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# Wheat Gluten Causes Dendritic Cell Maturation and Chemokine Secretion<sup>1</sup>

Marina Nikulina,\* Christiane Habich,\* Stefanie B. Flohé,\* Fraser W. Scott,<sup>†</sup> and Hubert Kolb<sup>2\*</sup>

**Wheat gluten causes gut inflammation in genetically predisposed individuals. We tested the hypothesis that wheat gluten is not only a target of adaptive immunity, but also modulates the function of APC. Dendritic cells (DC) derived from the bone marrow of BALB/c mice were exposed to chymotrypsin-treated wheat gluten. This induced DC maturation as estimated by all surface markers tested (MHC class II, CD40, CD54, and CD86). The effect was dose dependent, and, at 100  $\mu\text{g/ml}$  gluten matched that caused by 10  $\text{ng/ml}$  LPS. A role of endotoxin contamination was ruled out by demonstrating the resistance of wheat gluten effects to LPS antagonist polymyxin B. DC from LPS nonresponder strain C3H/HeJ were affected by wheat gluten, but not by LPS. Proteinase K-digested wheat gluten was unable to stimulate DC maturation. Wheat gluten induced a unique secretion pattern of selected cytokines and chemokines in DC. Classic pro- or anti-inflammatory mediators were not produced, in contrast to LPS. Rather, chemokines MIP-2 and keratinocyte-derived cytokine were secreted in large amounts. We conclude that wheat gluten lowers the threshold for immune responses by causing maturation of APC, by attracting leukocytes and increasing their reactivity state. In the presence of an appropriate genetic predisposition, this is expected to increase the risk of adverse immune reactions to wheat gluten or to other Ags presented. *The Journal of Immunology*, 2004, 173: 1925–1933.**

Immune tolerance to food Ags is an important characteristic of healthy individuals. Its establishment and maintenance is complex and not fully understood. Broken immune unresponsiveness to dietary Ags causes allergic or chronic inflammatory diseases. A dietary component with considerable potency to induce an inflammatory condition in genetically predisposed individuals is wheat gluten. Intolerance to wheat gluten, a collection of storage proteins from endosperm cells, and to related proteins from rye and barley, leads to the development of celiac disease (CD),<sup>3</sup> in association with HLA DQ2 or to a lesser extent with HLA DQ8 (1, 2). Gluten consists of gliadin monomers ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\omega$ -type) and large disulfide-linked glutenin polymers with high- and low-molecular-mass subunits (3). Gluten, as well as barley- and rye-derived hordeins and secalins, are rich in glutamine (30–40%) and proline (20%)—a feature that makes these proteins good substrates for the calcium-dependent enzyme tissue transglutaminase (tTG) (4, 5). tTG, which is present in the epithelial cells that line the gastrointestinal tract, converts glutamine into negatively charged glutamate residues, which leads to increased binding affinity of gluten peptides to DQ2 and DQ8 molecules (5). The property of gluten to promote gut inflammation has been linked to its

ability to elicit cellular and humoral immune responses. Immuno-reactive T cell epitopes were found in  $\alpha$ -gliadin,  $\gamma$ -gliadin, and glutenin (6–11). Deamidation of gluten peptides by tTG usually increases the activation of gluten-specific T cells from CD patients, although deamidation-independent responses also exist (6, 7, 11).

Abs of IgA and IgG classes against tTG and gliadin are sensitive markers of CD, and they are also elevated in type 1 diabetes mellitus, which shares with CD the MHC class II-risk genes HLA-DQ2 and DQ8 (5, 12–16).

It has been shown that gluten-free diet prevents diabetes in nonobese diabetic (NOD) mice and improves insulin secretion, reduces IgG gliadin Abs, but does not change disease-associated autoantibody titers in human preclinical type 1 diabetes mellitus (17–19). Wheat-based diets promote the development of diabetes in BioBreeding (BB) rats, and IgG Abs to wheat storage salt-soluble globulin, G1b1, were recently found in diabetic patients and BB rats (20, 21). Furthermore, G1b1 Ab reactivity showed a strong correlation with pancreatic inflammation and insulinitis (21).

The induction of an immune response largely depends on the concomitant delivery of costimulatory signals from APC, notably dendritic cells (DC) (22, 23). An Ag would be particularly immunogenic if it would not only provide appropriate peptides for T cell recognition, but would also induce costimulatory signals in APC, such as has been reported for several heat shock proteins (24–28). There is some evidence that gluten can activate innate immunity. Thus, gliadin as well as its peptic-tryptic digest and peptic fragment stimulate IFN- $\gamma$ -treated macrophages (29, 30). Nonimmunodominant gliadin peptide, which is toxic to small intestine in CD, also induces a rapid expression of CD83 on duodenal biopsy samples from patients with CD (31).

We therefore found it of interest to study whether wheat gluten exerts any specific effect on DC maturation or function.

## Materials and Methods

### Generation and culture of bone marrow-derived DC (BMDC)

DC were generated according to the method described previously (32). Briefly, bone marrow cells ( $2 \times 10^6$ ) from tibiae and femurs of 7- to

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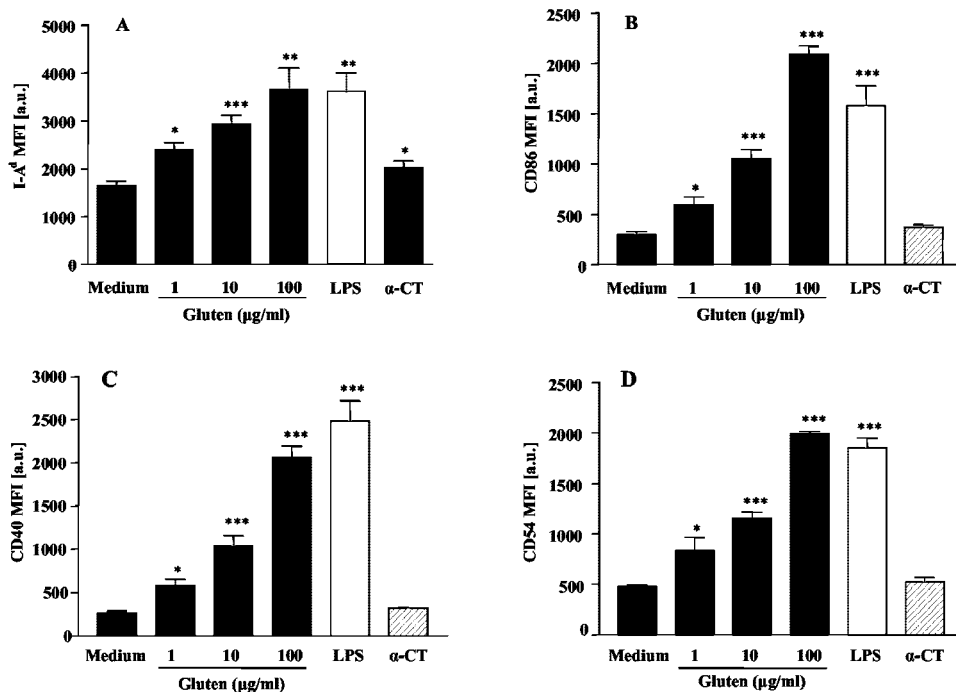
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<sup>3</sup> Abbreviations used in this paper: CD, celiac disease; tTG, tissue transglutaminase; DC, dendritic cell; BMDC, bone marrow-derived DC; PmB, polymyxin B;  $\alpha$ -CT,  $\alpha$ -chymotrypsin; KC, keratinocyte-derived cytokine; MFI, mean fluorescence intensity.

