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Histamine H4 Receptor Optimizes T Regulatory Cell Frequency and Facilitates Anti-Inflammatory Responses within the Central Nervous System

Roxana del Rio,*1 Rajkumar Noubade,*1 Naresha Saligrama,*, Emma H. Wall,* Dimitry N. Krementsov,*, Matthew E. Poynter,*,† James F. Zachary,‡ Robin L. Thurmond,§ and Cory Teuscher*‡,¶

Histamine is a biogenic amine that mediates multiple physiological processes, including immunomodulatory effects in allergic and inflammatory reactions, and also plays a key regulatory role in experimental allergic encephalomyelitis, the autoimmune model of multiple sclerosis. The pleiotropic effects of histamine are mediated by four G protein-coupled receptors, as follows: Hrh1/H1R, Hrh2/H2R, Hrh3/H3R, and Hrh4/H4R. H4R expression is primarily restricted to hematopoietic cells, and its role in autoimmune inflammatory demyelinating disease of the CNS has not been studied. In this study, we show that, compared with wild-type mice, animals with a disrupted Hrh4 (H4RKO) develop more severe myelin oligodendrocyte glycoprotein (MOG)35–55-induced experimental allergic encephalomyelitis. Mechanistically, we also show that H4R plays a role in determining the frequency of T regulatory (TReg) cells in secondary lymphoid tissues, and regulates TReg cell chemotaxis and suppressor activity. Moreover, the lack of H4R leads to an impairment of an anti-inflammatory response due to fewer TReg cells in the CNS during the acute phase of the disease and an increase in the proportion of Th17 cells. The Journal of Immunology, 2012, 188: 541–547.

Histamine is implicated in the pathogenesis of multiple sclerosis (MS), as well as experimental allergic encephalomyelitis (EAE). HA modulates blood-brain barrier (BBB) permeability, and enhances leukocyte rolling, adhesion, and vascular extravasation of inflammatory cells into the CNS (3, 4). Increased levels of HA in cerebrospinal fluid correlate with relapses in MS patients (5) and with the onset of EAE (6). In addition, transcriptional profiling of MS lesions revealed that H1R expression was upregulated relative to normal tissue (7). Moreover, epidemiological data indicate that use of sedating H1R antagonists is associated with decreased MS risk (8), and, in a small study, MS patients treated with a H1R antagonist remained stable or improved neurologically (9). Likewise, H1R and H2R transcripts are present in the brain lesions of mice with active EAE, and administration of pyrilamine, a H2R antagonist, reduces EAE severity (10). We previously identified Bondetella pertussis toxin-induced HA sensitization (Bphs) as a susceptibility locus for EAE and experimental allergic orchitis, and positional candidate gene cloning identified Bphs as Hrh1 (11). Furthermore, genetic studies have demonstrated that HA, H1R, H2R, and H3R play important roles in disease development and EAE susceptibility either by regulating APC function, the encephalitogenic T cell responses, or BBB permeability (11–14). However, the role of H4R in autoimmune inflammatory demyelinating disease of the CNS has not yet been studied.

H4R expression is mostly restricted to hematopoietic cells, including T cells (15). H4R is coupled to second messenger signaling pathways via the pertussis toxin (PTX)-sensitive heterotrimeric Gαi0 proteins (16) and to the β-arrestin pathway (17). The activation of H4R mediates intracellular calcium mobilization, cAMP inhibition, modulation of JAK-STAT, MAPK/ERK and PI3K pathways, and activation of the transcription factor AP-1 (15, 18). As a result, H4R signaling regulates cytokine production, dendritic cell (DC) function, and Th cell polarization (19). In addition, H4R activation induces actin polymerization, upregulation of adhesion molecules, changes in cell shape, and chemotaxis.
of different immune cells, including eosinophils, mast cells, Langerhans cells, and T cells (15, 20–22). The role of H4R in the integrated immune response, however, remains unclear. Moreover, the use of different models has led to conflicting results about the role of H4R in the immune response. In the murine model of alergic asthma, Morgan et al. (23) reported that the administration of 4-methyl HA (4-mHA), a H4R agonist, reduced airway hyper-responsiveness and inflammation, while increasing Tc2 cell recruitment to the lung, suggesting an anti-inflammatory and immunomodulatory role for H4R in this response. In contrast, studies using H4RKO mice and H4R antagonists, particularly JNJ 7777120 and its derivatives, suggest a proinflammatory role for this receptor in a variety of in vivo models (15, 20, 21). Furthermore, single nucleotide polymorphisms and copy number variations in human Hrh4 have been reported to be associated with atopic dermatitis (24) and systemic lupus erythematosus (25). Despite conflicting results, the findings of the experiments above underscore the role of H4R in modulating immune responses.

