Loss of H2R Signaling Disrupts Neutrophil Homeostasis and Promotes Inflammation-Associated Colonic Tumorigenesis in Mice

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SUMMARY

This work highlights the immunomodulatory role of neutrophil-based histamine type 2-receptor signaling in intestinal inflammation and inflammation-associated colonic tumorigenesis.

BACKGROUND & AIMS: We previously showed that histamine suppressed inflammation-associated colonic tumorigenesis through histamine type 2 receptor (H2R) signaling in mice. This study aimed to precisely elucidate the downstream effects of H2R activation in innate immune cells.

METHODS: Analyses using online databases of single-cell RNA sequencing of intestinal epithelial cells in mice and RNA sequencing of mouse immune cells were performed to determine the relative abundances of 4 histamine receptors among different cell types. Mouse neutrophils, which expressed greater amounts of H2R, were collected from the peritoneum of wild-type and H2R-deficient mice, of which low-density and high-density neutrophils were extracted by centrifugation and were subjected to RNA sequencing. The effects of H2R activation on neutrophil differentiation and its functions in colitis and inflammation-associated colon tumors were investigated in a mouse model of dextran sulfate sodium–induced colitis.

RESULTS: Data analysis of RNA sequencing and quantitative reverse-transcription polymerase chain reaction showed that Hrh2 is highly expressed in neutrophils, but barely detectable in intestinal epithelial cells. In mice, the absence of H2R activation promoted infiltration of neutrophils into both sites of inflammation and colonic tumors. H2R-deficient high-density neutrophils yielded proinflammatory features via nuclear factor-κB and mitogen-activated protein kinase signaling pathways, and suppressed T-cell proliferation. On the other hand, low-density neutrophils, which totally lack H2R activation, showed an immature phenotype compared with wild-type low-density neutrophils, with enhanced MYC pathway signaling and reduced expression of the maturation marker Toll-like receptor 4.

CONCLUSIONS: Blocking H2R signaling enhanced proinflammatory responses of mature neutrophils and suppressed neutrophil maturation, leading to accelerated progression of...
Histamine elicits a variety of physiological processes via activation of histamine receptors (HRs), namely H1R, H2R, H3R, and H4R. We previously showed that histamine suppresses intestinal inflammation and the development of inflammation-associated colon tumors through H2R. Consistent with the importance of histamine in cancer development, Yang et al. showed that loss of endogenous histamine significantly inhibited neutrophil maturation, resulting in the acceleration of inflammation-associated colon cancer in dextran sulfate sodium (DSS)-treated histidine decarboxylase–deficient mice. However, the detailed effects of H2R downstream signaling on immune cells, inflammation, and cancer remain unclear.

Infiltration of inflammatory immune cells is a common feature in human cancers. Although immune cells, in theory, should respond to tumor development and help control tumor growth, as the tumor develops the cancer cell mechanisms evolve to evade immune cells and promote tolerance. Tumor immune tolerance is driven by the expansion of suppressor-cell populations, including myeloid-derived suppressor cells (MDSCs), which consist of granulocytic MDSCs and monocytic MDSCs that can suppress T-cell functions and promote tumor angiogenesis and metastasis. Of these, granulocytic MDSCs (neutrophils) are the predominant MDSC population in solid tumors and play a critical role in tumor-associated immune suppression. MDSCs inhibit the antitumor T-cell effector phase through various mechanisms, including producing reactive oxygen species (ROS) and arginase-1, reducing cytokine in the environment, up-regulating expression of programmed death-ligand 1 (PD-L1), promoting secretion of Gal-9, and producing S100A8/S100A9 (the endogenous Toll-like receptor [TLR] ligands). As a result, many research and clinical efforts currently are underway trying to suppress MDSC numbers and function in cancers.

Histamine has been shown to suppress MDSC functions and intratumoral infiltration of MDSCs. However, it is not clear which histamine receptor is responsible for this effect in MDSCs. H2R activation by histamine has been shown to inhibit neutrophil chemotaxis and leukotriene synthesis through adenosine 3',5'-cyclic monophosphate–dependent protein kinase signaling and to suppress reduced nicotinamide adenine dinucleotide phosphate oxidase activation induced by WKYMVM and N-formyl-methionyl-leucyl-phenylalanine in human neutrophils. Because reduced nicotinamide adenine dinucleotide phosphate oxidae–mediated ROS production is a key factor to suppress T-cell functions, it is reasonable to hypothesize that H2R activation may modulate MDSC functions.

In this study, we show that disruption of H2R signaling enhanced MDSC functions in vitro and significantly aggravated inflammation-associated colonic carcinogenesis in mice. Gene signature analysis showed the mechanisms by which H2R signaling promotes neutrophil maturation during granulopoiesis and suppresses proinflammatory responses in mature neutrophils.

**Results**

*Intestinal Hrh2 Is Expressed Almost Exclusively in Neutrophils*

We previously reported that histamine may affect the development of inflammation-associated colon cancer through different histamine receptors, H1R and H2R. However, expression of histamine receptors in different cell types has not been thoroughly examined. To address this point, we performed quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to compare mRNA quantities of Hrh1 and Hrh2 among mouse macrophages, neutrophils, T cells, and mouse colonic epithelium–derived colonoids. Hrh1 is expressed in macrophages and colonic epithelial cells, while Hrh2 is expressed predominantly in neutrophils, but not in intestinal epithelial cells (Figure 1A).

