Identification and molecular characterization of oat peptides implicated on coeliac immune response

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Abbreviations

CD, coeliac disease; DC, dendritic cells; GFD, gluten-free diet; HC, healthy control; IEF, isoelectric focusing; LC-MS/MS, liquid chromatography-tandem mass spectrometry; moAbs, monoclonal antibodies; PAGE, polyacrylamide gel

electrophoresis; PCR, polymerase chain reaction; SD, standard deviation; RT, room temperature.

ABSTRACT

Background: Oats provide important nutritional and pharmacological properties, although their safety in coeliac patients remains controversial. Previous studies have confirmed that the reactivity of the anti-33-mer monoclonal antibody with different oat varieties is proportional to the immune responses in terms of T-cell proliferation. Although the impact of these varieties on the adaptive response has been studied, the role of the dendritic cells is still poorly understood. The aim of this study is to characterize different oat fractions and to study their effect on dendritic cells from coeliac patients.

Methods and results: Protein fractions were isolated from oat grains and analysed by SDS-PAGE. Several proteins were characterized in the prolamin fraction using immunological and proteomic tools, and by *Nano-LC-MS/MS*. These proteins, analogous to α- and γ-gliadin-like, showed reactive sequences to anti-33-mer antibody suggesting their immunogenic potential. That was further confirmed as some of the newly identified oat peptides had a differential stimulatory capacity on circulating dendritic cells from coeliac patients compared with healthy controls.

Conclusions : This is the first time, to our knowledge, where newly identified oat peptides have been shown to elicit a differential stimulatory capacity on circulating dendritic cells obtained from coeliac patients, potential identifying immunogenic properties of these oat peptides.

Keywords: coeliac disease, oats, immune response, gluten-free diet

INTRODUCTION

Coeliac disease (CD) is an autoimmune disorder developed in genetically (HLADQ2/8) predisposed individuals and caused by a permanent intolerance to gluten contained in some cereals, such wheat, rye and barley that leads to a chronic inflammation of the small intestine (1-3).

The most accepted model for explaining CD immunopathogenesis is the two-signal model (4) characterized by a first innate immune response followed by a secondary antigen-specific adaptive response. Some peptides like the 19-mer gliadin peptide trigger an innate immune response (5) mainly characterized by the production of IL-15 by epithelial cells. The result is the disruption of the epithelial barrier, by increasing the permeability and inducing enterocyte apoptosis (6). As a consequence, immuno-adaptive peptides, like the 33-mer gliadin peptide, can now reach the lamina propria where they are deaminated by the enzyme tissue transglutaminase. Such deamidation provides a negative load to gliadin peptides and hence enhancing their affinity to fit in the HLA-DQ2/8 bound, which is also the "susceptibility gene" in CD, expressed on the surface of dendritic cells (DC) (7-9). DC are indeed the most potent antigen presenting cells of the immune system as they promote differentiation of pro-inflammatory antigen-specific effector T-cell at the time that they also direct them to the target tissue via homing marker imprinting (10, 11). DC are therefore central in CD pathogenesis as they present gluten antigen to T-cells (12) driving progression of the pro-inflammatory antigen-specific adaptive immune response which will turn into the symptomatology of the disease.

The gluten-free diet (GFD) is the basis of all the present treatments for CD, after which the immune response is abrogated leading to clinical remission of the disease (13, 14). Recently, the gluten-free products market has witnessed a revolution due to an increased incidence of CD coupled with the fact that it is not only coeliac and gluten sensitive patients consuming these products, but also individuals not affected by those pathologies (15). Therefore, oats are of particular interest to all GFD consumers because they could improve the nutritional value of the GFD given their rich nutritional value and a considerably high protein concentration. Oats contain as well a number of important minerals, lipids, β-glucan, a mixed-linkage polysaccharide, which forms an important part of oat dietary fiber, and also contains various other phytoconstituents.

Finally, oats also have different pharmacological activities including antioxidant, anti-inflammatory, antidiabetic, anticholesterolaemic, and many others. Therefore, all these properties have led to wider appreciation of oats as valuable human food (16).

Studies on the suitability of oats for patients with CD showed contradictory results. While some studies suggested that oats are safe for CD patients (17-20), others have revealed that oats can trigger an immune reaction on these patients (18, 21-23) including activation of mucosal T-cells and subsequent gut inflammation ultimately leading to villous atrophy (22). Indeed, CD patients have circulating anti-avenin antibodies (24, 25) while, a recent study revealed that dietary oats altered the mRNA immune status of the intestinal mucosa suggesting T-cell activation and leaky tight-junctions (26). These contradictory results regarding oats safety for CD patients might be explained by the fact that the oat varieties used in the different studies were different. Oats include numerous varieties, differing in the prolamin genes and hence in the resulting amino acid sequences showing different immunoreactivities associated with toxic prolamins (27, 28). In previous studies, our group has reported the utility of the G12 monoclonal antibody (moAb) against the main immunogenic epitope of the α-2 gliadin, 33-mer peptide, for detecting oat varieties potentially toxic for CD patients. Indeed, the reactivity with the anti-33-mer moAb of the different oat varieties correlated with T-cell proliferation and interferon gamma production of blood T-cells isolated from such patients (29).

In the present study we have characterized the different protein fractions of oat prolamins and have identified several reactive sequences to anti-33-mer moAb, analogous to α- and γ-gliadin-like, with immunogenic potential for CD patients. Moreover, some of the peptides from these subunits, obtained from *Nano-LC-MS/MS* data, induced specific activation of circulating DC obtained from CD patients on the GFD, as opposed to those from healthy controls (HC), confirming therefore their immunogenic potential.

MATERIALS AND METHODS

Oat sample preparation

Oats (*Avena sativa* L.) from cultivars designated OE717, OA729, OM719, OC723, OH727, and OL715 (obtained from Spanish and Australian commercial sources) were used in this work. These cultivars were chosen based on their previously reported CD toxicity (29).

DNA extraction

DNA extraction from oat seeds was performed using a modified cetyltrimethylammonium bromide (CTAB) method. DNA concentrations were determined by UV absorption. The purity of the DNA solution was assessed by the 260/280 nm absorption ratio. Oligonucleotides from wheat, barley, rye and oats were used. This protocol and oligonucleotides have been characterized in detail in previous studies from our laboratory (29).

Protein extraction and quantification

Oat flours were prepared by grinding the dehusked kernels. One hundred mg of wholemeal flour was used for the sequential extraction of the albumins, globulins, avenins and glutenins according to Osborne (30) and Marion et al. (31).

Albumin extraction

The albumins were extracted with cold water with continuous mixing at 4ºC for 90 min. The mixture was centrifuged (8,000*g*, 20 min) and the proteins in the supernatant were precipitated with 2 volumes of acetone at -20ºC. The pellet was then washed three times with acetone and dried at room temperature.

Globulin extraction

Globulins were extracted from the pellet with a salt solution (Tris-HCl 50 mM, NaCl 1M, pH 8.5) in continuous mixing at 4°C for 1h 30 min. Similarly, the mixture was centrifuged and the proteins in the supernatant were recovered with acetone.

