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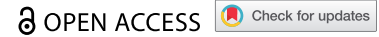
Translocation of *Helicobacter hepaticus* synergizes with myeloid-derived suppressor cells and contributes to breast carcinogenesis

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ABSTRACT

Microbial dysbiosis plays an important role in the development of intestinal diseases. Recent studies suggest a link between intestinal bacteria and mammary cancer. Here, we report that female Apc$^{Min/+}$ mice infected with *Helicobacter hepaticus* exhibited an increased mammary and small/large intestine tumor burden compared with uninfected littermates. *H. hepaticus* DNA was detected in small/large intestine, mammary tumors, and adjacent lymph nodes, suggesting a migration pathway. CD11b$^+$Gr1$^+$ myeloid-derived suppressor cells (MDSCs) infiltrated and expressed high levels of Wnts, likely enhancing tumorigenesis through activation of Wnt/β-catenin pathway. Our previous studies indicated that histidine decarboxylase (Hdc) marks a population of myeloid-biased hematopoietic stem cells and granulocytic MDSCs. Cytokines/chemokines secreted by IL-17-expressing mast cells and tumor tissues promoted Hdc$^+$ MDSCs expansion and trafficking toward mammary tumors. Adoptive transfer of MDSCs isolated from *H. hepaticus*-infected mice increased MDSCs frequencies in peripheral blood, mesenteric lymph nodes, mammary gland, and lymph nodes in recipient Apc$^{Min/+}$ mice. The adoptive transfer of *H. hepaticus* primed MDSCs also increased the size and number of mammary tumors. Our results demonstrate that *H. hepaticus* can translocate from the intestine to mammary tissues to promote mammary tumorigenesis with MDSCs. Targeting bacteria and MDSCs may be useful for the prevention and therapy of extraintestinal cancers.

Abbreviations: Helicobacter hepaticus, Hh; myeloid-derived suppressor cell, MDSC; histidine decarboxylase, Hdc; Breast cancer, BC; T regulatory, T$_R$; inflammatory bowel disease, IBD; fluorescence in situ hybridization, FISH; myeloid-biased hematopoietic stem cells, MB-HSCs; granulocytic MDSCs, PMN-MDSCs; Lipopolysaccharide, LPS; Toll-like receptors, TLRs; Mast cells, MCs; Granulocyte-macrophage colony-stimulating factor, GM-CSF; epithelial–mesenchymal transition, EMT; Intestinal epithelial cells, IECs.

Introduction

Breast cancer (BC) is the second-leading cause of cancer-associated mortality in women.[1] The incidence of breast cancer has continued to increase, highlighting that complicated molecular mechanisms underlie carcinogenesis of the breast. Not discounting that heritable influences are important in a subset of patients,[2] environmental factors arguably play a crucial role in most patients with mammary cancer.

Interactions between epithelium, stromal cells, and microbiota are essential to maintain homeostasis in normal tissues.[3] Accumulating evidence suggests that intestinal dysbiosis can trigger local or systemic diseases, even neoplasia.[4–6] The hygiene hypothesis was proposed to state that a positive correlation exists between decreasing microbial exposure and rising immune disorders in developed countries.[7] However, while there is strong evidence for a bacterium in directly promoting the development of gastric and colorectal cancer, the evidence for a role of intestinal microbiota in the carcinogenesis of extra-intestinal organs has been sparse.

Helicobacter hepaticus (*H. hepaticus*) is a bacterium that has been found in the liver and intestine of inbred mice.[8] Accumulating evidence indicates its important role in promoting typhlitis and colitis, raising the possibility that *H. hepaticus* and related species may contribute to intestinal dysbiosis. The use of *H. hepaticus* infection in diverse mouse models, including *IL10*$^{-/-}$ and *Rag*$^{-/-}$, has revealed that the innate immune system plays a critical role in the development of colitis and colitis-associated cancer.[9,10] *H. hepaticus* infection has also been demonstrated to trigger precancerous lesion of colon in Apc$^{Min/+}$ mice.[10–12] Of particular note is the fact that *H. hepaticus*-induced microbial dysbiosis can promote the development of mammary and prostate tumors in Rag$^{-/-}$; Apc$^{Min/+}$ murine model, presumably through modulation of innate immunity.[11,13–16] A recent study has supported this

view, as it was shown that alterations in intestinal microbiota are also involved in the development of malignant brain tumor.[17]

Breast cancer in FVB-Tg(C3-1-Tag)cJeg/JegJ female mice infected with *H. hepaticus* infection is characterized by extensive infiltration of tumor-associated myeloperoxidase[+] granulocytes or neutrophils. In a recent study, we found that the pro-tumorigenesis effect could be blocked by the administration of anti-Ly6G antibody,[15] given thrice weekly by intraperitoneal injections, resulting in systemic depletion of neutrophils. While this earlier study demonstrates a likely role for granulocytic myeloid cells in mammary tumorigenesis, the reason for tumor infiltration by these myeloid cells remained unaddressed. Nevertheless, disruption of normal host flora and intestinal homeostasis has been shown to contribute to the metastasis of breast cancer cells, and the blockade of intestinal dysbiosis by antibiotics or fecal microbiota transplantation can significantly attenuate tumor progression.[18]

Innate immune response to *H. hepaticus* infection is sufficient to induce mammary carcinogenesis of the breast at least in part through a TNFα-dependent pathway. In addition, the adoptive transfer of CD4^{+}CD25^{+} T regulatory (T$_R$) can inhibit mammary tumorigenesis.[11] However, in colorectal cancer mouse models, the increase in regulatory T cells is in many cases linked to the expansion of myeloid-derived suppressor cells (MDSCs), which are frequently observed in breast tumors.[15] MDSCs are CD11b^{+}Gr1^{+} immature myeloid cells, comprising both granulocytic and monocytic lineages, that in the setting of tumorigenesis acquire immunosuppressive properties. We previously reported that granulocytic myeloid-derived suppressor cells (MDSCs) expressing histidine decarboxylase (Hdc) were able to induce and recruit Foxp3^{+} regulatory T cells in murine colon cancer.[19] Aberrant expansion and recruitment of MDSCs can inhibit cytotoxic T cells and contribute to the progression of breast and colon cancer.[19,20] However, little progress has been made in elucidating some of the initial environmental triggers for MDSC recruitment in the earliest stages of cancer development.

Having established the *H. hepaticus* infected mouse model and having provided preliminary data to support the hypothesis that *H. hepaticus* can promote mammary tumor development, we next asked whether MDSCs are sufficient to induce mammary tumorigenesis in susceptible female mice and how they might be recruited to that site. In the current study, the data show that *H. hepaticus* can translocate from intestinal tract to breast with CD11b^{+}Gr1^{+} MDSCs in C57BL/6 Apc[Min/+] mice and the Wnt/β-catenin pathway is involved in the tumorigenesis of breast epithelium.

Results

H. hepaticus infection promotes mammary and small/large intestine tumorigenesis

Previous studies showed that gastric gavage of Rag2^{−/−}Apc[Min/+] female mice with *H. hepaticus* can increase the incidence of mammary cancer.[11] However, these studies in the background of Rag2-deficiency suggest that activation by *H. hepaticus* of the

innate immune system can directly promote the progression of the mammary tumor independent of its role in T cell suppression. In this study, we carried out *H. hepaticus*-infection of immune competent C57BL/6 Apc[Min/+] mice between 4 to 4.5 months, and found that *H. hepaticus* infection resulted in a larger number of mammary cancers compared with uninfected littermates [Figure 1a –b]. Consistent with our previous study, the histopathologic type of mammary cancer was primarily adenosquamous carcinoma, rather than pure infiltrating ductal carcinoma [Figure 1a].[11] Tumor histology revealed well-defined solid nests that consisted of squamous carcinoma cells. Unlike squamous cell carcinoma of the skin, tumor cells in the mammary gland exhibited cytoplasmic keratinization, rather than squamous pearls. Adenocarcinoma cells were also present that mimicked differentiated tubules. The tumorous epithelium in the small/large intestine of both infected and uninfected Apc[Min/+] mice exhibited low grade of atypia [Figure 1c]. However, we found that the number of adenomatous polyps in *H. hepaticus* infected mice was increased significantly compared with controls [Figure 1d].