Taking advantage of available online single-cell RNA sequencing data (available from: singlecell.broadinstitute.org), we confirmed that in mouse intestinal epithelial cells (GEO: GSE92332), less than 0.2% of total cells expressed Hrh1, and approximately 3% of enteroendocrine cells expressed Hrh3. Intestinal epithelial cells did not harbor Hrh2 or Hrh4 beyond negligible quantities (Figure 1B). Using another data set of human colonic epithelial cells from patients with ulcerative colitis [UC) and healthy controls (access: SCP259), we observed that approximately 4% of intestinal epithelial cells expressed HRRH1, while neither HRRH2 or HRRH4 were expressed in any cell type in the intestinal epithelium of healthy individuals. HRRH3 was not included in this study because to its relative absence of expression in human intestinal epithelial cells (Figure 1C).

Further analysis of Hrh2 expression levels in various immune cell types in mice was performed using the Gene Skyline database (available from: http://rstats.immgen.org/Skyline/skyline.html). Hrh2 was found to be highly expressed in neutrophils isolated from multiple tissues, including bone marrow, spleen, and thioglycolate-
Figure 1. H2R is highly expressed in neutrophils. (A) Relative expression levels of Hrh1 and Hrh2 in different cell types of female C57/B6 mice by qRT-PCR (n = 4). (B) The gene expression pattern of histamine receptors in mouse intestinal epithelial cells obtained from the single-cell portal (GEO: GSE92332). (C) The gene expression pattern of histamine receptors in human intestinal epithelial cells obtained from the single-cell portal (SCP259). (D) The relative gene expression of Hdc, histamine receptors, and surface markers in different immune cells obtained from the Gene Skyline database (http://rstats.immgen.org/Skyline/skyline.html). (E) The gene expression pattern of histamine receptors in human immune cells analyzed using the Human Immune Cell Gene Viewer. mDC, ■; mRNA, messenger RNA; NK, ■; PBMC, ■; pDC, ■; TA, ■; Th, ■; Treg, ■.
stabilized peritoneal cells, while Hrh1, Hrh3, and Hrh4 were not expressed in neutrophils (Figure 1D). Similar expression patterns of histamine-receptor genes also were observed in human neutrophils (Platform: Human Immune Cell Gene Viewer) (Figure 1E), suggesting that histamine signaling may influence neutrophil function through H2R. Increased Hdc expression, which promotes histamine production, was observed in neutrophils, suggesting that activation of H2R in neutrophils may be autocrine. It is worth noting that Hdc also is expressed in peritoneal macrophages, but at a much lower level compared with that in neutrophils.

**H2R Deficiency Promoted Development of Inflammation-Associated Colon Cancer**

To evaluate H2R functions in cancer development, we crossed H2R KO and Apcremin/+ mice to generate double-mutant mice. Using DSS treatment of Apcremin/+ mice, a widely used model to examine inflammation-associated colon cancer,26 we observed multiple tumors in the middle and distal colon of Apcremin/+ mice by colonoscopy after 2 cycles of DSS treatment (Figure 3A). We observed larger and more abundant tumors in H2R KO/Apcmin/+ double-mutant mice compared with WT Apcmin/+ mice (Figure 3A). Further examination under a dissecting microscope showed greater numbers of colonic tumors in double-mutant mice (19.43 ± 1.59 tumors/mouse) compared with control mice (5.71 ± 0.52 tumors/mouse) (Figure 3B), as well as increased tumor size (Figure 3C), suggesting that H2R signaling suppresses both tumorigenesis and tumor growth in chronic inflammation. Moreover, neutrophil infiltration was increased in colonic tumors from H2R KO/Apcmin/+ mice compared with Apcmin/+ control mice (Figure 3D and E).

Cancer cells commonly evade the immune system by aberrantly expressing the protein PD-L1. PD-L1 suppresses the adaptive immune system and promotes a worse outcome for many cancers. Increased neutrophil numbers are associated positively with PD-L1 expression in CRC.25 In Apcmin/+ control mice, PD-L1 was rarely expressed in the colonic tumor cells, with a rate of 12.5% (1 of 8 mice). In Apcmin/+ mice lacking H2R, PD-L1 expression was detected in approximately 90% (7 of 8) of colonic tumors (Figure 3F and G). Interestingly, neutrophils were found around tumor cells (red signals) where PD-L1 was expressed (Figure 3H). Tumor-associated PD-L1 expression might be related to increased numbers of H2R-deficient neutrophils in the local tumor environment.

The acquisition of invasive properties by tumor cells is a key event during tumor development. Immunofluorescence with proliferating tumor cells (PCNA in red) and smooth muscle cells (SMA in green) showed that colon tumor cells invaded into submucosa in more than 70% of the H2R KO/ Apcremin/+ mice (7 of 9), whereas no tumors in the Apcmin/+ control mice showed such invasive lesions (Figure 3I and J). These findings show that H2R signaling in mucosal neutrophils may suppress tumor cell invasion.

**RNA Sequencing Showed Distinct Gene Signatures for Low- and High-Density Neutrophils**

Neutrophils can be separated into high-density and low-density fractions by density gradient centrifugation. Most high-density cells are mature granulated neutrophils (high-density neutrophils [HDNs]), whereas low-density neutrophils (LDNs) are immature granulated neutrophils and mature degranulated neutrophils.23 We first separated casein-elicted mouse peritoneal cells into HDNs and low-density cells, and then further purified LDNs within the low-density cells using a negative selection kit (Figure 4A).

