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Gliadin Regulates the NK-Dendritic Cell Cross-Talk by HLA-E Surface Stabilization

Giuseppe Terrazzano,2* Michela Sica,*, Carmen Gianfrani,† Giuseppe Mazzarella,‡ Francesco Maurano,‡ Beatrice De Giulio,† Sophie de Saint-Mezard,‡ Delia Zanzi,§ Luigi Maiuri,¶ Marco Londei,¶ Bana Jabri,† Riccardo Troncone,§ Salvatore Auricchio,§ Serafino Zappacosta,* and Ennio Carbone*#**

We analyzed the autologous NK cell interaction with gliadin-presenting dendritic cells. Gliadin is the known Ag priming the celiac disease (CD) pathogenesis. We demonstrate that gliadin prevents immature dendritic cells (iDCs) elimination by NK cells. Furthermore, cooperation between human NK cells-iDCs and T cells increases IFN-γ production of anti-gliadin immune response. Gliadin fractions were analyzed for their capability to stabilize HLA-E molecules. The α and ω fractions conferred the protection from NK cell lysis to iDCs and increased their HLA-E expression. Gliadin pancreatic enzyme digest and a peptide derived from gliadin peptides to mucosa infiltrating T cells (2). Gliadin intake in celiac patients correlates with the presence of serum autoantibodies against tissue transglutaminase. Although CD has been classified recently among the autoimmune pathologies (3), the mechanisms of its autoimmune pathogenesis still appear to be poorly understood. Innate immunity preceding the adaptive response may be involved in the CD pathogenesis (4).

NK cells are found in the murine intestinal epithelium and in the lamina propria (5, 6) and NK T cells in the gut (7). Intestinal NK cells have cytotoxic (5) and IFN-γ production activity (6). The

Celiac disease (CD)3 is a small intestinal enteropathy triggered by the ingestion of gliadin in susceptible individuals (1). Genetic, environmental, and immunological factors play a central role in CD pathogenesis (1). The majority of patients are HLA-DQ2 positive, while a small group express DQ8. Both DQ2 and DQ8 molecules are involved in the presentation of gliadin peptides to mucosa infiltrating T cells (2). Gliadin intake in celiac patients correlates with the presence of serum autoantibodies against tissue transglutaminase. Although CD has been classified recently among the autoimmune pathologies (3), the mechanisms of its autoimmune pathogenesis still appear to be poorly understood. Innate immunity preceding the adaptive response may be involved in the CD pathogenesis (4).

NK cells are found in the murine intestinal epithelium and in the lamina propria (5, 6) and NK T cells in the gut (7). Intestinal NK cells have cytotoxic (5) and IFN-γ production activity (6). The expansion of intraepithelial lymphocytes expressing CD94, a typical NK cell receptor (8), and of a subset of gut epithelium CD3–CD56+ lymphocytes (9) has been described in CD. IL-15, a cytokine that controls NK cell homeostasis (10), is up-regulated in atrophic mucosa where most of the epithelial changes occur in the active stage of the disease (11). The p31–43 gliadin peptide triggers IL-15 production in the gut (12). IL-15, together with NKGD2 expression, induces CTL activity contributing to the tissue destruction in CD (12).

NK cells represent a distinct lymphocyte population of innate immunity characterized by cytotoxicity against tumors, virus-infected cells, and parasites (13). IFN-γ and TNF-α are secreted by NK cells in response to cytokines produced by macrophages and dendritic cells (DCs) (14, 15). Moreover, IFN-γ and TNF-α production by NK cells could promote DC maturation degree (16, 17). IFN-γ promotes Th1 differentiation (18) and is expressed in small intestinal mucosa biopsy from celiac patients (19). This cytokine could induce both the cytotoxic intraepithelial lymphocytes and macrophage activation, fostering the damage of intestinal mucosal extracellular matrix (20). The interaction between NK cells and target cells is modulated by several receptor-ligand interactions (21–23). NK cells express several MHC class I (MHC-I) recognizing receptors, mainly classified into two families: the killer Ig-like receptors (KIRs) and the lectin-like receptors (22). NK cell cytotoxicity is triggered by non-MHC-I receptors such as CD16, CD69, 2B4, NKGD2, NKp30, NKp44, NKp46, and NKp80 (22).

DCs play a key role in immune responses (24). Immature DCs (iDCs) exert Ag capture and processing ability in peripheral tissue (25). Inflammatory cytokines and microbial components (26) trigger DC migration into lymphoid tissues and promote their maturation. In this process, DCs acquire the ability to stimulate T cells (27). DCs, present in the gut (28), in Peyer’s patches (29), and in lamina propria (30), have been described to have a key role in activating naïve T cells in GALT (31).

NK cells are involved in several autoimmune diseases (32), and their cross-talk with iDCs leads to different outcomes (16): 1) NK cells recognize and eliminate immature but not mature DCs (33);
2) reciprocal activation (17, 34); and 3) NK cells comigrate with DCs in the lymph nodes (35).

