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A signaling cascade of IL-33 to IL-13 regulates metaplasia in the mouse stomach

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Abstract

Objective—Alternatively-activated macrophages (M2) are associated with the progression of spasmolytic polypeptide-expressing metaplasia (SPEM) in the stomach. However, the precise mechanism(s) and critical mediators that induce SPEM are unknown.

Design—To determine candidate genes important in these processes, macrophages from the stomach corpus of mice with SPEM (DMP-777-treated) or advanced SPEM (L635-treated) were isolated and RNA sequenced. Effects on metaplasia development after acute parietal cell loss induced by L635 were evaluated in IL-33, IL-33 receptor (ST2) and IL-13 knockout mice.

Results—Profiling of metaplasia-associated macrophages in the stomach identified an M2a-polarized macrophage population. Expression of IL-33 was significantly upregulated in

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macrophages associated with advanced SPEM. L635 induced metaplasia in the stomachs of wild type mice, but not in the stomachs of IL-33 and ST2 knockout mice. While IL-5 and IL-9 were not required for metaplasia induction, IL-13 KO mice did not develop metaplasia in response to L635. Administration of IL-13 to ST2 knockout mice re-established the induction of metaplasia following acute parietal cell loss

Conclusion—Metaplasia induction and macrophage polarization after parietal cell loss is coordinated through a cytokine signaling network of IL-33 and IL-13, linking a combined response to injury by both intrinsic mucosal mechanisms and infiltrating M2 macrophages.

Keywords

metaplasia; SPEM; macrophage polarization; IL-13; IL-33; ST2

Introduction

Gastric cancer is a leading cause of cancer-related deaths worldwide [1]. Intestinal-type gastric cancer, the most abundant type of gastric adenocarcinoma, is frequently detected at later stages due to poor early clinical indicators. *Helicobacter pylori* infection is the most common risk factor for developing gastric adenocarcinoma, by inducing a chronic inflammatory environment. *Helicobacter* infection results in the loss of acid-secreting parietal cells (oxyntic atrophy) within the corpus of the stomach, leading to the evolution of metaplasia in the gastric epithelium [2]. Two different types of metaplasia are typically observed in the atrophic human stomach: spasmolytic polypeptide expressing metaplasia (SPEM) and intestinal metaplasia [3]. SPEM arises from mature chief cells at the base of the gland that transdifferentiate into mucus- and TFF2-expressing cell lineages [3]. Under chronic inflammatory conditions, increasing evidence suggests that SPEM gives rise to intestinal metaplasia [4, 5, 6].

Little is known about the molecular pathways that induce or drive the progression of metaplasia towards gastric cancer. It is difficult to study the normal pathophysiologic process of gastric cancer development because mice do not typically develop goblet cell intestinal metaplasia or gastric cancer after extended *Helicobacter* infection [7]. Instead metaplasia advances towards an intestinalized phenotype with increased proliferation and elevated levels of intestinal gene expression, which we have defined as advanced SPEM [8]. Two drugs, DMP-777 and L635, have previously been utilized in our lab to induce acute oxyntic atrophy and study the progression of metaplasia in mice [9, 10]. DMP-777 is a parietal cell protonophore and neutrophil elastase inhibitor that causes oxyntic atrophy and metaplasia with minimal inflammation in the stomach after 10 days of treatment [9]. L635, an analog of DMP-777, causes oxyntic atrophy without inhibiting the inflammatory response [3]. Mice treated with L635 for three days develop a prominent inflammatory infiltrate that results in proliferative SPEM with increased expression of intestinal transcripts (advanced SPEM) that closely resembles lineage changes observed after 6–12 months of *Helicobacter felis* infection [8].

We recently reported that macrophages are necessary for the progression of metaplasia to a more advanced SPEM phenotype [11]. Macrophage-depleted L635-treated mice developed

fewer proliferative SPEM cells and less intestinal transcripts compared to control L635-treated mice. Thus macrophages that infiltrate into the stomach after parietal cell loss promote the advancement of metaplasia [11]. In addition, macrophages infiltrating into the mucosa after L635 treatment displayed M2-like markers [11]. M2 macrophages are characterized as anti-inflammatory, tumor-associated inflammatory cells driven by Th2 cytokines [12]. However, macrophages also specialize in response to local environmental stimuli, and therefore have diverse gene expression and function within different organ systems [13]. In addition, the generalized M1 and M2 classification, which relies on a limited number of markers, is overly simplified [14]. Thus an in depth characterization of metaplasia-associated macrophages is necessary to identify key inflammatory mediators that promote the induction and progression of metaplasia in the stomach.

We have now performed RNA sequencing of macrophages associated with SPEM (DMP-777-treated mice) or advanced SPEM (L635-treated mice) from the stomach corpus to generate a profile of metaplasia-associated macrophages. We found that IL33 is significantly upregulated in M2 macrophages from advanced metaplasia. Using IL33 and IL33 receptor (ST2) knockout mice, we determined that an IL33 signaling pathway is required for the development of metaplasia after parietal cell loss. Downstream pathway analysis confirmed that IL33 is necessary for Th2 cytokine induction in the stomach. Investigations to determine the role of IL5, IL9 and IL13 on metaplasia development revealed that IL13 is necessary and sufficient for chief cell transdifferentiation into metaplasia following parietal cell loss. These findings reveal a multicellular signaling pathway necessary to induce metaplasia in the stomach after parietal cell loss and mucosal injury.