To assess the role of H4R signaling in the regulation of auto-inflammatory demyelinating disease of the CNS, we studied myelin oligodendrocyte glycoprotein (MOG)35–55-induced EAE in H4RKO mice. The results of our study provide direct evidence that H4R modulates EAE severity. We show that H4RKO mice, despite having equivalent T effector (T eff) cell responses, develop more severe EAE, augmented neuroinflammation, and increased BBB permeability compared with wild-type (WT) mice. In addition, we show that H4R signaling exerts control over the frequency of Tc2 cell in secondary lymphoid tissues, as well as chemotaxis and suppressive ability of TR cells. Consistent with this, the lack of H4R leads to a lower proportion of these cells in the CNS during the acute effector phase of the disease, resulting in an increase in the proportion of CD4+IL-17+ cells and impairment of an anti-inflammatory response.

Materials and Methods

Animals

B6.C57BL/6J (B6/J, WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129P-Hrh4tm1Thr (H4RKO) mice were generated by Lexicon Genetics (Woodlands, TX), and were backcrossed onto B6/J. The N10 mice were intercrossed, and resulting mice were used in the experiments. B6- Foxp3gfp KI mice were provided by V. Kuchroo (Center of Neurological Diseases, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA). H4RKO-Foxp3Ko KI mice were generated by crossing breed H4RKO mice with H4RKO-Foxp3 KI mice. Mice were housed at 25°C with 12/12-h light-dark cycles and 40–60% humidity. The experimental procedures performed in this study were under the guidelines of the Animal Care and Use Committees of the University of Vermont (Burlington, VT).

Induction and evaluation of EAE

Mice were immunized for the induction of EAE using a single injection protocol. The animals were injected s.c. in the posterior right and left flank and the scruff of the neck with a sonicated PBS/oil emulsion containing 200 μg MOG35–55 and CFA (Sigma-Aldrich) accompanied with 200 μg Mycobacterium tuberculosis H37Ra (Difco Laboratories). Immediately after-treatment, each mouse received 200 μg PTX (List Biological Laboratories) in 0.2 ml Munoz buffer by i.v. injection (14). Mice were scored daily for clinical quantitative trait variables beginning at day 5 after injection, as follows: 0, no clinical expression of disease; 1, flaccid tail without hind limb weakness; 2, hind limb weakness; 3, complete hind limb paralysis and floppy tail; 4, hind leg paralysis accompanied by a floppy tail and urinary or fecal incontinence; and 5, moribund. Assessments of clinical quantitative trait variables, EAE pathology, and BBB permeability were performed, as previously described (14).

CNS-infiltrating mononuclear cell isolation

Mice were perfused with saline, and brain and spinal cord were removed. A single-cell suspension was obtained and passed through a 70-μm strainer. Mononuclear (MN) cells were obtained by Percoll gradient (37/70%) centrifugation, collected from the interphase, and washed. Cells were labeled with Live-Dead UV Blue dye (BD Pharmingen), followed by surface and intracellular staining.

Abs and flow cytometric analysis

The draining lymph node (DLN), spleen, and thymus were excised and dissociated into single-cell suspensions. For the identification and phenotypic analysis of Tc2 cells (CD4+CD8-TCRβ-Foxp3+), the following surface antibo-mouse mAb was used: anti-CD4 (MCDCD17; Caltag); anti-CD8 and anti-CD25 (53-6.7, PC61; BD Pharmingen); and anti-TCRβ, anti-CCR7, and anti-Foxp3 (H57-5987, 4B12, FJK-16s; eBioscience). Intracellular Foxp3 was stained with the mouse/rat Foxp3 staining set (eBioScience), according to the manufacturer’s instructions. For intracellular cytokine staining, CNS-infiltrating MN cells were stimulated with 5 ng/ml PMA, 250 ng/ml ionomycin, and 2 μg/ml monensin (Sigma-Aldrich) for 4 h. Cells were first stained with LIVE/DEAD fixable stain (Invitrogen) and anti-CD4–Pacific blue (GK1.5; BioLegend). Cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with buffer containing 0.1% saponin, and stained with anti–IL-17A-PE (TC11-18H10; BD Pharmingen) and anti–IL-10-Alexa Fluor 647 (JES5-16E7; BioLegend). Cells were collected using BD LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Software).