Because NK cells are present in the GALT (36) and the gut Ag-loaded DCs migrate from GALT to mesenteric lymph nodes (37), the interaction between NK cells and mature DCs could occur in these environments and could contribute to the priming of immune response and to Th1 polarization (18, 35, 39).

In the research reported here, we explored the potential role that gliadin plays in regulating NK cells–DCs cross-talk and its role in regulating T cell response.

Materials and Methods

Patients

A total of 24 celiac patients (mean age of 14.2 years) was enrolled for this study. All patients gave their informed consent, according to the ethical regulations of the University of Naples “Federico II.” Patients were diagnosed according to the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition criteria (40); all of them had been on gluten-free diet for at least 2 years and were serum negative for anti-endomysial Abs.

Culture of NK polyclonal lines, iDCs, and gliadin treatments

PBMCs and NK cells were isolated from celiac patients or from healthy donors as reported previously (33). NK cell purity was always $>$95%. All NK polyclonal cell lines were CD94/NKG2A positive in a percentage ranging between 70 and 80% of total CD3 CD56+ lymphocytes.

iDCs were generated as described originally (26). iDC population was found to be 85% pure expressing high levels of CD1a, with no detectable amount of CD14 by FACS staining. iDCs were incubated for 18 h with 50 µg/ml peptic-tryptic digest (PT) of gliadin (PT-gliadin), 50 µg/ml PT of β-lactoglobulin, lacto-albumin, and casein or with medium alone. Alternatively, the iDCs were treated with 50 µg/ml single-purified α-, β-, γ-, or ω-gliadin fractions in the same experimental conditions. The PT gliadin was prepared from gliadin extracted from San Pastore cultivars (41). Gliadin or gluten were digest with pepsin (50:1) in 5% formic acid for 4 h at 37°C while stirring, then the fraction of pepsin polypeptides was exposed to a sequential digestion with trypsin, chymotrypsin, and elastase (50:1) in 50 mM ammonium bicarbonate for 4 h at 37°C. Digest preparations were collected in RPMI 1640 (Sigma-Aldrich) to obtain a complete peptrinc-tryptic digestion (PTCT).

Fractionation of gliadin was performed as reported originally (41). α Gliadin was obtained as a unique fraction of all α gliadin subgroups. The proportion of protein was determined according to the Bradfor method (42). PT proteins and gliadin fractions were assayed for endo-toxin LPS by using a detection kit (BioWhittaker) and found LPS levels to be $<$0.5 EU/ml (43).

Generation of gliadin-specific, intestinal T cell lines and evaluation of DC phenotype in mononuclear cells from mucosal explants

Mucosal explants from two DQ2-positive CD patients (female, untreated, 24 years old; male, treated, 20 years old) were digested with collagenase A at 1 mg/ml for 1 h at 37°C. Cells were plated in 24-well plates at 2–3×10^6/ml and stimulated with 1×10^6 irradiated autologous PBMCs and 50 µg/ml PT-gliadin. Cultures were fed with 10 ng/ml IL-2. At days 7 and 21, gliadin-restricted intestinal T cell lines (g-iTCLs) were restimulated with irradiated autologous PBMCs and PT-gliadin/tissue transglutaminase as described above and thereafter with cyclic (14 days) restimulation with a feeder-cell mixture containing PHA (0.5 µg/ml) and IL-2 (100 U/ml). Ten to 14 days after the last restimulation when cells were in complete resting phase, g-iTCLs were assayed for responsiveness to gliadin by IFN-γ-ELISPOT assay.

After the digestion of mucosal explants by collagenase (1 mg/ml for 1 h at 37°C), the mononuclear cells obtained were stained by immunofluorescence and analyzed by FACS to evaluate DC phenotype.

IFN-γ-ELISPOT assay

Ninety-six nitrocellulose-backed plates (MAHAS4510; Millipore) were coated with 10 µg/ml anti-human IFN-γ mAb (BD Pharmingen) as described above. Gliadin (0.3 × 10^9) were plated in the presence of 0.6 × 10^9 irradiated autologous DCs (corresponding to a ratio g-iTCLs/DC of 1:2), which had been pulsed overnight with PT-gliadin or PT-β-lactoglobulin at 50 µg/ml. Ag-pulsed-DCs were incubated 6 h in presence of 0.3 × 10^6 autologous NK cells, corresponding to a ratio of DCs: NK cells 2:1 before adding responder g-iTCLs in a final volume of 200 µl. After 36 h, plates were incubated for 2 h with biotinylated anti-IFN-γ mAb, followed by streptavidin–HRP incubation (BD Pharmingen) for 1 h. IFN-γ Spot Count/well were counted by immunospot image analyzer (AEL VIS). To inhibit the IFN-γ secretion by NK cells, 1×10^6 autologous NK cells were pretreated for a 37°C overnight incubation with 10 µg/ml Golgi protein-traficking blocker brefeldin A (BFA) (Sigma-Aldrich).