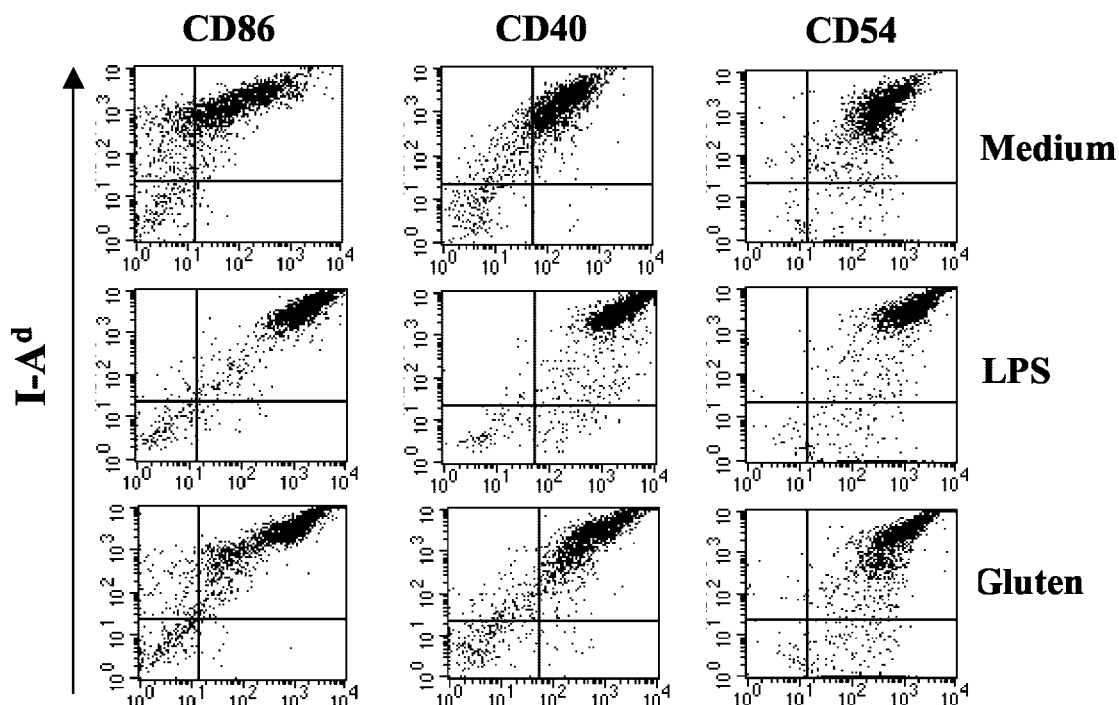
**FIGURE 1.** Expression of surface molecules on BMDC after treatment with wheat gluten, LPS, or  $\alpha$ -CT. BMDC from BALB/c mice were incubated for 20 h with 1, 10, or 100  $\mu$ g/ml gluten; 10 ng/ml LPS; or 28  $\mu$ g/ml  $\alpha$ -CT. Nonadherent cells were collected, stained for I-A<sup>d</sup> (A), CD86 (B), CD40 (C), or CD54 (D), and analyzed by FACS. The data represent MFI  $\pm$  SEM of three to six independent experiments. MFI is expressed in arbitrary units (a.u.). Significant differences to medium are indicated as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ ; \*\*\*,  $p < 0.0001$ ; Student's unpaired  $t$  test. One-way ANOVA for wheat gluten data shows  $p < 0.001$  (A),  $p < 0.0001$  (B),  $p < 0.0001$  (C), and  $p < 0.0001$  (D).



12-wk-old female BALB/c (M&B, Ry, Denmark), C3H/HeN, and C3H/HeJ (Harlan Winkelmann, Borcheln, Germany) mice were seeded in petri dishes (Falcon; BD Biosciences, Heidelberg, Germany) in 10 ml of culture medium containing 20 ng/ml GM-CSF (R&D Systems, Wiesbaden, Germany). As culture medium, very-low-endotoxin RPMI 1640 medium (Biochrom, Berlin, Germany), supplemented with 10 mM HEPES (Biochrom), 2 mM L-glutamine (Biochrom), 20  $\mu$ g/ml gentamicin, 60  $\mu$ g/ml penicillin, 50  $\mu$ M 2-ME (all from Sigma-Aldrich, Taufkirchen, Germany), and 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria), was used. On the third day, 10 ml of culture medium containing 20 ng/ml GM-CSF were

