Mucosal Overexpression of Thymic Stromal Lymphopoietin and Proinflammatory Cytokines in Patients With Autoimmune Atrophic Gastritis

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INTRODUCTION: **The immune mechanisms underlying human autoimmune atrophic gastritis (AAG) are poorly understood. We sought to assess immune mucosal alterations in patients with AAG.**

METHODS: **In 2017–2021, we collected gastric corpus biopsies from 24 patients with AAG (median age 62 years, interquartile range 56–67, 14 women), 26 age-matched and sex-matched healthy controls (HCs), and 14 patients with *Helicobacter pylori* infection (HP). We investigated the lamina propria mononuclear cell (LPMC) populations and the mucosal expression of thymic stromal lymphopoietin (TSLP) and nicotinamide phosphoribosyltransferase (NAMPT). *Ex vivo* cytokine production by organ culture biopsies, under different stimuli (short TSLP and zinc-l-carnosine), and the gastric vascular barrier through plasmalemma vesicle-associated protein-1 (PV1) were also assessed.**

RESULTS: **In the subset of CD19+ LPMC, CD38+ cells (plasma cells) were significantly higher in AAG compared with HC. *Ex vivo* production of tumor necrosis factor (TNF)-α, interleukin (IL)-15, and transforming growth factor β1 was significantly higher in AAG compared with HC. At immunofluorescence, both IL-7R and TSLP were more expressed in AAG compared with HC and HP, and short TSLP transcripts were significantly increased in AAG compared with HC. In the supernatants of AAG corpus mucosa, short TSLP significantly reduced TNF-α, while zinc-l-carnosine significantly reduced interferon-γ, TNF-α, IL-21, IL-6, and IL-15. NAMPT transcripts were significantly increased in AAG compared with HC. PV1 was almost absent in AAG, mildly expressed in HC, and overexpressed in HP.**

DISCUSSION: **Plasma cells, proinflammatory cytokines, and altered gastric vascular barrier may play a major role in AAG. TSLP and NAMPT may represent potential therapeutic targets, while zinc-l-carnosine may dampen mucosal inflammation.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A836, http://links.lww.com/CTG/A837, http://links.lww.com/CTG/A838, http://links.lww.com/CTG/A839, http://links.lww.com/CTG/A840, http://links.lww.com/CTG/A841, http://links.lww.com/CTG/A842, http://links.lww.com/CTG/A843, http://links.lww.com/CTG/A841, http://links.lww.com/CTG/A842, http://links.lww.com/CTG/A843, http://links.lww.com/CTG/A844, http://links.lww.com/CTG/A845, http://links.lww.com/CTG/A846

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INTRODUCTION

Autoimmune atrophic gastritis (AAG) is a slowly progressive, organ-specific disease characterized by the immune-mediated destruction of the gastric parietal cells, which results in atrophy of the oxyntic mucosa, hypo-achlorhydria, and intrinsic factor deficiency, hence determining over time iron, vitamin B12, and other micronutrient malabsorption (1–3). In addition, AAG predisposes to the development of gastric adenocarcinoma and type I neuroendocrine tumors; thus, early AAG diagnosis (4) and proper endoscopic follow-up are warranted (5,6).

Although improvements have been recently made in the clinical management of AAG (1), little is known regarding the immunological mechanisms underlying this condition. In particular, most of the knowledge concerning the molecular pathways leading to mucosal damage in AAG is based on experimental models (7,8), while few data regarding humans are available. From mice AAG models, we learned that a number of proinflammatory cytokines are overexpressed in the atrophic gastric corpus mucosa, including tumor necrosis factor (TNF)-α, interleukin (IL)-21, and IL-17A (9–11). Moreover, the thymic stromal lymphopoietin (TSLP) signaling seems to be altered in mice models of AAG, thus providing evidence for a regulatory role of this cytokine in this condition (12).

A major turning point in the understanding of the pathogenesis of human AAG was the discovery of the major target autoantigen recognized by the anti-parietal cell antibodies (PCAs), the serological marker of AAG, namely the gastric proton pump H+/K+ ATPase, located on parietal cells (13). Of note, although previous studies showed a cytotoxic role of PCA against parietal cells *in vitro* (14), this is unlikely to happen *in vivo* because parietal cells are difficult to be accessed by these antibodies. Indeed, in a mice model, transferred human PCA were not able to induce an inflammatory response in the gastric corpus mucosa (15). Regarding data in humans, the study by D'Elios et al. (2) showed that H+/K+ ATPase induced *in vitro* proliferation of CD4+ T-cell clones derived from the gastric corpus mucosa of 5 patients with AAG. These cells were also found to produce TNF-α and interferon (IFN)-γ, and they triggered immunoglobulin (Ig) production by activated B cells and plasma cells, causing cytotoxicity and apoptosis of gastric parietal cells. Nevertheless, a thorough characterization of the lamina propria mononuclear cells (LPMCs) infiltrating the gastric corpus mucosa of patients with AAG is still lacking, and a systematic dissection of the inflammatory pathways (i.e., cytokines) and tissue remodeling/damage pathways (i.e., matrix metalloproteinases [MMPs], transforming growth factor [TGF]-β1) implicated in the development of human AAG-related lesions is warranted.

Hence, starting from these premises, we aimed at characterizing LPMC infiltrating the gastric corpus mucosa of patients with AAG and exploring immune pathways driving gastric atrophy through *ex vivo* organ culture experiments.

METHODS

Patients

This study was conducted in January 2017–January 2021, coordinated by a gastroenterological outpatient clinic of a tertiary referral, university hospital of Northern Italy (IRCCS San Matteo Hospital Foundation, University of Pavia), with a peculiar expertise in the diagnosis and treatment of AAG. The main study population comprised of consecutive patients with an established diagnosis of AAG, undergoing upper gastrointestinal endoscopy during the study period as part of their routinary follow-up, enrolled at IRCCS San Matteo Hospital Foundation (Pavia) and Sant'Andrea Hospital (Rome). The diagnosis of AAG was made in all cases by expert gastroenterologists (E.M., E.L., M.V.L., and A.D.S.), according to internationally agreed criteria. More in depth, the diagnosis was based on histopathological grounds, according to the Sydney-Houston criteria (16). In all patients, at least 5 gastric biopsies were taken, including 2 from the antrum, 1 from the incisura angularis, and 2 from the corpus. Two expert pathologists (A.V. and G.A.) independently reviewed all biopsies, reaching a consensus on the diagnosis in all cases. In all patients with AAG, *Helicobacter pylori* was excluded by means of histopathology and stool *H. pylori* antigen test. Patients with atrophic pangastritis or uncertain diagnosis were not included. As control groups, we also enrolled non-AAG patients with active *H. pylori* gastritis (HP; positive control group), together with age-matched and sex-matched healthy controls (HCs; negative control group), that is, individuals undergoing upper gastrointestinal endoscopy for dyspepsia with a normal gastric mucosa at histology. In all patients and controls, serum fasting 17-gastrin, chromogranin A, and PCA were also assessed. PCA-positive controls and PCA-positive *H. pylori*-infected patients were excluded.

In all cases, biopsy specimens were taken by the endoscopists (L.R. and M.V.L.) in the upper third of the gastric corpus. Some of the biopsy samples were immediately fixed in a 3% buffered formalin, embedded in paraffin within 24 hours and processed according to the standard methods for histology, or embedded in OCT Tissue-Tek (Sakura Finetek, Torrance, CA), snap frozen, and processed for immunofluorescence; some other were homogenized and used for immunoblotting analysis. Finally, some biopsies were used for LPMC isolation and organ cultures or collected and stored in RNA *later* for RNA extraction as detailed below. Peripheral blood was taken from the same patients at the time of endoscopy to isolate peripheral blood mononuclear cells (PBMCs). Written informed consent was obtained in all cases, and the research was approved by the local ethics committee (Protocol No. P3599/2017). Data are reported according to the STROBE guidelines for quality assurance.

