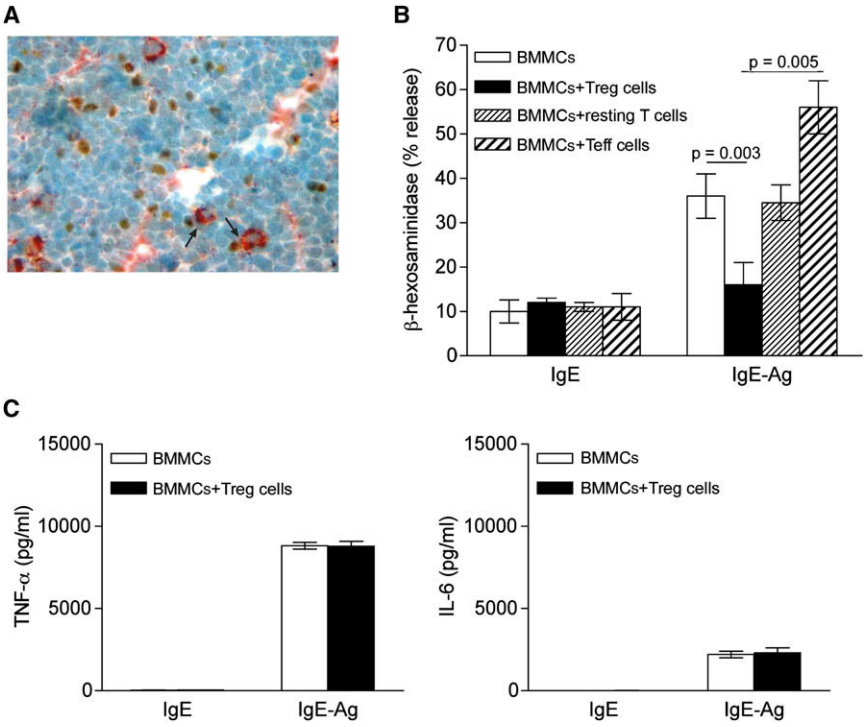

Figure 1. In Vivo Colocalization of Foxp3$^+$ Treg Cells and Mast Cells and In Vitro Impairment of IgE-Mediated Degranulation of BMMCs by Treg Cells

(A) Inguinal lymph node sections were stained with mouse anti-rat-FcεRIβ (red) and rat anti-mouse-Foxp3 (brown). Arrows indicate cell-cell contact. Original magnification was 400×.

(B) BMMCs sensitized with IgE anti-DNP (IgE) and challenged with Ag (IgE-Ag) in the absence or presence of equal amounts of CD4$^+$CD25$^+$ Treg cells (Treg cells), CD4$^+$CD25$^-$ T cells (resting T cells), or a-CD3- plus a-CD28-stimulated CD4$^+$CD25$^-$ effector T cells (Teff cells) were examined for release of β-hexosaminidase, expressed as a percentage of the cells' total mediator content. Shown are the means ± SD of four independent experiments, each performed in duplicate.

(C) TNF-α and IL-6 concentrations were evaluated in the supernatants of BMMC-Treg cell cocultures. Shown are the means ± SD of three independent experiments.

cytokines, thus indirectly inhibiting the effector functions of MCs and basophils.

In this study, we investigated the possibility that Treg cells might directly modulate the acute phase of allergic reactions by affecting the FcεRI-initiated MC degranulation. This was based on previous findings demonstrating that MCs can physically interact with T cells (Bhattacharyya et al., 1998) and are essential intermediaries in Treg cell tolerance (Lu et al., 2006). Our findings showed that CD4$^+$CD25$^+$Foxp3$^+$ Treg cells were able to dampen the release of prestored allergic mediators from MCs through an OX40-OX40L-dependent mechanism. The interaction of Treg cells with MCs impaired the influx of extracellular Ca^{2+} after FcεRI triggering. This was not a consequence of impaired phospholipase C-γ2 (PLC-γ2) activation or defective Ca^{2+} release from intracellular stores. The Treg cell-mediated suppression was accompanied by increased cyclic adenosine monophosphate (cAMP) in the suppressed MCs, and antagonism of cAMP reversed the inhibitory effect of Treg cells on MCs, demonstrating that cAMP increase in MCs is the likely mechanism for suppression of Ca^{2+} influx. Finally, in vivo depletion or inactivation of Treg cells enhanced the extent of histamine release in a mouse model of systemic anaphylaxis, a common IgE-mediated type I hypersensitivity reaction involving MC degranulation. These findings underscore the broad immunosuppressive efficacy of Treg cells by demonstrating their control on immediate allergic responses.

RESULTS

Treg Cells Impair FcεRI-Mediated MC Degranulation

MCs are activated in various T cell-mediated inflammatory processes, reside in physical proximity to T cells, and contribute to T cell recruitment, activation, and proliferation (Kashiwakura et al., 2004; Nakae et al., 2006). T cell-derived cytokines and adhesion-molecule-dependent contact between effector T cells (Teff cells) and MCs result in the release of both preformed granule contents and de novo-synthesized cytokines from the latter (Inamura et al., 1998). However, it is not known whether Treg cells can be found in contact with MCs in vivo and whether they can directly affect the immediate hypersensitivity response of MCs.

Immunohistochemical analysis of inguinal lymph node of C57BL/6 mice revealed FcεRIβ$^+$ MCs in close proximity to Foxp3$^+$ Treg cells, suggesting the possibility of crosstalk between these two cell types (Figure 1A). Our initial experiments explored the consequences of different T cell subsets on FcεRI-initiated degranulation of bone marrow-derived cultured MCs (BMMCs) from C57BL/6 mice (Figure 1B). Degranulation was measured by the release of the MC granule-associated enzyme β-hexosaminidase. As shown in Figure 1B, Treg cells significantly inhibited BMMC degranulation, with IgE-antigen (Ag)-stimulated MCs alone releasing 36% ± 5% of their granule contents compared with 16% ± 5% for MCs coincubated with Treg cells ($p = 0.003$). In contrast, anti-CD3 + anti-CD28-activated CD4$^+$ T cells (Teff) significantly enhanced MC IgE-Ag-dependent degranulation (56% ± 6% degranulation; $p = 0.005$), in agreement with previous findings (Inamura et al., 1998). Treg cells from BALB/c mice cocultured with syngenic BMMCs showed a similar ability to inhibit MC degranulation (Figure S1 available online). The Ag-induced release of TNF-α and IL-6 was not affected by the presence of Treg cells when compared with that for BMMCs cultured alone (Figure 1C).

