Mast cells promote small bowel cancer in a tumor stage-specific and cytokine-dependent manner

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Mast cells (MCs) are tissue resident sentinels that mature and orchestrate inflammation in response to infection and allergy. While they are also frequently observed in tumors, the contribution of MCs to carcinogenesis remains unclear. Here, we show that sequential oncogetic events in gut epithelia expand different types of MCs in a temporal-, spatial-, and cytokine-dependent manner. The first wave of MCs expands focally in benign adenomatous polyps, which have elevated levels of IL-10, IL-13, and IL-33, and are rich in type-2 innate lymphoid cells (ILC2s). These vanguard MCs adhere to the transformed epithelial cells and express murine mast cell protease 2 (mMCP2; a typical mucosal MC protease) and, to a lesser extent, the connective tissue mast cell (CTMC) protease mMCP6. Persistence of MCs is strictly dependent on T cell-derived IL-10, and their loss in the absence of IL-10 expression of MCs markedly delays small bowel (SB) polyposis. MCs expand profusely in polyp-prone mice when T cells overexpress IL-10. The frequency of polyp-associated MCs is unaltered in response to broad-spectrum antibiotics, arguing against a microbial component driving their recruitment. Intriguingly, when polyps become invasive, a second wave of mMCP5/mMCP6– CTMCs expands in the tumor stroma and at invasive tumor borders. Ablation of mMCP6 expression attenuates polyposis, but invasive properties of the remaining lesions remain intact. Our findings argue for a multistep process in SB carcinogenesis in which distinct MC subsets, and their elaborated proteases, guide disease progression.

Type-2 inflammation characterized by the abundance of IL-4, IL-13, IL-15, and IL-33, as well as associated cellular components, has been best studied in connection with asthma, allergy, and arthritis. In these situations, two types of tissue sentinel cells respond to cellular stress signals, or danger-associated molecular patterns, and orchestrate type-2 inflammation. These are the type-2 innate lymphoid cells (ILC2s) that seed tissues during embryonic development and mast cells (MCs), which partially reside in tissues but are also recruited from the bone marrow as progenitors. Communication between these two sentinel cell types can produce protective (1) or pathogenic type-2 inflammation (2, 3). Their activation involves IL-10 (4) and IL-33 (3).

In the gastrointestinal (GI) tract, MCs are classified based on their response to infection with nematodes, including Trichinella spiralis (5, 6). Two distinct subsets of MCs are identified by their expression of specific proteases, distribution, and kinetics of expansion and contraction (7, 8). Mucosal mast cells (MMCs) expand during the early phase of infection in small bowel (SB) crypts and contract during resolution of infection. This contraction coincides with the expansion of connective tissue mast cells (CTMCs) in the stroma and then in the submucosa of the infested SB (5, 6). Based on these and later molecular analyses, expression of the β-chymases murine mast cell protease 1 (mMCP1) and mMCP2 defines lymphocyte-dependent MCs, whereas the tetrameric tryptases mMCP6 and mMCP7, the β-chymase mMCP4, and the α-chymase mMCP5 define lymphocyte-independent CTMCs (9, 10).

There is clinical and experimental evidence linking MCs to carcinogenesis (11, 12). The protumorigenic properties of MCs appear, in part, attributed to their ability to alter regulatory T cell (Treg) properties (13) as well as to influence lipid metabolism (14). In earlier studies, we reported expansion of MCs during polyposis (11, 15). Here, we evaluate the significance of MCs in mouse models of multistep SB cancer, induced by sequential inactivation of the adenomatous polyposis (APC) gene and PTEN or activation of Kras in gut epithelial cells. Combining these models with T cell-specific IL-10 deficiency allows us to dissect cancer-driven inflammation in the course of tumor initiation and progression. We observe a tumor stage-specific expansion of distinct MC subsets with characteristics of MMCs and CTMCs, and elucidate their contribution to cancer initiation and progression. Our data suggest that maturation and lineage commitment of MCs to MMC and then CTMC subsets is inherently and etiologically intertwined with SB multistage carcinogenesis.

