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Histamine H_4 receptor optimizes T_R cell frequency and facilitates anti-inflammatory responses within the CNS

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Abstract

Histamine (HA) 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 (EAE), the autoimmune model of multiple sclerosis (MS). The pleiotropic effects of HA are mediated by four G protein-coupled receptors: *Hrh1*/H_1R, *Hrh2*/H_2R, *Hrh3*/H_3R, and *Hrh4*/H_4R. H_4R expression is primarily restricted to hematopoietic cells, and its role in autoimmune inflammatory demyelinating disease of the CNS has not been studied. In this report we show that, compared to wild type (WT) mice, animals with a disrupted *Hrh4* (H_4RKO) develop more severe myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}) peptide-induced EAE. Mechanistically, we also show that H_4R plays a role in determining the frequency of T regulatory (T_R) cells in secondary lymphoid tissues, and regulates T_R cell chemotaxis and suppressor activity. Moreover, the lack of H_4R leads to an impairment of an anti-inflammatory response due to fewer T_R cells in the CNS during the acute phase of the disease and an increase in the proportion of Th17 cells.

Introduction

Histamine [2-(4-imidazolyl)-ethylamine] (HA) is a biogenic amine that mediates multiple physiological processes, including neurotransmission and brain functions, secretion of pituitary hormones, and regulation of gastrointestinal and circulatory functions (1). Additionally, HA is an important mediator of inflammation and of innate and adaptive immune responses (1, 2). The pleiotropic effects of HA are mediated by four HA receptors (*Hrh1*/H_1R, *Hrh2*/H_2R, *Hrh3*/H_3R, and *Hrh4*/H_4R), all of which belong to the G protein-coupled receptor family (1, 2). These receptors are expressed on multiple cell types and

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signal through distinct intracellular pathways, which in part explains the diverse effects of HA on different cells and tissues.

Histamine is implicated in the pathogenesis of MS, as well as 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 H_1R expression was upregulated relative to normal tissue (7). Moreover, epidemiological data indicate that use of sedating H_1R antagonists is associated with decreased MS risk (8) and in a small study MS patients treated with an H_1R antagonist remained stable or improved neurologically (9). Likewise, H_1R and H_2R transcripts are present in the brain lesions of mice with active EAE, and administration of pyrilamine, a H_1R antagonist, reduces EAE severity (10). We previously identified *Bordetella 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). Further, genetic studies have demonstrated that HA, H_1R, H_2R and H_3R 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 H_4R in autoimmune inflammatory demyelinating disease of the CNS has not yet been studied.

H_4R expression is mostly restricted to hematopoietic cells, including T cells (15). H_4R is coupled to second messenger signaling pathways via the pertussis toxin (PTX)-sensitive heterotrimeric $G_{i/o}$ proteins (16) and to the β-arrestin pathway (17). The activation of H_4R 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, H_4R signaling regulates cytokine production, DC function, and Th cell polarization (19). In addition, H_4R 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 H_4R in the integrated immune response, however, remains unclear. Moreover, the use of different models has led to conflicting results about the role of H_4R in the immune response. In the murine model of allergic asthma, Morgan *et al* reported that the administration of 4-methyl HA (4-mHA), a H_4R agonist, reduced airway hyperreactivity and inflammation, while increasing T_R cell recruitment to the lung, suggesting an anti-inflammatory and immunomodulatory role for H_4R in this response (23). In contrast, studies using H_4RKO mice and H_4R antagonists, particularly JNJ 7777120 and its derivatives, suggest a pro-inflammatory 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 H_4R in modulating immune responses.

To assess the role of H_4R signaling in the regulation of autoimmune inflammatory demyelinating disease of the CNS, we studied $MOG_{35\text{-}55}$-induced EAE in H_4RKO mice. The results of our study provide direct evidence that H_4R modulates EAE severity. We show that H_4RKO mice, despite having equivalent T effector (T_E) cell responses, develop more severe EAE, augmented neuroinflammation, and increased BBB permeability compared to WT mice. In addition, we show that H_4R signaling exerts control over the frequency of T_R cell in secondary lymphoid tissues, as well as chemotaxis and suppressive ability of T_R cells. Consistent with this, the lack of H_4R leads to a lower proportion of these cells in the CNS during the acute effector phase of the disease, leading to an increase in the proportion of $CD4^+IL17^+$ cells and impairment of an anti-inflammatory response.

