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Gliadin Peptides Activate Blood Monocytes from Patients with Celiac Disease

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To elucidate the role of innate immune responses in celiac disease, we investigated the effect of gliadin on blood monocytes from patients with celiac disease. Gliadin induced substantial TNF-α and IL-8 production by monocytes from patients with active celiac disease, lower levels by monocytes from patients with inactive celiac disease, and even lower levels by monocytes from healthy donors. In healthy donor monocytes gliadin induced IL-8 from monocytes expressing HLA-DQ2 and increased monocyte expression of the costimulatory molecules CD80 and CD86, the dendritic cell marker CD83, and the activation marker CD40. Gliadin also increased DNA binding activity of NF-κB p50 and p65 subunits in monocytes from celiac patients, and NF-κB inhibitors reduced both DNA binding activity and cytokine production. Thus, gliadin activation of HLA-DQ2$^+$ monocytes leading to chemokine and proinflammatory cytokine production may contribute to the host innate immune response in celiac disease.

KEY WORDS: Celiac disease; Innate immunity; Blood monocytes.

INTRODUCTION

Celiac sprue is a chronic inflammatory disorder of the small intestine induced by dietary gluten or related rye and barley proteins in genetically predisposed individuals. More than 90% of patients with celiac disease express the HLA class II molecule HLA-DQ2, encoded by the DQA1*05 and DQB1*02 alleles, compared to 20–30% in the general population in Europe and Northern America. Symptoms and pathological changes in the small intestine, including villous atrophy, crypt hyperplasia, infiltration of inflammatory cells, and activation of the infiltrating inflammatory cells, are effectively treated by the exclusion of gluten from the diet (1–6).

Lamina propria CD4$^+$ T cells that produce interferon (IFN)-γ in response to gliadin and intraepithelial CD8$^+$ lymphocytes cytotoxic for epithelial cells expressing MHC class I polypeptide-related sequence A (MICA) appear to play fundamental roles in the pathogenesis of celiac disease (7–14). The activation of local as well as systemic humoral responses is reflected in the presence of circulating IgG and IgA antibodies to gliadin, endomysium, tissue transglutaminase (tTG), and other autoantigens (15–22).

Recent evidence indicates that innate immune response cells, including monocytes/macrophages and dendritic cells (DCs), also contribute to celiac disease pathogenesis. Gliadin fragments formed after pepsin digestion induce mouse peritoneal macrophages to produce TNF-α, IL-10, and RANTES and to release increased amounts of nitric oxide in the presence of IFN-γ (23–25). In addition, gliadin triggers NF-κB activation, interferon regulatory factor (IRF)-1 release, and STAT-1-mediated signal transduction in mouse monocytes (26). Importantly, gliadin-induced activation of human monocytes appears to be dependent on the stage of cell differentiation (23–25, 27–29). Gliadin fragments also induce phenotypic and functional maturation of human monocyte-derived dendritic cells (30).

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To further understand the possible role of innate immune cells in celiac disease, we investigated whether peptic fragments of gliadin activate blood monocytes and the mechanism of that activation, and whether the gliadin-induced response of monocytes from celiac patients with active disease differs from the response of monocytes from patients with inactive disease on a gluten-free diet.

METHODS

Food Proteins

Peptic fragments of gliadin and soya protein were prepared using pepsin-agarose gel (ICN, Biomedicals, Inc., Ohio), as previously described (24, 30). Protein concentrations were measured by Bicinchoninic acid assay (BCA Protein assay, Pierce, Rockford, IL). All reagents were tested by the E-toxate test for lipopolysaccharide (LPS) (Sigma, St. Louis, MO) and shown to be below the limit of detection (2 pg/mL).

Study Subjects

The study population consisted of 54 patients with biopsy-proven celiac disease, including 14 symptomatic, untreated subjects [ESPGAN criteria; (31)] and 40 asymptomatic, treated (gluten-free diet) subjects. The control group consisted of 45 healthy blood donors. Patients were recruited after appropriate local ethics committee approval, and informed consent was obtained from all subjects.

