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Deamidation and Enzymatic Hydrolysis of Gliadins Alter Their Processing by Dendritic Cells in Vitro

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Supporting Information

ABSTRACT: Gliadins are major wheat allergens. Their treatment by acid or enzymatic hydrolysis has been shown to modify their allergenic potential. As the interaction of food proteins with dendritic cells (DCs) is a key event in allergic sensitization, we wished to investigate whether deamidation and enzymatic hydrolysis influence gliadin processing by DC and to examine the capacity of gliadins to activate DCs. We compared the uptake and degradation of native and modified gliadins by DCs using mouse bone marrow-derived DCs. We also analyzed the effects of these interactions on the phenotypes of DCs and T helper (Th) lymphocytes. Modifying gliadins induced a change in physicochemical properties (molecular weight, hydrophobicity, and sequence) and also in the peptide size. These alterations in turn led to increased uptake and intracellular degradation of the proteins by DCs. Native gliadins (NGs) (100 μg/mL), but not modified gliadins, increased the frequency of DC expressing CD80 (15.41 ± 2.36% vs 6.81 ± 1.10%, $p < 0.001$), CCR7 (28.53 ± 8.17% vs 17.88 ± 2.53%, $p < 0.001$), CXCR4 (70.14 ± 4.63% vs 42.82 ± 1.96%, $p < 0.001$), and CCR7-dependent migration (2.46 ± 1.45 vs 1.00 ± 0.22, $p < 0.01$) compared with NGs. This was accompanied by Th lymphocyte activation (30.37 ± 3.87% vs 21.53 ± 3.14%, $p < 0.1$) and proliferation (16.39 ± 3.97% vs 9.31 ± 2.80%, $p > 0.1$). Moreover, hydrolysis decreases the peptide size and induces an increase in gliadin uptake and degradation. Deamidation and extensive enzymatic hydrolysis of gliadins modify their interaction with DCs, leading to alteration of their immunostimulatory capacity. These findings demonstrate the strong relationship between the biochemical characteristics of proteins and immune cell interactions.

KEYWORDS: *allergy, deamidation, dendritic cells, gliadins, hydrolysis*

INTRODUCTION

Dendritic cells (DCs) play a crucial role in initiating and orienting immune responses to food antigens because of their capacity to sense stimuli and to control the differentiation and proliferation of T helper (Th) lymphocytes. Food proteins are taken up by DCs, and endolysosomal processing of these proteins induces cell maturation.[1] During maturation, a decrease in endocytic ability and an increase in CCR7-dependent migration to mesenteric lymph nodes have been observed, together with an increase in the presentation capacity characterized by the expression of MHC class II molecules and costimulatory molecules CD80 and CD86.[2,3] Once DCs reach the lymph nodes, presentation of the allergen to naïve (unpolarized and unactivated) Th lymphocytes triggers their differentiation into various Th cells [Th1, Th2, Th17, and regulatory T cells (Tregs)] according to micro-environmental and genetic factors. Dietary proteins usually induce tolerance, characterized by T cell energy and differentiation into Treg cells.[4] However, the failure to establish oral tolerance may result in diseases by a Th1 response, such as celiac disease, or by a Th2 response, such as IgE-mediated food allergy. In the latter case, sensitization results, among other things, from naïve lymphocyte (Th0) differentiation into Th2, an IL-4-producer subpopulation.[5]

Wheat proteins, including gluten proteins such as gliadins, are well known and highly consumed food allergens. Food allergy to wheat affects around 0.11% of the European population on the basis of food challenges.[6] Wheat may also be responsible for other pathologies such as celiac disease, which afflicts around 1% of the general population.[7] To increase their solubility and application, wheat proteins are modified by industrial processes such as deamidation and enzymatic hydrolysis.[8] We and others have previously demonstrated that these changes to the physicochemical properties of wheat proteins affect their allergenicity.[9−11]

Enzymatic hydrolysis reduces the in vitro IgE binding of gliadins.[11] In mouse models, Alcalase hydrolysis of gliadins significantly reduced their sensitizing potential.[10,12] In contrast, acid hydrolysis induces deamidation, which results in conversion of some of the numerous glutamine residues found in gluten to glutamic acid residues. This modification creates new/neo-epitopes associated with severe allergic reactions to wheat-containing products.[9,13] In mice models of allergies, deamidation was shown to increase the sensitizing potential of gliadins.[10,14,15] This increase was associated with a change in the frequency of several allergy effectors, such as DCs, in mesenteric lymph nodes.[10]

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In an adverse outcome pathway proposed for allergic sensitization to food proteins, activation of DCs and their migration to mesenteric lymph nodes followed by Th0 lymphocyte priming were considered key events.[16] The potential of food proteins to activate DCs could be assessed by in vitro assays. Several in vitro studies have pointed out that certain modifications to allergens can alter their uptake or degradation by DCs, leading to a change in Th lymphocyte response.[17,18] However, no general biochemical characteristics of food proteins have been linked to a specific orientation of the immune response.

The potential of wheat proteins to activate DCs has mostly been studied in the context of celiac disease using digests of these proteins and cells from healthy human or mouse donors.[19−23] Neither the influence of undigested gliadins on DC properties nor the comparison of native and functionalized gliadins has been investigated. To address these questions, we modified gliadins by acid or enzymatic hydrolysis and compared native and modified samples for their biochemical characteristics, their processing by bone marrow-derived DCs (BMDCs), and their ability to influence the phenotype and functionality of DCs. We also examined whether phenotype modulation of DCs affected Th cell stimulation.

EXPERIMENTAL SECTION

Chemicals and Reagents. The following reagents were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA): Alcalase, fluorescein isothiocyanate (FITC), DNase I, dithiothreitol (DTT), 2-mercaptoethanol, acetic acid, dichloromethane, trifluoroacetyl (TFA), acetonitrile, Triton X100, Tris, and NaCl. Ethanol and HCl were purchased from Carlo Erba (Milano, Italy) and NaOH from VWR (Radnor, Pennsylvania, USA). Reagents used for native-polyacrylamide gel electrophoresis (PAGE) analysis were purchased from Bio-Rad (Hercules, California, USA). Cell culture reagents were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA) unless otherwise specified. Cytokines (GM-CSF and CCL19) were from Miltenyi (Bergisch Gladbach, Germany). All the reagents used for cell staining and for the preparation of cells were purchased from BD Biosciences (San Jose, California, USA).