Avenins extraction

To extract avenins, 3 washes were performed with cold water on the pellet described above for 5 min with continuous vortexing. The samples were treated with 70% ethanol (v/v) for 60 min with continuous mixing, followed by centrifugation (10,000*g*, 5 min). This step was repeated three times to remove most of the avenins. The supernatants were pooled and incubated overnight at 65°C to recover the avenins.

Glutenins extraction

The glutenins in the pellet were extracted and reduced with 50% propanol-1-ol solution containing 1% w/v dithiothreitol (DTT) for 30 min with continuous mixing at 65ºC, followed by centrifugation (10,000*g*, 5 min) (32).

SDS-PAGE

Proteins (albumins, globulins, avenins and glutenins) were resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (45 mM Tris-HCl, PH 6.8, 50 mM DTT, 1% SDS, 10% Glycerol, 0.001% bromophenol blue). Protein concentration was determined by Bradford assay (33). Proteins were separated by SDS-PAGE using 12.5% polyacrylamide gel using a Hoefer tank (GE Healthcare). Gels were stained with Coomassie Brilliant Blue (CBB).

Acid-PAGE

In order to identify a large number of avenins according to their relative mobility, an acid PAGE (A-PAGE) was performed following the method of Branlard et al. (34).

2-DE

For further characterization of avenins, two biological extracts with two replicates per extract were used. Isoelectric focusing (IEF) was performed using the IPGPhor II apparatus (GE Healthcare) on 13 cm Immobiline dry strips of 3–10 linear pH gradients. Passive re-hydration was performed overnight in a solution containing 7M urea, 2M thiourea, 70mM DTT, 1% IPG buffer (pH 3–10), 4% CHAPS, 0.34% anti-protease and 100 µg (analytical gel) or 1 mg (preparative gel) of the protein extract. IEF was carried

out by applying a cumulative voltage of 30 kVh and 60 kVh for analytical and preparative gel respectively.

Following IEF, proteins were reduced for 15 min in an equilibration buffer containing 0.05M Tris-HCl (pH 8.8), 6M urea, 30% glycerol, 2% SDS and 1% DTT, followed by alkylation for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. The second dimension was performed using SDS-PAGE gels (12% T, 2.1% C) sealed with 0.5% agarose in SDS buffer on Hoeffer vertical system (GE Healthcare). The migration conditions were 10 mA/gel for first 30 min then 35 mA/gel until the exit of the dye front. Gels were stained with CBB.

Immunoblotting

For immunoblot analysis, protein samples were separated on a 12 % SDS–PAGE or a 2-DE gel and then transferred onto a nitrocellulose membrane using a Hoefer TE77 semidry transfer blotter. The blotted membrane was incubated for 60 min at room temperature in blocking buffer consisting of 10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.01% Tween 20 and 5 % skim milk and then exposed to G12 moAb.

Anti-mouse IgG Alkaline phosphatase A3562 and kit SIGMA fast (ref F4523) were used for detection according to the manufacturer instructions.

Gel images of 300 dpi and 16-bit greyscale pixel depth were obtained with G-800 (GE Healthcare) scanner and were analyzed using SameSpots v3.2 (Nonlinear Dynamics, Newcastle, UK). SameSpots applies highly accurate pixel-level alignment so that 2D gels with secondary stained images including antibodies can be directly compared.

In-gel digestion

Protein spots were excised from gels and de-stained with a solution containing 25 mM NH_4HCO_3, 5% ACN for 30min and 25 mM NH_4HCO_3 50% ACN twice for 30 min. After dehydration in 100% ACN for 10 min, the spots were dried. Briefly, 100 ng of chymotrypsin (C6423, Sigma), solution in Tris-HCl 100 mM, pH 7.8, $CaCl_2$ 10 mM was added to the spots and digestion was performed at 37°C for 4–5h. After centrifugation, peptides were extracted by adding 8 µL of ACN**.**

Nano-LC-MS/MS **analysis and database searching**

For Nano-LC-ESI-MS/MS analysis, peptides mixtures were analyzed by online nanoflow liquid chromatography using the Ultimate 3000 RSLC (Dionex, Voisins le Bretonneux, France) with nanocapillary columns of 15 cm length x 75 µm I.D., 3 µm, 100Å (Acclaim PepMap100 C18, Dionex). The solvent gradient increased linearly from 4% to 50% acetonitrile in 0.5% formic acid at a flow rate of 300 nL/min for 30 min. The elute was then electrosprayed in positive-ion mode at 2.7 kV in a LTQ-VELOS mass spectrometer (Thermo Fisher Scientific, Courtaboeuf, France) through a nanoelectrospray ion source which was operated in a CID top 10 mode (i.e., 1 full scan MS and the 10 major peaks in the full scan were selected for MS/MS). Full-enhanced-scan MS spectra were acquired with 1 microscan (m/z 300 – 1800). Dynamic exclusion was used with 1 repeat counts and 50s exclusion duration. For MS/MS, isolation width for ion precursor was fixed at 2 m/z, single charged species were rejected; fragmentation used 37% normalized collision energy as the default activation of 0.25.

Protein and peptide sequence analysis

Raw data files were processed using version Peaks 5.3 software with the EBI database (Taxonomy viridiplantae, 1023819 entries). The following parameters were considered for the searches: Parent Mass Error Tolerance of 1.5 Da, Fragment Mass Error Tolerance of 0.8 Da, a maximum of one missed cleavage, partial methionine oxidation and partial carbamidomethylation of cysteine. If the peaks score was statistically significant ($p<0.05$), the protein was considered valid. When proteins were identified from only two peptides, spectra were checked to assess their validity.

Synthesis of peptides

Different peptides derived from avenin sequences obtained from the MSMS data (Table 1) were supplied by Biomedal S.L. (Seville, Spain).

Dendritic cells from peripheral blood

Human peripheral blood was collected from 3 HC with no known autoimmune or inflammatory diseases, allergies or malignancies and 3 patients with CD following informed consent. All CD patients had been on a GFD for at least 6 months and had no clinical symptoms or positive serology at the time of sample taking. The study was

approved by the ethic committee of St Thomas' Hospital, London (United Kingdom) and written informed consent was obtained.

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Chalfont St. Giles, UK). Human blood enriched DC were subsequently enriched following NycoPrep™ centrifugation of overnight cultured PBMC in complete medium Dutch modified RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 100u/mL penicillin/streptomycin, 2mM L-glutamine, 50µg/mL gentamicine (Sigma-Aldrich) and 10% foetal calf serum (TCS cellworks, Buckingham, UK). This protocol has been characterised in detail in previous studies as a way to obtain fresh human blood enriched DC (35-38). Blood enriched DC were further cultured for 24 hours in complete medium in the presence of oat peptides, 33-mer peptide, STp (peptide secreted by *Lactobacillus plantarum*) or LPS (lipopolysaccharide from *Escherichia coli*) (Sigma-Aldrich, St. Louis, USA). The same molar concentration was used for all peptides to avoid the problem of epitope load between large and small peptides. Results were referred with a paired culture in basal medium which acted as an internal control.