Increasing the Treg cells:MCs ratio or preincubating the two cell types for up to 30 min before Ag challenge did not cause a further decrease in MC degranulation (data not shown), indicating that a rapid mechanism for MC inhibition by Treg cells underlies the observed effect. This also suggested that cell-cell contact

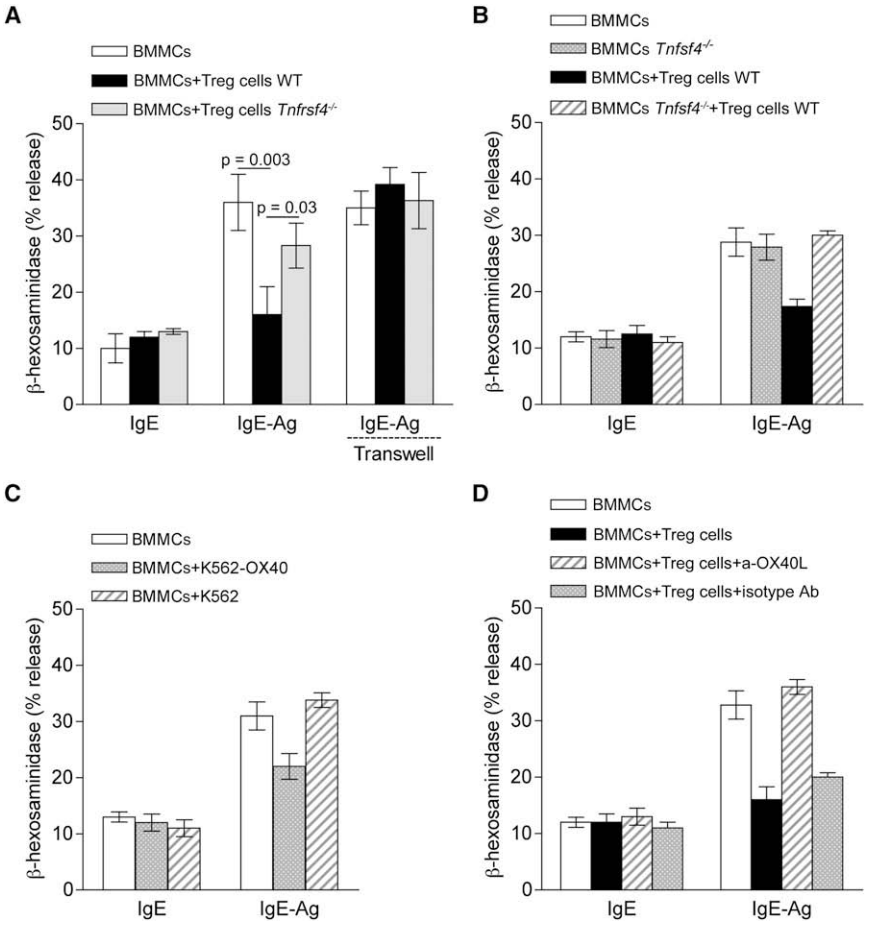

Figure 2. The Contact-Dependent Inhibitory Role of Treg Cells on MC Degranulation Depends on OX40 Expression by Treg Cells and Requires OX40L on BMMCs

(A) BMMCs sensitized with mouse IgE anti-DNP (IgE) and challenged with Ag (IgE-Ag) in the absence or presence of equal amounts of WT or *Tnfrsf4*$^{-/-}$ $CD4^+CD25^+$ Treg cells or separated by a transwell membrane (Transwell) were then examined for release of β-hexosaminidase.

(B) Same as (A), but BMMCs were obtained from WT or *Tnfsf4*$^{-/-}$ mice and cocultured with WT $CD4^+CD25^+$ Treg cells.

(C) IgE-sensitized BMMCs were challenged with Ag in the absence or presence of membranes from K562 cells expressing OX40 (K562-OX40) or empty vector (K562).

(D) IgE-sensitized BMMCs were challenged with Ag in presence of Treg cells, plus blocking anti-OX40L (clone MGP34) or isotype control (rat IgG2c). All the graphs show the means ± SD of three independent experiments, each performed in duplicate.

might be important because de novo production of cytokines normally requires a few hours poststimulation. To explore this possibility, we assayed degranulation with a transwell to separate Treg cells and MCs. Figure 2A shows that the inhibition of FcεRI-dependent MC degranulation by Treg cells was abolished when MCs and Treg cells were separated by the transwell membranes, thus revealing the requirement for cell-cell contact.

OX40-OX40L Interaction Is Required for Treg Cell-Mediated Inhibition of MC Degranulation

MCs constitutively express OX40L (encoded by *Tnfsf4*), which mediates MC-induced T cell proliferation in vitro (Kashiwakura et al., 2004; Nakae et al., 2006). OX40 (encoded by *Tnfrsf4*) is constitutively expressed on naive and activated Treg cells, and its signal can modulate Treg cells' suppression of Teff cells (Takeda et al., 2004; Valzasina et al., 2005). Thus, we investigated whether OX40L-OX40 interaction might function to mediate the inhibitory effect of Treg cells on MC activation. Treg cells isolated from *Tnfrsf4*$^{-/-}$ mice poorly inhibited MC degranulation, with responses of IgE-Ag-activated MCs at 36% ± 5% compared with 28% ± 4% degranulation in the presence of *Tnfrsf4*$^{-/-}$ Treg cells. However, this difference was not significant (Figure 2A). This suggested a dominant mechanism of inhibition mediated by OX40 on Treg cells, although other interactions or factors might contribute a minor component of the inhibitory effect on MCs.

The reverse experiment showed the importance of OX40L in MCs for Treg cell-mediated inhibition, given that BMMCs differentiated from OX40L-deficient mice (*Tnfsf4*$^{-/-}$) were completely resistant to the Treg cells' inhibitory effect (Figure 2B). The presence of OX40 on wild-type (WT) and its absence on *Tnfrsf4*$^{-/-}$ Treg cells, as well as the presence of OX40L on unstimulated or IgE-Ag-stimulated BMMCs or its absence in *Tnfsf4*$^{-/-}$ BMMCs, was demonstrated by flow cytometry (Figures S2 and S3A, respectively). To explore whether the triggering through OX40-OX40L was required for the inhibitory effect on MCs, we stimulated BMMCs in the presence of OX40-expressing membranes derived from the chronic myelogenous leukemia K562 cell line or incubated them with Treg cells in the presence of an OX40L-blocking antibody. OX40-expressing K562 membranes also elicited an inhibitory effect on MC degranulation, although one that was weaker than that in presence of Treg cells (Figure 2C). Additionally, when BMMCs were incubated with a blocking OX40L antibody, the inhibitory effect of Treg cells on MC degranulation was reversed (Figure 2D). FcεRI expression was not altered by OX40L deficiency, nor modulated in BMMCs in the presence of WT or *Tnfrsf4*$^{-/-}$ Treg cells (Figures S3B and S3C). No major differences were observed between *Tnfrsf4*$^{-/-}$ and WT Treg cells in expression of other costimulatory molecules (Figure S3D). These experiments demonstrated that OX40-OX40L interactions between Treg cells and MCs, respectively, appear to be the unique requirement for the dampening of MC degranulation.

