Published in final edited form as:
Eur J Immunol. 2012 June ; 42(6): 1536–1546. doi:10.1002/eji.201141859.

Combinatorial roles for histamine H_1–H_2 and H_3–H_4 receptors in autoimmune inflammatory disease of the central nervous system

Naresha Saligrama[1], **Rajkumar Noubade**[1], **Laure K Case**[1], **Roxana del Rio**[1], and **Cory Teuscher**[1,2,3]

[1]Department of Medicine, University of Vermont, Burlington, Vermont, USA

[2]Department of Pathology, University of Vermont, Burlington, Vermont, USA

[3]Center for Nuclear Receptors and Cell Signaling, The University of Houston, Houston, Texas, USA

Abstract

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system in which histamine (HA) and its receptors have been implicated in disease pathogenesis. HA exerts its effects through four different G protein-coupled receptors designated H_1–H_4. We previously examined the effects of traditional single HA receptor (HR) knockouts (KOs) in experimental allergic encephalomyelitis (EAE), the autoimmune model of MS. Our results revealed that H_1R and H_2R are propathogenic, while H_3R and H_4R are antipathogenic. This suggests that combinatorial targeting of HRs may be an effective disease-modifying therapy (DMT) in MS. To test this hypothesis, we generated H_1H_2RKO and H_3H_4RKO mice and studied them for susceptibility to EAE. Compared with wild-type (WT) mice, H_1H_2RKO mice developed a less severe clinical disease course, whereas the disease course of H_3H_4RKO mice was more severe. H_1H_2RKO mice also developed less neuropathology and disrupted blood brain barrier permeability compared with WT and H_3H_4RKO mice. Additionally, splenocytes from immunized H_1H_2RKO mice produced less interferon(IFN)-γ and interleukin(IL)-17. These findings support the concept that combined pharmacological targeting of HRs may be an appropriate ancillary DMT in MS and other immunopathologic diseases.

Keywords

Disease-modifying therapy; Experimental allergic encephalomyelitis; G protein-coupled receptors; Histamine; Multiple sclerosis

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) characterized by focal demyelination, axonal loss, and gliosis [1]. Although the etiology of MS remains ill-defined, susceptibility likely results from a combination of factors, including genetic/epigenetic, environmental, immunological, hormonal, and

Full correspondence: Dr. Cory Teuscher, Immunobiology Program, C331 Given Medical Building, University of Vermont, Burlington, VT 05405, USA, Fax: +1-802-656-3854, C.Teuscher@uvm.edu.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

infectious agents. Experimental allergic encephalomyelitis (EAE) is the autoimmune model of MS, in which disease pathogenesis is associated with major histocompatibility complex (MHC) class II-restricted $CD4^+$T cells capable of secreting either interferon(IFN)-γ (Th1) or interleukin(IL)-17 (Th17) [2].

Histamine (HA, 2-(4-imidazole) ethylamine) is a biogenic monoamine that mediates a variety of physiological processes including neurotransmission, gastrointestinal and circulatory functions, secretion of pituitary hormones, cell proliferation, and hematopoiesis [3]. In addition, HA is a potent mediator of inflammation and regulator of innate and adaptive immune responses [4]. HA is synthesized by decarboxylation of L-histidine by the rate-limiting enzyme histidine decarboxylase (HDC). Mast cells and basophils are the major sources of stored HA in the body [5]. However, induced or nascent secretion of HA can occur in other cell types including dendritic cells, T cells, neutrophils, macrophages, and immature myeloid cells [6–13]. HA exerts its effects by binding to four different G protein-coupled receptors designated H_1–H_4. H_1R and H_2R couple to second messenger signaling pathways via stimulatory G proteins ($G\alpha_{q/11}$ and G_s, respectively), whereas H_3R and H_4R couple via inhibitory G proteins ($G_{i/o}$) [14–16]. HA has a diverse effect on many cell types due to differential expression of HRs and signaling through distinct intracellular signaling pathways. H_1R and H_2R are expressed more widely, while H_4R expression is mostly restricted to hematopoietically derived cells. Recently, it has been shown that H_4R is also expressed functionally in the CNS [17]. H_3R is primarily expressed within the CNS presynaptically, where it is an inhibitory auto- and heteroreceptor [18].

The role of HA in the pathogenesis of MS and EAE has been well documented [19]. HA and agents causing its release from mast cells alter the permeability of the blood brain barrier (BBB) [20, 21]. The use of first-generation antihistamines, which can readily cross the BBB, is associated with a decrease in MS risk [22]. Patients with relapsing-remitting or relapsing-progressive MS given the H_1R antagonist hydroxyzine were reported to remain stable and improved neurologically [23]. In addition, microarray analysis on the chronic plaques of MS patients revealed increased levels of H_1R transcripts [24].

In EAE, higher levels of HA within the cerebrospinal fluid (CSF) correlate with the onset of disease and mast cell granule stabilizers and H_1R-specific antagonists reduce EAE severity [25–27]. Importantly, in EAE, Th1 and Th2 clones reactive to proteolipid protein 139–151 have increased levels of H_1R and H_2R transcripts, respectively. In addition, mice treated with the H_1R antagonist pyrilamine or the H_2R agonist dimaprit showed reduced clinical severity and pathology [28, 29]. We have previously demonstrated that *Bordetella pertussis* toxin-induced HA sensitization (*Bphs*) is a shared autoimmune disease susceptibility gene in EAE and experimental allergic orchitis, and positional candidate gene cloning identified *Bphs* to be *Hrh1* 27]. In addition, gene targeting studies from our lab and other groups demonstrated that HA, H_1R, H_2R, H_3R, and H_4R play important roles in EAE susceptibility and pathogenesis, either by regulating encephalitogenic T-cell responses, cytokine production by antigen-presenting cells (APCs), BBB permeability, or T regulatory (Treg)-cell activity [27, 30–34].

The current therapeutic mainstays for MS include IFN-β and glatiramer acetate; however, in most instances, these drugs are of limited efficacy [35]. Consequently, research efforts have been increasingly directed toward identifying new therapeutic modalities and disease-modifying therapies (DMTs). Previously, using individual H_1R-H_4RKO mice, we showed that H_1R and H_2R are propathogenic, whereas H_3R and H_4R are antipathogenic. This suggests that combinatorial pharmacological targeting of HRs may be an effective DMT in MS. To test this hypothesis, we generated H_1H_2RKO and H_3H_4RKO mice on the C57BL/6J (B6) background and studied them for susceptibility to EAE elicited by immunization with

myelin oligodendrocyte glycoprotein peptide 35–55 ($MOG_{35–55}$). The results of our study show that compared to B6 mice, H_1H_2RKO mice exhibit decreased susceptibility to EAE, whereas H_3H_4RKO mice develop more severe disease. The findings of our study support the concept that combined pharmacological targeting of HRs may be an appropriate DMT in the treatment of MS and other immunopathologic diseases, particularly given the recent development of highly selective agonists and antagonists for H_3R and H_4R [36].

Results

H_1H_2RKO and H_3H_4RKO mice exhibit differential susceptibility to EAE

EAE was induced in B6, H_1H_2RKO, and H_3H_4RKO mice by immunization using a 2× $MOG_{35–55}$-CFA protocol [37, 38]. The severity of the clinical disease courses differed significantly among the three strains (F=28.5; p< 0.0001) (Fig. 1A), with H_1H_2RKO mice exhibiting significantly less severe disease than both B6 (F = 17.3; p < 0.0001) and H_3H_4RKO (F = 57.3; p < 0.0001) mice. In contrast, the severity of the clinical disease course of H_3H_4RKO mice was significantly greater than B6 (F = 8.2; p< 0.0001) and H_1H_2RKO (F = 57.3; p < 0.0001) mice. Analysis of EAE-associated clinical quantitative trait variables [31] revealed that the percentage of animals affected, cumulative disease score, and days affected were significantly greater in H_3H_4RKO compared with B6 mice. In contrast, percentage of animals affected, cumulative disease score, and days affected were significantly less in H_1H_2RKO compared with B6 mice (Table 1).

Furthermore, histopathological analysis of the CNS revealed augmented inflammatory responses and pathology in the brains of H_3H_4RKO mice compared with H_1H_2RKO and B6 mice (Fig. 1B) with no significant difference in spinal cord pathology (Fig. 1C). Disruption of BBB permeability is another parameter used to assess the neuroinflammatory response in EAE and susceptibility to disease. At day 10 (d10) postimmunization, we found that B6 and H_3H_4RKO mice had increased BBB permeability during the acute early phase of the disease compared with that of H_1H_2RKO mice (Fig. 1D). These results indicate that the combined effect of disrupting H_1R and H_2R signaling is antipathogenic in EAE, whereas the combined effect of disrupting H_3R and H_4R signaling is propathogenic.

