JLB

Article

Histamine regulates autoreactive T cell activation and adhesiveness in inflamed brain microcirculation

Marilena Lapilla,[*,1] Barbara Gallo,*[*,1] Marianna Martinello,[†] Claudio Procaccini,[‡] Massimo Costanza,[*] Silvia Musio,[*] Barbara Rossi,[†] Stefano Angiari,[†] Cinthia Farina,[*] Lawrence Steinman,[§] Giuseppe Matarese,[‡] Gabriela Constantin,[†] and Rosetta Pedotti*[*,2]

[*]Neuroimmunology and Neuromuscular Disorders Unit, Neurological Institute Foundation, IRCSS Carlo Besta, Milan, Italy; [†]Department of Pathology, University of Verona, Verona, Italy; [‡]Istituto di Endocrinologia e Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, Naples, Italy; and [§]Department of Neurological Sciences and Interdepartmental Program in Immunology, Beckman Center for Molecular Medicine, Stanford University School of Medicine, Stanford, California, USA

RECEIVED SEPTEMBER 6, 2010; REVISED OCTOBER 18, 2010; ACCEPTED OCTOBER 23, 2010. DOI: 10.1189/jlb.0910486

ABSTRACT

Histamine may contribute to the pathology of MS and its animal model EAE. We explored the effects of histamine and specific HR agonists on activation and migratory capacity of myelin-autoreactive T cells. We show that histamine in vitro inhibits proliferation and IFN-γ production of mouse T cells activated against $PLP_{139–151}$. These effects were mimicked by the H1R agonist HTMT and the H2R agonist dimaprit and were associated with reduced activation of ERK1/2 kinase and with increased levels of cell cycle inhibitor p27Kip-1, both involved in T cell proliferation and anergy. H1R and H2R agonists reduced spontaneous and chemokine-induced adhesion of autoreactive T cells to ICAM-1 in vitro and blocked firm adhesion of these cells in inflamed brain microcirculation in vivo. Thus histamine, through H1R and H2R, inhibits activation of myelin-autoreactive T cells and their ability to traffic through the inflamed BBB. Strategies aimed at interfering with the histamine axis might have relevance in the therapy of autoimmune disease of the CNS. *J. Leukoc. Biol.* 89: 259–267; 2011.

Introduction

Histamine is an important mediator in several physiological and pathological processes, including neurotransmission and brain function, hormonal secretion, and gastrointestinal and circulatory function [1, 2]. Histamine is also critical in inflammation and a potent regulator of the innate and adaptive immune responses [3]. Synthesized from histidine by a unique enzymatic reaction mediated by HDC, histamine exerts its effects through four types of membrane HRs: H1R, H2R, H3R, and H4R. All HRs are heptahelical GPCRs with seven putative transmembrane domains and transduce extracellular signals through Gq (for H1R), Gs (for H2R), and Gi/o (for H3R and H4R) [1, 2]. In the immune system, histamine influences T cell polarization by having effects on monocytes and DCs, which promote a Th2 environment in humans and in mice [4–10]. Histamine can also influence T and B cell functions directly [11]. Indeed, in polarized human T cells, depending on the receptor engaged, histamine promotes Th1 responses through H1R and down-regulates Th1 and Th2 responses through H2R [12] with congruent data in mice lacking H1R and H2R [12, 13].

A role for histamine in MS and in EAE, an animal model for this disease associated with $CD4^+$ T cells reactive to myelin-secreting IFN-γ (Th1) and/or IL-17 (Th17) [14, 15], has been suggested (reviewed in refs. [3, 16]). We have shown previously that H1R as well as H2R are expressed on mononuclear cells within the inflammatory foci in the brain of mice with EAE, whereas encephalitogenic Th1 cell lines activated against $PLP_{139–151}$ expressed more H1R and less H2R compared with Th2 TCLs [17]. Inhibition of myelin oligodendrocyte $glycoprotein_{35–55}$-induced, chronic EAE has been reported in mice deficient for H1R [13, 18, 19], and treatment with H1R antagonists reduced the severity of $PLP_{139–151}$-induced, relapsing-remitting EAE [17, 20] and rat EAE [21]. Supporting the hypothesis of an important role for H1R in the development of EAE, it has been shown that *Bphs*, a gene that is associated

Abbreviations: ATTC=American Type Culture Collection, BBB=blood brain barrier, CMFDA=5-chloromethylfluorescein diacetate, CMTMR=5 (and 6-)-chloromethyl-benzoyl-amino-tetramethylrhodamine, C_T=comparative threshold cycle, EAE=experimental autoimmune encephalomyelitis, H1R=histamine receptor 1, HDC=histidine decarboxylase, $HDC^{-/-}$=histidine decarboxylase-deficient, histamine=2-(1*H*-imidazol-4-yl)ethanamine, HR=histamine receptor, HTMT=histaminetrifluoromethyl toluidide dimaleate, LNC=LN cell, MS=multiple sclerosis, PLP=myelin proteolipid protein, PSGL-1=P-selectin glycoprotein ligand-1, TCL=T cell line

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. These authors contributed equally to this study.

2. Correspondence: Neuroimmunology and Neuromuscular Disorders Unit, Neurological Institute Foundation, IRCCS Carlo Besta, Via Celoria 11, 20133 Milan, Italy. E-mail: rpedotti@istituto-besta.it

with susceptibility to EAE and other autoimmune diseases in animal models, is *H1R* [13]. Re-expression in T cells of the *H1R* allele from mice susceptible to EAE restores susceptibility to this disease in resistant H1R-deficient mice [18, 19]. However, histamine might also have an important role in limiting autoimmune brain inflammation, as $HDC^{-/-}$ mice, which lack histamine, exhibit exacerbation of chronic EAE with an increased T cell production of proinflammatory cytokines compared with WT mice, and a more diffuse inflammatory CNS infiltrates containing a large polymorphonuclear component and eosinophils [22]. Further, treatment with a specific agonist of H2R prevents chronic EAE [23]. These in vivo data emphasize a major regulatory role for histamine and its receptors in EAE. However, the direct effects of histamine on autoreactive myelin-specific T cells from nongenetically manipulated mice are largely unknown.

In this study, by using histamine and H1R and H2R agonists, we investigated the effects of these molecules on mouse T cells activated against the myelin peptide $PLP_{139-151}$. We show that these molecules modulate autoreactive T cells to reduce antigen-induced proliferation and IFN-γ production. HR agonists inhibit the adhesion of myelin-autoreactive T cells to mouse ICAM-1 in vitro and block firm adhesion of these cells on the activated brain vessels in an in vivo model of early EAE inflammation. The H1R agonist inhibits integrin activation of myelin-autoreactive T cells. These data show an unexpected role for histamine as an inhibitor of autoreactive T cell activation and reveal for the first time an important role for histamine in the regulation of the ability of myelin-activated T cells to traffic through the inflamed brain microcirculation.

MATERIALS AND METHODS

Mice

SJL mice (Charles River, Calco, Italy), female, 8–12 weeks old, were used in this study. All procedures involving animals were approved by the Ethical Committee of the Neurological Institute Foundation Carlo Besta (Milan, Italy) and performed according to the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC).

Peptide synthesis and immunization protocol

$PLP_{139-151}$ (HSLGKWLGHPDKF) was synthesized using standard 9-fluorenylmethyloxycarbonyl chemistry and purified by HPLC. The peptide purity was >95%, as assessed by analytical, reverse-phase HPLC. EAE was induced in mice by s.c. immunization in their flanks with $PLP_{139-151}$ (100 μg/mouse), emulsified in CFA containing 2 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI, USA), and assessed daily for neurological signs of EAE, according to a five-point scale [24].

