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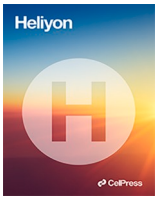

Research article

Analysis of the effect of hypusination in myeloid cells on colitis and colitis-associated cancer

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ABSTRACT

Hypusine is an amino acid synthesized by the enzyme deoxyhypusine synthase (DHPS). It is critical for the activity of eukaryotic translation initiation factor 5A (EIF5A). We reported that hypusination *i*) in macrophages supports the innate response towards pathogenic bacteria and *ii*) in epithelial cells maintains intestinal homeostasis. Herein, we investigated the effect of myeloid hypusination on the outcome of colitis and colitis-associated cancer. We found that patients with Crohn's disease exhibit increased levels of DHPS and EIF5A^{Hyp} in cells infiltrating the colon lamina propria. However, the specific deletion of *Dhps* in myeloid cells had no impact on clinical, histological, or inflammatory parameters in mice treated with dextran sulfate sodium (DSS). Further, tumorigenesis and level of dysplasia were not affected by myeloid deletion of *Dhps* in the azoxymethane-DSS model. The composition of the fecal and the mucosa-associated microbiome was similar in animals lacking or not DHPS in myeloid cells. Thus, hypusination in myeloid cells does not regulate colitis associated with epithelial injury and colitis-associated cancer. Enhancement of the DHPS/hypusine pathway in patients with inflammatory bowel disease could have therapeutic impact through epithelial effects, but modulation of hypusination in myeloid cells will be unlikely to affect the disease.

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1. Introduction

Inflammatory bowel disease (IBD) consisting of ulcerative colitis (UC) and Crohn's disease (CD) is a major public health problem and its incidence continues to increase worldwide [1,2]. Of importance, IBD increases risk for colorectal cancer (CRC), which has the 3rd highest incidence and the 2nd highest mortality rate of all cancers. It has been estimated that up to 20 % of patients with UC will develop colitis-associated cancer (CAC) [3,4]. While improvements in therapy for IBD have improved quality of life for patients [5], development of CAC remains an important issue. In this context, deciphering the molecular and cellular events that initiate and maintain colon inflammation and neoplastic transformation is important for IBD patients and could reduce risk for CAC, and potentially be useful for other forms of CRC.

Polyamines are organic cations with numerous biological effects [6,7], notably in the gastrointestinal tract [8–14]. There are 3 main polyamines in mammalian cells, namely putrescine, spermidine, and spermine. Putrescine is synthesized by the rate-limiting enzyme, ornithine decarboxylase (ODC), and is then converted to spermidine and to spermine by the enzymes spermidine synthase and spermine synthase, respectively. The regulation of polyamine levels also occurs through the back-conversion action of spermine to spermidine and putrescine by *i*) spermidine/spermine N1-acetyltransferase and polyamine oxidase, which acetylates spermine and spermidine and oxidizes N1-acetyl-spermine and N1-acetyl-spermidine into spermidine and putrescine, respectively, and *ii*) the enzyme spermine oxidase (SMOX) that oxidizes spermidine to produce spermine, 3-aminopropanal and hydrogen peroxide [6,7]. We reported decreased SMOX levels in the colon tissues of IBD patients [13] and that *Smox*$^{-/-}$ mice exhibit low colonic spermidine levels and exacerbated colitis [12,13]. Moreover, treatment with spermidine improves experimental colitis and reduces colon tumorigenesis in WT and *Smox*$^{-/-}$ mice [13].

Spermidine is the substrate for the generation of the non-proteinogenic amino acid hypusine by deoxyhypusine synthase (DHPS). This enzyme synthesizes deoxyhypusine, which corresponds to the N-terminal region of spermidine and the lysine-50 residue of the protein eukaryotic translation initiation factor 5A (EIF5A, also known as IF5A). Then, deoxyhypusine hydroxylase (DOHH) hydroxylates this intermediate [15] to complete the formation of hypusine. This unique post-translational modification of EIF5A [16,17], termed hypusination, leads to the binding and translation of specific mRNAs containing an AAAUGU consensus sequence [18–20]. In addition, hypusinated EIF5A (EIF5A^{Hyp}) suppresses ribosomal pausing during the translation of peptides enriched in diprolyl and diglycyl motifs [21–23]. Hypusination is essential for embryonic development and *Dhps*-deficient mice are not viable [24].

Hypusination in myeloid cells is a critical mediator of the innate response towards pathogenic bacteria [25]. DHPS is induced in macrophages by enteric pathogens and increases hypusination of EIF5A, thus facilitating the translation of proteins involved in autophagy and with antimicrobial properties [25]. Consequently, mice with specific deletion of *Dhps* in myeloid cells and infected with *Citrobacter rodentium*, the rodent equivalent of the human pathogen enteropathogenic *Escherichia coli*, show reduced EIF5A^{Hyp} in macrophages and more colonization and inflammation in the colon [25]. In the same way, integrated omics analysis of macrophages from animals with metabolic inflammation has shown myeloid EIF5A^{Hyp} supports M1 polarization and the development of a pro-inflammatory response [26]. In this context, we aimed to test the hypothesis that the DHPS/hypusine pathway in myeloid cells influences the outcome of colon inflammation and carcinogenesis under conditions of mucosal injury without infection.

