Circulating dendritic cells from celiac disease patients display a gut-homing profile and are differentially modulated by different gliadin-derived peptides

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Keywords:	Antigen presentation, autoimmune disease, gliadin, monocyte, T cell

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TITLE: Circulating dendritic cells from celiac disease patients display a gut-homing profile and are differentially modulated by different gliadin-derived peptides

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ABBREVIATIONS: c, conventional; CCR, C-C motif chemokine receptor; CeD, celiac disease; cDC, conventional dendritic cell; cl, classical; CLA, cutaneous leukocyte-associated antigen

receptor; DC, dendritic cell; GFD, gluten-free diet; int., intermediate; MHC, major histocompatibility complex; Mo, monocyte; MoDC, monocyte-derived dendritic cell; p, peptide; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; TGF, transforming growth factor; Th, T helper cell; TNF, tumor necrosis factor.

KEYWORDS: Antigen presentation, autoimmune disease, gliadin, monocyte, T cell

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ABSTRACT

Scope

Circulating dendritic cell (DC) and monocyte subsets contribute to the pool of intestinal DC and macrophages in celiac disease (CeD), an autoimmune gut disorder triggered by dietary gluten. Here, we aimed to characterize these circulating subsets in CeD and assess the effect of different gliadin-derived peptides on conventional DC (cDC).

Methods and results

Flow cytometry profiling of peripheral blood mononuclear cells revealed a slight decrease in the proportion of plasmacytoid and type 1 cDC in gluten-free diet (GFD)-treated CeD patients. In comparison to healthy donors, DC and monocyte subsets from active and GFD-treated CeD patients displayed an increased gut-homing profile. Type 2 cDC (cDC2) were sorted and stimulated with the gliadin-derived peptides 8-mer, 19-mer and 33-mer. All peptides induced cDC2 maturation, although the profile was different. While peptide 8-mer induced a Th1/Th17 pro-inflammatory cytokine profile in active CeD patients, cDC2 primed with peptide 33-mer displayed a higher capacity to promote gut-homing CCR9⁺ expression onto autologous T-cells.

Conclusion

Distinct gliadin-derived peptides elicit different effects on cDC2 phenotype and function. This effect is compatible with a model where diverse gliadin peptides might cooperate to promote full cDC2 activation and the subsequent T-cell response in genetically predisposed individuals.

1. Introduction

Celiac disease (CeD) is an increasingly common food sensitivity and gastrointestinal autoimmune disorder of the small intestine, affecting approximately 1% of the population.^[1] It is triggered in genetically predisposed individuals, carrying the human leukocyte antigen (HLA)-DQ2 and/or HLA-DQ8 molecules, following gluten consumption.^[2] Currently, the only reliable treatment is a lifelong strict gluten-free diet (GFD) after which all clinical symptoms usually disappear.^[1a, 3]

Gluten is a complex mixture of seed storage proteins found in wheat, rye, barley and certain oat grains that are highly resistant to proteolytic processes occurring during digestion.^[4] Among these resistant fractions, α - and ω -gliadins from wheat's gluten contain the most immunodominant peptides for CeD patients. The most studied gliadin peptides typically include peptide (p)31-49 (PGQQQPFPPQQPYPQPQPF, also known as 19-mer), which generates an innate immune response in intestinal epithelial cells and intraepithelial lymphocytes; and p56-88 (LQLQPFPPQQLPYPQPQLPYPQPQLPYPQPQPF, also known as 33-mer), that mediates an adaptive immune response.^[5] However, the latest technical improvements have allowed the identification of new gliadin-derived peptides that can be recognized by T-cells.^[6] This is the case of a small eight residue peptide, 8-mer (FPLQPQQP), which our group recovered from a gluten degrading band found after a duodenal extract from a CeD patient was incubated with gliadin by zymography.^[7] However, the mechanism of action of this novel 8-mer peptide remains unknown.

Dendritic cells (DC), the most potent antigen presenting cells, determine the antigen-specific immune outcome, i.e. pro-inflammatory or tolerogenic response.^[8] In the intestinal mucosa, DC control immune tolerance towards nutrients and commensals by promoting regulatory T-cell differentiation and B-cell IgA secretion via α V β 8 integrin and retinaldehyde dehydrogenase type (RALDH) 2 enzyme, respectively.^[9] DC also imprint homing properties over the cells that they stimulate by inducing small bowel-homing C-C motif chemokine receptor (CCR) 9 and