ApcMin/
The purity for HDNs and LDNs (CD11b⁺Ly6g⁺) was determined to be 98% and 92%, respectively, by flow cytometry (Figure 4B). We performed RNA sequencing on HDN and LDN cells from both WT and H2R-deficient mice (GSE162850). RNA sequencing identified 941 up-regulated genes in WT HDN populations (Padjusted < .05; average fold change, >1.4) (Figure 4C), and 800 up-regulated genes in WT LDN populations (Padjusted < .05; average fold change, >1.6). To fully explore the gene signature of the whole transcriptome, especially to capture low-amplified genes that may be below the signal-to-noise significance cut-off level, we applied unbiased Gene Set Enrichment Analysis using whole HDNs and LDN transcriptome. At a conventional threshold of $P < .05$ and a false-discovery rate of less than 0.25, 8 and 11 pathways were enriched significantly in LDNs and HDNs, respectively. We found that LDNs...
harbor enriched immature/pro-proliferative pathways, such as increased expression of MYC pathways, E2F targets, and G2M checkpoints (Figure 4D). Figure 4E and F show the enriched gene map and gene list of MYC targets, respectively. On the contrary, HDNs showed mature/proinflammatory features, such as increased expression of the genes involved in tumor necrosis factor (TNF) signaling via the nuclear factor-κB (NF-κB) pathway, interferon-γ...
pathway, and the interleukin (IL)6 Janus kinase signal transducer and activator of transcription pathway (Figure 4G). As an example, enrichment of TNF signaling via the NF-κB pathway and the corresponding gene lists are shown in Figure 4H and I. The relative expression levels of representative genes for MYC targets, NF-κB pathway, and surface markers were verified by qRT-PCR (Figure 5A–C). These unique gene signatures of mouse HDNs and LDNs showed the distinct functions of mature and immature neutrophils.

Although CXCR2 and TLR4 are known to be neutrophil differentiation markers,24,25 no reports at present have used these markers to differentiate mature and immature neutrophils. We performed immunofluorescence for S100A8, CXCR2, and TLR4 on HDNs and LDNs. As expected, most HDNs showed positive staining for both CXCR2 and TLR4, confirming their mature features. However, in some LDNs, low CXCR2 expression was detected with no detectable level of TLR4 (Figure 5D). Therefore, TLR4 should be used as a maturation marker rather than as a differentiation marker. In our RNA sequencing data, we also observed greater expression of the endogenous ligands for TLR4 S100a8 and S100a9 in HDNs than in LDNs (Figure 5E), suggesting that HDNs harbor more proinflammatory features.

**H2R Deficiency Inhibited Neutrophil Maturation**

Ly6G and CD11b are early neutrophil markers, expressed even in the promyelocyte stage.26 Flow cytometry analysis did not show a significant difference in the CD11b+Ly6G+ cell population between H2R KO and WT cells from bone marrow and in blood (Figure 6A), indicating that H2R deficiency did not affect the initial neutrophil lineage commitment. We next sought to determine whether H2R signaling was involved in neutrophil maturation. RNA sequencing showed 929 down-regulated genes (Padj < .05; average fold change, >1.4) and 496 up-regulated genes (Padj < .05; average fold change, >1.4) in H2R KO LDNs compared with WT LDNs. The top genes of each group are shown in the heatmap (Figure 6B). Strikingly, loss of H2R, confirmed by exon use (Figure 6C), in LDNs further enhanced the gene expression of proinflammatory pathways, such as the MYC and E2F target pathways (Figure 6D). The enrichment map of MYC targets and the corresponding gene list are included in Figure 6E and F, respectively.

Interestingly, WT LDNs expressed relatively greater levels of inflammatory pathway genes than H2R-deficient LDNs (Figure 6G). The enrichment map of inflammatory response and inflammatory pathway gene list indicated a proinflammatory phenotype of WT LDNs (Figure 6H and I). In H2R KO LDNs, we observed increased expression of MYC targets (Figure 6J), and decreased levels of proliferation transcription factors, such as CEBP family members (Figure 6K). We also found reduced cell surface markers, such as Cxcr2, Tlr4, and Itgam (Figure 6L), and increased quantities of growth factors such as Hdgf (Figure 6M) in H2R KO LDNs compared with WT LDNs. These findings suggest that H2R signaling plays a central role during neutrophil maturation. Based on our RNA sequencing data, we speculate that H2R activation suppresses pro-proliferative signals, such as MYC and MYC targets, thereby promoting cell maturation.

Our data identified that Hrh2 expression was correlated negatively with Myc expression in neutrophils. Moreover, a negative correlation was found between expression of Hrh2 and Myc in a spectrum of mouse and human immune cells (Figure 7A and B). This phenomenon also was found in pathologic settings, such as in leukocytes in pneumonia (Figure 7C) and in acute myeloid leukemia (Figure 7D). In fact, increased TCF4 gene expression, an important transcription factor of MYC, was shown in the H2R KO LDNs compared with WT LDNs by RNA sequencing (Figure 7E). We therefore speculate that H2R signaling might suppress MYC expression.

All neutrophils, both mature and immature, originate from bone marrow. By S100A8, TLR4 and CXCR2 staining, we found more TLR4-negative neutrophils in the bone marrow of H2R-deficient mice (Figure 6A and B), consistent with our pathway and gene expression data. Cell-cycle analysis showed a significant increase in S-phase cells and a decrease in G0/G1-phase cells in H2R-deficient bone marrow compared with WT bone marrow (Figure 8A), suggesting that H2R signaling activation may suppress cell proliferation. H2R-deficient mice showed similar pathologic phenotypes during colitis, as previously shown in Apcmin/- mice. Consistent with the results using H2R-deficient neutrophils, histidine decarboxylase deficiency also resulted in increased Myc expression in both HDNs and LDNs (Figure 8A). These findings suggest that histamine suppresses Myc expression and promotes neutrophil maturation through H2R signaling.