Treg Cells Inhibit Ca^{2+} Influx in MCs Independently of PLC-γ Activation or Ca^{2+} Mobilization

To explore the underlying mechanism for the inhibitory effect of Treg cells on MC degranulation, we investigated signaling events

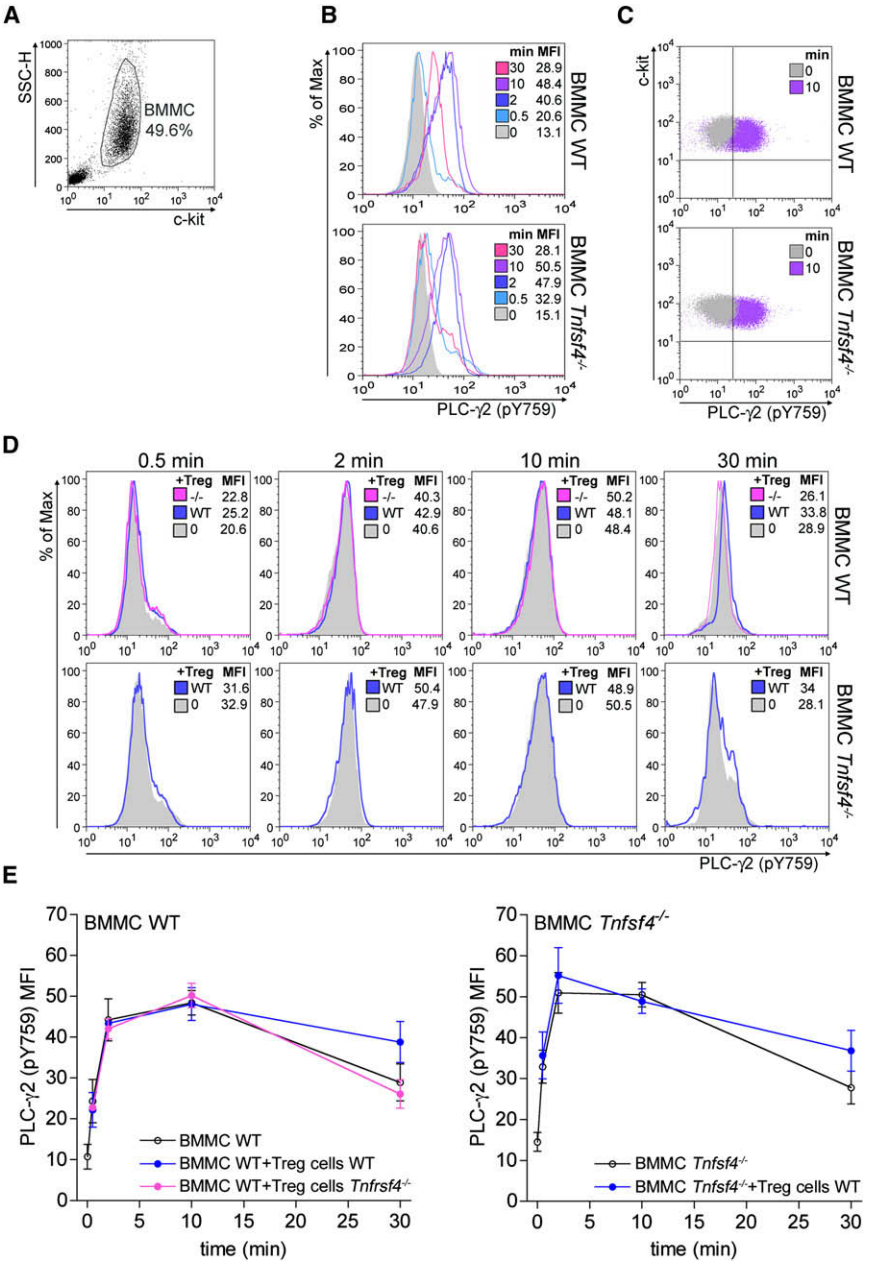

Figure 3. Treg Cells Do Not Affect FcεRI-Dependent PLC-γ Phosphorylation

WT or *Tnfsf4*$^{-/-}$ sensitized BMMCs were stimulated with Ag in the presence of WT or *Tnfrsf4*$^{-/-}$ Treg cells for the indicated times. Cells were immediately fixed and stained for c-kit and phosphorylated PLC-γ2. From c-kit^{+}-gated BMMCs (A), histogram overlays of phosphorylated PLC-γ2 at different time points were obtained from WT (upper panels) or *Tnfsf4*$^{-/-}$ (lower panels) BMMCs challenged in absence of Treg cells (B). Dot plot overlay of basal phosphorylated PLC-γ2 at 0 min (left, gray) and after 10 min (right, violet) is shown in (C). Histogram overlays of phosphorylated PLC-γ2 from WT (upper panels) or *Tnfsf4*$^{-/-}$ (lower panels) BMMCs challenged in the presence of Treg cells are shown in (D). Results shown are representative of three independent experiments. Kinetics of PLC-γ2 phosphorylation at different conditions are shown in (E) and are the mean ± SD of three independent experiments.

known to be essential for MC degranulation. Very little is known about the signals generated subsequent to OX40L stimulation, but it has been published that OX40L engagement results in the rapid translocation of the Ca^{2+}-dependent protein kinase C (PKC)-β to the membrane of human airway smooth muscle cells (Burgess et al., 2004). Ca^{2+} mobilization and PKC-β activation are known to be absolutely essential for MC degranulation (Blank and Rivera, 2004). Ca^{2+} mobilization is initiated by the phosphorylation of PLC-γ after FcεRI engagement, which leads to the hydrolysis of phosphatidylinositol-4,5-biphosphate to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) from endoplasmic reticular stores, whereas DAG and Ca^{2+} act in concert to promote activation of Ca^{2+}-dependent PKCs, like PKC-β. The emptying of internal stores triggers Ca^{2+} influx from external sources, a step required for MC degranulation (Gilfillan and Tkaczyk, 2006).

We first explored whether a defect in the activation of PLC-γ2 (as measured by phosphorylation at the Y759 site, known to be required for lipase activity) was present. The PLC-γ2 isoform was chosen for its essential role in MC degranulation (Wen et al., 2002). Rapid tyrosine phosphorylation of the PLC-γ2 isoform was detected upon FcεRI engagement in both WT and *Tnfsf4*$^{-/-}$ BMMCs (Figures 3A, 3B, and 3C). Although a trend toward a slightly more transient phosphorylation was observed in *Tnfsf4*$^{-/-}$ BMMCs, this was not significant. Moreover, the presence of WT or *Tnfrsf4*$^{-/-}$ Treg cells did not significantly alter the phosphorylation of PLC-γ2 in either WT or *Tnfsf4*$^{-/-}$ BMMCs (Figures 3D and 3E).

