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Jason C. Mills
et al

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Interleukin 33 Triggers Early Eosinophil-Dependent Events Leading to Metaplasia in a Chronic Model of Gastritis-Prone Mice

Carlo De Salvo,1 Luca Pastorelli,1,2,3 Christine P. Petersen,4 Ludovica F. Buttò,5 Kristine-Ann Buela,1 Sara Omenetti,1 Silviu A. Locovei,1,5 Shuvra Ray,1 Hannah R. Friedman,1 Jacob Duijser,1 Wei Xin,1 Abdullah Osme,1 Fabio Cominelli,5 Ganapati H. Mahabeleshwar,1 Jason C. Mills,6 James R. Goldenring,4 and Theresa T. Pizarro1

1Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, Ohio; 2Gastroenterology Unit, Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Donato, San Donato Milanese, Milan, Italy; 3Department of Biomedical Sciences for Health, University of Milan, Milan, Italy; 4Department of Surgery and the Epithelial Biology Center, Vanderbilt University, Nashville, Tennessee; 5Division of Gastroenterology & Liver Disease, Department Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio; and 6Gastroenterology Division, Department of Medicine, Washington University in St. Louis School of Medicine, St Louis, Missouri

BACKGROUND & AIMS: Interleukin (IL)33/IL1F11 is an important mediator for the development of type 2 T-helper cell (Th2)-driven inflammatory disorders and has also been implicated in the pathogenesis of gastrointestinal (GI)-related cancers, including gastric carcinoma. We therefore sought to mechanistically determine IL33’s potential role as a critical factor linking chronic inflammation and gastric carcinogenesis using gastritis-prone SAMP1/YitFc (SAMP) mice. METHODS: SAMP and (parental control) AKR mice were assessed for baseline gastritis and progression to metaplasia. Expression/localization of IL33 and its receptor, ST2/IL1R4, were characterized in corpus tissues, and activation and neutralization studies were both performed targeting the IL33/ST2 axis. Dissection of immune pathways leading to metaplasia was evaluated, including eosinophil depletion studies using anti-IL5/anti-CCR3 treatment. RESULTS: Progressive gastritis and, ultimately, intestinalized spasmylocytic polypeptide-expressing metaplasia (SPEM) was detected in SAMP stomachs, which was absent in AKR but could be moderately induced with exogenous, recombinant IL33. Robust peripheral (bone marrow) expansion of eosinophils and local recruitment of both eosinophils and IL33-expressing M2 macrophages into corpus tissues were evident in SAMP. Interestingly, IL33 blockade did not affect bone marrow-derived expansion and local infiltration of eosinophils, but markedly decreased M2 macrophages and SPEM features, while eosinophil depletion caused a significant reduction in both local IL33-producing M2 macrophages and SPEM in SAMP. CONCLUSIONS: IL33 promotes metaplasia and the sequelae of eosinophil-dependent downstream infiltration of IL33-producing M2 macrophages leading to intestinalized SPEM in SAMP, suggesting that IL33 represents a critical link between chronic gastritis and intestinalizing metaplasia that may serve as a potential therapeutic target for preneoplastic conditions of the GI tract.

Keywords: IL-33/ST2 Axis; M2 Macrophages; SPEM/Intestinalized SPEM; Gastric Cancer.
The link between chronic inflammation and cancer is well established, with inflammation-associated cancers among the most highly represented and frequently occurring neoplasias worldwide. Investigation over the last several years has focused on determining critical pathways involved in this process, with a number of candidate molecules identified, including members of the interleukin-1 (IL-1) family that are particularly important in cancers of the GI tract.

**NEW FINDINGS**

IL-33 (or IL-1F11), a member of the IL-1 family of cytokines, serves as an important mediator linking chronic inflammation and metaplasia by inducing the expansion and recruitment of activated eosinophils leading to advanced, intestinalized SPEM in gastripronc SAMP1/YitFc (SAMP) mice.

**LIMITATIONS**

Further studies are warranted to determine the precise inciting factors of increased IL-33 leading to intestinalized SPEM in SAMP mice, as well as in patients with gastric cancer.

**IMPACT**

The present manuscript contributes to a better understanding of potential mechanism(s) that promote the inflammation-metaplasia-dysplasia-carcinoma sequelae that can apply to several GI-related cancers.

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**WHAT YOU NEED TO KNOW**

**BACKGROUND AND CONTEXT**

The link between chronic, non-resolving inflammation and cancer is well established, with inflammation-associated cancers among the most highly represented and frequently occurring neoplasias worldwide. Investigation over the last several years has focused on determining critical pathways involved in this process, with a number of candidate molecules identified, including members of the interleukin-1 (IL-1) family that are particularly important in cancers of the GI tract.

IL-33 (or IL-1F11), a member of the IL-1 family of cytokines, serves as an important mediator linking chronic inflammation and metaplasia by inducing the expansion and recruitment of activated eosinophils leading to advanced, intestinalized SPEM in gastripronc SAMP1/YitFc (SAMP) mice.

Further studies are warranted to determine the precise inciting factors of increased IL-33 leading to intestinalized SPEM in SAMP mice, as well as in patients with gastric cancer.

The present manuscript contributes to a better understanding of potential mechanism(s) that promote the inflammation-metaplasia-dysplasia-carcinoma sequelae that can apply to several GI-related cancers.
polarized macrophages, and SPEM that can develop intestinal characteristics in mice. When this model is used, IL33 promotes IL13-dependent M2 polarization of recruited macrophages and the development of SPEM.

