

Mouse Colonic Epithelial Cells Functionally Express the Histamine H₄ Receptor^S

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Received December 4, 2019; accepted February 3, 2020

ABSTRACT

We hypothesized that, in mice, histamine via the histamine receptor subtype 4 (H₄R) on colon epithelial cells affects epithelial barrier integrity, perturbing physiologic function of the colonic mucosa and thus aggravating the severity of colitis. To test this hypothesis, bone marrow–chimeric mice were generated from H₄R knockout (H₄R^{-/-}) and wild-type (WT) BALB/cJ mice and subjected to the dextrane sodium sulfate (DSS)-induced acute colitis model. Clinical symptoms and pathohistological derangements were scored. Additionally, total RNA was extracted from either mouse whole-colon homogenates or primary cell preparations enriched for epithelial cells, and gene expression was analyzed by real-time quantitative polymerase chain reaction. The impact of the H₄R on epithelial barrier function was assessed by measurement of transepithelial electrical resistance of organoid-derived two-dimensional monolayers from H₄R^{-/-} and WT mice using chopstick electrodes. Bone marrow–chimeric mice with genetic depletion of the H₄R in nonhematopoietic cells exhibited less severe DSS-induced acute colitis symptoms compared with WT mice,

indicating a functional proinflammatory expression of H₄R in nonimmune cells of the colon. Analysis of H₄R expression revealed the presence of H₄R mRNA in colon epithelial cells. This expression could be confirmed and complemented by functional analyses in organoid-derived epithelial cell monolayers. Thus, we conclude that the H₄R is functionally expressed in mouse colon epithelial cells, potentially modulating mucosal barrier integrity and intestinal inflammatory reactions, as was demonstrated in the DSS-induced colitis model, in which presence of the H₄R on nonhematopoietic cells aggravated the inflammatory phenotype.

SIGNIFICANCE STATEMENT

The histamine H₄ receptor (H₄R) is functionally expressed on mouse colon epithelial cells, thereby aggravating dextrane sodium sulfate–induced colitis in BALB/cJ mice. Histamine via the H₄R reduces transepithelial electrical resistance of colon epithelial monolayers, indicating a function of H₄R in regulation of epithelial barrier integrity.

Introduction

Besides a series of other functions, the biogenic amine histamine serves as a proinflammatory mediator, commonly recognized in cases of allergic inflammation. With the exception of allergic asthma, symptoms of allergic diseases can be readily controlled using pharmacological inhibitors acting at the histamine H₁ receptor. This activity can be explained by the functional expression of histamine H₁ receptor on vascular

smooth muscle cells and on endothelial cells, on which histamine induces contraction and dilation, respectively (Ashina et al., 2015). The latest identified histamine receptor, H₄R, was initially described to be expressed on cells of hematopoietic origin, such as mast cells, eosinophils, T cells, and dendritic cells. H₄R is functionally involved in the pathogenesis of several models of inflammatory diseases, and this function has largely been attributed to its expression on hematopoietic, i.e., immune, cells. However, an increasing body of evidence now suggests that H₄R is functionally expressed on nonhematopoietic cells, too (Cianchi et al., 2005; Breunig et al., 2007; Morini et al., 2008; Connelly et al., 2009; Lethbridge and Chazot, 2010; Rossbach and Bäumer, 2014).

2,4,6-trinitrobenzenesulfonic acid–induced colitis and DSS-induced colitis in rodents are models resembling certain aspects of the major forms of human inflammatory bowel disease (IBD), Crohn's disease, and ulcerative colitis. In rats, symptoms of 2,4,6-trinitrobenzenesulfonic acid–induced colitis were ameliorated by administration of the H₄R-selective

This work was supported by a grant of the Deutsche Forschungsgemeinschaft [NE 647/8-1 to Detlef Neumann].

The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (German Animal Welfare Legislation) and were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), AZ 33.14-42502-04-12/1021. This article does not contain any studies with human participants performed by any of the authors.

<https://doi.org/10.1124/jpet.119.264408>.

 This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: DAI, disease activity index; DMEM, Dulbecco's modified Eagle's medium; DSS, dextrane sodium sulfate; H₄R, histamine H₄ receptor; H₄R^{-/-}, H₄R knockout; IBD, inflammatory bowel disease; PCR, polymerase chain reaction; qPCR, quantitative PCR; TEER, transepithelial electrical resistance; TJL, The Jackson Laboratory; TNBS, 2,4,6-trinitrobenzenesulfonic acid; WT, wild type; XTT, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy6-nitro) benzene sulfonic acid hydrate.

inhibitor JNJ7777120 (Varga et al., 2005), and in mice, ablation of endogenous histamine production as well as genetic or pharmacologic inhibition of H₄R function reduced the severity of DSS-induced colitis (Schirmer et al., 2015). Therefore, it is assumed that histamine, which can be detected in high concentrations in affected tissues of both human colitis patients and animals subjected to IBD models (Raithel et al., 1995; Rijniere et al., 2007; Kuefner et al., 2008), promotes the pathogenesis in colitis, most probably via H₄R.

Several studies addressed the expression of histamine receptors in the intestinal system (Sander et al., 2006; Breunig et al., 2007; Morini et al., 2008; Coruzzi et al., 2012; Deiteren et al., 2014, 2015; Sullivant et al., 2016). The results reported are inconsistent in some details, possibly because of the different species they were conducted in. Nevertheless, concerning H₄R, the studies consistently report its expression in the colon. Some also describe H₄R-expressing cell types of the colon; however, these studies relied on immunohistochemistry, and H₄R antibody-based analyses should be considered with caution because many commercial H₄R antibodies lack specificity (Beermann et al., 2012b; Neumann et al., 2012). Moreover, the expression of H₄R in mouse colon seems to be enhanced because of inflammation (Coruzzi et al., 2012; Deiteren et al., 2014), whereas in humans, the data reported so far are inconsistent (Sander et al., 2006; Deiteren et al., 2015). Thus, though H₄R certainly is involved in the pathogenesis of experimental colitis models, the mechanism beyond, i.e., the cellular source of histamine/H₄R function in colitis, remains elusive.

In this study, a variety of methodological approaches was used to collect data indicating that colonic epithelial cells are responsible for such effect. Most importantly, antibody-based detection of H₄R was consequently avoided in favor of highly specific and sensitive molecular biologic techniques complemented by functional analyses. In summary, we demonstrate that inflammation, at least colitis in mouse models, can be regulated by histamine via the H₄R, not only by an effect on immune cells but also via its effect on epithelial cells.

Materials and Methods

Materials. If not stated otherwise, all chemicals were obtained from Sigma-Aldrich (Munich, Germany).

