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Systemic Lack of Canonical Histamine Receptor Signaling Results in Increased Resistance to Autoimmune Encephalomyelitis

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Abstract

Histamine (HA) is a key regulator of experimental allergic encephalomyelitis (EAE), the autoimmune model of multiple sclerosis (MS). HA exerts its effects through four known G protein coupled receptors (GPCR): H_1, H_2, H_3, and H_4 ($H_{1-4}R$). Using HR deficient mice, our lab has demonstrated that H_1R, H_2R, H_3R, and H_4R play important roles in EAE pathogenesis, either by regulating encephalitogenic T-cell responses, cytokine production by APCs, blood brain barrier permeability, or T regulatory cell activity, respectively. Histidine decarboxylase deficient mice (HDCKO), which lack systemic HA, exhibit more severe EAE and increased Th1 effector cytokine production by splenocytes in response to myelin oligodendrocyte glycoprotein 35-55. In an inverse approach, we tested the effect of depleting systemic canonical HA signaling on susceptibility to EAE by generating mice lacking all four known GPC-HRs (H_{1-4}RKO mice). Here we report that in contrast to HDCKO mice, H_{1-4}RKO mice develop less severe EAE compared to WT animals. Furthermore, splenocytes from immunized H_{1-4}RKO mice produce less Th1/Th17 effector cytokines compared to WT mice. The opposing results seen between HDCKO and H_{1-4}RKO mice suggest that HA may signal independently of $H_{1-4}R$ and supports the existence of an alternative HAergic pathway in regulating EAE resistance. Understanding and exploiting this pathway has the potential to lead to new disease modifying therapies in MS and other autoimmune and allergic diseases.

Introduction

Histamine (HA) [2-(4-imidazole) ethylamine] is an important mediator involved in regulating various physiological processes like neurotransmission, secretion of pituitary hormones, and gastrointestinal and circulatory functions (1). Additionally, HA is a potent mediator of inflammation and regulates innate and adaptive immune responses (2). Histidine decarboxylase (HDC) synthesizes HA through the decarboxylation of histidine, and mast cells and basophils provide the major source of stored HA in the body (3). However, other cellular sources of HA have recently been identified, including dendritic cells (DCs), T cells, neutrophils, and macrophages (4) and induced or nascent HA secretion occurs in conjunction with increased HDC activity in these cell types. HA mediates its effect through binding to four distinct histamine receptors (HRs), namely H_1-H_4. All four HRs are 7-

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transmembrane G-protein-coupled receptors (GPCRs). H_1R and H_2R couples to $G\alpha_{q/11}$ and G_s class of G proteins, respectively, whereas H_3R and H_4R are coupled to $G_{i/o}$ (1).

HA plays an important role both in the development of allergic inflammation and autoimmune diseases such as multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE) the principal animal model of MS. HA and HA releasing agents from mast cells have a dramatic effect on the permeability of the blood brain barrier (BBB) (5, 6). The use of first generation H_1R antihistamines, which readily cross the BBB, is associated with a decrease in MS risk (7). MS patients given an H_1R antagonist remained stable and improved neurologically (6). In addition, microarray analysis on the chronic plaques of MS patients revealed increased levels of H_1R transcripts (8). Similarly, in EAE, T cell clones activated against myelin peptides have increased levels of H_1R and H_2R transcripts, respectively (9). Mast cell granule stabilizers and H_1R specific antagonists reduce EAE severity (10, 11) and mice treated with the H_2R agonist dimaprit showed reduced clinical severity and pathology (12). In contrast, the absence of HA leads to an elevation in proinflammatory cytokines and increased susceptibility to EAE in HDCKO mice (13). In both MS and EAE, it is well accepted that MHC class II-restricted $CD4^+$ T cells, which are capable of secreting either IFN-γ (Th1) or IL-17 (Th17) (14), are necessary and sufficient to induce neuropathology.

We have extensively studied the role of HRs in the development of EAE using HRKO mice (4, 15-18). H_1RKO mice show a significant delay in the development of EAE and have reduced clinical signs compared to their WT counterparts (15). During myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) induced EAE, T cells from H_1RKO mice produce significantly less IFN-γ and increased Th2 cytokines (19). H_2RKO mice are also less susceptible to EAE with a blunted Th1 cytokine response in *in vitro* recall assays (16). H_3R is an inhibitory auto/hetero receptor expressed presynaptically on neurons. H_3RKO mice develop severe acute early phase EAE and supports the existence of a novel H_3R mediated CNS component in the neurogenic control of BBB permeability and peripheral T cell responses (17). H_4R is predominantly expressed on hematopoietic cells and exhibits diverse functions (20). H_4RKO mice showed increased susceptibility to MOG_{35-55} induced EAE in association with decreased CNS Treg cell activity (18).

Although the majority of MS and EAE studies have focused on the role of HA signaling through the four known GPC-HRs, there is evidence for HA signaling through non-GPCRs, for example $GABA_AR$, which are ligand-gated ion channels named for their ability to bind the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (21-23). Therefore, to test the hypothesis that HA signaling through non-canonical GPC-HR signaling pathways plays a role in allergic inflammation and the immune responses, we generated mice deficient for the four known HRs ($H_{1-4}RKO$) and studied them for susceptibility to EAE. Here we report that $H_{1-4}RKO$ mice develop less severe EAE and neuropathology compared WT and HDCKO mice. Furthermore, splenocytes from immunized $H_{1-4}RKO$ mice produce significantly less IFN-γ and $H_{1-4}RKO$ Th1 effector cells are less encephalitogenic under adoptive transfer conditions. Therefore, our data supports the possible existence of an alternative HAergic pathway which in the absence of GPC-HRs significantly reduces susceptibility to EAE.

Materials and methods

Animals

B6.129P-*Hrh1*^{tm1Wat} (H_1RKO) (24), B6.129P-*Hrh2*^{tm1Wat} (H_2RKO) (16), B6.129P2-*Hrh3*^{tm1Twl} (H_3RKO) (17) and B6.129P-*Hrh4*^{tm1Thr} (H_4RKO) mice (Lexicon Genetics, Woodlands Park, TX) (25) were maintained at the University of Vermont, Burlington, VT. All the above strains were backcrossed to C57BL/6J background for >10 generations.

$H_{1\text{-}4}$RKO mice were generated by intercrossing individual HR knockout mice (H_1RKO X H_2RKO X H_3RKO X H_4RKO). The genetic background of $H_{1\text{-}4}$RKO mice was assessed by the DartMouse™ Speed Congenic Core Facility at the Geisel School of Medicine at Dartmouth (Lebanon, NH). DartMouse uses the Illumina, Inc. (San Diego, CA) GoldenGate Genotyping Assay to interrogate 1449 SNPs spread throughout the genome. The raw SNP data were analyzed using DartMouse's SNaP-Map™ and Map-Synth™ software, allowing for the determination of the SNP allele at each location. The SNP analysis revealed that the $H_{1\text{-}4}$RKO mice are 97% C57BL/6J, with minor carryover (3%) from 129 only at the retained KO loci. HDC $^{\Delta 6\text{-}8}$/HDC$^{\Delta 6\text{-}8}$ (HDCKO) mice were obtained from Dr. Paul J Bryce, Northwestern University, Division of Allergy-Immunology, Feinberg School of Medicine, Chicago, IL 60611, USA (26). 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 either 1× or 2× immunization protocol (27). For the 2× protocol, mice were injected subcutaneously in the posterior right and left flank with an emulsion containing 100 ug of myelin oligodendrocyte glycoprotein 35-55 ($MOG_{35\text{-}55}$) and an equal volume of complete Freund's adjuvant (CFA) (Sigma Aldrich, St. Louis, MO) having 200 ug of *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI); one week later all mice received an identical injection of $MOG_{35\text{-}55}$-CFA. For the 1× protocol, mice were immunized with an emulsion containing 200 ug of $MOG_{35\text{-}55}$ and an equal volume of CFA containing 200 ug of *Mycobacterium tuberculosis* H37RA. On the day of immunization each mouse received 200 ng of pertussis toxin (PTX) (List Biological Laboratories, Campbell, CA) by i.v.