In the present study, we evaluate a murine model, SAMP1/VitFc (SAMP) strain,32 that spontaneously develops chronic gastric inflammation to uncover potential IL33-dependent mechanism(s) leading to and downstream of parietal cell loss and chief cell transdifferentiation that can result in SPEM and an even more proliferative, intestinalized SPEM. We show that:

1. gastritis-prone SAMP mice progressively develop metaplasia leading to advanced SPEM-like features;
2. exogenous recombinant (r)IL33 administration induces moderate metaplasia and M2 macrophage infiltration in stomachs of healthy (AKR) mice, and under chronic inflammatory conditions, exacerbates and advances metaplasia-acquiring intestinal characteristics in SAMP;
3. neutralization of IL33 in SAMP with established disease dampens gastritis and progression of metaplasia/SPEM;
4. IL33 induces potent peripheral eosinophil expansion and subsequent recruitment into stomachs of treated mice; and
5. eosinophil depletion markedly diminishes M2 macrophage infiltration and SPEM-like features in SAMP.

Together, our data suggest that IL33 plays a central role in the early events leading to SPEM and that during chronic inflammation, or perhaps under other predisposing conditions that promote lack of immune tolerance, or both, IL33-dependent eosinophil activation and migration are essential for this process to occur. As such, targeting the IL33/ST2 axis for therapeutic purposes may be beneficial for the treatment or prevention, or both, of preneoplastic states leading to gastric cancer.

Materials and Methods

Mice

Mice were provided through core services supported by the Animal and Mouse Models Cores of National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases P01 DK091222 and P30 DK097948, respectively. SAMPxIl33−/− mice were developed by backcrossing male C57BL/6J (B6) mice homozygous for a null allele of Il33 (Il33−/−)5,24 with female SAMP for 10 generations and validated by microsatellite analysis comparing SAMP- vs B6-specific markers. Breeding of F1,N19 heterozygous offspring generated SAMP homozygous Il33−/−, heterozygous, and wild-type controls, identified by polymerase chain reaction (PCR).5,24 Mice were maintained as previously described,24 with all procedures approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Tissue Harvest and Histologic/Spasmolytic Polypeptide-Expressing Metaplasia Assessment

Mice were euthanized and whole stomachs removed and opened along the greater curvature. Forestomach and antrum were excised, and the corpus was dissected along the longitudinal plane, with 1 strip each placed in RNAlater (Ambion, Thermo Fisher Scientific, Waltham, MA) or radioimmunoprecipitation buffer (Pierce Biotechnology, Rockford, IL) and maintained at −20°C until later RNA and protein extraction, respectively. The remaining tissue strip was submerged in Bouin’s Fixative Solution (Ricca Chemical Company, Arlington, TX), 4% paraformaldehyde (PFA) (ChemCruz, Santa Cruz Biochemicals, Dallas, TX), or methacarn. Bouin’s/PFA- and methacarn-fixed tissues were rinsed in 70% ethanol after 24 hours or 20 minutes, respectively, processed, paraffin-embedded, and sectioned at 3 to 4 μm. Specimens were stained with H&E or Alcian blue/periodic acid–Schiff (PAS) with optional hematoxylin 7221 (Thermo Scientific Richard-Allan Scientific, Kalamazoo, MI).

Samples were evaluated by trained GI pathologists (W.X., A.O.) in a blinded fashion for gastritis/epithelial alterations and SPEM/intestinalized SPEM as previously described.30,32 SPEM was scored by calculating the percentage of cross-sectional involvement, defined by oxyntic atrophy with loss of parietal cells and replacement of gastric glands with mucous cell lineages staining positive for PAS/Alcian blue.

In Vivo Studies

Experiments were conducted in a blinded manner, with mice randomized to different interventions using progressive numeric labeling, the code only known to animal caretakers and revealed at end of each experiment. Scientific rigor, data reproducibility, and biological variables were followed, based on recently published guidelines.35 SAMP and sex- and age-matched AKR mice were evaluated for baseline gastritis and progression to SPEM/intestinalized SPEM.30,32 For exogenous IL33 experiments, 8- to-12-week-old mice were injected intraperitoneally with rIL33 (33 μg/kg) (Enzo Life Sciences, Farmingdale, NY) or Hank’s buffered salt solution (vehicle), daily for 1 week. IL33 neutralization was achieved using a fusion protein (5 mg/kg, intraperitoneally twice weekly for 4 weeks) consisting of the extracellular domain of mouse soluble (s)ST2 fused to IgG1-Fc (sSt2-Fc); controls were similarly administered IgG1-Fc (Dirk E. Smith, Amgen, Inc., Seattle, WA). Eosinophil depletion was performed using monoclonal antibodies against IL5 and C-C motif chemokine receptor 3 (CCR3) and controls treated with an isotype IgG.24

Quantitative Reverse-Transcription Polymerase Chain Reaction and Western Blots

Total RNA was isolated, reverse-transcribed, and quantitative polymerase chain reaction (qPCR) performed as previously described,24 using specific target gene primers (Supplementary Table 1) normalized to β-actin or 36B4 and reported as relative fold-difference among groups, with baseline/controls set arbitrarily at 1. Total protein extracts from the corpus were prepared and Western blotting was performed.30,32

Staining With 5-Bromo-2-Deoxyuridine and Immunohistochemistry

Mice were injected with 5-bromo-2-deoxyuridine (BrdU) labeling agent (Invitrogen, Carlsbad, CA) 2 hours before euthanization. Gastric tissues were harvested, fixed in 4% PFA, and stained following the manufacturer’s instructions (BrdU
Staining Kit; Invitrogen). Immunohistochemistry was performed using a polyclonal goat anti-mouse IL33 IgG (R&D Systems, Minneapolis, MN), biotinylated Dolichos biflorus agglutinin (DBA, Vector Laboratories, Burlingame, CA), or a monoclonal rat anti-mouse major basic protein (MBP) IgG (clone MT-14.7) (James J. Lee, Mayo Clinic, Scottsdale, AZ), with negative controls prepared under identical conditions in the absence of respective primary antibodies.