Immune profiling of H_1H_2RKO and H_3H_4RKO mice

HA and HRs have been shown to be important in regulating hematopoiesis [39–41]. We examined the frequency of different cell types in the primary and secondary lymphoid tissues of naïve B6, H_1H_2RKO, and H_3H_4RKO mice. There was no significant difference in the total number of cells in the thymus, lymph node, or spleen among the three strains (Fig. 2A). We also analyzed the frequency of $CD4^+$, $CD8^+$, $Foxp3^+$ Treg, B cell ($CD19^+$), $CD11b^+$, $CD11b^+Gr1^+$, NKT (CD1d tetramer$^+$), and NK ($NKp46^+$) cells in primary and secondary lymphoid tissues and found no significant differences in the frequency of these cell types (Fig. 2B, C, D, and E), with the only exception being the lymph node B-cell frequency, which was significantly decreased in H_3H_4RKO compared with H_1H_2RKO mice (Fig. 2C). These results strongly suggest that the differences in EAE susceptibility observed between H_1H_2RKO and H_3H_4RKO mice are not due to differences in the frequency of immune cells in the primary and secondary lymphoid tissues.

Changes in $CD4^+$ T-cell responses associated with EAE in H_1H_2RKO and H_3H_4RKO mice

Although the exact pathogenic mechanisms underlying MS and EAE are not known, it is thought to be highly dependent on $CD4^+$ T cells capable of producing IFN-γ and/or IL-17 [2]. HA and HRs play a role in T-cell polarization, proliferation, and cytokine production [4]. Therefore, to elucidate the immune mechanisms associated with differential EAE susceptibility observed in B6, H_1H_2RKO, and H_3H_4RKO mice, we compared their

$MOG_{35–55}$-specific T-cell responses on d10 post immunization. In ex vivo proliferation assays, splenocytes and draining lymph node (DLN) cells from all three strains responded equivalently in a dose-dependent fashion to $MOG_{35–55}$ (Fig. 3A). Splenic and DLN cells from H_1H_2RKO mice restimulated ex vivo with $MOG_{35–55}$ produced significantly less IFN-γ (Fig. 3B) and IL-17 (Fig. 3C) compared with restimulated cells from H_3H_4RKO mice. In addition, H_3H_4RKO mice produced significantly more IFN-γ and IL-17 compared with B6 mice. IL-4 was undetectable among the three strains. These results suggest that the differences in EAE susceptibility observed in H_3H_4RKO mice can, in part, be attributed to increased encephalitogenic Th1 and Th17 effector T-cell responses.

HRs influences the polarization of $CD4^+$T cells

Previously, we showed that H_1R regulates IFN-γ and IL-4 production by activated $CD4^+$ T cells and Th1/Th2 effector T-cell responses [31]. In the current study, we observed in ex vivo recall assays that splenic and DLN cells from H_1H_2RKO mice produce significantly less IFN-γ and IL-17 and undetectable levels of IL-4. Therefore, to address whether the lack of the two different classes of HRs have an intrinsic effect on cytokine production or differentiation of $CD4^+$ T cells, we stimulated purified $CD4^+$ T cells from the spleen and lymph nodes of naïve B6, H_1H_2RKO, and H_3H_4RKO mice with plate bound anti-CD3 and soluble anti-CD28 mAbs and screened the culture supernatants for IL-17, IFN-γ, IL-4, and IL-2 production by enzyme-linked immunosorbent assay (ELISA) at 24, 48, and 72 h. IL-17 was undetectable among the three strains. Interestingly, across the time points examined, $CD4^+$T cells from H_3H_4RKO mice produced significantly more IFN-γ compared with cells from H_1H_2RKO and B6 mice (Fig. 4A). In addition, IL-4 production by stimulated H_1H_2RKO $CD4^+$ T cells was significantly greater than that of $CD4^+$ T cells from H_3H_4RKO and B6 mice, which was undetectable (Fig. 4B). Among the strains, we observed no significant difference in the production of IL-2 by $CD4^+$T cells (Fig. 4C). These results indicate that $CD4^+$ T cells from H_3H_4RKO have an inherent bias toward IFN-γ production, while H_1H_2RKO are predisposed to produce IL-4. Therefore, the lack of H_1R-H_2R and H_3R-H_4R predisposes $CD4^+$T cells to differentiate into either Th2 or Th1 cells, respectively, and may account for the altered cytokine production and differences in disease severity seen among the strains of mice.

Differential cross-regulation of HR expression in H_1H_2RKO and H_3H_4RKO $CD4^+$T cells

The severity of EAE observed in H_1H_2RKO and H_3H_4RKO parallels that of the respective individual receptor knockout (KO) mice in that clinical EAE is less severe in both H_1RKO and H_2RKO mice and more severe in H_3RKO and H_4RKO mice. Similarly, EAE pathology was significantly less in H_1R, H_2R and H_1H_2RKO mice, whereas it was significantly greater in H_3RKO, H_4RKO, and H_3H_4RKO mice. The basis of this effect may be due to a compensatory upregulation of the remaining HRs in single HRKO, H_1H_2RKO, and H_3H_4RKO mice. With respect to T cells, we showed that HR expression is rapidly downregulated upon T-cell receptor activation, and HR signaling associated with $CD4^+$ T-cell differentiation and effector functions occurs during initial activation [31]. Therefore, we compared HR expression in naïve $CD4^+$ T cells of single HRKO, H_1H_2RKO and H_3H_4RKO mice by quantitative real-time polymerase chain reaction (qRT-PCR). H_3R expression was undetectable in naïve $CD4^+$ T cells from all single HRKO and H_1H_2RKO mice. Interestingly, in the absence of single HRs, the expression of the remaining HRs was increased above B6 levels in naïve $CD4^+$ T cells (Fig. 5A). Moreover, H_4R expression was increased in H_1RKO, H_2RKO, and H_1H_2RKO mice with H_1RKO<H_2RKO<H_1H_2RKOs (Fig. 5B). Compared with B6 $CD4^+$ T cells, H_1R expression was significantly increased in H_3RKO, H_4RKO, and H_3H_4RKO mice with H_3RKO< H_4RKO< H_3H_4RKOs (Fig. 5C). Similarly, compared with B6 cells H_2R expression was significantly increased in H_3RKO, H_4RKO, and H_3H_4RKO mice with H_3RKO = H_4RKO< H_3H_4RKOs (Fig. 5D). To address

whether compensation by the upregulated HRs in H_1H_2RKO and H_3H_4RKO $CD4^+$ T cells affects the expression of HDC or the production of HA, anti-CD3, and soluble anti-CD28 mAb stimulated $CD4^+$ T cells were analyzed for HDC expression (Fig. 5E) by qRT-PCR and screened for HA production by enzyme immunoassay (EIA) (Fig. 5F) at 24, 48, and 72 h. Surprisingly, we did not detect a significant strain effect or (strain × time) interaction for HA production or HDC expression. These data therefore do not support the existence of a compensatory loop with respect to HA production and HDC expression by H_1H_2RKO and H_3H_4RKO $CD4^+$ T cells. However, the HR expression studies clearly indicate that disease severity in H_1H_2RKO and H_3H_4RKO mice is associated with compensatory upregulation of the corresponding receptors.

Discussion

Here, we have assessed the overall contribution to EAE susceptibility imposed by H_1R and H_2R (couple via stimulatory G proteins) and the H_3R and H_4R (couple via inhibitory G proteins). The results of our study demonstrate that H_3H_4RKO mice develop a significantly more severe clinical disease course compared with B6 and H_1H_2RKO mice in association with greater pathology in the brain but not the spinal cord. In contrast, despite a significant difference in the severity of the clinical disease courses between B6 and H_1H_2RKO, a significant difference in pathology was not detected in either the brain or spinal cord, suggesting as in H_3RKO mice [18], H_1R and/or H_2R may also play a significant role in central functions related to the severity of clinical signs. Increased susceptibility to EAE in H_3H_4RKO mice is associated with significantly higher production of IFN-γ and IL-17 in $MOG_{35–55}$ specific ex vivo recall assays. In contrast, H_1H_2RKO mice exhibit decreased susceptibility to EAE and decreased BBB permeability. We have also shown that $CD4^+$ T cells from H_1H_2RKO mice, upon in vitro activation, have an intrinsic immune deviation toward the Th2 phenotype, while activated T cells from H_3H_4RKO mice have an intrinsic immune bias toward Th1 type cells.