2. Methods

2.1. Mice, models of colitis and CAC

We used co-housed littermate C57BL/6J *Dhps*$^{fl/fl}$ and *Dhps*$^{fl/fl}$;*Lyz2*$^{cre/cre}$ female and male mice (*Dhps*$^{\Delta mye}$) mice [25] fed with 5L0D chow (LabDiet).

For the colitis model, 8-12 wk-old mice were treated for 5 days with 4 % dextran sulfate sodium (DSS; TdB Labs) and then maintained on regular water until euthanasia at day 10 [12,13,27]. For the model of CAC, mice received one intraperitoneal injection of 12.5 mg/kg azoxymethane (AOM; Millipore) followed by 3 cycles of 4 % DSS for 4 days each, beginning at day 5, 26, and 47 [13,27]. Mice were euthanized at day 56.

Animals were monitored daily for the DSS model and weekly for the CAC model. After euthanasia, colons were harvested, measured, weighed, dissected, and Swiss-rolled for histology. In the CAC model, tumors were counted and measured in two dimensions in the distal colon with electronic calipers [13,27,28]. Tumor burden corresponds to the sum of areas of all tumors.

2.2. Histology

Colons were Swiss-rolled and fixed in formalin and embedded in paraffin, and sections (5 μm) were stained with hematoxylin and eosin (H&E) and examined by the gastrointestinal pathologist (M.K.W.) in a blinded manner. Histological injury score (0–40) was assessed for both the DSS and AOM-DSS models, and severity of dysplasia in the AOM-DSS model was scored as we reported [12,13,27,28].

2.3. Analysis of mRNA expression

RNeasy Mini kit (Qiagen) was used to extract total RNA. Reverse transcription was done with Superscript II Reverse Transcriptase and Oligo dT, and mRNAs were amplified using the PowerUp SYBR Green Master Mix and the following specific primers: Murine *Actb*: F, CCAGAGCAAGAGAGGTATCC and R, CTGTGGTGGTGAAGCTGTAG; murine *Cxcl1*: F, GCTGGGATTCACCTCAAGAA and R,

CTTGGGGACACCTTTTAGCA; murine *Il1b*: F, ACCTGCTGGTGTGTGACGTTCC and R, GGGTCCGACAGCACGAGGCT; murine *Il6*: F, AGTTGCCTTCTTGGGACTGA and R, TCCACGATTTCCCAGAGAAC; murine *Tnf*: F, CTGTGAAGGGAATGGGTGTT and R, GGTCACTGTCCCAGCATCTT; murine *Il17a*: F, ATCCCTCAAAGCTCAGCGTGTC and R, GGGTCTTCATTGCGGTGGAGAG; murine *Il22*: F, TTGAGGTGTCCAACTTCCAGCA and R, AGCCGGACGTCTGTGTTGTTA. We used the gene *Actb*, which encodes for β-actin, as the housekeeping gene. The semi-quantitative analysis was performed using the mean of all the untreated $Dhps^{fl/fl}$ mice as the reference sample.

2.4. Immunostaining

For CD3 and myeloperoxidase (MPO) immunohistochemistry (IHC), fixed tissues were deparaffinized, sequentially incubated with 3 % hydrogen peroxide and Protein Block Serum-free (Dako), and washed in Tris-HCl buffered saline-tween. Slides were then incubated *i*) overnight at 4 °C with a rabbit monoclonal anti-CD3 antibody (Ab; Abcam; 1:150) in Antibody Diluent (Dako) followed by EnVision + Single Reagent (HRP. Rabbit) from Dako for 30 min at room temperature, or *ii*) overnight at 4 °C with prediluted anti-MPO Ab (Biocare Medical; 1:500) and with MACH 2 Rabbit HRP-Polymer (Biocare Medical) for 30 min at room temperature. We used 3,3′-diaminobenzidine as chromogen, and tissues were counterstained by hematoxylin. The staining was analyzed in a blinded manner by our GI pathologist (M.B.P.).

For IF, deparaffinized tissues were treated with proteinase K (Dako) for 10 s, Universal Protein Block for 40 min, the anti-CD68 (Boster Biological Technology; 1:300) overnight incubation at 4 °C, and with the donkey anti-rabbit IgG (H + L) Highly Cross-Adsorbed secondary Ab, Alexa Fluor Plus 555 (Invitrogen; 1:600) for 45 min at room temperature. After washes, slides were sequentially treated with 5 % rabbit serum for 30 min and with 5 % AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch) for 30 min. Then, we used *i*) a rabbit anti-$EIF5A^{Hyp}$ polyclonal Ab (Millipore; 1:750) overnight at 4 °C followed by a donkey anti-rabbit IgG (H + L) Highly Cross-Adsorbed secondary Ab, Alexa Fluor Plus 488 (Invitrogen; 1:600) for 45 min at room temperature, or *ii*) a rabbit anti-DHPS polyclonal Ab (Proteintech; 1:800) overnight at 4 °C followed by a donkey anti-rabbit IgG (H +