Cell isolation and culture

LPMC and PBMC were isolated as previously described (17). Cells were not used if viability, as assessed by flow cytometry after vital die (Zombie; Biolegend, San Diego, CA) staining, did not exceed 90%. Human cells were stained with combinations of directly conjugated antibodies as specified in Supplementary Table 1 (see Supplementary Digital Content 6, http://links.lww.com/CTG/A841). Freshly isolated LPMCs were cultured (2×10^5 cells/well, in duplicate) in RPMI medium (Sigma, Poole, UK) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C, 5% CO_2 for 48 hours in 96-well plates (BD Biosciences, Oxford, UK) with soluble anti-CD45 (0.5 μg/mL; BD Biosciences) and anti-CD19 antibodies (0.5 μg/mL; Biolegend). Subsequently, purified CD19+ cells were cultured with 10 μg/mL anti-CD38, anti-IgD, anti-IgG, and anti-IgM antibodies (Immunological Sciences, Rome, Italy). Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) before the addition of the antibodies detecting the cytokine released.

Organ culture

Biopsy specimens were placed on iron grids in the central well of an organ culture dish and the dishes placed in a tight chamber with 95% O_2/5% CO_2 at 37°C. Biopsies were cultured for 24 hours in serum-free HL-1 medium (Cambrex Bio Science, Milan, Italy),

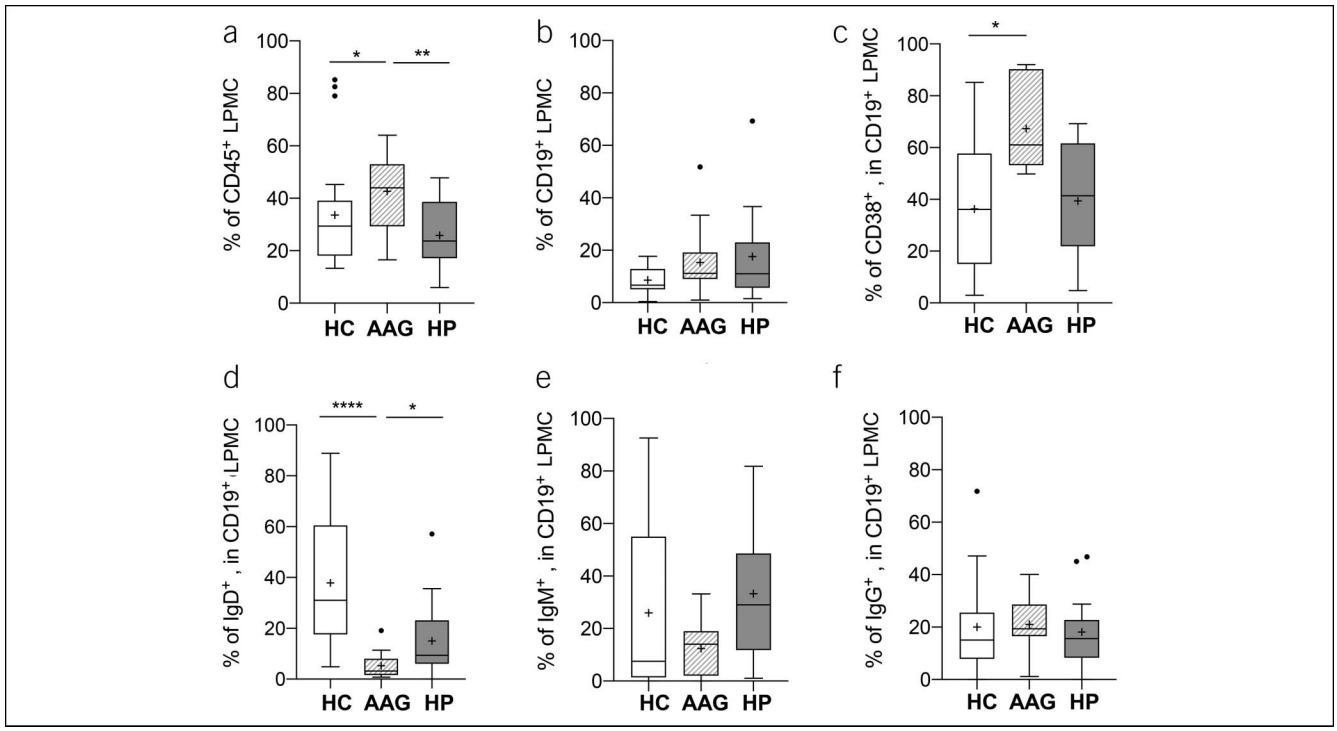

Figure 1. Whisker plot showing characterization by flow cytometry of CD45+ (**a**), CD19+ (**b**), CD38+ (**c**), immunoglobulin (Ig) D+ (**d**), IgM+ (**e**), and IgG+ (**f**) lamina propria mononuclear cells (LPMCs) isolated from the gastric corpus of 26 healthy controls (HCs), 24 patients with autoimmune atrophic gastritis (AAG), and 14 patients with *Helicobacter pylori* gastritis (HP). Box and whisker plot represents the median, upper and lower quartiles, and minimum/maximum value, while the symbol "+" is the mean. The dots, when present, represent the outliers. $^{*}P < 0.01$; $^{**}P < 0.001$; $^{****}P < 0.00001$.

added with antibiotics, with or without 10 ng/mL short TSLP (synthetized and provided by Maria Rescigno's laboratory) (18) or 10 ng/mL zinc-L-carnosine (Azienda Farmaceutica Italiana; Sant'Egidio alla Vibrata, Teramo, Italy) or human recombinant extracellular nicotinamide phosphoribosyltransferase (eNAMPT; Peprotech, London, UK) at the concentration of 500 ng/mL. After 24-hour culture, supernatants and tissues were used to assess the cytokine production.

Cytokine assay

The concentrations of TNF-α, IL-11, IL-15, IL-33, TGF-β1, IFN-γ, IL-17, IL-6, IL-21, and IL-22 were measured in organ culture supernatants using the Luminex x-MAP technology (Luminex, DiaSorin Company, Austin, TX). eNAMPT was evaluated with a commercially available sandwich enzyme-linked immunosorbent assay for human NAMPT (ELISA kit from AdipoGen, Seoul, Korea).

Western blotting

Western blotting was performed according to a modified method previously described (19). A rabbit anti-MMP-3 (10 ng/mL; Abcam, Cambridge, UK) and a sheep anti-MMP-12 (10 ng/mL; Abcam) were used as primary antibodies. Appropriate horseradish peroxidase-conjugated antibodies (DAKO, High Wycombe, UK) were used as secondary antibodies, and the reaction was developed with the ECL plus kit (Amersham Biosciences, Little Chalfont, UK). When required, blots were stripped and analyzed for internal loading controls, and bands were quantified using an LKB Ultrascan XL Laser Densitometer (Kodak, Hemel Hempstead, UK).

Flow cytometry

Freshly isolated LPMC and PBMC were stained with combinations of directly conjugated antibodies as specified in Supplementary Table 1 (see Supplementary Digital Content 6, http://links.lww.com/CTG/A841). Samples were analyzed with a FACS-Celesta flow cytometer (BD Bioscience), gated to exclude singlets based on a light scatter and nonviable cells by Zombie Dye labeling. Data were analyzed using FlowJo v10 software (BD Bioscience).

