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INNATE IMMUNITY AND COELIAC DISEASE

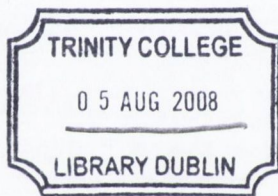
A thesis submitted for the degree of Doctor of Philosophy

by

Stacey Kelly, BSc (Hons)

**Trinity College,
University of Dublin**

**Trinity Term
2008.**



THESIS
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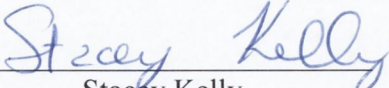

Stacey Kelly

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DEDICATION

This thesis is dedicated to Mam and Dad

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COMMUNICATIONS FROM THIS THESIS

Poster Presentations

Stacey Kelly, Dunne J, ten Bokum A, Abuzakouk M, Feighery C.

The effects of peptic tryptic gliadin on blood monocytes in control and coeliac subjects. The nineteenth annual meeting of the Irish Society of Immunology (National University of Ireland, Maynooth, 2004)

Stacey Kelly, Dempsey E, Bracken S, Byrne G, Weiser H, Jackson J, Kelly J, Feighery C.

PT gliadin and IL-15 do not up-regulate iNOS and COS-2 in Caco-2 cells. The twelfth international coeliac disease symposium (New York, U.S.A., 2006)

LIST OF ABBREVIATIONS

Abbreviation	Meaning
BSA	Bovine serum albumin
Caco-2	Colon adenocarcinoma 2 cell line
CAM	Cellular adhesion molecule
CD	Cluster of differentiation
CELISA	Cellular enzyme linked immunosorbent assay
COX	Cyclooxygenase
DAPI	4',6-Diamidino-2-phenylindole
dNTP	deoxy ribonucleotide triphosphate
DMEM	Dulbecco's modified eagle's medium
EATL	Enteropathy associated T cell lymphoma
EBAO	Ethidium bromide acridine orange
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FAE	Follicle associated epithelium
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GALT	Gut associated lymphoid tissue
GM CSF	Granulocyte macrophage colony stimulating factor
HLA	Human leukocyte antigen
HRP	Horse raddish peroxidase
ICAM	intracellular adhesion molecule
IELs	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IRF	Interferon regulatory factor

JAK	Janus kinase
JAM	Junction adhesion molecule
iNOS	Inducible nitric oxide synthase
IL	Interleukin
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MICA	Major Histocompatibility Complex Class I related chain A
NaOH	Sodium hydroxide
NFκB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
NO ₂	Nitrite
NO ₃	Nitrate
NOD	Nucleotide binding oligomerization domain
PCR	Polymerase chain reaction
PE	Phycoerytherin
PK	Protein kinase
PT	Peptic tryptic
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
RP HPLC	Reverse phase high pressure liquid chromatography
STAT	Signal transducers and activators of transcription
TAE	Tris acetate ethylenediamine tetraacetic acid
TCR	T cell receptor
TEER	Transepithelial electrical resistance
TG2	Tissue transglutaminase
TGF	Transforming growth factor
TLR	Toll like receptor
TNF	Tumour necrosis factor
ZO	Zonula occluden

SUMMARY

Coeliac disease is a gluten sensitive enteropathy in which well defined gliadin peptides initiate a T cell mediated inflammatory response within the small intestine. This disseminates into destruction of the villous architecture and results in lifelong complications upon continued inclusion of gliadin in the diet. Complete removal of gliadin and its related peptides remains the only form of treatment of coeliac disease. Recent evidence supports a role of gliadin and particular peptides in activation of an innate immune response and that these triggering events may influence the subsequent activation of the adaptive immune response. Intervention therapies targeting these initial triggering events of innate immunity offer an attractive alternative to gliadin exclusion from the diet. Numerous studies aimed at these initial events have provided invaluable information about how the disease is established in individuals with the disease susceptibility genes, despite the fact that 30% of the normal population remain unaffected.

The aims of this thesis were to investigate the direct role of gliadin in regulating aspects of the enterocyte and the monocyte, two key innate cells, in coeliac disease. As the cytokine IL-15 has been shown to mediate a number of gliadin induced innate effects, this cytokine was investigated for its expression within control and coeliac blood monocytes. Direct effects of IL-15 on enterocytes in relation to epithelial tight junction integrity and enzyme activity were also assessed. The regulation of the inducible nitric oxide synthase within enterocytes was determined along with its potential role in regulation of epithelial tight junction integrity through the generation of nitric oxide. Pro-inflammatory cytokines secreted by T cells during the later, adaptive immune response in coeliac disease were also included in this study, to determine the role of adaptive mediators versus the innate effects of gliadin and IL-15.

Confocal imaging of tight junctional proteins in Caco-2 and T 84 cell lines found disruption of key tight junctional proteins in response to IL-15 and a nitric oxide donor. Alterations in tight junction organisation were also found in response to a mixture of pro-inflammatory cytokines characteristic of an adaptive immune response. Electrophysiological characterisation of these changes found that they were accompanied by increased permeability through the epithelial monolayers. Gene expression within enterocytes as assessed by the Caco-2 cell line by real time PCR found that up-regulation of iNOS occurred upon IFN- γ stimulation but this was not observed following gliadin or IL-15 stimulation. Neither gliadin, IL-15 nor IFN- γ were found to regulate COX-2 gene expression or activity within Caco-2 cells. Gliadin induced iNOS activity within peripheral blood mononuclear cells as assessed by the measurement of the nitric oxide breakdown product nitrite but caused suppression of iNOS activity within the Caco-2 enterocytes cell line. This suppression was also seen in Caco-2 cells in response to IL-15. In contrast, IFN- γ was found to increase enterocyte iNOS activity but not peripheral blood mononuclear cell NOS activity. In flow cytometric analysis of blood monocytes derived from healthy controls and treated coeliac patients IFN- γ was shown to increase HLA-DR in both study groups though only reaching statistical significance in the control group. IFN- γ caused surface and intracellular IL-15 up-regulation within control blood monocytes in response to IFN- γ in healthy controls but not in the coeliac cohort. PT gliadin exposure caused an increase in HLA-DR expression in healthy control monocytes. However this response was not gliadin specific as similar findings were shown in the control BSA stimulated cells.

In conclusion, IL-15 and nitric oxide were shown to have direct effects on enterocytic cell barrier function. IL-15 and gliadin were shown to decrease iNOS activity in enterocytes whilst gliadin was found to increase peripheral blood mononuclear iNOS activity showing a cell specific response to gliadin stimulation. The pro-inflammatory cytokine IFN- γ appears central to all these

findings and supports a role for adaptive immunity along with innate immunity in these observations.

CHAPTER 1

GENERAL INTRODUCTION

1.1.0 The Gut Associated Lymphoid Tissue

1.1.1 The Gastrointestinal Immune System

The total mucosal surface in the adult gastrointestinal tract extends to 200-300m², the largest area of the body in contact with the external environment (Dommett, Zilbauer et al. 2005). The gut mucosal immune system is exposed to a wide variety of antigens derived from foods, resident bacteria and invading micro-organisms (Wittig and Zeitz 2003).

This single layer of gut epithelium is comprised of a number of specialised cells which enable nutrient uptake whilst also maintaining a mucosal defence against harmful pathogens by a number of different mechanisms. These specialised cells consist primarily of enterocytes (90%) that arise from stem cells in the crypt and contain a brush border (microvilli) at their apical surface. These differentiating cells move up the villi structures where they completely differentiate into absorptive cells and produce brush border enzymes (Kong, Heel et al. 1998). They also have a filamentous brush border glycocalyx to prevent penetration by pathogens (Acheson 2004). Tight junctions comprising of a meshwork of filaments form a selective seal in the intercellular space of the epithelium which regulates permeability (Fasano and Shea-Donohue 2005).

Within the epithelial layer are mucus producing goblet cells which secrete mucins to produce the protective gel layer that coats the mucosal surfaces and trefoil proteins that contribute to maintenance and repair of the mucosa. Paneth cells produce cell lysozyme and defensins involved in providing a mucosal barrier, thus providing a biochemically hostile environment for invading pathogens (Kong, Heel et al. 1998). Along with its barrier function, the epithelium has evolved a highly specialised immune system termed the gut associated lymphoid tissue which is divided into two main sites of induction and effector functions (Wittig and Zeitz 2003).

1.1.2 Induction Compartment

The primary inductive sites are organized lymphoid aggregates located in the wall of the small and large intestine (Wittig and Zeitz 2003). These include the mesenteric lymph nodes, Peyer's patches found in the small intestine and similar lymphoid aggregates in the large intestine (Cheroutre and Madakamutil 2004). The epithelium overlying these follicles is termed the follicle associated epithelium (FAE) and comprises of specialised epithelial cells called M cells whose function is to transport antigen into the dome of the follicle. These antigen presenting cells efficiently take up and transport various antigenic material to immune cells of the follicles (Neutra, Pringault et al. 1996).

Another important antigen presenting cell that allows passage of luminal antigen into lymphoid tissue is the dendritic cell. By extending its dendrites through the epithelium it can sample luminal antigen directly without damaging the tight junctional structures (Rescigno, Urbano et al. 2001). Macrophages and B cells also represent important antigen presenting cells for immune induction (Brandtzaeg 2001).

The lymphoid structures within the intestine have B cell follicles and germinal centres that are surrounded by areas that contain predominantly T cells (Nagler-Anderson 2001). When T and B cells are activated at these inductive sites by antigen presenting cells, they proliferate and express their antigen receptor, co-stimulatory molecules and various chemokine receptors and integrins such as the $\alpha 4 \beta 7$ integrin. Subsequent entry into the bloodstream is followed by tissue specific homing through the interaction of integrin and chemokine receptors with adhesion molecules on the epithelium e.g. MadCAM-1 on the small intestine. Transmigration through the epithelium enables activated T and B cells access to the underlying effector compartment (Campbell, Kim et al. 2003).

1.1.3 Effector Compartment

The lamina propria represents the cells beneath the basement membrane residing below the FAE and around lymphoid follicles. This region consists of a large number of terminally differentiated B cells, T cells, dendritic cells, macrophages, mast cells and polymorphonuclear leukocytes. Antigen presentation also occurs here with macrophages, dendritic cells and epithelial cells sampling and engulfing antigens to present to T cells (Acheson 2004).

In humans, 80-90% of the terminally differentiated B cells found in the lamina propria are IgA immunocytes that produce polymeric IgA (Brandtzaeg, Baekkevold et al. 2001). This polymeric form of IgA comprises two IgA antibodies linked by a polypeptide J chain along with a heavily glycosylated protein termed the secretory component. This secretory component is part of a cell surface polymeric Ig receptor which facilitates IgA transcytosis across the epithelial barrier during secretion (Kerr 1990). IgA maintains homeostasis of the epithelium by interacting with antigen within the gut lumen after epithelial transcytosis or inside infected epithelial cells or by formation of immune complexes with antigen within the lamina propria (Wittig and Zeitz 2003). Lamina propria T cells resemble other peripheral cells in that they virtually all express T cell receptor and show similar predominance of CD4⁺ (60-70%) over CD8⁺ T cells. Phenotypically they appear as memory/effector cells but they show a low level of proliferation and little expression of CD25. They represent cells of restricted reactivation (Brandtzaeg, Farstad et al. 1998; Helgeland 2000).

The epithelium represents another distinct effector site. The composition of intraepithelial lymphocytes (IELs) differ markedly from the lamina propria with a dominance of CD8 over CD4 positive T cells and the occurrence of unusual cell subpopulations (Helgeland 2000). These unusual subpopulations include CD8 $\alpha\alpha$ homodimers, CD4⁻CD8⁻ double negative and CD4 $\alpha\beta$ CD8 $\alpha\alpha$ double positive expressing cells. IELs also consist of a high number of TCR $\gamma\delta$ expressing cells. These unusual subpopulations can respond to antigens not restricted by conventional HLA molecules (Hayday, Theodoridis et al. 2001). It has been proposed that these $\gamma\delta$ TCR bearing cells

may play a role in repair processes through recognition of unique markers presented by epithelial cells such as MICA (Witherden, Rieder et al. 2000; Didierlaurent, Sirard et al. 2002).

To summarise, the intestine provides an efficient barrier to prevent unrestricted access of antigen whilst comprising of a highly organised immune system. Through its distinct functional organisation and cellular mechanisms this immune system maintains homeostasis within the gut.

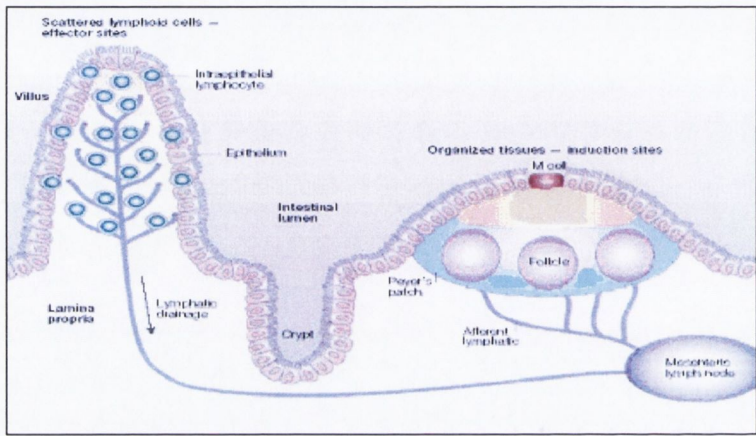


Figure 1.1: Anatomical structure of key elements of the intestinal immune system (Mowat 2003).

1.1.4 Pattern recognition receptors and mucosal immunity

Innate immunity initiates immediate defense mechanisms against pathogens on the basis of non-clonal recognition of conserved molecular motifs present on a wide range of different microbes. These motifs are termed pathogen associated molecular patterns and they are recognised by host receptors expressed on macrophages, neutrophils, dendritic cells and epithelial cells. These receptors are broadly termed pattern recognition receptors and are comprised of two main classes; Toll like receptors (TLRs) which are membrane bound and nucleotide binding oligomerization domain proteins (NODs) which reside within the cytoplasm. Recognition of microbes by this mechanism activates the NFκB signalling pathway triggering cytokine secretion and up-regulation of co-stimulatory molecules on antigen presenting cells and subsequent T cell activation (Athman and Philpott 2004; Tlaskalova-Hogenova, Tuckova et al. 2005).

Intestinal epithelial cells have been shown to express TLR 1 to 9 along with accessory signalling molecules and are responsive to stimulation by TLR ligands. Continuous exposure to ligands such as the TLR 4 ligand LPS may induce hyporesponsiveness allowing a state of tolerance (Otte, Cario et al. 2004). Some TLRs such as TLR5 are expressed at the basolateral surface of intestinal cells which may limit exposure to potential triggers. Deficiencies in the expression of NOD2 by intestinal epithelial cells causes decreased ability to eliminate intracellular pathogens such as *Salmonella* (Athman and Philpott 2004). Toll like receptors have also been shown to contribute to increasing intestinal barrier function through TLR2 activation. Induction of PKC through TLR2 signalling results in increased expression of the tight junctional protein ZO-1 at the apices of intestinal cells (Cario, Gerken et al. 2004).

1.2 Tolerance

Oral tolerance is defined as the induction of a state of systemic immune nonresponsiveness to orally administered antigen upon subsequent antigen exposure. This mechanism prevents the development of an immune reaction or allergy against intestinal intraluminal antigens (Wittig and Zeitz 2003). Apart from commensal bacteria, the main source of potential antigenic stimulation comes from food proteins (Nagler-Anderson 2001). The mesenteric lymph nodes of the GALT appear central in this tolerance (Spahn, Weiner et al. 2002). Oral tolerance is an active immunologic process that is mediated by more than one mechanism. A low dose of antigen favours the induction of active cellular regulation whereas higher doses favour the induction of clonal anergy or deletion of reactive cells (Whitacre, Gienapp et al. 1991; Friedman and Weiner 1994; Chen, Inobe et al. 1995).

Antigen driven bystander suppression can also induce tolerance. In this situation tolerance induced by oral administration of a specific antigen upon initial exposure can result in overall suppression upon subsequent encounter through release of regulatory T cell soluble factors (Miller, Lider et al. 1991). Factors that influence the outcome of an immune response to orally administered antigens include antigen availability, barrier integrity, the

immune environment, type of antigen presenting dendritic cell, dendritic cell maturation and activation, the level and form of co-stimulation and the actions of regulatory T cells and their cytokines, mucosal adjuvants and genetic background (van Wijk and Knippels 2007). Breakdown in oral tolerance by self peptides results in inflammation, autoimmunity or allergy. Cross reactivity between a self antigen and microbial antigen can induce inflammation in a process termed molecular mimicry (Christen and von Herrath 2004). Microbes can also expose self antigens to the immune system by directly damaging tissues during active infection inducing autoimmunity. This mechanism is referred to as a bystander effect (Fasano and Shea-Donohue 2005). Breakdown of oral tolerance may result in intestinal immunisation that leads to disease. A classic example of this is the food hypersensitivity reaction to gliadin in coeliac disease (Wittig and Zeitz 2003).

1.3 Coeliac Disease

1.3.1 Definition

Coeliac disease is a permanent gluten sensitive enteropathy characterised by reversible small bowel mucosal atrophy in a genetically predisposed person (Goddard and Gillett 2006). This inflammatory response is directed towards wheat gluten and related proteins in barley and rye and causes villous atrophy, hypertrophic crypts and infiltration of intraepithelial lymphocytes into the small intestine (Marsh 1992).

1.3.2 History

The disease was first broadly recognised in the second century AD by Arataeus who described a malabsorption syndrome which he called “the Coeliac Disease of a chronic nature” (Adams 1856). Samuel Gee published the first modern account of the disease in his paper “On the Coeliac Affection” in 1888 (Gee 1888). It was not until the late 1940s that Dicke, a Dutch paediatrician first recognised that the ingestion of wheat was responsible for the manifestations of the disease (Dicke, Weijers et al. 1953; Van De Kamer, Weijers et al. 1953).

1.3.3 Epidemiology

Coeliac disease is universally distributed to involve all races and is one of the most commonly known genetic diseases with a mean prevalence of 0.5%-2% in the general population (Rodrigo 2006; Stepniak and Koning 2006). Most studies report an approximate 2:1 to 3:1 predominance of women (Goddard and Gillett 2006).

1.3.4 Clinical Presentations

Coeliac disease is a multi-factorial disorder which presents with highly diverse clinical manifestations. These can be broadly subdivided into classical or symptomatic patients that present with gastrointestinal symptoms and atypical or asymptomatic patients who have either no gastrointestinal manifestations or these are less pronounced and extraintestinal manifestations can occur. Patients may be defined as “silent” in cases where enteropathy is present but the patient feels healthy and “potential” coeliac disease in cases where despite no obvious enteropathy, serology positivity and minimal histological changes are apparent (Fasano 2005; Rodrigo 2006; Torres, Lopez Casado et al. 2007).

The clinical manifestations of coeliac disease vary markedly with the age of the patient, the duration and extent of disease and the presence of extraintestinal pathologic conditions (Periolo and Chernavsky 2006). Symptoms range from vague systemic symptoms such as tiredness or moderate weight loss to having abdominal symptoms including abdominal pain, diarrhea, vomiting and constipation. In other patients extraintestinal manifestations such as anemia, dermatitis herpetiformis, arthritis, alopecia, neuromuscular disorders and osteoporosis predominate (Kennedy and Feighery 2000; Torres, Lopez Casado et al. 2007).

1.3.5 Pathology

Susceptibility to coeliac disease and its activation and perpetuation involve a combination of environmental factors,genetic factors and immunological factors.

1.3.5.1 Environmental factor: Gliadin

Wheat, barley and rye are closely related cereals which have all been shown to be toxic to patients with coeliac disease. Cereal seed proteins may be separated in to three broad fractions; albumins, globulins and prolamins. The prolamin proteins are thought to be responsible for toxicity and are known in wheat, barley and rye as gliadins, hordeins and secalins (Godkin and Jewell 1998). The prolamins of the Triticeae family which comprise wheat, barley and rye may be divided into three broad groups: sulphur rich, sulphur poor and high molecular weight prolamins (Shewry and Halford 2002). Whilst the ability of gliadin fractions to exacerbate coeliac disease is well documented, recent evidence supports a role for high molecular weight glutenin sub-units in disease toxicity (Dewar, Amato et al. 2006).

Table 1.1:
Contents of the
prolamin
proteins found in
wheat (Shewry
and Halford 2002)
The prolamin
proteins contain
extensive repeated
sequences based
on proline and
glutamine rich
motifs. The high
proline content

Components	M _r (%) Total)	M _r (%) Total)	Partial amino acid composition (mol %)
HMW Prolamins			
HMW subunits of glutenin	Polymers	65-90,000 (6-10%)	30-35% Gln, 10-16% Pro, 15-20% Gly, 0.1-1.5% Cys, 0.7-1.4% Lys
S-rich prolamins			
γ-gliadins	Monomers	30-45,000	
α-gliadins	Monomers	(70-80%)	
B- and C- type LMW subunits of glutenin	Polymers		30-40% Gln, 15-20% Pro, 2-3% Cys, <1% Lys
S- poor prolamins			
ω-gliadins	Monomers	30-75,000	40-50% Gln, 20-30% Pro, 8-9% Phe, 0-0.5%
D- type LMW subunits of glutenin	Polymers	(10-20%)	Lys, 0-<0.5% Cys
C-type LMW subunits are essentially polymeric forms of α- and γ- gliadins and D-type LMW subunits polymeric ω-gliadins. The B-type LMW subunits constitute a discrete group of S-rich prolamins. Cys is present in D-type LMW sub-units but not ω-gliadins.			

renders these proteins resistant to complete digestion by gastric, pancreatic and brush border enzymes which lack prolyl-endopeptidase activity resulting

in accumulation of relatively large peptides in the small intestine (Hausch, Shan et al. 2002; Shan, Qiao et al. 2005).

Recently, it has emerged that there are two different gliadin peptide sub groups that can initiate either an immune activating effect or alternatively a direct toxic effect. These immunogenic peptides are involved in the adaptive immune response in disease pathogenesis. These peptides have been mainly identified in α gliadins but can also be found in glutenin and γ gliadins (Jabri, Kasarda et al. 2005). A 33mer peptide, part of the α -2 gliadin peptide, has been described as a central immunodominant peptide in coeliac disease pathogenesis. This peptide incorporates three distinct specific HLA DQ2 T cell epitopes. This peptide is thought to play a key role in disease pathogenesis not only because it contains CD4 T cell specific epitopes but also is an excellent substrate for the enzyme tissue transglutaminase which is involved in increasing its antigenicity. It has also been found to be highly resistant to proteolytic cleavage (Dieterich, Ehnis et al. 1997; Anderson, Degano et al. 2000; Shan, Molberg et al. 2002; Shan, Qiao et al. 2005).

The second sub-group of peptides are termed innate peptides. These peptides are not recognised by T cells but induce an innate-like response in the epithelium and antigen presenting cells. The most studied peptide in this category is the α gliadin p31-43. The cytokine IL15 appears central to this innate response along with non classical MHC molecules such as MICA (Maiuri, Ciacci et al. 2003; Hue, Mention et al. 2004). This peptide has also been found to increase mucosal tissue transglutaminase expression and its enzymatic activity (Maiuri, Ciacci et al. 2005).

1.3.5.3 Genetic factors

Predisposition to coeliac disease is polygenic and multifactorial. Human leukocyte antigen (HLA) genes encoding the class II molecules DQ2 and DQ8 have been firmly identified as key genetic risk factors in disease. 90-

95% of coeliac patients have the DQ2 haplotype DQA1*05/DQB1*02 with 5-10% of patients expressing the DQ8 haplotype DQA1*0301/DQB1*0302. Most coeliac patients have the DR3-DQ2 haplotype or are DR5-DQ7/ DR7-DQ2 heterozygotes. Of the patients that are DQ2 negative, most express the DR4-DQ8 haplotype (Sollid and Thorsby 1993; Lundin, Gjertsen et al. 1994; Sollid 2002; Louka and Sollid 2003). Incidents of non DQ2 or DQ8 haplotypes are extremely rare (Karell, Louka et al. 2003).

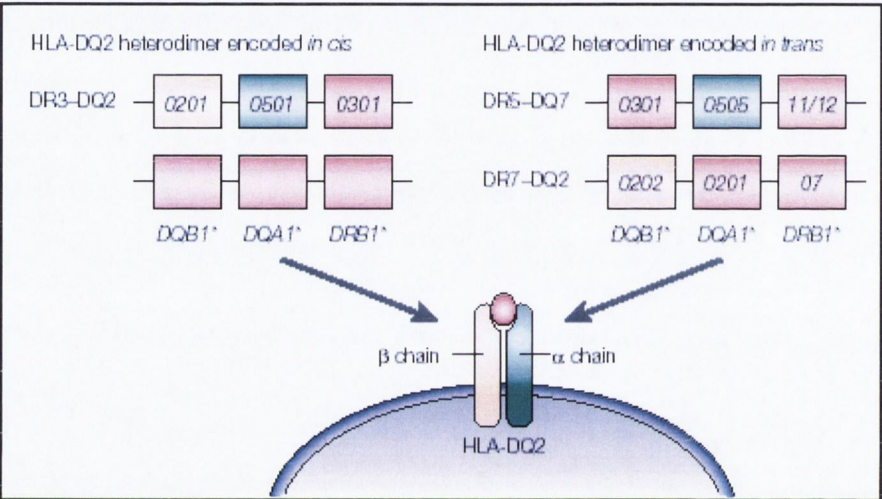


Figure 1.2: HLA-DQ2 haplotypes. Haplotypes may be either encoded *in cis* (on one chromosome) in individuals that have the DR3-DQ2 haplotype or *in trans* (across both chromosomes) in individuals who are DR5-DQ7/DR7-DQ2 heterozygotes (Sollid 2002).

The monozygotic twin concordance rate is high at around 75% with dizygotic concordance at 11% (Greco, Romino et al. 2002). This suggests a strong influence of genetic factors. Despite strong linkage to coeliac disease, HLA genes alone are not sufficient for disease development as up to 35% of the general population have been found to express the HLA DQ2 susceptibility genes (Sollid, Markussen et al. 1989). Both HLA-DQ2 and HLA-DQ8 have very characteristic peptide binding motifs characterised by a preference for hydrophobic and negatively charged amino acids at specific positions in bound peptides (Johansen, Vartdal et al. 1996; Godkin, Friede et al. 1997; Stepniak, Vader et al. 2005).

Gliadin derived peptides display only a low affinity for DQ alleles but up to 40% of their content is composed of glutamine residues which through a process of deamidation can convert them to negatively charged glutamic acid residues (van de Wal, Kooy et al. 1998). It was found that a key enzyme in coeliac disease pathogenesis resulted in increasing binding efficiency to HLA-DQ2 and HLA-DQ8 by deamidation of these peptides. The enzyme in question is tissue transglutaminase (TG2), a ubiquitous enzyme whose expression is increased in coeliac disease. TG2 is expressed at the epithelial brush border as well as in the subepithelial region (Molberg, McAdam et al. 1998). TG2 catalyses the covalent and irreversible cross linking of a protein with a glutamine residue (glutamine donor) to a second protein with a lysine residue (glutamine acceptor) resulting in the formation of a ϵ -(γ -glutamyl)-lysine isopeptide. This process is termed transamidation. Under certain conditions when no primary lysine residues are available as glutamine acceptors, deamidation of glutamines to glutamic acid residues may be favoured. (Dieterich, Esslinger et al. 2003). Below a pH of 7.3, deamidation is favoured over transamidation. The proximal intestinal brush border with a pH of 6.6 and endocytic vesicles (pH 5) which are thought to be involved in TG2 uptake would therefore favour the deamidation reaction (Fleckenstein, Molberg et al. 2002). The high glutamine content of gluten makes it a preferred glutamine donor substrate for TG2 (Molberg, McAdam et al. 1998; van de Wal, Kooy et al. 1998).

1.3.5.4 Immunological Mechanisms: Innate

A two signal model has been proposed to explain coeliac disease progression. These represent two integrated but principally different mechanisms. The first signal is thought to be generated by the innate immune response representing an initial “danger” signal to the immune system brought about by recognition of certain gliadin peptides (Matzinger 2002; Brandtzaeg 2006).

The direct toxic effects attributed to gliadin focussed on changes in cell morphology, cytoskeletal re-arrangement, growth and viability, apoptosis, agglutinating activity, alterations in DNA, RNA and protein synthesis and altered oxidative balance (Hudson, Cornell et al. 1976; Weiser and Douglas

1976; Sjolander and Magnusson 1988; De Vincenzi, Dessi et al. 1995; Giovannini, Luchetti et al. 1997; Giovannini, Sanchez et al. 2000; Dolfini, Elli et al. 2002; Clemente, De Virgiliis et al. 2003; Giovannini, Matarrese et al. 2003; Dolfini, Roncoroni et al. 2005). More recently it has been shown that the p31-43 “toxic” peptide of A gliadin is largely responsible for this innate effect. These peptides do not bind to HLA-DQ 2 and HLA-DQ8 molecules thus are not capable of activating an adaptive immune response. The induction of the innate cytokine IL-15 appears crucial to this innate response and has been shown to be responsible for a number of innate mechanisms in disease pathogenesis (Maiuri, Ciacci et al. 2000; Maiuri, Ciacci et al. 2003).

The cytokine IL-15 interacts with a number of innate cells and thus can mould the innate immune response in a number of ways to facilitate the subsequent adaptive pathogenic events. IL-15 has been shown to induce the expansion and survival of the CD8 TCR $\gamma\delta$ intra-epithelial lymphocytes whose increased infiltrating numbers in coeliac disease is a classic hallmark of the disease (Ebert 1998). The nature of the antigens recognised by intestinal TCR $\gamma\delta$ + IELs and the function of these cells remains elusive but they are thought to act as sentinels allowing an immediate response to epithelial damage and therefore participate in the innate immune response. IELs are thought to influence epithelial cell proliferation and differentiation. They do not require HLA expression for antigen recognition and instead respond to stress molecules (Groh, Steinle et al. 1998). IELs may contribute to epithelial cell damage via engagement of their ligand receptor to molecules expressed on enterocytic cells with subsequent release of perforin. The receptor for Fas (Fas ligand) has been shown to be up-regulated on the surface of IELs in active coeliac disease along with an increase in enterocyte Fas expression (Ciccocioppo, Di Sabatino et al. 2000; Ciccocioppo, Di Sabatino et al. 2001). Using *in vitro* organ culture, gliadin exposure has been found to increase FAS on enterocytes (Maiuri, Ciacci et al. 2000). Engagement of IEL Fas ligand with Fas expressed on enterocytes with subsequent enterocyte cytolysis would provide a further mechanism of villous atrophy (Maiuri, Ciacci et al. 2000; Ciccocioppo, Di Sabatino et al. 2001).

IL-15 has been found to increase the surface expression of an NKG2D receptor on IELs which participates in the cytolytic effector function of CD8 T cells. Following induction of the NKG2D receptor, these intra-epithelial cells can participate in a TCR-independent cytotoxicity through engagement with stress molecules located on enterocytic cells (Meresse, Chen et al. 2004). IL-15 increases the expression of the stress molecule MHC class I related protein A (MICA) on the surface of enterocytic cells. Engagement of these surface stress molecules with NKG2D receptors found on IELs ($\text{TCR}\alpha\beta^+\text{CD}^+$), results in cytotoxicity of the MICA positive enterocytes. This may result in the classical disease tissue atrophy and epithelial barrier destruction seen in coeliac disease (Hue, Mention et al. 2004).

IL-15 increases proliferation of intra-epithelial T cells and their production of IFN- γ and TNF- α . It also has been found to increase perforin/granzyme-dependent cytotoxicity and to reduce apoptosis of these cells (Di Sabatino, Ciccocioppo et al. 2006). Along with inducing proliferation of IELs, IL-15 also exerts its pro-inflammatory function through inhibition of the anti-inflammatory cytokine TGF- β by T cells. This could contribute to the promotion and sustained inflammation observed in coeliac disease (Benahmed, Meresse et al. 2007).

Gliadin has also been shown to cause structural alterations to the epithelium with resulting reduction in innate barrier function in coeliac disease. Disruption of the cytoskeleton through F-actin re-arrangement and junctional breakdown occur following gliadin exposure. This results in a leaky gut phenomenon allowing gliadin cross the epithelium (Clemente, De Virgiliis et al. 2003; Ciccocioppo, Finamore et al. 2006; Drago, El Asmar et al. 2006). Whether IL-15 plays a role in these phenomena has yet to be addressed. The p31-43 peptide of gliadin has been shown to prolong the effects of epidermal growth factor (EGF) by delaying the degradation of the epidermal growth factor and its receptor within endocytic vesicles resulting in sustained EGF activity. As a consequence the effects of EGF such as induction of cell proliferation and actin re-arrangement within enterocytes are prolonged in the presence of gliadin (Barone, Gimigliano et al. 2007).

Using cell lines and organ culture, gliadin has been demonstrated to have a direct effect on the monocytes, macrophages and dendritic cells. Gliadin stimulates human monocytes to produce IL-8 and TNF- α (Jelinkova, Tuckova et al. 2004). PT gliadin and to a lesser extent the p31-43 and 33-mer peptide stimulate murine macrophage gene expression of TNF- α , IL-12, IL-6, IFN- β and IL-15 mRNA. Gliadin also increases zonulin and TNF- α secretion (Jelinkova, Tuckova et al. 2004; Thomas, Sapone et al. 2006). PT digests of gliadin also increase iNOS enzyme activity and protein expression in cells pre-stimulated with IFN- γ (Maiuri, De Stefano et al. 2003).

Gliadin also plays a role in the functional maturation of dendritic cells, key antigen presenting cells which link the innate and adaptive immune responses. Gliadin fragments induce a mature phenotype within dendritic cells and induce cytokine and chemokine production and efficient antigen presentation to T cells (Nikulina, Habich et al. 2004; Palova-Jelinkova, Rozkova et al. 2005).

An increase in COX-2 mRNA, ICAM upregulation and p38 MAPK activation, along with an increase in CD83 cells, have also been observed in response to p31-43 in organ culture systems. IL-15 appears central in these observed phenomena. Thus the innate immune system is thought to provide the initial danger signal in coeliac disease with alterations in innate cells and induction of a number of mechanisms in response to gliadin which is thought to be necessary for a successful adaptive response. (Maiuri, Ciacchi et al. 2003).

1.3.5.5 Immunological Mechanisms: Adaptive

The toxicity of prolamins is largely attributed to their ability to generate a specific T cell response to gliadin peptides in the intestine of individuals at risk (van de Wal, Kooy et al. 1998; Sollid 2002). In an activated state, due to

innate activation signals, mucosal antigen presenting cells may induce mucosal gluten reactive Th1 cells within the lamina propria and perpetuate disease pathogenesis (Brandtzaeg 2006). Gliadin is taken up by macrophages and dendritic cells within the lamina propria and presented in the context of HLA-DQ molecules to T cells in the lamina propria resulting in T cell activation (Raki, Tollefsen et al. 2006). Upon uptake of gliadin, dendritic cells migrate to mesenteric lymph nodes and activate T cells which in turn can migrate back to the lamina propria as effector cells (Mowat 2003).

Active coeliac lesions show extensive induction of CD25 and HLA-DQ on sub-epithelial macrophages and dendritic cells. An important gluten dependent event is hyperactivation of CD4 T cells with increased expression of CD25 and CD3 (Brandtzaeg 2006). Release of IFN- γ and other cytokines such as TNF- α , perpetuates the ongoing response and alters key mucosal functions including intestinal permeability (Nilsen, Lundin et al. 1995; Bruewer, Luegering et al. 2003). Activation and release of enzymes such as matrix metalloproteases can also result in mucosal damage (Pender, Tickle et al. 1997).

The adaptive immune response involves not only T cells but also a serological B cell component. Despite considerable expansion of plasma cells, the Ig class pattern shows only little pro-inflammatory skewing in untreated patients. The IgA phenotype remains remarkably dominant in the lamina propria (Brandtzaeg 2006). IgA antibodies to gliadin and endomysium, a structure of the smooth muscle connective tissue are produced (Dieterich, Ehnis et al. 1997). Anti-gliadin antibodies, in particular IgG anti-gliadin can be found in normal individuals and are not specific for diagnosis (Rostom, Dube et al. 2005). IgA antibodies to endomysium are particularly specific indicators of coeliac disease suggesting that this structure contains one or more target autoantigens that play a role in the disease. Tissue transglutaminase (TG2) was identified as the actual if not major endomysial autoantigen. Serum autoantibodies directed against TG2 have therefore been found to be a specific marker for active coeliac disease (Dieterich, Ehnis et al. 1997; Brandtzaeg 2006).

Two mechanisms for the production of these TG2 antibodies have been proposed. The first mechanism suggests TG2 is crosslinked to gliadin peptides resulting in the formation of a neo-epitope. The recognition of these neo-epitopes likely favours inter-molecular epitope spreading from gliadin to TG2. The subsequent immune response is directed towards gliadin and TG2 (Dieterich, Ehnis et al. 1997). The second mechanism proposes that TG2 crosslinks with gliadin to form a complex which may be taken up by TG2 specific B cells and after intracellular gliadin degradation, gliadin is presented to gluten specific T cells in the context of HLA-DQ2 or HLA-DQ8. These T cells would in turn provide the necessary help for antibody production. When gliadin is removed from the diet, gliadin specific T cell help would cease stimulation of anti-TG2 specific B cells. This would result in a drop in TG2 antibody titres, which is observed upon introduction of a gluten free diet (Sollid, Molberg et al. 1997). The exact role of these autoantibodies in pathogenesis remains unclear (Maki 1995). Anti-TG2 antibodies do not seem to contribute to the formation of the intestinal lesions because disease symptoms disappear rapidly after the introduction of a gluten free diet and antibody titers drop much slower (Stepniak and Koning 2006).

1.3.6 Diagnosis

The diagnosis of coeliac disease is often missed because many individuals do not present with the classical gastrointestinal symptoms. Serological screening is often used as the first step in the diagnosis of coeliac disease, measuring endomysial TG2 antibodies (Monsuur and Wijmenga 2006). The presence of these antibodies correlates with the degree of villous atrophy though diagnosis of coeliac disease based solely on serologic markers is not accepted. The identification of the characteristic changes at the duodenal mucosa is necessary before complete certainty with respect to diagnosis (Rodrigo 2006). Traditionally, the diagnostic feature of coeliac disease is a flat mucosa with loss of villous architecture (Kennedy and Feighery 2000). Examination of duodenal biopsies is currently still the gold standard in the diagnosis of coeliac disease.

Marsh classified the characteristic pathologic findings as a continuum:

- Marsh 0 lesion is histologically normal
- Marsh I lesion consists of an intraepithelial lymphocytosis in normal appearing villi.
- Marsh II lesion consists of crypt hyperplasia in addition to the lymphocytosis.
- Marsh III lesion demonstrates villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis with partial, subtotal or total villous atrophy (Marsh 1992).

This latter sub-division of Marsh III was revised to facilitate diagnostic applications which subcategorised type III lesions based on reduced villous height as:

- Type IIIa mild atrophy
- Type IIIb marked atrophy
- Type IIIc as total villous atrophy

This is referred to as the Marsh-Oberhuber grading system (Oberhuber, Granditsch et al. 1999; Dickson, Streutker et al. 2006). The diagnosis is not established until there is an unequivocal response to gluten withdrawal, both histologic and symptomatic (Jabri, Kasarda et al. 2005).

Despite this, it is emerging that the absence of a mucosal lesion in routine histological examination does not exclude the diagnosis of coeliac disease. This reflects the importance of serological testing in the diagnosis of the disease (Mohamed, Feighery et al. 2007). Therefore a combination of serological testing with histological examination appears to be the most comprehensive method of disease diagnosis (Kennedy and Feighery 2000). Family members of known coeliac patients represent the most important group of subjects with an elevated risk of developing the disease. HLA typing is a first step to determine at risk family members, as it can exclude one third of first degree relatives (Torres, Lopez Casado et al. 2007).

1.3.7 Treatment and complications

The current treatment of coeliac disease involves lifelong dietary avoidance of wheat, rye, barley and oats (Rodrigo 2006). The toxicity of oats remains a

matter of controversy. The gluten free diet is a safe and efficient treatment but adherence is complicated and a true social burden for patients (Cerf-Bensussan, Cellier et al. 2003). The main problems associated with life long adherence to a gluten free diet are: the added cost of gluten free foods, the lack of convenience when eating outside the home and the difficulty of dietary compliance in asymptomatic patients. In these asymptomatic patients despite feeling well, dietary non-compliance may lead to long term complications associated with coeliac disease (Hourigan 2006). Whilst the gluten free diet is a very healthy and complete diet, it may be necessary to supplement the diet with iron, folic acid, vitamins and minerals until the intestinal mucosa recovers sufficiently, especially if deficiencies were noted on diagnosis. Adoption of this diet may reduce fibre, iron and calcium (Kennedy and Feighery 2000; Rodrigo 2006).