Results

Polyps Develop Type-2 Inflammation. Previously, we showed that type-3 cytokines are critical for polyp growth in the SB and colon small bowel | cancer | mast cells | ILC2 | inflammation

We show that distinct subsets of mast cells (MCs) expand with sequential oncogetic events in small bowel cancer. Mucosal mast cells (MMCs) previously detected early during Trichinella spiralis infection expand in adenomatous polyps in an IL-10–dependent manner. Connective tissue mast cells (CTMCs), earlier shown to expand during the resolution of inflammation following clearance of T. spiralis, are independent of IL-10 and associate with the transition of polyps to adenocarcinoma. IL-33 upregulates the CTMC lineage-specific protease murine mast cell protease 6 (mMCP6). Ablation of mMCP6 attenuates tumor growth. Thus, tissue sentinel cells respond to oncogenic events and cellular transformation in effect to help promote cancer. Delineating the types of MCs present at various stages of disease offers actionable cellular targets for therapeutic intervention in disease progression.

Significance


The authors declare no conflict of interest.

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polyp-infiltrating MCs is consistent with their response to IL-33. Next, we stained paraffin sections of fixed gut “jelly-rolls” for infiltrating T helper 2 cells and ILC2s. Both cell types expanded significantly within polyps compared with the healthy surrounding tissues (Fig. 1 D and E). There were parallel increases in IL-1p (inflammasome activity) and type-3 cytokines in line with ongoing type-3 inflammation (Fig. S1). The T cell tropic chemokines CXCL9, CXCL10, CCL5, and CCL11 and cytokines IL-2 and IL-15 were also increased (Fig. S1). In conclusion, the microenvironment of SB polyps was strongly supportive of type-2 and type-3 inflammation and of T lymphocyte recruitment.

IL-10 Promotes MC Expansion and Polyp Growth in the SB. In earlier studies, we showed that IL-10 deficiency attenuates SB polyposis (19). We reasoned that the initial attenuation of polyposis in IL-10 deficient APCΔ468 mice reflected a role for IL-10 in MC function. Therefore, we tested here whether polyp-infiltrating MCs depend on IL-10 using three strategies: (i) conditional ablation of IL-10 in T cells using CD4Cre (CD4CreIL10Δ) (ii) germine ablation of IL-10 (IL-10Δ) (19, 20), and (iii) transgenic overexpression of IL-10 in T cells under the control of the IL-2 gene promoter (IL2pIL10) (21). Mice were aged 4 mo to allow SB polyps to develop.