**Figure 3. (See previous page).** H2R deficiency promoted the development of inflammation-associated colon cancer in Apc<sup>min/+</sup> mice. (A) Representative images taken during colonoscopy of mice at the end of the second recovery phase. (B and C) Tumor numbers were counted and measured in the entire colon of Apc<sup>min/+</sup> (n = 10) and Apc<sup>min/-</sup>; H2R KO (n = 9) mice under a dissecting microscope. (D) Infiltrating neutrophils stained with S100A8 antibody (green) (magnification: 40×, 200×, and 1000×). (E) The mean numbers of neutrophils in 8 fields at 200× power for each Apc<sup>min/+</sup> (n = 4) and each Apc<sup>min/-</sup>; H2R KO (n = 4) mouse. (F) Immunofluorescence of S100A8 (green) and PD-L1 (red) for colon tumors (magnification: 40×, 200×, and 400×). The red arrow in the top panel indicates the Apc<sup>min/+</sup> tumor cells that do not express PD-L1, and the red arrow in the bottom panel indicates the tumor cells that express PD-L1 in Apc<sup>min/-</sup>; H2R KO mice. (G) Incidence of PD-L1–positive tumors for Apc<sup>min/+</sup> (n = 8) and Apc<sup>min/-</sup>; H2R KO (n = 8) mice. (H) Immunofluorescence (magnification: 40× and 200×) of S100A8 (green) and PD-L1 (red) for Apc<sup>min/+</sup>; H2R KO tumors. (I) Immunofluorescence (magnification: 40× and 200×) of proliferating cell nuclear antigen (PCNA) (red) and SMA (green). The arrow indicates invading tumor cells through the muscularis mucosa into the submucosa. (J) Percentage of invasion in tumors of Apc<sup>min/+</sup> (n = 9) and Apc<sup>min/-</sup>; H2R KO (n = 9) mice. In all panels, means ± SD are shown. *P < .01 and **P < .001. DAPI, 4′,6-diamidino-2-phenylindole.
Figure 4. The distinct gene signatures of LDNs and HDNs. (A) Schematic illustration showing how LDNs and HDNs are isolated from peritoneal cells. (B) The purity of HDNs and purified LDNs was determined by flow cytometry using CD11b and Ly6g antibodies. (C) The heat map of representative genes of LDNs and HDNs. (D) Identification of the top pathways enriched in LDNs were assessed by gene set enrichment analysis with Hallmark gene sets. (E and F) The enrichment plot and gene list of MYC targets in HDNs and LDNs. (G) Identification of the top pathways enriched in HDNs were assessed by gene set enrichment analysis with Hallmark gene sets. (H and I) The enrichment plot (H) and enriched genes (I) of TNFα signaling pathway via NF-κB. WH, ____; WL, ____.
H2R Signaling Modulated Neutrophil Chemotaxis and Mitogen-Activated Protein Kinase Signaling

As "first responders" during infection, neutrophils can be activated by different pathogen-associated molecular patterns through multiple highly expressed Toll-like receptors. Activation of pathogen-associated molecular patterns triggers downstream pathways, such as NF-κB and mitogen-activated protein kinase (MAPK) pathways, to induce proinflammatory cytokine production. The activation of NF-κB signaling and MAPKs in response to lipopolysaccharide (LPS) stimulation in neutrophils was determined by Western blot, using antibodies against phosphorylated iκB, phosphorylated ERK, and phosphorylated p38 MAPK. LPS treatment strongly induced the activation of NF-κB in H2R-deficient HDNs compared with control HDNs. On the other hand, LPS induced NF-κB activation in control LDNs, but not in H2R-deficient LDNs (Figure 9A), suggesting that H2R activation suppresses NF-κB signaling in mature and immature neutrophils during inflammation or cancer.

We previously showed that H2R activation suppresses phosphorylation of ERK and p38 in macrophages and colorectal cancer cells. Consistent with these findings, we found enhanced ERK phosphorylation in H2R-deficient neutrophils compared with WT neutrophils without LPS stimulation. Moreover, LPS treatment induced stronger p38 activation in H2R-deficient neutrophils than in the WT neutrophils, especially in HDNs (Figure 9A). The activation of these key proinflammatory pathways could explain more severe intestinal inflammation in H2R-deficient mice.

Consequently, MagPix (Luminex Corporation) was used to analyze the production of cytokines induced by LPS in HDNs and LDNs. Figure 5 confirms the distinct gene signatures of LDNs and HDNs. (A–C) Expression of representative genes in MYC target, NF-κB pathways, and surface markers of LDNs and HDNs by qRT-PCR (n=4). Means ± SD are shown. The P value is shown under each panel. (D) Immunofluorescence staining (magnification: 200× and 1000×) of S100A8 (green), CXCR2 (pink), and TLR4 (red) in LDNs and HDNs. (E) The expression levels of S100a8 and S100a9 in LDNs and HDNs by qRT-PCR (n=4). Means ± SD are shown. P values are shown on the panel. CXCR2, ______; DAPI, 4',6-diamidino-2-phenylindole.
HDNs and LDNs. The relative concentrations of IL1α, IL1β, IL6, IL10, and TNF were increased significantly after LPS treatment for 6 hours in the H2R-deficient HDNs than in control HDNs (Figure 9B). However, this phenotype was not observed in H2R-deficient LDNs (Figure 9C). We speculate that this result may be owing to reduced TLR4 expression in H2R KO LDNs compared with control LDNs. Increased amounts of IL1β also were found in neutrophils in mucosal inflammation (Figure 9D), as well as in colonic tumors (Figure 9E). In addition to IL1β protein, an increase in the...
expression of IL1β receptor 1 gene Il1r1 was observed in tumor samples (Figure 9F), suggesting that the IL1 pathway could be more potently activated in H2R-deficient mice. In human colorectal cancer, increased IL1R1 expression was associated with significantly worse outcomes (Figure 9G), suggesting the potential involvement of the IL1 pathway in the pathogenesis of colon cancer.