For passively induced disease, donor mice were immunized using 1× immunization protocol, d12 post-immunization single cell suspensions of DLNs prepared. Cells (10×10^6 cells/ml in 75 ml tissue culture flasks) were stimulated with $MOG_{35\text{-}55}$ (10 μg/ml) and IL-12 (0.5 ng/ml) for 72 h. Before transferring the cells into recipient mice, cell viability was assessed by trypan blue exclusion; cells stained for intracellular cytokines and culture supernatant also screened for IFN-γ, IL-17 and GM-CSF production by ELISA. After 72 h of culture, cells were washed twice at room temperature with PBS and 1×10^7 cells/200 μl PBS injected i.v. into WT recipient mice. The mice were monitored for the onset of EAE for 30 days.

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 (27).

Cytokine and Proliferation assays

For *ex vivo* cytokine assays, mice were immunized using 2× immunization protocol, spleens and DLNs were harvested on d10, and single cell suspensions were prepared (1×10^6 cells/ml) in RPMI 1640 (10% FBS) and restimulated with 50 μg/ml of $MOG_{35\text{-}55}$. Cell culture supernatants were recovered after 72 h and assayed for IFN-γ, IL-4 and IL-17 by ELISA using anti-IFN-γ, anti-IL-4 and anti-IL-17 mAbs and their respective biotinylated mAbs (BD Biosciences-Pharmingen, San Jose, CA). For proliferation assays, 5×10^5 cells/well in RPMI 1640 were plated on standard 96-well U-bottom tissue culture plates and stimulated with 0, 1, 2, 10 and 50 μg 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.

CNS-infiltrating mononuclear cell isolation

D15 post-immunization, animals were perfused with saline and brains and SCs removed. A single-cell suspension was obtained and passed through a 70 μm strainer. Mononuclear cells were obtained by Percoll gradient (37%/70%) centrifugation and collected from the interphase. Cells were washed and stimulated for 4 h with PMA + ionomycin in the presence of Brefeldin A (Golgi Plug; BD Biosciences). Cells were labeled with LIVE/DEAD UV-Blue dye (Invitrogen) followed by surface staining (CD45 from Invitrogen and CD4, CD8, TCRγΔ, CD11b, and TCRβ from BD Biosciences). Afterward, cells were fixed, permeabilized, and stained for intracellular IL-17A (BD Biosciences) and IFNγ (Invitrogen).

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 minutes on ice with the desired fluorochrome-conjugated mAbs or isotype control Ig 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 anti-mouse mAb were used: anti-CD4 (MCD0417, Caltag); anti-CD8, and anti-CD25 (53-6.7, PC61; BD Pharmingen); anti-TCRβ, and anti-Foxp3 staining set (H57-5987 and FJK-16s; eBioscience), 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).

Histamine assay

HA concentrations were assessed using an EIA-HA kit according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, Michigan). 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 histamine 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.

Assessment of antibody responses

Blood was collected from $H_{1\text{-}4}$RKO and C57BL/6J mice d30 post-immunization and sera were stored at −20°C until analyzed. $MOG_{35–55}$-specific IgG Ab was measured by ELISA as previously described (13). Briefly, 96-well microtiter plates were coated overnight at 4°C with 100 μl of $MOG_{35–55}$ (0.010 mg/ml) diluted in coating buffer (0.1 M $NaHCO_3$ pH 9.5). The plates were blocked with PBS/1% BSA (blocking buffer) for 2 h. Dilutions of mouse sera from C57BL/6J and $H_{1\text{-}4}$RKO were incubated in $MOG_{35\text{-}55}$ coated wells. Antibody binding was tested by the addition of peroxidase-conjugated monoclonal goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL), each at a 1/5000 dilution in blocking buffer. Enzyme substrate was added and plates were read at 450 nm on a microplate reader.

Cell preparation and culture conditions

From the lymph node and spleen, $CD4^+$ T cells were isolated by negative selection (Qiagen, Valencia, CA). 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). Supernatants were collected at different time points (24, 48 and 72 h) and analyzed for IFN-γ, IL-4 and IL-2 production by ELISA. $CD4^+$ T cells (1×10^6 cells/ml) were polarized towards Th1, Th2, and Th17 effector cells as previously described (15) analyzed for IFN-γ, IL-4 and IL-17 production by ELISA.

Statistics

Statistical analyses as indicated in the figure legends were performed using GraphPad Prism 5 software (GraphPad software Inc).

Results

$H_{1\text{-}4}$RKO mice exhibit increased resistance to EAE compared to HDCKO and WT animals

Previously, we demonstrated that mice lacking individual HRs display differential susceptibility to EAE elicited by immunization with a single injection of $MOG_{35\text{-}55}$+CFA +PTX (1× protocol) or two injections of $MOG_{35\text{-}55}$+CFA (2× protocol) (15-18). Additionally, 1× immunized HDCKO mice, which lack HA, exhibit exacerbated EAE (13). In the present study, we assessed susceptibility to $MOG_{35\text{-}55}$-induced EAE in $H_{1\text{-}4}$RKO mice, lacking all four known GPC-HRs, to that of WT and HDCKO mice using the 1× immunization protocol. The severity of the clinical disease courses differed significantly among the strains ($F = 277.7$; $P < 0.0001$). Surprisingly, we found that $H_{1\text{-}4}$RKO mice exhibited a significantly less severe clinical disease course than both WT ($F = 307.7$; $P < 0.0001$) and HDCKO ($F = 485.4$; $P < 0.0001$) mice. The severity of clinical disease in HDCKO mice was significantly greater than WT ($F = 74.6$; $P < 0.0001$) and $H_{1\text{-}4}$RKO ($F = 485.4$; $P < 0.0001$) mice (Fig. 1A).

Analysis of EAE-associated clinical quantitative trait variables revealed that the incidence, cumulative disease score, peak score, number of days affected, mean day of onset, overall severity index, and frequency of lethal disease were significantly lower in $H_{1\text{-}4}$RKO mice compared to both WT and HDCKO mice (Table I). Analysis of EAE susceptibility in $H_{1\text{-}4}$RKO and WT mice using the 2× immunization protocol yielded similar results. $H_{1\text{-}4}$RKO mice exhibit a less severe disease course ($F = 82.7$; $P < 0.0001$) (Fig. 1B) and lower incidence, cumulative disease score, peak score, and number of days affected compared to WT mice (Table II). Therefore, in contrast to the increase in EAE susceptibility observed in HDCKO mice, $H_{1\text{-}4}$RKO mice exhibited a dramatic resistance to EAE.

Immune profiling and HA production in $H_{1\text{-}4}$RKO mice

To determine whether the absence of $H_{1\text{-}4}$R inherently influenced immune cell profiles and HA production, we determined the frequency of different cell types in the central and peripheral immune compartments of naïve WT and $H_{1\text{-}4}$RKO mice. There was no significant difference in the total number of cells either in the lymph node or spleen, but $H_{1\text{-}4}$RKO mice had greater numbers of thymocytes compared to WT mice (Fig. S1A). Further analysis revealed a higher frequency of CD4 and CD8 double negative (DN) cells and a lower frequency of CD4 and CD8 double positive (DP) cells in the $H_{1\text{-}4}$RKO mice compared to WT mice (Fig. S1B and S1C). We did not observe any difference in the frequency of single positive (SP) CD4 and CD8 cells between $H_{1\text{-}4}$RKO and WT thymocytes (Fig. S1B and S1C). We also analyzed the frequency of different immune cell subtypes in the lymph node and spleen and found no significant differences (Fig. S1D-F). The only exception being the frequency of splenic B cells, which was decreased in $H_{1\text{-}4}$RKO compared to WT mice (Fig.