gut-homing $\alpha 4\beta 7$ integrin expression,^[10] hence restricting immune responses to the gastrointestinal compartment.^[11] In addition, DC can trigger immune responses against invading pathogens in the gut.^[12] Consequently, changes in this delicate balance between immunity and tolerance are related to progression of autoimmune diseases, including CeD.^[2b, 12d, 13] Intestinal macrophages also maintain the mechanisms of immune homeostasis in health, although they can also exacerbate immune responses in the presence of inflammation.^[14] Their main role in the gut is the clearance of microbes that cross the epithelial barrier in a CX3CR1-dependent manner.^[15] Thus, intestinal macrophages are abundant in the epithelial layer of the mucosa and contribute to the homeostasis and replacement of epithelial cells. To do so, macrophages remodel the extracellular matrix by producing metalloproteinases and tumor necrosis factor (TNF)- α .^[16] They also contribute to recruit monocytes, maintain and expand Th17 cells, and co-stimulate antigen-specific T cells *in situ*.^[14d] The last members of the mononuclear phagocyte system are monocytes, considered the precursors of intestinal macrophages.^[13, 17]

Recently, Guilliams *et al.* proposed a homogeneous system to classify all mononuclear phagocyte cells. Human DC are divided into plasmacytoid DCs (pDCs: CD11c-CD123⁺), which produce large amounts of type I interferons; and myeloid, classical or conventional DC (cDC: CD11c⁺), which perform antigen presentation. cDC can be further divided into type 1 (cDC1: CD141⁺, C-X-C motif chemokine receptor (CXCR) 1⁺), which present exogenous antigens on MHC-I molecules; and type 2 (cDC2: CD1c⁺, signal regulatory protein (SIRP) α ⁺), which present antigens in the classical major histocompatibility complex (MHC)-II molecules.^[18] On the other side, macrophages are self-maintaining cells derived from embryonic progenitors and are classified according to their particular location or historical name.^[18a] However, under inflammatory conditions, mucosal macrophages are originated from circulating monocytes,^[19] which are divided into classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺), and non-classical (CD14⁻CD16⁺) monocytes.^[13, 17]

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3 105 The celiac mucosa is characterized by low levels of pDCs and regulatory CD103⁺ DCs,
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5 106 together with low expression of interferon (IFN)- α .^[20] On the contrary, active cDCs and
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7 107 macrophages accumulate in the inflamed tissue, which leads to the activation of gluten-specific
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9 108 T-cells.^[20b, 21] These changes, however, are not restricted to the intestinal mucosa as active
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11 109 CeD patients also display low numbers of circulating cDC.^[22] Nevertheless, not much effort
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13 110 has been focused on characterizing circulating cDC and monocyte/macrophage populations
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15 111 in depth in CeD. In addition, most studies have focused on the characterization of monocyte-
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17 112 derived DC (MoDC) to describe antigen presentation and immune responses in CeD.^[23]
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19 113 However, MoDC do not truly resemble the properties of human cDC2.^[24] Thus, the specific
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21 114 effect that different gliadin-derived peptides may elicit over *bona fide* cDC phenotype and
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23 115 function remains to be analyzed.
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27 116 In order to address these limitations, we provide a comprehensive characterization of human
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29 117 circulating DC and monocyte subsets in healthy controls and CeD subjects. Moreover, we also
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31 118 unravel the effects that different gliadin peptides (8-mer, 19-mer and 33-mer) induce on human
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33 119 cDC2 phenotype and function, describing how distinct peptides activate cDC2 in different
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2. Experimental section

2.1 Study population

Blood samples (50mL) from adult CeD patients and healthy volunteers were obtained in heparin-litium tubes from the Digestive Disease Unit at “Hospital Clínico Universitario” (Valladolid, Spain). CeD was diagnosed following the current diagnosis guidelines, including clinical history, genetics, serology, upper gastroscopy and small intestinal biopsy (Marsh-Oberhuber classification).^[25] In total, 5 patients with active CeD and 11 GFD-treated CeD patients were enrolled. Additionally, 9 healthy donors with no known autoimmune diseases, inflammation or autoimmune malignancies were recruited as controls. Detailed patient characteristics can be found in table 1. Written informed consent was obtained from all subjects following approval by the local ethics committee (FO-P-07-03). All subjects were of European ancestry.