Recent studies demonstrated that in MCs, extracellular Ca^{2+} influx can be regulated independently of PLC-γ, for example, through the activity of sphingosine kinase 2 (Olivera et al., 2007). Thus, investigating the effects of WT and OX40-deficient Treg cells on the Ca^{2+} response of MCs was warranted. Interestingly, we observed that FcεRI-dependent Ca^{2+} mobilization in MCs was impaired in the presence of WT Treg cells, but not OX40-deficient Treg cells (Figure 4A). Given that PLC-γ2 activation was largely unaffected, this suggested that the effect on Ca^{2+} mobilization was probably independent from the emptying of intracellular stores. Indeed, the depletion of extracellular Ca^{2+} showed that MCs had a relatively normal mobilization of Ca^{2+} from intracellular stores in the presence of Treg cells. However, restoration of Ca^{2+} in the extracellular medium revealed a

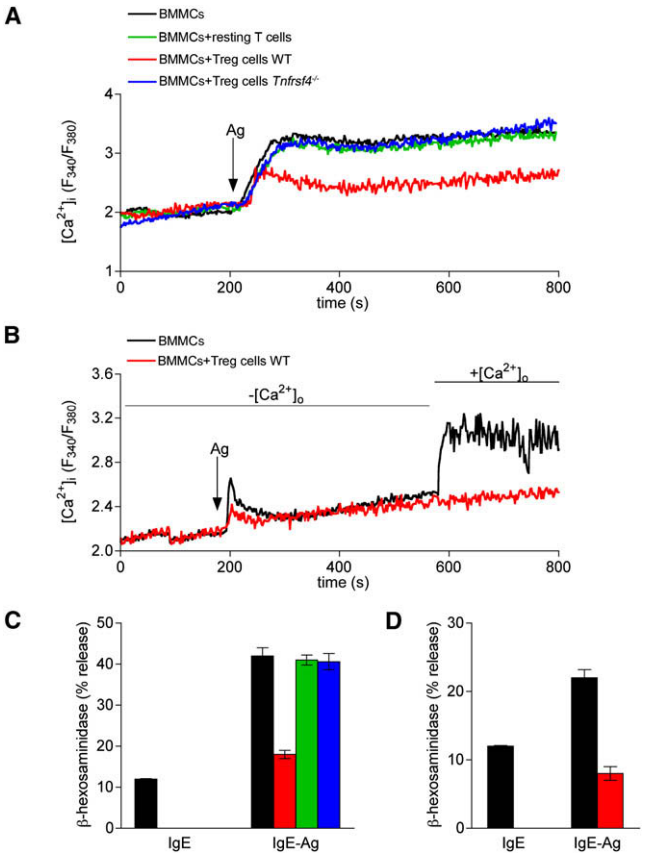

Figure 4. Reduced FcεRI-Dependent Ca^{2+} Influx after BMMC-Treg Cell Engagement, but Not Intracellular Ca^{2+} Mobilization

(A) BMMCs loaded with FURA-2AM were stimulated via FcεRI in the absence (BMMCs, black line) or presence of $CD4^+CD25^-$ T cells (resting T cells, green line), WT $CD4^+CD25^+$ Treg cells (red line), or *Tnfrsf4*$^{-/-}$ $CD4^+CD25^+$ Treg cells (blue line), and fluorescence emission was monitored.

(B) FURA-2AM-loaded BMMCs were stimulated via FcεRI and cocultured with WT $CD4^+CD25^+$ Treg cells (red line) in the absence of extracellular Ca^{2+}. 400 s after Ag stimulation, 2 μM Ca^{2+} was added to the medium, and fluorescence emission was monitored.

(C and D) At the end of each experiment, 14 min after Ag addition, the percentage of β-hexosaminidase release from each individual sample was measured. Results shown are the mean ± SD of one representative experiment from three independent experiments.

considerable defect in Ca^{2+} influx (Figure 4B). The ability or inability of these MCs to flux Ca^{2+} across the plasma membrane in the absence or presence of Treg cells was consistent with their normal or decreased MC degranulation, respectively (Figures 4C and 4D). Thus, when activated in the presence of WT Treg cells, but not OX40 null Treg cells, MCs showed reduced Ca^{2+} influx, independently of PLC-γ2 or Ca^{2+} release from intracellular stores.

cAMP Is Involved in Treg Cell-Mediated Suppression of BMMC Activation

Our data indicated that Treg cells directly inhibited MC degranulation by cell-cell contact through the interaction of OX40 on Treg cells with OX40L on MCs, resulting in the suppression of Ca^{2+} influx in MCs upon FcεRI stimulation. However, how these events are linked remained to be explored. Treg cells have been reported to block Teff cell functions by producing cAMP. Thus, one possibility was that Treg cell-mediated suppression of MCs might occur through Treg cells' production of cAMP, which could lead to decreased Ca^{2+} influx and suppression of degranulation in BMMCs, as recently shown (Hua et al., 2007). We first confirmed this effect by treating MCs with forskolin, which raises the intracellular cAMP concentration, and found that this treatment caused inhibition of MC degranulation (Figure S4) and induced a 1.5-fold increase in cAMP concentrations in BMMCs (Figure 5A). When IgE-Ag-activated BMMCs were incubated in the presence of Treg cells, cAMP concentrations in sorted BMMCs increased by approximately 3-fold (Figure 5A; $p = 0.005$). No increase in cAMP was observed when BMMCs were cocultured with *Tnfrsf4*$^{-/-}$ Treg cells or when *Tnfsf4*$^{-/-}$ BMMCs were cocultured with Treg cells (Figure 5A). No differences were found in the intracellular concentrations of cAMP in WT and *Tnfrsf4*$^{-/-}$ Treg cells alone or coincubated with BMMCs (Figure 5B). Therefore, these findings showed that constitutive OX40-OX40L interaction between Treg cells and MCs resulted in significant increase of cAMP only in the MCs. To address the possibility that OX40-OX40L interactions might enhance gap-junction formation, resulting in cAMP transfer from Treg cells to MCs as previously described for Treg cell-Teff cell interactions (Bopp et al., 2007), we first incubated BMMCs with OX40-expressing membranes from the K562 cell line and found that cAMP increased in MCs, albeit at lower amounts than with intact cells (Figure 5C). This was consistent with the more modest inhibitory effect of such OX40-expressing membranes on MC degranulation (Figure 2C), which likely reflects a less extensive engagement of OX40L by K562 OX40-expressing membranes relative to intact Treg cells. To fully exclude the passage of cAMP from Treg cells to MCs, we measured the transfer of calcein, a dye that diffuses only via gap junction (Fonseca et al., 2006), and found no transfer (Figure S5). These data suggest that the rise of cAMP in MCs is likely to result from intracellular signals induced by Treg cells to MCs through OX40L engagement. To determine whether the reversal of cAMP increase would cause reversal of the inhibitory effects, we pretreated MCs with the antagonist Rp-cAMP (shown to block cAMP-dependent PKA activity) and then tested MC degranulation and Ca^{2+} mobilization in the presence of Treg cells. Treatment with Rp-cAMP did not alter either cell viability or the threshold of MC activation (data not shown). Rp-cAMP-treated BMMCs were resistant to the inhibitory effects of Treg cells upon FcεRI stimulation, showing a degranulation response identical to that of FcεRI-stimulated MCs in the absence of Treg cells (Figure 5D). Moreover, in presence of the Rp-cAMP, the uptake of extracellular Ca^{2+} was unaffected by the presence of Treg cells (Figure 5E). Thus, cAMP increment within MCs is the mechanism responsible for Treg cells' inhibition of MC degranulation.