S1E). Analysis of the WBC differentials in the peripheral blood between $H_{1\text{-}4}$RKO and WT mice revealed no significant differences (Fig. S1G and S1H). Therefore, the absence of the four GPC-HRs neither inherently affects the frequency of T cell subsets nor inflammatory leukocytes in the peripheral immune system, but does exert an influence on total thymic cell numbers and the frequency of DN and DP cells. Lastly, we measured the level of HA in the plasma and found that $H_{1\text{-}4}$RKO mice have significantly higher levels of HA compared to WT mice (Fig. S2).

Impaired differentiation and cytokine production by $CD4^+$ T cells from $H_{1\text{-}4}$RKO mice

Previous reports indicated that HRs have a role in T cell differentiation and cytokine production (2). To address whether the lack of HRs has an intrinsic effect on the differentiation and/or cytokine production by $CD4^+$ T cells, we stimulated purified $CD4^+$ T cells from the spleen and lymph nodes of naïve WT and $H_{1\text{-}4}$RKO mice with plate bound anti-CD3 and soluble anti-CD28 mAb for 24, 48 and 72 h and screened the culture supernatants for IL-17, IFN-γ, IL-4, and IL-2 production by ELISA. IL-17 was undetectable among the strains. Interestingly, $CD4^+$ T cells from $H_{1\text{-}4}$RKO mice produced less IFN-γ compared to WT $CD4^+$ T cells at 48 and 72 h (Fig. 2A). However, IL-4 production from the stimulated cells was greater in $H_{1\text{-}4}$RKO compared to WT $CD4^+$ T cells (Fig. 2B). We observed no significant difference in the production of IL-2 by these mice (Fig. 2C). These results indicate that $CD4^+$ T cells from $H_{1\text{-}4}$RKO mice upon polyclonal stimulation have an inherent bias towards the Th2 phenotype.

To address whether the lack of HRs can influence $CD4^+$ T cell differentiation, we purified $CD4^+$ T cells from naïve WT and $H_{1\text{-}4}$RKO mice and *in vitro* differentiated them into different effector T helper cell subsets. *In vitro* differentiated Th1 effector cells from $H_{1\text{-}4}$RKO mice produced less IFN-γ compared to Th1 effectors from WT mice (Fig. 2D). We found no difference in the production of IL-4 (Fig. 2E) or IL-17 (Fig. 2F) from Th2 and Th17 effector cells, respectively. Thus, under *in vitro* nonpolarizing or polarizing conditions, $CD4^+$ T cells from $H_{1\text{-}4}$RKO mice produce less IFN-γ, indicating a deficiency in the Th1 response with a bias towards Th2 under nonpolarizing conditions.

Changes in the Immune Response associated with EAE in $H_{1\text{-}4}$RKO mice

EAE is primarily associated with pathogenic Th1 and Th17 cells.(14) HA and HRs play a role in T cell polarization, proliferation, and cytokine production (2), as well as Ab production by B cells (28). Therefore to elucidate the immune mechanisms associated with differential EAE susceptibility observed in WT and $H_{1\text{-}4}$RKO mice, we compared $MOG_{35\text{-}55}$ specific T cell responses on day 10 (d10) post-immunization. In *ex vivo* proliferation assays, splenocytes and draining lymph node (DLN) cells from both strains responded equivalently in a dose-dependent fashion to $MOG_{35\text{-}55}$ (Fig. 3A). Splenic and DLN cells from $H_{1\text{-}4}$RKO mice re-stimulated with $MOG_{35\text{-}55}$ produced less IFN-γ (Fig. 3C) and trended towards reduced IL-17 (Fig. 3D) compared to re-stimulated cells from WT mice. We also analyzed IL-4 production by these cells, which is an indicator of an EAE protective Th2 response, but the level was below the limit of detection. Lastly, as HA and HRs can influence Ab production and the frequency of B cells, (24, 28) and $H_{1\text{-}4}$RKO mice have slightly fewer B cells (Fig. S1E), we measured the $MOG_{35\text{-}55}$ specific IgG levels in the sera on d30 1× post-immunization and found no difference in the anti-MOG Ab titers between $H_{1\text{-}4}$RKO and the WT mice (Fig. 3B).

We also evaluated immune responses in the target organ of 1× immunized WT and $H_{1\text{-}4}$RKO mice at d15 post-immunization. Mononuclear cells from the CNS were isolated, stained for cell surface molecules, stimulated with PMA/ionomycin for 4 h in the presence of Brefeldin A, and analyzed by flow cytometry for intracellular cytokines. The total number

of infiltrating cells and the number and frequency of $CD45^+$ CNS-infiltrating cells were comparable between WT and $H_{1\text{-}4}$RKO mice (Fig. S3A and S3B). The frequency of $TCR\alpha\beta^+$, $TCR\gamma\Delta^+$, $CD4^+$, $CD8^+$ T cells, and $CD11b^+$ cells (Fig. S3C-E) was also similar. In contrast, the frequency of IFN-γ producing $CD4^+$ T cells was significantly decreased in $H_{1\text{-}4}$RKO mice compared to WT mice, while that of IL-17 producing cells did not change (Fig. 4A and B). We also evaluated the frequency of IFN-γ and IL-17 producing $TCR\gamma\Delta^+$ and $CD8^+$ T cells and found no difference (Fig. S3F and S3G).

In addition to the CNS, we also examined the peripheral T cell response d15 post-immunization. Compared to WT mice, no difference was detected in the frequency of $TCR\gamma\Delta^+$, $TCR\alpha\beta^+$, $CD4^+$, $CD8^+$ T cells, $Foxp3^+$ Treg, and $CD11b^+$ cells in the DLNs of $H_{1\text{-}4}$RKO mice (Fig. S3H-K). There was also no difference in the frequency of IFN-γ or IL-17 producing cells among $CD8^+$ T cells (Fig. S3L). Similar to the CNS, we found that the frequency of IFN-γ producing $CD4^+$ T cells was significantly decreased in $H_{1\text{-}4}$RKO mice compared to WT mice (Fig. 4A and C) with no effect on the frequency of IL-17 producing cells. Taken together, these results suggest that a reduced MOG-specific Th1 response underlies the increased EAE resistance observed in $H_{1\text{-}4}$RKO mice.

T cells from immunized $H_{1\text{-}4}$RKO mice are less encephalitogenic

In the current study, we observed that upon polyclonal stimulation, during *in vitro* differentiation, and in *ex vivo* recall assays, Th1 effector cells from $H_{1\text{-}4}$RKO mice produced significantly less IFN-γ. Therefore, to address whether the lack of all four GPC-HRs had any impact on the encephalitogenic potential of T cells, we immunized donor WT and $H_{1\text{-}4}$RKO mice using the 1× immunization protocol and at d12 post-immunization, DLN cells were *ex vivo* re-stimulated with $MOG_{35\text{-}55}$ and IL-12 for 72 h. Before adoptive transfer, representative cells were stimulated with PMA/ionomycin + Brefeldin A for 4 h, stained for intracellular IFN-γ and IL-17 and supernatants screened for IFN-γ, IL-17, and GM-CSF production. Compared to WT restimulated DLN cells, $H_{1\text{-}4}$RKO cells produced significantly less IFN-γ and IL-17 with no effect on GM-CSF production (Fig. 5A-E). At the end of *ex vivo* restimulation, an equal number of WT cells were transferred into naïve WT, HDCKO, and $H_{1\text{-}4}$RKO mice. In addition, we transferred restimulated $H_{1\text{-}4}$RKO cells into WT recipients and monitored them for the development of EAE. Interestingly, we found that restimulated cells from WT mice were fully capable of inducing EAE in both WT and $H_{1\text{-}4}$RKO recipients. In contrast, restimulated $H_{1\text{-}4}$RKO cells were unable to induce EAE in WT recipients. Furthermore, we observed that the restimulated WT cells transferred into HDCKO mice also induced EAE but with an intermediary disease course compared to WT recipients (Fig. 5F). These results demonstrate that in the absence of $H_{1\text{-}4}$Rs, T cells are markedly less encephalitogenic.