Parietal cell/eosinophil counts were calculated by average intact, nucleated cells positive for DBA/MBP in 10 randomly selected high-power fields. Immunofluorescence for confocal imaging was performed to detect Griffonia simplicifolia II (GSII), cluster of differentiation (CD) 44v, clusterin (Clu), CD163, IL33, gastric intrinsic factor (GIF), and imaged/analyzed by the Vanderbilt Digital Histology Shared Resource.

Flow Cytometry
Bone marrow (BM) from femurs/tibias was harvested and processed as previously described. Then, 2 \times 10^6 cells were stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain (Thermo Fisher Scientific) and specific antibodies to detect eosinophils and M2 macrophages (Supplementary Table 2). Samples were acquired with a BD LSR II flow cytometer and data analyzed with FlowJo Software (both, Becton Dickinson, Franklin Lakes, NJ).

Statistical Analyses
Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA). Selection of appropriate statistical tests was based on variance and underlying distribution of data. Global effects between groups were assessed using 1-way analysis of variance with Bonferroni’s correction for multiple comparisons. Differences between individual groups were directly compared using the 2-sample unpaired Student’s t test, and results are expressed as mean ± SEM, unless otherwise indicated, with \( P < .05 \) considered significant.

Results
Gastritis-Prone SAMP Exhibit Morphologic Features and a Molecular Profile Consistent With Intestinalized Spasmolytic Polypeptide-Expressing Metaplasia
SAMP mice develop chronic, immune-mediated gastritis with increased severity over time. Stomachs from 20-week-old SAMP show remarkable and progressive parietal cell loss compared with age-matched AKR controls, which possess abundant parietal cells (Supplementary Figure 1A). The emergence of prominent Alcian blue/PAS-stained hyperplastic mucous neck cells in SAMP highlight the presence of acidic mucins, commonly found in SPEM and adenocarcinoma, but are absent in AKR (Supplementary Figure 1A). Older SAMP display intense staining for the mucous marker, GSII lectin, while colocalization with CD44v or Clu, indicative of SPEM, shows more intense Clu expression along the gastric glands compared with AKRs. The presence, albeit subtle, of Cd44v at the base of gastric glands in 20-week-old SAMP is also detected but is virtually absent in the other experimental groups (Supplementary Figure 1B).

Molecular profiling of full-thickness corpus shows a striking decrease in the expression of genes associated with oxyntic atrophy and foveolar hyperplasia, including gastric intrinsic factor (Gif), adenosine-5’-triphosphatase H+/K+ transporting alpha subunit (Atp4a), and trefoil factor 1 (Tff1), comparing SAMP vs AKR, particularly in older mice (Supplementary Figure 2A). During acute oxyntic atrophy, as parietal cells die, chief cells show a rapid decrease in the zymogen granule maturation transcription factor (Mist1) and an increase in Tff2. Although no differences in Tff2 are detected among experimental groups, Mist1 is markedly decreased in SAMP vs AKR (Supplementary Figure 2B). Furthermore, the SPEM markers, human epididymis protein 4 (He4), Clu, lysozyme (Lyz), and glutathione peroxidase 2 (Gpx2), are all dramatically increased in SAMP with established disease vs AKR (Supplementary Figure 2C). Importantly, markers of more advanced, proliferative lesions (intestinalized SPEM), including cystic fibrosis transmembrane conductance regulator (Cftr) and deleted in malignant brain tumors protein 1 (Dmbt1), are strongly upregulated in 20-week-old SAMP vs AKR, while the early tumor shrinkage variant 5 (Etv5) transcription factor is unchanged (Supplementary Figure 2D).

Increased Circulating Interleukin 33, With Its Most Bioactive Form Localizing to Gastric M2 Macrophages in SAMP With Advanced Spasmolytic Polypeptide-Expressing Metaplasia
To test the hypothesis that increased and persistent exposure to IL33 may be needed for intestinalized SPEM to occur, we initially measured systemic/circulating IL33, which is substantially increased in SAMP, even before histologically evident gastritis (at 4 weeks) and dramatically rises as disease becomes more severe, compared with IL33 in AKR that remains relatively stable (Supplementary Figure 3). Locally, within nondiseased stomachs of 4-week-old mice, IL33 is primarily found in foveolar epithelium, almost exclusively localized to nuclei (Figure 1A). In older mice, AKR retain some epithelial-specific nuclear expression with scant IL33+ cells within the lamina propria (LP), while abundant and diffuse IL33 immunoreactivity is observed in SAMP, also within the LP, as well as at the base of the mucosa (Figure 1A).

Evaluation of IL33 isoforms reveals less abundant IL33 in 4-week-old SAMP compared with AKR; however, in older SAMP, only full-length IL33 (flIL33), representing its most bioactive 30-kD form, is prominently expressed, whereas other less bioactive forms are present in AKR (Figure 1B). In fact, SAMP express mainly flIL33, whereas the 20- to 22-kD cleaved form (cIL33) is virtually undetectable but clearly present in AKR (Figure 1B), suggesting increased release of bioactive flIL33 in SAMP with established disease. Surprisingly, IL33 in SAMP stomachs is decreased compared with AKR (Figure 1C), which may be due to high circulating IL33...
levels (Supplementary Figure 3) and potential negative feedback mechanism(s) downregulating IL33’s bioactivity.