Material and Methods

Animals

C57BL/6J (B6/J, WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129P-*Hrh4*^{tm1Thr} (H_4RKO) 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-*Foxp3*^{gfp} KI mice were kindly provided by Dr. Vijay Kuchroo (Center of Neurological Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA). H_4RKO-*Foxp3*^{gfp} KI mice were generated by crossbreeding B6-*Foxp3*^{gfp} KI mice and H_4RKO 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 of $MOG_{35\text{-}55}$ and CFA (Sigma-Aldrich) supplemented with 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). Immediately afterward, each mouse received 200 ng of PTX (List Biological Laboratories) in 0.2 ml of 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; 5, moribund. Assessments of clinical quantitative trait variables, EAE pathology, and BBB permeability were performed as previously described (14).

CNS-infiltrating MN 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. 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.

Antibodies and flow cytometric analysis

The DLN, spleen, and thymus were excised and dissociated into single cell suspensions. For the identification and phenotypic analysis of T_R cells ($CD4^+CD8^-TCR\beta^+Foxp3^+$), the following surface anti-mouse mAb were used: anti-CD4 (MCD0417, Caltag); anti-CD8, and anti-CD25 (53–6.7, PC61; BD Pharmingen); 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 of PMA, 250 ng/ml of ionomycin and 2 μM monensin (Sigma-Aldrich) for 4h. 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-IL17A-PE (TC11-18H10; BD Pharmingen) and anti-IL10-Alexa Fluor 647 (JES5-16E7; BioLegend). Cells were collected using BD LSR II cytometer (BD biosciences) and analyzed using FlowJo software (TreeStar Software, Inc).

Cell culture conditions and lymphokine assays

For *ex vivo* cytokine analysis, spleen and DLN were obtained from d10 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 of $MOG_{35\text{-}55}$. Cell culture 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 d10. 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 of $MOG_{35\text{-}55}$ for 72 h at 37°C. During the last 18 h of culture, 1 μCi of [^{3}H] 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_E) and $CD4^+CD25^+$ (T_R) T cells from LN and spleen were sorted using anti-CD4, anti-TCRβ, and anti-CD25 mAbs. $CD4^+CD25^+$ T cell purity was consistently >97%. $CD4^+CD25^-$ T_E cells were cultured for 3 d with irradiated spleen cells as APC (1×10^5/well) in the presence of anti-CD3 mAb (5 μg/ml), with or without $CD4^+CD25^+$ T_R cells at 0.5:1 (T_R:T_E) cell ratio. The cell cultures were pulsed with 0.5 μCi [^{3}H] thymidine for the last 18 hrs. T_E cell proliferation with WT T_R cells was set at 100%. Percentage of inhibition in the presence of H_4RKO T_R cells was calculated.

Cell migration assay

Migratory capacity of $CD4^+$ T cells or B6-*Foxp3*^{gfp} KI or H_4RKO-*Foxp3*^{gfp} KI T_R or T_E 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 T_R cells were sorted based on GFP expression. $CD4^+TCR^+$ cell purity was >85% and sorted T_R 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 T_R cells in RPMI 1640 with 1% BSA and medium containing either no HA, 10^{-4} M or 10^{-7} M HA or 100 ng/ml SDF-1α was added to the bottom chamber. After 4 h at 37°C in 5% CO_2, cells that migrated to the bottom chamber were harvested, counted, and stained for subsequent flow cytometric analysis.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from T_E cells or T_R cells from naïve WT and H_4RKO mice using RNeasy isolation reagent (Qiagen Inc.), and reverse transcribed using Superscript III reverse transcriptase (Invitrogen). The generated cDNA was used in qRT-PCR using the SYBR green method. The sequences of *Hrh4* primers used were as follows: forward, 5′ TGAGGAGAATTGCTTCACGA 3′; reverse, 5′ TGCATGTGGAGGGGTTTTAT 3′. β2-microglobulin was used as a reference gene and the relative expression levels were calculated using the comparative threshold cycle (C_T) method.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4 software (GraphPad software Inc, San Diego, CA). Significance of differences was determined using parametric and non-parametric tests as described in the Figure Legends. For all analyses, $p \leq 0.05$ was considered significant.

