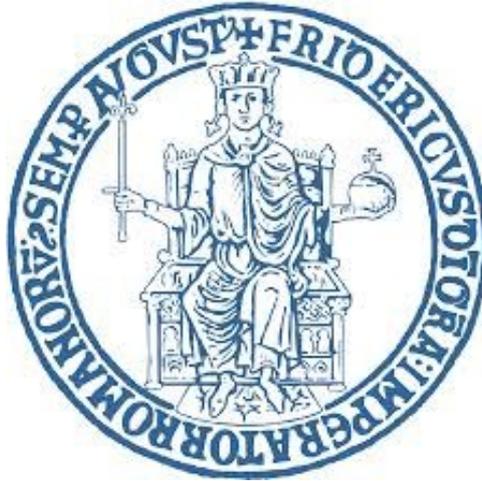


University of Naples “Federico II”



Polytechnic and Basic Sciences School

Doctorate in Applied Biology

XXVIIth Cycle

Bacillus subtilis spores as Mucosal Adjuvant

Coordinator

Ch.mo Prof. Ricca Ezio

PhD student

Dr. Bonavita Roberta

Tutor

Ch.mo Prof. Ricca Ezio

Supervisor

Dott. Rossi Mauro

<u>Index</u>	page 2
Summary	page 4
Chapter 1: Introduction	page 8
Bacillus subtilis	page 8
Celiac Disease	page 18
References	page 25
Chapter 2: Use of <i>Bacillus subtilis</i> spores to induce an intestinal immune response	
Introduction	page 29
Materials and Methods	page 32
Results	page 38
Discussion and Conclusion	page 49
References	page 53
Chapter 3: Biochemical characterization of transamidated gliadin	
Introduction	page 56
Materials and Methods	page 58
Results	page 62
Discussion and Conclusion	page 72
References	page 75

Chapter 4: Characterization of immunomodulatory molecules and their adsorption on Bacillus subtilis spores

Introduction	page 77
Materials and Methods	page 78
Results	page 83
Discussion and Conclusion	page 93
References	page 95

Summary

Recently, researches attention has been focused on the development of mucosal immunity strategies based on the use of probiotic spores as mucosal adjuvant. As described in the introduction (**Chapter 1**) *Bacillus subtilis* spores are a good system to surface display and vaccine delivery. An important advantage of a spore-based display system is its high stability.

Spores can survive under adverse environmental conditions and are resistant to proteolytic digestion. Therefore, they represent a good adjuvant to deliver antigens into the gastrointestinal tract and they could be used to mucosal vaccine. Also the mucosal vaccinology was used not only for responses toward pathogens but also for down-regulating inflammatory conditions. Among these conditions, celiac disease (CD) represents a very interesting model to test possible immunomodulatory strategies. So the aim of my research has been the use of *Bacillus subtilis* spores as a display system to development an immunomodulatory strategies for celiac disease. In particular, in **Chapter 2** I focused my attention on the adsorption reaction of *Bacillus subtilis* spores and gliadin. I preliminarily explored the possibility of adsorbing gliadin on *Bacillus subtilis* spores and then characterized the gliadin-specific T cell-mediated response elicited by the spore-adsorbed gliadin in DQ8 mice (a transgenic mouse model of gluten sensibility). A manuscript with the experiments described in this part of the thesis is in press (**Roberta Bonavita**, Rachele Isticato, Francesco Maurano, Ezio Ricca and Mauro Rossi, 2015. Mucosal immunity induced by gliadin-

presenting spores of *Bacillus subtilis* in HLA-DQ8-transgenic mice, Immunology letters 2015, *in press*).

In the second part of my research I focused my attention on the development of immunomodulatory strategy, based on the use of tolerogenic molecule to down regulate the inflammatory response induced by gliadin. Therefore, firstly I did biochemical analysis of soluble transamidated gliadin (spf), which is known to induce a decrease of INF γ into spleen cells and intestinal biopsies. I decided to characterize spf to identify similarity and differences with gliadin, in order to use transamidation products as alternative therapeutic strategies for celiac patients. The experiments of this biochemical characterization are described in the **Chapter 3**. Also, this part of my work has been published in 2013 (Maria F. Mazzeo, **Roberta Bonavita**, Francesco Maurano, Paolo Bergamo, Rosa A. Siciliano, Mauro Rossi, 2013. Biochemical modifications of gliadins induced by microbial transglutaminase on wheat flour. *Biochimica et Biophysica Acta* 1830:5166-74).

After this biochemical analysis I decided to use this modified gliadin to analyzed its immune response into DQ8 mice. So in the **Chapter 4** I put together experiments aimed at understanding immune response induced by spf and I compared this response with native gliadin. My experiments showed that spf is able to induce phenotypic shift of gliadin specific immune response. In fact it induced a regulatory response. This part of my research is the object of a publication that is still in preparation.

Finally, I verified the capability of spores to adsorb spf and other immunomodulatory molecules such as conjugated linoleic acid (CLA).

Chapter 1

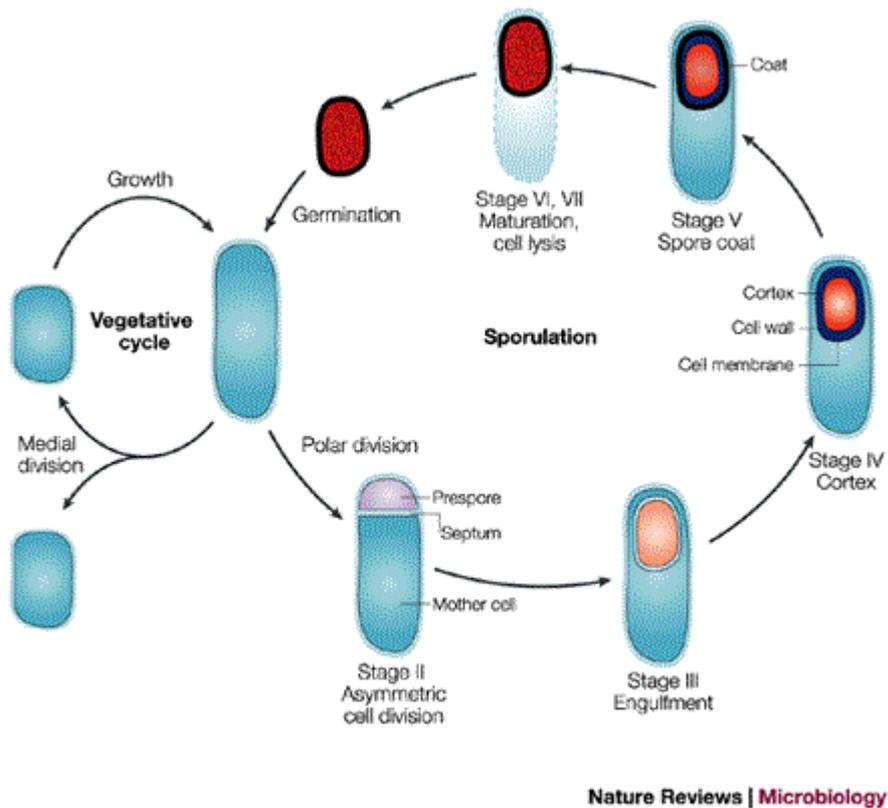
Introduction

Bacillus subtilis

The life cycle of *Bacillus subtilis*

Bacillus subtilis is a gram-positive bacterium. It is an aerobe with a doubling time of about 20 minutes when grown in rich medium at 37°C. During adverse environmental conditions, when either a nitrogen or carbon source becomes deficient, the vegetative cell initiates the process of sporulation. This process is characterized by an asymmetric division that gives rise to a larger mother cell and a smaller forespore, which matures into a spore (1, 2).

The bacterial spore is a dormant cell with high stability and it is able to survive in adverse conditions, such as high temperatures, pH extremes, detergents, hydrolytic enzymes and antibiotics (3). This dormant spore can survive for several years or until nutrients are available in the environment. In this condition spores go back to vegetative cell through the process of spore germination. The life cycle of *Bacillus subtilis* is summarized into Figure 1.1.



Nature Reviews | Microbiology

Figure 1.1: The sporulation cycle of *Bacillus subtilis* (Adapted from Errington J. Regulation of endospore formation in *Bacillus subtilis*. *Nat Rev Microbiol* 2003; 1: 117-26).

***Bacillus subtilis* sporulation**

When the nutrients are sufficient, *Bacillus subtilis* grows and divides into two genetically identical cells. Sporulation process is divided into seven stages based on morphological changes that give rise the two daughter cells different (Figure 1.1) (4). This is possible because they follow two different gene expression programmes controlled by four transcription factors.

Growing cells are in stage 0 and nuclear division precedes the initiation of sporulation. This is immediately followed by stage I, that is characterized by the presence of the two nucleoids in an axial filament. In stage II a septum is placed asymmetrically at one end of the cell such that it gives rise to two unequal compartments; a smaller forespore and a larger mother cell. The next stage is stage III, wherein the mother cell's membrane grows around the forespore until the forespore is engulfed. Therefore the forespore has two membranes that will give rise to the outer and inner spore membranes. During stage IV peptidoglycan is deposited between the inner and outer spore membranes forming the cortex. The germ cell wall is also formed under the cortex. Also during this stage the forespore becomes more acidic and core dehydration begins. The transition to state V is characterized by development of coat layer outside the outer forespore membrane and synthesis of many coat proteins. During stage VI the spore core becomes maximally dehydrated and spores become metabolically dormant. Finally, in stage VII, the spore is released by lysis of the mother cell.

Regulation of sporulation cycle

Different gene expression programme of two daughters cell is controlled by cascade of sigma factors. The first sigma factors to direct sporulation-specific gene expression is σ^H , which plays early role in sporulation and contributes to build the septum, together σ^A . Once the sporulation septum is formed, σ^F becomes active into forespore

compartment and activates transcription of genes involved in engulfment (5). When σ^F is active in prespore, σ^E becomes active into mother cell and the engulfment of forespore compartment is finished. After that another factor, σ^G becomes active into forespore, probably thanks to the presence of a channel. At this point the cytoplasm of the forespore dehydrates. Finally, last factor activated is σ^K , that it is required for the final events of sporulation, in particular for the synthesis of coat. The mechanism of regulation of gene expression into two cell compartments called criss-cross regulation and was born in 1992 (6) (Figure 1.2).

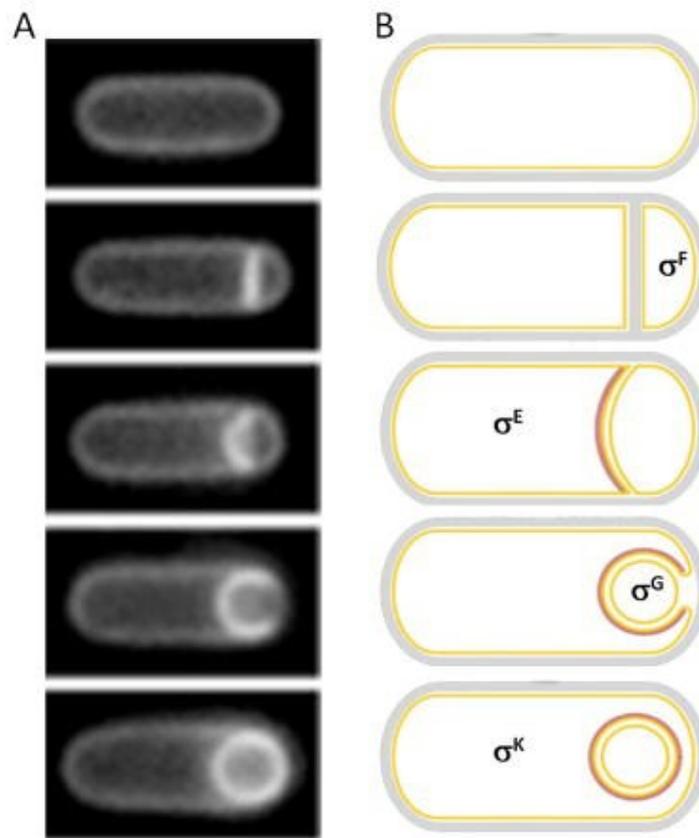


Figure 1.2: (A) Morphological asymmetry. During sporulation surrounds the cells form an asymmetric septum, which then surrounds the smaller cell. This process of engulfment is completed by the loss of attachment between the forespore and the mother cell. **(B) Genetic asymmetry.** These morphological changes are accompanied by activation of specific transcription factors in each compartment (Adapted from Higgins D, Dworkin J. Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiol Rev* 2012; 36: 131-48).

Spore structure

The bacterial spore consist in various layers: a core, surrounded by the inner membrane, the cortex, the outer membrane, the coat and spore crust (Figure 1.3).

The core is the innermost part of the spore. It contains the spore cytoplasm with all cellular components, such as cytoplasmic proteins, ribosomes and DNA. The water

content is 30–50 % of the core cytoplasm wet weight instead of the 70–88 % of the vegetative cytoplasm.

This dehydrated state plays an important role in spore longevity, dormancy and resistance. Other molecules that play an important role in spore resistance is dipicolinic acid (DPA) and acid-soluble spore proteins (SASP) associated with spore DNA. (7, 8, 9).

In coat formation the gene expression is regulated by coat proteins with morphogenetic activity. These proteins are: SpoIVA, SpoVID, CotE and CotH.

SpoIVA is produced in the mother cell, under the control of σ^E and it is essential for the synthesis of spore cortex and for the localization of SpoVID and CotE (10).

CotE is a 24 kDa produced in mother cell compartment and surrounds the engulfed forespore (11). The inner coat proteins collect in the space between CotE and the forespore surface, while the outer coat proteins assemble around the CotE layer. The formation of the outercoat involves CotE together with CotH (12). Also CotE interacts with many spore coat components (13).

CotH is a 42.8 kDa protein that has a morphogenetic role in the assembly of coat components and in the development of lysozyme resistance of spore (12). In the mother cell compartment CotH acts as a chaperone and also it is essential for stabilization of CotG and CotC.

The spore core is surrounded by the inner membrane, which is the site where spore germination receptors are located. Inner membrane is rounded by cortex. The cortex

is composed of peptidoglycan and it has a crucial importance for the maintenance of spore core dehydration. The inner part of the cortex is called the germ cell wall or primordial cell wall and it is also composed of peptidoglycan. The germ cell wall becomes cell wall of the freshly germinated spore following spore germination. Around the cortex there is the outer membrane. The outer membrane, deriving from engulfment, has opposite polarity with respect to the inner membrane (Figure 1.3).

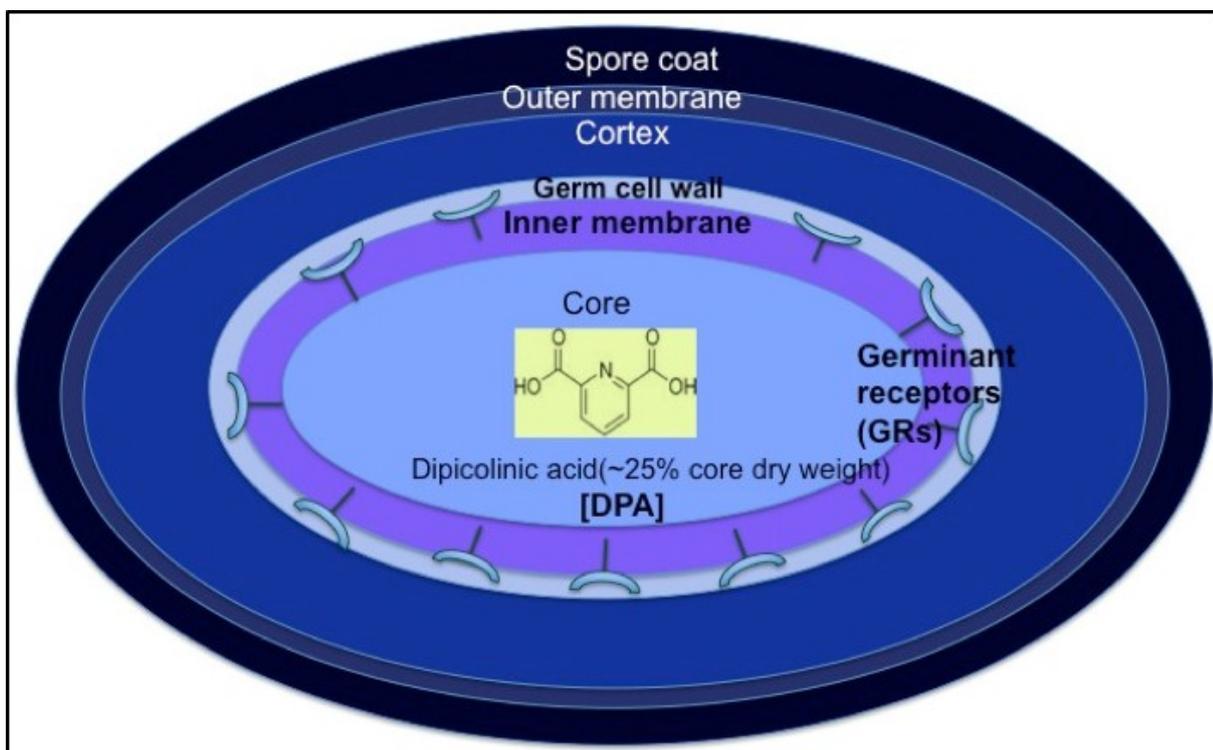


Figure 1.3: Structure of spore. An artistic representation of spore and its layers.

Use of Bacillus spores as a display system

Recent studies show a novel surface display system based on the use of bacterial spores. There are many advantages in the use of spores, such as stability even after a

prolonged storage, resistance to lysozyme so they can cross gastrointestinal tract and finally the possibility to display large multimeric protein because the protein are produced in the mother cell and assembled around the forming spore without transport through the membrane (14).

The spore-based display system requires the construction of a gene fusion between gene coding for spore surface protein (carrier) and the heterologous DNA coding for the protein to be displayed (Figure 1.4). With this process many heterologous protein are expressed on the surface of spores and they are used for many application, such as vaccine vehicles (15).

Two antigen were initially selected to bind spore coat protein: C-terminal fragment of the tetanus toxin (TTFC) and B subunit of the heat labile toxin (LTB). The strategy to obtain recombinant *Bacillus subtilis* spores expressing CotB-TTFC or CotB-LTB on their surface was based on use of the *cotB* gene and its promoter for the construction of translational fusions and chromosomal integration of the *cotB-tetC* and *cotB-eltB* gene fusions into the coding sequence of gene *amyE*. Another coat protein that it is used as a carrier is CotC (16).

