# Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4

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**Ingestion of wheat, barley, or rye triggers small intestinal inflammation in patients with celiac disease. Specifically, the storage proteins of these cereals (gluten) elicit an adaptive Th1-mediated immune response in individuals carrying HLA-DQ2 or HLA-DQ8 as major genetic predisposition. This well-defined role of adaptive immunity contrasts with an ill-defined component of innate immunity in celiac disease. We identify the α-amylase/trypsin inhibitors (ATIs) CM3 and 0.19, pest resistance molecules in wheat, as strong activators of innate immune responses in monocytes, macrophages, and dendritic cells. ATIs engage the TLR4–MD2–CD14 complex and lead to up-regulation of maturation markers and elicit release of proinflammatory cytokines in cells from celiac and nonceliac patients and in celiac patients' biopsies. Mice deficient in TLR4 or TLR4 signaling are protected from intestinal and systemic immune responses upon oral challenge with ATIs. These findings define cereal ATIs as novel contributors to celiac disease. Moreover, ATIs may fuel inflammation and immune reactions in other intestinal and nonintestinal immune disorders.**

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Abbreviations used: ATI, α-amylase/trypsin inhibitor; DTT, dithiothreitol; KC, keratinocyte-derived cytokine; PT, pepsin/trypsin.

Wheat has only recently been added to the human diet (since the Neolithic age, i.e., roughly 12,000 years ago; Charmet, 2011). Today wheat has become the world's major staple, and wheat products are widely used ingredients in processed foods. Wheat consumption correlates with certain disorders like wheat allergies and especially celiac disease (Shewry et al., 2003). Celiac disease is a common small intestinal enteropathy caused by dietary gluten in wheat, barley, and rye and affects ~1% of most populations (Fasano et al., 2003; Green and Cellier, 2007; Di Sabatino and Corazza, 2009; Schuppan et al., 2009) Wheat gluten represents a family of largely water-insoluble storage proteins, subdivided into gliadins and glutenins, whereas other proteins are extractable as water-soluble albumins and salt-soluble globulins (Shewry et al., 2003; Wieser, 2007). Because of their unusual structure, with a high proline and glutamine content, the gluten proteins are partly resistant to intestinal enzymes, which leads to several nondegraded immunogenic peptides that can be sensed by the intestinal immune system. These gluten peptides are bound by the human lymphocyte antigen HLA-DQ2 or HLA-DQ8 (the major and necessary genetic predisposition for celiac disease) on intestinal antigen-presenting cells, and this binding is potentiated

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by the ubiquitous enzyme tissue transglutaminase (TG2), the celiac disease autoantigen (Dieterich et al., 1997). TG2 deamidates certain glutamine residues in (immunodominant) gluten peptides to glutamic acid, thereby increasing the peptides' binding affinity to HLA-DQ2 and HLA-DQ8 and the subsequent T cell activation (Dieterich et al., 1997; van de Wal et al., 1998). This results in villus atrophy and crypt hyperplasia of the intestinal mucosa, the histological hallmarks of celiac disease, and frequent nutrient malabsorption and may promote certain celiac disease–associated autoimmune disorders (Green and Cellier, 2007; Di Sabatino and Corazza, 2009; Schuppan et al., 2009).

Although several HLA-DQ2– and HLA-DQ8–restricted gluten peptides that trigger the adaptive immune response in celiac disease have been identified (Molberg et al., 1998; Anderson et al., 2000; Shan et al., 2002), only 2–5% of individuals expressing these HLAs develop the disease, indicating additional mechanisms of celiac disease pathogenesis, especially innate immune activation. The innate immune system provides an early response to many microbial and chemical stimuli and is critical for successful priming of adaptive immunity. Responsive innate cells are primarily macrophages, monocytes, DCs, and polymorphonuclear leukocytes that by means of their pattern-recognition receptors, such as TLRs, induce the release of proinflammatory cytokines and chemokines, resulting in recruitment and activation of additional inflammatory cells (Medzhitov, 2007).

Thus, peptides p31-43 or p31-49 from α-gliadin that lack adaptive stimulatory capacity were incriminated as triggers of innate immunity as they induced IL-15 and Cox-2 expression in patients' biopsies (Maiuri et al., 2003) and MHC class I polypeptide–related sequence A (MICA) on intestinal epithelial cells (Hüe et al., 2004). However, these studies were difficult to reproduce in cell culture, and no receptor responsible for the observed effects could be identified.

In cell culture, gliadin was reported to induce increased expression of co-stimulatory molecules and the production of proinflammatory cytokines in monocytes and DCs (Nikulina et al., 2004; Cinova et al., 2007). In addition, the chemokine receptor CXCR3 was implicated in increased intestinal epithelial permeability upon gliadin challenge in a MyD88-dependent manner (Thomas et al., 2006; Lammers et al., 2008). However, no defined gliadin peptide was reproducibly identified. Collectively, a clear picture of the role of the innate immune system in celiac disease has not emerged.

In this study, we show that members of the nongluten α-amylase/trypsin inhibitor (ATI) family contained in wheat and related cereals are strong inducers of innate immune responses in human and murine macrophages, monocytes, and DCs. ATI family members activate the TLR4–MD2–CD14 complex and elicit strong innate immune effects not only in vitro but also in vivo after oral or systemic challenge. Our findings have broad implications not only for celiac disease but also for other intestinal inflammatory disorders of the gastrointestinal tract.

## RESULTS

### Gliadin digests induce innate immune responses

Human monocytic THP-1 and U937 cells were exposed to pepsin/trypsin (PT)-digested gliadin mimicking intestinal digestion. PT-digested zein, a mixture of storage proteins from corn which is functional homologous to gliadin but lacks immunogenic peptides, served as a negative control. PT gliadin but not zein caused a dose-dependent secretion of IL-8, TNF, and MCP-1 (monocyte chemotactic protein-1) in both cell lines (Fig. 1, A and B; and not depicted) in accordance with other studies (Nikulina et al., 2004; Cinova et al., 2007). To rule out LPS contamination as a trigger of innate responses, gliadin, LPS, and TNF were digested with proteinase K to eliminate peptide-induced responses. Both TNF and PT gliadin lost their stimulatory capacity, whereas LPS was still able to induce IL-8 (Fig. 1 C), indicating that the stimulatory effect of gliadin was caused by protein and not LPS contamination. Interestingly, exposure of primary human monocyte-derived DCs to PT gliadin and LPS led to a similar cytokine profile in response to gliadin. In contrast, the intestinal epithelial cell line HT29 showed only a minor response (not depicted).

Moreover, DCs from celiac patients, both on gluten-free and on regular diet, and DCs from healthy controls strongly secreted IL-8 upon gliadin stimulation, with no significant differences between these groups, which is in line with prior data (Fig. 1 D; Rakhimova et al., 2009). In addition, DCs upregulated the cell surface maturation markers CD25, CD80, CD83, and CD86 upon exposure to PT gliadin and LPS but not to zein (Fig. 1 E). α-Gliadin peptide p31-43, which was shown by others to increase IL-15 secretion in celiac biopsies (Maiuri et al., 2003), did not elicit secretion of IL-8, TNF, or MCP-1 in monocytic or intestinal epithelial cell lines even at high concentrations (Fig. 1 F and not depicted).

### Innate immune responses to gliadin are mediated via TLR4

A recent study suggested that gliadin signals via MyD88, a key adapter molecule in the TLR–IL-1β pathway (Thomas et al., 2006). We studied peritoneal macrophages of C3H/HeJ mice that lack TLR4 responses because of a spontaneous point mutation in the *Tlr4* gene. In these mice, keratinocyte-derived cytokine (KC; IL-8) and TNF secretion was reduced to baseline levels after stimulation with PT gliadin and LPS compared with syngenic C3H/HeOuJ TLR4-competent mice. In macrophages from both mouse strains, the specific TLR2 agonist Pam3CSK4 induced indistinguishable KC and TNF secretion, confirming otherwise intact MyD88 and TLR signaling, and cell viability (Fig. 2, A and B). In addition, HEK-293 cells transfected with the human TLR4–CD14–MD2 complex but not untransfected TLR4-deficient HEK-293 cells secreted IL-8 in response to gliadin and LPS but not to the TLR2 agonist Pam3CSK4 (Fig. 2, C and D). Finally, incubation with anti-TLR4 and CD14 blocking antibodies before addition of gliadin significantly reduced IL-8 production by human DCs, whereas blocking had no effect on TLR2 and TLR3 stimulation (Fig. 2 E).