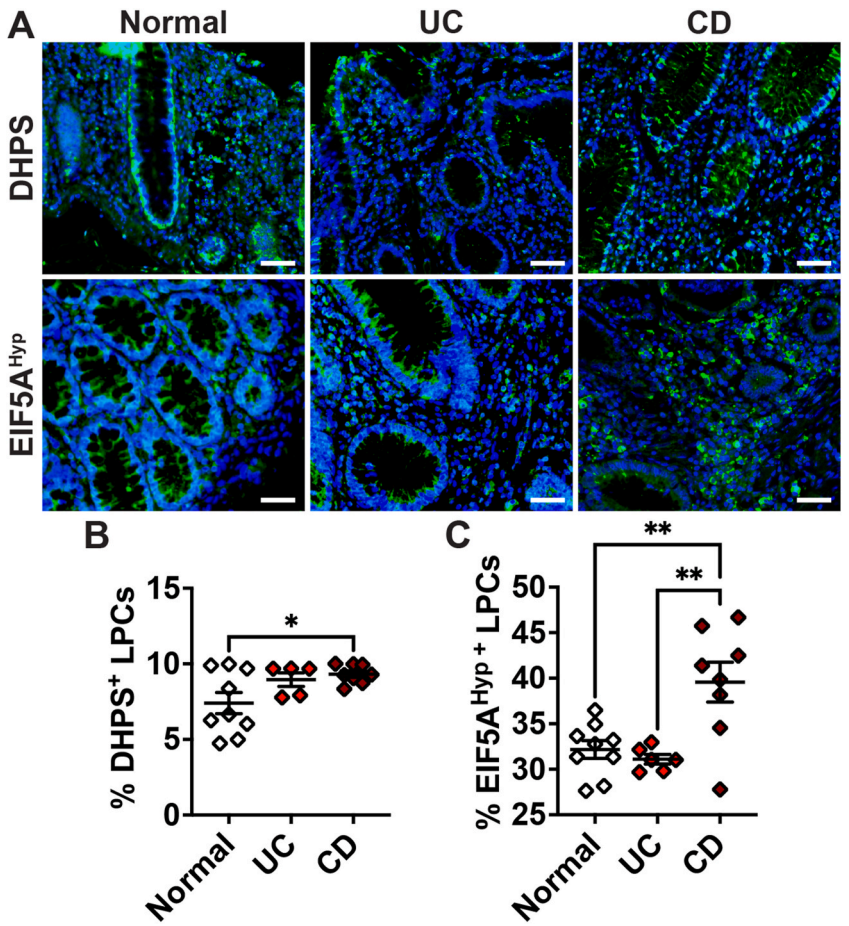

Fig. 1. Hypusination in LPCs from patients with colitis. Colon tissue sections of two TMAs were immunostained for DHPS or $EIF5A^{Hyp}$ and representative images of normal tissues ($n = 9$) or tissues from patients with UC ($n = 5$) or CD ($n = 8$) are shown (A); scale bar, 50 μm. Quantification of $DHPS^{+}$ (B) and $EIF5A^{Hyp+}$ (C) LPCs was performed using QuPath. $^{*}P < 0.05$, $^{**}P < 0.01$ by ANOVA and Tukey test.

L) Highly Cross-Adsorbed secondary Ab, Alexa Fluor Plus 488 (Invitrogen; 1:600) for 45 min at room temperature. We also stained the human colon IBD tissue microarrays (TMA; ref. CO245 and CO246, US Biomax Inc.). Tissue sections were blocked with Background Sniper (Biocare Medical; 45 min) at room temperature and then treated with the rabbit polyclonal anti-DHPS Ab (Abcam; 1:500) or the rabbit polyclonal anti-EIF5A^{Hyp} Ab (Millipore; 1:600) overnight at 4 °C. After washes, slides were treated with a donkey anti-rabbit IgG (H + L) Highly Cross-Adsorbed secondary Ab, Alexa Fluor Plus 488 (Invitrogen; 1:600) for 45 min at 37 °C. All the IF slides were then mounted with VECTASHIELD HardSet™ Antifade Mounting Medium with DAPI (Vector Laboratories). Slides were scanned in the Vanderbilt Digital Histology Shared Resource at Vanderbilt University Medical Center using the Leica Apiro Versa 200 platform and quantified using QuPath 0.3.2 [29].

2.5. Analysis of the fecal microbiota

Colon tissues and feces present in the colon were harvested from littermate *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta epi}$ mice (9 wks). DNA was extracted from the colon and the feces using the QIAGEN DNeasy Blood & Tissue kit and the QIAamp Fast DNA Stool kit, respectively. Sequencing of 16S rRNA genes, and analysis were performed as described [13,27].

2.6. Statistics

Figures were generated and statistics were performed using GraphPad Prism 10.1.0 software. Significance level was set as $P < 0.05$ and all statistical tests were two-sided. Data are depicted as the mean ± SEM. Data that were not normally distributed (D'Agostino & Pearson normality test) were transformed and distribution was re-assessed. The Student's *t*-test and the one-way/two-way ANOVA with the Tukey post-hoc test were used to determine significance between two or multiple groups, respectively.

For the analysis of the intestinal microbiota, the relative abundance of the genera was assessed by the Wilcoxon rank sum test after multiple comparison adjustment by the Benjamini & Hochberg method.