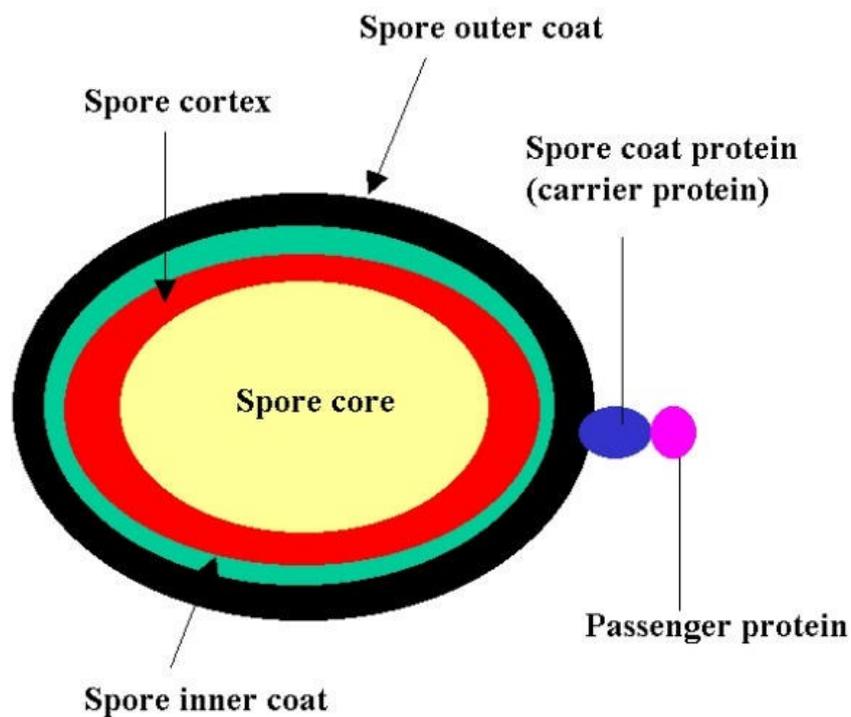


Figure 1.4: Spore surface-display. A schematic image of spore display system. (Adapted from Ricca E and Cutting SM: Emerging Applications of Bacterial Spores in Nanobiotechnology. *J Nanobiotechnology* 2003; 1: 6)

Spore surface-display system based on non-recombinant approach

The major disadvantages of recombinant approach are the release of live recombinant organisms into nature, raising concerns over the use and clearance of genetically modified microorganisms. To overcome this obstacle, a non-recombinant approach to use spores as a display system has been recently proposed. In the first study suggesting that heterologous proteins can be adsorbed on the spore surface, the mammalian NADPH-cytochrome P450 reductase (CPR), a diflavin-containing enzyme, was over-expressed in sporulating *Bacillus subtilis* cells and released into the culture medium after sporulation by autolysis of the mother cell and was found

associated to spore surface. So the protein was spontaneously bound to spore surface (17). In this first study the authors did not observe the mechanism involved into spontaneously adsorption. An essential characteristic of adsorption reaction is the pH of the binding buffer. In fact we have been shown that when the pH of the buffer is acid (pH 4) there is an increase of binding efficiency (18). Electrostatic and hydrophobic forces were suggested to drive the adsorption reaction.

Also recent studies shown that mutant spores with a strongly altered (*cotH*) or completely lacking (*cotE*) outer coat adsorbed heterologous protein more efficiently than wild-type (wt) spores do (19, 20). Interestingly, autoclaved spores were also found to be effective in adsorbing heterologous proteins and stimulating an immunological response towards an host antigen (21). So, the spores as antigen vehicle by nasal or oral route, represent a promising tool in mucosal vaccinology. Mucosal vaccinology could be instrumental not only to induce response toward antigen but also to down-regulate an inflammatory condition. In this case celiac disease represents an interesting model to test immunomodulatory strategies.

Celiac Disease

Celiac disease: definition and etiopathogenesis

Celiac disease is an immune-mediated enteropathy caused by ingestion of wheat gluten and prolamins, generally developed in genetically susceptible individuals.

Approximately 90% of patients with celiac disease have HLA DQ2 which is generally in tight linkage with the DR3 haplotype, and most of the remaining patients (8% to 10%) have HLA DQ8 associated with the DR4 (22). Therefore, individuals with celiac disease almost always have the DQ2 or DQ8 molecule: DQ2 is present in approximately 30% of the general population and DQ8 is present in 40% of the general population (23).

Celiac disease is a multifactorial disorder with genetic factor and environmental factor: gluten. Gluten ingestion caused villus atrophy, hypertrophic crypts, and infiltration of intraepithelial lymphocytes (IELs).

Gluten is a mixture of proteins: gliadins and glutenins. Gliadin is considered the toxic component responsible of celiac damages into intestinal mucosa of celiac patients.

Biochemical characteristic of gliadin

The gliadin fraction has been grouped in three classes named “ α ,” “ γ ,” and “ ω ” based on biochemical analysis that revealed a tight structural relationship among the various constituents. The numerous components of gliadin can be classified according to their primary structure into omega 5, omega 1,2, alpha- and gamma-type. Omega-types have almost entirely repetitive amino acid sequences consisting of glutamine, proline and phenylalanine. While alpha and gamma-type gliadins contain four and five different domains, respectively. Unique for each alpha and gamma-type is domain I, which consists mostly of repetitive sequences rich in glutamine, proline and aromatic amino acids. In *vitro* testing of gliadin peptides revealed that domain I of alpha-type gliadins is involved in activating celiac disease. The sequences -Pro-Ser-Gln-Gln- and -Gln-Gln-Gln-Pro- were demonstrated to be common for toxic gliadin peptides (24). Have been identified some of protein with “toxic” activity as listed in Table 1 (25).

Tab1 Toxic gliadin peptides

Amino acid sequences	Position	Toxicity	References
VPVQLQPQNPSQQQPQEQ	α 3–21	–	[22,36,37]
PGQQQPFPPQQPY	α 31–43	+	[39]
PGQQQPFPPQQPYQPQPF	α 31–49	+	[22,36,37]
PGQQQPFPPQQPYQPQPFPSQQPY	α 31–55	+	[36,39]
PQPQPFPSQQPY	α 44–55	+	[39]
SQQPYLQLQPFPPQQLPY	α 51–70	+	[40]
LQLQPFPPQQLPYQPQQLPY	α 56–75	+	[57]
QYPLGQGSFRPSQQNPQA	α 202–220	–	[37]
LGQGSFRPSQQN	α 206–217	+	[38]

A binding motif for the HLA-DQ8 molecule has been described by using a combination of computational sequencing data, in vitro binding assays (26, 27), and x-ray crystallography (28). The motif consists of five anchor regions: P1 and P9 anchors require negative or polar residues; P4 with aliphatic/aromatic, neutral residues; P6 with small aliphatic amino acids and any residue at P7 (Figure 1.5).

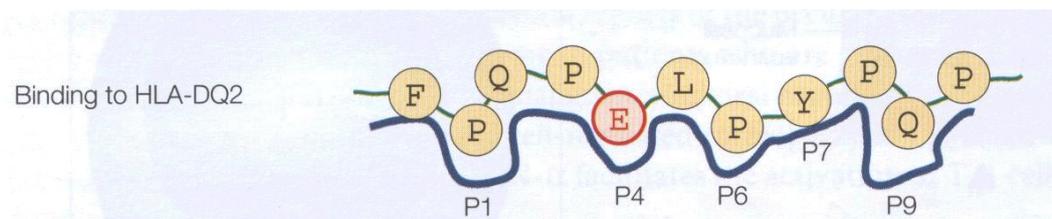


Figure 1.5: Binding motif of HLA-DQ. Schematic representation of binding motif of HLA-DQ with pocket anchors crucial for gliadin binding (Adapted by Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol*, 2002, 2: 647-55).

The high content of glutamine makes gluten a good substrate for tissue transglutaminase (tTG) (29).

This enzyme is constitutively expressed into lamina propria and it converts glutamine into the negatively charged glutamic acid, a process called deamidation. This process has a key role into activation of immune response toward gliadin.

Innate and Adaptive immune response

Tissue transglutaminase plays critical role in the development of immune response toward gluten. It deamidates gliadin in the lamina propria causing change of the some glutamine residues into glutamic acid. Deamidated peptides bind with far greater affinity to specific positively charged residues on the peptide-binding groove of HLA-DQ2 or HLA-DQ8 major histocompatibility complex (MHC) class II molecules. These peptides are then presented by HLA-DQ2 or HLA-DQ8 to activate gliadin-specific CD4⁺T cells thereby activating an intestinal inflammatory response (29).

This inflammatory disease is characterized by high production of IFN- γ and a decrease of IL10. So the phenotype of adaptive immune response is Th1. CD4⁺T cells stimulate cytotoxic T cells and fibroblasts to produce a particular matrix metalloproteinase pattern which is responsible for degradation of both extracellular matrix and basement membrane. Activated T cells also become able to trigger enterocyte apoptosis by producing molecules like Fas ligand and granzyme, which are responsible for cytotoxicity, leading to the characteristic mucosal lesions. Stimulated CD4⁺T cells are also able to induce lymphocyte B differentiation into plasma cells producing specific anti-gliadin and anti-tissue transglutaminase antibodies (25).

Also gluten stimulates IL-15 production, which characterizes innate response. IL-15 causes an upregulation of stress protein by enterocytes that are recognized by the

natural killer receptors present on intraepithelial lymphocytes. Therefore in CD are induced two effector immune responses: adaptive (a gluten specific CD4⁺Tcell response) and innate (mediated by intraepithelial lymphocyte) summarized into Figure 1.6.

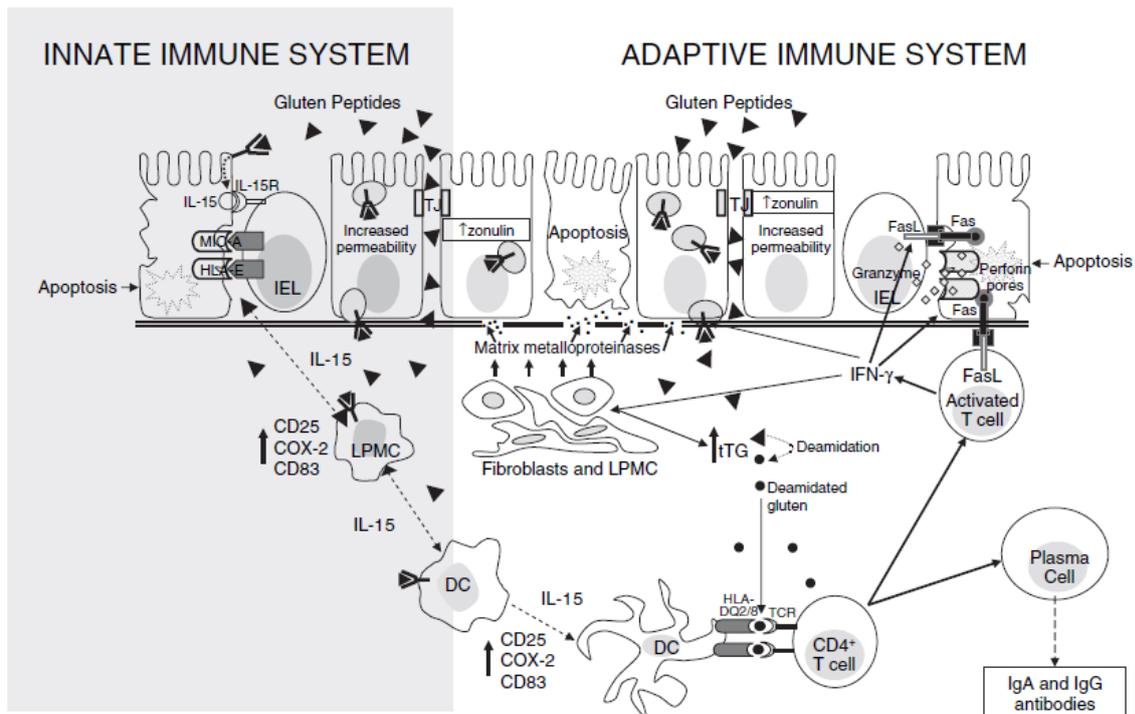


Figure 1.6: Schematic representation of immune response toward gluten (Adapted by Ciccocioppo R, Di Sabatino A, Corazza GR. The immune recognition of gluten in coeliac disease. Clin Exp Immunol 2005; 140: 408–16)

Therapeutical Strategies

The treatment for celiac disease is a gluten free diet. This involves elimination of the grains containing gluten, wheat, rye and barley, as well as food products and

additives derived from them (30). Maintaining a gluten free diet improves the health and quality of life, but there is low adherence to diet especially for adult patients.

There are numerous reasons why there is low adherence to the diet. They include palatability, cost and availability, inadequate food labeling as well as social pressures and quality-of-life issues (17).

Therefore many studies are focused to develop new therapeutical strategies alternative to the gluten free diet.

Non dietary therapies have focused on three main areas: to decrease gluten exposure, to modify intestinal permeability and to modulate immune activation.

Kapoerchan et al (31) have shown that gluten peptides can be modified at specific positions with an amino acid substitution, without affecting their affinity for HLA-DQ2, and that these constructs can compete with native gluten peptides and prohibit recognition by HLADQ2-specific T-cells. These antagonist peptides might be a therapeutic option to treat CD patients.

A major approach that has been explored is the enzymatic degradation of the large, immunogenic gliadin peptides into small non toxic fragments. This can be performed by prolyl endopeptidases (PEPs). These are proteases able to degrade the proline-rich gluten peptides into smaller, less immunogenic fragments. This can be achieved by bacterial, or fungal enzymes (29, 32). Alternatively, probiotics have been demonstrated to degrade gluten and exert a protective effect on the damage exerted by gluten on cell cultures (33).

Another therapeutic target is to prevent the migration of luminal gluten peptides across the intestinal epithelium. Therefore a peptide that inhibits the action of zonulin and the increase of intestinal permeability have been discovered (34).

Another potential target of drug therapy is modulation of the immune response to gluten. This may be achieved by preventing gliadin deamidation through the inhibition of tissue transglutaminase, by preventing HLA presentation through blocking the HLA DQ2 or DQ8 molecules, or by modulating cytokine production.

There are many studies that showed modulation of cytokine production and they are based on modification of wheat flour or immunomodulatory strategies to tolerize toward gluten. Gianfrani et al 2007 (35) and Lombardi et al 2013 (36) showed that the transamidation of wheat flour with microbial transglutaminase can be used to block the T-cell-mediated gliadin activity.

Also Rossi et al (37) showed that intranasal administration of gliadin in mice systematically immunized with gliadin induced an IFN γ decrease. This results highlighted the potential usefulness of ag delivery by nasal route as useful vaccine strategy.

References

- 1 Driks A. Overview: Development in bacteria: spore formation in *Bacillus subtilis*. *Cell Mol Life Sci* 2002; 59: 389-91.
- 2 Piggot PJ, Hilbert DW. Sporulation of *Bacillus subtilis*. *Curr Opin Microbiol* 2004; 7: 579-86.
- 3 Nicholson WL, Munakata N, Horneck G, Melosh HJ and Setlow P. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* 2000; 64: 548-72.
- 4 Stragier P, Losick R. Molecular genetics of sporulation in *Bacillus subtilis*. *Annu Rev Genet* 1996; 30: 297-341.
- 5 Sun D, Stragier P, Setlow P. Identification of a new σ -factor involved in compartmentalized gene expression during sporulation of *Bacillus subtilis*. *Genes Dev* 1989; 3: 141-49.
- 6 Stragier P. Establishment of forespore-specific gene expression during sporulation of *Bacillus subtilis*. In Cole JA, Mohan F, and Dow C editors. Prokaryotic structure and function. Society for General Microbiology, London, United Kingdom. 1992. p. 297-310.
- 7 Setlow P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol* 2006; 101: 514-25.
- 8 Setlow P. I will survive: protecting and repairing spore DNA. *J Bacteriol* 1992; 174: 2737-41.
- 9 Setlow P. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annu Rev Microbiol* 1995; 49: 29-54.
- 10 Driks A, Roels S, Beall B, Moran CP, Jr Losick R. Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. *Genes Dev* 1994; 8: 234-44.
- 11 Zheng L, Losick R. Cascade regulation of spore coat gene expression in *Bacillus subtilis*. *J Mol Biol* 1990; 212: 645-60.
- 12 Nacleiro G, Baccigalupi L, Zilhao R, De Felice M and Ricca E. *Bacillus subtilis* spore coat assembly require *cotH* gene expression. *J Bacteriol* 1996; 178: 4375-80.
- 13 Kim H, Hahn M, Grabowski P, McPherson DC, Otte MM, Wang R, Ferguson CC, Eichenberger P and Driks A. The *Bacillus subtilis* spore coat protein interaction network. *Mol Microbiol* 2006; 59: 487-502.
- 14 Istatico R, Cangiano G, Tran HT, Ciabattini A, Medagliani D, Oggioni R. M, De Felice M, Pozzi G and Ricca E. Surface display of recombinant proteins on *Bacillus subtilis* Spores. *J Bacteriol* 2001; 183: 6294-01.
- 15 Ricca E, Cutting S: Emerging Applications of Bacterial Spores in Nanobiotechnology. *J Nanobiotechnology* 2003; 1: 6
- 16 Mauriello EMF, Duc LH, Istatico R, Cangiano G, Hong HA, De Felice M, Ricca E, Cutting SM. Display of Heterologous Antigens on the *Bacillus subtilis* Spore Coat Using CotC as a Fusion Partner. *Vaccine* 2003; 22: 1177-88

- 17** Lee A. and Newman JM. Celiac diet: Its impact on quality of life. *J Am Diet Assoc* 2003; 103: 1533-35.
- 18** Huang JM, Hong HA, Van Tong H, Hoang TH, Brisson A, Cutting SM. Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine* 2010; 28: 1021-30
- 19** Sirec T, Strazzulli A, Isticato R, De Felice M, Moracci M, Ricca E. Adsorption of beta-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. *Microb Cell Fact* 2012; 11: 100.
- 20** Ricca E, Baccigalupi L, Cangiano G, De Felice M, Isticato R. Mucosal vaccine delivery by non-recombinant spores of *Bacillus subtilis*. *Microbial Cell Factories* 2014; 13: 115.
- 21** Song M, Hong HA, Huang JM, Colenutt C, Khang DD, Nguyend TVA, ParK SM, Shim BS, Song HH, Cheon IS, Jang JE, Choi JA, Choi YK, Stadler K and Cutting SM. Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. *Vaccine* 2012; 30: 3266-77.
- 22** Farre C, Humbert P, Vilar P, Aldequer X, Carnicer J, Carballo M, Gassul MA. Serological markers and HLA-DQ2 haplotype among first-degree relatives of celiac patients. Catalanian Coeliac Disease Study Group. *Dig Dis Sci* 1999; 44: 2344-49.
- 23** Sollid LM, Thorsby E. HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterol* 1993; 105: 910-22.
- 24** Wieser H, Modl A, Seilmeier W and Belitz HD. High-performance liquid chromatography of gliadins from different wheat varieties: amino acid composition and N-terminal amino acid sequence of components. *Z Lebens Unters Forsch* 1987; 185: 371-78.
- 25** Ciccocioppo R, Di Sabatino A and Corazza GR. The immune recognition of gluten in coeliac disease. *Clin Exp Immunol* 2005; 140: 408-16.
- 26** Godkin AJ, Davenport MP, Willis A, Jewell DP and Hill AV. Use of complete eluted peptide sequence data from HLA-DR and -DQ molecules to predict T cell epitopes, and the influence of the nonbinding terminal regions of ligands in epitope selection. *J Immunol* 1998; 161: 850-58.
- 27** Godkin A, Friede T, Davenport M, Stevanovic S, Willis A, Jewell D, Hill A, and Rammensee HG. Use of eluted peptide sequence data to identify the binding characteristics of peptides to the insulin-dependent diabetes susceptibility allele HLA-DQ8 (DQ 3.2). *Int Immunol* 1997; 9: 905-11.
- 28** Lee KH, Wucherpfennig KW and Wiley DC. Structure of a human insulin peptide-HLA-DQ8 complex and susceptibility to type 1 diabetes. *Nat Immunol* 2001; 2: 501-07.
- 29** Stepniak D and Koning F. Celiac Disease-Sandwiched between Innate and Adaptive Immunity. *Hum Immunol* 2006; 67: 460-68.
- 30** Green PH and Jabri B. Coeliac disease. *Lancet* 2003; 362: 383-91.