Proliferation assay

Freshly obtained PBMC from HC were suspended in MiniMACs buffer (PBS containing 0.5% BSA and 2 mM EDTA). T-cells were enriched by depletion of CD14, CD19 and HLA-DR positive cells with immunomagnetic beads (Miltenyi Biotech, Bisley, UK) following manufacturer's instructions. An average of 94.91% ± 1.06 (mean ± SD) T-cells was obtained following enrichment. T-cells were labeled with 10 µM 5-carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen Ltd, UK) following manufacturer's instructions. CFSE-labelled T-cells (4×10^5/well) were incubated for 5 days in U-bottomed 96 well microtitre plates with allogeneic blood DC at 0%, 1% or 3%. Cells were washed twice in FACS buffer, fixed with 1% paraformaldehyde in 0.85% saline and stored at 4^0C prior to acquisition on a FACSCantoII (BD Biosciences) flow cytometer (within 48 hours). Data was analysed using WinList 5.0 software (Verity, ME). The percentage of proliferating T-cells was assessed via CFSE dilution of viable cells based on their forward and size properties as previously described (11).

Statistical analysis

Results were analysed in the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) using Two-way and one-way repeated measures ANOVA, and two-tailed paired tests. $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Protein characterization in different oat varieties

Protein fractions from six oat varieties previously described as potentially reactive for CD patients (29), were studied by SDS-PAGE. The purity of the oat seeds was tested by a visual examination, and PCR experiments discarded the presence of wheat, rye and barley DNA in the samples. The protein patterns of these cereals were compared with those of gliadins (from wheat) and with a pre-stained molecular weight marker (Fig. 1A).

Proteins with a wide range of molecular weights ranging from <20 to 80 kDa were found in all albumin fractions of the different cultivars. Oat albumins, which are considered to be primarily enzymes, are a minor component with values ranging from 1-12% of total protein (39). Nevertheless, most of the protein fraction from the oat grains was soluble in buffered salt solutions and thus classified as globulins (40). These proteins (Fig. 1A) were mainly of two families, the first ranging from 20-37 kDa, and the second grouping around 50 kDa. Globulins appeared with the same distribution in all cultivars studied. Opposed to wheat gliadins, which have a typical size range of 29-70 kDa, oat avenins were smaller in size ranging from 20 kDa to 36 kDa with weaker bands around 50-70 kDa. Another important feature of these proteins is that their protein patterns were diverse; confirming that polymorphism of avenin patterns was more heterogeneous than in the globulin fraction.

Concerning the glutenin fraction, a wide range in the molecular weight, ranging from 50 to even lower than 20 kDa was also observed for these proteins. For all other accessions, the glutenin protein patterns showed a diversity and heterogeneity in both size and intensity of the subunits. Our findings confirmed therefore that oat grains

contain a significant protein fraction that is insoluble in alcohol and soluble in denaturing/reducing solution composed mainly low molecular weight glutenin subunits (LMW-GS)-like proteins (41).

Because of the diversity found in the alcohol-soluble fractions, in order to identify the relative mobility of these proteins, an acid PAGE (A-PAGE) was performed. The diversity of cereal prolamins is usually better resolved by using electrophoresis in aluminium lactate buffer (such as in A-PAGE). Moreover, A-PAGE allows oat prolamins to be separated according to the ratio ionic charge / molecular mass resulting in a better resolution as compared to the classic SDS PAGE procedure. A large number of avenin subunits were observed (Fig. 1B). Differences in the band patterns were found for the studied varieties, showing a great diversity in the composition of avenins among the different cultivars. These results confirm therefore the presence of different avenins in the cultivars as they differ in both their size and ionic charge in acid pH. However, Spanish varieties OH727 and OC723, shared the same pattern of bands by A-PAGE. Likewise, two Australian accessions (OE717 and OM719) kept the same prolamin pattern but differed in two protein bands obtained in the lower region of the gel. These accessions probably have the same progenitors or related progenitors, or they may have independently evolved from accessions with small differences between them.

Evaluating immunotoxic proteins in the alcohol-soluble fraction from oat seeds

In order to get further insights into the proteomic and immunological properties of the avenin proteins previously described, and their implications in CD pathogenesis, we focused on accession OE717 as it was described with high immunogenic effect of CD patients (29). The avenin extract from this accession was studied by Western blot using staining techniques which reveal in the membrane at the same time both the antibody-recognized proteins and the total proteins as separated by SDS-PAGE. A dual double staining, first with the moAb and then nigrosin, distinguished the total avenin fraction vs. specific proteins (Fig. 2). Reactive proteins appeared in the region of 25-37 kDa (major bands according to results obtained by SDS-PAGE); however, anti-33-mer moAb also recognized other minority oat prolamins with higher molecular weights. The latter may be avenin dimers and/or oat prolamins not yet labelled with higher molecular weights.

When avenins from OE717 variety were separated in more details by 2-DE, a single band on 1-DE typically yielded more than one protein spot (Fig. 3A). Avenin fraction was separated by 2-DE and made visible by CBB staining. The 2-DE gels of these proteins revealed spots with relative molecular masses ranging from 35 to 20 kDa and a p*I* between 3 to 10. Fig. 3B showed proteome map obtained after immunobloting by anti-33mer moAb. Immunoreactive spots were observed ranging from 40 to 25 kDa on the western blot.

Among all the identified bands and spots, we next focused on the most reactive one as revealed by their immunoblotting intensity (Figs. 2B and 3B). Following overlaying of the nigrosin-stained membrane, the immunoblot and the corresponding CBB stained gel, bands (1-DE) and spots (2-DE) (as revealed by G12 moAb from the CBB stained gel) were excised and mass spectrometry analysis subsequently applied. A total 16 reactive proteins were identified, all belonging to the family of prolamins, specifically avenin proteins (Table 2). We further confirmed by BLAST searches, using *Multiple Protein Alignment Tool* algorithms, that all the proteins identified belong to fractions previously termed α- and γ-gliadin-like. However, some bands and spots could not be identified due to lack of information from oat proteins in available databases.

Circulating dendritic cells from CD patients react to avenin peptides

CD pathogenesis is driven both by the innate and the adaptive immune system (1, 4, 42). Although the impact of gluten peptides on the adaptive immune system has been studied in much details; the role of the innate immune response cells, including monocytes/macrophages and dendritic cells (DC) is still poorly understood.

DC are key actors in the connection between innate immunity and adaptive immunity responses. Furthermore, they are described as “decision makers” to commit tolerance or immunity (12, 43), yet information on DC in CD pathogenesis is scarce (8, 44-47). Moreover, most of the studies which have investigated the effect of gliadin and/or its derived peptides on DC phenotype and/or function have usually focused on monocyte-derived DC, generated following 5-7 *in vitro* culture of monocytes in the presence of IL-4 and GM-CSF (48-53), which although essential to further our understanding of human DC, they do not always resemble the properties of circulating DC (54, 55).