LPS also has been shown to induce PD-L1 expression in human polymorphonuclear leukocytes. We found that LPS treatment promoted CD274 (PD-L1 gene) expression in all neutrophils, especially in LDNs by qRT-PCR (Figure 10A). This finding suggests that neutrophils might directly suppress T-cell proliferation through the PD-L1/PD1 axis. Next, we evaluated the suppressive effects of HDNs and LDNs on CD8+ T-cell proliferation in vitro. Similar to other studies, we found that HDNs, the mature neutrophils, can suppress CD8+ T-cell proliferation (Figure 10B). Interestingly, LDNs were not found to suppress T-cell proliferation. One of the mechanisms MDSCs use to suppress T-cell function is through generation of ROS. We found that the H2R-deficient HDNs enhanced suppression of T-cell proliferation compared with the WT HDNs (Figure 10C); however, this effect was not modified by the addition of a ROS scavenger, N-acetylcysteine. These findings suggest that

Figure 7. The reverse correlation between the expression levels of Hrh2 and Myc. (A and B) The expression patterns of Hrh2 (dashed green line) and Myc (dashed red line) in mouse immune cells obtained using the (A) Gene Skyline database, and in human immune cells using the (B) Human Immune Cell Gene Viewer platform. (C and D) The expression of Hrh2 and Myc in leukocytes of (C) pneumonia and in (D) acute myeloid leukemia (AML) through the R2 platform. (E) Tcf4 expression in LDNs of WT and H2R-deficient mice (n = 4) by qRT-PCR. All values are means ± SD. **P < 0.01, mDC, ; NK, ; PBMC, ; pDC, ; Th, ; Treg, .

Figure 6. (See previous page). H2R deficiency altered features of LDNs. (A) Neutrophil populations (CD11b+ Ly6g+) in bone marrow (n = 10) and in blood (n = 7) of WT and H2R-deficient mice. (B) A heat map of representative genes of WT and H2R-deficient LDNs. (C) The confirmation of Hrh2 gene deletion (exons 2 and 3) by exon use by RNA sequencing. (D) Identification of the top pathways that are enriched most significantly in H2R KO LDNs were assessed by gene set enrichment analysis using Hallmark gene sets. (E and F) The enrichment plot and gene list of MYC targets in WT and H2R-deficient LDNs. (G) Identification of the top pathways that are enriched most significantly in WT LDNs were assessed by gene set enrichment analysis using Hallmark gene sets. (H and I) The enrichment plot and gene list of the inflammatory response pathway in WT and H2R-deficient LDNs. (J-L) The expression of representative genes in MYC target, CEBP family members, and surface markers of WT and H2R-deficient LDNs by qRT-PCR (n = 4). (M) Hdhg expression in HDNs and LDNs of WT and H2R-deficient mice (n = 4) by qRT-PCR. All values are means ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001. CEBP, ; KL, ; WL, .
H2R-deficient HDN-mediated T-cell suppression is independent of ROS production. In contrast to N-acetylcysteine, T-cell suppression by H2R-deficient HDNs was enhanced significantly by adding N-formylmethionyl-leucyl-phenylalanine (fMLP), a compound that activates neutrophils (Figure 10D). It has been shown that histamine inhibits neutrophil mobility via H2R signaling.24 We observed that H2R deficiency promoted random migration, as well as fMLP-mediated chemotaxis, in both HDNs and LDNs (Figure 10E). This is in line with the in vivo phenotype, in that more neutrophils infiltrated the areas of intestinal inflammation and colonic tumors.

**H2R Antagonism Suppressed Neutrophil Maturation and Promoted Inflammation-Associated Colon Cancer**

Finally, we tested whether an H2R antagonist, cimetidine, also could aggravate colonic inflammation and promote colon tumor development. Compared with control neutrophils, LDNs from cimetidine-treated mice showed significantly increased levels of expression of MYC target genes, and reduced expression of surface markers, including Tlr4, Cxcr2, and Itgam (Figure 11A–C). Furthermore, an increase in the S phase and a decrease in the G0/G1 phase of bone marrow cells also were observed in cimetidine-treated mice (Figure 11D), suggesting that inhibition of H2R by cimetidine promotes cell proliferation in the bone marrow. In cimetidine-treated mice, more and larger tumors were visualized by colonoscopy and direct macroscopic observation compared with WT controls (Figure 11E and F). Consistent with the results of the H2R-deficient mice, most cimetidine-treated mice developed squamous metaplasia in the distal colon during DSS-induced chronic inflammation, which was not observed in the control mice (Figure 11G and H).

Importantly, cimetidine treatment promoted PD-L1 expression in the DSS-induced colon tumors in ApcMin/+ mice (Figure 11I and J), which is consistent with the phenotypes found in H2R-deficient mice. In line with the preceding in vitro results, cimetidine significantly enhanced the suppression of CD8+ T-cell proliferation by HDNs (Figure 11K). Collectively, our data showed that absence of H2R signaling shifts gene expression patterns of neutrophils toward creating a proinflammatory and protumorigenic microenvironment in the colon (Figure 12).

**Discussion**

Herein, we showed that H2R is highly expressed in neutrophils, and disruption of H2R signaling, either by genetic manipulation or by antagonists such as cimetidine, influences the maturation stage-specific features of neutrophils. Our study showed that H2R-deficient mature neutrophils harbored more proinflammatory features, which promoted increased colonic cancer pathophysiology. We also found that H2R-deficient immature neutrophils expressed growth factor–related genes, indicating a proproliferative phenotype. Our data indicate that H2R-inactivated neutrophils create a tumor-friendly microenvironment that may accelerate tumor development in mice. Our study details the role of H2R signaling in neutrophils and downstream consequences for inflammation-associated colon cancer development.