Cells and Their Activation

Peripheral blood mononuclear cells were isolated by Ficoll paque (Amersham Biosciences, Piscataway, NJ) and incubated for 24 h in 24-well plates (Nunc, Roskilde, Denmark). Nonadherent cells were removed by washing, and the adherent monocytes were recultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin (Sigma). The monocytes were removed by scraping and analyzed by flow cytometry (BD FACSVantage SE, San Jose, CA) after staining with the indicated monoclonal antibodies (mAbs). The monocytes (70–80% $CD14^+$) were cultured at a concentration of 1×10^6 cells/mL for 24 h in complete RPMI-1640 with gliadin (100–200 μg/mL) alone or with gliadin (100 μg/mL) plus IFN-γ (150 U/mL; R&D Biosciences, Minneapolis, MN), as described previously (7, 29, 32). In parallel monocyte cultures, the NF-κB inhibitors L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, 1.0–25.0 μM) or pyrrolidine dithiocarbamate (PDTC, 1.0–10.0 μM) (both Sigma) were added to the cells for 30 min prior to the addition of gliadin. Cultures were maintained at 37°C, 5% CO_2 in a humidified incubator.

Flow Cytometric Analysis

To evaluate the effect of gliadin fragments on monocyte surface antigen expression, blood monocytes (5×10^5) were incubated with optimal concentrations of FITC-labeled mouse mAbs to HLA-DR, CD40, CD80, CD83, CD86 (all from BD Biosciences Pharmingen), and CD14 and MEM-18 (kindly provided by V. Hořejší, Charles University, Prague). Cells also were incubated with FITC- or PE-conjugated irrelevant antibodies of the same concentration and isotype. After two washings, cells were resuspended in ice-cold PBS with 0.1% NaN_3 or fixed with 2% paraformaldehyde and analyzed by flow cytometry (FACSCalibur, BD Bioscience). Data were evaluated using CellQuest software (BD Biosciences). Staining with propidium iodide was performed to assess cell viability.

Preparation of Nuclear Extracts and Colorimetric NF-κB Assays

Nuclear extracts were prepared from purified monocytes stimulated for 90 min with gliadin digest (100 μg/mL) as mentioned earlier, with or without TPCK (1–25 μM) and PDTC (1–10 μM), using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). NF-κB DNA binding activity was determined using the TransAM NF-κB family transcription factor assay (Active Motif), according to the manufacturer's protocol. Briefly, microwells coated with a double-stranded oligonucleotide containing the NF-κB consensus sequence were incubated with nuclear extracts for 1 h at room temperature and washed with washing buffer. The wells containing captured active transcription factor were incubated for 1 h with mAb specific for p50 or p65 NF-κB subunits, then for 1 h with anti-rabbit IgG coupled to horseradish peroxidase and, after washing, exposed to developing solution for 10 min. Optical density was measured at 450 nm using a Titertec Multiscan MCC/340 (Flow Lab., Irvine, Scotland).

Measurement of IL-8 and TNF-α Proteins

The amounts of IL-8 and TNF-α in supernatants of monocytes (1×10^6 cells/mL) cultured for 24 h with or without gliadin (100 μg/mL) in the presence or absence of IFN-γ (150 U/mL) were determined by ELISA (R&D Systems, Europe, Germany) according to the manufacturer's instructions.

Preparation of Genomic DNA

QIAamp spin columns (QIAGEN, GmbH, Hilden, Germany) were used for rapid DNA purification according to the specifications of the manufacturer. Briefly, lysis buffer and proteinase K were added to the monocyte cultures, which were mixed by pulse-vortexing and incubated for 10 min at 56°C. After the addition of 100% ethanol, the mixture was applied to the QIAamp Spin Column, centrifuged, and the filtrate discarded. After the QIAamp Spin Column was washed with washing buffers, elution buffer was applied, and the column was incubated at 15–20°C for 1 min. The DNA filtrate was collected by centrifugation and stored at 4°C.

