Mast Cells Down-Regulate $CD4^+CD25^+$ T Regulatory Cell Suppressor Function via Histamine H1 Receptor Interaction[1]

Nicholas A. Forward,* Suzanne J. Furlong,* Yongjun Yang,† Tong-Jun Lin,*† and David W. Hoskin[2]*‡§

Mast cells promote both innate and acquired immune responses, but little is known about the effect of mast cells on T regulatory (T_{reg}) cell function. In this study, we show for the first time that the capacity of murine $CD4^+CD25^+$ T_{reg} cells to suppress in vitro proliferation by $CD4^+CD25^-$ T responder (T_{resp}) cells in response to anti-CD3/anti-CD28 mAb-coated beads was reduced in the presence of syngeneic bone marrow-derived mast cells (BMMC) activated by FcεR cross-linking. Activated BMMC culture supernatants or exogenous histamine also inhibited T_{reg} cell suppressor function while the histamine H1 receptor-specific antagonist loratadine, but not the H2 receptor-specific antagonist famotidine, restored T_{reg} cell suppressor function in the presence of activated BMMC or activated BMMC culture supernatants. Moreover, treatment of T_{reg} cells with loratadine, but not famotidine, rescued T_{reg} cell suppressor function in the presence of exogenous histamine. In addition, the H1 receptor-specific agonist 2-pyridylethylamine dihydrochloride inhibited T_{reg} cell suppressor function to an extent that was comparable to histamine, whereas the H2 receptor-specific agonist amthamine dihydrobromide was without effect. Both T_{reg} cells and T_{resp} cells expressed H1 receptors. Exposure to histamine caused T_{reg} cells to express lower levels of CD25 and the T_{reg} cell-specific transcription factor Foxp3. Taken together, these data indicate that BMMC-elaborated histamine inhibited T_{reg} cell suppressor function by signaling through the H1 receptor. We suggest that histamine released as a result of mast cell activation by microbial products might cause a transient decrease in T_{reg} cell suppressor function, thereby enhancing the development of protective immunity. ***The Journal of Immunology,*** **2009, 183: 3014–3022.**

In recent years, the importance of several different T regulatory (T_{reg})[3] cell subsets in the establishment and maintenance of immune tolerance have become increasingly apparent. Perhaps the best studied of these are "natural" T_{reg} cells that develop in the thymus and are important endogenous regulators of immune responses to self- and foreign Ags (1, 2). The vast majority of naturally occurring $CD4^+$ T_{reg} cells constitutively express CD25 (IL-2R α-chain) (3) and the Foxp3 transcription factor, which plays a critical role in T_{reg} cell development and effector function (4–6). Endogenous T_{reg} cells can potently suppress the in vitro activation of other T cell subsets through contact-dependent processes that have been suggested to involve CTLA-4 (7), membrane-bound TGF-β (8), and/or the release of granzyme B, without or with a requirement for perforin (9, 10). T_{reg} cells also express the ectonucleotidases CD39 and CD73, which allows T_{reg} cells to generate adenosine and thereby mediate immune suppression (11). Although T_{reg} cell suppressor function appears to be strictly contact-dependent in vitro, immunosuppressive cytokines such as IL-10 and TGF-β contribute to T_{reg} suppressor activity in vivo (12). The ability of T_{reg} cells to prevent the activation of autoreactive T cells and limit nonspecific bystander damage by modulating immune responses to foreign Ags is of obvious benefit; nevertheless, the initiation of protective immune responses toward pathogens must involve processes that overcome or at least diminish the immunosuppressive activity of endogenous T_{reg} cells.

Although mast cells are traditionally viewed in the context of allergy and asthma, the ability of mast cells to become activated by diverse stimuli and to produce a wide variety of mediators makes them important players in many other physiological processes (13). Mast cells are an important component of the innate immune system, acting as sentinel cells that detect infecting microorganisms via pattern recognition molecules such as TLR (14). The wide variety of cytokines, chemokines, and other mediators, including histamine, that are produced and released by mast cells following their activation are believed to promote the recruitment of other immune effector cells and modulate their activity. Since mast cells are first-line responders to microbial infections and are present in environments that may also contain endogenous T_{reg} cells, it seems likely that mast cells and T_{reg} cells might affect each others' function. However, only limited information is available on the interactions that take place between mast cells and T_{reg} cells. In a mouse model of sepsis, adoptive transfer of T_{reg} cells correlates with an increase in mast cell numbers in the peritoneum (15), whereas mast cell recruitment to skin allografts in response to IL-9 produced by T_{reg} cells is essential for the establishment of tolerance to alloantigens (16). In addition, a recent study (17) showed that T_{reg} cells down-regulate FcεRI expression by mast cells in vitro

Departments of *Microbiology and Immunology, †Pediatrics, ‡Pathology, and §Surgery, Dalhousie University, Halifax, Nova Scotia, Canada

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[2] Address correspondence and reprint requests to Dr. David W. Hoskin, Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia B3H 1X5, Canada. E-mail address: d.w.hoskin@dal.ca

[3] Abbreviations used in this paper: T_{reg}, T regulatory; 2-PEA, 2-pyridylethylamine dihydrochloride; BMMC, bone marrow-derived mast cell; MFI, mean fluorescence intensity; T_{resp}, T responder; ADHB, amthamine dihydrobromide.

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through a contact-dependent mechanism and also down-regulate IgE-mediated leukotriene C_4 production by mast cells. Although these studies indicate that T_{reg} cells possess the capacity to recruit mast cells and regulate their activation, the effect(s) that mast cells have on T_{reg} cell suppressor function has not yet been investigated.

In this study, we show for the first time that histamine released by activated murine bone marrow-derived mast cells (BMMC) inhibited the suppressor function of $CD4^+CD25^+$ T_{reg} cells by signaling through the H1 receptor. In addition, the histamine-induced decrease in T_{reg} cell suppressor function was associated with reduced expression of CD25 and the T_{reg} cell-specific transcription factor Foxp3. Activation-induced release of histamine by mast cells may promote the development of protective immune responses to infecting microorganisms by transiently down-regulating endogenous T_{reg} cell activity.

Materials and Methods

Mice

For all experiments, 6- to 8-wk-old female C57BL/6 mice (Charles River Laboratories Canada) were used. Animals were cared for and housed in the Carleton Animal Care Facility (Dalhousie University) in accordance with Canadian Council on Animal Care guidelines.

Isolation and culture of T cells

$CD4^+CD25^+$ T_{reg} cells were isolated from mouse spleen cell preparations by negative selection for $CD4^+$ cells and positive selection for $CD25^+$ cells using a MACS mouse T_{reg} cell isolation kit (Miltenyi Biotec). The $CD4^+CD25^-$ T cell population was retained for use as T responder (T_{resp}) cells in suppression assays. The purity of the MACS-isolated T cell fractions was confirmed by two-color flow cytometric analysis using anti-CD25-PE mAb (Miltenyi Biotec) and anti-CD4-FITC mAb (eBioscience). Purity of the $CD4^+CD25^+$ T_{reg} cell fraction and $CD4^+CD25^-$ T_{resp} cell fraction was typically 90 and 95%, respectively. For all experiments, T cells were cultured at 37°C/5% CO_2/95% humidity in RPMI 1640 medium (Sigma-Aldrich Canada) supplemented with 5% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5 mM HEPES (all from Invitrogen).