T cell proliferation assays

LNCs or spleen cells were cultured in vitro in 96-well microtiter plates at a density of 500×10^3 cells/well (for LNCs) or 350×10^3 cells/well (for splenocytes) in 200 μl RPMI 1640, supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5×10 5 M), HEPES buffer (0.01 M), and 10% FCS (enriched RPMI), and stimulated with $PLP_{139-151}$ (20 μg/ml), Con A (2 μg/ml), anti-CD3 mAb (1 μg/ml; clone 41452C11, BD PharMingen, San Diego, CA, USA), or medium alone. The amount of histamine contained in culture medium was 1.1×10^{-9} M, as measured by an enzyme immunoassay kit (ImmunoTech, Beckman Coulter, Brea, CA, USA), as described previously [22]. $CD3^+$ T cells were purified from suspensions of splenocytes depleted of B220, CD11b, CD49b, and Ter-119-positive cells by magnetic separation (Miltenyi Biotec, Germany). Cell purity was confirmed by flow cytometric analysis and was determined to be 94% or higher. $CD3^+$ T cells (50×10^3/well) were stimulated in a 96-well plate with $PLP_{139-151}$ (20 μg/ml), anti-CD3 mab (1 μg/ml), or medium alone in enriched RPMI in the presence of 500×10^3 γ-irradiated (3000 rad) splenocytes from naïve mice as an APC source [25]. After 72 h of incubation (37°C, 5% CO_2), cultures were pulsed for 18 h with 0.5 μCi/well [H^3]-thymidine, and proliferation was measured from triplicate cultures on a β-counter (Perkin Elmer, Waltham, MA, USA). Data are shown as mean cpm ± SEM. To test the effects of histamine on T cell proliferation, LNCs or purified $CD3^+$ T cells were treated with histamine dihydrochloride (Sigma-Aldrich, Seelze, Germany), HTMT dimaleate (Tocris Bioscience, Bristol, UK), dimaprit dihydrochloride (Tocris Bioscience), or medium alone for 2 h at 37°C in RPMI 1640, washed, and in vitro-stimulated as described above. The concentrations of histamine, HTMT, or dimaprit used in the different experiments are reported in figure legends.

Cytokine measurements

Supernatants from LNCs or purified $CD3^+$ T cells cultured in parallel with those cells used in proliferation assays were used for cytokine analysis. IFN-γ, IL-10, IL-6 (anti-mouse OptEIA ELISA set, BD PharMingen), and IL-17 (anti-mouse DuoSet ELISA, R&D Systems, Minneapolis, MN, USA) were analyzed by ELISA, according to the manufacturers's protocols. Supernatants were collected from cultured cells at 48 h for IFN-γ and IL-6 and at 96 h for IL-10 and IL-17. Results are shown as mean of triplicates ± SEM, and SEMs were within 10% of the mean.

Real-time PCR

Total RNA was isolated from LNCs or from magnetically purified $CD3^+$ T cells from LNCs of naïve or immunized mice ex vivo at different time-points during EAE or upon in vitro stimulation using Trizol reagent (Invitrogen, San Diego, CA, USA), following the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using Superscript II RT (Invitrogen) and random primers, as described by the manufacturers. The expression of H1R and H2R was quantified by using the following commercial primer-probe sets (Applied Biosystems, Foster City, CA, USA): H1R, Mm00434002_s1; H2R, Mm00434009_s1; GAPDH, Mm99999915_g1. A C_T was used to determine H1R and H2R mRNA expression relative to housekeeping GAPDH. The C_T value was normalized for each sample using the formula: $\Delta C_T = C_{T\ (target)} - C_{T\ (GAPDH)}$, and the relative expression of the receptors was calculated using the equation $2^{-\Delta CT}$.

Western blot and biochemical analyses

Total cell lysates were obtained from LNCs ex vivo (baseline) and at different time-points during in vitro stimulation in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 μg/ml each aprotinin, leupeptin, and pepstatin, as described previously [26]. Total proteins from each lysate were subjected to SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (Protan, Schleicher and Schuell BioScience, Dassel, Germany) using a Trans-Blot cell (Bio-Rad Laboratories, Milan, Italy) and transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were placed in 5% nonfat milk in PBS plus 0.5% Tween 20 at 4°C for 2 h to block nonspecific binding sites. Filters were incubated with specific antibodies before being washed and then incubated with a peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). Peroxidase activity was detected by using the ECL system (Amersham Biosciences). The antibodies used were the following: anti-p27Kip-1 (Cell Signaling Technology Inc., Beverly, MA, USA), anti-ERK1/2, and antiphosphorylated ERK1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The filters were also probed with an antitubulin antibody (Sigma-Aldrich) to normalize the amount of loaded protein. All filters were quantified by densitometric anal-

ysis of the bands using the program ScionImage (Version 1.63 for Mac, Scion Corp. Inc., Frederick, MD, USA).

Flow cytometry

A TCL activated against $PLP_{139-151}$, prepared as described previously [27], was antigen-stimulated for 4 days and then treated for 2 h with histamine, HTMT, dimaprit, or medium alone before labeling with fluorescent antibody for α4 integrin (VLA-4; PS/2 clone, kindly provided by Dr. Eugene Butcher, Stanford University, Palo Alto, CA, USA), LFA-1 (anti-αL chain; clone TIB-213 from ATCC, Manassas, VA, USA), PSGL-1 (clone 4RA10, kindly provided by Dr. Dietmar Vestweber, Max Plank Institute, Germany), L-selectin (Mel-14 clone, ATCC), and CD44 (IM/7 clone, ATCC). Isotype-matched antibodies were used as controls. Cell suspensions were incubated with 15% adult bovine serum before incubation with the specific mAb at 4°C for 30 min. At least 10,000 events were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA) using the CellQuest software.

In vitro T cell adhesion to mouse ICAM-1

A TCL activated to $PLP_{139-151}$ was treated with histamine, HTMT, or dimaprit or left untreated for 2 h at 37°C and then added in triplicates on slides coated overnight at 4°C with purified mouse ICAM-1 as described [28]. Binding assay medium was represented by DMEM without sodium bicarbonate containing 10 mM HEPES and 5% BCS (HyClone, Logan, UT, USA), pH 7.2. To determine spontaneous adhesion, 100×10^3 T cells in 25 μl/well were incubated on ICAM-1-coated slides for 10 min at 37°C, and then slides were washed in PBS and fixed. For chemokine induced adhesion, 50×10^3 T cells in 25 μl/well were incubated on ICAM-1-coated slides for 3 min at 37°C, CCL20 (MIP-3β; 1 μM) was added, and after an additional 3 min, incubation slides were washed in PBS and fixed. Computer-assisted enumeration was performed.

Intravital microscopy and image analysis

Lymphocytes of a $PLP_{139-151}$-activated TCL were labeled with green CMFDA (Molecular Probes, Eugene, OR, USA) or orange CMTMR (Molecular Probes). Cells were kept for 1–5 days in antigen-free medium before intravital microscopy experiments to reduce background adhesiveness and to allow inside-out signaling generated by local proadhesive agonists [29]. For the last 2 h, cells were treated with HTMT, dimaprit, or PBS. Mice were surgically treated and injected i.p. with 12 μg LPS (*Escherichia coli* 026:B6; Sigma-Aldrich) 5–6 h before starting the intravital experiment as described previously [29]. A total of 2×10^6 fluorescent-labeled cells/condition was injected slowly into the carotid artery by a digital pump. The images were visualized by using a silicon-intensified target video camera (VE-1000 SIT, Dage-MTI, Michigan City, IN, USA) and a Sony SSM-125CE monitor and recorded using a digital VCR (Panasonic NV-DV10000). Video analysis was performed by playback of digital videotapes as described [29]. No significant differences were observed in hemodynamic parameters during the injection of cells treated with histamine agonists or left untreated. Lymphocytes that remained stationary on a venular wall for ≥30 s were considered adherent. At least 140 consecutive cells/venule were examined. Rolling and firm-arrest fractions were determined as the percentage of cells that roll or firmly arrest within a given venule on the total number of cells that enter that venule during the same period of time.