Results

Analysis of the macrophage transcriptome from acute SPEM models

We have previously determined that M2-like macrophages are present in the corpus in association with advanced SPEM that demonstrated elevated intestinal gene expression, and increased proliferation, determined by increased nuclear Ki-67 staining [11]. Therefore, we sought to determine the characteristics of this macrophage population to identify factors that induce or promote the progression of metaplasia. F4/80-positive cells from the corpus of L635-treated mice and DMP-777-treated mice were isolated and RNA sequenced for transcriptome analysis (Supplemental Figure 1). The purity of the macrophage-enriched cell populations was established using the normalized expression values from the RNA sequencing (Figure 1A). Macrophage-specific genes (*Cd68*, *Cd11b*, *Cd11c*) were enriched as compared to other immune (*Cd3e*, *Ly6c*, *Ly6g*, *Prg2/Mbp*, *Cma1*) or gastric epithelial cell populations (*Dcl1*, *Chga*, *Cckbr*, *Wfdc2*, *Mist1*). We assessed macrophage polarization markers and determined that macrophages associated with advanced SPEM demonstrated more prominent upregulation of M2 polarization-associated markers (*Fizz1*, *Ym1*, *Klf4*, *Alox15*, *Il33*, *Mrc1*, *Ccl2*) as opposed to canonical M1-associated markers (*Il6*, *Il12*, *Nos2*) (Figure 1B). However, there was still prominent expression of some M1-associated genes (e.g. *Klf6*, *Irf5* and *Il18*). Previous studies relied upon only a few key markers to determine macrophage polarization. Our sequencing data confirms known polarization markers and

provides a more detailed analysis that illustrates that macrophages associated with advanced metaplasia may represent a subclass of M2a polarized macrophages (*Ccl17*). Our analysis concentrated primarily on secreted factors or receptors, as macrophages impact the local environment predominantly through paracrine and juxtacrine signaling [15, 16]. Genes fitting this description that were significantly upregulated in L635-associated macrophages were organized into groups: macrophage genes (*Csf1*, *Syk*), cytokines (*Il33*, *Il4*), chemokines (*Ccl24*, *Cxcl3*), matrix remodeling enzymes (*Mmp25*, *Mmp9*), growth factors (*Hgf*, *Areg*, *VegfA*), and cell adhesion molecules (*Itgb2*, *Itga4*) (Figure 1C).

IL-33 and the IL-33 receptor (ST2) are necessary for SPEM induction

Based on our RNA sequencing results, the IL1 family member IL-33 was the most significantly upregulated cytokine in macrophages associated with an advanced SPEM from L635-treated mice. IL-33 expression is localized to surface mucus cells in the normal stomach. However after parietal cell loss, macrophages expressing IL-33 are present within the corpus mucosa (Supplemental Figure 2). No IL33 staining was observed in either mucosal cells or macrophages in IL33 deficient mice (Supplemental Figure 2). Western blot analysis showed elevations in IL-33 expression in gastric mucosa from mice treated with L635 and mice infected with *H. felis* for 6 months (Supplemental Figure 2C). As observed in L635-treated mice, *H. felis*-infected mice showed expression of IL-33 in foveolar cells as well as in macrophages (Supplemental Figure 2D).

To investigate the role of IL33 in the initiation and progression of SPEM, we treated IL-33 knockout (IL33KO) mice with L635 to induce advanced metaplasia (Figure 2). Consistent with previous studies, acute parietal cell loss in wild-type mice resulted in the loss of the mature chief cell transcription factor, *Mist1*, in chief cells located at the base of the glands [17]. While *Mist1*-positive chief cells also significantly decreased after L635-treatment in IL33KO mice, there were significantly more *Mist1*-positive chief cells remaining at the base of glands compared to wild-type L635-treated mice (Figure 2C). SPEM is characterized by lineages co-expressing Muc6 (GSII-lectin), CD44 variant 9 (Cd44v9) and gastric intrinsic factor (GIF) [18]. The number of GIF-only positive cells (chief cells) and cells co-positive for GIF, GSII and Cd44v9 (SPEM cells) was quantified to determine the presence and amount of SPEM development. In wild type mice, L635-treatment induced GIF-staining cells co-expressing GSII and CD44v9, indicative of SPEM. L635-treated IL33KO mice showed significantly fewer SPEM cells as compared to wild-type L635-treated mice (Figure 2D). Further, a hallmark of inflammation-associated advanced metaplasia in the stomach is the development of a highly proliferative SPEM, visualized by cells triple-positive for GIF, Ki67, and GSII-lectin. In L635-treated wild-type mice, approximately 45% of SPEM cells were proliferative, indicative of advanced SPEM [8]. However, IL33KO L635-treated mice had almost no proliferative metaplastic cells (Figure 2E).

After acute parietal cell loss in wild-type L635-treated mice, F4/80 positive macrophages infiltrate into the corpus mucosa [11]. To determine if IL-33 is required for macrophage recruitment after parietal cell loss, F4/80 positive cells were counted in IL33KO L635-treated mice. No significant difference in macrophage infiltration was observed in IL33KO L635-treated mice compared to wild-type L635-treated mice (Figure 2F). Our RNA

sequencing data showed macrophages associated with advanced metaplasia have a M2 polarized phenotype (Figure 1B). Using co-labeling of macrophage marker F4/80 with the M2 macrophage marker CD163, we found that macrophages in L635-treated IL33KO mice had a significant reduction in M2 macrophages compared to L635-treated wild-type mice (Figure 2F). We confirmed a significant loss of M2 macrophage polarization in L635-treated IL33KO mice using quantitative real-time PCR for the M2 marker, Ym1 (Supplemental Figure 3).

We also examined the effects of IL-33-receptor (ST2) deletion on the evolution of SPEM induced by L635 treatment. Figure 3 demonstrates that ST2KO mice showed a similar pattern to IL33KO mice. L635-treated ST2KO mice have decreased Mist1 expression (Figure 3B), loss of SPEM (Figure 3C), and reduced proliferative SPEM (Figure 3D). In addition, macrophages infiltrating into the mucosa of L635-treated ST2KO mice did not express the M2 marker CD163 (Figure 3E). L635-treated ST2KO mice also had reduced Ym1 expression (Supplemental Figure 3). These data suggest a role for IL-33 signaling in the initiation of metaplasia and M2 macrophage polarization.

