# Gastric Mucosal Immune Profiling and Dysregulation in Idiopathic Gastroparesis

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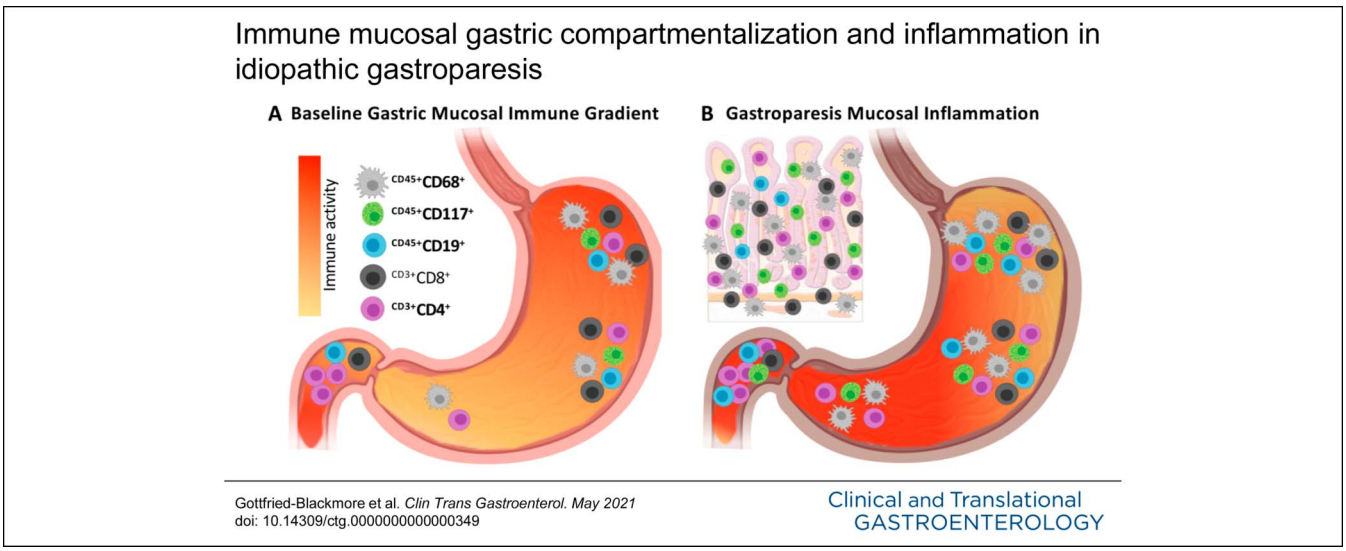
INTRODUCTION: **It is unclear how immune perturbations may influence the pathogenesis of idiopathic gastroparesis, a prevalent functional disorder of the stomach which lacks animal models. Several studies have noted altered immune characteristics in the deep gastric muscle layer associated with gastroparesis, but data are lacking for the mucosal layer, which is endoscopically accessible. We hypothesized that immune dysregulation is present in the gastroduodenal mucosa in idiopathic gastroparesis and that specific immune profiles are associated with gastroparesis clinical parameters.**

METHODS: **In this cross-sectional prospective case-control study, routine endoscopic biopsies were used for comprehensive immune profiling by flow cytometry, multicytokine array, and gene expression in 3 segments of the stomach and the duodenal bulb. Associations of immune endpoints with clinical parameters of gastroparesis were also explored.**

RESULTS: **The gastric mucosa displayed large regional variation of distinct immune profiles. Furthermore, several-fold increases in innate and adaptive immune cells were found in gastroparesis. Various immune cell types showed positive correlations with duration of disease, proton pump inhibitor dosing, and delayed gastric emptying.**

DISCUSSION: **This initial observational study showed immune compartmentalization of the human stomach mucosa and significant immune dysregulation at the level of leukocyte infiltration in idiopathic gastroparesis patients that extends to the duodenum. Select immune cells, such as macrophages, may correlate with clinicopathological traits of gastroparesis. This work supports further mucosal studies to advance our understanding of gastroparesis pathophysiology.**

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## INTRODUCTION

Gastroparesis is a functional motility disorder of gut-brain interaction associated with delayed gastric emptying in the absence of a gastric outlet obstruction and characterized by recurrent nausea, vomiting, abdominal pain, fullness, bloating, and early satiety. The most common etiology is idiopathic, followed by diabetes, postsurgical or postinfectious. Although it is considered a neuromuscular disorder of the stomach (1), questions remain about the causes, extent of organ involvement, and pathophysiology of idiopathic gastroparesis.

Immune dysregulation has been implicated in gastroparesis pathophysiology by direct or indirect association to abnormal function of the enteric nervous system (ENS) (2,3), pacemaker cells (4,5), or smooth muscle (6). In particular, studies have focused on the stomach muscularis propria, reporting various abnormalities such as increased oxidative stress with inflammatory cytokine burden (7); loss of c-Kit staining in interstitial cells of Cajal (ICC) that correlates with loss of anti-inflammatory muscularis macrophages (8,9); increased leukocyte infiltration in the myenteric plexus (5,10); increased cytokines (5,11); and broad changes in immune gene expression (12,13). However, the impact of immune dysregulation on gastroparesis symptoms and gastric emptying remains to be explained, and data are lacking for immune perturbations in the gastric mucosa.

The mucosal immune system, in direct contact with the gut lumen, can regulate every aspect of gastrointestinal function through its impact on epithelial barrier function, enteric nervous system modulation, blood flow, nociceptor thresholds, smooth muscle contractility, and gut-brain communication (rev. (14,15)). In fact, mucosal inflammation is implicated in visceral hypersensitivity and the generation of symptoms in irritable bowel syndrome and functional dyspepsia, the 2 most common disorders of gut-brain interaction (14,16–19). These studies have detailed increased numbers of mucosal inflammatory cells such as mast cells (17,20,21), eosinophils (21–24), and lymphocytes (21,25), as well as differentially expressed immune genes (26,27). It is unclear, however, whether mucosal immune dysregulation occurs and/or contributes to clinical symptoms and dysmotility in gastroparesis. We hypothesized that immune dysregulation is present in the gastric mucosa in idiopathic gastroparesis and that specific immune profiles are associated with gastroparesis clinical parameters. To address this hypothesis, upper endoscopy mucosal biopsies were used for gene expression, cytokine array, and flow cytometry immune profiling.