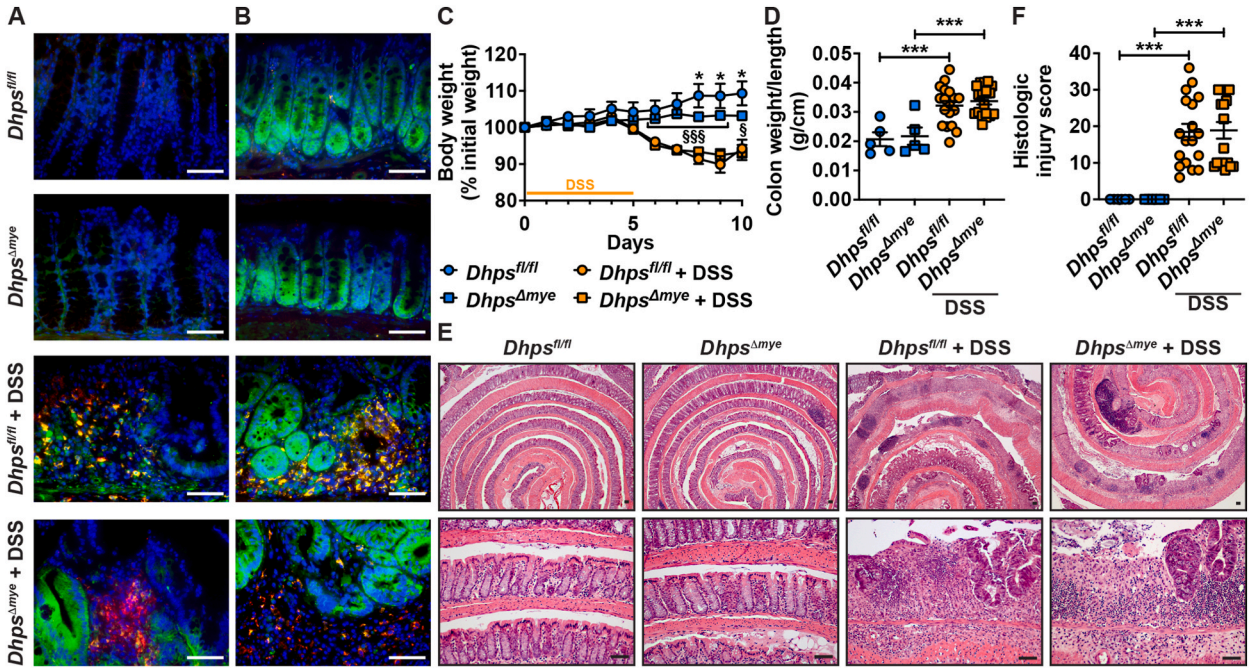

Fig. 2. DSS-induced colitis in *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice. Animals were treated or not with 4 % DSS. Expression of CD68 (red) and DHPS (green) (A) or CD68 (red) and EIF5A^{Hyp} (green) (B) was assessed in the colon by IF; merged, yellow; nucleus, blue. These pictures are representative of staining performed on 3 control mice and 4–5 DSS-treated mice per genotype. Body weights were measured daily and are shown as percentage of initial body weight (C); *$P < 0.05$ versus *Dhps*$^{fl/fl}$ mice + DSS and $^{§}P < 0.05$, $^{§§§}P < 0.001$ compared to DSS-treated *Dhps*$^{\Delta mye}$ mice, by two-way ANOVA and Tukey test. At sacrifice, colons were dissected, washed, weighed, and measured (D). Swiss-rolls were stained with H&E (E; scale bars, 50 μm) and analyzed to determine the histologic injury score (F). In (D) and (F), ***$P < 0.001$, ****$P < 0.0001$ by one-way ANOVA and Tukey test; *Dhps*$^{fl/fl}$, $n = 5$; *Dhps*$^{\Delta mye}$, $n = 4$; *Dhps*$^{fl/fl}$ + DSS, $n = 18$; *Dhps*$^{\Delta mye}$ + DSS, $n = 18$.

3. Results

3.1. Levels of DHPS and EIF5A^{Hyp} in patients with IBD

We previously reported that CD and UC patients show reduced levels of DHPS and EIF5A^{Hyp} in colonic epithelial cells [27]. We used the same TMA and staining to analyze the levels of these two proteins in lamina propria cells (LPCs) using QuPath 0.3.2. The images depicted in Fig. 1A show that DHPS and EIF5A^{Hyp} were less expressed in the epithelium of patients with UC and CD compared to normal individuals, as we reported [27]. However, we observed (Fig. 1A) and quantified that DHPS (Fig. 1B) and EIF5A^{Hyp} (Fig. 1C) levels were enhanced in colon LPCs from CD patients.

3.2. Myeloid cell-specific deletion of Dhps does not affect colitis severity

The deletion of *Dhps* in the colon of *Dhps*$^{\Delta mye}$ mice was previously reported by our laboratory [25]. We verified herein by IF the level of hypusination in the colon during DSS treatment. We found an increased number of cells double positive for the macrophage marker CD68 and for DHPS (Fig. 2A) or EIF5A^{Hyp} (Fig. 2B) in the colon of DSS-treated *Dhps*$^{fl/fl}$ mice, when compared to untreated animals. There was also less DHPS and EIF5A^{Hyp} staining in CD68^{+} cells from *Dhps*$^{\Delta mye}$ mice + DSS (Fig. 2A and B). The loss of *Dhps* in myeloid cells had no influence on the level of hypusination in CECs (Fig. 2A and B).