Analysis of the HLA Class II Molecule HLA-DQ2

Genes were typed by PCR with sequence-specific primers (33, 34). Oligonucleotide primers (Genovision, West Chester, PA) were designed to amplify the second exon of the class II genes. PCR amplification was carried out in a final volume of 10 μL containing: 6 ng/μL of genomic DNA, PCR Master Mix (200 μM of each dNTP, PCR buffer: 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 0.001% w/v gelatin, 5% glycerol, 100 μg/mL cresol red) complete with 0.4 U/μL Taq polymerase and specific primer mix. Thermal cycling was accomplished by heating the sample at 94°C for 2 min and then running 10 cycles of 10 s at 94°C (denaturation), 60 s at annealing temperature 65°C, and 20 cycles of 10 s at 94°C (denaturation), 50 s at annealing temperature 61°C, 30 s at 72°C (extension). After 30 cycles, the extension was completed, and the samples were stored at 4°C.

Statistical Analysis

The differences among healthy donors and active and treated celiac patients groups were evaluted by the Kruskal–Wallis test and Mann–Whitney test for pairwise comparison. The levels of significance of the Mann–Whitney tests were adjusted according to Holm's method (35). P values < 0.05 were considered significant.

RESULTS

Cytokine Production by Gliadin-Stimulated Monocytes

Since monocytes are a major source of the chemokine IL-8 and the proinflammatory cytokine TNF-α, we evaluated blood monocytes from celiac patients and healthy donors for spontaneous and inducible IL-8 and TNF-α production. Monocytes from patients with active celiac disease and patients with inactive celiac disease on a gluten-free diet spontaneously released low levels of IL-8 (525 $\pm$ 404 pg/mL and 965 $\pm$ 682 pg/mL, respectively, $P > 0.05$), which were significantly greater than the amounts spontaneously released by monocytes from healthy control subjects (131 $\pm$ 202 pg/mL) ($P < 0.001$) (Fig. 1A). The addition of IFN-γ (150 U/mL) to the cultures did not significantly enhance IL-8 production by monocytes from patients or healthy donors. In sharp contrast, the addition of gliadin fragments (100 μg/mL) to the cultures induced monocytes from all three groups to secrete markedly higher levels of IL-8; monocytes from patients with active celiac disease produced more IL-8 (3365 $\pm$ 2451 pg/mL than monocytes from patients with inactive disease on a gluten-free diet (2645 $\pm$ 1723 pg/mL), which in turn produced more IL-8 than monocytes from healthy donors (1046 $\pm$ 783 pg/mL) ($P < 0.001$). The production of