To better understand the kinetics of the disease, we examined mice at 2, 4, and 7 mo of age. Immune-competent APCΔ468 mice had few small polyps and mostly aberrant crypts at 2 mo but multiple visible polyps at 4 mo (Fig. 2A). In contrast, T cell-specific IL-10 ablation or germine IL-10 deficiency delayed SB polyposis, such that there was no significant change in polyp load at 4 mo compared with 2 mo (Fig. 2A). Microscopic polyps of 2-mo-old IL-10 deficient APCΔ468 mice and age-matched IL-10 competent APCΔ468 mice had comparable densities of intraepithelial mMCP2+ (Fig. 2B and Fig. S3B), but had very few mMCP6+ MCs in the stroma or submucosa (Fig. 2C and Fig. S3 C and D). However, by 4 mo of age, polyp-infiltrating MCs were absent or barely visible in the IL-10 deficient mice (Fig. 2 A and C and Fig. 3 B–D), suggesting that IL-10 was needed for MC persistence and expansion in the polyps. By 7 mo of age, the mice of APCΔ468 mice. These mice were born with a hereditary defect in the APC gene rendering them susceptible to polyposis (16); however, somewhat surprisingly, they also exhibited mastocytosis in polyps (11, 13–15) consistent with type-2 inflammation. To test whether expansion of MCs in polyps is indicative of local type-2 inflammation, we measured tissue cytokine levels in the intestines of polyp-ridden mice. IL-10, IL-13, and IL-5, as well as TGF-β, were strongly expressed (Fig. S1). Although we did not detect a significant increase in soluble IL-33, transformed epithelial cells of the polyps displayed strong nuclear IL-33 staining compared with epithelia of the underlying hyperproliferative crypts, and healthy neighboring tissues (Fig. 1A and Fig. S2A–C). IL-33 is a nuclear alarmin and maturation factor for both MCs and ILCs (17). IL-33 functions both as a cytokine and as a nuclear factor that regulates expression of ST2/IL-33 receptor (IL-33R) and its soluble decoy receptor counterpart (18). Therefore, we measured expression of the IL-33R (ST2/IL-33R) by polyp-infiltrating MCs and compared this with the healthy surrounding tissues. Microdissected polyps were enzymatically digested to prepare mononuclear cells (MNCs), which were then subjected to flow cytometric analyses. MCs were identified after negative gating for lineage-specific markers and positive gating for FcεRI and cKit. While all MCs expressed ST2/IL-33R, the mean fluorescence intensity of staining, which represents the density of receptors per cell, was significantly higher on polyp-infiltrating MCs compared with MCs infiltrating the healthy surrounding tissues (Fig. 1B and C and Fig. S2D). Up-regulation of ST2 on
developed invasive tumors (Fig. S3A). New waves of MCs infiltrated the stroma and the submucosa of the lesions, and notably accumulated in the invasive front of the tumors (Fig. S3C). These MCs had a strong preference to express mMCP6 relative to mMCP2 (Fig. 2B; compare with Fig. 2C). In addition to MCs, eosinophils were detected in and around the polyps, with significantly higher densities in IL-10-deficient than IL-10–competent APCΔ468 mice. Eosinophils showed a different distribution in the SB, where they accumulated at the margins of polyps, and relative to the colon, where they gathered in the submucosa beneath the polyps (Fig. S3D).

The outcome of IL-10 deficiency in the SB sharply contrasted with that in the colon. At 4 mo of age, the SB of the IL-10–deficient mice had normal levels of TGF-β and type-3 cytokines IL-17a, IL-23, and IL-22 but increased concentrations of IL-33, IFN-γ, and the T cell tropic chemokines CXCL9 and CXCL10 (Fig. S1). By contrast, IL-10 deficiency is known to increase microbial-initiated type-3 cytokines in the colon (20). While the APCΔ468 mice with IL-10–deficient T cells exhibited a striking slowdown in growth of SB polyps in the colon, polyposis was accelerated (Fig. 2A). Consistent with the delayed SB polyposis in IL-10–deficient APCΔ468 mice, we noted that mitotic activity of normal SB crypt epithelial cells was reduced (Fig. S4A). By contrast, there was enhanced crypt epithelial mitosis in the colon of IL-10–deficient APCΔ468 mice (Fig. S4B). It is well known that IL-10 deficiency worsens microbial-induced inflammation and colonic polyposis (20). This has to do with the indispensable function of IL-10–expressing T cells in suppressing bacteria-induced type-3 inflammation. Our findings suggested a different and potentially contrasting function for IL-10 dependence of polyp-infiltrating mMCP2<sup>+</sup> and mMCP5<sup>+</sup> MCs, we overexpressed IL-10 in T cells under the control of the IL-2 gene promoter (IL2pIL10 APCΔ468), and compared IL-10 mice with IL-10–deficient APCΔ468 mice. By far the majority of MCs in benign polyps were SB stained with CAE for MCs are T cells in the SB. (Magnification: 3 fields, ×400.)

Differentiation of MCs and increased densities of IL-10–expressing T cells in the SB. To test how T cell expression of IL-10 alters polyposis and MCs, we overexpressed IL-10 in T cells under the control of the IL-2 gene promoter (IL2pIL10 APCΔ468) and examined mice at 4 mo of age. Polyposis in both the small intestine and colon was reduced, with a larger impact on the small intestine (Fig. 3 A and B). The IL2pIL10 APCΔ468 mice did not have elevated levels of IL-10 in the SB, but TGF-β was reduced compared with the parental APCΔ468 mice (Fig. S1). There was extensive expansion of MCs in the SB of IL2pIL10 APCΔ468 mice (Fig. 3 C and D), both inside polyps and spreading beyond the polyps to the neighboring healthy SB tissues (Fig. 3 E and F). Furthermore, there were 40-fold more T cells expressing IL-10 in the SB of IL2pIL10 mice than control APCΔ468 mice (Fig. 3 G–I).