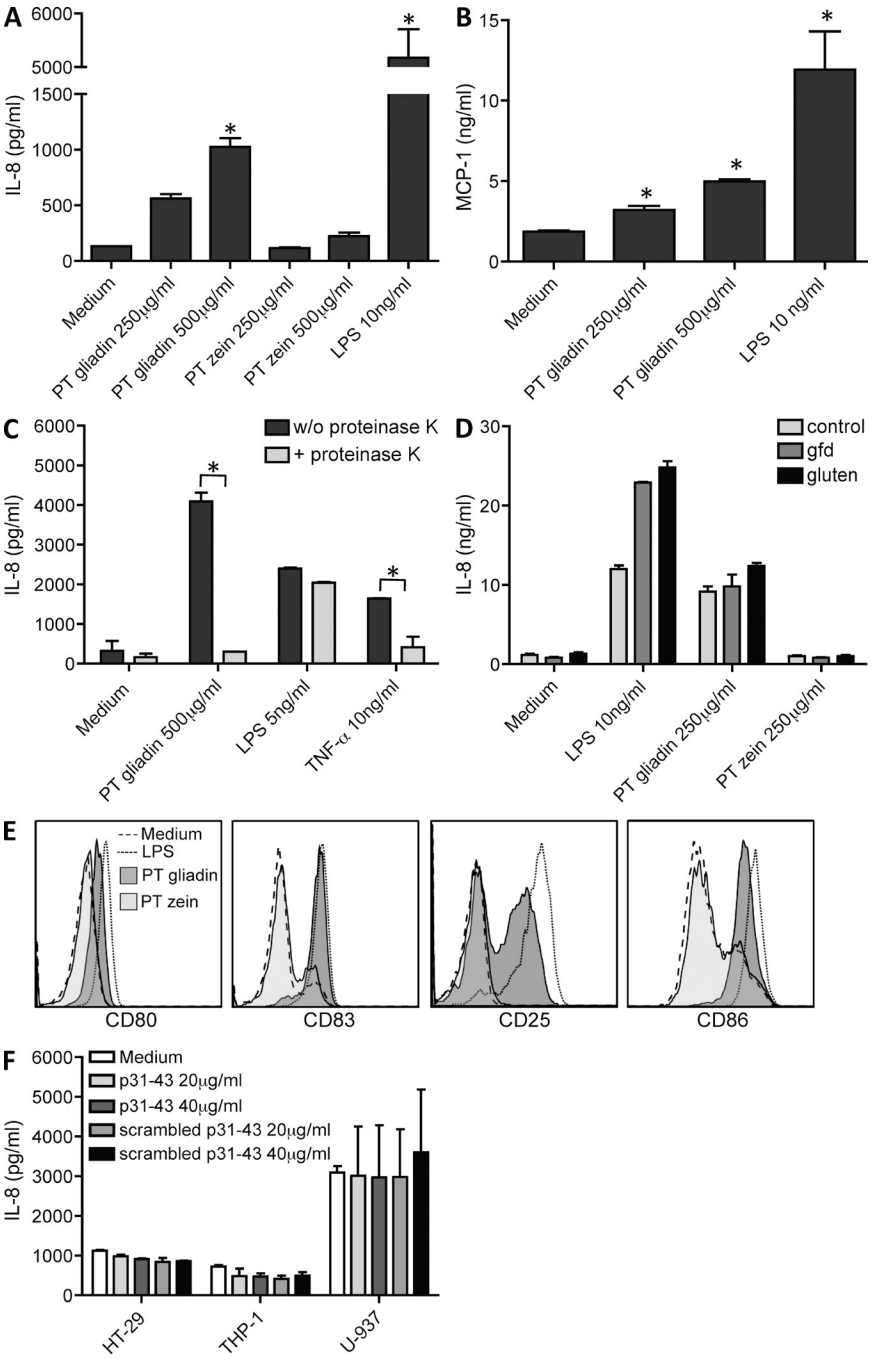


**Figure 1. Gliadin leads to secretion of inflammatory cytokines.** (A and B) THP-1 cells were treated with PT gliadin, and IL-8 (A) and MCP-1 (B) secretion was measured. LPS and PT zein served as positive and negative controls, respectively. (C) Proteinase K digests of PT gliadin, TNF, and LPS were added to THP-1 cells, and IL-8 secretion was used as read out of innate activation. (D) PT gliadin stimulation of monocyte-derived DCs of healthy controls ($n = 10$) and of patients with celiac disease on a gluten-free (gfd; $n = 8$) or regular diet ($n = 3$). Graph shows one representative patient per group. (E) DCs of healthy controls were stimulated with PT gliadin and subjected to analysis by flow cytometry. The dark gray and dotted histograms represent PT gliadin and LPS stimulation, respectively, the light gray histograms show PT zein stimulation, and the dashed lines represent the unstimulated control. (F) HT29, U937, and THP-1 cells were stimulated with gliadin peptide p31-43 or a scrambled control peptide, and IL-8 secretion was measured. *, $P < 0.05$ versus negative control (if not indicated otherwise); graphs in A–C, E, and F illustrate representative data from one of at least three independent experiments, all performed in triplicates. Error bars depict standard errors of the mean.

TLR4 is unique in its ability to mediate cellular activation via the adapter molecule MyD88 and IRF3 (interferon regulatory factor 3). Compared with C57BL/6J wild-type mice, peritoneal macrophages from MyD88 knockout mice displayed markedly reduced KC and TNF secretion after gliadin and LPS stimulation, indicating signaling via MyD88. The TLR3 agonist poly I:C served as a positive control because TLR3 does not use MyD88 as an adapter protein for signaling (Fig. 2 F and not depicted). RANTES, which is secreted upon activation of the alternative pathway, was also increased in supernatants of gliadin-stimulated macrophages from C57BL/6J wild-type mice, suggesting that both the MyD88-dependent and -independent pathways (Fitzgerald et al., 2003) are activated by gliadin (Fig. 2 G).

### Characterization of the TLR4 stimulatory activity in gliadin extracts

Gliadins from the pure wheat strain "Rektor" were separated into their α, γ, and ω fractions via HPLC, and their PT digests were tested on THP-1 monocytic cells. Neither the α- nor the