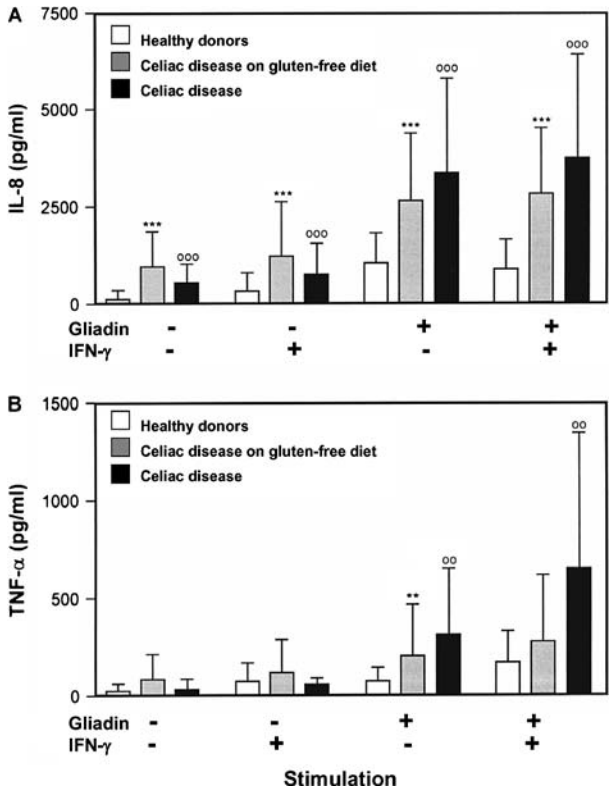

Fig. 1. IL-8 and TNF-α production by monocytes from patients with celiac disease. Monocytes isolated from patients with active celiac disease ($n = 14$), asymptomatic patients with celiac disease on a gluten-free diet ($n = 40$), and healthy donors ($n = 45$) were cultured for 24 h with gliadin (100 μg/mL) alone or with gliadin plus IFN-γ (150 U/mL). The amounts of (A) IL-8 and (B) TNF-α (mean $\pm$ SD) released into the supernatants were determined by ELISA. The level of significance for patients versus healthy donors are indicated as follows: $^{**}P < 0.01$, $^{***}P < 0.001$, $^{oo}P < 0.01$, $^{ooo}P < 0.001$.