Accumulation of CD11b^{+}Gr1^{+} MDSCs in mammary cancer

Previous studies indicate an association between the recruitment of MDSCs and tumorigenesis.[21,22] In the C3-1-Tag mouse model treated with *H. hepaticus*, morphologically typical MDSCs were found infiltrating the precancerous lesions on histologic examination.[15] Thus, we used FACS to analyze and characterize the myeloid infiltrate, which confirmed that in the small/large intestine and mesenteric lymph nodes, there was a significant increase in the percentage of CD11b^{+}Gr1^{+} MDSCs following *H. hepaticus* infection [Figure 2a–d]. Interestingly, a similar increase in CD11b^{+}Gr1^{+} MDSCs was detected in the mammary gland [Figure 2c –d]. Immature myeloid cells with similar surface markers (CD11b^{+}Gr1^{+}) can usually be detected in the spleen of cancer-free mice, but are generally non-immunosuppressive and morphologically distinct.[22] However, mice with cancer often show significant increases in CD11b^{+}Gr1^{+} MDSCs in both the circulation and spleen, and patients with cancer show similar increases in myeloid cells (although with different surface markers).[23–25] Consistent with previous studies, *H. hepaticus* infection led to an expansion of CD11b^{+}Gr1^{+} MDSCs in the spleen of Apc[Min/+] mouse [Figure 2c –d].

Translocation of *H. hepaticus* is accompanied by increased MDSCs trafficking

Intestinal dysbiosis is thought to disrupt normal host–microbiota interactions, leading to changes in the microenvironment of the intestinal epithelium.[26] Alterations in intestinal microbiota have been shown to facilitate the development of metabolic diseases,[4] inflammatory bowel disease (IBD),[5] and gastrointestinal cancer.[6,27] While our previous studies demonstrated that *H. hepaticus*-induced intestinal dysbiosis can promote carcinogenesis of the mammary gland, in part through myeloid cells, the precise mechanism by which *H. hepaticus* enhances immune cell recruitment to tumors is largely unknown.[11,15]
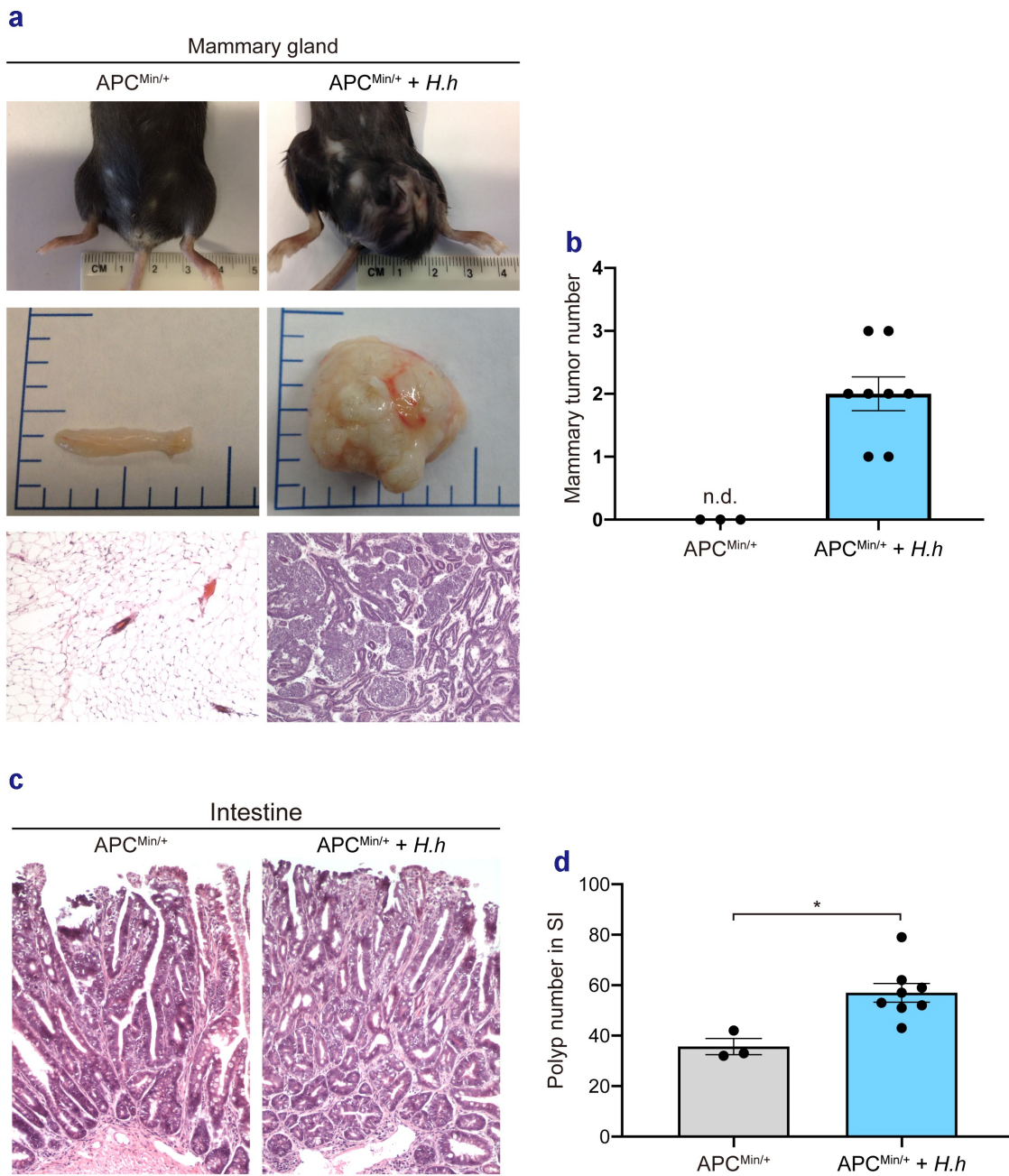
Figure 1. *H. hepaticus* infection increased the mammary and intestine tumor burden. A. Adenosquamous carcinoma of the mammary gland in Apc$^{Min/+}$ female mice infected (n = 4 per group) or uninfected (n = 3 per group) with *H. hepaticus*. B. Quantification of the number of the mammary tumor. C. Adenomous polyps observed in both infected (n = 4 per group) and uninfected mice (n = 3 per group). D. Infected mice showed larger number of polyps than that of uninfected mice. Data were analyzed with two-tailed Student's t test (b and d). For all panels, ±SEM is shown (*p < .05, **p < .01, and ***p < .001; n.s., not significant; n.d., not detectable; n indicates biological replicates). For infected groups, two independent experiments were performed unless otherwise indicated. A (H&E) and C, original magnification × 200.

We hypothesized that this might be mediated in part by direct infection of mammary tissue. To test whether *H. hepaticus* can translocate from the intestinal tract to mammary tissue, we used fluorescence in situ hybridization (FISH) and qPCR to detect *H. hepaticus* DNA. Positive signals were observed by FISH not only within the small/large intestine and mesenteric lymph nodes but also within the mammary gland and associated lymph nodes in infected mice, but not in uninfected controls [Figure 3a –b]. qRT-PCR further confirmed elevated DNA levels of *H. hepaticus* in mammary tissue and lymph nodes [Figure 3c].