Animals. BALB/cJrj (WT) mice (male, 6–10 weeks old) were purchased from Janvier Laboratories. H₄R knockout mice (H₄R^{-/-}; strain: C.129HrH₄^{tm1Lex}) were generated by Lexicon Genetics (Woodlands, TX) as described by Hofstra et al. (2003) and backcrossed for more than 10 generations onto the BALB/cJrj strain. Mice were housed in the animal facility of Hannover Medical School (temperature: 21 ± 1°C; 14/10-hour day/night cycle) with access to standard diet and drinking water ad libitum. All procedures involving animals were in accordance with the institutional ethical standards (German Animal Welfare Legislation) and were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (AZ 33.14-42502-04-12/1021).

Generation of Bone Marrow Chimera. Recipient mice (male, 6–8 weeks old) received antibiotics daily, starting at the day of fractionated γ -irradiation (2 × 3 Gy separated by 4 hours) and ending 4 weeks thereafter. Bone marrow cells from donor mice (male, 8–10 weeks old) were prepared by flushing the bone marrow out of aseptically treated hollow bones with sterile ice-cold PBS. Obtained cells were repeatedly washed in PBS and eventually resuspended in PBS at a density of 6.66 × 10⁷ cells/ml for transplantation. Transplantation into recipient mice was performed immediately after

irradiation by intravenous injection of 1 × 10⁷ cells/mouse into the tail vein (Fig. 1). The transplanted hematopoietic system was allowed to settle for 6 weeks, yielding chimeric mice carrying the WT H₄R on nonhematopoietic cells and no functional H₄R on hematopoietic cells or vice versa. Mice reconstituted with syngenic bone marrow cells served as controls (Fig. 1). Successful generation of chimerism was genetically verified by PCR of mH₄R locus-specific sequences using samples from blood cells (hematopoietic system) and from hair follicles or tail tips (nonhematopoietic system) (Supplemental Fig. 1).

Induction of Colitis by DSS and Animal Dissection. For induction of acute colitis, mice were fed with water charged with 3% (w/v) DSS from day 0 to day 7. Water only-fed mice served as control. Mice were inspected daily as detailed in the section *Evaluation of Disease Activity*. On day 7, the animals were euthanized with carbon dioxide insufflation and subsequent heart puncture to draw blood. Cola were resected, washed with PBS to remove remaining feces, divided into comparable sections, and either directly stored at -80°C or in RNeasy (Thermo Fisher, Waltham, MA) for mRNA analyses or immediately submitted to further processing as described in the sections *Histology, Excision of Colon Epithelial Layers by Laser Capture Microdissection, and Preparation of Colonic Crypts, Generation of Primary Epithelial Cells, and Isolation of EpCAM⁺ Cells*.

Evaluation of Disease Activity. Mice were examined at 24-hour intervals, and a disease activity index (DAI) (adopted from Alex et al., 2009) ranging from 0 to 14 was employed. The DAI was based on total body weight loss (0: no weight loss, 1: ≤5%, 2: ≤10%, 3: ≤15%, 4: >15%), stool consistency (0: normal, 2: soft, 4: diarrhea, 6: no defecation), and peranal bleeding (0: no bleeding, 2: little bleeding, 4: massive bleeding/no defecation).

Histology. Cola were fixed in 4% (v/v) formaldehyde (Merck, Darmstadt, Germany), embedded in paraffin, sliced, and stained with H&E. Stained tissue slices were analyzed in a blinded fashion by two independent researchers. A histologic severity score [The Jackson Laboratory (TJL) score] was calculated by evaluating the overall severity of inflammation (0: normal, 1: mild, 2: moderate, 3: severe), the hyperplasia (0: normal, 1: mild, 2: moderate, 3: severe), the degree of ulceration (0: no ulcers, 1: 1–2 ulcers involving up to 20 crypts, 2: 1–4 ulcers involving 20–40 crypts, 3: any ulceration exceeding the aforementioned criteria), and the area of affected tissue (0: 0%, 1: ≤30%, 2: >31% ≤70%, 3: >70%). The maximum score thus sums up to 12 per segment and 36 per the whole colon of a mouse.

Excision of Colon Epithelial Layers by Laser Capture Microdissection. Colon tissue slices were mounted on a poly-l-lysine-coated membrane attached to a metal frame. The tissue was deparaffinized and stained by hemalum staining, and then epithelial layers were excised by laser capture microdissection using the CellCut Plus system (MMI Molecular Machines & Industries AG, Glattbrugg, Switzerland). Samples were isolated using a notouch technique, as previously described (Jonigk et al., 2011). RNA was isolated from the microdissected tissue using NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany), essentially as described by the manufacturer.

Preparation of Colonic Crypts, Generation of Primary Epithelial Cells, and Isolation of EpCAM⁺ Cells. To prepare crypts, the colon segment was opened lengthwise, cut into small pieces, transferred into PBS containing 2 mM EDTA, and incubated for 1 hour at room temperature with gentle agitation. Then, fragments were washed three times with PBS supplemented with penicillin/streptomycin and thereafter moved into dissociation buffer (PBS containing 54.9 mM D-Sorbitol and 43.4 mM D-Sucrose) supplemented with 10 μM Y-27632. This suspension was vortexed for 10 seconds, fragments were sedimented, and the supernatant was collected and centrifuged for 10 minutes at 100g and 4°C. The pellet containing the crypts was washed five times by resuspension in dissociation buffer supplemented with Y-27632 followed by centrifugation as described above.

Crypts were processed to single cells by suspension of the crypts in TrypLE Express (Thermo Fisher Scientific), subsequent transfer into C-tubes (Miltenyi Biotec), and digestion using the GentleMACS

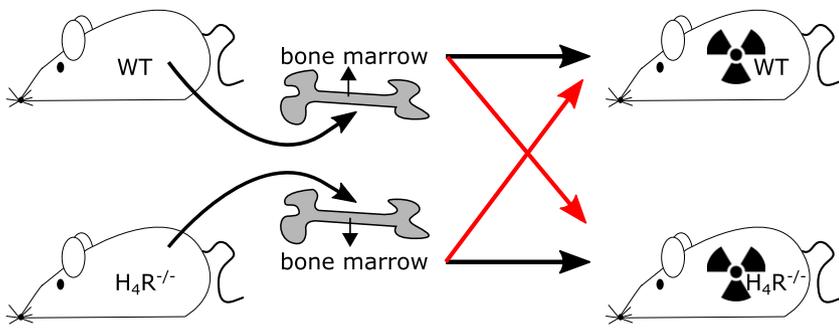


Fig. 1. Scheme of bone marrow chimera generation. Bone marrow was isolated from femora and tibiae of either BALB/cJ WT or H₄R^{-/-} mice and transferred to lethally irradiated WT or H₄R^{-/-} mice via tail vein injection to engraft the hematopoietic system of the donor mice within the recipient mice. After successful engraftment, mice carrying the wild-type H₄R on non-hematopoietic cells and no functional H₄R on hematopoietic cells (or vice versa) were obtained (red arrows). Mice reconstituted with bone marrow cells matching their own H₄R genotype served as controls (black arrows).

dissociator (Miltenyi Biotech) and running the program “m-intestine-1” two times, separated by a 5-minute incubation at 37°C. The obtained cellular suspension was added to ice-cold minigut medium (advanced DMEM/F12, 2 mM L-Glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 × N2, 1 × B27), filtered through a 100-µm cell strainer, and then centrifuged for 10 minutes at 300g and 4°C. The pellet was suspended in minigut medium to count the obtained single cells.