To assess the role of hypusination in myeloid cells on colon inflammation, we treated *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice with 4 % DSS, as a reliable model of epithelial injury-induced colitis. The kinetics of body weight loss induced by DSS treatment was similar between *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice (Fig. 2C). Macroscopically, the colon weight/length ratio was also similarly increased with DSS treatment in *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice (Fig. 2D). Histologically, contrary to mice with epithelial-specific deletion of *Dhps* that exhibit spontaneous colitis [27], the colon of naïve *Dhps*$^{\Delta mye}$ mice was identical to that of *Dhps*$^{fl/fl}$ animals, with no sign of inflammation (Fig. 2E and F). In DSS-treated mice, we observed a marked expansion of the colonic mucosa, loss of crypts, and infiltration of immune cells in the lamina propria and submucosa (Fig. 2E). The histologic injury score was similar in *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice (Fig. 2F).

Accordingly, the level of induction by DSS treatment of the genes encoding for the innate immune effectors CXCL1, TNF-α, IL-6, and IL-1β and for the prototype Th17 cytokines IL-17 and IL-22 was identical in the colon mucosa of *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice (Fig. 3A). Moreover, immunostaining and quantification of MPO (Fig. 3B and C) and CD3 (Fig. 3D and E) evidenced no difference in the recruitment of neutrophils/macrophages and T cells in the colonic mucosa between *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice.

3.3. Colon carcinogenesis is not altered by loss DHPS in myeloid cells

Mice with specific *Dhps* deletion in intestinal epithelial cells (IECs) are highly susceptible to CAC [27]. To test the role of

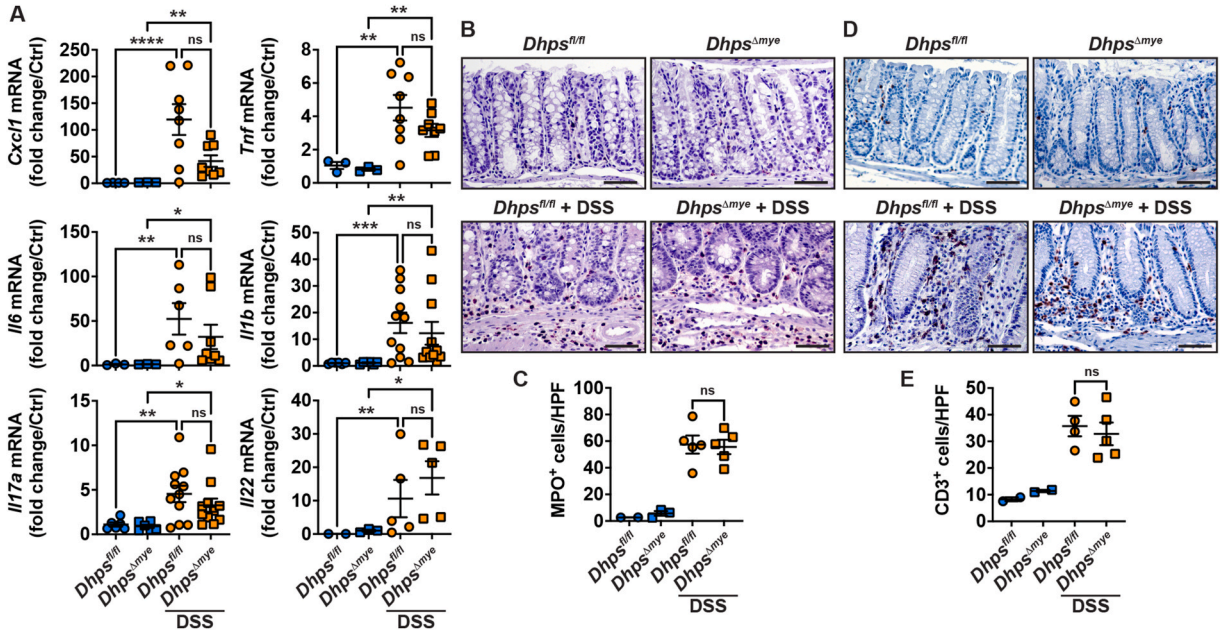

Fig. 3. Colonic immune response. *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice were treated or not with 4 % DSS. Gene expression in the colon was determined by RT-real time PCR (A). Tissues were also stained for MPO (B) or CD3 (D); scale bar, 50 μm. For quantification (C, E), each dot represents the mean of the number of positive cells determined per high power field (HPF; 40X) performed on 20 fields per mouse. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, by one-way ANOVA and Tukey test.

hypusination in myeloid cells during colon carcinogenesis, we treated *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice with AOM-DSS, as a model of CAC. We observed that mice from both genotypes lost weight in a similar way at each cycle of DSS (Fig. 4A). The number of polypoid tumors (Fig. 4B) and the total tumor burden (Fig. 4C) was identical in *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice treated with AOM-DSS. Fig. 4D shows representative photomicrographs of H&E staining of the colon from *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice, evidencing a mild inflammation characterized by infiltration of immune cells into the mucosa and crypt damage, as well as tumors with low grade dysplasia (LGD). The scoring of inflammation and epithelial damage (Fig. 4E) and dysplasia (Fig. 4F) did not evidence differences between *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice. In addition, the level of CD3$^+$ cells infiltrating the tumors was identical in *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice that were given AOM-DSS (Fig. 4G and H).