2.2 Dendritic and T-cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using 1.077 g/mL Ficoll (BioChrom, Berlin, Germany), and further washed with phosphate-buffered solution (PBS) (Lonza, Verviers, Belgium) supplemented with 2% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). Five million PBMCs were kept aside for flow cytometry analysis of fresh circulating DC and monocyte subsets. cDC2 cells were magnetically isolated from total PBMCs with CD1c (BDCA-1)⁺ dendritic cell isolation kit for human samples (Miltenyi Biotech, Bergisch Gladbach, Germany), following the manufacturer’s instructions. Briefly, PBMCs were firstly incubated with Fc Receptor blocking reagent and anti-CD19 microbeads in order to deplete B-cells. In a second magnetic isolation round with anti-CD1c-biotin microbeads, cDC2 and the remaining non-labelled cells were collected in separate tubes. An average purity of 91.30 ± 8.98 % cDC2 was confirmed by flow cytometry (supplementary figure 1). cDC2 were used for *in vitro* stimulation with gliadin-derived peptides (see below), while the B-cell and cDC2 depleted cell fraction was used to isolate naïve T-cells (CD3⁺CD4⁺CD45RA⁺)

by cell sorting in a FACS Aria instrument (Becton Dickinson, Franklin Lakes, NJ, USA). Purity of sorted naïve T-cells was higher than 95% in all cases. Details of all the antibodies used can be found in Supplementary Table 1.

2.3 *In vitro* assays and dosage information

A total of 70,000 viable cDC2 cells were seeded in complete medium (RPMI 1640 medium (Lonza) supplemented with 10% FBS (Invitrogen), 1% L-glutamine (Sigma-Aldrich, San Luis, MO, USA) and 1% penicillin-streptomycin (Sigma-Aldrich)). Cell viability was assessed with TrypanBlue (ThermoFisher Scientific). The gliadin-derived peptides 8-mer, 19-mer, and 33-mer were added at a concentration of 100 μ M to stimulate cDC2 for 18h (37°C, 5% CO₂). All the peptides were LPS-free and were synthesized by Biomedal S.L. (Sevilla, Spain) at a purity level of 98%. Unstimulated cells were kept in culture in resting conditions as negative controls. The dose was selected on the basis of previous studies on *in vitro* challenged cell lines with gliadin-derived peptides 19-mer and 33-mer.^[26] Following culture, cDC2 were recovered with TrypLE Express (ThermoFisher Scientific, Waltham, MA, USA). Cell viability – assessed by flow cytometry – after culture was over 80% in all cases with no changes between different patient groups or culture conditions (supplementary figure 2).

A total of 10,000 stimulated, viable cDC2 were seeded together with sorted naïve T-cells in a 1:10 proportion for 5 days in the supplemented RPMI 1640 medium described above (37°C, 5% CO₂). Naïve T-cells had been previously labelled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), following the manufacturer's instructions. In all cases, cell viability was assessed on cells before culture using TrypanBlue.

2.4 Flow cytometry

PBMCs were used to characterize monocyte and DC subset homing profile (C-C chemokine receptor (CCR) 9; β 7 integrin, and cutaneous leukocyte-associated antigen receptor (CLA)). After *in vitro* stimulation with gliadin-derived peptides, cDC2 were also labelled with

LIVE/DEAD fixable yellow dead cell stain kit (Invitrogen, following the manufacturer's instructions), and characterized for the expression of maturation (CD40, CD80, CD83, CD86, HLA-DR) and migration (CCR9, β 7, CLA) markers. Isolated naïve T-cells co-cultured with cDC2 were labelled with LIVE/DEAD fixable yellow dead cell stain kit, and used to assess the expression of migration markers (CCR9, β 7, CLA) and cytokine production (IL-10, IFN- γ , IL-17A). Antibody details can be found in Supplementary Table 1. In all cases, cells were stained for 30 min in the dark at 4°C and washed with FACS buffer (phosphate saline buffer with 1% FBS). Intracellular cytokine staining was performed on T-cells after fixation with 2% paraformaldehyde (PFA, Sigma-Aldrich) in PBS, and using a freshly home-made 0.1% saponin (Sigma-Aldrich) solution in FACS buffer for 30 min in the dark at room temperature with frequent agitation. For T-cell analysis, fluorochromes used for naïve T-cell sorting were avoided. Cells were always fixed in 2% PFA as mentioned above following antibody staining. Fluorescence minus one (FMO) samples were used as internal controls to set positive and negative gating. All flow cytometry assays were run in a Gallios flow cytometer and analyzed with Kaluza v2.1.1 software (all from Beckman Coulter Inc., Brea, CA, USA) and Fowjo v10.6.1 (BD, Franklin Lakes, NJ, USA).