Results

H_4R negatively regulates EAE severity

To investigate the role of H_4R in autoimmune inflammatory demyelinating disease of the CNS, EAE was induced in WT and H_4RKO mice by immunization with $MOG_{35\text{-}55}$-CFA-PTX. The clinical disease course of H_4RKO mice was more severe than WT mice (Figure 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 significantly greater in H_4RKO compared to WT mice (Table I). Furthermore, histopathological analysis revealed more extensive pathology in the brains and spinal cords of H_4RKO mice compared to WT mice (Figure 1B).

As an additional quantitative measure of differences in the neuroinflammatory response, we examined BBB permeability at d8, d10, d12, and d14 post-immunization. Compared to WT mice, the increase in BBB permeability during the early acute phase of the disease was significantly greater in H_4RKO mice (Figure 1C). Taken together, these data show that H_4R signaling negatively regulates EAE severity.

WT and H_4RKO ex vivo $CD4^+$ T_E cell responses are comparable

Although the precise pathogenic mechanism of EAE and MS is unknown, it is believed to be mediated by $CD4^+$ T cell-dependent activities (26). H_4R 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 H_4RKO mice, the $MOG_{35\text{-}55}$ specific T cell responses were compared on d10 post immunization. No significant differences in T cell proliferation (Figure 2A) or cytokine/chemokine production (Figure 2B) in response to $MOG_{35\text{-}55}$ re-stimulation were detected between H_4RKO and WT splenic and draining lymph node (DLN) cells.

H_4R influences the frequency of T_R cells in secondary lymphoid organs

$Foxp3^+$ T_R 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_R cells (29, 30). In addition, the H_4R agonist 4-mHA induces recruitment of T_R cells into the lung and inhibits development of allergic asthma (23). Although H_4R expression has been reported in T cells, it is unknown if it is expressed by T_R cells. We therefore compared the *Hrh4* mRNA levels between $CD4^+CD25^-Foxp3^-$ conventional T cells and $CD4^+CD25^+Foxp3^+$ T_R cells from naïve C57BL/6J mice. As shown in Figure 3A, *Hrh4* mRNA levels were higher in T_R cells compared to conventional $CD4^+$ T cells. Given this elevated expression of *Hrh4* in T_R cells and the importance of these cells in controlling inflammatory and autoimmune responses, we hypothesized that the deficiency of H_4R may affect T_R cell development and/or frequency. Therefore, we compared the proportion of $Foxp3^+$ T_R cells in the thymus of naïve WT and H_4RKO mice and found no difference among the single positive CD4 thymocytes (Figure 3B). However, the proportion and the absolute number of T_R cells in spleen and LN were significantly lower in H_4RKO mice compared to WT mice (Figure 3C and Suppl. Fig. 1A). Next, we examined the proportion of the CNS-resident T_R cells in naïve WT and H_4RKO mice and, in contrast to the periphery, no detectable difference was observed (Figure 3D).

H_4R controls T_R cell infiltration and inflammation in the CNS during acute EAE

Because H_4RKO mice have a lower frequency of T_R cells in secondary lymphoid organs compared with WT mice, we reasoned that the increased disease severity in H_4RKO mice may be due to a paucity of CNS-T_R cells during the induction- and/or acute effector-phase of the disease. Therefore, the frequency of T_R cells was determined in WT and H_4RKO mice following immunization. DLN cells and CNS-associated infiltrating mononuclear (MN) cells were isolated at different times after immunization, and the frequency of T_R cells among the $CD4^+TCR^+$ T cells analyzed. On d10 post-immunization, the proportion of T_R cells in the DLN comprised ~20% of the $CD4^+TCR^+$ T cells, representing an increase of ~2-fold over naïve mice. On d12 and d14 the proportion of T_R cells dropped to ~10–12% of the T cells. However, no significant difference in the proportion of T_R cells or T_E cells in the DLN was detectable between WT and H_4RKO mice at any of the time points examined (Figure 4A and Suppl. 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_R cells with mature DC in the paracortical area of the LN (31). H_4RKO mice have a lower proportion of T_R cells expressing CCR7 at d8 and d10 after immunization when compared to WT mice, with no differences by d12 (Figure 4B).