In recent studies, we showed that histidine decarboxylase (Hdc) marks a population of myeloid-biased hematopoietic stem cells (MB-HSCs) and granulocytic MDSCs (PMN-MDSCs).[19,28] Hdc-eGFP mice were crossed to Apc$^{Min/+}$ mice to generate Hdc-eGFP; Apc$^{Min/+}$ mice. Two-photon microscopy traced the migration of PMN-MDSCs in *H. hepaticus*-infected Hdc-eGFP; Apc$^{Min/+}$ mice and revealed that Hdc$^+$ MDSCs trafficked to the mammary gland and lymph nodes through vessels [Figure 3d]. This pattern of migration by granulocytic MDSCs was not observed in uninfected animals. Thus, taken together, these data indicate that
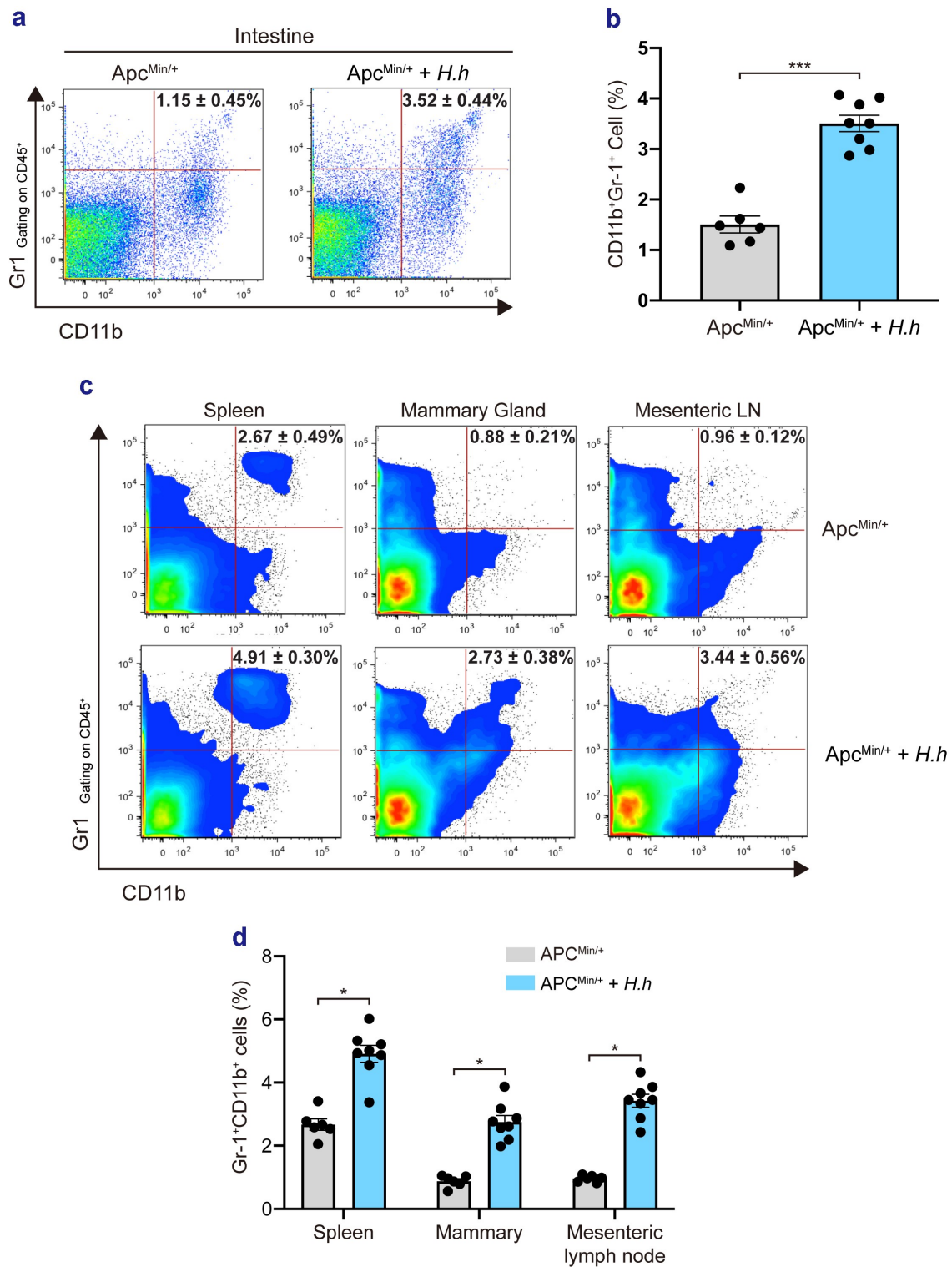
Figure 2. *H. hepaticus* infection recruited MDSCs. A. Inflammation response to *H. hepaticus* infection increased the number of MDSCs in the intesine (n = 3 to 4 per group). B. Quantification of the frequency of MDSCs in the intestine. C. MDSCs expansion in the spleen, mammary gland, and mesenteric lymph nodes (n = 3 to 4 per group). D. Frequencies of MDSCs increased significantly in infected mice compared with uninfected controls. MDSCs were gated on single cells/live/CD45+. Data were analyzed with two-tailed Student's t test (b and d). For all panels, ±SEM is shown (*p < .05, **p < .01, and ***p < .001; n.s., not significant; n.d., not detectable; n indicates biological replicates). For all experiments, greater than or equal to two independent experiments were performed unless otherwise indicated.
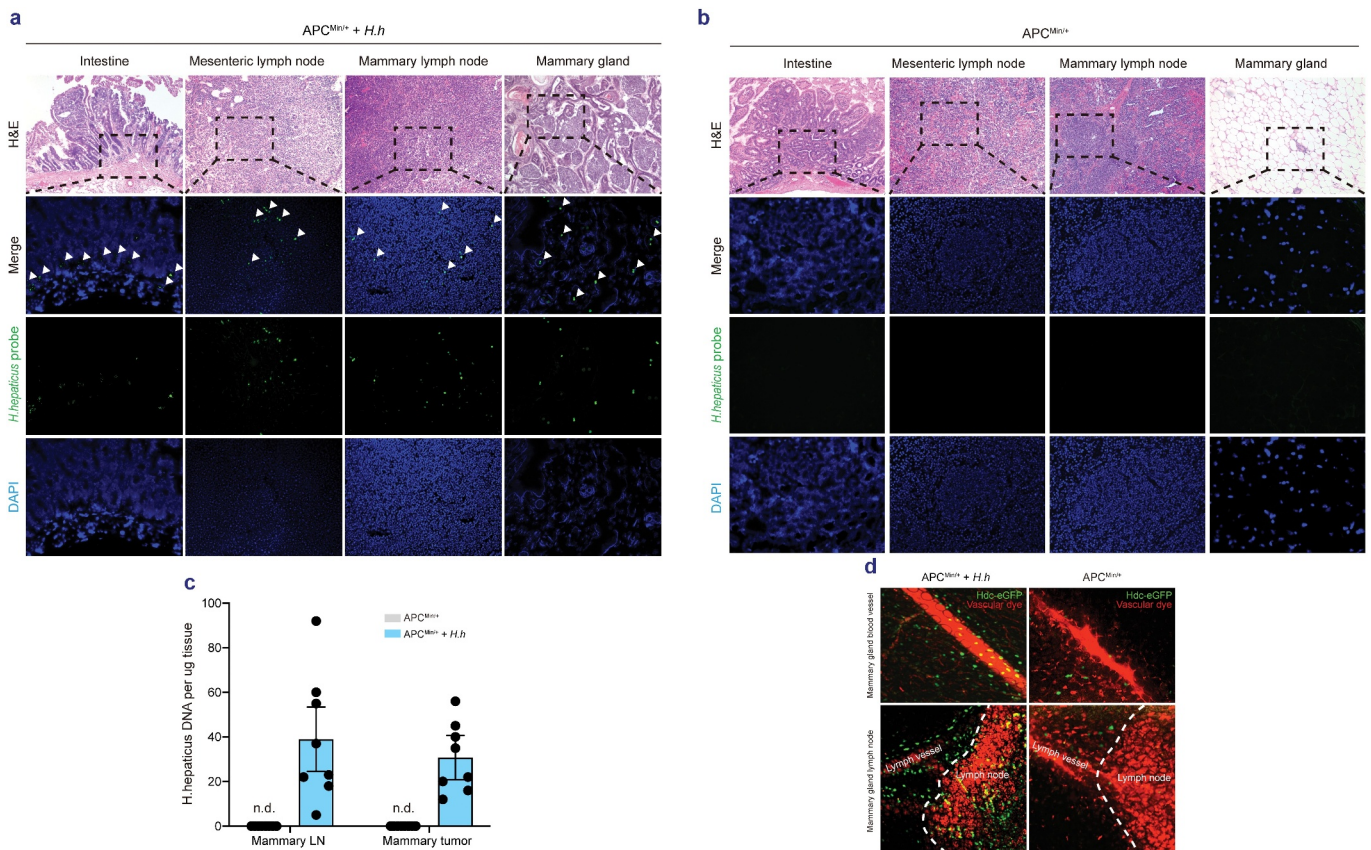
Figure 3. Translocation of *H. hepaticus* from the intestine to the mammary gland. A. DNA probe for *H. hepaticus* showed positive signals in the intestine, mesenteric lymph node, mammary gland, and mammary lymph node (n = 3 to 4 per group). B. Uninfected mice did not show positivity for *H. hepaticus* probe (n = 4). C. Levels of *H. hepaticus* DNA in the mammary tumor and lymph nodes (n = 4 per group). D. 2 photon microscope showed the traffic of Hdc$^+$ MDSCs toward the mammary (n = 4 per group). For all panels, ±SEM is shown (*p < .05, **p < .01, and ***p < .001; n.s., not significant; n.d., not detectable; n indicates biological replicates). For all experiments, greater than or equal to two independent experiments were performed unless otherwise indicated. A and B (H&E), original magnification × 200. A and B (Probe, DAPI, and Merge), original magnification × 400.