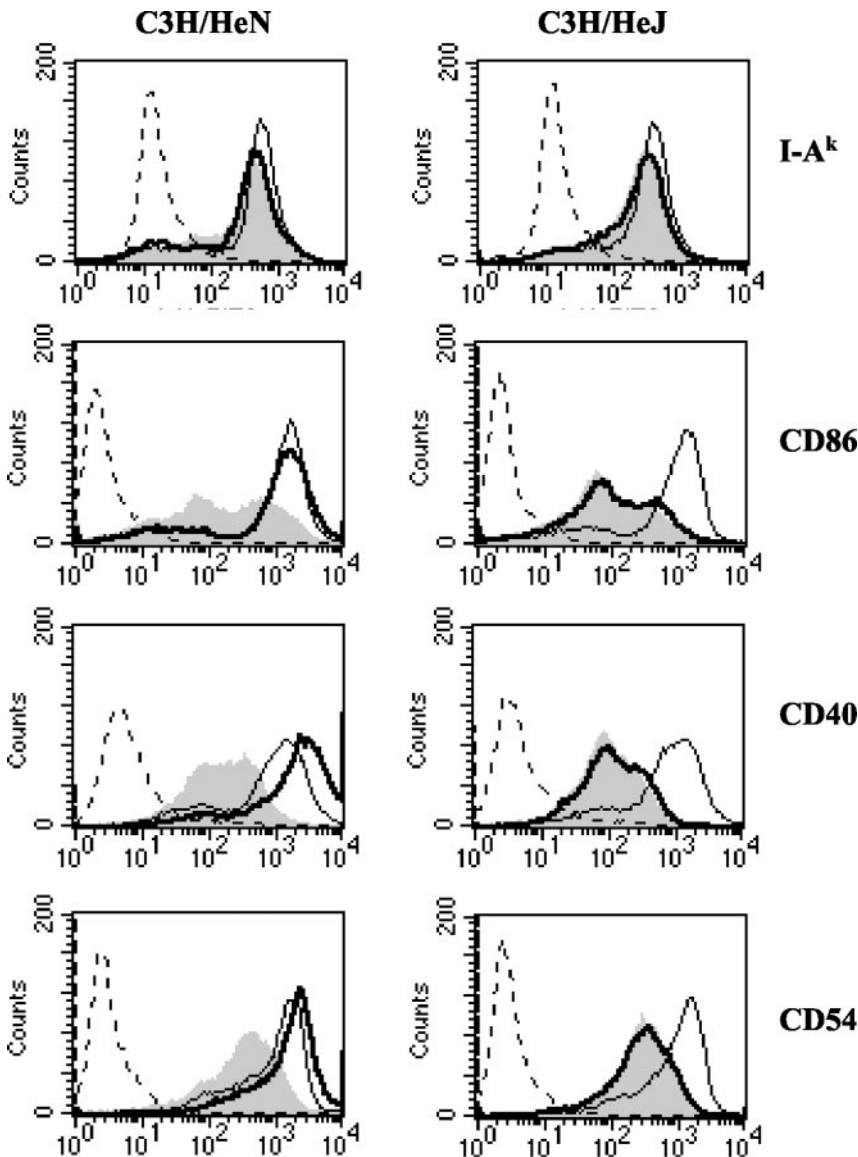
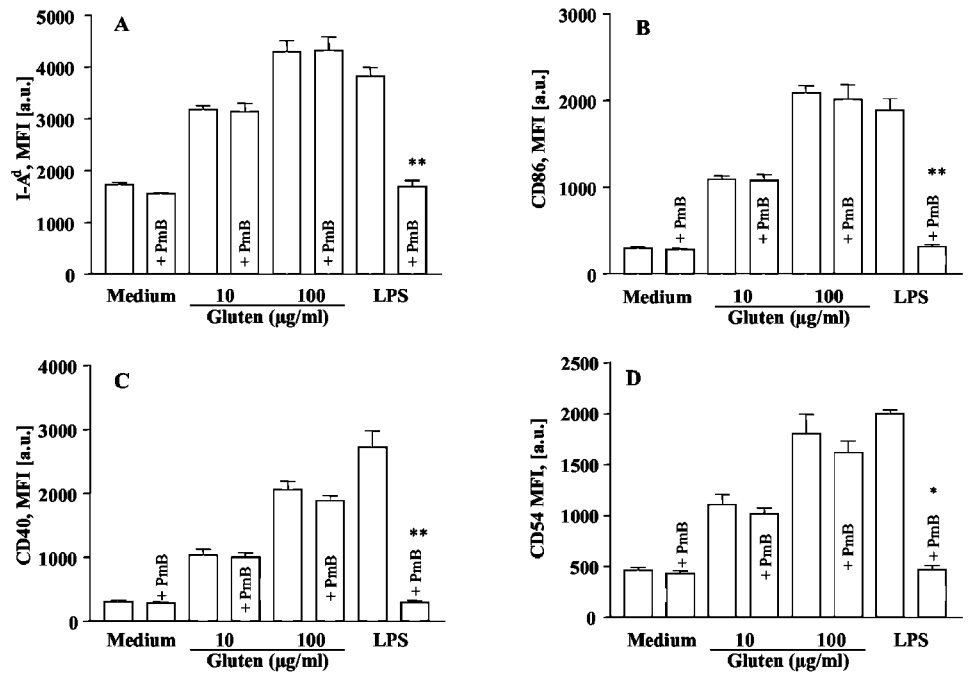
added to the dishes. On the seventh day, 10 ml of the medium was replaced by fresh culture medium supplemented with 20 ng/ml GM-CSF. On day 9, nonadherent cells contained 90–94% DC or their progenitors (CD11c<sup>+</sup>, <0.5% macrophages (CD14<sup>+</sup>CD11c<sup>-</sup>), <5% granulocytes (Ly6G<sup>+</sup>CD11c<sup>-</sup>), and <1% B cells (B220<sup>+</sup>CD11c<sup>-</sup>).

These cells ( $1 \times 10^6$ /ml) were seeded in 1 ml of culture medium supplemented with 10 ng/ml GM-CSF (Falcon; BD Biosciences) in 24-well plates (Falcon; BD Biosciences). After 20 h of stimulation with 1, 10, or 100  $\mu$ g/ml  $\alpha$ -chymotrypsin ( $\alpha$ -CT)-treated wheat gluten (ICN Biochemicals, Cleveland, OH) or 10 ng/ml LPS from *Escherichia coli* O26:B6 (Sigma-Aldrich) or 1, 10,



**FIGURE 2.** Analysis of BMDC maturation after exposure to wheat gluten or LPS. BMDC from BALB/c mice were cultured in medium or medium supplemented with 10  $\mu$ g/ml wheat gluten or 10 ng/ml LPS for 20 h. After incubation, nonadherent cells were collected, stained for I-A<sup>d</sup>, CD86, CD40, or CD54, and analyzed by FACS. Isotype controls were used to set quadrant lines (solid lines) in such a way that the number of false-positive cells was <5% of all cells tested.

**FIGURE 3.** The effect of PmB on wheat gluten- or LPS-induced maturation of BMDC. Wheat gluten (10 and 100  $\mu\text{g/ml}$ ) or LPS (10  $\text{ng/ml}$ ) was incubated with 10  $\mu\text{g/ml}$  PmB (1 h; 4°C) before the addition to BMDC from BALB/c mice for another 20 h. Thereafter, nonadherent cells were harvested, stained for I-A<sup>d</sup> (A), CD86 (B), CD40 (C), or CD54 (D), and analyzed by FACS. The data represent MFI  $\pm$  SEM of three independent experiments. Significant effects of PmB treatment are indicated as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ ; Student's unpaired  $t$  test.