The results of our current study indicate that HA signaling through H_1R and H_2R augments EAE susceptibility by influencing antigen-specific T-cell effector responses, immune deviation, and BBB permeability. It is well known that HA and HRs modulate the innate and adaptive immune systems [4]. Previously, we have shown that H_1RKO mice develop less severe EAE that was associated with an immune deviation of the $CD4^+$ T-cell population from an encephalitogenic Th1 response to a protective Th2 response [27, 31]. In addition, mice deficient for H_2R are significantly less susceptible to acute early phase EAE and T cells from immunized H_2RKO mice exhibit blunted Th1 effector cell responses [32]. Similar to H_1R, HA acting through H_2R determines T-cell effector functions and their polarization. T cells from H_2RKO mice stimulated in vitro with anti-CD3 and anti-CD28 mAbs produce higher intracellular levels of IFN-γ, IL-4, and IL-13 [42]. Although the mechanism by which H_1R and H_2R regulates T-cell effector function is poorly understood, possible mechanisms include multiple signaling through HRs, receptor density on a particular cell type, the use of different second messenger molecule/pathways or direct/indirect effect on T cells, APCs, or both. Therefore, while H_1R and H_2R signaling clearly influences $CD4^+$ T-cell differentiation and effector functions, HR signaling may also contribute to EAE pathogenesis by acting in other cells types associated with disease and remains the subject of future studies.

Pathophysiology associated with MS is thought to be initiated by peripheral autoreactive T cells that cross the BBB and elicit neuroinflammation or autoimmune responses that are secondary to the events initiated by the CNS tissue [43]. Unlike other HRs, H_3R is expressed primarily on nonhematopoietic cells. It is predominantly expressed presynaptically and regulates the release of HA and other neurotransmitters. H_3RKO mice

develop significantly more severe acute early phase disease, neuropathology, and increased BBB barrier permeability compared with B6 mice. T cells from H_3RKO mice restimulated ex vivo with $MOG_{35–55}$ had greater expression of MIP-2, IP-10, and CXCR3 with no significant difference in the Th1, Th2, or Th17 cytokine production [18]. H_4R expression is confined mainly on hematopoietic cells and its activation can result in actin polymerization, upregulation of adhesion molecules, and chemotaxis of many immune cells [44–46]. However, recently H_4R has been shown to be functionally expressed in the CNS [17]. H_4RKO mice develop more severe $MOG_{35–55}$ induced EAE, augmented neuroinflammation, and increased BBB permeability compared with B6 mice. Similar to H_3RKO mice, H_4RKO mice had no effect on the production of Th1, Th2, or Th17 cytokines in ex vivo recall assays [34].

Based on the phenotypes observed in the single HRKO animals, it was surprising for us to find no difference in the production of IFN-γ by H_1H_2RKO CD4 T cells in ex vivo recall assays, nor a difference in BBB permeability in H_3H_4RKO mice. Importantly, however, H_1H_2RKO mice had a significant decrease in BBB permeability while H_3H_4RKO mice had significantly increased production of IFN-γ and IL-17 compared with B6 mice. The observed phenotypes in H_1RKO and H_2RKO mice parallels the phenotypes seen in H_3H_4RKO mice while the H_3RKO and H_4RKO phenotypes mimic those of H_1H_2RKO mice. The basis of this yin-yang effect is unknown but may be due to differential cross-regulation of HR expression. Here, we show that in the absence of a single HR, the expression of the remaining HRs is increased above B6 levels in $CD4^+$ T cells. In addition, we demonstrate that in the absence of both H_1R and H_2R, there is a compensatory upregulation of H_4R expression, and in the absence of both H_3R and H_4R there was upregulation of the H_1R or H_2R. Importantly, the compensatory upregulation of single HRs in H_1H_2RKO and H_3H_4RKO mice may explain the opposing results obtained using pharmacological approaches, where agonists of H_1R and H_2R inhibited proliferation and cytokine production by antigen-specific T cells and the H_2R agonist dimaprit reduced the severity of EAE [29, 47]. In contrast, we can exclude an effect of a T-cell HDC-HA compensatory loop on the HRKO EAE phenotypes since HR expression does not affect HDC expression or HA production by activated $CD4^+$ T cells from B6, H_1H_2RKO, and H_3H_4RKO mice.

HA has a long history as a DMT in MS and is purported to improve electrical conductance through demyelinated axons, actively/passively enhance myelin repair and remyelination, and increase the oxygenation of affected CNS tissues by influencing cerebrovascular blood flow and perfusion [48, 49]. HA signaling through its receptors is highly complex and diverse because of the number of receptors, the relative proportion of the receptor subtypes on a given cell type, differences in receptor affinity, and due to the concentration of HA in the local microenvironment.

In this study, we used a dual-gene KO approach to understand the role of HRs coupled to second messenger signaling pathways via stimulatory and inhibitory G proteins as potential targets for effective DMT in MS. Previous epidemiological and clinical studies indicate that the use of H_1R-specific blockers is associated with decreased MS risk or stabilization of the disease in MS patients [22, 23]. HA, acting through H_2R, can regulate MHC class II expression on immunoreactive cells and the receptor antagonist ranitidine has been used as a long-term therapy in controlling autoimmune psoriasis [50]. Our results presented here indicate that administering antagonists of both H_1R and H_2R simultaneously may be protective in CNS disease due to the upregulation of the antipathogenic H_3R and H_4R. Results of the present study indicate that the absence of H_3R or H_4R signaling has a negative effect on EAE susceptibility and encephalitogenic T-cell activity, suggesting that agonists for this class of receptors may have a beneficial effect in the treatment of CNS autoimmune

diseases by overriding HA signaling through the propathogenic H_1R and H_2R. Therefore, the combined pharmacological targeting of each HR may prove to be an appropriate ancillary DMT in the treatment of MS.

There is an increasing need for new DMT in the treatment of MS and other immunopathologic diseases. Although the lack of specific and highly selective agonists or antagonists for H_3R and H_4R have precluded their targeting in the clinical treatment of disease, research in recent years has progressed to the point where their use in the clinic is highly likely. Our results, using HR KO mice that couple to two distinct classes of G proteins (stimulatory vs. inhibitory), support the concept of pharmacological targeting of all four HRs along with mainstream drugs like IFN-β or glatiramer acetate as an effective DMT in MS.

Materials and methods

Animals

C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6.129P-*Hrh1*^{tm1Wat} (H_1RKO) [51], B6.129P-*Hrh2*^{tm1Wat} (H_2RKO) [52], B6.129P2-*Hrh3*^{tm1Twl} (H_3RKO) [53], and B6.129P-*Hrh4*^{tm1Thr} (H_4RKO) mice (generated by Lexicon Genetics, Woodlands Park, TX) [54] were maintained at the University of Vermont (Burlington, VT). All strains were backcrossed to the C57BL/6J background for at least 10 generations. Individual HRKO mice were interbred and the resulting F_1 mice were intercrossed together to generate H_1H_2RKO and H_3H_4RKO mice. The experimental procedures used in this study were approved by the Animal Care and Use Committee of the University of Vermont.

Induction and evaluation of EAE

Mice were immunized for the induction of EAE using a 2× immunization protocol. The animals were injected subcutaneously in the posterior right and left flank with a sonicated phosphate-buffered saline (PBS)/oil emulsion containing 100 μg of $MOG_{35–55}$ and CFA (Sigma-Aldrich, St. Louis, MO) supplemented with 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). One week later, all mice received an identical injection of $MOG_{35–55}$-CFA [31]. Mice were ranked 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 were performed as previously described [31].

Histopathological evaluations were done as previously described [55]. Briefly, brains and spinal cords were dissected on 30th day postimmunization, from calvaria and vertebral columns, respectively, and fixed by immersion in 10% phosphate-buffered formalin (pH 7.2). After fixation, trimmed and representative transverse section-embedded in paraffin and mounted on glass slides. Sections were stained with hematoxylin and eosin for routine evaluation and Luxol fast blue-periodic acid-Schiff reagent for demyelination. Representative areas of the brain and spinal cords were selected for histopathological evaluation. The following components of the lesions were assessed: (i) severity and extent of the lesion; (ii) extent and degree of myelin loss and tissue injury (swollen axon sheaths, swollen axons, and reactive gliosis); (iii) severity of the acute inflammatory response (predominantly neutrophils); and (iv) severity of the chronic inflammatory response (lymphocytes/macrophages). Lesions in the brain and spinal cord (SC) were evaluated separately and assigned a numerical score based on a subjective scale ranging from 0 to 5. A

score of 0 indicates no lesions; 1 indicates minimal; 2, mild; 3, moderate; 4, marked; and 5, severe lesions.

