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Gliadin Induces an Increase in Intestinal Permeability and Zonulin Release by Binding to the Chemokine Receptor CXCR3

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Abstract

Background & Aims—Celiac disease is an immune-mediated enteropathy triggered by gliadin, a component of the grain protein gluten. Gliadin induces an MyD88-dependent zonulin release that leads to increased intestinal permeability, a postulated early element in the pathogenesis of celiac disease. We aimed to establish the molecular basis of gliadin interaction with intestinal mucosa leading to intestinal barrier impairment.

Methods—α-Gliadin affinity column was loaded with intestinal mucosal membrane lysates to identify the putative gliadin-binding moiety. In vitro experiments with chemokine receptor CXCR3 transfectants were performed to confirm binding of gliadin and/or 26 overlapping 20mer α-gliadin synthetic peptides to the receptor. CXCR3 protein and gene expression were studied in intestinal epithelial cell lines and human biopsy specimens. Gliadin-CXCR3 interaction was further analyzed by immunofluorescence microscopy, laser capture microscopy, real-time reverse-transcription polymerase chain reaction, and immunoprecipitation/Western blot analysis. Ex vivo experiments were performed using C57BL/6 wild-type and $CXCR3^{-/-}$ mouse small intestines to measure intestinal permeability and zonulin release.

Results—Affinity column and colocalization experiments showed that gliadin binds to CXCR3 and that at least 2 α-gliadin 20mer synthetic peptides are involved in this binding. CXCR3 is expressed in mouse and human intestinal epithelia and lamina propria. Mucosal CXCR3 expression was elevated in active celiac disease but returned to baseline levels following implementation of a gluten-free diet. Gliadin induced physical association between CXCR3 and MyD88 in enterocytes. Gliadin increased zonulin release and intestinal permeability in wild-type but not $CXCR3^{-/-}$ mouse small intestine.

Conclusions—Gliadin binds to CXCR3 and leads to MyD88-dependent zonulin release and increased intestinal permeability.

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Celiac disease (CD) is an autoimmune enteropathy triggered by ingestion of gluten-containing grains (eg, wheat, rye, and barley). The disease persists in the continued presence of gliadin, the toxic component of gluten.[1] Other characteristics of CD include a highly specific autoantibody response against tissue transglutaminase[2] and a strong association with specific major histocompatibility complex haplotypes. Greater than 90%–95% of CD patients carry the HLA-DQ2, with the remaining carrying the HLA-DQ8 haplotype; however, non-HLA genes have been implicated in the disease pathogenesis as well.[3]

Under physiologic conditions, access of gliadin to gutassociated lymphoid tissue is prevented by competent intercellular tight junctions (TJ) that limit passage of macromolecules (including gliadin peptides) across the intestinal epithelial barrier.[4] In susceptible individuals, however, the interplay between the initiating stimulus (eg, gliadin) and intestinal cells triggers TJ disassembly. It has been hypothesized that this is an early biologic change that precedes the onset of gliadin-induced immune events that eventually lead to the pathology associated with CD.[5]

One protein that induces TJ disassembly and therefore is thought to be involved in the early phase of CD is zonulin.[6] Increased and persistent production of this protein as determined by Western immunoblotting[7] and enzyme-linked immunosorbent assay (ELISA)[8] were observed in patients with active CD.[6] Furthermore, ex vivo studies, using intestinal biopsy specimens in the microsnapwell system, showed that intestinal biopsy specimens of CD patients mounted a more pronounced response to gliadin when compared with nonceliac controls, including an increased and persistent release of zonulin and a significant increase in intestinal permeability.[8] It is noteworthy that epithelial release of zonulin occurs after apical, but not basolateral, exposure to gliadin.[9] The latter finding implies that gliadin interacts with an intestinal luminal receptor and prompted us to seek the identity of this moiety.

In this paper, we provide evidence that the chemokine receptor CXCR3 serves as the target receptor for gliadin. Our data demonstrate that, in the intestinal epithelium, CXCR3 colocalizes with gliadin and that this interaction coincides with recruitment of the adapter protein, MyD88, to the receptor. We also demonstrated that binding of gliadin to CXCR3 is crucial for the release of zonulin and subsequent increase of intestinal permeability because CXCR3-deficient mice failed to respond to gliadin challenge in terms of zonulin release and TJ disassembly.

Materials and Methods

Reagents

Gliadin (crude wheat), pepsin, and trypsin were purchased from Sigma (St Louis, MO). Gliadin was pepsin/trypsin digested (PT-gliadin) as described previously[10] with minor modifications.[11] Recombinant α-gliadin was a gift from Dr D. Kasarda (USDA-ARS, Albany, CA). Recombinant interleukin (rIL)-1, monokine induced by interferon (IFN) γ (rMig/CXCL9), IFN-γ-inducible protein 10 (rIP-10/CXCL10), IFN-γ-inducible T-cell α-chemoattractant (rI-TAC/CXCL11), and tumor necrosis factor-α (rTNF-α) were purchased from R&D (Minneapolis, MN) and Calbiochem (San Diego, CA), respectively. Pertussis toxin or inactivated pertussis toxin were kindly provided by Dr N. Carbonetti (University of Maryland, Baltimore, MD).

α-Gliadin Affinity Column Chromatography

For the preparation of the affinity column, α-gliadin was dissolved in 70% alcohol, mixed with Affi-Gel 15 Gel, and gently shaken for 4 hours at 4°C. The reaction was terminated by ethanolamine. Soluble total membrane preparations[12] from rabbit small intestine were loaded on an Affi-gel 15-α-gliadin affinity column, incubated for 90 minutes at 25°C, washed with

phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Sigma), and eluted with PBS containing 0.1% Triton X-100 with increasing NaCl concentrations. Fractions were collected and subjected to SDS-PAGE. The eluted proteins were characterized by MALDI mass spectroscopy fingerprint analysis (Protein and Nucleic Acid Biotechnology Facility; Stanford University, Palo Alto, CA).

Transfection Studies

HEK293T cells (2.5×10^6, passages 1–9) were plated in 10-mL culture Petri dishes in complete culture medium (Dulbecco's modified Eagle medium [DMEM]; Cellgro, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin/ 50 g/mL streptomycin, and 2 mmol/L L-glutamine (Gibco, Carlsbad, CA) and incubated overnight at 37°C in 5% CO_2. Cells were transfected with either empty vector (pcDNA3.1; Invitrogen) or CXCR3 construct generated as previously described[13] with minor modifications at a concentration of 500 ng/well using Superfect transfection reagent (Qiagen, Valencia, CA). After transfection, fresh complete culture medium was added to the dishes, and cells were incubated overnight at 37°C in 5% CO_2.

α-Gliadin Peptide Library Design and Synthesis

Overlapping (every 10 amino acids), 20mer peptides were designed based on the amino acid sequence of α-gliadin and synthesized using solid phase synthesis, resulting in a 26 peptide library (see Supplementary data online at www.gastrojournal.org). Peptide synthesis was carried out using standard Fmoc chemistry on Rink resin. Peptides were isolated as tri-fluoro acetate salts at purity levels of greater than 80% by high-performance liquid chromatography.