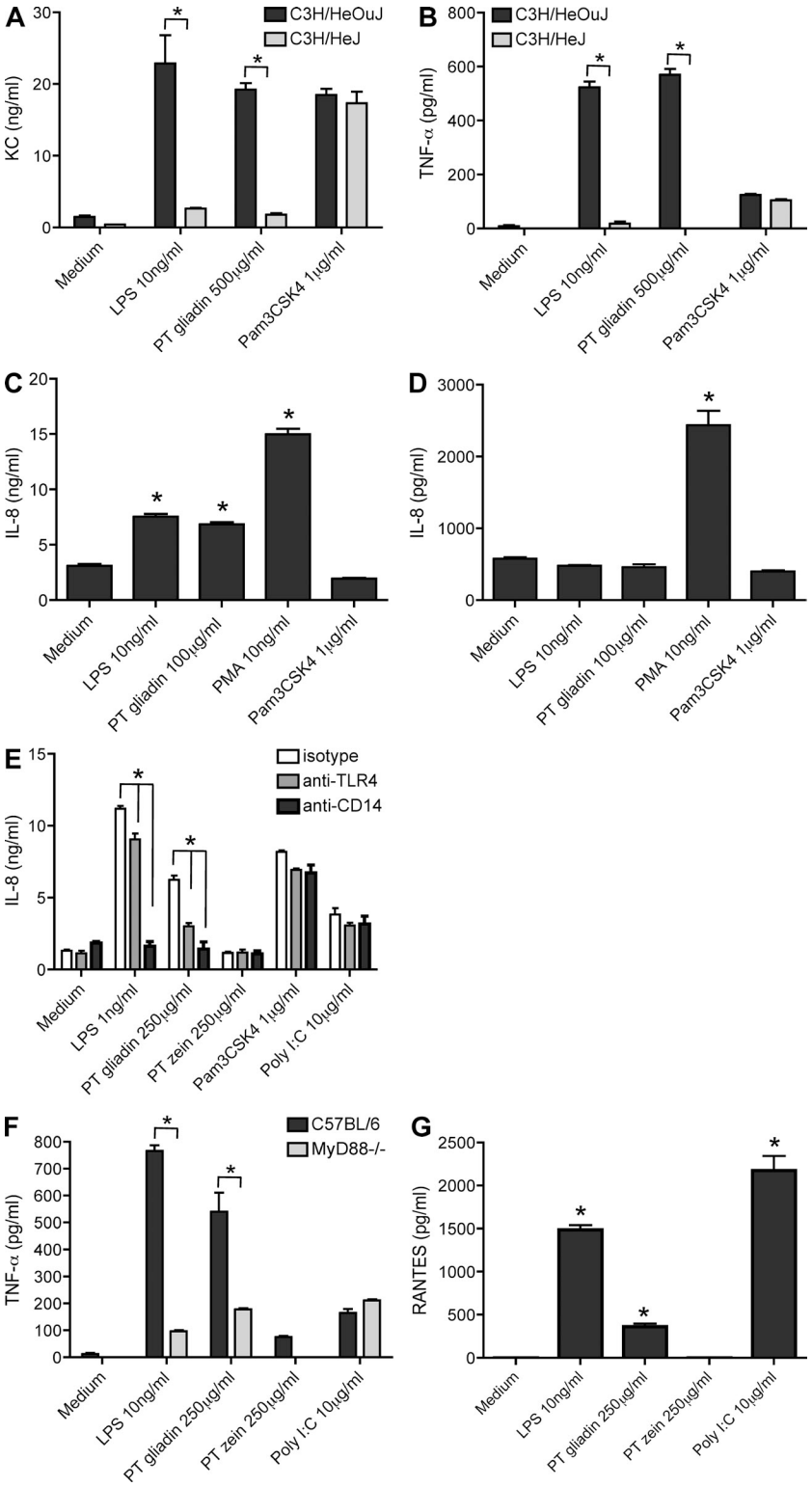


**Figure 2. Gliadin-induced innate immune responses are mediated via TLR4 and via the MyD88-dependent and -independent pathway.** (A and B) KC (murine IL-8; A) and TNF (B) secretion in peritoneal macrophages isolated from TLR4-deficient C3H/HeJ mice compared with C3H/HOuJ wild-type mice stimulated with LPS or PT gliadin. The TLR2 agonist Pam3CSK4 served as cell viability control. (C and D) IL-8 secretion upon PT gliadin and LPS stimulation in 293 cells transfected with the TLR4–MD2–CD14 complex (C) and in untransfected cells (D). LPS and PMA served as positive controls, and the TLR2 agonist Pam3CSK4 served as negative control. (E) IL-8 secretion after preincubation with anti-TLR4 and anti-CD14 antibodies in monocyte-derived DC stimulated with PT gliadin and LPS. TLR2 agonist Pam3CSK4 and TLR3 agonist Poly I:C served as positive controls. (F) TNF secretion in peritoneal macrophages isolated from MyD88$^{-/-}$ mice compared with C57BL/6J wild-type mice upon PT gliadin stimulation. LPS served as positive control for the MyD88 knockdown, and TLR3 agonist Poly I:C served as cell viability control. (G) RANTES secretion in peritoneal macrophages isolated from C57BL/6J mice upon LPS, Poly I:C, and PT gliadin stimulation. *, $P < 0.05$ versus negative (positive) control (if not indicated otherwise); all graphs illustrate representative data from one of at least three independent experiments, and all experiments were performed in triplicates. Error bars depict standard errors of the mean.

γ-gliadins, which represent >90% of total gliadin, harbored stimulatory activity, whereas IL-8 release was strongly induced by PT-digested ω1.2- and ω5-gliadins (Fig. 3 A). The lack of stimulation by α- and γ-gliadins was not caused by toxic effects because addition of LPS or whole PT gliadin fully restored the stimulatory capacity (Fig. 3 A). Furthermore, ω1.2- and ω5-gliadins strongly induced IL-8 secretion in TLR4-transfected but not untransfected HEK-293 cells (Fig. 3, B and C).

Next, we tested synthetic overlapping 20mers covering the ω5-gliadin sequence of 439 aa to identify TLR4-stimulating

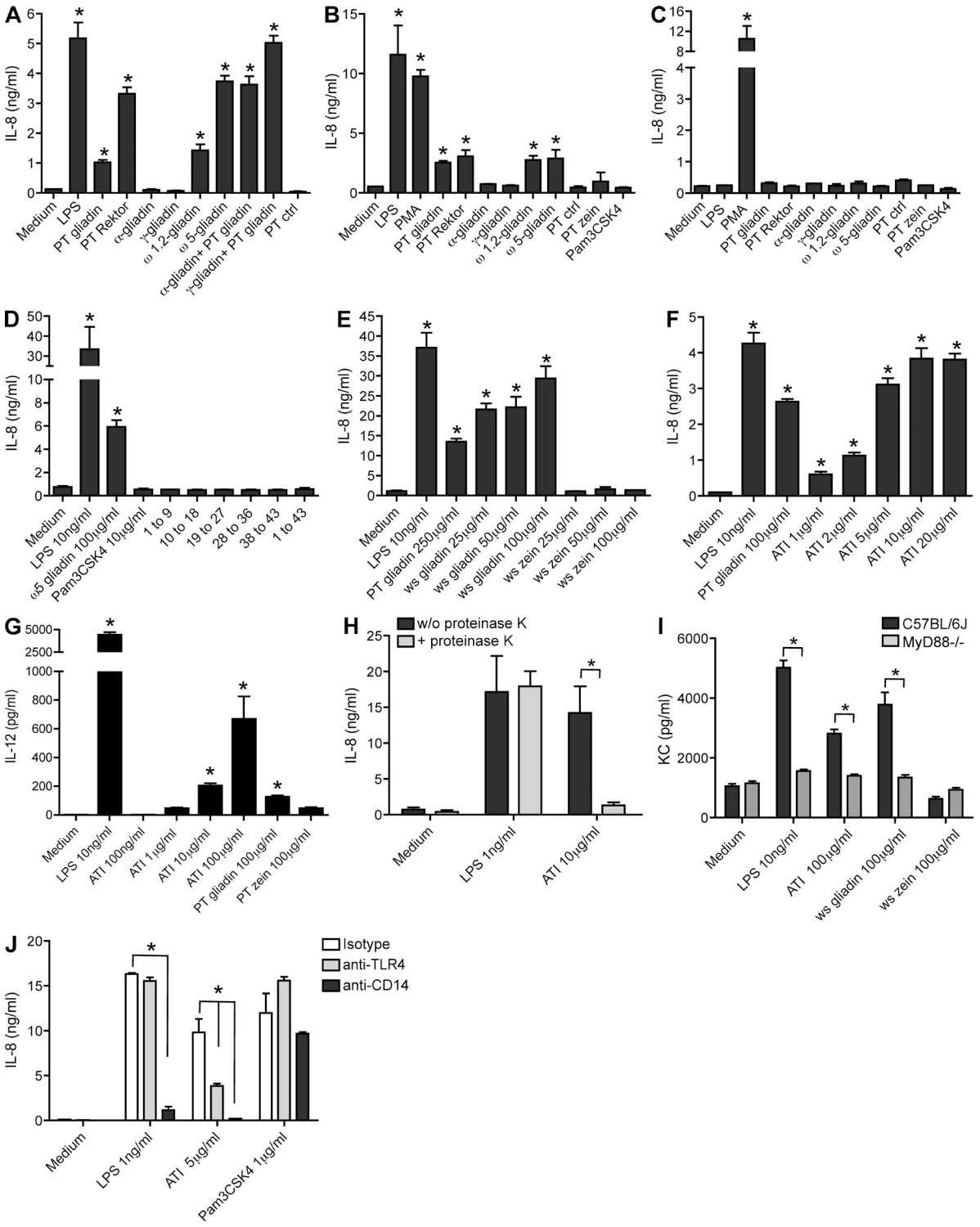


**Figure 3. Gliadin-induced innate immune responses are elicited by wheat ATI, a protein copurifying with ω-gliadins.** (A) Stimulation of THP-1 cells with α-, γ-, ω1.2-, and ω5-gliadin fractions (all 100 μg/ml) isolated from the pure wheat strain Rektor. Co-incubation of α- and γ-gliadin with 100 μg/ml of regular PT gliadin from Sigma-Aldrich served as cell viability control. LPS was used as positive control, whereas PT or PT zein served as negative control. (B and C) IL-8 secretion after stimulation with 100 μg/ml ω-gliadins in TLR4-transfected (B) and in untransfected HEK-293 cells (C). 10 ng/ml PMA served as cell viability control. 10 ng/ml LPS, 100 μg/ml PT gliadin, or 100 μg/ml of a PT digest of Rektor gliadin (PT Rektor) served as positive control, and 100 μg/ml PT zein, 1 μg/ml Pam3CSK4, or a PT mixture (PT ctrl) served as negative controls. (D) Stimulatory capacity of synthetic overlapping 20mers of ω5-gliadin in TLR4-transfected HEK-293 cells. For illustration purposes, 9 fractions each were pooled in the stimulation experiments (each fraction at a concentration of 100 μg/ml), while also all 43 fractions were tested individually. LPS served as positive and Pam3CSK4

peptide sequences. Surprisingly, none of the synthetic peptides triggered IL-8 secretion by TLR4–CD14–MD2-transfected HEK-293 cells (Fig. 3 D). This could have been caused by posttranslational modifications of the gliadin or a particular secondary structure that was not captured by the synthetic peptides. However, most gliadins are not modified posttranslationally, and their structure is well reflected even by smaller peptides (Wieser and Koehler, 2008), raising the possibility that other wheat proteins contained in the ω-gliadin preparation might be responsible for the observed effects. Coomassie staining revealed a 15-kD protein contained in the ω- but not the nonreactive α- and γ-gliadin fractions. Mass spectrometry characterized this protein as wheat ATI, containing mainly its family members CM3 and 0.19 (see sequences shown in Fig. 5). As part of the albumin fraction, wheat ATIs are highly disulfide-linked, water-soluble proteins that copurify specifically with ω-gliadins (Pastorello et al., 2007). In line with this, a water-soluble gliadin extract that contained a prominent 15-kD band (identified to be ATI) showed a higher stimulatory capacity than PT gliadin (Fig. 3 E, not depicted).