CSF collection and BBB permeability

BBB permeability was assessed as previously described [56]. Briefly, a 50 μg/g dose of FITC-labeled BSA (Sigma, St. Louis, MO) was injected i.v. into B6, H_1H_2RKO, and H_3H_4RKO mice on d10 post immunization. CSF and blood were collected after 4 h. Both CSF and plasma samples, prepared by centrifugation at 3000 rpm for 15 min, were diluted in PBS, and the fluorescence intensity was measured with a microplate fluorescence reader (Flx-800-I, Bio-Tek Instruments Inc., Winooski, VT) using the software KC-4, with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The BBB permeability index is expressed as the ratio of the fluorescence intensity of the CSF (I_{CSF}) divided by the fluorescence intensity of the plasma (I_{Blood}).

Cytokine assays

For ex vivo cytokine assays, spleen and DLNs were obtained from d10 immunized mice. Single-cell suspensions at 1×10^6 cells/mL in RPMI 1640 medium (Cellgro Mediatech, Manassas, VA) plus 10% FBS (HyClone) were stimulated with 50 μg of $MOG_{35–55}$. Cell culture supernatants were recovered at 72 h and assayed for IFN-γ, IL-4, and IL-17 by ELISA.

Proliferation assay

Mice were immunized for EAE induction, and spleen and DLNs were harvested on d10. Single-cell suspensions were prepared, and 5 × 105 cells/well in RPMI 1640 (10% FBS) were plated on standard 96-well U-bottom tissue culture plates and stimulated with 0, 1, 2, 10, and 50 μg of $MOG_{35–55}$ 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.

Cell preparation and in vitro activation/differentiation of $CD4^+$ effector T cells

From the DLNs and spleen, $CD4^+$ T cells were isolated by negative selection as previously described (Qiagen, Valencia, CA) [31]. In culture, purified $CD4^+$ T cells (1×10^6 cells/mL) were stimulated with anti-CD3 (5 μg/mL) and anti-CD28 (1 μg/mL) mAbs (BD Biosciences-Pharmingen, San Jose, CA) for different time points (24, 48, and 72 h) and supernatants were analyzed for IFN-γ, IL-2, IL-4, and IL-17 production by ELISA using anti-IFN-γ, anti-IL-2, anti-IL-4, and anti-IL-17 mAbs and their respective biotinylated mAbs (BD Biosciences-Pharmingen, San Jose, CA).

Antibodies and flow cytometric analysis

Single-cell suspensions of thymocytes, lymph node cells, and splenocytes were prepared and the red blood cells were lysed with ammonium chloride. Total numbers of cells were counted using the Advia 120 hematology analyzer (Bayer/Siemens, Tarrytown, NY). For flow cytometric analysis, the cells were washed twice and incubated for 30 min on ice with the desired fluorochrome-conjugated mAbs or isotype control immunoglobulin at 0.5 μg/10^6 cells. For the identification and phenotypic analysis of T_R cells ($CD4^+CD8^-TCR\beta^+Foxp3^+$), the following surface antimouse mAb were used: anti-CD4 (MCD0417, Caltag), anti-CD8, and anti-CD25 (53–6.7, PC61; BD Pharmingen, San Jose, CA); anti-TCRβ, and anti-Foxp3 staining set (H57-5987 and FJK-16s; eBioscience, San Diego, CA), according to the manufacturer's instructions. Viable cells were selected for flow cytometric analysis (LSR II, BD) based on forward and side scatter properties and analysis was performed using FlowJo software (TreeStar Software, Inc.).

RNA extraction and qRT-PCR analysis

Total RNA was extracted from naïve and anti-CD3 + anti-CD28 mAbs stimulated $CD4^+$ T cells from naïve wild-type (WT) and $H_{1–4}$RKO, H_1H_2RKO, and H_3H_4RKO mice using RNeasy isolation reagent (Qiagen Science, MD), and reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The generated cDNA was used in qRT-PCR using the SYBR green method. The sequences of primers used were as follows:

Hrh1—forward, 5′ TTGAACCGAGAGCGGA 3′; reverse, 5′ TGCCCTTAGGAACGAAT, *Hrh2*—forward, 5′ TGGCACGGTTCATTCC3′; reverse, GCAGTAGCGGTCCAAG3′, *Hrh3*—forward, 5′ TGCCTCCTCAGTCTTCAACA 3′; reverse, 5'CCTTCTACCGTGACCAC3′, *Hrh4*—forward, 5′ TGAGGAGAATTGCTTCACGA 3′; reverse, 5′ TGCATGTGGAGGGGTTTTAT 3′, and for *Hdc* TaqMan® primers and probes were from Applied Biosystems. β2-microglobulin was used as a reference gene and the relative expression levels were calculated using the comparative threshold cycle (C_T) method.

HA assay

HA concentrations were assessed using an EIA HA kit according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI). Briefly, 50 μL of derivatization buffer was added to 200 μL undiluted supernatants followed by the addition of 20 μL derivatization reagent. The samples, controls, and standards were added in duplicate to the plate, 100 μL of HA AChE tracer was added to each well, and the plate was incubated at 4°C for 24 h. The wells were washed and 200 μL Ellman's Reagent was added and incubated for 30 min in the dark at room temperature while shaking. The plate read at 405 nm when the maximum binding control wells reached an absorbance of 0.2–0.8.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad software Inc., San Diego, CA). Significance of differences was determined as described in the Figure legends.

Acknowledgments

We thank Dr. Dimitry N. Kremenstov and all the members of the Teuscher lab for helpful discussions; the staff at the University of Vermont DNA sequencing facility for assistance with qRT-PCR. We also thank Dr. Robin L Thurmond, Dr. Timothy W Lovenberg, and Johnson and Johnson Pharmaceutical Research and Development, LLC, San Diego, CA, USA for providing us with H_3RKO and H_4RKO mice. This work was supported by National Institute of Health Grants NS061014, AI041747, NS060901, NS036526, and NS069628 (to C. T).

Abbreviations

BBB	blood brain barrier
B6	C57BL/6J
CSF	cerebrospinal fluid
DLN	draining lymph node
DMT	disease-modifying therapy
HA	histamine
HDC	HA decarboxylase
HR	HA receptor

$MOG_{35–55}$ myelin oligodendrocyte glycoprotein peptide 35–55

References

1. Greenstein JI. Current concepts of the cellular and molecular pathophysiology of multiple sclerosis. Dev. Neurobiol. 2007; 67:1248–1265. [PubMed: 17514718]
2. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Clin. Exp. Immunol. 2010; 162:1–11. [PubMed: 20682002]
3. Parsons ME, Ganellin CR. Histamine and its receptors. Br. J. Pharmacol. 2006; 147(Suppl 1):S127–S135. [PubMed: 16402096]
4. Akdis CA, Simons FE. Histamine receptors are hot in immunopharmacology. Eur. J. Pharmacol. 2006; 533:69–76. [PubMed: 16448645]
5. Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol. Rev. 1997; 77:1033–1079. [PubMed: 9354811]
6. Taguchi Y, Tsuyama K, Watanabe T, Wada H, Kitamura Y. Increase in histidine decarboxylase activity in skin of genetically mast-cell-deficient W/Wv mice after application of phorbol 12-myristate 13-acetate: evidence for the presence of histamine-producing cells without basophilic granules. Proc. Natl. Acad. Sci. USA. 1982; 79:6837–6841. [PubMed: 6960352]
7. Aoi R, Nakashima I, Kitamura Y, Asai H, Nakano K. Histamine synthesis bymouse T lymphocytes through induced histidine decarboxylase. Immunology. 1989; 66:219–223. [PubMed: 2784410]
8. Takamatsu S, Nakashima I, Nakano K. Modulation of endotoxininduced histamine synthesis by cytokines in mouse bone marrow-derived macrophages. J. Immunol. 1996; 156:778–785. [PubMed: 8543833]
9. Shiraishi M, Hirasawa N, Oikawa S, Kobayashi Y, Ohuchi K. Analysis of histamine-producing cells at the late phase of allergic inflammation in rats. Immunology. 2000; 99:600–606. [PubMed: 10792508]
10. Ghosh AK, Hirasawa N, Ohtsu H, Watanabe T, Ohuchi K. Defective angiogenesis in the inflammatory granulation tissue in histidine decarboxylase-deficient mice but not in mast cell-deficient mice. J. Exp. Med. 2002; 195:973–982. [PubMed: 11956288]
11. Yokoyama M, Yokoyama A, Mori S, Takahashi HK, Yoshino T, Watanabe T, Ohtsu H, et al. Inducible histamine protects mice from P. acnes-primed and LPS-induced hepatitis through H2-receptor stimulation. Gastroenterology. 2004; 127:892–902. [PubMed: 15362044]
12. Tanaka S, Deai K, Konomi A, Takahashi K, Yamane H, Sugimoto Y, Ichikawa A. Expression of L-histidine decarboxylase in granules of elicited mouse polymorphonuclear leukocytes. Eur. J. Immunol. 2004; 34:1472–1482. [PubMed: 15114681]
13. Yang XD, Ai W, Asfaha S, Bhagat G, Friedman RA, Jin G, Park H, et al. Histamine deficiency promotes inflammation-associated carcinogenesis through reduced myeloid maturation and accumulation of CD11b+Ly6G+ immature myeloid cells. Nat. Med. 2011; 17:87–95. [PubMed: 21170045]
14. Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, et al. International Union of Pharmacology. XIII. Classification of histamine receptors. Pharmacol. Rev. 1997; 49:253–278. [PubMed: 9311023]
15. Leurs R, Smit MJ, Timmerman H. Molecular pharmacological aspects of histamine receptors. Pharmacol. Ther. 1995; 66:413–463. [PubMed: 7494855]
16. Liu C, Ma X, Jiang X, Wilson SJ, Hofstra CL, Blevitt J, Pyati J, et al. Cloning and pharmacological characterization of a fourth histamine receptor (H(4)) expressed in bone marrow. Mol. Pharmacol. 2001; 59:420–426. [PubMed: 11179434]
17. Lethbridge NL, Chazot PL. Immunological identification of the mouse H4 histamine receptor on spinal cordmotor neurons using a novel anti-mouse H4R antibody. Inflamm. Res. 2010; 59(Suppl 2):S197–S198. [PubMed: 20020316]

