IL-33-mediated mast cell activation promotes gastric cancer through macrophage mobilization

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The contribution of mast cells in the microenvironment of solid malignancies remains controversial. Here we functionally assess the impact of tumor-adjacent, submucosal mast cell accumulation in murine and human intestinal-type gastric cancer. We find that genetic ablation or therapeutic inactivation of mast cells suppresses accumulation of tumor-associated macrophages, reduces tumor cell proliferation and angiogenesis, and diminishes tumor burden. Mast cells are activated by interleukin (IL)-33, an alarmin produced by the tumor epithelium in response to the inflammatory cytokine IL-11, which is required for the growth of gastric cancers in mice. Accordingly, ablation of the cognate IL-33 receptor St2 limits tumor growth, and reduces mast cell-dependent production and release of the macrophage-attracting factors Csf2, Ccl3, and Il6. Conversely, genetic or therapeutic macrophage depletion reduces tumor burden without affecting mast cell abundance. Therefore, tumor-derived IL-33 sustains a mast cell and macrophage-dependent signaling cascade that is amenable for the treatment of gastric cancer.

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The interactions between cancer cells and their microenvironment can result in tumor progression as well as suppression and/or eradication of cancers. Besides extra-cellular matrix and stromal cells, the tumor microenvironment is composed of immune cells of the adaptive and innate immune system, with the latter comprising neutrophils, macrophages, mast cells, myeloid-derived suppressor cells, dendritic cells, innate lymphocytes, and natural killer cells. Often T-lymphocytes and tumor-associated macrophages (TAMs) account for the most abundant immune cell populations infiltrating established tumors.

Mast cells are long-lived secretory cells of hematopoietic origin that function as sentinels by responding to changes in their environment. They respond to extrinsic signals through a multitude of cell surface receptors to secrete histamine and proteases from prestored sources in cytoplasmic granules, alongside newly synthesized inflammatory mediators. Accordingly, mast cells can be part of innate and adaptive immune responses and therefore contribute to various pathophysiological conditions. In cancer, the presence of mast cells, both at the tumor periphery and in the tumor core can correlate with disease progression, increased metastasis and reduced survival of patients with melanoma, prostate cancer, pancreatic adenocarcinoma, squamous cell carcinoma, and gastric cancer. However, in other solid malignancies, mast cells have been associated with more favorable outcomes. The conflicting nature of these correlative findings are reminiscent of contradictory functional observations in mouse models claiming that mast cells are required for pancreatic islets tumorigenesis, while pancreatic adenocarcinoma occurs in a tumor microenvironment devoid of mast cells.

Interleukin (IL)-33 is a danger-associated signal that can serve as a molecular alarmin when released upon necrotic and necrotic cell death including death of cancer cells. However, cell death-independent IL-33 release can also occur. IL-33 signals through the heterodimeric ST2 receptor, encoded by Il1rl1 gene, which is constitutively expressed on the surface of some innate immune cells including mast cells, innate lymphoid cells type 2 (ILC2), and regulatory T-cells (Treg). IL-33/ST2 signaling is involved in triggering innate immune responses upon parasite and viral infections, and has been identified as an important mast cell activating factor in the context of allergic responses. Furthermore, elevated IL-33 expression was associated with poor outcomes in patients with gliomas, ovarian, as well as head and neck cancers. However, predicting the outcome of IL-33/ST2 signaling in malignancies remains uncertain with both tumor promoting as well as tumor restricting activities being reported in knockout mouse models.

Here, we employ preclinically validated mouse models of gastric cancer and corresponding patient biopsies to functionally elucidate the role of mast cells during gastric tumorigenesis. Our genetic analysis reveals a linear signaling axis initiated by tumor genetic analysis reveals a linear signaling axis initiated by tumor genotype, with tumorigenesis independently of the nature of the oncogenic driver mutation, we also assessed mast cell distribution in Tg (Tff1-CreERT2); Pika3caH1047R+/−; Pten+/− mice. In this model, conditional activation of a pan-glandular gastric epithelium-specific Cre-recombinase induces aberrant activation of phosphatidylinositol 3-kinase (PI3K), a pathway frequently mutated in human gastric cancer. Akin to our observations in gp130FF mice, mast cells also accumulated in the submucosa of tumors in Tg (Tff1-CreERT2); Pika3caH1047R+/−; Pten+/− mice (Supplementary Fig. 1a, b). Strikingly, toluidine blue, alcoin blue/safranine O, and May–Grunwald–Giemsa stains for granular content all consistently revealed that mast cells localized to the submucosal layers of the antrum and body of the stomach, rather than within the tumors (Supplementary Fig. 1c).

We next assessed the stromal abundance of mast cells in human biopsies collected from patients with either histopathologically classified diffuse or intestinal-type gastric cancer, or patients with preneoplastic intestinal metaplasia or chronic gastritis. Similar to our observation in mice, we observed a profound submucosal accumulation of toluidine-blue positive mast cells in intestinal-type gastric cancer. Strikingly, mast cells numbers were not elevated in the submucosa of patients with gastritis or intestinal metaplasia (Fig. 1c, d). Collectively, our results show increased mast cell abundance in the tumor-adjacent submucosa of intestinal-type gastric adenocarcinoma in human and mice, irrespective of the nature of the oncogenic driver mutation(s).

**Results**

Increased mast cell density in human intestinal-type gastric cancer and in corresponding mouse models. In order to characterize the role of mast cells in gastric cancer, we initially investigated the mast cell frequency in gp130FF mice, a preclinically validated model for spontaneously occurring intestinal-type gastric cancer. Gastric tumors in gp130FF mice, which harbor a knock in germine mutation in the shared IL-6/IL-11 receptor subunit gp130, arise from excessive IL-6/IL-11 dependent STAT3 activity; these tumors remain associated with chronic inflammation and immune cell infiltration. We used toluidine blue stains to quantify mast cells, and observed profound accumulation of mast cells in the submucosa of the gastric antrum in gp130FF mice compared to wild-type mice. Importantly, mast cells in the submucosa adjacent to gastric tumors were more abundant than the mast cells in the normal antrum-associated submucosa of gp130FF mice (Fig. 1a, b). In addition, mast cell numbers in gp130FF; Stat3+/−/− compound mutant mice, which neither develop gastric tumors nor the other pan-inflammatory changes observed in their Stat3-proficient gp130FF; Stat3+/+/− littermates, remained comparable to those in wild-type mice.