## METHODS

### Study cohort and sample collection

Adult subjects (age 18–65 years) seen at the Stanford Digestive Health Center with a diagnosis of idiopathic gastroparesis based on established guidelines (28) and previous gastric scintigraphy showing delayed emptying at 2 hours and/or 4 hours (29). Since there is significant overlap between idiopathic gastroparesis and functional dyspepsia (30), Rome IV criteria (31) were used to assess for functional dyspepsia. For purposes of this study, patients who met Rome IV criteria for functional dyspepsia and had normal gastric scintigraphy were categorized as functional dyspepsia, and those with delayed gastric emptying were characterized as idiopathic gastroparesis. Conversely, all gastroparesis subjects met Rome IV criteria for postprandial distress syndrome, and 73% (11/15) met criteria for epigastric pain syndrome, an overlap that has been reported before (32). Control subjects were patients with none of the cardinal symptoms of gastroparesis or dyspepsia, who presented for routine esophagogastroduodenoscopy (EGD) for conditions including Barrett's esophagus surveillance, iron deficiency, gastroesophageal reflux follow-up, prebariatric surgery evaluation, esophageal stricture, and heartburn. Subjects were excluded if they had diabetes, active *Helicobacter pylori*, active peptic ulcer disease, concomitant inflammatory/autoimmune disorder, active nonsteroidal anti-inflammatory drug use, or if they had a history of gastric surgery or gastric electric stimulator placement. Demographics and clinical information can be found in Table 1. Routine cold-forceps biopsies were randomly taken by trained endoscopists from the duodenal bulb, antrum, body, and fundus (see Figure 1A, Supplementary Digital Content 1, http://links.lww.com/CTG/A601). In some cases, biopsy data were not available for all the analyses; n for each experiment has been indicated in all figure legends. Gastroparesis symptoms were assessed using the gastroparesis cardinal symptom index daily diary (GCSI-dd) (33). Control subjects were queried for abdominal pain on a 0–5 scale in the 2–4 weeks preceding their endoscopy. All tissue collections, processing, and data collection were conducted between June 2017 and October 2019 at Stanford University.

### NanoString gene expression array

RNA was obtained from flash-frozen (−80 °C) EGD samples stored in RNAlater (Sigma) using the RNAmini with on-column DNA digestion (Qiagen) following manufacturer's instructions. RNA (250 ng, RIN >7.0) was submitted to NanoString for determination of transcript counts. Gene expression scores were obtained from a predefined subset of 288 immune-associated genes (see Table 1, Supplementary Digital Content 2, http://links.lww.com/CTG/A602) from the NanoString "Human Neuropathology" gene expression panel (Neuroinflammation panel was not available at the time of this study). Data were analyzed using nSolver software following manufacturer instructions.

### Luminex cytokine array

Plasma was obtained after a 10-minute centrifugation (800*g*) of fasting venous blood in heparin-sulfate collection tubes. For tissue protein extracts, flash-frozen endoscopy biopsy samples stored at −80 °C were thawed in NP-40 lysis buffer (Invitrogen) supplemented with protease and phosphatase inhibitors (cOmplete-Mini, Roche; Halt, Thermo), homogenized on ice (Tissue Master 125, Omni), centrifuged at 1000*g*, and supernatants aliquoted and frozen until further use. Bicinchoninic acid (BCA) protein quantification was performed (Thermo). Cytokine measurements in plasma and tissue protein extracts were obtained using the Human 62-multiplex array on the

**Table 1. Subject demographics**

| | Control | Idiopathic gastroparesis | | Functional dyspepsia | |
|---|---|---|---|---|---|
| n | 10 (range) | 15 (range) | | 5 (range) | |
| Median age | 57 (26–70) | 34 (22–59) | $P = 0.011$[a] | 43 (28–53) | ns[a] |
| Female | 53% | 80% | ns[b] | 80% | ns[b] |
| White race | 73% | 80% | ns[b] | 60% | ns[b] |
| BMI | 27.8 (19–49) | 22.3 (18–30) | ns[a] | 22.6 (21–29) | ns[a] |
| A1C | 5.1 (5.0–5.9) | 5.3 (4.7–5.5) | ns[a] | 5.0 (5.0–5.3) | ns[a] |
| On acid suppression therapy | 42% | 60% | ns[b] | 20% | ns[a] |
| Endoscopic gastritis[c] | 0.2 (0–1) | 0.2 (0–1) | ns[a] | 0.4 (0–1) | ns[a] |
| Abd pain[d] | 0.4 (0–1) | 3.5 (0.1–4.2) | $P < 0.0001$[a] | 1.4 (0.8–2.5) | $P = 0.018$[a] |
| Median 2-hr, 4-hr retention[e] | — | 53% (92–23) | | 47% (69–2) | |
| | — | 29% (61–9) | | 4% (9–2) | |
| GEBT $T^{1/2}$ (min) | — | 156 (205–59) | | 92 (103–54) | |

A1C, hemoglobin A1C; Abd, abdominal; BMI, body mass index; GEBT, gastric emptying breath test.
[a]False discovery rate–adjusted *P* value for multiple comparisons after paired 1-way ANOVA.
[b]$\chi^2$ test vs control group.
[c]2- to 4-hour solid meal gastric scintigraphy; gastric emptying breath test.
[d]Relative gastritis scale based on endoscopy and pathology findings: 0—normal; 1—erythema/gastropathy/mild gastritis; and 2—erosions/ulcers/active gastritis.
[e]Abd pain: scale as in gastroparesis cardinal symptom index daily diary from 0 to 5.

Luminex 200 IS system (Affymetrix) performed at the Stanford Human Immune Monitoring Core as previously described (34). Briefly, equal protein concentration of samples was tested in duplicate wells, and matched sets of cases and controls were always mixed in all plates to reduce confounding case status with plate artifacts. Few samples suffered from selective cytokine bead aggregation, leading to exclusion of these cytokine/sample pairs from the analysis. Median fluorescence intensity data were preprocessed for each cytokine through a sequence of averaging over duplicate wells, natural-logarithm transformation to reduce variance heterogeneity, and isolation and removal of plate effects as previously reported (34).

### Histological analyses

For this analysis, 2 gastroparesis samples and 4 controls with available tissue for histology were complemented with retrospectively collected and stored hematoxylin and eosin (H&E)-stained slides from 7 idiopathic gastroparesis subjects and 4 controls for analysis. For total leukocyte estimation, 4 high power fields (HPFs) (0.25 $mm^2$) were counted in a blinded fashion on H&E slides by a trained gastrointestinal pathologist and plotted as cells per HPF or cells/$mm^2$ (1 HPF = 0.25 $mm^2$). Immunohistochemistry was performed with antibodies to $CD117^+$ (Ventana, clone 9.7) using a standard autostainer according to manufacturer instructions (Ventana BenchMark ULTRA).

### Multicolor flow cytometry

Cell suspensions were obtained from fresh, weighed, upper endoscopy biopsy samples by type IV collagenase digestion and divided into 2 equal volumes for control and stimulation (Lipopolysaccharide (LPS) 1 μg/mL + phorbol myristate acetate (PMA) 50 ng/mL + ionomycin 1 μg/mL) before surface staining and subsequent intracellular cytokine staining as previously described (35). Fluorescence-conjugated antibodies are in Supplemental Table 2, Supplementary Digital Content 2, http://links.lww.com/CTG/A602. Nonviable cells were excluded using Zombie Aqua Fixable Viability Kit (BioLegend, San Diego, CA). Data were acquired on an LSRII or Fortessa cytometer (BD Biosciences) and analyzed with FlowJo software (Becton, Dickinson, and Company, Ashland, OR). Gating of putative major leukocyte populations (see Table 3, Supplementary Digital Content 2, http://links.lww.com/CTG/A602) is provided in Supplemental Figure 1B–C, Supplementary Digital Content 1, http://links.lww.com/CTG/A601. Total immune cell counts were log 10 transformed to reduce variance heterogeneity and normalized to total live cells to generate a measure of cell infiltration.