Cell culture conditions and lymphokine assays

For ex vivo cytokine analysis, spleen and DLN were obtained from day 10 immunized mice. Single-cell suspensions at 1 × 10^6 cells/ml in RPMI 1640 medium (Cellgro Mediatech) plus 5% FBS (HyClone) were stimulated with 50 μg/ml MOG35–55. Cell supernatants were recovered at 72 h, and concentrations of 23 different cytokines were quantified in duplicate by Bio-Plex multiplex cytokine assay (BD Biosciences).

Proliferation assay

Mice were immunized for EAE induction, and DLN and spleens were harvested on day 30. Single-cell suspensions were prepared, and 5 × 10^5 cells/well in RPMI 1640 (5% FBS) were plated on standard 96-well U-bottom tissue culture plates and stimulated with 0, 1, 2, 10, and 50 μg/ml MOG35–55 for 72 h at 37°C. During the last 18 h of culture, 1 μCi [3H]thymidine (PerkinElmer) was added. Cells were harvested onto glass fiber filters, and thymidine uptake was determined with a liquid scintillation counter.

Suppression assay

CD4+CD25+ (T eff) and CD4+CD25+ (T regulatory [TR]) T cells from lymph node (LN) and splenu were sorted using anti-CD4, anti-TCRβ, and anti-CD25 mAbs. CD4+CD25+ T cell purity was consistently >97%. CD4+CD25+ Tc2 cells were cultured for 3 d with irradiated spleen cells as APC (1 × 10^6/well) in the presence of anti-CD3 mAb (5 μg/ml), with or without CD4+CD25+ Tc2 cells at 0.5:1 (Tc2/Tc2) cell ratio. The cell cultures were pulsed with 0.5 μCi [3H]thymidine for the last 18 h. Tc2 cell proliferation with WT Tc2 cells was set at 100%. Percentage of inhibition in the presence of H4RKO Tc2 cells was calculated.

Cell migration assay

Migratory capacity of CD4+ T cells or B6-Foxp3 KI or H4RKO-Foxp3 KI Tc2 cells was evaluated using 24-well Transwell plates with a 3.0 μm pore size (Costar). Total CD4+ T cells were isolated by negative selection, and Tc2 cells were sorted based on GFP expression. CD4+TCRβ cell purity was >85%, and sorted Tc2 cell purity was >97%. One hundred microliters of cells were added to the top well at 1 × 10^6 CD4+ T cells or 0.5 × 10^6 Tc2 cells in RPMI 1640 with 1% BSA, and medium containing either no HA, 10, 100 ng/ml stromal cell-derived factor-1 (SDF-1) was added to the bottom chamber. After 4 h at 37°C in 5% CO2, cells that migrated to the bottom chamber were harvested, counted, and stained for subsequent flow cytometric analysis.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from Tc2 cells or Tc2 cells from naive WT and H4RKO mice using RNAesy isolation reagent (Qiagen), and reverse transcribed using Superscript III reverse transcriptase (Invitrogen). The generated cDNA was used in quantitative real-time PCR (qRT-PCR) using the SYBR green method. The sequences of Hrh4 primers used were as follows: forward, 5′-TGAGGAGAATTGCTTCACGA-3′; reverse, 5′-TGATGTTGGAGGGGTGTTAT-3′. β2-microglobulin was used as a reference gene, and the relative expression levels were calculated using the comparative threshold cycle method.
**Results**

**H4R negatively regulates EAE severity**

To investigate the role of H4R in autoimmune inflammatory demyelinating disease of the CNS, EAE was induced in WT and H4RKO mice by immunization with MOG35–55-CFA-PTX. The clinical disease course of H4RKO mice was more severe than WT mice (Fig. 1A). Analysis of EAE-associated clinical traits (14) revealed that the mean day of onset, mean cumulative disease score, days affected, overall severity index, and peak score were revealed that the mean day of onset, mean cumulative disease score, days affected, overall severity index, and peak score were significantly greater in H4RKO compared with WT mice (Table I). Furthermore, histopathological analysis revealed more extensive pathology in the brains and spinal cords of H4RKO mice compared with WT mice (Fig. 1B).