Immunofluorescence and immunohistochemistry

Four 5-μm cryostat sections were fixed in cold acetone or in paraformaldehyde 4% for 10 minutes. Primary antibodies were incubated overnight at 4°C, namely anti-total TSLP (#ab47943; Abcam), anti-TSLPR (#743961; BD Biosciences), and anti-CD127 (#351318; Biolegend). The secondary antibody for the detection of total TSLP, donkey anti-rabbit 555 (Life Technologies, Thermo Fisher, Waltham, MA), was incubated for 1 hour, and sections were counterstained with Sytox Orange (Thermo Fisher). Slides were mounted with Vectashield (Vector laboratories, Burlingame, CA) and visualized under a Leica TCS SP8 laser scanning confocal microscope. Three-millimeter-thick paraffin sections were used for immunohistochemistry by the Dako Omnis automatic platform (Agilent, Santa Clara, CA). Immunohistochemical detection of plasma cells was performed through an anti-CD138 antibody (36-2900; Thermo Fisher), while an anti-plasmalemma vesicle-associated protein-1 (PV1)/PLVAP antibody (clone 174/2; LSBio, Seattle, WA) was used to visualize the gastric vascular barrier. We evaluated PV1 in a semiquantitative manner by

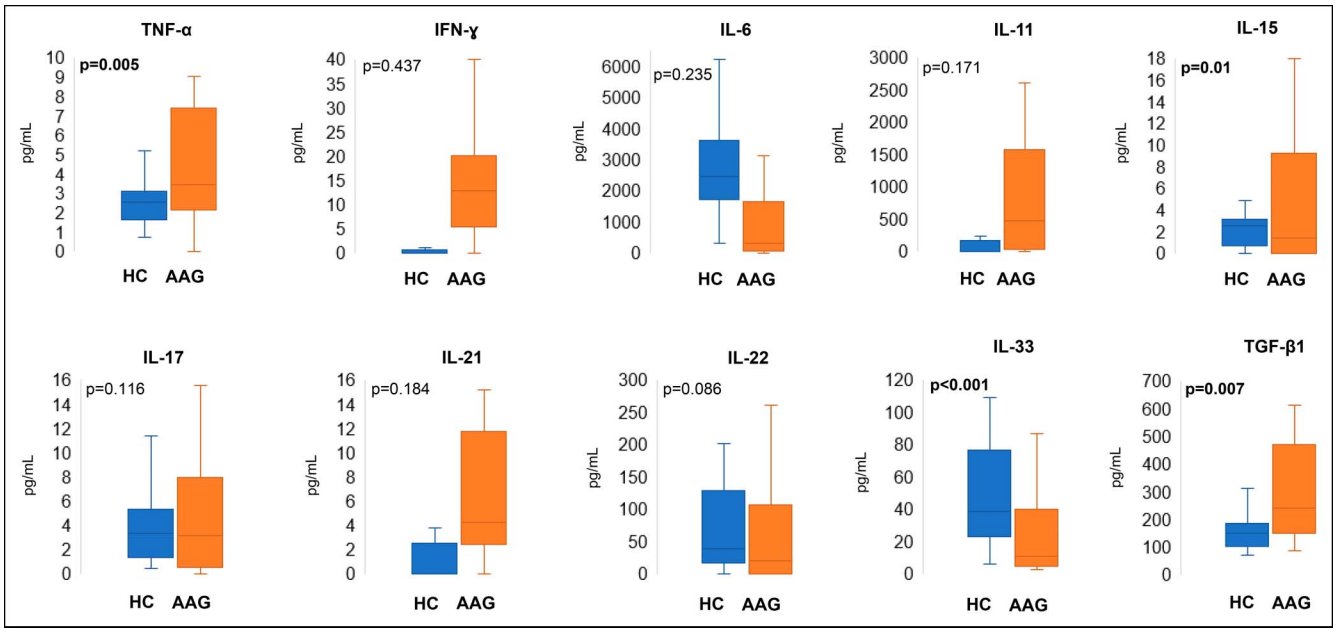

Figure 2. *Ex vivo* levels of cytokines, expressed in pg/mL, in the supernatants of gastric corpus mucosa biopsies collected from 24 patients with autoimmune atrophic gastritis (AAG) compared with 26 healthy controls (HCs), cultured for 24 hours in the absence of stimuli. Box and whisker plot represents the median, upper and lower quartiles, and minimum/maximum value.

using the following score: 0 (absence of PV1-positive vessels), 1 (rare PV1-positive vessels), and 2 (numerous PV1-positive vessels).

RNA extraction and analysis of mRNA expression by quantitative RT-PCR

Total RNA was extracted from human biopsies using the Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA). RNA was reverse transcribed with oligo(dT) and ImProm-II Reverse Transcriptase (Promega, Milan, Italy). cDNA expression was detected using Rotor-Gene Q 2Plex (Qiagen, Valencia, CA). TSLPR, IL-7R, NAMPT, TNF-α, IL-17A, IL-33, IL-6, IFN-γ, IL-15, and PV-1 primers (genome wide bioinformatically validated primers sets) were provided using Qiagen (QuantiTect Primer Assays). Real-time PCR reactions were performed using the Fast Sybr Green PCR kit (QuantiStudio 7 Flex R real time PCR, Applied Biosystems, Thermo Fisher). GAPDH, ACTIN, and S18 were used as an endogenous reference control, transcript quantification was performed with the 2-ΔCT method, and the results are expressed as "fold induction" in reference to the expression of the housekeeping gene. For short TSLP detection, we used the custom primers: forward -CCGCCTATGAGCAGCCAC- and reverse -CCTGAGTAGCATTTATCTGAG-.

Sample size calculation and statistical analysis

The calculation of the power of the sample size was performed by estimating a difference (delta) of approximately 20 pg/mL and an SD of 30 pg/mL of the cytokine TNF-α between HC and AAG or HP ($\alpha = 0.05$, power 90%; enrolment rate of at least 1:0.5) (10,20). This estimation was inferred from a study conducted in a mouse model of AAG (10) and a study conducted on patients with *H. pylori* gastritis (20). Among the various cytokines, we chose TNF-α as it has been the most studied in this condition. In particular, the Mann-Whitney *U* test was used for skewed data, with a 2-sided significance level of 0.05, estimating a number of 20 patients with AAG and at least 10 individuals for the control groups, when a probability equals 0.336. Data are presented as either mean and SD or median and interquartile range, using box and whisker plots when applicable. The normality of data distributions was assessed using the Shapiro-Wilk test. Parametric (unpaired *t* test and 1-way ANOVA followed by Tukey *post hoc*) or non-parametric (Mann-Whitney *U* test and 1-way Kruskal-Wallis *H* test followed by Dunn *post hoc*) statistical analysis was used for comparisons and performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

Overall, 26 HCs, 24 patients with AAG, and 14 patients with HP provided written consent and were enrolled over the study period. Clinical data from HCs, patients with AAG, and patients with HP are given in Supplementary Table 2 (see Supplementary Digital Content 7, http://links.lww.com/CTG/A842). All patients with AAG had severe atrophy of the gastric corpus and fundus mucosa.

Characterization of LPMC and PBMC

Flow cytometric characterization of LPMC is reported in Figure 1, Supplementary Figure 1 (see Supplementary Digital Content 1, http://links.lww.com/CTG/A836), and in Supplementary Table 3a (see Supplementary Digital Content 8, http://links.lww.com/CTG/A843). Figure 1 shows that the percentage of CD45+ LPMC was significantly higher in AAG in comparison with both HP and HC, with no significant difference between HC and HP (A). The percentage of B cells (CD19+) was comparable among the 3 groups (B). In the subset of CD19+ LPMC, the percentage of CD38+ cells (plasma cells) was significantly higher in AAG compared with HC, whereas no significant difference was found between AAG and HP, and