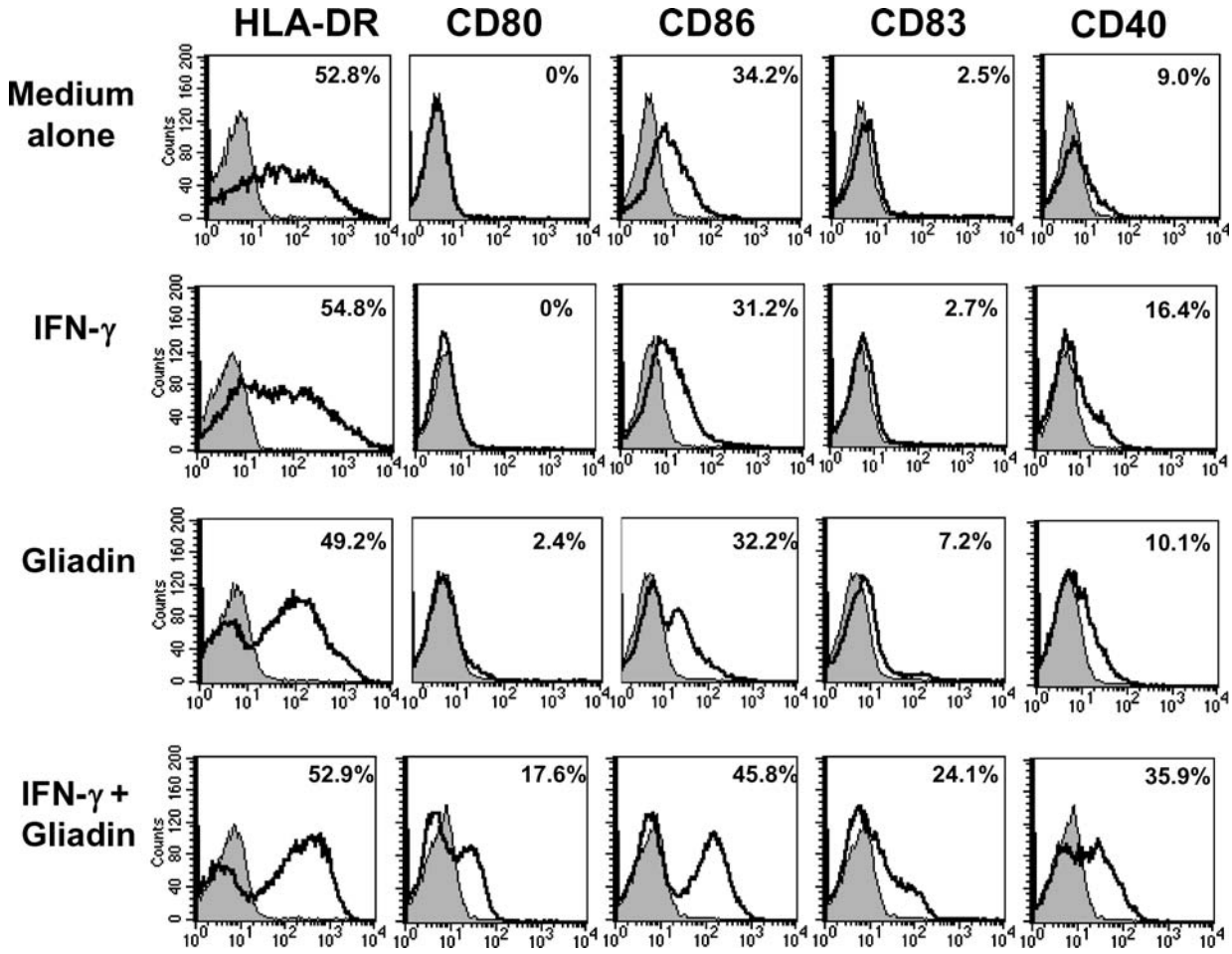

Fig. 2. Effect of gliadin on monocyte phenotype. Blood monocytes from healthy donors were incubated for 24 h in media or with IFN-γ alone, gliadin alone, or IFN-γ plus gliadin at the indicated concentrations and then analyzed by FACS for the expression of activation and differentiation markers. Staining after exposure to isotype-matched Ig is shown as a *shaded histogram*. Values are the percent monocytes that expressed the indicated marker from a representative donor ($n = 3$).

TNF-α by monocytes from the three groups paralleled that of IL-8 (Fig. 1B). The addition of IFN-γ did not further enhance gliadin-induced IL-8 production, but did enhance TNF-α production. Similar results were obtained for IL-8 and TNF-α production when the monocytes were preincubated with IFN-γ (data not shown). In contrast to gliadin, soya proteins (100 μg/mL) with or without IFN-γ did not induce production of IL-8 or TNF-α by monocytes from the three groups.

Gliadin Fragments Induce Differentiational Changes in Monocyte Surface Phenotype

Systemic immune activation is well documented in patients with celiac disease (36–39). In related studies (30), we have shown that gliadin fragments induce maturation of monocyte-derived dendritic cells. Therefore, we determined whether incubation of blood monocytes from healthy persons with gliadin fragments (100 μg/mL) induced changes in monocyte phenotype. Incubation of blood monocytes with gliadin, and to a lesser extent with IFN-γ (150 U/mL) alone, induced a slight increase in the percentage of cells expressing dendritic cell (DC) markers of maturation (CD83) and activation (CD80, CD40), compared to monocytes incubated in medium (Fig. 2). Importantly, incubation of monocytes with gliadin fragments plus IFN-γ induced a marked upregulation in the surface density and percentage of cells expressing CD80, CD86, CD83, and CD40 (Fig. 2). In contrast to the ability of gliadin to induce phenotypic changes, soya proteins (in the presence or absence of IFN-γ) did not alter

Table I. Interleukin (IL)-8 Production by HLA-DQ2-Positive and -Negative Monocytes from Healthy Donors

		Monocyte IL-8 Production (pg/mL)[a]					
HLA Type	Donors (n)	Medium	Gliadin 100 μg/mL	Gliadin 100 μg/mL + IFN-γ 150 U/mL	Gliadin 200 μg/mL	Gliadin 200 μg/mL + IFN-γ 150 U/mL	IFN-γ 150 U/mL
HLA-DQ2$^+$	11	228 ± 116	1792 ± 272	1463 ± 358	1959 ± 271	1801 ± 394	566 ± 222
HLA-DQ2$^-$	34	98 ± 24	788 ± 116	677 ± 97	1073 ± 121	809 ± 96	273 ± 68

[a]Values are expressed as mean ± SEM of IL-8 protein.

monocyte surface protein expression (data not shown). This altered phenotype suggests a synergistic effect of gliadin and IFN-γ on monocyte activation.