- 31** Kapoerchan VV, Wiesner M, Overhand M, van der Marel GA, Koning F and Overkleeft HS. Design of azidoproline containing gluten peptides to suppress CD4⁺T cell responses associated with celiac disease. *Bioorg Med Chem* 2008; 16: 2053–62.
- 32** Piper JL, Gray GM and Khosla C. Effect of prolyl endopeptidase on digestive-resistant gliadin peptides in vivo. *J Pharmacol Exp Ther* 2004; 311: 213-19
- 33** Lindfors K, Blomqvist T, Juuti-Uusitalo K, Stenman S, Venalainen J, Maki M, Kaukinen K. Live probiotic *Bifidobacterium lactis* bacteria inhibit the toxic effects induced by wheat gliadin in epithelial cell culture. *Clin Exp Immunol* 2008; 152: 552-8.
- 34** Paterson BM, Lammers KM, Arrieta MC, Fasano A and Meddings JB. The safety, tolerance, pharmacokinetic and pharmacodynamic effects of single doses of AT-1001 in coeliac disease subjects: a proof of concept study. *Aliment Pharmacol Ther* 2007; 26: 757-66.
- 35** Gianfrani C, Siciliano RA, Facchiano AM, Camarca A, Mazzeo MF, Costantini S, Salvati VM, Maurano F, Mazzarella G, Iaquinto G, Bergamo P and Rossi M. Transamidation of wheat flour inhibits the response to gliadin of intestinal T cells in celiac disease. *Gastroenterol* 2007; 133: 780-89.
- 36** Lombardi E, Maurano F, Bergamo P, Bozzella G, Luongo D, Mazzarella G, Rotondi Aufiero V, Iaquinto G, Rossi M. Selective inhibition of gliadin immune reactivity by transamidation of wheat flour with microbial transglutaminase. *J Leukoc Biol* 2013; 93: 479–88.
- 37** Rossi M, Maurano F, Caputo N, Auricchio S, Sette A, Capparelli R and Troncone R. Intravenous or Intranasal Administration of Gliadin is Able to Down-Regulate the Specific Immune Response in Mice. *Scand J Immunol* 1999; 50: 177-82.

Chapter 2

Use of Bacillus subtilis spores to induce an intestinal immune response

“published on Immunology Letters 2015 in press”

Introduction

As described in the Chapter 1, probiotic bacterial strains represent very promising new tool for active vaccination. They are used to vehicular antigen toward target cell, but also for down-regulating adverse immune responses and tolerance induction. The development of immunomodulatory strategy based on mucosal immunity generally requires efficient antigen delivery and adjuvant systems.

Initially, the adjuvant system used ADP-ribosylating enterotoxins, LT and CT (1). However, CT as well as LT are normally considered to be too toxic for human use. Recently, attention has been focused on spores produced by Gram positive bacteria, such as *Clostridium* or *Bacillus* genres (2). Specifically, *Bacillus subtilis* spores have been engineered to display antigens (3, 4) as well active enzymes (6). Spore display system has many advantages. An important advantage of a spore-based display system is its high stability. In fact spores can survive under adverse environmental conditions for a long period and are resistant to proteolytic digestion (6). Therefore, they potentially represent a good adjuvant to deliver antigens into the gastrointestinal tract and to induce a specific immune response (7).

A spore display system normally requires fusion between genes encoding for spore coat protein, called carrier and a DNA coding for heterologous passenger protein. This construct after transcription process is displayed on spore surface. Genetic manipulation has many obstacle, therefore more recently, a non recombinant approach has been developed. Non recombinant approach are based on the

spontaneously stable adsorption of an antigen or foreign protein on the spore surface. There are many studies that show this adsorption reaction and have been shown that mutant spores with a strongly altered (*cotH*) or completely lacking (*cotE*) outer coat adsorbed heterologous protein more efficiently than wild-type (wt) spores (8, 9). Interestingly, autoclaved spores were also found to be effective in adsorbing heterologous proteins and stimulating an immunological response towards an host antigen (10). So, it can be concluded that spores are a useful vaccine system.

Mucosal vaccinology could be instrumental not only for boosting responses towards pathogens but also for preventing/down-regulating ongoing intestinal inflammatory conditions. Among these conditions, celiac disease (CD) represents a very interesting model to test possible immunomodulatory strategies, considering that the eliciting antigen (wheat gliadin) and the immunopathogenic mechanisms are known.

CD is an immune-mediated enteropathy triggered by wheat gliadin in HLA-DQ2 or HLA-DQ8-expressing individuals. This disease is characterised by activation of intestinal gliadin-specific CD4⁺T cells with a Th1 phenotype, which appear to play a major role in the induction of villus atrophy and increased intestinal permeability (11). HLA-DQ8-transgenic mice have widely been used both to further dissect the molecular mechanisms underlying CD pathogenesis and to develop possible immunomodulatory strategies (12, 13, 14). So we preliminarily explored the possibility of adsorbing gliadin on *Bacillus subtilis* spores and then characterised the

gliadin-specific T cell-mediated response elicited by the spore-adsorbed gliadin in DQ8 mice.

Materials and Methods

Strains

The *Bacillus subtilis* strains used in this study were PY79 (wt) (7) and its isogenic mutant derivatives ER220 (*cotH*-null mutant) (15) and DL76 (*cotE*-null mutant) (16). Sporulation of *Bacillus subtilis* was induced by growth in Difco Sporulation Media (SPM) at 37°C for 30 h with strong shaking. After incubation at 37°C, spores were collected and centrifuged 10 min at 7000 g. Then, the pellet was resuspended in distilled water. This washing step was repeated several times. Spores were separated from vegetative cells by a renografin gradient purification step (17). Spore amount was determined by plate counting of spore serial dilutions.

Antigen preparation

Gliadin was extracted from wheat flour by a modified Osborne protocol (18). Briefly wheat flour was resuspended in 0.4 M NaCl and was centrifuged at 1000 rpm for 10 min. Then the pellet was washed for several times and gliadin was extracted in 70% ethanol. The supernatant, which contained gliadin, was recovered and freeze-dried. Protein content was measured by Bradford analysis (19). Gliadin stock solution was prepared by suspending 10 mg gliadin in 50 mM acetic acid.

Binding assay

In total, 2×10^9 spores were incubated with 125 μg native gliadin from the stock solution in 200 μl of 50 mM sodium acetate, pH 4.0 (adsorption solution), for 1 h at RT by continuous shaking (7). The spores were centrifuged at 13000 g for 10 min and washed three times to remove unbound gliadin. The pellet (spore-adsorbed gliadin) was used for *in vitro* and *in vivo* experiments. Gliadin adsorption was evaluated by dot blot and Bradford analyses (19). The dot blot analysis was performed as previously described (20). The membrane was probed with an in-house-produced mouse polyclonal antibody against wheat gliadin (1:5000 dilution) and then with biotinylated streptavidin peroxidase-conjugated anti-mouse IgGs (1:10000 dilution; Dako SpA, Milano, Italy).

Immunodetection was performed using ECL reagent (Amersham-GE Healthcare Europe GmbH, Glattbrugg, Switzerland) and a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA). For Bradford analysis, the amount of spore-adsorbed gliadin was calculated as the difference in the protein content of the adsorption solution before and after incubation.

Mice and immunization protocol

Transgenic mice expressing the HLA-DQ8 molecule were maintained under pathogen-free conditions at our animal facility (Accreditation No. DM.161/99). The animals were reared on a gluten-free diet for several generations and were used at the

age of 6-12 weeks. The experiments were approved by the National Institutional Review Committee and performed in accordance with European regulations (EU Directive 2010/63/EU). In tolerization experiments, mice were nasally administered multiple doses of spore-adsorbed gliadin (days: -6, -5, -4, and -3; 1, 2, 3, and 4; and 8, 9, 10, and 11; 2×10^9 spores/dose). The control groups were mice instilled with multiple doses of free gliadin (40 μ g) dissolved in water or with water alone. On days 0, 7 and 14 the mice were injected intraperitoneally (i.p.) with gliadin emulsified with FCA (day 0) or IFA (days 7 and 14). The mice were sacrificed on day 21. In immunisation experiments, mice were nasally administered seven doses of spore-adsorbed gliadin (2×10^9 spores/dose) or an equivalent amount of free gliadin at weekly intervals. The mice were sacrificed one week later to collect the spleens and mesenteric lymph nodes (MLNs). Also in another immunization experiment mice were intragastric immunized with spore-adsorbed gliadin (2×10^9 spores/dose) one dose a week for six weeks. The mice were sacrificed one week later to collect spleens and MLNs.

Isolation of T cells and dendritic cells (DCs) from DQ8 mice

The spleens and MLNs were passed through a stainless-steel wire mesh and subsequently through a 40 μ m cell strainer (Falcon, BD Biosciences, Erembodegem, Belgium) to separate the cells. The splenic suspension was further treated with Tris-buffered ammonium chloride solution to remove erythrocytes. Then untouched

CD4⁺T cells from the splenic and MLN cells of immunised mice were separated using a CD4⁺T Cell Isolation Kit (Miltenyi Biotec Srl, Calderara di Reno-BO, Italy). Finally, CD4⁺T cell aliquots (5×10^5) were added to plates containing DCs. Immature DCs (iDCs) were purified from the bone marrow of the femurs and tibiae of naïve DQ8 mice according to a published protocol (21). Briefly femurs and tibiae of mice were re-moved and purified from the surrounding muscle tissue. Then intact bones were left in 70% ethanol for 2–5 min for disinfection and the marrow flushed with PBS using a Syringe with a 0.45 mm diameter needle.

At day 0 cells were seeded at 5×10^6 100 mm dish in 10 ml complete RPMI-1640 with 20 ng/ml rmGM-CSF. Every two days half of the culture supernatant was collected, centrifuged, and the cell pellet resuspended in 10 ml fresh medium containing 20 ng/ml rmGMCSF and given back into the original plate. From day 8 to 10 cells can be used.

In vitro co-cultures

iDCs were placed in 48-well plates (2.5×10^5 cells/well), incubated with gliadin alone (100 µg/ml) or spore-adsorbed gliadin (DC:spore ratio: 1:30) for 18 h and finally pulsed with LPS for 4 h to stimulate maturation of the DCs. After removal of the spent medium, 5×10^5 CD4⁺T cells were added along with fresh medium, and the co-cultures incubated for 72 h. Supernatants were collected and analysed for cytokine secretion.

Western blotting

iDCs were incubated at 37°C for different times (0-18 h) with spore-adsorbed gliadin (DC:spore ratio: 1:30) or free gliadin to analyse the antigen-processing kinetics. After incubation, the iDCs were washed with phosphate buffer, recovered, centrifuged and resuspended in Laemmli sample buffer and boiled for 5 min. Then equivalent protein aliquots were fractioned by 12 % denaturing SDS-PAGE, after that blotted on Immobilon PVDF membranes (Millipore, Billerica, MA, USA) and probed with mouse anti-gliadin serum (1:5000 dilution). After washing, the membranes were incubated with peroxidase-conjugated anti-mouse IgG antibodies (1:10000 dilution; Dako SpA, Milano, Italy). Finally, immunodetection was performed using ECL reagent and Hyperfilm (GE Healthcare Europe GmbH - Filiale Italiana, 20 Milan, Italy).

Cytokine assay

Spleen and MLN cells isolated from immunised mice were cultured at 2.5×10^6 cells/ml in 24-well plates in the presence or absence of gliadin (100 $\mu\text{g/ml}$) or an equivalent amount of spore-adsorbed gliadin. After 72 h, supernatants were collected and analysed for IFN- γ and IL-10 protein levels by ELISA using commercial kits. Supernatants from co-cultures of DCs and CD4⁺T cells were similarly analysed.

Statistical analysis

Differences among the various experimental groups were determined by one-way ANOVA, followed by the Turkey test (for the spore adsorption test and co-culture experiments) or the Kruskal-Wallis non-parametric test and Dunn's multiple comparison test for post-test analysis (for the *in vivo* analyses). $P < 0.05$ was selected to denote a significant difference.

Results

Adsorption of gliadin to *Bacillus subtilis* spores

A preliminary test was performed to identify the optimal binding buffer conditions to adsorb gliadin on spore surface, taking into account the known hydrophobicity of this protein. Previous study showed that soluble proteins were more efficiently adsorbed on the surface of *Bacillus subtilis* spores in phosphate buffer at pH 4.0 than at pH 7.0 (7). We found that this condition were not suitable for gliadin, because there were protein precipitation. So we demonstrated that wheat gliadin was efficiently solubilised in 50 mM sodium acetate at pH 4 at a concentration of 625 µg/ml (adsorption solution). For adsorption reaction we used wt spores, CotH and CotE mutants spores at 2×10^9 spores because our previous data indicated that an optimal nasal dosage in mice is 2×10^9 spores/20 µl (22). Therefore 2×10^9 wt or mutant spores were incubated in 200 µl adsorption solution for 1 h.

To assess the amount of gliadin adsorbed on the spore surface, the adsorption mixture was centrifuged to separate spore-adsorbed gliadin from free gliadin. Then the pellet was washed for several times to remove unbound gliadin. Finally two-fold serial dilutions of the pelleted fractions were dot blotted and probed with anti-gliadin antibody.

The results showed that *CotH*, but not wt spores, bound gliadin (Figure 1). Gliadin was also similarly adsorbed by *CotE* spores and *CotH* spores (data not shown). Interestingly, autoclaved wt spores were more effective in adsorbing gliadin than untreated wt spores, but still less efficient than autoclaved mutant spores. By

densitometric analysis of dot blots (no densitometric software), we estimated that approximately 6, 10 and 24 ng gliadin/ 10^6 spores were adsorbed on autoclaved wt, *CotH* and *CotE* spores, respectively. Only faint residual positivity was observed in washes, suggesting the absence of substantial gliadin leakage. This was a qualitative determination.

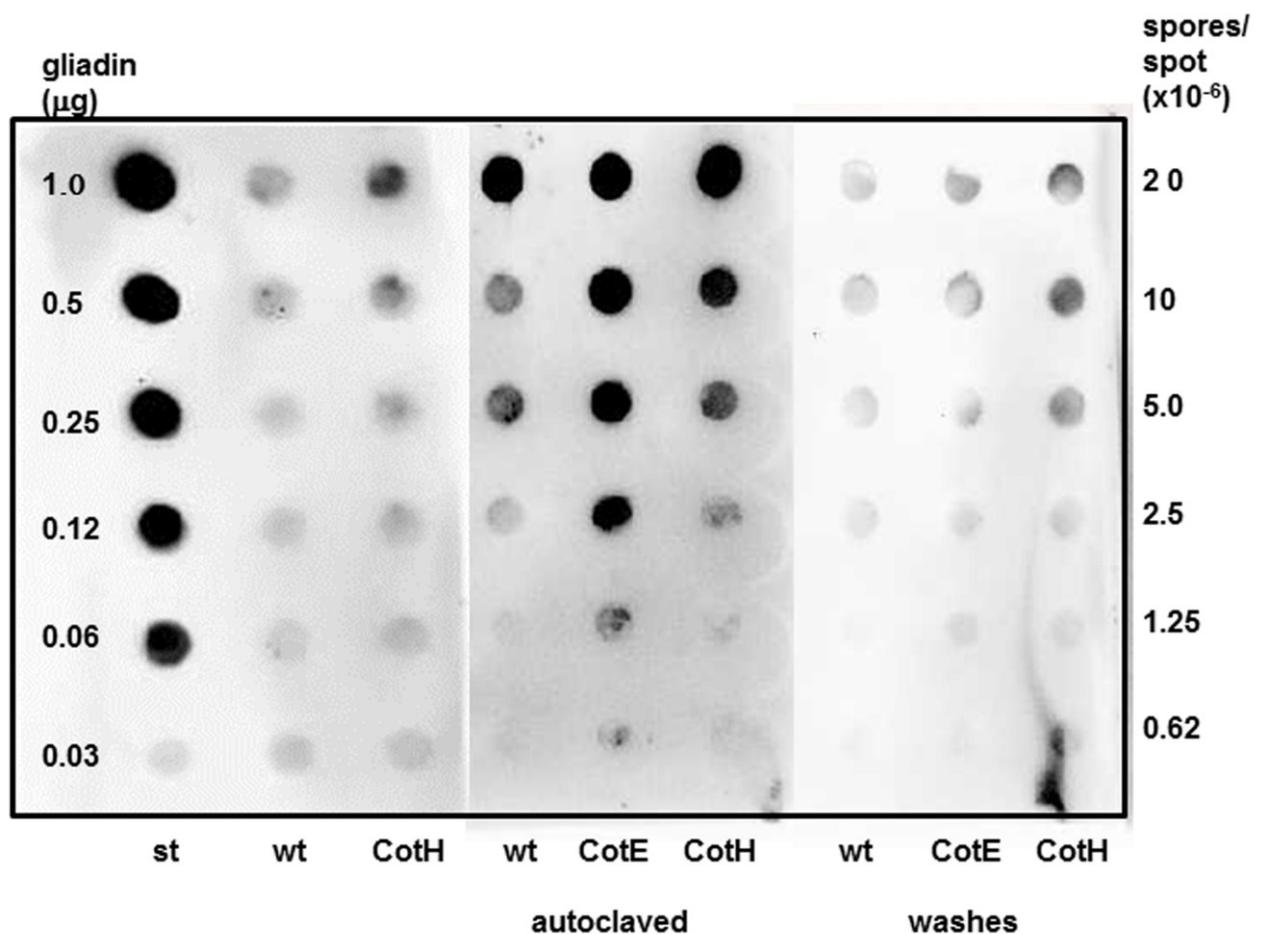


Figure 1: Dot blot analysis of gliadin adsorption by *B. subtilis* spores. Serial dilutions of untreated or autoclaved wt and mutant (*cotH* and *cotE*) spores were analysed following the adsorption reaction with gliadin.

To quantitatively assess spore-adsorbed gliadin, we determined the difference between the initial gliadin content and the residual gliadin in the adsorption solution following

the incubation step by Bradford Test. The results reported in Table 1 confirmed the better performance of autoclaved spores in terms of gliadin-binding activity. Interestingly, the gliadin content was higher than the content estimated by dot blot analysis for all spore types. Surprisingly, autoclaved wt spores showed the maximal binding capacity (41.1 ± 9.7 % for 2×10^9), followed by *CotH* (28.0 ± 1.4 %) and *CotE* (25.0 ± 5.7 %) spores.

Table 1: Percentage of adsorbed gliadin on wild type (wt) and mutant spores

Sample	Gliadin (%)	
	mean \pm SD	
	Adsorbed*	Washed**
Untreated spores		
wild type	18.0 ± 2.8	13.5 ± 0.7
<i>cotH</i>	30.0 ± 9.9	0.0 ± 0.0
<i>cotE</i>	34.0 ± 11.3	7.0 ± 1.4
Autoclaved spores		
wild type	41.1 ± 9.7	4.0 ± 2.8
<i>cotH</i>	28.0 ± 1.4	11.8 ± 0.2
<i>cotE</i>	25.0 ± 5.7	10.3 ± 0.9

* , determined as the difference in the protein content of the adsorption solution before (100%) and after incubation. **, residual protein content in the first wash following incubation.

Together, these data suggest that gliadin molecules were not only bound on the external surface, but probably it was incorporated into a network created by spores.

The absolute values of the amount of gliadin adsorbed by the different tested spores are shown in Figure 2.

Based on these results, autoclaved wt spores were used to analyse the induction of an immune response in DQ8-transgenic mice.