To prepare EpCAM⁺ cells, single cells were pelleted and suspended in autoMACS running buffer (Miltenyi Biotech). Biotin antimouse CD326 (EpCAM; Miltenyi Biotech) was added to the cell suspension and incubated for 15 minutes at 4°C. The cells were washed twice in autoMACS running buffer and then resuspended in running buffer containing α-biotin MicroBeads (Miltenyi Biotech) and incubated for 15 minutes at 4°C. After washing in running buffer, the cells were suspended in running buffer, and EpCAM⁺ cells were enriched using the autoMACS Pro Separator (Miltenyi Biotech). In the resulting cell suspension, EpCAM expression and absence of expression of CD45, CD19, CD3, CD11c, Ly6G, and F4/80 were verified by flow cytometry.

Generation of Colon Organoids (“Colonoids”) and Preparation of Monolayers. Crypts were isolated essentially according to the method described in Mahe et al. (2015). Approximately 500 crypts were suspended in 50 µl 3D-matrix (50% v/v Matrigel; Thermo Fisher Scientific), placed in the center of a well of a 24-well plate, and incubated for 20 minutes at 37°C. Then, 500 µl colonoid growth medium (50% DMEM/F12 plus 50% DMEM/F12 medium preconditioned using L-WRN cells, supplemented with 10% v/v fetal calf serum, 2 mM L-Glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 × N2, 1 × B27) supplemented with 1 µM epidermal growth factor and 10 µM Y-27632 were added. The medium was replaced with fresh medium every other day. After 7 days in culture at 37°C with 5% CO₂ in a humidified atmosphere, colonoid cultures were split by physical disintegration of the 3D-matrix, recovery of the colonoids by centrifugation, and resuspension in fresh 3D-matrix as described above.

Organoid-derived monolayers were generated by recovery of the organoid cells using Cell Recovery Solution (Corning, Wiesbaden, Germany) and placing the obtained cells on 24-well tissue culture inserts (Sarstedt, Nümbrecht, Germany) coated with diluted 3D-matrix. Culture conditions of monolayers were identical to that of colonoids, except that growth medium was replaced by differentiation medium (90% DMEM/F12 plus 10% DMEM/F12 preconditioned using L-WRN cells, supplemented with 10% v/v fetal calf serum, 2 mM L-Glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 × N2, 1 × B27, 1 µM epidermal growth factor).

mRNA Quantification. Organoids were released from the 3D-matrix using the Cell Recovery Solution (Corning). RNA from all samples was extracted using the Nucleospin RNA II kit (Macherey-Nagel) essentially according to the manufacturer’s instructions. The colon tissue specimens dedicated for mRNA analyses were lysed using the FastPrep 24 device (MP Biomedicals), whereas microdissected samples, crypts, and EpCAM⁺ cells were lysed directly in the corresponding buffer. RNA of each sample was reversely transcribed

for 30 minutes at 50°C into cDNA by means of Maxima Reverse Transcriptase (Thermo Fisher Scientific). H₄R-specific sequences were quantified proportionately to β-actin by real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). For microdissected samples, a preamplification step was introduced into the protocol.

Measurement of Transepithelial Electric Resistance. Trans-epithelial electrical resistance (TEER) was measured using the EVOM Epithelial VoltOhmmeter together with the STX2 Chopstick Electrode (World Precision Instruments, Sarasota, FL). Measurements were performed within 5 minutes after taking the culture plates out of the incubator to eliminate the disturbances caused by temperature variations. Electrodes were equilibrated and the measurements performed essentially as recommended by the manufacturer. The resistance of a blank (insert without cells) was measured in parallel to the samples, and the samples’ resistances were calculated by subtracting the blank value from the samples’ total resistances. Finally, by multiplying the samples’ resistances by the area of the inserts’ membrane, the resistance values were adjusted to the area, resulting in the unit Ω*cm².

Measurement of Cellular Metabolic Activity (XTT Assay). Confluent monolayer cell cultures derived from WT organoids were cultured for 1 week at 37°C/5% (v/v) CO₂ in a humidified atmosphere. Then, the cells were stimulated with 10 µM histamine. On day 5 after addition of histamine, XTT working reagent (Cell Proliferation Kit XTT; AppliChem, Darmstadt, Germany) was added to the wells according to the manufacturer’s instruction. A control well without cells served as background control. The absorption of the formazan dye formed was measured at 475 nm after incubation of the cells for 5 hours at 37°C/5% (v/v) CO₂ in a humidified atmosphere.

Statistical Analysis. Data are represented as arithmetic means ± S.D. for each parameter. Statistical analyses were performed with GraphPad Prism version 6.07 (GraphPad Software, La Jolla, CA) using one-way ANOVA with Dunnett’s post-test or nonparametric Kruskal-Wallis test with Dunnett’s post-test in case of DAI/TJL scores.

Results

Function of H₄R on Extra-Hematopoietic Cells. Recent data question the exclusive expression of H₄R on hematopoietic cells (Cianchi et al., 2005; Breunig et al., 2007; Morini et al., 2008; Connelly et al., 2009; Lethbridge and Chazot, 2010; Rossbach and Bäumer, 2014). To analyze a possible function of H₄R in DSS-induced colitis in extra-hematopoietic cells, we generated bone marrow chimera and submitted them to DSS-induced colitis. WT mice that received syngeneic bone marrow (WT→WT) demonstrated an increasing DAI during the observation period with an onset at day 3. The DAI of H₄R^{-/-} mice that had received a H₄R^{-/-} bone marrow (H₄R^{-/-}→H₄R^{-/-}) started to increase no earlier than day 4. At days 5–7, the DAI of H₄R^{-/-}→H₄R^{-/-} mice was

lower as compared with WT mice with a transplanted WT bone marrow (WT→WT) (Fig. 2A). In WT mice that received $H_4R^{-/-}$ bone marrow ($H_4R^{-/-}$ →WT), the DAI developed essentially as observed in WT→WT mice, whereas that of WT→ $H_4R^{-/-}$ mice essentially resembled that of $H_4R^{-/-}$ → $H_4R^{-/-}$ mice (Fig. 2A). Thus, the DSS-induced DAI in WT→WT mice and in $H_4R^{-/-}$ →WT mice resembled that observed in DSS-treated normal WT mice, whereas the DSS-induced DAI in $H_4R^{-/-}$ → $H_4R^{-/-}$ mice and in WT→ $H_4R^{-/-}$ mice resembled that observed in DSS-treated normal $H_4R^{-/-}$ mice.