To ascertain that submucosal mast cell accumulation correlated with tumorigenesis independently of the nature of the oncogenic driver mutation, we also assessed mast cell distribution in Tg (Tff1-CreERT2); Pika3caH1047R+/−; Pten+/− mice. In this model, conditional activation of a pan-glandular gastric epithelium-specific Cre-recombinase induces aberrant activation of phosphatidylinositol 3-kinase (PI3K), a pathway frequently mutated in human gastric cancer. Akin to our observations in gp130FF mice, mast cells also accumulated in the submucosa of tumors in Tg (Tff1-CreERT2); Pika3caH1047R+/−; Pten+/− mice (Supplementary Fig. 1a, b). Strikingly, toluidine blue, alcion blue/safranine O, and May–Grunwald–Giemsa stains for granular content all consistently revealed that mast cells localized to the submucosal layers of the antrum and body of the stomach, rather than within the tumors (Supplementary Fig. 1c).

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**Mast cell deficiency diminishes gp130FF-driven tumor growth.** Having established a reproducible correlation between submucosal mast cell density and gastric cancer in humans and mice, we next determined the functional role of mast cells during tumor initiation and progression. For this we exploited the gp130FF model because of its high disease concordance with the human GC subtype, reproducible penetration, onset, and progression of gastric tumorigenesis.

In order to establish whether submucosal mast cell accumulation preceded tumorigenesis, we analyzed stomach sections of 4-week-old gp130FF mice prior to onset of tumor formation. We detected increased mast cell density in the gastric submucosa of these tumor-free gp130FF mice when compared to the submucosa of age-matched WT littermates (Supplementary Fig. 2a). To functionally determine whether mast cells indeed promote gastric tumor growth, we crossed gp130FF mice with mast cell-deficient C57BL/6 c-KitW-sh/W-sh mice carrying an inversion of the 5′-regulatory region of the c-Kit gene that results in hypomorphic expression of the corresponding stem cell factor receptor protein.
Accordingly, \( c^{-}\text{Kit}^{W-sh/W-sh} \) mice exhibit a substantial reduction of mast cells in all tissues\(^4^1\)–\(^4^3\), but do not suffer from anemia, sterility and lethality associated with complete \( c^{-}\text{Kit} \) deficiency\(^4^1\). Likewise, we did not observe differences in the peripheral blood composition between \( gp130^{FF} \) and \( gp130^{FF}; c^{-}\text{Kit}^{W-sh/W-sh} \) compound mutants and we confirmed that EpCAM positive tumor cells lack c-Kit expression (Supplementary Figs. 2b, c). However, \( gp130^{FF}; c^{-}\text{Kit}^{W-sh/W-sh} \) compound mice had significantly smaller and fewer tumors than their age and sex-matched \( gp130^{FF} \) littermates (Fig. 2a–c). This reduction in tumor burden coincided with reduced tumor cell proliferation rather than increased apoptosis, and was associated with a reduction of CD31+ endothelial cells (Fig. 2d and Supplementary Fig. 2d).

To exclude that the anticancer effect of the \( c^{-}\text{Kit}^{W-sh} \) hypomorphic allele was not in part mediated by hematopoietic cells other than mast cells, we generated mast cell-deficient \( gp130^{FF}; \text{Cpa3-Cre}; \text{Mcl1}^{fl/fl} \) gastric cancer mice, where mast cell-specific carboxypeptidase A3 (Cpa3) promoter driven Cre recombinase activity leads to the deletion of Mcl1 prosurvival gene. As a consequence \( \text{Cpa3-Cre}; \text{Mcl1}^{fl/fl} \) mice retain less than 10% mast cells and have reduced numbers of basophils, while all other hematopoietic cell populations remain unaffected\(^4^4\). We

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**Fig. 1** Submucosal mast cell numbers are increased in gastric cancer in mice and humans. a Representative cross sections of stomachs of 100 day old \( gp130 \) mutant mice of the indicated genotype and stained with toluidine blue showing the affected antrum (AN) and antral tumor (AT), respectively. Mast cells appear purple (arrows). Scale bars = 50 μm. b Quantification of submucosal mast cell in sections depicted in (a), \( n = 7 \) mice per cohort obtained from two independent experiments, one-way ANOVA \( F \) (DF\( n \) = degree of freedom nominator, DFD = degree of freedom denominator) = 34.96 (3, 24).

c Representative sections of toluidine blue-stained biopsy cores of human gastric cancer (GC) and adjacent submucosa and of normal stomach submucosa. Scale bars = 50 μm. d Quantification of submucosal mast cell in sections depicted in c. Each symbol represents an individual patient biopsy from submucosa of normal (N; \( n = 22 \)), gastritis (Gas; \( n = 2 \)), intestinal metaplasia (IM; \( n = 4 \)), diffuse (Diff; \( n = 8 \)), mixed (Mix; \( n = 3 \)), and intestinal-type (Int; \( n = 8 \)) gastric cancer. One-way ANOVA \( F \) (DF\( n \), DFD) = 5.809 (5, 41). Data are represented as mean ± SEM, with \( p < 0.05 \) considered significant.
confirmed that gp130FF; Cpa3-Cre; Mcl1−/− mutant mice lack mast cells in their stomachs, while their gp130FF; Cpa3-Cre; Mcl1+/+ littermates display normal mast cell density (Supplementary Fig. 2e). Importantly, mast cell-deficient gp130FF; Cpa3-Cre; Mcl1−/− mice had significantly reduced tumor mass and tumor number compared to their mast cell-proficient controls (Fig. 2c, f), and this observation coincided with reduced angiogenic vessel density in the tumors of gp130FF; Cpa3-Cre; Mcl1−/− mice (Fig. 2g).