### Statistics

Data were analyzed using Prism 7.0 software (GraphPad, San Diego, CA). Flow cytometry, cytokine, and gene expression data were analyzed by 2-way ANOVA (diagnosis and anatomical location) and corrected for multiple comparisons (cell subsets, cytokines, etc.) by controlling the false discovery rate (FDR) by Benjamini, Krieger, and Yekutieli procedure, and adjusted $P < 0.05$ was considered significant. Pearson correlations and linear regressions (2-tailed) were performed to assess the relationship of immune endpoints to gastroparesis clinical characteristics. Spearman correlations were performed if data did not pass the D'Agostino and Pearson normality test. A 2-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli with Q = 5% was used to estimate the number of true null hypotheses for the various Pearson correlations analyzed (n = 32). A threshold of $P < 0.0015$ was considered significant in the gastric emptying comparison and $P < 0.0013$ for the GCSI-dd symptom comparison.

### Study approval

This observational study was conducted according to the Declaration of Helsinki, in accordance with good clinical practice

guidelines, and approved by Stanford's Institutional Review Board (41569). Written informed consent was received from participants before inclusion in the study. Patients or the public were involved in the dissemination and enrollment of our study, but they were not involved in the design, conduct, or reporting of our research.

## RESULTS

### Description of study cohort

The idiopathic gastroparesis cohort in this study comprised subjects involved in our previous study with vagal nerve stimulation (36) at their baseline before any intervention. Briefly, they were mostly white women (80%) with normal body mass index (BMI) and A1C (Table 1). All had delayed gastric emptying on scintigraphy (median retention at 4 hours, 29%, range 61%–9%), and 60% were on acid suppression therapy. The control group was older (median age 57 vs 34 years), 53% female, and slightly overweight (median BMI 28 vs 22), but otherwise had similar demographic and baseline characteristics. The small cohort of functional dyspepsia patients was well matched in age, race, BMI, and symptoms to the gastroparesis cohort, although had normal gastric emptying on scintigraphy (median retention at 4 hours 4%, range 9%–2%).

### Immune profiling in nongastroparesis controls reveals a distinct gastric mucosal gradient

The human gastric mucosa is compartmentalized into distinct functional regions, i.e., acid vs mucus production, etc.; however, little is known about its immune composition. We first compared the mucosal immune compartment in control subjects across the major anatomical segments of the stomach and duodenal bulb using a combination of gene expression, cytokine array, and flow cytometry (see Figure 1A, Supplementary Digital Content 1, http://links.lww.com/CTG/A601). Immune-associated transcripts from a NanoString expression panel distinguished the proximal stomach (fundus + body) from the antrum and duodenum by unsupervised hierarchical clustering and principal component analysis (Figure 1a). The major pathways differentiating the proximal stomach from the antrum/duodenum included adaptive immune response, neutrophil degranulation, antimicrobial peptides, infectious disease, signaling by vascular endothelial growth factor (high in the duodenum and antrum), and extracellular matrix organization, endothelial nitric oxide synthase activation, unfolded protein response, and signaling by transforming growth factor (TGF)-β (high in the proximal stomach) (Figure 1b). Cytokine Luminex array in control subjects showed higher duodenal cytokine protein levels (C-X-C motif ligand (CXCL) 1, interleukin (IL)-12p40, IL-2, IL-17α, platelet-derived growth factor (PDGF)-β, and brain-derived neurotrophic factor) (Figure 1c), which was consistent with higher levels of $CD4^+$ T cells in this location by flow cytometry (Figure 1d). Select cytokines were more abundant in the proximal stomach (IL-31, TGFα, leptin, and chemokine ligand (CCL)-3) or showed an increased trend (FAS-ligand, IL-6, and IL-17 F) (Figure 1c). Flow cytometry showed that the gastric fundus and body had comparable total leukocyte densities to the duodenum and the highest density of $CD8^+$ T cells and macrophages (Figure 1d). There was overall good concordance of cell densities between the various gastric compartments and duodenal bulb (Figure 1e), except for $CD206^+$ macrophages in the fundus and the antrum/duodenum. In summary, the human proximal and distal stomach are quite distinct in their immune composition.

### Gastroparesis is characterized by dysregulated mucosal immune profiles

When compared at the gene expression level by NanoString, gastroparesis subjects maintained the same anatomical relationships as controls (Figure 2a) and had a small, but significant increase in duodenal global immune gene expression (Figure 2b). However, no individual genes reached statistical significance after controlling for multiple comparisons (data not shown). Luminex cytokine array of whole tissue lysates showed modest (1.3–1.1 fold), but significant, increases in select cytokines in the antrum (CXCL1, IL17F, resistin, and IL18) (Figure 2c). IL-31, a Th2 cytokine, was significantly reduced in the fundus (Figure 2c). These results showing increased cytokines in the antrum were supported by retrospective histologic analysis of a small subset of biopsies showing increased leukocytes and mast cells in the antrum, broadly distributed along the glands and pits of the lamina propria (Figure 2d–f).

Given that $CD45^+$ leukocytes only comprised a small fraction of total biopsy cells (3%–17%), biopsy bulk RNA/cytokine analysis may not be sensitive enough to detect immune cell-specific changes. To circumvent this, we leveraged our expertise in multicolor flow cytometry for single cell analysis. A smaller cohort of functional dyspepsia patients was included as an additional control of patients with gastroparesis-like symptoms, but normal gastric emptying (Table 1). Gastroparesis was associated with significantly increased mucosal $CD45^+$ leukocytes in all 3 major compartments of the stomach, with a similar trend in the duodenal bulb (Figure 3). All cell types examined were elevated except $CD8^+$ T cells and B cells (Figure 3). Increased immune gastric infiltrates were specific to idiopathic gastroparesis and not observed in the small functional dyspepsia group (Figure 3). These results show a significant and broad infiltration of innate and adaptive immune cells in the gastric mucosa occurring in gastroparesis, with the largest fold change occurring in the antrum.

Myeloid cells and B cells were stained with IL1α, TNFα, and TGFβ and lymphocytes with TNFα, IFNγ, IL-5, and IL-17 to assess inflammatory vs anti-inflammatory phenotypes. No change was seen for frequency of $TGF\beta^+$ or $TNF\alpha^+$ cells (see Figure 2A–B, Supplementary Digital Content 1, http://links.lww.com/CTG/A601), but there was a trend toward increased IL1α staining in $CD206^+$ macrophages (see Figure 2C, Supplementary Digital Content 1, http://links.lww.com/CTG/A601). Frequencies of major subsets of $CD4^+$ and $CD8^+$ T cells and regulatory $CD4^+$ T cells (see Figure 2D–E, Supplementary Digital Content 1, http://links.lww.com/CTG/A601) were similar between controls and gastroparesis. Given that total cell infiltration was greater for most leukocyte subsets (Figure 3), the number of $cytokine^+$ cells was higher in gastroparesis samples (not shown).