By d10 post-immunization, we observed robust recruitment of T_R cells into the CNS of both WT and H_4RKO mice compared to naïve controls (Figure 3D *vs.* Figure 4D). In contrast to the DLN of immunized mice, the frequency of T_R cells in the CNS of H_4RKO mice was lower than that detected in WT mice at d10, d12, and d17 post-immunization (Figure 4D). Consistent with the lower proportion of T_R cells in the CNS of H_4RKO mice, a decrease in the absolute number of T_R cells was also observed (Suppl. Fig. 1B). A role for histamine in regulating the adhesion and recruitment of immune cells has been suggested previously (32). Therefore, since T_R cells have been reported to express greater levels of ICAM-1/CD45 and P-selectin in comparison with non-T_R cells (33), and CCR6 expression has been shown to regulate EAE pathogenesis by controlling T_R cell recruitment to the CNS (33), we evaluated the expression levels of these in CNS infiltrating T_R cells of WT and H_4RKO mice. No differences in the expression levels of these molecules were detected between WT and H_4RKO CNS-infiltrating MN cells during EAE (data not shown). We also determined whether there was any difference in the overall infiltration of $CD4^+$ T cells into the CNS after immunization. As expected, the proportion of TCR^+CD4^+ T cells increased with disease progression, and by d14 post-immunization the CNS of H_4RKO mice exhibited a significantly greater proportion of these cells compared to WT mice (Figure 4C).

Th1 and Th17 cells have been shown to contribute to the pathogenesis of EAE, and T_R cells inhibit the induction of these pathogenic cells (26). Therefore, we compared autoreactive effector CD4 responses in the target organ of WT and H_4RKO mice during EAE by examining the frequency of encephalitogenic IFNγ– and IL17-producing Th1 and Th17 cells in the CNS. The frequency of Th17 cells in H_4RKO mice was higher than that of WT mice, whereas the frequency of Th1 cells was comparable between the two strains (Figure 4E).

H_4R regulates T_R cell chemotaxis and suppressor functions

It has been shown that the H_4R agonist 4-mHA reduces airway hyperreactivity and inflammation, and that this effect is associated with the recruitment of T_R cells into the lung (23). Additionally, H_4R 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_4RKO mice correlates with decreased numbers of infiltrating T_R cells into the CNS during the acute phase of disease (Figure 4D and Suppl. Fig. 1B), consistent with the requirement for T_R cells in the target tissue for adequate immune regulation (34). Because we observed differences in the number of T_R cells in the CNS between immunized WT and H_4RKO mice, we examined whether H_4R is required for optimal $CD4^+$ T cell chemotaxis. We performed *in vitro* migration assays using purified $CD4^+$ T cells from immunized WT and H_4RKO mice. WT and H_4RKO $CD4^+$ T cells responded equally to stromal cell-derived factor-1 (SDF-1), a known strong chemotactic factor for leukocytes (Figure 5A). However, WT $CD4^+$ T cells responded to HA-induced migratory signals, whereas H_4RKO $CD4^+$ T cells did not (Figure 5B). These results suggest that HA, acting through H_4R, functions as a chemotactic factor for T cells.

Interestingly, when the proportion of Foxp3$^+$ T_R 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$^+$ T_R cells compared to those from H_4RKO mice (Figure 5C). To test the H_4R-dependent chemotactic activity directly in T_R cells, we utilized WT- and H_4RKO-*Foxp3^{gfp}* knockin (KI) mice, sorted GFP$^+$ T_R cells from immunized mice and assessed the chemotactic response to HA. As with total CD4$^+$ T cells, the H_4RKO Foxp3$^+$ T_R cells had an impaired migratory response to HA (Figure 5D). Furthermore, we tested whether T_R cells from WT and H_4RKO mice exhibited differences in their *in vitro* suppressive function and found that d10 H_4R-deficient T_R cells have decreased ability to suppress anti-CD3 + APC-induced proliferation of CD4$^+$ T cells compared to WT T_R cells (Figure 5E). Importantly, compared to H_4RKO mice, the DLNs of WT mice contained significantly greater numbers of IL10$^+$ T cells. However, no difference in the number of IL10$^+$ cells infiltrating the CNS was seen between WT and H_4RKO mice (Suppl. Fig. 3). These data suggest that intrinsic H_4R signaling regulates T_R cell chemotaxis and suppressor activities, the absence of which leads to exacerbation of EAE in H_4RKO 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, H_1R, H_2R and H_3R have been shown to modulate susceptibility to EAE (11, 12, 36). The most recently characterized HR, H_4R, 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 H_4R in EAE, however, has not been studied. We show that the H_4R negatively regulates the severity of MOG_{35-55}-induced EAE, since 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 H_4R signaling modulates the anti-inflammatory response (23). Furthermore, we show that H_4R controls the frequency of T_R cells in secondary lymphoid tissues, as well as their chemotaxis and suppressive activities, and deficiency of this receptor leads to a reduction in the proportion of T_R cells in the CNS during the acute effector phase of disease and impairment of an anti-inflammatory response, leading to an increase in the proportion of encephalitogenic Th17 cells.