Moreover, these studies usually focus on the effect of gliadin and/or its derived peptides on monocyte-derived DC from healthy controls, usually avoiding a comparison with those obtained from CD patients. Contrary to those studies, here we decided to study the effect of these newly identified no-wheat oat peptides on circulating DC obtained from both HC and CD patients.

To determine the stimulatory capacity of the novel oat peptides, DC were pulsed with peptides QL6, QQ6, PV10, EF27, QL14 and QM27 of different sizes that were found having a homology to gliadin-like avenins (Table 1) and glutenin-like avenins. These peptides had proline-rich sequences and glutamine residues resembling wheat gluten sequences. Peptides PV10 and EF27 also carried a T-cell epitope recognized by $CD4^+$ T cells previously described by Sollid et al. (56): DQ2.5-ave-1a epitope, with glutamic acid to glutamine conversion at position 6. As positive control for stimulation, DC were pulsed with 33-mer peptide, the immunodominant antigen for CD (57) or with LPS. In addition to the basal (un-stimulated) internal negative controls, DC were also pulsed with microbiota-derived STp, previously described to induce regulatory effects on DC in HC without affecting their stimulatory capacity (58).

In order to exclude any potential effect of the ongoing inflammation on the profile of circulating DC, and therefore on their peptide response following *in vitro* challenge, experiments were performed with blood-enriched DC from HC and GFD-CD patients with no clinical symptoms and negative serology at the time of blood extraction. Following antigen-pulsing and subsequent co-culture with T-cells, DC from both HC and GFD-CD patients induced dose-dependent proliferative responses of CFSE-labelled allogeneic T-cells (determined as CFSE dilution by responding or dividing T-cells, Fig. 4) with no differential effect produced by blood DC from the groups irrespective of any differential basal stimulatory status between the groups.

The effect of the previously newly identified non-wheat oat peptides on DC from both HC and GFD-CD patients was studied next (Fig. 5). LPS increased DC stimulatory capacity from both HC and GFD-CD derived blood DC while STp did not induce any change in DC stimulatory status as previously described (Fig. 5) (58). When pulsed with the immunodominant 33-mer peptide, DC from both HC and GFD-CD patients increased their stimulatory capacity for T-cells in agreement with previous observations of an “ex-vivo” gluten-challenge biopsy-culture model (59). Having confirmed that *in*

vitro pulsing modulates DC stimulatory capacity from both HC and GFD-CD patients, we studied next whether the newly identified oat peptides displayed any differential effect on DC from the groups. Our findings revealed that oat peptides could be divided into 3 groups based on i) their lack of stimulatory effect on DC (peptides QL6 and QQ6); ii) increase of DC stimulatory capacity from both HC and GFD-CD patients (peptides EF27 and QM27); and iii) peptides which specifically up-regulated DC stimulatory capacity from GFD-CD patients but not from HC (peptides PV10 and QL14). A closer look into these oat peptides properties revealed that peptides QL6 and QQ6 (which did not have any stimulatory effect) were the smallest of the studied peptides (each with 6 residues). On the contrary, peptides EF27 and QM27 (which induced proliferative responses of T-cells stimulated by both HC and GFD-CD) were larger peptides (each with 27 residues); these peptides were of a size similar to the 33-mer peptide which had also activated DC from both HC and GFD-CD patients. This is in agreement with similar observations where large gliadin-derived peptides induced DC activation from non-CD patients (49, 50, 52). Finally, peptides PV10 and QL14 were unique in their capacity to specifically activate DC from GFD-CD patients but not those from HC. In contrast to the two other groups, peptides PV10 and QL14 had intermediate sizes (PV10: 10 residues; QL14: 14 residues). These differences between the differential stimulatory capacity of the peptides on DC from HC and GFD-CD are not likely to be due to different epitope load derived from their differential size as DC pulsing was performed at the same molar concentration. It seems, therefore, that DC capacity to trigger T-cell proliferative responses is not only dependent on the source of the peptides but also on their size and their possible differential intracellular processing. Thus, small peptides like QL6 and QQ6 (6 residues each) would fail to active DC while large gluten peptides (peptides EF27 and QM27 – of 27 and 33 residues respectively) would induce DC maturation in both HC and GFD-CD. Nevertheless, gluten peptides with the appropriate size and disposition of amino acids- peptides PV10 (10 residues) and/or QL14 (14 residues) are likely to go through a differential endocytic pathway which may end in a different peptide processing capacity elicited by DC from HC and CD patients. Future studies should identify this differential mechanism which makes blood DC from CD patients unique in their capacity to process these peptides increasing their stimulatory capacity for T-cells, and should identify whether this differential antigen processing mechanisms has an implications for the pathogenesis of CD.

CONCLUSIONS

Our findings exhibited the structural complexity and large differences among oat proteins. More specifically, we showed that oats is composed of a large number of avenin subunits. These proteins belong to fractions termed α- and γ-gliadin-like and some of which were reactive for anti-33-mer monoclonal antibody. Moreover, our study has shown the existence of new potentially toxic peptides for coeliac patients. These peptides were able to activate circulating dendritic cells from coeliac patients, identifying, therefore, their immunogenic properties.

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FIGURE LEGENDS

Fig. 1. **Protein pattern from six oat accessions.** (A) Oat proteins analysed by SDS-PAGE gel. (B) Avenins analysed by A-PAGE gel.

Fig. 2. **Electrophoresis and immunoblot analysis of avenin protein extracts from oat accession OE717.** (A) SDS-PAGE. (B) Immunoblot using G12 moAb. (C) Immunoblot using G12 moAb and Nigrosin staining.

Fig. 3. **Bi-dimensional analysis of avenin protein extracts from oat accession OE717.** (A) Image gel (IPG x SDS-PAGE). (B) Immunoblotting using G12 moAb.

Fig. 4. **Identification of dividing T-cells after dendritic cell stimulations.** Blood CFSE-labelled T-cells (400,000) from healthy controls (HC) were cultured for 5 days

with different doses (0%, 1%, 3%) of allogeneic blood enriched DC obtained from HC and gluten-free diet (GFD) coeliacdisease patients. Live T-cells were subsequently identified by flow cytometry based on the forward (FSC-A) and side (SSC-A) scatter properties. DC stimulatory capacity was assessed based on T-cell proliferation determined via CFSE dilution (determined on the FITC channel) compared with unstimulated T-cells (cultured in the absence of DC). A second negative control included the culture of CFSE-negative T-cells. Histograms are representative from several independent experiments performed with similar results. Pooled results are displayed on the right plot.

Fig. 5. **Effect of oat peptides on the stimulatory capacity of DCs for allogeneic T-cells in CD.** DC stimulatory capacity for CFSE-labelled allogeneic T-cells was determined as in Figure 4. DC stimulatory capacity from both gluten-free diet coeliacpatients (GFD, red lines) and healthy controls (HC, blue lines) was determined following 24h pulsing with different stimuli as detailed in the graphs. Two-way ANOVA repeated was applied on pulsed DC (GFD or HC) compared with their basal paired counterparts. P-value<0.05 were considered as statistically significant (*p<0.05; **p<0.01; ***p<0.001).

