Background: Celiac disease (CD) is an immune-mediated disorder characterized by an accumulation of immune cells in the duodenal mucosa as a consequence of both adaptive and innate immune responses to undigested gliadin peptides. Mast cells (MCs) are innate immune cells that are a major source of costimulatory signals and inflammatory mediators in the intestinal mucosa. Although MCs have previously been associated with CD, functional studies have never been performed.

Objective: We aimed at evaluating the role of MCs in the pathogenesis of CD.

Methods: Intestinal biopsy specimens of patients with CD were scored according to the Marsh classification and characterized for leukocyte infiltration and MC distribution. Moreover, MC reactivity to gliadin and its peptides was characterized by using in vitro assays.

Results: Infiltrating MCs were associated with the severity of mucosal damage, and their numbers were increased in patients with higher Marsh scores. MCs were found to directly respond to nonimmunodominant gliadin fragments by releasing proinflammatory mediators. Immunohistochemical characterization of infiltrating MCs and the effects of gliadin peptides on intestinal MCs indicated an increase in proinflammatory MC function in advanced stages of the disease. This was also associated with increased neutrophil accumulation, the prevalence of M1 macrophages, and the severity of tissue damage.

Conclusion: We provide a description of the progressive stages of CD, in which MCs are the hallmark of the inflammatory process. Thus the view of CD should be revised, and the contribution of MCs in the onset and progression of CD should be reconsidered in developing new therapeutic approaches. (J Allergy Clin Immunol 2017;139:1266-74.)

Key words: Celiac disease, mast cell, gliadin immunology, p31-43 fragment

Among immune-mediated diseases of the gastrointestinal tract, celiac disease (CD) is the prototypical model in which an antigen-specific immune response is entwined with the occurrence of mucosal damage driven by a T-cell receptor–biased T-cell response.Triggering by dietary gluten also involves the participation of innate immune cells, such as macrophages, dendritic cells, and granulocytic myeloid cells, and the instruction of T cell–dependent or T cell–independent B-cell responses. In such a scenario, a proinflammatory skewing of the cytokine milieu plays a role in priming immune effector cells and through bystander cell activation. The pathogenesis of tissue damage in patients with CD is related to cooperation between genetic and environmental determinants. However, the contribution of bystander immune elements in the induction and maintenance of tissue damage in patients with CD is unknown. These conditions share with CD the same immune cell infiltration pattern, although some differences in the topography of T-cell infiltrates have been reported. This suggests that the mechanisms related to jejunal mucosa damage in patients with these disorders might have common traits in terms of the quality and role of the infiltrating cells, despite potentially relevant differences in T-cell receptor recognition biases.

In this pathologic setting the actual contribution of diverse subsets of innate immune cells, including neutrophils, eosinophils, and mast cells (MCs), to the discrete phases of mucosal damage remain to be explained. Among these cells, MCs, which are classically considered mediators of IgE-dependent allergic responses, recently acquired a new role as pleiotropic cells acting at the interface between innate and adaptive immunity and in promoting and enhancing the effector capacity of T and B lymphocytes, as well as suppressing inflammatory responses, thus endorsing a regulatory role. MCs are present in the intestinal mucosa, and they are a major source of costimulatory signals and inflammatory mediators. Previous retrospective studies focusing on the accumulation of MCs in the intestinal mucosa of patients with CD produced conflicting results, which limited our knowledge of their involvement in CD pathobiology.

In this study intestinal biopsy specimens of patients with CD with a histologic diagnosis of jejunal mucosal damage were scored according to the Marsh classification and analyzed for the
density and distribution of immune cell subsets. We found that histologic damage progression was associated with the density of infiltrating MCs when expression of the overlying inflammatory MC phenotype and skewing of myeloid populations toward a T\textsubscript{H}1-polarizing environment were also observed. Notably, we demonstrated in vitro that MCs could be activated by the nonimmunogenic gliadin peptide p\textsubscript{31-43}.