Finally, to identify the cellular source of the considerable yet diffuse IL33 staining within the gastric submucosa of older SAMP (Figure 1A), immunofluorescent colocalization studies were performed, revealing abundant, infiltrating IL33-expressing CD163⁺ M2 macrophages that are virtually absent in noninflamed AKR but can be elicited with rIL33 (Figure 1D). Phenotypic characterization of SAMP gastric mucosal cells confirm the presence of IL33-producing M2 macrophages (Supplementary Figures 4 and 1E and F), with
an overall dominant M2 vs M1 profile vs AKR (Supplementary Figure 5).

**Exogenous Administration of Interleukin 33 Induces Spasmylic Polypeptide-Expressing Metaplasia in Normal (AKR) Mice**

One of the original observations regarding IL33’s bioactivity was its ability to promote epithelial hyperplasia, mainly in goblet cells, within GI and airway mucosae. These findings were confirmed in stomachs of IL33-treated B6 mice, which results in a Th2/signal transducer and activator of transcription 3 (STAT3)-driven gastric pathology. Aside from inducing infiltration of IL33-expressing CD163⁺ M2 macrophages (Figure 1D), acute systemic exposure of IL33 consistently promotes the gross anatomic appearance of thickened gastric mucosal folds that are absent in vehicle-treated mice (Figure 2A). Histologic evaluation of these folds reveals striking alterations, including overall hypertrophy of the gastric mucosa, epithelial hyperplasia, as well as oxyntic atrophy, while parietal and chief cells remain relatively intact in vehicle-treated AKR (Figure 2A). Interestingly, although exogenous IL33 results in only 25% of AKR displaying significant gastritis, increased epithelial hyperplasia suggests its contribution to inducing mucous neck cell hyperplasia and metaplasia (Figure 2B).

The presence of metaplasia is further characterized by prominent proliferation of cells within the gastric glands, demonstrated by a greater number of BrdU⁺ cells, along the neck and at the base of gastric glands (Figure 2A, right panel).
panels), and Alcian blue/PAS staining, highlighting the appearance of hyperplastic acidic mucin-producing neck cells, comparing IL33- vs vehicle-treated AKRs (Figure 2C).

Finally, accumulation of GSL lectin (a surrogate for MUC6 expression) colocalizing with CD44v at the base of glands in treated AKR (Figure 2C), indicating chief cells transdifferentiation, also supports the development of IL33-dependent SPEM (Figure 2D), but for the most part, in the absence of inflammation.

Consistent with oxyntic atrophy, molecular profiling shows that Gif and Atp4a are downregulated in corpus tissues from IL33-treated AKR (Figure 3A), and while Tff1 shows a trend toward decreased expression, Tff2 is strongly upregulated after short-term IL33 exposure (Figure 3A), further indicating the occurrence of IL33-induced chief cell transdifferentiation leading to SPEM. Additionally, He4 and Clu are consistently upregulated after rIL33 (Figure 3B); however, only Cfr is considerably increased for the intestinalized SPEM markers assayed (Figure 3C), suggesting that IL33 has a prominent effect on the development of SPEM, but to progress to a more advanced intestinalized phenotype, a longer, more chronic exposure to IL33 may be needed. Interestingly, exogenous IL33 does not affect Il1r11 (ST2L), encoding the signaling receptor for IL33, but Il1r11 (sST2), encoding soluble ST2, an IL33-specific decoy receptor, is markedly increased and IL33 is significantly decreased (Figure 3D), which is similar to what is observed in SAMP with established disease and may represent a negative feedback response after rIL33.

Figure 3. Molecular profiling indicates overt metaplasia in AKR corpus after acute exposure to IL33. Relative transcript levels of (A) Gif, Atp4a, Tff1, and Tff2, (B) He4 and Clu, (C) Cfr, Dmbt1, and Etv5, and (D) Il33, and Il1r11 variant 1 (ST2L) and 2 (sST2) after rIL33. Data are expressed as fold-change vs vehicle controls (N = 8). mRNA, messenger RNA. *P < .05, **P < .01, ***P < .001.
restorative epithelial processes toward normal homeostasis.

Histology images also show that overall gastric inflammation is visibly decreased after IL33 blockade vs control, confirmed by reduction in total inflammatory scores (Figure 4D). Of note, the primary component driving total inflammation downward in sST2-Fc-treated SAMP is chronic inflammation, which decreases by 41%, and not acute inflammation, which remains relatively unchanged between experimental groups (Figure 4D).

Peripheral Expansion and Local Recruitment of Eosinophils Are Dramatically Increased in SAMP With Advanced Spasmolytic Polypeptide-Expressing Metaplasia and Rely on Interleukin 33

We previously reported that eosinophils play a central role in mediating chronic, Th2-driven ileitis that is dependent on intestinal IL33 induced by the gut microbiome. In the present study, we found that expansion of peripheral BM-derived eosinophils, defined as CD11b⁺ Siglec-F⁺ cells (Supplementary Figure 6), is already present in 4-week-old SAMP, before the appearance of gastritis, compared with age-matched AKR (Figure 5A). This trend is consistent with what was previously observed in older SAMP, when intestinal-like metaplasia is fully established, and is increased vs 4-week-old SAMP. Locally, baseline presence of eosinophils identified by MBP, one of the most prevalent proteins specifically-produced by eosinophils, is detected in young SAMP and also found in equal numbers in age-matched AKR (Figure 5B). As disease progresses, the number of infiltrating MBP⁺ eosinophils greatly increases, first appearing at the margination between the submucosa and muscularis mucosa and then collecting at the base of the gastric glands with infiltrates percolating upwards throughout the LP vs AKRs, wherein eosinophils are scarce and sparsely detected (Figure 5B). Quantitatively, MBP⁺ eosinophils are clearly evident in SAMP corpus as intestinalizing SPEM progresses, showing a 4-fold increase by 20 weeks compared with AKR (Figure 5B).