We found that H_4R is highly expressed on T_R cells, which play an essential role in controlling autoimmune diseases, including EAE (28, 37). We show here that H_4R signaling has a significant impact on regulating the proportion and distribution of natural T_R 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 T_R cell biology (i.e., the frequency and distribution of T_R) (38, 39). Naïve H_4RKO mice have lower frequency of peripheral T_R cells than WT mice and, upon MOG_{35-55} 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 T_R cells present in naïve H_4RKO mice. Despite the observed differences in T_R numbers of naïve H_4RKO and WT mice, similar levels of peripheral T_E and T_R cells were detected in WT and H_4RKO mice during the acute phase of the disease. However, when we evaluated the *in vitro* capacity of T_R cells to inhibit the T_E cell proliferative response, d10 H_4RKO T_R cells were less potent than d10 WT T_R cells. Importantly, during disease progression, H_4RKO mice had fewer

IL10-producing T cells in the DLN, but not in the CNS. Taken together, these data indicate that the H_4R not only affects the frequency and/or localization of LN T_R cells but also influences their function. However, the lack of H_4R does not affect the numbers of induced T_R cells in periphery, which rules this out as a potential mechanism underlying the differences in severity to EAE between WT and H_4RKO mice. It is also possible that H_4R signaling influences the potency of the encephalitogenic T cell response and/or refractoriness to T_R cell suppression.

Our results show that immunized H_4RKO mice have a higher proportion of inflammatory Th17 cells within the CNS, consistent with fewer T_R cells infiltrating the CNS, despite the fact that no difference in CNS-resident T_R cells was observed between naïve WT and H_4RKO mice. These data may be explained by at least two mechanisms: a defect in the proliferation/expansion of T_R cells, or a deficit in the migratory capacity of these cells to enter the CNS. Since we observed a robust expansion of peripheral induced T_R cells during the effector phase of disease with no differences between WT and H_4RKO mice, a defect in T_R cell responsiveness is unlikely to be involved. We therefore hypothesize that a defect in migration may explain the reduced number of T_R cells in the CNS of immunized H_4RKO mice. Indeed, the pharmacological activation of H_4R with the agonist 4-mHA has been shown to influence T_R recruitment into the lung (23). Our current findings, using a genetic approach, further demonstrate that HA signals through the H_4R to induce migration of T_R cells.

In addition to our observation that there is a defect in migration/trafficking of T_R cells in immunized H_4RKO mice, we found that the proportion of peripheral T_R cells expressing the LN homing receptor CCR7 was decreased in H_4RKO compared to WT mice, on d8 and d10 post-immunization, but reached comparable levels by d12. This may have contributed to differences in disease severity, since one of the functions of this chemokine receptor is to promote the recruitment and interaction of T_R cells with mature DCs to ultimately regulate the T_E cell-immune response (31, 40). Indeed, *in vivo* studies show that $CD62L^+CCR7^+$ T_R cells delay adoptive transfer of diabetes (41). However, future studies will address whether CCR7 expression in T_R cells is directly regulated by H_4R signaling in our model. Additionally, the lack of H_4R may alter the ability of the other HRs to elicit migratory responses, i.e. through receptor desensitization.

Taken together, our results suggest that H_4R signaling, either directly or indirectly: 1) regulates the proportion of peripheral T_R cells, providing a checkpoint to regulate Ag-specific T_E expansion in the periphery, and 2) increases the proportion of T_R cells in the target tissue before the expansion and/or recruitment of encephalitogenic $CD4^+$ T cells into the CNS before the onset of EAE.

It has recently been shown that H_4R is functionally expressed in the CNS (42, 43); hence we cannot exclude the possibility that the absence of H_4R signaling also contributes to increased disease severity as a function of disrupted CNS-central functions. Our current findings suggest that H_4R signaling negatively regulates EAE by controlling the infiltration and suppressive activity of T_R 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 H_4RKO mice develop more severe EAE than WT mice highlights the importance of the temporal localization of T_R cells in the relevant tissue for controlling the inflammatory response. Moreover, our findings suggest that the use of both peripheral and central acting H_4R agonists may be useful in treating patients with clinically isolated syndrome, at the onset of MS, or upon relapse.