BMMC differentiation and IgE priming

BMMC were obtained by culturing bone marrow cells from murine femurs and tibias in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10% WEHI-3B conditioned medium, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-ME (Sigma-Aldrich Canada), and 200 nM PGE_2 (Sigma-Aldrich Canada) for 4–6 wk. BMMC purity of >98% was achieved, as determined by staining of fixed cytocentrifuged BMMC preparations with the mast cell-specific dye toluidine blue (18). BMMC were primed overnight with 1 μg/ml anti-TNP IgE obtained from cultures of IGEL b4 hybridoma cells (American Type Culture Collection).

T cell proliferation assays

$CD4^+CD25^-$ T_{resp} cells (1×10^5) and/or $CD4^+CD25^+$ T_{reg} cells (2×10^4) were cultured in quadruplicate wells of a 96-well microtiter plate with either anti-CD3/anti-CD28 mAb-coated T cell expander beads (5×10^4; Invitrogen) and/or anti-TNP IgE-primed BMMC (2×10^4) and 10 ng/ml TNP-BSA (Sigma-Aldrich Canada) for 48 h. In some experiments, anti-TNP IgE-primed BMMC were cultured for 24 h in the presence of 10 ng/ml TNP-BSA to generate activated BMMC culture supernatants, which were then harvested and added to T cell cultures at a concentration that was reflective of 2×10^4 BMMC. Histamine, loratadine, famotidine, 2-pyridylethylamine dihydrochloride (2-PEA) (all from Sigma-Aldrich Canada), or amthamine dihydrobromide (ADHB) (Tocris Biosciences) were added to T cell cultures as indicated. Cultures were pulsed with 0.25 μCi of [^{3}H]TdR (MP Biomedicals) for the last 6 h of culture. Alternatively, $CD4^+CD25^-$ T_{resp} cells were labeled with 2 μM Oregon Green 488 dye (Invitrogen) for 15 min at room temperature and seeded in 96-well microtiter plates as previously described without or with unlabeled $CD4^+CD25^+$ T_{reg} cells and/or BMMC. After 72 h, quadruplicate cultures were pooled, and T_{resp} cell proliferation was analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

IL-2 assay

$CD4^+CD25^-$ T_{resp} cells (1×10^5) and/or $CD4^+CD25^+$ T_{reg} cells (2×10^4) were cultured in quadruplicate wells of a 96-well microtiter plate with either anti-CD3/anti-CD28 mAb-coated T cell expander beads (5×10^4) and/or anti-TNP IgE-primed BMMC (2×10^4) and 10 ng/ml TNP-BSA for 24 h. Culture supernatants were then harvested, and IL-2 levels in quadruplicate samples were determined using an IL-2 OptEIA ELISA kit (BD Biosciences).

Flow cytometry

$CD4^+CD25^+$ T_{reg} cells were stained for 30 min at 4°C with 5 μg/ml anti-CD25-FITC mAb (Cedarlane Laboratories) or fixed, permeabilized, and stained with 1 μg/ml anti-Foxp3-FITC mAb (eBioscience), according to the manufacturers' specifications. Control cells were stained with the appropriate FITC-conjugated isotype control Ab. Cells were analyzed using a FACSCalibur flow cytometer and CellQuest software.

Real-time quantitative RT-PCR

Total RNA was extracted from equal numbers of T_{resp} cells and T_{reg} cells using TRIzol reagent and reverse transcribed using Superscript II RNase H reverse transcriptase (both from Invitrogen), according to the manufacturer's instructions. Histamine H1 receptor mRNA was quantified by TaqMan MGB Probe and TaqMan Master Mix (both from Applied Biosystems) on the ABI Prism 7000 sequence detection system (95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). GAPDH mRNA was analyzed using the TaqMan Rodent GAPDH Control Reagents (Applied Biosystems). Data were analyzed using the relative standard curve method, according to the manufacturer's protocol. The result was expressed as a ratio of histamine H1 receptor to GAPDH mRNA. In addition, PCR products were resolved on 2% agarose gel and visualized under UV light after staining with ethidium bromide.

Western blotting

T cells (2.5×10^6) were pelleted and lysed with ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM Na_2HPO_4, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 5 mM EDTA, and 5 mM EGTA) containing freshly added protease and phosphatase inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μM aprotinin, 1 mM PMSF, 10 mM NaF, 1 mM DTT, and 100 μM Na_3VO_4). Protein was quantified using Bio-Rad Protein Assay reagent (Bio-Rad) and diluted with SDS-PAGE sample buffer (200 mM Tris-HCl (pH 6.8), 30% glycerol, 6% SDS, 15% 2-ME, and 0.01% bromophenol blue). Samples were then boiled for 5 min and stored at −80°C. Frozen cell lysates were thawed on ice; proteins were resolved by SDS-PAGE (20 μg protein/lane) and electrotransferred onto nitrocellulose membranes. Membranes were blocked in TBST (20 mM Tris-HCl (pH 7.6), 200 mM NaCl, and 0.05% Tween 20) containing 5% fat-free milk overnight at 4°C, then washed with TBST and incubated with anti-histamine H1 receptor mAb (clone H-300; Santa Cruz Biotechnology) diluted 1/200 in blocking solution overnight at 4°C. Membranes were washed with fresh TBST and incubated with HRP-conjugated goat anti-rabbit Ab (Santa Cruz Biotechnology) diluted 1/1000 in blocking solution for 1 h at room temperature. Membranes were washed with TBST, reacted with ECL reagents (GE Healthcare) for 1 min, and exposed to x-ray film. To confirm equal protein loading, membranes were incubated in stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-ME) at 37°C for 30 min to remove Abs, then washed with fresh TBST and sequentially probed with anti-actin mAb (clone I-19; Santa Cruz Biotechnology) and HRP-conjugated bovine anti-goat IgG Ab (Santa Cruz Biotechnology), both at 1/1000 dilution.

Statistical analysis

Data were analyzed using the Instat statistics program (GraphPad Software). Statistical comparisons were performed using Student's *t* test or ANOVA with the Tukey-Kramer multiple comparisons posttest.