Secondary lactase deficiency is often associated with coeliac disease. Lactose intolerance is usually not severe enough to merit exclusion of lactose containing foods and usually regresses within three months of a gluten free diet (Kennedy and Feighery 2000; Rodrigo 2006). Autoimmune diseases occur 3-10 times more frequently in coeliac patients compared to the general population. These include: type I diabetes, primary biliary cirrhosis, Sjogren's syndrome and peripheral neuropathy (Jabri, Kasarda et al. 2005). Other associated diseases include dermatitis herpetiformis a relatively rare skin disorder characterised by IgA deposits in the upper dermis of uninvolved skin. Two thirds of patients with dermatitis herpetiformis have a small intestinal enteropathy with villous atrophy similar to coeliac disease with the remaining third showing no apparent intestinal lesion (Kennedy and Feighery 2000).

Due to poor absorption associated with the disease complications such as iron deficiency anemia and osteoporosis may occur. As dietary iron is absorbed by the proximal small intestine, the site of greatest damage in coeliac disease, iron deficiency anemia is not surprising but usually resolves with the institution of a gluten free diet (Rodrigo 2006). Macrocytic anemia can also occur with folate or vitamin B12 deficiency (Kennedy and Feighery 2000)

Coeliac disease is associated with a decreased bone mineral density in both children and adults (Kennedy and Feighery 2000; Goddard and Gillett 2006). Approximately one third of coeliac patients are osteopaenic, one third are osteoporotic and only one third have normal bone density at diagnosis. The risk increases with age at diagnosis, decreased body weight and in the postmenopausal state (Goddard and Gillett 2006). The mechanism for this may be due to malabsorption of vitamin D and calcium. The sex of the patient, malnutrition and reduced physical activity can also contribute to the low bone mineral density upon diagnosis (Rodrigo 2006). Osteopenia is reversible over time with a gluten free diet (Kennedy and Feighery 2000). Neurologic disorders associated with coeliac disease include peripheral neuropathy, cerebellar ataxia, epilepsy, multiple sclerosis and migraine (Rodrigo 2006).

Two serious complications unique to coeliac disease are refractory coeliac disease and enteropathy associated T cell lymphoma (EATL), both of which arise within the intra-epithelial compartment. There is a profound alteration in intra-epithelial lymphocyte homeostasis and the innate cytokine IL-15 appears central to these disorders which result in the production of clonal IELs. Refractory coeliac disease may be distinguished from uncomplicated coeliac disease by its failure to improve after 6 months on a gluten free diet. It is thought refractory coeliac disease may be the initial manifestation of overt EATL (Brousse and Meijer 2005).

Other malignancies associated with coeliac disease comprise of squamous carcinoma of the oropharynx and oesophagus, adenocarcinoma of the small intestine, as well as non-Hodgkin's lymphoma. The lymphomas are both T and B cell types and occur at intestinal and extra-intestinal sites (Jabri, Kasarda et al. 2005). Adapting a gluten free diet appears protective in preventing the development of these malignancies (Goddard and Gillett 2006).

1.3.8 Therapeutic potential

An increased understanding of disease pathophysiology enables novel therapeutic approaches to be developed. One such intervention is the restrictive introduction of gliadin into infants' diets. A gradual introduction of gliadin to the diet may help the immune system cope with the dietary proteins that are strong immunogens (Stepniak and Koning 2006). Another approach is the production of wheat seeds that are devoid of T cell epitopes either by traditional breeding programmes or by using transgenic technology (Sollid 2002). This is complicated by the large number of gliadin T cell epitopes and by the complexity of wheat genetics (Cerf-Bensussan, Cellier et al. 2003). Another approach would rely on the generation of artificial gluten genes from which T cell stimulatory epitopes have been removed and the introduction of such genes into non toxic cereals such as rice or maize (Stepniak and Koning 2006).

Another possible strategy is an enzyme supplement therapy which destroys T cell stimulating gluten peptides using exogenous bacterial proly endopeptidases (Sollid 2002; Dieterich, Esslinger et al. 2003). Problems encountered with these enzymes include their susceptibility to pepsin and lack of activity at low pH. Recently a novel extracellular prolyl endoprotease from *Aspergillus niger* that does not suffer from these limitations and could degrade gluten in the stomach preventing the activation of gluten specific T cells in the duodenum, has been described (Edens, Dekker et al. 2005).

Inhibition of TG2 raises another possibility as a therapeutic approach. Also if deamidation occurs in endosomes, inhibition of endosome acidification could prevent the deamidation reaction. However inhibiting TG2 may interfere with its downstream role in mucosal healing and other physiological processes such as programmed cell death (Sollid 2002; Kagnoff 2007).

Further approaches include blocking receptor-ligand interactions (either between the T cell receptor and HLA and/or MICA and NKG2D) (Hourigan 2006). Blocking HLA molecules poses a challenge as they are continuously synthesised by antigen presenting cells (Sollid 2002).

Blockade of IL-15 has been suggested to prevent MICA up-regulation and IL-15 signalling (Hourigan 2006). Abrogation of IL-15 signalling could prove useful in the malignant refractory coeliac disease where IL-15 plays an important role in intraepithelial lymphocyte expansion (Mention, Ben Ahmed et al. 2003). However, this may have potential side effects (Stepniak and Koning 2006). Nonetheless, delivery of therapeutic drugs to the intestine is relatively easy making therapeutic approaches even more attractive (Sollid 2002).

1.4 Interleukin 15 (IL-15)

1.4.1 Definition

Interleukin 15, with a molecular weight of 14-15 kilo-daltons, is a pleiotropic pro-inflammatory cytokine which has been found to increase in several inflammatory disorders. It exerts a broad effector function in both innate and acquired phases of the immune response. It belongs to the 4- α -helix bundle cytokine family that includes cytokines like IL-2, IL-3, IL-7, IL-9, IL-21 and growth factors such as GM-CSF (Bazan 1990; Bazan 1990; Burton, Bamford et al. 1994; Grabstein, Eisenman et al. 1994; Waldmann and Tagaya 1999).

1.4.2 Expression

IL-15 mRNA is ubiquitously expressed by multiple tissues including placenta, skeletal muscle, kidney, lung, heart, intestinal mucosa and skin within cells such as intestinal epithelial cells, fibroblasts, monocytes, macrophages, dendritic cells, bone marrow stromal cells, keratinocytes, microglial cells and mast cells. Freshly isolated PBMCs and normal T cells express IL-15 at very low levels. Due to tight regulation in translation, IL-15 protein expression is restricted to monocytes, epithelial cells, dendritic cells and fibroblasts (Tagaya, Bamford et al. 1996; Fehniger and Caligiuri 2001; Budagian, Bulanova et al. 2006; Bulfone-Paus 2006; Obermeier, Hausmann et al. 2006).

1.4.3 IL-15 Receptor

The IL-15 receptor (RIL-15) is composed of three sub-units, α , β and γ . The RIL-15 β chain is shared with the cytokine IL-2 and the RIL-15 γ chain is shared with the cytokines IL-2, IL-4, IL-7 and IL-9. The third chain, unique to the IL-15 receptor is the RIL-15 α sub-unit which binds IL-15 with high affinity. However the $\beta\gamma$ sub-units are sufficient for ligand binding and signal transduction. The α sub-unit is involved in binding IL-15 enabling *trans* presentation of IL-15 to RIL-15 $\beta\gamma$ expressing neighbouring cells along with *cis* presentation to the same cell and stabilisation of IL-15 with subsequent increased IL-15 signals. (Giri, Ahdieh et al. 1994; Giri, Kumaki et al. 1995; Tagaya, Bamford et al. 1996; Bulfone-Paus 2006; Olsen, Ota et al. 2007).

RIL-15 α mRNA is expressed by a number of immune and non-immune cells and tissues. These include T and B cells, NK cells, macrophages, thymic and bone marrow cells, PBMC, spleen, thymus, prostate, ovary, testis, brain, intestine, liver, skeletal muscle, lung, heart and kidney (Carson and Caligiuri 1998; Budagian, Bulanova et al. 2006).

Mast cells express a novel RIL-15 molecule that does not share any sub-units with the normal receptor. The widespread distribution of the of the IL-15 receptor underlies the pleiotropy of IL-15 (Tagaya, Burton et al. 1996).

A soluble form of the RIL-15 α , formed from proteolytic cleavage of the transmembrane receptor, is also found, through the action of TNF- α converting enzyme (Budagian, Bulanova et al. 2004).

1.4.4 Regulation

While transcriptional regulation of IL-15 occurs, the principal form of regulation is during translation, intracellular trafficking and secretion. IL-15 has been shown to co-localise with IL-15R α in the nucleus where a possible role in downregulating its own transcription has been described ((Fehniger and Caligiuri 2001; Nishimura, Fujimoto et al. 2005; Budagian, Bulanova et al. 2006). The IL-15 transcript is quite complex with multiple AUG codons upstream of the initiation codon causing inefficient translation (Bamford, Battiata et al. 1996).

Two isoforms of IL-15 exist which differ only in the length of their signal peptide, a long signal peptide (LSP) and a short signal peptide (SSP). The two distinct isoforms of IL-15 exhibit differential patterns of intracellular distribution, trafficking, secretion and endosomal localisation and translation efficiency. This points to an important role of the signal peptide in multiple mechanisms of control. The long signal peptide is found in the Golgi, ER system, and nucleus, is less efficiently translated and secreted at low levels. In contrast the short signal peptide is efficiently translated, found in the cytoplasm and nucleus and is not secreted. A region found in the C terminus of the mature IL-15 sequence elicits a negative constraint on IL-15 production also (Tagaya, Kurys et al. 1997; Gaggero, Azzarone et al. 1999).

Soluble IL-15R α may protect against excessive IL-15 activity by competing with membrane bound IL-15 receptors for IL-15 and thus sequestering the cytokine. Soluble IL-15R α may prolong the half-life of IL-15 and slow the release of bound IL-15 thus prolonging cytokine concentration. It may also act as an agonist or antagonist of IL-15 signalling through intermediate affinity with the $\beta\gamma$ subunit and acting therefore in a paracrine/ endocrine mode of signalling (Budagian, Bulanova et al. 2004). Eight different forms of the IL-15R α , with different tissue distributions may also account for regulated control of IL-15 effects in different tissues (Dubois, Magrangeas et al. 1999).

1.4.5 Signalling

IL-15 has been shown to recruit Janus kinase (JAK) 1 via its IL-15R β chain and JAK 3 via its IL-15R γ chain resulting in JAK cross-phosphorylation and activation, recruitment, phosphorylation and activation of signal transducers and activators of transcription (STAT) 3 and 5 which translocate to the nucleus (Johnston, Bacon et al. 1995). Phosphorylated IL-2R $\beta\gamma$ can also act as docking sites (Budagian, Bulanova et al. 2006). Additional signalling pathways through the IL-15 receptor include the src related kinase, induction of Bcl-2, and stimulation of the Ras/Raf/MAPK pathway that ultimately results in fos/jun activation. IL-15 can activate NF κ B in neutrophils and both

NF κ B and AP-1 in peripheral blood lymphocytes (Fehniger and Caligiuri 2001). IL-15R α may be involved in signalling independent of the $\beta\gamma$ sub-units which has been shown to signal via TRAF 2 and NF κ B (fibroblasts), Axl kinase, Syk kinase (in a B cell line and in neutrophils) (Bulfone-Paus 2006). Signalling of IL-15 in mast cells, in contrast, uses JAK2 and STAT 5 (Tagaya, Burton et al. 1996).

IL-15 signalling in cells occurs in a juxtacrine manner complexed to IL-15R α . This stable complex is constitutively maintained on the cell membrane via transendosomal internalisation, recycling and reappearance on the cell membrane (Dubois, Mariner et al. 2002; Bulfone-Paus 2006). IL-15 may also bind directly to the plasma membrane. IL-15 either bound directly or complexed to its receptor can generate a juxtacrine signal by interacting with an IL-15 receptor *in trans* (Bulfone-Paus 2006).

Reverse signalling of IL-15 also occurs, whereby binding of the IL-15 R α on a neighbouring cell with transmembrane IL-15 causes reverse signalling in the IL-15 expressing cell. This mediates the phosphorylation of the MAPK kinase family, ERK1 and 2 (extracellular signal regulated kinase) and p38 and focal adhesion kinase (FAK). Whilst IL-15 is phosphorylated in this process, no direct physical association of IL-15 with these kinases has been found (Budagian, Bulanov et al. 2006). These events increase cell adhesion and migration and induce IL-6, IL-8 and TNF- α expression by monocytes and prostate carcinoma cells. The short signal peptide IL-15 may participate in intracrine signalling, in the cell that it is produced in, without having to leave the cell (Bulfone-Paus 2006).

Type of signalling	Example	Targets	Effects
Juxtacrine	Transmembrane IL-15, IL-15 presented <i>in trans</i> on IL-15R α	Neighbouring cells expressing IL-15R β/γ chains (naïve T and B cells and NK cells)	Cell proliferation and differentiation
Intracrine	IL-15 with short signal peptide	Nucleus (macrophages)	Suppression of IL-15 transcription
Reverse	Transmembrane IL-15	Prostate carcinoma cells, macrophages monocytes	Activation of MAP kinases (ERK 1/2, p38), FAK, small Rho-GTPase Rac-3, induction of cytokine production (IL-6, IL-8, TNF- α), cell adhesion and migration

Table 1.2: Signalling pathways of IL-15 within and between neighbouring cells (Bulfone-Paus 2006).

1.4.6 IL-15 biological effects

IL-15 exerts a number of biological effects in both innate and adaptive responses, influencing the biological activity of both immune and non-immune cells. These include:

Polymorphonuclear cells

IL-15 increases the secretion of IL-8 and enhances phagocytosis in polymorphonuclear cells (Musso, Calosso et al. 1998). IL-15 induces morphological changes in neutrophils typical of activated cells. Actin is found to be induced upon IL-15 stimulation. IL-15 also delays apoptosis in neutrophils (Girard, Paquet et al. 1996).

Mast cells

In mast cells IL-15 acts as a growth factor and induces IL-4 production through STAT 6 signalling (Tagaya, Burton et al. 1996; Budagian, Bulanova et al. 2006).

Epithelial cells

Intestinal epithelial cells both express and respond to IL-15 which signal and proliferate in response to IL-15 (Reinecker, MacDermott et al. 1996).

IL-15 has been found to have an anti-apoptotic effect on human colon epithelial cells and this is thought to be mediated by inhibition of induced apoptosis (Obermeier, Hausmann et al. 2006). IL-15 up-regulates iNOS expression and NO production by gingival epithelial cells (Yanagita, Shimabukuro et al. 2002). IL-15 may be an important regulator of tight junction formation. Primary intestinal cells express the RIL-15 $\alpha\beta\gamma$ along with the cell lines Caco-2 and HT-29 (Reinecker and Podolsky 1995). Using the intestinal cell line T-84 which lacks the RIL-15 β chain, IL-15 was shown to up-regulate the tight junctional proteins ZO-1 and ZO-2 in the absence of the β chain. Upon transfection of the RIL-15 β into the T 84 cell line they are found to increase transepithelial electrical resistance properties and the expression of the tight junction proteins occludin and claudin-1 and claudin-2 demonstrating RIL-15 β dependent and independent mechanisms within this cell line (Nishiyama, Sakaguchi et al. 2001). IL-15 inhibits IL-8 and monocyte chemoattractant protein-1 in human colonic epithelial cells (Lugering, Kucharzik et al. 1999).

Monocytes, macrophages and dendritic cells

IL-15 has been shown to induce IFN- β and iNOS in a macrophage cell line (Liu, Zhai et al. 2004; Budagian, Bulanova et al. 2006). IL-15 is necessary for the functional maturation of dendritic cells and macrophages and for IL-12 dependent IFN- γ production by these cells. The IL-15/ IL-15 receptor is involved in nitric oxide production and up-regulation of MHC Class II (Ohteki, Suzue et al. 2001). IL-15 can trigger the production of IL-8 by monocytes and promotes IL-12 production in these cells via T cell dependent contact. IL-15 can also up-regulate the expression of CD40 on monocytes which, upon engagement with CD154 on CD4 T cells, release IL-12 (Avice, Demeure et al. 1998; Neely, Epelman et al. 2004).

Natural killer cells

IL-15 is a critical factor for natural killer (NK) cell differentiation and activates NK cell proliferation, cytotoxicity and up-regulates IFN- γ production in the presence of IL-12 (Carson, Ross et al. 1995; Bykovskaia, Buffo et al. 1999; Fehniger and Caligiuri 2001). IL-15 promotes the survival of NK cells, sustains their functional viability and prevents programmed cell death (Carson, Fehniger et al. 1997). IL-15 augments tumour surveillance mechanisms within these cells (Kobayashi, Dubois et al. 2005).

T cells

IL-15 synergises with IL-12 in the production of IFN- γ by CD4 T cells (Avice, Demeure et al. 1998). It plays an important positive role in lymphocyte homeostasis (Lodolce, Boone et al. 1998). IL-15 is a potent stimulant of intra-epithelial lymphocyte proliferation, IFN- γ production and cytotoxicity. It has been found to control the generation of $\gamma\delta$ intra-epithelial intestinal T cells through regulation of $\gamma\delta$ TCR gene rearrangement (Ebert 1998; Zhao, Nguyen et al. 2005). IL-15 enhances the activation of effector-memory CD4 and CD8 expressing T cells. IL-15 has been found to enhance TNF- α and IFN- γ of both these cell types and inhibits induced apoptosis (Mueller, Makar et al. 2003).

B cells

IL-15 in B cells in combination with CD40L stimulates IgA, IgG1 and IgM production. IL-15 has also been found to enhance germinal centre B cell proliferation (Armitage, Macduff et al. 1995; Park, Yoon et al. 2004).

Non immune cells

IL-15 is a major modulator of non-immune cells including fibroblasts, osteoclasts, epithelial and endothelial cells, myocytes, adipocytes and neuronal and glial cells (Budagian, Bulanov et al. 2006).

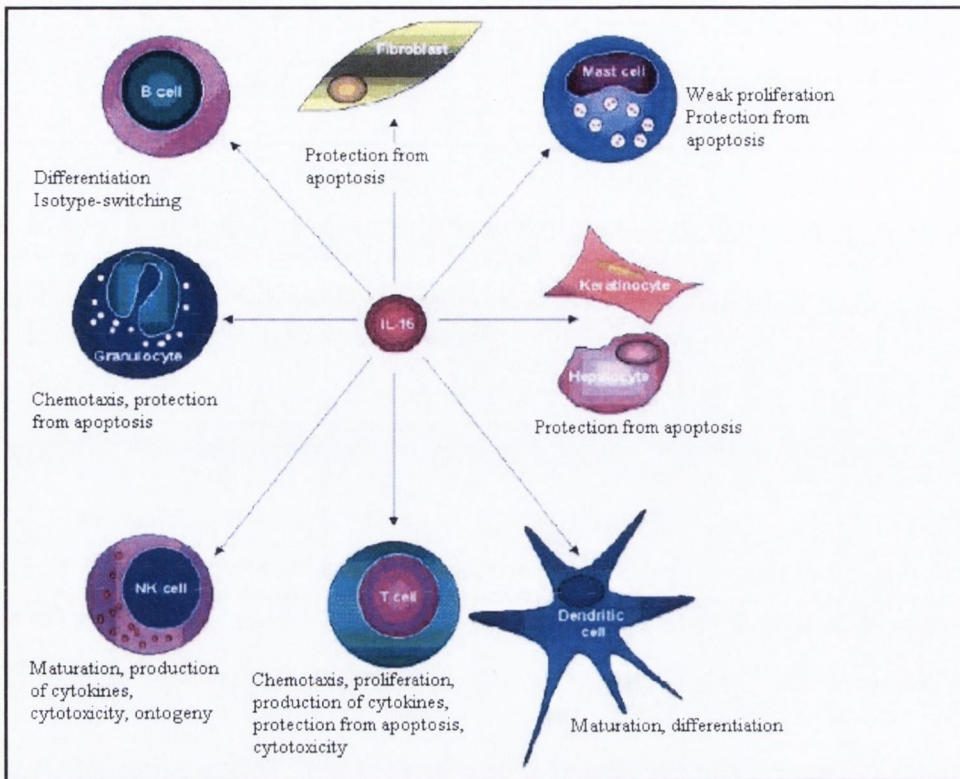


Figure 1.3: Summary of the different functions IL-15 exerts on a wide array of immune and non-immune cells (Bulfone-Paus 2006)

1.4.7 IL-15 and disease

1.4.7.1 Coeliac Disease

IL-15 has been found to play an important role in both the innate and adaptive arm of coeliac disease pathogenesis. Induction of IL-15 production has been found to occur in response to the innate p31-43 peptide (Maiuri, Ciacci et al. 2003). Upon exposure to gliadin peptides, IL-15 protein expression increases in both cells of the monocytic lineage and in epithelial cells (Maiuri, Ciacci et al. 2000; Mention, Ben Ahmed et al. 2003). The functional role of IL-15 in disease progression is multi-faceted with IL-15 using a number of mechanisms that may help to precipitate disease.

IL-15 $R\alpha$ is significantly up-regulated on intraepithelial lymphocytes in active coeliac disease thus it is not surprising that IL-15 exerts a number of effects on these cells during disease progression. IL-15 has been found to increase the secretion of IFN- γ and TNF- α by these cells. Intraepithelial

lymphocytes show enhanced proliferation in the presence of IL-15 along with a decreased propensity to undergo apoptosis.

IL-15 has also been shown to increase the cytolytic capacity of intraepithelial lymphocytes by a number of mechanisms. These include the induction and release of perforin and granzyme from intraepithelial cells which result in epithelial cell cytolysis (Hue, Mention et al. 2004; Di Sabatino, Ciccocioppo et al. 2006). Epithelial destruction may also occur through ligation of Fas found on its surface with its ligand FasL found on intraepithelial lymphocytes. This mechanism of cytolysis has been shown to occur in coeliac disease and IL-15 has been found to increase the expression of Fas on the surface of epithelial cells (Maiuri, Ciacci et al. 2000; Ciccocioppo, Di Sabatino et al. 2001).

IL-15 also arms the intraepithelial effector CD8 T lymphocyte population with cytotoxic capabilities by induction of NKG2D receptors on their cell surface. The target of intraepithelial cytolysis, the enterocyte is also regulated by IL-15. IL-15 increases stress markers such as the MHC class I chain related protein A (MICA) on epithelial cells and mononuclear cells of the lamina propria (Hue, Mention et al. 2004; Di Sabatino, Ciccocioppo et al. 2006) MICA is a non-conventional HLA class I molecules which can serve as a ligand for the receptor NKG2D expressed on all CD8 $\alpha\beta$ T cells and $\gamma\delta$ T cells and most NK cells. Engagement of the NKG2D on intraepithelial cells with MICA on enterocytes results in enterocyte destruction (Hue, Mention et al. 2004; Meresse, Chen et al. 2004).

In refractory coeliac disease, NKG2D engagement alone in the absence of T cell activation appears sufficient to arm the aberrant sCD3⁻CD7⁺ intraepithelial lymphocytes found in refractory coeliac disease with NKG2D cytolytic ability (Hue, Mention et al. 2004; Meresse, Chen et al. 2004). IL-15 also induces the selective growth and survival of the abnormal CD7⁺sCD3⁻ refractory intraepithelial lymphocytes. IL-15 induces granzyme B dependent cytotoxicity of refractory IELs against epithelial cells lines and induces IFN γ production (Mention, Ben Ahmed et al. 2003; Hue, Mention et al. 2004).

IL-15 has also been shown to influence T cells by inhibiting signalling by TGF- β within these cells. TGF- β is an anti-inflammatory cytokine and impairment of its function may promote and sustain intestinal inflammation by gliadin reactive T cells (Benahmed, Meresse et al. 2007). IL-15 also exerts its effects on lamina propria mononuclear cells where it is found to increase the expression of both MICA and the enzyme COX-2 (Maiuri, Ciacchi et al. 2003; Di Sabatino, Ciccocioppo et al. 2006).

Despite initial findings that IL-15 up-regulation seemed to only occur in active coeliac disease in response to gliadin, a recent report shows increases of IL-15 in cells and supernatants of normal, healthy individuals (Bernardo, Garrote et al. 2007). Thus IL-15 effects may not be specific to coeliac disease but the cytokine may provide the necessary signals and innate induction for a successful subsequent adaptive immune response which is characteristic of coeliac disease (Maiuri, Ciacchi et al. 2003).

1.4.7.2 Other inflammatory diseases

In rheumatoid arthritis patients, IL-15 promotes T cell proliferation and survival along with maintaining the proliferation of auto-reactive T cells *in vitro*. IL-15 in rheumatoid arthritis synovium recruits, activates and increases the trans-endothelial migration of synovial T cells (Bulfone-Paus 2006). IL-15 has been shown to increase COX-2 in rheumatoid synoviocytes and inhibit apoptosis of synovial fibroblasts (Min, Hwang et al. 2004; Budagian, Bulanova et al. 2006).

In inflammatory bowel disease, IL-15 is increased in peripheral blood mononuclear cells in ulcerative colitis, in particular in severe colitis. Lamina propria mononuclear cells proliferate in response to IL-15 (Fehniger and Caligiuri 2001). IL-15 has been found in the serum of ulcerative colitis and lamina propria T cells appear more responsive to IL-15 compared to controls. IL-15 mRNA is significantly increased in the inflamed rectal mucosa (Budagian, Bulanova et al. 2006). IFN- γ and TNF- α production was found to be greatly increased in inflammatory bowel disease T cells *in vitro* in response to IL-15 (Liu, Geboes et al. 2000).

IL-15 has also been linked to several other diseases including pulmonary inflammation, dermatological inflammatory disease, autoimmune diabetes (Fehniger and Caligiuri 2001; McInnes and Gracie 2004; Budagian, Bulanova et al. 2006).

1.4.8 Anti-IL-15 therapy

Therapeutic approaches include inhibition of IL-15 induced signalling using anti IL-15 antibodies, soluble IL-15R α and IL-15 R-Fc fusion proteins (Bulfone-Paus 2006). Such therapeutic approaches have been suggested in the treatment of coeliac disease and inflammatory bowel disease (Londei, Quarantino et al. 2003; van Heel 2006; Kagnoff 2007). In contrast, IL-15 agonists could be exploited to enhance T cell mediated immune responses so as to enhance the development of memory to pathogens or tumors or to enhance vaccination efficiency (Bulfone-Paus 2006).

1.5 Nitric Oxide

1.5.1 Definition

The Nobel prize for Medicine in 1998 was concurrently awarded to Robert Furchgott, Louis Ignarro and Ferid Murad in acknowledgement of their independent discoveries which led to the identification of nitric oxide (NO) formerly referred to as “endothelium derived relaxing factor” (Martin, Jimenez et al. 2001). Nitric oxide is a free radical gas derived from the amino acid L-arginine in a five electron oxidation reaction along with the production of L-citrulline (Marletta, Hurshman et al. 1998). It is regarded as a highly labile chemical substance with a very short half life of between 5 to 500 seconds depending on its surrounding environment (Ignarro, Fukuto et al. 1993). In an aqueous solution such as plasma, the reaction of nitric oxide and oxygen yields nitrite. Serum nitrite is rapidly oxidized to nitrate in the presence of oxyhemoproteins, which is subsequently eliminated by renal excretion. For this reason serum nitrate and nitrite are used as markers for NO synthesis (Ignarro, Fukuto et al. 1993; Veszelszky, Holford et al. 1995; ter Steege, Koster-Kamphuis et al. 1998).

Potential sources of NO in the gut include: intrinsic intestinal tissue (mast cells, epithelium, smooth muscle); resident and/or infiltrating leucocytes (neutrophils, monocytes and macrophages); and the action of commensal anaerobes within the intestine. Circulating nitrate derivatives from the diet may be reduced by the bacterial flora to nitrite which may subsequently be reduced to nitric oxide in the acidic environment of the stomach in a process termed denitrification (Salzman 1995; Martin, Jimenez et al. 2001).

1.5.2 Function of nitric oxide

Nitric oxide has been shown to exert both pathological and physiological effects. Nitric oxide may participate directly or indirectly to exert its function. The direct effects of nitric oxide involve its interaction with metal moieties and metal containing proteins. Two classic examples are the interaction of NO with heme groups on soluble guanyl cyclase and subsequent activation and production of cGMP and the interaction with heme groups on cytochrome p450 which forms part of the respiratory chain, resulting in its inhibition. Indirect effects involve its interaction with superoxide anions and results in production of radicals such as peroxynitrite. These reactive species can cause the alteration of proteins, DNA and lipids by inducing oxidative, nitrosative and nitrative stress through oxidation, nitrosation or nitration reactions respectively of proteins, DNA and lipids. Oxidation involves the removal of one or more electrons from a substrate, nitrosation involves the addition of NO^+ to an amine, thiol or hydroxyl aromatic group and nitration involves the addition of NO_2^+ to a molecule e.g tyrosine residues (Davis, Martin et al. 2001; Lirk, Hoffmann et al. 2002) This in turn can cause inhibition or activation of molecules involved in diverse roles e.g apoptosis, glycolysis, neurological activity and the modification of DNA, lipids and proteins. The diverse functions of NO are vast and include: increasing vasodilation, alteration of barrier function, activation and proliferation of lymphocytes, activation of cyclooxygenases, and regulation of dendritic cell maturation. These events often result in conflicting roles i.e. repair and damage of tissue, activation or inhibition of apoptosis. Nitric oxide concentration and physiological versus pathological conditions influence the effects of NO on different cell types (Davis, Martin et al. 2001; Martin, Jimenez et al. 2001; Fernandez-Ruiz, Gonzalez et al. 2004).

Cytotoxic	Cytoprotective
Antioxidant depletion Potentiation of reactive oxygen species/ reactive nitrogen species Nitrosation of amines and thiols Nitration of tyrosine residues Interaction with : • Transition metals • Intracellular calcium DNA damage by: • Deamination • Strand breakage • Alkylation • Oxidation • Inhibition of DNA repair enzymes Inactivation of key enzymes: • NAD dehydrogenase • Cytochrome p450 Lipid peroxidation Inhibition of membrane receptors	Antioxidant: • Free radical scavenger • Decreased lipid peroxidation Antibacterial Antiparasitic Tumoricidal Anti-apoptotic Regulation of vascular tone Activation of enzymes • Cyclooxygenase 1 and 2 • Soluble guanylate cyclase

Table 1.3: Summary of the effects of nitric oxide (Lirk, Hoffmann et al. 2002)

1.5.3 Enzymatic source of NO

NO production is related to the presence of an enzyme termed nitric oxide synthase which has been well characterised for at least three isoforms, two of which are constitutive and calcium dependent (cNOS) and one of which is calcium independent and found to be inducible in various tissues.

The endothelial and neural NOS as their name would suggest are expressed constitutively (though not solely) in endothelial and neural cells respectively and produce nanomolar levels of NO. These enzymes are thought to be involved in physiological processes. The inducible NOS is expressed in a number of cell types including macrophages, polymorphonuclear cells, intestinal and colonic epithelial cells. iNOS upon induction produces sustained, micromolar concentrations of NO and is thought to be primarily

involved in the pathophysiological function of nitric oxide in inflammatory diseases (Cavicchi and Whittle 1999; Murray, Daniels et al. 2002; Yanagita, Shimabukuro et al. 2002).

nNOS	iNOS	eNOS
Neural NOS	Inducible NOS	Endothelial NOS
Constitutive	Inducible	Constitutive
Calcium dependent	Calcium independent	Calcium dependent
Cystolic localisation	Cystolic and membrane localisation	Cystolic and membrane localisation

Table 1.4: Summary of the three isoforms of nitric oxide synthase (Lirk, Hoffmann et al. 2002; Rumbo, Courjault-Gautier et al. 2005).

1.5.4 iNOS induction and regulation

The cytokines IFN- γ , TNF- α , IL-1 β , IL-6 and IL-15 have all been shown to be important cytokines in iNOS regulation (Yanagita, Shimabukuro et al. 2002; Liu, Zhai et al. 2004; Jablonska, Puzewska et al. 2005).

In addition, the transcription factors NF κ B, IRF-1 and STAT-1 α have been found to be important transcription factors regulating iNOS production (Cavicchi and Whittle 1999; De Stefano, Maiuri et al. 2006).

Post transcriptional regulatory mechanisms include maintaining a rapid cellular turnover of iNOS, regulation of iNOS mRNA stability by RNA binding proteins and proteasomal targeting (Kleinert, Pautz et al. 2004; Kolodziejwski, Koo et al. 2004). Disruption of the interaction of iNOS with cytoskeletal proteins at the cell surface can result in loss of activity (Rumbo, Courjault-Gautier et al. 2005).

1.5.5 iNOS and disease

1.5.5.1 iNOS and Coeliac disease

iNOS protein and enzyme activity have found to be increased in coeliac disease. Increased plasma levels of nitrate and nitrite, markers of nitric oxide production have been found in active disease (ter Steege, Koster-Kamphuis et al. 1998; Murray, Bullimore et al. 2003). Nitrate and nitrite levels have also

been shown to be increased in the urine of children with active disease (Sundqvist, Laurin et al. 1998). Elevated levels of nitrotyrosine, a marker of NO activity is also elevated in active disease (ter Steege, Buurman et al. 1997). This increase in nitric oxide activity is not thought to represent a bystander effect of disease but may actually participate in disease progression and has been shown to be mediated by gliadin as has been found using *in vitro* organ culture systems (Beckett, Dell'Olio et al. 1999). Moreover, plasma levels of NO metabolites decrease upon introduction of a gluten free diet (Spencer, Daniels et al. 2004).

Both enterocytes and macrophages have been reported as potential nitric oxide sources in coeliac disease. Elevated levels of iNOS mRNA, enzyme activity (as assessed by NO metabolites) and protein have been found in coeliac disease enterocytes (Murray, Daniels et al. 2002; Daniels, Cavill et al. 2005). Increased iNOS labelled CD14 monocytes have been found in the small intestine (ter Steege, Buurman et al. 1997). Gliadin increases IFN γ induced iNOS gene expression and activity in the RAW 264.7 macrophage cell line (Maiuri, De Stefano et al. 2003). The exact role of increased iNOS activity in the small intestine of active disease is unclear but it could contribute to overall pathology.

1.5.5.2 iNOS and inflammatory bowel disease (IBD)

iNOS has also been implicated in inflammatory bowel disease. NO levels are increased in ulcerative colitis and to a greater extent in Crohn's disease. High levels of NO metabolites, nitrate and nitrite have been found in the plasma, stool and colonic lumen of inflammatory bowel disease patients. Increases in free radicals and cytoskeletal protein oxidation and nitration have been found in the colon of inflammatory bowel disease patients. Both oxidated and nitrated actin, oxidated tubulin and F-actin disruption have been seen in active diseases. The resulting cytoskeletal disruption could alter barrier dysfunction, a hallmark initiator and perpetuating factor in IBD (Keshavarzian, Banan et al. 2003). It has been suggested at early stages of inflammatory bowel disease, nitric oxide may have a protective effect on mucosal integrity but with sustained activity, high levels of NO and its

harmful derivatives may be pathogenic. How this molecule switches from homeostatic regulator to harmful destructor remains unknown (Kolios, Valatas et al. 2004).

1.6 Tight Junctions

1.6.1 Apical junctional complex

The integrity of the epithelial layer in multicellular organisms is maintained by intercellular junctional complexes composed of tight junctions, adherens junctions and desmosomes, whereas gap junctions provide for intercellular communication (Schneeberger and Lynch 2004). Gap junctions comprise of connexon channels which allow intercellular passage of ions and small molecular weight substances thereby metabolically and electrically coupling cells. Desmosomes form points of intercellular contact that rivet cells together and provide anchoring sites for intermediate filaments. Adherens junctions form a continuous belt around cells and function to hold neighbouring cells together through Ca^{2+} dependent cell-cell adhesion molecules (cadherins) that are linked to actin and myosin filaments. The tight junction occurs at the most apical portion of the cell and functions to separate apical and basolateral components within the cell and forms the paracellular barrier between cells (Denker and Nigam 1998; Bazzoni and Dejana 2004)

Junction	Function
Tight Junction	Regulate paracellular permeability and cell polarity
Adherens Junction	Hold cells together through calcium dependent cell adhesion molecules
Gap Junction	Allow intercellular passage of ions and small molecular weight molecules
Desmosome	Provide attachment points for intermediate filaments

Table 1.5: Summary of components of the apical junctional complex

1.6.2 Tight Junctions

Tight junctions which form at the most apical component of the intercellular junctional complex are regions where the outer leaflets of plasma membranes from adjacent cells fuse together and obliterate the intercellular space. They form continuous, circumferential belt like structures and may extend in depth from 0.2 to 0.5 μ m. (Harhaj and Antonetti 2004; Schneeberger and Lynch 2004). Tight junctions have two main functions: a gate/barrier and a fence function. The gate function regulates paracellular permeability by controlling the passage of ions, water and macromolecules through paracellular spaces. The fence function restricts the movement of lipids and membrane proteins between the apical and basolateral regions of cells, thus maintaining cell polarity (Sawada, Murata et al. 2003; Kohler and Zahraoui 2005). Polarity refers to the subdivision of the plasma membrane into apical and basolateral domains with distinct lipid and protein compositions and physiochemical properties (Hershberg and Mayer 2000; Wakabayashi, Chua et al. 2007).

1.6.3 Molecular components of Tight Junctions

Tight junctions are comprised of three cell adhesion molecules (CAMs) which are responsible for the mutual recognition and adhesion of similar cells in the epithelium (Miyoshi and Takai 2005).

1.6.3.1 Occludin

Occludin is a 60kDa tetra-span membrane protein that forms two extracellular loops with both amino and carboxy terminal domains terminating in the cytoplasm. Phosphorylated occludin appears to be preferentially expressed at tight junctions (Schneeberger and Lynch 2004). Occludin is not required to maintain the structurally intact tight junction. It interacts with a number of structural proteins e.g. ZO-1, ZO-2 and ZO-3 which may serve to link occludin to the actin cytoskeleton. Occludin interacts with a number of regulatory proteins e.g. VAP-33 which may serve to regulate vesicle targeting or occludin localisation to tight junctions (Harhaj and Antonetti 2004).

1.6.3.2 Claudins

Claudins are tetra-span proteins with relatively short cytoplasmic amino and carboxy termini with two extracellular loops. 24 members of the claudin family have been identified ranging in size from 20-27kDa (Schneeberger and Lynch 2004). Claudins interact with ZO-1, ZO-2 and ZO-3 molecules directly and indirectly with the myosin-binding molecule cingulin (Miyoshi and Takai 2005). Claudins are the major structural component of tight junctions and at least two or more species of the claudin family are found within the TJ of a single cell (Heiskala, Peterson et al. 2001).

1.6.3.3 Junctional adhesion molecules (JAM)

Junctional adhesion molecules (31-46kDa) have an extracellular domain with two immunoglobulin like domains, a single transmembrane segment and a short cytoplasmic tail. Five members within this family have been identified; JAM 1, JAM 2, JAM 3, JAM 4 and JAM like molecules (Weber, Fraemohs et al. 2007). JAM 1 is expressed on epithelium, monocytes, platelets, lymphocytes, neutrophils and antigen presenting cells and is thought to play a role in leukocyte migration through the paracellular pathway. JAM 2 expressed on the endothelium acts as a receptor for JAM 3 expressed by T cells which facilitates lymphocyte migration between endothelial cells. JAM 3 has a similar expression profile as JAM 1 and is also expressed by endothelial cells. JAM 4 is thought to have a role in cell adhesion. ZO molecules along with cingulin, PAR 3 and the adherens junction protein nectin have been found to bind to JAM (Schneeberger and Lynch 2004; Weber, Fraemohs et al. 2007). JAM is found to be up-regulated under inflammatory conditions. JAMs can also interact with leukocyte integrins (Weber, Fraemohs et al. 2007).

1.6.3.4 Adaptor molecules

Adaptor molecules are a heterogeneous class of cytoplasmic molecules that mediate functions as diverse as anchorage to actin, establishment of cell polarity, cell trafficking, cell signalling and control of gene expression (Bazzoni and Dejana 2004). Adaptor proteins usually bind to the C terminal tail of CAMs and connect them to the actin cytoskeleton either directly or by recruiting other proteins. They form a TJ “plaque” within cells (Miyoshi and Takai 2005).