Effect of HLA-DQ2 on Monocyte Cytokine Production

Since the majority of celiac patients are HLA-DQ2$^+$, we studied the relationship between HLA genotype and monocyte IL-8 production in healthy control subjects. Monocytes from HLA-DQ2$^+$ healthy donors secreted IL-8 spontaneously. However, in the presence of gliadin or IFN-γ, the monocytes released two- to three-fold more IL-8 than monocytes from HLA-DQ2$^-$ donors (Table I). These findings suggest that the HLA-DQ2$^+$ genotype predisposes monocytes to increased IL-8 secretion.

Gliadin Induces Monocyte Cytokine Production via NF-κB Activation

We have shown perviously that NF-κB activation is involved in gliadin-induced cytokine production by monocyte-derived DCs and THP-1 cells (30). Therefore, we next determined whether the NF-κB signal transduction pathway is also involved in gliadin-induced cytokine production by monocytes. Stimulation of monocytes from celiac patients with gliadin digest (200 μg/mL) resulted in a marked increase in the binding activity of the NF-κB p50 and p65 subunits ($P < 0.05$). Incubation of monocytes from celiac patients with the NF-κB inhibitor TPCK reduced p50 and p65 binding to 20% and 54%, respectively (Fig. 3). Supporting the role of NF-κB in gliadin-induced cytokine production by monocytes from patients with active celiac disease, NF-κB inhibitors also reduced gliadin-induced IL-8 and TNF-α production (Fig. 4). In contrast, stimulation of monocytes from healthy subjects with gliadin caused weak NF-κB binding, which was completely inhibited by TPCK (25 μM/mL), and reduced cytokine production to background levels.

DISCUSSION

We report for the first time that gliadin fragments stimulate cytokine production by blood monocytes from patients with celiac disease. The level of cytokine production varied with disease activity, as monocytes from patients with active celiac disease produced more gliadin-induced TNF-α and IL-8 than monocytes from patients with inactive disease, which in turn produced more inducible TNF-α and IL-8 than monocytes from healthy subjects. We also investigated whether IFN-γ further enhanced gliadin-induced TNF-α and IL-8 production by blood monocytes, since gliadin-induced T cells from patients with celiac disease produce increased levels of IFN-γ. IFN-γ plus gliadin upregulated TNF-α, but not IL-8,

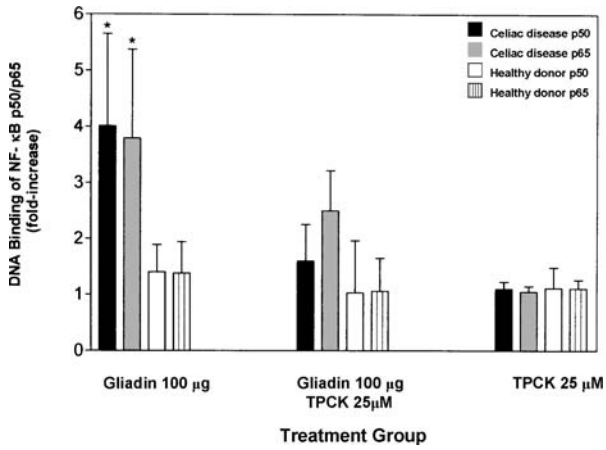

Fig. 3. Gliadin induction of monocyte NF-κB p50 and p65 subunit binding. Monocytes from patients with active celiac disease and healthy donors were analyzed for NF-κB DNA binding activity after a 90 min incubation with gliadin (in the presence or absence of the NF-κB inhibitor TPCK). Data are presented as the fold-increase in DNA binding activity by gliadin-stimulated versus nonstimulated monocytes (mean ± SD for three separate experiments). $^*P < 0.05$ corresponds to the binding activity of monocytes from celiac patients versus that of monocytes from healthy donors.

