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Gliadin Fragments Induce Phenotypic and Functional Maturation of Human Dendritic Cells[1]

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Celiac disease is a chronic inflammatory disease developing in genetically predisposed individuals. Ingested gliadin, the triggering agent of the disease, can cross the epithelial barrier and elicit a harmful T cell-mediated immune response. Dendritic cells (DC) are supposed to play a pivotal role in shaping the immune response. The direction of the immune response toward immunity or tolerance depends on the stage of maturation and the functional properties of the DC. DC become fully functional APC upon maturation by various stimuli. We investigated the effect of a peptic digest of gliadin on the maturation of human monocyte-derived DC. Stimulation of cells with gliadin, in contrast with other tested food proteins, led to enhanced expression of maturation markers (CD80, CD83, CD86, and HLA-DR molecules) and increased secretion of chemokines and cytokines (mainly of IL-6, IL-8, IL-10, TNF-α, growth-related oncogene, MCP-1, MCP-2, macrophage-derived chemokine, and RANTES). Maturation was accompanied by a greater capacity to stimulate proliferation of allogeneic T cells and significantly reduced endocytic activity. Furthermore, gliadin-induced phosphorylation of members of three MAPK families (ERK1/2, JNK, and p38 MAPK) was demonstrated. The largest contribution of p38 MAPK was confirmed using its inhibitor SB203580, which markedly down-regulated the gliadin-triggered up-regulation of maturation markers and cytokine production. Gliadin treatment also resulted in increased NF-κB/DNA binding activity of p50 and p65 subunits. Taken together, gliadin peptides can contribute to overcoming the stage of unresponsiveness of immature DC by inducing phenotypic and functional DC maturation, resulting in more efficient processing and presentation of gliadin peptides to specific T lymphocytes. *The Journal of Immunology,* **2005, 175: 7038–7045.**

Oral tolerance, i.e., the state of immune unresponsiveness to food Ags, is a complex process, and its establishment and maintenance are not completely elucidated. However, some food constituents, including wheat components, cause abnormal or adverse reactions in susceptible individuals. Wheat intolerance encompasses a broad spectrum of disorders with different pathologic mechanisms and clinical symptoms. The most frequent diseases caused by wheat ingestion are T cell-mediated disorder, celiac disease, and IgE-mediated allergic reactions.

Celiac disease develops in genetically susceptible individuals expressing HLA-DQ2 or HLA-DQ8 Ags. Gluten, a complex mixture of gliadin monomers (of α, β, γ, and ω type) and glutenin polymers, is subjected to the action of enzymes in the digestive tract and intestinal epithelial cells. Gliadin fragments cross the epithelium, access APC, and are presented to intestinal $CD4^+$ T cells together with simultaneous signaling mediated by costimulatory molecules. Afterward, activated gliadin-specific T lymphocytes secrete a spectrum of chemokines and cytokines affecting other cell types' participation in pathogenetic mechanisms of the disease (1).

Dendritic cells (DC)[3] are the most efficient APC, determining the balance between immunity and tolerance induction. DC are present in different compartments of the gut, including intestinal lamina propria, Peyer's patches, and mesenteric lymph nodes (2, 3). Recognition of pathogen-derived products (LPS, lipoteichoic acid, and bacterial CpG motif) mediated by specific receptors on DC initiates the process of maturation, which can be further modulated by inflammatory stimuli (TNF-α and IL-1β), or T cell-derived signals (CD40L (CD154) and IFN-γ) (4). Activated DC undergo morphological, phenotypic, and functional changes that culminate in the complete transition from Ag-capturing cells to fully mature APC. Maturation is characterized by increased expression of costimulatory molecules such as CD40, CD80, and CD86; MHC up-regulation; the loss of the capacity to take up and process Ags; and the production of a wide spectrum of inflammatory cytokines and chemokines (5). Mature DC migrate toward T cell-rich areas of secondary lymphoid organs and induce a primary immune response by stimulating naive T cells. The cytokine microenvironment generated at the moment of interaction of T cells and DC also affects the final outcome of the immune response (6). We previously showed that proteolytically treated gliadin can activate monocytes and macrophages in vitro (7, 8). Furthermore, stimulation of small intestine biopsies with gliadin resulted in an increase in costimulatory molecules on APC (9).

Several reports documented the involvement of NF-κB and MAPK in the DC signaling pathway and maturation (10–12). At least three main families of MAPK exist in mammals, including

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[3] Abbreviations used in this paper: DC, dendritic cell; CM, complete medium; MFI, mean fluorescence intensity; poly (I:C), polyinosinic-polycytidylic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

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ERK, JNK, and the p38 MAPK. These kinases are activated by phosphorylation of both tyrosine and threonine residues by distinct upstream kinases. The ERK pathway appears to regulate cell proliferation and differentiation in response to mitogens and growth factors, the JNK and p38 cascades are reported to be predominantly activated by stress-inducing agonists such as LPS, heat shock, osmotic changes, and inflammatory cytokines (13).

In this study we have investigated the effect of gliadin peptic digest on human monocyte-derived DC, with specific emphasis on the expression of maturation markers, production of chemokines and cytokines, and the appropriate functional changes. Moreover, the participation of the NF-κB complex and various MAPK in the DC maturation process was analyzed.

Materials and Methods

Abs and reagents

The following anti-human mAbs were purchased from Immunotech: CD83-FITC, CD80-FITC, CD86-PE, CD40-FITC, and CD14-PE. CD11c-PE and HLA-DR-FITC were obtained from BD Pharmingen. Anti-phospho-p38 MAPK Ab, anti-phospho-JNK1/2 Ab, anti-phospho-ERK1/2 Ab, and anti-ERK1/2 Ab were obtained from Cell Signaling Technology. The p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, the serine protease inhibitor L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK; Sigma-Aldrich), and the ERK inhibitor UO126 (Cell Signaling Technology) were dissolved in DMSO (Sigma-Aldrich).

Medium and cell culture

Cells were cultured in the complete RPMI 1640 medium (CM; Cambrex) supplemented with L-glutamine (2 mM; BioWhittaker), penicillin/streptomycin (100 U penicillin/ml and 100 μg of streptomycin/ml), and 10% FBS (Cambrex) at 37°C in a 5% CO_2 atmosphere.

Food proteins and proteolytic treatment

Peptic fragments of gliadin, soya protein, and OVA (Sigma-Aldrich) were prepared using the pepsin-agarose gel (ICN Biomedicals). Seven milliliters of 1% gliadin, soya protein, or OVA in 0.1 M HCl (pH 1.8), was incubated with 5 ml of pepsin-agarose gel at 37°C for 45 min. Removing the pepsin-agarose gel by centrifugation (1,500 × *g*, 10 min) stopped enzymatic cleavage. The supernatants were then centrifuged (12,000 × *g*, 10 min), and the pH of soluble protein fragments was adjusted to 7. Protein fragments were divided into aliquots and frozen at −20°C.