To assess whether therapeutic mast cell manipulation could reduce the burden of established tumors, we exploited sodium cromoglycate (cromolyn) as a blocking agent for mast cell degranulation in patients. We treated tumor-bearing gp130FF mice for 6 weeks with cromolyn, which significantly reduced tumor burden. The effect of cromolyn was particularly striking in mast cell-deficient mice, with a reduction of over 90% in tumor burden compared to vehicle control (Fig. 2h). These findings suggest that therapeutic targeting of mast cells may be a promising strategy for the treatment of gastric tumors.
tumor burden when compared to vehicle-treated mice (Supplementary Fig. 2f and Fig. 2h). Akin to our observations genetic observations in gp130FF; c-Kit−/−; W-sh/W-sh and gp130FF; Cpa3-Cre; Mc4rfl/fl mice, cromolyn treatment of gp130FF mice also reduced macrophage accumulation, tumor angiogenesis and proliferation (Supplementary Fig. 2g). These findings demonstrate the importance of mast cells and their released products for gastric tumor growth and suggest that mast cells promote tumor proliferation through mechanisms involving angiogenesis.

In the gp130FF mutant mouse all cells harbor the gp130 mutation. When expressed, the gp130 mutant protein increases Stat3 signaling in response to IL-6 family cytokines. Because mast cells express the gp130 coreceptor and can respond to IL-6 family cytokines, we next excluded the possibility that the gp130 allele in mast cells may augment their tumor-promoting ability. For this, we reconstituted the hematopoietic compartment of 6-week-old lethally irradiated gp130FF hosts with the bone marrow from WT (gp130+/+) or gp130FF mice (Supplementary Fig. 2h). Indeed, WT → gp130FF and gp130FF → gp130FF bone marrow chimeras showed not only similar mast cell frequencies in the gastric submucosa but also comparable tumor burden, and macrophage infiltration in the submucosa and tumor core (Supplementary Fig. 2i). These results indicate that the systemic presence of the gp130FF mutation did not skew hematopoietic cells towards a tumor-promoting role, nor did it increase the mast cell abundance within the gastric tumor microenvironment.

Macrophages are reduced in gastric tumors in a mast cell-dependent manner. Because we observed mast cells outside of the tumor cores, we surmised that tumor-promoting effect of mast cells might occur indirectly by affecting the composition and/or function of tumor infiltrating immune cells. We therefore enumerated tumor-free and tumor-containing stomach sections collected from gp130FF and mast cell-deficient gp130FF; c-Kit−/−; W-sh/W-sh mice for the F4/80 macrophage, CD3 T lymphocyte and B220 B-lymphocyte markers. While we observed a 30% reduction in macrophage frequency in the submucosa and cores of tumors from gp130FF; c-Kit−/−; W-sh/W-sh mice, the lymphocyte (CD3+), Treg (Foxp3+), and CD8+ T cell frequencies remained unchanged (Fig. 3a, b). Reduced macrophage abundance within the tumor core and submucosa was also observed in the mast cell-deficient gp130FF; Cpa3-Cre; Mc4rfl/fl mice (Fig. 3c) and cromolyn-treated gp130FF mice (Supplementary Fig. 2g). Because genetic or pharmacological interference with mast cells not only inhibited tumor growth but concomitantly also reduced the abundance of tumor-associated macrophages, we postulate that a hierarchical mast cell—macrophage relationship underpins the growth of gastric cancer.

Macrophage infiltration coincides with gastric tumorigenesis. To ascertain that the accumulation of tumor-associated macrophages was a common occurrence for intestinal-type gastric cancers, we assessed macrophage densities in the submucosa and mucosa of tumor-bearing gp130FF and Tg(Tff1-CreERT2); Plk3-caF1H1047R/+; Ptenfl/fl mice. We noted an increased abundance of macrophages in the submucosa and the mucosa of either model (Fig. 3d, e and Supplementary 3a, b), consistent with the increased abundance of submucosal mast cells in both models (Fig. 1a, b and Supplementary Fig. 1a, b). To establish whether the increased macrophage accumulation occurs prior to tumor formation, akin to our observations with mast cells, we analyzed naïve gp130FF mice at 4 weeks of age. In these mice macrophage numbers were increased in the submucosal layers, but remained similar between the precancerous mucosa of gp130FF and of WT mice (Supplementary Fig. 3c, d). Collectively, these findings positively correlate macrophage numbers with gastric tumor growth and suggest a potential macrophage contribution to tumor formation and progression.

Macrophages are required for gastric tumorigenesis in gp130FF mice. Macrophages have been established as drivers of tumor growth and cancer therapy targets for several tumor entities. To formally investigate a functional contribution of macrophages to gastric tumorigenesis, we generated macrophage-deficient gp130FF; Csf1r−/− mice, because expression of the Csf1r gene and its corresponding receptor protein for macrophage colony-stimulating factor is required for maturation of bone marrow-derived macrophages (BMDM) from their monocyctic precursors. Strikingly, the few surviving gp130FF; Csf1r−/− mice remained completely free of gastric tumors (Fig. 4a, b). The submucosal mast cell density was not altered in the macrophage-deficient animals (Fig. 4c). We next confirmed that this phenotype reversal was not related to the runted phenotype of gp130FF; Csf1r−/− mice associated with their feeding requirement for mashed chow to overcome the CsflR deficiency-dependent absence of teeth. We therefore depleted macrophages in adult tumor-bearing gp130FF mice by systemic administration of clodronate-loaded liposomes (clodrosomes) for 6 consecutive weeks and observed a significant reduction of tumor burden when compared to the vehicle control cohort (Fig. 4d). Although toluidine-staining confirmed that clodrosome treatment did not reduce the abundance of submucosal mast cells, gastric tumors and adjacent submucosa from the clodrosome cohort revealed reduced density of CD31-positive microvessels (Fig. 4e and Supplementary Fig. 4a).

The reduced tumor-burden and microvessel density observed in the clodrosome cohort were similar to our findings from the mast cell depletion experiments. We next aimed to exclude that they relate to off-target-effects associated with bisphosphonate (i.e., clodronate) administration. We therefore treated tumor-bearing gp130FF mice with the small molecule Csf1r/c-kit/Flt3 kinase inhibitor PLX3397 (Pexidartinib). We supplied PLX3397 in the food to tumor-bearing gp130FF mice for 4 weeks (acute therapeutic) and continued a cohort for a further 4 weeks without PLX3397 treatment (follow-up) (Supplementary Fig. 4b). Acute PLX3397 treatment revealed a significant reduction in tumor burden, although this antitumoral effect was reversible as the tumor burden increased once PLX3397 treatment ceased in the follow-up cohort (Fig. 5a). In the acute treatment cohort, we confirmed by immunohistochemical staining a significant reduction of F4/80-positive macrophages accompanied by reduced density of CD31-positive angiogenic vessels and increased staining for tumor-hypoxia (Fig. 5b and Supplementary Fig. 4c).