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Supplementary Material

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Abbreviations used

HA	histamine
4-mHA	4-methylHA
EAE	experimental allergic encephalomyelitis
MS	multiple sclerosis
***Hrh*/HR**	histamine receptor
WT	wild type
MOG_{35-55}	myelin oligodendrocyte glycoprotein 35–55
T_R	T regulatory cell
BBB	blood-brain barrier
T_E	T effector cell
PTX	pertussis toxin
LN	lymph node
DLN	draining LN
MN	mononuclear cells
KI	knockin

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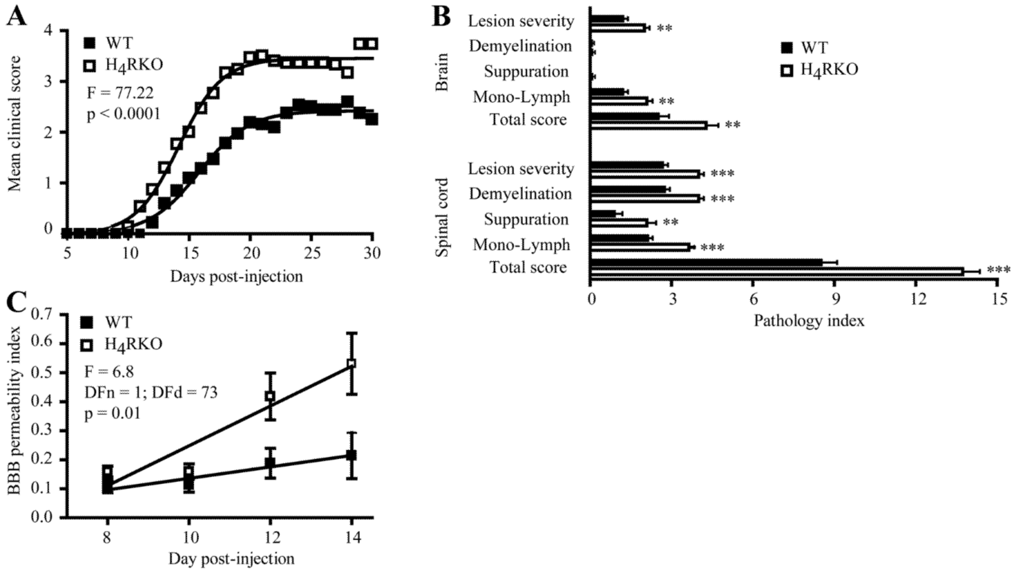

Figure 1. H_4R negatively regulates EAE severity and BBB permeability

Clinical score (A) and severity of CNS lesions (B) in WT (■, n=32) and H_4RKO (□, n=30) mice were compared following immunization with $MOG_{35\text{-}55}$-CFA-PTX. The significance of the differences between the clinical courses of disease was calculated by regression analysis and best-fit curve is shown. In (B), the significance of differences observed at d30 post-immunization was determined using the Mann-Whitney test (** $p<0.01$; *** $p<0.001$). (C) The significance of differences in BBB permeability indices of WT and H_4RKO mice across the early acute phase of disease was assessed by regression analyses (n=8 for each strain at each time point).

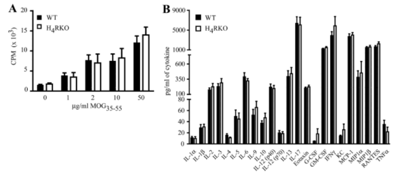

Figure 2. *Ex vivo* Ag-specific T cell proliferation and cytokine/chemokines profiles of $MOG_{35\text{-}55}$-CFA-PTX-immunized WT and H_4RKO mice

(A) Ag-specific T cell proliferative responses were evaluated by [^{3}H]-thymidine incorporation (n=6 per strain). Mean cpm ± SD were calculated from triplicate wells. The significance of differences was calculated by Two-Way Analysis of Variance. (B) Protein production by $MOG_{35\text{-}55}$-stimulated splenocytes from WT and H_4RKO mice. Spleen and DLN cells from d10-immunized mice were stimulated with $MOG_{35\text{-}55}$ for 72h and culture supernatants were analyzed for protein levels by Bio-Plex. Cytokine production in the absence of $MOG_{35\text{-}55}$ was below detection limits. Significance of differences in T cell proliferation or cytokine production was determined using Mann-Whitney test, and Bonferroni's corrected *p*-value for multiple comparisons.