ImageStream data acquisition and analysis

Lymphocytes of a $PLP_{139-151}$-activated TCL were treated with HTMT for 2 h or left untreated before chemokine stimulation with CXCL12. Cells (10^6) were then incubated with 10 μg/ml TIB-213 (anti-LFA-1; ATCC) for 30 min on ice. After washing, cells were stained with a secondary antibody (goat anti-rat IgG PE-conjugated; Caltag Laboratories, Burlingame, CA, USA). Stained cells were resuspended in PBS for the ImageStream analysis. Images were acquired on the ImageStream imaging cytometer System 100 (Amnis Corp., Seattle, WA, USA). Images of fixed cells were collected and analyzed using ImageStream data exploration and analysis software [30]. LFA-1 clustering was evaluated, analyzing the distribution on the cell surface of the fluorescence, associated with the PE staining specific for the integrin. Uniform (uniform distribution of fluorescence), clustered (small spots of fluorescence), and caps (big clusters of fluorescence) cells were gated using the Area feature versus the Delta Centroid XY feature [31]. The Area feature was calculated for Channel 4 (PE-specific emission; area of fluorescence), applying to the images of a threshold mask; this feature allows us to discriminate between cells with a larger fluorescence area (high Area values) and smaller fluorescence area. The Delta Centroid XY feature calculates the distance between the center of the PE fluorescence image and the center of the brightfield image for each image pair. This feature distinguished images with globally distributed staining (lower Delta Centroid values) from those with capped staining (higher Delta Centroid values). When plotted versus the Area feature, Delta Centroid XY permits distinguishing between punctate and uniform staining [31]. In our analysis, cells with Area values higher than 30 and Delta Centroid XY values lower than 180 were considered uniform cells for their fluorescence distribution. Cells with Area values lower than 30 and Delta Centroid XY values lower than 2 were considered clustered cells (small spots of fluorescence). Cells with Delta Centroid XY values higher than 2 were considered caps cells (highly polarized fluorescence).

Statistical analysis

Student's *t* test, two-tailed, was used to compare results between two groups. For intravital microscopy studies, multiple comparisons were performed using the Kruskall-Wallis test with the Bonferroni correction of *P*. Differences were regarded significant with a value of $P < 0.05$. Analysis was performed with SPSS software (Chicago, IL, USA). In all tests, $P < 0.05$ was considered statistically significant.

RESULTS

Histamine reduces in vitro proliferation and IFN-γ production of myelin-autoreactive T cells

Before exploring the effects of histamine on T cells activated against myelin, we examined the expression of H1R and H2R ex vivo on LNCs and on purified $CD3^+$ T cells derived from SJL mice ($H\text{-}2^s$ haplotype) in which relapsing-remitting EAE was induced with $PLP_{139-151}$. H1R and H2R were expressed at the mRNA level on LNCs (**Fig. 1A**) and on purified $CD3^+$ T cells (Fig. 1B) of mice with EAE, and transcripts for H2R were always over tenfold more elevated compared with those for H1R. The expression of both receptors varied during the different phases of EAE on LNCs and T cells, a fact that might be related to changes in the cytokine milieu [1] and/or in the activation state of T cells and of other immune cells during the disease. Indeed, it has been reported that H1R mRNA is down-regulated rapidly in unprimed $CD4^+$ T cells upon TCR activation with anti-CD3/CD28 antibody [18]. The reduced transcripts of H1R on LNCs and on purified T cells at the onset of EAE might reflect the activation of T cells during disease priming. Also, as autoreactive T cells after priming migrate rapidly from the LNs toward the CNS, differences in mRNA expression of HRs might also be influenced by the percentage of myelin-specific T cells that are present in LNs at a given time during EAE.

We then explored in vitro the effects of histamine on proliferation and cytokine production of LNCs derived from mice with EAE. As shown in **Fig. 2A**, treatment of LNCs with histamine for 2 h before in vitro stimulation with $PLP_{139-151}$ re-

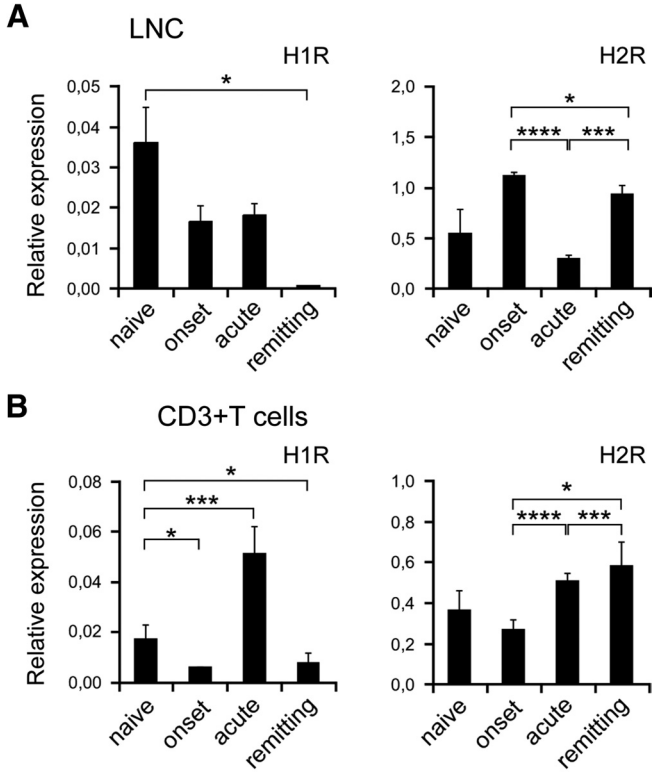

Figure 1. mRNA expression of H1R and H2R during the course of EAE. Freshly isolated LNCs (A) or LNC-purified $CD3^+$ T cells (B) from SJL mice at different phases of $PLP_{139-151}$-induced EAE were used to examine ex vivo mRNA expression of H1R and H2R. Mice were considered as remitting when the EAE score was decreased at least one point for at least two consecutive days. Data represent mean mRNA expression relative to GAPDH mRNA expression of three to four mice at each time-point ± SD and are representative of two independent experiments. $*P < 0.05$; $***P < 0.005$; $****P < 0.001$.

duced LNC proliferation and production of the Th1 cytokine IFN-γ, and no significant changes were observed in the production of the suppressor cytokine IL-10. As reported previously [11], we did not observe in these experiments a typical dose-response curve, a fact that could be related to the different affinity of histamine for H1R, H2R, and probably, for H4R (not evaluated in this study). Reduced proliferation and production of IFN-γ were even more pronounced when histamine was used to treat LNCs derived from mice at later stages of EAE, and IL-10 was also reduced by histamine treatment at those stages of the disease (Supplemental Fig. 1). To evaluate the contribution of H1R and H2R activation to the effects produced by histamine on these cells, we treated LNCs with HTMT, a selective H1R agonist, or dimaprit, a selective H2R agonist, for 2 h before antigen stimulation. As shown in Fig. 2B, HTMT inhibited proliferation and IFN-γ production significantly from LNCs, and IL-17 was also reduced by this treatment. All agonists inhibited the production of IL-6 in these cells, although HTMT was more effective in doing so, and IL-10 was slightly but significantly reduced by dimaprit.

A variety of immune cells expresses the receptors for histamine [1–3], and the effects of histamine that we observed on activated LNCs were likely to be the result of the triggering of this mediator on receptors expressed on different immune cells. We therefore wanted to ascertain the effects of histamine specifically on T cells by treating with histamine and its agonist $CD3^+$ T cells, which were purified magnetically from splenocytes of mice with EAE. As shown in Fig. 2C, treatment of $CD3^+$ T cells with histamine, HTMT, or dimaprit for 2 h before in vitro antigen stimulation inhibited T cell proliferation and IFN-γ production significantly and reduced the secretion of IL-17, IL-6, and IL-10.