These results indicate that systemic expansion of eosinophils and their recruitment into gastric tissues occurs early and is sustained in SAMP with advanced intestinalized SPEM. Significant abrogation of BM-derived eosinophils (Figure 5A) and subsequent decreased recruitment into the gastric mucosa of SAMP lacking IL33 (SAMPxIl33⁻/⁻)
compared with wild-type littermates (SAMPxIl33−/−) (Figure 5C) confirm the early reliance on IL33 for these effects.

Eosinophil expansion, in fact, can be dramatically elicited, particularly in the periphery, in healthy AKR after acute (1 week) IL33 administration. Likewise, aside from the striking mucosal epithelial alterations described above (Figure 2), IL33 also promotes a trend of eosinophil recruitment into the corpus of treated AKR (Figure 5D). These results indicate that in the absence of inflammation, exogenous IL33 induces robust systemic expansion, and to a lesser extent, local recruitment of eosinophils into the gastric mucosa. Interestingly, IL33 blockade in SAMP with established disease does not affect expansion of BM-derived eosinophils (Figure 5A) or local recruitment of MBP+ eosinophils (Figure 5E), indicating that once these processes are induced, particularly in mice exposed to chronic inflammatory conditions, they cannot be reversed.

**Eosinophil Depletion Effectively Reduces Gastritis, Infiltration of M2 Macrophages, and Intestinalized Spasmolytic Polypeptide-Expressing Metaplasia**

Because IL33 blockade is effective in dampening both infiltration of CD163+ M2 macrophages as well as intestinal-like metaplasia, but not recruitment of eosinophils in SAMP, we tested whether earlier intervention of eosinophil depletion has downstream effects on advanced intestinalized SPEM. As such, 14-week-old SAMP were treated with antibodies against CCR3 and IL5, alone and in combination, previously shown to effectively deplete eosinophils.34,47,48 Eosinophil depletion was confirmed in both peripheral BM and corpus tissues (Supplementary Figures 7A and 6A, left panels), with histologic evaluation showing global improvement in restoring normal epithelial architecture, reducing the presence of cellular immune infiltrates, and decreasing overall inflammation within the LP and hyperplasia of muscle wall layers in mice treated with anti-IL5 and anti-CCR3, alone and in combination, vs IgG-treated controls (Figure 6A and C). Eosinophil depletion is also able to effectively decrease the presence of infiltrating IL33-expressing M2 macrophages (Figure 6A and D and Supplementary Figure 7B) and potently reduces gene expression of M2-associated molecules (Supplementary Figure 7C), but does not affect epithelial-derived IL33 (Supplementary Figure 7D).

Importantly, CD44v colabeling with GSII and GIF reveals that while no dramatic differences are observed in GSII among experimental groups, increased intensity of GIF is noted, particularly in SAMP treated with anti-IL5 or combination anti-IL5/anti-CCR3 vs controls, indicating at least partial reversal of oxyntic atrophy (Figure 6A, right panels). Interestingly, the decreased Mist1 seen during SPEM is dramatically reversed after eosinophil depletion (Figure 6E), suggesting eosinophil-dependent chief cell transdifferentiation during SPEM. Taken together, these results indicate an essential role for eosinophils in the development of intestinalized SPEM.

**Discussion**

Increasing evidence confirms the importance of the IL33/ST2 axis in the development of gastritis and gastric cancer; in this context, IL33 is an ideal candidate to link these two processes. However, although several studies have reported associative findings in patients, correlating increased IL33 with intestinal-type gastric cancer18 and poor prognostic factors,15,49 less is known regarding specific IL33-dependent mechanisms leading to these pathologies. Based on the published literature and data from the present study, we propose a working hypothesis regarding the mechanistic role of IL33 in promoting SPEM progression and the gastritis-metaplasia-dysplasia-carcinoma sequelae in which eosinophils play a central role.

Some consider SPEM/intestinalized SPEM is a mandatory step for dysplasia, and eventually carcinoma, to proceed.50 Once established, intestinalized SPEM or intestinal metaplasia, or both, may not be reversible in the presence of inciting factors such as chronic inflammation.51 In fact, along with distinct gastric epithelial changes, an essential component to attain intestinal-like characteristics is non-resolving chronic inflammation, often thought to be dependent on H pylori status.51,52 In this setting, IL33 represents a critical mediator for SPEM/intestinalized SPEM to manifest, considering its ability to both potently stimulate epithelial proliferation and metaplasia as well as induce and sustain chronic Th2-driven inflammation.