As an additional quantitative measure of differences in the neuroinflammatory response, we examined BBB permeability at days 8, 10, 12, and 14 postimmunization. Compared with WT mice, the increase in BBB permeability during the early acute phase of the disease was significantly greater in H4RKO mice (Fig. 1C). Taken together, these data show that H4R signaling negatively regulates EAE severity.

**WT and H4RKO ex vivo CD4+ T<sub>e</sub> cell responses are comparable**

Although the precise pathogenic mechanism of EAE and MS is unknown, it is believed to be mediated by CD4<sup>+</sup> T cell-dependent activities (26). H4R is expressed by T cells (15) and has been implicated in immune regulatory functions (15, 20, 21). Therefore, to delineate the immune mechanism underlying increased EAE severity of H4RKO mice, the MOG35-55-specific T cell responses were compared on day 10 postimmunization. No significant differences in T cell proliferation (Fig. 2A) or cytokine/chemokine production (Fig. 2B) in response to MOG35-55 restimulation were detected between H4RKO and WT splenic and DLN cells.

**H4R influences the frequency of T<sub>reg</sub> cells in secondary lymphoid organs**

Foxp3<sup>+</sup> T<sub>reg</sub> cells play a fundamental role in controlling inflammatory responses and preventing autoimmune diseases, including EAE (27, 28), and mast cells and HA have been implicated in controlling peripheral tolerance via T<sub>reg</sub> cells (29, 30). In addition, the H4R agonist 4-mHA induces recruitment of T<sub>reg</sub> cells into the lung and inhibits development of allergic asthma (23). Although H4R expression has been reported in T cells, it is unknown whether it is expressed by T<sub>reg</sub> cells. We therefore compared Hrh4 mRNA levels between CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> conventional T cells and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells from naive C57BL/6J mice. As shown in Fig. 3A, Hrh4 mRNA levels were higher in T<sub>reg</sub> cells compared with conventional CD4<sup>+</sup> T cells. Given this elevated expression of Hrh4 in T<sub>reg</sub> cells and the importance of these cells in controlling inflammatory and autoimmune responses, we hypothesized that the deficiency of H4R may affect T<sub>reg</sub> cell development and/or frequency. Therefore, we compared the proportion of Foxp3<sup>+</sup> T<sub>reg</sub> cells in the thymus of naive WT and H4RKO mice and found no difference among the single-positive CD4<sup>+</sup> T lymphocytes (Fig. 3B). However, the proportion and absolute number of T<sub>reg</sub> cells in the spleen and LN were significantly lower in H4RKO mice compared with WT mice (Fig. 3C, Supplemental Fig. 1A). Next, we examined the proportion of CNS-resident T<sub>reg</sub> cells in naive WT and H4RKO mice, and, in contrast to the periphery, no detectable difference was observed (Fig. 3D).

**H4R controls T<sub>reg</sub> cell infiltration and inflammation in the CNS during acute EAE**

Because H4RKO mice have a lower frequency of T<sub>reg</sub> cells in secondary lymphoid organs compared with WT mice, we reasoned that the increased disease severity in H4RKO mice may be due to a paucity of CNS-T<sub>reg</sub> cells during the induction and/or acute ef-

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### Table I. Summary of EAE clinical trait variables in WT and H4RKO mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT</th>
<th>H4RKO</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>3:1/2</td>
<td>3:0/0</td>
<td></td>
</tr>
<tr>
<td>Mean day of onset</td>
<td>16.25 ± 3.74</td>
<td>13.63 ± 2.74</td>
<td>0.004</td>
</tr>
<tr>
<td>Cumulative score</td>
<td>36.00 ± 18.3</td>
<td>36.03 ± 20.72</td>
<td>0.0002</td>
</tr>
<tr>
<td>Days affected</td>
<td>14.00 ± 6.3</td>
<td>17.40 ± 2.77</td>
<td>0.002</td>
</tr>
<tr>
<td>Severity index</td>
<td>2.42 ± 0.88</td>
<td>3.21 ± 1.02</td>
<td>0.004</td>
</tr>
<tr>
<td>Peak score</td>
<td>3.37 ± 1.26</td>
<td>4.33 ± 0.84</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Animals were immunized with MOG35-55-CFA-PTX and scored daily for clinical signs starting on day 5. Mean trait values ± SD are shown. The significance of the difference in incidence was determined by χ<sup>2</sup> test, and the significance of the differences in EAE-quantitative trait variables was determined using the Mann–Whitney U test.