However, owing to the specificity of PLX3397, we also observed a decrease of tumor-adjacent submucosal mast cells. All these parameters reverted back to their pretreatment state following a drug-free period in the follow-up cohort (Supplementary Fig. 4c).

Based on our proposed role for macrophages to mediate the tumor promoting effect of mast cells, we hypothesized that the tumor-associated macrophages in the gastric lesions of gp130FF mice should have a tumor-promoting alternatively activated endotype. We therefore compared expression of macrophage endotype markers between naïve BMDM and tumor-associated macrophages, and used standard in vitro polarization protocols for BMDM to generate a reference population for alternative endotype markers. We therefore compared expression of macrophage endotype markers between naïve BMDM and tumor-associated macrophages, and used standard in vitro polarization protocols for BMDM to generate a reference population for alternative endotype markers. For the tumor-associated macrophage populations, this analysis revealed high expression of the alternative activation markers Arg1, Fizz1, and Mrc1, which coincided with reduced mRNA levels of the classically activated marker Nos2 (Fig. 5c). Consistent with the pronounced presence of microvasculature in tumors of these gp130FF mice, we also recorded elevated expression of angiogenic Vegfa in the tumor-associated
Fig. 3 Mast cell depletion reduces macrophage infiltration in gastric tumors of gp130T mice. a Representative images of tissue sections immunostained for macrophages (F4/80), B cells (B220) or T cells (CD3, CD8, Foxp3) in gastric tumor sections of gp130T; c-Kit+/+ and mast cell-deficient gp130T; c-KitW-sh/W-sh. Arrows indicate specific positive cell staining; scale bars = 100 μm. b Quantification of F4/80, B220, or CD3 expressing cells in tumor and tumor-associated submucosal layers of sections from (a). F4/80: n = 6 each group, one-way ANOVA F (DFn, Dfd) = 13.43 (3, 20); B220: n = 6 each group, one-way ANOVA F (DFn, Dfd) = 3.782 (3, 20); CD3: F4/80; c-Kit+/+ n = 6, FF; c-KitW-sh/W-sh n = 5, one-way ANOVA F (DFn, Dfd) = 11.55 (3, 18); CD8: FF, c-Kit+/+ n = 8, FF, c-KitW-sh/W-sh, n = 6, t-test t (df) = 0.71 (12); Foxp3: FF, c-Kit+/+ n = 6, FF, c-KitW-sh/W-sh n = 8, t-test t (df) = 0.33 (12). Data from two independent experiments were analyzed. c F4/80+ cells frequency in stomach submucosa and tumors of FF; MCwt mice (n = 9) and mast cell-deficient FF; MCdef mice (n = 7 FF; Cpa3-Cre; McIβ/β). One-way ANOVA F (DFn, Dfd) = 10.81 (3, 28). d Quantification of F4/80+ cells in unaffected antrum (AN) or antrum tumor (AT) in submucosa or mucosa of mice of the indicated genotype. All groups n = 4. Submucosal: one-way ANOVA F (DFn, Dfd) = 10.12 (3, 12); Mucosal: one-way ANOVA F (DFn, Dfd) = 5.086 (3, 12). e Quantification of F4/80+ cells in unaffected antrum (AN) or antrum tumors (AT) in submucosa or mucosa of mice from the Tg(Tfl-CreERT2); Pik3caH1047Rfl/fl strain that either harbor (Cre+) or lack (Cre−) the Tff1-CreERT2 driver. Cre− AN n = 4, Cre+ AN and Cre+ AT n = 5 for both. Analyses from two independent experiments. Submucosal: one-way ANOVA F (DFn, Dfd) = 10.52 (2, 11); Mucosal: one-way ANOVA F (DFn, Dfd) = 7.485 (2, 11). Data are represented as mean ± SEM, with p values p < 0.05 considered being significant. Source data are provided as a Source Data file. See also related Supplementary Fig. 3.
macrophage population when compared to the naïve BMDM population from the same mice (Fig. 5c). Given the alternative activated endotype of tumor-associated macrophages, our data collectively suggests that their abundance is likely to be a rate-limiting factor for the establishment of tumor microvasculature and the growth of gastric tumors.

**Tumor-derived IL-33 activates mast cell secretion and expression of macrophage-attracting chemokines.** Although our observations thus far suggested a functional mast cell–macrophage relationship, it remained to determine the molecular network underpinning this hierarchy including how its activation could occur. We therefore focused on IL-33 as a potential tumor-derived activator of mast cells, because IL-33 was reported to serve as an alarmin that is generated and released by necrotic cells, and that triggers mast cell degranulation. Indeed, we detected increased expression of IL33 in the EpCam+CD45– epithelial components of the tumors in gp130FF mice with a reciprocal expression of the Il1rl1 gene in the hematopoietic CD45+/EpCam– compartment of the antrum, which encodes the cognate St2 receptor subunit for IL-33 (Fig. 6a). In line with the reported storage of IL-33 protein in vesicles, we detected increased levels of Il33 mRNA and IL-33 protein in whole tumor tissue (Supplementary Figs. 5a, b). Immunofluorescence staining revealed that IL-33 protein localized most prominently to the peripheral regions of the epithelial components of the tumors (Fig. 6b) coinciding with the location of the highest level of cellular turn-over.

To assess whether tumor-associated mast cells could respond to IL-33, we FACs purified the c-Kit+/Fz.eR1+ mast cells from the submucosal antrum of WT and gp130FF mice using stomachs from gp130FF, c-Ki/W-sh mice as a specificity control for our purification strategy (Supplementary Fig. 5c). We confirmed the homogeneity of the c-Kit+/Fz.eR1+ mast cell population by toluidine blue staining (Supplementary Fig. 5d) and showed prominent expression of the St2 receptor in the c-Kit+/Fz.eR1+ mast cells isolated from the stomach of gp130FF mice (Supplementary Fig. 5e). We then stimulated this cell population for 3 h with IL-33 and detected by the multiplex cytokine assay increased amounts of IL-6 and IL-13 and to lesser extent of IL-1α, IL-4, alongside elevated secretion of the macrophage growth and attracting factors Gm-csf, Mcp1, Mip-1α, and Mip-1β (Supplementary Fig. 5f and Fig. 6c). Likewise, qPCR analysis of the IL-33 stimulated mast cells also revealed increased expression of Ccl2 (encoding Mcp1 protein), Ccl3 (encoding Mip-1α protein), and Ccl7 (Fig. 6d), which collectively exert chemotactic activity towards macrophages and neutrophils. Also, IL-33-stimulation induced elevated expression of angiogenic growth factor gene Vegfa (Fig. 6d). In order to confirm the chemotactic activity of the mast cell produced factors on macrophages, we performed migration assays with BMDM. Indeed, Mcp1 and Mip-1α alone induced migration significantly (Supplementary Fig. 5g).