Examination of the lengths of cola and caeca did not reveal any differences between the experimental groups (Supplemental Fig. 2). The quantitative analysis of histologic derangements in the cola mirrored the results of the DAI analysis; the degree of inflammatory signs in cola of $H_4R^{-/-}$ →WT mice was as high as that in WT→WT mice, whereas in WT→ $H_4R^{-/-}$ mice, the derangements were reduced similar to that in $H_4R^{-/-}$ → $H_4R^{-/-}$ mice (Fig. 2B). An essentially identical observation was made when evaluating the histology of the caeca (data not shown). Thus, the severity of clinical and pathohistologic parameters indicative for DSS-induced acute colitis were reduced because of the absence of H_4R expression in radio-resistant cells, indicating that the H_4R exerts its anti-inflammatory function in DSS-induced colitis on BALB/cJ mice on extra-hematopoietic cells.

H_4R mRNA Expression in Mouse Colon Epithelial Cells. The above-reported observation prompted us to investigate the cellular source for H_4R expression that is responsible for its function in acute DSS-induced colitis. Because of the local character of this inflammatory reaction, cells of the colon were supposed to be the primary candidates. The analysis of H_4R protein expression was hampered because of the unavailability of antibodies reliably recognizing the H_4R (Beermann et al., 2012b; Neumann et al., 2012). Thus, the present study relies on the relative quantification of H_4R mRNA by real-time qPCR, using β -actin as reference gene. This analysis verified the expression of H_4R in total cola preparations of BALB/cJ WT mice (Fig. 3A). The corresponding ΔCt_{WT} value (H_4R/β -actin) was calculated to be 13.39 ± 0.38 .

In samples from $H_4R^{-/-}$ mice, specific H_4R -derived sequences could not be detected (Fig. 3A; $\Delta Ct_{H_4R}^{-/-} = \infty$), demonstrating the specificity of the method.

Radio-resistant cells of the colon involved in inflammatory reactions are, besides others, epithelial cells. Thus, we hypothesized that colonic epithelial cells functionally express the H_4R . To test this, we initially took advantage of laser capture microdissection to access colonic epithelial cells (Fig. 3B, insert). In samples obtained from WT mice, but not from $H_4R^{-/-}$ mice, H_4R -encoding mRNA was observed (Fig. 3B and data not shown; ΔCt_{WT} : 17.44 ± 0.80). This observation was supported by a second assay system using another method to generate colonic epithelial cells, i.e., the preparation of colonic crypts (Fig. 4A). Indeed, H_4R expression was observed in the preparation of entire WT crypts (Fig. 4B; ΔCt_{WT} : 20.20 ± 0.28) as well as specifically in CD326 (EpCAM⁺) cells isolated thereof (Fig. 4C; ΔCt_{WT} : 21.15 ± 2.41). Moreover, the colonic crypts can be used to generate cultures of organo-typic structures ("organoids," Fig. 5A). If derived from cola of WT mice, these organoids retain the expression of H_4R mRNA (Fig. 5B; ΔCt_{WT} : 18.64 ± 0.17), whereas no H_4R expression was detectable in organoids from $H_4R^{-/-}$ mice.

H_4R is Functionally Expressed in Colonic Epithelial Cells. One of the major functions of the colonic epithelium is to provide a barrier between the luminal content of the colon and the body. This barrier function depends on a coherent cellular layer (i.e., tightly connected cells) that is able to provide an electric barrier, which can be measured as electric resistance. Colonic epithelial cells that were expanded in vitro by organoid culture and then processed to monolayers demonstrated an increasing TEER (Fig. 6A, days 0–15), concomitant with the cultures becoming a confluent layer (data not shown). Thereafter, the TEER remained at a rather constant level, which did not differ significantly between WT and H_4R organoids (Supplemental Fig. 3). Addition of histamine significantly reduced the TEER in the cultures compared with solvent (H_2O)-treated controls (Fig. 6A, days 16–35). The reduction of the TEER could be the consequence of reduced cellular

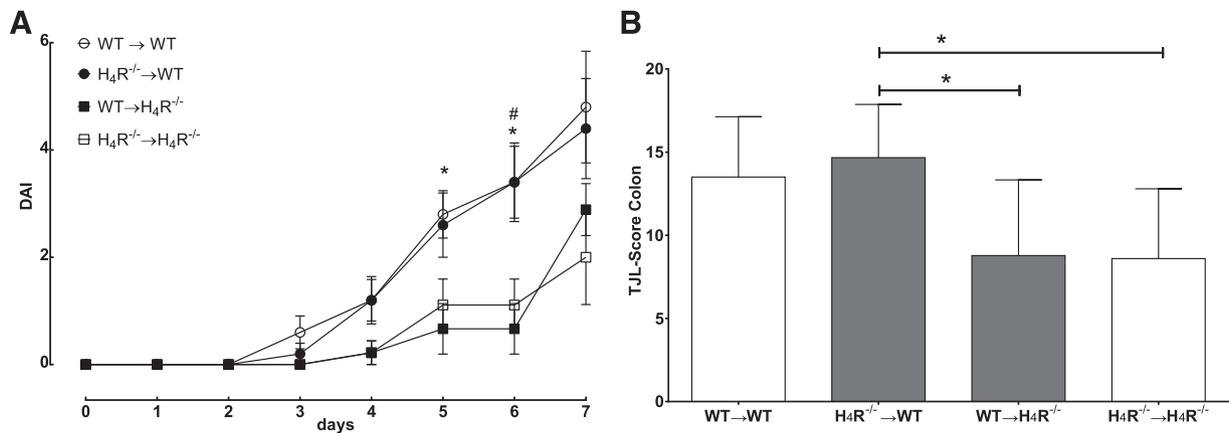


Fig. 2. DSS-induced colitis in bone marrow-chimeric mice generated from WT and $H_4R^{-/-}$ mice. Recipient mice were lethally irradiated, transplanted with bone marrow cells from syngenic or allogenic donors, and then subjected to DSS-induced colitis (start of DSS feeding = day 0). (A) Clinical symptoms of colitis (body weight, perianal bleeding, stool consistency) were evaluated daily in a quantitative manner and summed up into a disease activity index (DAI). *WT→WT vs. WT→ $H_4R^{-/-}$; #WT→ $H_4R^{-/-}$ vs. WT→ $H_4R^{-/-}$; */# $P < 0.05$; Kruskal-Wallis rank sum test with Dunnett's post test. (B) After the observation period, the cola of the mice were prepared and submitted to histologic analyses. Signs of inflammation were quantitatively evaluated and summed up into a TJL score. * $P < 0.05$; Kruskal-Wallis rank sum test with Dunnett's post test. WT→WT, WT donor, WT recipient; $H_4R^{-/-}$ →WT, $H_4R^{-/-}$ donor, WT recipient; WT→ $H_4R^{-/-}$, WT donor, $H_4R^{-/-}$ recipient; $H_4R^{-/-}$ → $H_4R^{-/-}$, $H_4R^{-/-}$ donor, $H_4R^{-/-}$ recipient; $n = 9-10$.