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**FIGURE 1. H4R negatively regulates EAE severity and BBB permeability.**

**A** Clinical score (A) and severity of CNS lesions (B) in WT (n = 32) and H4RKO (n = 30) mice were compared following immunization with MOG35-55-CFA-PTX. The significance of the differences between the clinical courses of disease was calculated by regression analysis, and best-fit curve is shown. B. The significance of differences observed at day 30 postimmunization was determined using the Mann–Whitney U test (**p < 0.01, ***p < 0.001). C. The significance of differences in BBB permeability indices of WT and H4RKO mice across the early acute phase of disease was assessed by regression analyses (n = 8 for each strain at each time point).
factor phase of the disease. Therefore, the frequency of T<sub>R</sub> cells was determined in WT and H4RKO mice following immunization. DLN cells and CNS-associated infiltrating MN cells were isolated at different times after immunization, and the frequency of T<sub>R</sub> cells among the CD<sup>4+</sup>TCR<sup>+</sup> T cells was analyzed. On day 10 postimmunization, the proportion of T<sub>R</sub> cells in the DLN comprised ∼20% of the CD<sup>4+</sup>TCR<sup>+</sup> T cells, representing an increase of ∼2-fold over naive mice. On days 12 and 14, the proportion of T<sub>R</sub> cells dropped to ∼10–12% of the T cells. However, no significant difference in the proportion of T<sub>R</sub> cells or TE cells in the DLN was detectable between WT and H4RKO mice at any of the time points examined (Fig. 4A, Supplemental Fig. 2). We then evaluated the expression of the chemokine receptor CCR7, which has been shown to be involved in the recruitment and interaction of T<sub>R</sub> cells with mature DC in the paracortical area of the LN (31). H4RKO mice have a lower proportion of T<sub>R</sub> cells expressing CCR7 at days 8 and 10 after immunization when compared with WT mice, with no differences by day 12 (Fig. 4B).

By day 10 postimmunization, we observed robust recruitment of T<sub>R</sub> cells into the CNS of both WT and H4RKO mice compared with naive controls (Fig. 3D versus Fig. 4D). In contrast to the DLN of immunized mice, the frequency of T<sub>R</sub> cells in the CNS of H4RKO mice was lower than that detected in WT mice at days 10, 12, and 17 postimmunization (Fig. 4D). Consistent with the lower proportion of T<sub>R</sub> cells in the CNS of H4RKO mice, a decrease in the absolute number of T<sub>R</sub> cells was also observed (Supplemental Fig. 1B). A role for histamine in regulating the adhesion and recruitment of immune cells has been suggested previously (32). Therefore, because T<sub>R</sub> cells have been reported to express greater levels of ICAM-1/CD45 and P-selectin in comparison with non-T<sub>R</sub> cells (33), and CCR6 expression has been shown to regulate EAE pathogenesis by controlling T<sub>R</sub> cell recruitment to the CNS (33), we evaluated the expression levels of these in CNS-infiltrating T<sub>R</sub> cells of WT and H4RKO mice. No differences in the expression levels of these molecules were detected between WT and H4RKO CNS-infiltrating MN cells during EAE (data not shown). We also determined whether there was any difference in the overall infiltration of CD<sup>4+</sup> T cells into the CNS after immunization. As expected, the proportion of TCR<sup>+</sup>CD<sup>4+</sup> T cells increased with disease progression, and by day 14 postimmunization the CNS of H4RKO mice exhibited a significantly greater proportion of these cells compared with WT mice (Fig. 4C).

Th1 and Th17 cells have been shown to contribute to the pathogenesis of EAE, and T<sub>R</sub> cells inhibit the induction of these pathogenic cells (26). Therefore, we compared autoreactive effector CD4 responses in the target organ of WT and H4RKO mice during EAE by examining the frequency of encephalitogenic IFN-γ– and IL-17–producing Th1 and Th17 cells in the CNS. The frequency of Th17 cells in H4RKO mice was higher than that of WT mice, whereas the frequency of Th1 cells was comparable between the two strains (Fig. 4E).