Detection of intracellular IFN-γ and TNF-α by flow cytometry

PT-gliadin- or PT-β-lactoglobulin- or medium-treated iDCs were mixed at a ratio of 1:1 with IL-2-activated NK effectors (2×10^5) and incubated in a sterile 96-well plate (Falcon) for 6 h at 37°C in 10% FCS-RPMI 1640 medium. BFA (Sigma-Aldrich) was added at 5 µg/ml during the incubation to induce cytokine intracellular retention, while it was not added in wells used to produce cytokine enriched supernatants. At the end of incubation, the 96-well plates were centrifuged at 800 × g for 5 min, the medium supernatants were recovered and the cells used to perform intracellular cytokine FACS staining. Alternatively, after the 6 h of incubation, 2×10^5 g-iTCLs (stimulated as described in IFN-γ-ELISPOT assay paragraph) were added to the DC-NK cells culture to perform intracellular cytokine FACS staining for an additional 36 h of incubation. For the cytokine-enriched supernatant transfer assay, 100 µl of medium supernatant from NK cell/ medium-DCs or NK cell/PT-gliadin-DCs or NK cell/PT-β-lactoglobulin-DC coculture were added to 2×10^5 g-iTCLs (stimulated as described in IFN-γ-ELISPOT assay paragraph) to perform intracellular cytokine FACS staining after a 36-h incubation. A cytokine staining kit (Caltag Laboratories) and FACS analysis were used to evaluate intracellular cytokine production in NK cells and g-iTCLs. IFN-γ and TNF-α production was stained by anti-IFN-γ (4SB3) and anti-TNF-α (MA911) mAbs (BD Pharmingen). Cytotoxicity assay

Cytotoxicity was measured in a conventional 4-h 51Cr release assay. Forty-eight-hour IL-2-activated polyclonal NK cell lines were used as effectors. iDC targets were labeled with Na2111In and used to evaluate intracellular cytokine production in NK cells and g-iTCLs.

IFN-γ-ELISPOT assay

Ninety-six nitrocellulose-backed plates (MAHAS4510; Millipore) were coated with 10 µg/ml anti-human IFN-γ mAb (BD Pharmingen) as described above. Gliadin (0.3 × 10^9) were plated in the presence of 0.6 × 10^9 irradiated autologous DCs (corresponding to a ratio g-iTCLs/DC of 1:2), which had been pulsed overnight with PT-gliadin or PT-β-lactoglobulin at 50 µg/ml. Ag-pulsed-DCs were incubated 6 h in presence of 0.3 × 10^6 autologous NK cells, corresponding to a ratio of DCs: NK cells 2:1 before adding responder g-iTCLs in a final volume of 200 µl. After 36 h, plates were incubated for 2 h with biotinylated anti-IFN-γ mAb, followed by streptavidin–HRP incubation (BD Pharmingen) for 1 h. IFN-γ Spot Count/well were counted by immunospot image analyzer (AEL VIS). To inhibit the IFN-γ secretion by NK cells, 1×10^6 autologous NK cells were pretreated for a 37°C overnight incubation with 10 µg/ml Golgi protein-traficking blocker brefeldin A (BFA) (Sigma-Aldrich).

Monoclonal Abs, immunofluorescence, and flow cytometry

Anti-HLA mAb (W6/32/second) was purchased from DakoCytomation. Anti-DQ2 mAb (AD5-14H12) and anti-DQ2 mAb (AC144) were purchased from Miltenyi Biotec. Anti-CD14 (M5E2), -CD1a (H149), -CD83 (HB15), -CD86 (IT2.2), -CD80 (BB1), -CD40 (5C3), -CD1 (UCHT1), -CD56 (B159) -CD94 (KP43), -CD11c (B-Ly6), FITC-, CyChrome-, and PE-labeled mAbs and isotype-matched mAb controls were purchased from BD Pharmingen and used as described previously (33). HLA-E expression was analyzed using two anti-HLA-E mAbs: the MEM-E08 (IgG1) from EXBIO and the 3D12 (IgG1), a gift from Dr. D. Geraghty (Fred Hutchinson Cancer Research Center, Seattle, WA). Anti-NKG2A/NKG2C (P25) mAb was a gift from Dr. L. Moretta (Istituto Gaslini, Genova, Italy).

HLA-E peptide design and stabilization studies

Gliadin chains were analyzed using Gene Jockey I software (Biossot). Gliadin polypeptide sequences were matched to canonical HLA-E-binding peptide sequence MAPARTL1L and compared with sequences generated by exchanging canonical amino acid moieties with other aa compatible with HLA-E binding and CD94 recognition (45). The gliadin-derived peptide SQPYPLQQL was used comparatively with positive control peptide MAPARTL1L in HLA-E molecule stabilization experiments by using the murine HLA-E transfectect RMA-S cell line (a gift from Dr. C. Moretta, Istituto Gaslini). HLA-A2-restricted influenza A virus protein (FLU-M1 58–66, GILGFVFTL) was used as negative control. Peptides were synthesized by PRIMM. HLA-E′ RMA-S cell line was incubated overnight at 37°C in presence of the synthetic peptides.
at the concentration of 200 \mu M. After incubation, the cells were recovered and analyzed by FACS for the expression of HLA-E.