Both untreated and PT-digested ATIs purified from wheat induced IL-8 secretion by human DCs (Fig. 3 F and not depicted). Furthermore, ATIs elicited up-regulation of the CD4 T cell–activating cytokine IL-12 in human DCs, thereby providing evidence that ATIs may contribute to Th1 polarization of gluten-specific CD4 T cells (Fig. 3 G). As for PT gliadin, proteinase K digestion abrogated its stimulatory capacity, ruling out LPS contamination (Fig. 3 H). Importantly, peritoneal macrophages from C57BL/6J but not MyD88$^{-/-}$ mice secreted KC (IL-8) upon stimulation with ATI (Fig. 3 I). Moreover, ATI induced up-regulation of IL-8 secretion in TLR4–MD2–CD14-transfected HEK-293 cells as compared with untransfected cells (not depicted), and incubation of human DCs with blocking TLR4 and CD14 antibodies before addition of ATI reduced IL-8 secretion (Fig. 3 J). Overall, these results confirmed the signaling pathways identified above with crude gliadin or ω-gliadin and demonstrated that these innate immune effects were mediated via the nongluten ATI.

To demonstrate that ATI directly interacts with TLR4, coimmunoprecipitation was performed. Biotinylated ATI was able to specifically pull down a soluble flag-tagged TLR4/MD2 fusion protein (Brandl et al., 2005), thereby proving their direct interaction (Fig. 4 A).

CM3 and 0.19, the main ATI family members detected by mass spectrometry, were recombinantly expressed in eukaryotic HEK-293 cells, to avoid potential bacterial contaminants (LPS) and to ensure correct protein folding (Fig. 4, B and C). Both affinity-purified ATIs stimulated TLR4–MD2–CD14-transfected but not untransfected HEK-293 cells, confirming their TLR4-stimulating activity. In line with eukaryotic expression, the stimulatory activity of recombinant CM3 and 0.19 was maintained after additional purification using an endotoxin depletion column (Fig. 4 D and not depicted). In addition, overexpression of both CM3 and 0.19 in TLR4–MD2–CD14-expressing HEK-293 cells strongly induced the canonical and the alternative TLR4 pathway (Fig. 4 E). Sequence comparison revealed that despite significant differences in primary sequence, both CM3 and 0.19 showed five stretches of highly conserved amino acid residues clustered around cysteines, indicative of a similar secondary structure of both ATIs (Fig. 5 A). Other ATI variants that occur in wheat or barley showed similar homology, suggestive of comparable biological activity (Fig. 5 B).

In addition, we could demonstrate that reduction and alkylation of wheat extracts that are enriched in ATIs completely abolished any stimulatory activity (Fig. 4 F). This demonstrates that the highly disulfide-linked secondary structure of ATIs is necessary to activate TLR4 and that indeed the unique ATIs (and no other, less disulfide-linked proteins, including the linear gliadins, or nonpeptidic components) are the main stimulants of wheat-induced TLR4-dependent innate immune reactions.

### ATI elicits innate immune responses in vivo

When injected intraperitoneally, water-soluble gliadin (containing ATI) but not zein led to an increase in peripheral KC and TNF levels comparable with LPS. In contrast, no response was observed in MyD88$^{-/-}$ mice (Fig. 6 A and not depicted). A recent study implicated the adaptive immune system in modulating innate immune responses (Kim et al., 2007). We therefore injected water-soluble gliadin into T cell– and B cell–deficient Rag1$^{-/-}$ mice. Rag1$^{-/-}$ mice showed cytokine levels similar to C57BL/6J mice, indicating that the innate immune response was not modulated by adaptive immunity (Fig. 6 B).

Next, we analyzed local intestinal effects of orally ingested ATI. C57BL/6J mice were first gavaged with water-soluble gliadin, LPS, or PBS, followed by analysis of transcripts of proinflammatory cytokines in the proximal duodenum. Interestingly, only water-soluble gliadin caused an up-regulation of duodenal KC, MCP-1, and IL-1β (but not TNF) transcripts (Fig. 6 C). LPS did not lead to increased cytokine transcription, which can be

or PT zein as negative controls. (E and F) Dose response of IL-8 release by monocyte-derived DCs stimulated with water-soluble (ws) gliadin (which is enriched in ATI; E) or with purified ATI (F). LPS and water-soluble zein served as positive and negative controls, respectively. (G) Secretion of IL-12 in monocyte-derived DCs from healthy subjects upon stimulation with ATI and PT gliadin in the presence of 1,000 U/ml Interferon-γ as co-stimulatory protein. LPS and PT zein served as positive and negative controls, respectively. (H) Effect of proteinase K digestion of ATI and LPS on IL-8 secretion in DCs. (I) KC secretion in peritoneal macrophages isolated from MyD88$^{-/-}$ mice compared with C57BL/6J wild-type mice upon ATI or water-soluble gliadin stimulation. LPS and water-soluble zein served as positive and negative controls, respectively. (J) IL-8 secretion of monocyte-derived DCs stimulated with ATI and LPS after preincubation with anti-TLR4 or anti-CD14 antibodies. TLR2 agonist Pam3CSK4 served as positive control. *, $P < 0.05$ versus negative (positive) control (if not indicated otherwise); all graphs illustrate representative data from one of at least three independent experiments, all performed in triplicates. Error bars depict standard errors of the mean.