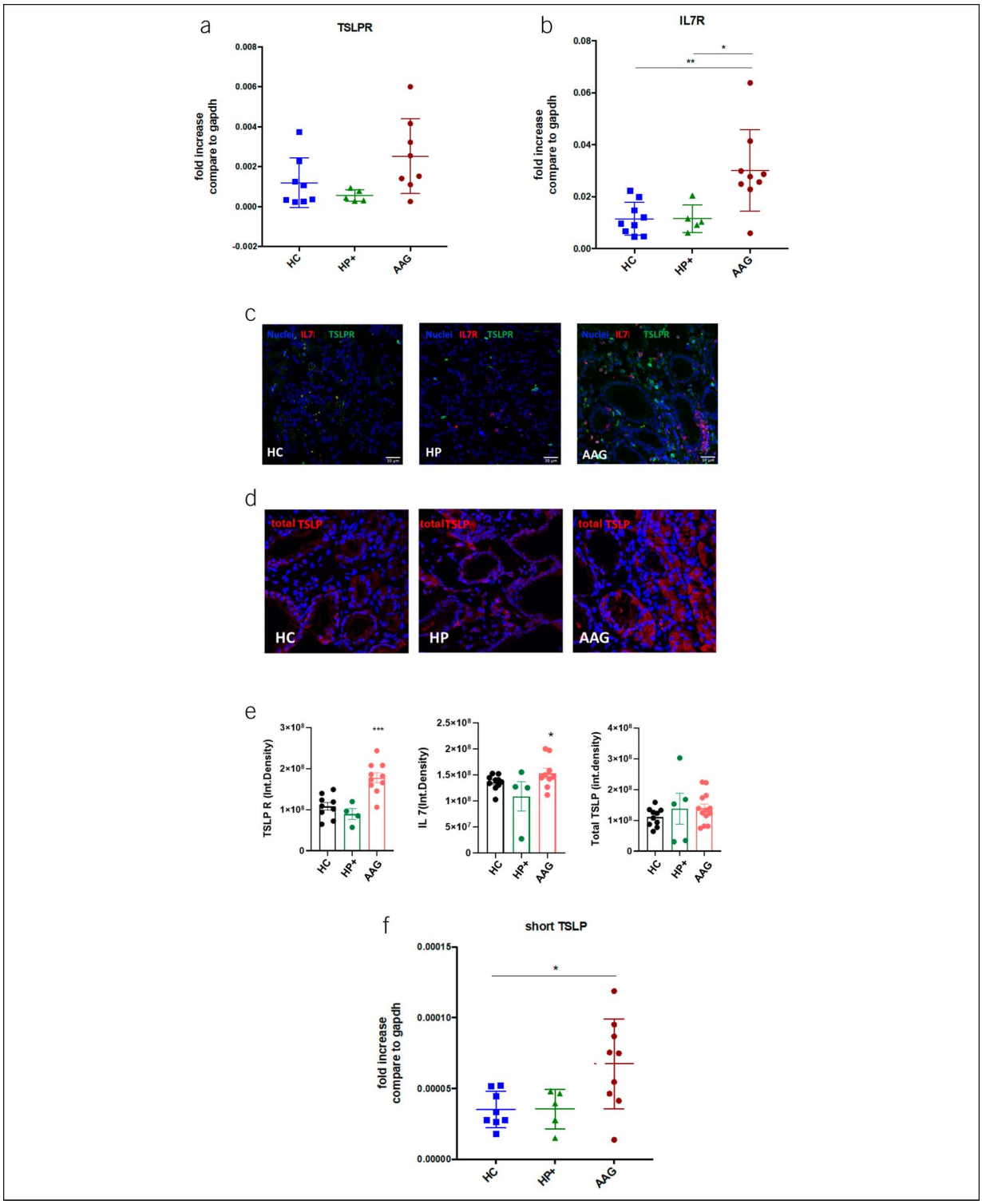

Figure 3. Transcripts of thymic stromal lymphopoietin receptor (TSLPR; **a**) and interleukin 7 receptor (IL-7R; **b**) in the corpus gastric mucosa of 9 healthy controls (HCs; squares), 5 patients with *Helicobacter pylori* gastritis (HP; triangles), and 9 patients with autoimmune atrophic gastritis (AAG; circles). Panel **c** shows immunofluorescence staining of interleukin (IL)-7R, TSLPR, and nuclei in a HC, a patient with HP, and a patient with AAG. In AAG, both IL-7R and TSLPR are significantly more expressed compared with both HC and HP. Results are representative of 12 HC, 12 HP, and 12 AAG. Panel **d** shows immunofluorescence staining of total TSLP in the gastric corpus mucosa in a HC, a patient with HP, and a patient with AAG. In AAG, total TSLP is more expressed compared with both HC and HP, although not significantly. Results are representative of 12 HC, 12 HP, and 12 AAG. Panel **e** shows the integrated density graphs for TSLPR, IL-7R, and total TSLP. Panel **f** shows the transcripts of short TSLP in the corpus gastric mucosa of 9 HCs (squares), 5 patients with HP (triangles), and 9 patients with AAG (circles). $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$.

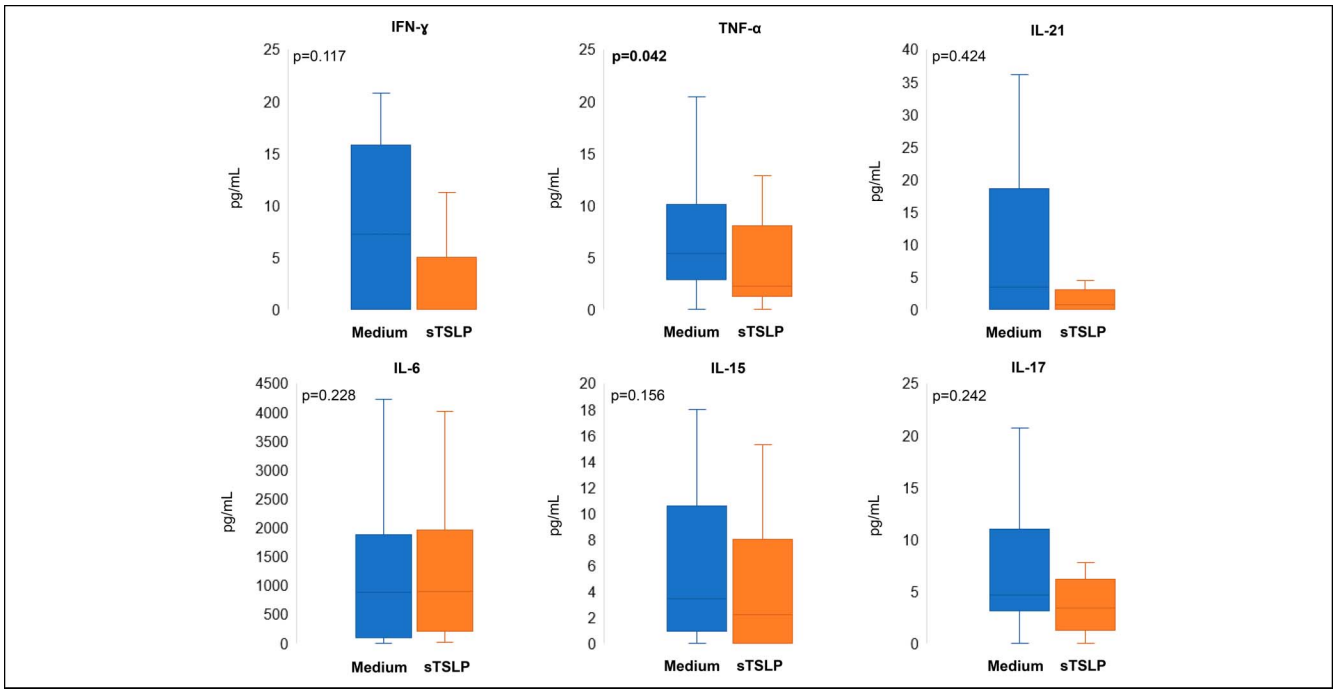

Figure 4. *Ex vivo* levels of cytokines, expressed in pg/mL, in the supernatants of gastric corpus mucosa biopsies collected from 18 patients with autoimmune atrophic gastritis (AAG), cultured for 24 hours in the absence of stimuli (medium) or with short thymic stromal lymphopoietin (sTSLP). Box and whisker plot represents the median, upper and lower quartiles, and minimum/maximum value.

between HC and HP (C). Accordingly, Supplementary Figure 2 (see Supplementary Digital Content 2, http://links.lww.com/CTG/A837) shows a high number of plasma cells (stained with CD138) infiltrating the atrophic gastric corpus mucosa of a patient with AAG. Within the subset of CD19+ LPMC, the percentage of IgD+ cells was significantly lower in AAG in comparison with HC and HP, with no significant difference between HC and HP (Figure 1d). On the contrary, the percentage of both IgM+ (E) and IgG+ (F) CD19+ LPMC did not significantly differ among the 3 groups. Supplementary Figure 1 (see Supplementary Digital Content 1, http://links.lww.com/CTG/A836) shows that the percentage of CD3+ (A), CD4+ (B), and CD8+ (C) LPMC did not differ among the 3 groups. On the contrary, the percentage of CD56+ LPMC was significantly lower in AAG in comparison with HP, with no significant difference between AAG and HC, and between HC and HP (D). Moreover, the percentages of CD11c+ (E) LPMC were significantly lower in AAG in comparison with HC, with no significant difference between HC and HP, and between AAG and HP. Single LPMC percentages are given in Supplementary Table 3a (see Supplementary Digital Content 8, http://links.lww.com/CTG/A843).