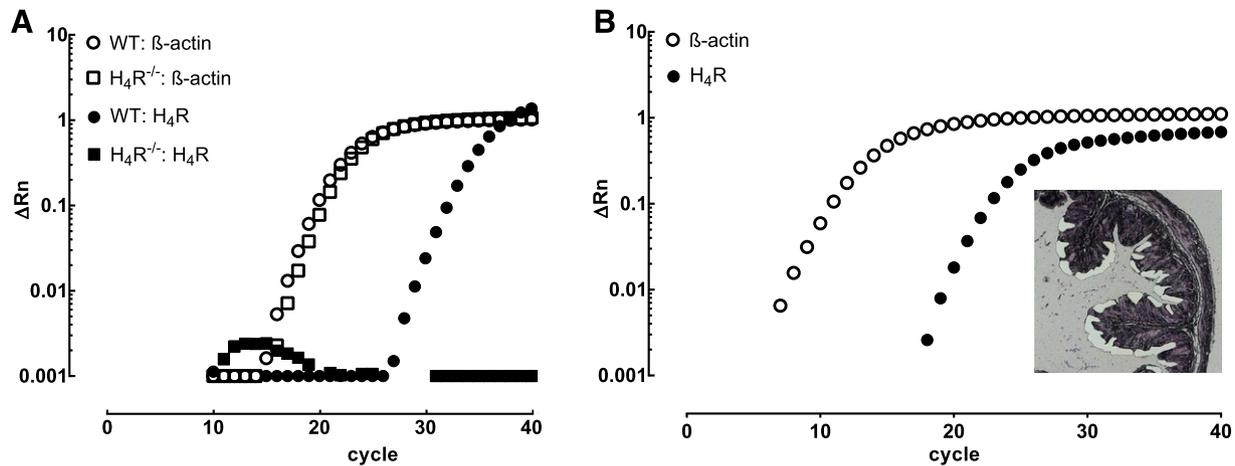


Fig. 3. H₄R expression in mouse colon epithelial cells. Cola were dissected from WT and from H₄R^{-/-} mice. (A) Out of the total colon, RNA was extracted and the abundance of H₄R-specific as well as β -actin-specific sequences was analyzed by real-time qPCR. (B) The cola were prepared for histologic analyses, and epithelial layers were collected by laser-captured microdissection. RNA was extracted, and the abundance of H₄R-specific as well as β -actin-specific sequences was analyzed by real-time qPCR. The inserted micrograph depicts a colon section and demonstrates the areas that have been collected (clear white areas). Shown are representative experiments out of at least three that have been independently performed.

proliferation and/or enhanced cell death. However, the metabolic activity in the monolayer culture, which is indicative for both proliferation and death, is not affected by the addition of histamine (Fig. 6B). Histamine not only reduces the TEER of colonic epithelial cell monolayers after having reached a maximum but also in cultures in which TEER is still increasing, more closely resembling the *in vivo* situation (Fig. 6C). This effect of histamine could be blocked by the specific H₄R antagonist JNJ7777120. Neither JNJ7777120 alone nor its solvent DMSO has any effect on the TEER (Fig. 6C). In addition, histamine is not able to reduce the TEER in epithelial monolayers obtained from

colonoids generated from genetically H₄R-deficient mice (Fig. 6D).

Discussion

The function of histamine in inflammatory diseases of the gut has long been discussed, but a cell type conveying this function via the H₄R has not been identified so far. Because of data obtained using models of inflammatory diseases of other organs, one can hypothesize that in colitis, the proinflammatory function of H₄R is mediated by cells of the immune system, e.g., macrophages, dendritic cells, mast cells, or

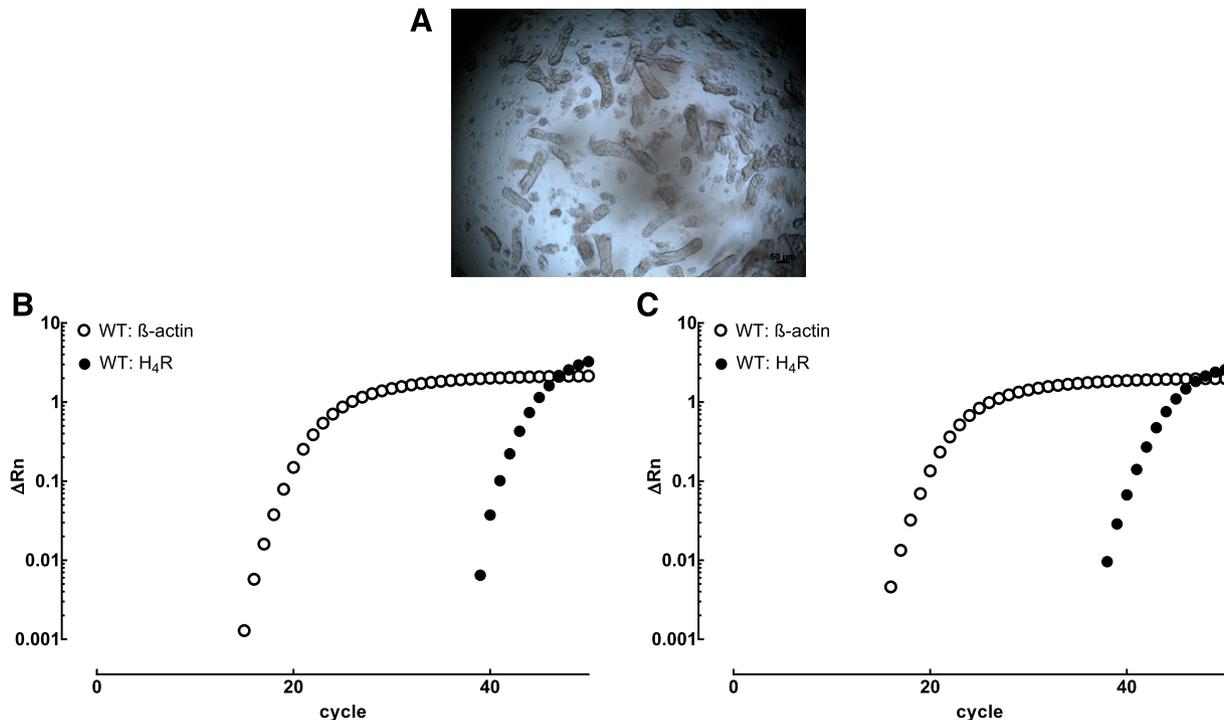


Fig. 4. H₄R expression in colonic crypts and EpCAM⁺ colon cells and colonoids. (A) Crypts were generated from cola of WT mice and analyzed for H₄R and β -actin transcript levels by real-time qPCR either (B) immediately or (C) after processing to single cells followed by isolation of EpCAM⁺ cells. Each plot is representative for at least three individual analyses performed.