Our data show that the most prevalent form of IL33 in SAMP with established disease is bioactive fIL33, which is released during chronic inflammation, with minimal presence of the less bioactive cleaved form (cIL33)38–40 that is more highly expressed in uninfamed AKR stomachs. In uninfamed young mice (AKR/SAMP) and older AKR, nuclear sequestration occurs within gastric epithelial cells, serving as reservoirs for IL33 that is readily available for immediate release upon exposure to appropriate stimuli.18 In fact, under homeostatic conditions, the primary role of IL33 in the GI tract is believed to be epithelial repair and restitution and to promote overall mucosal wound healing.33,53

Interestingly, we found that at the transcript level, IL33 is significantly decreased in SAMP compared with AKR, which likely reflects a negative feedback mechanism in response to high systemic IL33. In line with this finding, IL33 is also

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**Figure 5.** Early IL33-dependent peripheral expansion and increased eosinophil recruitment to SAMP stomachs. (A) Percentages of BM-derived eosinophils (EOS), and representative immunohistochemistry photomicrographs of corpus from (B) SAMP vs AKR, (C) SAMPxIl33−/− vs wild-type littermates, (D) IL-33− vs vehicle-treated AKR, and (E) sST2-Fc- vs IgG-treated SAMP, stained for MBP and eosinophil counts (N = 4-10). HPF, high-power field. Original magnification ×10+1.25; scale bars: 100 μm. **P < .01, ***P < .001.
Figure 6. Eosinophil depletion reduces gastritis, M2 macrophage recruitment, and SPEM in SAMP. (A) Representative photomicrographs of corpus stained (left-to-right): for MBP (eosinophils), with H&E, and by immunofluorescence for IL33- (red) expressing CD163⁺ (green) M2 macrophages (yellow), showing reduction after anti-IL5, anti-CCR3 or combination anti-IL5/CCR3, and for GSII (green), CD44v (red) and GIF (blue) showing specific increase in GIF (arrowheads), suggesting protection from oxyntic atrophy. Original magnification ×20 and ×40 (insets); scale bars: 100 μm, 50 μm (insets). Quantitation of gastric (B) eosinophils, (C) inflammation, (D) IL33-expressing M2 macrophages, and (E) Mist1 after eosinophil depletion. Data are representative of 3 experiments (N = 7-8 per experiment). *P < .05, **P < .01; ***P < .001; ****P < .0001.
decreased after exogenous IL33 administration, whereas the Il1rl1 variant coding for sST2 (soluble decoy receptor) is greatly increased, which together serve to downregulate overall IL33 bioactivity.

The present study also introduces the SAMP mouse strain as a model of intestinalized SPEM that develops spontaneously and progresses in severity, without chemical, genetic, or immunologic manipulation. These mice provide an ideal tool to investigate the natural course of disease and a convenient system to test potential preclinical therapies for preneoplastic conditions that may lead to gastric cancer, in which preventive and therapeutic strategies can both be effectively implemented.

Using a therapeutic approach, we found that IL33 neutralization by sST2-Fc administration is effective at reversing intestinalized SPEM, primarily by targeting the epithelial compartment and reducing IL33-dependent epithelial alterations, which are hallmark features of metaplasia. In addition, IL33 blockade decreases the severity of chronic gastritis and the presence of M2 macrophages, which are also characteristic of metaplasia and progression to intestinalized SPEM.13

Importantly, using SAMP, we not only demonstrate the essential role for IL33 in the development of intestinal-like metaplasia but also that the IL33/ST2 axis is important for several steps throughout this process. For example, IL33 clearly has direct effects on epithelial cells. Indeed, epithelial alterations within the GI tract were among the initial aberrancies described after healthy mice were exposed to high levels of IL33.9 We and others41 confirmed these observations in the stomach, showing that rIL33 induces severe oxyntic atrophy and upregulates specific markers of SPEM. Alternatively, IL33 also has profound effects on the gastric immune compartment, promoting, for example, vigorous infiltration of eosinophils and M2 macrophages,31 further perpetuating a chronic inflammatory state. Importantly, prolonged and elevated levels of IL33 have the ability to sustain chronic inflammation in the stomach, which is essential for intestinal metaplasia to advance to gastric cancer.

The results of our study also underscore the importance of eosinophils in the development of intestinal-like metaplasia and that IL33 plays a critical role in the systemic expansion and early recruitment of eosinophils into the gastric mucosa of SAMP as well as AKRs treated with rIL33. Previously, eosinophils in gastric metaplasia and cancer development have been proposed to have both pathogenic as well as protective functions. A prior study investigating early gastric cancers demonstrated the presence of tumor stromal eosinophils with morphologic evidence of activation as well as tumor cells in intimate contact with activated eosinophils with focal cytopathic changes.54

Gastric carcinomas have also been reported to express eosinophil-associated chemotactic cytokines, including IL2, IL5, and granulocyte-macrophage colony-stimulating factor, with expression of granulocyte-macrophage colony-stimulating factor appearing to be specific for signet ring carcinoma cells.25 On the other hand, in support of a protective function, high preoperative eosinophil counts were found to correlate with prolonged survival after surgery.56 and the-28G polymorphism in the promoter region of the eosinophil chemoattractant RANTES (regulated upon activation normal T cell expressed and secreted) is associated with a reduced risk of developing severe intestinal metaplasia.57

Nonetheless, while our results indicate that eosinophils play an important role during early events leading to gastritis/metaplasia, one can certainly speculate that other IL33-responsive innate immune cell populations may also contribute. Our data show that IL33 can promote local recruitment of M2 macrophages that themselves are potent producers of IL33. These findings are in line with previous reports demonstrating the ability of IL33 to increase overall macrophage numbers in the stomach and to polarize them to an alternatively activated M2 phenotype, albeit in the periphery (peritoneum).41 and that M2 macrophages are dramatically reduced in IL33-deficient mice in which advanced SPEM has been elicited, but that can also be rescued by administering rIL13.31

Our findings in SAMP, however, indicate that M2 macrophage infiltration is likely downstream of eosinophil activation and recruitment because eosinophil depletion, similar to systemic IL33 blockade, has a significant impact on reducing the infiltration of gastric M2 macrophages that also happen to produce IL33. Interestingly, however, eosinophil depletion does not appear to have an effect on IL33 expression in the gastric epithelium of SAMP. In contrast, L635-induced SPEM does not appear to require eosinophils,31 which may simply reflect differences in inciting factors between the 2 models (ie, chemical obliteration of parietal cells vs chronic, nonresolving inflammation).