3.4. *Dhps* deletion in myeloid cells does not modify the composition of the intestinal microbiota

We then analyzed the colon-associated and the fecal microbiome of littermate *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice by sequencing the V4 region of the 16S rRNA gene. Bacterial community diversity, assessed by Shannon and inverse Simpson indexes (Fig. 5A), and total richness, determined by the Chao1 index (Fig. 5B), were similar between both genotypes. The fecal microbiota of *Dhps*$^{fl/fl}$ mice was dominated by the Bacteroidetes phylum (Fig. 5C), including the genera *Bacteroides*, *Prevotella*, and *Porphyromonadaceae* (Fig. 5D), as previously shown in C57BL/6 mice [13,27]. The Firmicutes phylum (Fig. 5C) was also abundant in the fecal microbiota and was mainly represented by the *Lachnospiraceae* genus (Fig. 5D). Interestingly, most of the microbiota associated with the colon mucosa belonged to the Proteobacteria phylum (Fig. 5C), and more specifically to the *Helicobacter* genus and, to a lesser extent,

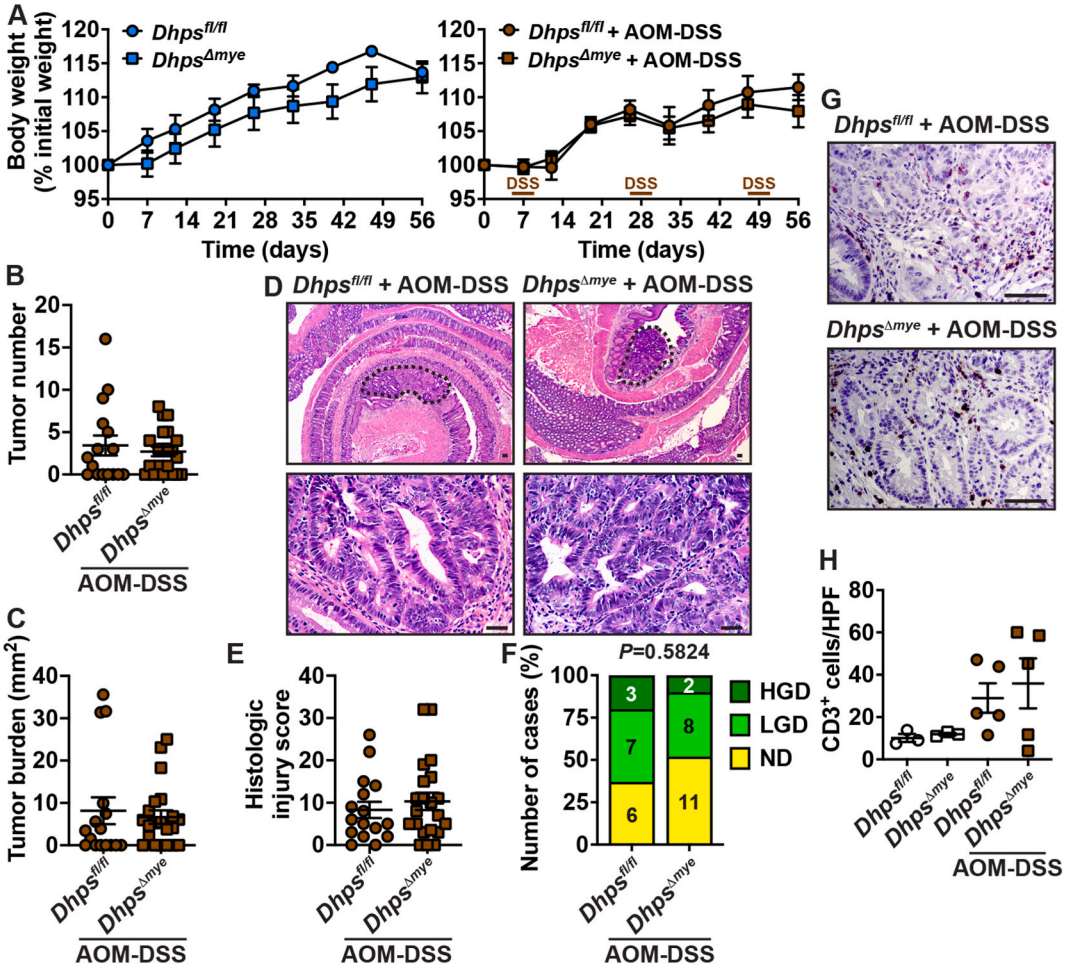

Fig. 4. Effect of myeloid *Dhps* deletion on colon carcinogenesis. *Dhps*$^{fl/fl}$ ($n = 16$) and *Dhps*$^{\Delta mye}$ ($n = 21$) mice were treated with AOM-DSS; there were also *Dhps*$^{fl/fl}$ ($n = 4$) and *Dhps*$^{\Delta mye}$ ($n = 7$) mice that did not receive AOM-DSS. Body weights are depicted as percentage of initial body weight (A). Colons were harvested, opened longitudinally, and tumors were counted (B) and measured in two dimension to determine the total tumor burden (C). H&E staining (D; scale bars, 50 μm) was used to assess histologic injury (E) and the grade of dysplasia (F); ND, no dysplasia. None of the sham-treated animals developed tumors. The number of CD3$^+$ cells infiltrating the tumors was determined by IHC (G) and quantification (H) in 2–10 HPF per mouse; these pictures are representative of staining performed on 3 AOM-DSS-treated mice per genotype. In (F), the *P* value was calculated by Chi-square test.