**Immunohistochemistry and confocal microscopy analysis**

Biopsy specimens from distal jejunum of 10 untreated CD patients (12–35 years old) were obtained during upper gastrointestinal endoscopies. Diagnosis was based on typical mucosal lesions with crypt cell hyperplasia and villous atrophy. All CD patients were positive for anti-endomysial Abs. Duodenal biopsies were also obtained from 8 treated CD patients (21–46 years old) and from 8 controls (23–54 years old) all having normal mucosal architecture with long villi and short crypts and all negative for endomysial Abs.

Tissue sections were prepared as described previously (46). Anti-HLA-E mAb (MEM-02; 4 \mu g/ml) was added at 4°C overnight. Primary Ab (4 \mu g/ml) was omitted in sections used as negative control samples. Slides were incubated with anti-mouse mAb conjugated to HRP (1/50 dilution; DakoCytomation) for 40 min at room temperature (RT). Immune-reactive cells were visualized by diaminobenzidine and hematoxylin (Sigma-Aldrich). Isotype control sections were prepared replacing anti-HLA-E mAb with purified mouse IgG1 mAb (Serotec). The number of cells expressing HLA-E was evaluated within a total area of 1 mm² of lamina propria. The counts were double-blind analyzed independently by two observers.

In experiments to detect NK cells (CD94/H11001 CD3/H11002) and HLA-A, -B, and -C Ags (G46-2.6 clone from BD Pharmingen), immunofluorescence combined with confocal microscopy was used. Cryosection were fixed in acetone and incubated for 1 h at room temperature with anti HLA-A, -B, and -C mAb (1/300), followed by 30 min of horse anti-mouse FITC-conjugated incubation. CD94⁺ and CD3⁺ immunofluorescence cells were imaged with a Leica SP confocal microscope.

**FIGURE 1.** Gliadin confers a NK cell autologous and allogeneic resistant iDC phenotype in both patients and normal donors. Human IL-2-activated polyclonal NK cell lines were tested in a cytotoxicity assay against human autologous and allogeneic DC targets. DCs were derived from two patients (A–D) and from one normal donor (E and F) and tested against autologous (A, C, and E) and allogeneic (B, D, and F) polyclonal NK cell lines. DCs were pretreated with PT-gliadin (□) or with PT-β-lactoglobulin (△) or with medium alone (○). Gliadin, but not other food-derived proteins, inhibits DC recognition by NK cells (G). iDCs were pretreated with PT-gliadin (□) or with PT-β-lactoglobulin (△), PT-lacto-albumin (△), PT-OVA (●), PT casein (■), or with medium alone (○). CD94/NKG2A blockade reverted the gliadin-dependent inhibition of the NK cell/iDC targets recognition (H). Autologous iDCs incubated with PT-gliadin (△, ■, and ●) or with medium alone (○). Before adding to PT-gliadin-treated DCs, NK cells were incubated with anti-CD94/ NKG2A (Z270) (∇) or with control 345.134 mAb (△). The percentage of lysis and E:T ratio were reported on the abscissa and ordinate axes, respectively. Data are representative of one of four independent experiments.

**FIGURE 2.** The effect of gliadin on DC phenotype in NK cell-DC coculture. NK cell-DC coculture were harvested after 6 h of incubation to evaluate the expression of CD1a, CD86, CD11c, BDCA3, and HLA-I Ags on DCs in the different condition of DC treatments (as indicated); y-values indicate the percentage of positive cells, and means ± SD were reported. The DCs were gated basing on their high forward scatter (data not shown). Results from three independent experiments were summarized.
Gliadin pretreatment conferred autologous and allogeneic NK cell lysis resistance to iDCs in patients as well as in healthy donors. The differences were statistically significant \((p < 0.05)\). To perform statistical analysis of results, we excluded from this study fresh, not IL-2-stimulated, NK cells because of their low cytotoxicity against autologous iDCs (data not shown). When NK cells were preincubated with anti-NKG2A mAb (Z270), their killing activity against PT-gliadin pulsed autologous target cells was restored at level similar to medium alone DCs (Fig. 1H). As previously described \((16, 33, 49–51)\), the anti-NKG2A mAb (Z270) pretreatment of NK effectors enhanced their killing against medium or PT-ß-lactoglobulin incubated DCs (data not shown). Similar results were observed by using another anti-NKG2A (Z199) mAb as well as with anti-CD94 mAbs (XA185 and HP3B1), while no effect was observed when NK cell treatment was performed by using mAbs against the CD158a or CD158b KIRs (data not shown). Notably, the celiac NK cell killing activity against the K562 cell line was comparable to the cytotoxic activity exerted by NK cells from the normal donors (data not shown). These suggest that the natural cytotoxicity baseline levels are unaffected in celiac patients.