Loss of Th2 cytokines correlates with the absence of M2a polarized macrophages in L635-treated IL33KO mice

Using a mouse-specific cytokine and chemokine array (Qiagen, PAMM-150Z), we examined the impact that IL33 loss has on downstream cytokines and chemokines after parietal cell loss. Whole stomach corpus from wild-type L635-treated mice and IL33KO L635-treated mice was assessed for 84 different chemokine and cytokine transcripts. Seven genes had significant differential expression ($p < 0.05$) between wild-type L635-treated mice and IL33KO L635-treated mice: *Ccl11*, *Ccl24*, *Ccl17*, *Il4*, *Il5*, *Il9* and *Il13* (Figure 4A and 4B). The genes *Ccl11*, *Ccl24* and *Ccl17* are characteristic of an M2a macrophage polarized environment, while *Il4*, *Il5*, *Il9* and *Il13* are Th2-related cytokines [19]. Thus, L635-treated IL33KO mice had a significant reduction in M2a macrophage polarizing chemokines and Th2-related cytokines compared to L635-treated wild-type mice.

IL-5 and IL-9 do not impact metaplasia development or macrophage polarization

IL-33 is known to induce the expression of Th2 cytokines in the stomach, lungs and intestine, and drive alternative activation of macrophages (M2) [20, 21, 22, 23]. However, little is understood about the specific influence of Th2 cytokines on metaplasia induction or progression in the stomach. IL-5 represents a major regulator of eosinophil activation. Eosinophils are present in the overall inflammatory milieu in the stomach mucosa during *Helicobacter* infection and are also implicated in gastrointestinal eosinophilia [24, 25]. While the F4/80 antibody is predominately used as a monocyte/macrophage and dendritic cell marker, it is also expressed at low levels on eosinophil granules [26]. Major basic protein (Prg2), an eosinophil granule protein, [27] and Ccr3, a chemokine receptor highly expressed on eosinophils, [19, 28] were upregulated over six-fold in the F4/80⁺ cells isolated from L635-treated mice (Supplemental Table 1). DMP-777 and L635-treated mice have a similar robust eosinophil infiltration into the mucosa during SPEM induction (Supplemental Figure 4A). We have previously identified macrophages as necessary for the progression to advanced SPEM, [11] and reexamination of L635-treated mice also treated with clodronate

to deplete macrophages revealed fewer eosinophils compared to control L635-treated mice (Supplemental Figure 4B). Thus, macrophages impact eosinophil recruitment to the stomach after parietal cell loss.

Since IL-5 and IL-9 were significantly decreased in L635-treated IL33KO mice, we investigated the role of these Th2 cytokines in metaplasia induction in the stomach corpus. To this end, IL-5 or IL-9 was depleted from mice followed by L635 administration. We evaluated IL-5 and IL-9-depleted mice as performed for IL33KO and ST2KO mice (Figure 5). Immunofluorescence staining for Mist1 expression was conducted in the following L635-treated groups: non-specific IgG-treated, anti-IL-5-treated and anti-IL-9-treated wild-type mice. We observed the loss of Mist1-expressing cells at the bases of the glands in non-specific IgG L635-treated wild-type mice. The loss of Mist1 was also observed in L635-treated anti-IL5 and L635-treated anti-IL-9 mice (Figure 5B). The presence of SPEM was evaluated by quantitation of the number of cells triple-positive for GSII-lectin, CD44v9 and GIF. We observed that L635-treated mice depleted of IL-5 or IL-9 developed SPEM similar to non-specific IgG L635-treated wild-type mice (Figure 5C). To determine whether SPEM was proliferative, we evaluated the number of Ki67 positive SPEM cells in each mouse model. L635-treated anti-IL-5 and anti-IL-9-treated mice did not have a significant difference in proliferative SPEM cells compared to non-specific IgG L635-treated wild-type mice (Figure 5D). These results demonstrate that neither IL-5 nor IL-9 is directly necessary for the development of SPEM. Furthermore, we examined the presence of eosinophils in L635-treated IL-5-depleted mice (Supplemental Figure 4C), and found an absence of eosinophils in the mucosa. Thus, eosinophils are not required for the induction of SPEM, as IL-5-depleted L635-treated mice develop SPEM normally.

Th2 cytokines promote the alternative activation of M2-polarized macrophages [29]. To determine the impact that loss of specific Th2 cytokines had on the polarization of infiltrating macrophages, we assessed the macrophage populations in non-specific IgG, anti-IL-5, and anti-IL-9 L635-treated mouse models. The number of F4/80-positive infiltrating macrophages was quantitated and we did not detect a significant difference in the number of F4/80-positive macrophages present after L635-treatment between either of the Th2 cytokine depleted mouse models (Figure 5E). M2 macrophage polarization was determined through dual- immunolabeling for F4/80 and CD163 from whole stomach corpus. We observed that almost all macrophages in non-specific IgG L635-treated control mice were co-positive for macrophage marker F4/80 and M2 macrophage marker CD163. Furthermore, both L635-treated anti-IL-5 and anti-IL-9-treated mice had similar high levels of CD163-positive macrophages, and M2-macrophage polarization after L635 treatment was confirmed by high expression of *Ym1* (Figure 5E and Supplemental Figure 3).