Blocking $GABA_AR$ in $H_{1\text{-}4}$RKO T cells alters functionality in vitro

HA mediates its effects by signaling through the four known GPC-HRs. However, it was shown that HA can signal through non-GPCRs, such as $GABA_AR$ (21-23). *Xenopus* oocytes or HEK-293T cells transfected with homomultimeric subunits of $GABA_AR$ can form functional HA-gated chloride channels (23). In addition, there is evidence for HA mediated chloride conductance in the mammalian brain, suggesting the existence of HA signaling through non GPC-HRs (22). We hypothesized that in the absence of HRs in $H_{1\text{-}4}$RKO mice HA may similarly signal through $GABA_AR$ expressed by $CD4^+$ T cells thereby diminishing their encephalitogenic capacity through altered cytokine production. To address this possibility, we stimulated purified $CD4^+$ T cells from the spleen and lymph nodes of naïve WT and $H_{1\text{-}4}$RKO mice with plate bound anti-CD3 and soluble anti-CD28 mAb for 72 h in presence or absence of the $GABA_AR$ antagonist, picrotoxin, and screened the culture supernatants for IL-2, IFN-γ, and IL-4 production by ELISA. We observed no difference in

the production of IL-2 (Fig. 6A) between WT and H_{1-4}RKO $CD4^+$ T cells stimulated in the presence or absence of picrotoxin. In the absence of picrotoxin, stimulated $CD4^+$ T cells from H_{1-4}RKO mice produced significantly less IFN-γ (Fig 2A) and more IL-4 (Fig. 2B) compared to WT $CD4^+$ T cells. Blocking $GABA_AR$ in H RKO $CD4^+$ 1-4 T cells with picrotoxin significantly increased IFN-γ (Fig. 6B) and decreased IL-4 production (Fig. 6C) supporting the concept that HA can exert its effect through $GABA_AR$ expressed in $CD4^+$ T cells and alter cytokine production.

Discussion

Here we have assessed the role of HA in the absence of all known GPC-HRs in EAE susceptibility to test the hypothesis that a non-canonical GPC-HR signaling pathway may influence allergic inflammation and immune responses. The results of our study demonstrate that compared to WT and HDCKO mice, H_{1-4}RKO mice are remarkably resistant to two different protocols of MOG_{35-55}-induced EAE. In contrast, HDCKO mice exhibit increased susceptibility to EAE compared to WT and H_{1-4}RKO mice. The absence of GPC-HRs results in an intrinsic inability of $CD4^+$ T cells to produce IFN-γ and differentiate into Th1 effector T cells. Consequently, decreased susceptibility to EAE in H_{1-4}RKO mice is associated with decreased IFN-γ production by both MOG_{35-55} specific peripheral and CNS infiltrating $CD4^+$ T cells. Furthermore, in passively induced disease, restimulated WT cells transferred into HDCKO mice also induced EAE, but with a reduced disease course compared to WT recipients, suggesting a role for HA in the effector phase of the disease. In addition, we show that compared to WT effector T cells, H_{1-4}RKO effector T cells, are markedly less encephalitogenic and produce less IFN-γ and IL-17 with no difference in the production of GM-CSF. Our results are in contrast to the observation that GM-CSF is required for the full encephalitogenic potential of T cells (29-31). Our results suggest that under these *in vitro* polarizing conditions, cells from H_{1-4}RKO mice are less encephalitogenic because of their diminished Th1 and Th17 cytokines. However, it is possible that *in vivo* within the CNS, infiltrating cells may produce less GM-CSF.

Our results confirm a previous observation showing increased EAE susceptibility in HDCKO mice (13). Since the H_{1-4}RKO mice lack all known GPC-HRs, and are therefore deficient in HA signaling, we anticipated that H_{1-4}RKO mice would be equally susceptible to EAE as HDCKO mice. Rather, these two models of impaired HA signaling have opposing EAE phenotypes. It is known that the prolonged lack of HA affects the level of HR expression, mast cell granule content, and the development of DCs, all of which are important in EAE. Compared to WT mice, HDCKO MOG_{35-55} specific T cells produce more IFN-γ, TNF-α, and IL-6 (13). Additionally, DCs from HDCKO mice have greater antigen presenting activity than their WT counterparts and tend to more readily polarize T cells toward a Th1 phenotype. Furthermore, HDCKO mice have reduced mast cell numbers with drastically decreased granularity (26).

The contrasting results between H_{1-4}RKO and HDCKO mice suggests a novel HAergic pathway capable of actively regulating resistance to EAE in H_{1-4}RKO mice or alternate ligand binding to canonical GPC-HRs in the absence of HA leads to increased disease severity in HDCKO mice. Support of the latter hypothesis comes from a single unreplicated study suggesting that CCL16 may be a low affinity functional ligand for the H_4R capable of eliciting chemotaxis in human and mouse eosinophils (32). Although CCL16 binding to H_4R in other immune cells expressing the gene has not been studied, it is theoretically possible that this mechanism leads to the increased lymphocyte chemotaxis, production of proinflammatory cytokines, and EAE susceptibility seen in HDCKO. However, we favor the existence of an alternative HAergic pathway capable of actively regulating increased resistance to EAE in H_{1-4}RKO mice.

We propose that this alternative HAergic pathway may reflect HA acting through either an additional as of yet unidentified high affinity GPC-HR or through less well characterized HA activation of known receptors and/or signaling pathways. Regarding the existence of an unidentified high affinity GPC-HR, it is unlikely that such a receptor has gone undiscovered given the extensive *in vitro* and *in vivo* based screens that led to the identification of H_3R and H_4R (33). Support for later hypothesis comes from the fact that biogenic amines, including HA, can activate ligand gated ion channels (34). Ligand gated ion channels can be either cation channels, which are activated by acetylcholine and serotonin, or anion channels, which are activated by GABA and glycine (35). There is evidence for HA signaling through $GABA_AR$, in that homomultimeric subunits of $GABA_AR$ expressed in *Xenopus* and HEK-293T cells form functional HA-gated chloride channels (23).

Additional evidence for HA activation and signaling through known receptors comes from studies in invertebrates (34, 36-38). Through immunohistochemistry, high levels of HA and HDC were seen in *Drosophila* photoreceptors (39, 40); however, the functional significance of HA localization in these receptors was not evident until the cloning of two novel HA-gated chloride channels, HisCl-α1 and HisCl-α2 (41). Importantly, the elements that are most important for HA binding and function of HisCl have the greatest mammalian sequence homology with particular subunits of $GABA_AR$ and glycine gated chloride channels (42). Additionally, there is evidence in mammalian brain for HA signaling through a non-canonical GPC-HR which is picrotoxin-sensitive and mediated by chloride conductance (22). Taken together these observations support the concept that HA can bind to and signal through GABA and/or glycine receptors. However, unlike the $GABA_AR$ β subunits, the glycine receptor β subunits expressed in HEK293 cells do not elicit gating of the channel in response to HA (42).