H. hepaticus can directly translocate from intestinal tract to mammary gland, and influence the behavior of the epithelium and surrounding stroma. Indeed, the migration of *H. hepaticus* was associated with a similar trafficking of Hdc$^+$ MDSCs to target tissue.

Inflammation increases myeloid expansion and recruitment

Immune responses to bacterial infection often lead to the expansion and recruitment of myeloid cells. Lipopolysaccharide (LPS), a characteristic cell wall component of Gram-negative bacteria, can trigger the activation and proliferation of myeloid lineage cells via Toll-like receptors (TLRs) expressed on myeloid-biased HSCs.[28,29] Thus, the translocation of *H. hepaticus* from the intestinal tract to mammary gland might serve as a strong driver for the further migration and recruitment of MDSCs.

Mast cells (MCs) are involved in carcinogenesis through the support of immune suppressive cells, such as regulatory T cell (Treg) and MDSCs.[30–33] Our previous study demonstrated that MCs can secrete proinflammatory cytokines and chemokines,

including IL-1β, IL-6, IL-17α, and IL-23α, to induce an expansion of MDSCs and suppression of CD8$^+$ T cells.[34] In *H. hepaticus*-infected Apc$^{Min/+}$ mice, but not uninfected littermates, IL-17-expressing MCs were observed in the tumorous tissue of the mammary gland and small/large intestine [Figure 4a]. qRT-PCR results further confirmed the upregulated expression of IL-17α and IL-23α in the mammary tumor and adjacent histologically normal tissue and small/large intestine [Figure 4b –c]. Furthermore, both the tumorous and adjacent normal mammary tissue in *H. hepaticus*-infected Apc$^{Min/+}$ mice exhibited high levels of chemokines, CXCL1 and CXCL2, which are known bind to their receptor CXCR2 on granulocytic MDSCs and induce their chemotaxis [Figure 4d].[35]

IL-6, a known stimulant of MDSCs, can also induce the proliferation of MB-HSCs.[28,36] The level of IL-6 in the small/large intestine and spleen increased significantly after gastric gavage with *H. hepaticus* [Figure 4e]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been linked to the differentiation of HSCs and progenitors into MDSCs.[37] The *H. hepaticus*-infected Apc$^{Min/+}$ mice showed higher level of GM-CSF in the spleen than uninfected mice [Figure 4e], which accounts in part for the expansion of MDSCs.
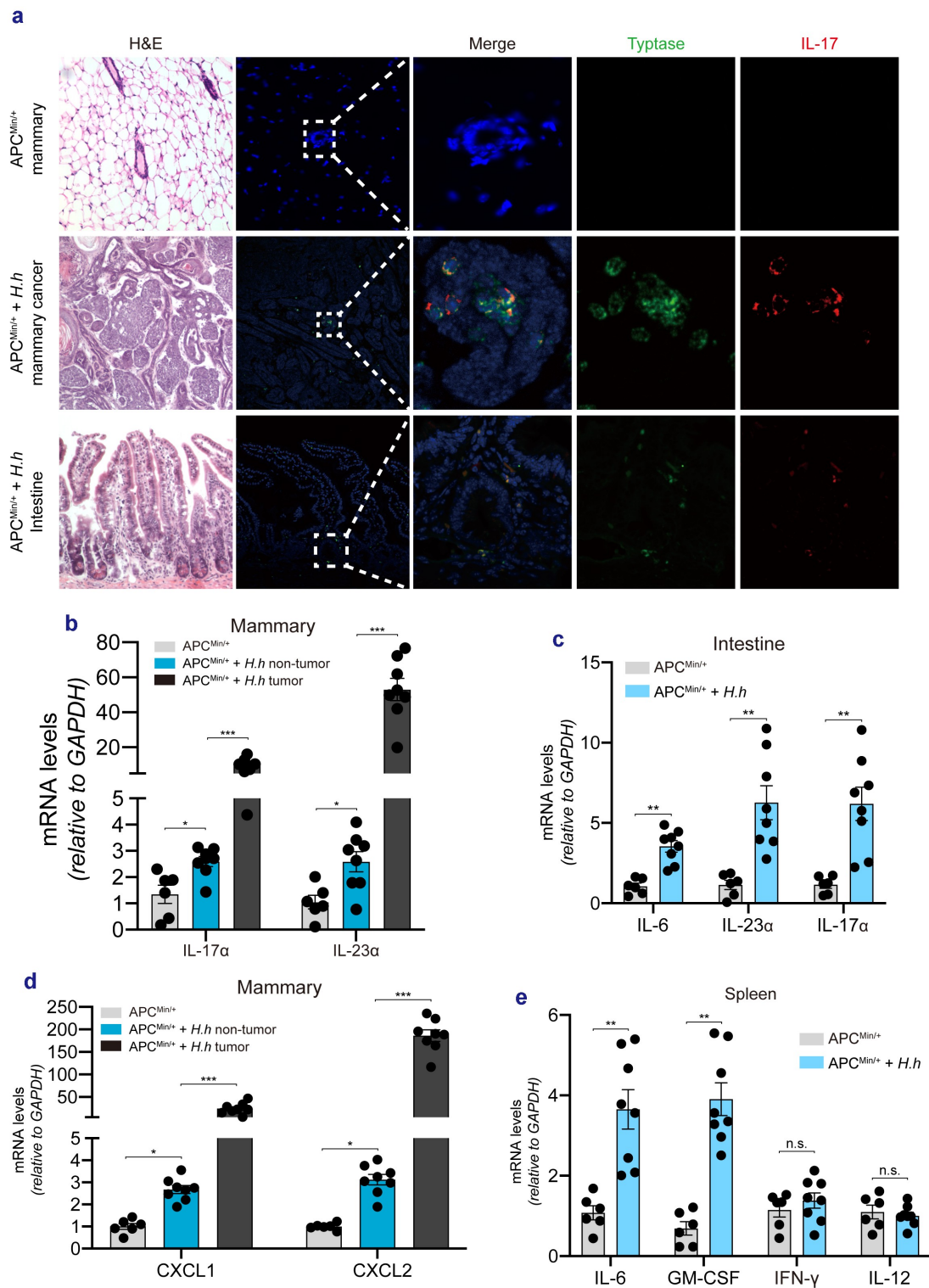
Figure 4. Proinflammatory cytokines and chemokines in *H. hepaticus*-infected mice. A. The number of IL-17+Typtase+ mast cells increased in the mammary and intesine of infected mice (n = 4 per group). B. Expression levels of IL-17α and IL-23α in the mammary tumor or adjacent normal tissue (n = 3 to 4 per group). C. Expression levels of IL-6, IL-17α, and IL-23α in the intestine (n = 3 to 4 per group). D. Expression levels of CXCL1 and CXCL2 in the mammary tumor or adjacent normal tissue (n = 3 to 4 per group). E. Expression levels of IL-6, GM-CSF, IFN-γ, and IL-12 in the spleen (n = 3 to 4 per group). Data were analyzed with two-tailed Student's t test (c and e) or one-way ANOVA with Bonferroni post hoc test (b and d). For all panels, ±SEM is shown (*p < .05, **p < .01, and ***p < .001; n.s., not significant; n.d., not detectable; n indicates biological replicates). For all experiments, greater than or equal to two independent experiments were performed unless otherwise indicated. A (H&E), original magnification × 200. A (Merge, Typtase, and IL-17), original magnification × 400.