Our results unveil a new role for MCs in shaping the inflammatory microenvironment in gluten-induced enteropathy in the onset, development, and resolution of the disease.

**METHODS**

**Reagents**

Gliadin peptides p\textsubscript{a-9} (p57-68) QLQPFPQPQLPY, p\textsubscript{e-2} (p62-75) PQQPQLPYQQPQLPY, p\textsubscript{31-43} LGQQQPFPPQQPY, and its respective N-terminal 5(6)-carboxyfluorescein homologue were synthesized by Primm (Milan, Italy). Peptides were used at 100 µg/mL final concentration. Gliadin and glutenin of wheat and rice were gifted by Domenico Lafiandra (University of Turin, Italy). Soybean proteins were from Sigma (St Louis, Mo). Recombinant human stem cell factor and mouse IL-3 were from PeproTech (London, United Kingdom).

**Patients and control subjects**

Four to 6 biopsy specimens were obtained from the distal duodenum during upper gastrointestinal endoscopy from 28 adult patients undergoing diagnostic protocols at the University Hospital of Palermo. The CD diagnosis was established according to standard criteria, including HLA genotyping, anti-TG2 serum titer measurement, and histologic analysis of small intestinal biopsy specimens.\textsuperscript{11} Intestinal biopsy specimens were evaluated for villous architecture, crypt height, and intraepithelial lymphocytes and scored according to the Marsh classification modified by Oberhuber et al.\textsuperscript{12} Control samples with normal intestinal histology were obtained from 4 subjects without CD undergoing biopsies for screening procedures for abdominal symptoms and excluded for CD diagnosis. Informed consensus was obtained in accordance with regulations and previous approval of the local ethics committee. Two biopsy specimens were used for histologic examination, and the others were used for in vitro experiments.

**Immunohistochemistry and immunofluorescence**

Immunohistochemistry was performed with the polymer detection kit (Novocastra, Newcastle upon Tyne, United Kingdom) and the following mouse primary antibodies form Novocastra: anti-human CD3, anti-human CD4, anti-human CD8, anti-human CD20 (Dako, Glostrup, Denmark), anti-human CD138, anti-human FOXP3 (Abcam, Cambridge, United Kingdom), anti-human myeloperoxidase, anti-human IgG, anti-human IgA, anti-human IL-17 (R&D Systems, Minneapolis, Minn), and anti-human MC tryptase (Dako). Aminoethylcarbazole (Dako) was used as a chromogranic substrate. Slides were evaluated under a Leica DM3000 optical microscope, and microphotographs were collected with a Leica DFC320 digital camera (Leica, Wetzlar, Germany). Intraepithelial CD3 lymphocytes every 100 epithelial cells were counted within villous or epithelial surfaces. All other cells were detected and counted out of 5 × 40 high-power microscopic fields in each case.

For double-marker immunofluorescence, sections underwent 2 sequential rounds of single-marker immunostaining. The following antibodies were used: anti-human IL-6 (R&D Systems), anti-human IL-17, anti-human tryptase, anti-human CD23 (Novocastra), anti-human CD68 (Novocastra), and anti-human arginase (Rabbit Polyclonal; GeneTex, Irvine, Calif). Alexa Fluor 488– and Alexa Fluor 568–conjugated specific secondary antibodies were used.

**MC cultures**

The human MC line LAD2, which closely resembles human CD34\textsuperscript{+}-derived MCs,\textsuperscript{13} was grown in serum-free medium StemPro-34 (Invitrogen, Carlsbad, Calif) containing 100 ng/mL human stem cell factor and periodically tested for c-Kit and Fc\textsubscript{ε}RI expression by using flow cytometry (FACScan; Becton Dickinson, San Diego, Calif).