To exclude contamination by LPS, Ag samples as well as all other reagents were tested by E-toxate test (Sigma-Aldrich), and only those below the LPS detection limit were used in the study.

Generation, maturation, and gliadin treatment of DCs

The sources of cells were buffy coats acquired from healthy donors (provided by Institute of Hematology and Blood Transfusion). PBMC were separated by Ficoll-Paque gradient centrifugation (Amersham Biosciences) according to the manufacturer instructions. PBMC at the concentration of 3×10^6 cells/ml were incubated 2 h in 75-cm^2 plastic culture flasks (Nunc). The nonadherent fraction was washed out thoroughly, and isolated adherent monocytes were incubated in CM in the presence of human GM-CSF (500 U/ml; Leucomax; Novartis) and human rIL-4 (20 ng/ml; PeproTech U.K.) for 5 days. The DC generated ($CD11c^+$,$CD14^-$) were harvested and seeded at a concentration of 0.5×10^6 cells/ml in 24-well plates. Gliadin digest (1, 10, or 100 μg/ml) and/or IFN-γ (75 U/ml; R&D Systems), LPS (1 μg/ml; *Escherichia coli*; Sigma-Aldrich), or polyinosinic-polycytidylic acid (poly (I:C); 50 μg/ml; Sigma-Aldrich) were added to cells for 24 h to induce DC maturation.

FACS staining

DC generated by in vitro culture were subjected to flow cytometric analysis using FACSCalibur (BD Biosciences), and the data were analyzed using CellQuest software (BD Biosciences). Cells (5×10^4) were incubated with FITC- or PE-conjugated mAbs on ice for 30 min at 4°C. After two washings, cells were resuspended in ice-cold PBS with 0.1% NaN_3. Staining with propidium iodide was performed to assess cell viability. FITC or PE fluorescence was measured using 530- and 575-nm filters with log-arrhythmic mode acquisition.

FITC-dextran endocytosis

DC (1×10^5) were harvested and resuspended in 100 μl of CM. Cells were incubated with 100 μg of dextran conjugated with FITC (Sigma-Aldrich) for 1.5 h at 37°C in 5% CO_2 or on ice at 0°C (control samples). After three washings in cold PBS, the cells were resuspended in PBS with 0.1% NaN_3, and mean fluorescence was measured by flow cytometry. Nonspecific binding of dextran to DC was determined by incubation at 0°C.

Mixed leukocyte reaction

T lymphocytes from the nonadherent fraction of PBMC were separated with microbead-conjugated CD4Ab (Miltenyi Biotec) and purified using midiMACS separation columns (Miltenyi Biotec). Then the lymphocyte population (95% of $CD4^+$ cells) was washed twice in PBS and labeled by CFSE as previously described (14), using the Vybrant CFDA SE Cell Trace kit (Molecular Probes). In brief, cells were resuspended in 1 μM CFSE in PBS. After 8 min of shaking at room temperature, cells were washed in pure FBS and twice in PBS with 10% FBS. DC (1×10^4) or DC treated for 24 h with gliadin fragments (100 μg/ml) or LPS (1 μg/ml) were cocultured with 1×10^5 allogeneic CFSE-labeled T lymphocytes in 200 μl of CM/well in 96-well, U-bottom plates (Nunc). A negative control ($CD4^+$ lymphocytes alone) and a positive control ($CD4^+$ lymphocytes with 1 μg of PHA) were performed in each experiment. After 5 days, the cells were harvested and washed in PBS. CSFE dilution in optically gated lymphocytes was assessed by flow cytometry.

Human cytokine array

The spectrum of cytokines produced by DC untreated and treated with gliadin fragments (100 μg/ml) was tested using Ab-based protein microarray (RayBio Human Cytokine Ab Array III and 3.1 Map; RayBiotech) designed to detect 42 growth factors, cytokines, or chemokines. Experiments were performed as recommended by manufacturer. Proteins were detected by enhanced chemiluminescence visualized using Fuji Film Las-1000.

ELISA

The concentrations of IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and TGF-β were determined in cell culture supernatants collected after 24 h of cultivation using an ELISA Duo Set kit (R&D Systems) or Cytoset (BioSource International) according to the manufacturer's instructions.

Preparation of nuclear extract and colorimetric NF-κB assay

Nuclear extracts were prepared from DC stimulated for 90 min with gliadin digest (100 μg/ml) alone or together with TPCK (25 μM) or LPS (1 μg/ml) using a nuclear extract kit (Active Motif). NF-κB DNA binding activity was detected using a TransAM NF-κB family transcription factor assay kit (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 the nuclear extract for 1 h at room temperature and washed three times with washing buffer. The captured active transcription factor was incubated for 1 h with Ab specific for p50, p65 (Rel-A), p52, Rel-B, and c-Rel NF-κB subunit, then for 1 h with anti-rabbit IgG-coupled HRP and after washing was exposed to developing solution for 10 min. The OD was measured at 450 nm using a Titertec Multiscan MCC/340 (Flow Laboratories).

Western blot analysis of cellular MAPKs

After treatment with MAPK inhibitors SP600125 (5–20 μM), SB203580 (5–10 μM), and U0126 (5–10 μM) for 30 min, DC (4×10^6) were stimulated with gliadin digest (100 μg/ml) for 15 or 30 min, then washed twice with cold PBS containing 6.25 mM sodium fluoride, 12.5 mM β-glycerophosphate, 12.5 mM para-nitrophenyl phosphate, and 1.25 mM $NaVO_3$ and incubated with 100 μl of lysis buffer (Active Motif). The homogenates were incubated on ice for 15 min, then passed 10 times through a 25-gauge needle and centrifuged at 15,000 rpm for 15 min at 4°C. Cell lysates were subjected to electrophoresis on 10% gel and transferred to nitrocellulose membranes for Western blot analysis. After blocking with 5% fat-free dried milk for 1 h at room temperature, the membranes were incubated overnight with Abs raised against phosphorylated p42/44 ERK, p38 MAPK, and p46/54 JNK. The membranes were washed and incubated with HRP-labeled goat anti-rabbit IgG (Cell Signaling Technology) for 1 h. Immunoreactive bands were visualized using ECL detection reagent (Amersham Biosciences). After stripping, the membranes were reprobed with Abs raised against ERK as loading controls. Immunoblots were quantified by using Kodak Digital Science 1D Image Analysis software (Eastman Kodak).