Taken together, these results suggest that the immune response to *in situ* or translocated *H. hepaticus* enhance the secretion of proinflammatory cytokines and chemokines, which contribute to the expansion and recruitment of MDSCs to the inflamed mammary gland and promote carcinogenesis.

MDSCs from H. hepaticus-infected Apc[Min/+] mice promote mammary tumorigenesis

To examine whether MDSCs harvested from *H. hepaticus*-infected Apc[Min/+] mice can directly promote mammary tumorigenesis in susceptible hosts, CD11b$^+$Gr1$^+$ MDSCs were isolated from infected or uninfected mice and injected into untreated Apc[Min/+] mice by tail vein injection. Four weeks after adoptive transfer, the frequencies of CD11b$^+$Gr1$^+$ MDSCs in mammary gland and lymph nodes, blood, and mesenteric lymph nodes were increased significantly compared with their counterparts transplanted with MDSCs from uninfected mice [Figure 5a – b], indicating the important role of *H. hepaticus* in the expansion and trafficking of MDSCs. In line with this, we found a significantly higher frequency of mammary tumors in female mice that had received transfers of *H. hepaticus*-related MDSCs compared to their age-matched littermates that received MDSCs collected from uninfected mice [Figure 5c –d]. These data reveal that MDSCs educated through *H. hepaticus* infection were sufficient to promote mammary tumor growth and progression.

MDSC-derived Wnts are increased in H. hepaticus-infected Apc[Min/+] mice

Wnt/β-catenin signaling plays an essential role in many cellular events. Aberrant activation of the Wnt/β-catenin pathway has been associated with many solid tumors, including breast and gastric cancer.[38,39] We next assessed whether MDSCs infiltrating in mammary tissue can influence carcinogenesis through the Wnt/β-catenin cascade. qRT-PCR on sorted CD11b$^+$Gr1$^+$ MDSCs obtained from both *H. hepaticus*-infected and uninfected Apc[Min/+] mice showed that several Wnt family members, including Wnt3, Wnt4, Wnt5b, and Wnt11, were significantly upregulated in *H. hepaticus*-infected mice [Figure 6a]. Wnt3 and Wnt5 promote epithelial–mesenchymal transition (EMT), a mechanism associated with tumor spread and resistance to chemotherapy.[40,41] Wnt4 mediates endocrine resistance of tumorous mammary cells.[42] Overexpression of Wnt11 have been shown to enhance the invasive capacity of breast cancer cells.[43]

The dysregulation of Wnt/β-catenin pathway is characterized by the translocation of β-catenin from the membrane into the nucleus. Immunostaining with antibodies to β-catenin decorated the membrane of non-cancerous tubular epithelium in uninfected Apc[Min/+] mice [Figure 6b]. After *H. hepaticus*
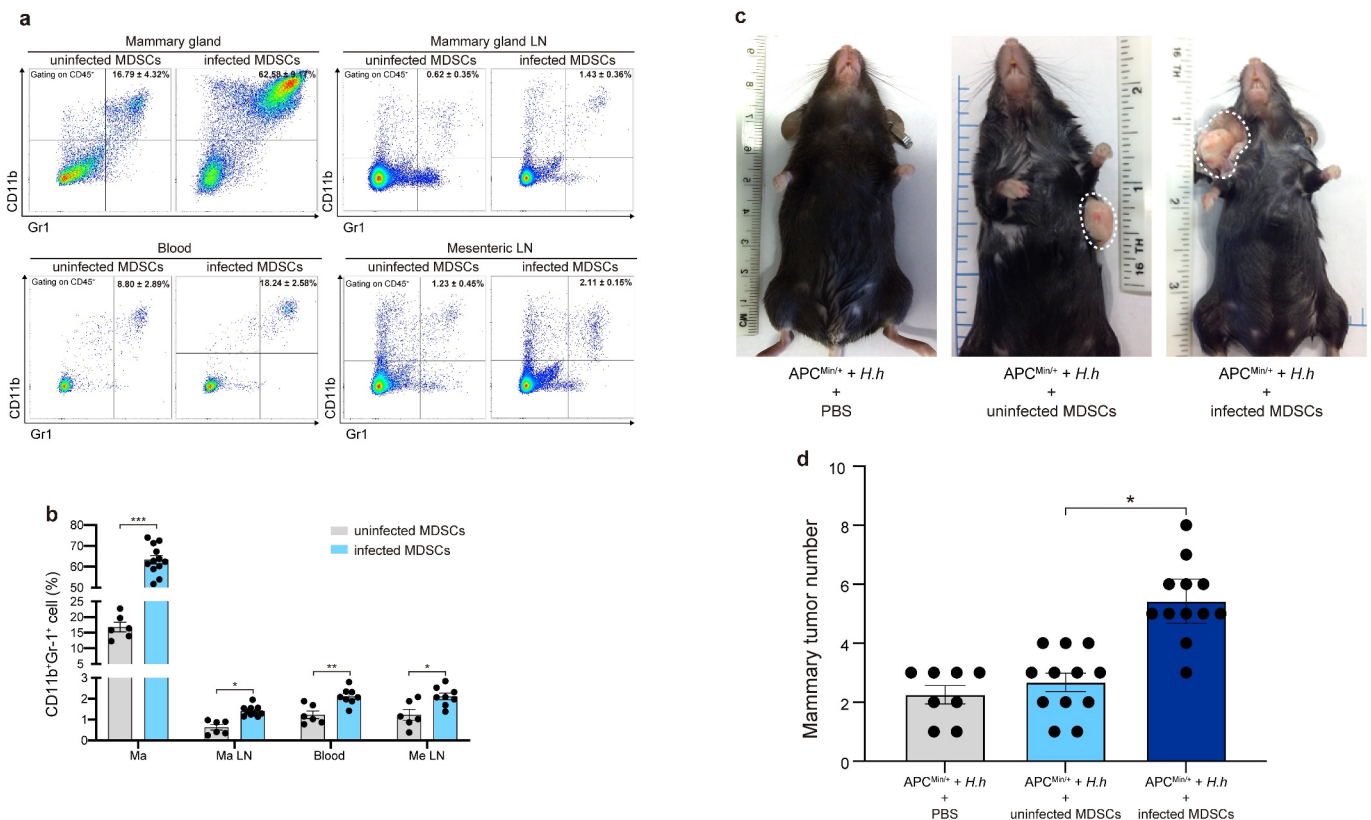