An alternative hypothesis is that ILC2s are also an IL33-responsive innate immune cell population that can affect SPEM. ILC2s have not been detected or isolated from gastric tissues until recently, when Li et al32 reported the prevalence of GATA-3^Lin^- cells in tumors of patients with H pylori-associated gastric cancer. Of note, and counterintuitive to this finding, Buzzelli et al41 showed that IL33 is rapidly induced after acute H pylori infection but suppressed in chronic infection. However, in the former study, it is unclear what the IL33 levels were, whether they correlated with GATA-3^Lin^- ILC2 frequency, and whether IL33 exhibited temporal changes during early vs late stages in different types of gastric cancers. In the latter study, acute vs chronic H pylori infection was defined as 1 and 7 days vs 2 months, which may not reflect chronic infection, especially in gastric cancer-susceptible individuals.

Although indirect evidence suggests emergence of gastric ILC2s in IL33-treated mice,41 we recently reported increased ILC2s after L635-dependent acute gastric injury.59 Similarly, preliminary data in SAMP mice also show an increased frequency, albeit quite low, of ILC2s vs AKR (Supplementary Figure 8); however, whether ILC2s contribute to the SAMP gastric phenotype is unknown. On the basis of studies characterizing SAMP lacking T/B cells, but with intact ILC function (ie, SAMPxRag2^/- strain), ILCs likely play a negligible role in SPEM development because these mice, without the ability to mount adaptive immune
responses and sustain a chronic inflammatory state, have virtually normal stomachs (Supplementary Figure 8).

Finally, yet another IL33-responsive innate immune cell population (ie, mast cells) has been recently implicated in the development of gastric cancer in gp130F/F mice, specifically through mobilization of macrophages.16 Although similar to early IL33-dependent activation of eosinophils upstream of macrophage recruitment, the precise temporal involvement of mast cells and their interaction(s) with other mucosal populations to promote events leading to intestinal metaplasia and gastric cancer is unclear.

Taken together, the present study underscores a critical role for IL33 in the development of chronic inflammation and metaplasia in the stomach and implicates eosinophils as an early IL33-responsive innate immune cell population that participates in the complex orchestration leading to SPEM/intestinalized SPEM. As such, targeting the IL33/ST2 axis for future therapeutic modalities to interrupt the early initial events leading to gastritis and metaplasia and potentially reversing intestinalized SPEM may prove to be beneficial for the prevention or treatment, or both, of patients susceptible to gastric cancer.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2020.09.040.

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Author names in bold designate shared co-first authorship.

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Correspondence
Address correspondence to: Theresa T. Pizarro, PhD, Department of Pathology, Case Western Reserve University School of Medicine, 2103 Cornell Road, WRB 5534, Cleveland, Ohio 44106. e-mail: theresa.pizarro@case.edu; fax: 216-368-0494.
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CRediT Authorship Contributions
Carlo De Salvo, PhD (Data curation: Lead; Formal analysis: Equal; Funding acquisition: Supporting; Investigation: Supporting; Methodology: Lead; Writing – original draft: Lead). Luca Pastorelli, MD, PhD (Data curation: Supporting; Formal analysis: Equal; Methodology: Equal; Writing – original draft: Equal). Christine P. Petersen, PhD (Data curation: Supporting; Formal analysis: Supporting; Methodology: Supporting). Sara Ormetti, PhD (Data curation: Supporting; Formal analysis: Supporting; Methodology: Supporting). Silvius A. Locovei, MD, PhD (Data curation: Supporting; Formal analysis: Supporting; Methodology: Supporting). Shuvra Ray, MD, PhD (Data curation: Supporting; Formal analysis: Supporting; Methodology: Supporting). Hannah R. Friedman, undergraduate student (Data curation: Supporting; Formal analysis: Supporting; Methodology: Supporting; Project administration: Lead; Writing – review & editing: Lead). Abdullah Osme, MD (Data curation: Supporting; Formal analysis: Supporting; Methodology: Lead). Fabio Cominelli, MD, PhD (Data curation: Supporting; Formal analysis: Supporting; Methodology: Lead; Supervision: Supporting). Ganapati H. Mahabeleshwar, PhD (Data curation: Supporting; Formal analysis: Supporting; Methodology: Supporting). James Goldenring, MD, PhD (Data curation: Supporting; Formal analysis: Supporting; Methodology: Supporting; Project administration: Supporting; Resources: Supporting; Supervision: Supporting; Writing – review & editing: Supporting). Theresa T. Pizarro, PhD (Conceptualization: Lead; Formal analysis: Lead; Funding acquisition: Lead; Investigation: Lead; Project administration: Lead; Resources: Supporting; Supervision: Lead; Visualization: Lead; Writing – review & editing: Lead).

Conflicts of interest
The authors disclose no conflicts.