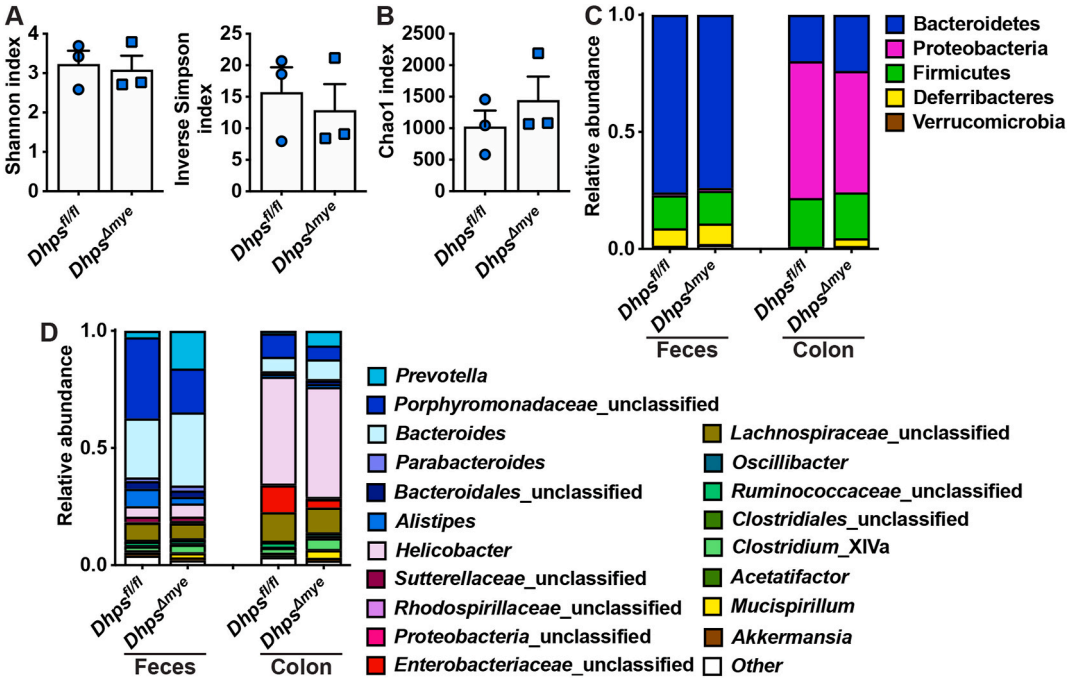

Fig. 5. Fecal and mucosa-associated microbiomes. DNA was extracted from colonic feces and from a piece of colon of 3 *Dhps*$^{fl/fl}$ and 3 *Dhps*$^{\Delta mye}$ mice. Subsequently, the 16S rRNA genes were sequenced. Alpha diversity was determined by the Shannon and Inverse Simpson indexes (A) and richness was assessed by the Chao1 index (B). Composition of the bacterial community at phylum (C) and genus (D) levels was expressed as a ratio to the total community.

Enterobacteriaceae (Fig. 5D). *Prevotella*, *Porphyromonadaceae*, *Bacteroides*, and *Lachnospiraceae* were also part of the dominant microbiota associated with the mucosa (Fig. 5D). Of importance, we did not find significant differences in the composition of the microbiota between *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice at the phylum or genus level (Fig. 5C and D).

4. Discussion

EIF5A hypusination is a highly conserved mechanism that regulates the translation of specific proteins. Innate immunity, which is also evolutionarily conserved, is the first line of defense against pathogens and is notably orchestrated by myeloid cells. Therefore, it is not surprising that DHPS is upregulated in macrophages by pathogenic bacteria and that EIF5A^{Hyp} supports the translation of proteins involved in the antimicrobial response and autophagy [25]. Using a model of intestinal infection by the pathogen *C. rodentium*, we found that *Dhps*$^{\Delta mye}$ mice exhibit reduced ability to kill phagocytized bacteria, increased colon colonization, and consequently enhanced infectious colitis [25]. Although we show that DHPS and EIF5A^{Hyp} levels are enhanced in cells infiltrating the lamina propria of CD patients, we found in the present report that the specific deletion of *Dhps* in myeloid cells has no impact on clinical and histological parameters during experimental colitis caused by DSS, an agent that initiates disease by injuring the epithelium. Moreover, the recruitment of MPO$^+$ and CD3$^+$ cells and level of expression of the genes involved in the pro-inflammatory response in the colon is similar between *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice. Further, we also evidenced that loss of DHPS in myeloid cells does not affect tumorigenesis in the AOM-DSS model.