Flow cytometric characterization of PBMC is reported in Supplementary Figures 3 and 4 (see Supplementary Digital Contents 3, http://links.lww.com/CTG/A838, and 4 http://links.lww.com/CTG/A839), and in Supplementary Table 3b (see Supplementary Digital Content 9, http://links.lww.com/CTG/A844). Supplementary Figure 3 (see Supplementary Digital Content 3, http://links.lww.com/CTG/A838) shows that the percentage of CD45+ PBMC was significantly higher in AAG and HC in comparison with HP, with no significant difference between HC and AAG (A). The percentage of B cells (CD19+) was significantly lower in AAG and HC in comparison with HP, with no significant difference between HC and AAG (B). In the subset of CD19+ PBMC, the percentage of CD38+ cells was significantly higher in AAG compared with HP, whereas no significant difference was found between AAG and HC, and between HC and HP (C). Within the subset of CD19+ PBMC, the percentage of IgD+ (D), IgM+ (E), and IgG+ (F) cells did not significantly differ among the 3 groups. Supplementary Figure 4 (see Supplementary Digital Content 4, http://links.lww.com/CTG/A839) shows that the percentage of CD3+ (A), CD4+ (B), and CD8+ (C) PBMC did not differ among the 3 groups. On the contrary, the percentage of CD56+ PBMC was significantly lower in AAG in comparison with HP, with no significant difference between AAG and HC, and between HC and HP (D). Moreover, the percentage of CD11c+ was significantly lower in AAG in comparison with HC, with no significant difference between AAG and HP, and between HC and HP (E). Single PBMC percentages are given in Supplementary Table 3b (see Supplementary Digital Content 9, http://links.lww.com/CTG/A844).

Ex vivo cytokine and MMP production by organ culture biopsies

As presented in Figure 2 and Supplementary Table 3c (see Supplementary Digital Content 10, http://links.lww.com/CTG/A845), the organ culture supernatant concentration of TNF-α, IL-15, and TGF-β1 was significantly higher in AAG compared with HC, whereas IL-33 was significantly lower in AAG in comparison with HC. No other significant differences were noticed regarding the remaining cytokines, namely IFN-γ, IL-6, IL-11, IL-17, IL-21, and IL-22.

As shown in Supplementary Figure 5 (see Supplementary Digital Content 5, http://links.lww.com/CTG/A840), when we evaluated MMP-3 and MMP-12 by western blotting in organ culture supernatants of biopsies collected from 7 patients with AAG and 6 HCs, we found that MMP-3 bands were markedly evident in all cases of AAG, whereas HC showed low or absence of

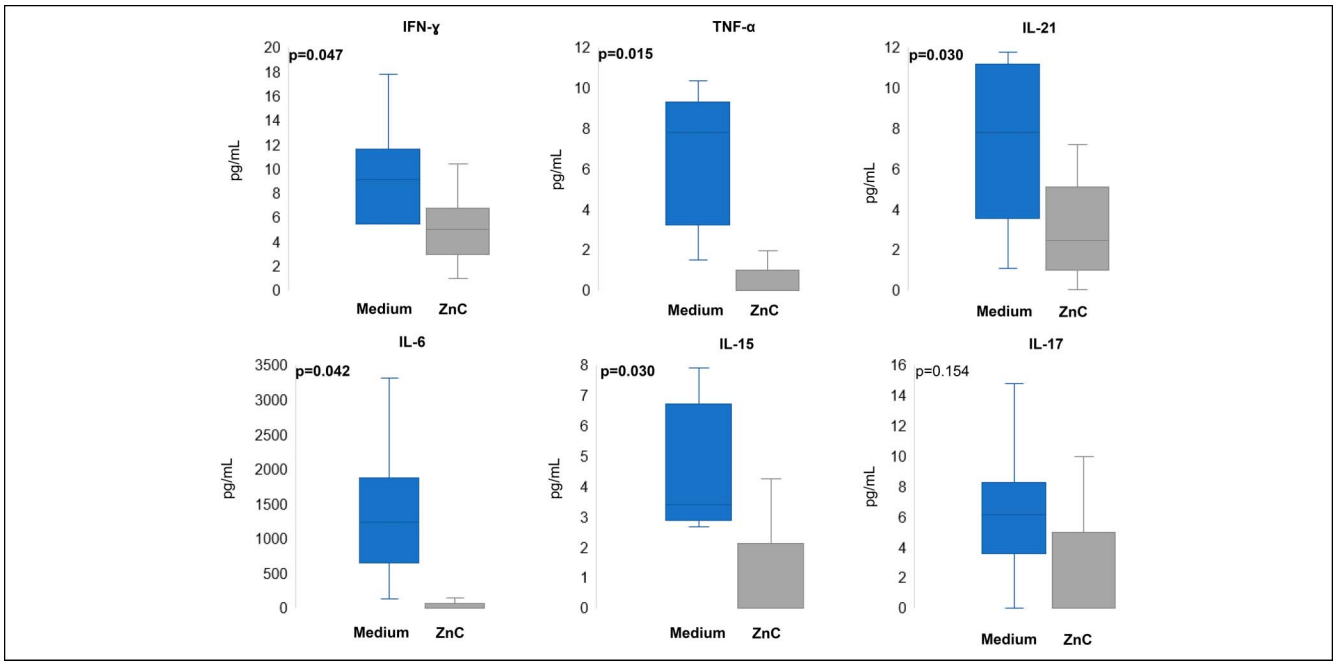

Figure 5. *Ex vivo* levels of cytokines, expressed in pg/mL, in the supernatants of gastric corpus mucosa biopsies collected from 18 patients with autoimmune atrophic gastritis (AAG), cultured for 24 hours in the absence of stimuli (medium) or with zinc-l-carnosine (ZnC). Box and whisker plot represents the median, upper and lower quartiles, and minimum/maximum value.

MMP-3 expression. On the contrary, MMP-12 production was absent in both AAG and HC.

TSLPR and IL-7R

Transcript levels of TSLPR and IL-7R in 9 HCs, 5 patients with HP, and 9 patients with AAG are shown in Figure 3 (a and b, respectively). No statistically significant difference was noticed among the 3 groups regarding TSLPR (although a trend for higher levels was noticed in AAG), whereas IL-7R was significantly more expressed in AAG compared with both HC and HP, and no difference was noticed between HC and HP. At immunofluorescence, both IL-7R and TSLPR were significantly more expressed in AAG compared with HC and HP (c). In addition, the total TSLP expression was more evident in AAG compared with HC and HP (d), although not significantly. The integrated density graphs, along with the statistical significance, are shown in (e). Finally, short TSLP transcripts were significantly increased in AAG compared with HC, whereas no difference between AAG and HP, and between HC and HP was noticed (f).

Ex vivo effect of short TSLP and zinc-l-carnosine on cytokine production in AAG

Figure 4 and Supplementary Table 3c (see Supplementary Digital Content 10, http://links.lww.com/CTG/A845) present e*x vivo* levels of cytokines, expressed in pg/mL, in the supernatants of gastric corpus mucosa biopsies collected from 18 patients with AAG, cultured for 24 hours in either the medium only or with short TSLP. Of note, short TSLP significantly reduced TNF-α, whereas it had no significant effect on the other tested cytokines (IFN-γ, IL-21, IL-6, IL-15, and IL-17). Figure 5 and Supplementary Table 3d (see Supplementary Digital Content 11, http://links.lww.com/CTG/A846) present *ex vivo* levels of cytokines, expressed in pg/mL, in the supernatants of gastric corpus mucosa biopsies collected from 18 patients with AAG, cultured for 24 hours in either the medium only or with zinc-l-carnosine. Of note, zinc-l-carnosine significantly down regulated the production of IFN-γ, TNF-α, IL-21, IL-6, and IL-15, whereas no significant effect was observed for IL-17.