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Supplementary Figure 1. Evidence of advanced intestinalized SPEM in stomachs of gastritis-prone SAMP mice. (A) Representative histologic image of 4-week-old SAMP shows early hyperproliferation of gastric glands compared with age-matched AKR (left panels), while in 20-week-old SAMP, Alcian blue/PAS staining highlights acidic mucin-secreting cells (arrows) replacing parietal (arrowheads) and chief cells as SPEM progresses, which is absent in age-matched AKR (right panels). Original magnification ×20 ± 1.25; scale bars: 100 μm. (B) Representative immunofluorescent images of full-thickness corpus from 4-week-old SAMP display early, aberrant staining of GSII (green) and Clu (red), characteristic of SPEM (left lower panels) compared with age-matched AKR (left upper panels) that becomes more evident in 20-week-old SAMP with established gastritis, with clear abundance of GSII⁺ cells and increased CD44v and Clu (both red), localizing to base of gastric glands (arrows, right lower panels) compared with age-matched AKR controls (right upper panels). Original magnification ×20; scale bars: 100 μm.
Supplementary Figure 2. Molecular profiling indicates advanced SPEM in SAMP corpus that progresses with age. Relative expression of (A) Gif, Atp4a, and Tff1, (B) Tff2 and Mist1, (C) He4, Clu, Lyz, and Gpx2, and (D) Cfr, Dmbt1, and Etv5 in young SAMP vs SAMP with established disease and vs age-matched AKR controls. Data are expressed as fold-change vs 4-week-old AKR (with mean arbitrarily set as 1) (N = 6-9). mRNA, messenger RNA. *P < .05, **P < .01, ***P < .001 vs age-matched AKR; ##P < .01, ###P < .001 vs 4-week-old AKR/SAMP.
Supplementary Figure 3. Increased circulating levels of IL33 in SAMP mice. Serum levels of total IL33 protein in 4-, 10- and 20-week-old SAMP vs age-matched control AKR (N = 3-6). *P < .05, ***P < .001 vs age-matched AKR; #P < .05 vs 4-week-old SAMP.
Supplementary Figure 4. Gating strategy for distinguishing M1 vs M2 macrophages by flow cytometry. Representative 2-dimensional dot plots (shown here for BM) for SAMP depict, from left-to-right: (1) side scatter (SSC) vs forward scatter (FSC) (gating on general cells), (2) FSC area (FAC-A) vs FSC height (FSC-H) (gating on singlets), (3) SSC area (SSC-A) vs live/dead (gating on live cells), (4) CD11b vs SSC-A (gating on granulocytes), (5) Ly6G vs SSC-A (gating on Ly6G- cells), (6) CD163 vs F4/80 (gating on macrophages, Mph), and (7) tumor necrosis factor (TNF) vs major histocompatibility complex (MHC) II (gating on M1 macrophages), and Arg1 vs CD163 (gating on M2 macrophages).
Supplementary Figure 5. Strong prominence of M2- vs M1-associated gene markers expressed in macrophages from SAMP vs AKR mice. Relative transcript levels of M1- vs M2-associated molecules (defined in Sica and Mantovani, Trends Immunol 2002 DOI: 10.1016/s1471-4906(02)02302-5; and Murray, Immunity 2017 DOI: 10.1146/annurev-physiol-022516-034339) in isolated macrophages from 10-week-old SAMP, normalized by 36B4 and expressed as percentage fold-change of age-matched AKR controls. Data are presented as mean ± SD (N = 6).

Supplementary Figure 6. Gating strategy for detecting eosinophils (EOS) by flow cytometry. Representative 2-dimensional dot plots (shown here for BM) for control AKR depict, from left-to-right: (1) side scatter (SSC) vs forward scatter (FSC) (P1, gating on general cells), (2) FSC area (FSC-A) vs FSC height (FSC-H) (P2, gating on singlets), (3) SSC area (SSC-A) vs live/dead (gating on live cells), and (4) CD11b vs Siglec-F (gating on eosinophils).
Supplementary Figure 7. Eosinophil (EOS) depletion is effective in decreasing peripheral (BM) and local (gastric) eosinophils, and reduces M2 macrophages and expression of M2-associated genes in SAMP stomachs. (A) Frequency of peripheral (BM)-derived eosinophils (left panel) and eosinophil count (right panel), (B) M2 macrophage frequency (left panel), and M2 macrophage count (right panel, defined as IL-33⁺CD163⁺ cells shown in Figure 6A, middle panels) in SAMP corpus after eosinophil depletion by administration of anti-IL5 and anti-CCR3, alone and in combination, vs IgG-treated controls (N = 4-9). (C) Relative transcript levels of M2-associated molecules, normalized by 36B4 and expressed as fold-change vs IgG-treated controls (with mean set arbitrarily as 1) (N = 6). mRNA, messenger RNA. (D) Representative immunohistochemistry images localizing IL33 (N = 4). Original magnification ×10×1.25; scale bars: 100 μm. *P < .05, **P < .01, ***P < .001, ****P < .0001.
Supplementary Figure 8. Evidence that aberrant adaptive immune responses, and not increased ILC2 frequency, is essential for development of gastritis/SPEM in SAMP mice. Corpus tissues were excised from stomachs of SAMP and AKR and processed into single-cell suspensions for flow cytometric analysis of ILC2s using the following gating strategy: (A) live cells were gated on CD45^+^, then on CD127^+^ cells negative for lineage markers CD3 (T cells), CD11c (dendritic cells), B220 (B cells), CD11b (myeloid cells), Ly6g, and Ter-119 (granulocytes), and positive for the transcription factor GATA3, (B) with ILC2s reported as percentages and absolute numbers (N = 3-7). *P < .05, **P < .01. (C) Total inflammation (left panel) and epithelial hyperplasia (right panel) in corpus from SAMP × Rag2^−/−^ mice vs wild type controls (N = 7-15). *P < .05, **P < .01, ***P < .001.