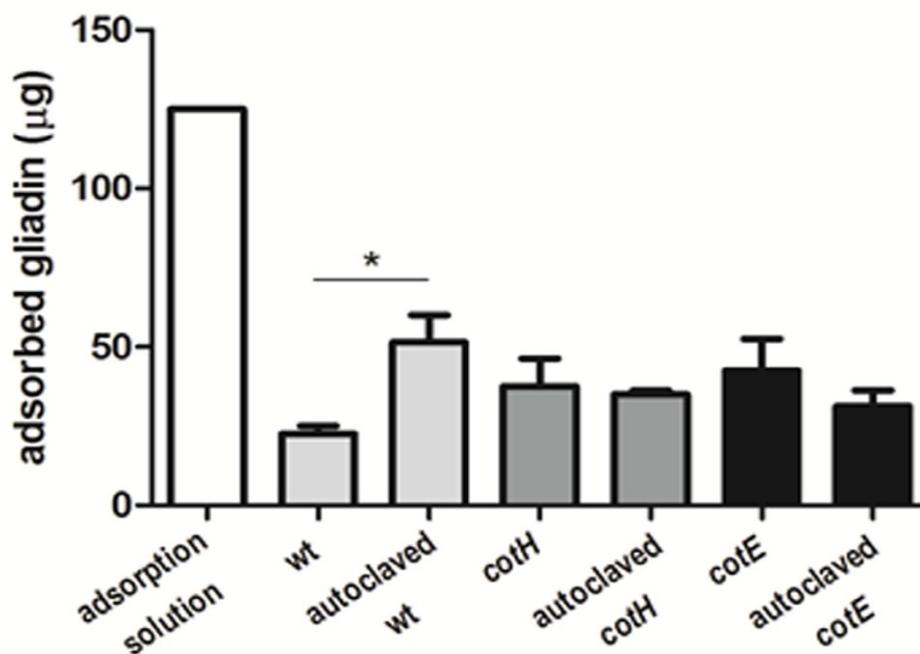


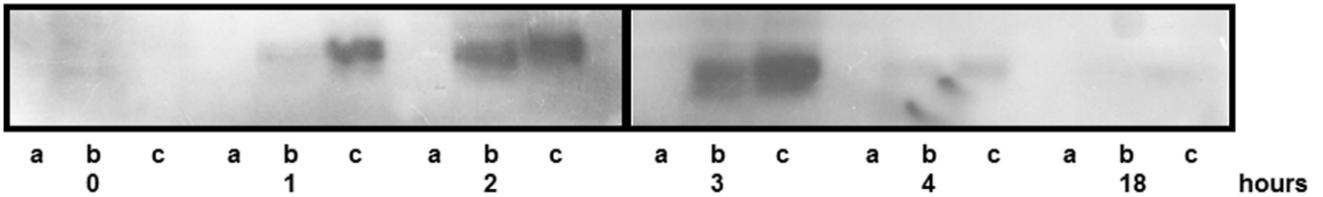
Figure 2: Quantitative analysis of conjugated gliadin. The amount of gliadin (μg) adsorbed on 2×10^9 spores was calculated as the difference in the protein concentration of the adsorption solution before and after incubation. Light grey columns, represented wt spores; dark grey columns, *coth* spores; black columns, *cotE* spores. The data represent the mean \pm SD of three different experiments. *, $P < 0.05$.

In vitro analysis of immune response to gliadin

To analyse immune response toward gliadin we firstly evaluated the influence of spores on gliadin processing by antigen-presenting cells (APCs) *in vitro*. Spore-adsorbed gliadin and an equivalent amount of free gliadin were incubated with bone marrow-derived iDCs (DC:spore ratio: 1:30) from DQ8 mice for 1-18 h. Protein samples were then analysed by Western blotting using anti-gliadin mouse serum as a probe. Interestingly, uptake of spore-adsorbed gliadin occurred faster than for free gliadin, in fact positive signals were observed after 1 and 2 h, respectively (Figure 3A). Also we observed that both signals disappeared after 4 h, suggesting similar kinetics of processing.

Then in the next experiment we wanted to analyse interaction between DCs and T cells. Therefore antigen-pulsed mature DCs (mDCs) were incubated with autologous gliadin-specific CD4⁺T cells for 72 h. The results of this experiment shown that gliadin-pulsed mDCs stimulated higher levels of IFN- γ in CD4⁺T cells than did mDCs challenged with spore-adsorbed gliadin (Figure 3B). These data were expected considering that, on the latter mDCs, a reduced number of DQ8 molecules could present gliadin peptides, probably because of steric obstruction or masking of the binding sites. Also we demonstrated that gliadin-pulsed mDCs were able to stimulate CD4⁺T cells isolated from mice immunised with spore-adsorbed gliadin. Under the various examined experimental conditions there were not significant levels of IL-10 (data not shown).

A



B

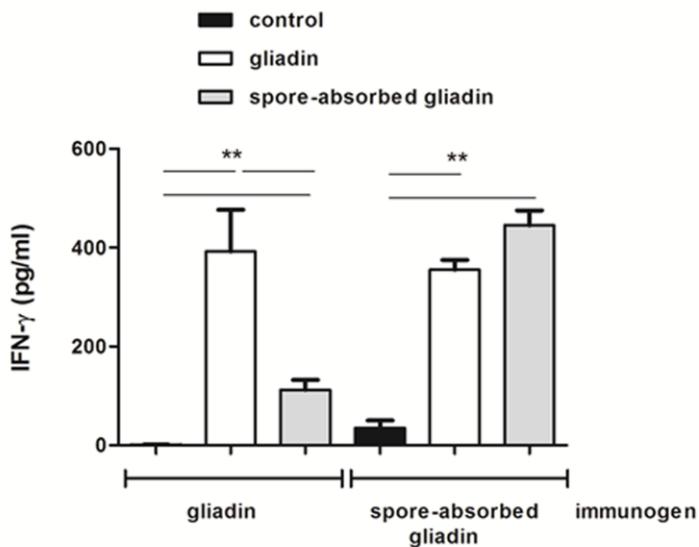


Figure 3: *In vitro* antigen processing of spore-adsorbed gliadin. A) Western blot analysis of antigen uptake and processing. iDCs were incubated with spores alone (lane a), gliadin (lane b) or spore adsorbed gliadin (lane c) at different times (from 0-18 h).

B) *In vitro* analysis of the gliadin-specific CD4⁺T cell-mediated response. mDCs (2×10^5 cells/well) that were not pulsed (black columns) or that were pulsed with gliadin (white columns) or spore-adsorbed gliadin (grey columns) were incubated with CD4⁺T cells (5×10^5 cells/well) isolated from DQ8 mice parenterally immunised with gliadin or spore adsorbed gliadin. IFN- γ levels secreted in the supernatant after 72 h of co-culture were detected by ELISA. The columns are the mean \pm SD of duplicate analyses. The results are representative of three different experiments. **, $P < 0.001$.

In vivo analysis of immune response induced by gliadin adsorbed spores

We previously reported that intranasal administration of gliadin in DQ8 mice was able to down-regulate the antigen-specific systemic response (13). Therefore we tested if intranasal administration of spore adsorbed gliadin similarly induced tolerance in mice systemically immunised with native gliadin. So mice received free gliadin or spore adsorbed gliadin before immunization. We observed that mice nasally treated with gliadin showed a significant increase in antigen-specific IL-10 secretion at the systemic level, compatible with the activation of T regulatory cells (Figure 4 panel C). In contrast, spore adsorbed gliadin failed to induce a similar phenotypic change; in fact the IL-10 secretion decreased both at systemic level and in the intestinal compartment (Figure 4 panel C and D). These results suggested that adsorbed gliadin lost its intrinsic tolerogenic properties.

Another important result was the capability of spore to vehiculate gliadin into the intestine by inducing an amplifying the IFN- γ production (Figure 4 panel B).

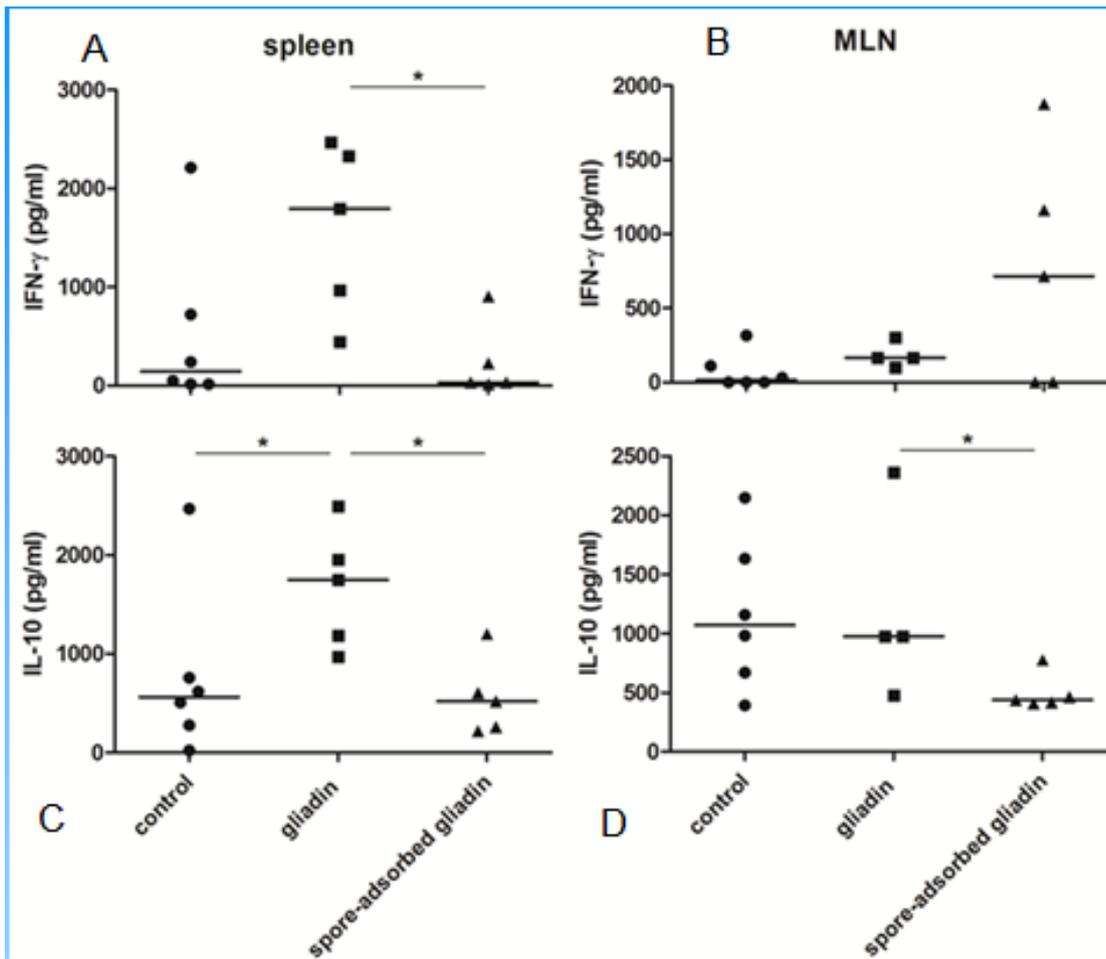


Figure 4: Effect of nasal administration of spore-adsorbed gliadin in immunised DQ8 mice. Analysis of cytokine pattern of spleen and MLN cells isolated from DQ8 mice i.n. treated with spore adsorbed gliadin (triangles), gliadin (squares) or water (circles) and immunised i.p. with wheat gliadin. The results represent one of three independent experiments, and the bars are the median values. There were 5-6 mice in each group. *, P < 0.05.

Then we analysed whether spore-adsorbed gliadin was able to directly induce a T cell-mediated mucosal response. Mice were nasally or intragastric immunised with spore-adsorbed gliadin (2.0×10^9 spores/dose) or were nasally immunized with free gliadin ($40.0 \mu\text{g}/\text{dose}$). The animals were dosed once per week for 7 weeks and sacrificed one week later, and their spleens and MLNs were analysed for gliadin-

specific production of IFN- γ and IL-10 *in vitro*. Both spleen and MLN cells from mice immunised with spore-adsorbed gliadin secreted significantly higher IFN- γ levels than those from mice challenged with free gliadin (Figure 5). In contrast, IL-10 was not significantly produced (data not shown).

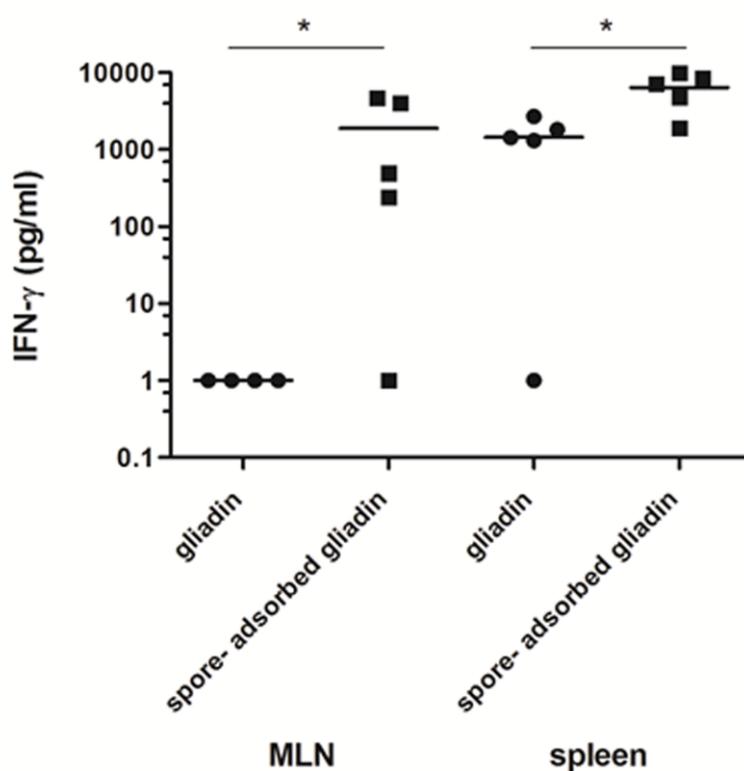


Figure 5: Induction of gliadin-specific cell immunity by nasal administration of spore-adsorbed gliadin in DQ8 mice. DQ8 mice were intranasally immunised with spore-adsorbed gliadin (square) or an equivalent amount of gliadin (circles). Gliadin-specific IFN- γ levels are reported after subtracting the value in the absence of antigen. The results are representative of three different experiments. There were 4-5 in each group. The bars represent the median values. *P <0.05.

Furthermore, intragastrically administered gliadin adsorbed spores did not induce an immune response (Figure 6). So the nasal route represents in our hand the optimal way to immunize with antigen adsorbed spores.

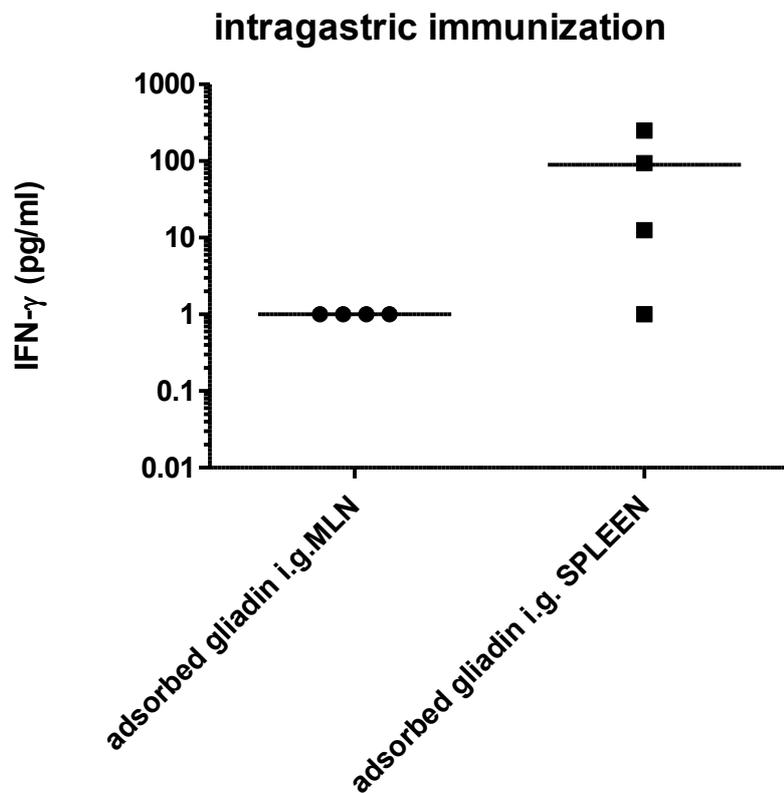


Figure 6: Effect of intragastric administration of spore-adsorbed gliadin in DQ8 mice. DQ8 mice were intragastrically immunized with spore-adsorbed gliadin for six weeks, one dose a week. Gliadin-specific IFN- γ levels are reported after subtracting the value in the absence of antigen.

We further analysed the response to gliadin in the intestinal CD4⁺T cell sub-population. As shown in Figure 7, spore-adsorbed gliadin induced an increased secretion of IFN- γ in CD4⁺T cells isolated from MLNs following *in vitro* stimulation with gliadin. IL-10 was not induced in gliadin-specific CD4⁺T cells, indicating that

nasal administration of spore-adsorbed gliadin promoted a Th1-biased immune response in the intestinal compartment of DQ8-transgenic mice.

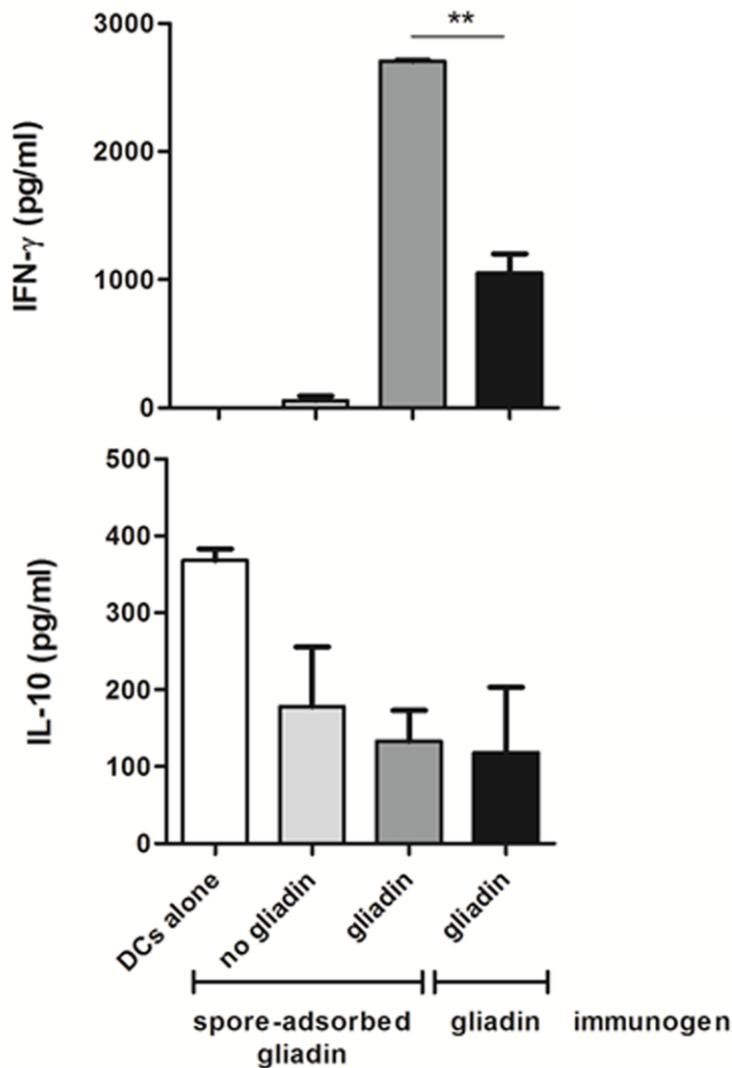


Figure 7: Characterization of the intestinal gliadin-specific response induced by nasal administration of spore-adsorbed gliadin in DQ8 mice. Mice were intranasally immunized. CD4⁺T cells were isolated from MLNs by immunomagnetic sorting and analysed *in vitro* for gliadin-specific IFN- γ (upper panel) and IL-10 secretions (bottom panel). The results are representative of three different experiments. The columns represent the mean + SD of triplicate cultures. **, $P < 0.001$.