Physiologically, neutrophil homeostasis is strictly maintained from granulopoiesis (release from the storage in the bone marrow) to migration into peripheral blood and tissues.21 In the setting of inflammation or cancer, neutrophils are released from the bone marrow before full maturation, which is enhanced by a loss of endogenous histamine.4 Our study showed that histamine signaling is involved in neutrophil maturation and contributes to the central role of neutrophils in innate immunity. Inactivation of H2R resulted in significant changes in the relative expression of genes involved in multiple pathways (eg, MYC and E2F) in neutrophils. Our data indicate that H2R activation may suppress proliferation of immature neutrophils. Our results are consistent with recent reports showing that H2R activation is required to promote quiescence of myeloid-biased
Considering that MYC is not expressed in the quiescent HSCs, we believe that H2R activation contributes to the maintenance of the quiescent status of HSCs through suppressing MYC expression. Moreover, immature neutrophils lacking H2R activation secrete more pro-proliferative factors, including HDGF, into the environment. Cancer cell-derived HDGF has been shown recently to promote carcinogenesis of colorectal cancer. We therefore speculate that release of HDGF from neutrophils may promote neighboring tumor cells to proliferate in a paracrine manner, creating an environment conducive to tumor proliferation. Conversely, increased H2R gene expression may result in tumor growth by suppressing MYC gene expression in tumor cells and the secretion of growth factors.

Figure 9. H2R deficiency activated the IL1 signaling pathway. (A) Western blot of HDNs and LDNs treated with LPS using antibodies against p-iKBa, p-ERK, total ERK, p-p38, total p38, adenosine 3',5'-cyclic monophosphate-dependent protein kinase (PKA) substrate, and β-actin. (B) The cytokine production of WT and H2R KO HDNs treated with LPS by MagPix (n = 3). (C) IL1β production in WT and H2R KO LDNs treated with LPS (100 ng/mL) by MagPix (n = 3). (D and E) Immunofluorescence staining of S100A8 and IL1β for inflammation and for inflammation-associated colon tumors (magnification: 40× and 400×). (F) The expression levels of Il1r1 in the colon tumors of ApcMin/+ and ApcMin/+; H2R KO mice by qRT-PCR (n = 4). Means ± SD are shown. *P < .05. (G) The association between IL1R1 expression in cancers and overall survival in CRC patients through the R2 database. Ctrl, control; DAPI, 4',6-diamidino-2-phenylindole; GM-CSF, __________; IFN, interferon.
factors from neutrophils. Our findings indicated that targeted strategies to suppress H2R signaling may be a potent therapeutic strategy for cancer.

Neutrophils are major contributors to both acute and chronic inflammation and commonly are present in patients with inflammatory bowel disease, especially UC. The colonic mucosa of UC patients who were hospitalized, received systemic corticosteroids, or underwent colectomy, were found to harbor an increased number of neutrophils. Neutrophil resolution has been associated with improvement of inflammation and long-term favorable outcomes for UC patients, suggesting that manipulation of neutrophil function via H2R is a potentially useful treatment strategy for UC. Consistent with our data, another study has shown that oral administration of cimetidine for 1–7 days increased neutrophil counts in the peripheral blood of healthy adults. In addition to yielding increased neutrophil counts, cimetidine treatment counteracts the suppressive effect of H2R on the proinflammatory features of mature neutrophils. Our results are consistent with the studies showing that administration of H2R antagonists increased the frequency of hospitalization and surgery in patients with UC.

The administration of cimetidine has been shown to be associated with an increased risk for multiple cancers, such as intestinal, lymphatic, and hematopoietic cancers, by several studies. In the tumor environment, in the absence of H2R activation, mature neutrophils produce more cytokines, chemokines, ROS, and enzymes, further strengthening MDSC activity and their impact on T-cell functions. We observed a link between H2R deficiency and neutrophil IL1β levels. IL1β may promote production of PD-L1 by cancer cells. Most PD-L1–expressing tumor cells were found in close proximity to H2R-deficient neutrophils in vivo, indicating that blocking H2R in neutrophils may enhance PD-L1 expression in tumor cells, promoting cancer.
progression. Some clinical studies have shown that after surgical removal of the primary tumor, CRC patients appeared to have an increased tumor-free survival when treated with combination therapy with cimetidine.\textsuperscript{40} However, the results were inconsistent, and, moreover, no study to date used cimetidine alone, making it difficult to conclude that cimetidine has any protective effect against CRC.

Figure 11. Cimetidine administration suppressed neutrophil maturation and promoted inflammation-associated colon cancer. (A and B) Gene expression of MYC targets and surface markers in the cimetidine-treated and control LDNs (n = 4). (C) Representative immunofluorescence staining (magnification: 400× and 1000×) of S100A8 (green) and TLR4 (red) in LDNs from the cimetidine-treated and control mice. (D) Cell-cycle analysis of bone marrow cells from the cimetidine-treated and control mice (n = 5). (E) Tumors in the middle colon examined by a colonoscopy (top), macroscopic observation of the colon (bottom). (F) Tumor numbers counted under a dissecting microscope (n = 8). (G and H) Rectal SM was observed (magnification: 40× and 200×) after 2 cycles of DSS treatment in the cimetidine-treated mice (n = 7), but not in the control mice (n = 9). (I and J) Immunofluorescence staining (magnification: 200× and 400×) of S100A8 (green) and PD-L1 (red) in the tumors of the cimetidine-treated (n = 6) and control (n = 6) mice. (K) Suppression of CD8$^+$ T-cell proliferation by cimetidine-treated and control HDNs (n = 3). All values are means ± SD. (A and B) P values are shown under the panels. *P < .05, **P < .01, and ***P < .001. Cim, cimetidine; Ctrl, control; DAPI, 4',6-diamidino-2-phenylindole; PC, positive control.
cells can promote mutations by producing ROS in colonic epithelial stem cells, possibly leading to CRC initiation. Based on these findings, we believe that neutrophil-mediated mucosal inflammation may be an initiating driver in colonic neoplasia. However, human cohort studies regarding H2R signaling and targeted interventions for CRC are needed in future studies.