CXCR3 Binding and Agonist Assays

Both assays were performed at Euroscreen S.A. according to the company protocols (www.euroscreen.com),[14] using increasing concentrations of PT-gliadin (for more details, see Supplementary data online at www.gastrojournal.org). To establish the binding affinity of the synthetic gliadin peptides to CXCR3, FITC-labeled CXCR3-binding peptide 4026 was incubated with CXCR3-transfected HEK293T cells and binding kinetic evaluated by flow cytometry analysis (for detailed information, see Supplementary data online at www.gastrojournal.org).

Immunofluorescence Microscopy

HEK293T cells transiently transfected with either pcDNA empty vector or CXCR3 gene-containing vector were detached by gentle scraping, seeded in Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY) at a density of 50,000 cells/well and allowed to attach to the wells overnight at 37°C in 5% CO_2. A separate small aliquot of detached cells was incubated with 5 μLof allophycocyanin-conjugated anti-human CXCR3 (clone 49801; R&D) or an isotype-matched control (clone 11711; R&D) mouse monoclonal antibody (mAb) and used for flow cytometry analysis to verify the expression of CXCR3 on transfectants. Experimental conditions and staining protocols are described in detail in the Supplementary data online (see Supplementary data online at www.gastrojournal.org).

CXCR3 Expression in Intestinal Cell Lines and Tissues

Intestinal epithelial cell lines, IEC6 (rat, passage 36–46) and CaCo-2 (human, passage 30–40), were grown on Lab-Tek I chamber slides and stained for CXCR3 as described above. To localize CXCR3 expression in intestinal tissues, 4-μm sections were prepared, and laser capture microdissection (mouse tissue) or immunohistochemistry (human tissue) were performed as previously described.[15,16] Human intestinal mucosa was obtained from non-CD patients who underwent a diagnostic upper endoscopy for dyspepsia (no duodenal damage) and CD patients

at the moment of diagnosis (active disease, with a Marsh IIIa–c lesion) during diagnostic endoscopy. RNA extraction and real-time polymerase chain reaction (PCR) protocols are described in the Supplementary data online (see Supplementary data online at www.gastrojournal.org).

Western Blot Analysis and Immunoprecipitation

IEC6 cells were grown in culture flasks and plated in Petri dishes (1×10^6 cells/mL). Confluent cells were stimulated with PT-gliadin at doses ranging from 100 μg/mL to 1 mg/mL at different time points (15, 45, and 60 minutes). At the end of stimulation, IEC6 cells were lysed in lysis buffer containing a cocktail of protease inhibitors. Total protein content was measured using the Lowry method (Pierce, Rockford, IL). CXCR3 coimmunoprecipitation with the adaptor molecule MyD88 was performed according to the protocol described in the Supplementary data online (see Supplementary data online at www.gastrojournal.org).

Microsnapwell System

Intestinal transepithelial electrical resistance (TEER) and changes in TEER in murine small intestine in response to gliadin exposure were measured using the microsnapwell system.[17] Intestinal segments isolated from either $CXCR3^{-/-}$[18] (backcrossed >10 generations onto a C57BL/6 background) or C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were incubated with PT-gliadin (1 mg/mL) added to the mucosal side of the tissue, and TEER was monitored every 30 minutes for the duration of the experiment. In selected experiments, medium alone or IP-10/CXCL10 (200 ng/mL), one of the known ligands for CXCR3, was added to the apical side of the tissue. In selected experiments, tissues were preincubated with IP-10/CXCL10 for 30 minutes, after which IP-10/CXCL10 was removed, and PT-gliadin was added to the tissue. In a third series of experiments, intestinal segments were preincubated with medium alone, pertussis toxin (10 ng/mL), or genetically modified (inactivated) pertussis toxin (10 ng/mL) for 30 minutes, followed by addition of PT-gliadin (1 mg/mL). Pertussis toxin or its inactive genetic mutant were present throughout the stimulation. In a fourth series of experiments, intestinal tissues were incubated with 4 different peptides (10 μg/mL) from the α-gliadin synthetic peptide library. TEER data were normalized to the initial value for that specific data set in each animal.

Zonulin ELISA

Zonulin was measured in the microsnapwell intestinal culture supernatants by ELISA as previously described.[17]

Statistical Analysis

Two-tailed Student *t* tests were used to test differences between 2 groups. Data were paired where appropriate. Values of $P < .05$ were regarded as significant.

Results

Identification of CXCR3 as the PT-Gliadin Intestinal Binding Protein

To identify the putative gliadin receptor, membrane fractions were prepared from rabbit small intestine and applied to an Affi-gel α-gliadin affinity column. Three main proteins with estimated molecular weights of 93, 100, and 107 kilodaltons were eluted from the affinity column and subjected to MALDI mass spectrometric fingerprint analysis following digestion with trypsin. The 100-kilodalton band was identified by mass spec/mass spec (MS/MS) as the chemokine receptor CXCR3, based on sequences derived from 21 peptides, whereas the other bands were identified as a heat shock protein (93 kilodaltons) and the glutamate receptor (107 kilodaltons) (data not shown).

CXCR3, a 7-transmembrane G-protein-coupled receptor, [19] is involved in cellular activation and cell migration (cytoskeleton rearrangement) into inflamed tissues, in particular of γ/δ T lymphocytes (as observed in CD).[13,20] In contrast, the glutamate receptor[21] and heat shock proteins[22] are involved in other cellular functions. Based on the observation that CXCR3 activities are potentially relevant to CD pathogenesis, we pursued the possible role of CXCR3 as a receptor for PT-gliadin.

Characterization of CXCR3 as the PT-Gliadin Intestinal Receptor

To establish whether CXCR3 is the receptor responsible for PT-gliadin-induced mucosal events leading to increased intestinal permeability, the following series of experiments were performed:

Gliadin Binds to the CXCR3 Receptor

Immunofluorescence microscopy experiments were performed to determine whether PT-gliadin and CXCR3 colocalize. Transiently transfected, CXCR3-expressing HEK293T cells were incubated with PT-gliadin and stained for CXCR3 and gliadin. FACS analysis revealed that >60% of transfected cells expressed CXCR3 (data not shown). After immunofluorescence staining, colocalization of CXCR3 and gliadin was observed in CXCR3-transfected (Figure 1*A*–*C*) but not in pcDNA3.1-transfected cells (Figure 1*D*). As additional controls for the specificity of the observed staining, PT-gliadin-treated CXCR3-transfected cells were stained with isotype control or secondary Ab alone (Figure 1*E*). Furthermore, CXCR3-transfected cells were incubated with the irrelevant protein bovine serum albumin (BSA) (1 mg/mL) and stained with a specific anti-BSA Ab (Figure 1*F*). None of the control stainings showed colocalization.

To further demonstrate direct and specific gliadin binding to CXCR3, a competitive binding assay was performed. Our results showed that PT-gliadin caused a concentration-dependent displacement of the radiolabeled CXCR3 ligand $^{[125]}$I-TAC from its target receptor on CHO-K1 host cells (Figure 2). However, contrary to the other CXCR3 ligands,[14] PT-gliadin binding to CXCR3 did not activate Ca^{2+} signaling (see Supplementary data online at www.gastrojournal.org). To define whether α-gliadin domain(s) are involved in CXCR3 binding, a synthetic peptide library consisting of 26, 10 AA overlapping, 20mer peptides was subjected to the binding assay. The results show that 2 of these peptides displaced radiolabeled I-TAC from CXCR3-expressing cells (Table 1). The specificity of this binding was confirmed by kinetic experiments performed on HEK293T cells transfected with human CXCR3 that showed a dissociation constant of peptide 4026 of 32 μmol/L (see Supplementary data online at www.gastrojournal.org).