2.5 Expression analyses

Total RNA was extracted from peptide-stimulated cDC2 with RNeasy mini kit following manufacturer's instructions (QIAGEN, Hilden, Germany). RNA was quantified in NanoDrop ND-1000, and retrotranscribed with High Capacity cDNA Reverse Transcription Kit (all from ThermoFisher Scientific). Complementary DNA was amplified using iTaq Universal SYBR Green Supermix or iTaq Universal Probes Supermix (all from BioRad, Hercules, CA, USA). Specific primers were designed with Primer Blast from the National Center for Biotechnology Information, NCBI (Supplementary table 2).^[27] A customized Universal Probe Library (UPL) was obtained for *IL6* (Roche Pharmaceuticals, Basel, Switzerland). Quantitative analysis was carried out in a LightCycler480 (Roche Pharmaceuticals) using the relative quantification - $\Delta\Delta$ Ct method.^[28] Glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) was used as

199 housekeeping gene. Non-stimulated cells from each CeD patient or healthy volunteer were
200 used as a control, and average population-based values were used to obtain $-\Delta\Delta C_t$ values.

201 2.6 Statistical analysis

202 Flow cytometry data was analyzed with ANOVA's analysis of variance to contrast data between
203 different patient groups, and two-tailed paired Student's t-test to contrast data from different
204 conditions within the same patient sample (i.e. treated vs untreated/control cells). Quantitative
205 PCR data were analyzed with Student's unpaired t-test. All statistical tests were performed in
206 SPSS software v15.0 (IBM, Armonk, NY, USA), and data was represented with GraphPad
207 Prism software v8.0.1 (GraphPad, San Diego, CA, USA). Results were considered significant
208 if p-value was less than 0.05.

3. Results

3.1 Circulating DC and monocyte subsets display a gut-homing profile in CeD patients

The contribution of circulating DC and monocyte subsets in CeD is not fully understood. However, it has been suggested that a mucosal immune cell misbalance is mirrored in the circulatory system.^[22] We therefore characterized the proportion and homing profile of both cell types in peripheral blood samples from active and GFD-treated CeD patients, as well as healthy donors. All cells were identified within singlet viable PBMCs as pDC (HLA-DR⁺CD123⁺), cDC1 (HLA-DR⁺CD11c⁺CD14⁺CD16⁺CD141⁺), cDC2 (HLA-DR⁺CD11c⁺CD14⁺CD16⁺CD1c⁺), classical monocytes (HLA-DR⁺CD11c⁺CD14⁺CD16⁻), non-classical monocytes (HLA-DR⁺CD11c⁺CD14⁻CD16⁺), and intermediate monocytes (HLA-DR⁺CD11c⁺CD14⁺CD16⁺) (Figure 1A). Although not statistically significant, GFD-treated CeD (but not patients with active disease) displayed a lower proportion of circulating pDC and cDC1 than healthy controls (Figure 1B-C). The proportion of circulating cDC2 and monocyte subsets was not altered in any case (Figure 1D-G). However, due to the low number of active CeD patients recruited, we are aware that these results should be considered carefully.

After identifying the different cell types, we next studied their homing profile by assessing the expression of the CCR9 and β 7 integrin gut-homing markers, and the CLA skin-homing marker (Figure 2A). CeD-circulating DC subsets mainly differed in CCR9 and CLA expression from healthy individuals (Figure 2B-D). In particular, pDC from GFD-treated CeD patients seemed to display higher levels of CCR9 and lower levels of CLA (Figure 2B). Similarly, cDC1 followed the same expression pattern trend as pDC, with slightly increased levels of CCR9 and lower levels of CLA in both active CeD and GFD-treated CeD patients (Figure 2C). On the other hand, cDC2 had higher CCR9 and lower average expression of CLA only in active CeD patients (Figure 2D). In contrast with DC, all monocyte subsets favored a higher expression of the β 7 integrin in patients with active inflammation, but not in those under a GFD (Figure 2E-G). Indeed, the expression of β 7 on classical monocytes from GFD-treated CeD patients was

even lower than in healthy controls (Figure 2E). In summary, these results demonstrate that antigen presenting cell subsets might be altered in active CeD as they display a preferential gut-homing profile during mucosal inflammation. However, this hypothesis deserves further analysis due to the low number of active CeD patients enrolled in this study.

3.2 Gliadin-peptides induce maturation on cDC2 from GFD-treated CeD patients

cDC have been considered as central drivers of CeD pathogenesis.^[20b, 20c, 29] Given their increased gut-homing capacity displayed in CeD patients,^[20b, 30] we next decided to characterize them. Since cDC1 proportion was scarce (Figure 1B), we focused on cDC2 to study the effect that several gliadin-derived peptides elicited over DC phenotype and function.

The classical immunogenic peptide 33-mer increased the expression of CD40 and CD80 in cDC2 from healthy controls, while it increased the expression of CD80, CD83, and CD86 in cDC2 from GFD-treated CeD patients (Figure 3A-D). On the other hand, cDC2 from GFD-treated CeD patients responded to peptide 8-mer by increasing the expression of CD40 and CD80; and to peptide 19-mer, by increasing CD80 and CD86 (Figure 3A-D). Of note, none of the peptides changed the expression of the activation markers on cDC2 enriched from patients with active disease (Figure 3A-D). Homing marker expression (CCR9, β 7 integrin and CLA) on cDC2 was not affected by these peptides in any of the three studied groups (supplementary figure 3). Altogether, these results suggest that gliadin-derived peptides induce cDC2 activation, being peptide 33-mer the most immunodominant peptide.