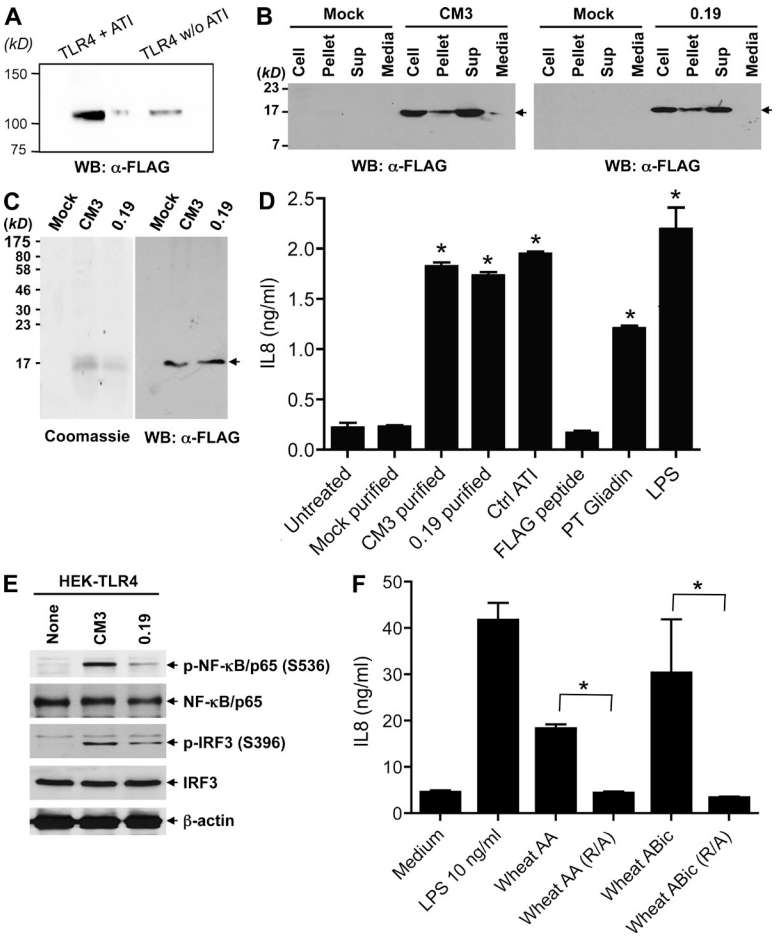


**Figure 4. Identification of ATI CM3 and 0.19 as inducers of innate immune responses.** (A) Coimmunoprecipitation of a soluble flag-tagged TLR4/MD2 fusion protein with or without biotinylated ATI. Pull-down was performed by streptavidin-agarose. Western blot (WB) analysis using a rabbit anti-flag antibody was performed to detect the TLR4/MD2 fusion protein. The image illustrates representative data from one of three independent experiments. (B) CM3 and 0.19 were expressed in HEK-293 cells and purified as Flag-tagged molecules, yielding expected molecular masses of 15 kD and 17 kD, respectively, after 15% SDS-PAGE and Western blotting with Flag antibody. There was no expression in mock (vector only)-transfected cells. 0.19 was not secreted into media but could be recovered in SDS/DTT buffer (Cell), deoxycholate and Triton X-100 buffer (Sup), or the remaining cell pellet. (C) Purity of CM3 and 0.19 ATI after affinity purification on Flag-agarose (15% SDS-PAGE) after staining with Coomassie blue or blotting with Flag antibody. (B and C) Arrows denote expected molecular masses of CM3 and 0.19 ATIs. (D) IL-8 secretion by TLR4–CD14–MD2-expressing HEK-293 cells after stimulation with recombinant ATI. Cells were left untreated or stimulated with affinity-purified detergent extracts from mock-transfected cells, from recombinant CM3, 0.19, or with ATI isolated from wheat (all 5 μg/ml). Controls were 5 μg/ml Flag peptide, 100 μg/ml PT gliadin, and 10 ng/ml LPS. (E) Effect of expression of CM3 and 0.19 in HEK-TLR4 cells on downstream signaling of the canonical (pNF-κB/p65) and the alternative (pIRF3) pathways. Western blots of whole cell lysates (50 μg protein). (F) Reduction and alkylation (R/A) of enriched ATIs from spring wheat (ABic: ammonium bicarbonate extract; AA: acetic acid extract). Stimulatory capacity was measured by IL-8 secretion in U-937 cells. LPS served as positive control. *, $P < 0.05$ versus negative (positive) control. All graphs illustrate representative data from one of at least three independent experiments. D and F were performed in triplicates. Error bars depict standard errors of the mean.

explained by its inactivation at low pH in the stomach and by intestinal epithelial alkaline phosphatase activity (Poelstra et al., 1997). In addition, KC transcripts were found to be increased only in wild-type but not TLR4-deficient mice. The lack of cytokine induction after ingestion of water-soluble zein largely excluded an unspecific reaction to uncommon nutritional antigens (Fig. 6 D).

Subsequently, C57BL/6 mice were gavaged with purified ATI, LPS, or water-soluble zein. As expected ATI increased duodenal transcript levels of KC, IL-1β, and IL-6 (Fig. 6 E). The less pronounced effects with purified ATI as compared with water-soluble gliadin are explained by the low ATI concentrations used in these experiments (0.075 mg of purified protein vs. 2 mg of ATI-containing gliadin extract per gram of mouse weight).

### ATI drives inflammatory responses in cultures of celiac intestinal biopsies

Human duodenal biopsies were incubated with PT gliadin (containing ATI), purified ATI, or a potent T cell stimulatory synthetic 33mer α-gliadin peptide (Shan et al., 2002) with or without purified ATI. Addition of PT gliadin, the 33mer, or of ATI alone to biopsies from patients with celiac disease in remission induced an increase in IL-8 mRNA expression. Moreover, the addition of ATI to the (ATI free) 33mer further elevated IL-8 expression (Fig. 6, F and G).

## DISCUSSION

Celiac disease is considered the best-characterized HLA-linked disease, as its nutritional trigger (gluten), its major genetic predisposition, and the adaptive immune responses to gluten are well defined. However, additional modulating factors, including cereal stimulants of innate immunity, were suspected, but their clear identity and signaling receptors remained elusive (Maiuri et al., 2003; Nikulina et al., 2004; Cinova et al., 2007). In these studies, only MyD88 was identified as a component of the signaling pathway in gliadin-induced innate immunity, whereas TLR4 did not seem to play a role as responsible receptor (Nikulina et al., 2004; Thomas et al., 2006).

Here, we identified the nongluten ATIs CM3 and 0.19 and likely other homologous ATI variants as potent activators

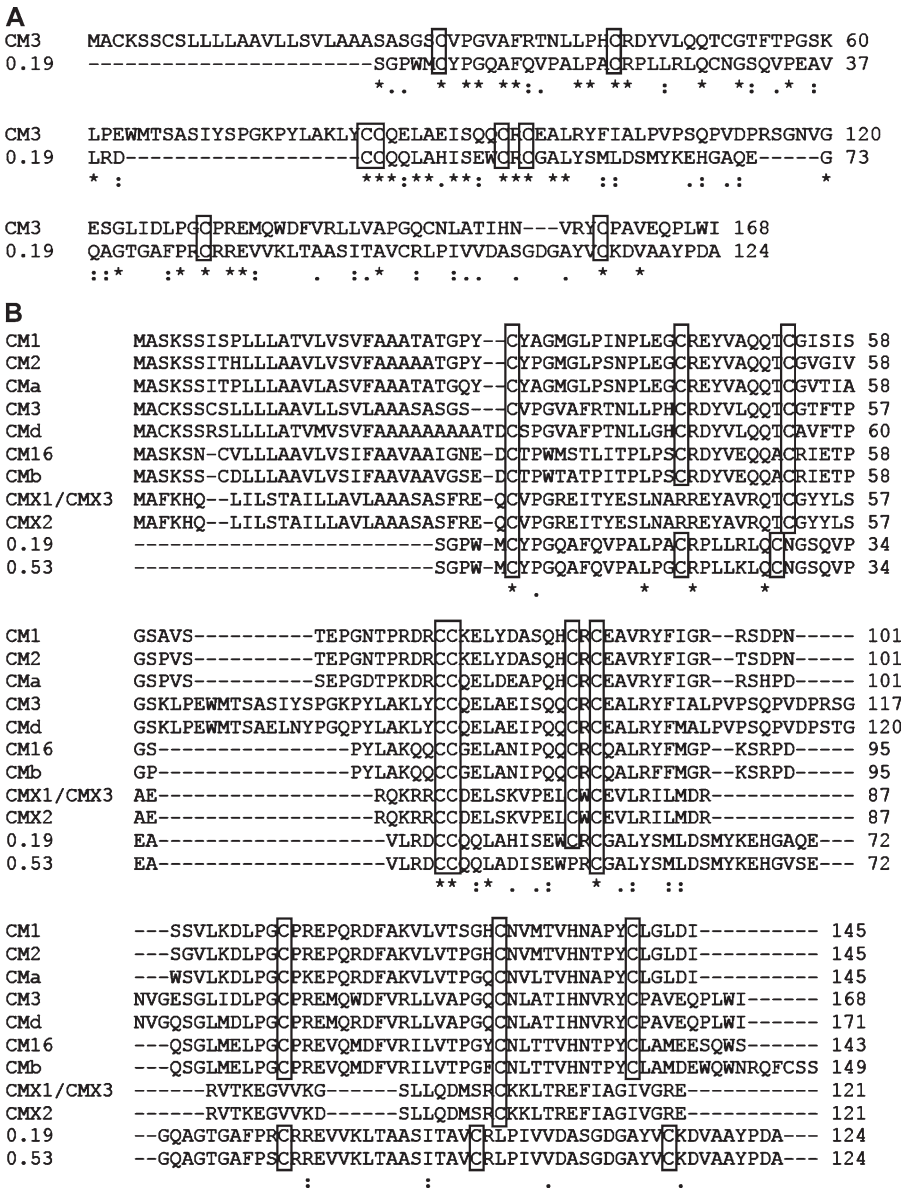


**Figure 5. Sequence alignment of distinct ATI variants from wheat and barley.** (A and B) Sequence alignment between CM3 and 0.19 (A) and between CM3 or 0.19 and other ATIs from wheat (CM1, CM2, CM3, CM16, CMX1, CMX2, 0.19, and 0.53) and barley (CMa, CMb, and CMd; B) using the ClustalW program (2.0.12 version). The highly conserved cysteine residues are boxed. Asterisks, colons, and periods denote identity, high homology, and low homology of amino acid residues, respectively. Similarities are much higher among individual ATIs.

of TLR4. Based on our data, we believe that this interaction of ATI with TLR4 is a major signaling pathway in innate immune reactions to wheat. We did not find activity in gliadins, and reduction of disulfide bonds completely abolished TLR4-stimulating activity in ATI-enriched fractions. However, our findings do not completely rule out other, gliadin-dependent signaling pathways that still remain to be characterized. The highly disulfide-linked ATIs stimulate monocytes, macrophages, and DCs in vitro to produce IL-8 and other inflammatory cytokines such as IL-12, TNF, MCP-1, or RANTES. Notably, feeding ATIs to C57BL/6J mice resulted in upregulation of proinflammatory cytokines in their duodenal mucosa, which confirms resistance of ATIs to enteric proteases and their stimulatory potential in vivo.