Effect of histamine on biochemical pathways involved in T cell activation and anergy

To understand the molecular mechanisms by which histamine modulated proliferation and cytokine production of myelin-activated T cells, we analyzed the ERK1/2 pathway, a known regulator of T cell proliferation and IFN-γ production. As shown in **Fig. 3A**, treatment with histamine, HTMT, or dimaprit before in vitro antigen stimulation of LNCs from mice with EAE reduced ERK1/2 phosphorylation, and HTMT induced the strongest inhibition. These data were in line with the reduction of IFN-γ production observed by LNCs upon treatment with histamine and its agonists. Also, we studied the modulation of cyclin-dependent kinase inhibitor p27Kip-1, a molecule involved in the control of the cell cycle and in the induction and maintenance of T cell anergy [32, 33]. The expression of p27Kip-1 was increased in antigen-stimulated LNCs that were pretreated with histamine, HTMT, or dimaprit, and dimaprit produced the strongest effect (Fig. 3B), suggesting the induction of a state of hyporesponsiveness in these treated cells.

Histamine reduces the adhesion capability of myelin-autoreactive T cells in vitro and in an in vivo model of early EAE inflammation

We then wanted to ascertain whether histamine affects the ability of myelin-autoreactive T cells to adhere to brain endothelium and thus, to influence their capacity to migrate through the BBB. We first explored the ability of histamine to modulate the adhesion of myelin-activated T cells to mouse ICAM-1 in vitro. As shown in **Fig. 4A** and **B**, treatment of a TCL activated against $PLP_{139-151}$ with histamine for 2 h significantly reduced spontaneous and chemokine-induced adhesion to ICAM-1. HTMT and dimaprit produced effects that were concordant with those produced by histamine, although the more profound inhibition of T cell adhesion to ICAM-1 was observed with the H1R agonist HTMT.

We next examined whether histamine triggering through H1R or H2R affects the ability of myelin-activated T cells to adhere to the endothelium in inflamed brain microcirculation in vivo by using intravital microscopy in an experimental model that mimics early EAE inflammation [29, 34]. As shown in Fig. 4C and D, pretreatment with HTMT and dimaprit of T cells of the TCL activated against $PLP_{139-151}$ had no significant effect on rolling but led to a significant inhibition of firm

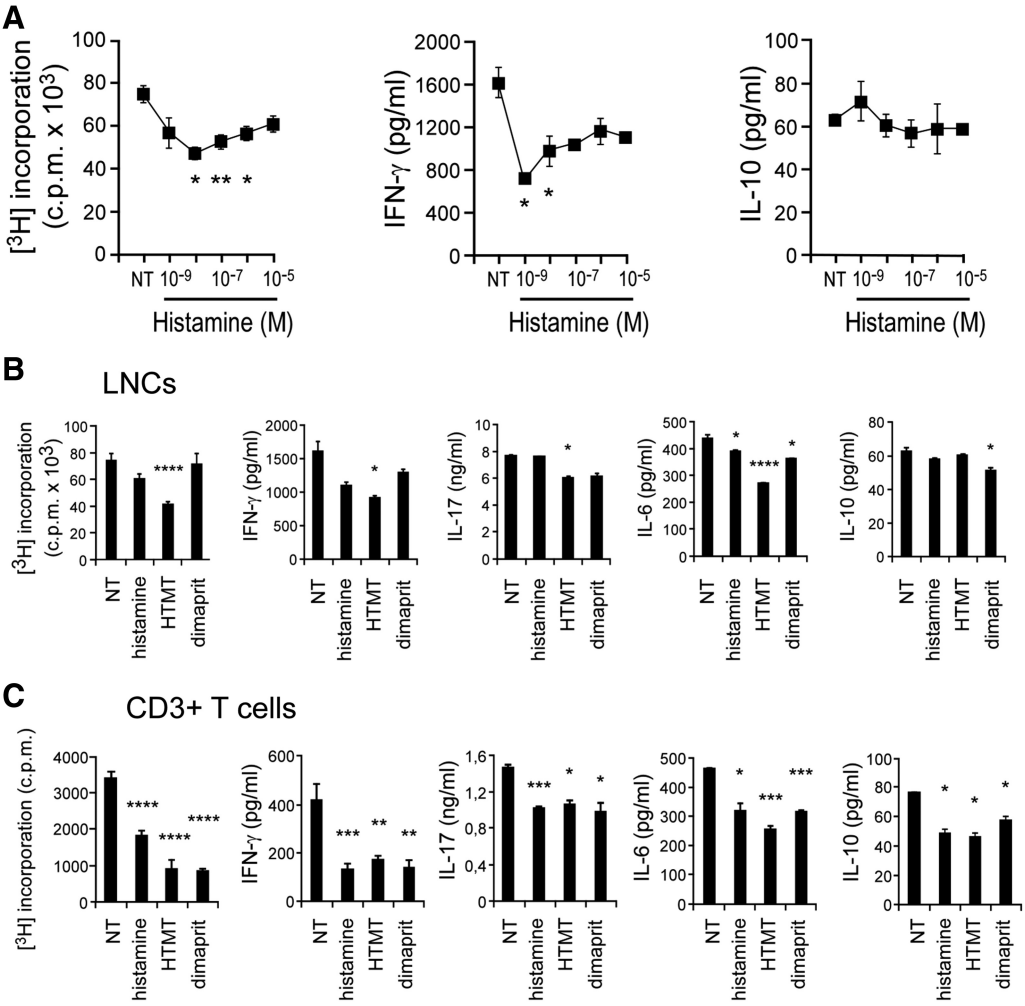

Figure 2. Histamine inhibits proliferation and IFN-γ cytokine production of LNCs and CD3$^+$ T cells activated against PLP$_{139-151}$. (A and B) LNCs obtained from SJL mice 10 days after the induction of EAE (n=3–5 mice) were pooled and left untreated (NT) for 2 h before in vitro stimulation with PLP$_{139-151}$ (20 μg/ml) or treated with increasing concentrations of histamine (A) or with histamine, H1R agonist HTMT or H2R agonist dimaprit, all agonists at a concentration of 10^{-5} M (B). (C) CD3$^+$ T cells magnetically purified from splenocytes of SJL mice 10 days after the induction of EAE were pooled (n=3–5 mice) and treated with histamine, H1R agonist HTMT or H2R agonist dimaprit (all at a concentration of 10^{-5} M) or left untreated for 2 h before in vitro stimulation with PLP$_{139-151}$ and γ-irradiated splenocytes. Proliferation and cytokine production were measured as described in Materials and Methods. Data are shown as mean ± SEM. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$; $^{****}P<0.0001$ versus untreated cells. Proliferation of unstimulated (=no antigen) cells was always below 7500 cpm in LNCs (A and B) and below 700 cpm in CD3$^+$ T cells (C) and was not modified by any of the pharmacological treatments used (data not shown). This is representative of similar results obtained in four to five independent experiments performed.

adhesion to inflamed brain microvessels of myelin-autoreactive T cells.

H1R agonist HTMT reduces LFA-1 distribution in clusters and caps

To explore the mechanisms by which histamine modulates the adhesion ability of myelin-autoreactive T cells, we first analyzed the expression of adhesion molecules on T cells of a TCL activated against PLP$_{139-151}$ upon treatment with histamine or its agonists. None of these compounds modified the expression of PSGL-1, VLA-4, L-selectin, LFA-1, or CD44 on PLP$_{139-151}$-activated T cells, as determined by flow cytometry analysis (**Fig. 5** and Supplemental Fig. 2).

We then wanted to ascertain if histamine interferes with signal transduction pathways leading to integrin activation and subsequent leukocyte arrest to inflamed brain venules. We analyzed LFA-1 integrin distribution on myelin-activated T cell membranes by using the ImageStream imaging cytometer, a powerful system that allows analysis of protein distribution and precise localization in different cellular compartments [30]. It has been shown previously that integrin distribution in small clusters or in larger areas ("big polar patches" or "caps") represents a modality of integrin activation leading to lymphocyte adhesion, whereas a more uniform distribution is indicative of a low-adhesion capacity [35, 36]. We observed that HTMT, which presented the highest inhibitory effect on the adhesion of activated T cells to mouse ICAM-1 in vitro and to inflamed brain vessels in vivo, significantly reduced LFA-1 distribution in caps on T cells of the TCL activated against PLP$_{139-151}$ under basal condition and after stimulation with chemokine CXCL12, suggesting a regulatory effect for this molecule on LFA-1 integrin activation (Fig. 4E and F).