3.3 Peptide 8-mer induces a Th1/Th17 cytokine profile on cDC2 from active CeD patients

Given that the gliadin-derived peptides induced cDC2 activation, we next addressed whether this was also followed with a differential cytokine profile. *IL4* and *IL12A* mRNA were not detected in any case, so they were excluded from the analysis (data not shown). Peptide 33-mer increased the expression of *IL6* and *IL23A* in healthy controls, whereas only the expression of *IL6* was increased in active CeD patients (Figure 4A-B). On cDC2 from GFD-

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treated CeD patients, however, 33-mer decreased the expression of *IFNG*, *IL10* and *IL23A* (Figure 4C). On the contrary, peptide 8-mer increased the expression of *IFNG*, *IL6* and *IL23A* on cDC2 from patients with active CeD (Figure 4B), and decreased the expression of *IFNG* and *IL10* in GFD-treated CeD patients (Figure 4C). However, 8-mer did not significantly changed the expression of any of the analyzed cytokines on cDC2 from healthy controls (Figure 4A). Finally, peptide 19-mer increased the expression of *IFNG* and decreased the expression of *IL23A* in cDC2 from active CeD patients, whereas it decreased the expression of *IL6*, *IL10* and *IL23A* in GFD-treated CeD patients (Figure 4B-C). Taken together, the cytokine expression profile induced on gliadin-peptide stimulated cDC2 suggests a pro-inflammatory response towards a Th1/Th17 profile in active CeD patients, which was more prominent when the cells had been primed with peptide 8-mer. On the other hand, gliadin-peptide stimulated cDC2 from GFD-treated CeD patients showed a decreased expression of proinflammatory cytokines, suggesting the need of additional stimuli to trigger the immune response to gluten.

3.4 Naïve T-cells increase CCR9 expression after co-culture with 33-mer primed autologous cDC2

Since these three gliadin-derived peptides induced changes on cDC2 activation markers (mainly peptide 33-mer) and cytokine profile (prominently peptide 8-mer), we next addressed whether this was resembled on a differential effect over autologous naïve T-cells. cDC2 did not alter T-cell proliferation –assessed by CFSE dilution on T-cells–, in any of the study groups when compared to non-stimulated cDC2 (data not shown). Moreover, despite the effects of the gliadin-derived peptides on cDC2 activation and cytokine expression, no changes were observed in the cytokine profile of stimulated T-cells (CFSE^{low}), neither from healthy controls nor from CeD patients (Figure 5A, B, C). However, 33-mer-primed cDC2 from all study groups (healthy controls, active CeD and GFD-treated CeD) increased the expression of the small bowel homing marker CCR9 on autologous responding T-cells (Figure 5D). In a similar

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3 286 manner, 19-mer primed cDC2 from patients with active CeD decreased CCR9 expression on
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5 287 stimulated T-cells (Figure 5D). The expression of β 7 integrin or skin homing marker CLA on
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7 288 stimulated T-cells was, however, not affected in any case (Figure 5E, F).
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4. Discussion

The mononuclear phagocyte system, including DCs, macrophages and monocytes, is of crucial relevance in autoimmune disorders. In CeD, mucosal pDCs and regulatory CD103⁺ DCs numbers are declined, while active cDCs and macrophages are increased.^[20-21] Here we demonstrate that GFD-treated CeD patients display a slight decrease of circulating pDC and cDC1 – which is in agreement with a previous report –,^[22a] while other DC or monocyte subsets are not altered referred to healthy controls. Both circulating DC and monocyte subsets display a gut-homing profile in patients with active CeD. Of note, although such gut-homing profile is characterized by a higher expression of the small-gut-homing CCR9 receptor and reduced expression of skin-homing marker CLA on DC subsets, the gut-homing profile on monocyte subsets is characterized by a higher expression of β 7 integrin. These differential mechanisms controlling DC and monocyte subsets migration to the intestinal mucosa may help to explain the higher DC and macrophage infiltrate described in the duodenal tissue of patients with active CeD.^[20b, 21, 30] However, additional research is needed to confirm our results since the number of active CeD patients recruited in this study was low.

