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Plasma Cells in the Mucosa of Patients with Inflammatory Bowel Disease Produce Granzyme B and Possess Cytotoxic Activities

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In both Crohn’s disease (CD) and ulcerative colitis (UC), the gut is massively infiltrated with B cells and plasma cells, but the role of these cell types in the pathogenesis of gut tissue damage remains largely unknown. Human B cells express granzyme B (GrB) when cultured with IL-21, a cytokine overproduced in CD and UC mucosa. We therefore examined whether mucosal B cells express GrB and have cytotoxic activity in inflammatory bowel disease (IBD). GrB-expressing CD19+ and IgA+ cells were seen in the normal intestinal mucosa, but they were significantly more frequent in both CD and UC. In contrast, only a minority of CD19+ B cells were CD38high and CD20 negative. CD19+ B cells from IBD patients induced HCT-116 cell death. IL-21 enhanced GrB expression when cultured with IL-21, a cytokine overproduced in CD and UC mucosa. We therefore examined whether mucosal B cells express GrB and have cytotoxic activity in inflammatory bowel disease (IBD). GrB-expressing CD19+ and IgA+ cells were seen in the normal intestinal mucosa, but they were significantly more frequent in both CD and UC. In contrast, only a minority of CD19+ and IgA+ cells expressed perforin with no difference between IBD and controls. GrB-producing CD19+ cells expressed CD27 and were CD38high and CD20 negative. CD19+ B cells from IBD patients induced HCT-116 cell death. IL-21 enhanced GrB expression in control CD19+ B cells and increased their cytotoxic activity. These data indicate that IBD-related inflammation is marked by mucosal accumulation of cytotoxic, GrB-expressing CD19+ and IgA+ cells, suggesting a role for these cells in IBD-associated epithelial damage. *The Journal of Immunology, 2014, 192: 6083–6091.

The cause of Crohn’s disease (CD) and ulcerative colitis (UC), the major forms of inflammatory bowel disease (IBD), remains unknown (1). However, accumulating evidence suggests that IBD results from the interaction of genetic and environmental factors that ultimately promote an abnormal immune response in the gut, leading to organ damage (1). Dysregulation of various components of the immune system can be seen in the gut of patients with IBD, but hyperactivity of T cells with excessive production of cytokines is perhaps the major immunologic sign of these disorders (2). The abnormal T cell response is thought to be directed against components of the luminal bacterial flora and sustained by increased local proinflammatory cytokine production and by defects in counterregulatory mechanisms (1–3).

The inflamed gut of patients with CD or UC is massively infiltrated with B cells and IgA+ and IgG+ plasma cells, with a remarkable skewing toward IgG production, depending on the severity of inflammation (4–6). In UC, plasma cells also produce non–organ-specific Abs, such as perinuclear anti–cytoplasmic neutrophil Abs (7). UC patient sera also contain Abs against tropomyosin 5 (8), an Ag expressed by epithelial cells in the colon and other sites (e.g., biliary tract, eyes) (9). Of interest, Abs to tropomyosin may induce complement deposition and destruction of colonic epithelial cells (8). Nonetheless, the exact contribution of B cells and plasma cells in the pathogenesis of IBD remains unknown.

B cell depletion therapy with rituximab, a mAb depleting CD20-positive B cells, has been used with success in chronic inflammatory disorders characterized by excessive B cell/plasma cell activation, such as rheumatoid arthritis (10) and systemic lupus erythematosus (SLE) (11), Sjögren syndrome (12), dermatomyositis (13), and autoimmune hemolytic anemia (14). On the basis of this evidence, Leiper and colleagues (15) have recently performed a clinical trial with rituximab in active UC patients. Unfortunately, however, rituximab had no significant effect on inducing remission in steroid-unresponsive moderately active UC.

Upon activation, B cells can differentiate into various subpopulations, bearing specific surface markers and exhibiting either regulatory or inflammatory properties. For example, it has been recently demonstrated that some subsets of CD19+ B cells express granzyme B (GrB), exert cytotoxic activity against various cell types, and are abundant in the systemic circulation of SLE patients (16) and subjects vaccinated against viral infections (17). Differentiation of naive B cells into GrB-expressing B cells is promoted by CD4+ T cells, through a mechanism that is strictly dependent...