18. Teuscher C, Subramanian M, Noubade R, Gao JF, Offner H, Zachary JF, Blankenhorn EP. Central histamine H3 receptor signaling negatively regulates susceptibility to autoimmune inflammatory disease of the CNS. Proc. Natl. Acad. Sci. USA. 2007; 104:10146–10151. [PubMed: 17548817]

19. Jadidi-Niaragh F, Mirshafiey A. Histamine and histamine receptors in pathogenesis and treatment of multiple sclerosis. Neuropharmacology. 2010; 59:180–189. [PubMed: 20493888]

20. El Behi M, Dubucquoi S, Lefranc D, Zephir H, De Seze J, Vermersch P, Prin L. New insights into cell responses involved in experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol. Lett. 2005; 96:11–26. [PubMed: 15585303]

21. Theoharides TC, Cochrane DE. Critical role of mast cells in inflammatory diseases and the effect of acute stress. J. Neuroimmunol. 2004; 146:1–12. [PubMed: 14698841]

22. Alonso A, Jick SS, Hernan MA. Allergy, histamine 1 receptor blockers, and the risk of multiple sclerosis. Neurology. 2006; 66:572–575. [PubMed: 16505314]

23. Logothetis L, Mylonas IA, Baloyannis S, Pashalidou M, Orologas A, Zafeiropoulos A, Kosta V, et al. A pilot, open label, clinical trial using hydroxyzine in multiple sclerosis. Int. J. Immunopathol. Pharmacol. 2005; 18:771–778. [PubMed: 16388727]

24. Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, Langer-Gould A, et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nat. Med. 2002; 8:500–508. [PubMed: 11984595]

25. Bebo BF Jr, Yong T, Orr EL, Linthicum DS. Hypothesis: a possible role for mast cells and their inflammatory mediators in the pathogenesis of autoimmune encephalomyelitis. J. Neurosci. Res. 1996; 45:340–348. [PubMed: 8872894]

26. Gregory GD, Robbie-Ryan M, Secor VH, Sabatino JJ Jr, Brown MA. Mast cells are required for optimal autoreactive T cell responses in amurine model ofmultiple sclerosis. Eur. J. Immunol. 2005; 35:3478–3486. [PubMed: 16285014]

27. Ma RZ, Gao J, Meeker ND, Fillmore PD, Tung KS, Watanabe T, Zachary JF, et al. Identification of Bphs, an autoimmune disease locus, as histamine receptor H1. Science. 2002; 297:620–623. [PubMed: 12142541]

28. Pedotti R, De Voss JJ, Steinman L, Galli SJ. Involvement of both ‘allergic’ and ‘autoimmune’ mechanisms in EAE, MS and other autoimmune diseases. Trends Immunol. 2003; 24:479–484. [PubMed: 12967671]

29. Emerson MR, Orentas DM, Lynch SG, LeVine SM. Activation of histamine H2 receptors ameliorates experimental allergic encephalomyelitis. Neuroreport. 2002; 13:1407–1410. [PubMed: 12167763]

30. Musio S, Gallo B, Scabeni S, Lapilla M, Poliani PL, Matarese G, Ohtsu H, et al. A key regulatory role for histamine in experimental autoimmune encephalomyelitis: disease exacerbation in histidine decarboxylase-deficient mice. J. Immunol. 2006; 176:17–26. [PubMed: 16365391]

31. Noubade R, Milligan G, Zachary JF, Blankenhorn EP, del Rio R, Rincon M, Teuscher C. Histamine receptor H1 is required for TCR-mediated p38 MAPK activation and optimal IFN-gamma production in mice. J. Clin. Invest. 2007; 117:3507–3518. [PubMed: 17965772]

32. Teuscher C, Poynter ME, Offner H, Zamora A, Watanabe T, Fillmore PD, Zachary JF, et al. Attenuation of Th1 effector cell responses and susceptibility to experimental allergic encephalomyelitis in histamine H2 receptor knockoutmice is due to dysregulation of cytokine production by antigen-presenting cells. Am. J. Pathol. 2004; 164:883–892. [PubMed: 14982842]

33. Lu C, Diehl SA, Noubade R, Ledoux J, Nelson MT, Spach K, Zachary JF, et al. Endothelial histamine H1 receptor signaling reduces blood-brain barrier permeability and susceptibility to autoimmune encephalomyelitis. Proc. Natl. Acad. Sci. USA. 2010; 107:18967–18972. [PubMed: 20956310]

34. Del Rio R, Noubade R, Saligrama N, Wall EH, Krementsov DN, Poynter ME, Zachary JF, et al. Histamine H4 receptor optimizes T regulatory cell frequency and facilitates anti-inflammatory responses within the central nervous system. J. Immunol. 2012; 188:541–547. [PubMed: 22147765]

35. Goodin DS, Frohman EM, Garmany GP Jr, Halper J, Likosky WH, Lublin FD, Silberberg DH, et al. Disease modifying therapies in multiple sclerosis: report of the Therapeutics and Technology

Assessment Subcommittee of the American Academy of Neurology and the MS Council for Clinical Practice Guidelines. Neurology. 2002; 58:169–178. [PubMed: 11805241]