GABA is a major neurotransmitter in the CNS but is also known to have immunomodulatory activity (43). $GABA_AR$ subunits are expressed by immune cells including $CD4^+$ T cells (44) and the production of IL-6 and IL-12 by peritoneal macrophages can be inhibited by exogenous GABA treatment (45). GABA can also modulate $CD8^+$ T cell cytotoxicity and decrease cutaneous delayed-type hypersensitivity reactions (46, 47). Importantly, GABA was shown to reduce myelin basic protein specific T cell proliferative responses (48), and treatment of SJL/J mice with GABAergic agents delays the onset of EAE in association with decreased production of Th1 and Th17 cytokines (49) which is similar to H_{1-4}RKO T cell effector response reported herein. Similarly, low dose GABA treatment of type 1 diabetes prone NOD mice inhibits the development of diabetogenic T cells and suppresses disease progression (44). Furthermore, in the present study, we show that purified $CD4^+$ T cells from H_{1-4}RKO mice stimulated with anti-CD3 + anti-CD28 in the presence of the $GABA_AR$ antagonist, picrotoxin, significantly decreased IFN-γ and increased IL-4 production, suggesting, that HA can exert its effect through $GABA_AR$ expressed in $CD4^+$ T cells leading to altered cytokine production. Therefore, we anticipate that HA binding to GABA receptors expressed by T cells has the potential to mediate the increased resistance to EAE in H_{1-4}RKO mice. Moreover, we observed that H_{1-4}RKO mice had significantly higher plasma levels of HA compared to WT mice. HRs, especially H_2R and H_3R, are known to regulate the levels of HA by feedback mechanisms in various systems (50-52). It is likely that higher plasma levels of HA observed in the absence of canonical HRs in H_{1-4}RKO mice may bind to low affinity receptors like $GABA_AR$. Additionally, HA may also act as an endogenous ligand at an unknown allosteric site on the $GABA_AR$ subunit potentiating the action of GABA (23).

An additional mechanism whereby HA may mediate disease resistant in H_{1-4}RKO mice is through HA transport. HA transporters are organic cation transporters (OCT) with polyspecificity for transporting molecules across cell membranes. One of these, OCT3 is

ubiquitously expressed and HA uptake through OCT3 has been studied in basophils (53). Once inside the cell HA is thought to play a role in cell signaling as a second messenger by binding to cytochrome P450 (P450). P450 is a member of a large and diverse group of heme-containing enzymes involved in the metabolism of xenobiotics, lipids, and hormones (54). In liver microsomes, polyamines, hormones, biogenic amines, and antihistamines can inhibit or displace HA binding to P450 (55). Furthermore, HA binding to P450 has been suggested to modulate its catalytic activity and influence cell growth (56). While the functional significance of HA transport and binding to P450 in the pathogenesis of EAE are unclear, this pathway nevertheless provides for an additional mechanism whereby HA may influence T cells responses and actively suppress EAE.

In summary we have studied the function of endogenous HA on EAE susceptibility in $H_{1\text{-}4}$RKO and HDCKO mice both of which are deficient in HA signaling. Rather than being equivalent in EAE susceptibility and immune responses as predicted, we found $H_{1\text{-}4}$RKO mice to be significantly less susceptible to $MOG_{35\text{-}55}$ induced EAE while HDCKO mice were highly susceptible. Taken together, our findings strongly support the concept that HA acting through mechanisms independent of the four known GPC-HRs mediates increased resistance to EAE. Clearly, delineating the mechanism(s) whereby this alternative inhibitory pathway(s) leads to immune deviation and EAE resistance has the potential to lead to the development of new disease modifying therapies for both allergic and autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

SC	Spinal cord
MS	Multiple sclerosis
EAE	Experimental allergic encephalomyelitis
HA	Histamine
HDC	Histidine decarboxylase
DC	Dendritic cell
HR	Histamine receptor
BBB	Blood brain barrier
GPCR	G-protein-coupled receptor
$MOG_{35\text{-}55}$	Myelin oligodendrocyte glycoprotein 35-55
GABA	γ-aminobutyric acid

DLN	Draining lymph node
PMA	Phorbol 12-myristate 13-acetate
WT	Wild-type
OCT	organic cation transporters

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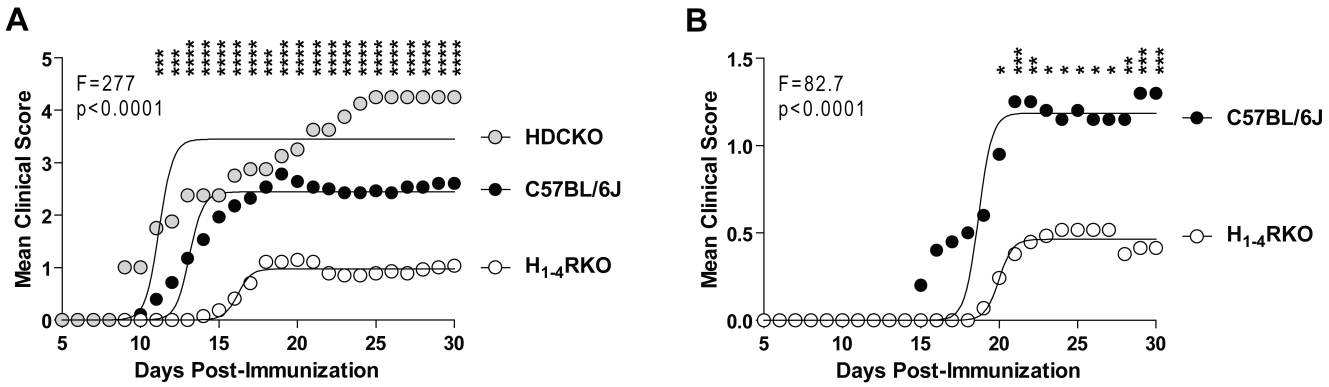

Figure 1. $H_{1\text{-}4}$RKO mice exhibit increased resistance to EAE

(A) WT (n = 28), HDCKO (n = 8) and $H_{1\text{-}4}$RKO (n = 27) mice were immunized using the 1× immunization protocol. (B) WT (n = 20), and $H_{1\text{-}4}$RKO (n = 29) mice were immunized using the 2× immunization protocol. (A and B) The clinical scores following immunization were recorded and the significance of differences between clinical courses was calculated by regression analysis with the best fit curve shown and two-way ANOVA followed by Bonferroni post-hoc multiple comparison test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