NAMPT in AAG corpus mucosa

Transcript levels of NAMPT were significantly increased in AAG gastric corpus biopsies compared with HC and in HP compared with HC, whereas no significant difference was found between AAG and HP (Figure 6a). When we evaluated the extracellular form of NAMPT, i.e., eNAMPT, in the supernatants of organ culture biopsies, we found that it was significantly increased in AAG in comparison with HC (Figure 6b). To verify whether NAMPT exerted a proinflammatory effect on normal gastric mucosa, we cultured HC gastric corpus biopsies with recombinant human eNAMPT, and we found that it significantly increased the IFN-γ and IL-6 mucosal transcripts. However, no significant effect was noticed regarding TNF-α, IL-15, IL-17A, and IL-33 (Figure 6c). Single cytokine transcripts are reported in Supplementary Table 3d (see Supplementary Digital Content 11, http://links.lww.com/CTG/A846).

Gastric vascular barrier

Figure 7a shows the immunohistochemical detection of PV1 in the gastric corpus of representative individuals from the HC, HP, or AAG subgroups. PV1 was almost absent in AAG (score 0), mildly expressed in HC (score 1), and overexpressed in HP (score 2). Figure 7b shows significantly higher levels of PV1 transcripts in HP in comparison with both AAG and HP. PV1 transcript

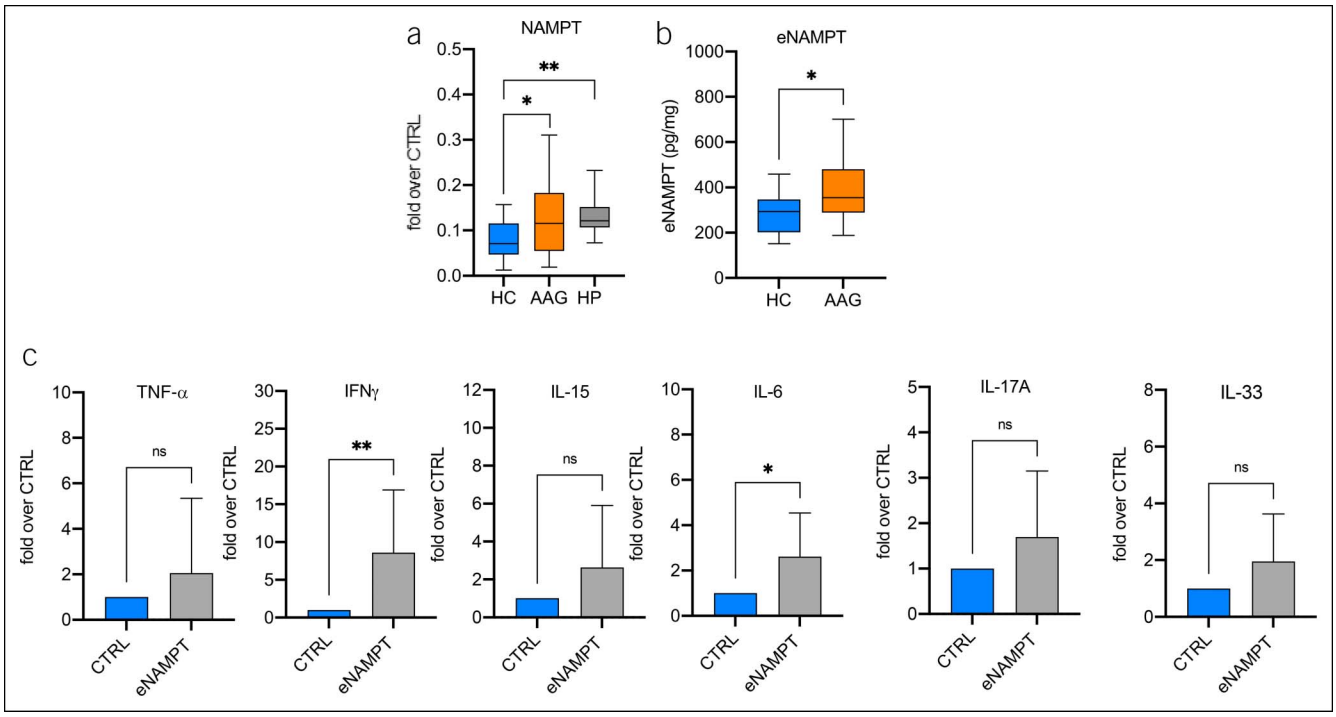

Figure 6. (**a**) Mucosal nicotinamide phosphoribosyltransferase (NAMPT) transcripts evaluated by RT-PCR in 26 healthy controls (HCs), 24 autoimmune atrophic gastritis (AAG), and 8 *Helicobacter pylori* (HP) gastric biopsies; (**b**) eNAMPT concentration (right) evaluated by ELISA assay in the supernatants of 15 HC and 13 AAG gastric biopsies; (**c**) mucosal transcripts of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-33, IL-17A, and transforming growth factor (TGF)-β evaluated with RT-PCR in organ culture of gastric corpus biopsies of 10 HC treated for 24 hours with recombinant eNAMPT (500 ng/mL). $*P < 0.01$; $**P < 0.001$.

levels were reduced in AAG in comparison with HC, although the difference did not reach a statistical significance.

DISCUSSION

In this study, we demonstrated that among the LPMC populations infiltrating the gastric corpus mucosa of patients with AAG, the only cell subset increased is represented by plasma cells, being CD19+ cells and all T-cell subsets comparable with those of control individuals. The increased *ex vivo* production by AAG organ culture biopsies of proinflammatory cytokines, including TNF-α and IL-15, of the metabokine NAMPT and of the extracellular matrix-degrading enzyme MMP-3 might sustain the inflammatory process leading to gastric atrophy, while lamina propria overexpression of TSLP could function as a compensatory—although ineffective—mechanism. The anti-inflammatory compound zinc-l-carnosine, which was shown to restore gastric healing in peptic ulcer disease (21), induced a significant *ex vivo* downregulation of the proinflammatory cytokines produced by AAG biopsies. Further studies are needed to clarify the significance of the less permeable gastric vascular barrier in AAG, as shown by the reduced mucosal expression of PV1.

The marked increase of CD38+ LPMC in AAG gastric corpus is consistent with previous observations showing a higher number of IgG4-producing plasma cells in this condition (22). In addition, plasma cells have been found in all AAG stages, from the earliest phase, involving the full thickness of the lamina propria, with a top-down gradient, and in the late phase, forming a dense mucosal infiltrate (23). The increased number of plasma cells in atrophic lesions might sustain the florid production of autoantibodies, which are the hallmark of AAG. However, we cannot exclude that plasma cells might have a role in driving mucosal damage by producing MMP-3, which we found to be upregulated in AAG gastric corpus mucosa, as it has been demonstrated in other immune-mediated gastrointestinal disorders, namely ulcerative colitis (24). Indeed, further studies are being conducted in our laboratory to assess the function of mucosal plasma cells in AAG.

Although in our study, the overall population of B cells was unchanged in AAG gastric mucosa, their expression of IgD was reduced, and this may be a sign of B-cell maturation, or it may just reflect an impaired immune response (25). The reduced number of CD11c+ LPMC infiltrating the AAG gastric corpus is consistent with the previous finding of mucosal dendritic cell depletion in a mouse model of AAG, especially in case of more severe mucosal damage (26). Regarding CD56+ LPMC, their number did not differ between AAG and HC. Again, a similar finding was reported in an experimental AAG model, in which AAG mice had similar levels of nonactivated natural killer cells in comparison with non-AAG mice (27). Instead, in our study, CD56+ cells were increased in HP compared with AAG, but not to HC. In the only study conducted in humans, natural killer cells did not differ between 6 HCs and 6 patients with HP, while no data are available regarding human AAG (28).