CXCR3 Is Expressed Both in Intestinal Epithelial Cells and Intestinal Immune Cells

To study the receptor expression in intestinal epithelial cells, we measured CXCR3 steady-state messenger RNA (mRNA) and protein expression in various human and murine intestinal cell lines and small intestinal tissues. Immunofluorescence analysis of human CaCo-2 cells showed constitutive CXCR3 expression (Figure 3*A* and *B*). Cross-reactivity of the antihuman CXCR3 mAb with rat CXCR3 permitted visualization of CXCR3 expression on the surface of rat IEC6 cells (Figure 3*C* and *D*). Real-time reverse-transcription (RT)-PCR analysis of Caco-2 cells confirmed CXCR3 mRNA expression (Figure 3*E*).

To confirm intestinal epithelial expression of CXCR3 in vivo, both murine and human intestinal tissues were analyzed. Murine small intestine was subjected to laser capture microdissection followed by real-time RT-PCR analysis. Although CXCR3 mRNA expression was more abundant in the lamina propria (probably because of the large number of CXCR3-positive immune cells present at this site), measurable expression of the receptor was detected also in murine intestinal epithelial cells (Figure 4). Immunohistochemical analysis of human small

intestinal tissue stained both for $CD3^+$ cells and CXCR3 confirmed that the receptor is expressed not only by immune cells but also by enterocytes (Figure 5*A*–*C*).

CXCR3 Is Up-Regulated During the Active Phase of CD

To investigate whether CXCR3 expression is altered in CD, human small intestinal biopsy specimens obtained from both non-CD and CD patients were subjected to immunohistochemical and real-time RT-PCR analysis. CXCR3 staining was detected at higher levels in the lamina propria and the epithelium of CD patients (Figure 5*E*) as compared with non-CD controls (Figure 5*D*). CXCR3 mRNA expression in biopsy specimens revealed a 9.6-fold increase in CXCR3 mRNA expression in CD patients with active disease compared with CXCR3 gene expression in non-CD patients ($P = .004$). This disease-associated enhanced mRNA expression returned to levels seen in non-CD intestinal tissue in CD patients in remission after implementation of a gluten-free diet (Figure 5*G*).

PT-Gliadin Activates the Zonulin Innate Immune Pathway Through CXCR3

We next established whether CXCR3 is required for the PT-gliadin-induced increase in zonulin release and subsequent changes in intestinal permeability previously described.[8] Intestinal tissues of wild-type C57BL/6 and $CXCR3^{-/-}$ mice were mounted in microsnapwell chambers, and PT-gliadin was added to the mucosal (eg, apical) side of the tissue. No differences in intestinal mucosal morphology or baseline TEER were noted between C57BL/6 and $CXCR3^{-/-}$ mice (data not shown). An initial series of experiments was designed to evaluate whether the effect of PT-gliadin on zonulin release and intestinal permeability is CXCR3 dependent. Intestinal segments from wild-type mice showed a significant 30% drop in TEER (Figure 6*A*). These TEER changes were preceded temporally by the release of zonulin following mucosal PT-gliadin challenge (Figure 6*B*). Conversely, $CXCR3^{-/-}$ mice did not exhibit changes in either intestinal TEER or zonulin release in response to gliadin (Figure 6*A* and *B*). To establish whether the zonulin pathway is operative in $CXCR3^{-/-}$ mice, we repeated the permeability experiments using the zonulin agonist AT1002. A significant drop in TEER was observed when $CXCR3^{-/-}$ tissues were challenged with the AT1002 compared with baseline (Figure 6*C*).

In addition, we chose 2 CXCR3-binding peptides and 2 peptides that did not show binding to CXCR3 from the α-gliadin synthetic peptide library and applied them to the luminal side of wild-type intestinal segments. Only the 2 CXCR3-binding peptides A (4026) and B (4022) induced a significant decrease in TEER, whereas the nonbinding peptides C (4018) and D (4030) did not alter intestinal permeability (Figure 6*D*).

A second set of experiments was performed on intestinal tissue from wild-type mice to assess whether the effects after gliadin binding to CXCR3 could be induced by other CXCR3 ligands. One of 3 previously described CXCR3 ligands, IP-10/CXCL10, was applied to the mucosal side of the intestinal tissue, and TEER w as measured. IP-10/CXCL10 did not cause significant changes in either TEER or zonulin release compared with medium alone (data not shown).

From these experiments emerges that IP-10/CXCL10 and PT-gliadin induce different cellular activation patterns after binding to CXCR3. We next evaluated whether IP-10/CXCL10 binding to CXCR3 affects PT-gliadin-induced changes in intestinal permeability as a result of the receptor tachyphylaxis. Pretreatment of wild-type intestinal segments with IP-10/CXCL10 for 30 minutes did not overall prevent the effects of PT-gliadin on TEER. However, the time in which the TEER started to drop following PT-gliadin exposure was delayed by 30 minutes in tissues pretreated with IP-10/CXCL10 (Figure 6*E*). These results suggest that CXCR3 receptors could be temporally unavailable secondary to IP-10/CXCL10 and needed to shuttle back to the cell surface before PT-gliadin could bind and exert its effects on TEER.

A third series of experiments was performed to examine whether gliadin binding to CXCR3, a G-protein-coupled receptor, requires G-protein signaling. For these experiments, intestinal tissue of wild-type mice was mounted in microsnapwells, and PT-gliadin was added to the mucosal side after pretreatment with medium alone, pertussis toxin (a G protein-coupled receptor inhibitor), or an inactive genetic mutant of pertussis toxin. PT-gliadin induced the expected drop in TEER in tissues preincubated with medium alone, and this was prevented by preincubation with pertussis toxin but not with its inactive mutant (Figure 6*F*).

PT-Gliadin Binding to CXCR3 Recruits MyD88

We recently reported that PT-gliadin-induced zonulin release is MyD88-dependent.[11] To investigate whether PT-gliadin binding to CXCR3 induces recruitment of the adapter protein MyD88, IEC6 intestinal epithelial cells were stimulated with PT-gliadin and subjected to coimmunoprecipitation assays. These assays revealed an association of CXCR3 and MyD88 after PT-gliadin challenge that was concentration and time dependent (Figure 7). This association was optimal when PT-gliadin was present at a concentration of 1 mg/mL (Figure 7*A*) and reached a plateau after 45 minutes of incubation (Figure 7*B*).

Discussion

TJs are central to the regulation of intestinal permeability because they maintain the contiguity of intestinal epithelial cells and are capable of prompt and coordinated responses to the many physiologic challenges to the intestinal epithelial barrier.[4] Increased intestinal permeability appears to be an early biologic change that precedes the onset of autoimmune diseases, including CD and type I diabetes.[8,23,24] The peculiarity of CD is that it is the only autoimmune disease for which the triggering environmental factor gliadin is known. This offers a unique opportunity to study the cellular and molecular basis of the autoimmune process using enzymatically digested gliadin as a stimulus in experimental assays.