Gluten is known to promote migration and activation of monocytes and MoDC, including the expression of maturation markers HLA-DR, CD40, CD80, CD83, and CD86; and cytokines IL-6, IL-8, IL-10, and TNF- α .^[23b, 31] MoDC have been traditionally considered as cDC2 analogues. However, recent studies have revealed that the phenotype and function of MoDC are different to that of cDC2,^[24] which questions the conclusions from previous MoDC models. Therefore, our work crucially contributes to understand the specific effects of gliadin-derived peptides on *bona fide* cDC2 and the autologous naïve T-cells that they stimulate, hence getting a deeper insight into the specific contribution of these cells towards CeD pathogenesis. Even if cDC2 might be less relevant than cDC1 for CeD, given the decreased number of cDC2 found in active CeD mucosa,^[20b] we focused our study on these cells due to the scarce presence of cDC1 (less than 0.04% of PBMCs, which is in agreement with previous reports^[32]).

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3 315 The peptide 33-mer has been characterized as the most immunodominant peptide for CeD
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5 316 patients due to high affinity for HLA-DQ molecules, especially after deamidation by tissue
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7 317 transglutaminase (TG) 2.^[5c] Indeed, Rakhimova *et. al.* reported that gluten promoted the
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9 318 expression of maturity markers on MoDC from healthy subjects, regardless of their
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11 319 genotype.^[23a] Consistently, we observed that peptide 33-mer activates cDC2 from both healthy
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13 320 controls and GFD-treated CeD patients. On the other hand, peptides 8-mer and 19-mer
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15 321 exclusively activated cDC2 from GFD-CeD patients, suggesting that their effects may be
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17 322 restricted to CeD patients. It should be noted, however, that none of the peptides affected
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19 323 cDC2 from patients with active disease. One possibility to explain this lack of cDC2 activation
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21 324 is that cDC2 from active CeD patients are already primed with gut-homing capacity. Thus, they
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23 325 may already display a pro-inflammatory phenotype, reason why the peptides cannot further
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25 326 increase the expression of activation markers. In agreement with this, the cytokine profile of
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27 327 cDC2 was affected by all peptides, preferentially in patients with active CeD – but not with
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29 328 GFD-treated CeD –, suggesting that these cDC2 are ready to respond to peptides when gliadin
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31 329 is present in the mucosa of active CeD patients (i.e. expressing pro-inflammatory cytokines).
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33 330 Interestingly, each gliadin-derived peptide promoted different cytokine profiles on cDC2, being
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35 331 peptide 8-mer the best inducer of a pro-inflammatory Th1/Th17 profile. Nevertheless, despite
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37 332 these effects on cDC2 phenotype and cytokine expression, gliadin-derived peptides were
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39 333 unable to translate the response to autologous naïve T-cells (i.e. cytokine production). Instead,
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41 334 cDC2 primed with the peptide 33-mer efficiently induced CCR9 expression on T-cells. This
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43 335 increased gut-homing imprinting capacity suggests that 33-mer might also contribute to boost
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45 336 T-cell infiltration into the duodenal mucosa, and the subsequent inflammation and tissue
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47 337 remodelling.
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52 338 The inability of immunodominant epitopes to activate mucosal cells has been previously
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54 339 observed, suggesting that a synergistic effect between different peptides might be needed for
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56 340 cDC to promote T-cell activation.^[5b] In their study, Maiuri *et. al.* found that the immunodominant
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58 341 gliadin epitopes p α -2 and p α -9 required a previous stimulation with the non-immunodominant
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342 p31-43 in order to induce an adaptive immune response in cultured biopsies from CeD
343 patients.^[5b] Consistently with their observations, we found that the peptide 8-mer induced a
344 pro-inflammatory cytokine expression profile in cDC2, while the immunodominant peptide 33-
345 mer enhanced the gut-homing capacity of T-cells. Thus, if both peptides were simultaneously
346 used to stimulate cDC2, they might contribute to develop a complete and functional adaptive
347 T-cell immune response.

348 On the other side, cDC1 and cDC2 process antigens and activates T-cells in different ways.
349 While cDC1 contribute to a CD8+ T-cell driven response, cDC2 efficiently stimulate CD4+ T-
350 cell responses.^[33] This, together with a possibly combined antigen presentation of native
351 peptides by other cells (e.g. B-cells),^[34] might even induce a greater immune response to
352 gliadin-derived peptides than the response shown here since we could only study the cDC2
353 outcomes. In addition, recent research has shown a relationship between bacterial proteolytic
354 activity and the infection with reoviruses with an increased sensitivity to dietary antigens such
355 as gluten.^[35] Therefore, alternative non-dietary triggers might alter the DC response and could
356 contribute to strengthen the signal transduction of antigen-presenting cells into T-cells.