Gliadin Preparation. Gluten was extracted from wheat (*Triticum aestivum*) flour (Recital cultivar) by a Martin-type process in a preparative scale, washed in water, and freeze-dried.[24] It was ground and defatted with dichloromethane for 2 h at room temperature (RT) (gluten/solvent ratio: 1/4). After filtration, two rinsing steps with the same gluten/solvent ratio were performed. The solvent was evaporated under vacuum, and defatted gluten was freeze-dried. Native gliadins (NGs) were extracted from dried gluten powder with ethanol 70% (v/v) (gluten/solvent ratio: 1/6) for 3 h.[25] The suspension was centrifuged at 10 000*g* for 30 min (Avanti J-26 XP, Beckman Coulter), and the supernatant (containing NGs) was extensively dialyzed and freeze-dried. Deamidation was carried out by heating a 10 mg/mL solution of NGs in 0.1 N hydrochloric acid at 90 °C for 1 h deamidated gliadins (1HDG) or 2 h deamidated gliadins (2HDG) and stopped by neutralizing pH with 0.1 N NaOH. The solutions were then dialyzed against Milli-Q water for 5 days. NGs were hydrolyzed gliadins (HGs) using Alcalase (110 U/g of NGs; Sigma-Aldrich, Saint Louis, USA) at 55 °C for 2 h. The reaction was stopped by 10 min of heating at 85 °C followed by cooling on ice. All the solutions were freeze-dried. The protein contents of the samples were measured according to the Dumas method with a corrective factor of 5.7.[26] Stock solutions for cell culture were prepared as follows: HGs were solubilized at 5 mg/mL in endotoxin-free water, while NGs, 1HDG, and 2HDG were solubilized at 50 mg/mL in endotoxin-free water with 50% ethanol and further diluted to 5 mg/mL in endotoxin-free water. Endotoxin contents were quantified using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, USA). Endotoxin contents were below 0.1 EU/mL (quantification limit) for all samples.

Gliadin Characterization. The distribution of the sample molecular weight (MW) was studied by size exclusion high-performance liquid chromatography (SE-HPLC) using a procedure adapted from Morel et al.[27] Briefly, all the gliadins were dispersed at 1 mg/mL in 1% sodium dodecyl sulfate (SDS) and 0.1 M sodium phosphate buffer (pH 6.9) and incubated at 60 °C for 80 min under rotary shaking. The shaking was continued overnight at RT. Samples were injected onto a Superose 6 10/300 prep grade column (GE Healthcare, Chicago, USA), and the absorbance was recorded at 215 nm. The degree of hydrolysis of our samples was estimated using the *ortho*-phthalaldehyde method according to Frister et al.[28] The degree of hydrophobicity was measured as described.[29,30] Modifications in sample charges were characterized by native-PAGE analysis.[8] Gliadins were solubilized in SDS-free Laemmli buffer and loaded onto an acrylamide gel (15%) and stained with Coomassie blue. Reverse Phase High Performance Liquid chromatography (RP-HPLC) was used to evaluate the hydrophobicity of the samples. Gliadins were dispersed at 1 mg/mL in 0.11% TFA + 5% acetonitrile and shaken for 1 h at RT. Samples were applied on a C18 300 Å 5 μm column (MACHEREY-NAGEL, Düren, Germany). A gradient elution of 0.08% TFA and 85% acetonitrile was employed, and the absorbance was recorded at 220 nm. The extent of gliadin sequence modification was studied by inhibition of ELISA using antibodies directed to repeat sequences of either native (Ab INRA-PQQ3B4) or deamidated (mAb INRA-DG1) gliadins as previously described.[31] Inhibition of antibody binding was expressed as the percentage of the maximal response obtained with the antibody in the absence of the competitor according to the following formula: % inhibition = 1 − (sample OD/blank sample OD) × 100.

Animals. Three to eight week old female BALB/cJRj mice (Janvier Labs, Le Genest-Saint-Isle, France) and five week old C3H/HeOuJ mice (Charles River, Wilmington, USA) were housed in a ventilated cage system and in specific pathogen-free conditions, respectively. Experiments were approved by the ethics committee of the INRA.

Bone Marrow-Derived Dendritic Cells. The protocol was adapted from an existing protocol.[32] Cells were extracted from the femoral and tibial bone marrow of the mice, and red blood cells were lysed using RBC lysis buffer (eBioscience, Thermo Fisher Scientific). Nucleated cells were cultured at 37 °C with 5% CO_2 in BMDC medium: RPMI 1640 medium-GlutaMAX with 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Thermo Fisher Scientific), 10% heat-inactivated fetal bovine serum (FBS) (Eurobio Scientific, Les Ulis, France), and 100 μM 2-mercaptoethanol supplemented with 10−50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miltenyi, Bergisch Gladbach, Germany). Fresh medium was added on day 3. On day 6, cells were collected using cold medium [phosphate-buffered saline (PBS), 10% FBS, 0.7 mM EDTA] and used for the following experiments.

Gliadin Uptake. The protein uptake was studied as previously described.[18] Briefly, gliadins were solubilized at 25 mg/mL in 50% ethanol in Milli-Q water or in Milli-Q water (HGs), diluted to 1 mg/mL in carbonate-bicarbonate buffer (pH 9.9), FITC-labeled, and purified on PD-10 Desalting Columns (GE Healthcare). The efficacy of the labeling was measured to confirm that the same ratio of moles of FITC/mole of gliadins was obtained for all the samples. BMDCs (1×10^6/mL) were incubated at 37 °C with 10 μg/mL of FITC-gliadins, and aliquots were collected at different time points. The percentage of FITC-positive cells was analyzed using a BD Accuri C6 cytometer (BD Biosciences, San Jose, USA).