The membrane associated guanylate kinases (MAGUK) comprising of zona occludin 1, ZO-2 and ZO-3 contain multiple protein binding domains which facilitate their binding to each other, to occludin, claudins, JAMs, actin and other TJ associated proteins (Bazzoni and Dejana 2004); (Harhaj and Antonetti 2004). Cellular adhesion molecules of the tight junction may bind indirectly to one another via the adaptor molecules ZO-1, ZO-2 and ZO-3. ZO molecules can also associate with one another along with binding to several other cytoplasmic molecules. Adaptor molecules recruit regulatory proteins such as protein kinases, phosphatases and small GTPases, signalling molecules such as G proteins and transcription factors. As a result, structural (actin and spectrin) and regulatory proteins are juxtaposed with transmembrane proteins. This protein scaffold facilitates the assembly of highly ordered junctional complexes (Bazzoni and Dejana 2004; Kohler and Zahraoui 2005).

1.6.4 TJ and the Peri-junctional actomyosin ring

In intestinal epithelial cells actin microfilaments form the core of apical microvilli and, in association with myosin II and other proteins, also form a circumferential belt known as the peri-junctional actomyosin ring around cells (Turner 2000). This in turn can bind to tight junctional proteins via ZO and occludin. Cytoskeletal and tight junction disruption is associated with retraction of microfilaments from the plasma membrane, altered cell shape, local accumulations of condensed actin, disruption of the parallel strands that comprise the tight junction and redistribution of the tight junction plaque proteins such as ZO-1 (Shen and Turner 2005).

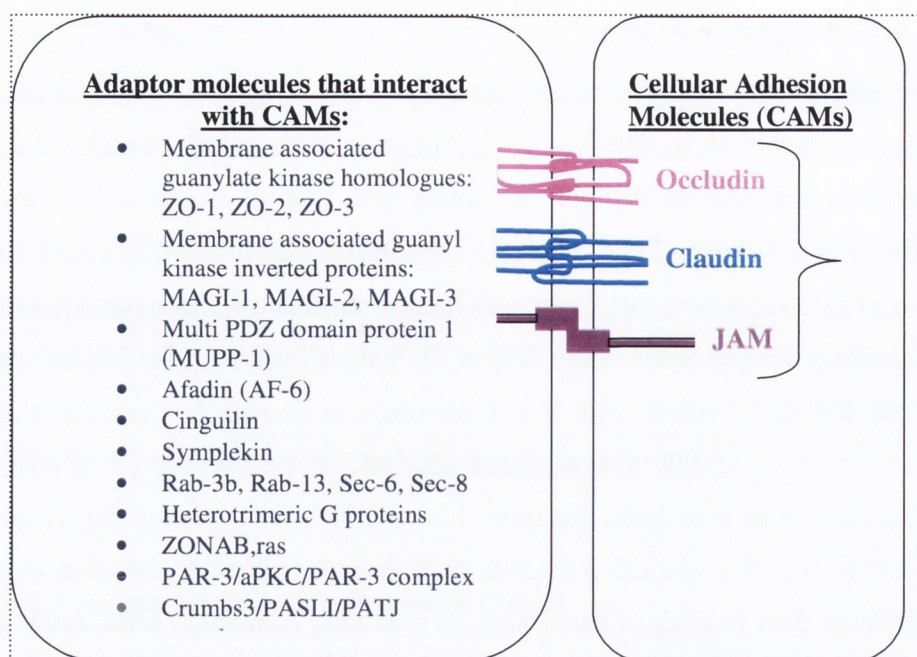


Figure 1.4: Summary of the cell adhesion molecules and associated adaptor/plaque proteins at the tight junction.

1.6.5 Functions of components of the tight junction

Along with its role in providing a physical barrier to regulate paracellular permeability, tight junctional components are also involved in maintaining cell polarity. Two complexes are involved: PAR-3/aPKC/PAR-6 and Crumbs-3/PALS-1/PATJ. The former is recruited to the tight junction through interaction with JAM; the latter binds to ZO-3 and claudin. Tight junctional proteins may also participate in signalling through interaction of adaptor proteins such as ZO and AF-6 with signalling molecules and transcription factors such as Ras, PKC, ZONAB. The tight junction complex participates in the structural association with the cell cytoskeleton through interaction of ZO, AF-6, α -catenin, α -actinin and cingulin with F-actin and myosin II heavy chain. Components of the tight junction also participate in membrane trafficking such as Rab-13, Rab-3b, Sec-6 and Sec-8 which may be involved in vesicle trafficking (Bazzoni and Dejana 2004; Harhaj and Antonetti 2004; Schneeberger and Lynch 2004).

1.6.6 Tight Junctions and disease

1.6.6.1 Tight junctions and coeliac disease

Gliadin has been shown to cause disruption and re-distribution of a number of tight junctional proteins including ZO-1 and occludin in a number of *in vitro* cell line and organ culture systems, along with disrupted F-actin filaments. The resulting alterations in permeability, caused by decreasing trans-epithelial resistance, have also been noted (Clemente, De Virgiliis et al. 2003; Dolfini, Roncoroni et al. 2005; Sander, Cummins et al. 2005; Drago, El Asmar et al. 2006). Biopsy specimens from active coeliac disease patients also show marked differences in the localisation and phosphorylation of cytoskeletal proteins (Ciccocioppo, Finamore et al. 2006). TG2 has been suggested to play a role in these gliadin induced modifications (Maiuri, Ciacci et al. 2005). Along with suggested routes of paracellular entry of gliadin, a transcellular route has recently been proposed. In this model secretory IgA bound to gliadin has been shown to bind to the transferrin receptor (CD71) the latter of which is up-regulated in active coeliac disease. Binding of the IgA-gliadin complex to the transferrin receptor on the apical side of the epithelium allows the passage of intact peptides to the basolateral compartment through a proposed transcellular route (Matysiak-Budnik, Moura et al. 2008).

Zonulin has also been suggested to be a key modulator of tight junctional integrity and alterations in its expression have been noted in coeliac disease. Zonulin a 47kDa protein was identified based on its homology to a *Vibrio cholera* toxin (Zot) which has been found to increase permeability and disrupt F-actin. Following appropriate stimulation of the intestine, zonulin is secreted into the luminal region of the small intestine (Fasano, Fiorentini et al. 1995; Wang, Uzzau et al. 2000). The zonulin receptor is detectable in the jejunum and distal ileum but not in the colon and decreases along the villous-crypt axis which localises zonulin effects to these distinct regions. The receptor is up-regulated with enterocyte differentiation. Binding to the zonulin receptor results in internalisation of zonulin and subsequent phospholipase C and protein kinase C activation and actin polymerisation (Wang, Uzzau et al. 2000; Fasano 2001). Zonulin may have a number of

physiological roles including regulation of the tight junction and protection against micro-organism colonisation (Fasano 2001). Zonulin is thought to be secreted from the epithelium in response to gliadin into the lumen where it interacts with its receptor. Upon binding it activates protein kinase C and causes displacement of ZO-1 and TJ disassembly. Zonulin activity appears to occur in both normal and active disease tissue upon gliadin exposure, though is more marked in the latter. The increased levels of zonulin found in coeliac patients suggest a role for this protein in tight junction defects seen in coeliac disease (Fasano, Not et al. 2000; Drago, El Asmar et al. 2006).

IL-15 which increases in response to gliadin stimulation in coeliac disease has been found to increase barrier function through increasing trans-epithelial resistance and the recruitment and increased production of key tight junctional proteins such as ZO, claudins and occludin (Nishiyama, Sakaguchi et al. 2001). A possible role for IL-15 in barrier function in coeliac disease has yet to be identified. Nitric oxide also found to be up-regulated in coeliac disease pathogenesis and has also been implicated in regulating barrier function. In this incidence NO has been shown to disrupt tight junctional localisation of key tight junction proteins (Han, Fink et al. 2003). Whether NO participates in villous atrophy and increased permeability by disruption of barrier integrity remains unclear.

1.6.6.2 Tight junctions and inflammatory bowel disease

A compromise in barrier integrity occurs in both ulcerative colitis and Crohn's disease (Bruewer, Samarin et al. 2006). Abnormal expression of occludin and ZO-1 has been found in the inflamed mucosa (Gassler, Rohr et al. 2001). Increased claudin 2 expression is seen in active Crohn's disease and ulcerative colitis which has pore forming properties facilitating increased permeability (Zeissig, Burgel et al. 2007).

1.7 Purpose and rationale of thesis

Gliadin, the triggering environmental factor of coeliac disease is transported through the epithelium where its peptide fragments become modified by deamidation in the presence of tissue transglutaminase. These modified peptides are presented in the context of HLA-DQ2 and HLA-DQ8 to CD4⁺ T cells and initiate an inflammatory cascade that is characteristic of coeliac disease. However, a growing body of evidence suggests that gliadin may cause direct toxic effects in the absence of modification through unique peptide fragments which can activate an innate response. The initial events which enable the paracellular uptake of gliadin through the epithelial barrier and the initial consequence of this barrier breachment are of central interest in understanding how the subsequent inflammation is established. This study aimed to investigate the critical factors that cause disruption of the intestinal barrier function which has been proposed to enable the entry of gliadin into the underlying mucosa. This study also focussed on the regulation of enzymes whose end products have been shown to correlate with active disease. A further study was performed to investigate differences in blood monocyte expression of markers of disease activity in a control and coeliac disease study group. Details of these experiments are presented in this thesis in the following chapters.

Chapter 2: Electrophysiological characterisation and immunofluorescent localisation of tight junctional proteins in response to stimulation by gliadin and associated inflammatory mediators using cell lines

In this chapter, the direct effects of gliadin, the cytokine IL-15, a nitric oxide donor and a mixture of pro-inflammatory cytokines (IFN- γ , TNF- α and IL-1 β) were assessed using two intestinal cell lines, T 84 and Caco-2 cells. Changes in permeability were assessed using electrophysiological methods through trans-epithelial electrical resistance determinations. Changes in tight junction protein localisation were assessed by immunofluorescent staining.

Chapter 3: Quantification of gene expression and enzyme activity of the inducible nitric oxide synthase enzyme (iNOS) and the cyclooxygenase 2 (COX-2) enzyme and iNOS protein production using an enterocyte cell line in response to gliadin, to the innate cytokine IL-15 and to the cytokine IFN- γ

In this chapter, the role of gliadin, IL-15 and IFN- γ on gene expression of the iNOS and COX-2 gene in Caco-2 cells was assessed using real time PCR. Enzyme activity assays for both enzymes were performed by measuring the end products of enzyme activity. For COX-2 activity this involved the measurement of prostaglandin E₂ using a competitive ELISA. iNOS activity was assessed by measuring the end product of the labile nitric oxide gas, nitrite, using a spectrophotometric and fluorimetric method for nitrite determination. Protein expression was also assessed for iNOS using flow cytometry.

Chapter 4: A comparative study of blood monocytes derived from a healthy cohort and a treated coeliac cohort and analysis of Caco-2 cell HLA expression in response to stimulation with gliadin and IFN- γ

In this chapter, peripheral blood mononuclear cells were isolated from both a control group and a treated coeliac disease group. Cells were stimulated with gliadin or IFN- γ and then analysed for surface HLA-DR and IL-15 expression and intracellular IL-15 levels by gating on the CD14 positive monocytes cells. Supernatants were assessed for nitrite levels using a nitrite fluorimetric kit for determination. Surface HLA expression was also assessed on Caco-2 cells in response to gliadin and IFN- γ stimulation using a cellular enzyme linked immunosorbent assay.

CHAPTER 2

**ELECTROPHYSIOLOGICAL CHARACTERISATION AND
IMMUNOFLUORESCENT LOCALISATION OF TIGHT
JUNCTIONAL PROTEINS IN RESPONSE TO
STIMULATION USING CELL LINES**

2.1 Introduction

Coeliac disease is a model immunological disorder as the environmental trigger gliadin has been well established (Dieterich, Esslinger et al. 2003). A number of advances in elucidation of disease including T cell mediated events and modification of gliadin within the mucosa by TG2 have been defined (van de Wal, Kooy et al. 1998; Anderson, Degano et al. 2000; Fleckenstein, Molberg et al. 2002). Initiation of the innate immune response has become a primary focus with a number of novel findings. These include the identification of distinct innate gliadin peptides e.g. p31-43 and findings in relation to the innate cytokine IL-15 and its central role in mediation of these initial events (Maiuri, Ciacchi et al. 2003; Mention, Ben Ahmed et al. 2003; Hue, Mention et al. 2004). Despite these advances, the precise mechanism by which gliadin breeches the intact epithelial barrier to gain access to the underlying mucosa still remains unclear.

Coeliac disease is associated with increased intestinal permeability (van Elburg, Uil et al. 1993; Schulzke, Schulzke et al. 1995). The entry of gliadin via a paracellular route has thus been suggested though as yet not directly shown. Tight junctions represent key modulators of intestinal paracellular transport (Schneeberger and Lynch 2004). Alteration in tight junction assembly has been proposed in a number of diseases including coeliac disease (Clayburgh, Shen et al. 2004). The disruption of tight junctions in disease may occur in response to a number of factors including pro-inflammatory cytokines such as IFN- γ (Bruewer, Luegering et al. 2003). In coeliac disease gliadin has been proposed to induce barrier disruption. Direct mechanisms have been suggested along with indirect methods in which gliadin induces a second molecule e.g. zonulin which mediates subsequent events (Dolfini, Roncoroni et al. 2005; Sander, Cummins et al. 2005; Drago, El Asmar et al. 2006).

A number of *in vitro* systems have been used to study these events. The epithelium includes multiple transport systems enacted by various cell types and conducted under the modulating influence of neural and lamina propria cells (Madara, Stafford et al. 1987). The ability to study permeability across

cultured monolayers of single species of epithelial cells would enable study of tight junctional events in a more controlled manner.

Primary intestinal cell cultures isolated from the small intestine display a number of disadvantages including difficulty in culturing, limited viability, variable degree of differentiation, loss of their differentiation characteristics upon isolation from underlying mesenchymal tissue, inability to sub-culture and are difficult to obtain in a reproducible and quantitative manner (Le Ferrec, Chesne et al. 2001; Sambruy, Ferruzza et al. 2001). Established intestinal cell lines of tumoral origin provide an attractive alternative for studies of intestinal barrier function. To usefully serve as models for intestinal function, such cell lines should demonstrate both structural and functional similarities to intestinal epithelial cells (Madara, Stafford et al. 1987). Among the numerous cell lines derived from human intestinal adenocarcinomas, two lines, Caco-2 and T-84 have been shown to express morphological and functional characteristics of differentiated cells of the intestinal mucosa (Sambruy, Ferruzza et al. 2001).

Caco-2 cell line

The Caco-2 cell line was isolated from a human adenocarcinoma. Caco-2 cells, despite their colonic origin, express the majority of the morphological and functional characteristics of small intestinal absorptive cells. In culture these cells differentiate spontaneously into polarised small intestinal cells possessing an apical brush border, well developed and organised micro-villi on their apical membrane. These cells express hydrolase activities associated with the apical membrane such as sucrase-isomaltase, lactase and aminopeptidase normally expressed on the microvilli of the absorptive enterocyte of the small intestine and are joined by functional tight junctions which form between adjacent cells. This process of differentiation starts when cells reach confluency and lead in 2 to 3 weeks to the formation of a monolayer of highly polarised cells (Pinto 1983; Le Ferrec, Chesne et al. 2001; Sambruy, Ferruzza et al. 2001).

T 84 cell line

This cell line is derived from a pulmonary metastasis of a human colon carcinoma. These cells form a cell monolayer with a highly polarised morphology, structural similarity to intestinal crypt cells, few apical microvilli and a very high trans-epithelial resistance which indicates well differentiated tight junctions. T 84 cells show regulated chloride secretion typical of colon crypt cells. The T 84 cell line represents the best available model of differentiated human colon cells (Madara, Stafford et al. 1987; Sambruy, Ferruzza et al. 2001).

Whilst T-84 cells, unlike Caco-2 cells do not display the spontaneous small intestinal phenotype, they offer an excellent model of tight junction assembly due to their well defined tight junctions. Distinct differences between Caco-2 cell lines and T 84 cells offer advantages in the investigation of alternative signalling pathways affecting tight junctional integrity. An example of this is the effect of zonulin on permeability, as T 84 are unresponsive to zonulin (Drago, El Asmar et al. 2006).

Caco-2 and T 84 cell lines are typically cultured on microporous culture supports where they differentiate into polarised monolayers as shown in figure 2.1. Cells grown on these culture supports form a two compartment system where the apical medium is separated from the basolateral by the cells and microporous filter. This arrangement reproduces the steric organisation of the intestinal mucosa *in vivo* and favours the development of cell polarity and vectorial transport of nutrients (Sambruy, Ferruzza et al. 2001). This method of cell culture offers attractive possibilities such as selective stimulation of apical surfaces (representing the lumen of the intestine) versus basolateral surfaces (the surface of the cell that faces the interstitial space and the vascular and lymphatic systems). It also offers the ability to manipulate cells through their growth cycle such as the measurement of resistance across cell monolayers.

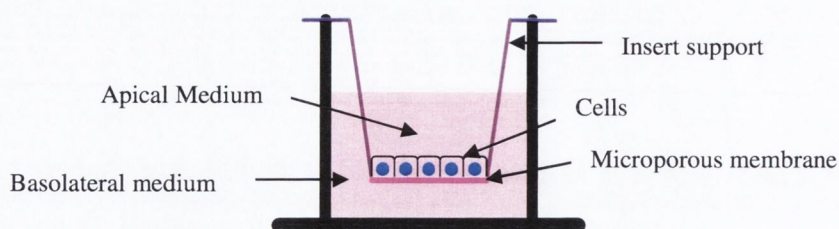


Figure 2.1. Microporous membrane supports used to culture Caco-2 and T 84 cell lines

Two mechanisms are typically used to assess tight junction integrity. The first of these involves measurement of resistance across monolayers; trans-epithelial electrical resistance (TEER). TEER is measured using a pair of Ag/AgCl electrodes suspended in the apical and basolateral medium above and below the cell monolayer. Upon application of a pulse of known amplitude across the epithelium, ohm's law principals are applied and resistance calculated which is read from an epithelial volthohmmeter. Electrical resistance across a monolayer represents the sum of the resistance of the tight junction and the intercellular space and the transcellular resistance which consists of the resistance of the apical and basolateral membrane. As the tight junction is often rate limiting to paracellular solute movement, alteration in TEER is often used as an index of tight junctional permeability. Despite this however, decreases in TEER may also be due to changes in the activity of ion channels or pumps in the plasma membrane upon activation by cAMP (Madara 1998; Harhaj and Antonetti 2004).

TEER is expressed as ohms $\times$ cm² ($\Omega \times$ cm²) and is obtained by the following equation: (Resistance of cells - resistance of blank filter) $\times$ growth area of filter.

During the establishment of confluent cell monolayers and the subsequent differentiation process, TEER values reach a peak before achieving steady state values corresponding to the formation of fully developed and functional tight junctions. TEER represents an instantaneous measurement (Sambruy, Ferruzza et al. 2001).

The other mechanism to assess barrier integrity involves the determination of paracellular tracer flux. In this method, permeability to hydrophilic

uncharged paracellular tracers is measured using radioactive or fluorescently conjugated tracers such as mannitol or dextran. Tight junction permeability to particular solutes depends on the size, shape and charge of the solute. *In vivo*, the epithelium is subjected to hydrostatic pressures and convective fluxes which may alter the response to permeabilising agents (Harhaj and Antonetti 2004).

In general increased flux of paracellular tracers is accompanied by decreased TEER though this relationship is not linear (Sambruy, Ferruzza et al. 2001).

Thus whilst TEER and paracellular tracer flux represents good indicators of tight junction integrity, further analysis of tight junction assembly e.g. immunofluorescent staining of individual tight junction components is necessary to determine the effects of stimulation on tight junction function.

The aim of this chapter

The aim of this study was to determine the effects of gliadin on tight junction integrity using the Caco-2 and T 84 cell lines. Direct effects of gliadin were investigated along with potential indirect effects. Coeliac disease is also characterised by increases in pro-inflammatory cytokines such as IFN- γ and TNF- α , increased nitric oxide production and recently described IL-15 production. We investigated the effects of these stimuli shown to be up-regulated in response to gliadin, on tight junction integrity, providing an indirect role of gliadin in alteration of barrier function. We used the combined methods of TEER assessment and immunofluorescent staining of key tight junction proteins at 24hours following stimulation.

2.2 Materials and Methods

2.2.1 Cell Culture

The human colon adeno-carcinoma cell line Caco-2 was used between passages 30-40 (a kind gift of Dr. Ann Hopkins, UCD) and passages 55-65 (a kind gift of Dr Ana Terres, IMM). Cells were maintained in high glucose Dulbeccos Modified Eagles Medium (Gibco BRL, UK) supplemented with 10% FCS (Sigma, USA), 2mM L-glutamine, 100 U penicillin and 0.1mg streptomycin (Gibco) and 0.250ug/ml amphotericin B (Gibco BRL, UK).

The human colon carcinoma cell line T 84 was used between passages 5-12. Cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagles Medium and F12 supplemented with 10% FCS (Sigma, USA), 0.2mM L-glutamine, 1,000 U penicillin and 1mg streptomycin (Gibco BRL, UK) and 0.250ug/ml amphotericin B (Gibco, BRL, UK).

Cells were grown in T-25cm² culture flasks. Upon reaching 80-90% confluency, Caco-2 and T 84 cells were washed free of FCS containing medium using PBS and removed from flasks with a 1X solution of trypsin EDTA (Gibco BRL, UK) by incubating at 37 °C for 10mins. Trypsin was inactivated by FCS containing DMEM and cells spun at 1,200rpm to wash away any remaining trypsin. Cell pellets were re-suspended in DMEM and placed in fresh T25cm² flasks. Cells were split in a ratio of 1:5 prior to reaching confluency and maintained in a 37°C incubator, 5% CO₂.

2.2.2 Labtek chamber slides

For the purpose of antibody optimisation, Caco-2 and T 84 cell lines were cultured on 8 well labtek chamber slides (NUNC, Thermo Fisher Scientific, USA). Cells were grown in T-75cm² flasks until 90% confluent. Cells were removed by trypsinisation as previously described and re-suspended in 3mls of medium. A cell count was performed by diluting cells 1:25 with EBAO and visualised under UV light using a Neubauer counter. A cell count was performed in all 9 squares of the counter and from this the average cell number per square calculated. This represents the number of cells per 0.1µl.

The total number of cells in 1ml was determined taking into account the dilution factor of cells in EBAO. Cell number was expressed per ml. Cells were diluted with medium to obtain a final concentration of between 1×10^5 - 5×10^5 cells/ml. 500µls of the cell suspension was added to each of 8 wells. Labtek chamber slides were incubated at 37°C. Medium was replaced every 2-3 days. Cells were stained after 10-15 days.

2.2.3 Double Immunofluorescent staining of labtek chamber slides

Upon reaching confluency, cells were cultured for 10-15 days to enable tight junction formation. Medium was removed from each chamber and cells were washed three times with PBS. Cells were fixed with 100% absolute ethanol for 20mins at -20°C. Cells were washed with PBS and blocked with 5% normal goat serum (Dako, Denmark) prepared in PBS (blocking buffer) for 1hr at room temperature. Cells were then washed with PBS.

Each chamber slide was double stained with a mouse and a rabbit anti-human antibody for two of the tight junctional proteins. Cells were double stained for mouse anti-human ZO-1 (Zymed, USA) and rabbit anti-human JAM-A (Zymed, USA). In separate wells, cells were double stained with mouse anti-human claudin-4 (Zymed, USA) and rabbit anti-human occludin (Zymed, USA). Antibody titrations were prepared for each antibody.

After incubating for 1hr, cells were washed three times with PBS and incubated with 1/500 mixture of a goat anti-mouse Alexa Fluor 568nm antibody (Molecular Probes, USA) and a goat anti-rabbit Alexa Fluor 488nm antibody (Molecular Probes, USA). Cells were incubated for 1hr at room temperature protected from light.

Antibody	Dilution	Supplier	Animal source	Corresponding secondary antibody
ZO-1	1:400	Zymed	Mouse	Goat anti-mouse Alexa Fluor 568
Occludin	1:400	Zymed	Rabbit	Goat anti-rabbit Alexa Fluor 633
JAM-A	1:50	Zymed	Rabbit	Goat anti-rabbit Alexa Fluor 633
Cl\udin-4	1:50	Zymed	Mouse	Goat anti-mouse Alexa Fluor 568

Table 2.1: Primary and secondary antibodies used in immunofluorescent staining

Cells were washed three times with PBS. Nuclei were stained with a 1/40,000 dilution of DAPI (Molecular Probes, USA) for 10mins at room temperature. Cells were washed again with PBS and chambers removed from slides by using the key and holder tool provided. Cells were mounted with coverslips using fluorescent mounting medium (Dako, Denmark) and visualised using a fluorescent microscope and Leica software.

2.2.4 Seeding cell culture inserts

Cells were cultured in T-75cm² flasks and confluency assessed using the inverted microscope. Upon reaching 90% confluency, cells were removed from the flask, resuspended in 3mls of medium and a cell count performed. Cells were diluted to a final concentration of 5×10^5 cells/ml. Cells were grown on transwell permeable polyester supports (Corning, USA) to enable differentiation of cells and the formation of tight junctions.

Prior to seeding of insert plates, an equilibration period of one hour was used to improve cell attachment. Medium (either DMEM for Caco-2 cells or DMEM: F:12 for T 84 cells) was added to insert wells and inserts. The plate was incubated at 37°C for 1hr. Medium was removed from each insert and well. 900µls of fresh medium was added per well. 200µls of the re-suspended cell pellet (at a concentration of 5×10^5 cells/ml) was added into each of insert in a 24 well plate and the plate was returned to the 37°C incubator.

2.2.5 Electrophysiology measurements

Cell differentiation and tight junction formation was assessed by measuring resistance values across the cell monolayer. Resistance was measured every second day-from day 3 of seeding, using an EndOhm 6 measurement chamber in conjunction with an EVOM-X epithelial Voltohmmeter (WPI Inc, UK). The EndOhm chamber was attached to the EVOM-X resistance meter via a connector lead. The height of the top electrode of the endohm was adjusted so that the clearance between the top electrode and the membrane was 1-2mm.

Cell inserts were placed into the EndOhm chamber which contained the same medium as used to culture cells. The height of the fluid in the chamber was approximately the same as that within the insert to avoid hydrostatic pressure on the membrane. The cap of the measurement chamber was replaced. The resistance toggle on the meter was switched to measure and resistance readings were read from the digital LCD display. Resistance readings were recorded and medium replaced in well and within each insert. Tight junction formation was monitored by a gradual increase in resistance values. Stimulation of cells was performed once resistance values had reached a plateau which occurred between days 10 to 20 of plate seeding.

2.2.6 Preparation of peptic tryptic digests of gliadin and ovalbumin

The peptic tryptic preparations were a gift from Herbert Weiser and were produced as follows. Kernels of the German wheat cultivar Rektor which contain α -, ϕ -, and γ type gliadins, was milled to flour with an ash content of 0.55 % using a laboratory mill (Brabender Quadrumat Junior)(Weiser 2001). The flour was defatted with light petroleum (boiling range 40 – 60°C) and air-dried. The defatted flour (50g) was extracted stepwise three-times with 200 ml of a salt solution (0.4 mol/l NaCl + 0.067 mol/l NaK phosphate, pH 7.6) and three-times with 200ml of 60 % (v/v) aqueous ethanol using an Ultra Turrax homogeniser for 5 min at room temperature (RT). The suspensions were centrifuged at 40,000g for 15 min at RT and the supernatants of the three ethanol extracts were combined. These were concentrated to approximately 200ml using a vacuum evaporator at 40°C, dialysed against 0.01mol/l acetic acid until free from chloride and freeze-dried.

The stepwise enzymatic hydrolysis of gliadin was performed with pepsin (Sigma P3286) and trypsin (Sigma T1763) both attached to agarose (Bolte, Osman et al. 1996). Gliadin (1g) was dissolved in 50ml of diluted HCl (pH 2.0) and 100mg (4000 U) of pepsin was added. The mixture was magnetically stirred at 37°C for 2 hours then centrifuged at 6,000g for 20 min at RT. The pH of the supernatant was adjusted to 7.8 with 0.1mol/l NaOH and 1ml of the trypsin suspension (25 U) was added. The mixture was magnetically stirred at 37°C for a further 2 hours and the pH was adjusted to 7.0 with 0.1mol/l HCl. Centrifugation followed for 20 min at 6,000g at RT.

The supernatant was then freeze-dried. Control RP-HPLC on C18 silica gel indicated complex peptide patterns for the gliadin. Peptic tryptic digests of ovalbumin were also prepared as described in the above method.

2.2.7 Stimulation of cells

Following differentiation, cells were stimulated for 24hrs and resistance measured at defined time points. Each insert was stimulated in duplicate for each stimulus:

- **Unstimulated cells (Control)**

600µls of medium (DMEM for Caco-2 cells; DMEM F:12 for T 84 cells) was added to each insert well and 200µls of medium added to each of the two inserts.

- **PT ovalbumin stimulated cells**

Cells stimulated with 1mg/ml PT ovalbumin. A 20mg/ml stock of PT ovalbumin was prepared in Dulbeccos PBS (Gibco). A 1 in 20 dilution of stock was prepared to get a final concentration of 1mg/ml and placed apically into each of the two inserts and basolaterally into the well of both inserts.

- **PT gliadin stimulated cells**

Cells stimulated with 1mg/ml PT gliadin. A 20mg/ml stock of PT gliadin was prepared in Dulbeccos PBS (Gibco). A 1 in 20 dilution of stock was prepared to get a final concentration of 1mg/ml and was placed apically into each of the two inserts and basolaterally into the well of both inserts.

- **Interleukin-15 stimulated cells**

Cells stimulated with 250ng/ml of Interleukin-15 (R and D Systems, USA). A 5 ug/ml stock of interleukin 15 was prepared in PBS. A 1 in 20 dilution of stock IL-15 was prepared in medium to get a final concentration of 250ng/ml and was placed apically into each of the two inserts and basolaterally into the well of both inserts.

- **Spermine NONOate stimulated cells**

Cells stimulated with 0.05mM of the Nitric Oxide donor Spermine NONOate (Molecular Probes, USA). A 20mM stock of Spermine

NONOate was prepared in 0.01M NaOH. A 1 in 400 dilution of stock was prepared to get a final concentration of 0.05mM which was placed apically into each of the two inserts and basolaterally into the well of both inserts.

- **IFN- γ , TNF- α and IL-1 β (cytomix preparation) stimulated cells**

Cells were stimulated with a mixture of cytokines referred to as cytomix containing 1000U/ml IFN γ (Roche, Switzerland), 10ng/ml TNF α (R and D Systems, USA) and 10ng/ml IL1 β (R and D Systems, USA). Stock TNF α and IL1 β were prepared by adding PBS containing 0.1% BSA to get 10ug/ml of each cytokine. A 1 in 100 dilution of stock IFN γ at 100,000U/ml was prepared. A 1 in 1000 dilution of a 10ug/ml TNF α stock and a 10ug/ml IL1 β stock were prepared to get a final concentration of 1000U/ml IFN γ , 10ng/ml TNF α and 10ng/ml IL1 β which was placed apically into each of the two inserts and basolaterally into the well of both inserts.

- **Forskolin stimulated cells**

Cells were stimulated with 0.05mM of the cAMP activator Forskolin (Sigma, USA). A 100mM stock solution of forskolin was prepared in ethanol. A 1 in 2000 dilution of stock was prepared in medium to get a final concentration of .05mM which was placed apically into each of the two inserts and basolaterally into the well of both inserts.

- **Crude gliadin stimulated cells**

In some instances cells were stimulated with 1mg/ml of crude gliadin (Sigma) prepared in 0.1M acetic acid.

2. 2.8 TEER measurement of tight junction integrity upon stimulation

Medium was changed in insert plates prior to stimulation and incubated at room temperature for 30mins to allow temperature equilibration. Initial resistance readings were taken at time zero prior to stimulation.

Following stimulation, resistance was measured at 3hrs, 6hrs, 9hrs, 12hrs and 24hrs allowing the insert plate to equilibrate to room temperature for 30mins prior to each reading. Changes in TEER were recorded from the LCD display on the EVOM X meter.

2.2.9 Immunofluorescent staining of inserts

Following stimulation for 24hrs, supernatants from each insert was removed and stored at -80°C. Inserts were washed three times with PBS. Cells were fixed for 20mins at -20°C with absolute ethanol. Cells were washed with PBS and then blocked for 1hr with 5% normal goat serum prepared in PBS (blocking buffer). Cells were then washed three times with PBS.

A humidity chamber was constructed using a square bioassay dish (using a method described by Dr. Ann Hopkins, UCD). Parafilm was placed in two strips at the base of the dish and etched onto the base of the plate using a tweezers. Paper towels saturated in water were placed on the lid. Paper towels were maintained wet throughout the staining procedure to ensure uniform staining.

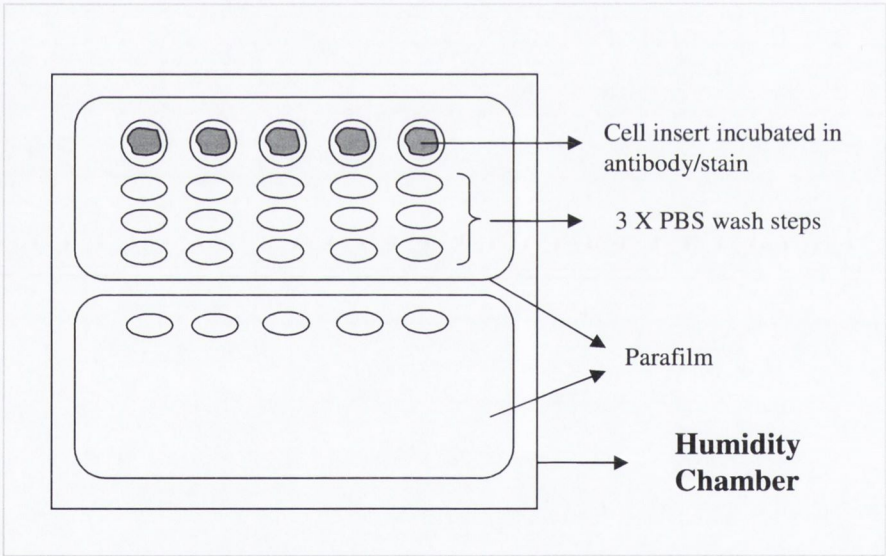


Figure 2.2 Humidity chamber used to immunofluorescently stain cell inserts following stimulation.

Each polyester membrane containing the cells of interest were removed from the insert support using a scalpel blade and placed in a drop of PBS aligned on the parafilm strips in the humidity chamber and stained as described in figure 2.2.

Inserts incubated with each of the defined stimulants as described in section 2.2.6 were stained in duplicate: the first set of stimulated inserts was stained for the tight junctional proteins ZO-1 and JAM-A. The second set of stimulated inserts was stained with occludin and claudin-4 as described in table 2.2. Antibodies were diluted in blocking buffer.

Primary antibody	Dilution	Final conc.	Animal source
ZO-1	1:400	1.25µg/ml	Mouse
Jam-A	1:50	25µg/ml	Rabbit
Occludin	1:400	50µg/ml	Rabbit
Claudin-4	1:50	0.625µg/ml	Mouse

Table 2.2: Primary antibodies used in immunofluorescent staining of cell inserts

80µls of primary antibody was used to stain each insert. Slides were immersed in primary antibody for 1 hour at room temperature within the humidity chamber. After 1 hr cell inserts were washed with PBS and stained with secondary antibody. A 1 in 500 dilution (2µg/ml) of goat-anti mouse Alexa Fluor 568nm and goat anti-rabbit Alexa Fluor 633nm was prepared in blocking buffer. Cells were incubated for 1hour at room temperature in 80µls of secondary antibody.

Following incubation, cells were washed with PBS and were stained for F-F-actin using an Alexa Fluor 488 phalloidin dye (Molecular Probes). A 1 in 500 dilution of phalloidin was prepared in PBS and inserts were stained for 1hr at room temperature.

Cells were washed with PBS and a nuclear stain was applied. A 1 in 40,000 dilution of the nuclear stain DAPI (Molecular Probes) was prepared in PBS and inserts were incubated for 10mins at room temperature. Cells were washed three times with PBS and inserts mounted on slides using anti-fade fluorescent mounting medium (Dako).

2.2.10 Analysis of staining

Staining was analysed using a Zeiss 510 Meta Confocal microscope using LSM software. Each insert was analysed at 4 different excitation wavelengths: DAPI 360nm with an excitation of 346nm and an emission of 442nm, Alexa Fluor phalloidin 488nm with an excitation at 495nm and emission at 519nm, goat anti mouse IgG Alexa fluor 568nm with an excitation at 578nm and emission at 603nm and goat anti rabbit IgG Alexa Fluor 633nm with an excitation of 632nm and an emission of 647nm. Expert mode was used along with the multi-track configuration and frame scan mode with a pixel depth of 8 bits. Adobe Photoshop Elements 6.0 was used in some instances for image manipulation.

2.2.11 Statistical analysis

Analysis of variance was performed by a two way ANOVA test followed by the BonFerroni *post hoc* test. Statistical significance was reached when p values were less than 0.05.

2.3.0 Results

2.3.1 Growth characteristics of intestinal cell line

Initially Caco-2 cell lines were used with high passage numbers ranging from 60 to 70. Increasing trans-epithelial electrical resistance occurred during the development of confluency and stabilised upon differentiation and tight junction formation. Their electrical resistance patterns can be seen in Figure 2.3 over a 15 day period. These cells were used for antibody concentration optimisation and to investigate the effects of a crude gliadin preparation and the effects of varying concentrations of PT gliadin on resistance and tight junction organisation. These cells were referred to as high passage number Caco-2 cells. Cells were grown on 0.4µm translucent polyethylene terephthalate inserts (BD Falcon; 0.3cm² growth areas).

Caco-2 cells with a lower passage number were also utilised. As can be seen from figure 2.3 their TEER values were much higher than previous Caco-2 cells. These cells were used to assess the effects of PT gliadin, IL-15, an NO donor and a mixed cytokine preparation of IFNγ, TNF-α and IL-1β on resistance and tight junction integrity. These cells were referred to as low passage number Caco-2 cells. These Caco-2 cells along with the T 84 cell line were cultured on 0.4µm transparent polyethylene terephthalate filters (Corning, 0.3cm² growth area) to facilitate confocal imaging. T 84 TEER values are also highlighted in Figure 2.3. As can be seen from graph, T 84 cells and low passage number Caco-2 cells show high resistance values indicative of well differentiated tight junctions.

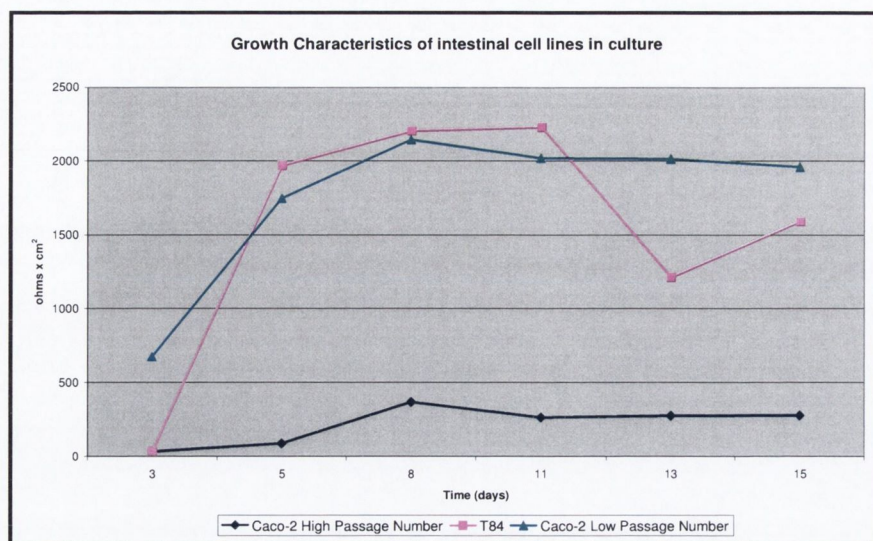


Figure 2.3 Growth Characteristics of Caco-2 and T 84 intestinal cell lines in culture: Transepithelial electrical resistance values obtained over a 15 day period.