Bone marrow–derived mast cells (BMMCs) were obtained by means of in vitro differentiation of bone marrow from wild-type (WT) or myeloid differentiation primary response gene--88 (MyD88)--deficient C57BL/6 mouse cells, as previously described.\textsuperscript{14}

**Degranulation response and cytokine production**

Cells (1 × 10\textsuperscript{7}/mL) were incubated in Tyrode buffer (10 mM/L HEPES buffer [pH 7.4], 130 mM/L NaCl, 5 mM/L KCl, 1.4 mM/L CaCl\textsubscript{2}, 1 mM/L MgCl\textsubscript{2}, 5.6 mM/L glucose, and 0.1% BSA) for 30 minutes at 37°C with 100 µg/mL glutenin, gliadin, soya proteins, or gliadin fragments or left untreated to assess degranulation response as a percentage of β-hexosaminidase release. We used 16 mM/L phorbol 12-myristate 13-acetate and 1 µmol/L 1-iodomycin as positive controls to induce MC degranulation. The enzymatic activity of the released β-hexosaminidase was assessed, as previously published.\textsuperscript{15}

Cytokine levels in cell supernatants after 24 hours of incubation with each peptide were measured with a Human Inflammatory Cytokine CBA Assay and a CBA Mouse T\textsubscript{H}1/T\textsubscript{H}2 Cytokine Kit (Becton Dickinson) according to the manufacturer’s instructions. Human CCL\textsubscript{2}, murine IL-6, IL-17, and monocyte chemoattractant protein 1 were evaluated by using specific ELISAs (eBioscience, San Diego, Calif). Human IL-17 ELISA was from Ray Biotech (Norcross, Ga). In some experiments 10 µmol/L of the antioxidant N-acetyl cysteine (NAC) or the nuclear factor-κB (NF-κB) inhibitors L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, 25 µmol/L) and pyrrolidinedithiocarbamate (PDTC; 10 µmol/L) were added to MCs 30 minutes before challenge with p\textsubscript{31-43}. Both inhibitors were from Sigma-Aldrich and were previously used to assess gliadin-induced NF-κB activation.\textsuperscript{15}

**p31-43 binding and competition assay**

LAD2 cells were incubated with fluorescein-labeled peptide for 30 minutes at room temperature, washed, and analyzed by using flow cytometry for positivity on the FL1-H channel. LAD2 cells were preincubated with increasing amounts (1 × and 50 ×, respectively) of unlabeled p\textsubscript{31-43} (comp) and then challenged with the labeled peptide to ensure binding specificity of p\textsubscript{31-43}. Data were acquired on a FACScan (Becton Dickinson) and analyzed with FlowJo software (TreeStar, Ashland, Ore), and the mean fluorescence intensity (MFI) for each condition was calculated. The mean MFIs from 4 independent experiments are shown.

**Evaluation of intracellular reactive oxygen species production by using flow cytometry**

Intracellular reactive oxygen species (ROS) were detected by incubating LAD2 cells with 5 µmol/L 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein (CM-H\textsubscript{2}DCFDA) and incubating for 30 minutes at 37°C in 1 mol/L HC\textsubscript{2}, 5.6 mol/L glucose, and 0.1% BSA for 30 minutes at 37°C with 100 µg/mL glutenin, gliadin, soya proteins, or gliadin fragments or left untreated to assess degranulation response as a percentage of β-hexosaminidase release. We used 16 nmol/L phorbol 12-myristate 13-acetate and 1 µmol/L 1-iodomycin as positive controls to induce MC degranulation. The enzymatic activity of the released β-hexosaminidase was assessed, as previously published.\textsuperscript{13}

Cytokine levels in cell supernatants after 24 hours of incubation with each peptide were measured with a Human Inflammatory Cytokine CBA Assay and a CBA Mouse T\textsubscript{H}1/T\textsubscript{H}2 Cytokine Kit (Becton Dickinson) according to the manufacturer’s instructions. Human CCL\textsubscript{2}, murine IL-6, IL-17, and monocyte chemoattractant protein 1 were evaluated by using specific ELISAs (eBioscience, San Diego, Calif). Human IL-17 ELISA was from Ray Biotech (Norcross, Ga). In some experiments 10 µmol/L of the antioxidant N-acetyl cysteine (NAC) or the nuclear factor-κB (NF-κB) inhibitors L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, 25 µmol/L) and pyrrolidinedithiocarbamate (PDTC; 10 µmol/L) were added to MCs 30 minutes before challenge with p\textsubscript{31-43}. Both inhibitors were from Sigma-Aldrich and were previously used to assess gliadin-induced NF-κB activation.\textsuperscript{15}