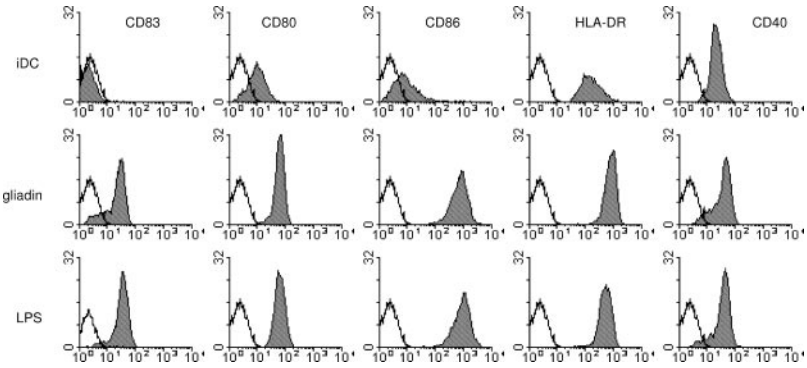


FIGURE 1. Flow cytometric analysis of DC maturation after exposure to gliadin digest and LPS. Monocyte-derived DC were stimulated with gliadin digest (100 μg/ml) or LPS (1 μg/ml) or were left untreated (iDC) for 24 h and immunostained with mAbs against CD83, CD80, CD86, HLA-DR, and CD40 molecules (shaded histograms). Open histograms represent the isotype control mAb staining of the cells. Representative histograms of at least three independent experiments are shown.

Statistical analysis

Data were calculated as the arithmetic mean of at least three independent experiments ± SD. Statistical analysis was performed using a nonparametric, two-tailed Mann-Whitney *U* test from GraphPad (PRISM 3.0). A value of $p < 0.05$ was considered significant.

Results

Peptic fragments of gliadin induce maturation of human DC

The treatment of DC generated from blood monocytes with peptic fragments of gliadin (100 μg/ml) for 24 h led to up-regulation of surface maturation markers, including CD83, CD80, CD86, CD40, and HLA-DR Ags, as documented in Fig. 1.

The effect of gliadin digest was dose dependent and was still observed when using gliadin at 1 μg/ml. After challenge with gliadin peptides (100 μg/ml), the mean fluorescence intensities (MFIs) increased from ~2.0- to 9.0-fold for various markers. The stimulatory effect elicited by gliadin peptides at a concentration of 100 μg/ml was similar to that induced by LPS (1 μg/ml) or poly (I:C) (50 μg/ml). The addition of gliadin in combination with the second, T cell-derived signal IFN-γ (75 U/ml) to cell cultures did not further increase the expression of any activation marker tested (data not shown). Proteolytic fragments of two other food proteins, OVA and soya proteins, were prepared in the same way and used as a control. Soya protein had no effect on DC maturation; however, addition of the highest dose of OVA digest (100 μg/ml) caused a mild up-regulation of the CD86 molecule (Fig. 2).

Gliadin fragments stimulate chemokine and cytokine production by DC

The maturation of DC is a process associated with the secretion of a wide spectrum of chemokines and cytokines (15). The profile of chemokines and cytokines secreted by gliadin-stimulated DC was tested using human cytokine arrays and compared with that of untreated cells (Fig. 3). Clearly visible dark spots on the map corresponded to positive controls and to IL-6, IL-8, MCP-1, and growth-related oncogene production; less intensive spots corresponded to macrophage-derived chemokine, TNF-α, RANTES, and growth-related oncogene-α; and spots of thymus and activation-regulated chemokine, MCP-2, and epidermal growth factor had a low, but detectable, intensity. Only a low production of IL-8 was detected in culture medium from untreated, immature DC.

The cytokine secretion by DC in response to various doses of gliadin fragments (10 and 100 μg/ml) was further quantified by ELISA. As shown in Table I, gliadin markedly increased the release of IL-6, IL-8, and TNF-α elicited in a dose-dependent manner. Moreover, slightly increased secretion of IL-10 and IL-4 was detected. Interestingly, we did not find any production of IL-12 p70 after gliadin treatment, in contrast with the effect of LPS. In comparison with untreated DC, cultivation with gliadin decreased TGF-β1 production. Fragments of soya protein and OVA applied in the same doses as gliadin had no effect on either IL-8 or TNF-α secretion by DC (data not shown).

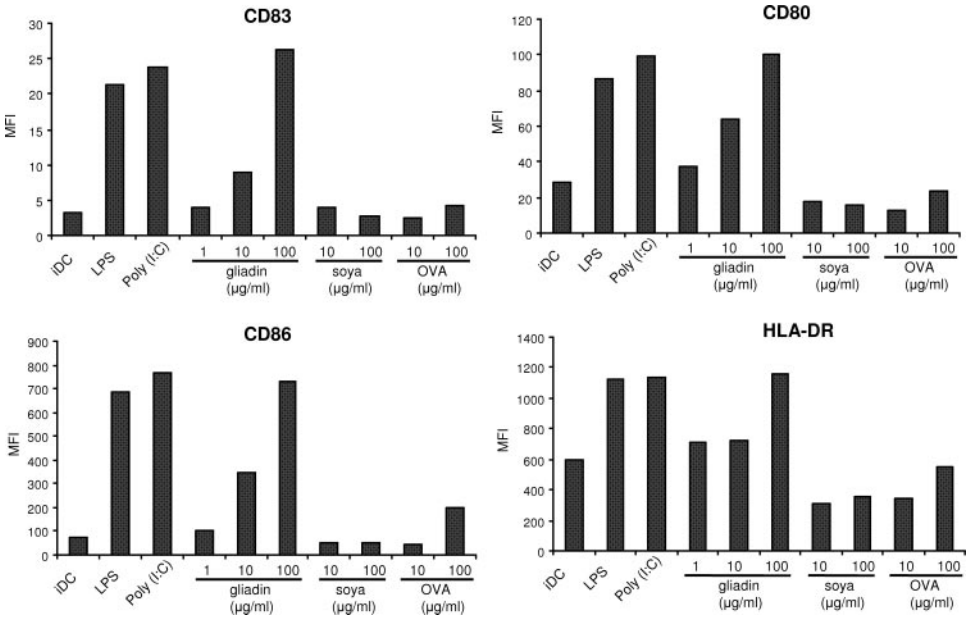
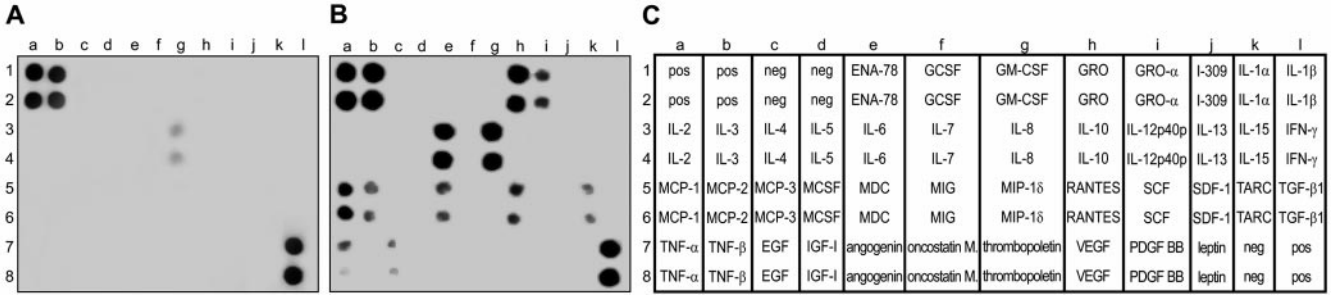