Results

BMMC inhibit $CD4^+CD25^+$ T_{reg} cell suppressor function

To determine the capacity of mast cells to modulate the function of $CD4^+CD25^+$ T_{reg} cells, $CD4^+CD25^-$ T_{resp} cells were stimulated with anti-CD3/anti-CD28 mAb-coated beads alone or in combination with $CD4^+CD25^+$ T_{reg} cells (5:1 ratio) and/or anti-TNP IgE-primed BMMC and TNP-BSA. As expected, T_{reg} cells inhibited T_{resp} cell proliferation ($p < 0.001$), as determined by [^{3}H]TdR

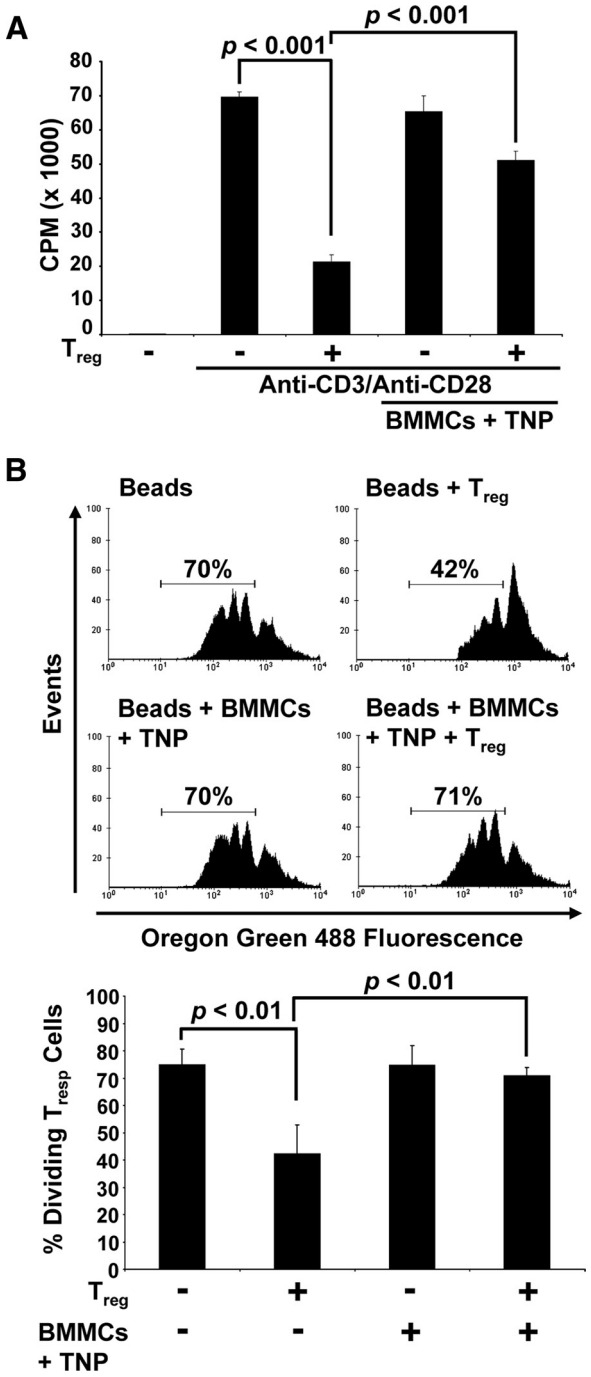

FIGURE 1. Inhibition of $CD4^+CD25^+$ T_{reg} cell suppressor function by activated BMMC. *A*, $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with $CD4^+CD25^+$ T_{reg} cells (2×10^4). Where indicated, anti-TNP-IgE-primed BMMC (2×10^4) and TNP-BSA (10 ng/ml) were also added to the cultures. After 48 h of incubation, cultures were pulsed with [^{3}H]TdR for the last 6 h of culture. Data from an experiment that is representative of three independent experiments are shown as mean cpm ± SD of quadruplicate cultures. *B*, Oregon Green 488-labeled $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with unlabeled $CD4^+CD25^+$ T_{reg} cells (2×10^4). Where indicated, anti-TNP IgE-primed BMMC (2×10^4) and TNP-BSA (10 ng/ml) were added to the cultures. After 72 h of incubation, T_{resp} cell proliferation was determined by flow cytometry. Data are from a representative experiment. Cumulative data from three independent experiments are shown as percent dividing T_{resp} cells ± SEM. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons posttest.

incorporation (Fig. 1*A*). TNP-activated BMMC did not affect T_{resp} cell proliferation. Importantly, there was a marked and significant ($p < 0.001$) reduction in T_{reg} cell-mediated suppression of T_{resp} cell proliferation in the presence of TNP-activated BMMC. Neither T_{resp} cell proliferation nor T_{reg} cell-mediated suppression of T_{resp} cell proliferation was altered in the presence of unactivated BMMC (data not shown). Because as many as three different cell populations were present in our assay system, it was important to confirm that changes in [^{3}H]TdR incorporation were due to increased T_{resp} cell proliferation and were not caused by increased proliferation of T_{reg} cells and/or BMMC. T_{resp} cells were therefore labeled with Oregon Green 488 dye, which allowed us to specifically measure T_{resp} cell division by flow cytometry. As shown in Fig. 1*B*, anti-CD3/anti-CD28 mAb-coated bead-stimulated T_{resp} cells that were cocultured with T_{reg} cells showed a smaller percentage of proliferating T_{resp} cells and fewer rounds of cell division over a 72-h period in comparison to T_{resp} cells activated in the absence of T_{reg} cells. Furthermore, the addition of anti-TNP IgE-primed BMMC and TNP-BSA to T_{reg} cell-T_{resp} cell cocultures restored T_{resp} cell proliferation to levels that were equivalent to that seen when T_{resp} cells were activated in the absence of T_{reg} cells. TNP-activated BMMC had no effect on T_{resp} cell division in the absence of T_{reg} cells. Unactivated BMMC did not affect T_{resp} cell division in the absence or presence of T_{reg} cells (data not shown). Taken together, these data show that activated BMMC down-regulated the suppressor function of $CD4^+CD25^+$ T_{reg} cells.

Supernatant from activated BMMC cultures abrogates T_{reg} cell suppressor function

To determine whether the inhibitory effect of mast cells on $CD4^+CD25^+$ T_{reg} cell function was due to a soluble factor, we next examined the effect of activated BMMC culture supernatant on $CD4^+CD25^+$ T_{reg} cell function. Cell-free culture supernatants were harvested after exposing anti-TNP IgE-primed BMMC to TNP-BSA for 24 h. $CD4^+CD25^-$ T_{resp} cells were then stimulated with anti-CD3/anti-CD28 mAb-coated beads either alone or in combination with $CD4^+CD25^+$ T_{reg} cells (5:1 ratio) in the absence or presence of supernatant from cultures of activated BMMC. As shown in Fig. 2*A*, T_{reg} cell suppressor function in a [^{3}H]TdR incorporation assay was abrogated in the presence of activated BMMC culture supernatant ($p < 0.001$). BMMC culture supernatant by itself did not affect T_{resp} cell proliferation. Similarly, the addition of activated BMMC culture supernatant to cocultures of Oregon Green 488 dye-labeled T_{resp} cells and unlabeled T_{reg} cells resulted in reduced T_{reg} cell-mediated suppression of T_{resp} cell division (Fig. 2*B*). Collectively, these data demonstrate that the inhibitory effect of BMMC on T_{reg} cell function was mediated by a BMMC-derived soluble factor.