In conclusion, disruption of H2R signaling enhanced the tumorigenic microenvironment by supporting the relative proliferation of immature, proinflammatory, and actively proliferating neutrophils. These studies support the potential importance of innate immunity in the initiation and development of colonic neoplasia. Therefore, H2R signaling and corresponding antagonism of these pathways should be carefully considered in the context of prevention and treatment strategies for inflammatory bowel disease and CRC.

Materials and Methods

Mouse Models

Hrh2 KO mice were generated as previously described by the Genetically Modified Mouse Core at Baylor College of Medicine using CRISPR-Cas9 method.49 Hrh2 KO; Apcmin+/+ mice were generated by crossbreeding Apcmin−/− mice (C57BL/6J background) and Hrh2 KO mice (C57BL/6J background). All mice were maintained under specific pathogen-free conditions with a 12-hour light and 12-hour dark cycle in the Feigin Tower vivarium at Texas Children’s Hospital. To induce colonic inflammation, 2-month-old female mice were given 2% DSS (molecular weight, 36,000−50,000; MP Biomedicals, Solon, OH) in drinking water for 5 days followed by a 14-day recovery period, and a second cycle of DSS for 4 days followed by a 14-day recovery period. To test the effects of an H2R antagonist on tumor development, Apcmin−/− mice were given cimetidine (H2R antagonist, 200 mg/L) in drinking water from the start of the first cycle of DSS treatment until the end of the second recovery period. After the second recovery period, a mouse colonoscopy was performed and imaged. Mice were then killed, and the tumors in the colon were counted under a dissecting microscope. The intestinal tissues were fixed in formalin and embedded in paraffin for histologic analysis. Inflammation scores were calculated using a scoring system by combining tissue damage and inflammatory cell infiltration.49 All procedures were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

Mouse Neutrophil Isolation and Purification

Mouse neutrophils were elicited, and high- and low-density neutrophils were collected according to a published protocol. Briefly, 1.0 mL sterile casein solution (9%) was injected twice into the peritoneal cavity of each mouse with a 12-hour interval. Three hours after the second injection, mice were killed and neutrophils in the peritoneal cavity were recovered with 10 mL phosphate-buffered saline (PBS). The peritoneal cells were layered on a discontinuous Histopaque gradient (1.077 g/mL and 1.119 g/mL;
Sigma, St. Louis, MO). High-density neutrophils were harvested from the 1.077–1.119 interface. Low-density cells, which are a heterogeneous population including neutrophils, macrophages, and lymphocytes, were harvested from the PBS-1.077 interface. The low-density neutrophils were purified further using a MojoSort Mouse Neutrophil Isolation Kit (BioLegend, San Diego, CA). The neutrophil purity was confirmed by flow cytometry using anti-CD11b, Ly6G antibodies (Table 1). Neutrophils were identified as CD11b+ Ly6G+ cells.

**T-Cell Suppression**

Mouse CD3+ T cells were purified from splenocytes of WT mice using a negative selection kit (BioLegend). Cells were stained with 10 umol/L CFSE (Sigma) in PBS containing 0.1% bovine serum albumin at 37°C for 10 minutes, washed with cold RPMI-1640, and resuspended with X-VIVO 15 Serum-free Hematopoietic Cell Medium. Cells then were seeded onto 96-well plates coated with CD3 (5 µg/mL) and CD28 (5 µg/mL) at 5 x 10^4/well. The same number of neutrophils was added to each well. Cells were treated with N-acetylcysteine (1 mmol/L; Sigma), fMLP (1 µmol/L; Sigma), or left untreated as a control. After 5 days of incubation at 37°C, the proliferative capacity of T cells (CD4+ and CD8+) was analyzed by flow cytometry based on CFSE signal in a fluorescein isothiocyanate channel.

**Neutrophil Chemotaxis**

Neutrophil chemotaxis was determined using a disposable chemotaxis system (Neuro Probe, Bethesda, MD) with a 3-µm pore filter membrane. fMLP (1 µmol/L) in Dulbecco’s modified Eagle medium or media control was added to the lower chamber compartment. Mouse neutrophils (1 x 10^5 cells in 30 µL) were seeded on the top of the 3-µm pore filter membrane. The plate was incubated for 1 hour at 37°C in a 5% CO2 incubator. The neutrophils that had migrated into the lower chamber were counted using an automated cell counter (Invitrogen, Grand Island, NY).

**Cell-Cycle Analysis**

Bone marrow cells from 2-month-old female mice were obtained by flushing the femurs with sterile PBS, and then the bone marrow cells were passed through 70-µm filters. Cells were fixed with 70% cold ethanol at 4°C overnight. After being washed with cold PBS twice, the cells were treated with 0.2 µg/mL RNase A (Qiagen, Valencia, CA), and then stained with 0.5 µg/mL propidium iodide (Sigma) in the dark for 30 minutes at room temperature. DNA content was determined by a FACSCanto flow cytometer (BD Biosciences, San Jose, CA) and percentages of cells in different phases of the cell cycle were assessed. The distribution of cells at different cell-cycle phases was analyzed using the model included in the ModFit LTTM (Verity Software House, Topsham, ME) software program.