Expansion of MCs and increased densities of IL-10–expressing T cells, but no significant increase in soluble IL-10, are in line with T cell dependence of MMC expansion. However, these observations also reflect the complex biology of polyposis and multifaceted functions of IL-10. IL-10 is naturally elevated in polyps; therefore, when polyposis is attenuated, IL-10 levels go down, which neutralizes the contribution of overexpression of IL-10 by IL2pIL10 T cells. Type-I response and establishment of CD8 T cell memory, as well as antitumor cytotoxicity (19), depend on IL-10–expressing T cells and Tregs (22). Thus, we cannot rule out that IL2pIL10 mice have stronger immune surveillance. Expression of IL-10 by T cells also could help stabilize the antiinflammatory properties of Tregs, which are protective in polyposis (23). It is also possible that polyp growth requires a threshold MC density above which it does not get any worse. Interestingly, as delineated below, tumor progression is more related to the subtype of expanded MCs rather than overall MC numbers.

IL-10–dependent expansion of MC was independent of microbiota. Only colonic polyps responded to treatment with broad-spectrum antibiotics; SB polyps showed little change (Fig. S5 A and B). Interestingly, while antibiotic treatment did not curtail MC expansion in SB polyps, it did hinder the expansion of eosinophils (Fig. S5 C and D). Thus, expansion of MCs and the associated inflammation driven by IL-10–expressing T cells were independent of microbiota. It is possible that intraepithelial MCs differentiate in response to sterile danger signals from the transformed epithelial cells. Indeed, SB polyposis has been reported to be less dependent on microbiota (24), and the growth of polyps in the SB was reported in germfree mice (25).

Altogether, these observations provide evidence for the existence of distinct IL-10–dependent, as well as IL-10–independent, MC populations in the SB with mucosal and connective tissue characteristics, based on expression of MC-specific proteases and localization. The differential kinetics of their expansion are consistent with each population being associated with and potentially contributing to distinct stages of tumor initiation and progression. Expansion of eosinophils in the absence, but not the presence, of IL-10 is consistent with these cells being suppressed by IL-10. In our model, the response of eosinophils to antibiotics, in contrast to MCs, points to microbiota driving their expansion.

**The Second Wave of MCs Marks the Polyp-to-Carcinoma Transition.** Next, we modeled the genetic cascade of multistage carcinogenesis to delineate the nature of MC expansion in progressive cancer. We used a modified fatty acid-binding promoter Cre mouse (26) in combination with conditional APC (cAPC) (27) to initiate polyposis in the distal ileum and colon (28). To study the polyptc-carcinoma transition, we also activated Kras or inactivated PTEN, superimposed onto the loss of function of APC. The resulting Ts4CrecAPC<sup>Kras</sup> and Ts4CrecAPC<sup>PTEN</sup> mice developed invasive tumors similar to those in older IL-10–deficient APCΔ468 mice. By far the majority of MCs in benign polyps were mucosal, adhered to the aberrant crypt epithelia, and expressed mMCP2 (Fig. 4 A and C). MC distribution and proteases changed in invasive lesions, with new waves of mMCP<sup>+</sup> and mMCP<sup>+</sup> MCs expanding in the stroma and submucosa (Fig. 4 B and D–F).
Submucosal MCs adhered to the smooth muscle at the tumor-invasive border, and preferentially expressed mMCP5 (Fig. 4 D and F). Thus, sequential oncogenic events led to expansion of distinct MC types with characteristic spatial distributions.