H<sub>4</sub>R regulates T<sub>R</sub> cell chemotaxis and suppressor functions

It has been shown that the H<sub>4</sub>R agonist 4-mHA reduces airway hyperresponsiveness and inflammation, and that this effect is as-
associated with the recruitment of T<sub>R</sub> cells into the lung (23). Additionally, H<sub>4</sub>R signaling is involved in the migration of DC and mast cells to sites of inflammation (15, 19, 21). We have shown that increased EAE severity in H<sub>4</sub>RKO mice correlates with decreased numbers of infiltrating T<sub>R</sub> cells into the CNS during the acute phase of disease (Fig. 4D, Supplemental Fig. 1B), consistent with the requirement for T<sub>R</sub> cells in the target tissue for adequate immune regulation (34). Because we observed differences in the number of T<sub>R</sub> cells in the CNS between immunized WT and H<sub>4</sub>RKO mice, we examined whether H<sub>4</sub>R is required for optimal CD4<sup>+</sup> T cell chemotaxis. We performed in vitro migration assays using purified CD4<sup>+</sup> T cells from immunized WT and H<sub>4</sub>RKO mice. WT and H<sub>4</sub>RKO CD4<sup>+</sup> T cells responded equally to SDF-1, a known strong chemotactic factor for leukocytes (Fig. 5A). However, WT CD4<sup>+</sup> T cells responded to HA-induced migratory signals, whereas H<sub>4</sub>RKO CD4<sup>+</sup> T cells did not (Fig. 5B). These results suggest that HA, acting through H<sub>4</sub>R, functions as a chemotactic factor for T cells.

Interestingly, when the proportion of Foxp3<sup>+</sup> T<sub>R</sub> cells within the total T cell population that migrated in response to HA was analyzed, the cells from WT mice contained a significantly greater proportion of Foxp3<sup>+</sup> T<sub>R</sub> cells compared with those from H<sub>4</sub>RKO mice (Fig. 5C). To test the H<sub>4</sub>R-dependent chemotactic activity directly in T<sub>R</sub> cells, we used WT- and H<sub>4</sub>RKO-Foxp3<sup>gfp</sup> knockin (KI) mice, sorted GFP<sup>+</sup> T<sub>R</sub> cells from immunized mice, and assessed the chemotactic response to HA. As with total CD4<sup>+</sup> T cells, the H<sub>4</sub>RKO Foxp3<sup>+</sup> T<sub>R</sub> cells had an impaired migratory response to HA (Fig. 5D). Furthermore, we tested whether T<sub>R</sub> cells from WT and H<sub>4</sub>RKO mice exhibited differences in their in vitro suppressive function and found that day 10 H<sub>4</sub>R-deficient T<sub>R</sub> cells have decreased ability to suppress anti-CD3 plus APC-induced proliferation of CD4<sup>+</sup> T cells compared with WT T<sub>R</sub> cells (Fig. 5E). Importantly, compared with H<sub>4</sub>RKO mice, the DLNs of WT mice contained significantly greater numbers of IL-10<sup>+</sup> T cells. However, no difference in the number of IL-10<sup>+</sup> cells infiltrating the CNS was seen between WT and H<sub>4</sub>RKO mice (Supplemental Fig. 3). These data suggest that intrinsic H<sub>4</sub>R signaling regulates T<sub>R</sub> cell chemotaxis and suppressor activities, the absence of which leads to exacerbation of EAE in H<sub>4</sub>RKO mice.
Discussion

Histamine and its receptors have been implicated in the pathogenesis of autoimmune inflammatory demyelinating diseases of the CNS such as MS and its autoimmune model EAE (35). To date, H1R, H2R, and H3R have been shown to modulate susceptibility to EAE (11, 12, 36). The most recently characterized HR, H4R, is mainly expressed on hematopoietic cells (15), and it is postulated to have an immunomodulatory function during inflammatory and allergic conditions (15, 20, 21). The role of H4R in EAE, however, has not been studied. We show that the H4R negatively regulates the severity of MOG35–55-induced EAE, because mice lacking this receptor exhibit an exacerbated disease and immunopathology, as well as an increase in BBB permeability during the early acute phase of the disease. Our results are consistent with the studies on airway inflammation in which H4R signaling modulates the anti-inflammatory response (23). Furthermore, we show that H4R controls the frequency of TR cells in secondary lymphoid tissues, as well as their chemotaxis and suppressive activities. Deficiency of this receptor leads to a reduction in the proportion of TR cells in the CNS during the acute effector phase of disease and impairment of an anti-inflammatory response in association with an increase in the proportion of encephalitogenic Th17 cells.