2.3.2 Optimisation of tight junction immunofluorescence staining

Caco-2 (high passage number) and T 84 cell lines were cultured in 8 well labtek chamber slides for 10-15 days to enable tight junctions to form. Primary antibody titrations were performed for each of the four tight junctional proteins: ZO-1, occludin, claudin-4 and JAM-A. Cells were initially double stained using the secondary antibodies goat anti-mouse Alexa fluor 568nm (ZO-1, claudin 4) and Alexa Fluor goat anti-rabbit 488nm (occludin, JAM) to enable visualization using a conventional fluorescent microscope. Once primary antibody concentrations had been determined, Caco-2 cells were grown on inserts and stained using Alexa Fluor 568nm and Alexa Fluor 633nm, the latter of which excites in the Far Red region and requires specific lasers to excite and detect the fluorochrome. Using confocal microscopy, 4 colours could be detected: thus, we performed titration experiments to determine optimal concentrations for each of the four fluorochromes to be used: Alexa Fluor 488nm (phalloidin F-actin), DAPI 350nm (nuclear), Alexa Fluor 568nm (goat anti-mouse for ZO-1 and claudin-4 detection) and Alexa Fluor 633nm (goat anti-rabbit for occludin and JAM detection). Results can be seen in Figure 2.4. Occludin, JAM and ZO-1 showed a classical honeycomb pattern of staining at the cell membrane. F-actin staining showed a peripheral band of actin staining at cell margins with a continuous dotted lawn of micro villous staining across the cell.

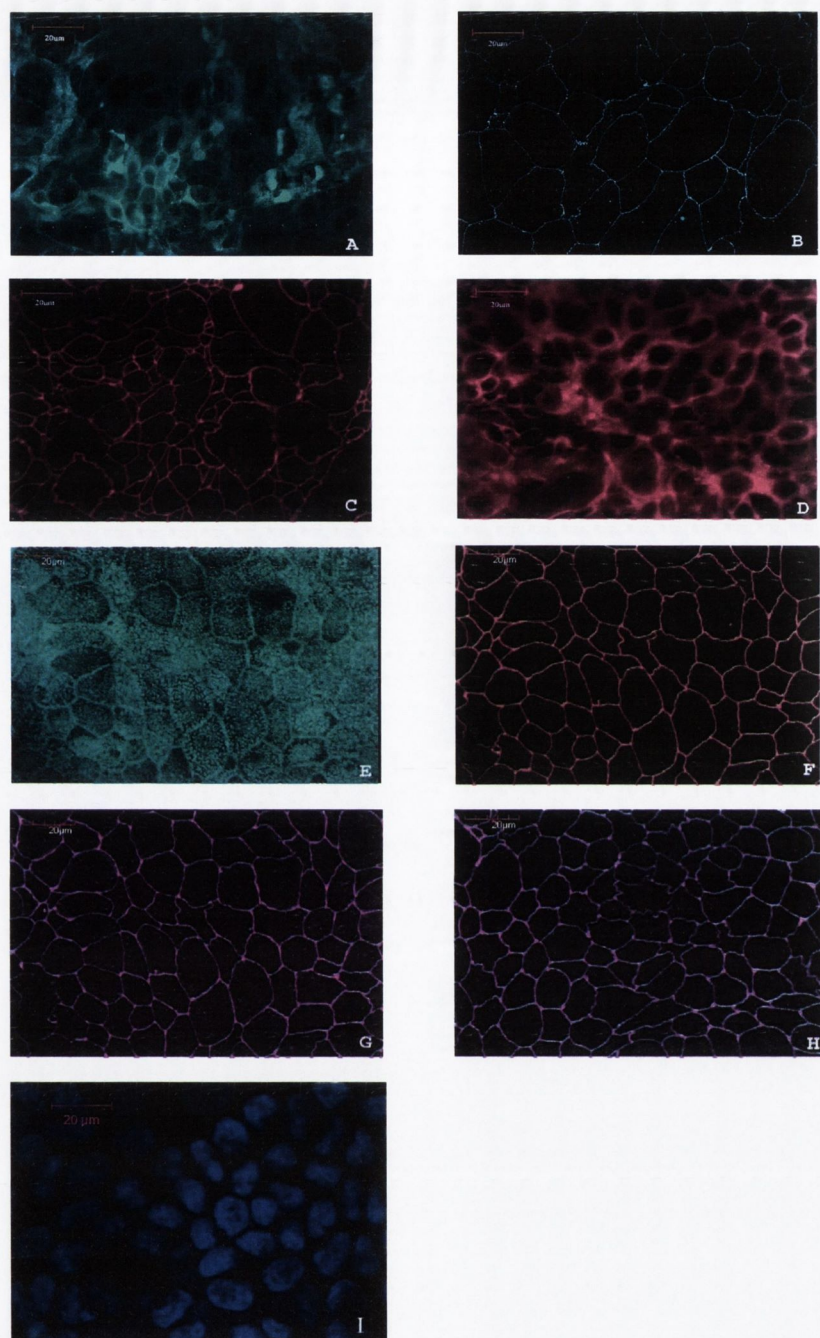


Figure 2.4: Staining of T-84 and Caco-2 cells for tight junction proteins:

T 84 cells were grown on chamber slides and stained for F-actin (A), Occludin (B), ZO-1 (C) and Claudin-4 (D). Cells were visualised using a Nikon fluorescent microscope with 488nm and 568nm filters (magnification X 40). Caco-2 cells were stained for F-actin (E), ZO-1 (F), JAM A (G), Occludin (H) and DAPI (I) and visualised using oil immersion on an Olympus Confocal microscope with 488nm, 568nm and 633nm filters.

2.3.3 Effect of a crude gliadin preparation on Caco-2 resistance values

A crude gliadin preparation dissolved in 0.1M acetic acid was used to assess changes in resistance in high passage number Caco-2 cells. Cells were incubated for 8hrs and resistance measured at set intervals. A peptic tryptic digest of crude gliadin has previously been shown to cause a reduction in TEER (Drago, El Asmar et al. 2006). The effect of 0.1M acetic acid (crude gliadin diluent) was also assessed (figure 2.5A). Whilst a significant difference was found between crude gliadin and control, this is due to the acetic acid used to prepare gliadin as similar statistical significant differences could be seen between acetic acid and control cells.

Forskolin was used as a positive control as it is known to activate Protein kinase A with resulting decreases in TEER (D'Souza, Agarwal et al. 2005).

The effect of forskolin and 97% ethanol (forskolin diluent) on TEER can be seen in figure 2.5B. Whilst forskolin caused a significant decrease in resistance over time, this effect was not due to its diluent (ethanol).

Inserts were also immunofluorescently stained for the tight junctional protein occludin and results can be seen in Figure 2.6. Unstimulated cells showed the classical honeycomb pattern of occludin staining with well defined F-actin structures. No difference was seen in cells stimulated with 1mg/ml of the crude gliadin preparation as occludin distribution remained intact as can be seen from figure 2.6 with well organised F-actin. On the other hand acetic acid (crude gliadin solvent) showed clear distinct dot like organisation of F-actin micro-villous structures with "punctuate" F-actin staining despite well organised occludin. Forskolin stimulated cells had some punctuate, less defined F-actin in areas. Thus, it would appear crude gliadin does not cause alterations in tight junctions and that the changes in resistance were due to 0.01M acetic acid used in its preparation.

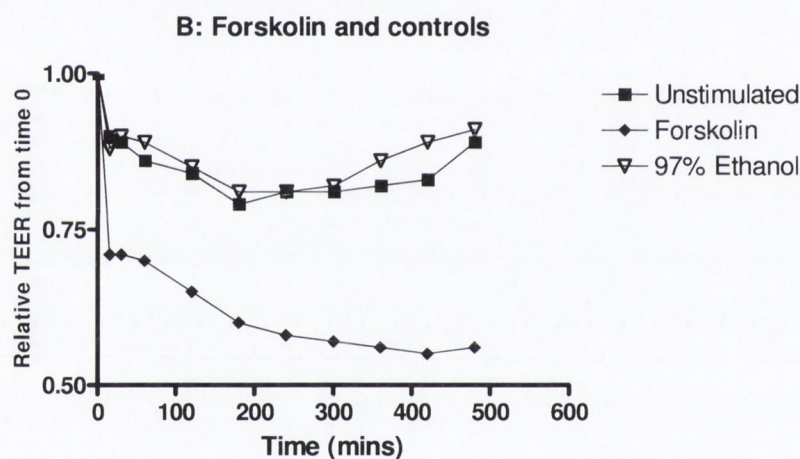
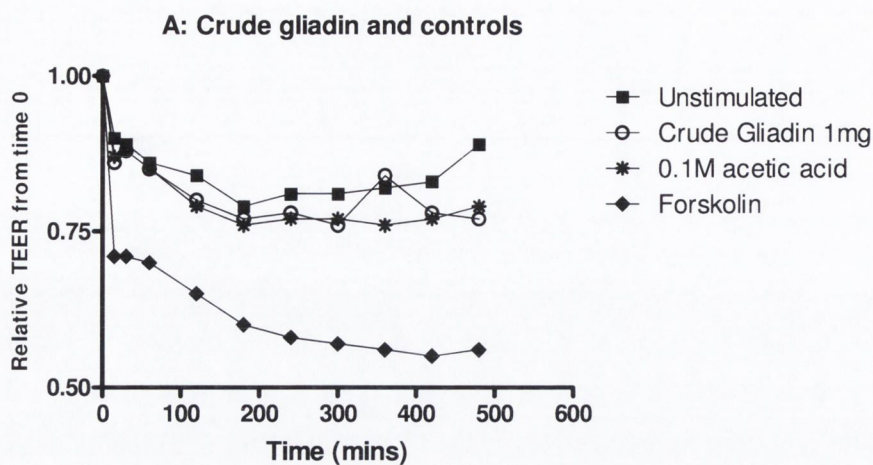


Figure 2.5: Effect of Crude gliadin and Forskolin on TEER

Figure 2.5A shows the effect of crude gliadin and its diluent (0.1M acetic acid) on TEER over 8hrs in Caco-2 cells. Figure 2.5B shows the direct effect of forskolin on TEER which is not due to its diluent (ethanol)

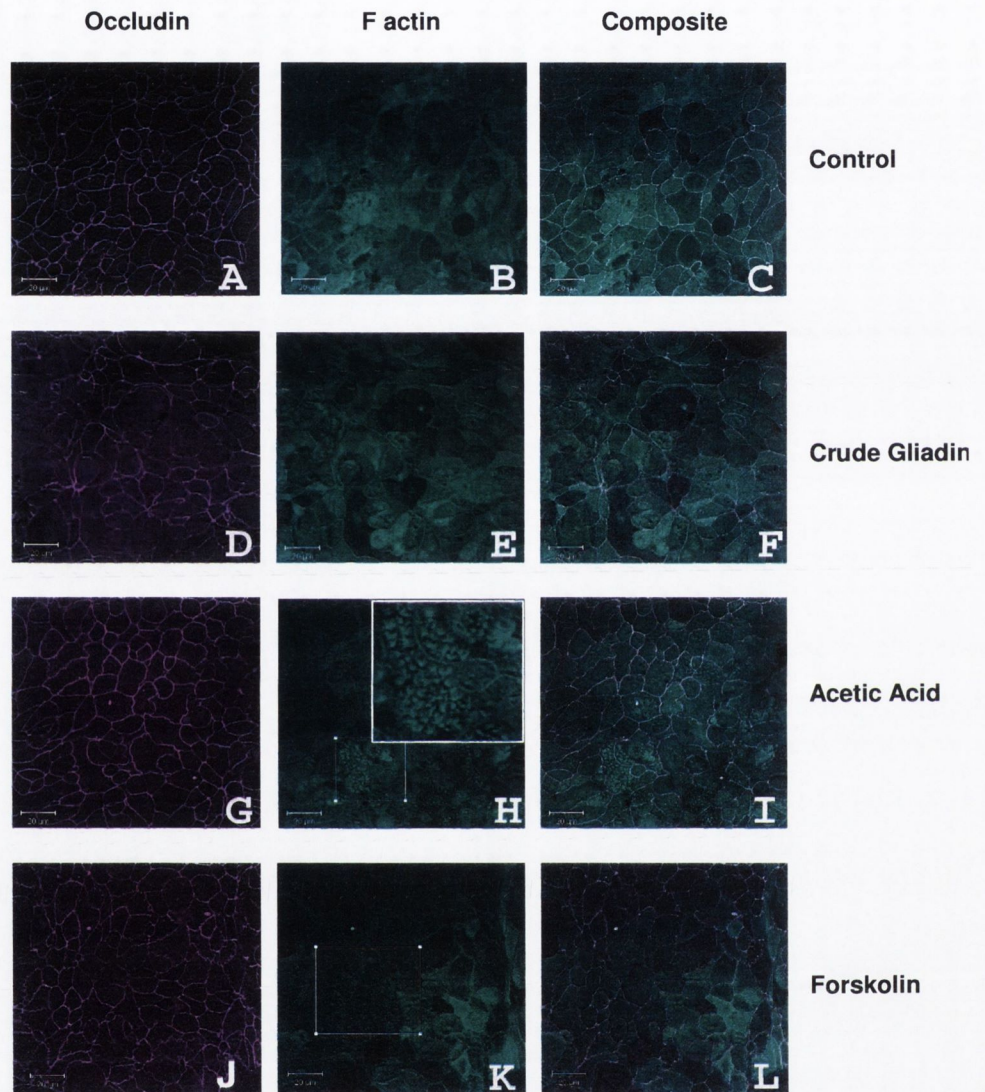


Figure 2.6 Immunofluorescent staining of Caco-2 cells: Cells were either left unstimulated (A, B, C) or stimulated with 1mg/ml crude gliadin (D, E, F) or 0.1M Acetic acid (G, H, I) or Forskolin (J, K, L). Caco 2 cells are stained with occludin (purple) and F-actin (green). A composite of both colours can be seen in also (C, F, I, L). Scale bars= 20um

2.3.4. Effect of PT gliadin at different concentrations on Caco-2 resistance values and the tight junction protein ZO-1

Previous studies have reported a decrease in trans epithelial resistance upon PT gliadin challenge of Caco-2 cells and intestinal tissue (Clemente, De Virgiliis et al. 2003; Sander, Cummins et al. 2005; Drago, El Asmar et al. 2006). High passage number caco-2 cells were cultured in the presence of increasing concentrations of PT gliadin and TEER assessed over 8hrs. The effect of each concentration can be seen in Figure 2.7 A-D. No significant difference was seen in cells stimulated with PT gliadin at any of the concentrations used compared to unstimulated cells despite a significant drop in resistance in forskolin stimulated cells. Inserts were subsequently stained for ZO-1 as seen in Figure 2.8. As can be seen from immunofluorescence results, there was no evidence of alterations in tight junctional integrity within the concentration of PT gliadin used.

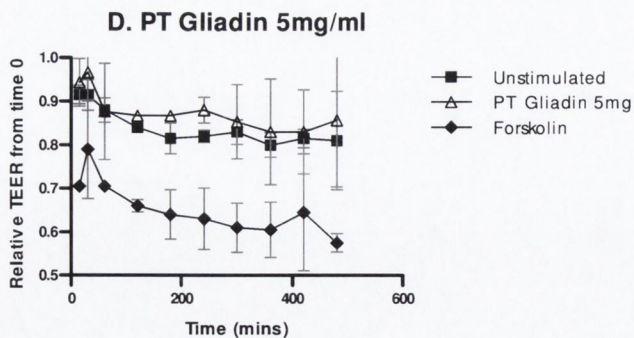
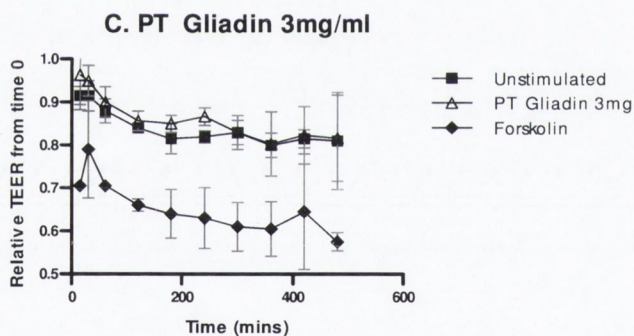
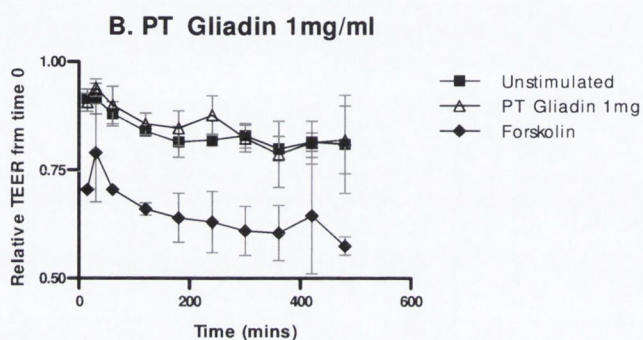
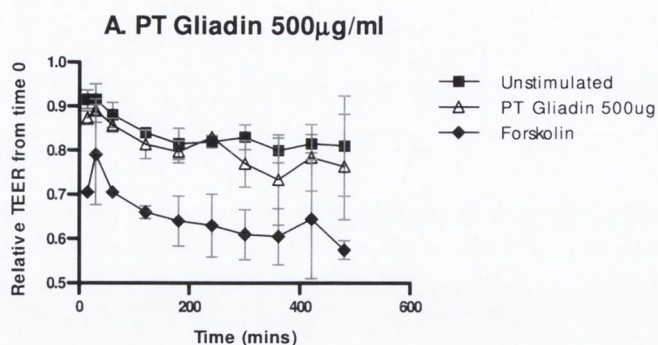
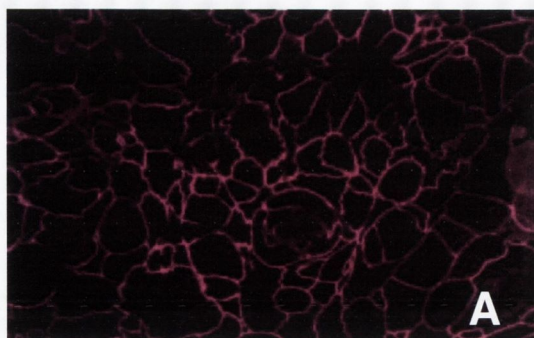
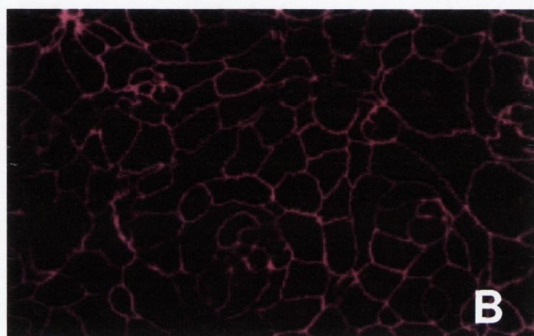


Figure 2.7 Effect of increasing PT gliadin concentration on TEER Graphs represent the mean $\pm$ the standard deviation of 3 individual experiments. Figure A, B, C, and D represent PT gliadin at increasing concentrations of 500 μ g/ml, 1mg/ml, 3mg/ml and 5mg/ml respectively.

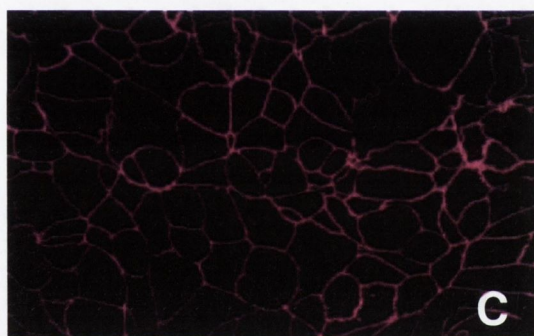
ZO-1



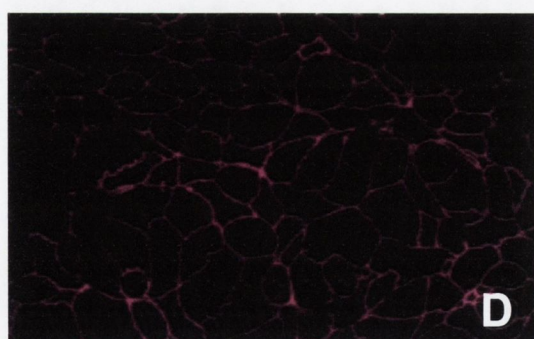
**500 ug
PT gliadin**



**1mg
PT gliadin**



**3mg
PT gliadin**



**5mg
PT gliadin**

Figure 2.8: Immunofluorescent staining of Caco-2 cells in response to different PT gliadin concentrations. Following stimulation for 8hrs, cells were stained for ZO-1. PT gliadin concentrations were as follows; 500 μ g/ml (A.); 1mg/ml (B); 3mg/ml (C) and 5mg /ml (D). (Magnification X 60).

2.3.5 Effect of stimulation on Caco-2 resistance characteristics and tight junctional proteins

The low passage number Caco-2 cells (Passage 30-35) were used to assess changes in tight junction integrity in response to stimulation with either PT gliadin, the control peptide PT ovalbumin, IL-15, an NO donor, a mixture of pro-inflammatory cytokines and forskolin. Low passage number Caco-2 cells were used in conjunction with T 84 cells in these experiments as their resistance profile during differentiation were comparable to the T 84 cell line as shown in figure 2.3.

2.3.5.1 Effect of PT ovalbumin and PT gliadin on resistance using the Caco-2 cell line

Caco-2 cells were incubated with PT ovalbumin (1mg) or PT gliadin (1mg) as with T 84 cells and resistance measured over 24hrs. Results can be seen in Figure 2.9. No significance between peptide stimulated cells and control cells was found over the duration of the experiments. These results are in agreement with results obtained in section 2.3.4 using the high passage number Caco-2 cells in which incubation with different concentrations of PT gliadin did not cause a significant difference in resistance compared to unstimulated cells.

2.3.5.2 Effect of PT ovalbumin and PT gliadin on tight junctional proteins using the Caco-2 cell line

Following 24hrs of challenge with either the control peptide PT ovalbumin or the test peptide PT gliadin, cells were immunofluorescently stained for key tight junction proteins. As can be seen in Figure 2.10 both PT ovalbumin and PT gliadin showed the classical honeycomb pattern of Occludin, ZO-1 and Claudin-4 distribution. Phalloidin staining highlighted patches of unusual F-actin staining in PT gliadin stimulated cells however this was also noted in cells in the absence of gliadin. Therefore this change is not specific to the gliadin peptide. From the immunofluorescence staining it does not appear that gliadin caused any remarkable alterations to barrier function. This is in agreement with earlier findings in section 2.3.4 using the high passage number Caco-2 cell line.

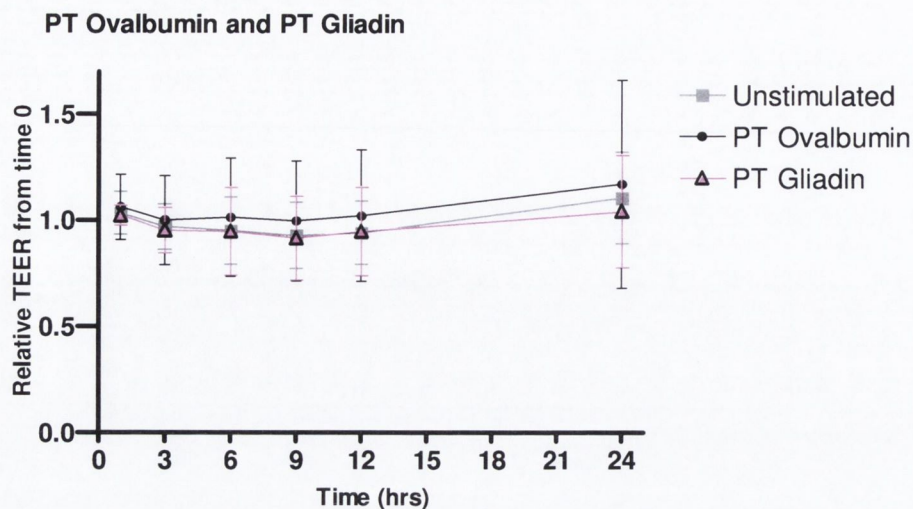


Figure 2.9: Effect of PT gliadin on Caco-2 resistance values over time: Caco-2 cells were either left unstimulated (■) or stimulated with 1mg PT ovalbumin (●) or 1mg PT gliadin (▲). No significant decrease was noted with PT gliadin incubation over the time course studied. Each point represents the mean and SD of 5 experiments.

Figure 2.10: Immunofluorescence staining of the Caco-2 cell line. A, D, G and J represent control cells; B, E, H and K represent cells stimulated with 1mg PT ovalbumin which served as a peptide control and C, F, I and L represent cells stimulated with 1mg PT gliadin. Images are representative of five independent experiments. Cells were stained for the tight junctional proteins ZO-1, Occludin, F-actin and Claudin-4 as marked in figure. Cells were visualised at 63X magnification (zoom 2) on a Zeiss confocal microscope. Bar = 20um.

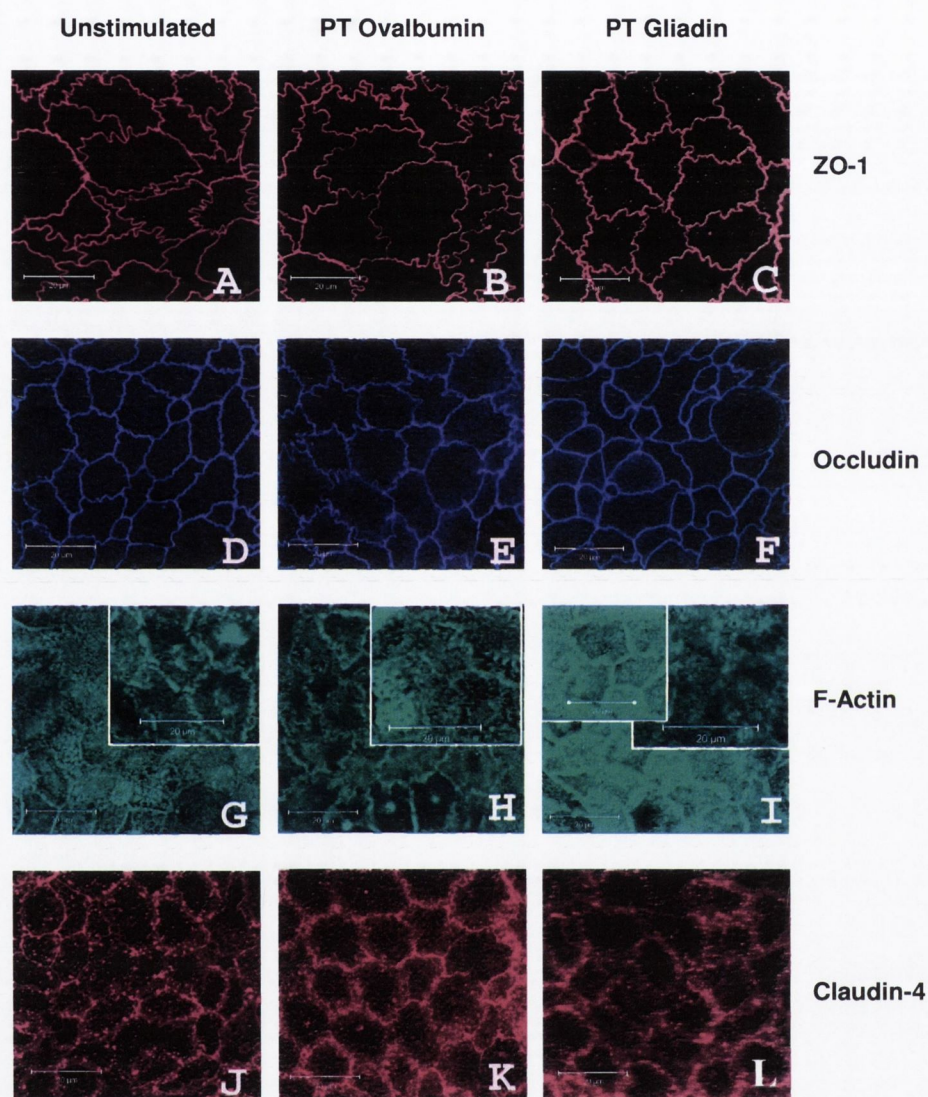


Figure 2.10: Immunofluorescence staining of the Caco-2 cell line in response to PT Ovalbumin and PT Gliadin.

2.3.5.3 Effect of IL-15 and cytomix on resistance using the Caco-2 cell line

The effect of IL-15 and the pro-inflammatory cytomix preparation of IFN- γ , TNF- α and IL-1 β were also assessed. Changes in resistance over 24 hours can be seen in Figure 2.11. No significant difference was seen between IL-15 stimulated cells and unstimulated cells for each of the individual time points examined using a two way ANOVA and BonFerroni *post hoc* test (n=5). Cytomix caused a significant decrease in resistance which was seen at 24hrs using a Two way ANOVA and BonFerroni statistical test with $P < 0.05$ (n=5). A decrease in response to all cytokines have been described in the literature (Madara and Stafford 1989; Ma, Iwamoto et al. 2004; Al-Sadi and Ma 2007).

2.3.5.4 Effect of IL-15 and Cytomix on tight junctional proteins using the Caco-2 cell line

Cells that had been stimulated for 24 hours with the cytokine IL-15 showed a cell separation of ZO-1 at cell contact points in all five experiments in localised areas of the cell monolayer. As can be seen in Figure 2.12, individual cells captured on confocal microscopy showed clear detachment of ZO-1 from one another as seen in cells which were dual stained with the ZO-antibody and DAPI to depict nuclear location. Complete separation of cells could be seen in some areas. Occludin distribution remained normal but a breakdown in localised areas of the classical honeycomb pattern of claudin-4 staining in response to IL-15 could be seen in some experiments in regions of the monolayer. Uneven phalloidin staining was noted in IL-15 stimulated cells but this was also evident in unstimulated cells. Cytomix stimulated cells also seemed to be breaking away from one another at tri-cellular corners when stained with ZO-1. This ZO-1 separation has previously been seen in IFN- γ stimulated airway epithelial cells co-administered with IL-4 and also seen in response to TNF- α in Caco-2 cells (Ma, Iwamoto et al. 2004; Relova, Shahana et al. 2005). These ZO-1 effects may be speculated to be an effect therefore of both these cytokines within the cytomix preparation. Occludin distribution at the cell surface was maintained in cytomix stimulated cells. Unusual phalloidin staining in cells stimulated with cytomix was also evident

in some areas areas with what appeared to be large aggregation of F-actin fibres.

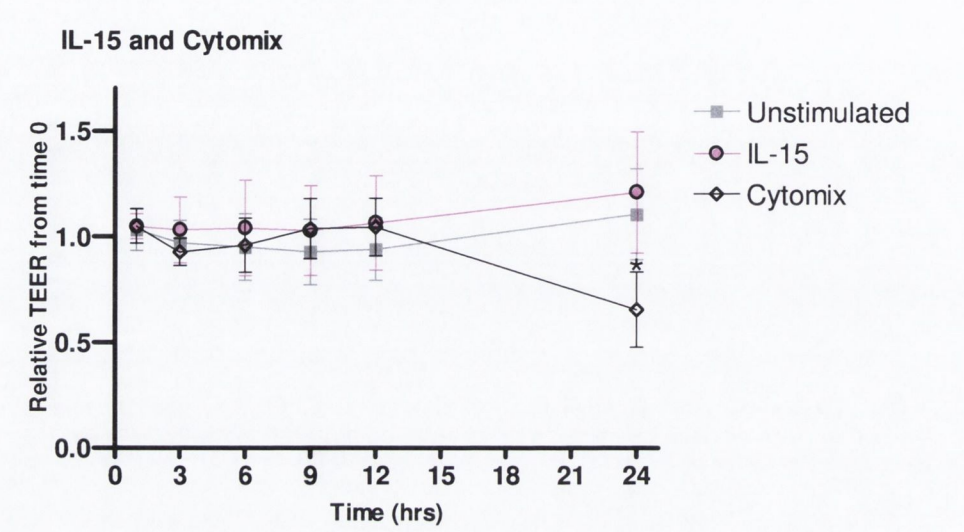


Figure 2.11: Effect of IL-15 and cytomix on Caco-2 resistance values over time: Caco-2 cells were either left unstimulated (■) or stimulated with 250ng IL-15 (●) or a mixture of the pro-inflammatory cytokines containing IFN-γ (1,000U), TNF-α (10ng) and IL-1β (10ng) (◊). * indicates a statistical significant difference compared to unstimulated cells (p<0.05). Each point represents the mean and SD of 5 experiments.

Figure 2.12: Immunofluorescence staining of the Caco-2 cell line. A, D, G and J represent control cells; B, E, H and K represent cells stained with 250ng of Interleukin-15 and C, F, I and L represent cells stained with a combination of pro-inflammatory cytokines, IFN-γ (1000U). TNF-α (10ng) and IL-1β (10ng) Images are representative of five independent experiments. Cells were stained for the tight junctional proteins ZO-1 (inserts show marked separation of ZO-1 contact points between neighbouring cells in IL-15 stimulated cells. Separation was also noted in response to cytomix), Occludin, F-actin (uneven distribution of actin noted in all cells treated and untreated) and Claudin-4 (breakdown in the classical honeycomb pattern in response to IL-15) as marked in the figure. Cells were visualised at 63X magnification (zoom 2) on a Zeiss confocal microscope. Bar = 20µm.

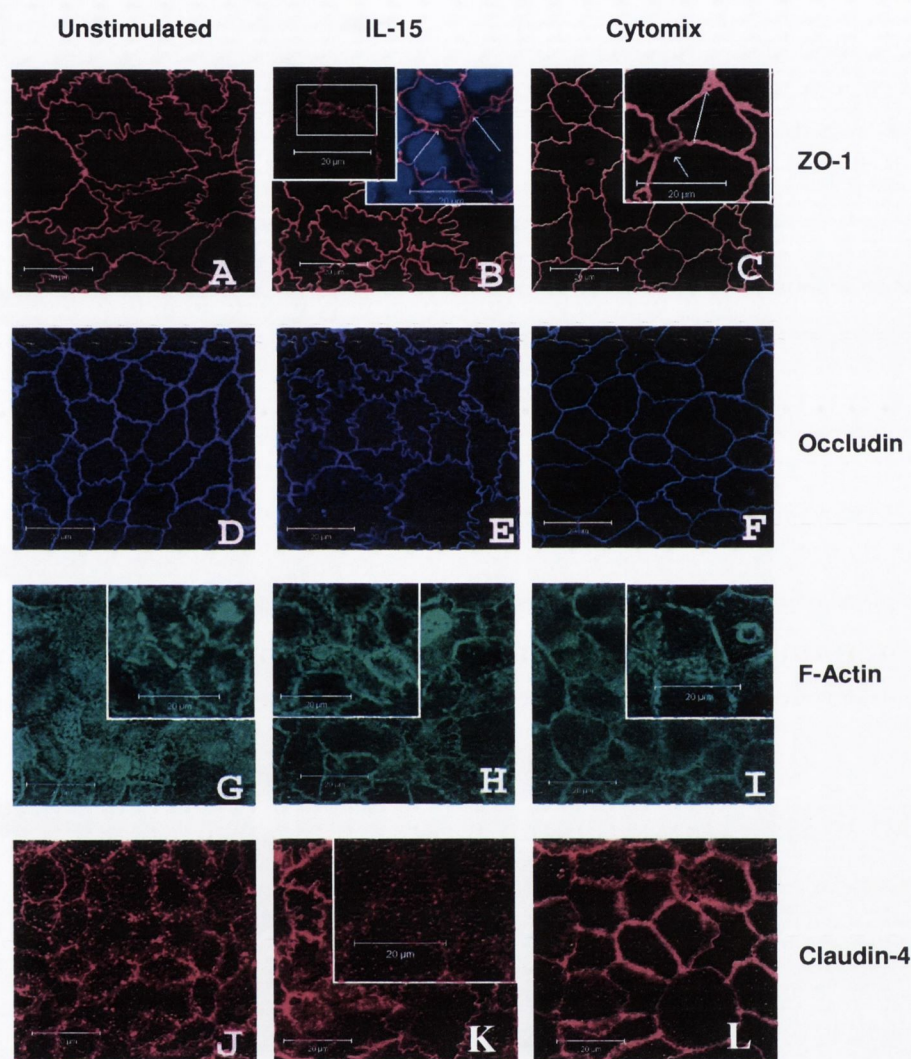


Figure 2.12: Immunofluorescence staining of the Caco-2 cell line in response to IL-15 and Cytomix.

2.3.5.5 Effect of Spermine NONOate (NO Donor) and Forskolin on resistance using the Caco-2 cell line

Nitric oxide was also generated by means of the nitric oxide donor spermine NONOate to assess potential effects on barrier function. Spermine NONOate produces a controlled release of nitric oxide under physiological conditions. As can be seen from figure 2.13, nitric oxide caused a statistically significant difference in resistance compared to unstimulated cells at 24 hours using a Two way ANOVA and BonFerroni post test ($n=5$). Further analysis of results found that this marked decrease in nitric oxide stimulated cells was only seen in 2 experiments as seen in figure 2.14 (replicates shown) making it difficult to interpret this significance. Forskolin stimulated cells induced a significant decreased in resistance compared to unstimulated cells with a s value less than 0.05 ($n=5$) using a two way ANOVA and BonFerroni statistical test with decreases in resistance noted as early as 15mins. To ensure changes in nitric oxide stimulated cells were not caused by spermine NONOate diluent, cells were incubated for 24 hrs in 0.01M NaOH (spermine NONOate diluent) As can be seen from figure 2.15 NaOH did not cause the drop in the TEER observed with spermine NONOate stimulated cells. However within the same experiment a marked decrease in response to NO donor was seen as shown in Fig. 2.14.

2.3.5.6 Effect of Spermine NONOate (NO Donor) and Forskolin on tight junctional proteins using the Caco-2 cell line

Normal patterns of ZO-1, occludin, F-actin and claudin 4 could be seen in response to the nitric oxide donor in three experiments. However in two experiments visually marked alterations in tight junction localisation was found in cells stimulated with Spermine NONOate. These cells corresponded to the above NO stimulated cells showing a marked decrease in resistance at 24 hours compared to unstimulated cells. ZO-1 remained at the cell surface of each cell but there was a clear break-down in its association with ZO-1 on neighbouring cells. This resulted in clusters of individual cells with marked separation from one another in areas of the cell monolayer. Occludin appeared to become internalised with loss of distinct occludin staining at the cell surface. Occludin showed uniform cytoplasmic staining. In F-actin

stained cells, large vacuoles with complete absence of phalloidin staining were noted. Claudin-4 remained intense at the cell surface but claudin-4 within the cytoplasm of the cell could be clearly demonstrated. These altered features were not seen in any of the unstimulated cells.

In forskolin stimulated cells, ZO-1 accumulation was evident at the tri-cellular corners as seen in figure 2.16. An unusual co-localisation of F-actin and ZO-1 within some cells could be seen in forskolin stimulated cells. Whilst these aggregations could be seen in other stimulated cells they were not to same degree or size. Despite alterations in ZO-1 and F-actin, occludin and claudin-4 staining showed the classical pattern of staining as unstimulated cells..

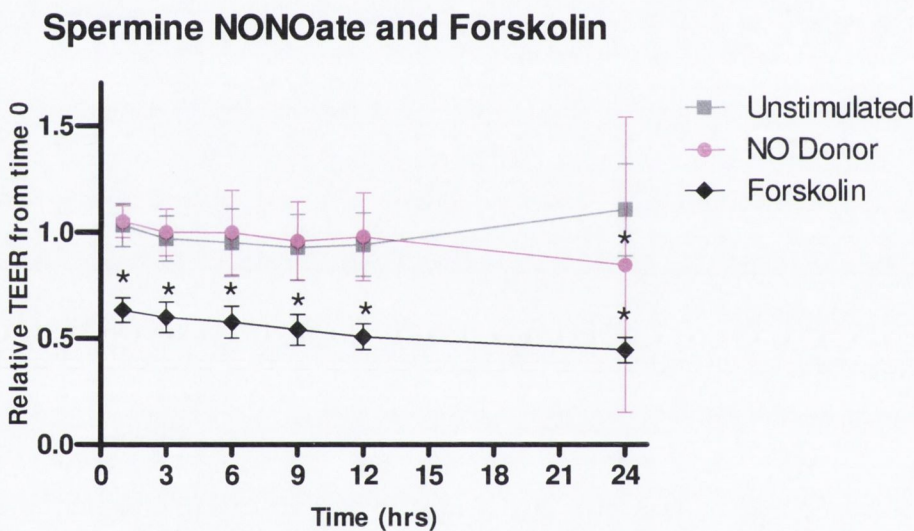


Figure 2.13 Effect of nitric oxide on Caco-2 resistance values over time: Caco-2 cells were incubated for 24 hours with the nitric oxide donor 0.05mM Spermine NONOate (●) or 0.05mM forskolin (◆), a cAMP stimulant. Each point represents the mean $\pm$ standard deviation of 5 experiments. * Significance from controls is indicated for forskolin stimulated cells (p values <0.05).