Treg Cells Control MCs' Ability to Release Histamine In Vivo through Constitutive OX40 Expression

To evaluate the role of Treg cells on the in vivo function of MCs, we employed an FcεRI-mediated acute-systemic-anaphylaxis model. This model depends on MCs, given that the observed increase in circulating histamine upon IgE-Ag stimulation is minimal in MC-deficient, c-kit-deficient mice (W/W^v) and absent in MC-deficient, stem-cell-factor-deficient mice (Sl/Sl^d). We first explored the effect of OX40 deficiency on the anaphylactic

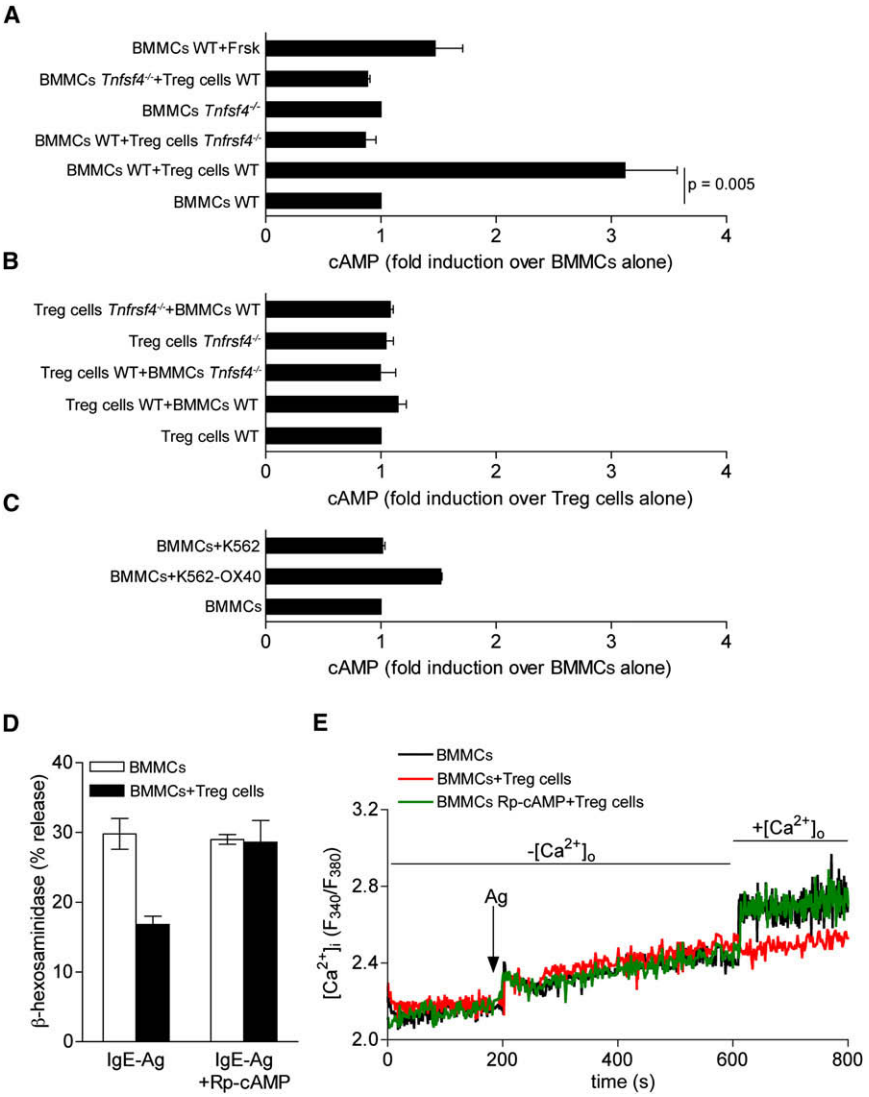

Figure 5. Treg Cells Inhibit MC Functions, Inducing the Increase of Intracellular cAMP in MCs

Sensitized WT and *Tnfrsf4*$^{-/-}$ BMMCs were CFSE labeled and Ag stimulated alone or with WT or *Tnfrsf4*$^{-/-}$ Treg cells. BMMCs and Treg cells were separated with FACS-based cell sorting, and cytosolic cAMP concentrations were measured with a cAMP-specific enzyme-linked immunosorbent assay (ELISA). As a positive control, sensitized BMMCs were incubated with forskolin and challenged with Ag.

(A) BMMCs' baseline [cAMP] was 10 pmoles/1 × 10^5 BMMCs. Results are expressed as fold induction over BMMCs alone.

(B) Treg cells' baseline [cAMP] was 50 pmoles/1 × 10^5 Treg cells. Results are expressed as fold induction over Treg cells alone.

(C) Sensitized BMMCs were activated with Ag plus K562 or K562-OX40 membranes. The means ± SD of three independent experiments are shown.

(D) Anti-DNP-IgE-preloaded BMMCs were preincubated for 30 min with 1 mM of the specific cAMP antagonist Rp-cAMP. Cells were washed and activated with Ag separately (BMMCs) or in coculture with $CD4^+CD25^+$ Treg cells (BMMCs + Treg cells). After 30 min, samples were examined for release of β-hexosaminidase as described. Shown are the means ± SD of three independent experiments, each performed in duplicate.

(E) IgE-sensitized BMMCs were preincubated for 30 min with 1 mM of the specific cAMP antagonist Rp-cAMP, and Ca^{2+} mobilization was assessed as described in Figure 4B. Results shown are representative of three independent experiments.

response by use of C57BL/6 *Tnfrsf4*$^{-/-}$ mice and the appropriate WT controls. As shown in Figure 6, *Tnfrsf4*$^{-/-}$ mice had significantly ($p = 0.001$) higher amounts of circulating histamine after challenge than did WT mice. The increase in circulating histamine concentrations ranged from 20%–35% of that seen in WT mice, with a mean increase of approximately 25%. To directly assess the importance of Treg cells, we used the approach of selectively depleting or inactivating these cells. C57BL/6 WT mice were treated with a CD25 antibody (PC61 Ab) 7 and 8 days prior to IgE sensitization for depletion of Treg cells. PC61 Ab was demonstrated to diminish Treg cell numbers but also decrease CD25 expression (Kohm et al., 2006; Simon et al., 2007). Thus, we measured both CD25 and Foxp3 expression in PC61-treated mice. We observed that more than 50% of $Foxp3^+$ T cells were decreased in both circulating blood cells and lymph nodes, and we also observed a more marked decrease (greater than 80% in the lymph nodes) in CD25 expression by $Foxp3^+$ T cells (Figure S6). Upon systemic anaphylactic challenge of Treg cell-depleted mice, circulating histamine concentrations mirrored those of OX40-deficient mice, showing a significant ($p < 0.005$) increase relative to those of WT controls (Figure 6A). No significant difference in FcεRI expression was detected in peritoneal MCs ($CD45^+$ c-kit^+) from non-IgE-sensitized mice, thus excluding the possibility that CD25 antibody treatment or OX40 deficiency might cause increased FcεRI expression and contribute to the observed in vivo effects (Figure 6B). Because differences in circulating IgE concentrations could affect the in vivo response of MCs by saturating FcεRI and increasing its expression (Yamashita et al., 2007), we measured serum IgE concentrations among *Tnfrsf4*$^{-/-}$ mice, PC61-treated mice, and WT controls and found them to be similar among all mice used in these experiments (data not shown). To more definitely show the role of OX40 expressed by Treg cells in controlling MC degranulation, we measured anaphylactic response in Treg cell-depleted thymectomized mice that had been left unreconstituted or reconstituted with WT or OX40-deficient Treg cells (Figure 6C). Strikingly, exogenous WT cells, but not *Tnfrsf4*$^{-/-}$ Treg cells, were able to revert in vivo the increased MC degranulation occurring in Treg cell-depleted hosts, thus proving the essential role of OX40 in mediating MC suppression by Treg cells.