TABLES

Table 1. Peptides derived from avenin sequences

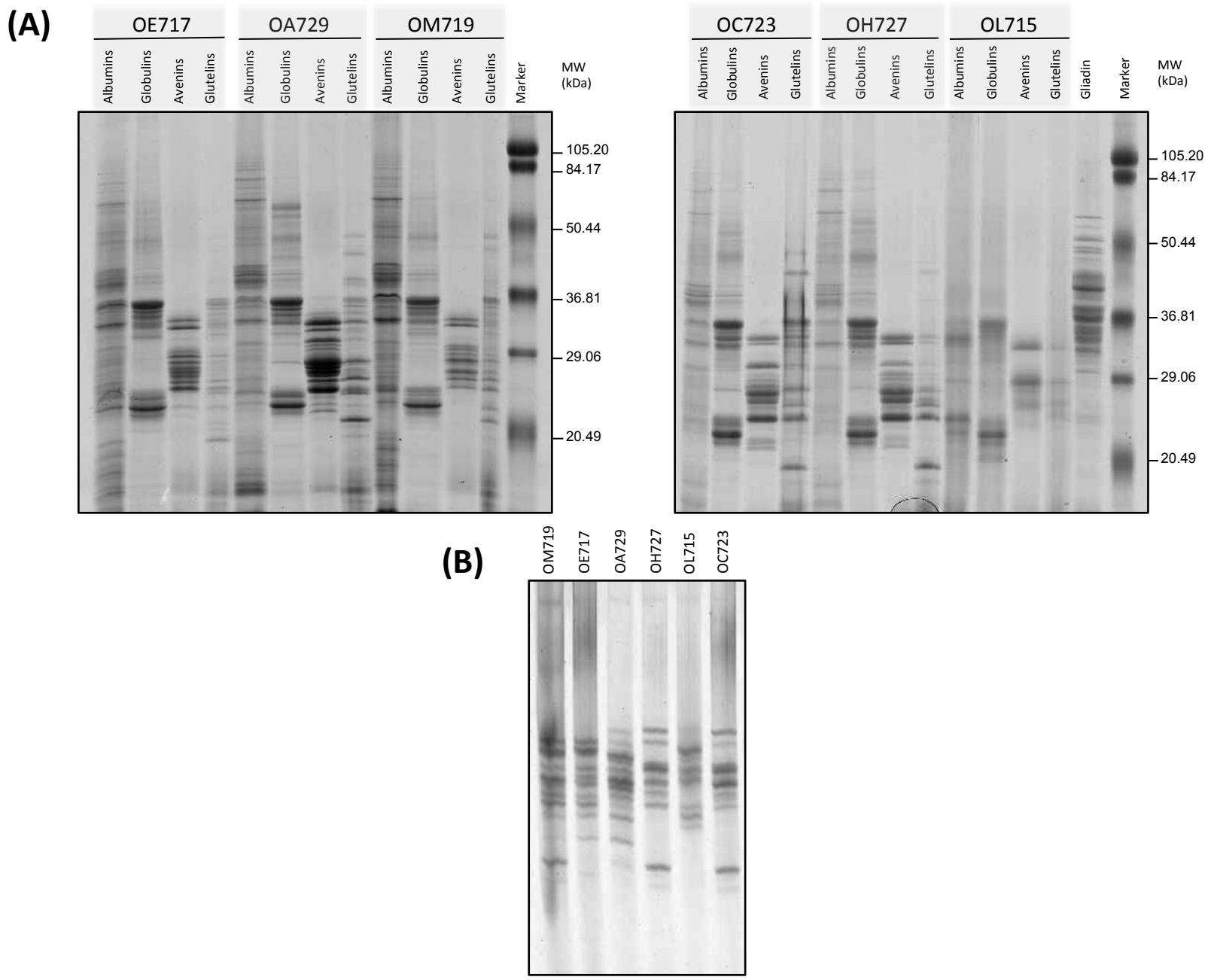
Gliadin-like avenins	
Peptide	*Sequence*
QL6	QPQLQL
QQ6	QPQLQQ
PV10	PYPEQQEPFV
EF27	EQYQPYPEQQEPFVQQQPPFVQQEQPF
Glutenin-like avenins	
Peptide	*Sequence*
QL14	QQPFMQQQPFMQPL
QM27	QYQPYPEQQPFMQQQQPFMQPLLQQQM

Table 2. List of identified proteins from 1D and 2D gels of avenin protein extract from oat accession OE717