**Abbreviations used**

BMMC: Bone marrow–derived mast cell
CD: Celiac disease
CM-H\textsubscript{2}DCFDA: 5-(And-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester
Foxp3: Forkhead box p3
MC: Mast cell
MFI: Mean fluorescence intensity
MyD88: Myeloid differentiation primary response gene--88
NAC: N-acetyl cysteine
NF-κB: Nuclear factor κB
ROS: Reactive oxygen species
TLR: Toll-like receptor
Treg: Regulatory T
WT: Wild-type

**MC cultures**

The human MC line LAD2, which closely resembles human CD34\textsuperscript{+}-derived MCs,\textsuperscript{13} was grown in serum-free medium StemPro-34 (Invitrogen, Carlsbad, Calif) containing 100 ng/mL human stem cell factor and periodically tested for c-Kit and Fc\textsubscript{ε}RI expression by using flow cytometry (FACScan; Becton Dickinson, San Diego, Calif).

Bone marrow–derived mast cells (BMMCs) were obtained by means of in vitro differentiation of bone marrow from wild-type (WT) or myeloid differentiation primary response gene--88 (MyD88)--deficient C57BL/6 mouse cells, as previously described.\textsuperscript{14}
Intestinal MC isolation and histamine release

Briefly, tissue fragments from intestinal biopsy specimens were incubated with 1.5 mg/mL collagenase and 0.75 mg/mL hyaluronidase in minimum essential medium containing 2% FCS for 70 minutes at 37°C. Dispersed cells were filtered through a polyamide Nybolt filter (pore size, 300 μm diameter) and suspended in minimum essential medium containing 10% FCS, 200 U/mL penicillin, and 200 μg/mL streptomycin. MC numbers were determined by means of light microscopy after cytocentrifugation and stained with a 0.5% toluidine blue at pH 1.16 Aliquots of 100 μL containing 5 × 10^3 MCs were incubated in Tyrode buffer with p31-43 for 30 minutes at 37°C. Reactions were terminated by the addition of 100 μL of ice-cold Tyrode buffer, and tubes were centrifuged immediately (500 g, 10 minutes, 4°C). For measurement of total histamine concentration, the cell suspension in some tubes was boiled for 9 minutes. Histamine levels were measured by means of ELISA, according to the manufacturer’s instructions (DRG Instruments GmBH, Marburg, Germany). All experiments were performed in triplicate. Histamine release induced by p31-43 was expressed as a percentage of total cellular histamine levels corrected for the spontaneous release measured in tubes in which cells had been incubated in Tyrode buffer alone.

Statistical analysis

Results are expressed as means ± SEMs. Data were analyzed with an ANOVA test by using the post hoc Bonferroni correction for paired multiple comparisons (Prism; GraphPad Software, La Jolla, Calif).

RESULTS

Severity of intestinal lesions of biopsy specimens from patients with CD is associated with increased MC infiltration

Twenty-eight adult patients with CD were enrolled to define the relationship between leukocyte infiltration and the degree of mucosal damage. Intestinal biopsy specimens were scored according to the Marsh classification modified by Oberhuber et al12: 10 patients classified as Marsh 1, 9 patients classified as Marsh 2, and 9 patients classified as Marsh 3 underwent immunohistochemical analysis of leucocyte microinfiltrate, as well as inflammatory products (Fig 1, A), and were associated with the degree of disease severity (Fig 1, B).

The extent of histologic tissue damage was significantly associated with the increase in total numbers of CD3^+ cells (P = .001). Indeed, the number of CD3^+ T cells in the lamina propria was significantly increased in patients with CD with high Marsh scores (P = .0006); however, no significant association was found between numbers of intraepithelial lymphocytes positive for CD3 and numbers of CD4^+ or CD8^+ T cells and the severity of tissue lesions.