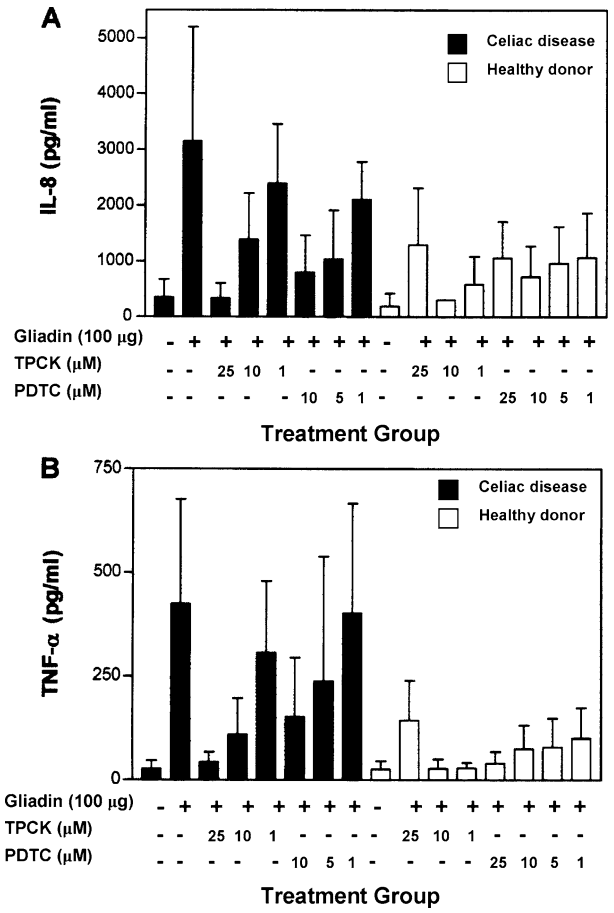

Fig. 4. NF-κB inhibitors block gliadin-induced monocyte IL-8 and TNF-α production. Monocytes from patients with celiac disease and healthy donors were cultured for 24 h with gliadin in the presence or absence of the NF-κB inhibitors TPCK (1–25 μM) or PDTC (1–10 μM), and the amount of IL-8 and TNF-α released into the culture supernatants were measured by ELISA. Results are expressed as mean $\pm$ SD of cytokine production of monocytes from three patients with active celiac disease and six healthy donors.

production. These data suggest that monocytes from celiac patients are activated *in vivo*, consistent with the increased levels of other cytokines, i.e., IL-6, that have been detected in patients with celiac disease (38, 39). Coincident with gliadin-induced monocyte activation, gliadin also induced a DC phenotype in blood monocytes, consistent with our earlier observation that gliadin promoted maturation of monocyte-derived DCs (30). Moreover, coincubation of gliadin with IFN-γ further enhanced the upregulation in the surface density and percentage of cells expressing CD80, CD86, CD83, and CD40, suggesting a synergistic effect of gliadin and IFN-γ on monocyte activation. Since our data indicates that gliadin enhances monocyte IL-8 release, and the majority of celiac patients are HLA-DQ2$^+$ (1–6), we examined the ability of monocytes from HLA-DQ2$^+$ and HLA-DQ2$^-$ healthy subjects to release IL-8 following exposure to gliadin. Monocytes from HLA-DQ2$^+$, but not HLA-DQ2$^-$, healthy subjects spontaneously released IL-8, and monocytes from HLA-DQ2$^+$ persons released two- to threefold more IL-8 than monocytes from HLA-DQ2$^-$ persons. These findings suggest that the enhanced cytokine production by monocytes from patients with celiac disease is due, at least in part, to the activated HLA-DQ2$^+$ population of monocytes.