Histamine inhibits $CD4^+CD25^+$ T_{reg} cell suppressor function

FcεR cross-linking causes mast cells to release large amounts of histamine, which is a soluble factor with numerous effects on the immune system (19). To determine whether histamine was able to inhibit $CD4^+CD25^+$ T_{reg} cell suppressor function, $CD4^+CD25^-$ T_{resp} cells were stimulated with anti-CD3/anti-CD28 mAb-coated beads in the presence of increasing doses of histamine (0–10 μM), either alone or in coculture with $CD4^+CD25^+$ T_{reg} cells (5:1 ratio). As shown in Fig. 3*A*, T_{reg} cell-mediated suppression of T_{resp} cell proliferation decreased with increasing doses of histamine, whereas histamine had no effect on T_{resp} cell proliferation in the absence of T_{reg} cells. Histamine (10 μM) did not affect the proliferation or viability of T_{reg} cells (data not shown). Since histamine could interfere with T_{reg} cell suppressor function either by

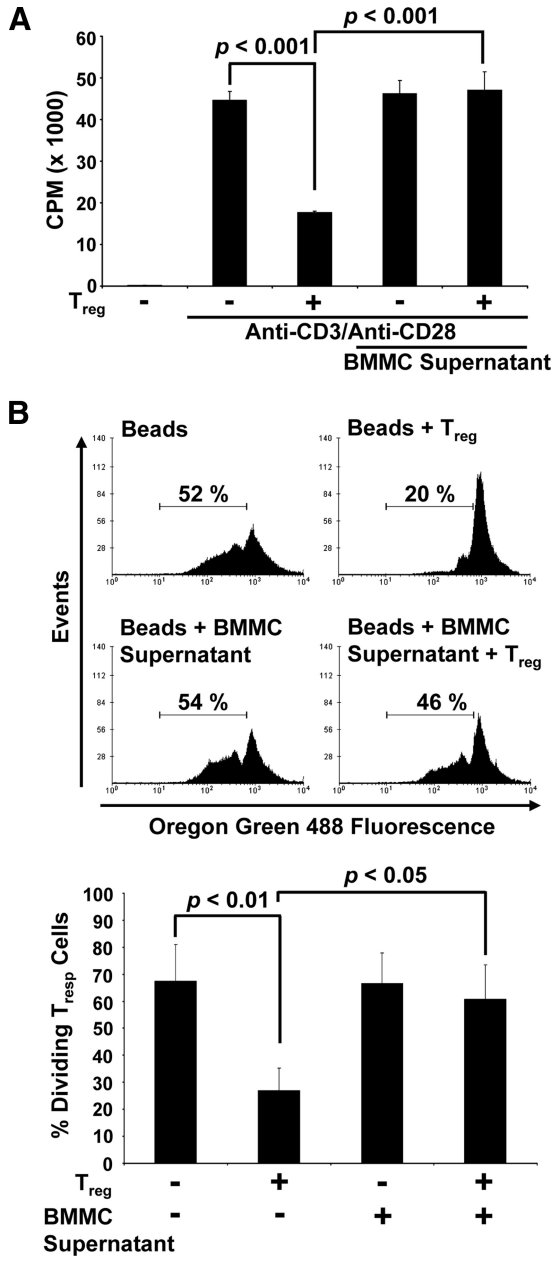

FIGURE 2. Inhibition of $CD4^+CD25^+$ T_{reg} cell suppressor function by activated BMMC culture supernatant. *A*, Anti-TNP IgE-primed BMMC were cultured for 24 h in the presence of TNP-BSA (10 ng/ml), after which cell-free culture supernatants were harvested. $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with $CD4^+CD25^+$ T_{reg} cells (2×10^4). Where indicated, activated BMMC culture supernatant was added to the cultures at a concentration that was reflective of 2×10^4 BMMC. After 48 h of incubation, cultures were pulsed with [^{3}H]TdR for the last 6 h of culture. Data from an experiment that is representative of three independent experiments are shown as mean cpm ± SD of quadruplicate cultures. *B*, Oregon Green 488-labeled $CD4^+CD25^+$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with $CD4^+CD25^+$ T_{reg} cells (2×10^4). Where indicated, activated BMMC culture supernatant was added to the cultures at a concentration that was reflective of 2×10^4 BMMC. After 72 h of incubation, T_{resp} cell proliferation was determined by flow cytometry. Data are from a representative experiment. Cumulative data from three independent experiments are shown as percent dividing T_{resp} cells ± SEM. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons posttest.

directly inhibiting the T_{reg} cells or by rendering T_{resp} cells refractory to T_{reg} cell-mediated suppression, we pretreated T_{resp} cells or T_{reg} cells with histamine (10 μM) for 30 min and then washed extensively before assaying cell proliferation by [^{3}H]TdR incorporation. T_{resp} cells that were pretreated with histamine proliferated normally following stimulation with anti-CD3/anti-CD28 mAb-coated beads and were suppressed by untreated $CD4^+CD25^+$ T_{reg} cells (Fig. 3*B*). In contrast, T_{reg} cells that were pretreated with histamine failed to inhibit the proliferation of untreated or histamine-pretreated T_{resp} cells. These data indicate that histamine directly inhibited T_{reg} cell suppressor function rather than rendering T_{resp} cells refractory to T_{reg} cell-mediated suppression. We also investigated the effect of histamine on T_{reg} cell-mediated inhibition of IL-2 production by anti-CD3/anti-CD28 mAb-coated bead-stimulated T_{resp} cells. Although histamine had no effect on IL-2 production by T_{resp} cells in the absence of T_{reg} cells, T_{reg} cell-mediated suppression of IL-2 production by T_{resp} cells was abrogated in the presence of histamine ($p < 0.001$; Fig. 3*C*).

H1 receptor antagonism prevents BMMC-mediated inhibition of T_{reg} cell suppressor function

To determine whether histamine was responsible for BMMC-mediated inhibition of $CD4^+CD25^+$ T_{reg} cell suppressor function, we treated cocultures of $CD4^+CD25^-$ T_{resp} cells, $CD4^+CD25^+$ T_{reg} cells, and anti-TNP IgE-primed BMMC with the histamine H1 receptor antagonist loratadine (20) or the histamine H2 receptor antagonist famotidine (21) before the addition of TNP-BSA and anti-CD3/anti-CD28 mAb-coated beads. Fig. 4*A* shows significant ($p < 0.001$) rescue of T_{reg} suppressor function in the presence of activated BMMC when loratadine was also present; however, there was no rescue by famotidine. Neither loratadine nor famotidine affected T_{resp} cell proliferation in the absence of T_{reg} cells. Similarly, treatment with loratadine, but not famotidine, significantly ($p < 0.001$) reduced the inhibitory effect of activated BMMC culture supernatant on T_{reg} cell suppressor function (Fig. 4*B*). Taken together, these data suggest that histamine was responsible for the inhibitory effect of activated BMMC on T_{reg} cell suppressor function and that BMMC-derived histamine acts on T_{reg} cells via the H1 receptor.