Gliadin Endolysosomal Degradation. NGs, 1HDG, and 2HDG were solubilized in 0.01 M acetic acid, while HGs were solubilized in 0.1 M carbonate-bicarbonate buffer (pH 10.08). All gliadins were washed and coupled to amino-activated Polybead microspheres (3.0 μm; Polysciences, Warrington, USA). Coupling efficacy was verified to ensure that the gliadins were well coupled to the beads. The endolysosomal degradation assay was performed using BMDCs as previously described.[18,33] Briefly, BMDCs were incubated with

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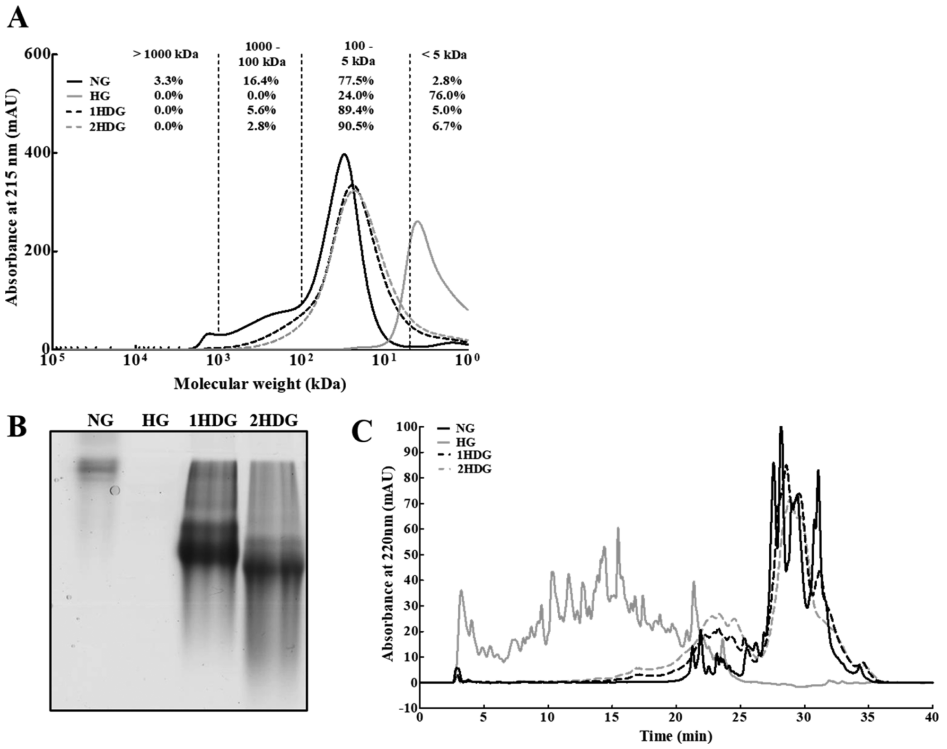

Figure 1. MW and hydrophobicity of proteins were decreased more by enzymatic hydrolysis than by deamidation. MWs were measured by (A) SE-HPLC. Charge and hydrophobicity modifications were measured by (B) native-PAGE migration and by (C) Retention time High performance Liquid Chromatography (RE-HPLC), respectively. NGs (black curve); HGs (gray curve); 1HDG (black dashed curve); and 2HDG (gray dashed curve).

gliadin-coated beads for 15 min. The uptake was stopped by adding ice-cold PBS, and noninternalized beads were removed using FBS flotation. Cells were resuspended in BMDC medium and incubated at 37 °C, and aliquots were collected at 0, 30, 120, and 240 min. The BMDCs were then lysed using a buffer composed of 50 mM Tris, 150 mM NaCl, 0.5% Triton X100, 1 mM DTT, and 10 μg/mL DNase I (pH 7.4) with complete protease inhibitor cocktail (Roche, Lewes, UK). Nondegraded gliadins coupled to beads were detected using INRA-DG1 (deamidated gliadins) or INRA-S1EH10B7 mAbs (NGs) and a secondary antibody coupled to Alexa Fluor 488 (Invitrogen, Carlsbad, USA). The percentage of degradation was calculated according to the following formula: [1 − (median fluorescence intensity (MFI) Alexa Fluor 488 of the beads in the sample/MFI Alexa Fluor 488 of the beads in the sample at t0)] × 100. We were unable to study HG degradation, as the hydrolysis cleaved nearly all the epitopes and we had no antibodies to detect them.

Flow Cytometry Analysis of BMDCs. BMDCs (1×10^6 per well) were cultured on six-well plates (Corning, Corning, USA) with 100 μg/mL gliadins, 1 μg/mL Lipopolysaccharide (LPS) (positive control, InvivoGen, San Diego, USA) or medium only (CTL) for 48 h. Cells were harvested and stained with antibodies directed to surface markers (CXCR4-PE, CD103-PerCp-Cy5.5, CCR7-PE-Cy7, CD80-APC, CD86-APC-Cy7, MHCII-BV421, and CD11c-BV510; BD Biosciences) in the presence of mouse BD Fc Block (BD Biosciences). Cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences). Data were acquired using Diva 8.0 software and analyzed with FlowJo X (Tree Star, Ashland, USA).

DC Migration in Transwell Assays. BMDCs (1×10^6/mL) were cultured with or without 100 μg/mL gliadins, 1 μg/mL LPS, or medium only (CTL). After 48 h, they were collected and loaded (5×10^5 cells) in the upper chamber of a transwell apparatus (5 μm pore size, Corning) in serum-free RPMI 1640. The lower chambers were filled with serum-free RPMI with or without CCL19 (200 ng/mL; Miltenyi). After 2 h at 37 °C, migrated cells were harvested from the lower chambers and counted by flow cytometry using Precision Count Beads (BioLegend, San Diego, USA). In each case, the number of migrated BMDCs was corrected by subtracting the number of migrated BMDCs in the absence of CCL19. The migration index was used to quantify cell migration and was calculated by dividing the number of migrated BMDCs by the number of untreated migrated BMDCs.

BMDC-T CD4$^+$ Lymphocyte Coculture. BMDCs (1×10^6/mL) were primed with or without 100 μg/mL gliadins, 1 μg/mL LPS (positive control, InvivoGen), or medium only (CTL). The next day, they were collected and transferred to 96-well plates. T CD4$^+$ (Th) lymphocytes were isolated from naïve BALB/cJRj mice, labeled with carboxyfluorescein succinimidyl ester (BD Biosciences) in order to study their proliferation, and added to the 96-well plates containing BMDCs (BMDC/Th lymphocyte ratio: 1:2) for 5 days. The protocol was adapted from an existing protocol.[34] Cells were restimulated 5 h before the end of incubation with the respective gliadins used for priming (100 μg/mL) and Brefeldin A and Monensin (BD Biosciences). Cells were harvested and stained with antibodies directed to surface markers (CD3-APC-Cy7, CD44-BV421, and CD4-BV510; BD Biosciences) in the presence of mouse BD Fc Block (BD Biosciences). Cells were fixed and permeabilized by BD Cytofix/Cytoperm (BD Biosciences) and stained for intracellular markers (IL-17-PE, IL-4-PE-Cy7, and IFNγ-APC; BD Biosciences). Cells were analyzed as previously described.