IL-13 is necessary for M2 macrophage polarization and SPEM development in the stomach

We next evaluated the role of IL-13 as a putative Th2 cytokine influencing the polarization of M2 macrophages and SPEM development in the stomach corpus. We examined the ability of L635 to induce SPEM and macrophage polarization in IL-13 deficient (IL13KO) mice (Figure 6). Untreated IL13KO mice demonstrated a normal compendium of chief cells. L635 induced parietal cell loss similar to that observed in wild type mice (data not shown).

Following L635-treatment, there was a reduction in Mist1-positive chief cells in IL13KO mice, however IL13KO mice maintained some Mist1-positive chief cells at the gland bases (Figure 6B). In addition, L635-treated IL13KO mice exhibited few GIF⁺GSII-lectin⁺CD44v9⁺ SPEM cells (Figure 6C) and very few proliferative SPEM cells (Figure 6D). L635-treated IL13KO mice also showed few CD163 and F4/80 co-positive macrophages consistent with low expression of Ym1 (Figure 6E and Supplemental Figure 3). Together, these data implicate a role for IL13 in the polarization of macrophages and the progression of metaplasia following parietal cell loss.

IL-13 is sufficient to induce metaplasia following parietal cell loss in the absence of IL33 signaling

IL-13 binds the heterodimer of IL4 α and IL13 α 1 receptor subunits to activate downstream signaling [30]. Two published microarray data sets from microdissected chief cells indicate that IL13 α 1 is expressed on both chief cells and SPEM cells in the murine stomach [8, 31]. We have further evaluated the presence of IL13 α 1 protein in the gastric oxyntic mucosa. Immunostaining demonstrated strong staining for IL13 α 1 in chief cells in the gastric corpus (Supplemental Figure 5). We therefore sought to ascertain if IL-13 was sufficient to restore SPEM induction after parietal cell loss in the absence of IL-33 signaling. ST2KO mice were treated with 10 μ g of recombinant mouse IL-13 during L635-treatment (ST2KO^{L635-IL13}). IL-13-treated ST2KO mice (ST2KO^{IL13}) and non-specific IgG L635-treated ST2KO mice (ST2KO^{L635}) were also assessed for Mist1 expression, SPEM development, proliferative SPEM, and M2 macrophage polarization (Figure 7). We did not observe any phenotypic changes in ST2KO^{IL13} mice compared to non-specific IgG-treated ST2KO mice (Figure 7A–E). In agreement with our previous findings, ST2KO^{L635} mice did not develop SPEM or proliferative SPEM in response to parietal cell loss (Figure 7C and 7D). ST2KO^{L635-IL13} mice did not have a significant change in the number of Mist1 positive cells compared to ST2KO^{L635} mice (Figure 7B). However, using CD44v9 co-labeling with GSII and GIF to identify SPEM cells, we found that ST2KO^{L635-IL13} mice had a significantly increased ($p < 0.0001$) number of SPEM cells compared to ST2KO^{L635} mice (Figure 7C). To determine if the metaplasia present in ST2KO^{L635-IL13} mice was proliferative, we immunolabeled with Ki67 and SPEM markers and found that ST2KO^{L635-IL13} mice did not develop proliferative SPEM (Figure 7D). Macrophage infiltration and M2 macrophage polarization were also assessed in ST2KO^{L635-IL13} mice. There was significant F4/80-expressing macrophage infiltration, of which approximately half were co-positive for the M2 marker, CD163 (Figure 7E). These findings suggest that IL13 is sufficient for the induction of SPEM, but progression to proliferative SPEM as well as complete M2 macrophage polarization may accrue from another IL-33-dependent effector.

Discussion

The present study identifies that the IL-33/IL-13 cytokine-signaling network is required and sufficient for the induction of metaplasia following parietal cell loss. Using *in vivo* mouse models, we established that IL-33 signaling via IL-13 drives chief cell transdifferentiation into a mucus-producing metaplastic lineage. In the absence of IL-33 metaplasia does not develop, which is a necessary process for gastric epithelial repair [32]. In addition to being

necessary for the induction of metaplasia, IL-33 signaling drives M2 macrophage polarization that is associated with the progression towards a more advanced metaplasia [33].

Previous studies in the stomach have attempted to ascertain the mechanism of metaplasia induction and progression. We recently identified infiltrating macrophages as necessary for the proliferation and intestinalization of metaplasia in mice [11]. Macrophage depletion in L635-treated mice resulted in a less advanced metaplasia. Our model established that macrophages promoted the progression of metaplasia, but is not required for metaplasia induction. Efforts to circumvent metaplasia development through manipulation of EGF ligands/receptors (Areg [34], EGFR [34, 35]), endocrine pathways (gastrin [10, 36], histamine [37]) and cytokines (IFN γ [38, 39, 40], IL-11 [41]) have yet to identify factor(s) necessary for the induction of metaplasia in response to parietal cell loss in either an acute drug induced system or chronic *Helicobacter* infection. Previous studies found that administration of IL-33 is sufficient to induce hypertrophy and mucous metaplasia in the airway, stomach, and intestine through the induction Th2 cytokines and infiltration of myeloid cells and eosinophils [20, 21, 42, 43, 44, 45]. Furthermore, it is well-established that IL-33 signaling results in up-regulation of the expression of IL-13, which is required for the development of mucous metaplasia in the airway allergic response. Our findings demonstrate an analogous role for IL-33 as an inducer of IL-13 in the promotion of metaplasia in the stomach following parietal cell loss. Recent investigations have suggested that SPEM is involved in the process of repair after acute mucosal injury [32, 46]. It therefore seems likely that an IL-33/IL-13 pathway leading to metaplasia induction is important not only for gastric pre-neoplasia, but also for gastric epithelial repair. These findings are consistent with previous studies that have shown that decreased IL-33 in TFF2-deficient mice may contribute to defective metaplasia development that delays gastric epithelial repair [32, 46], [21].