357 In summary, circulating DC and monocyte subsets display a gut-homing profile in CeD patients.
358 Therefore, monitoring peripheral DC and monocyte subsets may be useful, novel biomarkers
359 for the diagnosis of CeD or the assessment of the compliance to the GFD. Our data also
360 revealed that distinct gliadin-derived peptides elicit differential and complementary effects on
361 cDC2 phenotype and function. Given that CeD patients do not ingest single peptides but a
362 mixed pool of gluten proteins, our results are therefore compatible with a model where different
363 gluten peptides might cooperate independently to the full activation of cDC2 – and possibly
364 other antigen-presenting cells such as cDC1 and B-cells –, and the subsequent promotion of
365 T-cell activation in genetically predisposed individuals with altered microbiota and/or
366 gastrointestinal exposure to reovirus.

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Conflict of interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contributions

CEH, JAG, DB, and EA conceived and designed the experiments. LFS recruited all the patients and obtained the biological samples for the study. CEH, AM and RPA performed the experiments. CEH and DB contributed to data analysis. Manuscript was drafted by CEH and DB, and subsequently forwarded to all authors for corrections. EA obtained the funding to develop the study. All authors reviewed and approved the final version of the manuscript.

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Figure legends

Figure 1: Circulating plasmacytoid dendritic cell (DC) and type 1 conventional DC subsets are slightly decreased in gluten-free diet treated celiac disease patients.

A) Human circulating dendritic cells (DC) and monocyte subsets were identified within singlet viable peripheral blood mononuclear cells (PBMC). Plasmacytoid DC (pDC) were identified as HLA-DR⁺CD123⁺ cells within the HA-DR⁺CD11c⁻ fraction (gate 1). Monocytes and conventional DC (cDC) were identified as HLA-DR⁺CD11c⁺ cells (gate 2). Monocytes were further divided into classical (CD14⁺CD16⁻), non-classical (CD14⁻CD16⁺) and intermediate (CD14⁺CD16⁺) monocytes (gate 2). cDC were identified within the CD14⁻CD16⁻ fraction (gate 3) as cDC1 (CD141⁺) and cDC2 (CD1c⁺). Data are shown as percentage of gated cells. B-G) Pooled results, determined as percentage within singlet PBMC, from healthy controls, patients with active celiac disease (aCeD), and gluten-free diet treated (GFD)-CeD are shown for pDC (B), cDC1 (C), cDC2 (D), and classical monocytes (E), non-classical monocytes (F), and intermediate monocytes (G). Data are represented as mean±SD. One-way ANOVA was applied. Ad-hoc comparisons were, in all cases, compared with the healthy controls. P-values <0.05 were considered significant.

Figure 2: Circulating dendritic cells and monocytes display a gut-homing profile in celiac disease.

A) Dendritic cell (DC) and monocyte subsets were identified as in Figure 1A, and analysed for the expression of CLA as in the example, as well as β 7 and CCR9 (not shown). The percentage of positive cells was determined by the fluorescence minus one (FMO) method. B-G) Pooled results of positive cells for each marker within each cell type from healthy controls, patients with active celiac disease (aCeD), and gluten-free diet treated (GFD)-CeD are shown for plasmacytoid DC (pDC, B), type 1 conventional DC (cDC1, C), type 2 conventional DC (cDC2, D), classical monocytes (cl.Mo, E), non-classical monocytes (non-cl.Mo, F) and intermediate

monocytes (int.Mo, G). Data are shown as mean $\pm$ SD of the percentage of positive cells. One-way ANOVA was applied. Differences relative to Hc samples are shown as * $p < 0.1$; and differences relative to aCeD samples are shown as # $p < 0.1$ and ## $p < 0.05$.

Figure 3: Gliadin-derived peptides induce maturation in type 2 conventional dendritic cells from gluten-free diet treated celiac disease patients.

Type 2 conventional dendritic cells (cDC2) from healthy controls, patients with active celiac disease (active CeD) and patients with gluten-free diet treated (GFD)-CeD were challenged *ex vivo* with gliadin-derived 33-mer, 8-mer and 19-mer peptides. Cells cultured in resting conditions acted as internal negative controls (C). Percentage of positive cells for each marker was determined as in Figure 2A. Pooled results for the expression of CD40 (A), CD80 (B), CD83 (C), and CD86 (D) are shown. Data are shown as mean $\pm$ SD of the percentage of positive cells. Paired t-test was applied within each patient group referred to the culture in resting conditions. P-values < 0.05 were considered significant (* $p < 0.05$).

Figure 4: Peptide 8-mer induces a Th1/Th17 cytokine profile on type 2 conventional dendritic cells from active celiac disease patients.