Cytokine Release. Cytokine secretion (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, and TNF-α) was determined in the coculture supernatants by multiplex analysis using the Bio-Plex Pro cytokine assay (Bio-Rad). The concentration of CCL2 (MCP-1) and IL-12p40 in the BMDC supernatants was measured by ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Statistical Analysis. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA). Values were expressed as mean ± SD and compared using the Kruskal−Wallis test followed by Dunn's multiple comparison test or using two-way ANOVA with a Bonferroni multiple comparison test (curves). A p-value below 0.05 was considered significant.

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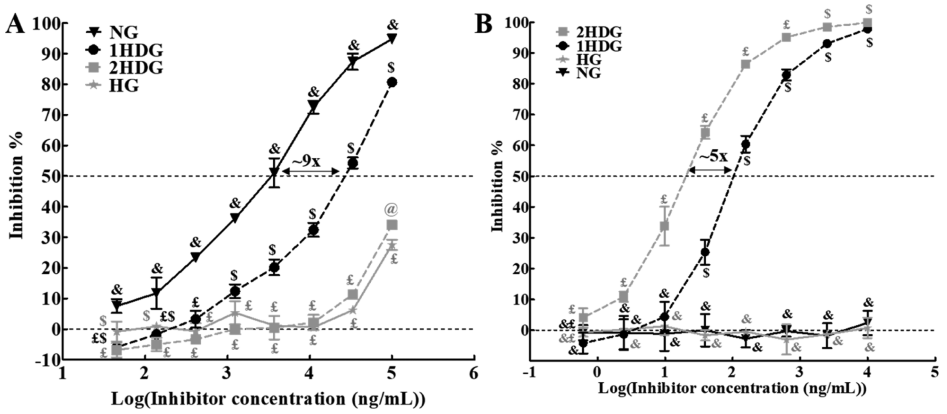

Figure 2. Gliadin deamidation induced loss of native epitopes and creation of deamidated epitopes. The composition of (A) native and (B) deamidated epitopes was measured by inhibition ELISA. NGs (black curve, inverted triangle); HGs (gray curve, star); 1HDG (black dashed curve, circle); and 2HDG (gray dashed curve, square). Values are mean ± SD, $n = 3$. Data with different symbols are significantly different [two-way ANOVA with the Bonferroni post test ($p < 0.05$)].

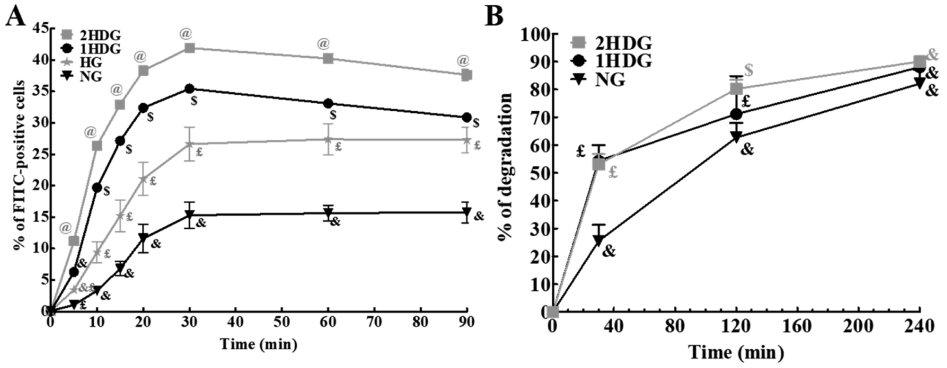

Figure 3. Modifications increase gliadin uptake and degradation rate by BMDCs. (A) FITC-gliadin uptake kinetics by BMDCs from C3H/HeOuJ mice. Data are expressed as % of FITC-positive cells ($n = 3$). (B) NGs, 1HDG, and 2HDG degradation kinetics by BMDCs from C3H/HeOuJ mice ($n = 7$). NGs (black curve, inverted triangle); HGs (gray curve, star); 1HDG (black curve, circle); and 2HDG (gray curve, square). Values are mean ± SD. Data with different symbols are significantly different [two-way ANOVA with the Bonferroni post test ($p < 0.05$)].

RESULTS

Acid and Enzymatic Hydrolysis-Reduced Gliadin MW, Hydrophobicity, and Typical Repeat Motifs. In order to evaluate the impact of our treatments, we characterized the biochemical properties of gliadins (Figure 1). We used SE-HPLC to analyze the MW distribution of the SDS-soluble fractions from the native and modified gliadins (Figure 1A). Most of the NG proteins had an MW between 100 and 5 kDa. NGs also contained fractions larger than 100 kDa or smaller than 5 kDa. Proteins in the range of 100−5 kDa and smaller than 5 kDa increased in 1HDG and 2HDG compared with NGs. Furthermore, the fraction between 100 and 1000 kDa decreased and that over 1000 kDa disappeared. Most of HG proteins had an MW lower than 5 kDa, and fractions larger than 100 kDa were not detected. Alcalase treatment induced a degree of hydrolysis of 12.4% (**data not shown**), while that of deamidated samples was only 0.1% (1HDG) and 0.3% (2HDG).

We also used migration in native-PAGE to characterize our samples (Figure 1B). We observed that NGs did not migrate in the absence of SDS, 2HDG migrated further than 1HDG, and HGs was not visible. Finally, we compared sample hydrophobicity using RP-HPLC (Figure 1C). The NG chromatogram presented minor peaks between 20 and 25 min and major peaks between 25 and 35 min. We observed a decrease in the elution of the constituent after 30 min and an increase under 30 min in 1HDG and 2HDG compared with NGs. Hydrophilicity increased in the order 2HDG > 1HDG > NG. HGs contained a high number of hydrophilic polypeptides eluted under 25 min.