Conversely, noticeable and statistically relevant differences were found in the number and distribution of B lymphocytes (CD20^+ cells), plasma cells (CD138^+ cells), and B-cell products, such as IgA, in patients compared with healthy control subjects, with no association at different Marsh scores. Moreover, high numbers of myeloperoxidase-positive cells were detected in intestinal lesions of patients with an advanced state of disease but completely absent in patients with Marsh scores of either 1 or 2.

Staining for IL-17 expression was found to inversely correlate with T-lymphocyte infiltrate and disease severity, whereas a positive association was observed between forhead box p3 (Foxp3)^+ cells and a high Marsh score. Interestingly, the Marsh classification was directly associated with an increase in infiltrating tryptase-positive MC numbers (Fig 2). The majority of these cells were randomly distributed within the lamina propria (Fig 2, A), and their numbers increased in tissues with a higher Marsh score, where statistically significant differences occurred between patients with CD with a Marsh score of 3 and patients with CD with a Marsh score of 1 or 2 (Fig 2, B), as well as healthy subjects. Thus MCs are a distinctive trait of the severity of intestinal lesions in patients with CD.

In vitro characterization of MC response to gliadin

Taking into account that MC numbers correlate with disease severity and the lack of studies aimed at proving their role in the pathogenesis of CD, we investigated whether gluten and its derivatives could affect MC reactivity in in vitro assays.

First, crude gluten and gliadin of wheat, together with rice gluten and soya proteins, were used to stimulate in vitro MC degranulation. Both wheat gluten and gliadin, as other food antigens, appeared to be ineffective by themselves at inducing β-hexosaminidase release from the human MC line LAD2 (Fig 3, A). Cytokine production was also investigated, but no cytokine or chemokine secretion was observed in response to any food antigens used (data not shown).

Different fragments of digested gliadin were found to modulate innate immune responses.17 Among these, p31-43 induced apoptosis of epithelial cell lines and activated both human and mouse monocyte/macrophage cell lines in terms of cytokine production and zonulin release.18 Given the above, LAD2 cells were incubated with the immunodominant (p30-2 and p9-9) and nonimmunodominant (p31-43) gliadin peptides, and proliferation, degranulation response, chemotaxis, and cytokine secretion were evaluated. However, none of the peptides significantly influenced apoptosis, necrosis, or cell proliferation (data not shown). All peptides increased basal levels of degranulation (Fig 3, B), but this effect was not dose dependent because increasing amounts of gliadin peptides did not induce a significant increase in β-hexosaminidase release (data not shown).

Interestingly, p31-43 was found to induce cytokine and chemokine expression, especially that of proinflammatory cytokines (Fig 3, C). A slight increase in CCL2 secretion was also observed after p9-9 stimulation.

p31-43 binds to MCs and induces ROS generation and NF-κB activation

It has been demonstrated that peptide p31-p43 enters Caco-2 epithelial cells through endocytosis, localizes to endocytic vesicles,19 and induces oxidative stress.20 Moreover, ROS generation and the NF-κB pathway were reported to be involved in response to p31-43 in IFN-γ–stimulated mouse RAW 264.7 in human THP-1 and U-937 monocyte/macrophage cell lines.15,21,22
LAD2 cells were incubated with fluorescein-labeled p31-43, washed, and analyzed by means of cytofluorimetry to investigate whether peptide p31-43–induced MC activation is associated with endocytosis of peptide (Fig 4, A). Our data clearly show that p31-43 binds to LAD2 cells, and binding specificity was confirmed by pretreating the cells with increasing amounts of