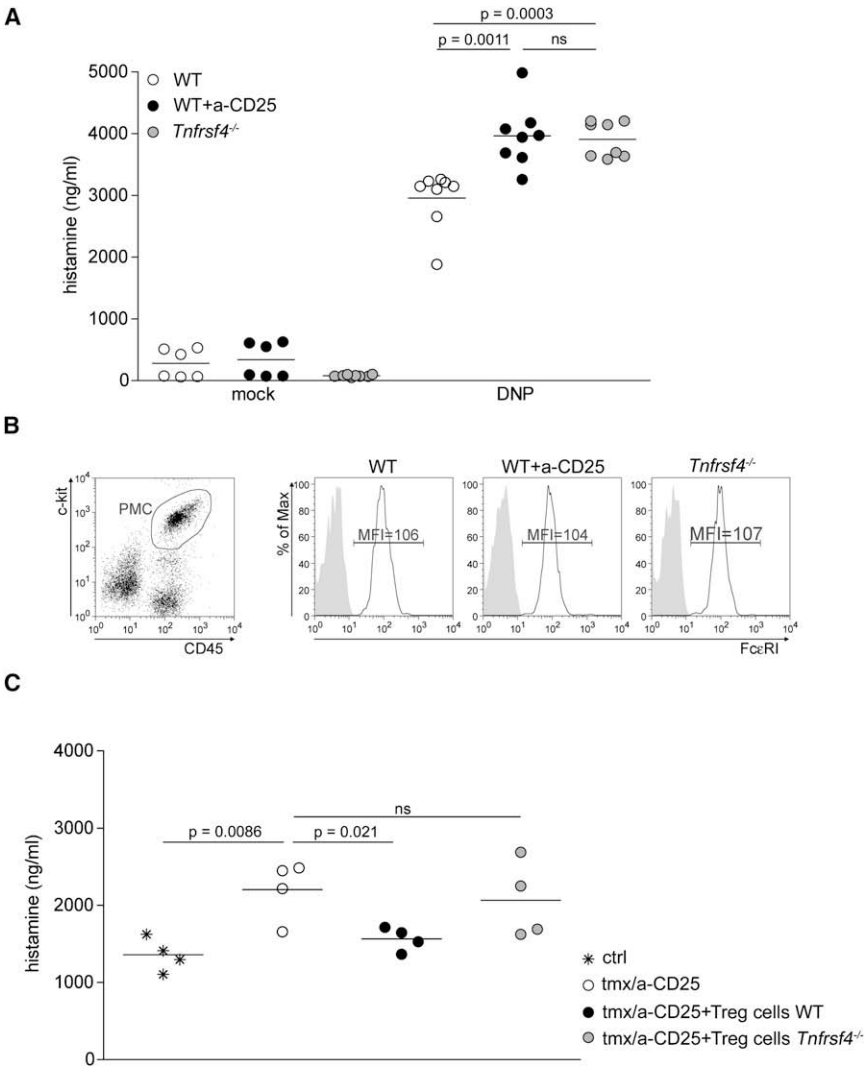

Figure 6. Treg Cells In Vivo Reduce via OX40 the Systemic Release of Histamine by MCs

(A) Plasma histamine levels were measured 1.5 min after challenge with PBS (mock) or DNP-HSA (DNP) in anti-CD25-treated mice, *Tnfrsf4*$^{-/-}$ mice, and control WT littermates that were presensitized with IgE anti-DNP (n = 8 in each group). Results shown are pooled from two independent experiments.

(B) CD45^{+} c-kit^{+} peritoneal MCs (PMC, left panel) from unsensitized mice in each experimental group were stained for FcεRI (black lines) or isotype control (shaded areas). Mean fluorescence intensity (MFI) is indicated.

(C) Thymectomized (tmx) and anti-CD25-treated mice were left unreconstituted or received i.v. 3 weeks after 1.5 × 10^{6} Treg cells obtained from WT or *Tnfrsf4*$^{-/-}$ donors. After 3 days, passive systemic anaphylaxis was induced, and plasma histamine was measured as in (A).

Collectively, the findings demonstrate the importance of Treg cells in suppressing FcεRI-induced MC degranulation in vivo in an OX40-dependent manner.

DISCUSSION

In recent years, MCs have been recognized to influence or be influenced by dendritic cells, T cells, and B cells, thus functioning as regulatory and/or effector cells (Sayed and Brown, 2007). However, little is known about this communication and the involvement of direct cell-cell contact. Because we found Treg cells and MCs in close proximity in vivo, we investigated the consequence of their interaction on MC effector responses. Here, we reported that Treg cells (but not other T cell populations) can inhibit MC degranulation through cell-cell contact. Specifically, we found that the interaction of OX40-expressing Treg cells with OX40L-expressing MCs inhibited the extent of MC degranulation in vitro and of the immediate hypersensitivity response in vivo. These findings established a previously unrecognized Treg cell-dependent regulation of MCs, the alteration of which might result in pathology. The functional presence of OX40L on both human and mouse MCs (Kashiwakura et al., 2004; Nakae et al., 2006) suggests that this regulatory mechanism is conserved across species.

Tnfrsf4$^{-/-}$ (OX40-deficient) mice do not have a higher incidence of spontaneous allergic disease, but rather, they show impaired development of allergic inflammation due to the requirement for OX40 in the development of Th2 cells (Jember et al., 2001; Salek-Ardakani et al., 2003). Bypassing this requirement for Th2 cell polarization and directly challenging the effector arm (MCs) of an allergic response, we revealed a role for Treg cell-expressed OX40 as a negative regulator dampening the immediate hypersensitivity caused by MCs. Whether this control of MC function extends beyond allergic responses is unclear. However, given the increasing evidence of a role for MCs in autoimmune diseases (Christy and Brown, 2007) and the demonstration that some organ-specific autoimmune disease can be mediated through MC-derived histamine and serotonin (Binstadt et al., 2006), the Treg cell-MC interplay herein described is potentially relevant to the development of autoimmunity. Notably, mutations in the X chromosome-encoded *Foxp3* gene (leading to Treg cell loss) were identified as the cause of the early-onset fatal autoimmune disorder observed in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and in *scurfy* mutant mice. Interestingly, in both humans and mice, this immune dysregulation is associated with increased asthma and allergy (Patel, 2001), thus arguing for the need of Treg cells in controlling allergic responses.

Tnfsf4$^{-/-}$ (OX40L-deficient) mice also display marked reduction of Th2 cell responses (Arestides et al., 2002; Hoshino et al., 2003). However, like in *Tnfrsf4*$^{-/-}$ mice, these effects can all be associated with the presence of OX40L, on activated APC, triggering Th2 cell polarization (Linton et al., 2003). Thus, although

OX40-OX40L interaction is essential for Th2 cell activation, our study showed that it also controlled the allergic response. Consistent with this view is the finding that induction of allergen-specific tolerant T cells caused a decrease in circulating histamine after allergen challenge in a mouse model of bronchial asthma (Treter and Luqman, 2000). This suppressive effect could not be entirely explained by reduced amounts of allergen-specific IgE, suggesting that tolerant T cells might act in another manner to modulate the FcεRI-dependent histamine release.