Figure 5. Procancerous effects of MDSCs obtained from infected mice. A. Transfer of MDSCs from *H. hepaticus*-infected mice increased frequencies of MDSCs in the mammary gland, mammary gland lymph node, peripheral blood, and mesenteric lymph node (n = 4 to 6 per group). B. Quantification of frequencies of MDSCs in different tissues. C. MDSCs isolated from infected mice promoted carcinogenesis of the mammary (n = 4 to 6 per group). D. Quantification of the number of the mammary cancer. MDSCs were gated on single cells/live/CD45$^+$. Data were analyzed with two-tailed Student's t test (b and d). For all panels, ±SEM is shown (*p < .05, **p < .01, and ***p < .001; n.s., not significant; n.d., not detectable; n indicates biological replicates). For all experiments, greater than or equal to two independent experiments were performed unless otherwise indicated.
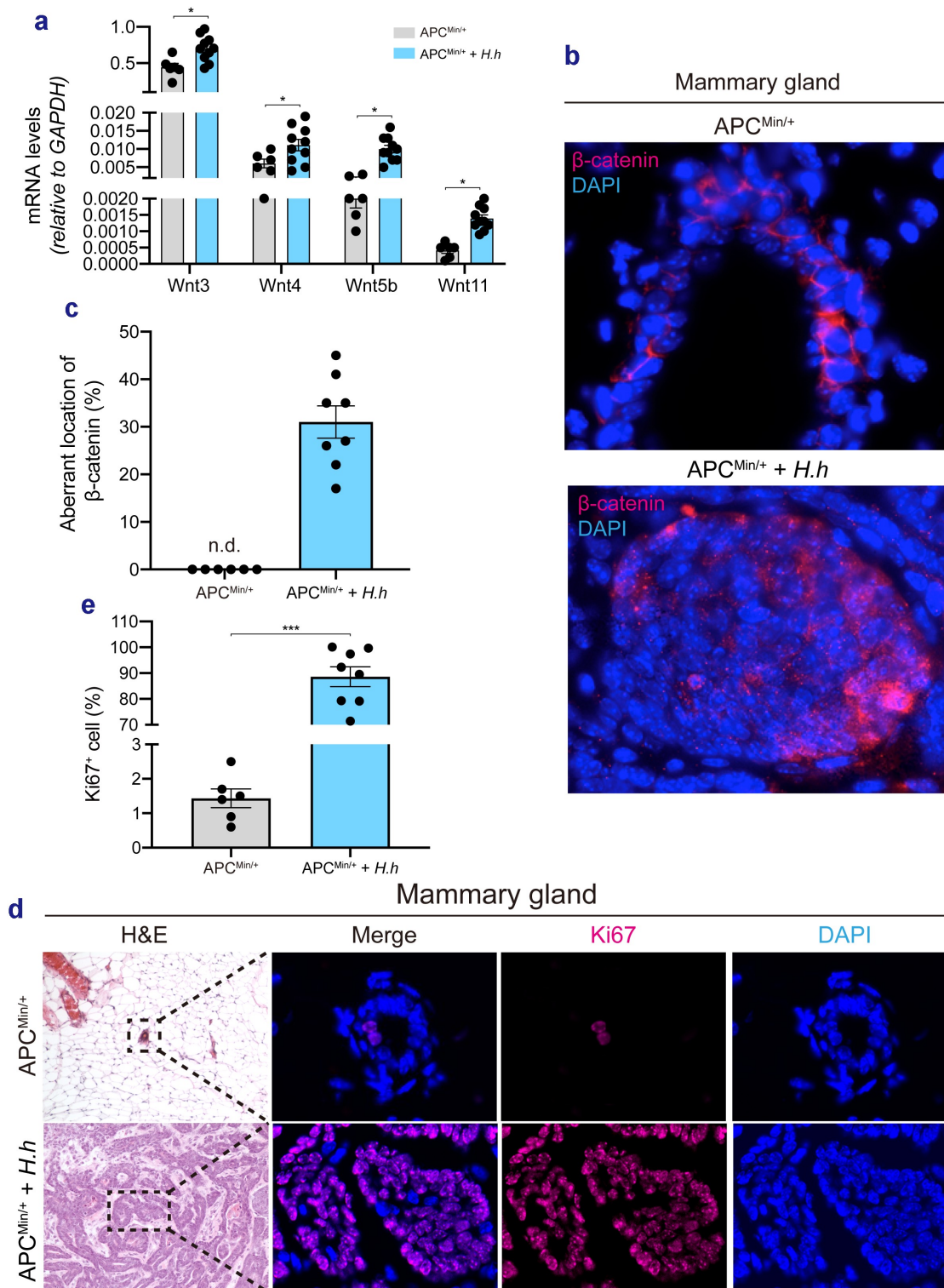
Figure 6. MDSCs promoted mammary tumorigenesis via Wnt/β-catenin pathway. A. MDSCs in infected mice expressed high levels of wnts (n = 3 to 5 per group). B. Expression pattern of β-catenin in the cancerous (Apc$^{Min/+}$ + H. h) and non-cancerous (Apc$^{Min/+}$) mammary tubular epithelium (n = 4 per group). C. Percent of the mammary duct cells with aberrant location of β-catenin. D. Ki67 level of the mammary epithelium in infected or uninfected mice (n = 4 per group). E. The frequency of Ki67$^+$ cells in different groups. Data were analyzed with two-tailed Student's t test (A, C, and E). For all panels, ±SEM is shown (*p < .05, **p < .01, and ***p < .001; n.s., not significant; n.d., not detectable; n indicates biological replicates). For all experiments, greater than or equal to two independent experiments were performed unless otherwise indicated. B and D (Merge, Ki67, and DAPI), original magnification × 400. D (H&E), original magnification × 200.

infection, the majority of mammary cancer cells showed aberrant nuclear positivity for β-catenin [Figure 6b]. The ratio of β-catenin$^+$ cells to total epithelial cells was increased markedly in tumor and adjacent mammary tissue [Figure 6c]. These tumorous areas also showed increased staining for Ki67, a nuclear marker indicating proliferation [Figure 6d]. In uninfected Apc^{Min}+ mice, we observed that only a small portion of non-cancerous epithelial cells showed Ki67 positivity and

located at the border of terminal ducts, which has been suggested as the mammary stem cell niche [Figure 6d –e].[44] Taken together, these data indicate that H. hepaticus-induced intestinal dysbiosis can result in the aberrant upregulation of Wnts in MDSCs and subsequent nuclear translocation of β-catenin in tubular epithelium to promote mammary gland tumorigenesis.