To confirm that histamine inhibited T_{reg} cell function by acting through H1 receptors, we showed that exposure to increasing doses (2.5–10 μM) of loratadine resulted in a dose-dependent rescue of T_{reg} cell suppressor function in the presence of histamine (Fig. 5*A*). In contrast, the highest dose of famotidine (10 μM) had only a slight effect on T_{reg} cell suppressor function in the presence of histamine. We also examined the effects of 2-PEA, a highly selective H1 receptor agonist (22), and ADHB, a highly selective H2 receptor agonist (23), on T_{reg} cell-mediated inhibition of T_{resp} cell proliferation in response to anti-CD3/anti-CD28 mAb-coated bead stimulation. Exposure to increasing doses of 2-PEA to T_{reg} cell-T_{resp} cell cocultures resulted in a dose-dependent decrease in T_{reg} cell-mediated suppression, whereas ADHB, even at the highest dose used, had little effect on T_{reg} cell function (Fig. 5*B*). Collectively, these data indicate that the inhibitory effect of histamine on T_{reg} cell suppressor function was mediated exclusively through H1 receptors.

H1 receptor expression by T_{reg} cells and T_{resp} cells

Although mouse T cells are known to express H1 receptors (24), H1 receptor expression by $CD4^+CD25^+$ T_{reg} cells has not yet been examined and compared with $CD4^+CD25^-$ T_{resp} cells. Real-time quantitative RT-PCR showed that H1 receptor mRNA expression by T_{resp} cells exceeded that of T_{reg} cells by ~2-fold

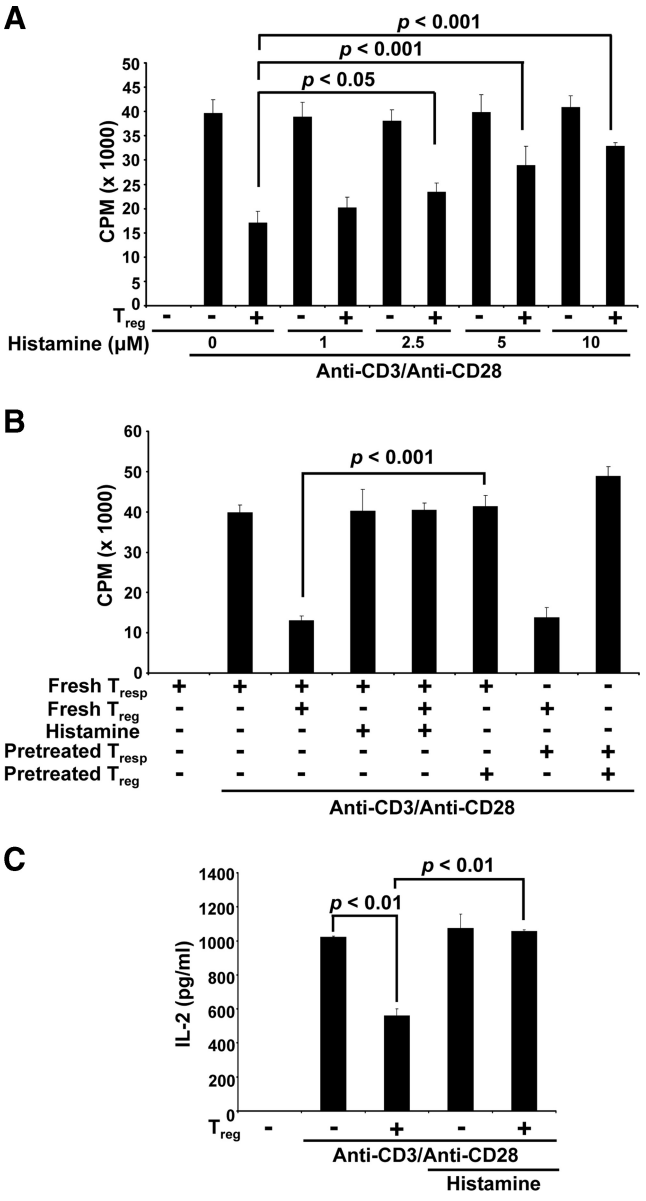

FIGURE 3. Inhibition of T_{reg} cell suppressor function by histamine. *A*, $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with $CD4^+CD25^+$ T_{reg} cells (2×10^4) and/or the indicated doses of histamine. After 48 h of incubation, cultures were pulsed with [^{3}H]TdR for the final 6 h of culture. Data from an experiment that is representative of three independent experiments are shown as mean cpm ± SD of quadruplicate cultures. *B*, $CD4^+CD25^-$ T_{resp} cells (1×10^5) were exposed to medium or 10 μM histamine for 30 min, washed, and then stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with untreated $CD4^+CD25^+$ T_{reg} cells or $CD4^+CD25^+$ T_{reg} cells (both at 2×10^4) that were pretreated with 10 μM histamine for 30 min and then washed. Exogenous histamine (10 μM) was also added to some cultures. After 48 h of incubation, cultures were pulsed with [^{3}H]TdR for the final 6 h of culture. Data from an experiment that is representative of three independent experiments are shown as mean cpm ± SD of quadruplicate cultures. *C*, $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with $CD4^+CD25^+$ T_{reg} cells (2×10^4) and/or 10 μM histamine. After 24 h of incubation, cell-free culture supernatants were harvested, and IL-2 levels were determined by ELISA. Data from an experiment that is representative of three independent experiments are shown as mean pg/ml ± SD of quadruplicate samples. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons posttest.