**DC phenotype: gliadin and its fractions effect on HLA-E expression**

DC maturation was analyzed and no significant differences in the expression of CD14, MHC-I, MHC class II, CD1a, CD40, CD83, CD86, CD11c, CD1c, and BDCA3 molecules were found in healthy donor or celiac patient-derived iDCs preincubated with PT-gliadin or with PT-ß-lactoglobulin if compared with medium alone incubation. DC phenotype evaluation was performed either in absence or in presence of NK cells in coculture (Fig. 2 and data not shown). Notably, DCs generated in vitro for our experiments share CD11c- CD86+ BDCA3+ phenotype (Fig. 2) with the recently described DCs that accumulate in duodenal sections from celiac-active stage patients \((53)\).

**Table I. In silico prediction of gliadin fraction HLA E-binding peptides**

<table>
<thead>
<tr>
<th>Peptide Sequence Analysis</th>
<th>VMAPRTLLL</th>
<th>VQQPYAILF</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha) Gliadin 1</td>
<td>None sequence with more than 2 aa identity</td>
<td>2 sequences with 4 aa identity</td>
</tr>
<tr>
<td>(\alpha) Gliadin 2</td>
<td>2 sequences with 3 aa identity</td>
<td>3 sequences with 5 aa identity</td>
</tr>
<tr>
<td>(\gamma) Gliadin</td>
<td>None sequence with more than 3 aa identity</td>
<td>None sequence with more than 3 aa identity</td>
</tr>
<tr>
<td>(\omega) Gliadin</td>
<td>1 sequence with 4 aa identity</td>
<td>6 sequences with more than 2 aa identity</td>
</tr>
<tr>
<td>Glutenin</td>
<td>1 sequence with more than 2 aa identity</td>
<td>None sequence with more than 2 aa identity</td>
</tr>
<tr>
<td>OVA A chain</td>
<td>None sequence with more than 2 aa identity</td>
<td>None sequence with more than 2 aa identity</td>
</tr>
<tr>
<td>OVA B chain</td>
<td>None sequence with more than 2 aa identity</td>
<td>None sequence with more than 2 aa identity</td>
</tr>
<tr>
<td>OVA C chain</td>
<td>None sequence with more than 2 aa identity</td>
<td>None sequence with more than 2 aa identity</td>
</tr>
<tr>
<td>OVA D chain</td>
<td>None sequence with more than 2 aa identity</td>
<td>None sequence with more than 2 aa identity</td>
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FIGURE 4. The purified gliadin fractions induce differential effects on DC susceptibility to NK cell lysis. IL-2-activated polyclonal NK cell lines were used as effectors in a cytotoxicity assay against human allogeneic DC targets (A) incubated with medium alone (○) or PT-β-lactoglobulin (△) or PT-gliadin (□) or with α (●), γ (▲), and ω (○) fractions. CD94/NKG2A blocking on NK effectors reverted α fraction-dependent lysis inhibition on DC targets (B). Autologous iDCs incubated with α gliadin (○, △, and ●), with PT-β-lactoglobulin (△) or with medium alone (○). Before adding to α gliadin-incubated DCs, NK cells were pretreated with anti-CD94/NKG2A (Z270) (△) or with control 345.134 mAb (●) or with medium alone (○). The percentage of lysis and E:T ratio were reported on the abscissas and ordinate axes, respectively. Data are representative of one of four independent experiments. NK cells and DCs were obtained from healthy donors.

Therefore, we suggest that gliadin did not interfere with DC phenotype maturation in NK cells/DC cross-talk (Fig. 2). A significant (p < 0.05) higher expression of HLA-E was observed in celiac patient-derived, PT-gliadin-treated iDCs compared with their PT-β-lactoglobulin-treated counterpart (Fig. 3A) or with the other food protein-derived PT-digest treatments (data not shown).

These data suggest that the increasing of HLA-E on PT-gliadin-treated DCs was a gliadin-specific effect.

Among the described (54), four principal gliadin-fractions (α1, α2, γ, and ω), we tested the effect of the most represented: the α (all α fractions, see Materials and Methods), γ and ω fractions. Fig. 3B shows the overexpression of HLA-E on healthy donor and celiac patient-derived iDCs, treated with α and ω gliadins. This effect was comparable to result obtained with the whole PT-gliadin incubation, and it was statistically significant (p < 0.05) in four independent experiments (Fig. 3C). In contrast, γ gliadin conferred a weaker expression of HLA-E on iDCs.

To investigate the possibility that gliadin fraction DC incubation could generate HLA-E-binding peptides, we predict in silico the homology in the amino acid sequences of known HLA-E-binding peptides (VMAPRTLLL and VQQPYAILF) (45), α1-, α2-, γ-, and ω- gliadin fractions and the OVA, by using Gene Jockey I software (Table I).

Notably, aa 52–60 of α2 chain contains a nonamer SQQQPYLQLQ carrying glutamine at P2 and P9 positions, known to be an amino acidic substitution compatible with the HLA-E cleft loading (52), although, we cannot exclude that other peptides with potential HLA-E binding motif are present in the gliadin fractions, see Table I.