Because gp130FF-driven gastric tumorigenesis has an absolute requirement for IL-11 signaling,38 we predicted that IL-11 could stimulate IL-33 expression in tumor-derived epithelium. To test
this, we exposed epithelial organoids grown from gp130FF tumors to recombinant IL-11 in vitro. While organoid growth and morphology were not altered (Fig. 6e and Supplementary Fig. 5h), organoid growth and expression upon IL-11 stimulation, which was comparable to the extent of induction of the bona fide IL-11/Stat3-target gene Socs3 (Fig. 6f). Additionally, we conducted a chromatin immunoprecipitation (ChIP) experiment on DNA from gastric tumors of gp130FF mice 60 min after acute systemic administration of IL-11. The ChIP experiment revealed a Stat3-specific binding peak on chromosome 19, which maps to the 5’-region of the Il33 gene and which contains a Stat3-binding site motif (Supplementary Fig. 5i, j). Collectively, these data indicate that tumor-derived IL-11 induces Il33 gene expression in gastric tumor cells, which triggers the secretion as well as the de novo synthesis of cytokines and chemokines by mast cells to produce a tumorigenic immune environment.

IL-33/St2 signaling deficiency decreases gp130FF-mediated gastric tumor growth. To validate a functional contribution of IL-33 signaling as a master regulator of mast cell activation in the gastric tumor microenvironment, we generated IL-33 signaling deficient gp130FF; St2−/− mice. Compared to their St2+/+ and St2+/−/progeny compound mutant litters, gp130FF; St2−/− mice harbored a significantly lower overall tumor burden at 100 days of age (Fig. 7a), which arose from reduced tumor growth rather than reduced tumor incidence (Fig. 7b). These observations coincided with a decrease in mast cell numbers in the tumor-adjacent submucosal layers and reduced density of F4/80+ macrophages in the tumors of gp130FF; St2−/− mice as well as reduced abundance of CD31-positive microvessels (Fig. 7c).

We next confirmed that c-Kit+/F4R1+ mast cells isolated from gp130FF; St2−/− mice showed significantly decreased expression of the macrophage recruiting and inflammatory mediators Csf2 (encoding Gm-csf), Ccl3, and Il6 (Fig. 7d). Because IL-33 signaling is involved in expansion and activation of ILC2 and Tregs, which could also afford a mechanism for IL-33 to promote gastric tumorigenesis in gp130FF mice, we assessed the relative abundance of ILC2 and Tregs among all cells harboring the pan-hematopoietic surface marker CD45. We observed comparable frequency of these cells between unaffected antrum and tumors of gp130FF and gp130FF; St2−/− mice. Moreover, less than 30% of all tumor-associated ILC2 cells, and less than 15% of all Tregs, expressed the St2 receptor (Fig. 7e, f).

To investigate the involvement of mast cells in the antitumoral effect of St2-deficiency, we established bone marrow-derived mast cells (BMMC) from gp130FF; St2−/− and gp130FF, St2+/− mice, confirmed their purity (Supplementary Figs. 6a–c) and performed adaptive transfer of BMMC into gp130FF; St2−/− hosts (Supplementary Fig. 6d). Reconstitution with St2+ wild-type BMMC (gp130FF; St2+/+) increased tumor burden compared to littermates injected with gp130FF; St2−/− BMMCs (Fig. 7g and Supplementary Fig. 6e).
IL-33/mast cells-activity gene expression signature predicts poor survival for human intestinal-type gastric cancer. In order to translate our preclinical findings in mice to a relevant setting in humans, we hypothesized that the extent of mast cell activation might provide retrospective prediction of patient survival. Based on the mast cell activation gene expression signature derived from our in vitro mast cell stimulation with IL-33, we analyzed a publically available data set (DErrico Gastric data set; GEO:
GSE13911) for differential expression of the mast cell signature comprising CCL2, CCL3, CCL4, IL1α, IL4, IL6, IL13, CSF2, and CCL7. With the exception of IL-4 and IL-13, we found that all other factors were at least 1.6-fold increase in the cancer samples (p ≤ 0.05) (Fig. 8a). We therefore assigned the differentially expressed factors (i.e., CCL2, CCL3, CCL4, IL1α, IL6, CSF2, and CCL7) to an IL-33/mast cell activation gene expression signature for a Kaplan–Meier survival analysis (KMplot.com)53.
Remarkably, we found a lower overall survival for intestinal-type gastric cancer patients with high expression of the entire signature (HR = 1.61; logRank P = 0.043) (Fig. 8b). Similar to our IL-33/mast cell activation gene expression signature, a trend for better survival probability was found for high expression of a classical mast cell marker gene signature consisting of KIT, FCER1G, and HDC (encoding Histidine Decarboxylase) (HR = 1.42; logRank P = 0.067) (Supplementary Fig. 7a) as well as for ST2-receptor encoding gene IL1RL1 (HR = 1.57; logRank P = 0.049) (Supplementary Fig. 7b). In accordance with our mast cell–macrophage hierarchy model, Kaplan–Meier survival analysis for macrophages revealed a survival disadvantage for intestinal-type gastric cancer patients with high expression of an alternatively activated macrophage gene signature (Fig. 8c). On the other hand, high expression of a classically activated macrophage gene signature correlates with a higher survival probability (Fig. 8d).