Sample *	Accession	Cov (%)	Peptides	Mass	Description
A6	TR:G8ZCU1_9POAL	60	4	27344.3008	G8ZCU1 Avenin protein (Fragment) OS=Avena prostrata GN=avenin PE=4 SV=1
	TR:G8ZCT4_9POAL	59	4	29672.8789	G8ZCT4 Avenin protein (Fragment) OS=Avena longiglumis GN=avenin PE=4 SV=1
	TR:F2Q9W5_AVESA	53	3	31613.2422	F2Q9W5 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=4 SV=1
	SP:AVEF_AVESA	100	2	5213.6143	Q09097 Avenin-F (Fragment) OS=Avena sativa PE=1 SV=1
A7	TR:F2Q9W5_AVESA	55	6	31613.2422	F2Q9W5 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=4 SV=1
	TR:Q09072_AVESA	61	6	25471.4941	Q09072 Avenin (Precursor) OS=Avena sativa PE=2 SV=1
	TR:G8ZCU1_9POAL	57	6	27344.3008	G8ZCU1 Avenin protein (Fragment) OS=Avena prostrata GN=avenin PE=4 SV=1
	TR:G8ZCT1_9POAL	51	4	28834.0137	G8ZCT1 Avenin protein (Fragment) OS=Avena strigosa GN=avenin PE=4 SV=1
	SP:AVEE_AVESA	66	3	21036.1562	Q09114 Avenin-E OS=Avena sativa PE=1 SV=1
	TR:G8ZCT5_9POAL	53	4	28018.0723	G8ZCT5 Avenin protein (Fragment) OS=Avena damascena GN=avenin PE=4 SV=1
	TR:G8ZCT6_9POAL	53	4	28018.0723	G8ZCT6 Avenin protein (Fragment) OS=Avena strigosa GN=avenin PE=4 SV=1
	TR:G8ZCT7_9POAL	53	4	28018.0723	G8ZCT7 Avenin protein (Fragment) OS=Avena canariensis GN=avenin PE=4 SV=1
	TR:G8ZCV9_9POAL	57	4	26660.6230	G8ZCV9 Avenin protein (Fragment) OS=Avena murphyi GN=avenin PE=4 SV=1
	TR:G8ZCW4_9POAL	57	3	24318.9219	G8ZCW4 Avenin protein (Fragment) OS=Avena macrostachya GN=avenin PE=4 SV=1
	TR:F2Q9W3_AVESA	52	2	25732.9043	F2Q9W3 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=4 SV=1
	TR:G8ZCW2_9POAL	16	2	23778.5078	G8ZCW2 Avenin protein (Fragment) OS=Avena ventricosa GN=avenin PE=4 SV=1
	TR:G8ZCW5_9POAL	16	2	23778.5078	G8ZCW5 Avenin protein (Fragment) OS=Avena murphyi GN=avenin PE=4 SV=1
	TR:G8ZCV7_9POAL	16	2	24500.3652	G8ZCV7 Avenin protein (Fragment) OS=Avena magna GN=avenin PE=4 SV=1
	TR:G8ZCV8_9POAL	16	2	24500.3652	G8ZCV8 Avenin protein (Fragment) OS=Avena murphyi GN=avenin PE=4 SV=1
A8	TR:G8ZCW3_9POAL	52	6	27387.3730	G8ZCW3 Avenin protein (Fragment) OS=Avena insularis GN=avenin PE=4 SV=1
	TR:G8ZCT8_9POAL	50	6	28205.2715	G8ZCT8 Avenin protein (Fragment) OS=Avena canariensis GN=avenin PE=4 SV=1
	TR:G8ZCT9_9POAL	50	6	28205.2715	G8ZCT9 Avenin protein (Fragment) OS=Avena strigosa GN=avenin PE=4 SV=1
	TR:F2Q9W5_AVESA	45	6	31613.2422	F2Q9W5 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=4 SV=1
	TR:G8ZCU1_9POAL	45	6	27344.3008	G8ZCU1 Avenin protein (Fragment) OS=Avena prostrata GN=avenin PE=4 SV=1
	TR:G8ZCU6_9POAL	45	4	24307.0723	G8ZCU6 Avenin protein (Fragment) OS=Avena prostrata GN=avenin PE=4 SV=1
	TR:G8ZCT2_9POAL	44	4	24435.2031	G8ZCT2 Avenin protein (Fragment) OS=Avena prostrata GN=avenin PE=4 SV=1
	TR:F2Q9W4_AVESA	48	4	24631.5215	F2Q9W4 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=4 SV=1
	SP:AVEE_AVESA	52	4	21036.1562	Q09114 Avenin-E OS=Avena sativa PE=1 SV=1
	TR:Q09072_AVESA	53	4	25471.4941	Q09072 Avenin (Precursor) OS=Avena sativa PE=2 SV=1
	TR:G8ZCW4_9POAL	42	4	24318.9219	G8ZCW4 Avenin protein (Fragment) OS=Avena macrostachya GN=avenin PE=4 SV=1
	TR:F4MJY1_AVESA	40	4	25600.3555	F4MJY1 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=2 SV=1
	TR:G8ZCW0_9POAL	37	4	26011.8594	G8ZCW0 Avenin protein (Fragment) OS=Avena murphyi GN=avenin PE=4 SV=1
	TR:G8ZCU2_9POAL	55	4	24137.8320	G8ZCU2 Avenin protein (Fragment) OS=Avena longiglumis GN=avenin PE=4 SV=1
	TR:G8ZCU3_9POAL	55	4	24137.8320	G8ZCU3 Avenin protein (Fragment) OS=Avena strigosa GN=avenin PE=4 SV=1
	TR:G8ZCU4_9POAL	55	4	24111.7949	G8ZCU4 Avenin protein (Fragment) OS=Avena strigosa GN=avenin PE=4 SV=1
	TR:G8ZCU5_9POAL	44	4	23093.7402	G8ZCU5 Avenin protein (Fragment) OS=Avena longiglumis GN=avenin PE=4 SV=1
A9	SP:AVE3_AVESA	60	7	25275.4844	P80356 Avenin-3 OS=Avena sativa PE=1 SV=1
	TR:Q2EPY2_AVESA	65	7	24258.3320	Q2EPY2 Avenin OS=Avena sativa PE=4 SV=1
	TR:G8ZCU1_9POAL	51	6	27344.3008	G8ZCU1 Avenin protein (Fragment) OS=Avena prostrata GN=avenin PE=4 SV=1
	TR:G8ZCW3_9POAL	54	6	27387.3730	G8ZCW3 Avenin protein (Fragment) OS=Avena insularis GN=avenin PE=4 SV=1
	SP:AVEE_AVESA	66	5	21036.1562	Q09114 Avenin-E OS=Avena sativa PE=1 SV=1
	TR:Q09072_AVESA	51	4	25471.4941	Q09072 Avenin (Precursor) OS=Avena sativa PE=2 SV=1
	TR:G8ZCV7_9POAL	62	4	24500.3652	G8ZCV7 Avenin protein (Fragment) OS=Avena magna GN=avenin PE=4 SV=1
	TR:G8ZCV8_9POAL	53	4	24500.3652	G8ZCV8 Avenin protein (Fragment) OS=Avena murphyi GN=avenin PE=4 SV=1
	TR:G8ZCU0_9POAL	54	4	23039.7891	G8ZCU0 Avenin protein (Fragment) OS=Avena damascena GN=avenin PE=4 SV=1
	TR:G8ZCW4_9POAL	51	5	24318.9219	G8ZCW4 Avenin protein (Fragment) OS=Avena macrostachya GN=avenin PE=4 SV=1
	TR:G8ZCW0_9POAL	45	5	26011.8594	G8ZCW0 Avenin protein (Fragment) OS=Avena murphyi GN=avenin PE=4 SV=1
AS1	TR:G8ZCU1_9POAL	57	10	27344.3008	G8ZCU1 Avenin protein (Fragment) OS=Avena prostrata GN=avenin PE=4 SV=1
	TR:F2Q9W5_AVESA	50	9	31613.2422	F2Q9W5 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=4 SV=1
	TR:G8ZCT1_9POAL	53	6	28834.0137	G8ZCT1 Avenin protein (Fragment) OS=Avena strigosa GN=avenin PE=4 SV=1
	SP:AVEF_AVESA	84	3	5213.6143	Q09097 Avenin-F (Fragment) OS=Avena sativa PE=1 SV=1
AS3	TR:F2Q9W5_AVESA	48	6	31613.2422	F2Q9W5 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=4 SV=1
	TR:G8ZCU1_9POAL	50	6	27344.3008	G8ZCU1 Avenin protein (Fragment) OS=Avena prostrata GN=avenin PE=4 SV=1
	TR:G8ZCW4_9POAL	51	6	24318.9219	G8ZCW4 Avenin protein (Fragment) OS=Avena macrostachya GN=avenin PE=4 SV=1
	TR:F4MJY1_AVESA	57	6	25600.3555	F4MJY1 Avenin protein (Fragment) OS=Avena sativa GN=avenin PE=2 SV=1
	TR:G8ZCW0_9POAL	56	6	26011.8594	G8ZCW0 Avenin protein (Fragment) OS=Avena murphyi GN=avenin PE=4 SV=1
	TR:Q09072_AVESA	55	4	25471.4941	Q09072 Avenin (Precursor) OS=Avena sativa PE=2 SV=1
	SP:AVEE_AVESA	55	5	21036.1562	Q09114 Avenin-E OS=Avena sativa PE=1 SV=1
	SP:AVEF_AVESA	67	3	5213.6143	Q09097 Avenin-F (Fragment) OS=Avena sativa PE=1 SV=1