**FIGURE 4.** TLR4-independent maturation of BMDC in response to wheat gluten. BMDC from C3H/HeN and C3H/HeJ mice were incubated with or without 10  $\mu\text{g/ml}$  wheat gluten (standard solid line) or 1  $\text{ng/ml}$  LPS (thick solid line) for 20 h. Thereafter, nonadherent cells were harvested, stained for I-A<sup>k</sup>, CD86, CD40, or CD54, and analyzed by FACS. The untreated medium control is shown as a shaded gray area and the isotype control as broken line, respectively.

or 100 ng/ml recombinant mouse IL-1 $\beta$  (R&D Systems), BMDC were harvested and used for FACS analysis. Cell culture supernatants were collected and stored at  $-20^{\circ}\text{C}$  before the determination of cytokines and chemokines.

In experiments using polymyxin B (PmB; Sigma-Aldrich), wheat gluten and/or LPS were incubated with 10  $\mu\text{g}/\text{ml}$  PmB for 1 h at  $4^{\circ}\text{C}$  before addition to the cells. For protein digestion, wheat gluten or LPS was incubated in culture medium without FCS with 30 U/mg of proteinase K—or a corresponding amount of protein G-coupled agarose beads (Sigma-Aldrich) for 3.5 h at  $37^{\circ}\text{C}$ . After centrifugation at  $8000 \times g$  for 1 min and filtration of supernatant through 0.2- $\mu\text{m}$  Acrodisc Syringe Filter (Gelman Laboratory, Ann Arbor, MI), digested wheat gluten or LPS was used for BMDC stimulation.

#### Solubilization of wheat gluten

Wheat gluten was digested with  $\alpha$ -CT (Sigma-Aldrich) according to the method described previously (33). Briefly, wheat gluten powder and  $\alpha$ -CT were mixed 100:1 (w/w) in 174 mM Tris base (pH 7.8) and incubated overnight at  $37^{\circ}\text{C}$ . After centrifugation at  $1500 \times g$  for 15 min at room temperature, the supernatant was sterilized by filtering through 0.2- $\mu\text{m}$  Acrodisc Syringe Filter. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany), and aliquots of digested gluten were stored at  $-20^{\circ}\text{C}$ . The endotoxin content of wheat gluten preparation was 0.016 endotoxin U (equivalent to 1.2 pg of LPS) per microgram of protein as determined by *Limulus* amoebocyte lysate assay (BioWhittaker, Verviers, Belgium).

#### FACS analysis

The staining of surface molecules on BMDC with fluorochrome-conjugated mAbs was performed on ice. After preincubation for 5 min with FcBlock (BD Biosciences) to avoid nonspecific binding,  $2\text{--}4 \times 10^5$  BMDC were stained for 30 min with saturating amounts of the following Abs (all purchased from BD Biosciences): FITC-conjugated anti-I-A<sup>d</sup> (catalog no. 553623) or anti-I-A<sup>k</sup> (catalog no. 553540); R-PE-conjugated anti-CD86 (catalog no. 553692), anti-CD54 (catalog no. 553253), or anti-CD14 (catalog no. 553740); biotinylated anti-CD40 (catalog no. 553789) or anti-CD11c (catalog no. 550261); and allophycocyanin-conjugated anti-B220 (catalog no. 553092). After washing twice with CellWash (BD Biosciences), BMDC were incubated for 30 min with streptavidin-conjugated CyChrome (catalog no. 554062; BD Biosciences) for staining of biotinylated primary Abs. Cells were washed twice with CellWash, fixed with 1% paraformaldehyde, and analyzed by cytofluorometry, using a FACSCalibur

(BD Biosciences) and CellQuest (BD Biosciences) software. For each Ab, an isotype control of appropriate subclass (BD Biosciences) was used.

#### Determination of cytokines or chemokines

Cytokine or chemokine content in cell culture supernatants was determined by ELISA for TNF- $\alpha$ , IL-10, MCP-1 (OptEIA Sets; BD Pharmingen, Heidelberg, Germany), and for IL-1 $\beta$ , MIP-2, MIP-1 $\alpha$ , and keratinocyte-derived cytokine (KC) (DuoSets; R&D Systems).

#### Statistical analysis

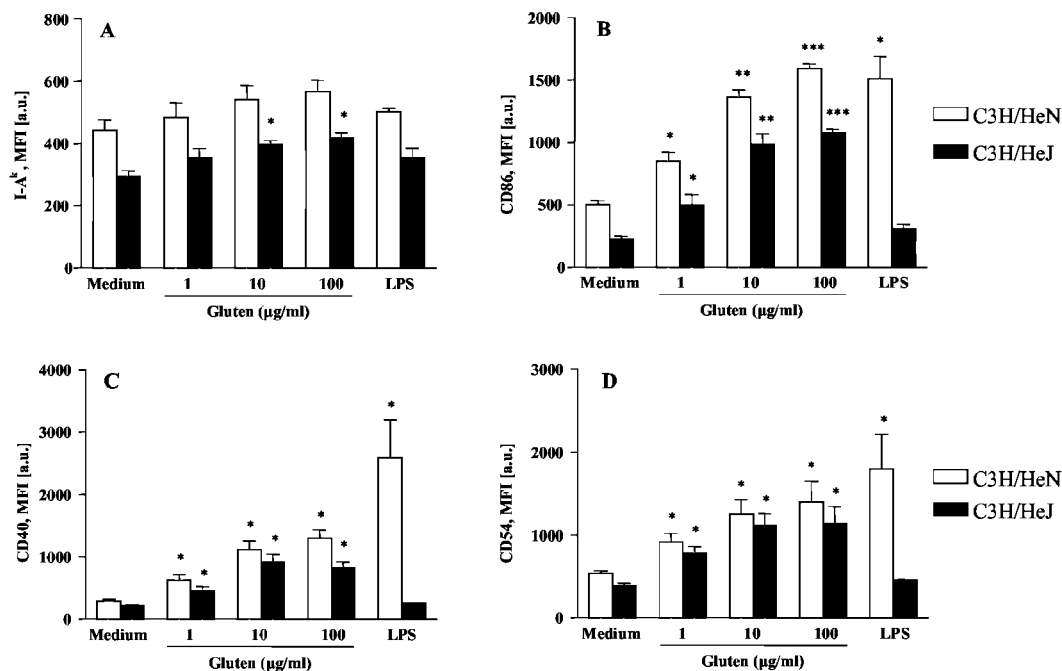
Data were expressed as mean  $\pm$  SEM. Statistical analysis was performed by using one- and two-way ANOVA and Student's two-tailed unpaired *t* test from GraphPad (San Diego, CA) Prism 3.0 software. Differences were considered statistically significant at  $p < 0.05$ .