We found that IL-33 signaling was necessary for metaplasia induction. In IL33KO and ST2KO mice, chief cells were able to down-regulate Mist1 but were unable to transdifferentiate into mucus-producing SPEM cells. Interestingly, the loss of IL-33 or ST2 receptor did not impact macrophage recruitment, although we detected decreased expression of M2a-associated cytokines (CCL17, CCL22, CCL24) and confirmed a loss of M2-polarized macrophages in both L635-treated IL-33 and ST2 knockout mice. Thus, IL-33 was not necessary for macrophage recruitment, nor was macrophage recruitment alone sufficient to induce transdifferentiation of chief cells in SPEM. IL-33 induces Th2 cytokine expression from ILC2 cells and T helper cells [20, 47, 48, 49]. A recent investigation has implicated ILC2 cells in the promotion of neoplasia in a model of diffuse gastric carcinogenesis [50]. Indeed, IL-33 knockout mice treated with L635 have reduced Th2 cytokine expression, further illustrating the relationship between IL-33 and Th2 cytokines induction.

We investigated the role of Th2 cytokines downstream of IL-33 and found that IL-5 and IL-9 depleted mice treated with L635 developed metaplasia normally after parietal cell loss. However, IL-13 knockout mice treated with L635 did not develop metaplasia, indicating that IL-13, downstream of IL-33, induces metaplasia after acute injury and parietal cell loss. It seems likely that IL-13 can act directly on chief cells to promote the transdifferentiation of

metaplasia due to the localization of the IL-13 receptor on chief cells. Thus, we present a novel pathway in the stomach required for metaplasia induction where IL-33 stimulates IL-13 release, likely from ILC2 cells, which then acts as a key initiator of metaplasia development. ST2KO mice treated with IL-13 concurrently with L635 were able to develop metaplasia in response to parietal cell loss, further supporting this finding. Therefore, IL-13 is sufficient to induce metaplasia after parietal cell loss in the absence of IL-33 signaling. Nevertheless, since treatment of ST2KO mice with IL-13 did not induce proliferation in SPEM after L635-treatment, a factor other than IL-13 must be required for IL-33 dependent induction of proliferative SPEM.

In summary, our investigations of the induction of metaplasia after acute parietal cell loss revealed the requirement of IL-33 in the development of SPEM. We further demonstrated that IL-33 is necessary to promote a Th2 inflammatory response and M2a polarization of recruited macrophages, which drives advancement of SPEM [11]. The identification of IL-13 downstream of IL-33 signaling as a critical regulator of metaplasia development supports a central role for cytokine and immune regulation of the epithelial response to acute injury. It is important to note that while IL33KO, ST2KO and IL13KO mice all show profound parietal cell loss after L635 treatment and reductions in *Mist1* expression, the chief cells fail to complete transdifferentiation. Thus, parietal cell loss alone is not sufficient to induce chief cell transdifferentiation. Rather cytokine responses after parietal cell loss are also required to promote transdifferentiation as part of repair and regeneration of the gastric epithelium. Therapeutic modalities currently used to modulate IL-13 could potentially promote epithelial regeneration. Similarly, inhibiting IL-33 or IL-13 cytokine pathways can modulate M2a macrophage polarization, a critical step in the progression of metaplasia towards gastric neoplasia.

Methods

All authors had access to the study data and reviewed and approved the final manuscript.

Mice

C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, ME). IL-33 knock out (IL33KO) mice were generated as previously described and maintained on the C57BL/6J background [51]. ST2 knock out (ST2KO) mice were developed as previously described [52] and backcrossed to a Balb/C background [53]. IL-13 knock out (IL13KO) mice used are previously described [54] and backcrossed to a Balb/C background [55]. Regular mouse chow and water *ad libitum* was provided during experiments in a temperature-controlled room with 12-hour light-dark cycles. All treatment maintenance and care of animals in these studies followed protocols approved by the Institutional Animal Care and Use Committees of Vanderbilt University and Case Western Reserve University.

Drug Treatment

Three to six mice were used per group. L635 and DMP-777 treatment and dosage was conducted as previously described [11]. Clodronate depletion of macrophages was performed as previously described [11]. Anti-IL-5 (1 mg/kg, R&D Systems, clone TRFK5)

and anti-IL-9 (1 mg/kg, R&D Systems, clone #222622) was administered via intraperitoneal injection to deplete IL-5 and IL-9, respectively. Depletion protocols were determined based upon previous studies [56, 57]. Anti-IL5 was given daily for two days prior to L635-treatment, and once on day 2 of L635-treatment protocol. Anti-IL9 antibody was given to mice on day 1 and 2 of L635 treatment. Recombinant mouse IL-13 dosage (5 µg/mouse, Shenandoah, St. Louis, MO) was determined based upon prior studies and administered once daily for two days with sacrifice occurring two hours after final dose [57, 58]. IL-13 administration was given on day 2 and day 3 of L635-treatment. Vehicle control non-specific IgG antibodies (Purified Rat IgG2a, Leaf; BioLegend, San Diego, CA) were administered to control mice at a similar dosage and timing as for experimental mice.