Discussion
Although conflicting data indicate a dichotomous role of mast cells to either restrict or support tumorigenesis, compelling evidence implicates mast cells in promoting progression and metastasis in many solid malignancies. Retrospective studies on human gastric cancers have suggested an involvement of tumor-adjacent submucosal mast cells to late stage disease and metastasis formation. However, the molecular mechanism by which submucosal mast cells contribute to gastric tumorigenesis, and indeed early stage tumorigenesis, is not understood. Here we demonstrate that submucosal accumulation of mast cells in two

Fig. 8 Kaplan–Meier analysis for an IL-33 - mast cell activation gene expression signature. a Expression analysis of genes associated with IL-33 dependent mast cell activation derived from the DERRICO gastric data set (GEO: GSE13911, extracted from oncomine.org) and comparing normal gastric mucosa (n = 31) and gastric intestinal-type adenocarcinoma (n = 29). The p-value for the genes is p < 0.05 except where indicated. b–d Kaplan–Meyer survival analysis was performed for human intestinal-type gastric cancer (iGC) with IL-33/mast cell activation signature consisting of CCL2, CCL3, CCL4, IL1a, IL6, CSF2, and CCL7 (b), with alternative activation macrophage expression signature (CD163, CD204, MARCO, ARG1) (c) and classical activation macrophage expression signature (NOS2A, HLA-DRA, CD80, CD86, CD69) (d). e Schematic illustration of the proposed gastric cancer growth promoting IL-11/IL-33/mast cell/TAM signaling axis. Source data are provided as a Source Data file. See also related Supplementary Fig. 7.
independent murine models of intestinal-type gastric cancer functionally contributes to tumorgenesis and the maintenance of established tumors and that this coincides with the presence of alternatively activated protumorigenic tumor-associated macrophages. We provide evidence that tumor cell-derived IL-33 stands on the apex of a cascade by which mast cells, through recruitment of tumor-associated macrophages and their support of a vascular network, ensures the growth and maintenance of gastric tumors. Indeed, ablating mast cells or macrophages in tumor-bearing mice either individually with cromolyn or clodrosomes, or together with PLX3397, were associated with a vascular collapse and tumor hypoxia. Importantly, we support the relevance of our functional findings in mice with a correlation in humans where a mast cell activation signature associates with poor patient outcome for intestinal-type gastric cancer.

Within the tumor microenvironment, myeloid cells are among the most important innate immune cells that promote tumor formation and restrict the effect of many forms of therapy, most notable immunotherapy. In particular, the plasticity of tumor-associated macrophages has attracted interest as therapeutic opportunities to limit the angiogenic and immune suppressive functions conferred by their alternatively activated endotype. On the other hand, mast cells have been proposed to be an abundant source of VEGF, TGFβ, and other angiogenic factors, as well as matrix metalloproteinase 9 and related stromal remodeling enzymes. Indeed, mast cells are one of the earliest cell types recruited into the tumor microenvironment, and progression of gastric cancer correlates with both the accumulation of chymase-positive mast cells and increased microvessel density. Functionally, these observations are mirrored by an angiogenic switch proposed to be orchestrated by mast cells during early stages of pancreatic cancer in mice required for the extensive vascular elaboration to prevent hypoxia during expansion of tumors. Surprisingly, the authors in this early study had argued against a role for macrophages in mast cell-dependent induction of early tumor angiogenesis, based on their observations that macrophage recruitment to pancreatic islets remained unaffected by mast cell deficiency. Indeed, our observations that IL-33 result in the induction of Vegfa expression in isolated mast cells supports this view and this is corroborated by reduced microvessel density in tumors of gp130FF; Stz2−/− mice. However, our observations presented here following either genetic ablation of mast cells or macrophages, or their respective pharmacological inhibition by cromolyn and cladronate or PLX3397 strongly suggest that the endothelial cell support provided by mast cells also depends in part on the recruitment of macrophages.

Because we functionally link tumor growth to the presence of mast cells in two independent genetic models, it is highly unlikely that this arises from independent serendipitous effects of the two models other than mast cells. Thus, it is highly likely that the incompletely understood mechanisms by which cromolyn blocks mast cell degranulation mediated the anti-tumor effect observed here, rather than cromolyn also affecting monocytes, the production of the neutrophil product myeloperoxidase, and circulating growth. Akin to tumor-associated macrophages being induced by tumor cells to adopt an alternatively activated endotype optimized for wound healing, neoplastic epithelia appears to also corrupt the wound healing function of mast cells to facilitate tumor growth. Accordingly, tumor cell-specific overexpression of the master-regulator c-Myc not only results in epithelial hyperproliferation (when coinciding with increased expression of BH3 survival proteins) but also in expression of chemo-attracting CCL2, CCL3, and CCL5 and concomitant accumulation of mast cells. Here we identify a second mechanism that depends on the IL-1 cytokine family member IL-33, which acts as a chief regulator of innate immunity and inflammation, and maintains epithelial barrier functions of the intestine. Indeed, IL-33 serves a bona fide alarmin and is released by damaged epithelium as an endogenous danger signal to activate innate immune responses. Accordingly, mast cells and ILC2s respond through the IL-33/ST2 axis to the damaged intestinal mucosa arising from acute injury or infection. Therefore mast cells are found at sites of epithelial recovery, and ILC2 cells release IL-13 to stimulate the intestinal stem cell compartment, respectively. Intriguingly, our experimental lines of evidence from tumors of gp130FF; Stz2−/− mice show that their reduced tumor burden correlates with decreased mast cell frequency rather than a change in ILC2 abundance. While not ruling out minor contributions by ILC2, regulatory T-cells or other IL-33 responsive cells, the increased tumor mass in gp130FF; Stz2−/− mice following adoptive transfer of ST2-proficient mast cells, provides definitive functional evidence that mast cells act as the major cell population through which IL-33 promotes tumor growth. Accordingly tumor growth of gp130FF mice was susceptible to the highly mast cell-selective cellular deficiency in gp130FF; Cpa3−Cre; Mc1Rfl/fl mice, or to cromolyn-dependent inhibition of mast cell degranulation. Meanwhile, and consistent with the capacity of IL-33 to promote tissue repair and wound healing, IL-33 can also mediate metaplasia of the gastric epithelium.