To investigate the effect of treatment on the gliadin sequences, we analyzed the presence of native and deamidated repeat epitopes by inhibition ELISA (Figure 2). Among the modified gliadins, only 1HDG inhibited the binding of INRA-PQQ3B4 mAb (directed to native epitopes) to NGs coated on the plate. 2HDG and HGs displayed very low inhibition capacities, indicating a greater loss of native epitopes (Figure 2A). The amount of residual native epitopes was of the same order of magnitude, as their inhibition curves were equivalent. The highly deamidated epitope detected by mAb INRA-DG1 was nondetectable in NGs and HGs (Figure 2B). As expected, the deamidation process generated a high amount of this epitope. Moreover, the longer the deamidation process, the more the deamidated epitopes created.

Deamidation Increases Gliadin Uptake and Degradation by BMDCs. The physicochemical properties of proteins influence their interactions with DCs and, in turn, immune responses.[17,18,35] To determine the extent of this influence in the case of gliadins, we measured gliadin uptake and endolysosomal degradation in vitro (Figure 3). Modified and unmodified gliadins were taken up and reached a plateau

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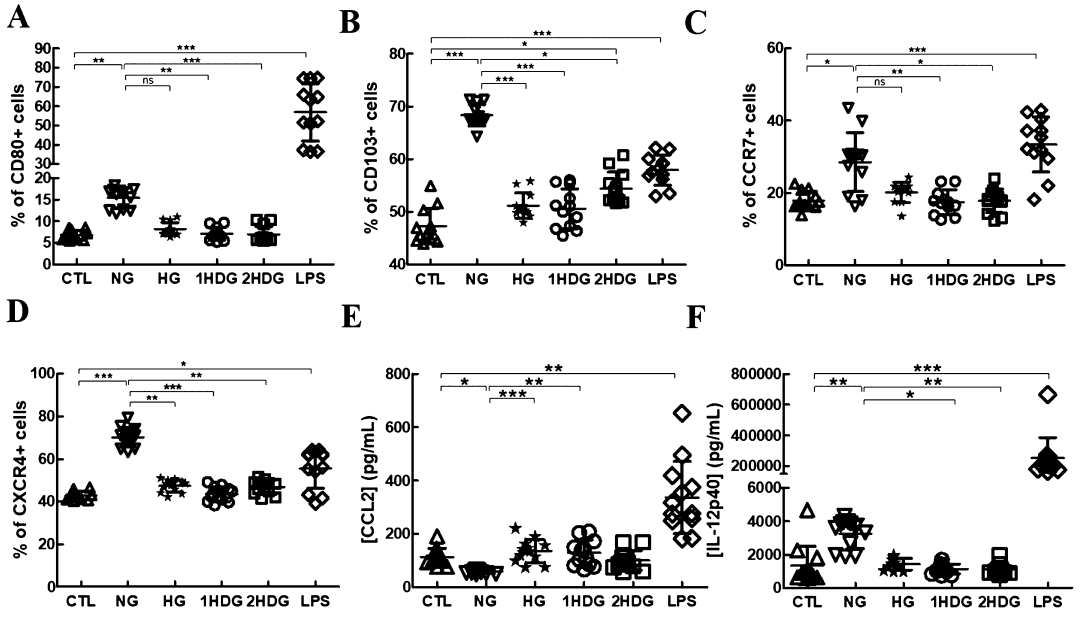

Figure 4. NG increase the % of DCs expressing CD80, CD103, CCR7, and CXCR4 and production of IL-12p40. After 48 h of exposure to gliadins (100 μg/mL) or LPS (1 μg/mL), the % of $CD80^+$ (A), $CD103^+$ (B), $CCR7^+$ (C), and $CXCR4^+$ (D) DCs ($CD11c^+$ $MHCII^+$) was monitored by flow cytometry and IL-12p40 (E) and CCL2 (F) secretion was monitored by ELISA. Values are mean ± SD. n = 12, Kruskal–Wallis test with Dunn's post test (*p < 0.05, **p < 0.01, ***p < 0.001).

at 30 min (Figure 3A). Surprisingly, modified gliadins were taken up more efficiently than NGs (p < 0.05). In fact, the NG uptake reached a maximum of 15.75 (±0.83)% of FITC-positive cells, while uptake of HGs, 1HDG, and 2HDG reached maxima of 27.51 (±1.29), 33.15 (±0.52), and 39.88 (±0.79)%, respectively. Endolysosomal degradation was measured with gliadins coupled to latex beads (Figure 3B). The degradation kinetics of deamidated gliadins were higher than those of NGs. Indeed, the percentage of degradation of deamidated gliadins was twice that of NGs at 30 min and still differed at 120 min. A difference in the percentage of degradation was observed between 2HDG and 1HDG (80.24 ± 3.21% vs 71.14 ± 13.72%; p < 0.05) at 120 min. At 240 min, the degradation of all the samples reached the same level.

NGs Increased the Frequency of BMDCs Expressing Activation and Homing Receptors as Well as Their Capacity to Migrate. We also investigated BMDC marker expression on gliadin exposure and especially activation markers (CD80 and CD86), homing markers (CCR7 and CXCR4), and the integrin CD103, which are essential in establishing tolerance or sensitization[2,3,36] (Figure 4). The number of $CD80^+$ cells increased after 48 h of exposure to NGs (15.41 ± 2.36% vs 6.81 ± 1.10%; p < 0.01) or to LPS (56.87 ± 14.86% vs 6.81 ± 1.10%; p < 0.001) (Figure 4A). In contrast, exposure to modified gliadins had no such effect. $CD86^+$ cells showed no change, regardless of the form of gliadins used, but were increased by LPS (**data not shown**). $CD103^+$ cells were increased by NGs (68.33 ± 2.03% vs 47.24 ± 3.36%; p < 0.001), LPS (57.93 ± 2.88% vs 47.24 ± 3.36%; p < 0.001), and to a lesser extent by 2HDG (54.46 ± 3.07% vs 47.24 ± 3.36%; p < 0.05), while they were unaffected by the other forms of gliadins (Figure 4B). NGs and LPS also induced an increase of the frequency of $CCR7^+$ cells (28.53 ± 8.17% vs 17.88 ± 2.53%; p < 0.05 and 33.36 ± 7.61% vs 17.88 ± 2.53%; p < 0.001, respectively) (Figure 4C). In addition, NGs increased the $CXCR4^+$ cells, while their augmentation was less pronounced with LPS (70.14 ± 4.63% vs 42.82 ± 1.96%; p < 0.001 and 55.51 ± 9.23% vs 42.82 ± 1.96%; p < 0.05, respectively) (Figure 4D).