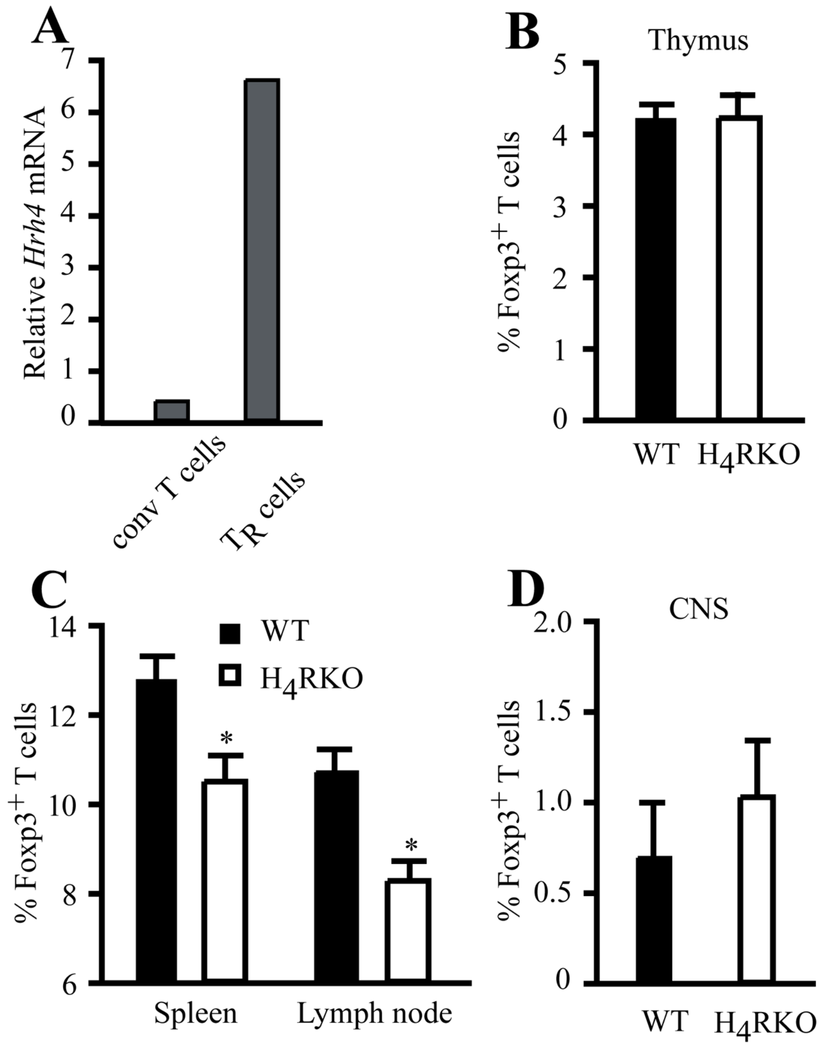

Figure 3. Analysis of Foxp3$^+$ T cell frequency in lymphoid tissues of naïve WT and H_4RKO mice

(A) mRNA expression of *Hrh4* was measured from sorted naïve CD4$^+$CD25$^+$ T cells (T_R cells) and compared to conventional CD4$^+$CD25$^-$ T cells (conv T cells). Expression levels were determined by qRT-PCR and analyzed using comparative Ct method with β2-microglobulin as an endogenous control. Data are representative of 3 independent experiments. (B–C) Flow cytometric analysis of CD4$^+$CD8$^-$TCRβ$^+$Foxp3$^+$ T_R cell frequency in thymus (B), spleen and LN (C), and CNS (D) of naive WT and H_4RKO mice. Statistical significance was determined using the Mann-Whitney test (*p<0.05). Flow cytometric data represent the mean ± SEM of 8 individual mice (B and C), or 3 experiments (pool of 5 mice per experiment) (D).