FIGURE 2. Expression of maturation markers on DC after treatment with food proteins. Monocyte-derived DC were cultured with medium or stimulated with gliadin (1–100 μg/ml), soya protein (10–100 μg/ml), and OVA (10–100 μg/ml) for 24 h. LPS (1 μg/ml) and poly (I:C) (50 μg/ml) were used as positive controls. Then the cells were immunostained with mAbs against CD80, CD83, CD86, and HLA-DR molecules. Data are expressed as the MFI of CD80-, CD83-, CD86-, and HLA-DR-positive cells. The data are from a single experiment and are representative of at least three experiments performed with similar results.



	a	b	c	d	e	f	g	h	i	j	k	l
1	pos	pos	neg	neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1β
2	pos	pos	neg	neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1β
3	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12p40p	IL-13	IL-15	IFN-γ
4	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12p40p	IL-13	IL-15	IFN-γ
5	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1
6	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1
7	TNF-α	TNF-β	EGF	IGF-I	angogenin	oncostatin M.	thrombopoletin	VEGF	PDGF BB	leptin	neg	pos
8	TNF-α	TNF-β	EGF	IGF-I	angogenin	oncostatin M.	thrombopoletin	VEGF	PDGF BB	leptin	neg	pos

FIGURE 3. The profile of cytokines and growth factors secreted by untreated human DC (*A*) and DC treated with gliadin digest (100 μg/ml) for 24 h (*B*). Cytokine production was evaluated using the RayBio Human Cytokine Ab Array III. Data are from a single experiment and are representative of at least three independent experiments. A key to cytokine coordinates is shown at the *right* (*C*).

DC endocytosis is reduced by gliadin

Because DC lose their high endocytic activity during the maturation process, we assessed the effect of gliadin on the mannose receptor-mediated endocytosis of FITC-dextran. The endocytic activity of untreated DC was significantly decreased after exposure to gliadin fragments (Fig. 4*A*). Exposure of DC to 1, 10, or 100 μg/ml gliadin reduced the activity to 84, 43, or 20%, respectively. Addition of soya or OVA peptides, even at the highest dose (100 μg/ml), only slightly reduced the uptake of FITC-dextran.

Gliadin enhances the allostimulatory capacity of DC

We evaluated the allogeneic T cell stimulatory capacity of untreated DC and DC treated with gliadin (100 μg/ml), soya protein (100 μg/ml), or LPS (1 μg/ml). DC were cocultured with purified CFSE-stained allogenic $CD4^+$ T lymphocytes. Gliadin- or LPS-stimulated DC exhibited a higher allostimulatory capacity (36.9 and 41.3%, respectively) compared with untreated DC (25.8%). Stimulation with soya proteins did not increase the allostimulatory capacity of DC. The mitogen-triggered proliferation (of 86.5% lymphocytes) and spontaneous proliferation of untreated lymphocytes (0.2% cells) were used as positive and negative controls (Fig. 4*B*).

Gliadin stimulation of DC is mediated via NF-κB pathway

To analyze the role of NF-κB in gliadin-induced maturation of DC, the NF-κB/DNA-binding activity was estimated in nuclear extracts prepared from DC stimulated with gliadin fragments (100 μg/ml) by colorimetric NF-κB assay. Stimulation of DC with gliadin fragments resulted in a marked increase in the binding activities of NF-κB subunits p50 and p65/Rel-A, whereas DNA binding by the other three NF-κB subunits, Rel-B, c-Rel, and p52, was not detected. The involvement of NF-κB complex in DC maturation was confirmed using the serine protease inhibitor TPCK, which is known to inhibit NF-κB translocation by preventing IκB-α degradation (16). Treatment with TPCK (25 μM) markedly inhibited gliadin-stimulated binding activity of p50 (70%) and completely abolished the binding activity of p65 subunits (Fig. 5*A*).

Moreover, FACS analyses of gliadin-treated DC revealed that addition of TPCK resulted in a strong inhibition of up-regulation of CD83 (90% inhibition), CD80 (78%), CD86 (81.2%), and HLA-DR (98%) molecules (Fig. 5*B*). The down-regulation of endocytosis by gliadin fragments was inhibited by the same dose of TPCK (to 77% the activity of immature DC; Fig. 5*C*). TPCK also markedly (up to 98%) diminished TNF-α secretion by gliadin-stimulated DC. We observed no effect of TPCK on proliferation of allogeneic T lymphocytes induced by gliadin-stimulated DC (data not shown).

Gliadin induces phosphorylation of members of the three families of MAPK

Because MAPK participates in the regulation of cell growth and differentiation, we examined by Western blot analysis the effect of gliadin treatment on the activity of these molecules in DC lysates. As shown in Fig. 6, increased phosphorylation of ERK1/2, JNK1/2, and p38 MAPK (ranging from 1.6- to 3.3-fold for various kinases and various gliadin doses) was observed in cells treated with gliadin compared with controls. The ERK inhibitor U1026 (17) inhibited the gliadin-induced phosphorylation of ERK, and the JNK inhibitor SP600125 (18) decreased the gliadin-induced phosphorylation of JNK, both in a dose-dependent fashion. In contrast, the p38 inhibitor SB203580 had no effect on p38 phosphorylation. This observation could be explained by the findings that SB203580 binds to the ATP-binding pocket of p38 kinase and inhibits its activity, but not its own phosphorylation (19).

Table I. *Cytokine production by DC stimulated with gliadin and LPS*[a]

	Cytokine Production (pg/ml) by DC		
	Stimulated with gliadin		Stimulated with LPS
Cytokine	10 μg/ml	100 μg/ml	1 μg/ml
IL-4	7.6 ± 9.1	31.0 ± 31.7	64.5 ± 12.0
IL-6	1,488.2 ± 325.4	5,055.5 ± 927.2	5,284.0 ± 432.5
IL-8	10,860.6 ± 5,006.2	13,400.7 ± 3,751.8	10,420.2 ± 3,557.0
IL-10	nd	310.5 ± 42.1	2,364.0 ± 246.5
IL-12 p70	0.0 ± 0.0	0.0 ± 0.0	645.0 ± 87.4
TNF-α	874.1 ± 200.0	3,783.3 ± 1,198.5	4,600.5 ± 781.0
TGF-β	447.0 ± 185.3	849.4 ± 546.3	1,760.5 ± 608.1

[a] Data are shown as the mean ± SD from at least three independent experiments. nd, Not done.