To verify the functional effect of gliadin fractions on NK cell lysis, we performed cytotoxicity assays using as target iDCs incubated with either the whole gliadin or with its fractions (Fig. 4A). A direct correlation was found between α and ω gliadin fractions in inducing high levels of HLA-E on presenting iDCs and their inhibitory effect on autologous NK cell cytotoxicity. In addition, blocking of CD94/NKG2A receptor, by using Z270 mAb, was able to revert the NK cell lysis inhibition against iDCs treated with α (Fig. 4B) and ω (data not shown) gliadin fractions.

These data suggest that gliadin fractions, mainly α and ω, could contain peptides able to load HLA-E cleft and preventing the killing by autologous NK.

To verify the effect of gliadin-derived peptides to increase the HLA-E surface expression, HLA class I stabilization experiments using the RMA-S/HLA-E-transfected cell system were performed, as described elsewhere (55). We have compared the HLA-E stabilization capacity of canonical VMAPRTLLL peptide with gluten, gliadin pancreatic digest (PTCE), and the gliadin-derived peptide SQQQPYLQLQ (Fig. 5). Gluten, PTCE, and SQQQPYLQLQ peptide potently increase the HLA-E levels on the cell membrane. In addition, the up-regulation of HLA-E molecules did not occur in RMA-S/HLA-E line performing experiment at low temperature (26°C), confirming previously reported results by Borrego et al. (56) and data not shown. This result suggests a role
for active metabolism, as at 37°C, in processing and uploading peptides into HLA-E.

**Cytokine production in the interplay between gliadin-treated DCs, NK cells, and gliadin-specific intestinal CD4⁺ T cells**

No significant differences were found in the percentage of CD3⁺CD56⁺ NK cells in infiltrating lymphocytes between CD patients and healthy donor mucosa (mean ± SD): 1.7 ± 0.7% in untreated, 1.85 ± 0.27% in treated patients, and 1.67 ± 0.57% in control mucosa. In contrast, T lymphocytes (CD56⁻ CD3⁺) were largely increased in acute CD compared with treated CD and healthy donor mucosa: 15.85 ± 10.59, 6.45 ± 4.4, and 10.3 ± 7.9%, respectively.

The gliadin-specific, T-dependent IFN-γ response was analyzed using two different methods: ELISPOT, to preserve a more physiological conditions in absence of any treatment to induce cellular cytokine retention, and FACS intracellular staining, to detect the cytokine in the producing cell subsets.

Two CD3⁺CD4⁺CD69⁺CD94⁻, NKG2A⁻, and NKG2C⁻CD56⁻ g-iTCLs were obtained from mucosal explants from HLA DQ2 patients (see Materials and Methods). IFN-γ production by g-iTCLs was tested by ELISPOT (Fig. 6A) and by intracellular FACS staining (Fig. 6B) using autologous DCs pulsed with PT-gliadin- or PT-digitonin-pulsed DCs.

To address the possible regulatory effect of NK cells on IFN-γ production by g-iTCLs, DCs were preincubated 6 h with autologous NK cells at g-iTCLs:APC:NK ratio of 1:2:1. A significant increased production was observed adding autologous NK cells in the PT-gliadin DC/g-iTCLs coculture, as scored using ELISPOT assays (Fig. 6A) and intracellular FACS staining (Fig. 6B).

The increasing of IFN-γ production was significantly (p = 0.0450) prevented when NK cells were pretreated with a high dose (10 µg/ml) of Golgi protein trafficking blocker (BFA) to prevent their cytokines secretion (Fig. 6A).

Intriguingly, PT-gliadin treatment of DCs did not influence the NK cell-dependent IFN-γ (Fig. 6, A and C) and TNF-α production (Fig. 6D) in the DC-NK cell coculture. In contrast, the addition of...
g-iTCLs to the NK cell/gliadin-treated DCs coculture prompted NK cells to an increased cytokine secretion (Fig. 6, C and D). In addition, the transfer of cytokine-enriched medium supernatant from coculture between NK cells and gliadin-pretreated DCs to the coculture between g-iTCLs and autologous gliadin-treated DCs did not enhance the IFN-γ/H9253 production by g-iTCLs (Fig. 6B). Such data suggested that the “cell-to-cell contact” and the reciprocal cytokine-generated signal by NK cells/g-iTCLs interplay are crucial for the increased IFN-γ response to gliadin.

As expected, in NK cell/DC/g-iTCLs coculture, we observed the presence of a higher number of the Ag-presenting, gliadin-treated DCs (ranging from 30 to 40% of surviving DCs) than medium- or PT-β-lactoglobulin DCs (ranging from 15 to 25% of surviving DCs) (data not shown). We do not rule out that the rescue of PT-gliadin-DCs from NK cell killing could provide cell-to-cell contact and/or other soluble stimuli-inducing T and NK cells to cytokine secretion.

Mononuclear cells expressing high levels of HLA-E and of CD3⁺/CD94/NKG2A⁺ lymphocytes are present in the lamina propria from celiac patient. The number of mononuclear cells/mm² expressing HLA-E was significantly higher (p < 0.01) in the lamina propria biopsy from untreated celiac patients (Fig. 7B) than from control (Fig. 7A) (mean ± SD: 153 ± 43 and 64 ± 39, respectively). No significant differences were found in biopsy specimens from treated CD patients (90 ± 53) when compared with healthy donors.