Celiac disease is characterized by increased infiltration of lymphocytes into the lamina propria and epithelium. Increased production of IL-8 and TNF-α by cells of the innate immune system (monocytes, macrophages, DCs) within the lamina propria could contribute to the pathogenesis of celiac disease through their ability to recruit lymphocytes to the mucosa. In this regard, IL-8 is reported chemotactic for T lymphocytes (40, 41) at concentrations equivalent to those reported in our study (40). Moreover, TNF-α induces the upregulation of vascular ICAM-1, a receptor for the adhesion molecule LFA-1 on memory T cells (42–44). Thus, the production of TNF-α by gliadin-stimulated monocytes in the lamina propria would increase adhesion properties on mucosal vessels and thereby promote T-lymphocyte infiltration into the adjacent mucosa (in celiac disease). In this regard, increased mucosal expression of ICAM-1 has been reported in the intestinal mucosa of patients with celiac disease (42, 43). In addition, IFN-γ, which is abundant in the mucosa of patients with celiac disease (44, 45) and has been implicated in T-lymphocyte recruitment (42–44) (via ICAM-1 upregulation) also has an additive effect on TNF-α-induced T-cell migration (43, 46). Thus, IFN-γ alone, and together with TNF-α, could contribute to T-lymphocyte recruitment in celiac disease. Our findings, therefore, suggest an important potential mechanism whereby gliadin-stimulated release of IL-8 and TNF-α from mucosal innate immune cells contribute to the recruitment of T cells to the mucosa.

While our report has focused on the potential inflammatory effects of gliadin on innate immune cells of the monocytic lineage, other cells within the mucosa, such as epithelial cells, also likely contribute to the innate mucosal response to gliadin in celiac disease. To our knowledge, there are no data available concerning gliadin-induced IL-8 production by primary intestinal epithelial cells, but gliadin is known to alter intercellular tight junction and barrier function in intestinal epithelial cell lines (47, 48), possibly through the induction of zonulin from gliadin-stimulated epithelial cells (47) and macrophages (49).

Members of the NF-κB/Rel family, including the p50 and p65 subunits, regulate inflammatory and immune

responses by inducing the expression of specific genes (50). We show here that exposure of monocytes to gliadin increased the binding activity of the p50 and p65 subunits. Increased p50 and p65 binding was more pronounced in monocytes from celiac patients with active disease than in monocytes from healthy subjects. This finding is consistent with our previous observations that gliadin activates the NF-κB p50/p65 complex in human monocyte-derived DCs (30) and THP-1 monocytes (29) and with observations by others that gliadin activation of macrophages is MyD88- and NF-κB-dependent (26, 49). In this regard, both NF-κB/DNA binding activity and p50/p65 nuclear levels are reported to be elevated in the inflamed mucosa of celiac patients (51). The involvement of the NF-κB/IκB complex in gliadin-induced activation of monocytes was confirmed using the NF-κB inhibitors TPCK, which prevents degradation of IκB inhibitor, and PDTC, which blocks dissociation of the NF-κB/IκB complex (52, 53). Both NF-κB inhibitors substantially reduced gliadin-induced NF-κB binding as well as TNF-α and IL-8 secretion by monocytes. Taken together, these findings underscore the likely involvement of monocyte/macrophage-derived innate immune reponses in celiac disease and suggest that NF-κB regulation should be considered in designing future therapeutic strategies.

In normal persons, wheat gluten, the major source of dietary gliadin, does not induce the T-cell activation or intestinal inflammation characteristic of celiac disease. In addition, intestinal macrophages isolated from healthy subjects do not respond to gliadin *in vitro* (Smythies, unpublished observation), which is consistent with the profound inflammation anergy characteristic of intestinal macrophages (54). Since intestinal macrophages are derived from blood monocytes (55), our findings suggest that local mucosal factors likely downregulate the ability of monocytes newly recruited to the mucosa to respond to gliadin. Since the "normal" downregulation of gliadin-induced monocyte activation and differentiation may be impaired in celiac disease, indentification of these mucosa-derived factors could have important therapeutic implications.

CONCLUSIONS

In summary, gliadin-derived fragments activate monocytes from celiac patients and HLA-DQ2$^+$ monocytes from healthy donors. The higher activity of monocytes in celiac patients could augment gliadin-specific immune responses and thus contribute to the pathogenesis of celiac disease.

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