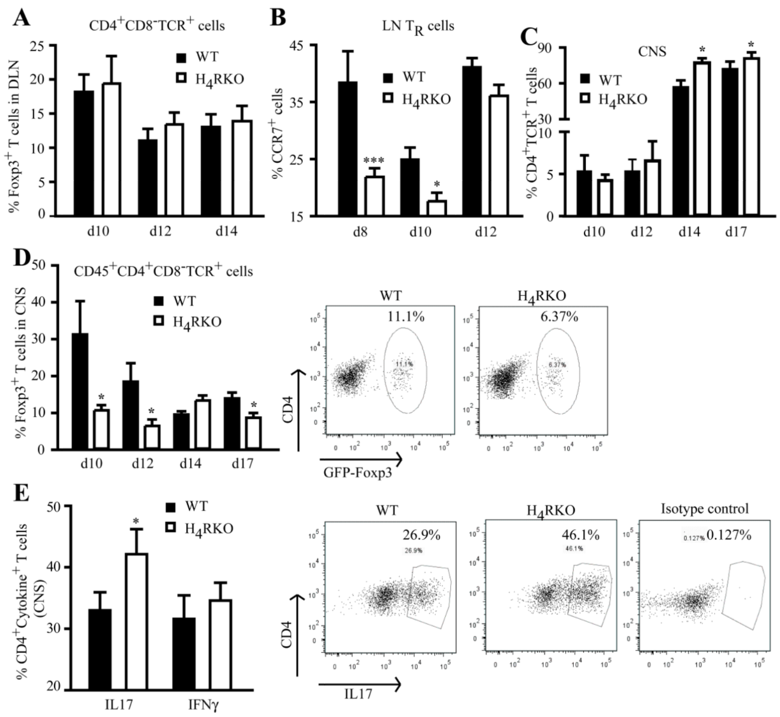

Figure 4. Reduced frequency of Foxp3⁺ T_R cell and increased Th17 cells in the CNS of immunized H_4RKO mice

Flow cytometric analysis of the frequency of $CD4^+CD8^-TCR\beta^+Foxp3^+$ T_R cell in DLN and spleen (A) and percentage of T_R cells expressing CCR7 (B), and percentage of T_R cells (D) or Th17 (E) in CNS-infiltrating MN cells of immunized WT and H_4RKO mice. (C) Percentage of total $CD4^+TCR^+$ T cells infiltrating the CNS of immunized mice. Statistical significance was determined using the Mann-Whitney test (*p<0.05). Flow cytometric data represent the mean ± SEM of 5–8 individual mice (at each time point).

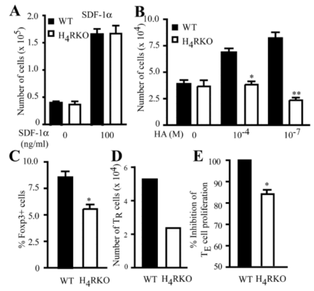

Figure 5. H_4R positively regulates T_R cell chemotaxis and suppressive activity

Total $CD4^+$ T cells (A–C) or sorted T_R cells (D) from d10 immunized WT (black bars) and H_4RKO (white bars) mice were subjected to migration assay using either 100 ng/ml SDF-1α(A) or 10^{-4} M and 10^{-7}HA (B). (C) Migrated cells were stained for identification of $Foxp3^+$ T_R cells and % of T_R cells from the total migrating cells is shown. (E) WT- or H_4RKO-$CD4^+GFP^-Foxp3^-$ responder cells were cultured with WT irradiated spleen cells and co-cultured at 0.5:1 (T_R:T_E) ratio with WT- or H_4RKO-$CD4^+GFP^+Foxp3^+$ T_R cells, respectively. Significance of differences was calculated using Mann-Whitney test (*p<0.05; **p<0.01). (A) and (B) are the average of 3 independent experiments. (D) is representative of 2 independent experiments. (E) is the average of 3 independent experiments.

Table I

Summary of EAE clinical trait variables in WT and H_4RKO mice

Animals were immunized with $MOG_{35\text{-}55}$-CFA-PTX and scored daily for clinical signs starting on d5. Mean trait values ± SD are shown. The significance of the difference in incidence was determined by Chi-square test and the significance of the difference in EAE-quantitative trait variables was determined using the Mann-Whitney test.

	WT	**H_4RKO**	***p* value**
Incidence	31/32	30/30	
Mean day of onset	16.25 ± 3.74	13.63 ± 2.74	0.004
Cumulative score	36.00 ± 18.3	56.03 ± 20.72	0.0002
Day affected	14.00 ± 6.3	17.40 ± 2.77	0.002
Severity index	2.42 ± 0.88	3.21 ± 1.02	0.004
Peak score	3.37 ± 1.26	4.33 ± 0.84	0.001