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M.L.C. performed experiments and flow cytometry studies, cultures with human cells, real-time PCR, and immunofluorescence, and contributed to writing the paper; M.S. performed experiments and flow cytometry studies and contributed to writing the paper; I. Marafini and E.F. contributed to isolating intestinal mucosal cells; A.O. and A.C. contributed to performing immunofluorescence studies; I. Monteleone and M.M.R. performed flow cytometry analysis; G.S. and P.S. provided mucosal specimens; R.C., E.P., and T.T.M. contributed to supervising parts of the project and to writing the paper; G.M. designed the research, analyzed data, supervised the project, and wrote the paper.

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; CD, Crohn’s disease; GrB, granzyme B; IBD, inflammatory bowel disease; LPMC, lamina propria mononuclear cell; SLE, systemic lupus erythematosus; UC, ulcerative colitis.

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on IL-21 (18). Because IL-21 is overproduced in the gut of patients with IBD (19), we hypothesized that, in IBD, some subsets of B cells/plasma cells can express GrB and have cytotoxic function.

Materials and Methods

Patients and samples

Mucosal specimens were taken from 11 patients with colonic CD and 7 patients with UC undergoing surgery for chronic active disease poorly responsive to medical treatment with steroids and/or immunosuppressive drugs. Paired mucosal samples were also taken from both involved and uninvolved areas of three patients with IBD. Controls included mucosal specimens taken from macroscopically and microscopically unaffected areas of 14 patients who underwent surgery for colon cancer. PBMCs were isolated from EDTA-stabilized peripheral blood samples of three CD patients, three UC patients, and three normal controls. Informed consent was obtained from all patients, and the study protocol was approved by the local ethics committee. At the time of surgery, one patient with CD was receiving mesalazine, three patients (one with CD and two with UC) were treated with mesalazine and corticosteroids, three CD patients were receiving corticosteroids and antibiotics, four patients (two CD and two UC) were treated with corticosteroids and anti-TNF Ab, four CD patients were treated with anti-TNF Ab, one UC patient was receiving immunosuppressive drugs and anti-TNF Ab, one patient with UC was on immunosuppressive drugs, and one UC patient was receiving no drugs.

Cell isolation and culture

All reagents were from Sigma-Aldrich (Milan, Italy) unless otherwise specified. The number of lamina propria mononuclear cells (LPMCs) isolated from mucosal specimens, as previously described (20), ranged from 10 to 50 × 10^6. LPMCs were resuspended (1 × 10^8/ml) in RPMI 1640 supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml; Lonza, Milan, Italy). LPMCs were used to purify CD19+, CD4+, and CD56+ cells using commercial isolation kits according to the manufacturer’s instruction (Miltenyi Biotec, Bergish Gladbach, Germany). Purity of the resulting cell preparations, checked by flow cytometry, was >85%. CD19+, CD4+, and CD56+ cells were cocultured with the human HCT-116 epithelial colon cancer cell line (American Type Culture Collection) labeled with CFSE (ratio of HCT-116/CD19+ or CD4+ or CD56+, 1:10) for 12 h. Apoptosis was then evaluated by flow cytometry using a commercially available Ab against Annexin V and 7-aminoactinomycin D (7-AAD). To examine the effect of IL-21 on the differentiation of GrB-expressing cells, intestinal CD19+ cells isolated from controls were cultured with IL-21 (25 ng/ml, 50 ng/ml, and 100 ng/ml; Life Technologies, Milan, Italy). IL-23 (50 ng/ml; R&D Systems, Minneapolis, MN), TNF-α (25 ng/ml; R&D Systems) and IFN-γ (100 ng/ml; Peprotech, Hamburg, Germany) for 48 h and then analyzed by flow cytometry. In parallel, an aliquot of cells was washed and cocultured in the presence of HCT-116 for a further 12 h. The percentage of IgA+ and GrB+ cells was assessed by flow cytometry.

To examine whether GrB-expressing CD19+ cells are killed by rituximab, IBD and control LPMCs were cultured with rituximab/MabThera (2 μg/ml; Roche, Milan, Italy) or control IgG for 48 h, and then the fraction of GrB-expressing CD19+ cells was evaluated by flow cytometry.