We showed a direct effect of gliadin on intestinal barrier function,[9] which was confirmed by others.[25] This effect of gliadin is polarized, eg, gliadin increases intestinal permeability only when administered on the luminal side of the intestinal tissue.[9] These data formed the basis for the present study because a missing link has been the identification of the luminal structure to which gliadin binds and through which gliadin induces epithelial zonulin release and TJ disassembly.

Our MS/MS data identified the chemokine receptor CXCR3 and 2 other proteins, a glutamate receptor and a heat shock protein, as the proteins that bound to α-gliadin. We chose to investigate the possible role of CXCR3 as a receptor for gliadin because of its function in recruiting γ/δ lymphocytes, a marker of early stage in CD pathogenesis.[26] In contrast, the glutamate receptor is an intrinsic transmembrane ion channel that is opened in response to binding of a chemical messenger but has not been described to be involved in cell activation and rearrangement of the cytoskeleton.[21] Heat shock proteins are cytoplasmic proteins involved in intracellular processes including protein folding and protein conformation and are found extracellularly only as shed contents from necrotic cells providing a strong danger signal to the immune system.[22]

The identification of CXCR3 as a receptor for gliadin is important for several reasons. The chemokine receptor CXCR3 is involved in various pathophysiologic conditions. Its biologic role is to provide a mechanism for cells that express this receptor to migrate to its ligands, the chemokines Mig/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11, which share the receptor, but exert different and nonredundant effects.[27] CXCR3 is associated with leukocyte recruitment to target organs and subsequent T helper cell 1 immune-mediated tissue damage in viral and bacterial infections[28,29] and autoimmune disease states.[30,31] CXCR3 is predominantly

expressed on different T-cell subsets, including activated T helper cell 1 (Th1) cells,[32] T lymphocytes,[33] a newly identified E-cadherin-bearing $CD8^+$ T-cell subset that specifically homes to the gut,[34] and natural killer cells,[35] but its expression has been reported on other cell types as well.[36,37]

The phenomenon that ligands other than chemokines can bind to chemokine receptors has been reported previously; for example, human immunodeficiency virus uses the CCR5 chemokine receptor for cell entry,[38] and PGP, a peptide derived from the extracellular matrix, signals through the CXCR2 receptor on neutrophils causing neutrophil recruitment into the lungs and production of superoxide.[39]

With this paper, we report for the first time CXCR3 expression in intestinal epithelium. CXCR3 expression showed the same qualitative distribution in both CD and non-CD intestinal tissues, but its expression was higher in CD. These differences were paralleled by higher CXCR3 gene transcription in CD patients with active disease that returned to baseline levels when the disease was in remission following the implementation of a gluten-free diet. The enhanced CXCR3 mRNA expression in intestinal tissue from active CD patients reflects the importance of CXCR3 expression on both intestinal epithelial cells and intraepithelial lymphocytes and its distinct regulation in CD. Our immunohistochemical staining studies show that epithelial CXCR3 expression is predominantly related to enterocytes and not to the large number of intraepithelial CXCR3-expressing γ/δ-positive T lymphocytes that typically infiltrate the intestinal mucosa during the acute phase of CD.[20]

The role of CXCR3 in mediating the PT-gliadin-induced zonulin release and subsequent increase in intestinal permeability was confirmed using $CXCR3^{-/-}$ mice in which PT-gliadin failed to release zonulin and, consequently, to reduce TEER. Our observation that pretreatment of C57BL/6 wild-type intestinal tissue with the G-protein inhibitor pertussis toxin prevented the effect of PT-gliadin on intestinal permeability is consistent with activation through CXCR3 that leads to subsequent TJ disassembly.

Interestingly, PT-gliadin, as well as 2 α-gliadin synthetic peptides, bound to CXCR3 but did not cause Ca^{2+} release (see Supplementary data online at www.gastrojournal.org) as reported for the 3 known natural CXCR3 ligands, Mig/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11.[14,27] The fact that IP-10/CXCL10 failed to cause TEER changes suggests that other intracellular signaling pathway(s) could be responsible for the PT-gliadin-induced zonulin release and TJ disassembly. Pretreatment with IP-10/CXCL10 caused a delay of TJ disassembly but was not able to inhibit the effects of PT-gliadin on intestinal permeability, indicating that both proteins act via binding to CXCR3 but exert different effects with regard to TJ disassembly and zonulin release. Zonulin characterization revealed that it belongs to a family of serine proteases with structure similarities with a series of growth hormones, including epidermal growth factor. One can hypothesize that the effect of gliadin on epidermal growth factor-related signaling as was recently reported[40] could eventually be mediated by zonulin.

Our data suggest that recruitment of the adapter protein MyD88 to CXCR3 is involved. Until recently, MyD88 has been described to be associated uniquely with signalin via Toll-like receptors (TLR) and the interleukin (IL)-1R family. TLRs are a family of pattern recognition receptors that recognize evolutionary highly conserved structures on microorganisms and give rise to nuclear factor-κB activation and proinflammatory gene transcription.[41] This knowledge was extended recently with the finding that MyD88 can associate with the IFN-γ receptor, providing an alternative way by which IFN-γ can enhance proinflammatory gene expression.[42] In our experiments, CXCR3 activation by PT-gliadin failed to activate nuclear factor-κB, IRF-3, or p38 (data not shown). This observation could indicate that CXCR3 associates with

another receptor that, in turn, leads to recruitment of MyD88 “by proxy.” This concept would exclude both TLR2 and TLR4 as coreceptors because our previous studies ruled out the involvement of these 2 TLRs in zonulin signalling and increased permeability.[11] Alternately, PT-gliadin-dependent CXCR3 activation signals leading to zonulin release may be mediated by a yet undefined pathway downstream of the recruitment of MyD88. Support for a direct interaction was suggested by our Clustal W analysis that identified a TIR-like region within the C-terminus of CXCR3 (Quan Nhu, unpublished observation).

In conclusion, using biochemical, genetic, and physiologic approaches, we identified the chemokine receptor CXCR3 as the receptor that binds gliadin. Our data suggest that gliadin binds to CXCR3 on epithelial cells to initiate an increase in intestinal permeability through an MyD88-dependent release of zonulin that enables the paracellular passage of gliadin (and possibly other non-self antigens) from the intestinal lumen to the gut mucosa. In genetically predisposed individuals, gliadin may attract and stimulate other CXCR3-expressing cells, including T cells, $CD3^+CD8^+$ T cells, and natural killer cells,[33,34,43] leading to the early activation of the innate immune arm of the CD inflammatory response.[44]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used in this paper

CD, celiac disease; Mig/CXCL9, monokine induced by interferon γ; IP-10/CXCL10, interferon-γ-inducible protein 10; I-TAC/CXCL11, interferon-inducible T-cell α-chemoattractant; PT-gliadin, pepsin/trypsin-digested gliadin; TEER, transepithelial electrical resistance; TJ, tight junctions.