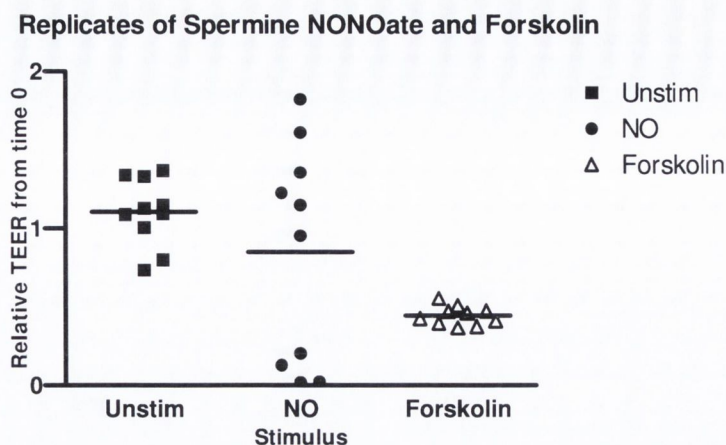


Figure 2.14 Effect of nitric oxide at 24hrs on Caco-2 resistance

Results shown represent duplicate resistance values obtained in response to cells stimulated in the presence of spermine NONOate or forskolin at 24 hours compared to unstimulated cells for 5 experiments.

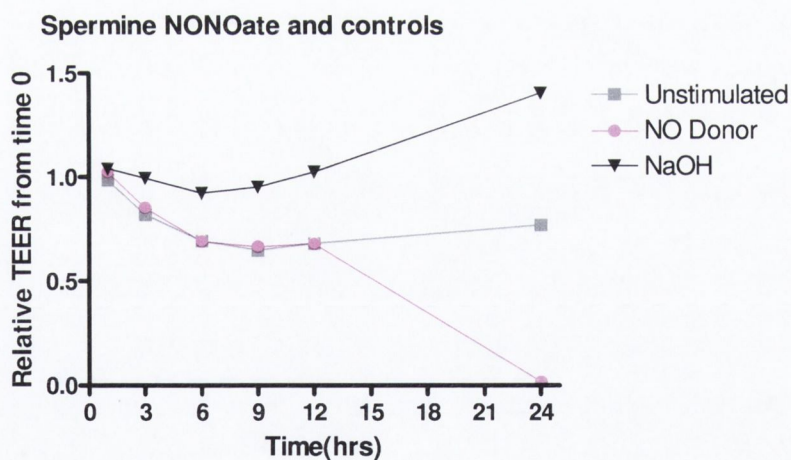


Figure 2.15 Effect of NaOH on Caco-2 resistance over time: Caco-2 cells were either left unstimulated or stimulated with spermine NONOate or 0.01M NaOH which was used in its preparation (n=1).

Figure 2.16: Immunofluorescent staining of the Caco-2 cell line. A, D, G and J represent control cells, B, E, H and K represent cells stained with 0.05mM Spermine NONOate. C, F, I and L represent cells stained with 0.05 mM Forskolin. Images are representative of five independent experiments. Cells were stained for the tight junctional proteins ZO-1 (insert shows marked separation of ZO-1 contact points with neighbouring cells in No donor stimulated cells; ZO-1 accumulates at tri-cellular corners in forskolin stimulated cells along with large aggregates of internal ZO-1, Occludin (insert shows separation of Occludin contact points with neighbouring cells in two expts and internalisation of occluding within the cell in response to NO donor), F-actin (separation of actin contact points and absence of actin within cells in response to NO donor, accumulation of actin aggregates in response to forskolin), and Claudin-4 (insert shows separation of claudin-4 between cell contact points between neighbouring cells and some internalisation in response to NO donor) as marked in figure. Cells were visualised at 63X magnification (zoom 2) on a Zeiss confocal microscope. Bar = 20um. Dual staining of ZO-1 and phalloidin can be seen in figure 2.16 B. Dual staining of phalloidin and the nuclear stain, DAPI can be seen in figure 2.16 H. Dual staining of claudin-4 and the nuclear stain, DAPI can be seen in figure 2.16K.

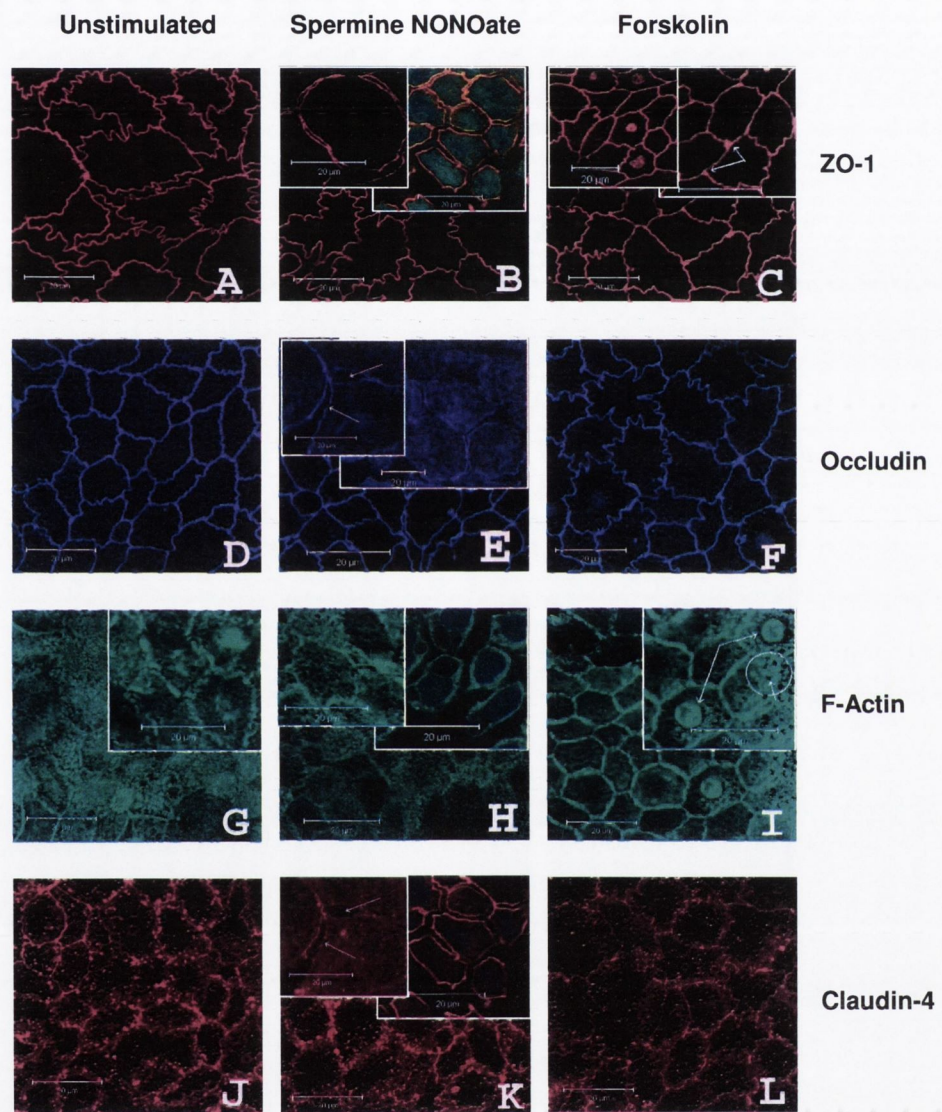


Figure 2.16: Immunofluorescent staining of the Caco-2 cell line in response to Spermine NONOate and Forskolin.

2.3.5.7 Effect of stimulation with PT ovalbumin, PT gliadin, IL-15, Spermine NONOate, cytomix and forskolin on ZO-1- F-actin aggregate formation in the Caco-2 cell line

Dual staining of Caco-2 cells for ZO-1 and F-actin highlighted aggregates of co-localised ZO-1 and F-actin internalised within cells. Enumeration of aggregates was performed for each stimulus to investigate the effect of stimulation on aggregate number and the results are shown in Figure 2.17. Enumeration of these co-localised “aggregates” was done by counting the immunofluorescently stained aggregates for 3 fields at 63 X magnification for each of five experiments and obtaining the average number of aggregates in response to stimulation. Whilst no statistical significance was noted for any of the stimuli, marginally elevated numbers in response to IL-15 and spermine NONOate were found. Both cytomix and forskolin had a much greater number of aggregates than unstimulated cells. However due to low sample number these figures did not reach statistical significance. These aggregates were found in both unstimulated and stimulated cells as can be seen in Figure 2.18.

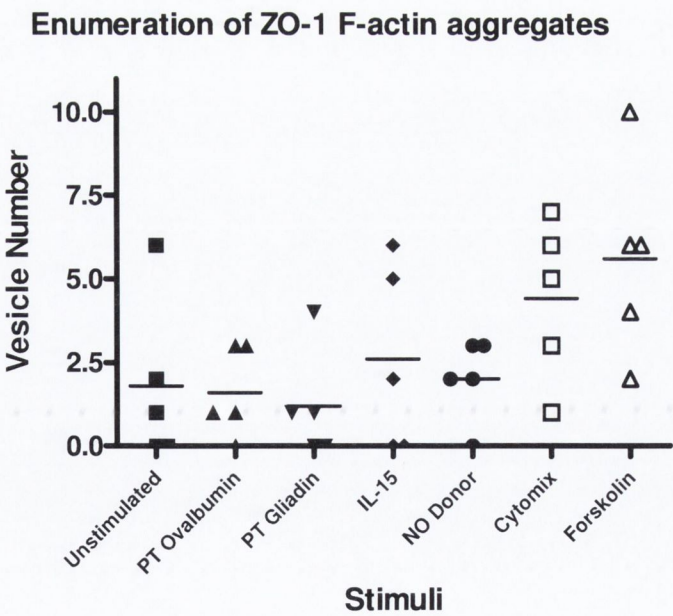


Figure 2.17 Enumeration of ZO-1, F-actin aggregates in the Caco-2 cell line. Immunofluorescently stained cells from five independent experiments were visualised at 63 X magnification and the number of aggregates counted per field and averaged for each experiment

Figure 2.18 Immunofluorescent staining of ZO-1 and F-actin aggregates in the Caco-2 cell line. Control cells (A), PT ovalbumin(B), PT gliadin (C), IL-15 (D), Spermine NONOate (E), Cytomix (F) and Forskolin (G) stimulated cells were stained with ZO-1 and phalloidin. Co-localisation can be seen in yellow. Cells were visualised at 63X magnification zoom 2. Scale bar = 20um.

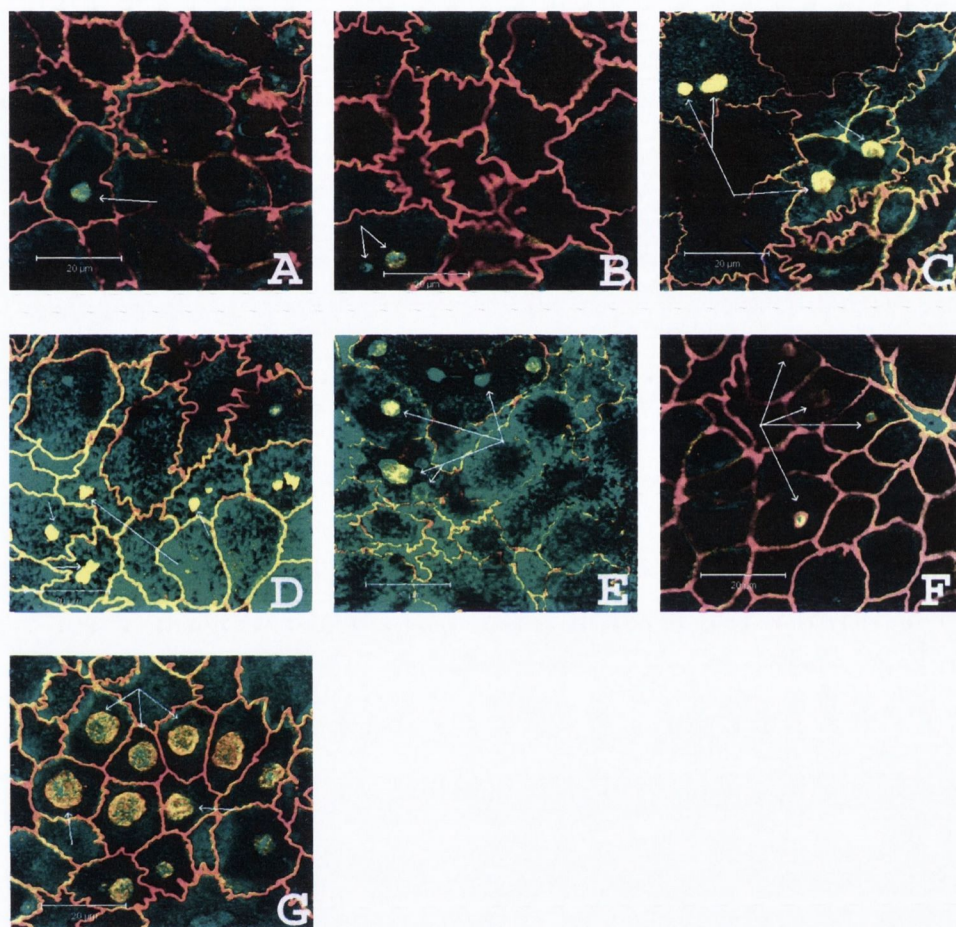


Figure 2.18 Immunofluorescent staining of ZO-1 and F-actin aggregates in the Caco-2 cell line.

2.3.6 Effect of stimulation on T 84 resistance characteristics and tight junctional proteins

To determine whether the effects seen in Caco-2 cells could be replicated, another enterocyte cell line, the T 84 cell line was also used.

2.3.6.1 Effect of PT ovalbumin and PT gliadin on resistance using the T 84 cell line

T 84 cells were either left unstimulated or incubated with 1mg of PT gliadin or PT ovalbumin, the latter serving as a peptide control. Stimulus was added to both apical and basolateral compartments and resistance values measured using an EndOhm system at defined time points. Changes in TEER from the initial readings were plotted against time as seen in Figure 2.19. No significant difference was found between PT gliadin stimulated cells and controls over the time course studied.

2.3.6.2 Effect of PT ovalbumin and PT gliadin on tight junctional proteins using the T 84 cell line

To further investigate the effects of PT gliadin incubation, cells were stained at 24hrs for some of the main components of the tight junction; ZO-1, occludin, claudin-4 and JAM-A, along with the cytoskeletal protein F-actin by immunofluorescence. Results are shown in Figure 2.20. Unstimulated cells and cells incubated with PT ovalbumin which served as a peptide control, showed the classical honeycomb staining for each of the tight junctional proteins. Tight junctional proteins were found to be localised to the border of each cell. In cells stimulated with PT gliadin, ZO-1, claudin-4 and JAM showed an intact junctional network. In one of the three experiments, occludin showed an unusual staining pattern with cells appearing to separate from one another with resultant separation of occludin contact points between cells. This was found to occur in localised areas and was insufficient to cause marked decreases in resistance over the time course studied. This PT gliadin associated alteration was not seen in any of the control experiments. Phalloidin staining of F-actin showed unusual areas of staining in PT gliadin stimulated cells, with aggregation of micro-villous structures. However this observation was also seen in one experiment in control cells which makes it

difficult to interpret the finding. Overall PT gliadin within 24hrs does not appear to cause significant alterations to T 84 barrier function.

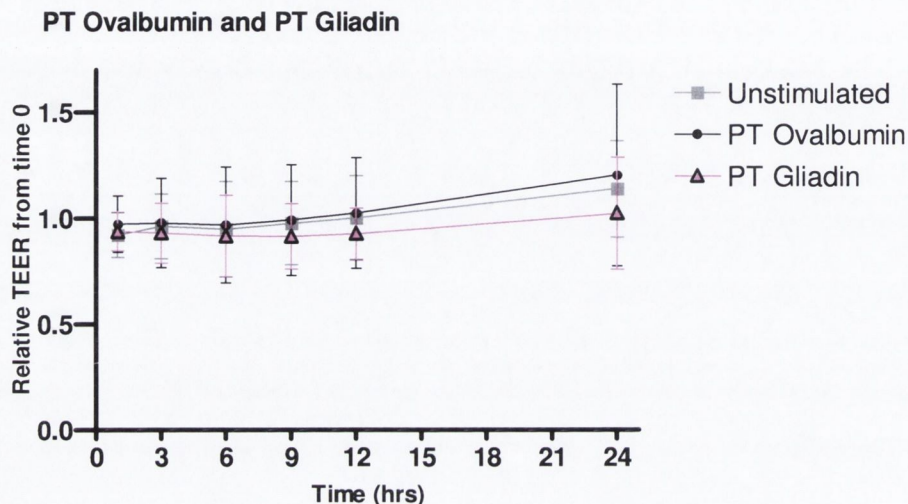


Figure 2.19 Effect of PT ovalbumin or PT gliadin on T 84 resistance values over time: T 84 cells were incubated for 24 hours in DMEM either alone (■) or in the presence of 1mg PT ovalbumin (●) or 1mg PT gliadin (▲). Each value represents the mean $\pm$ standard deviation of 3 experiments.

Figure 2.20: Immunofluorescence staining of the T 84 cell lines. A, D, G, J and M represent unstimulated cells, B, E, H, K, N represent cells stained with 1mg PT ovalbumin which served as a peptide control. C, F, I, L and O represent cells stimulated with 1mg PT gliadin. Images are representative of three independent experiments. Cells were stained for the tight junctional proteins ZO-1, occludin, F-actin, JAM and claudin-4. Cells were visualised at 63X magnification (zoom 2) on a Zeiss Confocal microscope. Scale bar = 20um.

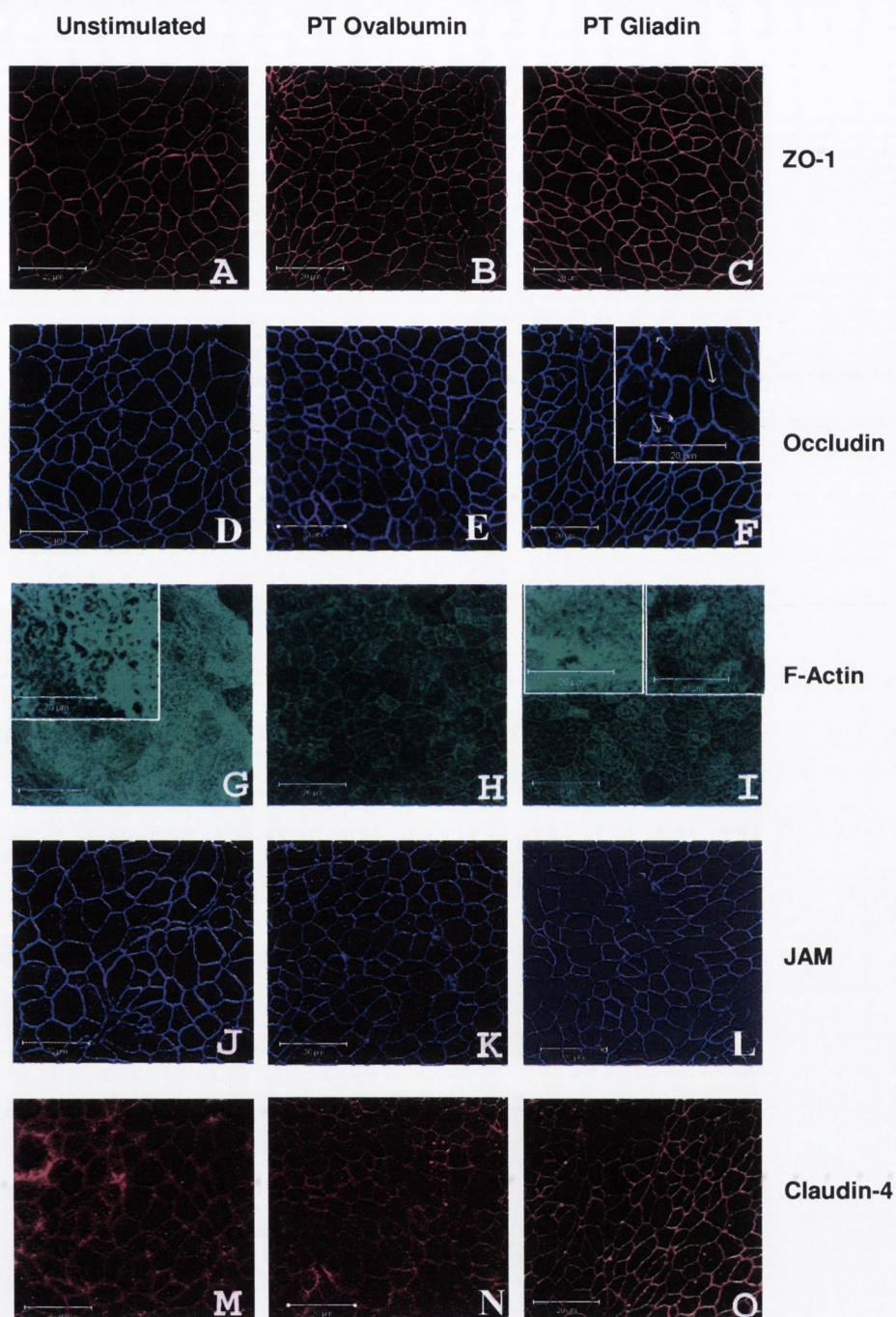


Figure 2.20: Immunofluorescence staining of the T 84 cell line in response to PT Ovalbumin and PT Gliadin.

2.3.6.3 Effect of IL-15 and cytomix on resistance using the T 84 cell line

To assess the effect of IL-15, a key cytokine of the innate immune response in coeliac disease, cells were incubated for 24hrs with 250ng/ml of IL-15 and resistance measured over time. Cells were also incubated with a mixture of pro-inflammatory cytokines, IFN- γ (1,000U/ml), TNF- α (10ng/ml) and IL-1 β (10ng/ml) referred to as cytomix, to assess their effects on resistance. Changes in resistance were plotted against time and results can be seen in Figure 2.21. No significant difference in resistance was found between cells in the absence of IL-15 and T 84 cells stimulated with IL-15 at individual time points using a two way ANOVA and BonFerroni statistical test. Resistance values of cytomix stimulated cells were significant compared to unstimulated cells ($p < 0.05$) at 9, 12 and 24 hours using a two way ANOVA followed by a BonFerroni statistical test ($n=3$). Thus in an inflammatory milieu of cytokines such as IFN- γ and TNF- α found in coeliac disease, this could cause an increase in permeability of intestinal cells .

3.3.6.4 Effect of IL-15 and Cytomix on tight junctional proteins using the T 84 cell line

Cells that had been incubated with the cytokine IL-15 and the mixture of pro-inflammatory cytokines IFN- γ , TNF- α and IL-1 β were also stained after 24 hours to assess alterations in tight junction integrity. Results representative of three independent experiments can be seen in Figure 2.22. In IL-15 stimulated cells, occludin and claudin-4 showed the classical honeycomb arrangement. However on analysis of ZO-1, marked separation of cells at contact points were noted in two of three experiments in regions of the cell monolayer along with accumulation of ZO-1 at tri-cellular corners. Due to low experimental number these results are difficult to interpret. A number of areas showed clear gaps between cell contact points. Unusual phalloidin stainingis was seen in IL-15 treated and also in the untreated cells.. Thus these actin morphological changes do not appear specific to treatment. Despite JAM appearing more diffuse in some areas of the IL-15 stimulated T 84 cells, overall JAM patterns of staining remained intact.

Inflammatory cytokines associated with a number of inflammatory disorders including coeliac disease was also used to investigate tight junctional alterations.. Areas of clear separation of ZO-1 staining could be seen at tri-

cellular corners with areas of complete loss of contact between cells in some experiments. JAM staining was found to be diffuse in one experiment. Aggregation of F-actin occurred in response to the cytomix stimulation with vacuole like structures forming within the F-actin meshwork. Claudin-4 disruption was also noted in some areas despite intact occludin staining.

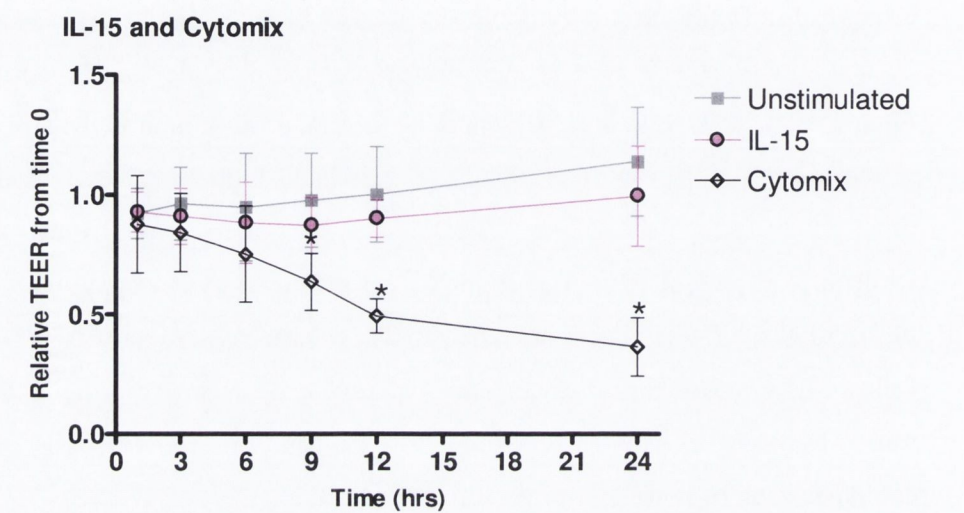


Figure 2.21 Effect of IL-15 and cytomix on T 84 resistance values over time. T 84 cells were incubated either with 250 ng of IL-15 (●) or a mixture of pro-inflammatory cytokines (◇); IFN-γ (1,000U), TNF-α (10ng) and IL-1β (10ng) and resistance measured over time. Each value represents the mean ± standard deviation of 3 experiments.

Figure 2.22: Immunofluorescence staining of T 84 cell lines. A, D, G, J and M represent unstimulated cells; B, E, H, K, N represent cells stimulated with 250ng of Interleukin-15 and C, F, I, L and O represent cells stimulated with a combination of pro-inflammatory cytokines, IFN-γ (1000U). TNF-α (10ng) and IL-1β (10ng) Images are representative of three independent experiments. Cells were stained for the tight junctional proteins ZO-1 (inserts show separation of ZO-1 contact points in response to Il-15 and accumulation of ZO-1 at tri-cellular corners in response to forskolin), Occludin, F-actin, JAM (inserts show breakdown in classical honeycomb pattern in response to both Il-15 and cytomix stimulation) and Claudin-4 (insert shows breakdown in the classical honeycomb pattern of claudin-4 staining in some expts. In response to cytomix) as marked in figure. Cells were visualised at 63X magnification (zoom 2) on a Zeiss Confocal microscope. Scale bar = 20uM

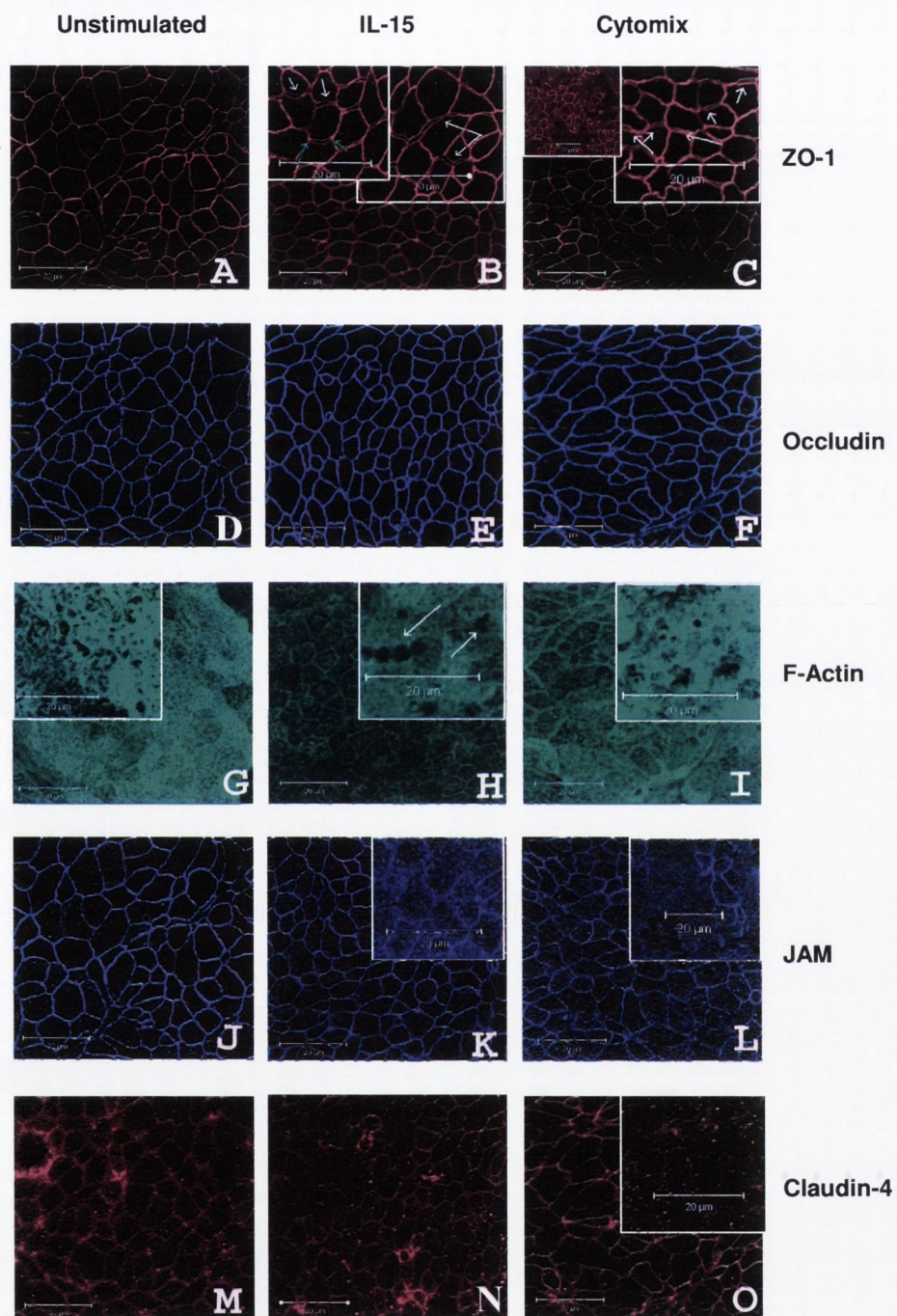


Figure 2.22: Immunofluorescence staining of the T 84 cell line in response to IL-15 and Cytomix.

2.3.6.5 Effect of Spermine NONOate (NO Donor) and Forskolin on resistance using the T 84 cell line

To assess the effect of nitric oxide on barrier function an NO donor, Spermine NONOate was incubated for 24hrs with T 84 cells. 0.05mM of spermine NONOate was incubated with cells and resistance measured for up to 24hrs. Resistance values were plotted against time as can be seen in figure 2.23. Nitric oxide is known to be involved in signalling pathways resulting in the generation of cGMP. Forkolin is a known cAMP activator and was included as a positive control, as alterations in nucleotidal signalling molecules have been shown to influence permeability.

No significant difference was seen for each of the individual time points tested between NO stimulated cells and unstimulated cells using a two way ANOVA and a BonFerroni statistical test. Whilst NO caused a decrease in TEER at 24hrs compared to unstimulated cells this did not reach statistical significance. Forskolin caused a statistically significant decrease in resistance compared to unstimulated cells at all time points.

2.3.6.6 Effect of Spermine NONOate and Forskolin on tight junctional proteins using the T 84 cell line

Cells incubated with the nitric oxide donor, Spermine NONOate and cells incubated with the cAMP activator, forskolin were also stained to investigate alterations in tight junctional proteins. As can be seen from Figure 2.24 Spermine NONOate caused separation of ZO-1 cell contact points between adjacent cells which could be clearly visualised at distinct areas of the cell monolayer in some experiments. A significant difference in phalloidin staining was clearly seen with loss of perijunctional actin. Dual staining of ZO-1 and F-actin can be seen in figure 2.24 H for visualisation purposes to show the position of F-actin fibres in relation to the cell contact points. Diffuse JAM and claudin-4 staining was also noted in some areas in some experiments. Forskolin stimulated cells showed ZO-1 separation though not as marked as that seen with nitric oxide. ZO-1 could be seen to increase at focal points between cells. F-actin redistribution was also noted though again not as obvious as seen with nitric oxide. Patches of JAM and claudin-4 breakdown was also evident with complete loss of classical honeycomb

distribution in some experiments. Occludin remained intact throughout all experiments in response to both stimuli.

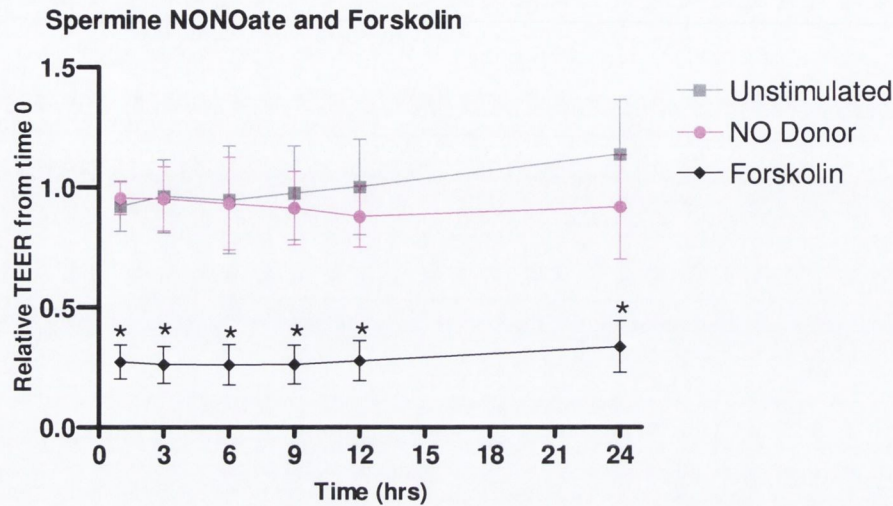


Figure 2.23 Effect of nitric oxide on T 84 resistance values over time: T 84 cells were incubated for 24 hours with 0.05mM Spermine NONOate(●), a nitric oxide donor or 0.05mM forskolin (◆), a cAMP stimulant. Each point represents the mean $\pm$ standard deviation 3 experiments. * Significance from controls is indicated for forskolin stimulated cells (p values <0.05).

Figure 2.24: Immunofluorescence staining of T 84 cell lines. A, D, G, J and M represent control cells; B, E, H, K, N represent cells stimulated with 0.05mM Spermine NONOate and C, F, I, L and O represent cells stimulated with 0.05 mM Forskolin. Images are representative of three independent experiments. Cells were stained for the tight junctional proteins ZO-1 (9 inserts show ZO-1 separation from neighbouring contact points in response to the NO donor and accumulation of ZO-1 at the tri-cellular corners in response to forskolin), Occludin, F-actin (inserts show absence of peripheral actin in response to the NO donor and uneven actin localisation in response to forskolin), JAM (inserts show breakdown in honeycomb pattern in some expts. in response to both treatments) and Claudin-4 (inserts show breakdown in honeycomb pattern in some expts.) as marked in figure. Cells were visualised at 63X magnification (zoom 2) on a Zeiss confocal microscope. Bar = 20um.

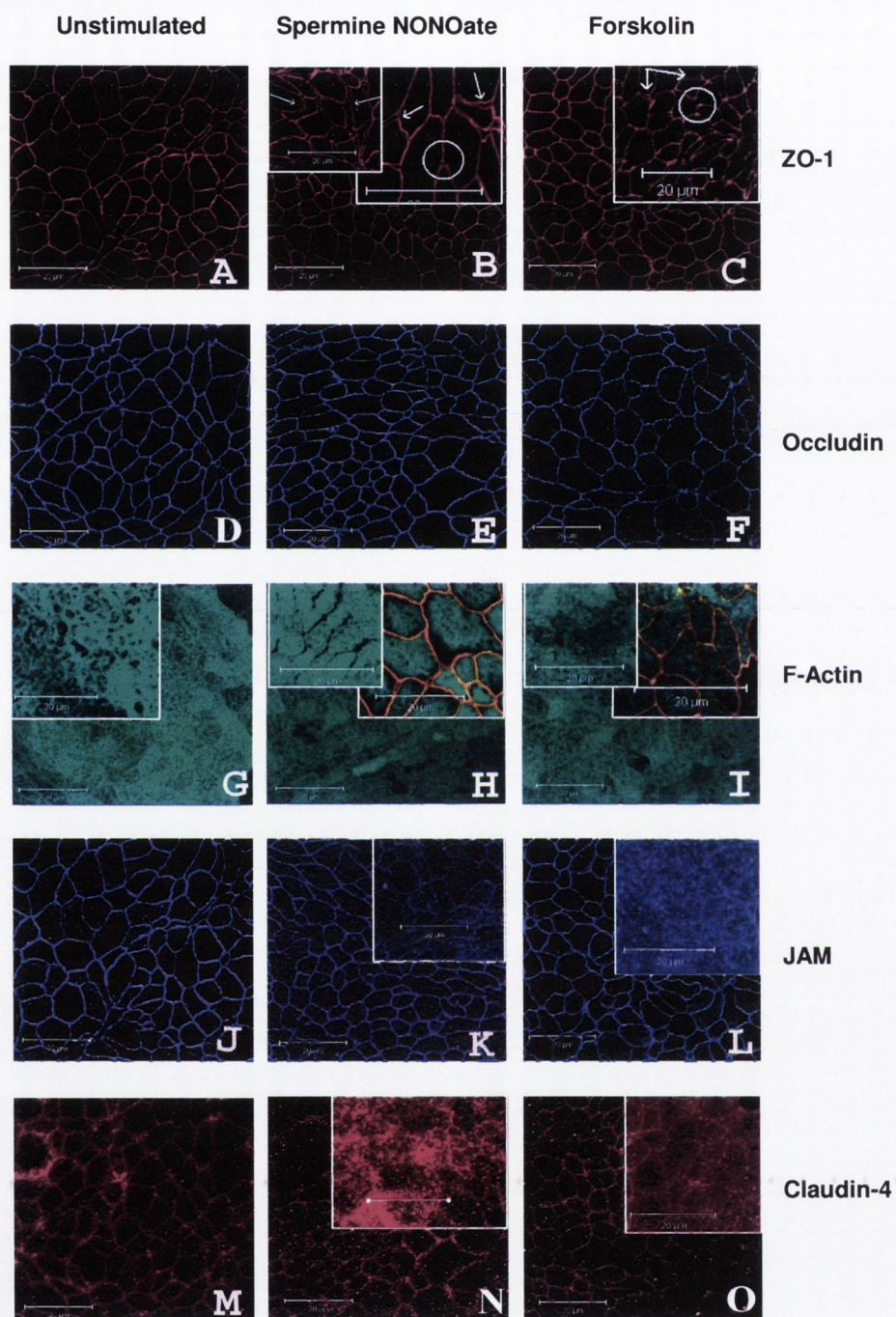


Figure 2.24: Immunofluorescence staining of the T 84 cell line in response to Spermine NONOate and Forskolin.

2.4 Discussion

Tight junctions represent critical gate-keepers of intestinal barrier permeability and disruption of this regulated process inevitably results in uncontrolled entry of potential immunogenic material to the underlying mucosa. In coeliac disease, transport of gliadin as relatively intact peptides across the mucosa is enhanced and this initiates inflammation associated with the disease upon entry through the epithelium (Matysiak-Budnik, Candalh et al. 2003). Transcellular and paracellular routes of gliadin entry have been proposed. Recently an IgA mediated transport of gliadin entry through the transcellular route involving the transferrin receptor has been shown (Matysiak-Budnik, Moura et al. 2008). Transcytosis of the 33mer gliadin peptide across the epithelium within endocytic vesicles has also been suggested (Schumann, Richter et al. 2008). Other studies support a paracellular route of entry for gliadin with a number of elegant studies demonstrating decreased resistance and alterations in tight junction components following gliadin exposure (Clemente, De Virgiliis et al. 2003; Dolfini, Roncoroni et al. 2005; Sander, Cummins et al. 2005; Ciccocioppo, Finamore et al. 2006; Drago, El Asmar et al. 2006). Understanding the initial events which enable gliadin entry into the mucosa is important for both treatment and possible preventative therapy.

Whilst studies on tight junctional integrity have focussed on the role of gliadin in mediating barrier function, the contribution of other innate factors up-regulated upon initial gliadin exposure e.g. IL-15, remain unknown. IL-15 up-regulation occurs as an early innate event upon gliadin exposure in both enterocytes and monocytic cells and has been suggested to be the key mediator of the observed gliadin effects (Maiuri, Ciacci et al. 2000). Due to the close proximity of IL-15 to the epithelium and its rapid up-regulation upon gliadin exposure, it is not unreasonable to suggest a direct role for this cytokine in altering barrier function thus allowing entry of gliadin fragments into the underlying mucosa to activate the T cell mediated events.