ATI, a "contaminant" in gluten preparations, rather than gluten itself might explain observations that in celiac patients in remission a duodenal and rectal gluten challenge increased proinflammatory chemokines and cytokines after only 1–4 h (Kontakou et al., 1995; Chowers et al., 1997), a time frame which is too short to induce adaptive immune responses. Of note, the local gluten doses applied in these studies (6–10 g) already come close to the doses that we used in mice in vivo. Moreover, we can assume that there is increased sensitivity toward ATI in patients with celiac disease or gluten sensitivity compared with healthy controls.

Apart from LPS, other (lower affinity) TLR4 ligands have emerged, i.e., heme, fusion proteins of respiratory syncytial virus, myeloid-related protein 8, heparan sulfate, tenascin C, hyaluronan, and fibronectin (Erridge, 2010). Tenascin C and hyaluronan do not require the same coreceptors as LPS, and heme does not trigger release of cytokines typical for LPS, indicating that the TLR4 complex harbors different

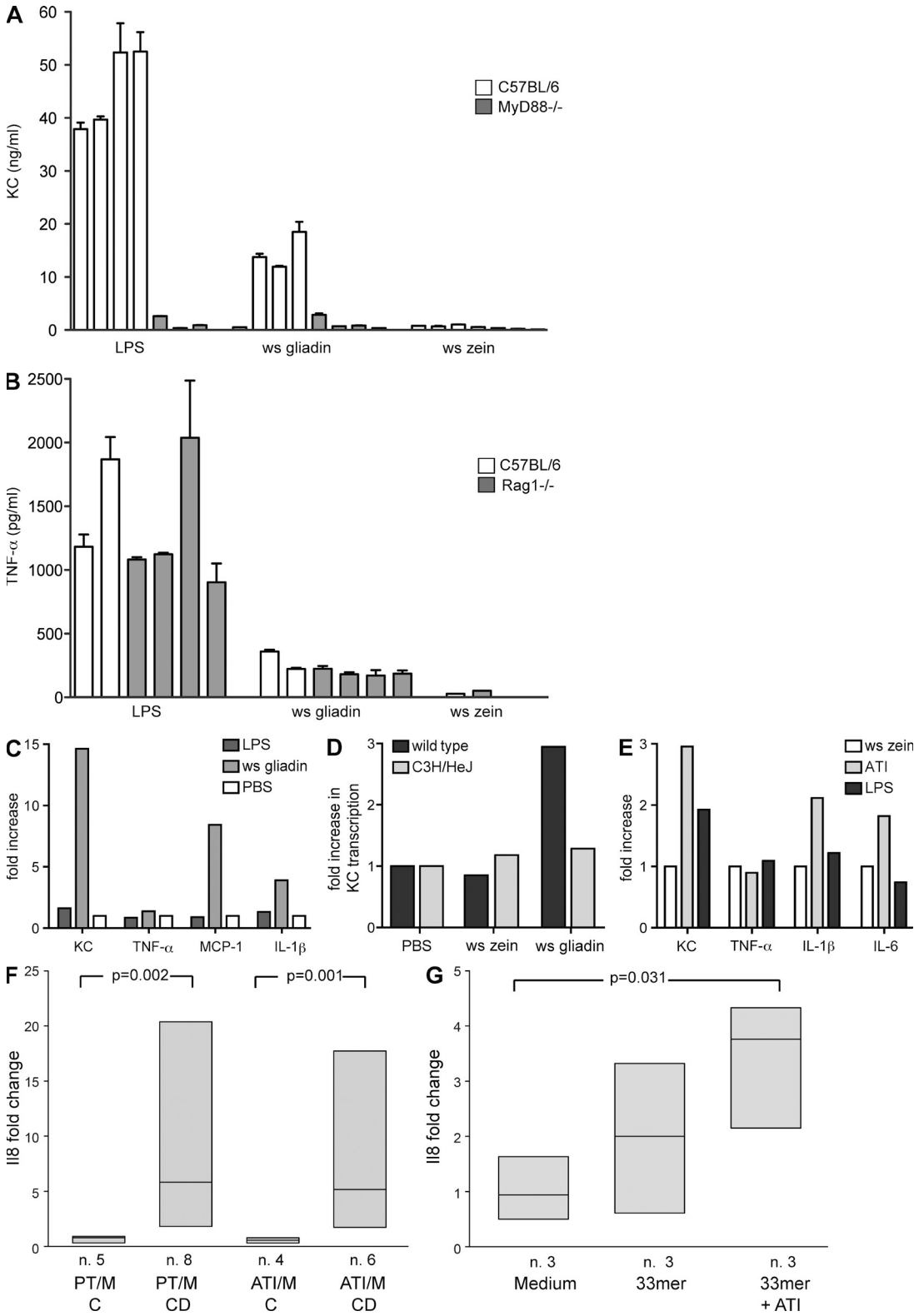


**Figure 6. ATI induces innate immune responses in vitro, in vivo, and in duodenal biopsies.** (A and B) Intraperitoneal injection of LPS (1 μg/g mouse weight), water-soluble (ws) gliadin (500 μg/g mouse weight), or water-soluble zein (500 μg/g mouse weight) into C57BL/6J, MyD88$^{-/-}$ (A) and Rag1$^{-/-}$ (B) mice ($n$ = 4 animals per group). Serum was taken 2 h after injection, and serum cytokine levels were measured by ELISA. (C–E) C57BL/6J (C and E), C3H/HeJ (TLR4 deficient; D), and C3H/HOuJ mice (D) were gavaged with LPS (20 μg/g mouse weight), water-soluble gliadin (2 mg/g mouse weight), ATI (0.075 mg/g mouse weight), water-soluble zein (2 mg/g mouse weight), or PBS. 4 h after gavage, the mice were euthanized, and duodenal

ligand-binding sites that can activate variant signaling pathways (Erridge, 2010).

LPS-mediated TLR4 receptor signaling involves the TLR4–MD2 complex and CD14, a glycosylphosphatidylinositol-anchored cell surface protein that binds LPS, thereby amplifying the signal. Downstream signaling can then engage two different pathways: either activation of the MyD88 pathway leading to activation of NF-κB and transcriptional upregulation of proinflammatory chemokines and cytokines such as IL-8, TNF, and MCP-1 or the TIR domain–containing adapter-inducing interferon-β (TRIF)–dependent cascade driving secretion of type I interferons and RANTES (Fitzgerald et al., 2003; Kawai and Akira, 2005; McGettrick et al., 2006). We demonstrated that TLR4 activation by ATI resembles that induced by LPS because it is CD14 dependent and engages downstream signaling by MyD88 as well as TRIF. However, in contrast to macrophages, in human DCs the cytokine profile induced by ATI differed from that induced by LPS (only modest secretion of TNF and RANTES; unpublished data).

To induce sufficient adaptive immune responses, antigens have to be recognized in the context of an activated innate immune system (Iwasaki and Medzhitov, 2004). Bystander inflammation, e.g., by viral infections, was implicated as innate immune trigger in celiac disease (Schuppan et al., 2009). Therefore, the discovery of ATIs as potent nutritional TLR4 ligands that, in contrast to LPS withstand gastrointestinal degradation, adds an important piece to the puzzle of celiac disease pathogenesis. Of note, we could not confirm gliadins or gliadin peptides, such as p31-43, as innate immune stimulators in our in vitro and in vivo experiments.

As is expected for innate responses, we found a similar extent of activation of DCs from healthy controls and celiac patients in vitro. This raises the question of how this reaction is controlled in the human intestine that generally favors tolerance toward food antigens. Several possibilities can be considered. First, patients with the celiac predisposition may require a lower threshold for immune activation and mucosal inflammation. Second, the TLR4 pathway is tightly regulated and onset of celiac disease therefore could result either from dysregulation of endogenous inhibitory processes or from overactivation (Liew et al., 2005). Thus, intestinal mucosal TLR4 expression in active or remitting celiac is increased (Szebeni et al., 2007), which could lead to hypersensitivity toward stimulation with ATI. Moreover, TLR4 can be negatively regulated by intracellular inhibitors, membrane-bound suppressors, receptor degradation, TLR-induced apoptosis, and microRNAs (Liew et al., 2005; Sheedy et al., 2010). It will therefore be important to determine whether patients with celiac disease (or other inflammatory gastrointestinal diseases) have specific defects in negative regulation of TLR4, which might affect the subtle balance between intestinal tolerance and inflammation.