The three polyamines are critical regulators of gastrointestinal inflammation and carcinogenesis. Notably, mice lacking *Odc1*, the gene encoding for ODC, in myeloid cells exhibit better clinical and histologic parameters compared to floxed mice when treated with DSS [11]. Myeloid *Odc1* deletion is also associated with a reduction of tumorigenesis and development of polyps with HGD [11]. These data demonstrate that the activity of myeloid ODC, which generates the first polyamine putrescine, augments epithelial injury-associated colitis and CAC. Importantly, we also found that specific deletion of *Odc1* in IECs has no effect on DSS colitis [11]. Additionally, our group has demonstrated that DSS colitis and AOM-DSS-induced CAC are worsened in mice lacking SMOX [12,13], an enzyme that generates spermidine from spermine. Moreover, treatment of WT and *Smox*$^{-/-}$ mice with spermidine protects from colitis, CAC, and sporadic CRC induced by specific deletion of the gene encoding for the adenomatous polyposis coli (APC) protein in colonic epithelial cells [13]. These data support the contention that, contrary to putrescine, spermidine protects from colon inflammation and neoplasia. Lastly, we recently published that specific deletion of *Dhps* in IECs results in dysregulation of colon homeostasis and development of spontaneous intestinal inflammation in mice [27]. Further, these mice are extremely susceptible to DSS treatment and exhibit more tumors when treated with the carcinogen AOM [27]. Importantly, we observed that spermidine supplementation in AOM-DSS-treated mice increases the level of EIF5A^{Hyp} in the colon [27]. Therefore, all these data from previous works and from the present study suggest that synthesis of putrescine in macrophages supports colitis and CAC independently of hypusine formation,

whereas epithelial spermidine/hypusine protects from intestinal inflammation and carcinogenesis independently of ODC. The discovery that the DHPS-EIF5A^{Hyp} axis in CD4^{+} T cells prevents colon inflammation [30] makes the scenario even more complex. This polyamine- and cell-specific network highlights that treatment of colitis and/or prevention of colon carcinogenesis using polyamines themselves or global inhibitors of polyamine/hypusine require more targeted approaches but remains a promising field of investigation.

We have reported that total *Smox* deletion dysregulates of the fecal microbiome, with a decrease of *Prevotella* prevalence and an increase of *Porphyromonadaceae* and the mucin-degrading bacterium *Mucispirillum schaedleri* [13], which has been associated with colitis [31] and colon tumorigenesis [32,33]. This dysbiosis was reversed towards the WT microbiome in mice that were given spermidine, indicating that this polyamine plays a key role in affecting the composition of the microbiota and is critical for its resilience. However, the fecal microbiome of mice lacking DHPS in IECs exhibits the same diversity and richness, and overall similar abundance of the classical phyla and genera as *Dhps*$^{fl/fl}$ mice [27]. Herein, we found that both the fecal and the colon-associated microbiota are similar in littermates of *Dhps*$^{fl/fl}$ and *Dhps*$^{\Delta mye}$ mice. We can then propose that if spermidine supports the establishment of a healthy microbiota, the hypusine pathway in the colon does not affect its composition.

To conclude, this current study shows that hypusination of EIF5A in myeloid cells does not affect the outcome of epithelial injury-induced colitis and the development of CAC. It is probable that increased hypusination has an impact on myeloid cells, as we reported [25], but our data suggest that these modifications of the myeloid proteome orchestrated by DHPS has no major impact on colon inflammation associated with epithelial damage. One limitation of our study is that we did not assess the effect of myeloid hypusine in a model of T cell-mediated colitis, which is comparable to CD, or in a model of genetically-mediated CRC. We are currently developing mice with inducible and specific deletion of both *Dhps* and *Apc* in colonic epithelial cells. However, it is more challenging to create mice that lack APC in colonic epithelial cells and DHPS in myeloid cells. Nonetheless, SMOX and DHPS levels are decreased in colonic epithelial cells from patients with IBD [13,27]. Thus, restoring hypusination by spermidine supplementation represents a therapeutic strategy to limit colitis and risk for neoplasia. The present study establishes that modulation of hypusination in myeloid cells will be unlikely to affect the outcome of these diseases.

Ethics statement

For this project, mice were utilized under protocol M20000047 approved by the Institutional Animal Care and Use Committee at Vanderbilt University, the Vanderbilt University Medical Center Institutional Biosafety Committee, and the Research and Development Committee of the Veterans Affairs Tennessee Valley Healthcare System (Nashville VAMC). All the mouse procedures were performed in accordance with Vanderbilt University and VA institutional policies, and the guidelines of the AAALAC and AVMA, and NIH regulations (specifically the Guide for the Care and Use of Laboratory Animals), and the US Animal Welfare Act of 1966.

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Data availability statement

The 16S rRNA sequencing of the intestinal microbiota has been deposited on the Sequence Read Archive website with the BioProject accession number PRJNA1062468.

CRediT authorship contribution statement

Alain P. Gobert: Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Conceptualization. **Jordan Finley:** Investigation. **Mohammad Asim:** Investigation. **Daniel P. Barry:** Investigation. **Margaret M. Allaman:** Investigation. **Caroline V. Hawkins:** Investigation. **Kamery J. Williams:** Investigation. **Alberto G. Delagado:** Investigation. **Raghavendra G. Mirmira:** Resources. **Shilin Zhao:** Formal analysis. **M. Blanca Piazuelo:** Investigation. **M. Kay Washington:** Investigation. **Lori A. Coburn:** Investigation. **Keith T. Wilson:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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