Finally, the impact of clinical parameters on leukocyte infiltration was analyzed. Total leukocytes and mast cells in the gastric antrum were positively correlated with duration of disease (Figure 4a). Other cell types did not show this correlation (not shown). Although there was no difference in acid suppression therapy use between groups (Table 1), the gastroparesis cohort showed a significant correlation between proton pump inhibitor (PPI) dosing and gastric, but not duodenal, myeloid cell infiltrates (Figure 4b). This was not the case for lymphocyte populations studied (not shown). Of note, no PPI correlations were found in

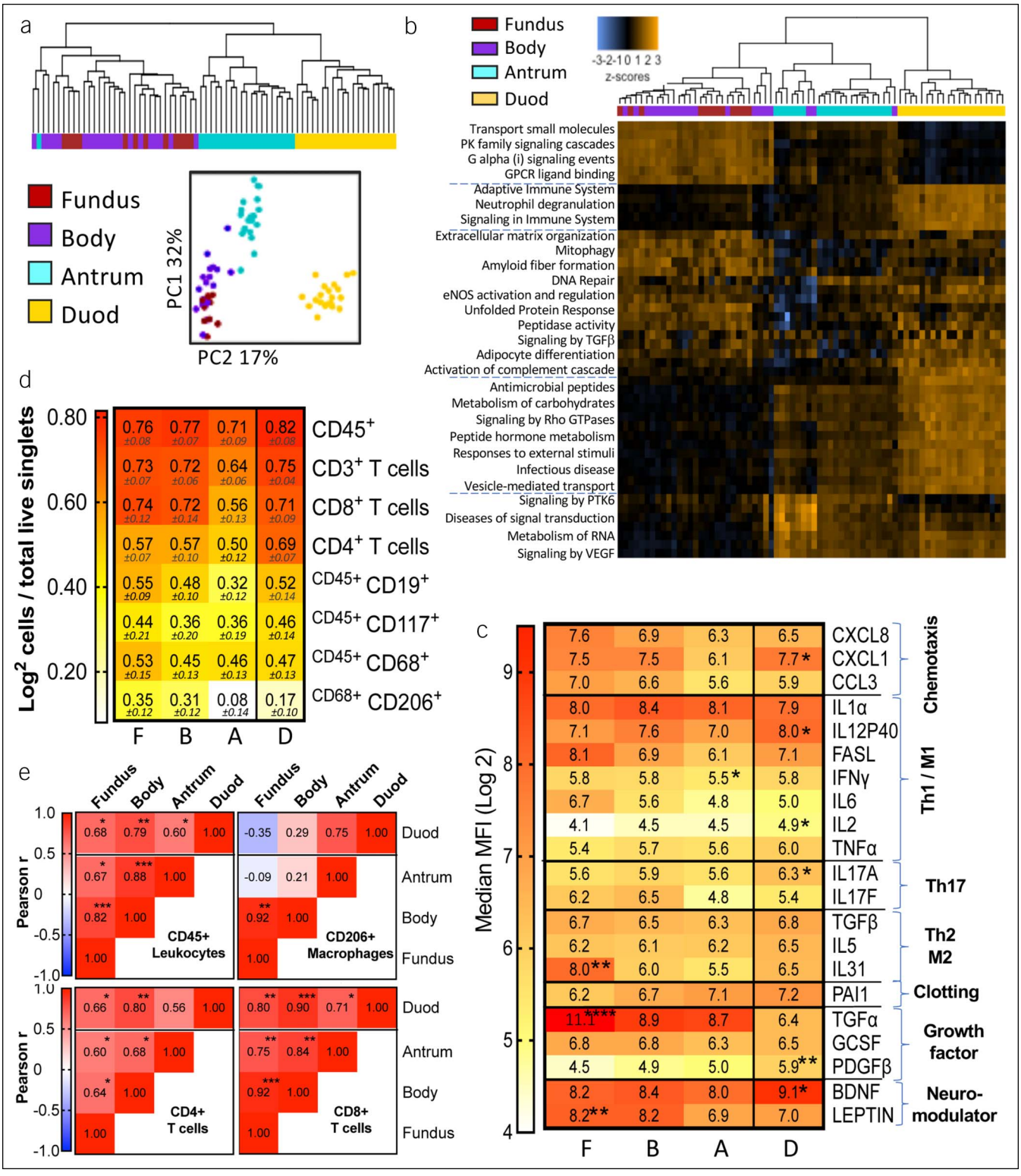


**Figure 1.** Immune profiling reveals a distinct gastric mucosal gradient. (**a**) (Top) Unsupervised hierarchical clustering by NanoString gene expression of 288 immune-related genes from each anatomical site (n = 7 for fundus; n = 10 for the body, antrum, and duodenum [Duod]). (Bottom) Principal component (PC) analysis for anatomy, showing distinct segregation of gastric compartments and duodenum. (**b**) Hierarchical clustering analysis of NanoString immune-related pathways generated by nSolver analysis software. (**c**) Heatmap showing median mucosal tissue cytokine levels in controls (n = 10 per group) for representative differentially abundant cytokines. * = $P < 0.05$, ** = $P < 0.01$, and **** = $P < 0.0001$ (FDR-adjusted $P$ values for multiple comparisons after 2-way ANOVA). (**d**) Heatmap showing median mucosal total immune cells by flow cytometry normalized to total live cells, with SD below in italics (n = 7) in each site. (**e**) Concordance of leukocyte populations across the various biopsy sites. * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$ (2-tailed $P$ value by Pearson correlation). F, fundus; FDR, false discovery rate; B, body; A, antrum; D, duodenum.