The direct role of gliadin and IL-15 was assessed in this study to determine the contribution of each factor to tight junction integrity. However no significant difference in tight junction permeability or organisation was noted in gliadin stimulated cells. In contrast, this study highlights subtle changes specific to IL-15 in tight junction proteins particularly demonstrated with ZO-1 separation.

Another possible innate contributor to disruption of tight junctional organisation is derived from the inducible nitric oxide synthase enzyme and this enzyme has also been shown to be up-regulated in the small intestine during coeliac disease in enterocytes and mononuclear cells (ter Steege, Buurman et al. 1997; Beckett, Dell'Olio et al. 1998; Murray, Daniels et al. 2002). The production of nitric oxide as a consequence of increased iNOS enzyme activity may also contribute to disease pathogenesis. Nitric oxide produced by macrophages represents an important innate mechanism in anti-bacterial host defence following antigenic challenge (Tsai, Strieter et al. 1997). Studies to-date using the murine macrophage cell line RAW 264.7 suggest nitric oxide up-regulation in coeliac disease is part of an adaptive response requiring IFN- γ which is further up-regulated by gliadin (Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003). However induction by other stimuli distinct from IFN- γ such as IL-15 has also been shown in this murine macrophage cell line (Liu, Zhai et al. 2004). Thus nitric oxide production may occur as an early event in coeliac disease in response to IL-15. Nitric oxide has also been shown to participate in tight junction reorganisation (Han, Fink et al. 2003). This study investigated the possible role of nitric oxide production on barrier function in coeliac disease. The results showed variable changes in response to nitric oxide ranging from no effect to severely compromised tight junction integrity.

This study also investigated the role of T cell derived cytokines produced during the adaptive phase of inflammation in coeliac disease to compare alterations in tight junction integrity caused by innate immune contributors compared to T cell derived adaptive responses. The study showed a significant alteration in intestinal permeability in response to the adaptive cytokine milieu with resultant altered tight junctional organisation.

Enterocyte cell lines

Caco-2 cells were used at two passage numbers to determine the effects of passage number on experimental findings. Low passage number Caco-2 cells had much higher resistance values upon differentiation compared to high passage number cells. Disparate transepithelial electrical resistance measurements has previously been noted in Caco-2 cells with differing passage numbers although paracellular transport properties were found to be unaltered (Lu, Gough et al. 1996). In this study the effect of PT gliadin was assessed in both high and low passage number cells. No significant change in transepithelial resistance was observed in cells stimulated with PT gliadin compared to unstimulated cells, in either high or low passage number Caco-2 cells. Forskolin, a cAMP activator which served as a positive control caused a significant decrease in transepithelial resistance and this was seen in both high and low passage number Caco-2 cells. Thus using Caco-2 cells derived from different laboratory sources (Institute of Molecular Medicine and Dr. Ann Hopkins, UCD) which displayed different resistance patterns, did not influence the subsequent results. As the low passage number Caco-2 cells had similar TEER profiles as the T 84 cell line, these low passage number cells were used to directly compare the effects of PT gliadin, IL-15, NO and cytomix between the Caco-2 and T 84 enterocyte cell lines. High TEER values indicate the presence of well differentiated tight junctions (Sambruy, Ferruzza et al. 2001).

Gliadin

In this study, experiments with a peptic/tryptic preparation of gliadin in both T 84 and Caco-2 cell lines (high passage number and low passage number) failed to show any significant decrease in response to gliadin as assessed by continuous measurement of TEER for up to 24 hours. This is in contrast to previous studies which have shown changes in TEER as early as 15 minutes following gliadin stimulation in Caco-2 cell lines. Changes in TEER in both control subjects and subjects with coeliac disease has been found (Sander, Cummins et al. 2005; Drago, El Asmar et al. 2006). In this study, initial decreases in response to a crude gliadin preparation were found to be due to the acetic acid used in its preparation. Moreover, immunofluorescent staining

showed intact honeycomb patterns in gliadin stimulated cells similar to that found in controls.

TEER did not decrease in response to PT gliadin stimulation in T 84 cells. Other groups have suggested that decreases in response to gliadin are zonulin mediated and T 84 cells do not express zonulin receptors on their surface in contrast to Caco-2 cells. Alterations in F-actin organisation seen in gliadin stimulated Caco-2 cells but not in T 84 cells have been explained by the zonulin non-responsiveness of T 84 cells (Drago, El Asmar et al. 2006). This may be why no difference in response to gliadin was found in this cell line.

Conflicting data between this study and previous findings in relation to the effect of gliadin on TEER in Caco-2 cells might be explained in part by variations in PT gliadin preparation and sources of wheat gliadin. As was seen in this study, the effects noted by crude gliadin were due to the solvent used in its preparation. The Caco-2 cell line is a highly heterogeneous cell line. Whilst this study found no difference in paracellular permeability as assessed by TEER between different laboratory cell sources with different passage numbers, other reports have found variability in the Caco-2 cell lines. Differences in membrane supports, time in culture, seeding density and medium supplements have all been shown to affect cell morphology as have alterations in F-actin distribution, paracellular permeability, TEER and expression of peptide transporters (Behrens and Kissel 2003; Sambuy, De Angelis et al. 2005). This inter-laboratory variability of Caco-2 cell morphology could explain different responses to gliadin stimulation. Results using cell lines must be interpreted with care and highlights the necessity for inclusion of several different cell lines within a study. In this study two different laboratory sources of Caco-2 cell lines along with the T-84 colonic cell line are used to investigate effects on tight junction integrity and comparable results were found in all cell lines studied. This study did not show alterations in response to PT gliadin on tight junction integrity by a zonulin dependent or independent mechanism.

Innate cytokine IL-15

Negative changes in tight junction integrity in response to gliadin prompted this study to investigate other potential stimuli, found within the coeliac lesion, on tight junction integrity. Hence, IL-15 induction of barrier dysfunction was investigated as a possible early event in coeliac disease. No significant alteration in TEER was seen in both cell lines within the time course of the experiment. Administration of exogenous IL-15 to T 84 cell lines has previously been shown to take three to four days before any appreciable change in TEER (depicted by an increase in TEER) was shown (Nishiyama, Sakaguchi et al. 2001). As the present study was performed for 24 hours, this may have been too short a time frame to observe significant changes in TEER. Both cell lines have previously been shown to express high levels of endogenous IL-15, this may also explain the non-responsiveness of both cell lines to exogenously administered IL-15 on TEER at 24 hours (Hue, Mention et al. 2004). Previous reports have shown positive regulation of IL-15 on TEER in T 84 cells. The T-84 cell line which lacks the RIL-15 β , upon transfection with RIL-15 β shows an increase in TEER compared to the parental T 84 cell line lacking the β chain. This increase however has been shown to occur in the absence of exogenous IL-15. Administering exogenous IL-15 did not significantly increase TEER suggesting that over-expression of the RIL-15 β is the cause of increasing TEER in response to endogenous IL-15 and not due to the exogenous IL-15. (Nishiyama, Sakaguchi et al. 2001).

Subtle changes in tight junction patterns were found to occur in both cell lines in response to IL-15 in this study. Alterations in T84 cell lines in response to exogenous IL-15 have previously been reported by signalling via RIL-15 β dependent and independent mechanisms (Nishiyama, Sakaguchi et al. 2001). Separation at ZO-1 contact points was found to occur in all five Caco-2 experiments following stimulation with IL-15. T-84 cells have been reported to lack the β chain of the IL-15 receptor despite being able to signal via IL-15 (Reinecker and Podolsky 1995; Nishiyama, Sakaguchi et al. 2001). This may explain the less pronounced effects of IL-15 on T 84 cells with respect to ZO-1 separation: ZO-1 separation was noted in 2 of 3 experiments as seen in section 3.3.6.4. This subtle cell separation has previously been

described in airway epithelial cells in response to IL-4 and IFN- γ (Relova, Shahana et al. 2005). Alterations in claudin-4 in the Caco-2 cell line and JAM staining in the T 84 cell line was also noted. In summary, these findings suggest a role for IL-15 in inducing subtle changes in tight junction paracellular permeability and tight junctional integrity especially with the ZO-1 tight junction protein over 24 hours. However at this early stage of altered cell morphology in response to IL-15, overall cell contacts are still largely retained.

Previous studies by other groups have shown IL-15 induction of stress molecules such as MICA on the epithelial surface (Hue, Mention et al. 2004). MICA are MHC I related proteins up-regulated in response to stress such as oxidative stress and in response to heat shock proteins (Mistry and O'Callaghan 2007). It is interesting that in the presence of IL-15, epithelial cells recognise this cytokine as a stress, danger molecule and increase the expression of the stress markers MICA on their surface (Hue, Mention et al. 2004). In the current study, findings with IL-15 of subtle tight junction disruption offer a potential role of IL-15 in inducing another form of stress response in enterocytes through alteration of tight junction networks between these cells during coeliac disease. However as TEER remained unaltered in response to IL-15 in both cell lines, the effect of these tight junction alterations on intestinal permeability appear negligible. High endogenous production of IL-15 by both cell lines may also explain the lack of effect of administration of exogenous IL-15.

The effect of a cytomix preparation of IFN- γ , TNF- α and IL-1 β

To investigate the possible role of cytokines produced during the adaptive phase of coeliac disease, experiments were performed on both the T 84 and high passage number Caco-2 cells when stimulated with IFN- γ , TNF- α and IL-1 β collectively termed a cytomix. Cytomix in contrast to IL-15 showed a marked decrease in TEER in both cell lines indicative of increased paracellular permeability. Subtle differences were found between both cell lines as TEER decreased much quicker in T 84 cells with a significant drop observed as early as 9 hours. Caco-2 cells required up to 24 hours before

statistically significant decreases in TEER were found. Some subtle changes were noted in some experiments with both cell lines with early separation of ZO-1 found in two of three T 84 stimulated cells and 3 of 5 stimulated Caco-2 experiments. However this effect was not found to occur in all instances unlike experiments stimulated with IL-15 and seemed less pronounced compared to IL-15 stimulated cells. Less defined JAM staining was noted in two of the T 84 experiments along with a single instance of irregular claudin-4 staining in T 84 cells incubated with cytomix. These pro-inflammatory cytokines have been shown by other groups to disrupt barrier function with alterations often reported following 30-48 hours of incubation (Bruewer, Utech et al. 2005; Wang, Graham et al. 2005; Al-Sadi and Ma 2007). Our study may have been too brief in detecting gross pathology in response to the cytomix. TEER is an early indicator of changes in permeability with alterations in tight junctions occurring at a later time point as shown by other groups (Bruewer, Luegering et al. 2003). Tight junctional proteins distinct from the ones investigated in this study may be altered during incubation with the cytomix preparation in conjunction with decreased TEER findings.

NO donor

Nitric oxide and its metabolites are increased in active coeliac disease but its precise role in disease pathology is unclear. This study investigated the direct effect of nitric oxide on tight junctional integrity. Nitric oxide has previously been found to disrupt barrier function directly and in conjunction with pro-inflammatory cytokines (Banan, Fields et al. 2001; Han, Fink et al. 2003). Nitric oxide was found to be up-regulated by IL-15 in a macrophage cell line by other groups (Liu, Zhai et al. 2004). Thus early induction of nitric oxide at the coeliac lesion in the presence of IL-15 may occur and could contribute to barrier disruption. In the T 84 cell line, nitric oxide caused a decrease in TEER at 24 hours however failed to reach significance compared to unstimulated cells. A statistically significant difference was seen in TEER in the Caco-2 cell line at 24 hours however on closer analysis this marked decrease in TEER was only found to occur in 2 of the 5 experiments performed. As seen in figure 2.14, T-84 cells showed subtle changes to nitric oxide in ZO-1 and JAM staining in two of three experiments with ZO-1 separation and areas of diffuse JAM staining. In one experiment disruption to

claudin-4 localisation was evident. In three of the Caco-2 cell experiments stimulated with nitric oxide, no significant alterations in tight junction localisation were found at 24 hours. However, in the two experiments which showed a dramatic drop in TEER, large scale separation of cells with internalisation of tight junctional proteins were noted. Whilst ZO-1 remained localised at the cell surface, clear separation of cells at contact points were seen. Occludin and claudin-4 breakdown showed intracellular staining of both proteins within the cytoplasm of the cells. Large vacuoles within the F-actin structure of the cell were seen in some areas. As alterations in TEER in Caco-2 cells were not found until 24 hours following NO stimulation, it is possible that if incubation times were extended these alterations in TEER and tight junction distribution would have been seen in the other 3 experiments. Previous findings by other groups of NO disruption of tight junctional proteins required 24 to 48 hours to show significant differences in TEER and disruption of ZO-1 in response to nitric oxide donors (Han, Fink et al. 2003). Caco-2 cells required a longer incubation time than T 84 cells for changes in TEER to reach significance in cytomix stimulated cells as seen in figure 2.11 and 2.21 respectively. As previously discussed, despite a significant fall in TEER in T 84 cells, the decrease seen in TEER in Caco-2 cells did not reach significance within the time course studied. Overall these results indicate a potential role for NO in alteration of intestinal permeability and show effects on tight junctional proteins ranging from subtle cell separation from one another as seen with T 84 cells, to gross alterations in tight junction localisation and complete separation as seen in the Caco-2 cell line. However due to low sample number and morphological changes occurring in only some of the NO treated cells, it is difficult to interpret the NO effect. It would appear NO causes subtle changes in tight junctional proteins compared to untreated cells but that this effect is not always reproducible.

Forskolin

Forskolin is an activator of adenylate cyclase with resulting increases in cAMP. It has been shown to cause a rapid decrease in TEER which may be mediated via paracellular disruption of tight junctions caused by cAMP or also by its regulation of chloride channels causing transcellular movement of ions (Le Varlet 1995; Madara 1998; Gabriel, Davenport et al. 1999). In all

experiments within this current study, forskolin induced a significant decrease in TEER as early as ten minutes following incubation with both T 84 and Caco-2 cells. Accumulation of ZO-1 at tri-cellular corners was seen in 2 of three T 84 experiments and 3 of the 5 Caco2 cell experiments in response to forskolin. Diffuse JAM staining was found in one of the forskolin stimulated T 84 cell experiments and diffuse claudin-4 was seen in response to forskolin in another T 84 stimulated cell line. However the honeycomb pattern of tight junctional staining was maintained overall in all experiments. Forskolin was incorporated into experiments as a positive control for TEER reduction and was shown cause a statistical significant difference in TEER in both T 84 and Caco-2 cell lines at all time points studied. Forskolin has been shown to disrupt transcellular passage of chloride ions via a cAMP dependent mechanism. Thus alterations in TEER may be due to transcellular disruption of ions (Gabriel, Davenport et al. 1999). This may explain the relatively intact tight junctional network in response to TEER despite a significant reduction in TEER in both cell lines.

Aggregates of ZO-1 and F-actin in Caco-2 cells

Co-localisation of F-actin and ZO-1 was noted in the Caco-2 cell line in both unstimulated cells and cells stimulated with PT ovalbumin, PT gliadin, IL-15, spermine NONOate, cytomix and forskolin. Enumeration of these co-localised “aggregates” was done by counting the immunofluorescently stained aggregates for 3 fields at 63 X magnification for each of five experiments and obtaining the average number of aggregates in response to stimulation. A marginal increase in co-localised proteins was shown in both IL-15 and NO donor stimulated Caco-2 cells as seen in figure 2.18. Co-localisation was most prominent in cytomix and forskolin stimulated cells, with noticeably large co-localisation in forskolin stimulated cells where proteins seemed to occupy a substantial area of the cell. Thus, in cells showing greatest permeability, these clusters of intracellular F-actin and ZO-1 appeared more prominent. The presence of these “aggregates” in unstimulated cells indicate this co-localisation may represent a normal physiological store of tight junctional proteins (Matsuda, Kubo et al. 2004). Tight junctional proteins have also been shown to become internalised in response to cytokines by endocytosis (Bruewer, Utech et al. 2005). ZO-1

internalisation has been shown to occur in response to re-modelling at cell-cell contacts (Gottardi, Arpin et al. 1996). While a possible endocytic uptake of ZO-1 has been shown below this is speculative as more extensive investigations would be necessary to ascertain the exact formation and role of these aggregates such as intensity profiling at cell corners. These “aggregates” may represent internal stores and may increase in response to stimulation.

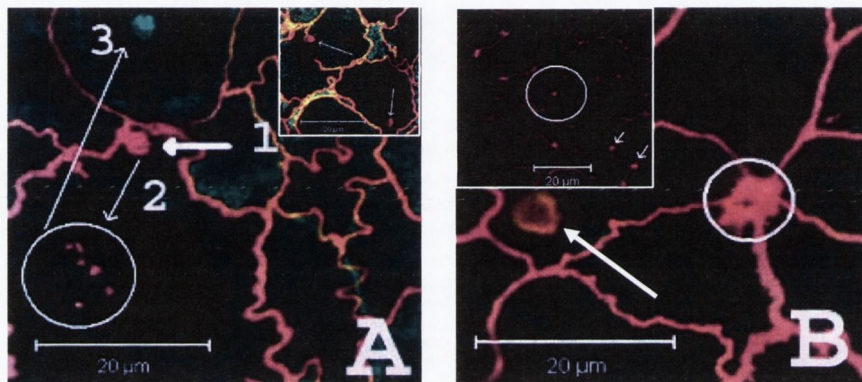


Figure 2.25 Immunofluorescent staining of aggregates in the Caco-2 cell line. Image A depicts ZO-1 which may become internalised at tri-cellular corners (1), internalisation of individual clusters of ZO-1 within the cell (2) and clustering together with F-actin to form large aggregates (3). Image B shows examples of ZO-2 build-up at tri-cellular corners along with clear co-localised ZO-1 and F-actin (yellow aggregate). Images are representative of both unstimulated cells and cells stimulated with either PT Ovalbumin, PT gliadin, IL-15, Spermine NONOate, cytomix and forskolin. Scale bar = 20μm.

Summary

In summary this study demonstrates subtle morphological changes in tight junction proteins in response to IL-15 and nitric oxide which could potentially enable entry of gliadin into the underlying mucosa and thus triggering adaptive immune responses. As has been reported in the literature, cytokines of the adaptive response do play a major role in tight junction dysfunction. However, IL-15 produced upon initial exposure to gliadin in the absence of an adaptive immune response may provide an attractive mechanism for altering intestinal integrity. As no specific role has been shown for nitric oxide during disease, this study provides an interesting potential pathological role in breachment of the intestinal barrier. Subtle alterations in tight junction organisation as has been shown in this study, by partial ZO-1 separation of cells in response to IL-15 and nitric oxide in localised regions of cell monolayers, may be sufficient to allow gliadin peptides into the underlying mucosa as a triggering event. Subsequent deamidation of gliadin and activation of the adaptive immune response would provide an extensive pro-inflammatory array of cytokines like IFN- γ and TNF- α which would further act on the epithelium and produce a cyclic effect of gliadin entry and immune activation. If IL-15 is the triggering event in gliadin entry, anti IL-15 therapy would provide an attractive preventative therapy.

CHAPTER 3

**QUANTIFICATION OF GENE EXPRESSION AND ENZYME
ACTIVITY OF THE iNOS ENZYME and THE COX-2
ENZYME AND iNOS PROTEIN PRODUCTION USING AN
ENTEROCYTE CELL LINE IN RESPONSE TO GLIADIN, TO
THE INNATE CYTOKINE IL-15 AND TO THE CYTOKINE
IFN- γ**

3.1 Introduction

3.1.1 Inducible nitric oxide synthase and cyclooxygenase 2

Coeliac disease is associated with multi-cellular interactions of both the innate and adaptive branch of the immune response. Focus on innate participation has highlighted both monocytic and enterocytic lineages as important sources of inflammatory stimuli in coeliac disease. Two enzymes iNOS and COX-2, shown to be increased in coeliac disease have been proposed to derive from these innate cells using a number of *in vitro* studies (Tuckova, Flegelova et al. 2000; Maiuri, Ciacci et al. 2003; Maiuri, De Stefano et al. 2003; Daniels, Cavill et al. 2005).

iNOS

iNOS is an enzyme involved in the production of nitric oxide from the oxidation of the amino acid L-arginine (Marletta, Hurshman et al. 1998). Its expression is dependent upon induction by inflammatory cytokines such as IFN- γ , TNF- α , IL-1 β and endotoxins such as lipopolysaccharides (Lirk, Hoffmann et al. 2002). Nitric oxide is a highly labile gas and becomes rapidly converted primarily to the end products nitrite and nitrate which are commonly used as markers of iNOS enzyme activity (Ignarro, Fukuto et al. 1993; Veszelszky, Holford et al. 1995; ter Steege, Koster-Kamphuis et al. 1998). Both increased serum and urine levels of nitrate and nitrite have been found in patients with active coeliac disease along with increased iNOS activity and protein expression in the small intestine (ter Steege, Buurman et al. 1997; Laurin, Falth-Magnusson et al. 2003; Murray, Bullimore et al. 2003; Spencer, Daniels et al. 2004; Daniels, Cavill et al. 2005). Whilst a number of studies propose monocytes as the main source of iNOS activity, other studies clearly demonstrate iNOS in the enterocytic compartment with both elevated levels of iNOS mRNA, protein and activity within these cells (Murray, Daniels et al. 2002; Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003; Daniels, Cavill et al. 2005; De Stefano, Maiuri et al. 2006).

Mechanisms of iNOS induction in coeliac disease remain unclear. Some groups have demonstrated a synergistic effect of PT gliadin with IFN- γ on iNOS in macrophages though a direct role of PT gliadin in the absence of

IFN- γ has not been seen (Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003). Recent evidence supports a role of IL-15 in iNOS regulation using distinct cell model types such as macrophages, gingival epithelial cells and tumour cells (Yanagita, Shimabukuro et al. 2002; Figueras, Busquets et al. 2004; Liu, Zhai et al. 2004). Despite these findings no study investigating the effect of PT gliadin or IL-15 on enterocytes in relation to coeliac disease has been reported.

COX-2

Cyclooxygenase 2 (COX-2) is a membrane bound glycoprotein involved in the conversion of the polyunsaturated fatty acid of the cell membrane, arachidonic acid to the inflammatory mediators prostaglandins. COX-2 catalyses the oxidation of arachidonic acid to form prostaglandin G₂ which it subsequently reduces to the unstable intermediate prostaglandin H₂. Through the action of other synthase enzymes this prostaglandin H₂ is converted to more stable prostanoids such as prostaglandin E₂, prostaglandin I₂ and thromboxane A₂ (Park, Pillinger et al. 2006). COX-2 is not constitutively expressed and is induced by a number of inflammatory stimuli including cytokines such as IL-1 β , TNF- α , IL-6, growth factors such as platelet derived growth factor and transforming growth factor- β and endotoxins such as lipopolysaccharide (Dubois, Abramson et al. 1998). COX-2 is predominantly expressed in cells involved in inflammatory reactions including macrophages along with fibroblasts, synoviocytes, endothelial cells, T cells and intestinal epithelial cells (Roy, Polgar et al. 1996; Barrios-Rodiles and Chadee 1998; Houchen and Stenson 1999; Min, Hwang et al. 2004; Yang, Zhang et al. 2007). COX-2 has been shown to be expressed at low levels in the intestinal epithelium but increase during active inflammation (Houchen and Stenson 1999).

In coeliac disease prostaglandin levels have been shown to be significantly higher in active disease patients compared to control groups (Branski, Karmeli et al. 1984; Lavo, Knutson et al. 1990; Friis, Anthonsen et al. 1994). *In vitro* studies have shown a distinct role of the innate peptide p31-43 in COX-2 mRNA induction and this is mediated by the cytokine IL-15. Recently IL-15 induction of COX-2 has been shown in rheumatoid

synoviocytes but no extensive studies on IL-15 and COX-2 regulation in coeliac disease have been published to date (Min, Hwang et al. 2004). While studies show COX-2 activity in mononuclear and T cell populations, no extensive studies have been carried out in relation to enterocytes and COX-2 induction in coeliac disease (Kainulainen, Rantala et al. 2002; Maiuri, Ciacchi et al. 2003).

3.1.2 Principals of iNOS activity assays

Spectrophotometric method

This method is based on the measurement of the stable oxidation product of nitric oxide, nitrite. The determination of nitrite by the colorimetric griess reaction is based on a two step diazotization reaction. Under the acidic reaction conditions, nitrite produces a nitrosating agent which reacts with sulphanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form a chromophoric azo derivative which can be detected spectrophotometrically at a wavelength of 550nm. This assay can be used to measure nitrite concentrations as low as 0.5uM (Nagano 1999; Sun 2003).

Fluorimetric method

In order to increase the sensitivity of nitrite determination under physiological conditions, a fluorometric method has been developed that exploits the ability of nitric oxide to produce N-nitrosating agents. The method employs the use of the aromatic diamino compound 2, 3-diaminonaphthalene. The relatively non fluorescent 2, 3-diaminonaphthalene reacts rapidly with the N-nitrosating nitrite to yield the highly fluorescent product 2, 3-naphthotriazole with an excitation and emission spectrum of 360nm and 430nm respectively. The sensitivity of this assay is much superior to the colorimetric method with detection limits of 0.01uM(Nagano 1999).

Reduction of nitrate to nitrite

Although nitrite is the main stable nitrogen oxide formed following decomposition in an aqueous solution *in vitro*, nitrate is found in extracellular fluids such as plasma or urine via its oxidation by certain oxy-hemoproteins such as oxy-hemoglobin or oxy-myoglobin. Due to a lack in detection methods equivalent in sensitivity and specificity as nitrite detection, nitrate is firstly reduced to nitrite via reaction with metallic cadmium or enzymatically via nitrate reductase and total nitrite can then be determined via methods such as the colorimetric or fluorometric as described (Miles 1995; Casey and Hilderman 2000).

3.1.3 Principals of Real Time PCR

In real time PCR, each PCR reaction reaches a plateau phase in amplification of each target gene in which the amount of end product does not change due to the consumption of primers and nucleotides. Therefore reactions with differing initial target concentrations are not distinguished by end product accumulation but by the number of cycles of amplification after which the exponential phase begins. This is termed the threshold cycle value or more commonly Ct. A fluorescent probe is attached to the target primer sequence for each gene of interest. During the real time PCR reaction, the taq polymerase enzyme used to generate the amplified PCR products uses its 5'-3' exonuclease activity to destroy the probe, during each amplification step, which emits fluorescence. The amount of fluorescence released is proportional to the amount of amplified target DNA present. The Ct value for each gene is determined from the curve plotted with cycles of amplification against fluorescence emitted. The threshold cycle value is defined as the point at which the fluorescence rises above the background fluorescence during the exponential phase of PCR amplification as can be seen in figure 3.1. Ct values characterise reactions in the early phase of the exponential growth in which low Ct values indicate a high initial target amount and low values are indicative of the opposite (Pfaffl 2001; Whelan, Russell et al. 2003; Bubner and Baldwin 2004). Thus samples of unknown concentration may be determined using Ct values obtained during their amplification by

real time PCR as described in figure 3.1. Quantification can occur via an absolute or relative method.

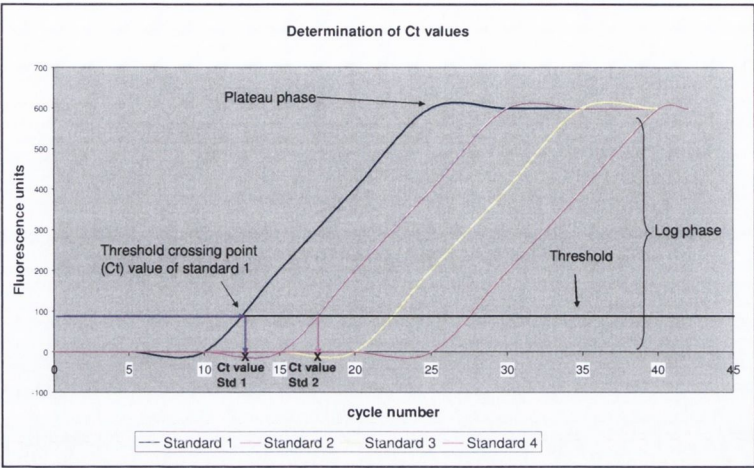


Figure 3.1: Determination of Ct values from real time PCR

Absolute Quantification

This method of mRNA quantification involves the preparation of a standard curve with an amplified sequence of known size and concentration. From this information the copy number per μl of the standard can be determined and a ten fold dilution series prepared. Standards are amplified using real time PCR and Ct values obtained for each known standard concentration. A standard curve is constructed with Ct values against the log concentration of standards. Corresponding Ct values for samples of unknown concentration may be used to determine their copy number per micro-litre using the standard curve (Lee, Kim et al. 2006). Fold changes between treated and untreated sample may be determined by calculating the ratio of treated copy numbers with untreated copy numbers.

Relative Quantification

In each real time PCR reaction, specific errors will be introduced due to minor differences in starting amount of RNA or differences in efficiency of cDNA synthesis and PCR amplification. These experimental variations may be corrected for by using a relative means of quantification. This is performed by normalisation of target DNA to a housekeeping gene whose expression should remain constant during the experimental conditions. This is termed the reference gene. It is critical in all methods of relative

quantification that the reference gene does not change its expression levels under differing experimental conditions. (Overbergh, Giulietti et al. 2003).

There are a number of different methods of relative quantification. One method involves the use of standard curves. If the amplification efficiency of both the target and reference gene is identical, one standard curve can be generated for both genes. However, if the efficiency is not identical standard curves are generated as in absolute quantification for the target gene and separately for the reference gene with a dilution series of target and reference genes of known concentrations. From the obtained Ct values, a separate standard curve can be plotted for both the target and reference gene and sample concentrations in copy numbers can be determined from their Ct values. The expression in reference gene should not change under different experimental conditions. Fold changes between stimulated and un-stimulated samples are calculated as follows. The ratio of the copy number of stimulated samples for the target gene and the reference gene are determined by dividing stimulated target gene copy number with the reference gene copy number to get a normalised target gene value. This is also done for unstimulated samples also and fold changes between stimulated and unstimulated obtained by dividing the normalised stimulated target gene samples with the normalised un-stimulated target gene samples. (Overbergh, Giulietti et al. 2003; Qiagen 2004).

Alternatively when amplification efficiency is identical for the target and reference gene, a comparative $\Delta\Delta Ct$ (delta delta Ct) method can be employed using the formula:

$$2^{-\Delta\Delta Ct}$$

where $\Delta\Delta Ct$ is equal to (Ct stimulated target-Ct stimulated reference)-(Ct un-stimulated target -Ct unstimulated reference). This gives the normalised fold difference in stimulated target gene expression compared to un-stimulated target gene expression levels. For this method to be successful amplification efficiencies must be identical between target and reference genes (Lehmann and Kreipe 2001; Qiagen 2004).

Another method of relative quantification based on a mathematical model for data analysis may also be used. The relative expression ratio of a target gene is calculated based on the efficiency and crossing point deviations of an unknown sample versus a control and expressed in comparison to a reference gene. The following formula is used to determine the fold changes in stimulated versus unstimulated samples which are normalised by using a reference gene.

$$\text{Ratio} = \frac{(\text{E target gene})^{\Delta\text{Ct (unstimulated-stimulated)}}}{(\text{E reference gene})^{\Delta\text{Ct (unstimulated-stimulated)}}$$

where E is the efficiency of the target and reference gene as determined from the formula $E = 10^{(-1/S)} - 1$, where S is the slope of the line obtained from a standard curve constructed for the target and reference gene.

This mathematical analysis is more accurate than the comparative method when differences in amplification efficiencies of target and reference genes occur (Pfaffl 2001).

The aim of this chapter

The aim of the chapter was to determine changes in expression and activity of iNOS and COX-2 in the Caco-2 cell line in response to stimulation with PT gliadin, IL-15 and IFN- γ .

- The gene expression of iNOS and COX-2 in response to stimulation was determined by real time PCR and data analysed using absolute quantification and using the three methods of relative quantification as described.
- iNOS activity was assessed by the measurement of the stable end-product nitrite using a colorimetric and fluorimetric method.
- COX-2 activity was assessed by the measurement of prostaglandin E₂ using an enzyme linked immunosorbent assay.
- iNOS protein levels in response to stimulation was assessed by flow cytometry.

3.2 Materials and Methods

3.2.1 Cell Culture

The colon adeno-carcinoma cell line, Caco-2 was used between passages 50-60. Cells were cultured in DMEM (Gibco BRL, UK) supplemented with 10% FCS (Sigma, USA), 2mM L-glutamine, 100 U penicillin and 0.1mg streptomycin (Gibco BRL, UK) and 0.250ug/ml amphotericin B (Gibco BRL, UK). Cells were maintained in T-25cm² flasks at 37°C, 5% CO₂. Cells were washed free of FCS containing medium using PBS and removed from flasks with a solution of trypsin/EDTA (Gibco BRL, UK) by incubating at 37 °C for 10mins. Trypsin was inactivated by FCS containing DMEM and cells spun at 1,200rpm to wash away any remaining trypsin. Cell pellets were resuspended in DMEM and placed in fresh T-25cm² flasks. Cells were split in a ratio of 1:5 prior to reaching confluency.

3.2.2 Culturing cells on insert plates

Caco-2 cells were cultured in T-75cm² flasks and confluency assessed using the inverted microscope. Upon reaching 90% confluency, cells were trypsinised from the flask, resuspended in 3mls of medium and a cell count performed with ethidium bromide/ acridine orange. A cell count was performed by diluting cells 1:25 with EBAO and visualised under UV light using a Neubauer counter. A cell count was performed in all 9 squares of the counter and from this the average cell number per square calculated. This represents the number of cells per 0.1µl. The total number of cells in 1ml was determined taking into account the dilution factor of cells in EBAO. Cell number was expressed per ml. Cells were diluted with medium to obtain a final cell number of 5×10^5 cells/ml. Cells were grown on polyethylene terephthalate permeable supports (BD Biosciences, UK) with a pore size of 0.4µm to enable differentiation of cells.

900µls of DMEM was added per well of a 24 well plate. 400µls of the resuspended cell pellet (at a concentration of 5×10^5 cells/ml) was added into each of 24 inserts and the plate was returned to a 37°C incubator. Cells were grown for 21 days to enable full differentiation.

3.2.3 Electrophysiology

Cell differentiation was assessed by measuring resistance values across the cell monolayer until cells became polarised and resistance measurements stabilised. Resistance was measured every second day-from day 3 of seeding using an Endohm 6 measurement chamber in conjunction with an EVOM-X epithelial Voltohmmeter (WPI Inc, UK) as described in section 2.2.5. Resistance readings were recorded and medium replaced in each well and within each insert.

3.2.4 Preparation of iNOS, COX-2 and 18s rRNA standards

3.2.4.1 Isolation of mRNA

Caco-2 cells were cultured on 24 well inserts for 21 days and removed by trypsinisation as previously described. Cells were resuspended in DMEM and a cell count performed using ethidium bromide/ acridine orange. RNA was extracted from cells using the NucleoSpin RNA II kit (Machery Nagel, Germany). Cells were spun at 1200rpm and DMEM removed. Lysis buffer was prepared by adding 3.5ul of β mercaptoethanol to 350ul of RA1 buffer. This was added to the cell pellet to lyse the cells. This was left overnight at -20°C to facilitate lysis. The cell lysate was filtered through NucleoSpin filter units to reduce viscosity and clear the lysate. The lysate was applied to the filters and placed in collecting tubes. Filters were spun for 1 min at 11,000g. Filter units were then discarded and 350ul of a 70% ethanol solution was added to the lysate to optimise RNA binding conditions and then vortexed. Lysate was then loaded onto a NucleoSpin RNA II column placed in a collecting tube and allowed to incubate for 1 min before spinning for 30sec at 8,000g. RNA was now bound to the column. Each column was then placed into a fresh collecting tube. 350ul of membrane desalting buffer was added to each column and centrifuged at 11,000g for 1 min to dry the membrane. DNase reaction mixture was prepared in a sterile microcentrifuge tube. For each isolation 10ul of reconstituted DNase was added to 90ul of DNase reaction buffer and mixed. 95ul of this DNase reaction mixture was added directly onto the membrane of each column and incubated for 15mins at room temperature. 200ul of RA2 buffer was added to each column to

inactivate the DNase and to wash the silica membrane. Columns were centrifuged for 30sec at 8,000 x g and columns placed in fresh collecting tubes. 600ul of RA3 buffer was added to each column to wash membranes and spun at 8,000 x g for 30secs. 250ul of RA3 was added to each column for a final wash step and spun at 11,000 x g for 2mins to dry the membrane. Highly pure RNA was eluted with 60ul of RNase free H₂O by spinning at 11,000 x g for 1min and RNA was stored at -20°C.

3.2.4.2 Quantification of mRNA standards and assessment of RNA purity

RNA purity and quantity was assessed using a nanodrop spectrophotometer ND-1000 (NanoDrop Technologies, USA). Once the spectrophotometer was blanked with distilled water, 1ul of total RNA was placed on the spectrophotometer stage and total quantity of RNA assessed at 260nm. Protein contamination was determined by calculating the 260:280nm ratio.

A 1% agarose gel (0.5g agarose in 50mls 1X TBE buffer containing 5ul of ethidium bromide) was prepared to assess RNA quality. 5ul of RNA was loaded per well with bromophenol blue loading dye (0.1% bromophenol blue in 50% glycerol solution). Gels were run at 120V for 1hr and visualised under UV light using a UV transilluminator. RNA was stored at -80°C for further analysis.

3.2.4.3 Reverse transcription of standards

10ul of total RNA was heated to 85°C for 2mins to denature and placed on ice. A reaction mix was prepared containing a final concentration of 5mM MgCl₂, reverse transcriptase 1X avian myeloblastosis virus reaction buffer (50mM Tris HCL pH8.3, 50mM KCl, 10mM MgCl₂, 10mM DTT, 0.5mM spermidine), 10mM of each deoxy nucleotide triphosphate, 1U/ul recombinant RNasin (ribonuclease inhibitor), 5ng/ul random hexamers, 1U/ul avian myeloblastosis virus reverse transcriptase (Promega, USA). 0.5ug of denatured total RNA was added to the reaction mix which was made up to a final volume of 25ul using DNase and RNase free H₂O (Sigma, USA). mRNA was reverse transcribed in a PTC-100 thermocycler for 1hr at 42°C. The resulting cDNA was stored at -80°C.

3.2.4.4 Polymerase Chain Reaction of iNOS, COX-2 and 18s rRNA standards

For the preparation of iNOS, COX-2 and 18s rRNA standards, cDNA was amplified by conventional PCR using specific primer pairs (Assays on Demand™, Applied Biosystems). These primer/probe pairs consist of a 20X mix of unlabeled PCR primers and TaqMan MGB probe (FAM dye labelled) for each gene. Briefly a reaction mix was prepared containing a final concentration of 1X SYBR Green PCR master mix (10mM Tris-HCl, pH8.3, 50mM KCl, 3.5mM MgCl₂, 0.2mM of each dNTP (dATP, dCTP, dGTP, dTTP), stabilisers, 0.025 U/ul Taq DNA Polymerase, Jumpstart Taq antibody and Sybr Green I (Sigma, USA), 1X Assays on demand primer/probe pairs for each gene of interest and distilled H₂O. 5uls of cDNA was added to separate reaction mixes for each of the three genes. The PCR was carried out in a thermocycler using the following conditions:

Temp.	Time	No. of cycles	Mechanism of Action
95°C	5mins	1	Activation of Taq DNA Polymerase
95°C	15secs	35	Denaturing of cDNA
60°C	1min		Annealing of primer pairs
72°C	1min		Extension
72°C	5mins	1	Completion of reaction
4°C	indefinite		Stable short term storage

Table 3.1: PCR thermal cycling parameters used to prepare iNOS, COX-2 and 18s rRNA standards

3.2.4.5 Gel extraction and purification of PCR products for the preparation of iNOS, COX-2 and 18s rRNA standards

PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen Ltd, UK). Products were run on a 1% agarose gel containing ethidium bromide in 1X TAE. PCR products were visualised on a UV transilluminator and PCR bands excised from the gel using a scalpel blade. PCR bands for

each gene were placed into individual 1.5ml eppendorffs. Each PCR band was weighed in pre-weighed 1.5ml eppendorffs and 3 volumes of Buffer QG (containing guanidine thiocyanate) was added to one volume of gel (300ul of buffer added to 100mg of PCR containing gel slice).