Flow cytometry

Cells were immunostained with the following anti-human mAbs: FITC anti-CD21, FITC, PerCP 5.5 and allophycocyanin-H7 anti-CD19, FITC anti-CD20, allophycocyanin-H7 anti-CD45,PeCy7 anti-CD5,PeCy7 anti-CD27, PerCP Cy5.5 anti-CD38, V500 anti-CD27, PE Annexin V, 7-AAD (all from Becton Dickinson, Milan, Italy); FITC anti-IgA (Miltenyi Biotec) and FITC Annexin V (ImmunoTools, Friesoythe, Germany); and Alexa Fluor 647 anti-perforin, PE anti-granzyme B (Life Technologies, Monza, Italy). In all experiments, appropriate isotype control IgG (Becton Dickinson) was used. All Abs were used at 1:50 final dilution. For intracellular immunostaining, cells were fixed and permeabilized using IC Fixation Buffer and Permeabilization Buffer (both from e Bioscience, San Diego, CA) according to the manufacturer’s instructions. Cells were analyzed by flow cytometry (FACSVerse and FACSComp, both from Becton Dickinson). Analysis was performed by acquiring 10,000 events in the gate of CD45+ cells.

RNA extraction, cDNA preparation, and real-time PCR

RNA was extracted using QIAGEN’s RNA Mini Kit (QIAGEN, Milan, Italy), according to the manufacturer’s instruction. A constant amount of RNA (0.5 μg) was retrotranscribed into cDNA, and this was amplified using a SYBR Green Master Mix (Bio-Rad, Milan, Italy) with the following conditions: denaturation for 1 min at 95°C, annealing for 30 s at 60°C for β-actin, and 58°C for GrB, followed by 30 s of extension at 72°C. Primer sequence was compared as follows: GrB: FWD 5’-CAGTACCATGGAT-TGTCG-3’ and REV 5’-GCCTTGGATTCCAATGAC-3’; β-actin: FWD 5’-AAATGACCACATGTATGTTGAGACC-3’ and REV 5’-AGCCAGCTCCAGCGAGAT-3’. β-Actin was used as a housekeeping gene, and gene expression was calculated using the ΔΔCt algorithm.

Immunofluorescence

Immunofluorescence was performed on frozen sections of mucosal samples taken from three non-IBD controls, three CD patients, and three UC patients. Tissue sections were embedded in a cryostat-mounting medium (Neg-50 Frozen Section Medium; Thermo Scientific, Langenselbold, Germany), snap frozen, and stored at −80°C. Samples were cut, mounted onto Superfrost Plus glass slides (Thermo Scientific), and immunostained with anti-human GrB (1/100 final dilution; Abcam, Cambridge, U.K.) followed by incubation with a highly sensitive biotinylated secondary Ab (1/200 final dilution; Dako Glostrup, Denmark) and Tyrinade Signal Amplification Kit (PerkinElmer, Waltham, MA). Following extensive washing, sections were immunostained with anti-human IgA-FITC (1/100 final dilution; Dako Glostrup). The nuclei were counterstained with Hoechst 33422 (1/2000 final dilution; Life Technologies). Additional frozen sections of mucosal samples taken from three non-IBD controls, three CD patients, and three UC patients were immunostained with anti-human CD19 (1/25 final dilution, Becton Dickinson), followed by incubation with Alexa Fluor 488-conjugated secondary Ab (1/1000 final dilution; Life Technologies, Monza, Italy). The nuclei were counterstained with Hoechst 3342 (1/2000 final dilution; Invitrogen). Isotype control sections were prepared under identical conditions, replacing the primary Ab with a purified normal mouse IgG2a control Ab (R&D Systems).

Statistical analysis

Differences between groups were compared using the Student t test or Mann–Whitney U test whether data were either normally or not normally distributed.