Ablation of mMCP6 Attenuates Polyposis but Does Not Hinder the Polypt-to-Carcinoma Transition. The expansion of mMCP6+ MCs in invasive tumors prompted us to examine the role of mMCP6 in SB cancer. Earlier studies suggested that expression of mMCP6 by MCs is controlled by IL-33 (29), whereas expression of mMCP2 is stimulated by IL-10 (30). We cultured MC progenitors in the presence of IL-33 or IL-10. MCs that matured with medium containing IL-33 had stronger expression of mMCP6, whereas IL-10 inhibited IL-33 expression, in agreement with earlier findings (29) (Fig. S6). Next, we generated chimeric TS4CrecAPC-Pten mice with mMCP6-deficient bone marrow or control wt bone marrow. The chimeric mice had reduced frequencies of mMCP6+ MCs in SB polyps (Fig. 5A and Fig. S7) and increased numbers of mMCP2+ MCs (Fig. S8). Polyposis was attenuated in mice with mMCP6-deficient bone marrow compared with control mice (Fig. 5C and D). Our findings are consistent with a tumor-promoting role for mMCP6. Interestingly, the few remaining lesions were still invasive, suggesting mMCP6 has a role in promoting polypl growth, but not invasion.

Discussion

MC and Polyposis. In the current study, we find that MCs which expand in benign polyps share characteristics with MMCs. These characteristics include expression of mMCP2 and adherence to gut epithelial cells. These MCs expanded in a tissue environment containing elevated levels of type-2 cytokines and ILC2s. A striking finding was that the expansion of this wave of mMCP2+ MCs was IL-10-dependent. IL-10 is generally thought to be an antiinflammatory cytokine. However, in some circumstances, IL-10 may function as a proinflammatory cytokine. Earlier studies suggest that IL-10 is involved in the recruitment and maturation of MMCs (30–32). IL-10 stimulates expression of the MMC-specific proteases mMCP1 and mMCP2 (30, 33), and promotes the influx and development of MMCs in a food allergy model (4). Thus, IL-10 is directly or indirectly a cofactor in the development and maturation of MMCs. The persistence of MMCs in benign polyps required expression of IL-10 by T cells. IL-10 deficiency eliminated MMCs and delayed SB polyposis. Conversely, overexpression of IL-10 by T cells expanded MMCs within polyps and in the neighboring tissues, confirming that IL-10–expressing T cells have a rate-limiting role in the maturation of MMCs in the SB. MCs did appear in microscopic polyps of young mice with IL-10–deficient T cells but were lost with time, suggesting that IL-10 may be needed for the persistence of MMCs. We conclude that IL-10 shapes the immune environment of the SB by changing the density of IL-10–dependent MCs. The coincident loss of MCs in IL-10-deficient mice and delay in polyposis suggest that MCs are required for polyposis in the SB, consistent with our earlier findings (13–15). However, further expansion of MMCs through overexpression of IL-10 did not enhance SB polyposis. This reflects the complex biology of IL-10 and polyposis, since IL-10 also enhances T cell memory and antitumor immune surveillance (19), as well as help recovery of Treg antiinflammatory T cells, and hence protective, functions (23). These simultaneous events complicate interpretation of how expansion of MCs over and above what naturally happens during polyposis contributed to polypl growth. The lack of response of polyp-infiltrating MMCs to broad-spectrum antibiotics strongly argues that MC expansion is not bacterially driven.