Discussion and Conclusion

Our results showed that gliadin-pulsed DCs stimulated gliadin adsorbed spores specific CD4⁺T cells to induce a Th1-biased antigen-specific intestinal response in DQ8 mice.

An adaptive response to gliadin-derived peptides caused by activation of CD4⁺T cells, occurs in the lamina propria of the small intestinal mucosa (23). These cells also represent the main immunological target for tolerance strategies. The classical protocol of oral tolerance with native antigen is not applicable in CD because local administration of gliadin peptides could develop the risk of exacerbating the disease. So in this part of the study we wanted to use nasal delivery of spore-adsorbed gliadin for inducing an antigen specific intestinal response to verify the ability of *Bacillus subtilis* spores to act as a mucosal adjuvant and antigen vehicle. Recently, a non-recombinant approach has been proposed based on non-covalent antigen adsorption, which involves electrostatic and hydrophobic forces that are not yet fully characterised (9). Acidic pH and antigen solubility (8) appear to play major roles in this mechanism. For these reasons, the use of hydrophobic antigenic molecules such as wheat gliadin carries the risk of stochastic aggregation, with unpredictable biological outcomes. So we tested the optimal binding buffer.

Moreover, to maximise the adsorbed gliadin molecules:spore ratio, we tested wt *Bacillus subtilis* and two mutant strains to identify which spores showed the highest

binding efficiency for gliadin. CotH and CotE belong to a small subset of coat proteins that play a regulatory role in the formation of the spore coat.

Dot blot experiments clearly indicated that surface binding of gliadin essentially occurred when mutant spores were used. In addition to differences in coat architecture, *Bacillus subtilis* wt spores are known to be negatively charged in water, whereas both CotH and CotE mutant spores are not or are less charged (8, 24).

Whether structural and/or chemical peculiarities are involved in determining the adsorption properties of spores remains to be fully addressed but an important result was that binding efficiency increased following drastic physical treatment via autoclaving. The treatment was particularly effective for wt spores. Furthermore, gliadin adsorption occurred at an acidic pH, a condition in which the number of negative surface charges is further reduced. Taken together, these data suggest a prevalent role of the structural features of spores in determining the loading capacity for gliadin.

Notably, comparison between dot blot and spectrophotometric protein analysis clearly indicated that the amount of bound gliadin was higher than shown by dot blot data. Therefore we suggest that most gliadin avoided from antibody recognition, probably because it was incorporated inside structural niches of spores.

Another important result was the high stability of gliadin-spore interactions. Based on the biochemical results, autoclaved wt spores were chosen for subsequent immunological studies.

Next, we studied spore-adsorbed gliadin processing by DCs *in vitro*. We initially analysed gliadin uptake in iDCs by Western blotting. Interestingly, we found that spore-adsorbed gliadin was incorporated more rapidly than free gliadin was, suggesting that antigen uptake occurred through different mechanisms. Whereas endocytosis of free gliadin is receptor mediated, the spore-adsorbed gliadin presumably was internalised by phagocytosis (25), but independently of the specific routing, both antigens were degraded at same time. In fact, we observed that spore-adsorbed gliadin and free gliadin were processed by DCs with similar kinetics. Notably, by using mDCs as APCs, we found that gliadin-pulsed mDCs stimulated not only gliadin-specific CD4⁺T cells but also CD4⁺T cells isolated from mice immunised with spore-adsorbed gliadin.

Together, the biochemical and immunological data indicated that gliadin bound to spores was correctly processed and was able to induce a T cell-mediated response with the same phenotype.

These results encouraged the study of the immunological properties of spore-bound gliadin *in vivo*. We initially tested the potential of this antigen to modulate the ongoing intestinal response to wheat gliadin. We used previously established protocol of nasal administration (13) and we have shown that spore-adsorbed gliadin were unable to induce down-regulation of immune response, but this antigen administered in multiple doses activated a Th1- intestinal response towards gliadin.

In conclusion, we demonstrated that nasal delivery of a spore-adsorbed food protein can induce an antigen-specific cell-mediated response. In particular, spore-adsorbed gliadin was able to induce an intestinal CD4⁺T cell response. Further studies are required to better characterise the phenotype of the induced response and, more particularly, to develop an immunomodulatory strategy based on nasal delivery of a spore-adsorbed protein with tolerogenic properties.

References

- 1 Lycke N, Tsuji T, Holmgren J. The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP ribosyltransferase activity. *Eur J Immunol* 1992; 22: 2277–81.
- 2 Kim JH, Roh C, Lee CW, Kyung D, Choi SK, Jung HC, Pan JG and Kim BG. Bacterial surface display of GFP(uv) on *Bacillus subtilis* spores. *J Microbiol Biotechnol* 2007; 17: 677–80.
- 3 Duc LH, Cutting SM. Bacterial spores as heat stable vaccine vehicles. *Expert Opin Biol Ther* 2003; 3: 1263-70.
- 4 Mauriello EM, Duc LH, Istickato R, Cangiano G, Hong HA, De Felice M, Ricca E and Cutting SM. Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. *Vaccine* 2004; 22: 1177–87.
- 5 Kwon SJ, Jung HC, Pan JG. Transgalactosylation in a water-solvent biphasic reaction system with beta-galactosidase displayed on the surfaces of *Bacillus subtilis* spores. *Appl Environ Microbiol* 2007; 73: 2251–6.
- 6 Mc Kenney PT, Driks A, Eichemberger P. The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nat Rev Microbiol* 2013; 11: 33-44.
- 7 Huang JM, Hong HA, Van Tong H, Hoang TH, Brisson A, Cutting SM. Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine* 2010; 28: 1021-30.
- 8 Sirec T, Strazzulli A, Istickato R, De Felice M, Moracci M, Ricca E. Adsorption of beta galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. *Microb Cell Fact* 2012; 11: 100.
- 9 Ricca E, Baccigalupi L, Cangiano G, De Felice M, Istickato R. Mucosal vaccine delivery by non-recombinant spores of *Bacillus subtilis*. *Microbial Cell Factories* 2014, 13: 115.
- 10 Song M, Hong HA, Huang JM, Colenutt C, Khang DD, 1 Nguyend TVA, ParK SM, Shim BS, Song HH, Cheon IS, Jang JE, Choi JA, Choi YK, Stadler K and Cutting SM. Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. *Vaccine* 2012; 30: 3266-77.
- 11 Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol* 2002; 2: 647–55.
- 12 Black KE, Murray JA, David CS. HLA-DQ determines the response to exogenous wheat proteins: a model of gluten sensitivity in transgenic knockout mice. *J Immunol* 2002; 169: 5595–5600.
- 13 Senger S, Luongo D, Maurano F, Mazzeo MF, Siciliano RA, David C, Troncone R, Auricchio S and Rossi M. Intranasal administration of a recombinant α -gliadin down-regulates the immune response to wheat gliadin in DQ8 transgenic mice. *Immunol Lett* 2003; 88: 127-34.
- 14 Senger S, Maurano F, Mazzeo MF, Gaita M, Fierro O, David CS, Troncone R, Auricchio S, Siciliano RA and Rossi M. Identification of immunodominant epitopes

of alpha-gliadin in HLA-DQ8 tg mice following oral immunisation. *J Immunol* 2005; 175: 8087-95.

15 Naclerio G, Baccigalupi L, Zilhao R, De Felice M, Ricca E. Bacillus subtilis spore coat assembly requires cotH gene expression. *J Bacteriol* 1996; 178: 4375-80.

16 Zheng L, Donovan WP, Fitz-James PC, Losik R. Gene encoding a morphogenic protein required in the assembly of the outer coat of the Bacillus subtilis endospore. *Genes Dev* 1988; 2: 1047-54.

17 Nicholson WL, Setlow P. Sporulation, germination and outgrowth. In: Harwood CR, Cutting SM, editors, Molecular biological methods for Bacillus. Chichester, UK: John Wiley & Sons Ltd; 1990, p. 391-450.

18 Weegels PL, Hamer RJ, Schofield JD. RP-HPLC and capillary electrophoresis of subunits from glutenin isolated by SDS and Osborne fraction. *J Cereal Sci* 1995; 22: 211-4.

19 Bradford MM. A rapid and sensitive method for the 1 quantitation of microgram quantities of protein utilizing the principlexin of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.

20 Hinc K, Isticato R, Dembek M, Karczewska J, Iwanicki A, Peszyńska-Sularz G, et al. Expression and display of UreA of *Helicobacter acinonychis* on the surface of *Bacillus subtilis* spores. *Microb Cell Fact* 2010; 9: 2.

21 Lutz MB, Kukutsch N, Ogilvie ALJ, Roßner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999; 223: 77-92.

22 Isticato R, Sirec T, Treppiccione L, Maurano F, De Felice M, Rossi M and Ricca E. Non recombinant display of the B subunit of the heat labile toxin of *Escherichia coli* on wild type and mutant spores of Bacillus subtilis. *Microb Cell Fact* 2013; 12: 98.

23 Nilsen EM, Jahnsen FL, Lundin KE, Johansen FE, Fausa O, Sollid LM, Jahnsen J, Scott H, Brandtzaeg P. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gut* 1998; 115: 551-63.

24 Pesce G, Rusciano G, Sirec T, Isticato R, Sasso A, Ricca E. Surface charge and hydrodynamic coefficient measurements of *Bacillus subtilis* spore by optical tweezers. *Colloids Surf B Biointerfaces* 2014; 116: 568-75.

25 Yu LH, Cutting SM. The effect of anti-spore antibody responses on the use of spores for vaccine delivery. *Vaccine* 2009; 27: 4576-84.

Chapter 3

Biochemical characterization of transamidated gliadin

“published on Biochimica et Biophysica Acta 2013, 1830: 5166-74”

Introduction

As described in Chapter 2 we demonstrated that nasal delivery of a spore-adsorbed food protein can induce an antigen-specific cell-mediated response. Next, we planned to bind a protein with immunomodulatory activity to spores. Firstly we focused our attention on transamidated gliadin because it has been shown to cause a dramatic down-regulation in IFN γ production *in vitro* in the intestinal T cells of CD patients (1).

In the small intestine of CD patients, specific glutamine residues are converted to glutamic acid by tissue transglutaminase (tTGase); this post-translational modification plays a major role in intestinal T cell activation. Tissue transglutaminase (TG2) modifies proteins and peptides by transamidation or deamidation of specific glutamine residues. During transamidation reaction, an acyl residue, derived from the γ -carboxamide group of a peptide-bound glutamine (acyl donor), is transferred to an appropriate primary amine, most commonly the ϵ -amino group of lysine residues (acyl acceptor). In the first step, the side chain of a glutamine residue forms a thioester with the active site cysteine, and ammonia is released (acylation). In the following transamidation step, the activated acyl group is transferred to the acyl acceptor amine, forming an isopeptide bond (deacylation). Alternatively, hydrolysis of the thioester bond leads to deamidation, converting the glutamine into a glutamic acid residue (2). The transamidation, using food-grade microbial transglutaminase (mTGase), masks glutamine residues of gliadin causing a down-regulation of immune

response and a positive changes in the phenotype of the antigen-specific immune response in models of gluten sensitivity (3).

Interestingly, mTGase was shown to exhibit a similar site specificity as tTGase on synthetic peptides, but lacked deamidase activity (1). Therefore in this study, we examined the biochemical features of gluten following the two-step transamidation reaction of wheat flour.

This enzymatic treatment was associated with a progressive production of new forms of gliadins with higher molecular weights that became soluble in water. We confirmed that transamidation reactions performed on wheat flour could successfully prevent the formation of immunodominant gluten peptides. Furthermore, we evidenced for the first time the ability of mTGase to inhibit the activity of the celiacogenic p31–49 in Caco-2 cells.

Materials and Methods

Transamidation reaction of wheat flour

A commercial preparation of bread wheat flour was used. A total of 100 g of flour was suspended in 8 volumes of 0.4 M NaCl and stirred for 10 min to extract albumin/globulins. The flour suspension was then centrifuged at 1000 g for 10 min, and the supernatant was discarded. The recovered pellet was exhaustively washed with water to eliminate any residual soluble protein, suspended in 1 volume of water containing 20 mM pharmaceutical grade lysine ethyl ester (K-C₂H₅; NutraBio.com, Middlesex, NJ, USA) and 8 U/g flour mTGase (ACTIVA®WM, specific activity: 81-135 U/g, Ajinomoto Foods Hamburg, Germany), and incubated for 2 hrs at 30°C. The suspension was then centrifuged at 3000 g for 10 min, and the soluble protein fraction (spf) was recovered in the supernatant. In the two-step process, the pellet was suspended again in 1 volume of water containing a final concentration of 20 mM K-C₂H₅ and fresh mTGase (8 U/g flour) and incubated for 3 hrs at 30°C. For the control, the sample was prepared using the same protocol in the absence of mTGase.

Purification of protein fractions

Gliadin was extracted from wheat flour by a modified Osborne protocol (4). Briefly wheat flour was resuspended in 0.4 M NaCl and was centrifuged at 1000 rpm for 10 min. Then the pellet was washed for several times and gliadin was extracted in 70% ethanol. The supernatant, which contained gliadin, was recovered and freeze-dried.

Protein content was measured by Bradford analysis (5) and qualitatively analyzed by 8-16 % denaturing SDS-PAGE and Coomassie R-250 blue staining.

Gliadin stock solution was prepared by suspending 10 mg gliadin in 50 mM acetic acid.

Peptide synthesis and transamidation reaction

Peptide p31–49 was synthesized by GeneCust Europe Laboratoire de Biotechnologie du Luxembourg S.A (Dudelange, Luxembourg). Purity (N95%) was assessed by the manufacturer by means of RP-HPLC and mass spectrometric analyses. Transamidation reaction of the peptide was carried out in 25 mM ammonium bicarbonate (pH 8.3) containing 20 mM K-C₂H₅ and 0.015 U/10 µg peptide mTGase at 37 °C for 30 min.

Western blotting

Equivalent protein aliquots (50 µg) were fractionated by 15% denaturing SDS-PAGE, blotted onto Immobilon™ PVDF membranes (Millipore SpA, Vimodrone-MI, Italy) and probed with in-house produced mouse anti-sera toward native gliadins (1:10,000 dilution), transamidated gliadins (K-gliadins; 1:10,000 dilution) or spf (1:10,000 dilution). After washing, the membranes were incubated with biotinylated streptavidin-peroxidase-conjugated antibodies against mouse IgGs (Dako SpA, Milano, Italy, 1:5,000 dilution). Finally, immunodetection was performed using

Hyperfilm and ECL reagents (Amersham-GE Healthcare Europe GmbH, Glattbrugg, Switzerland).

Chymotryptic digestion of native gliadin, spf and transamidated gliadins

Enzymatic digestion was performed in 50 mM Tris-HCl (pH 8.5), at 37°C for 4 hrs using an enzyme to substrate ratio of 1:50 w/w.

Cell culture and in *vitro* experiments

Caco-2 cells obtained from the American Type Culture Collection (ATCC, Gaithersburg, MD, USA) were used for experiments between passages 16 and 35. Cells were cultivated in DMEM (GIBCO—Life Technologies, Grand Island, NY, USA) containing 10% FCS (GIBCO), 100 units/mL penicillin–streptomycin (GIBCO) and 1 mM glutamine at 37 °C in a humidified 5% CO₂ atmosphere. For the experiments, the cells were seeded at a density of $30 \times 10^4/\text{cm}^2$ and used on the third day post-confluence.

Analysis of tTGase transamidating activity

Caco-2 cells were incubated for 90 min with 0.5 μ M EZ-Link Pentylamine-Biotin (ThermoFisher Scientific, Erembodegem, Belgium) and treated for the last 60 min with either native or transamidated peptide p31–49 (25–100 μ g/mL). The positive control was cells treated with 10 μ M ionomycin (Sigma-Aldrich, St. Louis, MO, USA). After incubation cells were lysed and protein aliquots (100 μ g) were coated into the wells of a 96-well plate. The wells were blocked with 10% bovine serum albumin, then incubated with peroxidase-conjugated streptavidin (1:3000 dilution, BioLegend, London, UK) in 5% bovine serum albumin. To reveal peroxidase activity, 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) was added to each well and, after stopping the reaction with H₂SO₄, absorbance was read at 450 nm. Data were evaluated by one-way analysis of variance (ANOVA) and Tukey test post-hoc analysis. P <0.05 was selected as the level denoting a statistically significant difference. Proteins from cell lysates were also analysed to SDS-PAGE, blotted onto Immobilon™ PVDF membranes, probed with peroxidase-conjugated streptavidin and visualized by ECL reagents.

Analysis of tTGase protein expression

Caco-2 cells were incubated for 48 h with either native or transamidated peptide p31–49 (25–100 μ g/mL). Equivalent protein aliquots were fractionated by 12% SDS-PAGE, blotted onto Immobilon™ PVDF membranes (Millipore) and probed with

anti-tTGase monoclonal antibody (TG II, clone CUB7402, Lab Vision Corporation, Fremont, CA, USA) followed by incubation with secondary anti-mouse antibodies conjugated with peroxidase (Sigma-Aldrich). Finally, immunodetection was performed using Hyperfilm and ECL reagents (Amersham). Blotted membranes were subsequently stained with Coomassie blue R-250 as loading control.

Results

Different solubilization of gluten components following transamidation of wheat flour

During the transamidation process the production of isopeptide bonds by catalytic activity of mTGase dramatically decreased the gliadin content of wheat flour to $17.4 \pm 0.7\%$ (mean \pm SD; Table 1), in agreement with previous results (3). Interestingly, glutenins were also partially recovered following transamidation treatment ($47.4 \pm 2.5\%$, Table 1). The residual insoluble gliadins and glutenins were further reduced to $7.6 \pm 0.5\%$ and $7.5 \pm 0.3\%$, respectively, after a second transamidation step (Figure 1A, upper panel). In particular, gliadin content essentially decreased to 40% during the first enzyme step, whereas glutenins resulted more markedly reduced during the second transamidation step. In line with these results, the water-soluble protein fraction (spf) increased during the first enzyme step and peaked at the end of this step (Figure 1A, lower panel). To identify molecular changes produced in the residual alcohol-soluble fraction (gliadins) during the transamidation reaction, protein aliquots

were collected at different times of transamidation process and analyzed by SDS-PAGE. An interesting result was distinct bands in the 29-45 kDa mw range, that characterize native gliadins. This native gliadin decreased during the two-step reaction, substituted by a protein with high molecular weight, in fact we observed a transient appearance of new components having a MW>200 kDa (Figure 1B).