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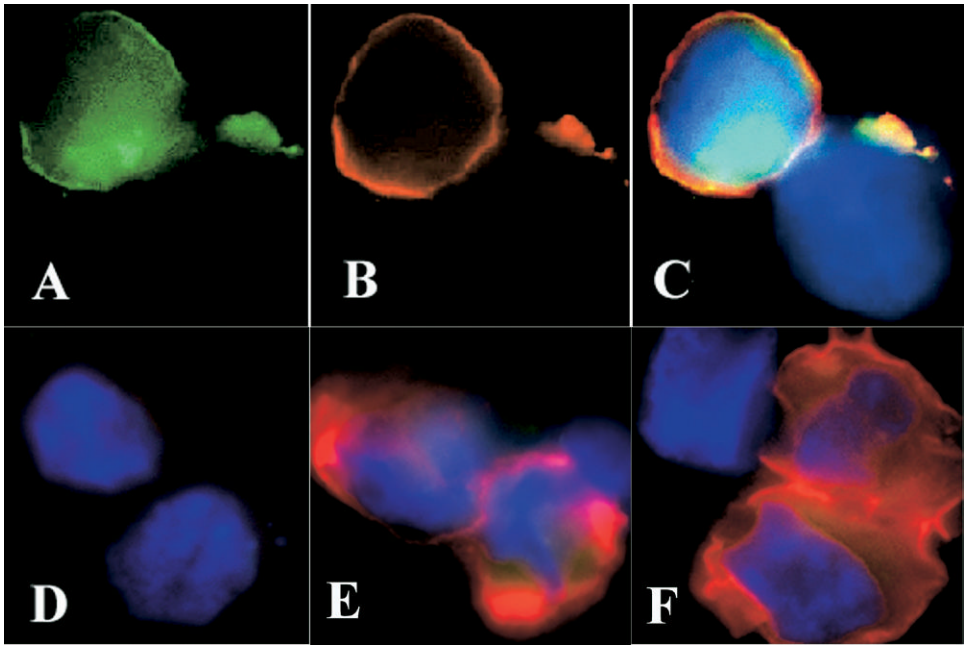

Figure 1. Colocalization of CXCR3 and PT-gliadin in CXCR3-transfected HEK293T cells

(*A–C*) Representative photomicrographs of PT-gliadin-stimulated CXCR3-expressing HEK293T cells. In CXCR3-transfected cells, CXCR3 (*B*) colocalized with gliadin (*A*) as indicated by a *yellow* appearance in the merged picture (*C*). A second cell expressing CXCR3 only in its upper pole showed gliadin binding only where CXCR3 was present. For details on immunofluorescence staining protocol, see Materials and Methods section. (*D–F*) Merged pictures of PT-gliadin-treated pcDNA vector-transfected cells stained with anti-gliadin and anti-CXCR3 specific Ab (*D*), gliadin-treated CXCR3-transfected cells stained with secondary anti-rabbit IgG-FITC and anti-CXCR3 specific Ab (*E*) and (*F*) BSA-treated CXCR3-transfected cells stained with anti-BSA and anti-CXCR3 specific Ab. None of the control stainings showed colocalization. Nuclei are in DAPI (*blue*). Original magnification, × 100. Photomicrographs are representative of 3 experiments.

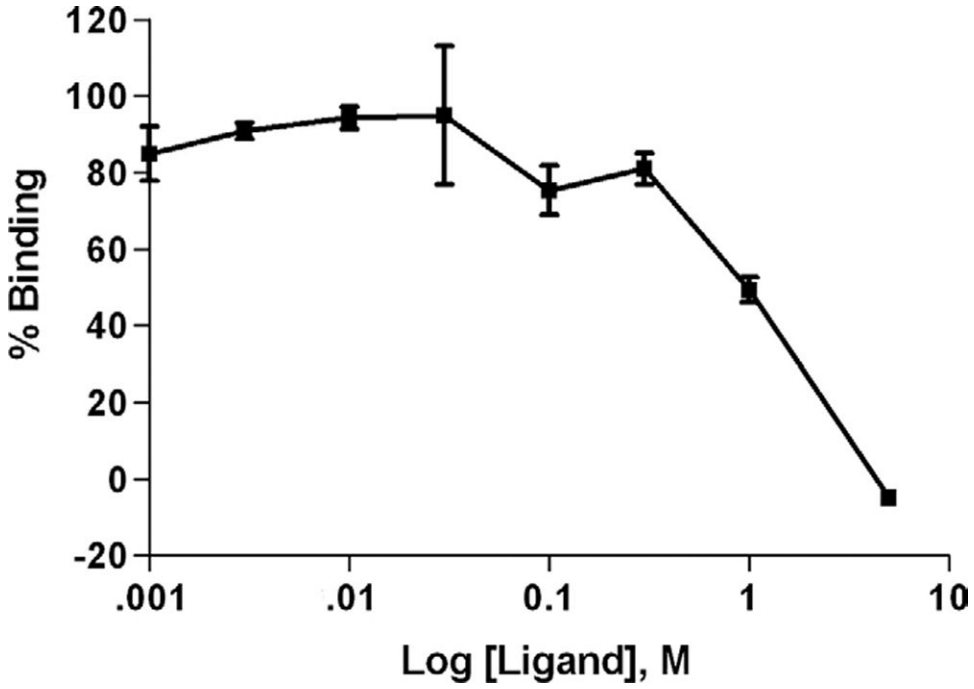

Figure 2. Dose-response binding curve of PT-gliadin. Concentration-response curve of PT-gliadin binding to CXCR3

PT-gliadin caused a concentration-dependent displacement of $^{[125]}$I-TAC binding from CXCR3, with 50% of ligand displacement obtained with PT-gliadin concentration of 1 mg/mL. The *solid boxes* represent the percentage of $^{[125]}$I-TAC bound to CXCR3 at the indicated concentrations of PT-gliadin.