Although epithelial cells of barrier tissues exposed to the environment are major sources of IL-33, and IL-33 expression is further increased during inflammation and in the tumor epithelium, the exact nature of the factors that induce IL-33 expression and its release remain unclear. For instance TNF, IL-1β, TGFβ, prostaglandins, lipopolysaccharides, and other pathogen-associated molecular patterns have all been suggested as drivers for IL-33 expression during intestinal adenomatous polyposis. Here, we provide evidence for IL-33 expression in gastric tumor epithelium to also be stimulated by an IL-11/Stat3 signaling cascade, which we and others have identified as an absolute requirement for effective growth of gastrointestinal tumors. However, at this stage we can only speculate that subepithelial myofibroblasts and possibly tumor-associated endothelial cells, which have been proposed as sources of IL-11, may also contribute to epithelial expression of IL-33. Furthermore, because mast cells express gp130, we could not formally exclude the possibility that the presence of the hypermorphic gp130 allele in mast cells may promote their proliferation and survival, as this receptor allele augments Stat3 signaling in response to IL-6 family cytokines. However, we noted similar submucosal mast cell accumulation adjacent to tumors in gp130FF and in Tg(Tgf1-CreERT2); Pik3caH1047Rfl/+; Ptenfl/fl mice, where the latter cohorts express WT gp130. Likewise, reconstitution of gp130FF mice with WT bone marrow did not affect mast cell numbers and tumor burden, as would be expected if the gp130 allele augmented mast cell activity.

Therapeutic inhibition of IL-33 signaling is gaining interest with anti-ST2 antibodies being trialed for asthma. Given the dichotomous activity of IL-33 in tumor biology, timing of anti-IL-33 signaling therapy will be crucial. In the context of gastric cancer, mast cells may not only promote metastasis but also modulate the immunosuppressive tumor environment through the release of IL-17. Thus, IL-33 signaling provides a target to restrict the tumor promoting activities of the myeloid compartment and may ultimately enable rational combination therapies to alleviate the activity by which myeloid cells limit the antitumor immune response. The linear signaling cascade identified here comprising IL-11/IL-33/mast cells/macrophages/tumor cells should provide complementary molecular and cellular targets for the development of improved cancer therapies.
Methods

Study approval. All animal studies were conducted in accordance with all relevant ethical regulations for animal testing and research including the Australian code for the care and use of animals for scientific purposes. All animal studies were approved by the Animal Ethics Committee of the Ludwig Institute for Cancer Research, the Walter and Eliza Hall Institute of Medical Research, or Austin Health.

We have complied with all relevant ethical regulations for work with human participants. Collection and usage of human gastric cancer tissues was approved by the Peter MacCullum Cancer Center Ethics Committee and informed consent was obtained from all subjects.

Mice. Knockout mice (gp130<sup>−/−</sup>, alias gp130<sup>F77</sup> mice), the mutant compound mice (gp130<sup>−/−</sup> c-Kit<sup>−/−</sup>):PLX3397 (Plexxicon), supplied at 800 mg/kg of chow, was given to mice for 4 consecutive weeks represents an estimated dose of 100 mg per kg body weight daily. Vehicle cohorts receivedPBS.

Liposomes containing an estimated dose of 75 mg/kg body weight via the intraperitoneal route (100 µl) three times per week for 6 consecutive weeks. Control cohorts received PBS.

Histological and immunohistochemical analysis. Hematoxylin-Eosin, toluidine blue, Alcian blue, Safranin O, and May-Grünewald-Giemsa staining of formalin-fixed paraffin-embedded stomach slides were performed according to theory and practice of histological technique from JD Bancroft48.

For in vivo assessment of proliferation by anti-BrdU staining, tissues were collected, fixed in 4% formaldehyde after stimulation with 100 µg/ml of BrdU (Amersham Biosciences, GE Healthcare). Stainings for apoptosis (Cell Death Detection Kit, Roche) and tissue hypoxia (60 mg/kg HP1 i.p. injection 30 min prior to tissue collection, detection with Hypoxyprobe-1 Kit, from Hypoxyprobe Inc., USA) were performed according to manufacturers’ instructions.

All other immunostaining procedures were conducted as follows. For antigen retrieval, paraffin-embedded sections were either heated in citrate buffer in a microwave pressure cooker (pH 6 for 15 min) or were incubated in 0.1% trypsin, 3% acetic acid solution at 37°C for 10 min. Sections were then blocked in 10% (v/v) normal goat serum or incubated overnight at 4°C in a humidified chamber. Biotin-labeled secondary antibodies from the Avidin Biotin Complex ABC-kit (Vector Laboratories) were used according to the manufacturer’s instructions. Visualization was achieved using 3,3-Diaminobenzine (DAB, DAKO). Images were generated with Aperio ImageScope V11.2.6.780 software. Primary antibodies used: rabbit anti-mouse CD3 (1:200 dilution, Abcam, Cat# 28364), rabbit anti-mouse CD11b (1:200 dilution, Abcam, Cat# 6640, RRID: AB_726362), rat anti-mouse F4/80 (1:200 dilution, Abcam, Cat# 6640, RRID: AB_1140040), rat anti-mouse B220 (1:200 dilution, BD Biosciences, Cat# 550286, RRID:AB_393581), rabbit anti-mouse CD3 (1:200 dilution, Abcam, Cat# ab5690, RRID:AB_305055), rat anti-mouse CD8 (1:150 dilution, eBioscience, Cat# 14-0080-82, RRID: AB_2572861), and rat anti-mouse Foxp3 (1:100 dilution, eBioscience, Cat# 12–5773–80, RRID: AB_465935).

Immunofluorescence staining. Paraffin-embedded stomach sections were dehydrated, rehydrated and antigen-retrieval was performed by heating in EDTA buffer (pH 9) for 15 min in microwave pressure cooker. Section were fixed in 1 h at room temperature with goat anti-mouse IL-33 (1:150 dilution, R&D Systems, Cat# AF3626, RRID:AB_884269) and then for 1 h with AlexaFlour 568 conjugated donkey anti-goat secondary antibody (Molecular Probes Cat # A-11037, RRID: AB_142581). After counter staining with spectral DAPI (1:200 dilution, Perki- nElmer, Cat# FP1490) for 5 min, sections were mounted with Vectashield mounting solution (VECTORlabs, Cat# H-1400). Imaging was performed with a Vector A 3.0 Automated Qualitative Pathology Imaging System (PerkinElmer, Cat# CLS142338) and representative images were produced using Phenochart<sup>TM</sup> v1.0.4 software (PerkinElmer).

Quantification. For quantification of mast cell numbers at least three fields of view (10x ocular) of three toluidine blue section per mouse were counted, submucosal area was measured and data represented as mast cells number/area tissue. Similarly, for quantification of CD3, BrdU, and ApopTag staining, either entire tumors or three fields of view of three sections were counted and analyzed comparing to tissue area.