DISCUSSION

The signaling of histamine occurs through each of its four known receptors, and each of these receptors might exert a variety of different effects within the immune response leading to brain inflammation and demyelination of EAE. In this study, we explored the ability of histamine, H1R agonist HTMT, and H2R agonist dimaprit to modulate two important steps of the autoimmune attack leading to EAE: T cell activation and adhesiveness to the inflamed microcirculation, an event that precedes T cell penetration in the brain parenchyma. Our results show that histamine in vitro inhibits antigen-induced proliferation and IFN-γ cytokine secretion of T

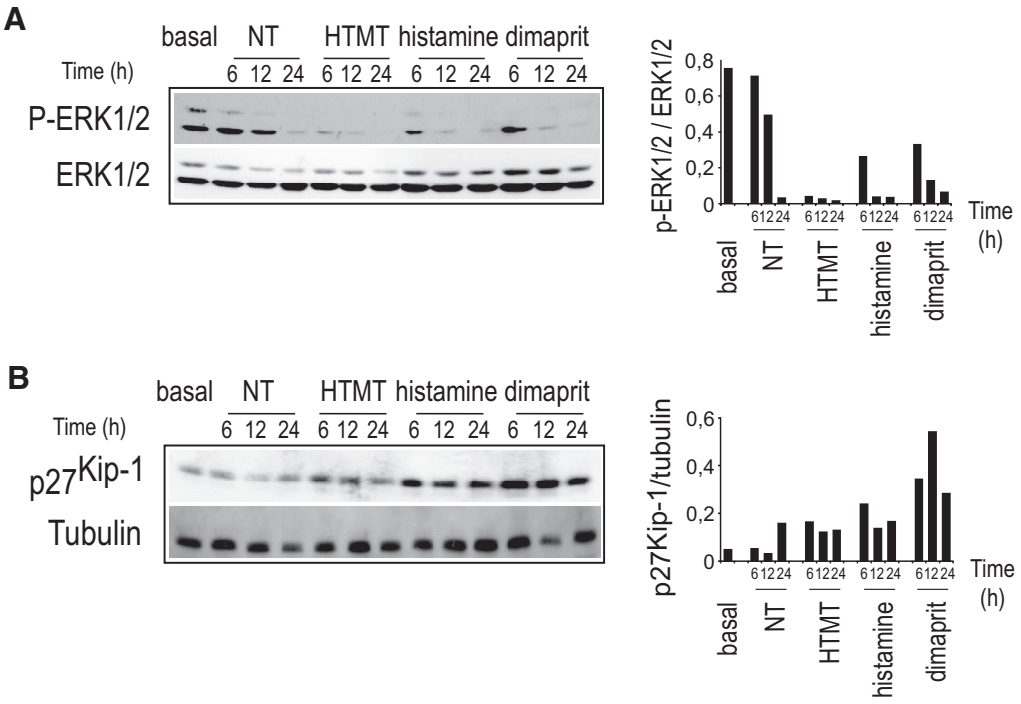

Figure 3. Effect of histamine on biochemical pathways involved in T cell activation and anergy. LNCs obtained from SJL mice 10 days after the induction of EAE (n=5 mice) were pooled and treated with histamine, H1R agonist HTMT, or H2R agonist dimaprit (all at a concentration of 10^{-5}) or left untreated for 2 h before in vitro stimulation with $PLP_{139-151}$ (20 μg/ml). Whole cell lysates were prepared from ex vivo untreated cells (basal) and at 6, 12, and 24 h after antigen stimulation and analyzed by Western blot analysis for phospho (P)-ERK1/2 and total ERK1/2 (A) and for p27Kip-1 and tubulin (B). The graphs on the right show quantitation of each specific protein.

cells activated against myelin. These effects were induced by HTMT and dimaprit, suggesting that the inhibitory effects induced by histamine on T cell proliferation and cytokine production are associated with histamine triggering of H1R or H2R. Our results were also corroborated by molecular analyses of intracellular pathways involved in differentiation, proliferation, and cell cycle control: ERK1/2 phosphorylation was reduced by histamine treatment, and p27Kip-1 expression increased, suggesting that histamine functionally modulates the activation state and responsiveness of myelin-activated T cells. Our data also indicate that histamine, through H1R and H2R, blocks spontaneous and chemokine-induced adhesion of myelin-autoreactive T cells to mouse ICAM-1 in vitro and reduces firm arrest of these cells to inflamed brain circulation in an in vivo model of early EAE inflammation. Although neither histamine nor its agonists modulated the expression of adhesion molecules on myelin-autoreactive T cells, HTMT inhibited LFA-1 distribution in clusters and caps, an important mechanism by which histamine might modulate the ability of T cells to adhere to inflamed brain vessels.

Our observation of a reduction of IFN-γ production by LNCs and $CD3^+$ T cells induced by histamine is consistent with the results of the work of Osna and colleagues [10], obtained on mouse splenocytes and Th1 cells with histamine and histamine antagonists, and with those of Sonobe and colleagues [37], obtained on splenocytes from H1R- and H2R-deficient mice. However, in apparent contrast with our data, recent work obtained in H1R-deficient mice has shown that H1R is important for optimal IFN-γ production by $CD4^+$ T cells [18]. It must be noted that in our experimental setting, we explored the effects of histamine and its H1R and H2R agonists on $CD3^+$ T cells purified from mice with EAE and thus activated in vivo with $PLP_{139-151}$, and the work by Noubade and colleagues [18] has been conducted on $CD4^+$ T cells purified from nonimmunized, H1R-deficient mice and activated polyclonally in vitro with the anti-CD3/28 antibody. It is possible that histamine has different effects on in vitro versus in vivo activated T cells. Also, it has to be considered that histamine can affect IFN-γ production by $CD8^+$ T cells [37], so that in our model the effects of histamine on $CD8^+$ T cells might have contributed to the reduced production of IFN-γ that we observed on $CD3^+$ T cells. Last, as discussed by the authors themselves in the work by Noubade et al. [18], it cannot be ruled out that in H1R-deficient mice, differences in H2R (and H4R) expression caused by loss of H1R-dependent cross-regulation may also influence IFN-γ production.

A role for histamine in the adhesion of leukocytes during inflammation and recruitment of immune cells into inflamed tissues has been suggested previously. Indeed, histamine can up-regulate the expression of vascular endothelial cell-associated molecules that promote trafficking of leukocytes through postcapillary venules [38, 39], an effect probably associated with the ability of this mediator to activate NF-κB through H1R (reviewed in ref. [40]). At the BBB level, histamine can increase vascular permeability by having direct effects on tight junctions (reviewed in ref. [41]). However, in an air pouch-type allergic inflammation model, histamine has been shown to down-regulate leukocyte infiltration through H2R [42], suggesting that depending on the specific receptor engaged, histamine might provide fine-tuning of leukocyte traffic through endothelial cells. The results shown here reveal an inhibitory effect of histamine, HTMT, and dimaprit on the adherence ability of myelin-autoreactive T cells on endothelial cells in vitro and in an in vivo model of EAE early brain inflammation. These effects did not appear to be associated with an effect of histamine on the expression of adhesion molecules on T cells.