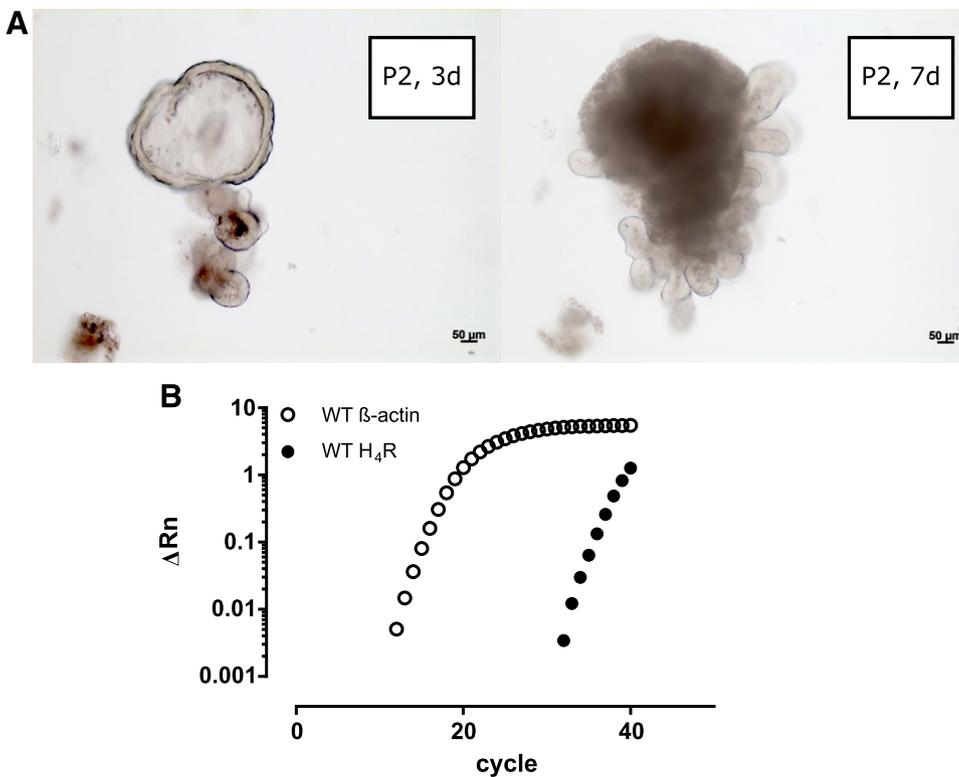


Fig. 5. H₄R expression in colon epithelial organoids. (A) After incubation of crypts in the presence of 3D-matrix and growth factors, organo-typic structures (organoids) develop and start to grow. (B) Cells obtained out of the organoid cultures were analyzed for H₄R and β-actin transcript levels by real-time qPCR. Each plot is representative for at least three individual analyses performed.

eosinophils (Cowden et al., 2010; Beermann et al., 2012a; Yamaura et al., 2012; Mahapatra et al., 2014; Hartwig et al., 2015). To test this hypothesis, we generated bone marrow–chimeric mice and subjected them to DSS-induced colitis. Although the experimental setup is quite complex and time-consuming, it is well-suited to analyze the role of radio-sensitive and radio-resistant cells in DSS-induced colitis (Iwasaki, 2006). The obtained data indicate that radio-resistant cells are necessary to provide the proinflammatory function of H₄R, whereas radio-sensitive cells only play a minor role. The radio-resistant cell compartment is composed of mostly nonhematopoietic, thus nonimmune, cells but also contains some tissue-resident immune cells, such as macrophages and dendritic cells. Because macrophages and dendritic cells contribute to colitis (Dunford et al., 2006; Muzaki et al., 2016) and functionally express H₄R (Yamaura et al., 2012; Bain and Mowat, 2014), their involvement in the H₄R's proinflammatory function in colitis formally cannot be excluded. Both these cell types reside within the colon wall, and indeed, in whole-colon samples, we verified the expression of H₄R. Because of the lack of antibodies specifically recognizing H₄R (Beermann et al., 2012b; Seifert et al., 2013), immune histochemical methods to detect H₄R expression in colon samples were excluded. Alternatively, we applied laser capture microdissection in combination with real-time qPCR and focused on the colonic epithelial layer. Indeed, in colon epithelial cells, mRNA expression of H₄R was identified and verified by a second sample preparation method, i.e., the generation of colonic crypts. The colonic epithelial layer is composed of several cell types, such as epithelial stem cells and terminally differentiated absorptive enterocytes, enteroendocrine cells, and goblet cells; however, macrophages and dendritic cells are also closely located (Czerner et al., 2014).

Still, the question remained whether hematopoietic or non-hematopoietic cells within the colon are responsible for H₄R mRNA expression. To exclude hematopoietic cells from our analyses, we sorted EpCAM⁺ epithelial cells (Litvinov et al., 1994) out of the pool of crypt-derived single cells. In the obtained cell population that lacked CD45⁺ hematopoietic cells (data not shown), H₄R mRNA still was detected. Thus, all these data in combination conclusively indicate the H₄R expression in colon epithelial cells, thereby adding an additional example to the list of H₄R⁺ cell types of extra-hematopoietic origin.

The expression of H₄R mRNA does not necessarily signify functional protein expression. To meet this issue, organoids were generated out of colonic crypts (colonoids), enabling the *in vitro* expansion of colon epithelial cells. Cells obtained thereof can be seeded in cultures on permeable tissue culture insert membranes, where they differentiate into a coherent polarized monolayer that allows for experimentally distinguishing between the apical and the basolateral membranes (Moon et al., 2014). This system resembles the epithelial layer in the colon, including its barrier function, which is defined by the ability for a directed and selected transport of molecules and ions (i.e., charges), resulting in the generation of a physicochemical gradient that can be measured as TEER. Using this system, it is demonstrated that histamine reduces the TEER to about 50% of the untreated layer. This effect is mediated by the H₄R because it is totally blocked by the addition of the H₄R-specific antagonist JNJ7777120 (Thurmond et al., 2004) and because it is absent in epithelial layers generated from H₄R-deficient mice. Thus, mouse colon epithelial cells functionally express the H₄R. This conclusion is in direct contrast to that of the study by Wechsler et al. (2018) who did not detect any H₄R mRNA expression in EpCAM⁺