In order to better characterize our cells, we measured BMDC cytokine secretion by ELISA. The secretion of IL-12p40, a pro-inflammatory cytokine, increased when BMDCs were stimulated by NGs (3250 ± 893.80 pg/mL vs 1337 ± 1163 pg/mL; p < 0.01) and with LPS (253 097 ± 134 087 pg/mL vs 1337 ± 1163 pg/mL; p < 0.001) (Figure 4E). In contrast, CCL2 secretion decreased under NG-stimulated BMDC conditions (58.74 ± 6.80 pg/mL vs 113.50 ± 32.35 pg/mL; p < 0.05) but increased in the presence of LPS (336.20 ± 135.90 pg/mL vs 113.50 ± 32.35 pg/mL; p < 0.01) (Figure 4F). Modified gliadins had no effect on the frequency of CCR7- and CXCR4-positive cells nor on cytokine secretion by BMDCs.

The migration of primed DCs to draining lymph nodes during an infection or in a steady-state environment is CCR7-dependent[2,3] and driven by the two CCR7 ligands, CCL19 and CCL21. To investigate the relation between CCR7 expression and the migration capacity of DCs, we used an in vitro transwell chemotactic assay. The results showed that NGs significantly increased (2.46 ± 1.45 vs 1.00 ± 0.22; p < 0.01) the number of migrating BMDCs, whereas modified gliadins and LPS did not (Figure 5).

BMDCs Exposed to NGs Promote Th Cell Proliferation and Activation. Allergic reaction is associated with a T cell response, and to this end, interaction between DCs and T cells is required. Because we showed that NGs induce BMDC activation, we aimed to analyze the consequences of this activation at the T cell level. To do so, we measured T $CD4^+$ (Th) lymphocyte proliferation and activation in a BMDC–Th lymphocyte coculture (Figure 6). Our results showed that BMDCs exposed to NGs increased T cell proliferation in the same way as LPS (16.39 ± 3.97% vs 9.31 ± 2.80%; p < 0.01 and 15.72 ± 5.12% vs 9.31 ± 2.80%; p < 0.05, respectively) (Figure 6A). In contrast, coculture with BMDCs primed with

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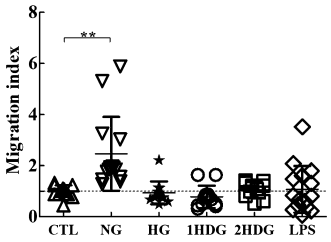

Figure 5. NGs promote BMDC migration. BMDC chemotactic migration to CCL19 (200 ng/mL) was measured after 48 h of exposure to gliadins (100 μg/mL) or LPS (1 μg/mL). Values are mean ± SD and are represented as a migration index. n = 9−12, Kruskal−Wallis test with Dunn's post test (**p < 0.01).

modified gliadins resulted in no modulation. The frequency of activated CD44$^+$ Th lymphocytes also increased when primed with NGs (30.37 ± 3.87% vs 21.53 ± 3.14%; p < 0.001) but not under the other experimental conditions (Figure 6B). The frequency of IL-4-producing Th lymphocytes decreased in all conditions primed with gliadins [3.50 ± 0.71% (HG), 4.17 ± 1.92% (NG), 3.79 ± 1.07% (1HDG), and 3.71 ± 0.84% (2HDG) vs 7.11 ± 1.49% (CTL); p < 0.05] but did not change in the presence of LPS (Figure 6C). The frequency of IL-17$^+$ Th cells also decreased slightly with modified gliadins [4.38 ± 0.77% (HG), 4.31 ± 0.64% (1HDG), and 4.36 ± 0.71% (2HDG) vs 6.71 ± 1.98% (CTL); p < 0.05] but did not change with NGs or LPS (Figure 6D). Neither modified or unmodified gliadins nor LPS had any effect on IFN-γ^+ Th cells (Figure 6E). No change in IL-4, IFN-γ, or IL-2 concentrations was observed in supernatants of cocultures primed with gliadins (Figure S1A−C, Supporting Information). Nevertheless, IFN-γ and IL-2 secretion increased in the supernatants of the coculture experiments stimulated by LPS (42.77 ± 33.53% vs 4.17 ± 2.87%; p < 0.001 and 77.38 ± 45.85% vs 22.87 ± 9.67%; p < 0.05, respectively) (Figure S1B,C, Supporting Information). Other cytokines measured (GM-CSF, IL-1β, IL-5, IL-10, and TNF-α) were not detectable (**data not shown**).

DISCUSSION

Despite the crucial role of DCs in oral tolerance and immune response orientation, little is known about how DCs sense and process food proteins or how these proteins enable DCs to activate specific Th cells. In addition, we lack data on the ways in which the biochemical characteristics of proteins affect these mechanisms. Some protein modifications, such as glycation, glycosylation, and aggregation, have been shown to alter their interactions with DCs, which could lead to modified immune responses.[16,18,35] Other protein treatments provide the food industry with valuable ingredients. Functionalization of gluten proteins by deamidation and enzymatic hydrolysis has been explored in several in vivo studies to assess and compare their sensitizing potential.[10,12,37] However, it has never been explored at the level of DC-initiating events. In the present study, we investigated the effect of gliadin functionalization by deamidation and Alcalase hydrolysis on gliadin processing by DCs and on the phenotype and immunostimulatory capacity of DCs in vitro.