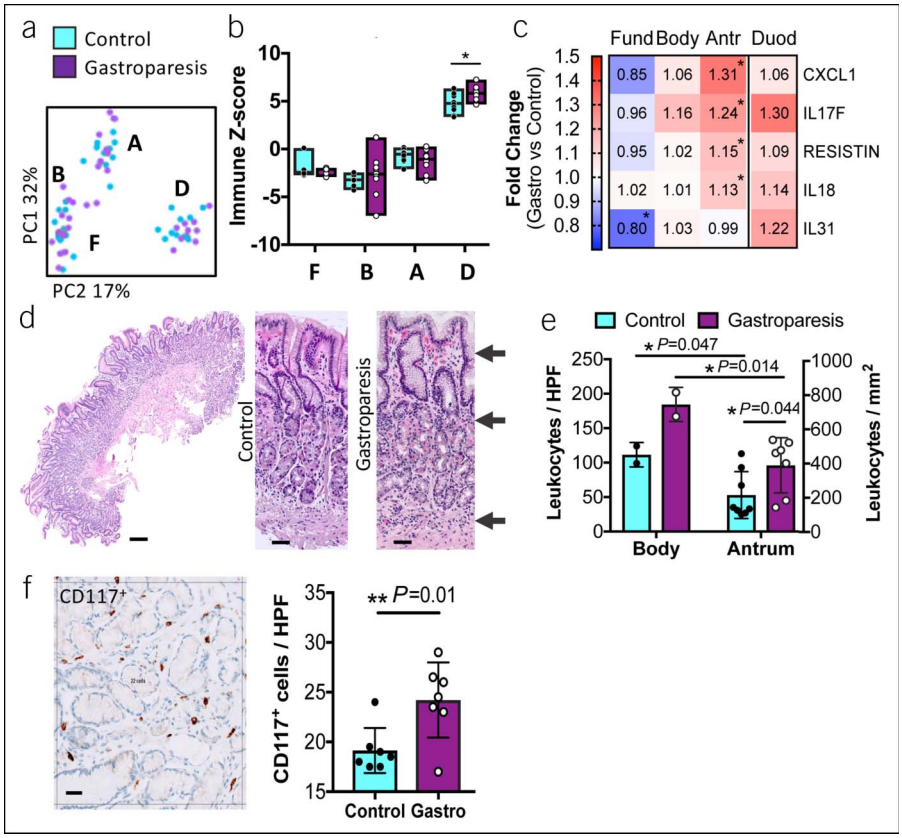


**Figure 2.** Targeted immune profiling reveals increased immune activity in idiopathic gastroparesis. (**a**) Principal component (PC) analysis for controls and gastroparesis from NanoString gene expression of 288 immune-related genes. (**b**) Calculated immune Z-scores based on global immune gene expression for each site, n = 7 for F; n = 10 for B, A, D. * = $P < 0.05$ (Bonferroni post hoc–adjusted $P$ values for multiple comparisons after 2-way ANOVA). (**c**) Heatmap showing median fold change in tissue cytokine levels (log2 Median fluorescence intensity) (n = 10 controls and n = 6–15 in the gastroparesis group). * = $P < 0.05$ (FDR-adjusted $P$ values for multiple comparisons after 2-way ANOVA). (**d**) (Left) Representative gastric biopsy on H&E staining to denote tissue depth (bar = 200 μm) spanning mostly mucosa and a small amount of muscularis mucosae and submucosa. (Right) H&E representative gastric biopsy (antrum) with arrows pointing to lamina propria glands and pits (bar = 50 μm; n = 8–7 per group). (**e**) Leukocyte counts per 4 high power fields (HPF = 0.25 $mm^2$) from biopsies of gastroparesis subjects and controls (n = 2 and 9 in the body and antrum, respectively). FDR-adjusted $P$ values for multiple comparisons after 2-way ANOVA. (**f**) (Left) CD117 immunoperoxidase representative staining and (right) cell counts by HPF in the antrum (bar = 15 μm; n = 7 per group). The unpaired Student $t$ test. A, antrum/Antr; B, body; D, duodenum/Duod; F, fundus/fund; FDR, false discovery rate; H&E, hematoxylin and eosin.

the control group (Figure 4b). During endoscopy, some gastroparesis subjects had bile/food stasis noted, and/or erythema, and/or reported antral gastropathy or chronic gastritis on biopsy pathology report. None of these endoscopic findings (pooled) was associated with higher leukocyte or lymphocyte counts (Figure 4c). Finally, although the control group was significantly older (Table 1), age did not correlate with leukocyte numbers in controls or gastroparesis (Figure 4d). That disease duration and PPI dosing correlate with leukocyte infiltration in the stomach is intriguing and requires further studies to assess causality.

### Gastric macrophages and CD8⁺ T cells may correlate with gastric emptying delay, but not with gastroparesis symptoms

From among the various immune cells studied, $CD45^+CD68^+$ macrophages, $CD8^+$ T cells, and B cells showed associations with gastric emptying when using percentage retention at 4 hours (Figure 5a,b), and a similar trend when using emptying half time ($T^{1/2}$ minutes, not shown). Gastric macrophages and $CD8^+$ T cells, specifically $IFN\gamma^+$ $CD8^+$ T cells, were positively correlated with gastric emptying delay, while duodenal B cells were inversely correlated. Cytokine positive macrophages (IL1α, TGFβ, or TNFα) did not show any correlations with gastric emptying (data not shown). Of note, only duodenal B cells (Figure 5) passed FDR criteria for significance after adjusting for multiple comparisons.

The aggregate GCSI-dd measures a composite of abdominal fullness, satiety, nausea, vomiting, bloating, and pain. Of the immune cells measured, only $CD3^+$ gastric lymphocytes and $CD8^+$ T cells showed a moderate negative correlation with the GCSI-dd fullness/satiety subscale (not passing FDR) with similar trends in the other symptom subscales (Figure 5c). When the 5 functional dyspepsia subjects with GCSI-dd scoring were added to the analysis, no significant correlations were found between GCSI-dd scores and leukocyte infiltration (not shown). These results suggest that mucosal immune cell infiltration per se does not correlate with symptom severity in gastroparesis, as has been reported for myenteric plexus $CD45^+$ cell counts (5).

### Idiopathic gastroparesis is not associated with markers of systemic inflammation, but with select increased plasma cytokines

To exclude systemic inflammation as a potential contributor of mucosal inflammation in our gastroparesis cohort, common

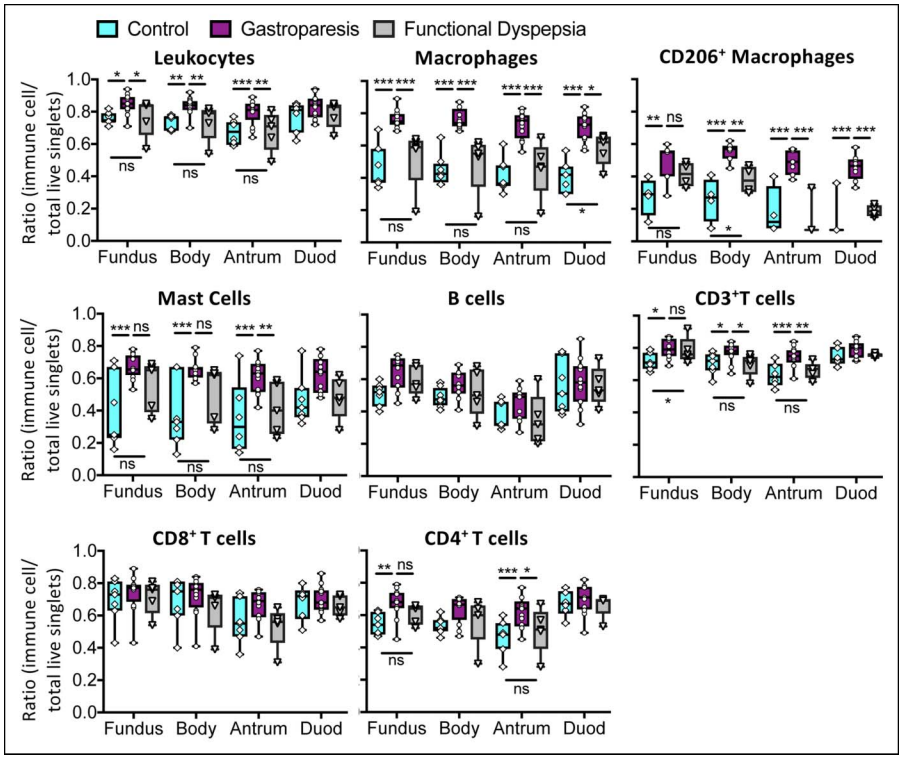


**Figure 3.** Flow cytometry shows distinct mucosal inflammation in idiopathic gastroparesis. Flow cytometry cell counts and total viable singlets were Log10 converted and then ratio taken between them. n = 7 controls, 13 GP, 5 FD. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (false discovery rate–adjusted $P$ values for multiple comparisons after 2-way ANOVA). Duod, duodenum, GP, gastroparesis.

blood inflammatory markers were evaluated. Complete blood counts and differential, C-reactive protein, erythrocyte sedimentation rate, and liver function tests were all within normal range in our gastroparesis cohort (see Figure 3, Supplementary Digital Content 1, http://links.lww.com/CTG/A601). The sole exception being one subject with a transaminitis (see Figure 3F, Supplementary Digital Content 1, http://links.lww.com/CTG/A601) secondary to ongoing alcohol abuse, who was excluded from our flow cytometry, cytokine, and NanoString immune analyses. Although within the normal range, it was of interest to note that erythrocyte sedimentation rate and C-reactive protein were moderately correlated with gastric $CD4^+$ T cells expressing TNF (see Figure 3C, Supplementary Digital Content 1, http://links.lww.com/CTG/A601).