Immunohistochemical analysis demonstrated a very high HLA-E expression on enterocytes and monocytes in lamina propria of active celiac patient (Fig. 7C). In addition, overnight stimulation with gliadin of duodenal mucosa organ cultures highly up-regulated HLA-E expression in lamina propria mononuclear cells from a gluten-free diet patient (Fig. 7E); no HLA-E was detected after an overnight incubation with complete medium (Fig. 7D).

**FIGURE 7.** Lamina propria from celiac patient shows the presence of mononuclear cells expressing high levels of HLA-E and of CD3⁺/CD94/NKG2A⁺ lymphocytes. Mononuclear cells expressing HLA-E of both duodenal mucosa sections from healthy donor (A) and from celiac patient (B). Immunohistochemical analysis of HLA-E expression on enterocytes and in lamina propria of an active celiac patient (C) and a gluten-free diet patient stimulated overnight with medium (D) or gliadin (E). Magnification, ×10. One representative experiment of three is shown. Immunofluorescence staining revealed similar levels of HLA-A, -B, and -C Ag expression in the basolateral membrane of epithelial and lamina propria mononuclear cells of duodenal mucosa of both CD patients (F) and normal control (G). In green the HLA-A, -B, and -C molecule staining by using G46-26 mAb, and in red the nuclei counterstaining by using ToPro-3 tracker (see Materials and Methods). One representative experiment of two is shown. H, CD3⁺/CD94⁺ NK cells (red stained), CD3⁺/CD94⁻ T lymphocytes (green stained), and CD3⁺/CD94⁻ T cells (yellow stained) was detected by confocal microscopy of jejunal mucosa from an untreated celiac patient. One representative experiment of four is shown.

**FIGURE 8.** Celiac patient intestinal DC phenotype. Mononuclear cells from digestion of celiac patient duodenal mucosa explants were stained by FACS analysis with anti-CD45 and anti-CD1a mAbs. A live gate (R1 in A) was set to collect only CD45⁺/CD1a⁺ mucosal explanted cells for the subsequent analysis of anti-HLA-E, -CD1c, -CD11c, -CD14, -BDCA3, and color isotype control mAb staining (B). One representative experiment of two is reported.
Because the induction of HLA-E could be dependent on the availability of leader sequences form HLA class I (45), we evaluated the expression of classical HLA-A, -B, and -C molecules in both untreated CD and normal duodenal section. Staining for HLA-A, -B, and -C molecules was intense on epithelial cells and in particular on basolateral membranes, as well as in lamina propria mononuclear cells (Fig. 7, F and G). Notably, no intensity difference in the jejunal mucosa was observed between untreated celiac patient (Fig. 7F) and control donor (Fig. 7G). This result is in agreement with previous data (57) and suggests a direct specific role for gliadin in the induction of high HLA-E expression level.

Confocal microscopy of jejunal mucosa from an untreated celiac patient (Fig. 7H) shows the simultaneous presence in the lamina propria of CD94/NKG2A^+CD3^- NK cells, CD94/NKG2A^+CD3" T lymphocytes, and CD94/NKG2A^+CD3" T cells. This result point to a potential role in vivo for CD94/NKG2A NK cells in the peripheral tissue control of surveillance regulation.

To evaluate the presence in the celiac gut of cells ascribing to DC phenotype and expressing HLA-E molecules, we stained for CD1a, CD1c, CD11c, CD14, BDCA3, and HLA-E molecules the mononuclear cells obtained from digestion of mucosal explants of untreated celiac patient (Fig. 8). We found the 3–4% of all mucosal explants mononuclear cells were CD45 CD1a double positive (Fig. 8A). In addition, CD45^+CD1a^- cells were also positive for HLA-E, CD1c, and CD11c and negative for CD14 and BDCA3 (Fig. 8B). To this regard, it is relevant that vast majority of ascribed duodenal mucosa myeloid DCs has been recently described as positive for CD11c, a typical myeloid DC marker (58), and negative for BDCA3, a marker differentially expressed on DC sub-sets (53, 59). These results could suggest the expression of HLA-E molecule on myeloid DC resident in intestinal mucosa of untreated CD patient.

**Discussion**

Our data reveal gliadin peculiar biological features in interfering within the NK cell-DC cross-talk. We addressed the potential role for NK cell-DC cross-talk in the pathogenesis of CD, and we hypothesized that the CD94/NKG2A^- NK cell-dependent killing of iDCs could be part of the peripheral tolerance mechanisms preventing the immune system priming by not fully mature DCs (33).

In this regard, it is relevant that CD94/NKG2A^- NK cell subset could regulate the maturation of new DC precursors from the inflamed tissue to the lymphoid secondary organs (18), and it could prevent the aberrant priming activity of iDCs in the lymph nodes (35, 49, 50).