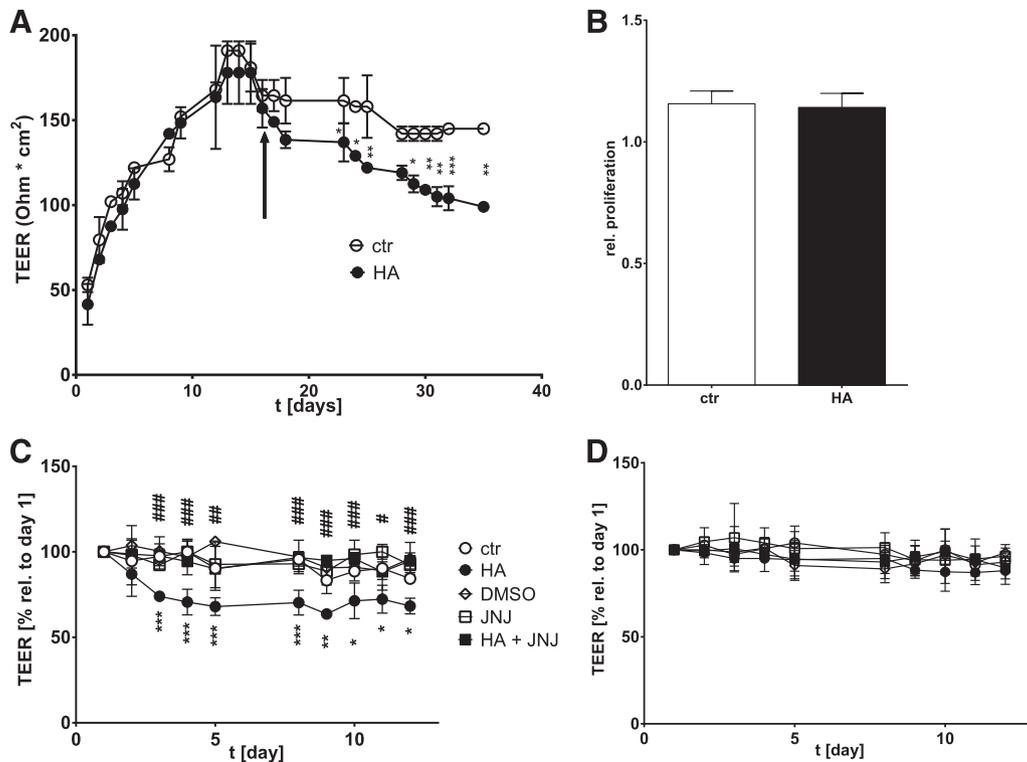


Fig. 6. Histamine reduces TEER in WT colonic epithelial cells through the H₄R. (A) Cells obtained from WT organoids were seeded at day 0 on permeable tissue culture inserts to generate cellular monolayers. TEER was measured at the indicated days, and 10 μ M HA was added to one half of the cultures at day 16 (vertical arrow). (B) Cells from WT organoids were seeded on permeable tissue culture inserts to generate cellular monolayers. After 1 week, 10 μ M HA was added, and after another 5 days, the cellular proliferation was analyzed (XTT assay). Cells obtained from WT (C) or H₄R^{-/-} (D) organoids were seeded on permeable tissue culture inserts to generate cellular monolayers. After 1 week in culture, the indicated agents (HA: 10 μ M, JNJ777120: 10 μ M, DMSO: 0.1% v/v) were added and TEER was followed over a period of 12 days. $n = 3$; *ctr vs. HA; #HA vs. HA + JNJ; *# $P < 0.05$; **## $P < 0.01$; ***### $P < 0.001$; two-way ANOVA with Tukey's post-test. HA, histamine; JNJ, JNJ777120.

mouse colon epithelial cells. A possible reason is the low abundance of H₄R mRNA in mouse colon epithelial cells. Although we have optimized the detection method in terms of sensitivity and specificity, Δ Rn values in our qPCR assays are still only at about 40. One might argue that such low Δ Rn values result from a false-positive detection, but the combination with the functional data rendered the H₄R expression conclusive. Lastly, this conclusion fits well to the proinflammatory function of the H₄R in DSS-induced acute colitis in mice (Schirmer et al., 2015, 2018). The H₄R-mediated reduction of the epithelial barrier function may promote antigenic spread into the mucosal tissue and subsequently the granulocytic infiltration.

Reduction of epithelial barrier function measured in vitro as decline of TEER induced by, for example, bacterial infection is a rapid process, occurring within hours (Ch Stratakos et al., 2017). The histamine-induced reduction of TEER in mouse colonoid-derived monolayers, however, is rather slow, reaching its maximum effect after an incubation of 2 to 3 days. The barrier function and thus TEER is regulated by the cellular cohesion within the epithelial layer. Tight junction proteins, which seal the paracellular space between epithelial cells, are responsible for this function (Bhat et al., 2019). A fast change of TEER within minutes to hours can be attributed to the redistribution of tight junction proteins toward the cell periphery (Barreau and Hugot, 2014). In the present study, the H₄R-driven decline in the TEER of colon-derived epithelial monolayers has manifested within a period of several days, indicating a transcriptional regulation instead of a spatial

reorganization of proteins. Thus, the expression of a series of 16 genes encoding junctional proteins that are described to be involved in colonic barrier function was assessed but without revealing any histamine-induced alterations (data not shown).

In conclusion, this study provides evidence that colon epithelial cells obtained from BALB/cJ mice express the H₄R, which probably mediates the proinflammatory function of histamine in experimental colitis by affecting the epithelial barrier function. Thus, targeting the H₄R in IBD with the growing repertoire of H₄R antagonists (Thurmond et al., 2017) is a promising therapeutic approach. In this context, it will be of particular importance to show a functional expression of the H₄R in human colon epithelial cells, too. Finally, based on these data, the molecular mechanisms at the bases of the inflammatory effects of the H₄R still have to be elucidated in an appropriate epithelial model system, as has been done before, such as for analysis of G-protein coupling in Sf9 cells (Schneider and Seifert, 2017).

Acknowledgments

The authors are grateful for excellent support and suggestions by Dr. D. Jonigk (Institute of Pathology, Hannover Medical School) and Dr. G. Graßl (Institute of Medicinal Microbiology, Hannover Medical School).

Authorship Contributions

Participated in research design: Schirmer, Neumann.
Conducted experiments: Schirmer, Lindemann, Bittkau, Isaev, Bösche, Juchem.

Performed data analysis: Schirmer, Lindemann, Bittkau, Isaev, Bösche, Seifert, Neumann.

Wrote or contributed to the writing of the manuscript: Schirmer, Seifert, Neumann.

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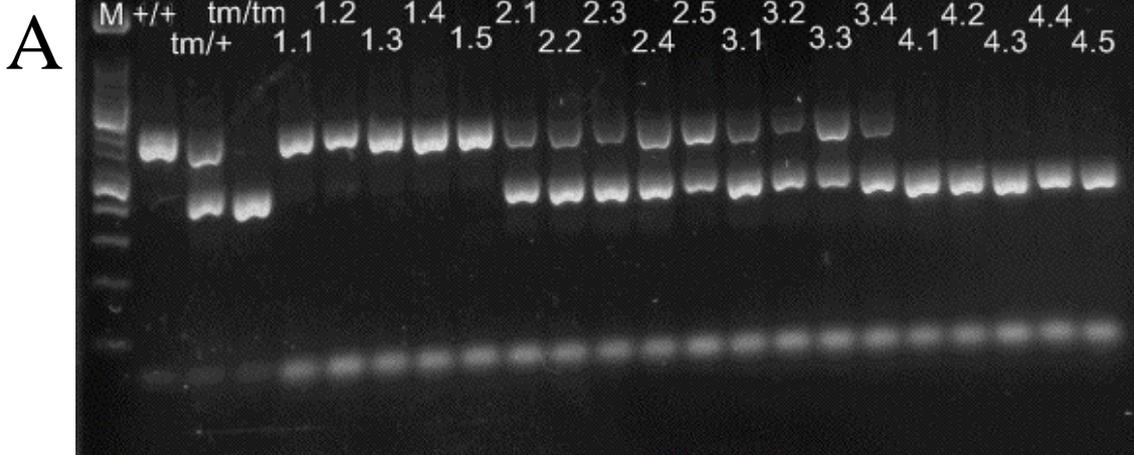
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**MOUSE COLONIC EPITHELIAL CELLS FUNCTIONALLY EXPRESS THE
HISTAMINE H₄ RECEPTOR**