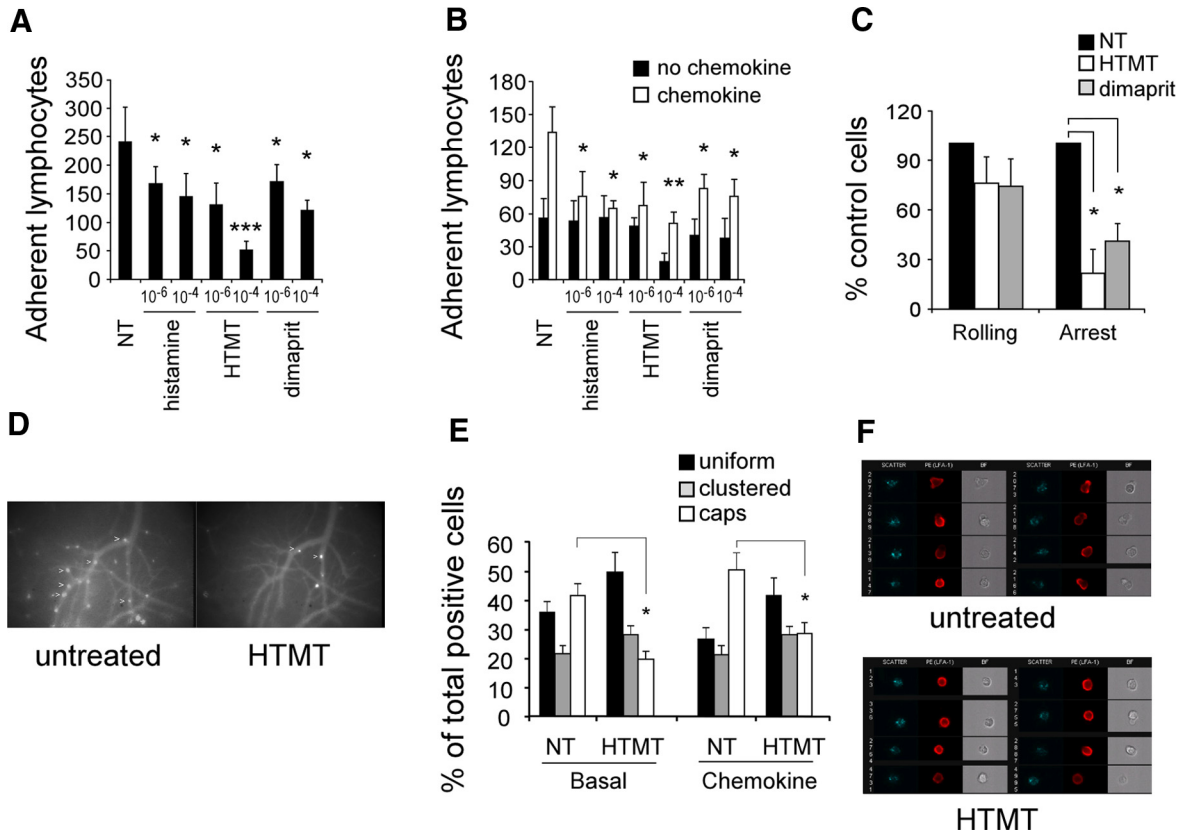

Figure 4. Histamine reduces the adhesion capability of myelin-autoreactive T cells. T lymphocytes of a TCL activated against $PLP_{139-151}$ were used to perform these experiments. (A and B) T cells were treated with histamine, H1R agonist HTMT, or H2R agonist dimaprit (used at concentrations of 10^{-6} or 10^{-4} M) or left untreated (NT) for 2 h at 37°C and then added in triplicates on slides precoated with purified mouse ICAM-1. (A) For evaluation of spontaneous adhesion, 100×10^3 T cells were incubated in medium culture for 10 min at 37°C. (B) For chemokine-induced adhesion, 50×10^3 T cells were incubated in medium for 3 min at 37°C (no chemokine), followed by an additional 3 min in the presence of CCL20 (1 μM; chemokine). Data are shown as mean counts of adherent T cells/0.2 mm^2 ± SD. *P <0.05; **P <0.005; ***P <0.001 versus untreated cells (A) or versus chemokine-stimulated, untreated cells (B). Data are representative of at least three independent experiments. (C and D) Fluorescently labeled T cells were treated for 2 h with HTMT or dimaprit (at a concentration of 10^{-5}) or left untreated before the intravital microscopy experiments. (C) Rolling and arrest fractions were calculated as described in Materials and Methods in five to seven venules from three to four animals for each experimental condition. Data are shown as mean ± SD. *P <0.05 versus untreated cells. (D) Adherent $PLP_{139-151}$-activated T cells (arrows) that were left untreated (CMFDA-labeled) or treated with HTMT (CMTMR-labeled) in the same brain venules are shown. (E and F) Analysis of LFA-1 clustering was performed on acquired cells with the ImageStream system software as described in Materials and Methods. (E) Mean percentages ± SD of cells presenting LFA-1 integrin distribution in uniform, clustered, and caps patterns in unstimulated (Basal) or chemokine (CXCL12)-stimulated cells in the presence or absence of HTMT from three experiments are shown. *P < 0.05 for distribution in caps in HTMT-treated cells versus untreated cells. (F) Representative cells from the most frequent pattern of LFA-1 distribution in unstimulated cells in the presence or absence of HTMT.

Instead, we observed that the H1R agonist HTMT reduced LFA-1 distribution significantly in clusters and caps (big polar patches) on activated T cells (Fig. 4), an event that facilitates cell–cell contacts and adhesive interactions, which are required for efficient migration through the endothelium [36]. Integrin distribution in clusters indicates increased lateral mobility and higher binding avidity [35, 36], and it has been shown recently that integrin clusters are associated with integrin-increased affinity, further emphasizing the importance of adhesion molecule distribution on cell surface [43]. Also, the distribution of adhesion molecules in larger areas (caps/clusters vs. round distribution) allows a higher outside-in signaling and a higher stabilization of cell adhesion required for adhesion strengthening during the migration through the endothelium [44]. Thus, the effects of H1R engagement on integrin activation and distribution on T cells might represent an important mechanism by which histamine modulates myelin-activated T cell adhesiveness in inflamed brain vessels. Further studies are required to explore the effects of HR-generated signaling on integrin affinity, another modality of integrin activation. Supporting our results, it has been shown previously that H2R signals through a PKA-dependent signaling pathway [45], whereas H1R signals through PKC as well as PKA in certain experimental conditions [46, 47]. Indeed, cAMP and PKA activation are able to block integrin activation and leukocyte adhesion through blockade of RhoA small G proteins [48], thus supporting the hypothesis that H1R activation can interfere with the signaling machinery controlling integrin activation and T cell migration into the brain.

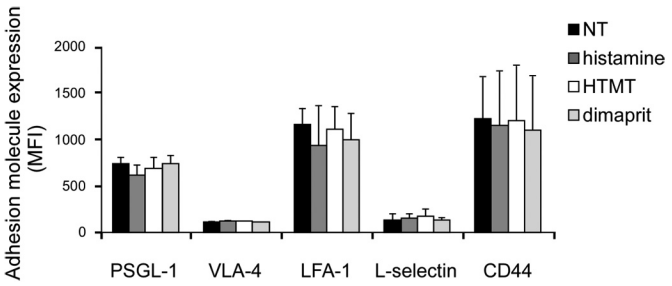

Figure 5. Flow cytometry analysis of the expression of adhesion molecules on myelin-autoreactive T cells upon treatment with histamine, H1R agonist HTMT, and H2R agonist dimaprit. T lymphocytes of a TCL activated against $PLP_{139-151}$ were treated with histamine, HTMT, or dimaprit (all used at concentrations of 10^{-5}) or left untreated for 2 h before labeling with adhesion molecule-specific fluorescent antibodies. Data represent mean fluorescence intensity (MFI) ± SEM from three experiments.

The results presented here have been obtained from in vitro studies and from an in vivo model of early brain inflammation and might help explain the exacerbation of EAE observed in vivo in histamine-deficient $HDC^{-/-}$ mice, including some of the features of the disease expressed by these mice, such as increased T cell production of proinflammatory cytokines and more diffuse inflammatory CNS infiltrates [22]. In this regard, it must be considered that within the four known receptors for histamine, we focused our study on the effects of H1R and H2R on myelin-autoreactive T cells because of the in vivo evidences supporting a role for these two receptors in EAE and because these receptors are expressed on T cells. Other receptors for histamine are known or are likely to play roles in EAE. Indeed, disruption of H3R, which is expressed on neural cells but not normally on hematopoietic cells [49, 50], leads to exacerbation of EAE associated with dysregulation of the BBB permeability [50], and the role in this disease of H4R, which is expressed on a variety of immune cell types and has been shown recently to be involved in Th2 polarization [51, 52], still needs to be investigated.