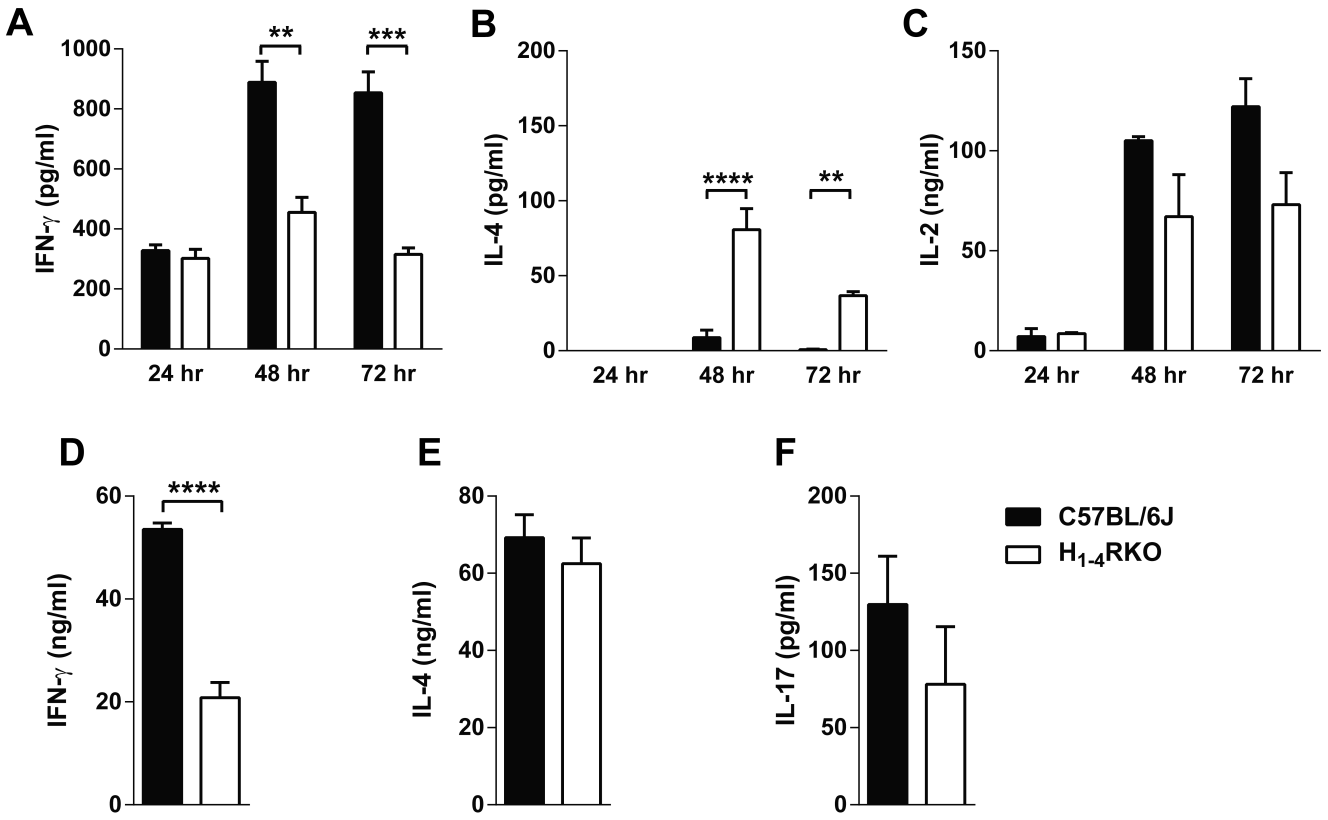

Figure 2. ***In vitro*** **activated CD4$^+$ T cells from $H_{1\text{-}4}$RKO mice produce decreased IFN-γ**

CD4$^+$ T cells from WT and $H_{1\text{-}4}$RKO mice were activated with anti-CD3 and anti-CD28 mAbs and (A) IFN-γ, (B) IL-4, and (C) IL-2 production at 24, 48, and 72 h post stimulation was determined by ELISA and shown as mean ± standard error of the mean (SEM) of n = 5 per strain. (D, E, and F) CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 mAbs in the presence of Th1, Th2 and, Th17 polarizing cytokines for 4 days. Cells were restimulated with anti-CD3 mAb and the supernatants were collected after 24 h. (D) IFN-γ (E) IL-4, and (F) IL-17 production was analyzed by ELISA and shown as mean ± SEM of n = 5 per strain. In (A, B, and C), significance of differences in cytokine production was determined by two-way ANOVA followed by Bonferroni post-hoc multiple comparison test. In (D, E, and F), significance of differences in cytokine production was determined using the Mann Whitney test (**, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

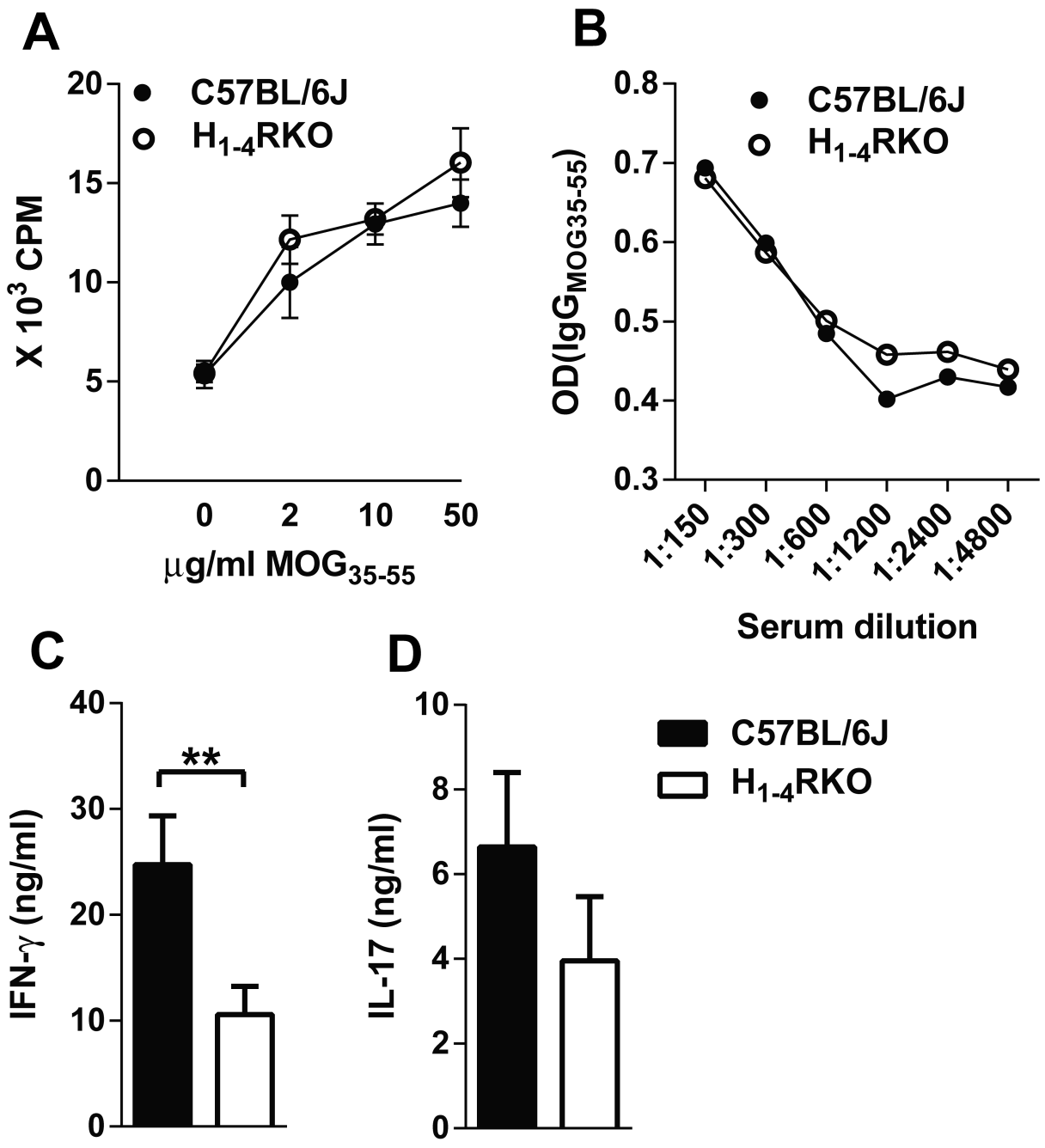

Figure 3. *Ex vivo* MOG_{35-55}-specific T cell cytokine profiles and serum MOG_{35-55} specific IgG antibody response in immunized WT and H_{1-4}RKO mice

(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. (B) Serum $MOG_{35–55}$-specific IgG Ab was measured by ELISA. The significance of differences in proliferation and antibody production were determined by two-way ANOVA followed by Bonferroni post-hoc multiple comparison test. (C) IFN-γ and (D) IL-17 production by $MOG_{35–55}$ stimulated DLNs and splenocytes from WT and H_{1-4}RKO 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 Mann Whitney test (**, $p<0.01$).