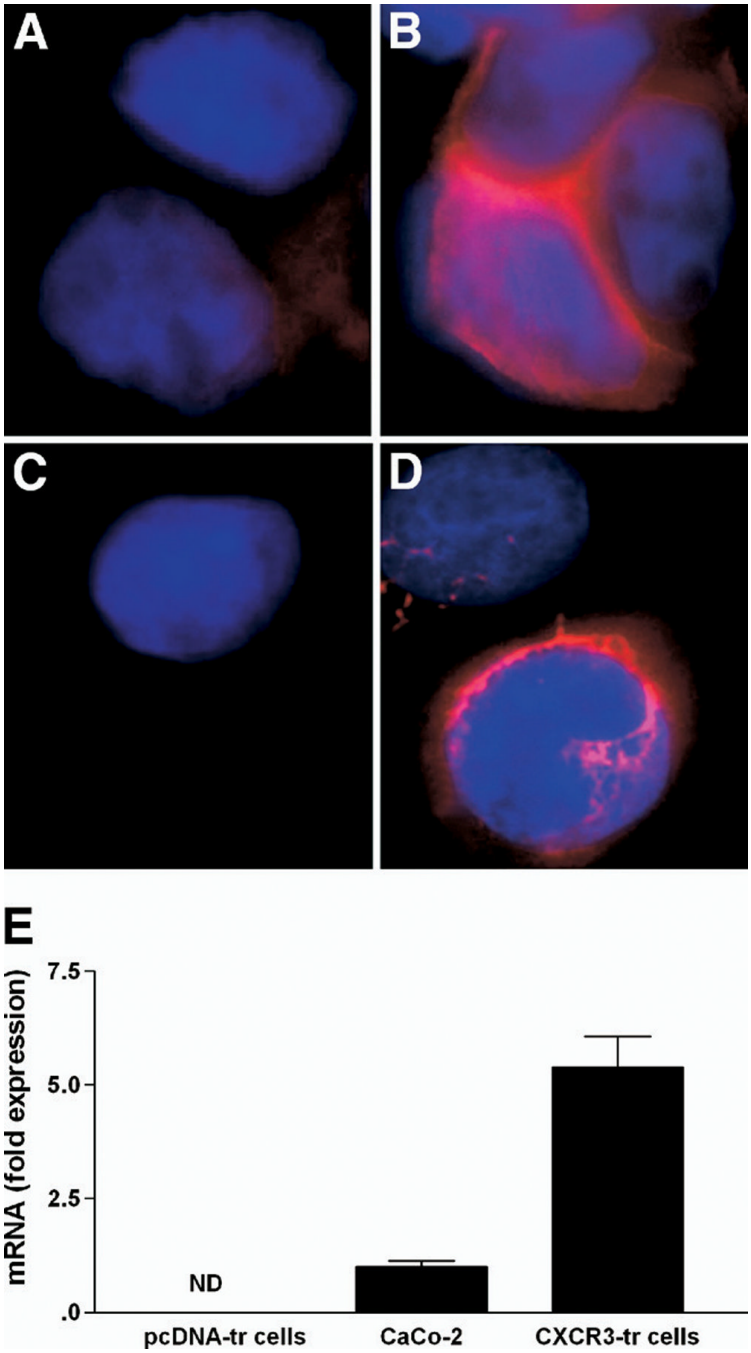

Figure 3. CXCR3 is expressed on intestinal epithelial cell lines

Isotype-matched control stainings in *panels A* and *C*, respectively. Basal CXCR3 (*red*) expression was detected on human CaCo-2 (*B*) and rat IEC6 cells (*D*). Nuclei are in DAPI (*blue*). Original magnification, × 100 (n = 2). Basal CXCR3 mRNA expression in CaCo-2 cells (1 ± 0.13) (n = 4) is shown in comparison with CXCR3 expression in HEK293T transfectants. No detectable (*ND*) CXCR3 mRNA was found in pcDNA-transfected cells. Expression in CXCR3-transfected cells was almost 6-fold higher (5.39 ± 0.68) (n = 9).

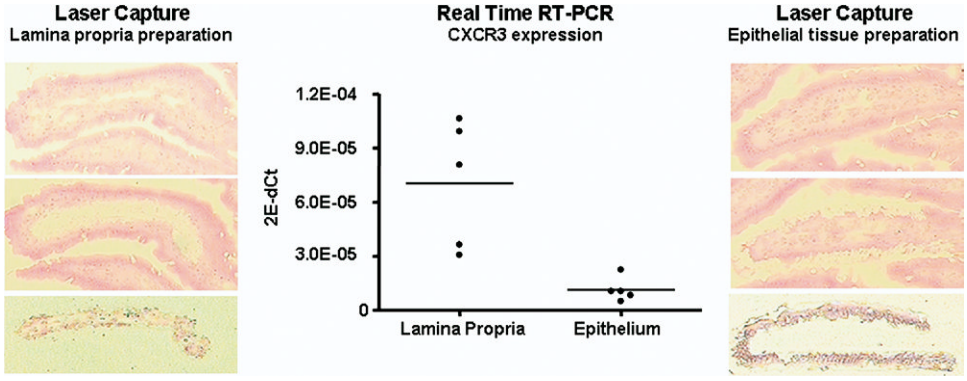

Figure 4. CXCR3 expression in mouse intestinal tissues

Laser capture microdissection followed by realtime RT-PCR revealed that CXCR3 mRNA was detectable in the epithelium. As expected, strong CXCR3 expression was detected in the lamina propria where immune cells are localized. *Horizontal bars* in the *graphs* indicate median values (n = 5).