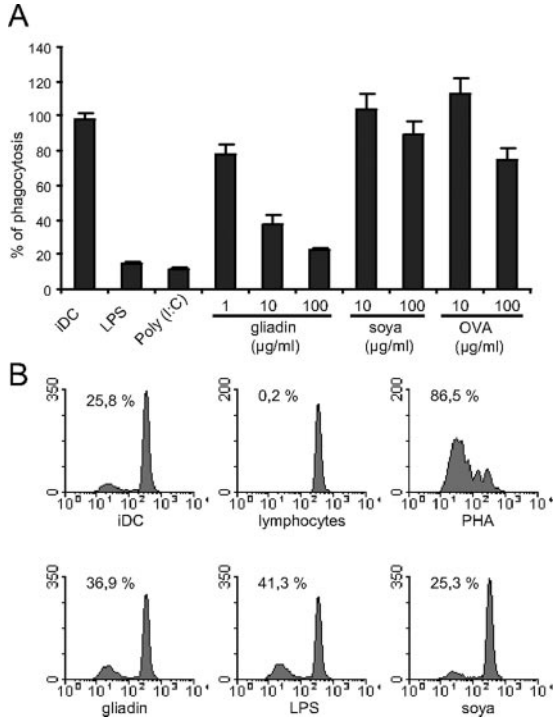


FIGURE 4. Functional changes in DC exposed to gliadin fragments. *A*, Endocytosis of FITC-labeled dextran. Immature DC (iDC) were stimulated with gliadin (1–100 μg/ml), LPS (1 μg/ml), poly (I:C) (50 μg/ml), and soya protein or OVA (10–100 μg/ml) for 24 h; incubated with FITC-labeled dextran for 1.5 h at 37°C; and analyzed by a flow cytometry. The data are expressed as the mean ± SD percentage of phagocytosis from three independent experiments. The percentage of phagocytosis of untreated DC was arbitrarily considered to be 100%. *B*, MLRs. DC were treated, or not, with gliadin fragments (100 μg/ml), soya proteins (100 μg/ml), or LPS (1 μg/ml), and proliferation of CFSE-labeled allogeneic $CD4^+$ T cells after 5 days of culture was measured using flow cytometry. Negative controls (lymphocytes alone) and positive controls (lymphocytes treated with 1 μg of PHA) were performed in each experiment. Data are from a single experiment and are representative of three independent experiments.

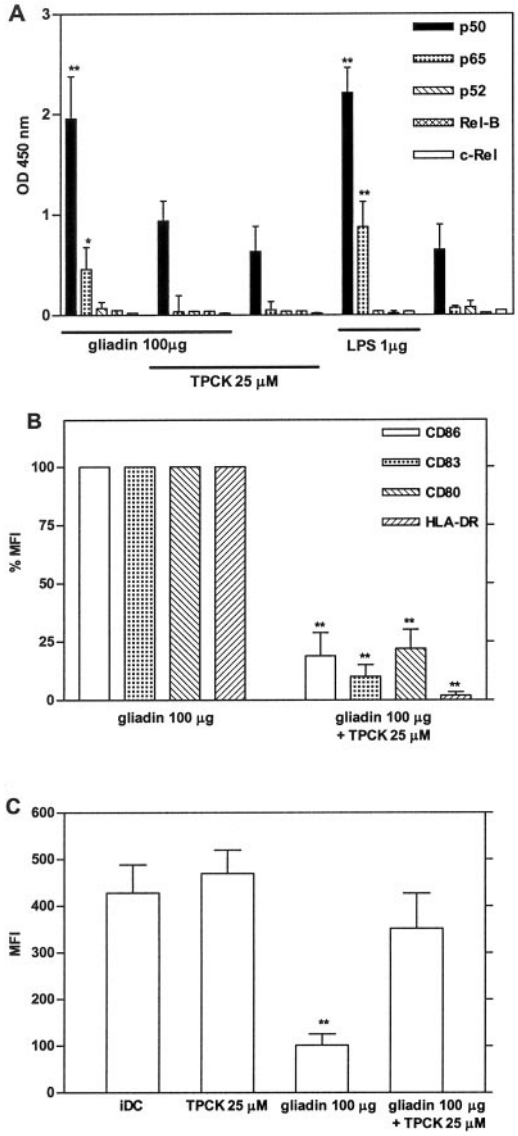


FIGURE 5. Effect of the NF-κB inhibitor TPCK on gliadin-induced DC maturation. *A*, Gliadin-induced activation of NF-κB subunits p50 and p65 in human DC is blocked by the NF-κB inhibitor TPCK. DC were left untreated or were stimulated with gliadin (100 μg/ml) and/or TPCK (25 mM) or LPS (1 μg/ml) for 90 min, then the DNA-binding activity of NF-κB subunits p50, p65, p52, Rel-B, and c-Rel was analyzed by colorimetric assay. Data are presented as the mean ± SD from at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (vs untreated cells). *B*, Gliadin-induced maturation of DC is inhibited by TPCK. DC were stimulated with gliadin alone or in combination with TPCK for 24 h, and the surface expression of CD86, CD83, CD80, and HLA-DR was analyzed by flow cytometry. The results are shown as the percentage of the MFI ± SD from at least three independent experiments. The data were normalized to the result from cells treated with gliadin alone, which was set at 100%. **, $p < 0.01$ compared with gliadin alone. *C*, TPCK prevented gliadin-induced down-regulation of FITC-dextran endocytosis. Endocytosis of FITC-dextran by DC treated with gliadin alone or with TPCK was analyzed by flow cytometry, and data are expressed as the MFI ± SD from at least three independent experiments. **, $p < 0.01$ compared with nonstimulated DC.

Inhibition of p38 MAPK activity prevents the gliadin-induced maturation of DC

In additional experiments we evaluated the effects of specific inhibitors of MAPK activation on the expression of DC maturation markers. SB203580 (5–20 μM) markedly reduced gliadin-triggered CD86, CD83, as well as CD80 expression and slightly inhibited HLA-DR expression on DC. On the contrary, maturation of DC in the presence of SP600125 (5–20 μM) significantly enhanced HLA-DR expression, had no effect on CD80 and CD83 expression, and caused significant inhibition only of CD86 expression. Interestingly, U1026 (5–20 μM) down-regulated only CD86 and exhibited mild, but not significant, effects on CD83, CD80, and HLA expression (Fig. 7).