36. Leurs R, Vischer HF, Wijtmans M, de Esch IJ. En route to new blockbuster anti-histamines: surveying the offspring of the expanding histamine receptor family. Trends Pharmacol. Sci. 2011; 32:250–257. [PubMed: 21414671]
37. Butterfield RJ, Sudweeks JD, Blankenhorn EP, Korngold R, Marini JC, Todd JA, Roper RJ, et al. New genetic loci that control susceptibility and symptoms of experimental allergic encephalomyelitis in inbred mice. J. Immunol. 1998; 161:1860–1867. [PubMed: 9712054]
38. Teuscher C, Noubade R, Spach K, McElvany B, Bunn JY, Fillmore PD, Zachary JF, et al. Evidence that the Y chromosome influences autoimmune disease in male and female mice. Proc. Natl. Acad. Sci. USA. 2006; 103:8024–8029. [PubMed: 16702550]
39. Schneider E, Bertron AF, Dy M. Modulation of hematopoiesis through histamine receptor signaling. Front Biosci. (Schol Ed). 2011; 3:467–473. [PubMed: 21196390]
40. Horvath Z, Pallinger E, Horvath G, Jelinek I, Falus A, Buzas EI. Histamine H1 and H2 receptors but not H4 receptors are upregulated during bone marrow regeneration. Cell Immunol. 2006; 244:110–115. [PubMed: 17420012]
41. Horvath Z, Pallinger E, Horvath G, Jelinek I, Veszely G, Furesz J, Falus A, et al. Extramedullary hematopoiesis is dysregulated in histamine-free histidine decarboxylase knockout (HDC-/-) mice. Inflamm. Res. 2010; 59:429–436. [PubMed: 19921486]
42. Jutel M, Watanabe T, Klunker S, Akdis M, Thomet OA, Malolepszy J, Zak-Nejmark T, et al. Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. Nature. 2001; 413:420–425. [PubMed: 11574888]
43. Prat A, Antel J. Pathogenesis of multiple sclerosis. Curr. Opin. Neurol. 2005; 18:225–230. [PubMed: 15891404]
44. Zampeli E, Tiligada E. The role of histamine H4 receptor in immune and inflammatory disorders. Br. J. Pharmacol. 2009; 157:24–33. [PubMed: 19309354]
45. Schneider E, Rolli-Derkinderen M, Arock M, Dy M. Trends in histamine research: new functions during immune responses and hematopoiesis. Trends Immunol. 2002; 23:255–263. [PubMed: 12102747]
46. Huang JF, Thurmond RL. The new biology of histamine receptors. Curr. Allergy Asthma Rep. 2008; 8:21–27. [PubMed: 18377770]
47. Lapilla M, Gallo B, Martinello M, Procaccini C, Costanza M, Musio S, Rossi B, et al. Histamine regulates autoreactive T cell activation and adhesiveness in inflamed brain microcirculation. J. Leukoc. Biol. 2011; 89:259–267. [PubMed: 21071626]
48. Gillson G, Wright JV, DeLack E, Ballasiotes G. Transdermal histamine in multiple sclerosis: part one — clinical experience. Altern. Med. Rev. 1999; 4:424–428. [PubMed: 10608915]
49. Gillson G, Wright JV, DeLack E, Ballasiotes G. Transdermal histamine in multiple sclerosis, part two: a proposed theoretical basis for its use. Altern. Med. Rev. 2000; 5:224–248. [PubMed: 10869103]
50. Nielsen HJ, Hammer JH. Possible role of histamine in pathogenesis of autoimmune diseases: implications for immunotherapy with histamine-2 receptor antagonists. Med. Hypotheses. 1992; 39:349–355. [PubMed: 1362972]
51. Banu Y, Watanabe T. Augmentation of antigen receptor-mediated responses by histamine H1 receptor signaling. J. Exp. Med. 1999; 189:673–682. [PubMed: 9989982]
52. Kobayashi T, Tonai S, Ishihara Y, Koga R, Okabe S, Watanabe T. Abnormal functional and morphological regulation of the gastric mucosa in histamine H2 receptor-deficient mice. J. Clin. Invest. 2000; 105:1741–1749. [PubMed: 10862789]
53. Toyota H, Dugovic C, Koehl M, Laposky AD, Weber C, Ngo K, Wu Y, et al. Behavioral characterization of mice lacking histamine H(3) receptors. Mol. Pharmacol. 2002; 62:389–397. [PubMed: 12130692]
54. Hofstra CL, Desai PJ, Thurmond RL, Fung-Leung WP. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. J. Pharmacol. Exp. Ther. 2003; 305:1212–1221. [PubMed: 12626656]

55. Teuscher C, Bunn JY, Fillmore PD, Butterfield RJ, Zachary JF, Blankenhorn EP, Gender age. season at immunization uniquely influence the genetic control of susceptibility to histopathological lesions and clinical signs of experimental allergic encephalomyelitis: implications for the genetics ofmultiple sclerosis. Am. J. Pathol. 2004; 165:1593–1602. [PubMed: 15509529]

56. Noubade R, del Rio R, McElvany B, Zachary JF, Millward JM, Wagner DD, Offner H, et al. von-Willebrand factor influences blood brain barrier permeability and brain inflammation in experimental allergic encephalomyelitis. Am. J. Pathol. 2008; 173:892–900. [PubMed: 18688020]

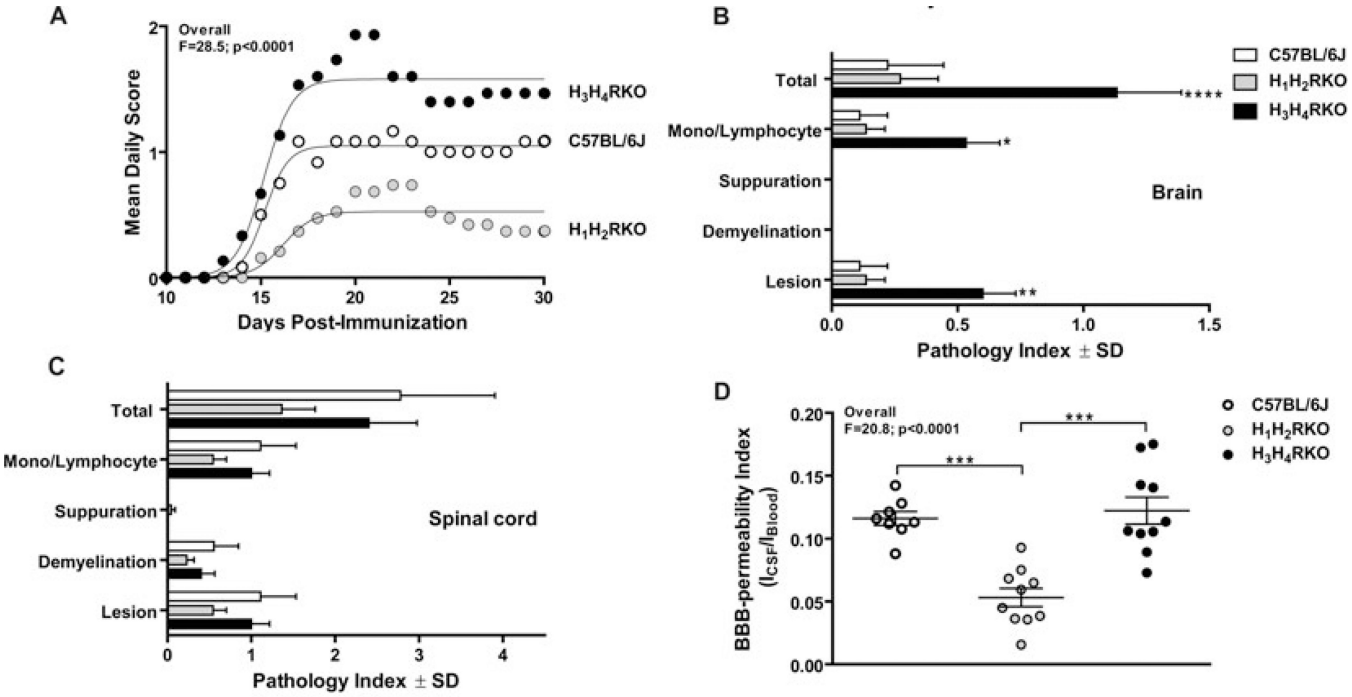

Figure 1.

H_1H_2RKO and H_3H_4RKO mice exhibit differential susceptibility to EAE. B6 ($n = 12$), H_1H_2RKO ($n = 23$), and H_3H_4RKO ($n = 15$) were immunized twice with $MOG_{35–55}$-CFA. (A) The clinical EAE scores following immunization were recorded and the significance of the differences between the clinical courses of disease was calculated by regression analysis. The best fit curve is shown. (B and C) The severity of CNS pathology in (B) brain and (C) spinal cord was determined and shown as mean + standard deviation. The significance of differences observed was determined using the Kruskal–Walis test followed by Dunns post-hoc multiple comparison test (*p < 0.05; **$p < 0.01$; ***$p < 0.001$). (D) BBB permeability was compared and expressed as mean + standard error of the mean (SEM) of $n = 10$ mice per strain. The significance of differences in BBB permeability indexes of B6, H_1H_2RKO, and H_3H_4RKO mice at d10 post immunization was calculated by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc multiple comparison test (***$p < 0.001$). (I_{CSF}/I_{Blood} - Permeability index, expressed as the ratio of the fluorescence intensity of the CSF divided by the fluorescence intensity of the plasma). Data are representative of two independent experiments.