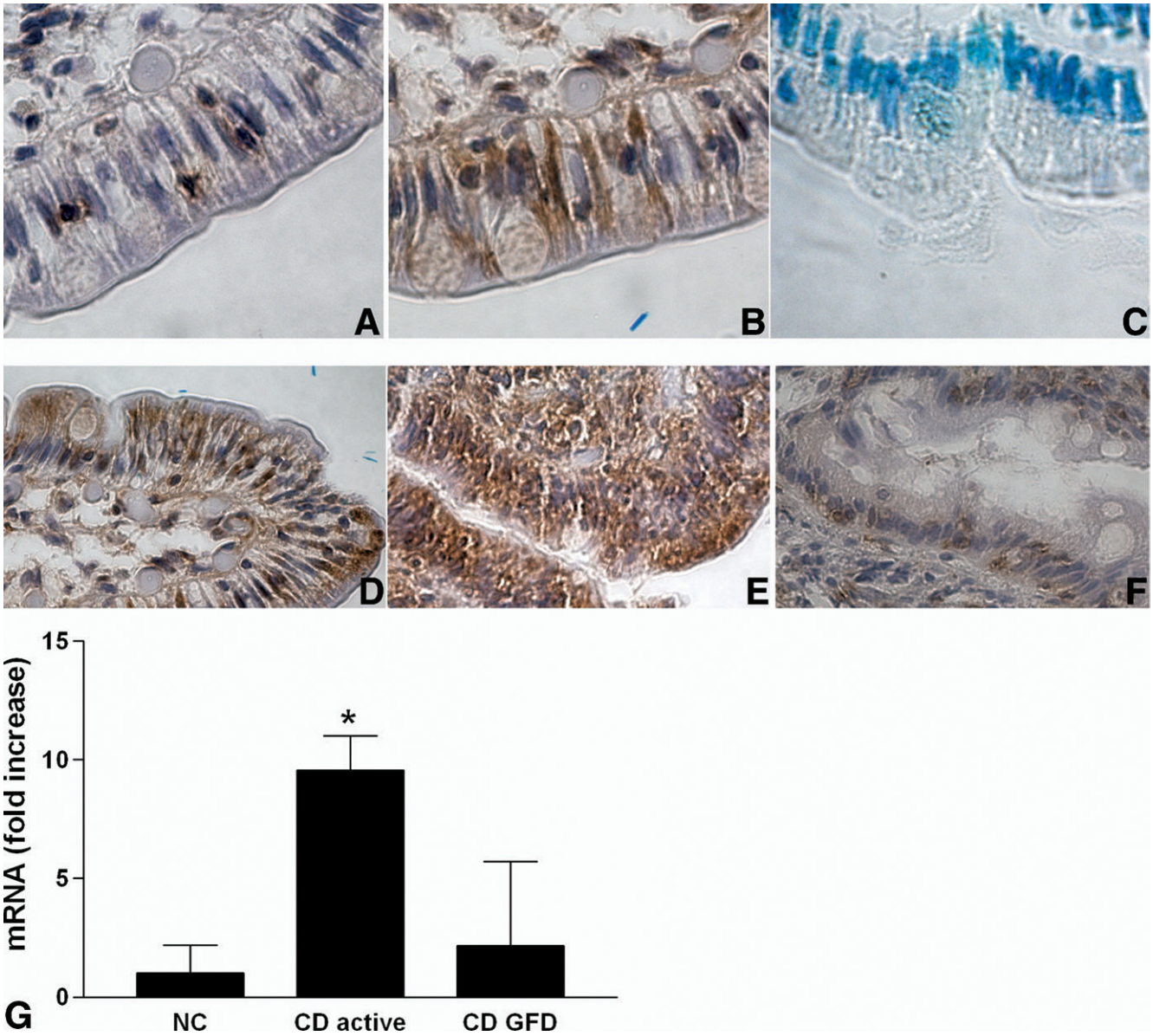

Figure 5. Differential mucosal CXCR3 expression in nonceliac and CD patients

In normal subjects in whom the number of intraepithelial lymphocytes is limited (*A*), CXCR3 is expressed both by immune cells and enterocytes (*B*). Incubation of the tissue with secondary antibodies alone (*C*) was performed to show specificity of the staining. Compared with controls (*D*), CXCR3 expression in CD subjects is highly increased both at the epithelial level and in the lamina propria (*E*). The increase in intraepithelial lymphocytes typical of CD (*F*) clearly cannot entirely account for the diffuse over expression of CXCR3. Original magnification, × 60. (*G*) In active CD, CXCR3 gene expression as determined by real-time PCR was elevated significantly compared to non-CD patients (**P* = .004), and this expression returned to baseline after the implementation of a gluten-free diet (n = 3–9).

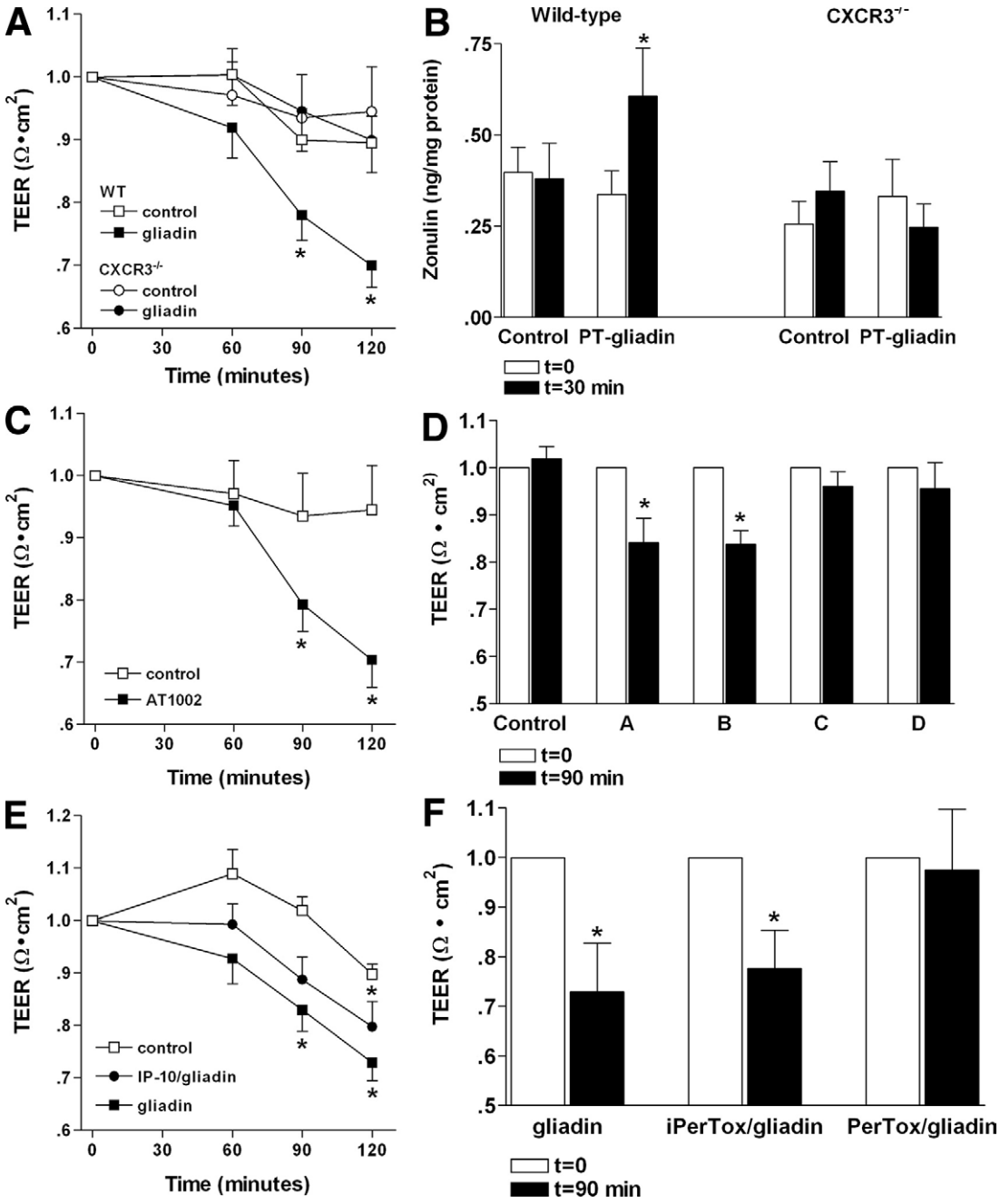

Figure 6. The increased intestinal permeability and zonulin release in response to PT-gliadin challenge is CXCR3-dependent

(*A*) Apical application of PT-gliadin increased intestinal permeability in wild-type mice but not in CXCR3$^{-/-}$ mice. In wild-type mice, a significant decrease in TEER (a measure of increased intestinal permeability) was observed after 90 minutes of PT-gliadin exposure (**P* = .001) and decreased further over time (t = 120 minutes, **P* = .001) (n = 9). (*B*) Wild-type C57BL/6 mice responded to PT-gliadin challenge by an increase in the zonulin release that was significant after 30 minutes (*solid bar*) as compared with baseline values (*open bars*) (**P* < .05). Conversely, zonulin release was unchanged after PT-gliadin challenge in CXCR3$^{-/-}$ mice. Baseline and post-PT-gliadin exposure TEER values are shown in *open* and *solid bars*, respectively (n = 9). (*C*) Zonulin pathway was intact in CXCR3$^{-/-}$ mice. Apical

challenge of CXCR3$^{-/-}$ intestinal segments with AT1002, a synthetic peptide derived from *Vibrio cholerae* protein Zot (the eukaryotic analog of zonulin), induced a decrease in TEER that became significant after 90 minutes (**P* = .001) and continued to decrease up to 120 minutes (**P* < .001) (n = 7). (*D*) Four peptides from the α-gliadin synthetic peptide library were applied to the luminal side of wild-type C57BL/6 intestinal segments. A significant decrease in TEER was observed 90 minutes after challenge with the 2 CXCR3-binding peptides A (**P* = .05) and B (**P* = .02), whereas the nonbinding peptides C and D failed to do so. Baseline and post-PT-gliadin exposure TEER values are shown in *open* and *solid bars*, respectively (n = 4–8). (*E*) Pretreatment of wild-type intestinal segments with IP-10/CXCL10 partially induced tachyphylaxis. In these tissues, PT-gliadin induced a decrease in TEER comparable with tissue that was not pretreated, but the onset was delayed 30 minutes. *P* values IP-10/gliadin, *P* = .08 (t = 60 minutes); *P* = .02 (t = 90 minutes); gliadin, *P* = .004 (t = 60 minutes), *P* = .001 (t = 90 minutes) (n = 4). (*F*) The PT-gliadin-induced TEER changes required G-protein signaling because pertussis toxin (a G-protein-coupled receptor inhibitor) pretreatment abrogated responsiveness to PT-gliadin challenge. Under control conditions, eg, pretreatment with medium alone (**P* = .016) or inactivated pertussis toxin (**P* = .018), PT-gliadin challenge reduced TEER (n = 10).