Moreover, we studied the effects of SB203580, SP600125, and U1026 on the secretion of gliadin-induced IL-6, IL-8, and TNF-α. Although SP600125 and U0126 inhibited TNF-α and IL-6 production in a dose-dependent manner, they did not affect IL-8 production. In contrast, SB203580 down-regulated the production of all three cytokines (Table II).

Discussion

The immune system associated with mucosal surfaces evolved mechanisms discriminating between harmless (e.g., food) Ags and dangerous pathogens. Oral tolerance is one of the forms of peripheral tolerance in which the mucosal administration of food Ags induces a systemic immunological hyporesponsiveness. DC are involved in mechanisms of oral tolerance by regulating T cell

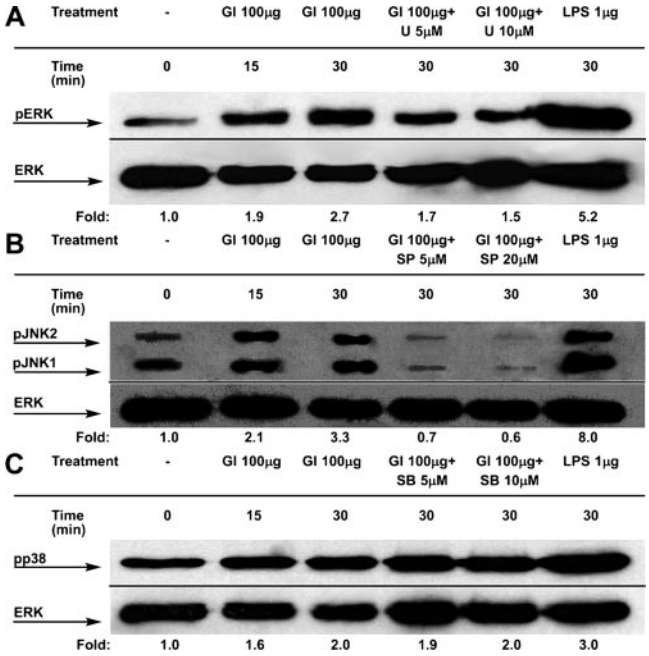


FIGURE 6. Western blot of phosphorylated ERK (pERK; *A*), phosphorylated JNK1/2 (pJNK1/2; *B*), and phosphorylated p38 (pp38; *C*). Cells were cultured in medium (*lane 1*) or with addition of 100 μg/ml gliadin digest alone or in combination with SP600125 (5–20 μM), SB203580 (5–10 μM), or U0126 (5–10 μM), which were added 30 min before the addition of gliadin. Nonphosphorylated ERK served as the control for protein loading. The fold induction (densitometric analysis) of protein phosphorylation, normalized to nonactivated cells and corrected for the amount of protein in each sample, is also indicated. Representative blots from three independent experiments are shown.

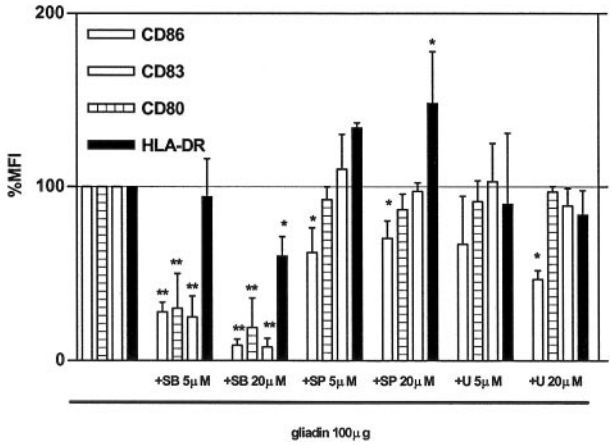


FIGURE 7. Effects of SP600125, SB203580, and U1026 on the phenotypic maturation of DC after gliadin stimulation. DC were stimulated with gliadin (100 μg/ml) for 24 h in the presence or the absence of SP600125 (5–20 μM), SB203580 (5–20 μM), or U0126 (5–20 μM), and the surface expression of CD86, CD83, CD80, and HLA-DR was analyzed by flow cytometry. The results are shown as the percentage of MFI ± SD from at least three independent experiments. The data were normalized to the result from cells treated with gliadin alone, which was set at 100%. *, $p < 0.05$; **, $p < 0.01$ (compared with gliadin alone).

functions, either by insufficient expression of costimulatory molecules or by production of immunosuppressive cytokines such as IL-10 and TGF-β. The current hypothesis is that intestinal DC remain relatively immature and thus tolerize, rather than activate, T cells. Therefore, one way of breaking the tolerance is to provide signals promoting DC maturation (20, 21).

Celiac disease is characterized as an abnormal (or adverse) response to gluten, and it is conceivable that in this case, the tolerance to gluten either is not established properly or is broken. A key question for understanding celiac disease pathogenesis is why only gluten, among so many food-derived Ags, induces a harmful T cell-mediated immune response. Presentation of peptide-derived epitopes on the surface of mature APC is a prerequisite for the development of the primary immune response. To investigate whether gluten has a stimulatory effect on APC, we tested the consequences of the treatment of DC by gliadin peptic fragments.

In this paper we show that peptic fragments of gliadin induce phenotypic and functional maturation of human monocyte-derived DC. The doses of gliadin used for DC stimulation seem to correspond to the expected concentrations of gliadin present in the small intestine after a gluten-containing meal (22, 23) and used by other authors in the in vitro experiments (24, 25). Treatment of DC with gliadin peptic fragments led to up-regulation of maturation and Ag presentation-associated markers (CD80, CD83, CD86, and HLA-DR), down-regulation of the phagocytic capacity, and, simultaneously, increased secretion of IL-8 and homologous chemokines and proinflammatory cytokines, IL-6 and TNF-α. Secretion of regulatory cytokines, IL-4 and IL-10, was low, and gliadin treatment decreased TGF-β production by DC compared with untreated cells. In contrast with the responses to LPS and poly (I:C), gliadin-matured DC did not secrete IL-12. The production of IL-12 by gliadin-stimulated DC could be below the detection limit, and other members of the IL-12 family (IL-23 and IL-27) and/or the IL-18 molecule could contribute to Th1 polarization. These data seem to be supported by the finding of increased levels of IL-18 in

Table II. *Effects of MAPK inhibitors SP600125, SB203580, and U1026 on gliadin-induced cytokine production*[a]