Similar to previous experimental AAG models (7–11), also in our study, some proinflammatory cytokines were found to be increased in the gastric mucosa of patients with AAG, namely TNF-α and IL-15. It is already known that TNF-α is implicated in acute gastric epithelial injury through the activation of the arachidonic acid pathway at the epithelial level (29). Moreover,

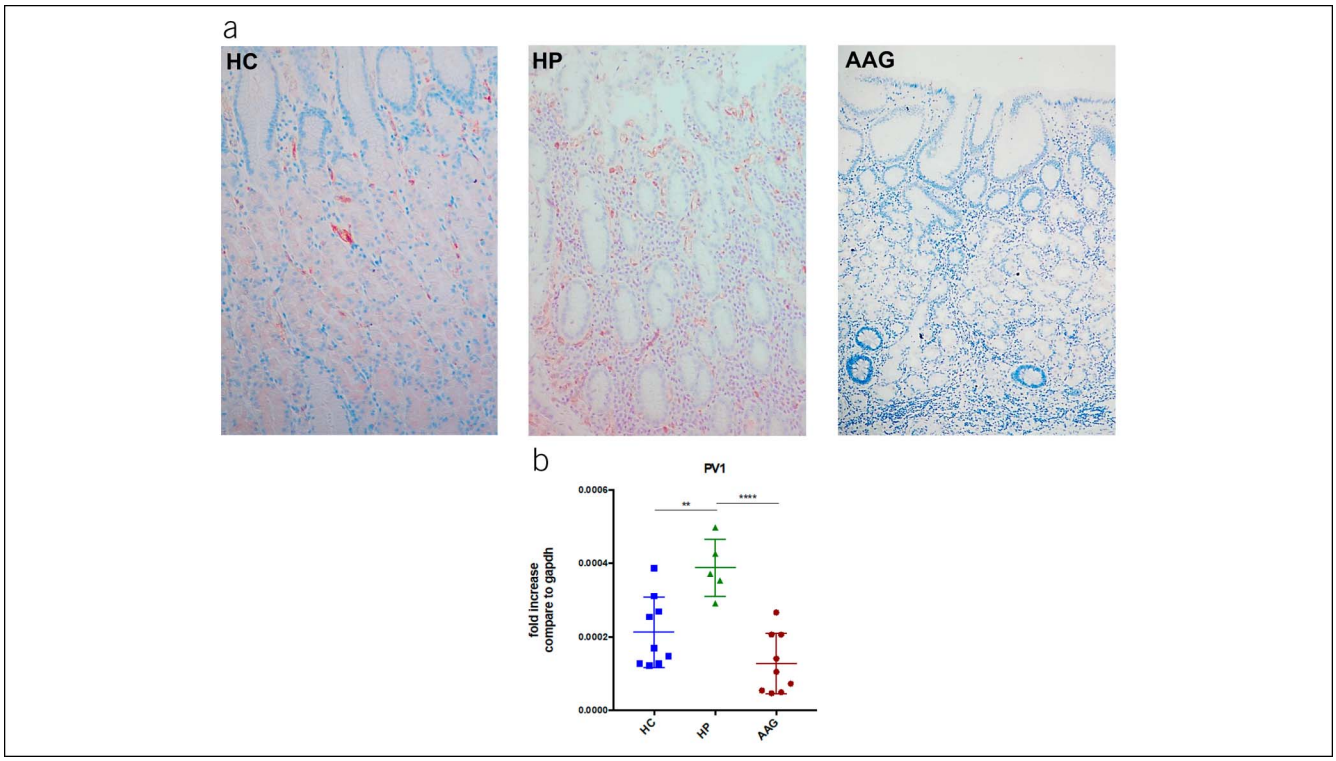

Figure 7. (**a**) Immunohistochemical detection of plasmalemma vesicle 1 (PV1) in gastric corpus of a representative patient with autoimmune atrophic gastritis (AAG), healthy control (HC), or patient with *Helicobacter pylori* gastritis (HP). PV1 is mildly expressed in HC, overexpressed in HP, and almost absent in AAG. Results are representative of 12 HC, 12 HP, and 12 AAG. (**b**) Mucosal transcript levels of PV1 in 9 HCs, 9 patients with AAG, and 5 patients with HP.

TNF-α has been shown to promote the processes of chronic inflammation and carcinogenesis in the stomach (30). IL-15, which is a pleiotropic cytokine able to activate both the innate and adaptive immune branches, is known to trigger Ig production and to stimulate the proliferation of CD4+ and CD8+ T cells, thus promoting apoptosis (31). Although IL-15 has been widely studied in other inflammatory or immune-mediated conditions of the gastrointestinal tract (32,33), no data are available regarding its function in gastric disorders. A similar effect may be exerted in the stomach epithelium in AAG, and future studies are needed to confirm this assumption. IL-33 production instead was found to be markedly decreased in AAG, and this may be implicated in the progression of mucosal atrophy and even adenocarcinoma. Indeed, IL-33 is a pivotal alarmin, which promotes immunosurveillance against mucosal injury and carcinogenesis, that is normally expressed in the gastric mucosa of healthy individuals (34). Chronic inflammation may, over time, lead instead to IL-33 reduction, thus increasing the risk of progression to precancerous lesions, including gastric metaplasia, atrophy, and dysplasia.

TGF-β1 was found to be increased in AAG compared with HC. This datum, which is novel for human AAG, may point to this pleiotropic cytokine as a central player in the deep mucosal changes and tissue remodeling process occurring during the natural history of AAG (35). This cytokine has been found to promote inflammation and the development of gastric lesions in *H. pylori*-infected patients, including gastric adenocarcinoma (36). In AAG, TGF-β1 overproduction might be related to the florid extracellular matrix remodeling process occurring in the atrophic AAG mucosa. Notably, MMP-3, also known as stromelysin-1, was overexpressed in the mucosa of patients with AAG. MMP-3 has been found to promote several congenital, inflammatory, or degenerative conditions. Its role in AAG is currently unknown but may be related to the progression of gastric atrophy into cancer (37).

In agreement with what was found in a mice model of AAG,[12] TSLP was found to be overexpressed in AAG atrophic lesions. However, in the same study, mice that were genetically deficient of TSLPR also had a more severe form of AAG. The authors concluded that TSLP may act as an anti-inflammatory mechanism, which, however, is not enough for blocking the development of gastritis. Similarly, in humans, the upregulation of TSLP may be a physiological attempt to counteract the inflammation within the gastric mucosa. Indeed, this is supported by the finding that short TSLP was able to significantly reduce the production of TNF-α in the gastric biopsies of our patients. This mechanism could be exploited in future trials assessing potential AAG treatments.

Zinc-l-carnosine has already been shown to reduce inflammation in several conditions, including peptic ulcer disease and radiation mucositis (21). Previous studies showed that this compound may have pleiotropic properties, and in particular, it may have a direct effect on polymorphonuclear cells, inhibiting their proinflammatory action, and on gastric epithelial cells, by stimulating mucus production (38). Here, we have shown that zinc-l-carnosine has a deep anti-inflammatory effect by inhibiting most of the tested proinflammatory cytokines, including IFN-γ, TNF-α, IL-21, IL-6, and IL-15. Indeed, this effect, on both a short and long term, should also be tested *in vivo* in a clinical trial.