## Results

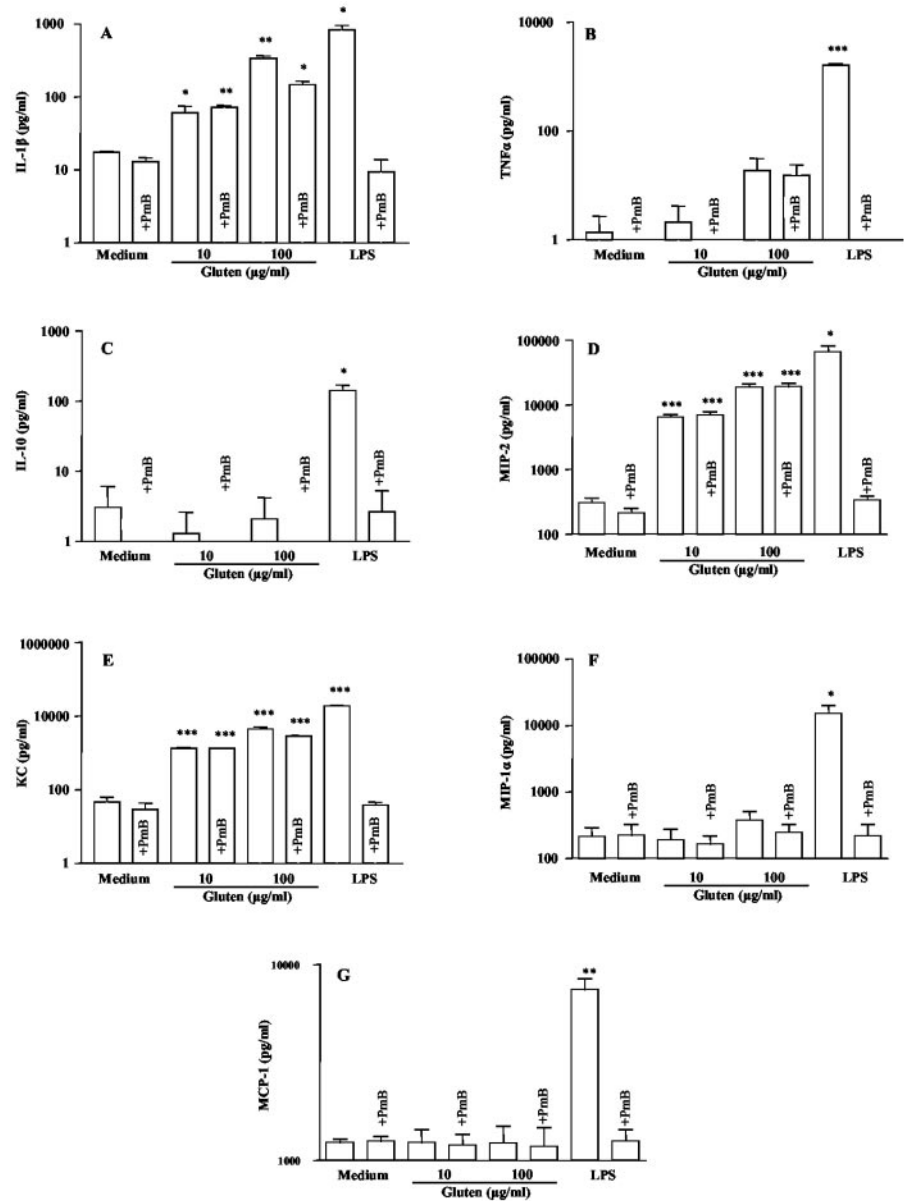
### Wheat gluten stimulates maturation of BMDC

Incubation of BMDC from BALB/c mice with 1, 10, or 100  $\mu\text{g}/\text{ml}$  gluten preparation resulted in a significant dose-dependent increase of the surface expression of MHC class II molecule I-A<sup>d</sup>, CD86, CD40, and CD54 on BMDC (Fig. 1). The mean fluorescence intensities (MFI) of these maturation markers after exposure to 100  $\mu\text{g}/\text{ml}$  gluten were 2.2-, 7.0-, 7.8-, and 4.1-fold higher, respectively, compared with medium control (Fig. 1). Up-regulation of surface expression of maturation markers was similar to that seen in the presence of 10 ng/ml LPS (2.2-, 5.3-, 9.3-, and 3.9-fold increase, respectively; Fig. 1).  $\alpha$ -CT at the concentration of 28  $\mu\text{g}/\text{ml}$ , corresponding to its content in 100  $\mu\text{g}/\text{ml}$  gluten preparation, did not enhance the expression of the investigated surface molecules on BMDC with the exception of I-A<sup>d</sup> (23% increase; Fig. 1).

Fig. 2 shows the result of double staining for MHC class II and costimulatory molecules, which are regulated partially independently. Stimulation of BMDC with LPS caused parallel increase of I-A<sup>d</sup> and costimulatory molecules CD86, CD40, or CD54. Incubation of BMDCs with gluten also induced coordinated up-regulation of



**FIGURE 5.** Dose dependency of wheat gluten-induced BMDC maturation in C3H/HeN vs C3H/HeJ mice. BMDC were incubated with 1, 10, or 100  $\mu\text{g}/\text{ml}$  wheat gluten, or 1 ng/ml LPS for 20 h. Thereafter, nonadherent cells were harvested, stained for I-A<sup>k</sup> (A), CD86 (B), CD40 (C), or CD54 (D), and analyzed by FACS. The data represent mean MFI  $\pm$  SEM of three independent experiments. Significant differences comparing with the medium control of corresponding strains are indicated as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ ; \*\*\*,  $p < 0.0001$ ; Student's unpaired *t* test.