Stimulated with Gliadin	Dose 100 μg/ml	% of Cytokine Production by DC		
		IL-6 100	IL-8 100	TNF-α 100
Gliadin + SP600125	5 μM	81.5 ± 31.2	123.3 ± 45.2	108.0 ± 41.8
	10 μM	50.7 ± 27.5	89.3 ± 42.1	37.7 ± 22.2
	20 μM	37.7 ± 14.9	75.1 ± 39.1	12.2 ± 9.1
Gliadin + SB203580	10 μM	15.3 ± 5.5	58.7 ± 14.0	25.8 ± 4.4
	20 μM	0.9 ± 0.8	42.2 ± 25.5	13.3 ± 15.3
	50 μM	23.0 ± 21.0	39.9 ± 7.3	22.5 ± 18.9
Gliadin + U1026	5 μM	86.1 ± 26.4	85.5 ± 50.2	44.3 ± 20.5
	20 μM	86.4 ± 15.7	86.2 ± 43.1	27.5 ± 19.8
	50 μM	44.6 ± 31.5	90.7 ± 45.0	2.8 ± 2.8

[a] Data are shown as the mean ± SD percentage of cytokine production. The percentage of cytokine production stimulated by gliadin was arbitrarily considered to be 100%.

intestinal mucosa as well as in the peripheral blood of celiac patients. Alternatively, a bystander effect of IFN-α and IFN-β, independently of IL-12, associated with some sort of intestinal infection can promote Th1 cytokine production. Interestingly, these data are in accordance with the studies showing that only minute amounts of IL-12 p40 mRNA expression were found in biopsy specimens from both patients and healthy controls (24, 26–30). Gliadin-treated DC also had a significantly higher T cell stimulatory potential than untreated DC or DC treated with OVA or soya. Although the same DC population derived from different tissues could respond differentially to the same stimuli (5, 21), similar induction of DC maturation in intestinal tissue of celiac patients was suggested in the studies by Maiuri et al. (9) and Nikulina et al. (31). A detailed study of in vivo DC maturation in tissue of celiac patients is the subject of our ongoing studies.

To understand the molecular mechanisms involved in DC maturation, we studied the signaling pathways activated by gliadin. We show that p38 kinase, ERK1/2, and JNK1/2 are all phosphorylated when DC are exposed to gliadin digest. Although the phosphorylation of ERK and JNK was markedly diminished using their specific inhibitors in a dose-dependent fashion, the ERK inhibitor had little or no effect on gliadin-induced DC maturation. These data are consistent with the finding that ERK activation is not involved or has little effect on DC maturation (12, 32, 33). In contrast, treatment of DC with the p38 inhibitor SB203580 markedly down-regulated the ability of gliadin to increase the surface expression of costimulatory molecules and, to a lesser extent, of HLA molecules. Similar effects of SB203580 were documented in DC maturation induced by LPS, TNF-α, HIV, and contact sensitizers (11, 33, 34).

Not only the MAPK, but also other signal transduction pathways, may be involved in cellular responses to external stimuli. In mammals, members of NF-κB/Rel family, including p50, p65 (RelA), p52, RelB, and c-Rel, regulate many genes involved in immune and inflammatory responses (35). NF-κB molecules were shown to be involved in DC maturation and are highly expressed in mature DC (10, 36). We have shown that treatment of DC with gliadin results in activation of p50 and p65 subunits of NF-κB. The role of NF-κB in gliadin-induced phenotypic and functional DC maturation was confirmed using the NF-κB inhibitor TPCK. The inhibitory effect of TPCK on the expression of maturation markers was also reported by Rescigno et al. (10) in LPS-induced maturation of DC.

The nature of the receptor triggered by gliadin remains to be identified. We can only suggest that pattern recognition receptors such as TLRs interacting with microbe-associated molecular patterns and/or other molecules of host or synthetic origin could be involved in gliadin recognition. However, a recent report showed that the stimulatory potential of gliadin was preserved in TLR 4-defective mice (31). Other receptors of the innate immunity system known to modify DC could also be relevant candidates.

Identification of the DC stimulatory potential of gliadin contributes to an understanding of the etiology of this multifactorial disease. Although the genetic factors play a dominant role in the predisposition for the disease, other modifying factors participate in its initiation. It can be suggested that intestinal inflammation could lead to disruption of the epithelial barrier and the increased influx of gluten peptides in the lamina propria. The higher local expression of tissue transglutaminase 2 causes the generation of more immunogenic gliadin peptides that are subsequently presented by fully mature DC. Interestingly, the attempts to detect gluten-directed T cell responses in healthy individuals failed, and T cell responses in celiac patients are preferentially directed to deamidated gluten fragments. The process of inflammation can be amplified by the proinflammatory cytokines produced by activated DC and macrophages (1, 37). In genetically predisposed individuals, this could lead to the breakdown of peripheral tolerance and the induction of an overt disease. In contrast, in healthy subjects, the unique ability of gliadin to activate DC may not be sufficient to induce the disease, because the physiological regulatory mechanisms maintain the unresponsiveness of innate as well as specific mucosal immune system (32). Several down-regulatory mechanisms participating in an analogous situation, i.e., nonresponsiveness to commensal bacteria, were recently described (33, 34).

In conclusion, this work documents that among dietary Ags, gliadin peptides have a unique ability to promote phenotypic and functional maturation of human DC. Moreover, gliadin stimulates monocyte-derived DC to produce a spectrum of cytokines and chemokines that differs in profile from that induced by LPS, mainly in IL-12 secretion. Furthermore, we have shown that the NF-κB molecule and p38 MAPK participate in the activation pathway triggered by gliadin in DC. Conceivably, modification of DC function in the tissue and circulation of celiac patients in terms of increased activation can promote mechanisms leading to an immune response rather than to T cell anergy/tolerance. This ability of gliadin fragments provides new insight into the role of innate immunity cells in celiac disease pathogenesis.

Disclosures

The authors have no financial conflict of interest.