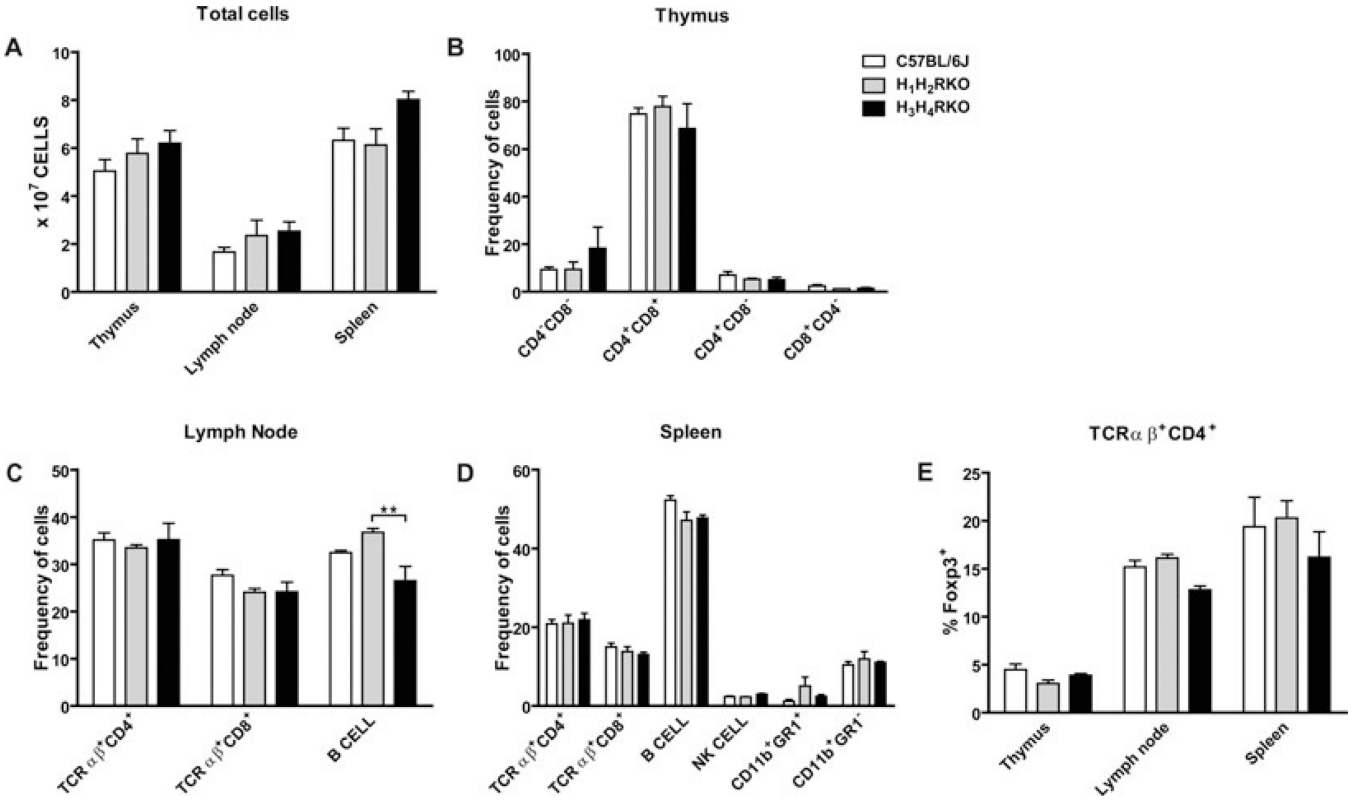

Figure 2.

Naïve H_1H_2RKO and H_3H_4RKO mice exhibit normal immune systems. Single-cell suspensions of thymus, lymph node, and spleen were generated from B6, H_1H_2RKO and H_3H_4RKO mice. (A) The total number of thymic, lymph node, and spleen cells was enumerated and shown as mean + SEM of $n = 10$ per strain. Flow cytometric analysis of immune cell subpopulations in (B) thymus, (C) lymph node and (D) spleen of B6, H_1H_2RKO, and H_3H_4RKO mice was also performed and shown as mean + SEM of $n = 10$ per strain. (E) The frequency of $Foxp3^+$ cells in thymus, spleen, and lymph node was also determined and shown as mean + SEM of $n = 10$ per strain. Significance of differences was determined by ANOVA followed by Bonferroni post-hoc multiple comparison (** $p < 0.01$; *** $p < 0.001$). Data are representative of two independent experiments.

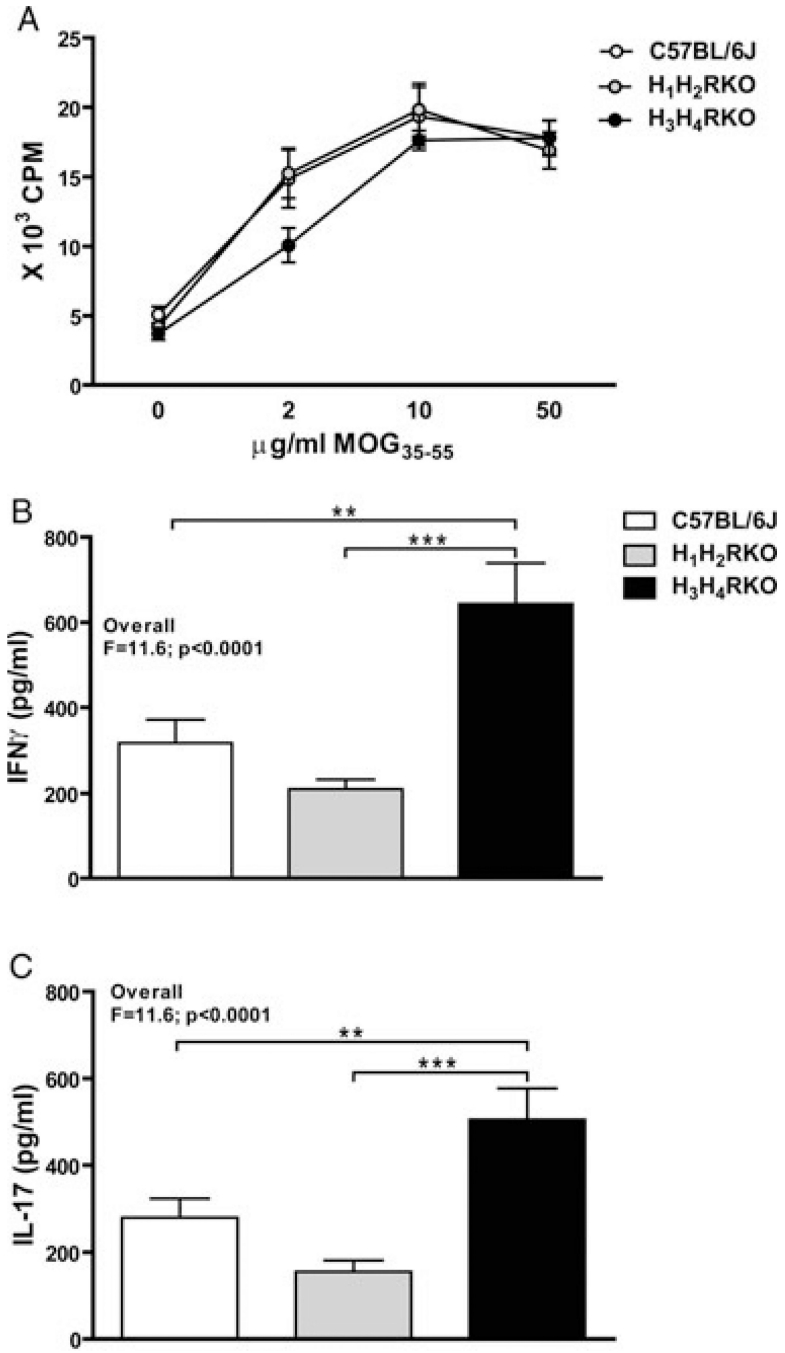

Figure 3.

Ex vivo $MOG_{35–55}$-specific T-cell proliferation and cytokine profiles of immunized B6, H_1H_2RKO, and H_3H_4RKO mice. (A) $MOG_{35–55}$-specific T-cell proliferative responses were evaluated by ^{3}H-thymidine incorporation. Mean counts per minute + standard deviation were calculated from triplicate wells of $n = 10$ per strain. The significance of differences was calculated by ANOVA followed by Bonferroni post-hoc multiple comparisons. (B) IFN-γ and (C) IL-17 production by $MOG_{35–55}$-stimulated DLNs and splenocytes from B6, H_1H_2RKO, and H_3H_4RKO mice were evaluated by ELISA and shown as mean + SEM of $n = 10$ per strain. Significance of differences observed in cytokine production was determined

by one-way ANOVA followed by Bonferroni's post-hoc multiple comparison test (**$p < 0.01$; ***$p < 0.001$). Data are representative of two independent experiments.