In this study, we have also identified the metabokine NAMPT as a novel pathway altered in AAG. NAMPT is secreted by many

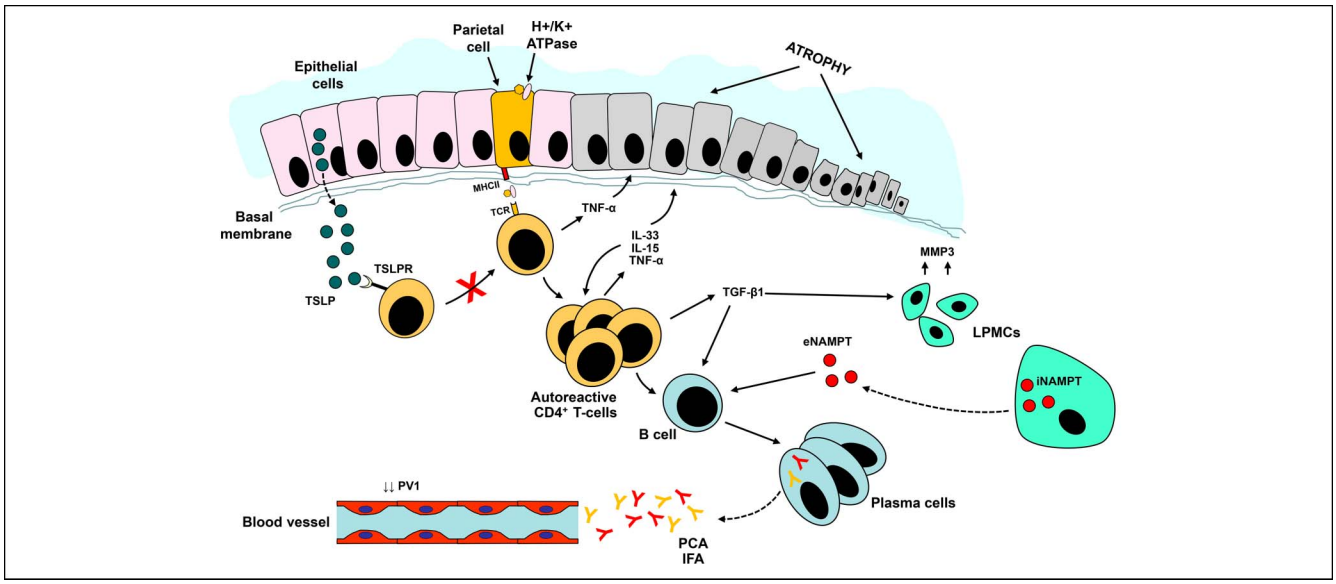

Figure 8. Summary of the potential pathogenetic mechanisms in autoimmune atrophic gastritis (AAG), according to the results found in the study. The oxyntic glands are made of different cell types including epithelial cells (e.g., mucous neck cells and chief cells) and parietal cells. The H+/K+ ATPase can be found on parietal cells, and its beta subunit constitutes the major autoantigen recognized by the T-cell receptor (TCR) located on T cells, through the major histocompatibility complex (MHC) II. Subsequently, autoreactive $CD4^+$ T cells produce tumor necrosis factor (TNF)-α, interleukin (IL)-33, and IL-15, all promoting a cascade which determines epithelial cell apoptosis and atrophy. In addition, autoreactive T cells also stimulate B cells that differentiate into plasma cells producing anti-parietal cell antibodies (PCAs) and anti-intrinsic factor antibodies (IFAs). Transforming growth factor (TGF)-β1 is also produced by T cells; it stimulates different lamina propria mononuclear cells (LPMCs) that in turn release matrix metalloproteinase 3 (MMP3), favoring fibrosis and tissue remodeling. Intracellular nicotinamide phosphoribosyltransferase (iNAMPT) is released by LPMCs and, in the form of extracellular NAMPT (eNAMPT), further enhances the B-cell immune response. The whole inflammatory process may potentially be counteracted by thymic stromal lymphopoietin (TSLP), which is produced by epithelial cells, acting on the TSLP receptor (TSLPR) located on T cells. Finally, the reduced expression of plasmalemma vesicle 1 (PV1) may lead to a lower gastric vascular permeability, maybe as a consequence of hypo-achlorhydria that favors bacterial overgrowth.

cell types, and it affects myeloid cell activation and differentiation (39,40). Indeed, our data indicate that NAMPT transcripts are higher in AAG compared with HC, and eNAMPT is overproduced *ex vivo* by AAG gastric biopsies. Notably, the incubation of HC gastric corpus biopsies with recombinant human eNAMPT induced the upregulation of some proinflammatory cytokines, such as IFN-γ and IL-6. These data prompt eNAMPT as a key cytokine directly involved in the regulation of the inflammatory niche in mucosal immunity.

Finally, we have here tested whether the gastric vascular barrier, as assessed by the expression of PV1, was altered in AAG. Interestingly, although we found that the gastric vascular barrier was disrupted in HP, as indicated by PV1 overexpression, in AAG it seemed to be less permeable. This finding may have different explanations. The vascular barrier has recently been implied in the systemic dissemination of bacteria from the intestine to the bloodstream (41). The local microbiota is believed to play a major role in maintaining the immunological homeostasis. In AAG, it has been recently found that hypochlorhydria is responsible for deep changes in the stomach microbiota (42). This may cause in turn the closure of the gastric vascular barrier as a mechanism of defense toward the systemic spreading of bacteria. Indeed, this assumption needs confirmation in future studies.

To summarize, on the basis of all our results, several inflammatory pathways, or regulatory molecules, including TNF-α, TSLP, and NAMPT, seem to have a crucial role in the development of AAG. A tentative, schematic representation of the possible contribution of the abovementioned pathways to the pathogenesis of AAG is shown in Figure 8. Indeed, it would be interesting in future studies to assess the same pathways in patients with potential AAG, which represent the very early, preatrophic phase of this disease.

Our study has some limitations that should be mentioned. We did not perform here a functional characterization of the LPMC nor we studied other cell types infiltrating the gastric mucosa, such as the eosinophils or mast cells. All our patients with AAG had severe gastric atrophy, which means that they had a severe disease stage, and hence, our data may not be transferable to the setting of mild or potential AAG. Nonetheless, our study also has several strengths. We have reported here for the first time in human AAG several immunological abnormalities, only some of which confirms the previous findings in mouse models, and which should be further investigated.

To conclude, AAG is an increasingly recognized condition worldwide, the pathogenesis of which remains elusive. We herein found several immunological alterations underlying AAG. The gastric mucosa in these patients is characterized by an increased number of plasma cells and by an exaggerated production of proinflammatory cytokines. TSLP and NAMPT may be future therapeutic targets, and zinc-l-carnosine could have a role in dampening mucosal inflammation. Further studies are needed to dissect more in depth the mechanisms causing AAG and to identify other potential therapeutic targets.

CONFLICTS OF INTEREST

Guarantor of the article: Antonio Di Sabatino, MD.
Statement of author contributions: All authors significantly participated in the drafting of the manuscript or critical revision of

the manuscript for important intellectual content and provided approval of the final submitted version. A.D.S., F.F., and M.V.L. designed the study. M.V.L., E.M., E.L., and A.D.S. enrolled and followed-up patients, collected and analyzed data. C.K. made and interpreted all statistical analyses. M.V.L. and F.F. collated, interpreted data, and wrote the manuscript. A.V., M.P., and G.A. reviewed all histopathological specimens. F.F., G.F., I.S., P.G., A.P., C.T., G.L., and M.R. made the experiment reported in the paper. A.D.S., G.R.C., E.L., and M.V.L. made critical revision of the manuscript for important intellectual contents. All other authors enrolled patients, collected data, and reviewed the paper.

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Potential competing interests: None to report.

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Study Highlights

WHAT IS KNOWN

- ✓ Autoimmune atrophic gastritis is a non-self-limiting, chronic, immune-mediated disorder of the stomach which predisposes to the development of gastric cancer.
- ✓ Immune mucosal alterations in patients with autoimmune atrophic gastritis have been poorly described.

WHAT IS NEW HERE

- ✓ Patients with autoimmune atrophic gastritis display in the gastric corpus mucosa an increased number of plasma cells, along with an increased expression of proinflammatory cytokines, transforming growth factor beta, and matrix metalloproteinase 3.
- ✓ Thymic stromal lymphopoietin and nicotinamide phosphoribosyl transferase are overexpressed, and gastric vascular barrier is altered.
- ✓ Zinc-l-carnosine exerts an anti-inflammatory action by reducing the expression of several cytokines.

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