In our study, functionalized gliadins displayed significantly different biochemical properties. Enzymatic hydrolysis by Alcalase resulted in strong proteolysis of gliadins and in a fraction mainly composed of constituents under 5 kDa. In contrast, the deamidation conditions used in this study caused limited variations in the gliadin size. However, all the functionalized gliadin samples contained a low residual number of typical gliadin repeat motifs. Li et al.[11] also found that treatment of gliadins by Alcalase reduced their MW and that those modifications were associated with reduced IgE binding in patients allergic to wheat. We thus conclude that the loss of native epitopes that we observed for our HG product is due to their cleavage by Alcalase, an enzyme with broad specificity, whereas deamidation changes the gliadin sequence and

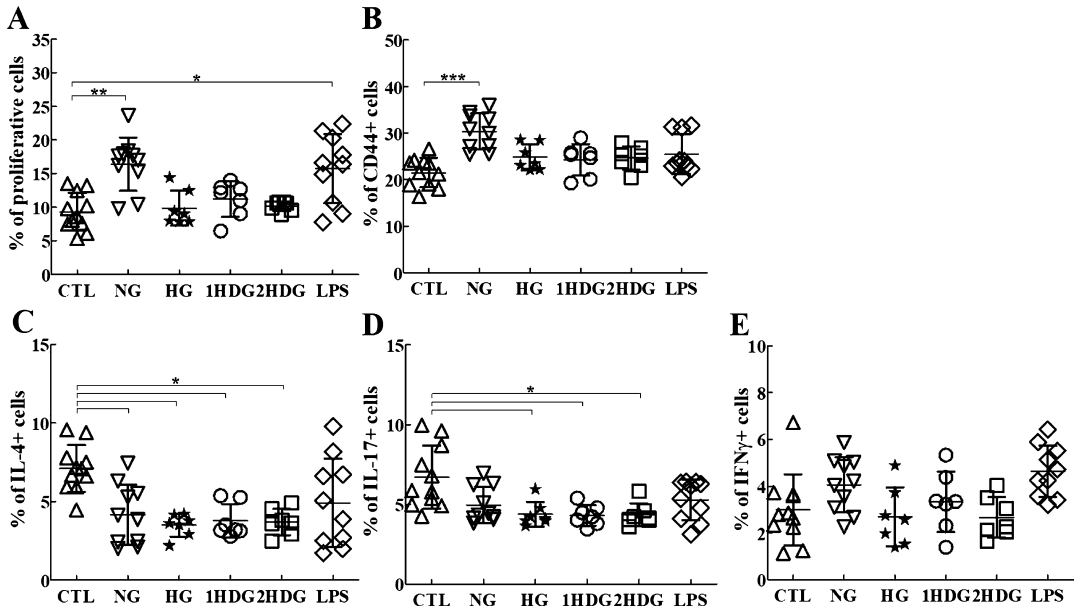

Figure 6. NGs enhance the capacity of BMDCs to activate and stimulate proliferation of Th lymphocytes. After 24 h of exposure to gliadins (100 μg/mL) or LPS (1 μg/mL), BMDCs were cocultured with Th lymphocytes (ratio BMDC/Th lymphocytes 1:2) for 5 days. % of proliferative cells (A), CD44$^+$ cells (B), IL-4$^+$ cells (C), IL-17$^+$ cells (D), and IFNγ^+ cells (E) among Th lymphocytes (CD3$^+$ CD4$^+$) was monitored by flow cytometry. Values are mean ± SD. n = 7−11, Kruskal−Wallis test with Dunn's post test (*p < 0.05, **p < 0.01, ***p < 0.001).

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transforms most repeat motifs into deamidated repeat motifs. Treatment also reduced gliadin hydrophobicity, although to different extents. Deamidation converted some of the glutamine residues found in gliadins to glutamic acid residues, leading to an increase in negative charges and decreased hydrophobicity. Alcalase hydrolysis led to an increase in charged N- and C-terminal functions and hence to greater hydrophilicity.

Biochemical properties of allergens such as protein size have been shown to influence their uptake.[17,18,35,38] For example, the cross-linking of β-lactoglobulin, a major milk allergen, led to an increase in its molecular size and slower uptake by BMDCs.[18,35] Along the same lines, in our work, NGs were taken up less efficiently by DCs than were the three modified gliadin samples. The hydrolyzed and deamidated samples either completely lack or have a much lower proportion of the high MW constituent (>100 kDa). These constituents represent about 20% of NG composition and might partly limit internalization. The poor solubility of NGs may also be partially responsible for this difference in uptake. Nevertheless, HGs, which are composed of peptides with high hydrophilicity, were taken up less efficiently than the deamidated samples. Consequently, other mechanisms, such as modification of the endocytic pathway, must be considered. Studies have shown that increased uptake of β-lactoglobulin and ovalbumin modified by the Maillard reaction in comparison with the native forms is accompanied by a shift in uptake of the glycated proteins to receptor-mediated endocytosis and particularly to scavenger receptors.[39,40] By modifying biochemical features (size, hydrophobicity, or sequence), our treatments could have created new gliadin properties, leading to modification of the endocytic pathways of these proteins. Moreover, the endolysosomal degradation of proteins following their uptake could also be influenced by their properties.[35] In our experiments, the degradation speed was higher for the deamidated forms. As deamidation leads not only to modification of gliadin sequences but also to conformational changes such as protein unfolding,[41] the protein backbone might be more reachable by lysosomal enzymes.

Hydrolysis and deamidation also alter the capacity of gliadins to activate BMDCs. Indeed, in our study, only NGs increased the frequency of cells expressing activation (CD80) and homing (CCR7 and CXCR4) markers. These two homing receptors have been shown to play an important role in migration of DCs to draining lymph nodes and, thus, in the initiation of immune responses.[42] NGs also increased the percentage of $CD103^+$ cells. In vivo, these cells represent most of the DCs migrating to mesenteric lymph nodes.[43] One of our deamidated samples (2HDG) also stimulated this cell phenotype, although to a lesser extent than NGs did. Moreover, because they stimulated CCR7 expression, NGs increased the number of cells migrating to CCL19, a CCR7 ligand. These results are consistent with those of Larsen et al., who showed that mice fed a gluten-containing diet contained more $CD40^+$, $MHCII^+$, and $CCR7^+$ cells among $CD11c^+$ cells in their lymph nodes compared with mice fed a gluten-free diet.[44] Similar results were found in vitro with digested gliadins and glutens (α-chymotrypsin, pepsin, or pepsin−trypsin digestion) on activation (CD80, CD86, CD40, and HLA-DR) and CCR7-dependent migration of murine and human DCs.[19−23] In line with BMDC stimulation, NGs were able to induce proliferation and activation of Th lymphocytes. Other studies have shown that increased DC activation by pepsin/trypsin-gliadins or gliadin peptic fragments is associated with increased proliferation of total or Th lymphocytes.[20,21] The pro-inflammatory potential of gliadins was difficult to evaluate because NGs, respectively, increased and decreased secretion of IL-12p40 and CCL2, two pro-inflammatory molecules. CCL2 may have an inhibitory effect on production of IL-12 by human monocytes in vitro.[45] Accordingly, decreased secretion of CCL2 by NG-stimulated BMDCs could have led to increased IL-12p40 secretion.