Treg cell-mediated control of degranulation transcends the MCs, given that granule exocytosis from cytotoxic T lymphocytes is inhibited in the presence of Treg cells through their physical interaction (Mempel et al., 2006). Notably, the inhibitory effect of Treg cells on MC degranulation, although considerable, was not complete and did not affect cytokine production. This selective inhibition of MC responses may underlie the complex relationship between Treg cells and MCs.

Considerable emphasis has recently been placed on the role of MCs as effectors in Treg cell tolerance. Unlike what is described here, soluble factors like Treg cell-derived IL-9 play a key role in mediating MC recruitment, and MC-secreted IL-10 and/or TGF-β may be involved in mediating the suppressive effects (Hawrylowicz and O'Garra, 2005; Lu et al., 2006). However, it remains to be determined whether MC degranulation is impaired when these cells act as effectors in Treg cell-mediated tolerance. Although it is well known that MCs can produce cytokines in the absence of degranulation (Theoharides et al., 2007), less is known about the impairment of MC degranulation when cytokine production is unaffected, but this type of effect is likely to require the selective dampening of signals.

The mechanism by which OX40-OX40L interaction drives inhibition of MC degranulation involves increased cAMP content within MCs. MC degranulation requires Ca^{2+} influx through store-operated Ca^{2+} channels that are sensitive to membrane potentials, which can be influenced by the ion balance across the plasma membrane. cAMP is known to alter the membrane potential, as observed in rat peritoneal MCs (Bradding, 2005; Penner et al., 1988). High cAMP concentrations within MCs were shown to cause decreased Ca^{2+} influx and inhibit degranulation (Hua et al., 2007). This inhibition is likely to result from the absolute requirement of the MC secretory granule fusion machinery for Ca^{2+} influx, given that the release of Ca^{2+} from intracellular stores alone is not sufficient to properly activate secretory fusion proteins (Blank and Rivera, 2004). Importantly, we found that drug-mediated antagonism of cAMP in OX40L-stimulated MCs reversed the decrease in Ca^{2+} influx and the inhibition of degranulation.

The inhibitory effect of cAMP also transcends the MCs, given that Treg cell suppression on Teff cells is wielded through cAMP transfer via gap junctions (Bopp et al., 2007). MCs express connexins and can form hexameric hemichannels, which do align in neighboring cells, forming gap junctions during cell contact (Vliagoftis et al., 1999). Thus, cAMP transfer was plausible between Treg cells and MCs. However, after the Treg cell-MC coincubation, cAMP increase was detected ìn MCs without the requirement of an intact $OX40^+$ cell, given that OX40-expressing K562 cell membranes could also elicit cAMP production in MCs. Moreover, no decrease in intracellular cAMP concentration was apparent in Treg cells after coincubation, excluding the translocation of cAMP from Treg cells to MCs. Finally, we failed to observe the transfer of calcein from Treg cells to MCs, suggesting that the increased cAMP elicited by OX40-OX40L interactions must be a result of OX40L signal in MCs triggering cAMP production. This argument is well supported by use of the antagonist Rp-cAMP, which reversed the effects of increased cAMP, namely inhibition of Ca^{2+} influx and of MC degranulation.

Direct antibody triggering of OX40L on MCs was not possible in this study because the available antibodies are crossreactive with the FcγR on MCs. Thus, signaling and function of OX40L on MCs remain to be elucidated. More is known about the effects of OX40 triggering on Treg cells. Virtually all Treg cells constitutively express OX40 at the naive stage, and OX40 engagement abolishes Treg cell suppression in vitro and in vivo (Takeda et al., 2004; Valzasina et al., 2005). This would suggest that OX40 engagement by OX40L-expressing MCs might reverse Treg cells' suppressive function, even though the complex consequences of this effect require further investigation. There is mounting evidence that deficiency in Treg cell number or function contributes to common allergic diseases and asthma. Here, we find that the decrease in Treg cells and/or their loss of function increases the responsiveness of MCs in vivo. Glucocorticoids, the most effective treatment for allergy, as well as the agonist of histamine receptor 4, induce the activation of Treg cells (Karagiannidis et al., 2004; Morgan et al., 2007). These findings suggest that the contribution of Treg cells toward the reduction of allergy could be mediated not only by inhibition of T cell-driven inflammation, but also by direct regulation of the release of preformed proinflammatory mediators by MCs. Therefore, induction and expansion of Treg cells could be a useful strategy in controlling allergen-mediated hypersensitivity.

EXPERIMENTAL PROCEDURES

Mice, Treatments, and Reagents

C57BL/6 and BALB/c mice were purchased from Harlan. C57BL/6 OX40-deficient mice (*Tnfrsf4*$^{-/-}$) (Pippig et al., 1999) were from the University of California at San Francisco. Bone marrow from C57BL/6 OX40L-deficient mice (*Tnfsf4*$^{-/-}$) was kindly provided by A.H. Sharpe, Harvard Medical School, Boston, USA. Mice were maintained under pathogen-free conditions at the animal facility of Fondazione IRCCS Istituto Nazionale dei Tumori Milano. Animal experiments were authorized by the Institute Ethical Committee and performed in accordance with institutional guidelines and national law (DL116/92). For in vivo experiments, mice intraperitoneally received 0.5 mg/0.2 ml of anti-CD25 (clone PC61, rat IgG1, ATCC, LGC) 7 days and 8 days prior to the systemic anaphylaxis induction. Anesthetized mice were thymectomized by suction method 4 days before PC61 injection and 3 weeks before i.v. transfer of 1.5×10^6 Treg cells. After 3 days, systemic anaphylaxis was induced. Murine DNP-specific IgE was produced as described (Liu et al., 1980). DNP-human serum albumin (DNP_{36}-HSA, Ag) and forskolin were from Sigma-Aldrich. Adenosine 3′,5′-cyclic monophosphorothioate, Rp-isomer, triethylammonium salt (Rp-cAMP) was from Calbiochem (Merck Biosciences).

Lymph Node Immunolabeling

For double immunohistochemical staining, 4 μm-thick sections were cut from formalin-fixed, paraffin-embedded inguinal lymph node samples from 8-week-old C57BL/6. Slides were preincubated with protein block (Novocastra), incubated with mouse anti-rat-FcεR (clone IRK) (Rivera et al., 1988) and then with biotinilated swine anti-mouse Ab and streptavidin-conjugated alkaline phosphatase (LSAB+ kit, Dako), and labeled with the fast red chromogenic substrate (Dako). Sections were incubated with the primary rat anti-mouse Foxp3 (clone FJK-16s, eBioscience) and secondary horseradish peroxidase (HRP)-conjugated anti-rat Ig and labeled with hydrogen peroxide-diaminobenzidine

(DAB+) (Dako). Imunohistochemical evaluation was performed with a Leica DM2000 optical microscope, and microphotographs were collected with a Leica DFC320 digital camera (Leica Microsystems).