We found that H4R is highly expressed on TR cells, which play an essential role in controlling autoimmune diseases, including EAE (28, 37). We show in this study that H4R signaling has a significant impact on regulating the proportion and distribution of natural TR cells in secondary lymphoid organs, but not on their thymic development. These results support the concept that specific factors in the microenvironment of peripheral lymphoid tissues may dictate the fate of the immune response by influencing TR cell biology (i.e., the frequency and distribution of TR) (38, 39). Naive H4RKO mice have a lower frequency of peripheral TRg cells than WT mice and, upon MOG35–55 immunization, exhibit more severe EAE. Given this result, peripheral activation/expansion of Ag-specific autoreactive T cells may be ineffectively controlled by the limited number of peripheral TRg cells present in naive H4RKO mice. Despite the observed differences in TRg numbers of naive H4RKO and WT mice, similar levels of peripheral TRg and TR cells were detected in WT and H4RKO mice during the acute phase of the disease. However, we evaluated the in vitro capacity of TRg cells to inhibit the TRg cell proliferative response, day 10 H4RKO TRg cells were less potent than day 10 WT TRg cells. Importantly, during disease progression, H4RKO mice had fewer IL-10–producing T cells in the DLN, but not in the CNS. Taken together, these data indicate that the H4R not only affects the frequency and/or localization of LN TRg cells, but also influences their function. However, the lack of H4R does not affect the numbers of induced TRg cells in the periphery, which rules this out as a potential mechanism underlying the differences in severity to EAE between WT and H4RKO mice. It is also possible that H4R signaling influences the potency of the encephalitogenic T cell response and/or refractoriness to TRg cell suppression.

Our results show that immunized H4RKO mice have a higher proportion of inflammatory Th17 cells within the CNS, consistent with fewer TRg cells infiltrating the CNS, despite the fact that no difference in CNS-resident TRg cells was observed between naive WT and H4RKO mice. These data may be explained by at least two mechanisms: a defect in the proliferation/expansion of TRg cells, or a deficit in the migratory capacity of these cells to enter the CNS. Because we observed a robust expansion of peripheral induced TRg cells during the effector phase of disease with no differences between WT and H4RKO mice, a defect in TRg cell responsiveness is unlikely to be involved. We therefore hypothesize that a defect in migration may explain the reduced number of TRg cells in the CNS of immunized H4RKO mice. Indeed, the pharmacological activation of H4R with the agonist 4-mHA has been shown to influence TRg recruitment into the lung (23). Our current findings, using a genetic approach, further demonstrate that HA signals through the H4R to induce migration of TRg cells.

In addition to our observation that there is a defect in migration/trafficking of TRg cells in immunized H4RKO mice, we found that the proportion of peripheral TRg cells expressing the LN homing receptor CCR7 was decreased in H4RKO compared with WT mice, on days 8 and 10 postimmunization, but reached comparable levels by day 12. This may have contributed to differences in disease severity, because one of the functions of this chemokine receptor is to promote the recruitment and interaction of TRg cells with mature DCs to ultimately regulate TRg cell-immune response (31, 40). Indeed, in vivo studies show that CD62L+CCR7+ TRg cells delay adoptive transfer of diabetes (41). However, future studies will address whether CCR7 expression in TRg cells is directly regulated by H4R signaling in our model. Additionally, the lack of H4R may alter the ability of the other HRs to elicit migratory responses, that is, through receptor desensitization.

Taken together, our results suggest that H4R signaling, either directly or indirectly, regulates the proportion of peripheral TRg cells, providing a checkpoint to regulate Ag-specific Tg expansion in the periphery, and increases the proportion of TRg cells in the target tissue before the expansion and/or recruitment of encephalitogenic CD4+ T cells into the CNS prior to the onset of EAE.

It has recently been shown that H4R is functionally expressed in the CNS (42, 43); hence, we cannot exclude the possibility that the absence of H4R signaling also contributes to increased disease severity as a function of disrupted CNS-central functions. Our current findings suggest that H4R signaling negatively regulates EAE by controlling the infiltration and suppressive activity of TRg cells within the CNS during the early acute effector phase of the disease, a critical time point in regulating the proliferation and expansion of autoreactive pathogenic T cells (26). Our observation that H4RKO mice develop more severe EAE than WT mice highlights the importance of the temporal localization of TRg cells in the relevant tissue for controlling the inflammatory response. Moreover, our findings suggest that the use of both peripheral and central acting H4R agonists may be useful in treating patients with clinically isolated syndrome, at the onset of MS, or upon relapse.

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Disclosures

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