Macrophage Isolation and Fluorescence Activated Cell Sorting (FACS)

Three mice were pooled for each macrophage preparation, and three preparations were used for RNA sequencing. L635 and DMP-777-treated mice were sacrificed on the final day of treatment. The stomach was removed and opened along the greater curvature. Stomach contents were rinsed with PBS. The antrum and forestomach were removed with a razor blade and discarded, and the corpus was diced into pieces roughly one mm³ in size, and washed with 0.07% DTT in PBS three times for five minutes. Stomach corpus pieces were then enzymatically digested using 1 U/mL of Dispase in DMEM/F-12 (Stem Cell Technologies, Vancouver BC) for 7 min at 37°C and subsequently diluted using an equal volume of macrophage growth media, Iscove's Modified Dulbecco's Medium (ThermoFisher), with 10% FBS. Pieces were sieved through a 100 µm filter (Corning) and crushed with a rubber syringe plunger into a single cell suspension over a 50 mL conical tube. Cells were spun down twice at 500g for 10 minutes at 4°C and resuspended in PBS with 10% FBS to remove excess cell debris and media. Cells were blocked using Mouse BD Fc Block (1:100, BD Pharmingen) for 15 minutes on ice and incubated with rat anti-mouse F4/80 conjugated to PE (1:25, BD Bioscience) for 30 minutes on ice. Prior to cell sorting, cells were washed twice in PBS with 10% FBS and incubated with 4',6-diamidino-2-phenylindol (DAPI) (1:10,000). Cells were sorted using a BD FACS Aria III (BD Biosciences, San Jose, California) and initially segregated from debris using forward scatter (FSC) and side scatter (SSC) properties of the 488 nm laser. Single cells were selected using the voltage pulse geometries of the FSC diode and SSC photomultiplier tube (PMT) detectors. Dead cells were excluded based on their DAPI staining. Macrophages expressing F4/80 were sorted directly into Trizol (Invitrogen) using a 100 µm nozzle. RNA was extracted from Trizol and DNase-treated (Promega) using the manufacturer's protocol. RNA sequencing was performed as detailed in Supplemental Methods and results were deposited in the GEO database (GSE77195).

Quantitative Real-time PCR analysis

The stomach corpus was isolated using a razor blade and placed into *RNAlater* or frozen on dry ice. Trizol was used to extract RNA from the tissue. One µg of DNase-treated (Promega) RNA was transcribed into complementary DNA using Superscript III Reverse Transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction was conducted and analyzed as previously described using SYBR Green Supermix [11]. Ym1 primers detecting M2 macrophage transcripts were obtained from Qiagen (QuantiTect) and followed

manufacturer protocol. Results were normalized to *GAPDH* and displayed as expression level (2^{-C_t}) [11].

Immunocytochemistry

Mouse stomachs were fixed in 4% paraformaldehyde overnight and transferred into 70% ethanol for paraffin embedding. The protocol for subsequent processing and sectioning was previously reported [11]. Tissue sections were blocked in Protein Block Serum-Free (DakoCytomation) for 1 hour at room temperature. The following primary antibodies were incubated overnight at 4°C in Antibody Diluent with Background Reducing Components (DakoCytomation): goat anti-intrinsic factor (1:1000, a gift from Dr. David Alpers, Washington University, St. Louis, MO), rat anti-Ki67 (1:50, BioLegend clone 16A8), rabbit anti-Ki67 (1:500, Cell Signaling), rat anti-F4/80 (1:500; Invitrogen, Grand Island, NY), rabbit anti-Mist1 (1:1000, a gift from Jason Mills, Washington University, St. Louis, MO), rabbit anti-IL-13ra (1:250, Abcam, 1:500), rat anti-Cd44v9 (1:25,000; Cosmo Bio, Japan), mouse anti-CD163 (1:200, NeoMarkers, Fremont, CA and goat anti-IL-33 (1:200; R&D Systems). Fluorescent secondary antibodies (1:500) and Alexa 488 conjugated *Griffonia simplicifolia* lectin II (GSII-lectin) (1:1000; Molecular Probes, Eugene, OR), and were incubated at room temperature for one hour. Zeiss Axio Imager M2 microscope with Axiovision digital imaging system (Zeiss, Jena GmbH, Germany) or an Ariol SL-200 automated slide scanner (Leica Biosystems, Buffalo Grove, IL) in the Vanderbilt Digital Histology Shared Resource was used to analyze sections.

Image Quantitation

Images were analyzed using CellProfiler or manually counted [59]. Experimental groups contained three or six mice, with three to five representative images taken from each mouse at 20X. Mist1 or gastric intrinsic factor positive cells denoted chief cells, while SPEM lineages were identified as gastric intrinsic factor, GSII-lectin and CD44v9 triple-positive cells. Proliferative SPEM was identified as gastric intrinsic factor, GSII lectin, and Ki67 triple-positive cells. Macrophages were counted using the marker F4/80. M2 macrophages were identified and counted as CD163 and F4/80 dual-positive cells. A one-way analysis of variance with post hoc examination of significant means with Bonferroni's test was used to determine statistical significance among multiple groups and Mann-Whitney test was employed for comparison between two group paradigms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance of this study

What is already known on this subject?

- Gastric cancer evolves in a field of pre-cancerous metaplasia.
- Spasmolytic polypeptide-expressing metaplasia (SPEM) and intestinal metaplasia in the human stomach are associated with chronic inflammation.
- Mouse models of SPEM have shown that M2-macrophages promote the progression of metaplasia towards a more proliferative and advanced phenotype.

What are the new findings?

- Transcriptional profiling of isolated metaplasia-associated gastric mucosal macrophages in the stomach shows that they have predominantly M2a phenotype.
- The primarily epithelial-associated cytokine IL-33, is also expressed in M2 macrophages.
- Mice that are null for IL-33, the IL-33 receptor (ST2) or IL13 do not develop metaplasia following acute parietal cell loss.
- IL-13 treatment in ST2 null mice restored the development of SPEM following acute parietal cell loss, thus identifying a novel pathway required for metaplasia development.

How might it impact on clinical practice in the foreseeable future?

The results suggest that intrinsic mucosal and macrophage cytokines are responsible for the induction of metaplasia in the atrophic stomach. Thus, antagonism against IL-33 and IL-13 may represent viable approaches for reversal of metaplastic changes in the stomach.