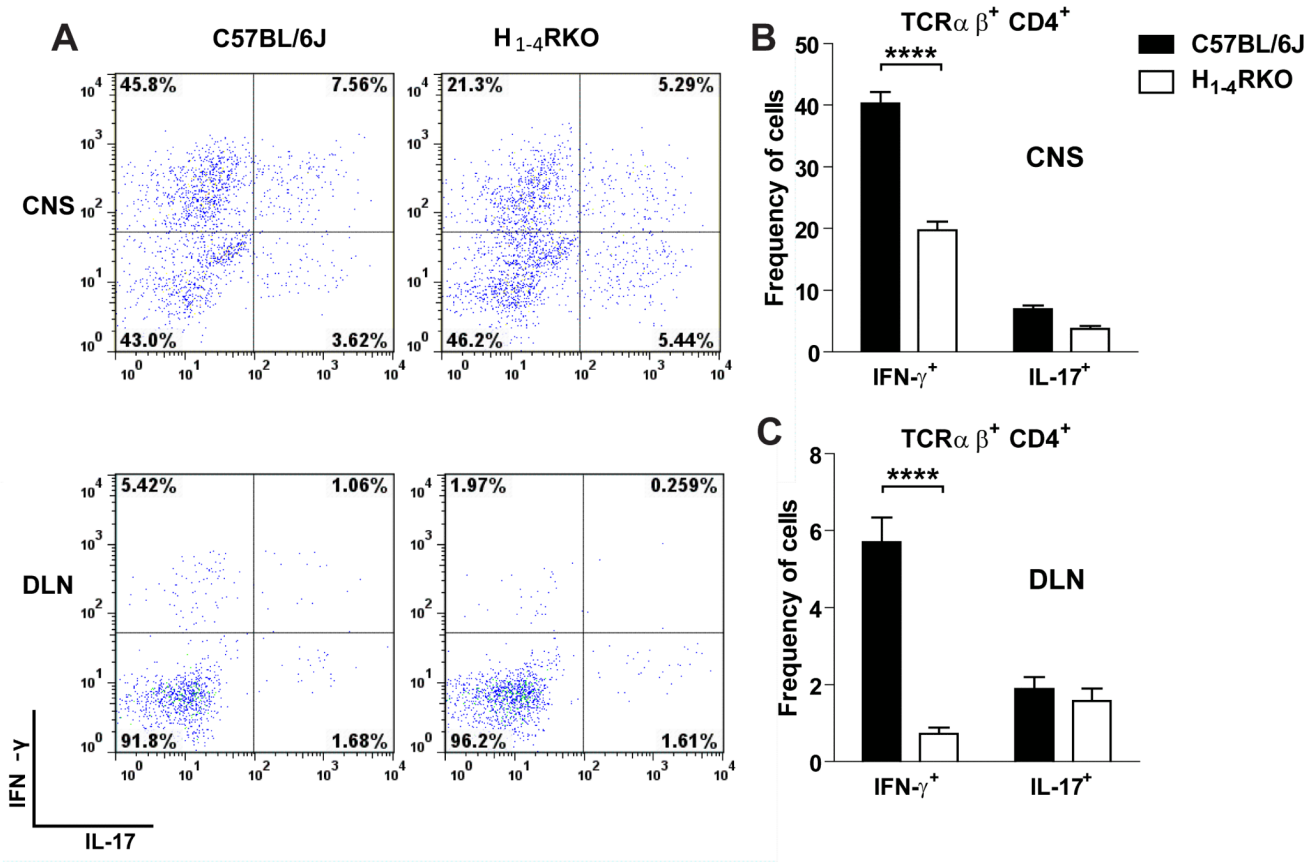

Figure 4. Reduced frequency of IFN-γ^+ CD4 T cells in the CNS and DLN of immunized $H_{1\text{-}4}$RKO mice

Mononuclear cells were isolated from the CNS and DLN of 1× immunized mice on d15 post-immunization, stimulated with PMA/ionomycin for 4 hours in the presence of Brefeldin A, stained, and analyzed by flow cytometry. (A) Representative flow cytometric data of the frequency of IFN-γ^+ and IL-17^+ cells from CNS and DLN, gated on TCRβ^+CD4^+. (B and C) Percentage of IFN-γ^+ and IL-17^+ cells from CNS and DLN, gated on TCRβ^+CD$4^+$of WT and $H_{1\text{-}4}$RKO mice. Data are shown as mean ± SEM of n = 8 per strain. Significance of differences observed in the percentage of cytokine positive cells were determined by two way ANOVA followed by Bonferroni's post hoc multiple comparison test (****, p<0.0001).