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**FIG 1.** Analysis of leukocyte infiltrate in intestinal lesions of patients with CD. A, Immunohistochemical staining for intraepithelial CD3, CD3 within lamina propria, CD3, CD4, CD8, Foxp3, CD20, CD138, myeloperoxidase (MPO), IgA, IgG, and IL-17 in duodenal biopsy specimens of patient with Marsh 1 and Marsh 3 scores. B, Association between cell counts and Marsh score. Each symbol is the average cell count from a single patient. CD3 I.E., Intraepithelial CD3; CD3 L.P., CD3 within the lamina propria. *P < .05, **P < .01, and ***P < .001.
activity was impaired (Fig 4, k). In response to gliadin significantly decreased when NF-κB and IL-17 release. Indeed, IL-6 and IL-17 levels produced by BMMC cultures showed a similar pattern of degranulation to the role of the MyD88 adapter. In response to p31-43, levels of which decreased when cells were preincubated with the antioxidant NAC (Fig 4, B and C). In contrast, α2- and pα-9 did not induce ROS generation (data not shown).

The contribution of ROS and NF-κB in p31-34–induced cytokine expression was confirmed by activating LAD2 cells with p31-43 in the presence of NAC and 2 different NF-κB–specific inhibitors. NAC antioxidant activity reduced IL-6 and IL-17 release. Indeed, IL-6 and IL-17 levels produced in response to gliadin significantly decreased when NF-κB activity was impaired (Fig 4, D).

In murine macrophages gliadin fragments have been reported to signal through Toll-like receptors (TLRs) because MyD88-deficient macrophages show a reduced capacity to secrete cytokines in response to gliadin. 

Primary cultures of mouse MCs (BMMC) derived from WT and MyD88-deficient mice were challenged in vitro with gliadin fragments to assess the role of the MyD88 adapter. In response to p31-43, both BMMC cultures showed a similar pattern of degranulation (Fig 5, A). Concerning cytokine production, p31-43–treated WT BMMC secreted several proinflammatory cytokines, including IL-6, IL-17, TNF-α, and monocyte chemoattractant protein 1 (Fig 5, B) but not IL-4 and IL-10 (data not shown). Instead, MyD88-deficient BMMC cultures were poorly responsive to p31-43 stimulation (Fig 5, B). Thus cytokine production in response to p31-43–gliadin in MCs is MyD88 dependent.

**MCs within intestinal lesions show different response to in vitro challenge with p31-43 and a different pattern of cytokine expression ex vivo that correlates with disease severity**

Human MCs were isolated from duodenal biopsy specimens, challenged in vitro with p31-43, and evaluated for histamine release. Intestinal MCs were found to degranulate in vitro in response to p31-43 similar to the human MC line LAD2. Interestingly, MCs from patients with CD and healthy subjects showed the same total histamine content (Fig 6, A); however, patients with CD with high Marsh scores were found to be prone to release a higher amount of histamine (Fig 6, B).

To define MC contributions to the cytokine milieu in the course of CD progression, intestinal biopsy specimens were
stained for tryptase and IL-6 or IL-17 (Fig 6, C), and the percentages of double-positive cells (IL-6+/tryptase-positive and IL-17+/tryptase-positive) were associated with the patient’s Marsh score. In the lamina propria of healthy subjects, only few tryptase-positive cells were identified, and they did not show significant coexpression of IL-6 or IL-17 (see Fig E1, A and B, in this article’s Online Repository at www.jacionline.org). However, MCs are an important source of the proinflammatory cytokine during all CD phases, and increases in the numbers of IL-6+/tryptase-positive and IL-17+/tryptase-positive cells in patients with high Marsh scores indicate that MCs contribute to create a microenvironment responsible for disease progression (Fig 6, C and D).

FIG 4. p31-43 binds to MC membranes and induces ROS generation and NF-κB activity. A, LAD2 cells were incubated with fluorescein-labeled p31-43 in the presence or absence of NAC, stained with CM-H2DCFDA, and analyzed by means of flow cytometry. B, LAD2 cells were treated with p31-43 in the presence or absence of NAC, stained with CM-H2DCFDA, and analyzed by means of flow cytometry. In Fig 4, B and C, 1 representative histogram plot and MFI average from 3 experiments are shown. C, LAD2 cells were pretreated with NAC or with TPCK and PDTC, challenged with p31-43, and tested for cytokine production. Averages from 3 experiments performed in duplicate are shown. ***P < .001.