* Samples obtained from the 1D and 2D gel spots

Figure 1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OL715
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Globulins
Avenins
Glutelins
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105.20
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20.49
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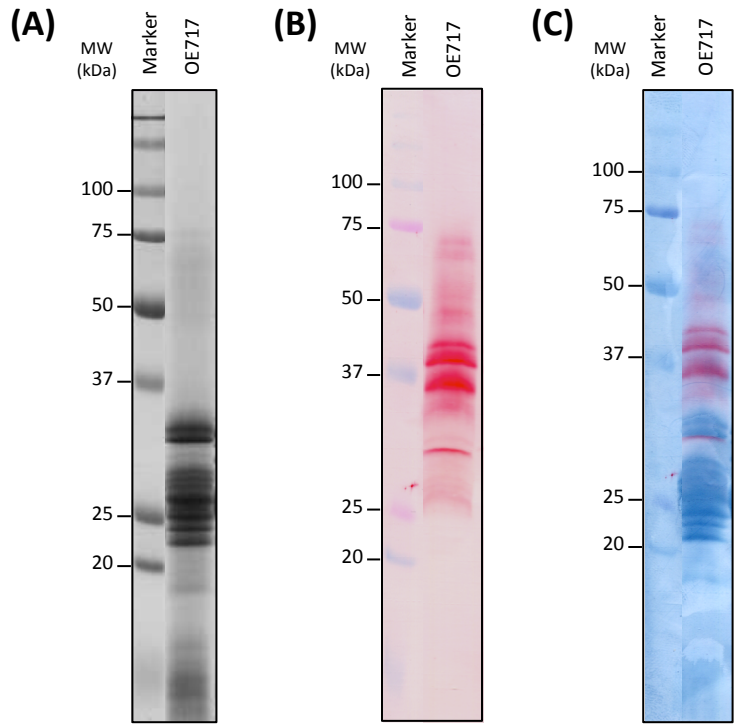
OE717
OA729
OH727
OL715
OC723

Figure 2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(C)
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OE717
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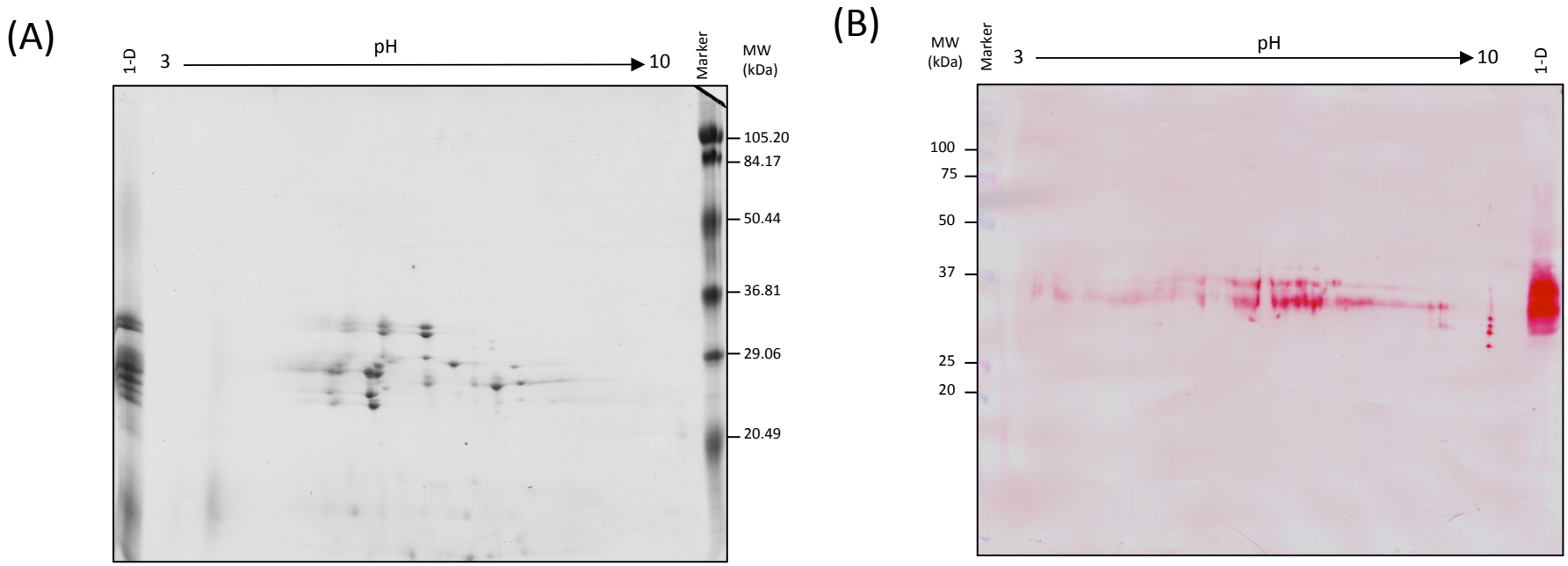
50
37
25
20

Figure 3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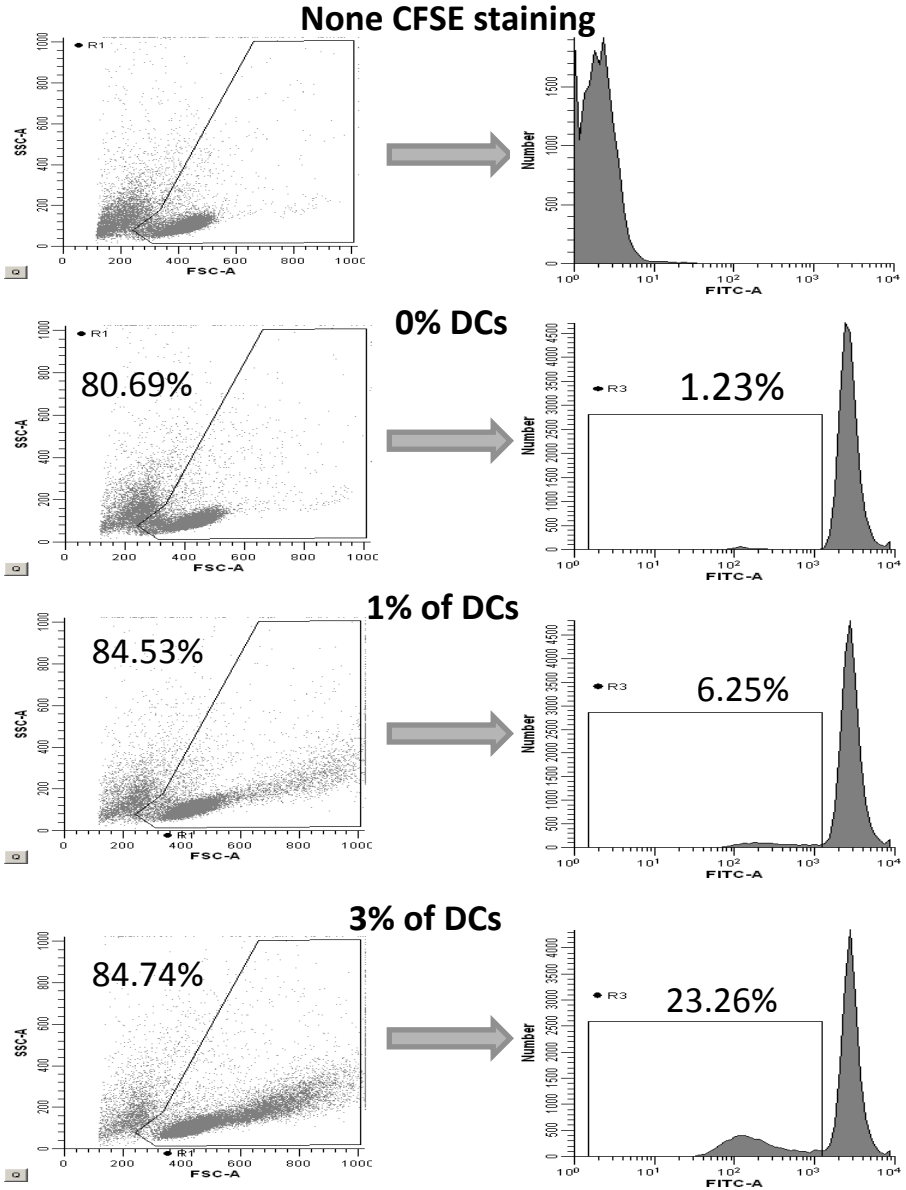
20