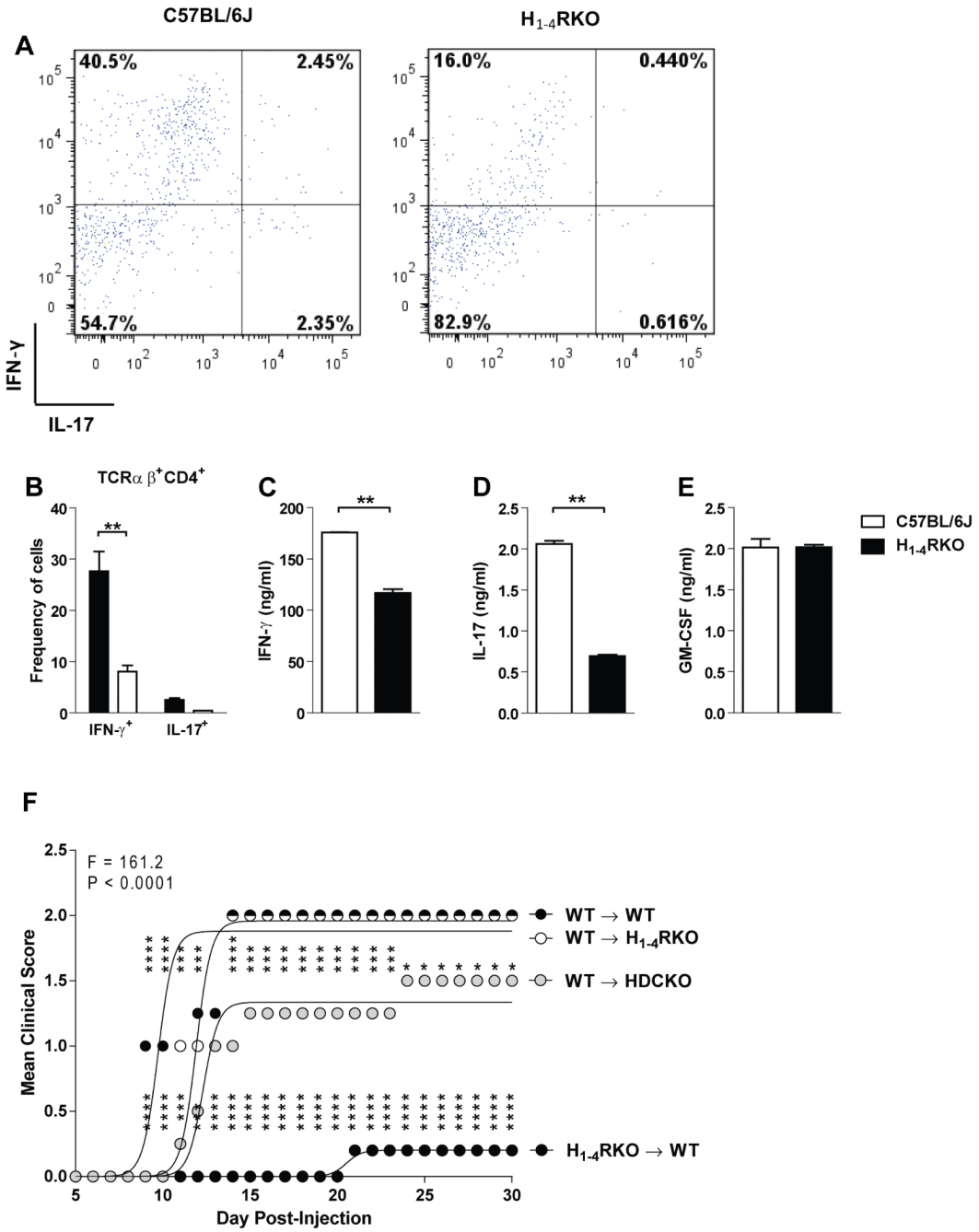

Figure 5. T cells from $H_{1\text{-}4}$RKO mice are less encephalitogenic

WT and $H_{1\text{-}4}$RKO mice were immunized using 1× immunization protocol, d12 post-immunization DLN cells were restimulated with $MOG_{35\text{-}55}$ and IL-12 for 72 h. (A) Flow cytometric analysis of WT and $H_{1\text{-}4}$RKO cells stimulated with PMA/ionomycin + Brefeldin A for 4 h and stained intracellularly for IFN-γ and IL-17, gated on CD4 and TCRβ. (B) The percentage of IFN-γ^+ and IL-17^+CD4^+ cells from the DLN of WT and $H_{1\text{-}4}$RKO mice and shown as mean ± SEM of n = 5 per strain. In (B) significance of differences observed in the percentage of cytokine positive cells were determined by two way ANOVA followed by Bonferroni's post hoc multiple comparison test (**, p<0.01). Cell Culture supernatants were screened for (C) IFN-γ, (D) IL-17, and (E) GM-CSF by ELISA and shown as mean ± SEM

of n = 5 per strain. In (C, D, and E) significance of differences in cytokine production was determined using the Mann Whitney test (**, $p<0.01$). Equal numbers (10×10^6 cells/animal) of restimulated cells were transferred into WT (n = 5/strain) recipients. (F) The clinical scores following immunization were recorded and the significance of differences between clinical courses was calculated by regression analysis with the best fit curve shown and two-way ANOVA followed by Bonferroni post-hoc multiple comparison test (*, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$).

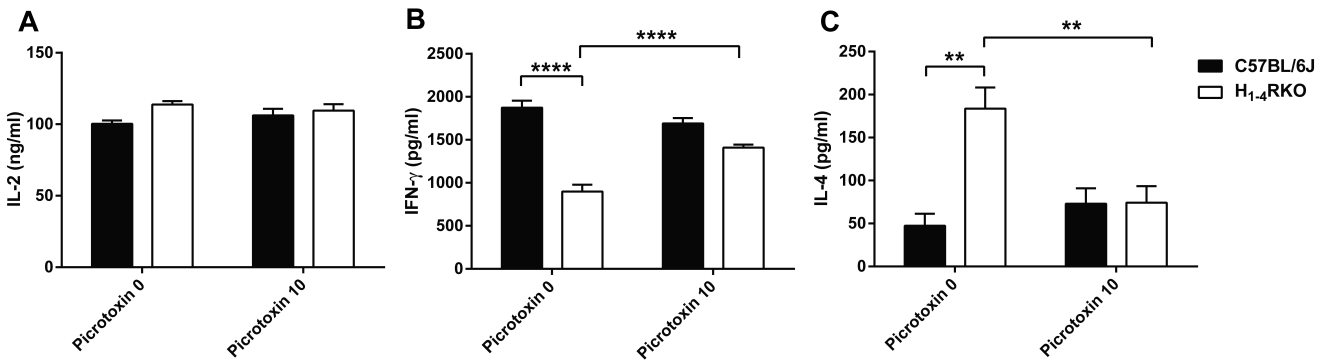

Figure 6. Blocking $GABA_AR$ in $H_{1\text{-}4}$RKO T cells alters cytokine production *in vitro*

$CD4^+$ T cells from WT and $H_{1\text{-}4}$RKO mice were activated with anti-CD3 and anti-CD28 mAbs in the presence or absence of picrotoxin and (A) IL-2, (B) IFN-γ, and (C) IL-4 production 72 h post stimulation was determined by ELISA and shown as mean ± SEM of n = 5 per strain. In (A, B, and C), significance of differences in cytokine production was determined by two-way ANOVA followed by Bonferroni post-hoc multiple comparison test (**, $p<0.01$; ****, $p<0.0001$).

Table I

Clinical disease traits following immunization of C57BL/6J, $H_{1\text{-}4}$RKO, and HDCKO mice with 1× $MOG_{35–55}$ + CFA + PTX.

Strain	Incidence[a]	CDS	PS	DA	DO	SI	LD
B6	28/28 (100)	43.4 ± 3.8	3.0 ± 0.2	16.8 ± 0.8	13.9 ± 0.7	2.5 ± 0.2	2/28 (7)
$H_{1\text{-}4}$RKO	16/27 (59)	14.2 ± 2.9	1.7 ± 0.3	6.3 ± 1.2	17.9 ± 1.0	2.2 ± 0.1	0/16 (0)
HDCKO	8/8 (100)	68.4± 3.6	4.3 ± 0.4	22.0	9	3.1 ± 0.2	5/8 (63)
Overall	X^2=17.8, 2 p=0.0001	H=36.5 p<0.0001	H=19.4 p<0.0001	H=42.0 p<0.0001	H=28.7 p<0.0001	H=10.8 p=0.005	X^2=20.0 p<0.0001
Post-hoc	B6=HDCKO >$H_{1\text{-}4}$RKO	HDCKO>B6 >$H_{1\text{-}4}$RKO	HDCKO>B6 >$H_{1\text{-}4}$RKO	HDCKO>B6 >$H_{1\text{-}4}$RKO	HDCKO<B6 <$H_{1\text{-}4}$RKO	HDCKO>B6 =$H_{1\text{-}4}$RKO	HDCKO>B6 =$H_{1\text{-}4}$RKO

[a]Animals were considered affected if clinical scores ≥1 were apparent for 2 or more consecutive days (percent affected). Cumulative disease score (CDS), peak score (PS) days affected (DA), day of onset (DO), severity index (SI) and lethal disease (LD). Mean trait values ± SE are shown. The significance of differences for the trait values among the strains was assessed by χ^2 analysis (overall incidence) and the Kruskal–Wallis test (H), followed by Dunn's post hoc multiple comparisons.

Table II

Clinical disease traits following immunization of C57BL/6J, $H_{1\text{-}4}$RKO with 2× $MOG_{35–55}$ + CFA.

Strain	Incidence[a]	CDS	PS	DA	DO	SI	LD
B6	16/20 (80)	14.6 ± 2.9	1.7 ± 0.2	8.4 ± 1.3	19.5 ± 1.0	1.7 ± 0.2	0/16
$H_{1\text{-}4}$RKO	6/29 (21)	5.0 ± 1.9	0.6 ± 0.2	2.2 ± 0.8	20.2 ± 0.6	2.2 ± 0.2	0/6
p-value	<0.0001	0.0007	0.0007	0.0002			

[a]Animals were considered affected if clinical scores ±1 were apparent for 2 or more consecutive days (percent affected). Cumulative disease score (CDS), peak score (PS) days affected (DA), day of onset (DO), severity index (SI), and lethal disease (LD). Mean trait values ± SE are shown. Significance of differences in trait values between the strains was assessed using the Fisher's exact test (incidence) and Mann-Whitney test (clinical disease parameters).