FIG 5. p31-43 induces MC activation partially through MyD88. A, BMMCs from WT and Myd88−/− mice were incubated with 100 μg/mL gliadin peptides and tested for β-hexosaminidase release. Cells treated with phorbol 12-myristate 13-acetate and ionomycin (P/I) were included as a positive control. B, Supernatants of WT and Myd88−/− BMMCs treated with or without p31-43 for 24 hours were assessed by means of ELISA for the indicated cytokines. ns, Not stimulated. All data were obtained from 2 independent experiments performed in triplicate. *P < .05 and ***P < .001. nd, Not detected.

MC accumulation within intestinal lesions in patients with CD with high Marsh scores correlates with M1 and neutrophil infiltration

The pattern of mediators that MCs can release in response to gliadin challenge could influence the local cytokine milieu, leading to accumulation of inflammatory cells and development of a local proinflammatory microenvironment. Double-marker immunofluorescence analysis of M1 and M2 macrophages in sections of duodenal biopsy specimens of patients classified by different Marsh scores was performed to better characterize the microinfiltrate. M1 macrophages identified as double-positive IL-23+/CD68+ cells were found to be unmodified in patients with moderate or severe intestinal lesions (Marsh 3), whereas numbers of Arg1+/CD68+ M2 macrophages strongly decreased in patient with Marsh 3 lesions (Fig 7). Macrophages, which are highlighted in the intestinal biopsy specimens of healthy subjects through CD68 immunolabeling, did not display an inflammatory phenotype, being negative for IL-23 and expressing arginase in a subset of cells (see Fig E1, C and D). Thus, during worsening of intestinal damage, a progressive skew toward a Th1-polarizing environment characterized by MC accumulation, reduction
of M2 macrophage numbers, and infiltration of neutrophils (Fig 1, B, last panel) was observed.

**DISCUSSION**

Profound changes in lymphocyte homeostasis characterize the intestinal mucosae of patients with CD, such as increasing CD8⁷ T-cell numbers in the epithelium and accumulating antigen-presenting cells, as well as CD4⁺ T cells and plasma cells, in the lamina propria. In addition to the increase in numbers of resident B and T cells, inflammatory cells, namely macrophages, neutrophils, and MCs, are also recruited to these tissues. However, systematic staining for immune markers of activation in duodenal biopsy specimens has not been validated in large cohorts of patients.

To elucidate the immune system’s effect on the homeostatic functions of gut epithelium and on CD progression, we extensively investigated the leukocyte infiltrate in patients with CD and associated it with Marsh scores modified by Oberhuber et al. Despite the overall increase in T- and B-cell compartments, no significant association between cell subsets and tissue damage was observed. However, we found that the number of resident MCs increased progressively within intestinal lesions and that, compared with other leukocyte subpopulations, their increased numbers are closely associated with disease severity.

In the past increased MC numbers and release of 5-hydroxytryptamine have been observed in jejunal biopsy specimens from patients with Marsh 1 and Marsh 3 scores. **FIG 7.** Accumulation of M1 macrophages and eosinophils within intestinal lesions in patients with CD with high Marsh scores. A, Double-marker immunofluorescence analysis of M1 and M2 macrophages in duodenal biopsy specimens from patients with Marsh 1 and Marsh 3 scores identified as double-positive IL-23/CD68 and Arg1/CD68, respectively (double staining IL-23/CD68: green, IL-23; red, CD68; double staining Arg1/CD68: green, Arg1; red, CD68). B, IL-23/CD68 and Arg1/CD68 double-positive cell counts in duodenal biopsy specimens from 4 healthy subjects and 5 patients with Marsh 1 and Marsh 3 scores. ***P < .001.

However, functional studies on the role of MCs in the pathogenesis of CD have never been performed.