References

1. Sollid, L. M. 2002. Coeliac disease: dissecting a complex inflammatory disorder. *Nat. Rev. Immunol.* 2: 647–655.
2. Pavli, P., C. E. Woodhams, W. F. Doe, and D. A. Hume. 1990. Isolation and characterization of antigen-presenting dendritic cells from the mouse intestinal lamina propria. *Immunology* 70: 40–47.
3. Ruedl, C., C. Rieser, G. Bock, G. Wick, and H. Wolf. 1996. Phenotypic and functional characterization of CD11c$^+$ dendritic cell population in mouse Peyer's patches. *Eur. J. Immunol.* 26: 1801–1806.
4. Reis e Sousa, C. 2004. Toll-like receptors and dendritic cells: for whom the bug tolls. *Semin. Immunol.* 16: 27–34.
5. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18: 767–811.
6. Chehade, M., and L. Mayer. 2005. Oral tolerance and its relation to food hypersensitivities. *J. Allergy Clin. Immunol.* 115: 3–12.
7. Tuckova, L., J. Novotna, P. Novak, Z. Flegelova, T. Kveton, L. Jelinkova, Z. Zidek, P. Man, and H. Tlaskalova-Hogenova. 2002. Activation of macrophages by gliadin fragments: isolation and characterization of active peptide. *J. Leukocyte Biol.* 71: 625–631.
8. Jelinkova, L., L. Tuckova, J. Cinova, Z. Flegelova, and H. Tlaskalova-Hogenova. 2004. Gliadin stimulates human monocytes to production of IL-8 and TNF-α through a mechanism involving NF-κB. *FEBS Lett.* 571: 81–85.
9. Maiuri, L., A. Picarelli, M. Boirivant, S. Coletta, M. C. Mazzilli, M. De Vincenzi, M. Londei, and S. Auricchio. 1996. Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients. *Gastroenterology* 110: 1368–1378.
10. Rescigno, M., M. Martino, C. L. Sutherland, M. R. Goldm, and P. Ricciardi-Castagnoli. 1998. Dendritic cells survival and maturation are regulated by different signaling pathway. *J. Exp. Med.* 188: 2175–2180.
11. Arrighi, J. F., M. Rebsamen, F. Rousset, V. Kindler, and C. Hauser. 2001. A critical role for p38 mitogen-activated protein kinase on the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-α, and contact sensitisers. *J. Immunol.* 166: 3837–3845.
12. Nakahara, T., H. Uchi. K. Urabe, Q. Chen, M. Furue, and Y. Moroi. 2004. Role of c-Jun N-terminal kinase on lipopolysaccharide induced maturation of human monocyte-derived dendritic cells. *Int. Immunol.* 16: 1701–1709.
13. Chang, L., and M. Karin. 2001. Mammalian MAP kinase signaling cascades. *Nature* 410: 37–40.
14. Langenkamp, A., M. Messi, A. Lanzavecchia, and F. Sallusto. 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat. Immunol.* 1: 311–316.
15. McColl, S. R. 2002. Chemokines and dendritic cells: a crucial alliance. *Immunol. Cell Biol.* 80: 489–496.
16. Baldwin, A. S., Jr. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14: 649–683.
17. Favata, M. F., K. Y. Horiuchi, E. J. Manos, A. J. Daulerio, D. A. Stradley, W. S. Feeser, D. E. Van Dyk, W. J. Pitts, R. A. Earl, F. Hobbs, et al. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273: 18623–18632.

18. Bennett, B. L., D. T. Sasaki, B. W. Murray, E. C. O'Leary, S. T. Sakata, W. Xu, J. C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, et al. 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. USA* 98: 13681–13686.
19. Tong, L., S. Pav, D. M. White, S. Rogers, K. M. Crane, C. L. Cywin, M. L. Brown, and C. A. Pargellis. 1997. A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. *Nat. Struct. Biol.* 4: 311–316.
20. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21: 685–711.
21. Bilsborough, J., and J. L. Viney. 2004. Gastrointestinal dendritic cells play a role in immunity, tolerance, and disease. *Gastroenterology* 127: 300–309.
22. Chartrand, L. J., P. A. Russo, A. G. Duhaime, and E. G. Seidman. 1997. Wheat starch intolerance in patients with celiac disease. *J. Am. Diet. Assoc.* 97: 612–618.
23. Matysiak-Budnik, T., C. C., C. Dugave, A. Namane, C. Cellier, N. Cerf-Bensussan, and M. Heyman. 2003. Alterations of the intestinal transport and processing of gliadin peptides in celiac disease. *Gastroenterology* 125: 696–707.
24. Nilsen, E. M., F. L. Jahnsen, K. E. Lundin, F. E. Johansen, O. Fausa, L. M. Sollid, J. Jahnsen, H. Scott, and P. Brandtzaeg. 1998. Gluten induces an intestinal cytokine response strongly dominated by interferon γ in patients with celiac disease. *Gastroenterology* 115: 551–563.
25. Mazzarella, G., M. Maglio, F. Paparo, G. Nardone, R. Stefanile, L. Greco, Y. van de Wal, Y. Kooy, F. Koning, S. Auricchio, et al. 2003. An immunodominant DQ8 restricted gliadin peptide activates small intestinal immune response in in vitro cultured mucosa from HLA-DQ8 positive but not HLA-DQ8 negative coeliac patients. *Gut* 52: 57–62.
26. Salvati, V. M., T. T. MacDonald, M. Bajaj-Elliott, M. Borrelli, A. Staiano, S. Auricchio, R. Troncone, and G. Monteleone. 2002. Interleukin 18 and associated markers of T helper cell type 1 activity in coeliac disease. *Gut* 50: 186–190.
27. Monteleone, G., S. L. Pender, E. Alstead, A. C. Hauer, P. Lionetti, C. McKenzie, and T. T. MacDonald. 2001. Role of interferon α in promoting T helper cell type 1 responses in the small intestine in coeliac disease. *Gut* 48: 425–429.
28. Merendino, R. A., G. Di Pasquale, G. C. Sturniolo, A. Ruello, V. Albanese, P. L. Minciullo, S. Di Mauro, and S. Gangemi. 2003. Relationship between IL-18 and sICAM-1 serum levels in patients affected by coeliac disease: preliminary considerations. *Immunol. Lett.* 85: 257–260.
29. Lettesjo, H., T. Hansson, A. Berqvist, J. Gronlund, and A. Dannaeus. 2005. Enhanced interleukin-18 levels in the peripheral blood of children with coeliac disease. *Clin. Exp. Immunol.* 139: 138–143.
30. Watford, W. T., M. Moriguchi, A. Morinobu, and J. J. O'Shea. 2003. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev.* 14: 361–368.
31. Nikulina, M., C. Habich, S. B. Flohe, F. W. Scott, and H. Kolb. 2004. Wheat gluten causes dendritic cell maturation and chemokine secretion. *J. Immunol.* 173: 1925–1933.
32. Ardeshna, K. M., A. R. Pizzey, S. Devereux, and A. Khwaja. 2000. The PI3 kinase, p38 SAP kinase, and NF-κB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived cells. *Blood* 96: 1039–1046.
33. Puig-Kroger, A., M. Rellosso, O. Fernandez-Capetillo, A. Zubiaga, A. Silva, C. Bernabeu, and A. L. Corbi. 2001. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. *Blood* 98: 2175–2182.
34. Wilflingseder, D., B. Mullauer, H. Schramek, Z. Banki, M. Pruenster, M. P. Dierich, and H. Stoiber. 2004. HIV-1-induced migration of monocyte-derived dendritic cells is associated with differential activation of MAPK pathways. *J. Immunol.* 173: 7494–7505.
35. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-κB and Rel proteins: evolutionary conserved mediators of immune responses. *Annu. Rev. Immunol.* 16: 225–260.
36. Saccani, M., S. Pantano, and G. Natoli. 2003. Modulation of NF-kB activity by exchange of dimers. *Mol. Cell* 11: 1563–1574.
37. Schuppan, D., B. Esslinger, and W. Dieterich. 2003. Innate immunity and coeliac disease. *Lancet* 362: 3–4.