Importantly, ATIs are the primary resistance molecules of cereals to fend off pests and parasites such as the meal bug (Ryan, 1990; Cordain, 1999), and more recent breeding of high yielding and highly pest-resistant wheat (Ryan, 1990; Cordain, 1999; Sands et al., 2009) has led to a drastic increase of ATI content (Ryan, 1990). Our finding of ATI as a potent stimulator of TLR4 in the intestine might not only be relevant to celiac disease, but is likely to have implications for patients with so-called gluten sensitivity and possibly for patients with irritable bowel syndrome, inflammatory bowel disease, and even nonintestinal inflammation. Patients with gluten sensitivity do not have celiac disease, but their symptoms improve on a gluten-free (i.e., cereal and therefore ATI free) diet, and several case reports documented that other intestinal inflammatory conditions improved on a gluten-free diet (Verdu et al., 2009; Biesiekierski et al., 2011). Moreover, members of the ATI family have been previously characterized as allergens in baker's asthma and gastrointestinal hypersensitivity to wheat (Tatham and Shewry, 2008). It is therefore tempting to speculate that their ability to stimulate TLR4 contributes to their allergenicity, as has been shown recently for the main house dust mite allergen Der p2 (Trompette et al., 2009). In conclusion, we identify ATIs, nutritional proteins from wheat and related cereals, as activators of TLR4 and inducers of innate immune responses in vitro and in vivo, with broad implications not only for the onset and course of celiac disease, but also for other intestinal and possibly nonintestinal inflammatory diseases.

## MATERIALS AND METHODS

**Isolation of gliadin fractions and PT digestion of gliadin (PT gliadin).** Unless otherwise stated, gliadin purchased from Sigma-Aldrich was used for experiments. PT-digested gliadin was generated as originally described (Frazer et al., 1959), with minor modifications. In brief, gliadin was digested with pepsin in 0.1 M HCl, pH 1.8, at 37°C for 4 h, followed by pH adjustment to 7.8 and digestion with trypsin at 37°C for 4 h (substrate/enzyme ratio of 200:1 for both reactions). Adjustment of the pH to 4.5 resulted in a precipitate, which was removed by centrifugation at 2,500 rpm, and *N*-tosyl-chloromethylketone was added to inhibit residual trypsin activity. The supernatant containing the PT gliadin was dialyzed against 10 mM ammonium carbonate, pH 7.8 (molecular mass exclusion 1,000 D; Spectra/Por; Serva), sterile filtered, and lyophilized. A parallel digest of zein from corn (Sigma-Aldrich) was used as negative control.

The water-soluble fraction of gliadin was obtained by incubating 10 g gliadin in 50 ml sterile water at 37°C for 8 h under continuous stirring. Insoluble material was removed by centrifugation, and the supernatant was sterile filtered and lyophilized.

samples were snap frozen in liquid nitrogen. Duodenal cytokine mRNA levels were measured by quantitative RT-PCR ($n$ = 4 animals per group). (F and G) IL-8 transcript levels after incubation of duodenal biopsies with PT gliadin (PT), α-gliadin 33mer, ATI, or 33mer plus ATI (results expressed as medians and quartiles). For F, a biopsy obtained from the same patient and incubated with medium alone (M) served as control; for G, an external calibrator (cDNA derived from RNA extracted from human duodenum) was used. P-values were derived by Student's $t$ test: *, $P < 0.05$ versus negative control. Error bars depict standard errors of the mean.

Gliadin from the pure wheat strain Rektor was subfractionated as described previously (Wieser et al., 1998). In brief, α-, γ-, ω1.2-, and ω5-gliadins were obtained by HPLC purification on a Nucleosil C8 column (4.6 × 240 mm) at 50°C, using 0.1% trifluoroacetic acid as phase A and 99.9% acetonitrile plus 0.1% trifluoroacetic acid as phase B and a gradient from 24% B to 56% B in 30 min. Detection was at 210 nm. Purity of the fractions was confirmed by SDS-PAGE, amino-terminal sequence analysis, and their characteristic amino acid composition.

**Synthetic peptides.** Peptide p31-43 of α-gliadin (sequence LGQQQPFP-PQQPY; Maiuri et al., 2003), a scrambled control peptide (sequence GLQPFQQPQPPQY), and the α-gliadin 33mer (sequence LQLQPF-PQPQLPYPQPQLPYPQPQPF), a potent stimulator of adaptive immunity (Shan et al., 2002), were synthesized by AnaSpec Inc. Purity of the peptides was >80% according to HPLC and mass spectrometry analysis. 43 20mer peptides with an overlap of 10 aa covering the 439-residue ω5-gliadin were synthesized at 60–80% purity by Primm Biotechnology.

**Cell culture and in vitro stimulation experiments.** THP-1, U937, HEK-293 (all from American Type Culture Collection), and HEK-293 cells stably transfected with the TLR4–CD14–MD2 complex (InvivoGen) were cultured in complete RPMI or DMEM (Cellgro) supplemented with 100 IU/ml penicillin/100 μg/ml streptomycin and 10% fetal calf serum at 37°C in a 5% $CO_2$ atmosphere. All cell lines were tested for mycoplasma contamination on a regular basis and proved to be mycoplasma free.

Peripheral monocytes were isolated from 40 ml blood obtained from 11 patients with celiac disease (8 on a gluten-free and 3 on a gluten-containing diet) during their diagnostic workup (median 37 yr, range 18–59 yr) after prior informed consent, and healthy control monocytes were from buffy coats of leukopheresis concentrates of anonymous blood donors (approval #2006-P-000117/4, Institutional Review Board of the Beth Israel Deaconess Medical Center). Peripheral blood mononuclear cells were isolated as described previously (Vissers et al., 1988), and CD14-positive monocytes were purified by MACS separation according to the manufacturer's protocol (Miltenyi Biotec). For generation of DCs, monocytes were cultured in RPMI supplemented with 10% fetal calf serum, 200 U/ml rhIL-4, and 300 U/ml rhGM-CSF (both from PeproTech) for 6–8 d.

Murine resident peritoneal macrophages were isolated by peritoneal lavage using a 3-ml syringe and 18G needles. 3 × 3 ml of sterile PBS was injected into the peritoneal cavity and reaspirated after gentle massage of the abdomen (Fortier et al., 1982). For further purification, MACS separation for CD11b-positive cells was performed according to the manufacturer's protocol. All animal experiments were approved by the review committee of the Beth Israel Deaconess Medical Center (protocol 031-2008).

To elicit IL-12 secretion, human DCs were stimulated with PT gliadin or ATI in the presence of 1,000 U/ml Interferon-γ as co-stimulatory protein. For stimulation, $10^6$/ml cells were seeded in triplicates on polystyrene wells. Unless stated otherwise, supernatants were harvested 16 h after addition of the stimulants.

**Exclusion of LPS contamination.** To prove that stimulatory effects were caused by protein, PT gliadin, wheat ATI, LPS, or TNF was incubated with or without 20 μg/ml proteinase K (Promega) for 4 h at 56°C. After proteinase K inactivation by boiling for 5 min, the digests were used for cell stimulation.

**Coimmunoprecipitation.** A soluble flag-tagged TLR4/MD2 construct (gift of W. Falk, University of Regensburg, Regensburg, Germany; Brandl et al., 2005) was expressed in HEK-293 cells. The supernatant was harvested, and protein was concentrated using a 100-kD size exclusion cartridge (EMD Millipore). Purified wheat ATI was biotinylated using 10 mM *N*-Hydroxy-sulfosuccinimide (NHS) esters of biotin solution according to the manufacturer's protocol (Thermo Fisher Scientific). 1 μg of flag-tagged TLR4/MD2 was then incubated with or without 10 μg ATI for 2 h at 4°C, followed by incubation with streptavidin-agarose for 1 h. Precipitates were boiled at 100°C, and eluates were run on a 4–20% gradient SDS gel followed by Western blot analysis using a horseradish peroxidase–labeled rabbit anti-flag antibody (Sigma-Aldrich).

**Reduction and alkylation on enriched ATIs from spring wheat.** 1 mg of a neutral extract enriched in ATIs from spring wheat (extracted with 50 mM ammonium bicarbonate or 0.05% acetic acid) was reconstituted in 1 ml of 100 mM Tris, pH 8.5, and incubated with 100 mM dithiothreitol (DTT; Promega) at 37°C for 1 h. Samples were alkylated with iodoacetamide (Sigma-Aldrich) at a final concentration of 250 mM for 30 min at room temperature in the dark. Finally, 200 mM DTT was added to quench iodoacetamide, and protein was purified using spin columns (Amicon-Ultra 3K; EMD Millipore). ATI treated in the same way but without the reducing and alkylating agents was used as control. Samples were freeze dried, reconstituted in PBS, and filtrated. Then they were added to the monocytic cell line U937 ($10^6$/ml) in triplicates at a concentration of 50 μg/ml and incubated overnight. IL-8 from cell supernatants was measured by ELISA according to the manufacturer's protocol.