Heatmap displaying cytokine gene expression changes in type 2 conventional dendritic cells from healthy controls, patients with active and gluten-free diet treated (GFD) (C) celiac disease (CeD) after *in vitro*-priming with peptides 33-mer, 8-mer, and 19-mer (A-C). Results are referred to those obtained from resting conditions for each patient group. Data are displayed as mean $\log_2$ -transformed fold-changes of the $-\Delta\Delta C_t$ values, with *GADPH* as housekeeping control. Paired t-test was applied within each patient group referred to the culture in resting conditions.

Figure 5: Type 2 conventional dendritic cells primed with the peptide 33-mer induce a gut-homing profile on responding T-cells.

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3 545 Type 2 conventional dendritic cells (cDC2) from healthy controls or patients with active or
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5 546 gluten-free diet (GFD)-treated celiac disease (CeD) were cultured in resting conditions (C) or
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7 547 in the presence of gliadin-derived peptides 33-mer (33m), 8-mer (8m) or 19-mer (19m). cDC2
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9 548 were subsequently used to stimulate autologous T-cells. The percentage of positive T-cells for
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11 549 the expression of intracellular cytokines IFN- γ (A), IL-17A (B), and IL-10 (C), and the surface
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14 550 homing markers CCR9 (D), β 7 integrin (E), and CLA (F) were determined. Data are shown as
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16 551 mean $\pm$ SD of the percentage of positive cells. Paired t-test was applied within each patient
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18 552 group referred to the culture in resting conditions. P-values <0.05 were considered significant
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20 553 (* p<0.05, and *** p<0.001).
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554 **Tables**

Table 1 Clinical and demographic characteristics of CeD patients and controls

Variable	Hc	aCeD	GFD CeD ^{a)}
Total number of subjects	9	5	11
Average age (range)	38 (20-57)	47 (31-61)	40 (19-56)
Gender (% females)	44.44	80.00	63.64
On GFD treatment	No	No	Yes
Months on GFD, (range)	-	-	96.64 (26-180)
HLA-DQ2 (% positive)	N.D.	100	91 ^{b)}
Anti-tTG (% positive)	N.D.	100	0
Marsh classification, number (% positive)	N.D.		N.D.
Marsh I	-	1 (20%)	-
Marsh II	-	0 (0%)	-
Marsh III	-	4 (80%)	-

a) GFD CeD patients were diagnosed after positive genetics, serology, and villous atrophy according to pathological analysis. After following a GFD, patients achieved mucosal healing and negative serology values of anti-tTG. b) Data was not available for one patient.

CeD, celiac disease; GFD, gluten-free diet; Hc, healthy controls; N.D., not determined; tTG, tissue transglutaminase.

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Gliadin-derived peptides (i.e. 8-mer, 19-mer, 33-mer) might cooperate to promote full dendritic cell activation to develop a subsequent T-cell response in genetically predisposed individuals (HLA-DQ2⁺)

254x190mm (96 x 96 DPI)

Graphical abstract text

Gliadin-derived peptides (i.e. 8-mer, 19-mer, 33-mer) might cooperate to promote full dendritic cell activation to develop a subsequent T-cell response in genetically predisposed individuals (HLA-DQ2⁺)

For Peer Review

Figure 1

Figure 1: Circulating plasmacytoid dendritic cell (DC) and type 1 conventional DC subsets are slightly decreased in gluten-free diet treated celiac disease patients.

210x297mm (300 x 300 DPI)

Figure 2

Figure 2: Circulating dendritic cells and monocytes display a gut-homing profile in celiac disease.

210x297mm (300 x 300 DPI)

Figure 3

Figure 3: Gliadin-derived peptides induce maturation in type 2 conventional dendritic cells from gluten-free diet treated celiac disease patients.

172x98mm (300 x 300 DPI)

Figure 4: Peptide 8-mer induces a Th1/Th17 cytokine profile on type 2 conventional dendritic cells from active celiac disease patients.

179x52mm (300 x 300 DPI)

Figure 5

Figure 5: Type 2 conventional dendritic cells primed with the peptide 33-mer induce a gut-homing profile on responding T-cells.

172x144mm (300 x 300 DPI)

Supplementary figure 1

Supplementary figure 1: Purity of viable type 2 conventional dendritic cells after magnetic cell isolation.

86x53mm (300 x 300 DPI)

Supplementary figure 2

Supplementary figure 2: Gliadin-derived peptides do not alter type 2 conventional dendritic cell viability following in vitro challenge.

87x58mm (300 x 300 DPI)

Supplementary figure 3

Supplementary figure 3: Gliadin-derived peptides do not modify type 2 conventional dendritic cells homing profile.

87x144mm (300 x 300 DPI)