Results

Granzyme B–expressing CD19+/IgA+ cells are abundant in the gut of IBD patients

To examine whether intestinal mucosal B cells express GrB or perforin (well-known cytotoxic molecules) (21, 22), LPMCs isolated from intestinal mucosal samples of IBD patients and controls were immunostained for CD19, GrB, and perforin, and then analyzed by flow cytometry. GrB-expressing CD19+ B cells were seen in all the LPMCs samples, but their percentage was significantly increased in LPMCs isolated from both CD and UC patients compared with controls (Fig. 1A). In IBD, the fraction of GrB-expressing CD19+ B cells was increased in LPMCs isolated from involved mucosal areas (23.3 ± 7), compared with cells isolated from uninvolved areas (15 ± 4), and no difference was noted between patients receiving mesalazine and those taking other drugs (i.e., steroids, immunosuppressive drugs, or anti-TNF) (not shown). In contrast, few perforin-expressing CD19+ cells were seen in colonic LPMCs, with no significant difference between IBD and controls (Fig. 1B). To determine whether, in IBD, GrB-expressing CD19+ cells are present exclusively in the gut, PBMCs were isolated from IBD patients and controls and analyzed for GrB and perforin. The percentages of CD19+ cells expressing either GrB or perforin did not differ between IBD and controls (Fig. 1C, 1D). Similarly, the percentages of CD19+ LPMCs and PBMCs coexpressing GrB and perforin in IBD were not different from those in controls (Supplemental Fig. 1A, 1B). The percentages of total CD19+ cells and IgA+ cells, as determined by flow cytometry, were 6.5 ± 2 and 1.65 ± 0.6, respectively, in non-IBD controls, 8.5 ± 1 and 5 ± 1, respectively, in CD, and 11.3 ± 5 and 5 ± 3, respectively, in UC. The number of IgA+ and CD19+ expressing cells in the intestinal lamina propria was also evaluated by immunofluorescence assay (Supplemental Fig. 2A, 2B).
Next, we analyzed GrB RNA expression in CD19+ cells purified from IBD and control LPMCs. GrB transcripts were significantly increased in both CD and UC, compared with controls (p < 0.01) (Supplemental Fig. 3).

IBD and control LPMCs were also immunostained for IgA, GrB, and perforin and then analyzed by flow cytometry. The percentage of GrB-expressing IgA+ cells was increased in the colonic mucosa of both CD and UC patients compared with controls (Fig. 2A).

Moreover, in IBD, the percentage of GrB-expressing IgA+ cells was increased in inflamed areas (16.6 ± 11) compared with uninflamed areas (11 ± 10). In contrast, a small number of IgA+ LPMCs expressed perforin, with no significant differences among the groups (Fig. 2B). Double immunofluorescence analysis of colonic tissues showed that GrB-expressing IgA+ cells were more frequent in the intestinal mucosa of CD and UC patients, compared with controls (Fig. 2C).
Altogether, these data indicate that intestinal CD19⁺/IgA⁺ cells express GrB, and such cells are abundant in the inflamed mucosa of IBD patients.

**GrB-expressing CD19⁺ cells are CD38high and CD27⁺**

Next we characterized the phenotype of GrB-expressing IgA⁺ and CD19⁺ cells. LPMCs were immunostained for CD21, CD5, CD27, and CD38 and the fractions of positive cells analyzed by flow cytometry. Nearly all the GrB-expressing CD19⁺ cells isolated from IBD patients expressed CD27 and CD38 and, to a lesser extent, CD5 and CD21 (Fig. 3A). Further analysis revealed that GrB-expressing CD19⁺ cells were CD38high and CD27⁺ in both CD (Fig. 3B) and UC (Fig. 3C) as well as in non-IBD (Fig. 3D). Because mucosal plasma cells are usually identified as CD38high and CD27⁺ cells by flow cytometry, our data indicate that GrB-expressing CD19⁺ B cells are plasma cells.

**CD19⁺ cells from IBD patients promote intestinal epithelial cell apoptosis**

To determine whether GrB-expressing CD19⁺ cells are cytotoxic, we cocultured CFSE-labeled HCT-116 cells with CD19⁺ LPMCs purified from IBD LPMCs. Because IBD CD19⁺ LPMCs express low levels of perforin, we measured target cell death not by standard 51Cr release assay, but rather by Annexin V/7-AAD immunostaining and flow cytometry analysis. This method allows detection of apoptosis in target cells, in contrast to the 51Cr release assay, which detects rapid perforin-mediated lysis. We found that IBD CD19⁺ cells, but not non-IBD CD19⁺ cells, significantly...
CD19+ cells were purified from control LPMCs and cultured in the presence or absence of increasing doses of IL-21. The expression of GrB was then assessed by flow cytometry. IL-21 dose-dependently enhanced the percentage of GrB-expressing CD19+ cells (Fig. 6A). By contrast, no significant change in the fraction of GrB-expressing CD19/IgA-positive cells was seen following stimulation with IL-23, TNF-α, and IFN-γ (not shown). Next we assessed whether IL-21 enhanced the ability of CD19+ LPMCs to kill intestinal epithelial cells. To this end, CD19+ cells were purified from control LPMCs and cultured in the presence or absence of IL-21. After 48 h, cells were washed and cocultured with HCT-116 for a further 12 h. CD19+ LPMCs cultured with IL-21 showed a greater ability to induce epithelial cell apoptosis than did cells cultured in the absence of IL-21 (Fig. 6B).