**Luminex MagPix Analysis of Cytokines**

Supernatants from neutrophils were assayed using a cytokine magnetic bead panel (cat. MCYTOMAG-70K; Millipore, Billerica, MA) with a MagPix instrument according to the manufacturer’s protocol. Raw data were obtained with a

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**Table 1. Antibody Information**

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<thead>
<tr>
<th>Antibody</th>
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Immunoﬂuorescence Staining

Immunoﬂuorescence was performed on 5-μm-thick, formalin-ﬁxed, parafﬁn-embedded slides that were deparafﬁnized with xylene, hydrated with a series of washes using graded alcohols, followed by heat-induced antigen retrieval with Tris-EDTA buffer (pH 8.0, 1 mmol/L EDTA, 10 mmol/L Tris) for 30 minutes. After a PBS wash, slides were blocked with 10% goat sera for 30 minutes, and then incubated with primary antibodies (Table 1) in TBS-T buffer at 4°C overnight. After washing with TBS-T, slides were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) (Table 1) for 60 minutes, followed by 4',6-diamidino-2-phenylindole counterstaining and imaging by ﬂuorescence microscopy (Nikon, Tokyo, Japan). Antibodies are listed in Table 1.

Immunoblosts

Neutrophils were lysed with RIPA buffer (Thermo Fisher Scientiﬁc, Waltham, MA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). After centrifugation, the supernatants were resolved by sodium dodecyl sulfate–polyacylamide gel electrophoresis and transferred to polyvinylidene diﬂuoride membranes (Millipore). The membranes were blocked with 5% dry milk in PBS-T for 30 minutes, washed with PBS-T, and then incubated with primary antibodies overnight at 4°C. After 3 washes with TBS-T, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature, and developed with ECL substrate (GE Health Care, Buckinghamshire, UK) after TBS-T washes.

RNA Extraction, RNA Sequencing, and qRT-PCR Analysis

Total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD) followed by puriﬁcation with a RNeasy Mini Kit (Qiagen). RNA sequencing was performed at Genewiz (South Plainﬁeld, NJ). The RNA samples that met the criteria for sequencing were processed for library preparation using standard Illumina protocols. High-throughput paired-end (2 × 150 bp) sequencing was performed on a HiSeq platform (Illumina). Sequencing reads that detected splice junctions and incorporated them to help align the entire read sequences. BAM ﬁles were generated as a result. Complementary DNA was prepared with 1 μg total RNA using the SensiFAST Complementary DNA Synthesis kit (Bioline, Taunton, MA). Quantitative PCR then was performed using Fast SYBR Green (Life Technologies) on an Applied Biosystems 7900HT instrument. Ribosomal RNA (18S) was used as a control. Primers are included in Table 2.

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F, forward; R, reverse.

Table 2. Primer Information
Relative levels of gene expression were analyzed using the comparative CT method (2-ΔΔCT method).

**Flow Cytometry**

Neutrophils were incubated first with Fc-block (BD Biosciences) in 100 μL PBS at 4°C for 15 minutes and then with antibodies (Table 1) at 4°C for 30 minutes in the dark. The cells were washed in PBS, spun down, and resuspended with 200 μL PBS. The cell samples were examined using a FACSCanto flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (version10.5.3; FlowJo LLC, Ashland, OR).

**Colonoid Culture**

The procedure of colonoid culture was modified from the protocol described by Fernando et al. Briefly, mouse colon was cut into approximately 1-cm pieces and washed with cold PBS for 5 minutes at 4°C with end-to-end shaking. The tissues then were incubated in cold PBS containing 3 mmol/L EDTA, dithiothreitol, and sucrose for 30 minutes at 4°C with shaking. After vigorous shaking with cold PBS, the crypts were pelleted and embedded in Matrigel (Corning, Tewksbury, MA). After incubation for 20 minutes at 37°C, the solidified Matrigel was overlaid with complete crypt culture media containing EGF (50 ng/mL; R&D Systems, Minneapolis, MN) and 10 μmol/L Y-27632 (Sigma), an inhibitor of Rho-associated protein kinase, to block apoptosis.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism version 5.04, and statistical significance was determined by an unpaired, 2-tailed, Student t-test or 1-way analysis of variance as follows: *P < .05, **P < .01, and ***P < .001.

**References**


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Yuko Mori-Akiyama, MD (Conceptualization: Equal; Formal analysis: Equal; Supervision: Lead; Writing – original draft: Lead; Writing – review & editing: Lead);
Wa Du, PhD (Data curation: Supporting; Formal analysis: Supporting; Investigation: Supporting; Methodology: Supporting; Software: Lead; Writing – original draft: Supporting; Writing – review & editing: Supporting);
Robert Fultz, PhD (Data curation: Supporting; Investigation: Supporting; Methodology: Supporting; Writing – original draft: Supporting);
Yanling Zhao, PhD (Data curation: Supporting; Investigation: Supporting; Writing – original draft: Supporting);
Wenly Ruan, MD (Data curation: Supporting; Investigation: Supporting; Writing – review & editing: Supporting);
Susan Venable (Data curation: Supporting; Investigation: Supporting; Writing – original draft: Supporting);
Melinda Anne Engevik, PhD (Data curation: Supporting; Formal analysis: Supporting; Funding acquisition: Supporting; Investigation: Supporting; Methodology: Supporting; Writing – original draft: Supporting; Writing – review & editing: Supporting);
James Versalovic, MD, PhD (Conceptualization: Equal; Funding acquisition: Lead; Investigation: Equal; Resources: Lead; Supervision: Lead; Writing – review & editing: Supporting).

Conflicts of interest
This author discloses the following: James Versalovic has received unrestricted research support from BioGaia AB, and serves on the scientific advisory boards of Seed Health, Biomica, and Plexus Worldwide. The remaining authors disclose no conflicts.

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