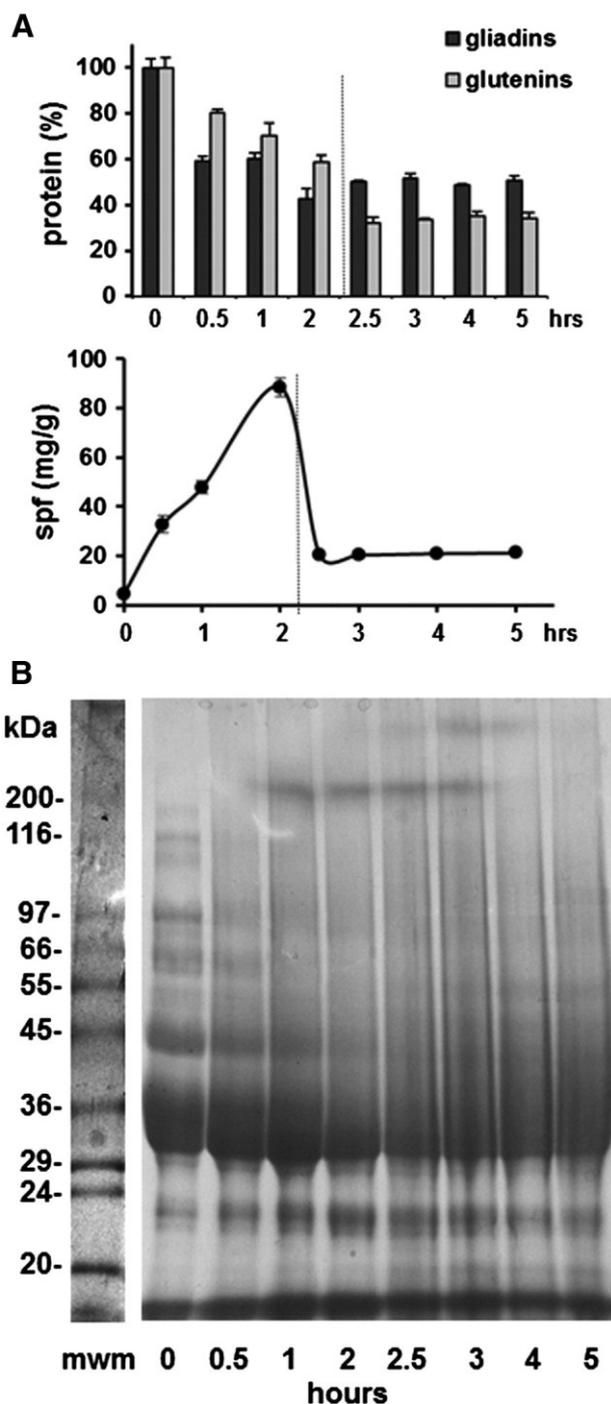


Figure 1: Biochemical and physical modifications of gliadins and glutenins after transamidation. A, Upper panel, percentage of residual insoluble prolamins at different times of a two-step transamidation reaction; lower panel, relative soluble protein fraction (spf) yield expressed in mg protein/g flour; the dotted line identifies the two enzymatic steps. B, SDS-PAGE analysis of proteins isolated from flour after removal of the albumin/globulin fraction and at different times of a two-step enzymatic reaction. Molecular weights of protein markers are indicated (kDa).

Immune cross-reactivity of different gliadin forms after flour transamidation

To analyze transamidation products and establish the relationship among gliadins, spf and new hmw alcohol-soluble components, we produced mouse anti-sera toward spf and transamidated insoluble gliadins (K-gliadins). Then we examined cross-reactivity among different forms. Western blot analysis performed by probing protein samples with an anti-native gliadin antiserum confirmed the loss of immune cross-reactivity in K-gliadins and spf, in agreement with previous findings (4) (Figure 2A). On the contrary, anti-K-gliadins antibodies identified epitopes in all examined samples. These findings confirmed the presence of native gliadin-specific determinants in K-gliadins. (Figure 2B). Adsorption of serum with native gliadins caused a strongly reduced reactivity with native gliadin in all samples but, interestingly, pre-treated serum still recognized high molecular weight (hmw) bands suggesting that this new epitopes were generated following transamidation of gliadin with $K-C_2H_5$ (Figure 2C). Probing with anti-spf serum also showed cross-reactivity of spf with native gliadins. Interestingly, this antiserum weakly cross-reacted with proteins in K-gliadins of similar hmw (Figure 2D). Coomassie blue staining of electrophoresed proteins confirmed that Western blot results were not due to differences in protein content (Figure 2E).

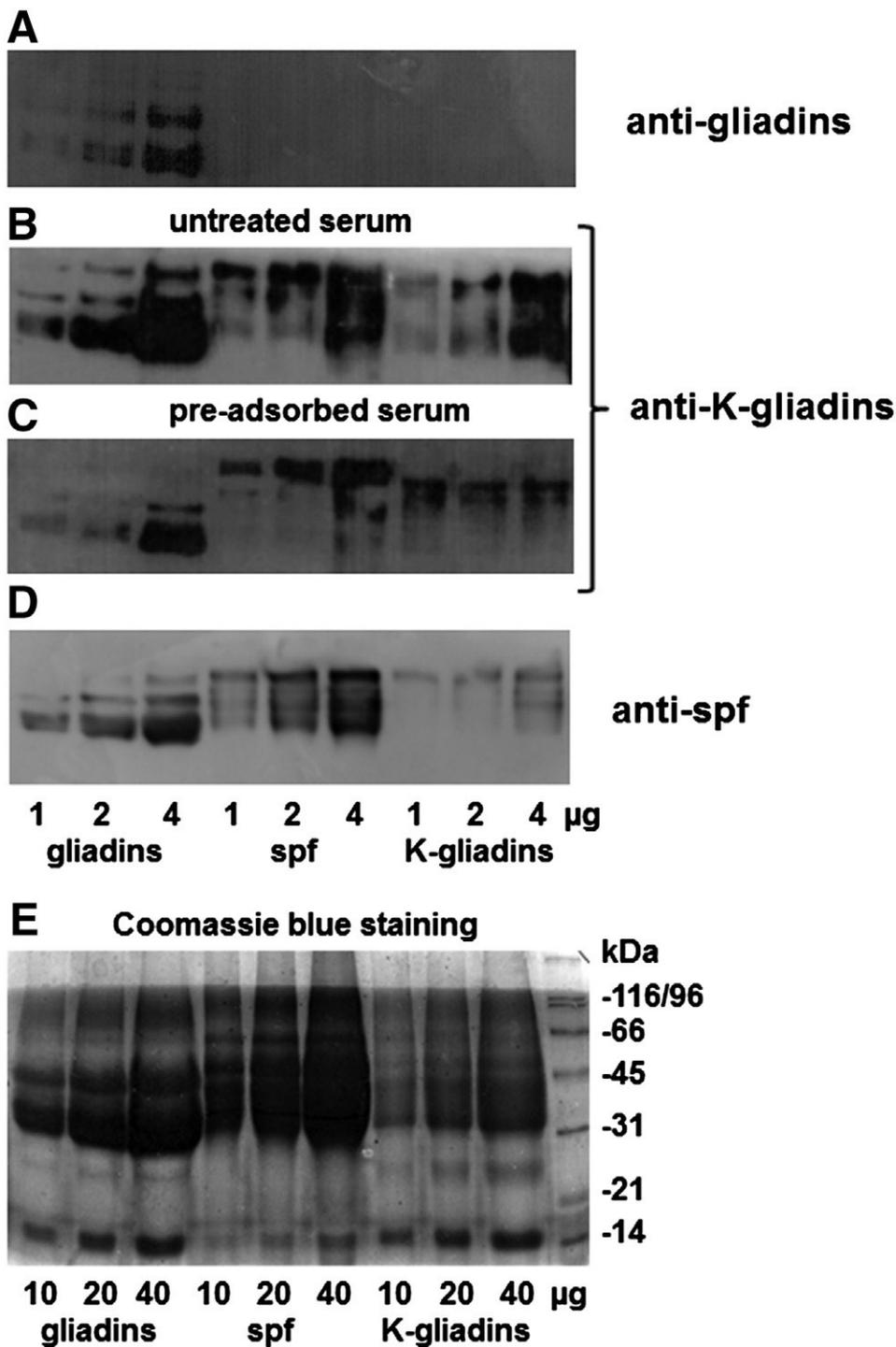


Figure 2: Cross-reactivity of different transamidated form.

A-D Western Blot of different forms of transamidated gliadin, probing with anti-native gliadin **A**; **B** anti K gliadin untreated serum; **C** anti K gliadin preadsorbed with native gliadin; **D** anti-spf. **E** SDS-PAGE of different amount of native gliadin, spf and K-gliadin.

We found that this feature was not a technical artifact, as it was confirmed by using two different preparations of immunogens and anti-sera (Figure 3A).

Next, we examined the kinetic of transamidation process by probing with anti-K-gliadins antibodies. Results reported in Figure 3B showed the appearance of hmw proteins after only 0.5 hrs of treatment. In agreement with data reported in Figure 1B, positive staining of hmw bands in the alcohol-soluble fraction was short-lived and there was a progressive reduction of cross-reacting proteins in the 45-29 kDa mw range.

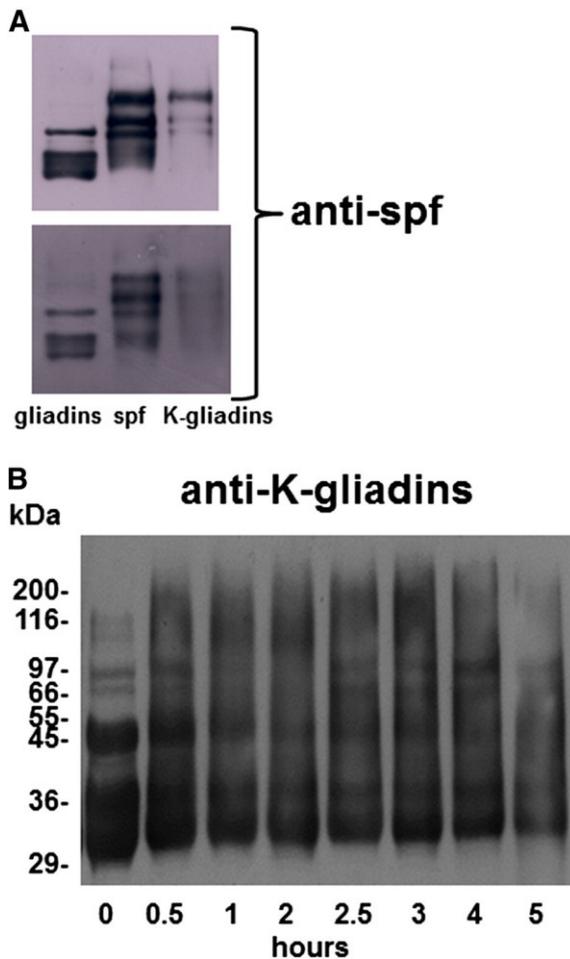


Figure 3: Cross-reactivity of transaminated gliadin forms. **A**, Western blot analysis of high molecular weight (hmv) components in spf and K-gliadin fractions by comparing two different preparations of anti-spf serum. **B**, Western blot analysis of proteins isolated from flour at different times of a two-step enzymatic reaction and probed with anti-K-gliadin antiserum.

Transamidation modified gliadin activity

Mass spectrometric analysis of native gliadin digest identified four known celiacogenic peptides, α -gliadin p56–68 (6), α -glia-33-mer (7), peptide #19(8), and α -gliadin p31–49, which contained peptide fragment p31-43 known to induce intestinal damage into CD patients (9).

Analysis of transamidation reaction mixture by MALDI-TOF-MS clearly indicated that celiacogenic peptide, p31-49 was modified by the addition of one K-C₂H₅ moiety and this modified form accounted for more than 90% (data not shown).

To specifically evaluate the biological effects of transamidation products we analyzed α -gliadin p31-49, using a synthetic peptide p31-49.

It was previously shown that Ca²⁺ mobilization induced by gliadin peptide p31-43 activated the crosslinking function of intracellular tTGase in Caco-2 cells. Therefore, we tested the ability of p31-49 to activate tTGase in this cell line and how this activity was modified on transamidation. We used pentylamine-biotin as tTGase substrate and quantify the intracellular transamidating activity in Caco-2 cells by a microplate assay on cell homogenates obtained after incubation with different amounts of p31-49. As previously reported for p31-43 (10), we found that tTGase activity was higher in p31-49-treated cells than in cells exposed to pentylamine-biotin alone (Figure 6A), in particular at a dose of 25 μ g/mL. Interestingly, in cells treated with transamidated p31-49 there was not increase of tTGase activity. Also protein aliquots were fractionated by SDS-PAGE, blotted and probed with peroxidase-conjugated streptavidin. Results showed a 78 kDa band, presumably tTGase, that resulted stronger in samples incubated with native p31-49 than in those treated with transamidated peptide at the doses of 25 and 50 μ g/mL (Figure 6B).

Finally, we evaluated the effect of modified p31-49 on tTGase expression in Caco-2 cells. Western blot analysis of different amounts (1-10 μ g) of lysates from cells

incubated with 25 $\mu\text{g}/\text{mL}$ native/transamidated p31–49 indicated that there was not differences in tTGase expression into examined samples (Figure 6C).

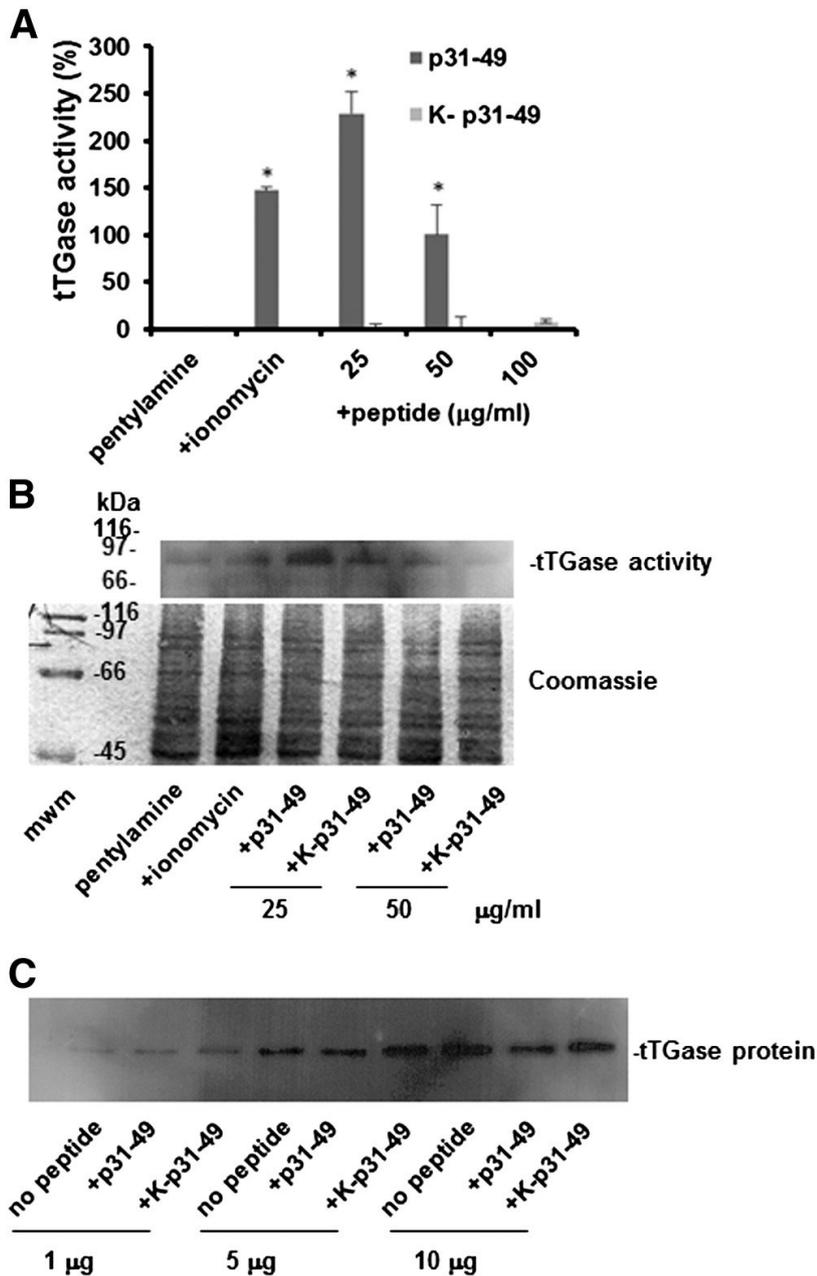


Figure 6: Biological activity of peptide p31-49 and its transamidated form in Caco-2 cells. **A**, Quantification of tTGase activity from lysates of Caco-2 cells incubated with 25–100 µg/mL native or transamidated p31-43 in the presence of pentylamine-biotin. Values are the means ± SD of at least 3 independent experiments performed in duplicate; * P < 0.05. **B**, tTGase activity of protein fractions from Caco-2 cells incubated with 25 and 50 µg/mL peptide and analyzed following SDS-PAGE and blotting by using peroxidase-streptavidin as probe and ECL detection (upper); post-Coomassie staining of the blotted membrane (bottom), **C**, Western blot analysis of different protein fractions from Caco-2 cells incubated with 25 µg/mL peptide using an anti-tTGase monoclonal antibody as probe. K-p31-49: transamidated p31-49.

Discussion and Conclusion

In this part of the thesis we studied transamidation reaction and characterized transamidated products to identify their immunological property. We showed that the two-step transamidation reaction of wheat flour with lysine ethyl ester produced water-soluble gluten and modified immunogenic and not immunogenic epitopes of gliadins.

Gluten proteins are divided into two classes based on their solubility in alcohol–water solutions: gliadins (soluble) and glutenins (insoluble).

In this study firstly we found that the solubility of both gliadins and glutenins was drastically changed following mTGase treatment of wheat flour in the presence of K-C₂H₅. Also during transamidation step there was different yields of solubilized gliadins and glutenins.

More specifically, we found that in the first step of transamidation was produced the soluble protein fraction (spf) which resulted mainly enriched in gliadins, indicating that these prolamins represent a favorite substrate for mTGase.

We reported that hmw forms (>200 kDa) were transiently produced in the residual insoluble gliadins during the enzymatic treatment. In parallel, we observed a decrease of 29–45 kDa gliadins. The appearance of hmw forms was confirmed by Western blot analysis and indicated the formation of neo-epitopes following transamidation in both K-gliadins and spf.

Interestingly, these experiments also revealed traces of hmw spf forms in the alcohol-soluble fraction, further indicating that they are mainly formed by gliadin monomers and $K-C_2H_5$, though the contribution of glutenins cannot be completely excluded.

In this study we found that gliadin peptides containing immunogenic epitopes were completely modified by the transamidation reaction. mTGase modified the same glutamine residues that are involved into celiacogenic epitopes.

Taken together, biochemical and structural data pointed out the existence of a mixed population of transamidated gliadins that included partially transamidated gliadins showing the classical mw range; discrete hmw gliadins, fully transamidated, that progressively acquired water-solubility. Interestingly, the transamidated forms were characterized by a complete loss of immune cross-reactivity toward anti native gliadin antibodies. So the transamidation of wheat flour in the presence of $K-C_2H_5$ causes effective epitope masking of gliadin.

To demonstrate that transamidation reaction mediated by mTGase modified gliadin activity we used a syntetic celiacogenic peptides p31–49.

It is known that p31–43 induced interleukin (IL)-15 secretion in enterocytes which caused the disappearance of the villi in CD. Subsequent studies showed that p31–43 altered the trafficking of vesicular compartments in the intestinal Caco-2 cell and induced cell proliferation and crypts hyperplasia in CD (11). More recently, gliadin peptide p31–43 was reported to induce tTGase activation in Caco-2 cells thought

Ca²⁺ release (10). This activation could contribute to the inflammatory response in CD.