Discussion

Recent studies have shown that CD19+ B cells have the ability to secrete GrB under certain circumstances and to kill target cells (16–18). GrB is a 32-kDa protein released from cytotoxic cells via granule exocytosis, which initiates perforin-independent death in target cells by cleaving caspase-3, as well as by triggering additional cytotoxic pathways (26, 27). Because B cells recognize Ags in an MHC-independent manner, they are able to respond more rapidly than T cells, which require Ag presentation by professional APCs (28). Moreover, the variety of Ags potentially recognized by the BCR is broader than that of Ags recognized by the TCR, being not limited to peptides, but also including carbohydrates, nucleic acids, and other types of Ags (29). Therefore, B cells could play an important role in early cell-mediated immune responses during inflammatory and neoplastic processes (30, 31). As IBD-related inflammation is associated with intestinal epithelial damage (3), in this article we examined whether B cells/plasma cells infiltrating the IBD mucosa express GrB and are able to kill epithelial cells.

Our data show that GrB-expressing CD19+ cells are present in the intestinal lamina propria of normal controls, and their frequency is markedly increased in inflamed mucosa of patients with IBD. In particular, we evaluated whether these cells express CD20 and are sensitive to rituximab. Flow cytometric analysis of IBD intestinal LPMCs revealed that the majority of GrB-expressing CD19+ cells were CD20+ (Fig. 5A). Treatment of LPMCs with rituximab did not change the fractions of GrB-expressing CD19+ cells in both IBD and control samples (Fig. 5B–D).

IL-21 enhances the ability of GrB-expressing cells to induce intestinal epithelial cell death

Activation and differentiation of B cells and plasma cells are tightly regulated by T cell-derived cytokines (23). One such cytokine is IL-21, which is considered a key regulator of B cell and plasma cell activity (24). Recently, it was demonstrated that, under certain circumstances, IL-21 can induce CD19+ cells to produce GrB (17, 18). Because IL-21 is overproduced in the inflamed intestine of patients with IBD (19, 25), we evaluated whether GrB-expressing intestinal B cells/plasma cells were regulated by IL-21. Initially, we purified CD19+ LPMCs from the intestine of non-IBD patients and cultured them in the presence or absence of increasing doses of IL-21. The expression of GrB was then assessed by flow cytometry. IL-21 dose-dependently enhanced the percentage of GrB-expressing CD19+ cells (Fig. 6A). By contrast, no significant change in the fraction of GrB-expressing CD19/IgA-positive cells was seen following stimulation with IL-23, TNF-α, and IFN-γ (not shown). Next we assessed whether IL-21 enhanced the ability of CD19+ LPMCs to kill intestinal epithelial cells. To this end, CD19+ cells were purified from control LPMCs and cultured in the presence or absence of IL-21. After 48 h, cells were washed and cocultured with HCT-116 for a further 12 h. CD19+ LPMCs cultured with IL-21 showed a greater ability to induce epithelial cell apoptosis than did cells cultured in the absence of IL-21 (Fig. 6B).

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CD or UC. Analysis of GrB RNA transcripts in purified CD19+ LPMCs confirmed the abundance of these cells in IBD, raising the possibility that GrB expression in B cells may be also regulated at the transcriptional level. Stimulation of normal LPMCs with IL-21 increased the fractions of GrB-expressing CD19+ cells. It is thus conceivable that IL-21 is one of the positive regulators of GrB-expressing CD19+ cell differentiation in IBD, as this cytokine is overproduced in both CD and UC tissues (19).