Gel fragments were incubated at 50°C vortexing occasionally for 10mins until the gel slice completely dissolved. After the gel had dissolved the colour of the solution was assessed to determine pH. In tubes where the solution was not yellow i.e. orange or violet, 10ul of 3M sodium acetate pH 5 was added and mixed to optimise DNA binding to the QIAquick membranes. 100ul of isopropanol (equivalent to 1 gel volume) was added to each solution and placed into a QIAquick spin column with a collection tube. Columns were centrifuged for 1min at 13,000 rpm. Flow-through was discarded and 0.75ml of buffer PE was added to wash the column by spinning at 13,000 rpm for 1 min. Flow-through was discarded and column spun again for 1 min at 13,000 rpm to remove any residual ethanol from the buffer PE. The QIAquick column was placed into a clean 1.5ml microcentrifuge tube. To elute DNA, 50ul of buffer EB (10mM Tris-Cl, pH 8.5) was added to each column and incubated for 1 min. Columns were spun at 13,000 rpm for 1 min and purified DNA stored at -20°C.

3.2.4.6 Quantification of PCR products

PCR products were quantified using a Fluorescent DNA Quantitation Kit (Bio Rad, USA). A standard curve was generated using the standard calf thymus DNA provided. Standards were prepared at concentrations ranging between 5ng to 500ng (100ng to 2,500ng/ml) using the TEN assay buffer and sterile H₂O. A 2ug/ml solution of the fluorochrome Hoechst 33258 dye was prepared. 10ul of each DNA standard was added (in triplicate) to a 96 well microplate along with 10ul of the PCR amplified iNOS/COX-2/18s rRNA standards (in duplicate). 200ul of Hoechst dye was added to each well. A blank containing just dye was also included. The plate was read using a TECAN fluorimeter at an excitation wavelength of 360nm and emission of 460nm. The concentration of each PCR standard was determined from a calibration curve by plotting the DNA concentrations of the DNA standards to the relative fluorescent units obtained.

3.2.4.7 Determination of the base pair length of each standard

The length of each PCR product was determined by gel electrophoresis. A 2% agarose gel (1g agarose in 50mls TAE, 5µls ethidium bromide) was prepared. 5-10µls of PCR product for each gene was added along with 5µls bromophenol blue loading dye to the gel, along with a 1/10 diluted 50bp ladder. The gel was visualised using a UV transilluminator and PCR product size determined with reference to the 50bp ladder.

3.2.4.8 Determination of the copy number of each PCR amplified standard

The copy number for each gene that had been amplified and purified was calculated. Using the values obtained from the determination of PCR concentration (section 3.2.4.6) and PCR base pair length (section 3.2.4.7), the copy number of each PCR product was determined using the following formula:

$$\text{Copy number}/\mu\text{l} = \frac{\text{Avagadro's Number } (6.02 \times 10^{23}) \times \text{PCR product concentration (grams/ml)}}{\text{DNA length (base pairs)} \times 660 \text{ (grams/mole/ base pair)} \times 10^3}$$

Figure 3.2: Formula for calculating the copy number/ul of each PCR standard (Johnston, Zhang et al. 2005; Lee, Kim et al. 2006).

3.2.4.9 Preperation of standard curves for the iNOS, COX-2 and 18s rRNA standards using real time PCR

A standard curve was prepared for each gene by doing a 10 fold dilution series on each PCR product using the copy no/ul. Standard curves were generated within the range of 1×10^0 to 1×10^7 copy numbers per ul. Standards were run on an ABI TaqMan system (Applied Biosystems, UK) to generate standard curves.

For each gene a Taqman reaction mix was prepared containing 1X primer probe, 1X Taqman Universal PCR master mix and water. 3uls of each standard (in triplicate) within the range of 1X 10⁰ to 1X 10⁷ copy numbers per ul. were added to a 96 well reaction plate To this, Taqman reaction mix was added to a final volume of 20uls.

The plate was sealed with an optical adhesive cover and was analysed in an ABI Taqman 7000 using the following parameters:

<u>Temp.</u>	<u>Time</u>	<u>No. of cycles</u>	
50°C	2mins	1	Decontamination of carry over PCR via UNG
95°C	10mins	1	Activation of Taq Polymerase
95°C	15 secs	35-40	Denaturation
60°C	1 min		Annealing/ Extension

Table 3.2: Taqman thermocyler conditions for real time PCR

Standard curves were plotted with threshold values (Ct) against the log of the known copy number for each standard. Ct values were obtained for each standard concentration in triplicate. The quality of the standard curve was assessed on the basis of the tightness of the replicates and the amplification efficiency. An amplification efficiency of 1 represented 100% efficiency and was determined for each gene using the following equation:

Efficiency of Amplification = 10^{-(1/slope)} - 1

Figure 3.3: Equation for the determination of amplification efficiency (Lee, Kim et al. 2006).

3.2.5 Stimulation of cells to determine the effects of PT gliadin, IL-15 and IFN- γ on iNOS and COX-2 gene expression

Caco-2 cells were grown on insert plates for 21 days (as previously described in section 2.2.6) after which cells were stimulated at various time points with 500ug/ml PT gliadin, 200ng/ml IL-15 (R and D Systems, UK) and 1000U/ml of IFN γ (Roche Diagnostics, Switzerland). Cells were also left unstimulated to assess basal gene expression. Phenol red free DMEM was used, supplemented with 10% FCS (Sigma, USA), 2mM L-glutamine, 100 U penicillin and 0.1mg streptomycin (Gibco BRL, UK), 1mM sodium pyruvate (Sigma, USA). Following stimulation, supernatants were stored at -20°C for further analysis. Cells were removed by trypsinisation as previously described in section 3.2.1.

3.2.5.1 Isolation, quantification and reverse transcription of sample mRNA

Total RNA was extracted from the stimulated cells using the Nucleospin RNA II kit and quantified as previously described in sections 3.2.4.1 and 3.2.4.2. Reverse transcription was carried out as described in section 3.2.4.3.

3.2.5.2 Preparation of samples for quantification

cDNA samples from stimulated cells were quantified using the fluorescent DNA Quantification kit as previously described in section 3.2.4.6. Samples were diluted with sterile H₂O to get a final concentration of 500pgms per Taqman reaction. A Taqman reaction mix was prepared as previously described in section 3.2.4.9. Each gene was quantified on a separate microplate. 3uls of the 10 fold diluted standards for each gene was added to each plate in triplicate along with 3uls of diluted sample cDNA (from stimulated and unstimulated cells) in duplicate (500pgms). Taqman reaction mix was added to a final volume of 20uls. Quantification was assessed using the ABI 7000 analyser. Sample quantity was analysed using two methods.

3.2.5.3 Analysis via Absolute Quantification

Samples were quantified using an absolute quantification method. Using standard curves generated for each gene the absolute copy number was determined for each gene in response to stimulation. A standard curve for each gene was plotted with a dilution series of each standards copy number against obtained Ct values by real time PCR. Ct values for each sample were used to determine corresponding sample copy numbers from curve. Fold changes between control and stimulated cells were determined to assess changes in iNOS and COX-2 expression upon stimulation.

3.2.5.4 Analysis via Relative Quantification

Samples were also quantified by three relative methods. Gene expression levels were calculated by determining the ratio between the amount of a target gene and an endogenous reference gene to give a normalised value for each sample. The 18s rRNA gene was chosen as the endogenous reference gene. The reference gene is normally a housekeeper or maintenance gene whose expression remains constant under a wide variety of physiological conditions and must not vary under different experimental conditions.

For the standard curve method of relative quantification a standard curve was prepared for the 18s rRNA gene by plotting Ct values obtained by real time PCR against known copy numbers per ul. From the 18s rRNA Ct values obtained for each sample, the corresponding copy number per ul was assessed. iNOS and COX-2 copy numbers were divided by the 18s rRNA copy numbers to normalise iNOS and COX-2 values.

The efficiency of amplification was obtained from the standard curve generated as described in figure 3.3 and was used in determination of gene expression using the second method of relative quantification as described in section 3.1.3 and described by Pfaffl using a mathematical model.

The third method of relative quantification employed used the Ct values directly to calculate gene expression using the delta delta Ct method.

Using all three relative methods, a ratio of the target gene (iNOS, COX-2) and the reference gene was obtained to produce a normalised result for each target gene. Fold changes between control and stimulated cells were determined to assess changes in iNOS and COX-2 expression upon stimulation. Fold changes between normalised stimulated and control cells were then determined.

3.2.6 Determination of iNOS activity

3.2.6.1 Spectrophotometric method

The concentrations of nitrite in cell supernatants were assessed using a commercial Griess reagent (Sigma) reconstituted in H₂O. Nitrite levels in cell supernatants were determined using this griess reagent as follows. A standard curve of sodium nitrite was prepared from 1uM to 30uM performed in triplicate. A 1:1 ratio of griess reagent and standard/sample solutions were placed in a 96 well plate and incubated for 15 mins at room temperature. Absorbance readings were obtained using a spectrometer at 550nm wavelength. Sample nitrite was determined from the standard curve generated. Nitrite concentrations within each sample were calculated with reference to the standard curve.

3.2.6.2 Fluorometric method

A fluorescent method of iNOS activity was also employed, as published detection limits were reported to be lower than that detected with the absorbance method. A Nitrite Fluorimetric assay kit (Cayman Chemicals, USA) was used to detect nitrite in each sample. For nitrite determination, a standard curve was prepared with final nitrite concentrations between 0.06uM to 3.5uM. Standards were prepared with a mixture of buffer and cell culture medium so that the amount of medium in standard and sample wells remained constant. 100uls of each standard was added to a 96 well microtitre plate in duplicate. 20uls of sample made up to a final volume of 100uls with assay buffer, were also added to the plate in pre-defined wells. Blank control wells containing just buffer were also included. 10uls of DAN was added per well, the plate covered and incubated for 10mins at room temperature. The

reaction was stopped by the addition of 20ul NaOH and the plate read on a TECAN fluorimeter using an excitation wavelength of 360nm and an emission wavelength of 430nm. Nitrite concentrations in samples could be extrapolated from the nitrite standard curve.

3.2.7 Prostaglandin ELISA

For the determination of COX-2 activity, a PGE₂ Parameter ELISA for prostaglandin system was used (R and D Systems, UK). A PGE₂ high sensitivity standard curve was prepared in the range of 19.6pg/ml to 1250pg/ml in calibrator diluent containing medium to normalise the quantity of medium in standard and sample wells. All samples were diluted 1:2 with calibrator diluent. A microplate with pre-coated goat anti-mouse polyclonal antibody was used. 200ul of calibrator diluent was included in the experiment as a non-specific binding control (NSB). 150ul of calibrator diluent was added to the zero standard wells. 150ul of each standard and diluted sample in duplicate were added. 50ul of the primary antibody solution (mouse monoclonal anti human PGE₂) was added to each well except the NSB well. 50ul of the PGE₂ conjugate (HRP conjugated) was added to each well and covered with an adhesive strip and the plate was incubated overnight at 4°C. Following the overnight incubation, each well was aspirated and washed 4 times with 400ul of wash buffer using a plate washer. After the final wash step, the plate was inverted and blotted dry. 200ul of substrate solution (containing tetramethylbenzidine chromagen and hydrogen peroxide) was added to each well and incubated for 30mins at room temperature, protected from light. 50ul of stop solution (2N sulphuric acid) was added to each well and the optical density determined using a spectrophotometer at a wavelength of 450nm. Results were calculated by averaging the duplicates of each standard and sample and subtracting the NSB from each value. A standard curve was plotted with the mean absorbance for each standard on a linear y axis against the concentration on a logarithmic x axis from which the concentration of PGE₂ in each sample was extrapolated.

3.2.8 Determination of iNOS protein

iNOS protein expression was examined using flow cytometry. Cells were stimulated as described in 3.2.5 for 48 hours and removed from inserts via trypsinisation. The cells were resuspended in DMEM and were then spun at 1200rpm for 5mins and supernatant removed. The cells were fixed with 2% paraformaldehyde for 5mins and then washed with PBS containing 5% FCS, 1mM EDTA and 0.05% saponin to permeabilise cell membranes. The cells were then spun at 1200 rpm for 5mins. A blocking step was then carried out for 10mins to prevent non specific binding of antibody with 5% normal rabbit serum prepared in the PBS containing FCS, EDTA and saponin.

Cells were washed and stained with one of the following unlabelled antibodies at a 1 in 20 dilution. Antibodies were prepared in the intracellular buffer.

- Mouse anti-human IgG_{2A} isotype control (Dako, Denmark)
- Mouse anti-human iNOS (BD PharMingen, USA)
- Mouse anti-human HLA ABC (Dako, Denmark)

Cells stained for intracellular HLA-ABC served as a positive control as Caco-2 cells have previously been shown to express HLA-ABC which increases significantly following IFN- γ stimulation (Siavoshian, Blottiere et al. 1996; Imanishi, Kamigaki et al. 2006).

The cells were incubated for 15mins at room temperature and then washed with PBS containing FCS, EDTA and saponin. The cells were stained with a 1 in 100 dilution of rabbit anti-mouse FITC conjugated secondary antibody for 15mins protected from the light. Cells were then washed and resuspended in 1X cell fix and analysed on a FACS Cell Sort (BD Biosciences, UK). The cells were gated based on their forward (FSC) and granularity (SSC) scatter plot. As cells were found to be quite large, settings were adjusted accordingly. Cells were gated and analysed for FITC expression (FL1 channel) using a SSC: FL1 scatter plot and the results were extrapolated in the form of a histogram.

3.2.9 Statistical analysis

A two way ANOVA was performed for analysis of gene and protein expression and activity in response to stimulation. A BonFerroni *post hoc* test was subsequently performed and statistical significance was reported when p values were less than 0.05

3.3 Results

3.3.1 iNOS and COX-2 gene expression following stimulation using both absolute and relative methods of gene quantification

3.3.1.1 Determination of iNOS, COX-2 and 18s rRNA standard copy number

mRNA was isolated from Caco-2 cells, quantified and reverse transcribed as described in Materials and Methods section for each gene. cDNA products were subsequently amplified by PCR using iNOS, COX-2 and the housekeeper gene 18s rRNA specific primers. Purified PCR standards for each gene were quantified using the Fluorescent DNA Quantification Kit (Bio-Rad, UK). Using the generated absorbance values for the thymus DNA standards provided in the kit, a calibration curve was created as seen in figure 3.4 from which the standard concentration of the iNOS, COX-2 and 18s rRNA PCR standards could be determined. The size of each PCR standard was determined by electrophoresis as can be seen in figure 3.5. Using the concentration and base pair length of each PCR standard, the copy number/ μ l for each gene was determined using the formula described in section 3.2.4.8. A summary of the PCR concentrations, base pair lengths and corresponding copy numbers for each gene can be seen in table 3.3.

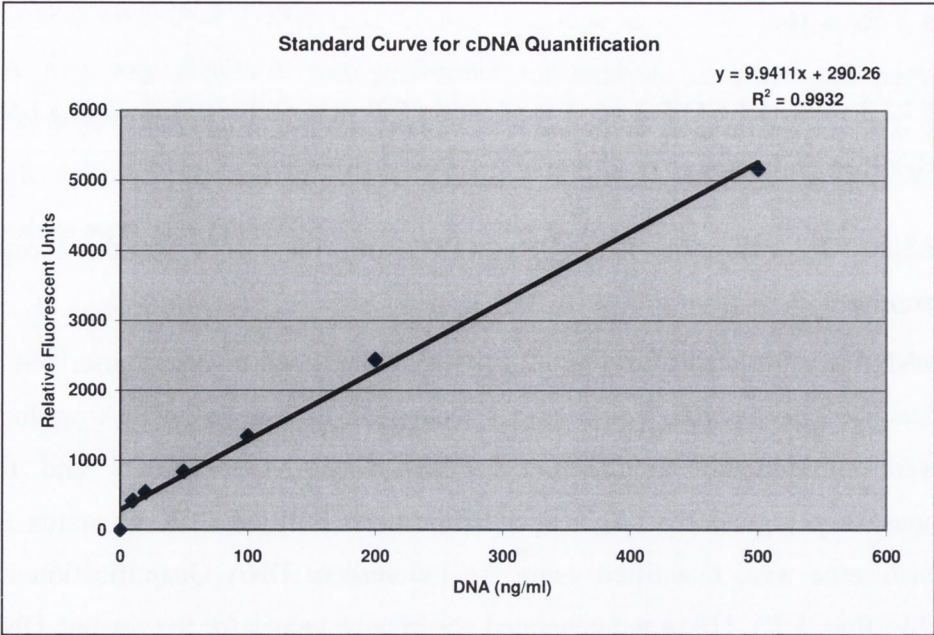


Figure 3.4: Standard curve for the quantification of iNOS, COX-2 and 18s rRNA standards.

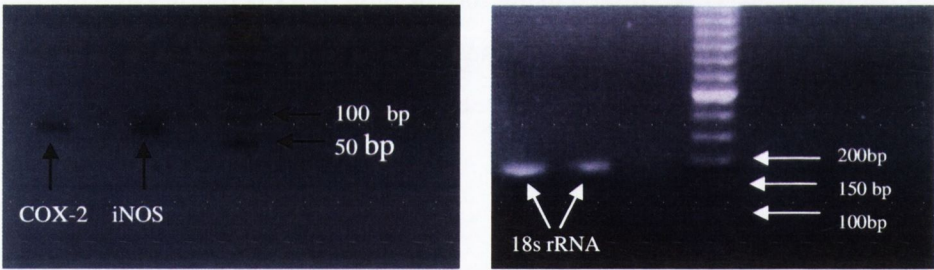


Figure 3.5: Electrophoresis of iNOS, COX-2 and 18s rRNA PCR products to determine base pair size

Sample Name	Average OD	Calculated conc. from graph (ng/ml)	cDNA conc. (pg/ul)	Size (bp)	copy no./ul
iNOS standard	656	37.4	747.98	80	9.00E+09
COX-2 standard	583.5	30.16	603.12	80	7.00E+09
18s rRNA standard	1089.5	80.71	1614.15	180	8.00E+09

Table 3.3: Summary of the concentration, base pair length and copy number of the iNOS, COX-2 and 18s rRNA standards.

3.3.1.2 Efficiency of amplification for each standard

A ten fold dilution series of the iNOS, COX-2 and 18s rRNA standards were prepared and the corresponding crossing point value for each standard was determined by real time PCR. A standard curve for each gene was generated of the crossing point values obtained by real time against the log of the known pre-determined copy numbers. The efficiency of amplification was determined from the slope of each standard curve using the formula described in figure 3.3 of the methods section. Results can be seen in Figure 3.6 and table 3.4. As the amplification efficiencies for each gene were not identical, a separate standard curve was prepared for each gene used was subsequently used for the quantification of sample gene expression.

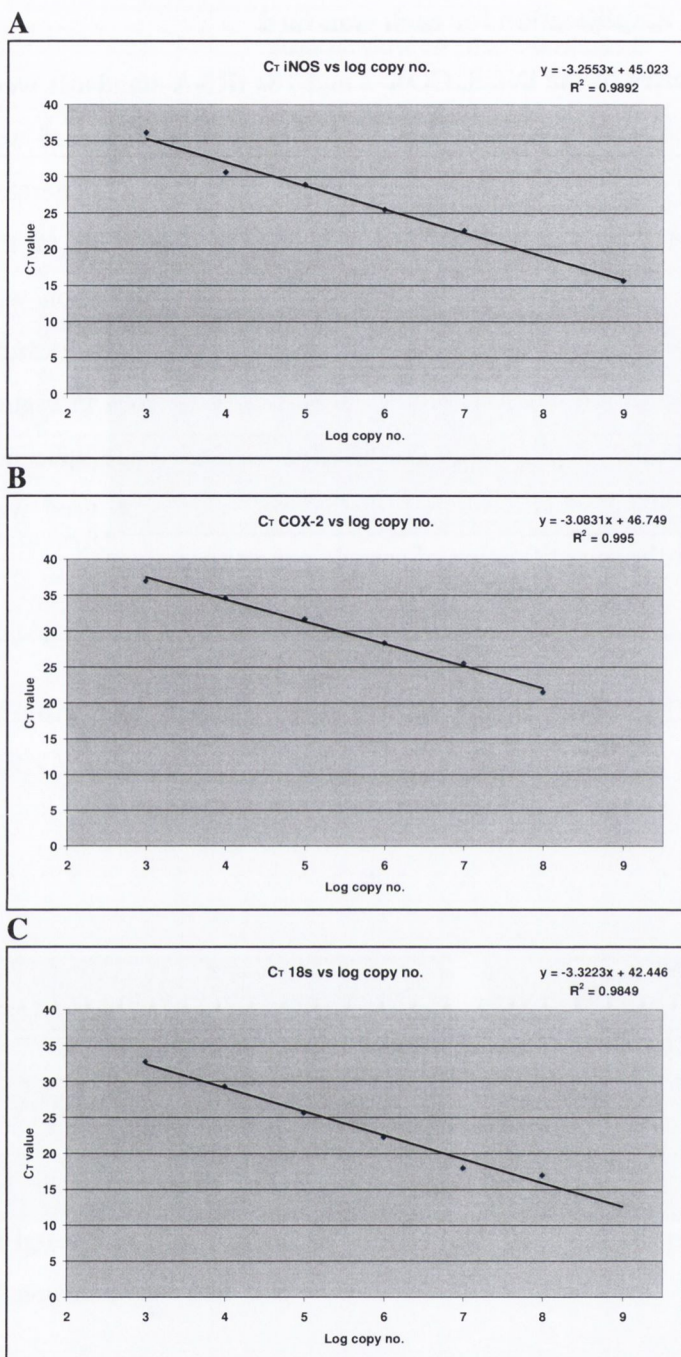


Figure 3.6: Standard curves for iNOS (A), COX-2 (B) and 18s rRNA (C) genes for determination of amplification efficiency.

Gene	Slope from graph	Efficiency of amplification $E=10^{(-1/\text{slope})}-1$	% Efficiency
iNOS	-3.2553	1.03	103%
COX-2	-3.0831	1.11	111%
18s rRNA	-3.3223	1	100%

Table 3.4: Calculated amplification efficiencies

3.3.1.3 Preparation of samples for gene quantification

mRNA was extracted from stimulated and unstimulated cells and quantified by a nanodrop spectrometer as described in section 3.2.4.2.. All samples were free of protein contamination as assessed by an absorbance ratio A260/A280 greater than 2. The mean mRNA concentration extracted from both stimulated and unstimulated cells was 121.7 ng/ul. 500ng of each sample mRNA was reverse transcribed and the subsequent cDNA quantified fluorescently as described in section 3.2.5.2. The mean concentration of cDNA was found to be 974.7pg/ul. Raw data of mRNA and cDNA concentrations for each sample can be seen in Appendix II. 500pgms of each sample cDNA was quantified by real time PCR using the iNOS, COX-2 and 18s rRNA primers to assess changes in gene expression following stimulation. A standard curve using the standards generated in section 3.3.1.1 for each gene was incorporated in to each real time PCR reaction to enable quantification of iNOS and COX-2 by both absolute and relative methods.

3.3.1.4 Quantification of gene expression using Absolute and Relative method of analysis

Differences in iNOS and COX-2 gene expression in response to stimulation with PT gliadin, IL-15 and IFN- γ was assessed by using an absolute method of quantification and by using three methods of relative quantification as described in section 3.1.3 and sections 3.2.5.3 and 3.2.5.4. Results were graphed using fold changes determined by a standard curve method of absolute and relative gene quantification for comparison. As can be seen from figure 3.7 and figure 3.8 both methods of quantification gave comparable results.

3.3.1.4.1 iNOS gene expression

iNOS mRNA levels were assessed in response to PT Gliadin, IL-15 and IFN- γ stimulation. The results from the absolute and relative method using standard curves are shown in figure 3.7. Cells stimulated for 4 hours are shown in figure 3.7 A and B. Cells stimulated for 30 hours are shown in figure 3.7 C and D. Fold changes for each of the four experiments were calculated using an absolute method and three relative methods of gene

quantification. As can be seen in table 3.5 for each experiment at both time points, all four methods of gene quantification gave comparable results. Results from the absolute method of quantification were used to calculate the mean fold changes in response to stimulation. Statistical significance was assessed using results from all four methods of gene quantification.

PT Gliadin stimulation

As can be seen in figure 3.7A and B, PT Gliadin did not induce a fold change difference compared to controls at 4hours (confidence interval range of -2.4 to 4.2). mRNA levels remained unchanged at 30 hours also with a mean fold increase of 1.4 (confidence interval range of -1.1 to 1.7) compared to unstimulated cells. In summary, PT gliadin does not have a significant effect on iNOS gene expression.

IL-15 stimulation

As can be seen from Figure 3.7 A and B IL-15 failed to elicit any change in iNOS mRNA at 4hrs (confidence interval range -3.38 to 4.4). At 30hrs, one of four experiments showed <15 fold difference in iNOS compared to unstimulated. However repetition of experiment failed to elicit the same response. Mean fold changes at 30hours were found to be 4.5 (confidence interval range -7.9 to 17.28) and were not significant compared to unstimulated cells.

IFN- γ stimulation

As can be seen from figure 3.7 A and B, IFN- γ did not significantly induce iNOS at the earlier time point of 4 hours with mean fold increase of 1.3 (95% confidence interval range of -1.77 to 4.29) compared to unstimulated cells but induced a significant fold increase in iNOS at 30hours compared to unstimulated cells ($P < 0.05$) as assessed using a two way ANOVA analysis of variance followed by the BonFerroni *post hoc* test ($n=4$) with a mean fold increase of 17.6 (confidence interval range of 3.64 to 31.6) as is seen in figure 3.7 C and D.

iNOS

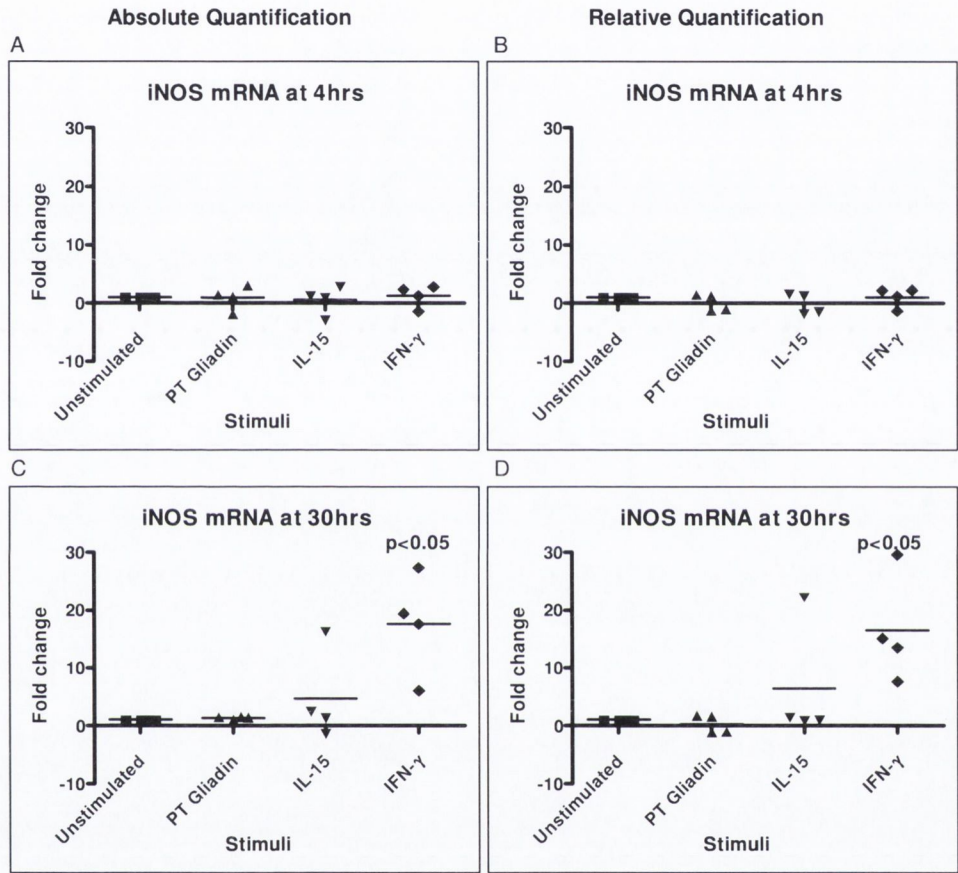


Figure 3.7: iNOS gene expression. iNOS gene expression at 4hrs (A and B) and 30hrs (C and D) in response to stimulation with PT Gliadin, IL-15 and IFN- γ . Gene expression was analysed using a two way ANOVA and Bonferroni *post hoc* test (n=4).

Stimulus	Incubation Time (hrs)	Expt. No.	Absolute fold changes using a standard curve method	Relative fold changes using a standard curve method	Relative fold changes using the $\Delta\Delta Ct$ method	Relative fold changes using the Pfaffl method
Unstimulated	4	1				
PT Gliadin	4	1	1.44	1.45	1.44	1.46
IL-15	4	1	1.24	1.27	1.27	1.27
IFN- γ	4	1	2.35	2.24	2.22	2.26
Unstimulated	4	2				
PT Gliadin	4	2	3.00	-1.02	-1.09	-1.06
IL-15	4	2	2.84	1.57	1.51	1.54
IFN- γ	4	2	2.82	2.18	2.13	2.18
Unstimulated	4	3				
PT Gliadin	4	3	-1.92	-1.29	-1.27	-1.28
IL-15	4	3	-2.90	-1.78	-1.72	-1.75
IFN- γ	4	3	-1.43	-1.28	-1.27	-1.28
Unstimulated	4	4				
PT Gliadin	4	4	1.07	1.23	1.24	1.24
IL-15	4	4	-1.04	-1.45	-1.49	-1.49
IFN- γ	4	4	1.29	1.20	1.20	1.20
Unstimulated	30	1				
PT Gliadin	30	1	1.51	1.54	1.54	1.55
IL-15	30	1	16.31	22.24	22.01	23.36
IFN- γ	30	1	6.08	7.66	7.62	7.92
Unstimulated	30	2				
PT Gliadin	30	2	1.06	-1.11	-1.11	-1.11
IL-15	30	2	-1.37	1.46	1.52	1.51
IFN- γ	30	2	17.62	13.54	13.00	13.81
Unstimulated)	30	3				
PT Gliadin	30	3	1.43	-1.04	-1.06	-1.05
IL-15	30	3	2.47	1.02	-1.03	-1.02
IFN- γ	30	3	19.43	15.13	14.52	15.47
Unstimulated	30	4				
PT Gliadin	30	4	1.46	1.72	1.73	1.74
IL-15	30	4	1.34	-1.04	-1.06	-1.05
IFN- γ	30	4	27.38	29.64	28.84	30.94

Table 3.5:iNOS gene expression. Comparison of fold changes in iNOS gene expression using an absolute method and three relative methods of quantification

3.3.1.4.2 COX-2 gene expression

COX-2 mRNA levels were assessed in response to PT Gliadin, IL-15 and IFN- γ stimulation. The results are shown in figure 3.8. Cells stimulated for 4 hours are shown in figure 3.8 A and B. Cells stimulated for 30 hours are shown in figure 3.8 C and D. Fold changes for each of the four experiments were calculated using an absolute method and three relative methods of gene quantification. As can be seen in table 3.6 for each experiment at both time points, all four methods of gene quantification gave comparable results. Results from the absolute method of quantification were used to calculate the mean fold changes in response to stimulation. Statistical significance was assessed using results from all four methods of gene quantification.

PT Gliadin stimulation

Caco-2 cells were assessed for COX-2 mRNA induction in response to PT Gliadin at 4hrs and 30hrs. As can be seen in figure 3.8 there was a small fold increase in some experiments at 4hours with a mean fold increase of 1.5 compared to unstimulated cells (95% confidence interval -3.65 to 6.59) and also at 30hours with a mean fold increase of 3 (95% confidence interval range between -0.12 to 6.1). However this increase failed to reach significance over the time course examined due to the large variance seen between experiments.

IL-15 stimulation

IL-15 regulation of the COX-2 gene was also assessed at 4hrs and 30hrs. At 4hrs a 20 fold increase in COX-2 expression could be seen in one experiment. This decrease was not seen when the experiment was repeated three other times. The mean fold change was found to be 6.4 (95% confidence interval range of -8.1 to 20.99) and was not significant compared to unstimulated cells. This experiment gave similar high increases in iNOS gene expression at 30 hours which was not seen in subsequent experiments. A mean fold increase of 2.5 (95% confidence interval range of -1.5 to 6.48) was seen at 30 hours as can be seen in Figure C and D which was not significant compared to unstimulated cells.

IFN- γ stimulation

IFN- γ did not induce an increase in COX-2 gene expression at 4hrs as can be seen in Figure 3.8A and B with a mean fold increase of 1.3 (95% confidence interval range of 1.14 to 1.55). After 30 hours of incubation with the Caco-2 cell line, increases in COX-2 gene expression compared to controls were seen with a mean fold increase of 3.3 (95% confidence interval range between -5.0 to 7.1) similar to the fold increase seen in both PT Gliadin and IL-15. This was not significant compared to cells not treated with IFN- γ . Thus it would appear using this enterocyte cell model IFN- γ does not play a significant role in COX-2 regulation.

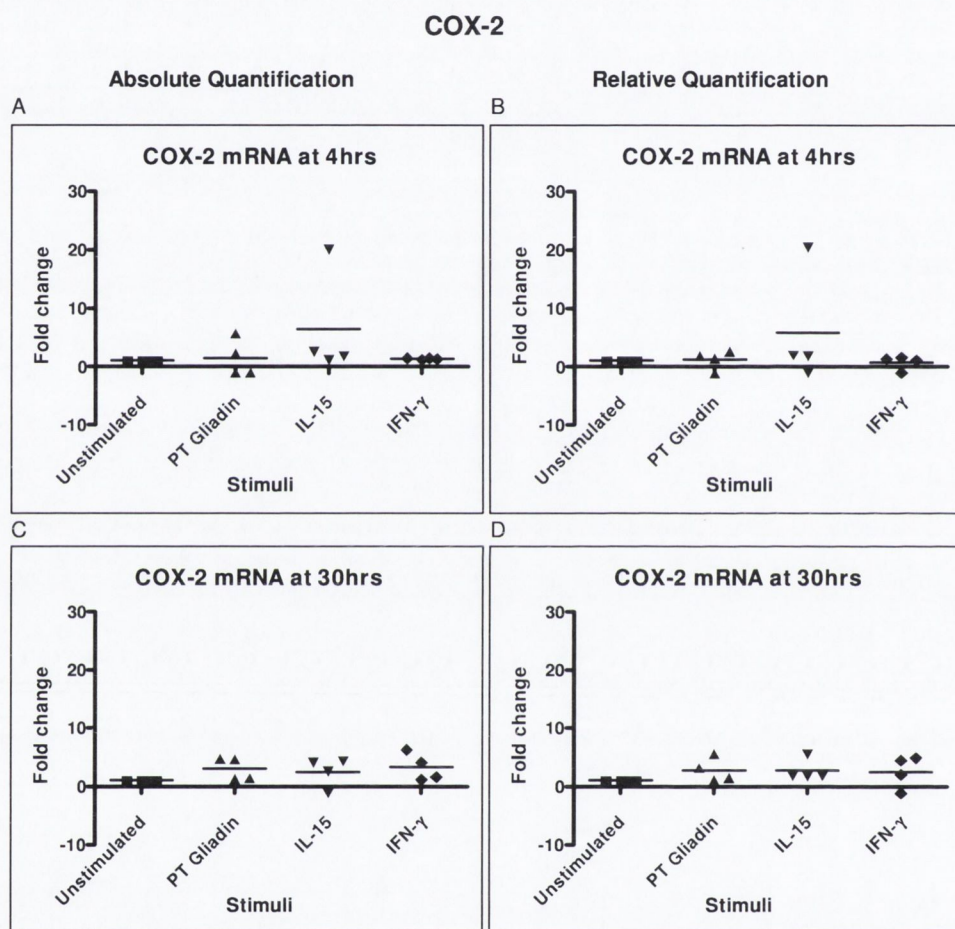


Figure 3.8: COX-2 gene expression. COX-2 gene expression at 4hrs (A and B) and 30hrs (C and D) in response to stimulation with PT Gliadin, IL-15 and IFN- γ . Gene expression was analysed using a two way ANOVA and Bonferroni *post hoc* test ($n=4$).

Stimulus	Incubation Time (hrs)	Expt. No.	Absolute fold changes using a standard curve method	Relative fold changes using a standard curve method	Relative fold changes using the $\Delta\Delta Ct$ method	Relative fold changes using the Pfaffl method
Unstimulated	4	1				
PT Gliadin	4	1	-1.04	-1.04	-1.03	-1.04
IL-15	4	1	20.13	20.53	16.80	20.85
IFN- γ	4	1	1.45	1.38	1.35	1.38
Unstimulated	4	2				
PT Gliadin	4	2	5.69	1.86	1.56	1.77
IL-15	4	2	1.80	-1.01	-1.07	-1.03
IFN- γ	4	2	1.28	-1.01	-1.04	-1.02
Unstimulated	4	3				
PT Gliadin	4	3	-1.04	1.41	1.44	1.44
IL-15	4	3	1.19	1.98	2.01	2.04
IFN- γ	4	3	1.46	1.63	1.60	1.65
Unstimulated	4	4				
PT Gliadin	4	4	2.26	2.62	2.50	2.65
IL-15	4	4	2.61	1.86	1.72	1.84
IFN- γ	4	4	1.20	1.12	1.10	1.12
Unstimulated	30	1				
PT Gliadin	30	1	1.39	1.41	1.39	1.42
IL-15	30	1	4.07	5.55	5.13	5.68
IFN- γ	30	1	1.61	2.03	1.99	2.06
Unstimulated	30	2				
PT Gliadin	30	2	1.22	1.04	1.02	1.04
IL-15	30	2	-1.12	1.77	1.85	1.84
IFN- γ	30	2	1.15	-1.14	-1.16	-1.15
Unstimulated)	30	3				
PT Gliadin	30	3	4.66	3.14	2.77	3.09
IL-15	30	3	4.36	1.80	1.56	1.73
IFN- γ	30	3	6.27	4.88	4.26	4.86
Unstimulated	30	4				
PT Gliadin	30	4	4.73	5.57	5.06	5.66
IL-15	30	4	2.59	1.85	1.71	1.83
IFN- γ	30	4	4.13	4.47	4.08	4.52

Table 3.6: COX-2 gene expression.Comparison of fold changes in COX-2 gene expression using an absolute method and three relative methods of quantification

3.3.2 iNOS Enzyme Activity

3.3.2.1 Spectrophotometric determination of iNOS activity

iNOS activity was assessed by the determination of nitrite in cell supernatant following stimulated with 0.5 μ g PT Gliadin, 250ng IL-15 and 1000U IFN- γ as described in the Methods section. A nitrite standard curve was prepared as shown in figure 3.9 using a dilution series of sodium nitrite and nitrite levels detected at 550nm by reacting with a griess reagent. Results of nitrite levels in the samples can be seen in figure 3.10. The detection limit using this spectrophotometric method is between 0.43-65uM . As can be seen from graph, most samples lie out of the range of detection with many showing negative results. IFN- γ stimulated cells showed detectable levels of nitrite from 24hours to 36hours of stimulation supporting the earlier results showing iNOS mRNA induction in response to IFN- γ stimulation.