Figure 4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6.25%
3% of DCs
84.74%
23.26%

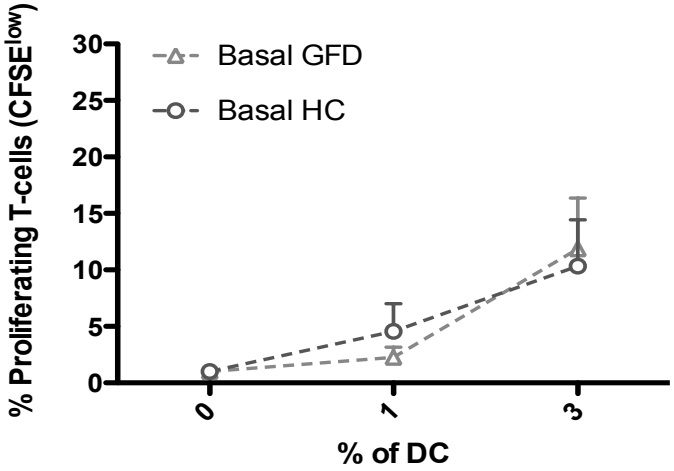
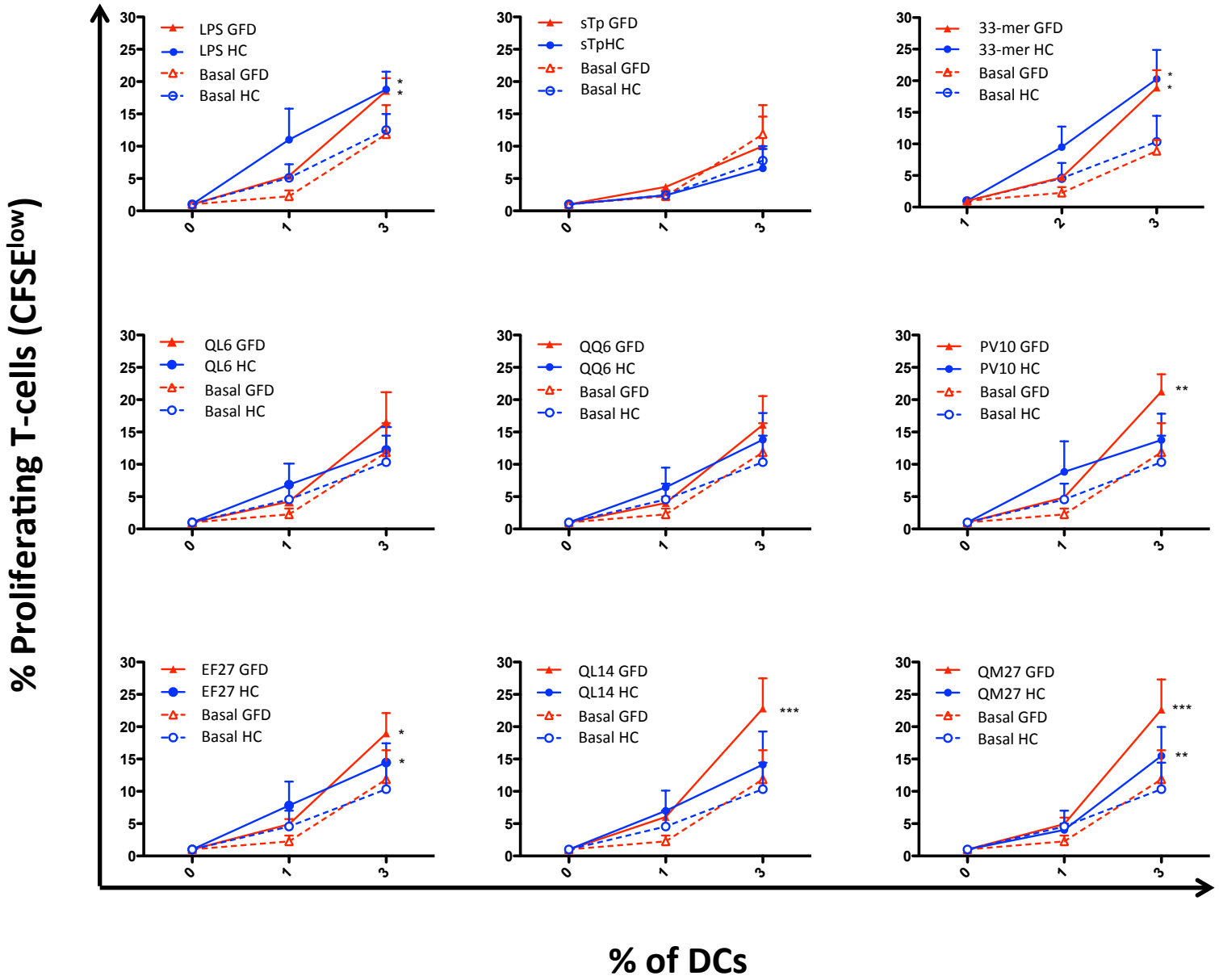

% Proliferating T-cells (CFSE^low)
Basal GFD
Basal HC
30
25
20
15
10
5
0
0
1
3
% of DC

Figure 5


% Proliferating T-cells (CFSE^low)
% of DCs
LPS GFD
LPS HC
Basal GFD
Basal HC
sTp GFD
sTpHC
Basal GFD
Basal HC
33-mer GFD
33-mer HC
Basal GFD
Basal HC
QL6 GFD
QL6 HC
Basal GFD
Basal HC
QQ6 GFD
QQ6 HC
Basal GFD
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QL14 HC
Basal GFD
Basal HC
QM27 GFD
QM27 HC
Basal GFD
Basal HC