In conclusion, although the role of histamine in a disease as complex as EAE, let alone human MS, requires further investigation, our study confirms an important role for this mediator in the activation of myelin-autoreactive T cells and reveals for the first time an important role for histamine in the regulation of the ability of these autoreactive cells to adhere to the inflamed brain endothelium.

AUTHORSHIP

M.L. and B.G. performed pharmacological and expression studies, analyzed and interpreted data, and contributed to write the manuscript. M.C. and S.M. helped with pharmacological in vitro experiments and analyzed data. M.M., B.R., and S.A. performed in vitro adhesion, intravital microscopy, and ImageStream studies. C.P. performed intracellular signaling studies and analyzed and interpreted the results. C.F. contributed to the discussion and interpretation of the results. L.S. contributed to the discussion of the results and to writing the paper. G.M. supervised, analyzed, and interpreted the results of the intercellular signaling studies and contributed to write the paper. G.C. supervised, analyzed, and interpreted the results of adhesion, intravital microscopy, and ImageStream studies and contributed to write the paper. R.P. designed and directed the study and wrote the paper.

ACKNOWLEDGMENTS

This investigation was supported by grants from National Multiple Sclerosis Society-New York (RG3858-A-2 to R.P. and RG3703-A-3 to G.C.), Fondazione Italiana Sclerosi Multipla (AISM-FISM; 2002/R/41 and 2003/R/42 to R.P. and 2004/R/4 and 2008/R/6 to G.C.), Italian National Institute of Health (to R.P.), Italian Ministry of Education and Research (MIUR, PRIN; to G.C.), University of Verona (ex-60% MIUR; to G.C.), Fondazione Cariverona (to G.C.), European Research Council (ERC) starting grant Leptin MS n. 202579 (to G.M.), and the JDRF-Telethon grant n. GJT08004 (to G.M.). M.C. is supported by a fellowship from AISM-FISM. We thank Stephen J. Galli for fruitful discussion of the results, Renato Longhi for kindly providing the peptides used in this study and Cariverona Foundation and the Center for Biomedical Computing from the University of Verona for the ImageStream facility.

REFERENCES

1. Dy, M., Schneider, E. (2004) Histamine-cytokine connection in immunity and hematopoiesis. *Cytokine Growth Factor Rev.* **15,** 393–410.
2. Jutel, M., Watanabe, T., Akdis, M., Blaser, K., Akdis, C. A. (2002) Immune regulation by histamine. *Curr. Opin. Immunol.* **14,** 735–740.
3. Pedotti, R., Steinman, L. (2005) Histamine in immune regulation: possible roles in autoimmune demyelinating disease of the central nervous system. *Curr. Med. Chem. Anti-Inflammatory and Anti-Allergy Agents* **4,** 637–643.
4. Elenkov, I. J., Webster, E., Papanicolaou, D. A., Fleisher, T. A., Chrousos, G. P., Wilder, R. L. (1998) Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. *J. Immunol.* **161,** 2586–2593.
5. Van der Pouw Kraan, T. C., Snijders, A., Boeije, L. C., de Groot, E. R., Alewijnse, A. E., Leurs, R., Aarden, L. A. (1998) Histamine inhibits the production of interleukin-12 through interaction with H2 receptors. *J. Clin. Invest.* **102,** 1866–1873.
6. Idzko, M., la Sala, A., Ferrari, D., Panther, E., Herouy, Y., Dichmann, S., Mockenhaupt, M., Di Virgilio, F., Girolomoni, G., Norgauer, J. (2002) Expression and function of histamine receptors in human monocyte-derived dendritic cells. *J. Allergy Clin. Immunol.* **109,** 839–846.
7. Caron, G., Delneste, Y., Roelandts, E., Duez, C., Bonnefoy, J. Y., Pestel, J., Jeannin, P. (2001) Histamine polarizes human dendritic cells into Th2 cell-promoting effector dendritic cells. *J. Immunol.* **167,** 3682–3686.
8. Mazzoni, A., Young, H. A., Spitzer, J. H., Visintin, A., Segal, D. M. (2001) Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. *J. Clin. Invest.* **108,** 1865–1873.
9. Vannier, E., Miller, L. C., Dinarello, C. A. (1991) Histamine suppresses gene expression and synthesis of tumor necrosis factor α via histamine H2 receptors. *J. Exp. Med.* **174,** 281–284.
10. Osna, N., Elliott, K., Khan, M. M. (2001) The effects of histamine on interferon γ production are dependent on the stimulatory signals. *Int. Immunopharmacol.* **1,** 135–145.
11. Banu, Y., Watanabe, T. (1999) Augmentation of antigen receptor-mediated responses by histamine H1 receptor signaling. *J. Exp. Med.* **189,** 673–682.
12. Jutel, M., Watanabe, T., Klunker, S., Akdis, M., Thomet, O. A., Malolepszy, J., Zak-Nejmark, T., Koga, R., Kobayashi, T., Blaser, K., Akdis, C. A. (2001) Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. *Nature* **413,** 420–425.
13. Ma, R. Z., Gao, J., Meeker, N. D., Fillmore, P. D., Tung, K. S., Watanabe, T., Zachary, J. F., Offner, H., Blankenhorn, E. P., Teuscher, C. (2002)