Studies looking into the interaction of gliadins and immune cells have used digested gliadins (α-chymotrypsin, pepsin, or pepsin−trypsin digestion). Therefore, when gliadins are ingested, most of them undergo gastro-intestinal digestion before encountering immune cells. Nevertheless, none of the gliadins underwent a complete gastro-intestinal digestion, but rather partial digestion. Moreover, gastric digestion is often overestimated. Thus, it is difficult to conclude on which type of gliadins is the best model to mimic at best the biological phenomenon. Nevertheless, to be immunologically active, a protein has to be insufficiently digested or undigested.[46] Furthermore, gliadins can induce immune reactions in the absence of digestion, for example, by dermal exposure.[15] For these reasons, we used undigested gliadins as allergens. However, the use of only undigested gliadins may represent a limitation of our model in order to measure the impact of gliadin modification on biological properties especially regarding the dose used and the ratio between undigested and digested gliadins.

Under our experimental conditions, NGs had a stimulatory effect on BMDCs, favoring their activation, their capacity to migrate, and their ability to induce proliferation of Th lymphocytes. Functionalization of gliadins both by extensive proteolysis and by deamidation almost completely suppressed these capacities. The absence of a stimulatory effect in Alcalase-HGs is consistent with studies of mouse models that show a significantly reduced gliadin sensitizing potential following this treatment.[10,12] In contrast, gluten subjected to limited enzymatic hydrolysis did not differ from unmodified gluten in its sensitizing ability in a rat model.[47] The similarity of DC stimulatory effects observed in vitro with undigested gliadins in our work and with digested gliadins in work by others may be explained by low hydrolysis degrees of gliadin digests.[48] Although we were unable to investigate HG degradation kinetics, we assume that they are faster than those of NGs because of the small size of their components and because the peptide backbone is within easy reach of lysosomal enzymes. This supposition could explain the absence of HG stimulatory effects observed in BMDCs and Th lymphocytes and the decreased sensitizing potential observed in mice.[12] It has been postulated that proteins that are more resistant to degradation could be more efficiently presented by DCs and promote greater proliferation of T lymphocytes.[49,50] The observed absence of the effect of deamidated gliadins on BMDCs and on Th lymphocyte activation and proliferation may also be explained by their faster uptake and degradation compared with NGs. Another investigation has shown that a Bet v 1 mutant that was less susceptible to endolysosomal degradation than wild-type protein proved to be a more potent immunogen and allergen in vivo.[51] Nonetheless, the weak or null capacity of deamidated gliadins to stimulate BMDCs and Th lymphocytes in vitro appears to contradict some in vivo studies. Deamidated gliadins have been shown to be potent allergens in mouse models, and deamidated glutens were

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responsible for severe allergic reactions. Exposure to new epitopes resulting from gluten deamidation and a cutaneous route of sensitization appear to promote the breakdown of the already established tolerance to gluten in humans and rats.[8,37]

In summary, our investigation shows that modification of gliadins by extensive proteolysis and by deamidation alters certain biochemical properties of the proteins and results in increased efficiency of their uptake and degradation by DCs. Functionalized gliadins exhibited almost no capacity to promote activation and migration of BMDCs or to increase their Th lymphocyte priming abilities, in contrast to native proteins. Differences observed in the assessed allergenic potential of deamidated gliadins in vivo and in vitro remind us that allergy is a complex disease orchestrated by many cells and that such a phenomenon cannot properly be reproduced in vitro without all the cellular actors involved. A multicellular tool such as organoids would enable an overview of the broad range of these effects. Such an overview would constitute a crucial step toward gaining greater understanding of the relationship between the biochemical characteristics of allergens and immune cell response to them.

After decades of gluten analysis by immune methods, there is no consensus reference material for gluten. The amount of cereal varieties, proteins, peptide sequences, hydrolytic and other industrial processes, and the diverse individual response to gluten peptides make the detailed analysis of the potential risk of allergy extremely complex. However, it is reasonable to propose that the concept should be linked to the immunogenic activity. In the past years, the advance in the scientific knowledge and pathology of allergy has allowed to identify gluten peptide that triggers the allergic reaction and impact of protein properties. There are immune in vivo methods with reported characterization of relation between allergen properties and allergic reaction. However, there is a lack of global methods demonstrating the effect of protein properties on allergenicity. Here, we demonstrate the impact of processing in vitro using DCs. Future research should focus on generalization of this kind of test using may be human cells. However, there is no enough research in this field to date. In addition, the cell models still have some contradictions, so it is necessary to further improve and develop the present and novel food allergy models.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b06075.

Native and modified gliadins do not alter secretion of cytokines by Th lymphocytes (PDF)

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Notes

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C.V., S.D.-P., and G.B. designed the study. J.J.S. and R.H.H.P. helped to design the study. C.V., O.T., and V.S.-J. carried out the characterization experiments. C.V. performed the in vitro cellular experiments and analyzed the data. C.V., G.B., and S.D.-P. wrote the manuscript. All authors read and approved the final manuscript. We thank Michel Lopez from Improve SAS, Fabrice Battais, all the members of the toxicology team of the INRS (Institut National de Recherche et de Sécurité) for their constructive input, and Florence Pineau for her technical support.

■ ABBREVIATIONS

DCs, dendritic cells; BMDCs, bone marrow-derived DCs; NGs, native gliadins; HGs, hydrolyzed gliadins; 1HDG, 1 h deamidated gliadins; 2HDG, 2 h deamidated gliadins; MW, molecular weight; MFI, median fluorescence intensity; Th, T helper; Treg, regulatory T cells; SE, size exclusion; RT, room temperature; FBS, fetal bovine serum; CFSE, carboxyfluorescein succinimidyl ester

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