When analyzing plasma cytokine levels in the same gastroparesis cohort compared with nongastroparesis controls, significant upregulation of 10 plasma cytokines was noted (resistin, IL2, EGF, IL-5, CCL2, IL7, IL1RA, PDGFβ, TGFβ, and LIF) (Figure 6a). Several of these cytokines and chemokines showed a positive correlation with gastric emptying delay (Figure 6b), but did not meet FDR criteria ($P < 0.0007$). Cytokines did not correlate with gastroparesis symptoms (not shown).

Altogether, these results show that our cohort of idiopathic gastroparesis does not have elevated markers of systemic inflammation, despite having increased mucosal immune infiltrates and small, yet significant, increases in plasma cytokines. How these circulating cytokines may affect gastroparesis remains to be studied.

## DISCUSSION

This study suggests that idiopathic gastroparesis is associated with mucosal immune dysregulation, predominantly reflected by increased leukocyte infiltration in the lamina propria (graphical abstract). As with previous human gastroparesis studies (11–13,37), there was not a cohesive result when analyzing gene expression pathways or cytokine arrays. This highlights the limitations of bulk RNA/proteomic analyses, which are unable to detect changes in key minority gut cell populations such as immune cells. This limitation was overcome by complementing this study with a detailed flow cytometry analysis.

Immune infiltration correlated with disease duration and PPI dosage, but was independent of age or findings at the time of endoscopy. Chronic treatment with PPIs has been reported to promote leukocyte infiltration through increased gastrin signaling (38,39), but it is unclear why we only observed a positive association between PPI dose and leukocyte infiltration in the gastroparesis cohort and not in controls. Furthermore, all gastroparesis subjects had increased immune cell counts compared with controls despite PPI use.

Gastric stasis may be an additional factor because reduced immune infiltration occurred in control and functional dyspepsia subjects with normal gastric emptying. Also, the greatest increase of immune cell infiltration was observed in the distal stomach, where enhanced antral stasis has been noted in gastroparesis (40). Furthermore, our results suggest gastric delay may be correlated with both macrophage and $CD8^+$ T-cell infiltration. A major question remaining for future studies is whether mucosal immune dysregulation is a passive consequence of delayed gastric emptying or an active contributor to gastroparesis pathophysiology. These results suggest immune changes in the gastric mucosa may reflect immune dysregulation in the deeper muscle layers of gastroparesis subjects. Mouse models have demonstrated that luminal/mucosal events such as microbiota changes

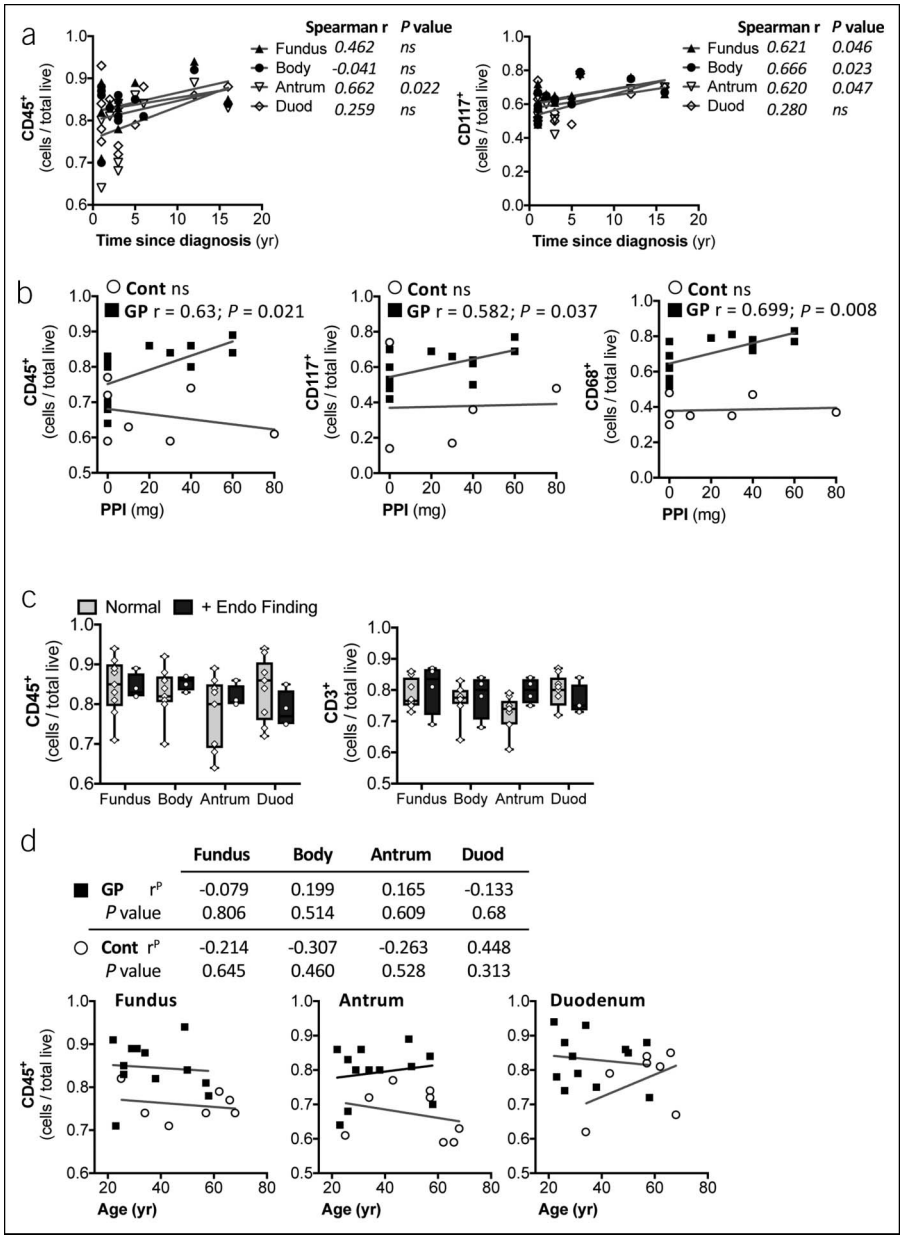


**Figure 4.** Antral mucosal leukocytes correlate with duration of disease and PPI use, but not by age or endoscopic findings. (**a**) Linear regressions and Spearman correlations between $CD45^+$ (left) and mast (right) cells in each compartment and years since diagnosis in gastroparesis subjects. (**b**) Linear regression and Pearson correlations between various myeloid antral cells and PPI dosing in controls and GP (2-tailed *P* value). (**c**) Major immune cell types per anatomic location in gastroparesis cohort grouped by endoscopic findings (as described in text). (**d**) Table (top) and representative graphs (bottom) of linear regressions and Pearson correlations between $CD45^+$ leukocytes and age in controls and GP. n = 7 controls, 13 GP. Duod, duodenum; GP, gastroparesis; ns, nonsignificant; PPI, protein pump inhibitor.