Our data indicated that p31–49, like p31–43, increased the intracellular specific activity of tTGase in Caco-2 cells. Importantly, transamidated p31–49 was unable to induce a similar activity, probably it was unable to evoke an increase of Ca²⁺ in the cytosol.

In summary, the detoxification protocol of wheat flour based on the use of food-grade mTGase and K-C₂H₅ largely produced fully transamidated water-soluble gliadins that lost their bio-immune activity.

References

- 1 Gianfrani C, Siciliano RA, Facchiano AM, Camarca A, Mazzeo MF, Costantini S, Salvati VM, Maurano F, Mazzarella G, Iaquinto G, Bergamo P and Rossi M. Transamidation of wheat flour inhibits the response to gliadin of intestinal T cells in celiac disease. *Gastroenterol* 2007; 133: 780–89.
- 2 Fleckenstein B, Qiao SW, Larsen MR, Jung G, Roepstorff P and Sollid LM. Molecular characterization of covalent complexes between tissue transglutaminase and gliadin peptides. *J Biol Chem* 2004; 279: 17607-16.
- 3 Lombardi E, Maurano F, Bergamo P, Bozzella G, Luongo D, Mazzarella G, Rotondi Aufiero V, Iaquinto G, Rossi M. Selective inhibition of gliadin immune reactivity by transamidation of wheat flour with microbial transglutaminase. *J Leukoc Biol* 2013; 93: 479–88.
- 4 Weegels PL, Hamer RJ, Schofield JD. RP-HPLC and capillary electrophoresis of subunits from glutenin isolated by SDS and Osborne fractionation. *J Cereal Sci* 1995; 22: 211–24.
- 5 Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 1976; 72: 248–54.
- 6 Arentz-Hansen H, Körner R, Arntzen MO, Quarsten H, Vader W, Kooy YM, Lundin KE, Koning F, Roepstorff P, Sollid LM, McAdam SN. The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J Exp Med* 2000; 191: 603–12.
- 7 Qiao SW, Bergseng E, Arntzen MØ, Xia J, Fleckenstein B, Khosla C, Sollid LM. Antigen presentation to celiac lesion-derived T cells of a 33-mer gliadin peptide naturally formed by gastrointestinal digestion. *J Immunol* 2004; 173: 1757–62.
- 8 Dørum S, Arntzen MØ, Qiao SW, Holm A, Koehler CJ, Thiede B, Sollid LM, Fleckenstein B. The preferred substrates for transglutaminase 2 in a complex wheat gluten digest are peptide fragments harboring celiac disease T-cell epitopes. *PLoS One* 2010; 5: 14056.
- 9 Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Auricchio S, Picard J, Osman M, Quarantino S, Londei M. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet* 2003; 362: 30–37.
- 10 Caputo I, Secondo A, Lepretti M, Paoletta G, Auricchio S, Barone MV, Esposito C. Gliadin peptides induce tissue transglutaminase activation and ER-stress through Ca²⁺ mobilization in Caco-2 cells. *PLoS One* 2012; 7: 45209.
- 11 Barone MV, Zanzi D, Maglio M, Nanayakkara M, Santagata S, Lania G Miele E, Ribocco MT, Maurano F, Auricchio R, Gianfrani C, Ferrini S, Troncone R, Auricchio S. Gliadin-mediated proliferation and innate immune activation in celiac disease are due to alterations in vesicular trafficking. *PLoS One* 2011; 6: 17039.

Chapter 4

Characterization of immunomodulatory molecules and their adsorption on Bacillus subtilis spores

“manuscript still in preparation”

Introduction

As described in the Chapter 1 only gluten free diet alleviates the symptoms of CD and normalizes antibody levels and the intestinal mucosa (1). On the other hand, the research of alternative therapeutic approaches is very active. In particular, we focused our attention on possible immunomodulatory strategies based on the use of modified gliadin by food-grade microbial transglutaminase (mTGase), which caused positive change of immune response.

Recently, as described in Chapter 3 we characterized the products of a two-step transamidation process and demonstrated that this enzymatic treatment prevents the formation of immunodominant gluten peptides (2).

Therefore in this part of the study we wanted to immunologically characterize modified gliadin to identify a specific components responsible of immunomodulatory properties. Firstly, we characterized soluble transamidated gliadin (spf) and used its to induce an immune response into DQ8 mice to verify the phenotype of immune response. Then, we compared responses of native and modified gliadin and concluded that spf induced a phenotypic shift of the immune response. Another molecule with previously characterized beneficial property in celiac disease was conjugated linoleic acid (CLA). In particular, it CLA caused a decrease of gliadin toxicity and had a protective effect against oxidative stress induced by gliadin (3). Therefore, in the second part of the study we adsorbed spf and CLA on spores surface to evaluate the effectiveness of these tools as immunomodulatory strategies.

Materials and Methods

Gliadin preparation and transamidation reaction

Native and modified gliadin were extracted from wheat flour by a modified Osborne protocol (4). In briefly, a total of 100 g of flour was suspended in 8 volumes of 0.4 M NaCl and stirred for 10 min to extract albumin/globulins. The flour suspension was then centrifuged at 1000 g for 10 min, and the supernatant was discarded. Then, the pellet was washed several times to remove any residual soluble protein. To obtain modified gliadin the pellet was suspended in a solution containing 20 mM pharmaceutical grade lysine ethyl ester (K-C₂H₅; NutraBio.com, Middlesex, NJ, USA) and 8 U/g flour mTGase (ACTIVA®WM, specific activity: 81-135 U/g, Ajinomoto Foods Hamburg, Germany), and incubated for 2 hrs at 30°C. The suspension was then centrifuged at 3,000 g for 10 min, and the soluble protein fraction (spf) was recovered in the supernatant. Protein content was measured by Bradford analysis (5).

Gliadin and Spf stock solution were prepared by suspending 10 mg protein in 50 mM acetic acid.

Reverse Phase Chromatography (RP-HPLC)

The components of native gliadin and soluble transamidated gliadin were separated by micro-RP-HPLC (column μ RPC C2/C18 PC 3.2/3, SMART, Pharmacia). 2,5 mg of native gliadin was suspended in 1 ml of 10% buffer B according Wieser et al 1994

(6) while 2,5 mg of soluble transamidated gliadin (spf) was suspended in 1ml buffer A. The elution system were: (A) water with 0.1% trifluoric acid and (B) acetonitrile (90% v/v), water (10%), 0.1% trifluoric acid. Elution was performed by a linear gradient from 25 to 80% solvent B at a flow rate 100 µl/min and monitored at 280nm. The eluates were collected in fractions that were dried in a Speed-Vac centrifuge (Savant instruments, Inc. Farmingdale, NY, USA), lyophilized and stored at -20°C. The amount of protein in each fraction was determined by Bradford Test (5). The spf fractions were analysed by 12.5% SDS-PAGE (7).

Immunization Protocol

Transgenic mice expressing the HLA-DQ8 molecule were maintained in pathogen-free conditions at our animal facility (Accreditation No. DM.161/99). DQ8 mice maintained for several generations on a gluten free diet and used at the age of 6-12 wk. These experiments were approved by the National Institutional Review Committee and performed in accordance with European regulations (EU Directive 2010/63/EU). In immunization experiments, mice were injected intraperitoneally (i.p.) with gliadin or spf (100 µg) emulsified with FCA (day 0) or IFA (days 7 and 14). Mice were sacrificed on day 21 to collect spleens.

Isolation of T cells from spleen of DQ8 mice

Spleens were passed through a 40 µm cell strainer (Falcon, BD BIOSCIENCES, Erembodegem, Belgium) to separate cells. The splenic suspension was further treated with Tris-buffered ammonium chloride solution to remove erythrocytes. Then CD4⁺T cells from spleens of immunized mice were separated by using the CD4⁺T Cell Isolation Kit (Miltenyi Biotec Srl, Calderara di Reno-BO, Italy). Finally, CD4⁺T cells aliquots (5×10^5) were added to plates with mature dendritic cells (mDCs).

DCs and CD4⁺T cells co-cultures

Immature dendritic cells (iDCs) were purified by bone-marrow of femurs and tibiae of naïve DQ8 mice according with a published protocol (8). On day 8 non-adherent DCs were harvested by gentle pipetting and were placed in 48-well plates (2.5×10^5 cells/well), incubated with gliadin or protein fractions of gliadin (28 $\mu\text{g/ml}$); spf or protein fractions of spf (50 $\mu\text{g/ml}$) for 18 h and finally pulsed with LPS for 4 h to stimulate maturation of DCs. After 5×10^5 CD4⁺ T cells were added to mDCs and co-cultures for 72 h. Supernatants were collected and analyzed for IFN- γ and IL-10 protein levels by ELISA using commercial kits.

Binding assay

In total, 2×10^9 spores were incubated with 125 μg spf from the stock solution in 200 μl of 50 mM sodium acetate, pH 4.0 (adsorption solution), for 1 h at RT by continuous shaking (9). The spores were centrifuged at 13000 g for 10 min and washed three times to remove unbound spf. Also 2×10^9 spores were incubated with different concentration (90 μg ; 47 μg and 19 μg) of conjugated linoleic acid (CLA), isomers's mixture (Tonalin TG80; Cognis) in the same buffer used for spf. The amount of CLA bound to spores was determined by measuring absorbance at 236 nm (max absorbance value for CLA) (10).

Spf adsorption was evaluated by dot blot and Bradford analyses (5). The dot blot analysis was performed as previously described (11). The membrane was probed with

an in-house-produced mouse polyclonal antibody against spf (1:5000 dilution) and then with biotinylated streptavidin peroxidase-conjugated anti-mouse IgGs (1:10000 dilution; Dako SpA, Milano, Italy). Immunodetection was performed using ECL reagent (Amersham-GE Healthcare Europe GmbH, Glattbrugg, Switzerland) and a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA).

The amount of spf or CLA adsorbed spores was calculated as the difference in the protein content or fatty acid content of the adsorption solution before and after incubation.

Statistical analysis

To determine statistically significant differences among the various experimental groups were used one-way ANOVA. $P < 0.05$ was selected to denote a significant difference.

Results

Analysis of immune response induced by native gliadin and transamidated gliadin (spf)

In the first experiment mDCs were pulsed with gliadin or spf and then were co-cultured with gliadin or spf specific CD4⁺T cells. We observed that only gliadin-pulsed mDCs, as previously described, induced production of IFN- γ in gliadin CD4⁺T cells (Figure 1 panel A). Interestingly, we showed that spf specific CD4⁺T cells also recognized gliadin (Figure 1 panel C) but they induced an increase of gliadin-specific IL-10 production. Also, spf specific CD4⁺T cells produced IL-10 when DCs was pulsed with spf (Figure 1 panel D).

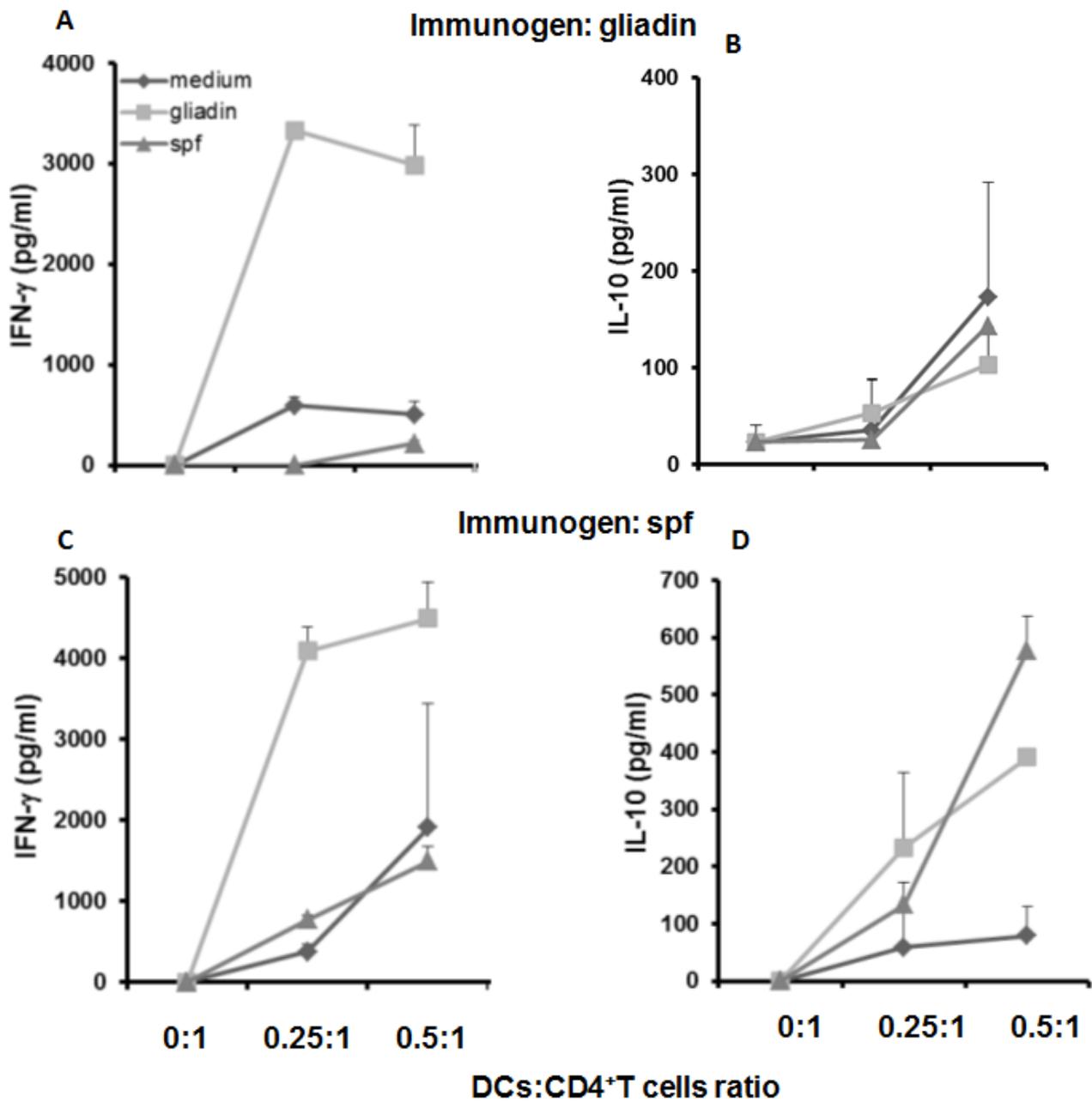


Figure 1: *In vitro* analysis of the gliadin or spf-specific CD4⁺T cell mediated response. mDCs (2×10^5 cells/well) that were not pulsed (diamond) or that were pulsed with gliadin (square) or spf (triangle) were incubated with CD4⁺T cells (5×10^5 cells/well) isolated from DQ8 mice parenterally immunised with gliadin or spf.

Isolation of gliadin and spf fractions

The different components of gliadin and spf were separated by RP-HPLC. We observed that in the elution profile of gliadin there were seven majority peaks that were separately collected (Figure 2A). Interestingly, in the elution profile of spf there were only three different majority peaks (Figure 2B) that were analysed by SDS-PAGE. Electrophoresis analysis of spf fractions showed that each fraction was characterized by discrete protein bands with different migration rates (Figure 2B).

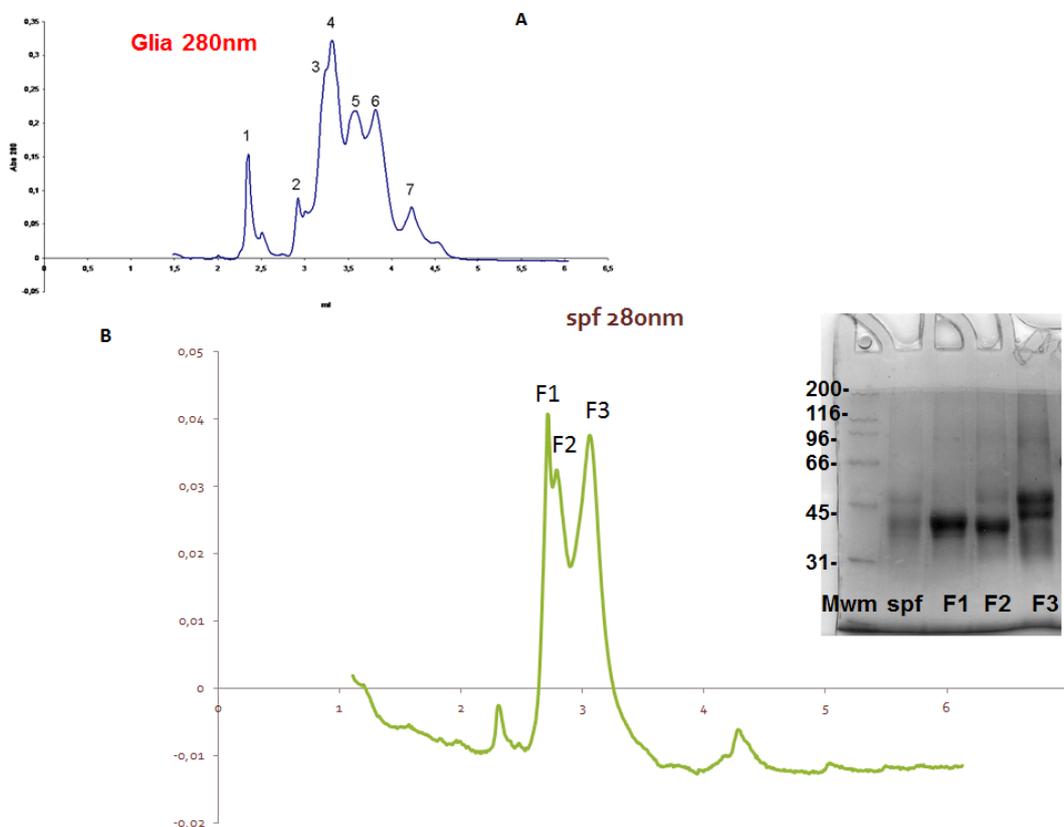


Figure 2: Reverse-Phase Chromatography. Purification of gliadin and spf by Reverse-Phase Chromatography with SMART System. A) Gliadin chromatogram at 280 nm. B) Spf chromatogram at 280 nm and SDS-PAGE of three protein fragments identified.

In *vitro* analysis of immune response to different gliadin fractions

In the next experiment we incubated iDCs with different fractions of gliadin and then pulsed with LPS to induce their maturation (mDCs). mDCs were incubated with autologous gliadin- or spf-specific CD4⁺T cells for 72 h. To evaluate the phenotype of the induced immune response we assessed the secretion of IL-10 and IFN- γ in the supernatant and expressed these values as IL-10/IFN- γ ratio. We observed that all gliadin fractions stimulated higher production of IFN- γ and a lower production of IL-10 in gliadin-specific CD4⁺T cells (Figure 3 left panel). Interestingly, we showed that these fractions induced an increase of IL-10 production in spf-specific CD4⁺T cells, as a higher IL-10/IFN- γ ratio was reported. In particular, we identified fractions 3 and 4 as components that significantly increased this ratio in comparison with the other fractions and the same whole gliadin (Figure 3 right panel).