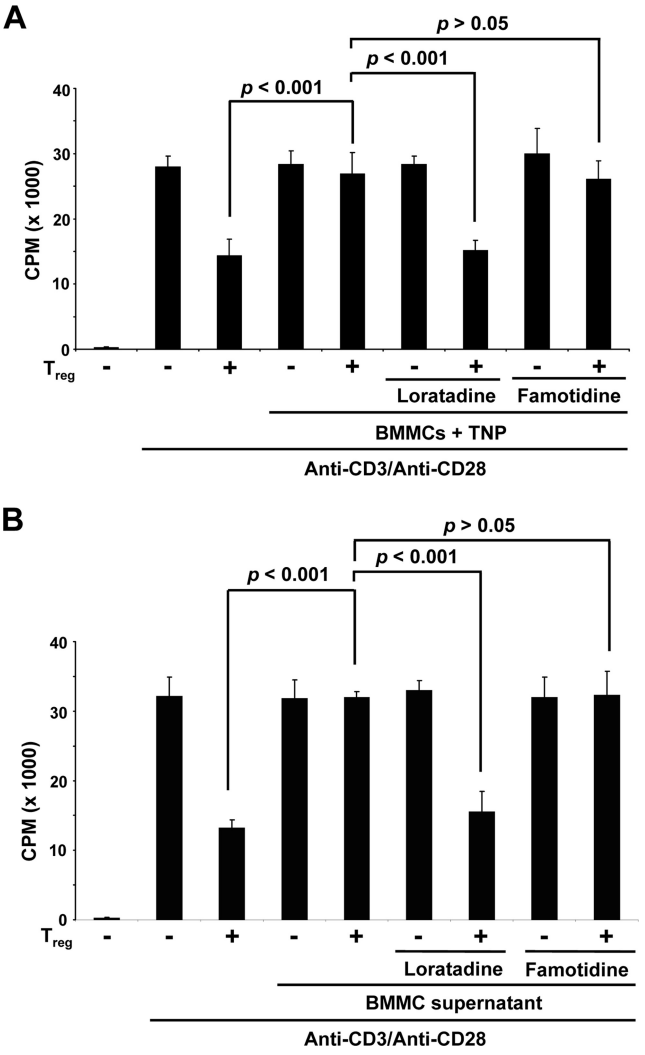

FIGURE 4. Rescue of T_{reg} cell suppressor function by the H1 receptor antagonist loratidine in the presence of activated BMMC or activated BMMC culture supernatant. *A*, $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with $CD4^+CD25^+$ T_{reg} cells (2×10^4) and/or the H1 receptor antagonist loratidine (10 μM) or the H2 receptor antagonist famotidine (10 μM). Where indicated, anti-TNP IgE-primed BMMC (2×10^4) and TNP-BSA (10 ng/ml) were also added to the cultures 30 min later. Cultures were incubated for 48 h and pulsed with [^{3}H]TdR for the last 6 h of culture. Data from an experiment that is representative of three independent experiments are shown as mean cpm ± SD of quadruplicate cultures. *B*, Anti-TNP IgE-primed BMMC were cultured for 24 h in the presence of TNP-BSA (10 ng/ml), at which point cell-free culture supernatant was harvested. $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 Ab-coated beads (5×10^4) alone or in coculture with $CD4^+CD25^+$ T_{reg} cells and/or loratadine or famotidine. Where indicated, activated BMMC culture supernatant was added 30 min later to the cocultures at a concentration that was reflective of 2×10^4 BMMC. Cultures were incubated for 48 h and pulsed with [^{3}H]TdR for the last 6 h of culture. Data from an experiment that is representative of three independent experiments are shown as mean cpm ± SD of quadruplicate cultures. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons posttest.

($p < 0.01$; Fig. 6*A*); however, similar levels of H1 receptor protein were expressed by T_{resp} cells and T_{reg} cells ($p > 0.05$; Fig. 6*B*).

Histamine down-regulates CD25 and Foxp3 expression by T_{reg} cells

$CD4^+CD25^+$ T_{reg} cells are characterized by the constitutive expression of the high-affinity IL-2R α-chain, CD25 (3). Moreover,

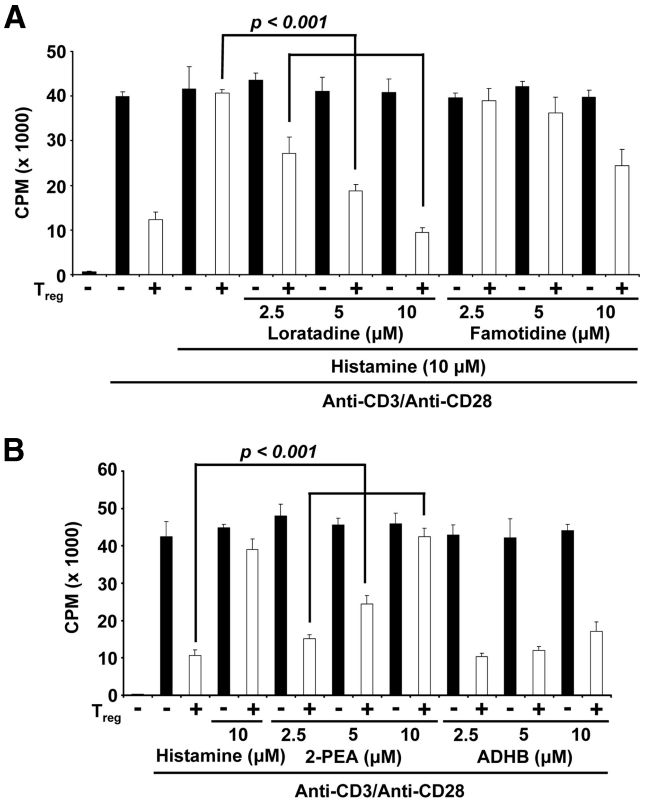

FIGURE 5. Histamine signals through H1 receptors to inhibit T_{reg} cell suppressor function. *A*, $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with $CD4^+CD25^+$ T_{reg} cells (2×10^4) and/or 10 μM histamine. The indicated doses of the H1 receptor antagonist loratadine or the H2 receptor antagonist famotidine were added 30 min before histamine treatment. After 48 h of incubation, cultures were pulsed with [^{3}H]TdR for the final 6 h of culture. Data from an experiment that is representative of three independent experiments are shown as mean cpm ± SD of quadruplicate cultures. *B*, $CD4^+CD25^-$ T_{resp} cells (1×10^5) were stimulated with anti-CD3/anti-CD28 mAb-coated beads (5×10^4) alone or in combination with $CD4^+CD25^+$ T_{reg} cells (2×10^4) and/or the indicated doses of histamine, the H1 receptor agonist 2-PEA, or the H2 receptor agonist ADHB. After 48 h of incubation, cultures were pulsed with [^{3}H]TdR for the last 6 h of culture. Data from an experiment that is representative of three independent experiments are shown as mean cpm ± SD of quadruplicate cultures. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons posttest.