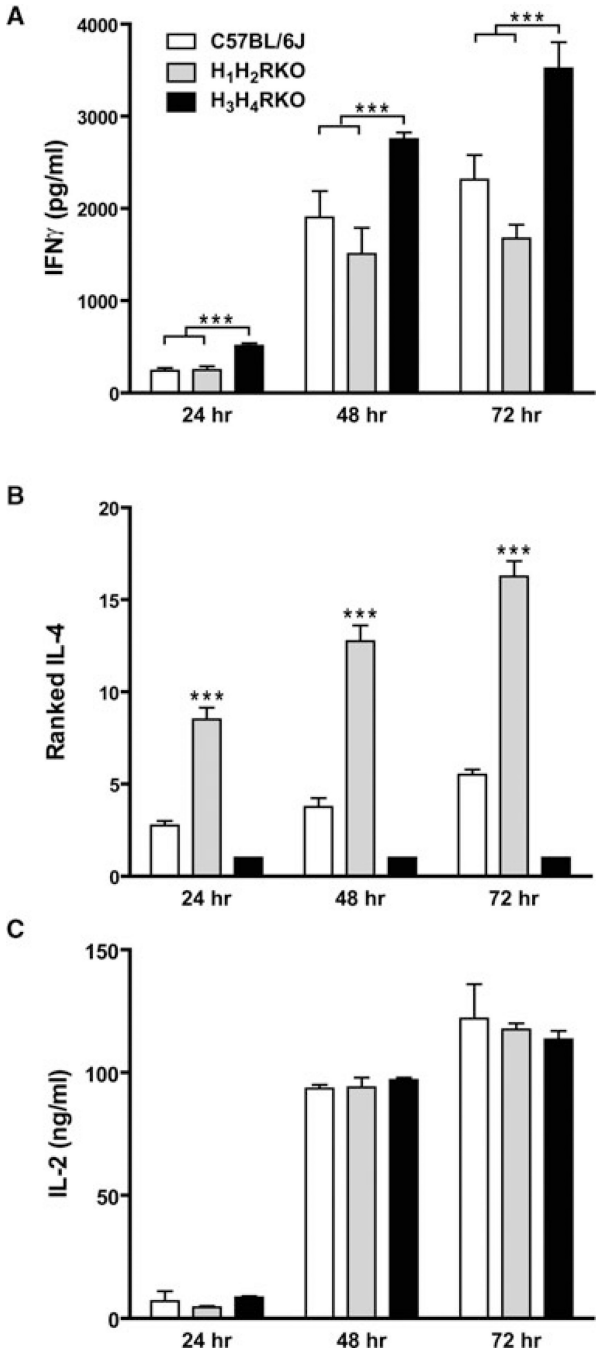

Figure 4.

HRs influence the polarization of in vitro activated $CD4^+$ T cells. $CD4^+$T cells from B6, H_1H_2RKO, and H_3H_4RKO mice were activated with anti-CD3 and anti-CD28 mAbs. (A) IFN-γ, (B) IL-4, and (C) IL-2 production at 24, 48, and 72 h poststimulation was determined by ELISA and shown as mean + SEM of $n = 4$ per strain. In (A) and (C) significance of differences in cytokine production was determined by ANOVA followed by Bonferroni post-hoc multiple comparison. In (B) the significance of differences in IL-4 production was determined using a rank transformed nonparametric two-way ANOVA followed by Bonferroni post-hoc multiple comparison (**$p < 0.01$; ***$p < 0.001$). Data are representative of two independent experiments.

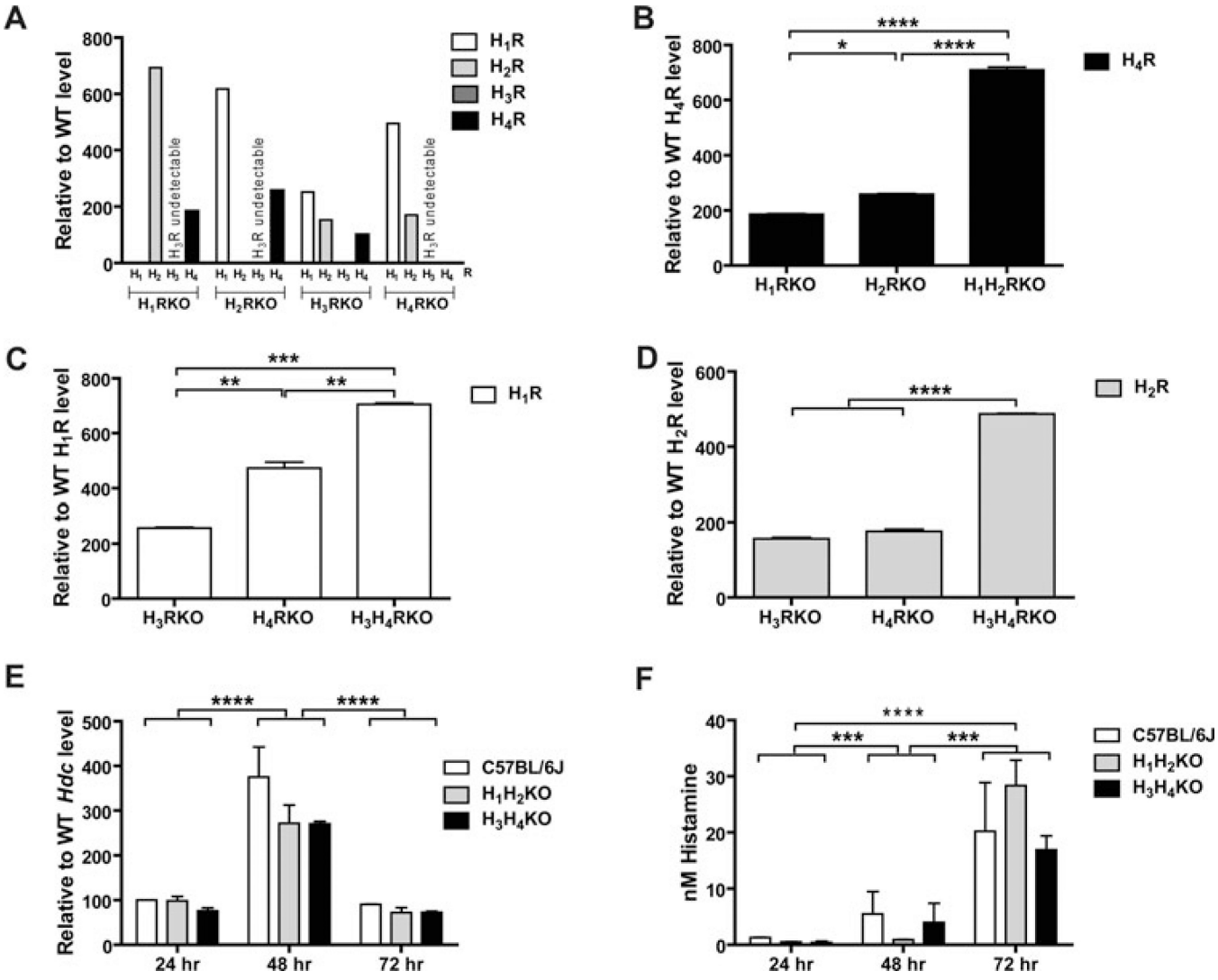

Figure 5.

Compensatory upregulation of HRs in H_1H_2RKO and H_3H_4RKO CD4$^+$T cells. *Hrh1 Hrh2 Hrh3 Hrh4* and *Hdc* mRNA expression levels were determined by qRT-PCR and analyzed using comparative threshold cycle method with β2-microglobulin as an endogenous control. Receptor expression levels are normalized to that of WT levels. (A) HR expression was evaluated in individual HRKO CD4$^+$T cells. (B) *Hrh4* expression was evaluated in H_1RKO, H_2RKO, and H_1H_2RKO CD4$^+$ T cells. (C) *Hrh1* and (D) *Hrh2* expression was evaluated in H_3RKO, H_4RKO, and H_3H_4RKO CD4$^+$ T cells. (E and F) CD4$^+$T cells from B6, H_1H_2RKO, and H_3H_4RKO mice were activated with anti-CD3 and anti-CD28 mAbs. (E) *Hdc* expression was analyzed by qRT-PCR and (F) HA production was determined by EIA at 24, 48, and 72 h poststimulation. Data are representative of two independent experiments. In (B), (C), and (D), significance of differences in HR expression levels was determined by one-way ANOVA followed by Bonferroni post-hoc multiple comparison. In (E and F) significance of differences in HA production was determined by two-way ANOVA followed by Bonferroni post-hoc multiple comparison (*$p = 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). All data are shown as mean + standard error of the mean of $n = 3$ per strain and are representative of two independent experiments.

Table 1

Summary of EAE clinical trait variables in B6, H_1H_2RKO, and H_3H_4RKO mice

Variable	B6	H_1H_2RKO	H_3H_4RKO
Percentage of affected [a)]	66.7 ± 14.2	42.5 ± 10.6 (0.03)[b)]	93.3 ± 6.7 (0.001)
Mean day of onset	15.4 ± 0.3	17.0 ± 0.7 (0.03)	15.9 ± 0.5
Cumulative score	15.3 ± 4.2	8.0 ± 2.4 (0.006)	28.5 ± 6.0 (0.04)
Days affected	8.4 ± 2.2	4.4 ± 1.3 (0.005)	12.4 ± 1.6 (0.02)
Peak score	2.0	1.8 ± 0.1	2.5 ± 0.3
Severity index	1.8 ± 0.3	1.7 ± 0.2	2.2 ± 0.3

[a)] Animals were considered affected if clinical scores ≥1 were apparent for 2 or more consecutive days.

[b)] The significance of the observed differences in clinical quantitative trait variables was determined using the one sample *t*-test against the control mean. *p*-values are as indicated.