Purification of $CD4^+CD25^+$ and $CD4^+CD25^-$ Subsets

$CD4^+CD25^+$ cells were purified with the $CD25^+$ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Flow cytometry showed that the separated fractions were more than 90% $Foxp3^+$ (Figure S2). For some experiments, $CD4^+CD25^-$ T cells were stimulated for 72 hr with 1 μg/ml of plate-coated anti-CD3 plus 2.5 μg/ml of soluble anti-CD28 (both from eBioscience).

Bone Marrow-Derived Mast Cell Differentiation, Activation, and FcεRI Expression

BMMCs were obtained by in vitro differentiation of bone marrow cells taken from mouse femur as described (Frossi et al., 2007). After 5 weeks, BMMCs were monitored for FcεRI expression by flow cytometry. Purity was usually more than 97%. BMMCs were obtained from three to four mice, and all experiments were performed with at least three individual BMMC cultures. Before experiments, 1×10^6 BMMCs per ml were sensitized in medium without IL-3 for 4 hr with 1 μg/ml of DNP-specific IgE and challenged with DNP-HSA in Tyrode's buffer (10 mM HEPES buffer [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA). For measurement of FcεRI expression ex vivo, MCs were enriched from peritoneal lavage by 70% Percoll Gradient (GE Healthcare) and stained with APC anti-CD45 (clone 104), FITC anti-c-kit (clone 2B8), and PE anti- FcεRI (clone MAR-1), all from eBioscience.

β-hexosaminidase- and Cytokine-Release Assay and PLC-γ2 Phosphorilation

IgE-presensitized BMMCs were challenged in Tyrode's buffer with Ag (100 ng/ml) for 30 min in the presence or absence of equal amounts of the indicated cell types. In some experiments, BMMCs and T cells were separated by a 3.0 μm transwell membrane (Corning Life Sciences). In some experiments, presensitized BMMCs were incubated for 30 min with 10 μg/ml blocking anti-OX40L (clone MGP34) (Murata et al., 2000) or isotype control (rat IgG2c) before Ag challenge in presence of $CD4^+CD25^+$ Treg cells. Samples were placed on ice and immediately centrifuged to pellet cells. The enzymatic activities of β-hexosaminidase in supernatants and in the cell pellets, after solubilizing with 0.5% Triton X-100 in Tyrode's buffer, were measured with *p*-nitrophenyl N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 60 min at 37°C. The reaction was stopped by addition of 0.2 M glycine (pH 10.7). The release of the product 4-*p*-nitrophenol was detected by absorbance at 405 nm. The extent of degranulation was calculated as the percentage of 4-*p*-nitrophenol absorbance in the supernatants over the sum of absorbance in the supernatants and in cell pellets solubilized in detergent. For cytokine analysis, IgE-sensitized BMMCs and Treg cells were cultured alone or together for 16 hr in presence of 100 ng/ml Ag. Concentrations of TNF-α and IL-6 were determined in supernatants with Mouse Inflammation Kit (BD Biosciences). For assessment of PLC-γ2 phosphorilation, BMMCs were Ag stimulated in the presence of Treg cells and fixed after the indicated time, then immediately stained with a PE-conjugated anti-PLC-γ2 (pY759) (BD Biosciences) and FITC anti-c-kit (clone 2B8, eBioscience). Flow-cytometry data were acquired on a FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (version 8.5.2; Treestar).

Expression of Mouse OX40 by K562 Cells and Preparation of Membranes

Human chronic myelogenous leukemia K562 cells were stably transfected with empty pCDNA3 plasmid or pCDNA3 expressing murine OX40 molecule (OX40Dir 5′GCGAATTCAGAAAGCAGACAAGG3′; OX40Rev 5′CACTCGA GTACTAATGCTCAGAT 3′). OX40-positive K562 cell clones were identified by flow cytometry with anti-mouse OX40 (OX86, BD Biosciences). Membranes from K562 and K562-OX40 cell clones were prepared as previously described (Merluzzi et al., 2008) and resuspended at a final concentration of 5×10^7 cell equivalents per ml on the basis of the starting cell numbers. Membranes were added to IgE-sensitized BMMCs at dilution of 1:125 v/v together with the Ag.

Intracellular Ca^{2+} Determination

For Ca^{2+} measurements, 1×10^6 IgE-sensitized BMMCs were loaded with 3 μM FURA-2AM (Molecular Probes) in RPMI 2% fetal bovine serum for 45 min at 37°C. Cells were washed in Tyrode's-BSA buffer (Saitoh et al., 2000), incubated in the presence of equal amounts of the indicated cell types, and challenged with Ag (20 ng/ml). All fluorescence measurements (excitation and emission wavelengths, 340/380 and 505 nm, respectively) were performed in a Perkin-Elmer LS-50B spectrofluorimeter (Perkin-Elmer) equipped with a thermostatically controlled cuvette holder and magnetic stirring. During the experiment, temperature was kept at 37°C. The changes in $[Ca2^+]_i$ are expressed as a ratio of the light emitted at 505 nm upon excitation at the two wavelengths, 340 and 380 nm (F340/F380).

FACS-Based Cell Sort and cAMP ELISA

For evaluation of the intracellular levels of cAMP, presensitized BMMCs were labeled for 15 min with 5 μM carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; CFSE) (Molecular Probes, Invitrogen) and Ag challenged in presence of Treg cells. After coculture, CFSE-labeled BMMCs and CD25PE-labeled Treg cells were isolated with a MoFlo cell sorter (Dako). The purity of the sorted populations was >99%. As positive control, sensitized BMMCs were incubated with 25 μM forskolin for 1 hr before Ag stimulation. Cells were washed twice in PBS and lysed in 0.1 M HCl/0.1% Triton X-100 (10^7 cells/ml). cAMP levels were measured with Correlate EIA Direct cAMP assay (Assay Design).

Systemic Anaphylaxis

Mice were sensitized with 3 μg of mouse DNP-specific IgE by tail vein injection. 24 hr later, mice were challenged i.v. with 0.5 mg of Ag or vehicle (PBS). After 1.5 min, mice were euthanized with CO_2, and blood was withdrawn by cardiac puncture. Plasma histamine concentration was determined by ELISA according to the producer's instruction (DRG Instruments).

Statistical Analysis

Results are expressed as the means ± SD. Data were analyzed with a nonpaired Student's t test (Prism software, GraphPad Software).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00462-7.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministero dell'Istruzione Università e Ricerca (PRIN 2005), Agenzia Spaziale Italiana (Progetto OSMA), Associazione Italiana Ricerca sul Cancro, and LR.11 del Friuli Venezia Giulia. S.P. is supported by a fellowship from Fondazione Italiana Ricerca sul Cancro. The work of J.R. and S.O. was supported by the National Institute of Arthritis, Musculoskeletal and Skin Diseases of the National Institutes of Health. We thank A.H. Sharpe (Harvard University) for providing *Tnfsf4*$^{-/-}$ bone marrow and I. Arioli for technical assistance. We also thank D. Cesselli and E. Puppato for cell sorting.

Received: October 8, 2007
Revised: July 7, 2008
Accepted: August 27, 2008
Published online: November 6, 2008

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