We suggest that gliadin could have a detrimental role in the regulation of NK cell-iDC cross-talk with consequences on the adaptive immunity activation. iDC gliadin treatment inhibits NK cell cytotoxicity against iDCs via the interaction between CD49/ NKG2A and HLA-E. Indeed, gliadin increases the HLA-E expression on iDCs and the blocking of CD94/NKG2A receptor on NK cells restores their killing against gliadin-treated iDCs.

Autologous interaction between gliadin-presenting iDCs, NK, and celiac patient-derived, T-restricted lines potently increases the T-dependent IFN-γ response to gliadin. This effect appears to be dependent on cytokine production by NK cells since a secretion-inhibiting drug (BFA) pretreatment of NK cells strongly reduces the IFN-γ CD4^+ T response to gliadin. It is worth noting that the direct interplay between NK and T cells appears to generate a cytokine environment able to induce reciprocal activation. Indeed, the addition of both T and NK cells in the coculture with PT-gliadin-pretreated DCs strongly enhanced T and NK cell-dependent cytokine secretion, whereas IFN-γ and TNF-α by NK cells was unaltered in the interaction with PT-gliadin-DCs alone. In addition, the presence of NK cells strongly increased the anti-gliadin-specific IFN-γ T cell response primed by gliadin-presenting DCs. We observed that the NK cell/g-iTCLs interplay could increase cytokines production by NK cells and could contribute to foster IFN-γ production by g-iTCLs in triple interaction between NK cell/iDCs/g-iTCLs. We do not exclude that other cytokines could contribute to this effect. In this context, it remains to be solved the nature of all NK cell and T cytokine secretion patterns when cocultured with gliadin-treated iDCs. Because the 20–30% of our NK cell preparations are CD94/NKG2A negative, we do not rule out that other NK cell subsets, expressing the activating receptors, could establish different relationship with gliadin-presenting DCs and therefore contribute in a different way to celiac pathogenesis.

Moreover, we demonstrated the presence of HLA-E-expressing mononuclear cells and CD3^-CD94/NKG2A^- NK lymphocytes in lamina propria from celiac patient. In addition, we found a coexpression of CD11c, a marker recently used to identify intestinal myeloid DCs (53) and HLA-E in CD45^-CD1a^-CD14^- BDCA3^-BDCA2^- mononuclear cells from gut mucosa of CD patients. Such evidence points to the in vivo role for HLA-E-mediated myeloid iDC-NK cell interaction in celiac disease, as previously described for interaction between NK cell like-CTL and HLA-E-expressing enterocytes (60).

Given the multichain composition of the gliadin, we analyzed also which fraction was responsible for NK cell protection and which for the HLA-E up-regulation. Both biological activities were found to cluster in α2 and ω fractions. Gene Jockey software reveals a nonamers potentially able to bind HLA-E. We focused on SQQPYLQLQ peptide derived from α2 chain. Even if this peptide increased the HLA-E molecule levels, the presence of a tyrosine makes it potentially susceptible to pancreatic enzymatic degradation. In this regard, the strong ability of gliadin-pancreatic digests to induce HLA-E expression on RMA-S suggests that pancreatic enzyme digestion could generate a large unknown and heterogeneous peptide repertoire binding to HLA-E molecule.

It is conceivable to speculate that gliadin could switch NK cell-iDC cross-talk from a tolerogenic pathway, based on iDC elimination, to a pathogenic one in which gliadin drives iDC-NK cell interaction to restricted CD4^-T cells activation; either by proinflammatory cytokines production or by gliadin-presenting iDC maturation. In this respect, other studies suggested that NK cell-iDC interaction provide a regulatory feedback of immune response during infectious and neoplastic diseases (51, 61) and could have a role in the disease pathogenesis (62). In addition, gliadin up-regulates IL-15 production (63); its 31–43 peptide stimulates IL-15 production from macrophage and DCs (11); and IL-15 induces MICA expression on epithelial cells and NK cell activation (10, 64).

Moreover, we demonstrated that gliadin could increase the expression of HLA-E on iDCs, while it does not affect the DC maturation degree.

Gliadin could shape the innate response toward a proinflammatory condition and together with other genetic features trigger the CD pathogenesis.

Preincubation of NK cells with anti-CD94/NKG2A mAb restores their killing against autologous celiac PT-gliadin-treated DCs, indicating that the gliadin processing could produce peptides increasing HLA-E expression on the APC. We speculate that gliadin processing could occur in a DC compartment where MHC-I and MHC class II are converging (65).

Immunohistochemical study in the celiac patient mucosa confirms in vitro data and suggests that HLA-E is up-regulated on APCs during the active stage of the disease and after overnight.
intestinal mucosa incubation with gliadin. Moreover, an extended immunohistochemical analysis of the CD patient showed that EC express high HLA-E levels after gliadin uptake (data not shown). It is conceivable that NK cell–gliadin presenting DC interactions can be considered as pathogenetic cofactor operating in vivo together with other immunological mechanisms, all contributing in giving the final pathological treat.

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Disclosures

The authors have no financial conflict of interest.

References


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