Isolation of gastric epithelial cells, immune cells, and flow cytometric analysis. Glandular stomachs or antral tumors were dissected, cut into very small pieces and incubated at 37°C for 30 min in Ca<sup>2+</sup> - and Mg<sup>2+</sup>-free HBSS medium plus 2.5% FCS and 1 mM EDTA with gentle shaking. Then samples were vortexed for 30 s and the supernatants containing intraepithelial lymphocytes were separated from the tissue fragments and kept on ice. The remaining tissue samples were further digested in Collagenase/Dispase (Roche) and DNase I (Roche) in Ca<sup>2+</sup> - and Mg<sup>2+</sup>-free HBSS medium plus 2% FCS for 45 min at 37°C under continuous rotation. Samples were vortexed for 30 s once during incubation and once after the incubation. Afterwards, cells suspensions from both incubations were pooled, filtered and washed in PBS plus 5% FCS for analysis by flow cytometry.

Single cell suspensions were stained for cell surface markers (listed below) and cell viability was controlled with propidium iodide (ThermoFisher Scientific, Cat# P3566) or STOXYBlue (ThermoFisher Scientific, Cat# S48457) staining. Cell sorting was performed with an Aria II cell sorter (BD Bioscience). All cell-type specific flow cytometric gating strategies are presented as a supplementary figure (Supplementary Fig. 8a–e). Mast cells sorting: from CD45<sup>−</sup> EpCam<sup>−</sup> cell population the CD11b<sup>−</sup> cells were selected, then finally the c-Kit<sup>−</sup>FceRI<sup>−</sup> population represents the mast cells. Macrophage sorting: from CD45<sup>−</sup> EpCam<sup>−</sup> cells, the F4/80<sup>−</sup>CD11b<sup>−</sup> population was back-gated to confirm that the selected macrophage population was Ly6C<sup>−</sup>Ly6G<sup>−</sup>.

The following fluorochrome-conjugated antibodies were used for flow cytometric cell sorting and analysis: CD16/CD32 (1:100 dilution, clone 93, Cat# 14-0161-86). EpCAM-FITC (1:1000 dilution, clone 9CA, Cat# 11-5791-82). Ly6c- ELCH7G (1:30 dilution, clone H1-4, Cat# 48-5932-82). F4/80-PE-Cy7 (1:200 dilution, BM8, Cat# 25-4801-80), St2-PerCP-Cy5.5 (1:2000 dilution, clone RMST2-2, Cat# 46-9335-82), F4/80-PE (1:200 dilution, clone FJK-165, Cat# 12-5773-82), Gaet3-PE (1:200 dilution, clone TWAJ, Cat# 12-9966-42), FcRε-PE-Cy7 (1:300, clone 36951, Cat# 25-5898-82), and CD5-PE-Cy7 (1:1000, 145-2C11, Cat# 25-0031-82) all from Ebioscience; CD11b-PE (1:400 dilution, M170, Cat# 553311), Ly6g (1:300 dilution, IAB, Cat# S56002) from BD PharMingen and CD45-2-A700 (1:400 dilution, clone 450-15-2), CD4 (1:50 dilution, clone GK 1.5), CD11b-PE (1:400 dilution, M170), and a-Ki-APC (1:200, ACK-2) from WEHI monoclonal antibody facility.

Isolation and stimulation of BMDM. Bone marrow was collected from the femur of mice by flushing with sterile PBS. Cells were washed with PBS twice and were filtered through a 100 µm sieve. Resulting cell suspensions were cultured in DMEM supplemented with 10% (v/v) FCS and 1,929 conditioned medium for 7 days with fresh media changes every second day until fully differentiated into BMDM. After reseding of cells, alternative macrophage polarization was conducted by stimulation with IL-4 (20 ng/ml, Preprotech) and IL-13 (20 ng/ml, Preprotech) for one day.

Cytokine bead array. A total of 50,000 isolated gastric gp130<sup>F77</sup> mast cells were cultured in 96-well plates for 3 h in 30 ng/ml IL-33. Supernatants were collected, diluted 1:2 in assay buffer and assayed for cytokine concentration using the Bio-Rad Bioplex cytokine bead assay (Bio-Rad Mouse 23-Plex Panel M60009/RDPP) strictly according to manufacturer’s instructions.
**BMMC adaptive transfer assay.** Mast cells were generated from bone marrow from either gp130FF, ST2−, or gp130FF, ST2+/− mice. Bone marrow was extracted and cells were maintained in IL-3-containing culture medium for several weeks until >95% pure BMMC cultures were established. Purity of BMCMCs was determined via flow cytometry. For the BMMC transplantation assay, syngeneic BMMC from the same colony as the host mice were FACS sorted, transected, and injected into tissue samples using Trizol. RNA isolation and quantitative RT-PCR (qPCR) was performed with the ThermoScript™ RT-PCR System (Invitrogen, Cat# 11146-024) according to the manufacturer’s protocol. From isolated gastric mast cells and from FACS sorted macrophages, RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Cat# 74034) and cDNA synthesis was performed with the ThermoScript™ RT-PCR System (Invitrogen, Cat# 11146-024) according to the manufacturer’s instructions. Quantitative RT-PCR analyses were performed in technical triplicates with SensiMix SYBR kit (Bioline, Cat# QT605®). Expression and regulation of interleukin-33 in human monocytes. The Stat3 chromatin immunoprecipitation-sequencing (ChIP-Seq) data have been deposited in the NCBI gene expression omnibus (GEO) under the accession code GSE48285. The source data underlying Figs. 1b, d, 2b–c, 4b–e, 5a, 6a, d, f, 7a–g, and 8a and Supplementary Figs 1b, 2a, b, i, 3d, 5a, b, g, h, and 6e are provided as a source data file. All the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file.

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Experimental work: M.F.E., A.J., M.B., R.J.J.O.D., T.P., C.D., J.B., S.T., E.T., N.D.H., N.E., A.R.P., and F.M. Data interpretation: M.F.E., A.J., M.B., R.J.J.O.D., A.B., M.L.H., M.A.G., F.M., and M.E. Writing and editing of manuscript: M.F.E., M.L.H., F.M., and M.E. Study concept: M.F.E., A.J., and M.E. Funding: M.E.
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