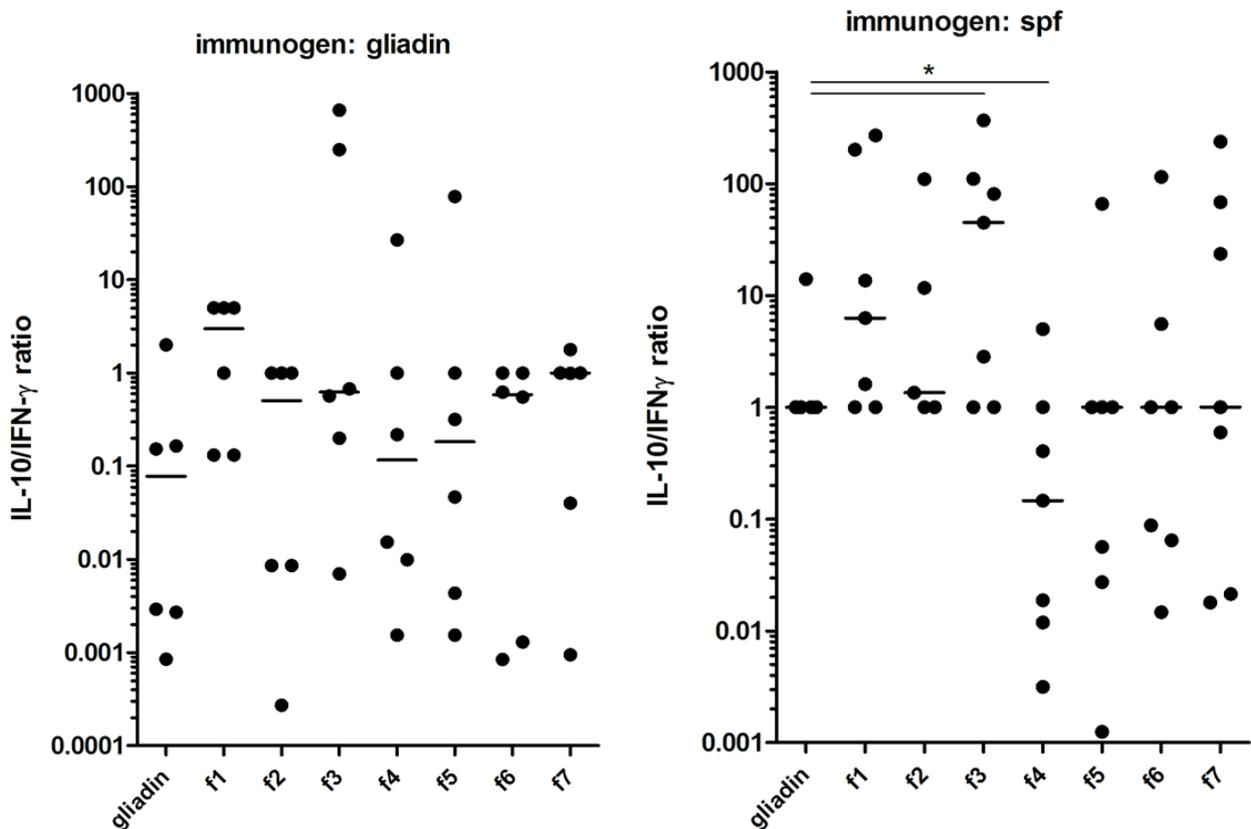


Figure 3: In vitro analysis of gliadin fraction immune response. mDCs (2×10^5 cells/well) that were pulsed with gliadin or its fractions were incubated with $CD4^+$ T cells (5×10^5 cells/well) isolated from DQ8 mice parenterally immunised with gliadin or spf. We calculated IL10/IFN- γ to define phenotypic of immune response. The results represent one of three independent experiments, and the bars are the median values. There were 5-6 mice in each group. *, $P < 0.05$.

Next, mDCs pulsed with the different spf fractions were incubated with autologous spf-specific $CD4^+$ T cells. We observed that IL-10 production in spf-specific $CD4^+$ T cell was mainly related to the components in fraction 3 of spf (Figure 4).

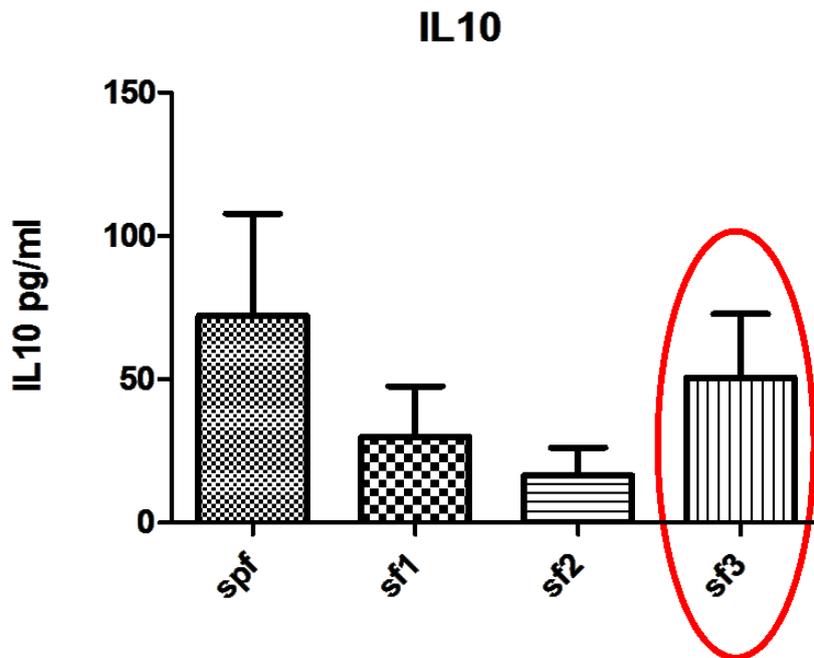


Figure 4: In vitro analysis of spf peptide fragments's immune response. mDCs (2×10^5 cells/well) that were pulsed with spf or its fractions were incubated with $CD4^+$ T cells (5×10^5 cells/well) isolated from DQ8 mice parenterally immunised with spf. The columns are the mean \pm SD of duplicate analyses.

Adsorption of spf on *Bacillus subtilis* spores

As previously described, gliadin was adsorbed to *Bacillus subtilis* spores in a 50 mM sodium acetate pH 4. Accordingly, we used the same conditions to adsorb spf to spores. We tested different strains of spores: autoclaved wt, autoclaved or not CotH and autoclaved or not CotE. To verify binding efficiency we did a dot blot analysis using two strains wt and CotH and we observed that mutant CotH bound spf with major efficiency. Also in this case we observed that the adsorption was stable (Figure 5).

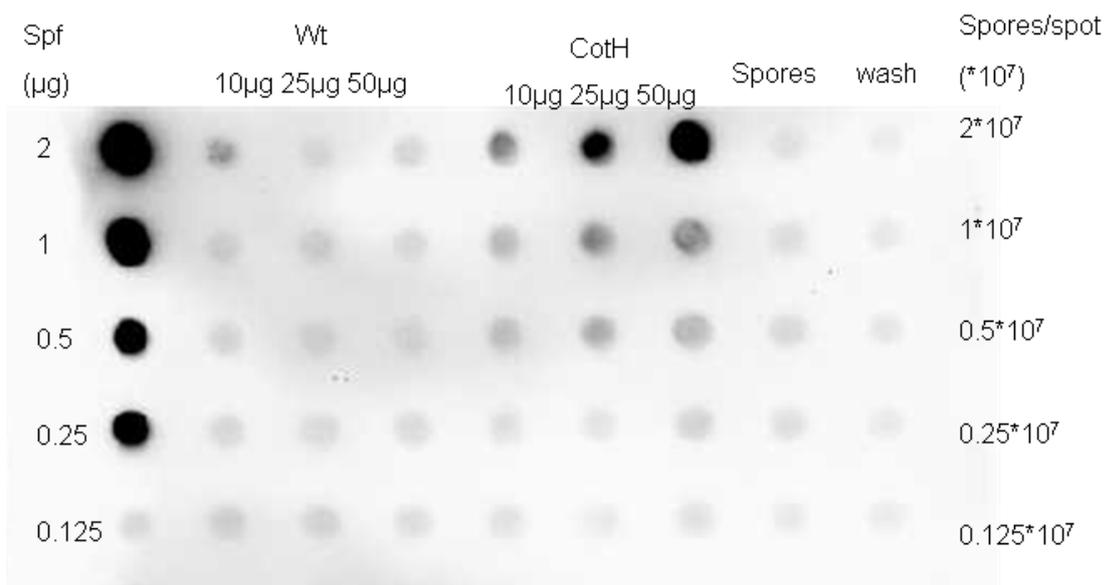


Figure 5: Dot Blot analysis of spf adsorbed spores. Serial dilutions of untreated wt and CotH spores were analyzed following the adsorption reaction of spf's different amount (10 μg , 25 μg and 50 μg).

Then, to determine the amount of spf adsorbed spores we did Bradford test and we showed that CotE, autoclaved or not, bound the highest amount of proteins (Figure 6).

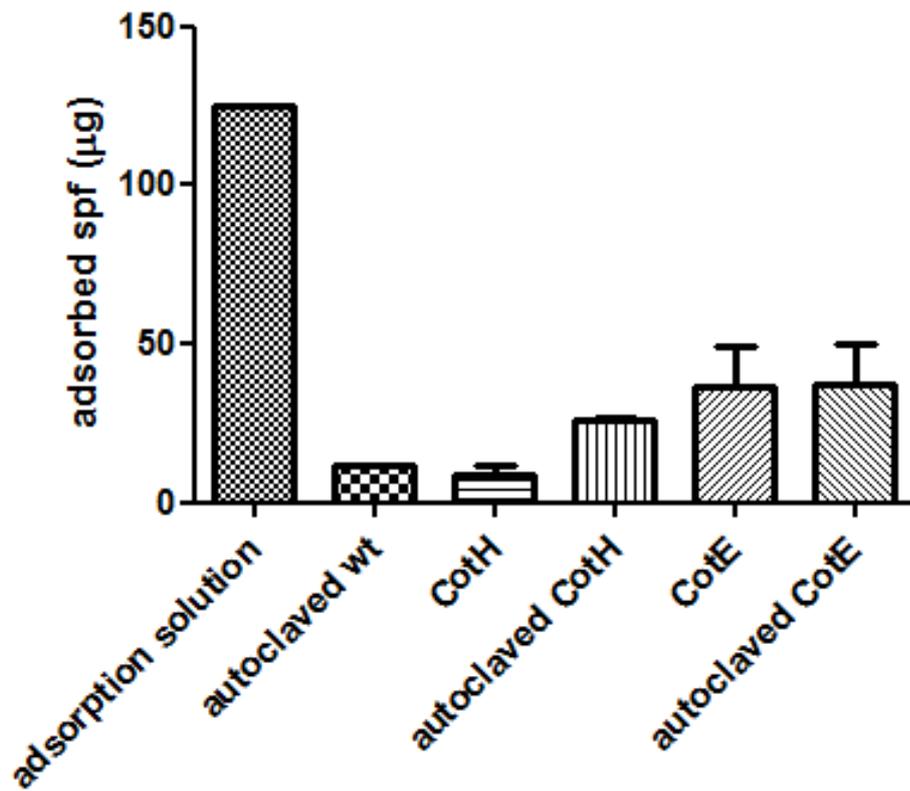


Figure 6: Quantitative analysis of spf adsorbed spores. The amount of spf (μg) adsorbed on 2×10^9 spores was calculated as the difference in the protein concentration of the adsorption solution before and after incubation. The data represent the mean \pm SD of three different experiments.

The exact amounts of spf adsorbed spores were reported in Table 1.

sample	% spf			
	Adsorbed*	DS	Wash**	DS
autoclavedwt	7,3	4,3	3,1	3,0
CotH	7,3	3,8	22,3	8,8
autoclavedCotH	21	2,6	2,0	1,8
CotE	27,1	10,9	12,7	11,2
autoclavedCotE	26,8	9,7	2,4	3,4

*, determined as the difference in the protein content of the adsorption solution before (100%) and after incubation. **, residual protein content in the first wash following incubation.

Table 1: Percentage of spf adsorbed on wt and mutant spores

Adsorption of CLA on *Bacillus subtilis* spores

As described previously, for adsorption reaction 2×10^9 spores were used in 50 mM sodium acetate pH 4. The strain used for this reaction was mutant CotE and we tested different concentration of CLA isomers's mixture. We showed that CLA isomers bound spores with high efficiency when the concentration of fatty acid in the adsorption buffer decreases (Figure 7 left panel). In fact we observed about 100% adsorption by incubating 19 μg of CLA isomers with spores (Figure 7 right panel). Also, we demonstrated that the adsorption of CLA to spores was stable.

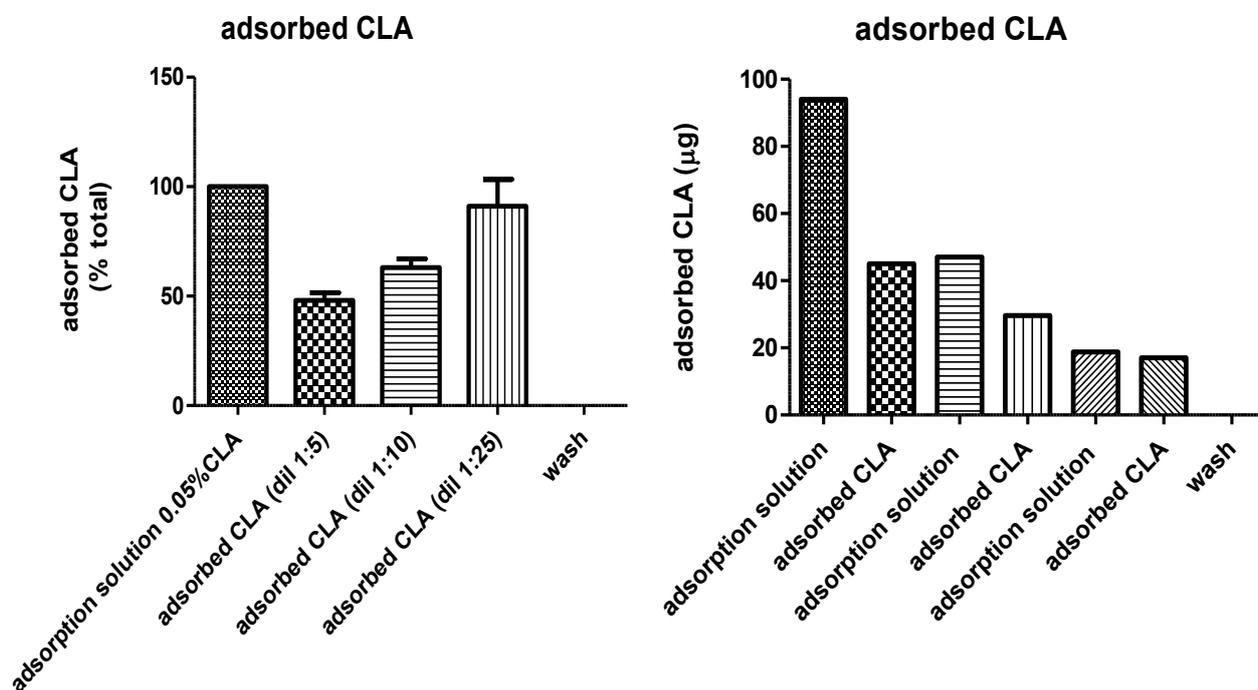


Figure 7: Quantitative analysis of CLA adsorbed spores. Percentage of adsorbed CLA (Left panel) or µg CLA (right panel). The amount of CLA adsorbed on $2 \cdot 10^9$ spores was calculated as the differences of fatty acid content between before and after incubation.

Discussion and Conclusion

In this study we shown that spf specific CD4⁺T cells were able to induce a phenotypic shift of gliadin dependent immune response. In a previous study it has been demonstrated that when spleen cells, isolated from immunized mice, were challenged with spf there was a down-regulation of gliadin specific immune response (12). So, we further characterized spf and used this protein to induce a regulatory response into DQ8 mice.

Firstly we confirmed that in *vitro* challenge with spf induced a down regulation of the gliadin specific CD4⁺T cells response. Interestingly, we demonstrated that spf specific CD4⁺T cells recognized also native gliadin. So we confirmed that spf derived from modification of native gliadin still harboring gliadin T cell epitopes. Also, we showed that spf specific CD4⁺T cells induced an increase of IL-10 produced by native gliadin. Another interesting data was the ability of spf specific CD4⁺T cells to induce an IL-10 dependent immune response. Notably, the IL10/IFN- γ ratio confirmed that spf induced a regulative response and we showed that fraction 3, separated by reverse phase chromatography, gave a major contribution to this production. So, we concluded that spf was an important molecule with a potential immunomodulatory activity, able to down-regulate the inflammatory response.

Then, we analysed the immune response of seven different gliadin protein fractions separated by reverse phase chromatography. We observed that all protein fractions stimulated gliadin specific CD4⁺T cells to produce high level of IFN- γ but,

interestingly, when gliadin fraction- pulsed mDCs interacted with spf specific CD4⁺T cells, there was an increase of IL-10 production. Also interestingly two fraction significantly induced a phenotype shift toward a regulative response. So, we have further confirmed the immunomodulatory ability of spf. Then, we adsorbed this molecule to spores in the perspective to induce a regulative response into DQ8 mice. We showed that spf stably bound spores and that strain CotE had a major binding efficiency for spf. This strain has also been used to bind CLA, another immunomodulatory molecule. We found that also this molecule was stably adsorbed to spores.

We concluded that this system represent a promising tool to induce an immunomodulatory response in inflammatory conditions.

Experiments are planned to use bacterial spores adsorbed with the characterized molecules as mucosal adjuvants aimed at restoring the regulatory response toward gliadin.

References

- 1 Green PH, Cellier C. Celiac disease. *N Engl J Med* 2007; 357: 1731-43
- 2 Mazzeo MF, Bonavita R, Maurano F, Bergamo P, Siciliano RA and Rossi M. Biochemical modifications of gliadin induced by microbial transglutaminase on wheat flour. *Bioch et Biophys Acta (BBA)-General Subjects* 2013; 1830: 5166-74
- 3 Bergamo P, Gogliettino M, Palmieri G, Cocca E, Maurano F, Stefanile R, Balestrieri M, Mazzarella G, David C and Rossi M. Conjugated linoleic acid protects against gliadin-induced depletion of intestinal defenses. *Mol Nutr Food Res* 2011; 55: 248-56
- 4 Weegels PL, Hamer RJ, Schofield JD. RP-HPLC and capillary electrophoresis of subunits from glutenin isolated by SDS and Osborne fraction. *J Cereal Sci* 1995; 22: 211-4.
- 5 Bradford MM. A rapid and sensitive method for the 1 quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.
- 6 Wieser H, Seilmeier W and Belitz HD. Quantitative Determination of Gliadin Subgroups from Different Wheat Cultivars. *J Cereal Sci* 1994; 19: 149-55
- 7 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nat* 1970; 227: 680-5
- 8 Lutz MB, Kukutsch N, Ogilvie ALJ, Roßner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999; 223: 77-92.
- 9 Huang JM, Hong HA, Van Tong H, Hoang TH, Brisson A, Cutting SM. Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine* 2010; 28: 1021-30.
- 10 Kappor R, Reaney M and Westcott ND. Conjugated linoleic acid oils. Bailey Industrial Oil and Fat Products. 6th ed; Canada 2005.
- 11 Hinc K, Isticato R, Dembek M, Karczewska J, Iwanicki A, Peszyńska-Sularz G, De Felice M, Obuchowski M, Ricca E. Expression and display of UreA of *Helicobacter acinonychis* on the surface of *Bacillus subtilis* spores. *Microb Cell Fact* 2010; 9: 2.
- 12 Lombardi E, Maurano F, Bergamo P, Bozzella G, Luongo D, Mazzarella G, Rotondi Aufiero V, Iaquinto G, Rossi M. Selective inhibition of gliadin immune reactivity by transamidation of wheat flour with microbial transglutaminase. *J Leukoc Biol* 2013; 93: 479-88.