**FIGURE 6.** Cytokine and chemokine secretion from BMDC in response to LPS or wheat gluten. BMDC of BALB/c mice were incubated for 20 h with 1, 10, or 100  $\mu\text{g/ml}$  wheat gluten, or 10 ng/ml LPS. Thereafter, the cells were collected and centrifuged at  $300 \times g$  for 5 min, and the concentrations of IL-1 $\beta$  (A), TNF- $\alpha$  (B), IL-10 (C), MIP-2 (D), KC (E), MIP-1 $\alpha$  (F), or MCP-1 (G) were determined in the supernatant by ELISA. All of the test compounds were incubated with 10  $\mu\text{g/ml}$  PmB (1 h; 4°C) before their addition to BMDC. The data represent mean values  $\pm$  SEM of three to five independent experiments. Significant differences from medium control are indicated as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ ; \*\*\*,  $p < 0.0001$ ; Student's unpaired  $t$  test.

I-A<sup>d</sup> and costimulatory molecules, i.e., cells with moderate- or high-density expression of MHC class II also exhibited moderate or high expression of costimulatory molecules, respectively.

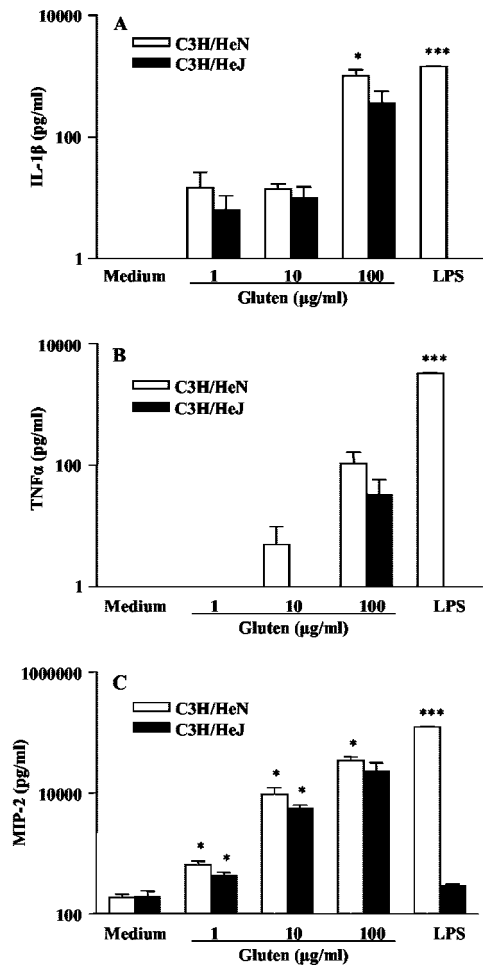
#### Wheat gluten-induced maturation of BMDC is not due to LPS contamination

To analyze whether the observed effect of gluten is due to LPS contamination, we preincubated gluten with PmB for neutralization of endotoxin. LPS plus PmB served as control. As expected, preincubation with PmB completely abrogated LPS-induced expression of I-A<sup>d</sup>, CD86, CD40, and CD54 molecules. When gluten was preincubated with PmB, no suppression of BMDC maturation was observed. This held true for all four maturation markers analyzed (Fig. 3). Next, we investigated the effect of gluten on the maturation of BMDC from C3H/HeJ mice, which express a mutated, largely nonreactive LPS receptor TLR4 (34). BMDC from wild-type C3H/HeN mice served as control. BMDC isolated from the two strains were incubated with gluten or LPS for 20 h. As shown in Fig. 4, fluorescence intensity curves for gluten- vs LPS-stimulated BMDC largely overlapped in C3H/HeN mice. By contrast, BMDC from

C3H/HeJ were unresponsive to LPS, whereas gluten retained its property to induce cell maturation. In BMDC from both strains, MHC class II expression on nonstimulated cells was already high and could not be further enhanced by LPS or gluten treatment. As in BALB/c mice, the effect of gluten was dose dependent in both strains (Fig. 5). Both background and induced expression of maturation markers were consistently higher in BMDC from C3H/HeN mice compared with BMDC from TLR4-defective mice. These results indicate that TLR4 is not involved in gluten-induced BMDC maturation.

#### Wheat gluten induces cytokine and chemokine production in BMDC

We analyzed whether gluten induces the production of cytokines or chemokines in BMDC, in addition to its effect on maturation. Gluten stimulated the dose-dependent release of IL-1 $\beta$  from BMDC. Maximal IL-1 $\beta$  release was seen at the gluten dose of 100  $\mu\text{g/ml}$  ( $337.3 \pm 32.1$  pg/ml), which was  $\sim 40\%$  of the IL-1 $\beta$  levels seen in response to LPS (Fig. 6A). TNF- $\alpha$  secretion was only minimally induced by the highest concentration of gluten, although it was strongly induced by LPS (Fig. 6B). An even more striking

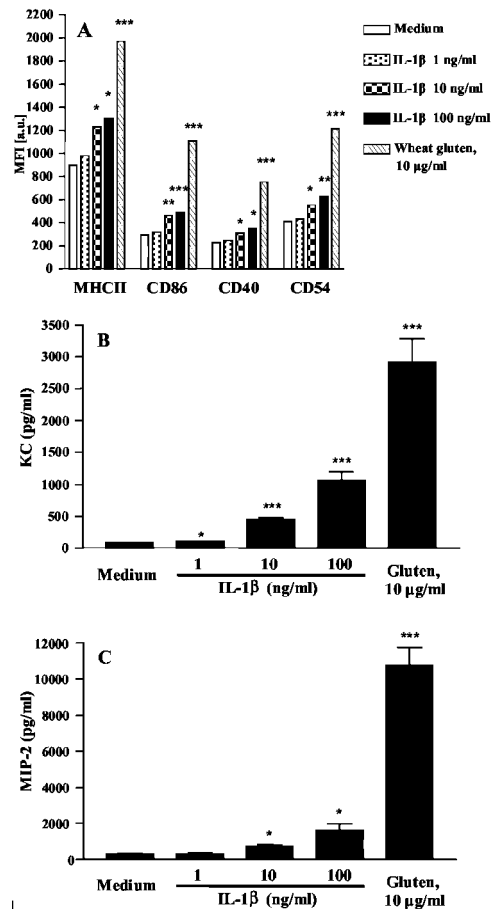


**FIGURE 7.** Role of TLR4 in cytokine and chemokine production from BMDC in response to gluten. BMDC from C3H/HeN or C3H/HeJ mice were incubated with 1, 10, or 100  $\mu\text{g/ml}$  wheat gluten, or 1 ng/ml LPS for 20 h. The content of IL-1 $\beta$  (A), TNF- $\alpha$  (B), or MIP-2 (C) in supernatant was determined by ELISA. The data represent mean values  $\pm$  SEM of two to three independent experiments. Significant differences from medium control are indicated as follows: \*,  $p < 0.05$ ; \*\*\*,  $p < 0.0001$ ; Student's unpaired  $t$  test.

disparity between LPS and gluten was observed for IL-10 production, which was only induced by LPS (Fig. 6C). Interestingly, gluten induced the production of large amounts of the chemokines MIP-2 (Fig. 6D) and KC (E), whereas MIP-1 $\alpha$  and MCP-1 were not induced (F and G). By contrast, LPS caused the secretion of all four chemokines from BMDC (Fig. 6, D–G).