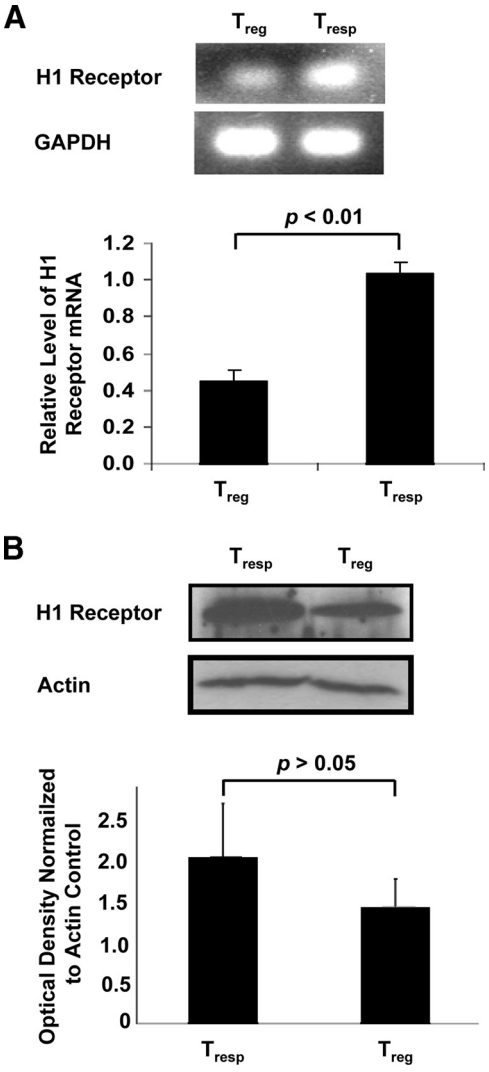

FIGURE 6. T_{reg} and T_{resp} cells express H1 receptor mRNA and protein. *A*, mRNA was extracted from equal numbers of $CD4^+CD25^-$ T_{resp} cells and $CD4^+CD25^+$ T_{reg} cells. H1 receptor expression relative to GAPDH was measured by quantitative real-time RT-PCR (*lower panel*) and visualized by ethidium bromide staining of the PCR product (*upper panel*). *B*, Cell lysates were prepared from 2.5×10^6 $CD4^+CD25^-$ T_{resp} cells or an equal number of $CD4^+CD25^+$ T_{reg} cells. Western blot analysis was performed to determine levels of H1 receptor protein (56 kDa; *upper panel*). Actin (42 kDa) expression was determined to confirm equal protein loading. Expression of H1 receptor relative to actin was quantified by densitometric analysis and expressed in arbitrary units (*lower panel*). Data represent the mean of three separate experiments ± SEM. Statistical significance was determined by Student's *t* test.

IL-2 is important for the development, maintenance, and activation of T_{reg} cells (25–27). We therefore examined the effect of histamine on CD25 expression by T_{reg} cells. After 24 h of culture in the absence or presence of 10 μM histamine, T_{reg} cells were stained for CD25 and analyzed by flow cytometry. In comparison to untreated T_{reg} cells, histamine-treated T_{reg} cells showed a marked decrease in CD25 expression (mean fluorescence intensity (MFI) = 105 ± 24 vs 27 ± 13, $p < 0.01$; Fig. 7*A*). We also determined the effect of histamine on T_{reg} cell expression of the forkhead box/winged helix transcription factor Foxp3, which is important for T_{reg} cell development and programming T_{reg} cell suppressor function (4–6). Fig. 7*B* shows that 24 h of exposure to 10 μM histamine caused a marked decrease in Foxp3 expression in comparison to untreated T_{reg} cells (MFI = 20 ± 9 vs 62 ± 8, $p < 0.01$). T_{reg} cell expression of CD25 and Foxp3 was therefore down-regulated by histamine. The inhibitory effect of histamine on CD25 and Foxp3 expression by T_{reg} cells was not evident at earlier time points (2 or 10 h), nor was it transient since washing out histamine from T_{reg} cell cultures after 30 min of exposure did not prevent CD25 and Foxp3 down-regulation at the 24-h time point (data not shown).

Discussion

There has been a paucity of information to date regarding the effect that mast cells have on T_{reg} cell function. We show here, for the first time, that histamine released by FcεR-activated BMMC abrogated the suppressor function of $CD4^+CD25^+$ T_{reg} cells. Activated BMMC potently inhibited the suppressor function of $CD4^+CD25^+$ T_{reg} cells through a mechanism that involved H1 histamine receptor signaling, because the H1 receptor antagonist loratadine blocked the inhibitory effect of activated BMMC,

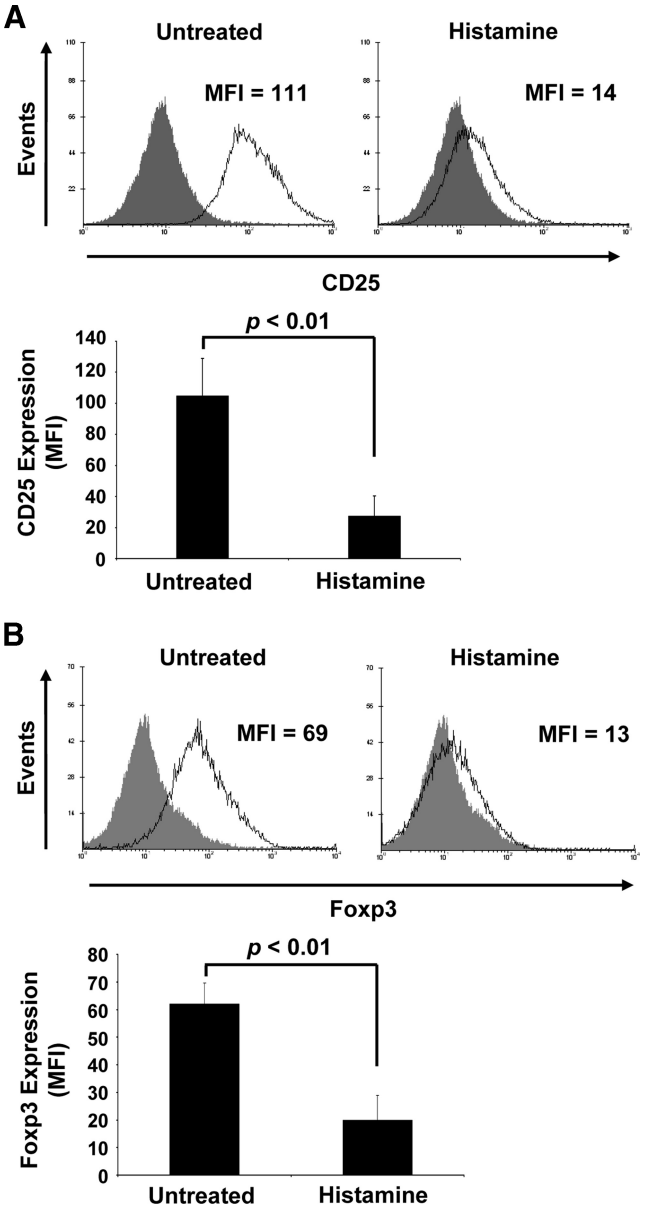

FIGURE 7. Histamine inhibits T_{reg} cell expression of CD25 and Foxp3. *A* and *B*, $CD4^+CD25^+$ T_{reg} cells were cultured for 24 h in the absence or presence of 10 μM histamine. Percent $CD4^+CD25^+$ T_{reg} cell viability at the end of 24 h was 24 $\pm$ 8 and 23 $\pm$ 8 in the absence or presence of histamine, respectively. T_{reg} cells were then stained with FITC-conjugated anti-CD25 mAb or FITC-conjugated isotype control Ab and CD25 expression (open peak) in comparison to background fluorescence (filled peak) was analyzed by flow cytometry (*A*). Alternatively, $CD4^+CD25^+$ T_{reg} cells were fixed, permeabilized, and stained with FITC-conjugated anti-Foxp3 mAb or FITC-conjugated isotype control Ab and Foxp3 expression (open peak) in comparison to background fluorescence (filled peak) was analyzed by flow cytometry (*B*). Flow cytometry histograms are from a representative experiment. Cumulative data from three independent experiments are shown as MFI $\pm$ SEM. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons posttest.

activated BMMC culture supernatant, or histamine on T_{reg} cell function. Furthermore, the H1 receptor agonist 2-PEA mimicked histamine-mediated inhibition of T_{reg} cell function. In contrast, H2 receptor antagonism with famotidine did not substantially block activated BMMC, activated BMMC culture supernatant, or histamine-mediated inhibition of T_{reg} cell function, and the H2 receptor agonist ADHB had little effect on T_{reg} cell function. Although both $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T_{resp} cells expressed H1 receptors, histamine acted on T_{reg} cells rather than on T_{resp} cells to render them refractory to T_{reg} cell-mediated suppression because the proliferation of T_{resp} cells that were pretreated with histamine and then washed was potently suppressed by untreated T_{reg} cells, whereas T_{reg} cells that were pretreated with histamine and then washed did not inhibit the proliferation of untreated T_{resp} cells.