and/or gastroenteritis impact gut motility/peristalsis through a gut-brain-gut circuit that involves vagal sensory neurons, sympathetic motor neurons, and adrenergic receptor signaling in muscularis macrophages (41,42). Furthermore, in models of mucosal inflammation such as TNBS-induced colitis, activation of lamina propria immune cells has been associated with both loss of anti-inflammatory macrophage phenotypes (reviewed in reference (43)) and muscularis macrophage activation/infiltration of myenteric ganglia with impaired ICC-myenteric contractility (44). Therefore, mucosal immune activation can affect the function of deeper muscularis macrophages with consequent dysmotility. These potential mechanisms need to be further validated prospectively in animal models. Further studies are needed to explore whether gastric mucosal immune dysregulation affects the gut-brain-gut motility axis and how it may play a role in gastroparesis. Particularly as immune dysregulation in gastroparesis is evident (11–13,37), but not yet clear, with no cohesive model yet proposed in humans (1). Examples of future studies addressing these questions would include evaluating the function of immune cells infiltrating the gastric mucosa, studying the impact of inflammatory cytokines on stomach vagal and ENS signaling, and assessment of how mucosal immune dysregulation modulates key cell types in the myenteric layer regulating gastric motility.

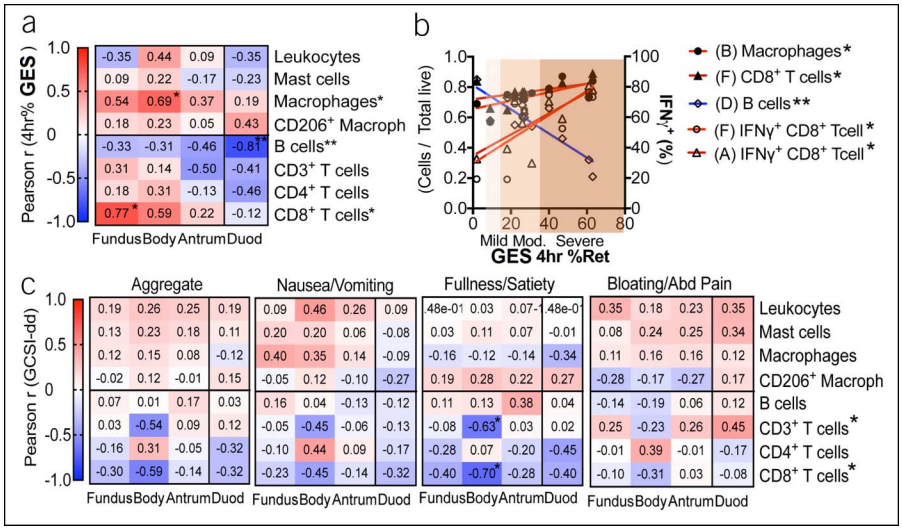


**Figure 5.** Gastric delay positively correlates with stomach, but not duodenal, immune cells nor with gastroparesis cardinal symptoms. (**a**) Heatmap showing Pearson correlation coefficients between distinct immune cells types (rows) and gastric delay by 4 hour % retention in gastric emptying scintigraphy (GES), grouped by anatomic location (columns). n = 10 per site. (**b**) Representative linear regressions for cell types (left axis) or % of IFNγ+ cells (right axis) with significant Pearson correlations * = $P < 0.05$, ** = $P < 0.01$ (2-tailed $P$ value). n = 10 per site. (**c**) Heatmap showing Pearson correlation coefficients between distinct immune cells types (rows) and gastroparesis cardinal symptom index daily diary (GCSI-dd) scores (aggregate, left) and subscores (middle/right), grouped by anatomic location (columns). n = 11 per site. * = $P < 0.05$ (2-tailed $P$ value). A, antrum; B, body; D, duodenum/Duod; F, fundus.

Abnormalities in muscularis macrophages have been reported in gastroparesis (9), and here, we report mucosal macrophages may correlate positively with gastric delay. We further found that idiopathic gastroparesis is associated with an increase in mucosal CD206$^+$macrophages. This is in contrast to the reported loss (9) or lack of change (8) in CD206 staining by histological analysis of muscularis macrophages in gastroparesis or diabetic mouse models. In fact, our results show that besides being increased in gastroparesis, mucosal CD206$^+$ macrophages may have higher levels of IL-1α, suggesting they are more reactive/inflammatory. This is in agreement with data reported recently by human gastric muscularis tissue transcriptomic profiling (12) and proteomics analysis (37), where transcripts associated with M1 macrophage phenotype were increased in idiopathic gastroparesis, and a reduction in anti-inflammatory M2 phenotype-promoting proteins was associated with both diabetic and idiopathic gastroparesis.

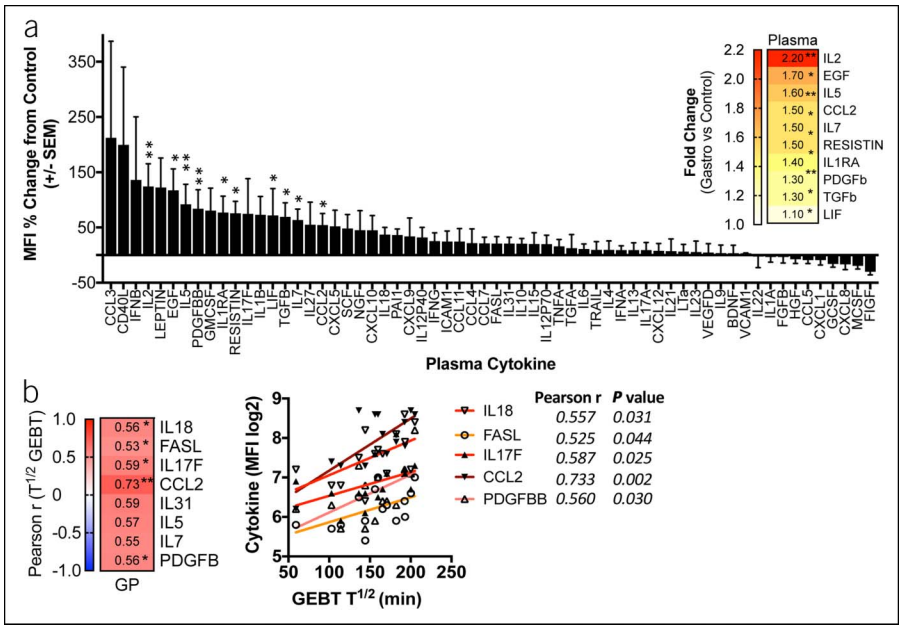


**Figure 6.** Specific circulating cytokines are increased in idiopathic gastroparesis, and associated with gastric delay. (**a**) Bar plot showing % change from median control levels of plasma cytokines by Luminex. Insert heatmap (right) showing fold change levels for significantly increased cytokines. n = 15 per group. * = $P < 0.05$, ** = $P < 0.01$ (FDR-adjusted $P$ values for multiple comparisons after 2-way ANOVA). (**b**) Heatmap (left) and linear regressions (right) showing Pearson correlation coefficients between distinct plasma cytokines (rows) and gastric delay half time ($T^{1/2}$) by the gastric emptying breath test (GEBT). n = 15. * = $P < 0.05$, ** = $P < 0.01$ (2-tailed $P$ value). FDR, false discovery rate.