We further investigated the effect of PmB on gluten-induced cytokine and chemokine production in BMDC. PmB partially suppressed the production of IL-1 $\beta$  (Fig. 6A) and KC (E) in response to the highest dose of gluten ( $p < 0.05$ ), but not for lower gluten concentrations or for MIP-2 or TNF- $\alpha$  (A, B, and D). In contrast to gluten, LPS-induced secretion of all investigated cytokines and chemokines from BMDC was completely abolished by PmB (10  $\mu\text{g/ml}$ ) treatment (Fig. 6).

To study the role of TLR4 in gluten-stimulated cytokine and chemokine production, BMDC from control C3H/HeN and *Tlr4*-defective C3H/HeJ mice were incubated with 1, 10, or 100  $\mu\text{g/ml}$  gluten. Gluten up-regulated the release of IL-1 $\beta$ , TNF- $\alpha$ , and MIP-2 from BMDCs of both strains (Fig. 7). As seen in the BALB/c strain, there was good response for IL-1 $\beta$  and MIP-2, and only little TNF- $\alpha$  production. LPS caused cytokine and chemokine

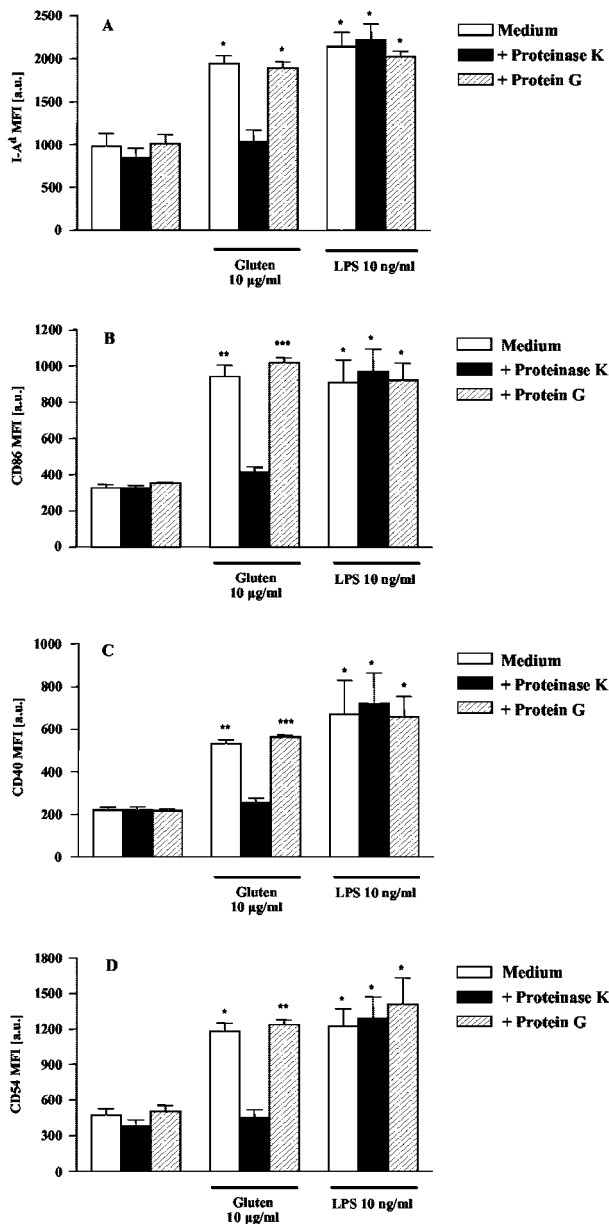


**FIGURE 8.** Maturation of BMDC and chemokine secretion in response to IL-1 $\beta$ . BMDC of BALB/c mice were incubated for 20 h with 1, 10, and 100 ng/ml IL-1 $\beta$ , or 10  $\mu\text{g/ml}$  wheat gluten. Thereafter, nonadherent cells were harvested, centrifuged at  $300 \times g$  for 5 min, stained for I-A<sup>d</sup>, CD86, CD40, or CD54, and analyzed by FACS (A). The concentrations of MIP-2 (B) and KC (C) were determined in the supernatant by ELISA. The data represent mean values  $\pm$  SEM of two to five independent experiments. Significant differences from medium control are indicated as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ ; \*\*\*,  $p < 0.0001$ ; Student's unpaired  $t$  test.

secretion from BMDC of C3H/HeN, but not from C3H/HeJ mice (Fig. 7). Again, BMDC of the mutant C3H/HeJ strain showed consistently lower reactivity.

#### Wheat gluten-stimulated chemokine production in BMDC is not secondary to IL-1 $\beta$ secretion

IL-1 $\beta$  was previously shown to increase the production of MIP-2 and KC, the expression of cytokine and costimulatory receptors on immune cells, and CD40L-mediated maturation of DC (35–40). Therefore, we addressed the question whether wheat gluten-induced increase in chemokine secretion from BMDC was secondary to IL-1 $\beta$  release seen in our system. In response to the highest wheat gluten concentration, the level of IL-1 $\beta$  secreted from DC was  $<0.4$  ng/ml (Fig. 7). At doses of 1–100 ng/ml, recombinant mouse IL-1 $\beta$  caused moderate maturation of BMDC, and at 1 ng/ml IL-1 $\beta$ , the expression of maturation markers on BMDC and MIP-2 level were not different from that of the control (Fig. 8, A and B). KC production at 1 ng/ml IL-1 $\beta$  was 33% higher than in the control ( $110.7 \pm 1.5$  vs  $82.8 \pm 6.4$ ;  $p < 0.05$ ; Fig. 8C). This increase was negligible compared with the 5.4-, 12.9-, and 35.1-fold enhancement after BMDC exposure to 10 and 100 ng/ml IL-1 $\beta$  and 10  $\mu\text{g/ml}$  wheat gluten, respectively. Therefore, IL-1 $\beta$



**FIGURE 9.** The effect of proteinase K-digested wheat gluten on BMDC maturation. Wheat gluten or LPS were treated with 30 U/ml proteinase K coupled to agarose beads and thereafter incubated with BMDC of BALB/c mice for 20 h. Nonadherent cells were harvested, centrifuged at  $300 \times g$  for 5 min, stained for I-A<sup>d</sup>, CD86, CD40, or CD54, and analyzed by FACS (A–D). The data represent mean values  $\pm$  SEM of three independent experiments. Significant differences from medium control are indicated as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ ; Student's unpaired  $t$  test.

at a concentration of  $<1$  ng/ml cannot stimulate BMDC, and MIP-2 and KC production in BMDC after cell incubation with 10  $\mu$ g/ml wheat gluten is not IL-1 $\beta$  inducible. In accordance with these data, Abs against IL-1RI did not interfere with wheat gluten-mediated expression of maturation markers on BMDC (data not shown).