Identification of Bphs, an autoimmune disease locus, as histamine receptor H1. *Science* **297,** 620–623.
14. Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* **6,** 1133–1141.
15. Steinman, L. (2008) A rush to judgment on Th17. *J. Exp. Med.* **205,** 1517–1522.
16. Pedotti, R., De Voss, J. J., Steinman, L., Galli, S. J. (2003) Involvement of both "allergic" and "autoimmune" mechanisms in EAE, MS and other autoimmune diseases. *Trends Immunol.* **24,** 479–484.
17. Pedotti, R., DeVoss, J. J., Youssef, S., Mitchell, D., Wedemeyer, J., Madanat, R., Garren, H., Fontoura, P., Tsai, M., Galli, S. J., Sobel, R. A., Steinman, L. (2003) Multiple elements of the allergic arm of the immune response modulate autoimmune demyelination. *Proc. Natl. Acad. Sci. USA* **100,** 1867–1872.
18. Noubade, R., Milligan, G., Zachary, J. F., Blankenhorn, E. P., del Rio, R., Rincon, M., Teuscher, C. (2007) Histamine receptor H1 is required for TCR-mediated p38 MAPK activation and optimal IFN-γ production in mice. *J. Clin. Invest.* **117,** 3507–3518.
19. Noubade, R., Saligrama, N., Spach, K., Del Rio, R., Blankenhorn, E. P., Kantidakis, T., Milligan, G., Rincon, M., Teuscher, C. (2008) Autoimmune disease-associated histamine receptor H1 alleles exhibit differential protein trafficking and cell surface expression. *J. Immunol.* **180,** 7471–7479.
20. El Behi, M., Zephir, H., Lefranc, D., Dutoit, V., Dussart, P., Devos, P., Dessaint, J. P., Vermersch, P., Prin, L. (2007) Changes in self-reactive IgG antibody repertoire after treatment of experimental autoimmune encephalomyelitis with anti-allergic drugs. *J. Neuroimmunol.* **182,** 80–88.
21. Dimitriadou, V., Pang, X., Theoharides, T. C. (2000) Hydroxyzine inhibits experimental allergic encephalomyelitis (EAE) and associated brain mast cell activation. *Int. J. Immunopharmacol.* **22,** 673–684.
22. Musio, S., Gallo, B., Scabeni, S., Lapilla, M., Poliani, P. L., Matarese, G., Ohtsu, H., Galli, S. J., Mantegazza, R., Steinman, L., Pedotti, R. (2006) A key regulatory role for histamine in experimental autoimmune encephalomyelitis: disease exacerbation in histidine decarboxylase-deficient mice. *J. Immunol.* **176,** 17–26.
23. Emerson, M. R., Orentas, D. M., Lynch, S. G., LeVine, S. M. (2002) Activation of histamine H2 receptors ameliorates experimental allergic encephalomyelitis. *Neuroreport* **13,** 1407–1410.
24. Pedotti, R., Mitchell, D., Wedemeyer, J., Karpuj, M., Chabas, D., Hattab, E. M., Tsai, M., Galli, S. J., Steinman, L. (2001) An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat. Immunol.* **2,** 216–222.
25. Scabeni, S., Lapilla, M., Musio, S., Gallo, B., Ciusani, E., Steinman, L., Mantegazza, R., Pedotti, R. (2008) CD4+CD25+ regulatory T cells specific for a thymus-expressed antigen prevent the development of anaphylaxis to self. *J. Immunol.* **180,** 4433–4440.
26. De Rosa, V., Procaccini, C., Cali, G., Pirozzi, G., Fontana, S., Zappacosta, S., La Cava, A., Matarese, G. (2007) A key role of leptin in the control of regulatory T cell proliferation. *Immunity* **26,** 241–255.
27. Constantin, G., Laudanna, C., Brocke, S., Butcher, E. C. (1999) Inhibition of experimental autoimmune encephalomyelitis by a tyrosine kinase inhibitor. *J. Immunol.* **162,** 1144–1149.
28. Laudanna, C., Campbell, J. J., Butcher, E. C. (1996) Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science* **271,** 981–983.
29. Piccio, L., Rossi, B., Scarpini, E., Laudanna, C., Giagulli, C., Issekutz, A. C., Vestweber, D., Butcher, E. C., Constantin, G. (2002) Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric G(i)-linked receptors. *J. Immunol.* **168,** 1940–1949.
30. George, T. C., Fanning, S. L., Fitzgeral-Bocarsly, P., Medeiros, R. B., Highfill, S., Shimizu, Y., Hall, B. E., Frost, K., Basiji, D., Ortyn, W. E., Morrissey, P. J., Lynch, D. H. (2006) Quantitative measurement of nuclear translocation events using similarity analysis of multispectral cellular images obtained in flow. *J. Immunol. Methods* **311,** 117–129.
31. Constantin, G., Marconi, S., Rossi, B., Angiari, S., Calderan, L., Anghileri, E., Gini, B., Dorothea Bach, S., Martinello, M., Bifari, F., Galiè, M., Turano, E., Budui, S., Sbarbati, A., Krampera, M., Bonetti, B. (2009) Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. *Stem Cells* **27,** 2624–2635.
32. Boussiotis, V. A., Freeman, G. J., Taylor, P. A., Berezovskaya, A., Grass, I., Blazar, B. R., Nadler, L. M. (2000) p27Kip1 functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. *Nat. Med.* **6,** 290–297.
33. Wells, A. D., Walsh, M. C., Bluestone, J. A., Turka, L. A. (2001) Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *J. Clin. Invest.* **108,** 895–903.
34. Baron, J. L., Madri, J. A., Ruddle, N. H., Hashim, G., Janeway Jr., C. A. (1993) Surface expression of α 4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J. Exp. Med.* **177,** 57–68.
35. Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J. Y., Butcher, E. C., Laudanna, C. (2000) Chemokines trigger immediate β2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* **13,** 759–769.
36. Ley, K., Laudanna, C., Cybulsky, M. I., Nourshargh, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* **7,** 678–689.
37. Sonobe, Y., Nakane, H., Watanabe, T., Nakano, K. (2004) Regulation of Con A-dependent cytokine production from CD4+ and CD8+ T lymphocytes by autosecretion of histamine. *Inflamm. Res.* **53,** 87–92.
38. Saito, H., Shimizu, H., Mita, H., Maeda, Y., Akiyama, K. (1996) Histamine augments VCAM-1 expression on IL-4- and TNF-α-stimulated human umbilical vein endothelial cells. *Int. Arch. Allergy Immunol.* **111,** 126–132.
39. Miki, I., Kusano, A., Ohta, S., Hanai, N., Otoshi, M., Masaki, S., Sato, S., Ohmori, K. (1996) Histamine enhanced the TNF-α-induced expression of E-selectin and ICAM-1 on vascular endothelial cells. *Cell. Immunol.* **171,** 285–288.
40. Leurs, R., Church, M. K., Taglialatela, M. (2002) H1-antihistamines: inverse agonism, anti-inflammatory actions and cardiac effects. *Clin. Exp. Allergy* **32,** 489–498.
41. Abbott, N. J. (2000) Inflammatory mediators and modulation of blood-brain barrier permeability. *Cell. Mol. Neurobiol.* **20,** 131–147.
42. Hirasawa, N., Ohtsu, H., Watanabe, T., Ohuchi, K. (2002) Enhancement of neutrophil infiltration in histidine decarboxylase-deficient mice. *Immunology* **107,** 217–221.
43. Cairo, C. W., Mirchev, R., Golan, D. E. (2006) Cytoskeletal regulation couples LFA-1 conformational changes to receptor lateral mobility and clustering. *Immunity* **25,** 297–308.
44. Giagulli, C., Ottoboni, L., Caveggion, E., Rossi, B., Lowell, C., Constantin, G., Laudanna, C., Berton, G. (2006) The Src family kinases Hck and Fgr are dispensable for inside-out, chemoattractant-induced signaling regulating β 2 integrin affinity and valency in neutrophils, but are required for β 2 integrin-mediated outside-in signaling involved in sustained adhesion. *J. Immunol.* **177,** 604–611.
45. Hershenson, M. B., Chao, T. S., Abe, M. K., Gomes, I., Kelleher, M. D., Solway, J., Rosner, M. R. (1995) Histamine antagonizes serotonin and growth factor-induced mitogen-activated protein kinase activation in bovine tracheal smooth muscle cells. *J. Biol. Chem.* **270,** 19908–19913.
46. Sakhalkar, S. P., Patterson, E. B., Khan, M. M. (2005) Involvement of histamine H1 and H2 receptors in the regulation of STAT-1 phosphorylation: inverse agonism exhibited by the receptor antagonists. *Int. Immunopharmacol.* **5,** 1299–1309.
47. Moniri, N. H., Booth, R. G. (2006) Role of PKA and PKC in histamine H1 receptor-mediated activation of catecholamine neurotransmitter synthesis. *Neurosci. Lett.* **407,** 249–253.
48. Laudanna, C., Campbell, J. J., Butcher, E. C. (1997) Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. *J. Biol. Chem.* **272,** 24141–24144.
49. Lovenberg, T. W., Roland, B. L., Wilson, S. J., Jiang, X., Pyati, J., Huvar, A., Jackson, M. R., Erlander, M. G. (1999) Cloning and functional expression of the human histamine H3 receptor. *Mol. Pharmacol.* **55,** 1101–1107.
50. Teuscher, C., Subramanian, M., Noubade, R., Gao, J. F., Offner, H., Zachary, J. F., Blankenhorn, E. P. (2007) Central histamine H3 receptor signaling negatively regulates susceptibility to autoimmune inflammatory disease of the CNS. *Proc. Natl. Acad. Sci. USA* **104,** 10146–10151.
51. Dunford, P. J., O'Donnell, N., Riley, J. P., Williams, K. N., Karlsson, L., Thurmond, R. L. (2006) The histamine H4 receptor mediates allergic airway inflammation by regulating the activation of CD4+ T cells. *J. Immunol.* **176,** 7062–7070.
52. Cowden, J. M., Riley, J. P., Ma, J. Y., Thurmond, R. L., Dunford, P. J. (2010) Histamine H4 receptor antagonism diminishes existing airway inflammation and dysfunction via modulation of Th2 cytokines. *Respir. Res.* **11,** 86.

KEY WORDS:
autoimmunity · EAE · MS · cytokine · chemokine