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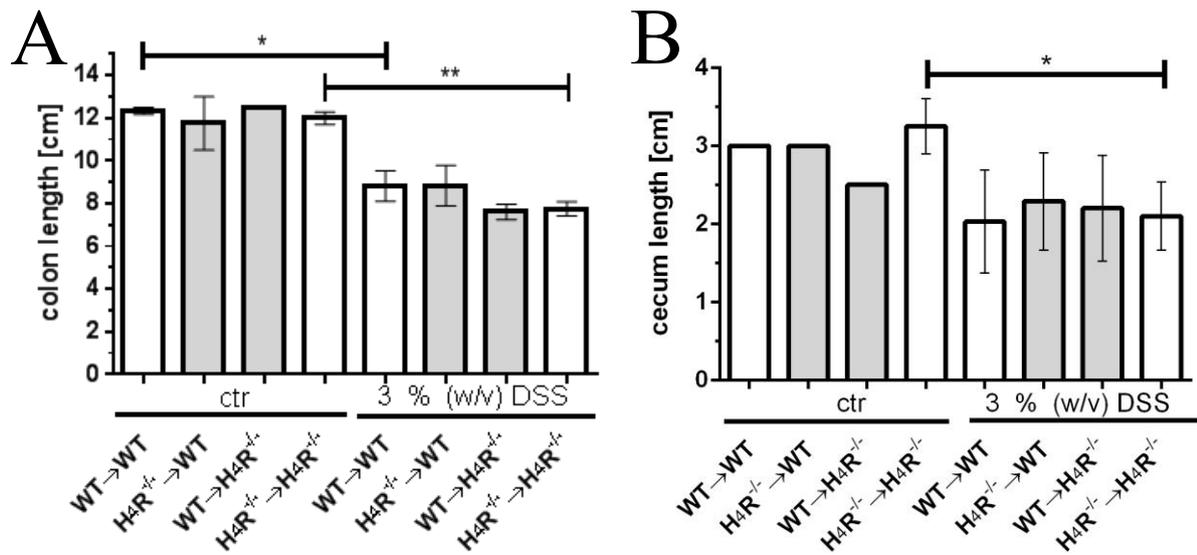
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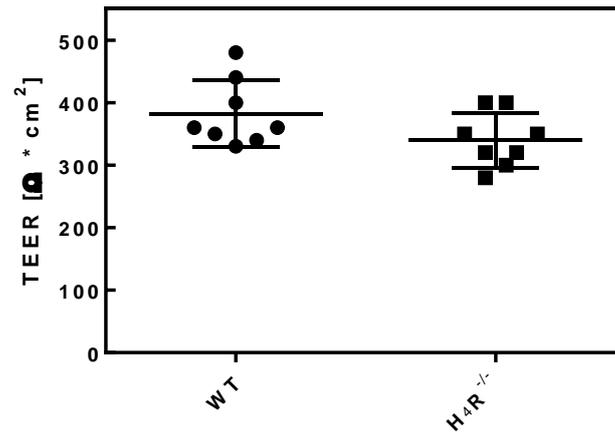
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| Animal No. | genotype (hair follicles/ tail tip) | genotype (blood) | experimental group |
|------------|-------------------------------------|------------------|--|
| 1.1 | +/+ | +/+ | WT→WT |
| 1.2 | +/+ | +/+ | WT→WT |
| 1.3 | +/+ | +/+ | WT→WT |
| 1.4 | +/+ | +/+ | WT→WT |
| 1.5 | +/+ | +/+ | WT→WT |
| 2.1 | tm/tm | tm/+ | WT→H ₄ R ^{-/-} |
| 2.2 | tm/tm | tm/+ | WT→H ₄ R ^{-/-} |
| 2.3 | tm/tm | tm/+ | WT→H ₄ R ^{-/-} |
| 2.4 | tm/tm | tm/+ | WT→H ₄ R ^{-/-} |
| 2.5 | tm/tm | tm/+ | WT→H ₄ R ^{-/-} |
| 3.1 | +/+ | tm/+ | H ₄ R ^{-/-} →WT |
| 3.2 | +/+ | tm/+ | H ₄ R ^{-/-} →WT |
| 3.3 | +/+ | tm/+ | H ₄ R ^{-/-} →WT |
| 3.4 | +/+ | tm/+ | H ₄ R ^{-/-} →WT |
| 4.1 | tm/tm | tm/tm | H ₄ R ^{-/-} →H ₄ R ^{-/-} |
| 4.2 | tm/tm | tm/tm | H ₄ R ^{-/-} →H ₄ R ^{-/-} |
| 4.3 | tm/tm | tm/tm | H ₄ R ^{-/-} →H ₄ R ^{-/-} |
| 4.4 | tm/tm | tm/tm | H ₄ R ^{-/-} →H ₄ R ^{-/-} |
| 4.5 | tm/tm | tm/tm | H ₄ R ^{-/-} →H ₄ R ^{-/-} |

Supplementary Figure 1: Validation of bone marrow chimerism in the mice. Analysis of chimerism was done by genotyping a whole blood sample of the mice 6 weeks after bone marrow transplantation. **(A)** Exemplary agarose gel analysis of PCR products with genotyping primers. +/+, tm/+, tm/tm: control lanes with PCR products from DNA derived from WT, heterozygous and H₄R^{-/-} mice, respectively. Further gel lanes are labeled according to **(B)**, which shows the assignment of animals to experimental groups based on their genotype established from the whole blood genotype assessment shown in **(A)** and the routine in-house genotype assessment of the mice by tail tip biopsies prior to irradiation.



Supplementary Figure 2: Lengths of cola (A) and ceca (B) dissected from mice after the observation period. WT→WT: WT donor, WT recipient; H4R^{-/-}→WT: H4R^{-/-} donor, WT recipient; WT→H4R^{-/-}: WT donor, H4R^{-/-} recipient; H4R^{-/-}→H4R^{-/-}: H4R^{-/-} donor, H4R^{-/-} recipient; n = 9 – 10 for DSS groups; n = 1 – 3 for water control groups. *, p < 0.05; **, p < 0.01; One-way ANOVA with Sidak's *post* test.



Supplementary Figure 3: Baseline TEER values do not differ significantly between WT and H4R^{-/-} mice. Cells obtained from WT or H4R^{-/-} organoids were seeded on permeable tissue culture inserts to generate cellular monolayers. After one week in culture, TEER was measured. (n = 8; no significant difference, p>0.05 with Student's t-test).