Discussion

Here, we report the direct translocation of the intestinal bacterium H. hepaticus to the mammary glands in Apc^Min/+ mice predisposed to mammary tumorigenesis. This process was accompanied by the migration of MDSCs, which can promote the mammary and intestinal tumorigenesis, as shown by adoptive transfer studies. These MDSCs, following H. hepaticus education, exhibit upregulation of numerous Wnt family members, and are associated with enhanced Wnt/β-catenin signaling within mammary tumors. Inflamed tissue and infiltrating mast cells express high level of cytokines and chemokines known to recruit MDSCs. Adoptive transfer of MDSCs predisposed to H. hepaticus infection led to increased MDSC trafficking and mammary tumors in uninfected Apc^Min/+ mice.

The contribution of bacterial dysbiosis to gastric and intestinal carcinogenesis has been widely studied, but a better understanding of the underlying mechanisms involved in the pathogenesis of non-intestinal tumors has been constrained by the complexity of innate immunity. Nevertheless, a number of studies suggest a link between bacterial infection and breast cancer. The administration of a carcinogen (3,2'-dlmethyl -4-amlnoblphenyl) in germ-free rats increased frequencies of several malignant tumors, including breast and colon, when compared with their normal housing littermates.[45] However, these data did not conclude whether the pro-tumorigenesis effects were derived from local breast microbiome or distant bacterial communities within gastrointestinal tract. A recent study provides substantial evidence to support the effects of intestinal dysbiosis on the pre-established commensal homeostasis of breast microenvironment, resulting in an enhanced ability of dissemination.[18] Other studies support the notion that intestinal dysbiosis serves as a risk factor for breast cancer development.[15,46] Mammary duct or intestine colonization with the gut commensal bacterium entero-toxigenic Bacteroides fragilis is sufficient to promote mammary tumorigenesis and progression.[47] However, whether intestinal bacteria can translocate to mammary tissue and become a central component of extraintestinal microenvironment is still debated.

Our group established a stable animal model via gastric gavage with H. hepaticus to provide insights into the role of intestinal dysbiosis in the tumorigenesis of non-gastrointestinal organs.[11,15,16] In the present study, we confirmed for the first time the existence of H. hepaticus in mammary cancer tissue following H. hepaticus inoculation of Apc^Min/+ mice, indicating that intestinal commensal bacteria can translocate to distant organ and promote the progression of cancer. Precisely how intestinal bacteria leave primary site and reach extra-intestinal tissues is largely unknown.

The integrity of the intestinal barrier requires an intact epithelial/endothelial lining and a mucus layer to protect the host from invasion by microbial pathogen. Bacterial-type VI

secretion system (T6SS) of H. hepaticus contributes to host-microbial interaction and limits its colonization in intestinal epithelial cells (IECs). T6SS mutations faciliates the internalization of H. hepaticus into mouse IECs and inhibits host immune reponse-related genes in the setting of in vitro culture.[48] Wild type H. hepaticus used in our studies may be primarily responsible for the results that FISH signals of H. hepaticus were detected mainly in the lumen of intestinal glands and mammary ducts.

Bacteria can be phagocyted by MDSCs and interrupt host immune responses.[49,50] MDSCs serve as a reservoir and support the survival of bacteria because of impaired pathogen elimination capability. In this study, H. hepaticus and Hdc^+ MDSCs have a remarkable tendency for invasion of breast, raising an intriguing possibility that Hdc^+ MDSCs vehiculate H. hepaticus from the intestine to the mammary tissue. While the FISH probe and wild-type bacterium have limitations, the temporal and spatial relationship between H. hepaticus and Hdc^+ MDSCs in vivo was largely unknown.

Normal breast tissue has low numbers of commensal bacteria, some of which have been found in the intestinal tract.[51] Accurately identifying the original sources of abundant bacteria in breast cancer tissues remains a big obstacle. A comparison between H. hepaticus and other intestinal bacteria was not the focus of this study. Although our previous study excluded the effects of a control bacterium on mammary carcinogenesis,[11] we did not obtain substantial evidence to clarify the exact mechanisms underlying H. hepaticus translocation and the role of other bacteria in this study.

E (epithelial)- or VE (vascular-endothelial)-cadherin/β-catenin complex decreases the permeability of epithelium or endothelium and prevents the systemic dissemination of intestinal bacteria.[52,53] Nevertheless, bacterial dysbiosis and/or aberrant activation of Wnt/β-catenin pathway could potentially disrupt this barrier, resulting in an increased level of bacterial DNA in circulation.[53,54] We found that MDSCs expressed high levels of Wnts, which was associated with increased evidence of nuclear translocation of β-catenin in intestinal epithelium and subsequent positivity for H. hepaticus DNA in intestinal mucous, mammary gland, and adjacent lymph nodes.

Bacterial translocation was accompanied by the migration of MDSCs. We employed histidine decarboxylase (Hdc) as a PMN-MDSCs marker and observed that MDSCs moved toward mammary gland and adjacent lymph nodes through the circulation in live H. hepaticus-infected mice. The inflammatory microenvironment of the mammary gland may recruit MDSCs in several ways. First, H. hepaticus-derived LPS is a strong stimulant for MDSCs expansion and chemotaxis.[28,29] The response of bone marrow HSCs to LPS via TLRs could promote the activation of MB-HSCs and differentiation into myeloid-lineage cells.[28] IL-6, another myeloproliferative stimuli, was upregulated in both mammary gland and small/large intestine in Apc^Min/+ mice. Second, the number of mast cells in H. hepaticus infected mice was increased significantly. Mast cells secrete several proinflammatory cytokines that are able to support MDSCs.[34] PMN-MDSCs are capable of suppressing tumoricidal immunity through the inhibition of CD8^+ T cells, indicating that interactions between lymphocytes and

myeloid cells regulate pro- and anti-tumor immunity.[19] However, the heterogeneity of both lymphocytes and myeloid cells is a real challenge when trying to unveil the pathogenic mechanisms. Finally, mammary tumor and adjacent normal tissue in infected mice expressed high levels of CXCL1 and CXCL2, which in turn can trigger the migration of MDSCs by binding to CXCR2 on MDSCs.[35]

It is widely recognized that inflammation is associated with the development of malignant tumors. In the context of *H. hepaticus*-related inflammation, the infiltration of tumor-associated neutrophils has been shown to increase in mammary tumors.[15] Of note is that neutrophils likely represent a subset of PMN-MDSCs, which have been demonstrated to express a high level of Hdc.[22,28] Intestinal dysbiosis has been demonstrated to induce the recruitment and expansion of tumor associated macrophage, another subset of myeloid cells, in the setting of breast cancer.[18] In this study, we observed large amounts of Hdc$^+$ MDSCs in the inflamed mammary gland, further supporting the hypothesis that MDSCs exert essential effects on the tumorigenesis of the mammary gland in mice with intestinal dysbiosis. Compared with their counterparts from uninfected mice, MDSCs from *H. hepaticus*-infected Apc$^{Min/+}$ mice enhanced the proportion of mammary epithelial cells entering the cell cycle. Thus, in addition to enhancing intestinal leakiness, MDSCs-derived Wnts further activated the translocation of β-catenin in mammary tubular epithelium, thus accelerating mammary gland carcinogenesis. A recent study further supports our conclusion that enterotoxigenic *Bacteroides fragilis* facilitates the aberrant translocation of β-catenin in MCF7 cells through *Bacteroides fragilis* toxin.[47] However, further studies are expected to gain more insights into the status of Wnt/β-catenin pathway in the pretumorous steps.