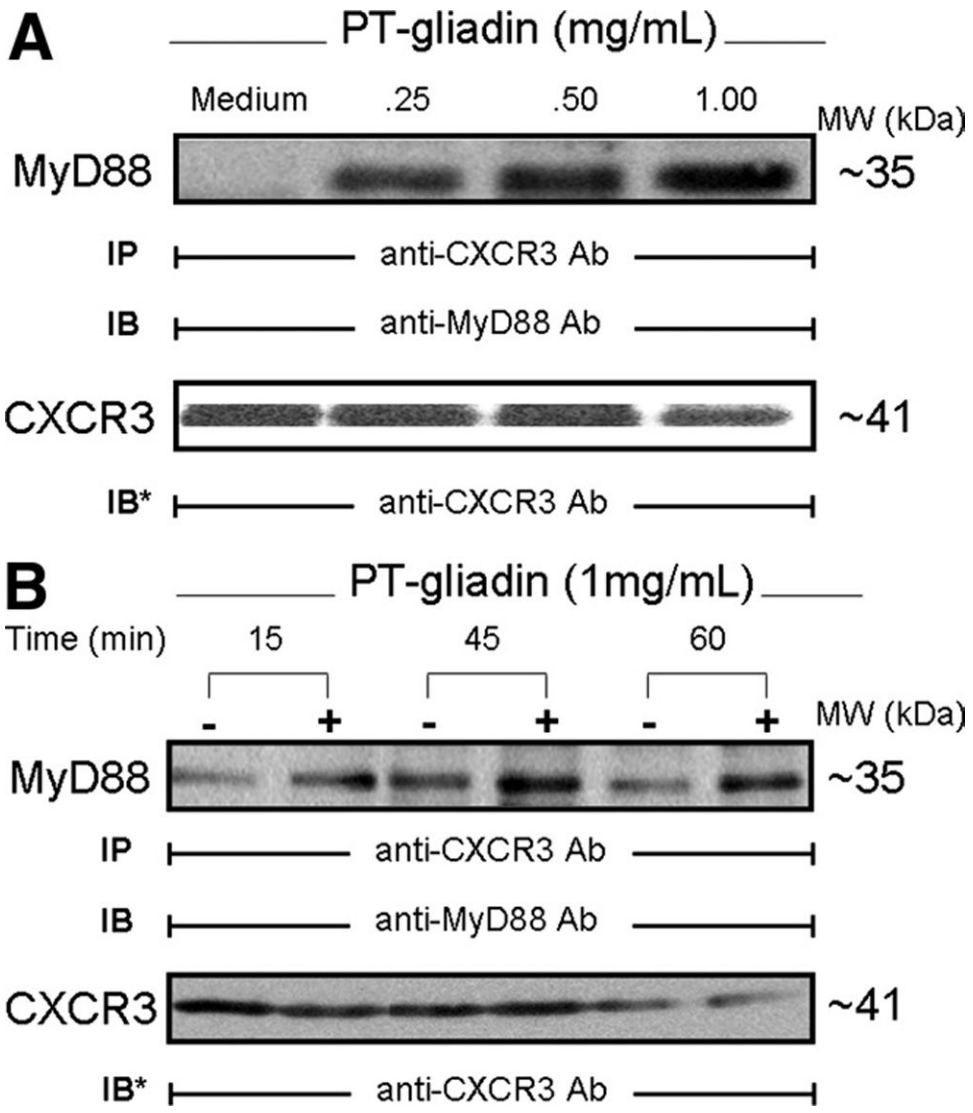

Figure 7. PT-gliadin binding to CXCR3 induces recruitment of MyD88

IEC6 cells were incubated at different concentrations (*A*) and for various times (*B*) with PT-gliadin. Immunoprecipitation with anti-CXCR3 mAb was performed, and the blot was probed for MyD88. Equal loading was checked by stripping and reprobing the blot for CXCR3. PT-gliadin induced the association of CXCR3 with MyD88, which was observed best when PT-gliadin was applied at a concentration of 1 mg/mL and after an incubation period of 45 minutes (n = 2–3).

Table 1

Synthetic Peptides

Peptide	Sequence	I-TAC binding[a]
	PT-gliadin	**69**
4012	MVRVPVPQLQPQNPSQQHPQ	98
4013	PQNPSQQHPQEQVPLVQQQQ	112
4014	EQVPLVQQQQFLGQQQSFPP	90
4015	FLGQQQSFPPQQPYPQPQPF	96
4016	QQPYPQPQPFPSQQPYLQLQ	103
4017	PSQQPYLQLQPFPQPQLPYL	94
4018	PFPQPQLPYLQPQPFRPQQP	93
4019	QPQPFRPQQPYPQPQPQYSQ	99
4020	YPQPQPQYSQPQQPISQQQQ	112
4021	PQQPISQQQQQQQQQQQQQQ	86
	QQQQQQQQQQQQQQQQQQQQ	—
4022	**QQQQQQQQQQQQILQQILQQ**	**55**
4023	QQILQQILQQQLIPCMDVVL	—
4024	QLIPCMDVVLQQHNIAHGRS	116
4025	QQHNIAHGRSQVLQQSTYQL	106
4026	**QVLQQSTYQLLQELCCQHLW**	36
4027	LQELCCQHLWQIPEQSQCQA	90
4028	QIPEQSQCQAIHNVVHAIIL	76
4029	IHNVVHAIILHQQQKQQQQP	93
4030	HQQQKQQQQPSSQVSFQQPL	126
4031	SSQVSFQQPLQQYPLGQGSF	100
4032	QQYPLGQGSFRPSQQNPLAQ	100
4033	RPSQQNPLAQGSVQPQQLPQ	108
4034	GSVQPQQLPQFEEIRNLALQ	95
4035	FEEIRNLALQTLPAMCNVYI	109
4036	TLPAMCNVYIPPYCTIVPFG	110
4037	PPYCTIVPFGIFGTNYR	108

NOTE. Designed peptides were subjected to a CXCR3 binding assay based on displacement of $^{[125]}$-radiolabeled CXCR3 ligand I-TAC. In addition to PT-gliadin, 2 of the synthetic peptides were identified to bind to CXCR3 (depicted in **bold**).

[a] Percentage binding average (n = 2).