Phenotypic analysis of intestinal GrB-expressing CD19+ cells revealed that almost all these cells express CD27 and are CD38 high, thus resembling the phenotype of plasma blasts/plasma cells. The demonstration that IBD plasma cells can produce additional molecules other than Iggs is supported by the fact that in IBD, plasma cells secrete matrix metalloproteinases, a family of proteases involved in tissue damage and remodeling (32).

Only a small percentage of GrB-expressing CD19+ LPMCs expressed CD5. This result contrasts with data from a recent study in patients with SLE showing that the majority of GrB-expressing CD19+ PBMCs are CD5+ (16). The reason for this apparent discrepancy remains unknown but could reflect differences in the type of B cells analyzed (e.g., blood versus mucosal cells) as well as in the factors/mechanisms that regulate GrB-expressing CD19+ cell differentiation.

CD19+ cells purified from IBD LPMCs induced HCT-116 cell apoptosis in a dose-dependent manner. Moreover, HCT-116 cell death was induced by normal CD19+ LPMCs cultured in the

**FIGURE 4.** IBD CD19-positive cells induce death of colon cancer cells. (A–C) CD19+ cells, purified from intestinal LPMCs of five patients with CD (A), four patients with UC (B), and four normal controls (non-IBD; C), were cocultured with CFSE-labeled HCT-116 cells at a 1:10 ratio (HCT-116/CD19+ cells) for 12 h. Cells were then analyzed for the expression of Annexin V (AV) and 7-AAD by flow cytometry. Histograms show the mean ± SD of the percentages of AV- and/or 7-AAD-positive HCT-116 cells cultured in the presence of medium or intestinal CD, UC, or non-IBD CD19+ cells. Right inset, Representative dot plots showing the percentage of AV- and/or 7-AAD-positive HCT-116 cells cultured in the presence of medium or intestinal CD, UC, or non-IBD CD19+ cells. Numbers in the quadrants indicate the percentage of positive cells. (D) Histograms show the mean ± SD of the percentages of AV- and/or 7-AAD-positive HCT-116 cells cultured in the presence of equivalent numbers of intestinal CD, UC or non-IBD CD19+ cells. Statistical analysis was performed comparing the total number of dead cells (one-tailed Student t test, *p < 0.05).
presence of IL-21, thus supporting the role of this cytokine in favoring GrB-expressing CD19+ cell differentiation. We are aware that studies in mouse models of colitis could help confirm the role of CD19+ cells in the pathogenesis of epithelial damage, but unfortunately murine B cells do not produce GrB (33).

Our data also show that few CD19+ LPMCs express perforin with no apparent difference between IBD and controls. This finding could appear surprising, as GrB-induced target cell death by NK cells and CD8+ lymphocytes is strictly dependent on perforin (34, 35). However, it has been demonstrated that GrB may...
enter the target cell by a perforin-independent pathway (36). Thus, the inability of CD19+ B LPMCs to produce high levels of perforin would not preclude their cytotoxic function. It remains, however, unclear how GrB produced by CD19+ LPMCs reaches the target cell cytoplasm in the absence of perforin, even though evidence exists that various classes of proteins (i.e., heat shock proteins) may mediate the uptake and endolysosomal release of GrB into the target cell (26).

The ability of intestinal CD19+ cells to produce GrB could have further implications in our understanding of mucosal homeostasis and IBD pathogenesis because B cells could exert cytotoxic effects toward additional cells other than epithelial cells (e.g., regulatory T cells, effector T cells, NK cells, dendritic cells), thus contributing to either suppression or amplification of inflammatory signals. In this context, it would be also noteworthy that GrB is produced not only by NK cells, CD8+ T lymphocytes, and B cells, but also by plasmacytoid dendritic cells, mast cells, basophils, and CD34 hematopoietic progenitor cells (26). Consequently, the spectrum of functions exhibited by GrB is much more diverse than originally thought and includes cytokine-like effects, immunosuppressive effects, Ag processing, matrix degradation, and cleavage of autoantigens.

In conclusion, this is, to our knowledge, the first study to show that intestinal plasma cells express GrB and have the ability to kill epithelial cells, thus suggesting their involvement in IBD-associated intestinal epithelial damage.

Disclosures
The authors have no financial conflicts of interest.

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