In summary, the results of the current study suggest that intestinal dysbiosis, as represented experimentally by *H. hepaticus* infection, promotes systemic dissemination of bacteria. Subsets of gut bacteria translocate from the intestinal tract via lymph nodes to the mammary and establish a proinflammatory microenvironment. Mast cells and tumorous tissue secreted several cytokines and chemokines to induce chemotaxis of MDSCs with the same tendency as bacterial translocation. Focal accumulation of MDSCs express high levels of Wnts to stimulate the dysregulation of Wnt/β-catenin pathway, contributing to carcinogenesis of mammary gland. Adoptive transfer of MDSCs isolated from infected mice facilitates the expansion of MDSCs and tumorigenesis of the mammary gland and small/large intestine in recipients. Targeting the Wnt/β-catenin pathway in MDSCs might be a promising strategy for the treatment or prevention of intestinal dysbiosis-related carcinogenesis.

Acknowledgments

Disclosure statement

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Appendix

Materials and Methods

Mice

C57BL/6 background Hdc-GFP and Apc$^{Min/+}$ have been described previously [9, 23]. In these transgenic mouse lines, the Hdc-GFP transgenic reporter indicates Hdc-expressing cells. In some experiments, Hdc-GFP was crossed with Apc$^{Min/+}$ mice to generate Hdc-GFP; Apc$^{Min/+}$ mice. Female mice were used in all studies. In order to minimize aging-related myeloid-biased effects, young (2 to 3 months of age) transgenic mice and their littermates were used in the majority of experiments.

Mice were observed carefully by laboratory staff and veterinarian personnel for health and activity. Mice were monitored to ensure that food and fluid intake meets their nutritional needs. Body weights were recorded at minimum weekly, and more often for animals requiring greater attention. Mice were maintained on wood chip bedding, and given ad libitum access to water and standard mouse chow, with 12-hour light/dark phase cycles. The colonies were specific pathogen free (SPF) and tested quarterly for known murine pathogens including enterohepatic *Helicobacter* spp. Mice in the barrier facilities are housed in cages with microisolator tops on ventilated or static racks. All caging materials and bedding are autoclaved. Food is irradiated and water is either RO, autoclaved or acidified, depending on the barrier. All manipulations are performed in laminar flow hoods. Once animals are removed from a barrier, they are not returned. All personnel wear shoe covers, gloves, hair bonnets, and gowns. All mouse studies were approved by the Massachusetts Institute of Technology (0912-093-18) and Columbia University Institutional Animal Care and Use Committee (AC-AAAH1603). All mice were housed and maintained in AAALAC International approved facilities.

Histopathological and immunohistochemical staining

Serial sections were cut into 3- to 4-μm slides followed by H&E and immunohistochemical staining. The histopathological evaluation of all sections was performed by independent pathologists, who were blinded regarding details. Primary antibodies for IL-17 (1:200; ab79056, Abcam), Typtase (1:150; ab2378, Abcam), ki67 (1:200; ab15580, Abcam), and β-catenin (1:200; ab32572, Abcam) were used to label targeted cells. A microscope (80i, Nikon) with a CCD camera (DS-Ri2, Nikon) was used to perform the image analysis. Counting of positive cells was conducted in nonoverlapping fields using the 40× objective. The average number of positive cells in each square centimeter was calculated for each specimen.

Targeted infection with H. hepaticus

Female mice at 2 to 3 months of age were housed in a separate area. *H. hepaticus* (strain 3B1, ATCC 51449) were cultured and prepared as previously described [9]. Each experimental mouse was received 0.2 mL of fresh inoculum by gastric gavage every other day for three doses. Mammary and intestine were harvested 4 to 4.5 months post-infection and underwent histopathological evaluation.

In situ hybridization

Fresh mouse mammary, mammary lymph node, intestine, and mesenteric lymph node were dissected and cut into 3- to 4-μm slides for in situ hybridization. 70% formamide solution was warmed up in circulating waterbath at 72°C. Chromosome slides were pre-treated with RNase and pepsin to digestive away cytoplasm. Denature slides were incubated in pre-warmed 70% formamide for 2 min and transferred to room temperature ethanol solutions in the order of 70%, 80%, 90%, and 100% for 2 min each. Specific probes were added into 10 μL formamide at 72°C for 5 min and chilled on ice. The mixture of denatured probe, formamide, and hybridization solution were added to the center of slides, which were covered with a plastic coverslip and incubated 16 hours in 37°C incubator. Slides were submerged in 50% formamide 2 × SSC and labeled by Dylight 488 Conjugate (Thermo Scientific). All slides were mounted with ProLong anti-fade mounting medium (Invitrogen) and evaluated by an A1 laser scanning confocal attachment on an Eclipse Ti microscope stand (Nikon Instruments, Melville, NY). The probe sequence is as follows: GGGGAGATAGCGTAA.

Flow cytometry analysis and cell sorting

Fresh tissues obtained from mammary, mammary lymph node, intestine, and mesenteric lymph node were manually minced and incubated in DMEM with collagenase A (Roche) and DNase I (Roche) for 45 min at 37°C. Suspensions were filtered three times using a 70 μm nylon mesh to remove dead cell debris and enrich leucocytes. TruStain FcX (10 μg/ml, BioLegend) were added to cell suspension (no more than 1×10^6 cells) on ice for 10 – 15 minutes to prevent nonspecific binding and background fluorescence, followed by the incubation with the primary antibody panel composed of CD45 (30-F11, eBioscience), CD11b (M1/70, eBioscience), Ly6G (1A8, eBioscience), and Fixable Viability Kit (Zombie Aqua, BioLegend). PMN-MDSCs were identified based on their phenotype: $CD45^+CD11b^+Ly6G^{hi}$. Hdc^+ cells were characterized by their high level of GFP expression (GFP^{hi}). $CD45^+CD11b^+Ly6G^{hi}GFP^{lo}$ cells harvested from eGFP wild-type littermates were used to set the gate. Stained cells were fixed with Cytofix (BD Bioscience) for 30 min on ice and analyzed by an LSR II flow cytometer (BD Bioscience).

Myeloid cell adoptive transfer

Both Hdc^+ and Hdc^- myeloid cells ($CD11b^+Gr1^+$) were sorted from infected or uninfected Hdc-GFP mice and injected i.v. into untreated mouse models at indicated time points.

Two-photon microscope

Mice were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg), followed by orotracheally intubated with angiocatheter and ventilated with room air at a rate of 120 breaths/min. The mammary, mammary gland, intestine, and mesenteric lymph node were exposed. To investigate the blood vessels, 100 μL of PBS with 20 μL of 655-nm nontargeted Q-dots were injected i.v. For time-lapse imaging of GFP^+ immune cells in the blood vessels, we averaged 0.5 s per slide during the acquisition. Sequential planes were acquired in the 2.5 μm z dimention.

Quantitative RT-PCR

Total mRNA of sorted cells was isolated using a RNeasy Micro Kit (Qiagen) and underwent reverse transcription using a SuperScript III First-Strand Synthesis System (Life Technologies). The PrimerQuest Tool (Integrated DNA Technologies) was used to design sequences of SYBR Green for IL-6, IL-12, IL-17α, IL-23α, CXCL1, CXCL2, GM-CSF, and IFN-γ. Quantitative PCR was performed with the StepOne Plus instrument (Applied Biosystems). Relative gene expression was normalized to Gapdh.

Statistical analysis

Experimental results were replicated at least once, unless otherwise indicated. Sample sizes for each study were estimated on the basis of the expected differences and previous experience with the particular assay. All data are shown as the mean ± SEM. The percentage of cytoplasmic or nuclear β-catenin positivity was compared by Fisher's exact test. Other statistical comparisons were evaluated with Student's t test or one-way ANOVA. Significance levels were set at *$p < .05$; **$p < .01$; ***$p < .001$; n. s., not significant. N indicates biological replicates. Data analyses were carried out using Prism 8 (GraphPad).