Figure 1. Transcriptome analysis of an enriched macrophage population associated with metaplasia in the stomach

Gene expression profile of macrophages from metaplasia, was determined for 3 replicates/each sample **A.** Expression values of macrophage genes (*Cd68*, *Cd11b*, *Fn1*), other immune cell types (*Cd3e*: T-cells, *Ly6c*: neutrophils, *Prg2*: eosinophils), and gastric epithelial cell lineages (*Dcl1*: tuft cells, *Chga*: endocrine cells, *Cckbr*: parietal cells, *Mist1*: chief cells), demonstrate a population enriched for macrophages. **B.** Expression levels of M1 (blue) and M2 (red)-associated genes, in macrophages isolated from advanced SPEM (L635-treated mice) plotted with SEM. Macrophages associated with advanced SPEM express increased M2-like transcripts (*Fizz1*, *Klf4*, *Il33*) compared to M1 genes (*Nos2*, *Il6*). **C.** Factors significantly upregulated in macrophages from advanced SPEM (L635-treated mice) ($p < 0.01$; fold change ≥ 2 compared with macrophages isolated from DMP-777-treated mice). Representative genes selected were grouped based on function.

Figure 2. IL-33 is necessary for induction of metaplasia and polarization of M2 macrophages

A. Immunofluorescence staining for chief cell transcription factor Mist1, mucus marker GSII-lectin, chief cell granule marker gastric intrinsic factor (GIF), SPEM marker CD44 variant isoform 9 (CD44v9), proliferation marker Ki67, macrophage marker F4/80, M2 macrophage marker CD163 and nuclei marker DAPI in untreated wild-type and L635-treated wild-type mice. **B.** Immunofluorescence staining for chief cell transcription factor Mist1, mucus marker GSII-lectin chief cell granule marker gastric intrinsic factor (GIF), SPEM marker CD44 variant isoform 9 (CD44v9), proliferation marker Ki67, macrophage

marker F4/80, M2 macrophage marker CD163 and nuclei marker DAPI in untreated IL33KO and L635-treated IL33KO mice. **C.** Quantification of Mist1 positive nuclei per 20× field. L635-treated IL33KO mice had significantly fewer Mist1-positive cells compared to untreated IL33KO mice, and significantly more Mist1-positive cells compared wild-type L635-treated mice. **D.** Quantification of SPEM cells determined by percent of GIF positive cells co-positive for CD44v9 and GSII-lectin. L635-treated IL33KO mice have significantly fewer SPEM cells compared to wild-type L635-treated mice. **E.** Quantification of proliferative SPEM cells determined by the percent of SPEM cells positive for Ki67. L635-treated IL33KO mice have significantly fewer proliferative SPEM cells compared to wild-type L635-treated mice. **F.** Quantification of F4/80-positive macrophages (red) and F4/80 and CD163 co-positive M2 macrophages (green) per 20× field. L635-treated IL33KO mice had similar F4/80-expressing macrophage infiltration as wild-type L635-treated mice, but significantly fewer F4/80 macrophages were co-positive for CD163. * denotes significant difference ($p < 0.05$) compared to untreated mice, + denotes significant difference ($p < 0.05$) compared to L635-treated wild-type mice. White dashed boxes are magnified in the insets. Scale bars = 100 μm .

Figure 3. IL-33 signaling drives SPEM development and M2 macrophage polarization

A. Immunofluorescence staining for chief cell transcription factor Mist1, mucus marker GSII-lectin, chief cell granule marker gastric intrinsic factor (GIF), SPEM marker CD44 variant isoform 9 (CD44v9), proliferation marker Ki67, macrophage marker F4/80, M2 macrophage marker CD163 and nuclei marker DAPI in untreated ST2 (IL33 receptor) knockout mice and L635-treated ST2 knockout mice. **B.** Quantification of Mist1 positive nuclei per 20× field. L635-treated ST2KO mice had significantly fewer Mist1-positive cells compared to untreated ST2KO mice, although some Mist1-positive cells remained. **C.** Quantification of SPEM cells determined by percent of GIF positive cells co-positive for CD44v9 and GSII-lectin. L635-treated ST2KO mice did not have significant changes in SPEM cell number compared to untreated ST2KO mice. **D.** Quantification of proliferative SPEM cells determined by the percent of SPEM cells positive for Ki67. Although L635-treated ST2KO had very few SPEM cells, some of the SPEM cells were proliferating so there is a significantly higher percent of proliferative SPEM cells in L635-treated ST2KO mice compared to untreated ST2KO mice. **E.** Quantification of F4/80-positive macrophages (red) and F4/80 and CD163 co-positive M2 macrophages (green) per 20× field. L635-treated ST2KO mice have significant F4/80-positive macrophage infiltration compared to untreated ST2KO mice. L635-treated ST2KO mice also have significantly more F4/80 and CD163 co-positive cells compared to untreated ST2KO mice, however the percent of F4/80

macrophages that are co-positive for CD163 is around 10% in both mouse models. * denotes significant difference ($p < 0.05$) compared to untreated mice. White dashed boxes are magnified in the insets. Scale bars = 100 μm .

A. Alternatively activated macrophage-associated cytokines

B. Th2-related M2 macrophage polarizing cytokines

Figure 4. Altered cytokine response in IL-33 KO mice

Analysis of 84 mouse chemokines and cytokines in whole stomach corpus RNA from L635-treated wild type mice and L635-treated IL33KO mice (n=3). **A.** Alternatively activated macrophage-related chemokines (Ccl11, Ccl24 and Ccl17) were significantly decreased in L635-treated IL33KO mice compared to L635-treated wild-type mice. **B.** Th2-related M2 macrophage polarizing cytokines were significantly decreased in L635-treated IL33KO mice compared to L635-treated wild-type mice. (*p<0.05).