*The active compound in wheat gluten preparation has a peptidic nature*

To further investigate the chemical structure of the stimulatory compound of wheat gluten, we digested it with proteinase K, a very stable and highly reactive serine protease. Proteinase

K-treated wheat gluten did not induce the maturation of BMDC from BALB/c mice as estimated by the expression of surface molecules I-A<sup>d</sup>, CD86, CD40, and CD54 (Fig. 9). In contrast, proteinase K did not influence the stimulatory capacity of LPS on BMDC. Protein G, which was used as a control for proteinase K, did not interfere with wheat gluten- or LPS-mediated maturation of BMDC. Taken together, these data strongly suggest that the active compound of wheat gluten is protein in nature.

## Discussion

The addition of wheat gluten to immature BMDC induced maturation for all four surface markers analyzed. Up-regulation of the surface density of MHC class II and costimulatory molecules occurred in parallel on the same cell, which was indicative of a physiologically regulated response. Up-regulation of the expression of MHC class II and costimulatory molecules CD86, CD40, and CD54 was dependent on the concentration of wheat gluten present, over the range of 1–100  $\mu$ g/ml. At the highest gluten dose, DC response was similar to that seen in response to 10 ng/ml LPS, which underscores the biological potency of wheat gluten. Because of the comparability with the effects of LPS, it was essential to exclude the contribution of a possible endotoxin contamination of wheat gluten preparation. Several lines of evidence excluded involvement of endotoxin. For one, experiments were repeated in the presence of an excess of PmB, a potent LPS antagonist, with virtually the same outcome for gluten, whereas the actions of LPS were completely blocked. In a second approach, we analyzed DC from the LPS nonresponder strain C3H/HeJ (34). DC from this strain were not affected by LPS, but preserved the ability to mature when exposed to wheat gluten. A third argument is that proteinase K-digested gluten lost the ability to stimulate DC, in contrast to LPS. In addition, the secretory response of DC to gluten was strikingly different from that to LPS.

Upon exposure to wheat gluten, DC produced both cytokines and chemokines. This production was dose dependent in that 100  $\mu$ g/ml gluten induced higher levels in the supernatant than 10  $\mu$ g/ml. The highest secretory responses were seen for the two chemokines MIP-2 and KC, whereas the two chemokines MIP-1 $\alpha$  and MCP-1 were not induced, in contrast to LPS-treated DC cultures. Again, in contrast to LPS, wheat gluten produced only minimal amounts of TNF- $\alpha$  and low amounts of IL-1 $\beta$ , but failed to stimulate IL-10 secretion.

It is noteworthy that wheat gluten in our study induced neither the two strongly proinflammatory mediators TNF- $\alpha$  and MIP-1 $\alpha$  (41–43) nor the potent anti-inflammatory cytokine IL-10 (44, 45). The induction of high MIP-2 and KC and low IL-1 $\beta$  secretion cannot easily be classified as pro- or anti-inflammatory. Rather, these immune mediators are expected to prepare the immune system for better reactivity, by attracting leukocytes and by increasing the reactive state of immune cells. The murine chemokines MIP-2 and KC, which are homologous to human IL-8 and share the receptor CXCR2, are the main attractants of polymorphonuclear neutrophils in mice (46). Chemokine-induced migration of neutrophils across the intestinal epithelium increases its permeability and might thereby enhance Ag influx (47). Interestingly, intracellular Ags from necrotic cells induce MIP-2 and KC production in macrophages, fibroblasts, and DC (48). Hence, wheat gluten appears to prepare the immune system for easier response by causing maturation of APCs, by attracting further leukocytes, and by increasing their reactive state. In the presence of an appropriate genetic predisposition, this may be expected to increase the risk of adverse immune reactions, to gluten itself or to other Ags presented. Although not detectable in our experimental system, a mild proinflammatory activity of wheat gluten cannot be excluded. It has



been reported that the addition of gliadin peptides to peritoneal macrophages up-regulated the already considerable spontaneous production of both TNF- $\alpha$  and IL-10 by these cells (30). The discrepancy between our observations in cytokines produced is more likely due to the use of different cell types (peritoneal macrophages vs BMDC) and culture conditions (IFN- $\gamma$  vs GM-CSF).

The finding that wheat gluten effects are dose dependent and quite selective in terms of the induced cytokine/chemokine pattern argues against a nonspecific irritation of DC. Rather, we assume the involvement of a single or a limited number of receptor types, such as scavenger receptors or other surface proteins. At present, the relevant epitopes of wheat gluten and the mechanism of the recognition by DC are not known. Recent observations support the complex nature of activation of the immune system by wheat gluten, which involves triggering of both innate and adaptive immunity (31). In addition to the discovery of immunodominant epitopes in gliadin and glutenin, nonimmunodominant peptides of  $\alpha$ -gliadin could induce the innate response in duodenal biopsy samples from patients with CD and increase T cell recognition of dominant gliadin epitopes (31). In addition, a normal trace component in wheat gluten, wheat storage globulin G1b1, is particularly antigenic in diabetic animals (21). Therefore, the wheat gluten preparation could include several candidate immunomodulatory polypeptides. These polypeptides are not necessarily rich in proline and glutamine residues and thereby sensitive to specific cleavage by prolyl-endopeptidase. This issue requires further investigation.

In summary, proteolytic fragments of wheat gluten induced maturation of BMDC and the secretion of selected cytokines and chemokines. In contrast to LPS, wheat gluten did not stimulate the production of classic proinflammatory mediators, such as TNF- $\alpha$  or MIP-1 $\alpha$ . It is conceivable that wheat gluten-based diets enhance immunological alertness in the gut, facilitating adverse immune responses to dietary Ags, particularly in genetically predisposed individuals.

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