MCs and SB Cancer. The ability to generate mice with a spectrum of benign and invasive lesions allowed us to identify different types of MCs with distinct spatiotemporal distributions as dysplasia progressed to cancer. We saw a striking expansion of MCs in the stroma and submucosa of tumors that arose by introduction of secondary oncogenic mutations, Kras, or PTEN. The new wave of MCs was distinct from the MCs detected in benign polyposis by their appearance in invasive lesions, location, and expression of CTMC-specific proteases mMCP6 and mMCP5. Here, we find an intriguing similarity in the sequential expansion of MMCs and CTMCs in SB cancer and the earlier reported sequence of MMC expansion leading to inflammation, followed by CTMC expansion and resolution of inflammation, in T. spiralis-infected mice. Interestingly, IL-10 deficiency, while delaying SB polyposis, eventually, by itself, led to cancer progression. This may be due to the absence of CD8 T cell memory in IL-10–deficient mice (19) or to the loss of Treg-dependent antiinflammatory properties (16, 23, 34), absence of cytotoxic CD4 T cells (35), or all of these. Loss of IL-10 eliminated polyp-infiltrating MCs that had MMC characteristics. However, it did not hinder MCs for long. New waves of MCs with CTMC properties vigorously expanded during the polypt-to-carcinoma transition. Thus, IL-10 deficiency in the long term reproduced the same sequel of events as in mouse
models of cancer based on the addition of secondary oncogenic events. The expression of MC proteases, mMCP6, mMCP5, and potentially also mMCP4, contributes to MC promotion of SB cancer. A glimpse into these mechanisms came from deletion of mMCP6 in the bone marrow of APCΔ468 mice, which led to attenuation of polyposis. Because of the preferential localization of mMCP5+ MCs to the tumor-invasive border, we predict that this protease may have a role in the transition from polyp to carcinoma. The role of mMCP4 also needs further investigation.

Region-Specific Effects of MC Expansion: Location, Location, Location. Interestingly, our data suggest that the inflammation-promoting properties of MCs are likely to be less important in the bacteria-rich colon. IL-10 is indispensable for Treg suppression of microbial-instigated type-3 inflammation (36–38), and down-regulation of IL-10 in Tregs is a characteristic of the immune pathology of colon cancer (16). We find that the colon of IL-10-deficient mice has exacerbated polyposis despite the loss of MCs. The role of the microbiota in colon polyposis is well established. In earlier studies, we showed that colon polyposis can be reversed by oral gavage of mice with strains of Lactobacillus acidophilus that lack cell surface expression of the bacterial lipoteichoic acid, a Toll-like receptor 2 ligand (28). However, we did not detect substantial changes in SB polyposis. Our observations clearly demonstrate striking differences between the immune environments of the SB and colon, and differential roles for IL-10 in these two sites. Since IL-10 deficiency increased colonic polyposis despite the loss of MCs, we can conclude that MCs are not required for polypl growth in the colon, where microbiota dominate regulation of inflammation.

In Conclusion
Together, our findings argue that type-2 inflammation, ILC2s, and MCs are etiologically linked to multistep SB carcinogenesis. Specifically, spatiotemporally regulated expansion of distinct MC subsets and expression of their elaborated proteases shape a supportive tumor environment as polyps grow and continue to progress to cancer. Targeted interruption of this pathway is likely to prevent or potentially interrupt the carcinogenesis process in the SB. We predict that this pathway is also important in other cancers of the upper GI tract where microbiotas are at low densities and not rate-limiting for inflammation.

Materials and Methods
SB tissue was fixed with formalin and paraffin-embedded, and then sectioned (5 μm) for immunofluorescent staining as reported before. A Leica light microscope mounted with a Zeiss Axioscan 503 camera was used for imaging immunohistochemistry staining. For fluorescent staining, a Zeiss Observer. 21 microscope mounted with an AxioCam 506 mono camera was used for imaging. Fiji software was used for image analysis. The ImmunoRatio plug-in was used for nuclear IL-33 ratio analysis. Additional information is available online as Supporting Information.

For fluorescence-activated cell sorting (FACS), SB tissue was digested with a mixture of collagenase (C5138-1G; Sigma), hyaluronidase (H6254, 500 mg; Sigma), and DNase (D5025-15KU; Sigma), and MCNs were prepared by Percoll gradient centrifugation as previously described. FACS analysis was done on a BD LSRII flow cytometer (BD Biosciences) and an Accuri BD flow cytometer (BD Biosciences), and was analyzed by FlowJo software (TreeStar). Detailed methods of antibodies used and staining conditions are available online as Supporting Information.

The statistical analyses were performed with the use of Prism7 (GraphPad). Unpaired one-tailed t tests with the Wells correction were used.

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