T_{reg} cells are dependent on IL-2 production by nonregulatory T cells because T_{reg} cell-expressed Foxp3 interacts with and prevents NFAT from binding to the *IL-2* promoter (28). Indeed, IL-2 is critical for the activation and function of $CD4^+CD25^+$ T_{reg} cells (12, 26). It has been suggested that constitutive high-level expression of CD25 may allow T_{reg} cells to inhibit the proliferation of T_{resp} cells by outcompeting T_{resp} cells for available IL-2 (27). Decreased CD25 expression by histamine-treated T_{reg} cells might therefore impact negatively on their ability to sequester T_{resp} cell-secreted IL-2. In line with this, IL-2 in culture supernatant was markedly decreased when T_{resp} cells were activated in the presence of T_{reg} cells but was restored to control levels following the addition of histamine to T_{resp} cell-T_{reg} cell cocultures. However, alternative interpretations for reduced IL-2 levels in the presence of $CD4^+CD25^+$ T_{reg} cells must be considered since $CD4^+CD25^+$ T_{reg} cells also suppress IL-2 mRNA synthesis by $CD4^+CD25^-$ T_{resp} cells (29), as well as interfering with IL-2R signal transduction (30). In this regard, it is noteworthy that the suppressor function of T_{reg} cells is programmed by the Foxp3 transcription factor (5, 6), which also controls constitutive expression of CD25 by T_{reg} cells (6, 31, 32). Moreover, Foxp3 levels correlate with the suppressor capacity of $CD4^+CD25^+$ T_{reg} cells (33). The virtual ablation of Foxp3 expression by T_{reg} cells in the presence of histamine might therefore account for the BMMC-mediated reduction in T_{reg} cell suppressor function. Interestingly, IL-2 is important for the maintenance of Foxp3 expression by T_{reg} cells (34). It follows then that reduced CD25 expression by T_{reg} cells in the presence of histamine may compromise their ability to use IL-2, thereby depriving T_{reg} cells of signals needed for the maintenance of Foxp3 expression. However, it is important to note that CD25-deficient T_{reg} cells that express IL-2R β- and γ-chains remain responsive to IL-2 (35). Moreover, IL-7 and IL-15 are sufficient to maintain Foxp3 expression by T_{reg} cells in the absence of IL-2 (36). Because CD25 expression is not always necessary for the induction of T_{reg} cell suppressor function, it is possible that histamine inhibited $CD4^+CD25^+$ T_{reg} cell function by direct suppression of Foxp3 expression rather than by down-regulating constitutive T_{reg} cell expression of CD25 with an attendant decrease in Foxp3. Interestingly, the inhibitory effect of histamine on CD25 and Foxp3 expression by $CD4^+CD25^+$ T_{reg} cells was not reversed when histamine was removed, which was consistent with the loss of suppressor function by T_{reg} cells that were pretreated with histamine and then washed before addition to culture.

A number of mast cell-derived mediators are known to regulate the activity of effector T cells. For instance, mast cell-produced TNF-α up-regulates vascular cell adhesion molecule expression by mouse endothelial cells, thereby promoting T cell accumulation in the inflamed footpads of mice treated with the mast cell-activating compound 48/80 (37). In addition, the proliferation of murine $CD4^+$ T cells that are suboptimally activated with anti-CD3 mAb in the absence of CD28 costimulation is enhanced in the presence of FcεRI-activated BMMC (38). On the basis of our findings, it is conceivable that BMMC-mediated inhibition of T_{reg} cell suppressor function contributed to enhanced $CD4^+$ T cell proliferation in the absence of costimulation because the authors did not remove

$CD4^+CD25^+$ T_{reg} cells from their responder population. Histamine signaling through the H1 receptor is reported to enhance proliferative responses and IFN-γ production by $CD4^+$ Th1 cells (39); however, increased $CD4^+$ T cell proliferation and cytokine production may have been caused by histamine H1 receptor-mediated inhibition of endogenous $CD4^+CD25^+$ T_{reg} cells contained within the $CD4^+$ responder T cell population. These observations underscore the need to consider the possible effect(s) of endogenous $CD4^+CD25^+$ T_{reg} cells when using $CD4^+$ T cells that are heterogeneous in terms of their CD25 expression as a readout system.

Although T_{reg} cells constitute an important mechanism for regulating immune responses to both self- and foreign Ags (1, 2), optimal development of protective immune responses against invading microorganisms requires transient modulation of T_{reg} cell activity. For example, TLR2 and TLR8 ligands directly inhibit T_{reg} cell suppressor activity (40, 41), whereas signaling via TLR9 renders rat $CD4^+CD25^-$ T_{resp} cells refractory to T_{reg} cell-mediated suppression (42). In addition, dendritic cell secretion of the proinflammatory cytokine IL-6 partially blocks T_{reg} cell suppressor function (43). Our findings suggest a possible role for mast cells in regulating the immunosuppressive activity of endogenous $CD4^+CD25^+$ T_{reg} cells. We suggest that at an early stage of infection-induced inflammation mast cells are activated and degranulate in response to microbial products. In this regard, streptococcal exotoxin B stimulates human mast cells to release histamine (44), whereas FimH from *Escherichia coli* is a potent stimulator of mouse mast cell degranulation (45). Histamine signaling through the H1 receptor leads to a transient reduction in the suppressor function of endogenous $CD4^+CD25^+$ T_{reg} cells, which allows for optimal activation of effector $CD4^+$ and $CD8^+$ T cells. As the infectious agent is cleared, mast cell stimulation by microbial products is reduced, and local histamine levels decline. At this point, T_{reg} cell suppressor function is restored, and the immune response is down-regulated to minimize bystander damage to healthy tissues. Interestingly, intratracheal administration of the H4 receptor agonist 4-methylhistamine is associated with the accumulation of $Foxp3^+$ T cells in the lung in a mouse model of airway hypersensitivity (46), suggesting that histamine may also act via H4 receptors to recruit T_{reg} cells to resolve acute inflammation.

Disclosures

The authors have no financial conflict of interest.

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