The dichotomy between CD206 expression and inflammatory phenotype may be due to the species, type, and location of macrophage in question. Generalization of the phagocytic mannose receptor CD206 being associated with an anti-inflammatory phenotype in macrophages is still debated (45). In mice, muscularis macrophages express high levels of CD206, but not lamina propria macrophages (46), and this indeed correlates with an anti-inflammatory phenotype. However, in humans, both lamina propria macrophages and muscularis macrophages have high levels of CD206, which are associated with muted responses to Toll-like receptor ligands (47). Extrapolating function between lamina propria macrophages and muscularis macrophages is not appropriate because these 2 macrophage populations have very distinct transcriptional programs (46–48). Our results, however, support the notion of altered macrophage function as a cardinal finding in gastroparesis.

The relationship between gastroparesis symptoms and leukocyte infiltration is less clear, and symptoms have not been found to correlate well with immune cell infiltration (5). This study was not powered to adequately address this question. Activated and/or increased lymphocytes, mast cells and/or macrophages, acting through pain neuropeptides (i.e., substance P) and neurotransmitters (i.e., serotonin), likely contribute to visceral hypersensitivity as suggested in studies of functional dyspepsia, irritable bowel syndrome, and inflammatory bowel disease (19,21,25). Therefore, future studies on downstream inflammatory neuropeptides and neurotransmitters known to modulate visceral sensation may be revealing.

Our results contrast those by Salicru et al., who showed no difference between controls and gastroparesis in prevalence of gastritis (6%–4%) in a large retrospective review of gastric biopsy pathology reports (49). This discrepancy likely reflects differences in quantification (flow cytometry vs retrospective histology reports) and gastritis grading among pathologists. Gastric mucosa increases in leukocyte infiltration by flow cytometry were specific to idiopathic gastroparesis and were not observed in our small cohort of functional dyspepsia. Our preliminary findings with functional dyspepsia are consistent with previous reports detailing no change in gastric or D1 mast cells and eosinophils (18,21,23,24,50). Although controls in this study were older (younger asymptomatic adults rarely present for endoscopy), no correlation between leukocyte infiltration and age was found, suggesting age was an unlikely confounder.

The stomach mucosa, in both controls and gastroparesis subjects, was surprisingly distinct in its immune composition, with relatively high leukocyte densities in the proximal stomach compared with the antrum and distinct cytokine and immune gene expression profiles. The implications are not yet clear, but may relate to the proximal stomach functioning as a reservoir of undigested food where immune barrier function should be robust. Other possibilities include differential gradients of specialized gastric epithelial cells (51). Further studies will be required to distinguish the functional specialization of mucosal immune cells in the proximal vs distal stomach.

To the best of our knowledge, this is the first comprehensive immune profiling of the human gastric mucosa in idiopathic gastroparesis. This human exploratory observational study was meant to be a hypothesis-generating investigation. This is particularly relevant for idiopathic gastroparesis, which has no animal models for mechanistic studies. Our study suggests that gastric mucosal immune profiling may reveal novel mechanistic insights into the pathophysiology of gastroparesis. This is significant given the limited access for research of gastric muscle tissue, which is heavily biased toward severe medically refractory gastroparesis and/or morbidly obese controls (13). Some limitations to this study include relatively small sample size, inability to assess acute gastritis assessed as neutrophil infiltrates, and exclusion of eosinophils given limited staining options on the flow cytometry panel. As a cross-sectional observational study, causation between immune profiles and gastroparesis clinical parameters cannot be established either. Importantly, correlations between immune profiles and clinical patient data need to be prospectively validated in appropriately powered studies because they did not meet FDR criteria in multiple comparison analyses.

In summary, the findings of this initial observational study suggest that mucosal immune dysregulation occurs in idiopathic gastroparesis and may directly or indirectly affect gastric motility. Care should be taken to standardize mucosal biopsy research sampling, given the significant differences in immune composition between the proximal and distal stomach. This study raises new interesting future questions, including (i) the role of impaired barrier permeability and/or luminal microbial composition in gastric mucosal inflammation, (ii) the basis for a marked immune gradient in the stomach, (iii) the differential roles of distinct mucosal immune cells and their secreted factors in gastroparesis, (iv) overlap of mucosal immune dysregulation in idiopathic and diabetic gastroparesis, and (v) the mechanisms tying mucosal immune dysregulation to visceral hypersensitivity and gastric dysmotility. Immune profiling of mucosal biopsies opens the door to a novel aspect of gastroparesis study and allows for further investigations to determine how mucosal immune dysregulation contributes to altered sensation and motility.

## CONFLICTS OF INTEREST

**Guarantor of the article:** Andres Gottfried-Blackmore, MD, PhD.
**Specific author contributions:** A.G.-B.: study concept, design and direction, sample collection protocol, data analyses and interpretation, manuscript writing, and approved the final draft submitted; H.N.: sample collection protocol, data analyses and interpretation, and approved the final draft submitted; B.M.: histology data generation and analysis, manuscript revision, and approved the final draft submitted; E.A.: sample collection protocol, subject enrollment and demographics, sample collection, and approved the final draft submitted; J.G.: data analysis and manuscript revision and approved the final draft submitted; N.F.-B.: subject enrollment, manuscript revision, and approved the final draft submitted; J.C.: subject enrollment, manuscript revision, and approved the final draft submitted; J.I.: data analysis, manuscript editing, and approved the final draft submitted; L.N.: study concept and design, subject enrollment, manuscript editing, and approved the final draft submitted; A.H.: study concept and design, data interpretation, edited manuscript, and approved the final draft submitted.
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## Study Highlights

### WHAT IS KNOWN

- ✓ Although the mucosa and submucosa have the greatest immune cell density, little is known about the regional distribution of immunological traits in the human gastric mucosa.
- ✓ Data are lacking in gastroparesis for immune characteristics of the gastric mucosal layer, which is endoscopically accessible.
- ✓ It is unclear how mucosal immune perturbations may influence gastroparesis.

### WHAT IS NEW HERE

- ✓ The gastric mucosa displays large regional variation of distinct immune profiles.
- ✓ Innate and adaptive immune cells are increased in gastroparesis mucosal biopsies compared with disease controls.
- ✓ Gastric mucosal macrophages and $CD8^+$ T cells may positively correlate with gastric emptying delay.

### TRANSLATIONAL IMPACT

- ✓ Significant differences in immune composition between the gastric mucosal regions has implications in the interpretation of endoscopic biopsy research sampling.
- ✓ Mucosal immune profiling may help to differentiate idiopathic gastroparesis from functional dyspepsia.
- ✓ Accessible mucosal biopsy analyses may advance future studies addressing the pathophysiology of gastroparesis and its treatment.

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