Mast cells are associated with the onset and progression of celiac disease

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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.[1] 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.[2,3] 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 environment determinants. However, the contribution of bystander immune elements in the induction and maintenance of tissue damage in patients with CD is unknown.[4] In terms of mucosal damage, CD has a considerable spectrum of overlap with other immune-mediated disorders that do not match CD serologic (anti-transglutaminase and anti-gliadin antibodies) and/or genetic criteria, such as nonceliac gluten sensitivity.[5,6] 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.[7,8]

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.[9] 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.[9,10]

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

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Abbreviations used

BMMC: Bone marrow–derived mast cell
CD: Celiac disease
CM-H_2DCFDA: 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

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_H1-polarizing environment were also observed. Notably, we demonstrated *in vitro* that MCs could be activated by the nonimmunogenic gliadin peptide p31-43.

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 pα-9 (p57-68) QLQPFPQPQLPY, pα-2 (p62-75) PQPQLPYPQPQLPY, p31-43 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 Tuscia, Italy). Soya 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.[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.[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 chromogenic 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 $CD34^+$-derived MCs,[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 FcεRI 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 C57/BL6 mouse cells, as previously described.[14]

Degranulation response and cytokine production

Cells (1×10^6/mL) were incubated in Tyrode buffer (10 mmol/L HEPES buffer [pH 7.4], 130 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5.6 mmol/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 ionomycin as positive controls to induce MC degranulation. The enzymatic activity of the released β-hexosaminidase was assessed, as previously published.[14]

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 $T_H1/T_H2/T_H17$ Cytokine Kit (Becton Dickinson), according to the manufacturer's instructions. Human CCL2, 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 mmol/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 pyrrolidine dithiocarbamate (PDTC; 10 μmol/L) were added to MCs 30 minutes before challenge with p31-43. Both inhibitors were from Sigma-Aldrich and were previously used to assess gliadin-induced NF-κB activation.[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 p31-43 (comp) and then challenged with the labeled peptide to ensure binding specificity of p31-43. 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