Figure 5. IL-5 and IL-9 are not required for SPEM development and M2 macrophage polarization in the atrophic stomach

A. Immunofluorescence staining for chief cell transcription factor Mist1, mucus marker GSII-lectin, chief cell granule marker gastric intrinsic factor (GIF), SPEM marker CD44 variant isoform 9 (CD44v9), proliferation marker Ki67, macrophage marker F4/80, M2 macrophage marker CD163 and nuclei marker DAPI in wild-type mice treated with non-specific IgG or the following L635 treated mice: non-specific IgG, anti-IL-5 and anti-IL-9. **B.** Quantification of Mist1 positive nuclei per 20× field. IL-5 or IL-9 depleted L635-treated

mice had significant loss of Mis1-positive cells similar to non-specific IgG L635-treated mice. **C.** Quantification of SPEM cells determined by percent of GIF positive cells co-positive for CD44v9 and GSII-lectin. IL-5 or IL-9 depleted L635-treated mice have a significant percent of SPEM cells similar to non-specific IgG L635-treated mice. **D.** Quantification of proliferative SPEM cells determined by the percent of SPEM cells positive for Ki67. IL-5 or IL-9 depleted mice have a significant percent of proliferating SPEM cells similar to non-specific IgG L635-treated mice. **E.** Quantification of F4/80-positive macrophages (red) and F4/80 and CD163 co-positive M2 macrophages (green) per 20× field. IL-5 or IL-9 depleted mice had significant infiltration of F4/80-positive macrophages, most of which were co-positive for CD163 similar to non-specific IgG L635-treated mice. * denotes significant difference ($p<0.05$) compared to non-specific IgG-treated mice without L635-treatment. White dashed boxes are magnified in the insets. Scale bars = 100 μm .

Figure 6. IL-13 signaling downstream of IL-33/ST2 drives metaplasia induction and M2 macrophage polarization

A. Immunofluorescence staining for chief cell transcription factor Mist1, mucus marker GSII-lectin, chief cell granule marker gastric intrinsic factor (GIF), SPEM marker CD44 variant isoform 9 (CD44v9), proliferation marker Ki67, macrophage marker F4/80, M2 macrophage marker CD163 and nuclei marker DAPI in untreated IL13KO mice and L635-treated IL13KO mice. **B.** Quantification of Mist1 positive nuclei per 20× field. L635-treated IL13KO mice had significantly fewer Mist1-positive cells compared to untreated IL13KO mice, although some Mist1-positive cells remained. **C.** Quantification of SPEM cells determined by percent of GIF positive cells co-positive for CD44v9 and GSII-lectin. L635-treated IL13KO mice have a significantly higher percent of SPEM cells, however this number is still relatively low. **D.** Quantification of proliferative SPEM cells determined by the percent of SPEM cells positive for Ki67. L635-treated IL13KO had a few SPEM cells and some of the SPEM cells were proliferating so there is a significantly higher percent of proliferative SPEM cells in L635-treated IL13KO mice compared to untreated IL13KO mice. **E.** Quantification of F4/80-positive macrophages (red) and F4/80 and CD163 co-positive M2 macrophages (green) per 20× field. L635-treated IL13KO mice have significant F4/80-positive macrophage infiltration compared to untreated IL13KO mice. L635-treated IL13KO mice also have significantly more F4/80 and CD163 co-positive cells compared to

untreated IL13KO mice, however the percent of F4/80 macrophages that are co-positive for CD163 is around 15% in both mouse models. * denotes significant difference ($p < 0.05$) compared to untreated mice. White dashed boxes are magnified in the insets. Scale bars = 100 μm .

Figure 7. IL-13 is sufficient to induce metaplasia after parietal cell loss in the absence of IL-33/ST2 signaling

A. Immunofluorescence staining for chief cell transcription factor Mist1, mucus marker GSII-lectin, chief cell granule marker gastric intrinsic factor (GIF), SPEM marker CD44 variant isoform 9 (CD44v9), proliferation marker Ki67, macrophage marker F4/80, M2 macrophage marker CD163 and nuclei marker DAPI in ST2KO mice treated with IL-13 (ST2KO^{IL13}), ST2KO mice treated with a non-specific IgG and L635 (ST2KO^{L635}), or ST2KO mice treated with IL-13 and L635 (ST2KO^{L635-IL13}). **B.** Quantification of Mist1 positive nuclei per 20× field. ST2KO^{L635-IL13} mice had significant loss of Mist1-positive cells similar to ST2KO^{L635}. **C.** Quantification of SPEM cells determined by percent of GIF

positive cells co-positive for CD44v9 and GSII-lectin. ST2KO^{L635-IL13} mice have a significantly higher percent of SPEM cells compared to ST2KO^{IL13} and ST2KO^{L635} mice.

D. Quantification of proliferative SPEM cells determined by the percent of SPEM cells positive for Ki67. Although there is more SPEM in ST2KO^{L635-IL13} mice, there is no significant difference in proliferative SPEM. **E.** Quantification of F4/80-positive macrophages (red) and F4/80 and CD163 co-positive M2 macrophages (green) per 20× field. ST2KO^{L635-IL13} mice exhibit similar levels of F4/80-positive macrophage infiltration to ST2KO^{L635} mice, but there are significantly more F4/80 and C163 co-positive M2 macrophages in ST2KO^{L635-IL13} mice. * denotes significant difference (p<0.05) compared to untreated mice, + denotes significant difference (p<0.05) compared to ST2KO non-specific IgG-treated L635-treated mice. White dashed boxes are magnified in the insets. Scale bars = 100 μm.