**Animals.** C3H/HeJ, C3H/HeOuJ, and $Rag1^{-/-}$ mice were obtained from the Jackson Laboratory. $MyD88^{-/-}$ mice were a gift from S. Akira (Osaka University, Osaka, Japan; Kawai et al., 1999). Congenic C57BL/6J mice served as experimental controls and were bred under the same conditions in the same facility. All experiments were performed with mice at age 5–7 wk.

**In vivo experiments.** Mice were injected intraperitoneally with water-soluble gliadin, zein (500 μg/g body weight), or LPS (1 μg/g body weight) in 200 μl PBS or PBS alone (negative control). 2 h after injection, mice were euthanized by ketamine/xylazine administration, and blood was drawn by retroorbital bleeding.

Mice were either raised on gluten-free diet (C57BL/6J and $MyD88^{-/-}$ mice) or put on gluten-free diet for at least 2 wk (C3H/HeJ and C3H/HeOuJ mice) and starved the night before the experiment. Gluten-free standardized diet AIN-76A was obtained from Research Diets. All stimulants were diluted in PBS. Mice were administered gliadin, zein (2 mg/g mouse weight), LPS (20 μg/g mouse weight), or ATI (0.075 mg/g mouse weight) in 200 μl PBS via gavage. Mice were euthanized after 4 h, and the duodenum was snap frozen in liquid nitrogen.

**Cytokine/chemokine assays.** The concentration of IL-8, TNF, IL-12, MCP-1, RANTES, and mouse IL-8 (KC) in cell culture supernatants and serum samples was determined using validated ELISAs (IL-8 and hTNF [BD]; MCP-1, hRANTES, mRANTES, KC, and IL-12 [R&D Systems]; and mTNF [eBioscience]) according to the manufacturers' protocols.

**RNA isolation and quantitative RT-PCR.** Samples from the small intestine (0.5-cm segment, 2 cm distal to the pylorus) were collected at sacrifice and snap frozen for further analysis. Total RNA isolation was performed using TRIzol (Invitrogen). Exon–exon boundary-spanning primer sequences were obtained from PrimerBank, and sequences are listed in Table 1. Real-time PCR was performed using 480 SYBR Green I master mix (Roche) and a LightCycler 480 system (Roche). Mouse GAPDH served as endogenous control. PCR was set up in triplicates, and threshold cycle (Ct) values of the target genes were normalized to the endogenous control. Differential expression was calculated according to the 2-ΔΔCT method.

**Blocking experiments.** Monocyte-derived DCs were seeded at a concentration of 1 × $10^6$/ml in 96-well plates. Cells were preincubated with blocking antibodies (10 μg/ml rat anti-TLR4 [InvivoGen] and 20 μg/ml goat anti-CD14 [R&D Systems]) for 3 h at 37°C before stimulation.

**Flow cytometry.** Human monocyte-derived DCs were stimulated with LPS, PT gliadin, and PT zein overnight. For flow cytometry, cells were preincubated with FcR blocking reagent (Miltenyi Biotec) for 15 min at 4°C before staining with monoclonal antibodies (final concentration 10 μg/ml; all from eBioscience) for 30 min at 4°C. Cells were then washed with staining

**Table 1.** Primers used in this study

| Cytokine/chemokine | Forward primer | Reverse primer |
|---|---|---|
| mKC | 5′-CTGGGATTCACCTCAAGAACATC-3′ | 5′-CAGGGTCAAGGCAAGCCTC-3′ |
| mTNF | 5′-CCCTCACACTCAGATCATCTTCT-3′ | 5′-GCTACGACGTGGGCTACAG-3′ |
| mIL-1β | 5′-GCAACTGTTCCTGAACTCAACT-3′ | 5′-ATCTTTTGGGGTCCGTCAACT-3′ |
| mIL-6 | 5′-TAGTCCTTCCTACCCCAATTTCC-3′ | 5′-TTGGTCCTTAGCCACTCCTTC-3′ |
| mMCP-1 | 5′-TTAAAAACCTGGATCGGAACCAA-3′ | 5′-GCATTAGCTTCAGATTTACGGGT-3′ |
| hGAPDH | 5′-TGGTAAAGTGGATATTGTTGCC-3′ | 5′-GGTGAAGACGCCAGTGGAC-3′ |
| hIL-8 | 5′-GGAAGGAACCATCCTCACTGT-3′ | 5′-CCACTCTCAATCACTCTCAG-3′ |

buffer (PBS and 1% BSA), cell viability was assessed by DAPI exclusion (0.1 μg/ml; Roche), and only viable cells were analyzed by flow cytometry using a four-laser LSRII (BD) and FlowJo software (Tree Star).

**Identification of ATI by mass spectrometry.** The 15-kD protein band that copurified with ω-gliadins was subjected to in-gel disulfide reduction and tryptic digestion, followed by nanoflow reverse phase chromatography and tandem mass spectrometry on a 4800Plus MALDI-TOF/TOF instrument (AB SCIEX) as described in detail elsewhere (Kornek et al., 2011; Krishnamurthy et al., 2011).

**Recombinant expression of ATI CM3 and 0.19 proteins.** To exclude bacterial contaminants, recombinant flag-tagged ATI CM3 and 0.19 were generated in eukaryotic mycoplasma-free HEK-293 cells, using cDNAs optimized to fit eukaryotic codon usage (Genscript; CM3 and 0.19 with GenBank accession numbers AY436554.1 and AY729672.1, respectively). Subconfluent HEK-293 cells cultured on 10-cm tissue culture dishes were transfected with 10 μg plasmid DNA encoding CM3 and 0.19 using Lipofectamine 2000 (Invitrogen), followed by incubation for 48 h. Media were obtained by centrifugation at 4,500 rpm for 10 min at 4°C. Cells were subjected to lysis and centrifuged at 14,000 rpm for 20 min at 4°C. Aliquots of the detergent-soluble and insoluble fractions were boiled at 100°C, separated on a SDS-15% polyacrylamide gel, and subjected to Western blot analysis. Protein lysates were probed with rabbit anti-flag antibody, followed by horseradish peroxidase–labeled anti–rabbit IgG (Vector Laboratories). Protein bands were visualized using enhanced chemiluminescence (Thermo Fisher Scientific) and X-Oat 2000A processor (Kodak).

Although sufficient CM3 protein was secreted into media, 0.19 ATI had to be isolated after cell lysis from the detergent-soluble fraction. 0.19 and CM3 were bound to Flag-M2 agarose (Sigma-Aldrich) and washed, and bound ATIs were eluted with TBS containing Flag peptide (Sigma-Aldrich). Eluted CM3 and 0.19 were also applied to an endotoxin removal column (Norgen Biotek Corp.). ATIs were used at 5 μg/ml to stimulate HEK-293 cells stably transfected with the TLR4–CD14–MD2 complex, and IL-8 secretion was quantified after 24 h.

**Downstream signaling pathways induced by CM3 and 0.19 overexpression.** HEK-293 cells stably expressing TLR4–CD14–MD2 were induced to express CM3 or 0.19, respectively, followed by cell lysis. 50 μg protein from cleared lysates was run on a 15% SDS-gel and subjected to Western blotting to analyze activation of the canonical and the alternative (p-IRF3) pathways using antibodies to phosphorylated and unphosphorylated NF-κB/p65 and IRF3 (Cell Signaling Technology).

**Stimulation of duodenal biopsies.** Duodenal biopsies were obtained after informed consent during diagnostic endoscopy from healthy controls and celiac disease patients in remission (on gluten-free diet for ≥24 mo). All experiments were approved by the Ethical Committee of the IRCCS–Ospedale Maggiore Policlinico, Milan, Italy (protocol 70/2004/584). Biopsies were incubated for 4 h in complete medium alone, with 1 mg/ml PT gliadin, 200 μg/ml ATI, 50 μg/ml of synthetic α-gliadin 33mer, or 33mer and ATI. RNA was extracted, reverse transcribed, and analyzed by quantitative RT-PCR as described in the section RNA isolation and quantitative RT-PCR using the SYBR Green method and the primers listed in Table 1.

**Statistical analysis.** Differences were tested for statistical significance by one-way ANOVA multivariate analysis followed by Dunnett's post-test using Prism 4.01 software (GraphPad Software). $P < 0.05$ was considered significant. In all graphs, error bars depict standard errors of the mean. For injection and gavage experiments, at least three mice per group were used. Graphs illustrate representative data from one of at least three independent experiments.

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Author contributions: Y. Junker and D. Schuppan designed the experiments and prepared the manuscript; Y. Junker performed most of the experiments; D. Schuppan conceived and supervised the experiments; S. Zeissig, S.-J. Kim, H. Wieser, D. Barisani, S. Dillon, and V. Zevallos designed and performed experiments; D.A. Leffler recruited patients and contributed to the design of the clinical studies; T.A. Libermann, T.L. Freitag, and C.P. Kelly contributed to the design of experiments. All authors discussed the results and implications and commented on the manuscript at all stages.

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