The nonimmunodominant (p31-43) gliadin fragment has been shown to induce epithelial cell activation and was found to activate both human and mouse monocyte/macrophage cell lines. Here we demonstrate that p31-43 specifically binds to the MC surface and induces degranulation, as well as selective release of proinflammatory cytokines, through ROS generation and NF-κB activation. Using WT and Myd88-deficient BMMCs, we found that cytokine production requires an adaptor protein, suggesting a potential involvement of TLRs in p31-43 signaling that, through TNF receptor–associated factor 6 and engagement of the protein evolutionarily conserved signaling intermediate in Toll pathways, are known to increase intracellular ROS levels.
with G protein–coupled receptors expressed on MCs and binding of cationic secretagogues, causing histamine release, or through internalization and ROS-mediated peroxisome proliferator–activated receptor γ downregulation, as demonstrated in enterocytes.

Nevertheless, the ability of MCs to respond to gliadin peptides could account for their direct role in the onset of CD. Furthermore, during CD progression, we found that MCs increase in number and acquire a more proinflammatory phenotype, as indicated by higher numbers of IL-6+/tryptase-positive and IL-17+/tryptase-positive cells in intestinal lesions of patients with high Marsh scores. In addition, intestinal MCs isolated from biopsy specimens with high Marsh scores were more prone to secrete histamine when challenged in vitro with p31-43. The few MCs identified by means of immunofluorescence for tryptase in the lamina propria of villi from control samples did not show significant coexpression of IL-6 or IL-17.

Thus the type and amount of mediators secreted by MCs could influence all phases of the inflammatory process, indicating a role in both the onset and progression of the disease. In fact, given that gliadin does not appear to act as a promoter of autoreactive T-cell activation through molecular mimicry and that HLA is necessary but not sufficient, there must be some other trigger that helps to initiate T-cell responses to gluten. Moreover, it has been shown that significant histologic, serologic, and symptomatic changes occur in adults with CD undergoing gluten challenge. Notably, change in intestinal histology was not correlated with serology or symptoms. The symptoms start shortly after gluten challenge, whereas anti-TG and anti-deaminated gluten peptides antibody titers do not increase appreciably until 14 days after gluten challenge. These data strengthen the idea that innate immune titters do not increase appreciably until 14 days after gluten whereas anti-TG and anti-deaminated gluten peptides antibody symptoms. The symptoms start shortly after gluten challenge, change in intestinal histology was not correlated with serology or symptoms. The symptoms start shortly after gluten challenge, whereas anti-TG and anti-deaminated gluten peptides antibody titers do not increase appreciably until 14 days after gluten challenge.

In this study we extensively characterized the leukocyte infiltrate of the intestinal mucosa from patients with CD and associated it with the severity of tissue lesions. Among innate immune cells, numbers of infiltrating MCs are associated with the patient’s Marsh score.

In conclusion, we found a common mechanism to explain the involvement of MCs not only in symptoms caused by CD but also in the onset and progression of the disease. Our data show that MCs could be considered one of the main features and a new potential diagnostic marker for CD progression. Thus the view of CD should be revised, and the contribution of MCs in the onset of CD should be considered in developing new approaches for disease therapy.

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**Key messages**

- In this study we extensively characterized the leukocyte infiltrate of the intestinal mucosa from patients with CD and associated it with the severity of tissue lesions. Among innate immune cells, numbers of infiltrating MCs are associated with the patient’s Marsh score.
- MCs can be directly activated by nonimmunogenic gliadin fragment p31-43 to release proinflammatory mediators.
- MCs from the small intestine in patients with CD show a different response to gliadin peptides than MCs from healthy donors.
- During CD progression, MCs contribute to an enhanced proinflammatory microenvironment. MC accumulation within intestinal lesions in patients with CD can be used to monitor disease progression.

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FIG E1. Intestinal mucosa of healthy subjects were stained for IL-6 (green) and tryptase (red; A), IL-17 (green) and tryptase (red; B), IL-23 (green) and CD68 (red; C), and arginase (green) and CD68 (red; D).