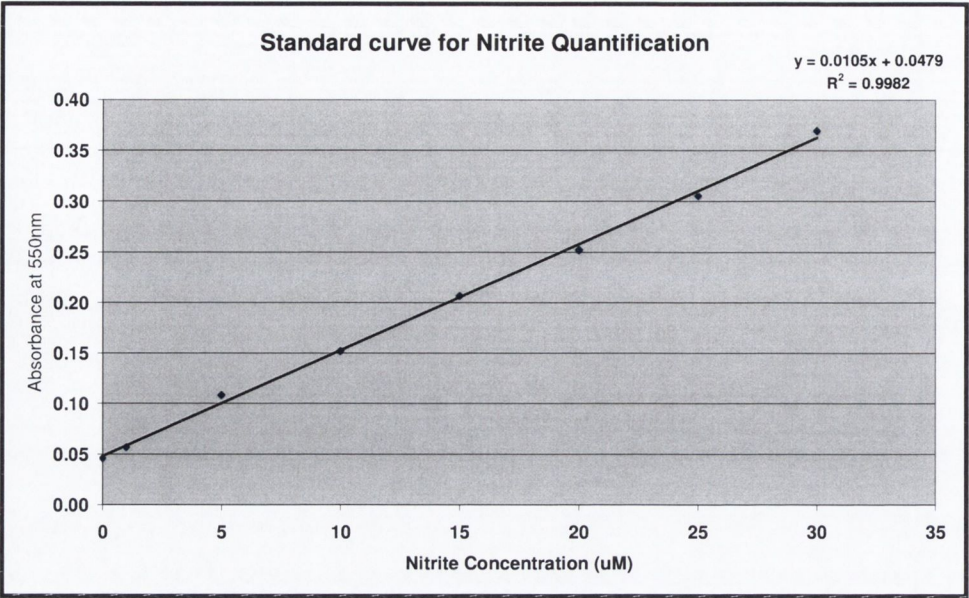


Figure 3.9: Standard curve for determination of nitrite concentration using a spectrophotometric method

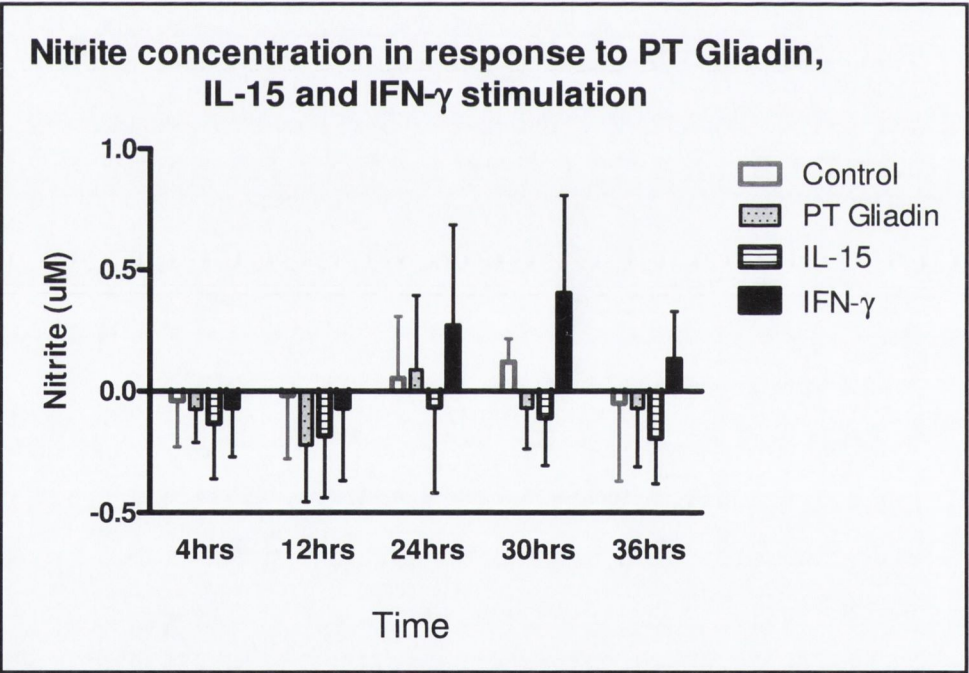


Figure 3.10: Nitrite concentrations. Nitrite concentrations in control cells and in cells stimulated with PT Gliadin, IL-15 and IFN- γ between 4hrs and 36 hrs. Bars represent the mean $\pm$ standard deviation of four independent experiments

3.3.2.2 Fluorometric determination of iNOS activity

A more sensitive method of nitrite detection was also employed to assess levels of nitrite in control and stimulated cells using a fluorometric assay kit as described in the Methods section. As no detectable difference in nitrite could be seen in the spectrophotometric results prior to 24 hours, samples were fluorometrically determined from 24 hours to 36 hours. A standard curve was constructed using sodium nitrite using concentrations in the range of 0 to 3.85 μ M as can be seen in figure 3.9. The concentration of nitrite was determined using the least squares regression equation for the line generated by the standards, which is $y = mx + b$ where: y is the instrument reading (RFU), x is the nitrite concentration (μ M), m is the slope of the line and b is the y -intercept. The calculated concentration was divided by the volume of sample (20 μ l). The mean and standard deviation of 4 independent experiments were graphed in figure 3.12 demonstrating the effect of PT Gliadin, IL-15 and IFN- γ stimulation on Caco-2 cells over time.

PT Gliadin

As can be seen in figure 3.12A nitrite levels are comparable in both control and PT Gliadin stimulated cells at 24 hours. However at 30 and 36 hours nitrite levels decrease in PT Gliadin stimulated cells compared to controls though this does not reach statistical significance using a two way ANOVA test.

IL-15

In IL-15 stimulated cell supernatants, a decrease in nitrite compared to controls was found at 24 hours which decreased further at 30 hours and 36 hours though failed to reach significance.

IFN- γ

In contrast to PT Gliadin and IL-15 stimulated cells, IFN- γ induced iNOS activity. Whilst this increase was seen at 24 hours, 30 hours and 36 hours, due to the variation between experiments this failed to reach significance. IFN- γ along with inducing iNOS mRNA also increases activity as assessed by its break-down product nitrite though the level of nitrite produced does not reach significance over the time course studied.

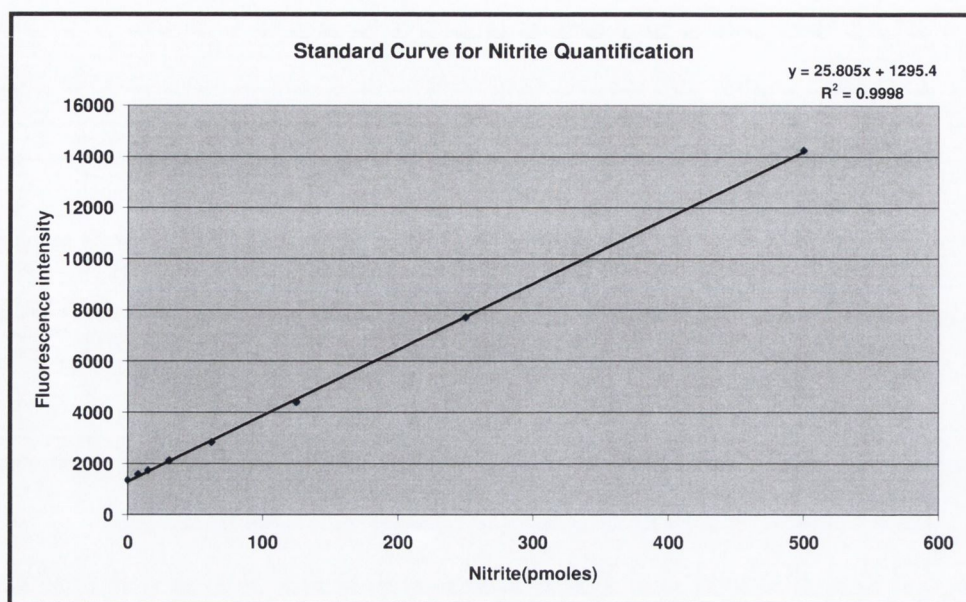


Figure 3.11: Standard Curve for determination of nitrite using a fluorometric method.

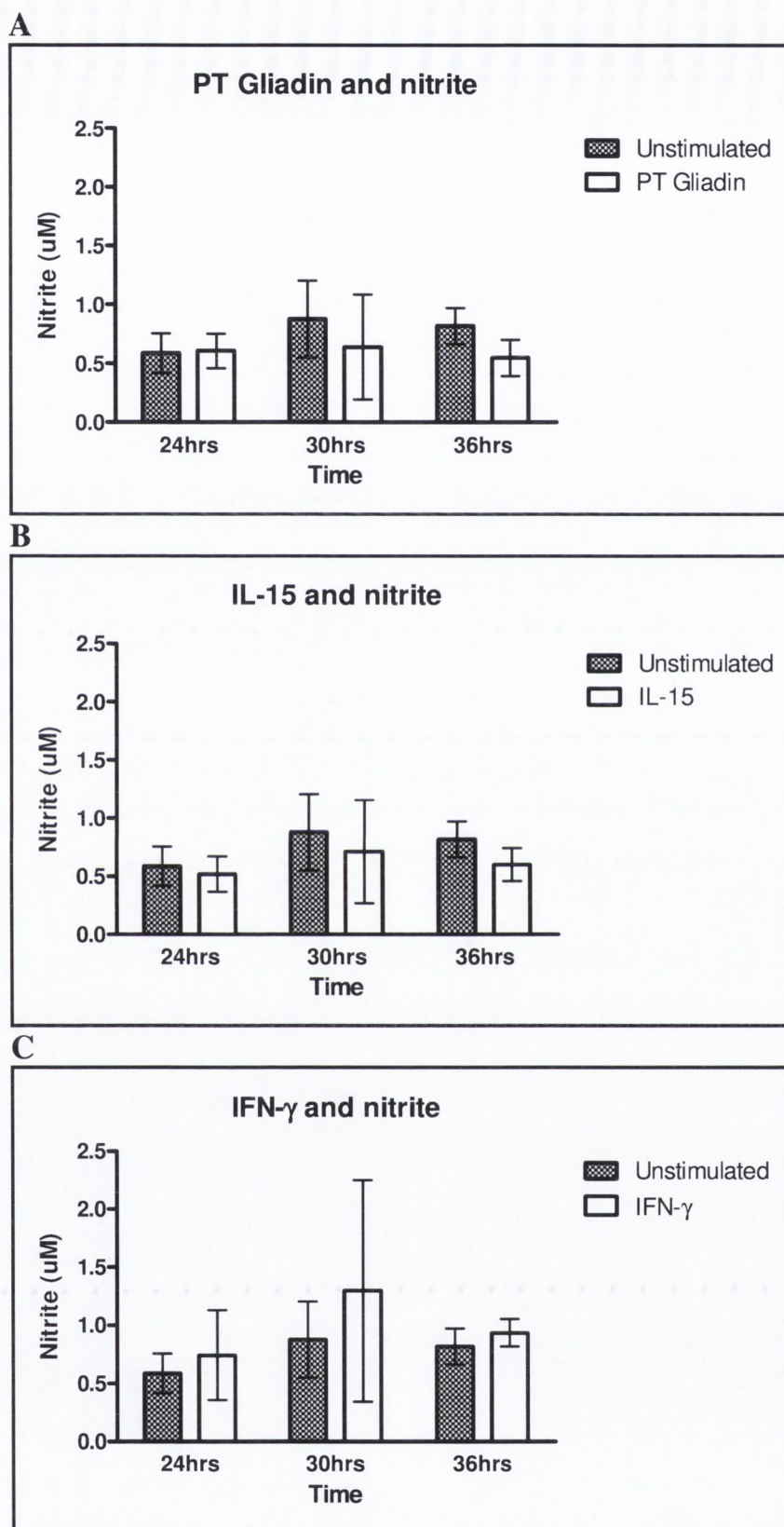


Figure 3.12: iNOS activity. iNOS activity in response to PT Gliadin (A), IL-15 (B) and IFN- γ (C) as assessed over a time course of 24 to 36 hours. Results are representative of at least 3 independent experiments. $\ast=p<0.05$

3.3.3 COX-2 Activity

COX-2 activity was investigated by analysis of prostaglandin E₂ in culture supernatants using a competitive ELISA technique. A standard curve was prepared by plotting the mean absorbance for each standard on a linear y axis against the concentration on a logarithmic axis to produce a non-linear standard curve. Standard concentrations were in the range of 19.6 to 625 picograms/ml. No detectable level of prostaglandin was found in either control or stimulated cell supernatants. Results are shown in Appendix III.

3.3.4 iNOS Protein

iNOS protein levels were assessed by flow cytometry following a 48 hour incubation with PT Gliadin, IL-15 and IFN- γ . The results can be seen in figure 3.13. The expression of HLA A, B and C MHC Class I antigens on Caco-2 cells and their up-regulation by IFN- γ have been previously described and were incorporated in to the experiment as a positive control (Siavoshian, Blottiere et al. 1996; Imanishi, Kamigaki et al. 2006). As can be seen from figure 3.13 both PT Gliadin and IL-15 had no effect on iNOS or HLA-ABC protein levels following 48 hours of stimulation. IFN- γ showed a marginal non significant increase in iNOS protein compared to unstimulated cells (mean 1.5 fold increase). HLA ABC which served as a positive control increased significantly following IFN- γ stimulation as expected.

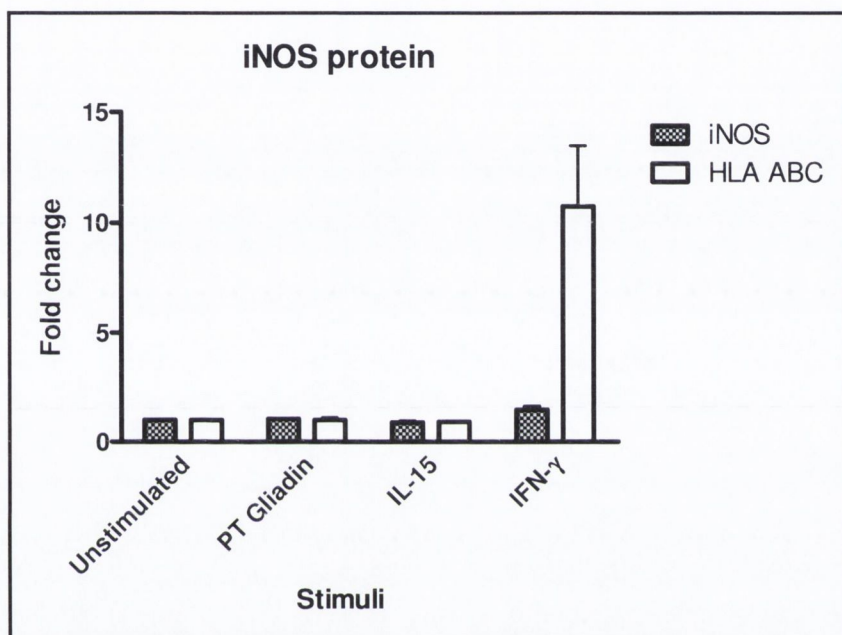


Figure 3.13: iNOS protein. Fold change of iNOS protein in response to stimulation with PT Gliadin, IL-15 and IFN- γ compared to control cells following a 48 hour incubation.

3.4 Discussion

The purpose of this study was to assess the regulation of the inflammatory enzymes iNOS and COX-2 in coeliac disease using an enterocyte cell line. The direct effect of gliadin, the causative agent in coeliac disease was investigated. The role of the innate cytokine IL-15 which is up-regulated upon initial exposure to gliadin and the role of IFN- γ which is up-regulated following induction of the T cell mediated response to gliadin were also determined. Gene expression along with enzyme activity and protein production was assessed in response to a short term exposure of 4 hours and a later exposure time of 30 hours. The major findings from this study were that PT gliadin and IL-15 do not regulate enterocyte iNOS gene expression or protein production as assessed by the Caco-2 cell line; however they do have a negative effect on iNOS activity. In contrast, IFN- γ produced in the latter phase of coeliac disease progression is a major inducer of iNOS gene expression and enzyme activity. Regulation of the COX-2 gene could not be determined in response to any of the stimuli tested.

iNOS and coeliac disease

iNOS expression is increased in a number of disease states including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, systemic lupus erythematosus, dermatitis and atherosclerotic plaques and coeliac disease (Kroncke, Fehsel et al. 1998). In coeliac disease iNOS activity is elevated in the small intestine and increased oxidation products of nitric oxide, nitrate and nitrite are found in the plasma and urine of coeliac patients (ter Steege, Buurman et al. 1997; Beckett, Dell'Olio et al. 1998; Sundqvist, Laurin et al. 1998; Spencer, Daniels et al. 2004). The major sources of iNOS in coeliac disease appear to be macrophages and enterocytes with elevated expression and activity noted in both cell types during disease (Murray, Daniels et al. 2002; Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003; Daniels, Cavill et al. 2005). The precise role of iNOS in disease is unclear but it may participate in altered chloride secretion through increasing cGMP levels resulting in diarrhoea or disruption of barrier function or generation of reactive oxygen species (Murray, Daniels et al. 2002; Resta-Lenert and Barrett 2002).

iNOS and PT gliadin

Results from this study show that PT gliadin does not directly induce the gene expression or protein production of iNOS within enterocytes as determined in the Caco-2 cell line. At both 4 hours and 30 hours, iNOS mRNA showed no significant change in PT Gliadin stimulated cells compared to control cells. When iNOS protein levels were assessed at 48 hours no difference in iNOS protein was found compared to control cells. PT Gliadin does not induce iNOS activity as assessed by nitrite production. Whilst iNOS gene expression, protein production and enzyme activity have been found to be regulated within macrophage cell lines by gliadin when administered in conjunction with IFN- γ , direct induction by gliadin has not been found (Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003; De Stefano, Maiuri et al. 2006). Elevated levels of iNOS activity have been found in enterocytes of coeliac patients, however the key regulators of iNOS expression have yet to be identified (Murray, Daniels et al. 2002; Daniels, Cavill et al. 2005). This study does not support a direct effect of PT gliadin on iNOS expression or activity.

iNOS and IL-15

In this study IL-15 showed no increase in iNOS mRNA at the early time point of 4 hours. Whilst induction was noted in one experiment at 30 hours this result could not be repeated in any of the subsequent three experiments. In conclusion IL-15 failed to regulate iNOS mRNA levels and this was reflected in the protein findings at 48 hours in which IL-15 had no effect on iNOS protein levels. IL-15 failed to stimulate iNOS activity over the time course studied as assessed by nitrite production. Nitrite levels were lower than those seen in unstimulated cells though did not reach significance using a two way ANOVA analysis of variance. IL-15 positive regulation of iNOS mRNA protein and activity has previously been found in a macrophage cell line and in gingival epithelial cells (Yanagita, Shimabukuro et al. 2002; Figueras, Busquets et al. 2004; Liu, Zhai et al. 2004). However negative regulation of iNOS by IL-15 has also been demonstrated with a significant reduction in iNOS protein levels following IL-15 stimulation in skeletal muscle of tumour bearing mice (Figueras, Busquets et al. 2004). This study

suggests IL-15 does not induce iNOS gene expression or activity within enterocytes.

iNOS and IFN- γ

IFN- γ was shown to positively regulate enterocyte iNOS in this study. Up-regulation of iNOS within Caco-2 cells has previously been demonstrated and this study supports these findings (Cavicchi and Whittle 1999; Vignoli, Srivastava et al. 2001). Whilst no increase in iNOS mRNA levels was seen at 4 hours, at 30 hours significant increases in response to IFN- γ were shown. Protein levels of iNOS did not significantly differ from controls but a small fold increase (mean fold increase of 1.5) in iNOS protein could be detected at 48 hours. When iNOS activity levels were assessed by measurement of nitrite levels, IFN- γ showed a positive induction of activity as assessed by nitrite production however this failed to reach significance compared to controls. Previous studies of macrophage iNOS regulation by IFN- γ in coeliac disease using murine cell lines have shown IFN- γ up-regulation of iNOS gene expression, protein production and activity and iNOS induction which was further amplified in the presence of gliadin (Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003; Maiuri, De Stefano et al. 2003). This study supports the role of IFN- γ in the regulation of iNOS derived from enterocytes also. Thus it would appear both macrophages and enterocytes within the coeliac lesion are likely to be regulated by IFN.

COX-2 and coeliac disease

COX-2 activity has been described in a number of diseases including rheumatoid arthritis, osteoarthritis, colorectal cancer, atherogenesis and thrombosis (Dubois, Abramson et al. 1998; Salinas, Rangasetty et al. 2007). In coeliac disease increased COX-2 activity has been found, reflected by increased levels of prostaglandin most notably PGE₂ which has been shown to be up-regulated following gliadin perfusion (Branski, Karmeli et al. 1984; Lavo, Knutson et al. 1990; Friis, Anthonsen et al. 1994). A speculative role of prostaglandins may be in the induction of chloride secretion by the epithelium which is related to diarrhea, a classical symptom of coeliac disease and altered barrier function which has also which has been described

in coeliac disease(Eckmann, Stenson et al. 1997; Resta-Lenert and Barrett 2002; Drago, El Asmar et al. 2006). COX-2 has also been suggested to play a role in resolution of inflammation by the restoration of the jejunal mucosa (Kainulainen, Rantala et al. 2002).

COX-2 and PT gliadin

PT Gliadin failed to elicit any direct effect on COX-2 gene expression as assessed at an early time point of 4 hours and at a later time point of 30 hours. A mean fold increase of 3 was found following PT gliadin stimulation at 30 hours however this increase was found in response to the other two stimuli and did not reach statistical significance. When COX-2 activity was assessed no detectable levels of prostaglandin was seen in the supernatants of PT gliadin stimulated cells. Thus PT gliadin does not have a direct effect on enterocyte COX-2 as shown in this study.

COX-2 and IL-15

In an initial experiment, IL-15 caused a 20-fold increase of 20 in COX-2 mRNA at 4 hours. However subsequent experiments failed to support this finding with no significant increase in COX-2 seen upon IL-15 stimulation. A small increase of COX-2 mRNA at 30 hours was shown similar to that seen in cells stimulated with PT gliadin and IFN- γ which did not reach significance. No detectable prostaglandin E₂ was found in IL-15 stimulated cell supernatants following 30 hours. Previous studies using *in vitro* organ culture have shown gliadin through its induction of IL-15 to be responsible for COX-2 induction in mononuclear cells (Maiuri, Ciacchi et al. 2003). Other studies have shown IL-15 regulation of COX-2 synoviocytes(Min, Hwang et al. 2004). In this study, investigation of the role of epithelial cells as sources of COX-2 activity and the regulation of COX-2 gene expression and activity found that IL-15 was not a major inducer of COX-2 gene expression and activity within enterocytic cells.

COX-2 and IFN- γ

IFN- γ failed to elicit any significant increase in COX-2 gene expression and enzyme activity. A non-specific increase of COX-2 gene expression at 30 hours could be seen in all three stimuli which were not statistically significant. When COX-2 activity was assessed no detectable levels of prostaglandin was seen in IFN- γ stimulated cell supernatants. Thus IFN- γ in these experiments did not regulate COX-2 activity. Previous studies have also found no induction of COX-2 protein or activity in response to IFN- γ (Pang and Knox 1997). In some instances IFN- γ has even been found to negatively regulate COX-2 activity induced by pro-inflammatory mediators (Deng, Montero et al. 2007). Low levels of COX-2 seen in Caco-2 cells both in unstimulated and stimulated cells may be in part due to the differentiation state of the Caco-2 cell line. Several studies have shown that the differentiation status of Caco-2 cells can reflect their COX-2 expression levels with decreased gene expression and activity occurring following differentiation even in response to stimulation (Di Popolo, Memoli et al. 2000; Martin-Venegas, Roig-Perez et al. 2006).

Summary

From this study it was shown that using an enterocyte cell model, COX-2 activity does not increase in response to PT Gliadin, IL-15 or IFN- γ . This study does not support a role of enterocytes in elevated COX-2 activity in response to key mediators of coeliac disease inflammation. Macrophages and T cells within the mucosa may represent the key sources of this enzyme as previously described (Kainulainen, Rantala et al. 2002).

Results from iNOS activity assays in this study show a novel inhibitory effect of PT gliadin and IL-15 on iNOS activity. Whilst direct gliadin effects on nitric oxide formation have not been demonstrated within macrophage cell lines, using this current cell model, clear down-regulation of nitrite levels over time in response to both PT gliadin and IL-15 was demonstrated (Maiuri, De Stefano et al. 2003). The iNOS enzyme is found at the apical portion of enterocytes within the intestine and thus inappropriate activation by antigenic material such as gliadin within the lumen would not be

conducive to the maintenance of homeostasis (Rumbo, Courjault-Gautier et al. 2005). The biological significance of IL-15 inhibition of nitric oxide is not clear. IL-15 has however been previously shown to negatively regulate iNOS protein production and these results support this IL-15 inhibitory role (Figueras, Busquets et al. 2004). As shown in previous studies, IFN- γ represents a potent inducer of iNOS gene expression and activity in enterocytes (Chavez, Morin et al. 1999; Vignoli, Srivastava et al. 2001).

Thus while the late induction of IFN- γ in the adaptive response of coeliac disease would result in induction of iNOS within the epithelium, it would appear at the innate phase of disease, PT Gliadin and IL-15 do not induce iNOS and may actually decrease its activity following induction from stimuli such as IFN- γ . This finding may provide novel insights into the role of PT Gliadin and IL-15 in the regulation of epithelial derived iNOS in coeliac disease.

CHAPTER 4

A COMPARATIVE STUDY OF BLOOD MONOCYTES DERIVED FROM A HEALTHY COHORT AND A TREATED COELIAC COHORT IN RESPONSE TO STIMULATION WITH GLIADIN AND IFN- γ

ANALYSIS OF CACO-2 HLA EXPRESSION IN RESPONSE TO STIMULATION

4.1 Introduction

Coeliac disease represents a persistent inflammatory condition of the small intestine characterised by activated gliadin specific CD4⁺ T lymphocytes and infiltrating intra-epithelial lymphocytes predominantly CD8⁺ (Brandtzaeg 2006). Activation of these HLA-dependent cells requires cells with antigen presenting function, primarily cells of the monocytic lineage. A growing body of evidence supports the role of gliadin in co-ordinating the development and activation of these antigen presenting cells in order to mount an effective immune response. Administration of gliadin alone to monocytic cell lines and blood monocytes from treated and untreated coeliac patients results in significant increases in cytokine secretion of IL-8 and TNF- α and this effect may also be further enhanced in the presence of IFN- γ (Jelinkova, Tuckova et al. 2004; Cinova, Palova-Jelinkova et al. 2007). Gliadin has also been shown to increase MHC Class II and co-stimulatory molecules on the surface of bone marrow derived murine dendritic cells along with the induction of IL-1 β secretion (Nikulina, Habich et al. 2004). Dendritic cell maturation has also been found in healthy human donors in response to gliadin. Gliadin causes the maturation of dendritic cells by up-regulation of surface maturation markers such as CD80, CD83, CD86, CD40 and HLA-DR. This effect is not augmented in the presence of IFN- γ (Palova-Jelinkova, Rozkova et al. 2005). Gliadin also increases cytokine secretion of IL-6, IL-8 and TNF- α in dendritic cells (Palova-Jelinkova, Rozkova et al. 2005). Gliadin has been found to induce the gene expression of a number of pro-inflammatory genes, in mouse macrophages, including TNF- α and IL-15 which are key cytokines in coeliac disease pathogenesis. Gliadin has also been shown to induce zonulin secretion from mouse macrophages which has been postulated to play an important role in intestinal permeability (Thomas, Sapone et al. 2006).

Gliadin administration along with IFN- γ increases the protein expression and activity of the iNOS enzyme, the latter demonstrated by increased levels of the nitric oxide end products, nitrate and nitrite, in supernatants of macrophage cell lines (Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003; De Stefano, Maiuri et al. 2006). *In vitro* organ culture studies of

control and coeliac biopsies in the presence of gliadin have shown marked increases in HLA-DR gene expression along with IL-15 protein up-regulation in coeliac patient samples (Maiuri, Ciacci et al. 2003). These combined findings suggest an important role for gliadin in triggering an innate immune response through the activation of cells of the monocytic lineage with subsequent effective activation of a T cell adaptive response characteristic of coeliac disease.

To further these findings this study aimed to investigate the effects of gliadin stimulation on surface HLA-DR expression on blood monocytes from control and treated coeliac subjects, to extend the previous findings using organ culture which showed increased HLA-DR mRNA and protein within the small intestine of coeliac patients in response to gliadin (Maiuri, Picarelli et al. 1996; Maiuri, Ciacci et al. 2003). As gliadin has been shown to induce IL-15 expression using a murine macrophage cell line and was found to up-regulate IL-15 protein expression in coeliac biopsies in culture, this study investigated the effect of gliadin incubation on surface and intra-cellular IL-15 levels in control and coeliac blood monocytes. The enterocyte may also play a role in the uptake and processing of gliadin as evidence supports the localisation of gliadin with MHC Class I and II molecules in coeliac subjects (Zimmer, Poremba et al. 1995; Zimmer 1997). Gliadin has also been shown to induce the MHC related protein MICA on the surface of coeliac enterocytes along with the induction of HLA-DR (Maiuri, Picarelli et al. 1996; Hue, Mention et al. 2004). To further these findings, this current study aimed to use the enterocyte cell line Caco-2 using a novel cell based enzyme linked immunosorbent assay (CELISA) to determine the effect of gliadin incubation on HLA expression.

CELISA (Cell enzyme linked immunosorbent assay)

CELISA represents an alternative method to conventional flow cytometry for the detection of cell surface molecules (Bishop and Hwang 1992; Parnas and Linial 1998). A pre-requisite for the use of flow cytometry is the requirement of cells to be in a single cell suspension and in the case of adherent cells such as enterocytes, this requires mechanical or chemical separation of cells e.g. trypsinisation procedures, which may alter protein surface expression on

cells. CELISA offers an attractive alternative to flow cytometry and correlation of data obtained using both methods has been shown to generate comparable results (Bishop and Hwang 1992; Erdile, Smith et al. 2001). The principal of CELISA is outlined below in figure 4.1. Cells grown on 96 well plates act as target antigens for the subsequent enzyme linked immunosorbent assay. CELISA has been used to screen a number of diverse cell surface molecules including HLA molecules, blood group antigens and tumour antigens. To normalize for variation in cell number between wells, cells are quantified using crystal violet and the absorbance value of the chromagen is divided by the absorbance obtained from cell number determination.

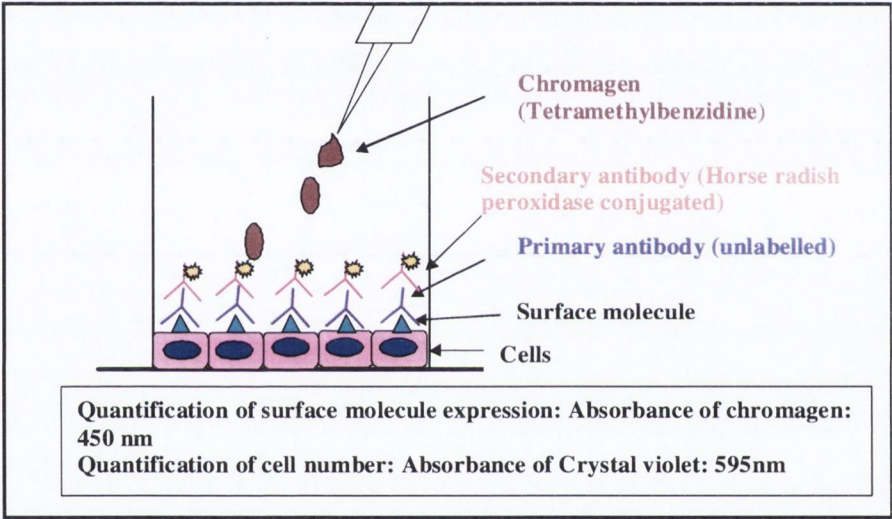


Figure 4.1 Principal of CELISA

The aim of this chapter

The aim of this study was to investigate the effect of PT Gliadin on surface HLA-DR expression on control and coeliac blood monocytes. Differences in both surface and intra-cellular IL-15 levels between the two study groups were also assessed. Alterations in iNOS activity in control and coeliac groups in response to PT Gliadin were assessed by measuring nitrite levels in supernatants. Finally, enterocyte HLA-DR and HLA-ABC was also investigated in response to PT Gliadin incubation.

4.2 Materials and Methods:

4.2.1 Study Subjects

Blood samples were taken from 10 treated coeliac patients and 10 control subjects. The mean age of the control group was 30. The mean age of the treated coeliac group was 35. The male to female ratio was 3:7 for the treated coeliac group and 1: 9 for the control group. Ethical approval for the study was granted by the Ethics Committee of the Board of St. James's Hospital, Dublin, Ireland.

4.2.2 Isolation of PBMC from whole blood

Whole blood samples were collected in lithium heparin tubes. A 1:1 dilution of whole blood was prepared in Hanks Balanced Salts Solution (HBSS) (Gibco BRL, UK). 5mls of Lymphoprep (Nycomed, Switzerland) was used to separate cells via density gradient centrifugation which was placed in a fresh 30ml universal tube. Gently, the blood: medium mixture was layered on top of the Lymphoprep solution. Tubes were centrifuged for 30mins at 1,200 rpm. Samples separated into three distinct layers. Using a pipette the purified PBMC layer was removed from the tube and placed into a fresh tube containing HBSS. Cells were centrifuged for 10mins at 1,600 rpm to wash the cells. The cell pellet was re-suspended in 10mls HBSS and centrifuged again at 1,600 rpm for a further 10mins. The cell pellet was re-suspended in RPMI (Gibco) and a cell count performed.

4.2.3 Cell count

25uls of the PBMC suspension was added to 475ul of EBAO. Using a haemocytometer, a cell count was performed as described in section 2.2.2.

4.2.4 Stimulation of PBMC preparation

1×10^6 cells were incubated in RPMI alone or in the presence of 500 ug PT gliadin, 500ug BSA or 600U IFN γ (Roche, Switzerland). A two point time experiment was performed at 6hrs and 24hrs. Cells were incubated in a 37°C incubator and at 6hrs the first set of stimulated cells removed for analysis.

Cells were spun at 1,200 rpm and supernatants from each stimulant were removed and stored at -20°C for further analysis. Remaining cells were left overnight and processed at 24hrs as described for the 6 hr samples.

4.2.5 Staining of PBMC cultures for Flow cytometry

Cells were washed with PBS and incubated in a concentrated solution of normal mouse serum (Dako, denmark) to block any subsequent non-specific antibody binding. Cells were stained for surface expression of HLA-DR and IL-15 using the directly labelled FITC mouse monoclonal antibodies. Monocyte cells were identified by the surface expression of CD14 using a directly labelled PE mouse monoclonal antibody. 5uls of antibody was used to stain cells in a 1: 20 ratio of antibody to cells. Cells were stained for surface expression of the following antibodies:

Mouse anti-human antibodies	Marker	Conjugate	Supplier
IL-15	IL-15 cytokine	FITC	R and D Systems (UK)
HLA-DR	MHC Class II Moleule	FITC	BD Biosciences (UK)
CD 14	Monocyte marker	PE	BD Biosciences (UK)

Table 4.1 Antibodies used in flow cytometry

Cells were subsequently fixed with Cell Fix (BD Biosciences) and analysed. For intracellular assessment of IL-15, cells were surface labelled with CD14 as described. Cells were permeabilised using 0.05% saponin (Sigma) in PBS and 5% FCS (Sigma). Cells were stained intracellularly using a directly labelled IL-15 antibody (R and D Systems, UK) and fixed.

4.2.6 Analysis by Flow Cytometry

Cells were analysed using a flow cytometer (FACS Sort, BD Biosystems, UK) . Monocytes were visualised initially based on their size and granularity using a FSC SSC scatter plot . Cells were gated using the CD14 (PE) marker

on SSC FL2 scatter plots. The mean fluorescence intensity of each antibody was determined using a FL2: FL1 scatter plot which plots the cells in accordance to their PE: FITC expression levels respectively. A histogram was prepared for each FITC antibody to determine the changes in the level of expression of HLA-DR and IL-15 following cell stimulation.

4.2.7 iNOS activity assays

Supernatants from control and coeliac PBMC subjects were used to assess iNOS activity based on the production of nitrite using a Fluorimetric Nitrite Determination Kit (Cayman Chemicals, USA). For the determination of nitrite, a nitrite standard curve was prepared at final nitrite concentrations between 0.156uM to 10uM and the assay was performed as previously described in section 3.2.6.2 for nitrite determination. Nitrite concentrations in samples could be extrapolated from the nitrite standard curve.

4.2.8 Cell Culture

The colon adeno-carcinoma cell line, Caco-2 was used between passages 50-60. Cells were cultured in DMEM (Gibco, BRL, UK) supplemented with 10% FCS (Sigma), 2mM L-glutamine, 100 U penicillin and 0.1mg streptomycin (Gibco) and 0.250ug/ml amphotercian B (Gibco) as described in section 2.2.1. Cells were maintained in T-25cm² flasks at 37°C, 5% CO₂.

4.2.9 Cell count and seeding on 96 well plates

Cells were removed from tissue culture flasks as previously described in section 2.2.1. Cell pellets were re-suspended in DMEM and a cell count performed using Ethidium Bromide/ Acridine Orange (EBAO) as described in section 2.2.2. Cells were diluted to a final concentration of. 1×10^6 cells/ml. 2×10^5 cells were added to each well of a 96 well plate. Cells were cultured until they reached 100% confluency. Medium was exchanged every 2 to 3 days.

4.2.10 Preparation of stimulants

A stock peptic/tryptic digest of gliadin was prepared in DMEM to a final concentration of 20mg/ml. This stock solution was diluted to a final working concentration of 500ug/ml. A stock solution of bovine serum albumin (BSA) (Sigma, USA) was prepared in DMEM to a final concentration of 20mg/ml. BSA was further diluted to a final working concentration of 500ug/ml. A stock solution of IFN- γ (Roche, Switzerland) of 100,000U/ml was diluted in DMEM to a final working concentration of 1000U/ml.

4.2.11 Cell Enzyme Linked Immuno- Sorbent Assay (CELISA)

Caco-2 cells grown on a 96 well plate were either cultured in the absence of stimulants or with the working concentrations of PT Gliadin, BSA or IFN- γ as outlined above, for 24hrs. Following 24hrs, supernatants were removed and cells were washed with PBS. Cells were then fixed onto the 96 well plate using 4% paraformaldehyde (VWR International) for 30minutes. Cells were washed twice with PBS. A 3% solution of hydrogen peroxide (Sigma) was added for 10 minutes to quench endogenous peroxidases. Cells were again washed twice with PBS. A blocking solution of 5% normal rabbit serum and 1% BSA was prepared in PBS and cells were incubated in this blocking solution for 1hour. Cells were washed once again with PBS and then incubated with one of the following primary antibodies.

Primary unlabelled antibody	Dilution	Supplier
Mouse anti-human isotype control IgG _{2A}	1:100	Dako
Mouse anti-human HLA-DR	1:100	Becton Dickinson
Mouse anti-human HLA-ABC	1: 100	Dako

Table:4.2: Primary unlabelled antibodies used in CELISA.

Control wells in which no primary antibodies were added were included in each experiment. Following incubation for 1 hr at room temperature with unlabelled primary antibody, cells were washed three times with PBS. Cells were incubated for 1 hr with rabbit-anti mouse horse raddish peroxidase (HRP) conjugated antibody (Dako, Denmark). Titration experiments were set up to determine optimal secondary antibody strengths. Secondary antibodies were diluted in the range of 1:100 to 1: 2000. Following incubation with secondary antibody, cells were washed four times in PBS.

Tetramethybenzidine (Sigma) was added to cells and incubated for 15 minutes protected from light. 1M sulphuric acid was then added to stop the reaction. The plate was read on a TECAN spectrafluorimeter at a 450nm wavelength. Cells were further washed with PBS and then incubated for 30 minutes with crystal violet to determine the cell number per well. Cells were then solubilised with 1% SDS and then results were read on the spectrafluorimeter at 595nm.

To normalize each well for cell number, the ODs at 450nm were divided by those obtained at 595nm. Background absorbance was determined from wells with no primary antibody and this was subtracted from each result.

4.2.12 Statistical analysis

The Mann-Whitney rank-sum test was used to evaluate statistical differences between patient groups, while the Wilcoxon signed-rank test was used to assess statistical differences between stimulated and unstimulated samples. A p-value < 0.05 was considered statistically significant

4.3 Results

4.3.1 Effect of stimulation of HLA-DR surface expression on blood monocytes

Control subjects

The Wilcoxon signed rank test was performed on the ten control subjects at 6 hours and 24 hours to assess changes in surface HLA-DR expression. A statistically significant difference was seen in HLA-DR expression in PT Gliadin stimulated cells (interquartile range of 403 to 744) compared to unstimulated cells (interquartile range of 250 to 507). However this increase was small as shown in figure 4.2 with a median fold increase of 1.6. This significant difference was also found in the control peptide (BSA) stimulated sample with a median fold increase of 1.7 (interquartile range of 345 to 810) as seen in figure 4.3. Thus this increase in HLA-DR is not specific to PT Gliadin with small fold changes shown compared to unstimulated cells. IFN- γ stimulated cells showed no significant difference at 6 hours (interquartile range of 262 to 712). At 24 hours PT Gliadin stimulated cells (interquartile range of 252 to 808) showed a statistically significant HLA-DR expression compared to unstimulated cells (interquartile range of 87 to 447) which was not found in the control peptide stimulated cells (interquartile range of 167 to 384). However the mean fold increase at 24 hours compared to unstimulated cells was very small with a median fold increase of 1.5 compared to unstimulated cells. Thus while PT gliadin cells showed a statistical significant increase compared to unstimulated cells at all time points, the median fold changes were very low compared to unstimulated cells. At 24 hours IFN- γ showed a significant increase in HLA-DR expression compared to unstimulated cells (interquartile range of 1516 to 2654) with a median fold increase of 8 ($p < 0.05$) as can be seen in figure 4.4

Coeliac Subjects

In the ten treated coeliac patients stimulated at 6 hours, no significant difference was found between unstimulated cells (interquartile range of 365 to 779) compared to cells stimulated with PT Gliadin (interquartile range of 377 to 796), BSA (interquartile range of 431 to 763) or IFN- γ (interquartile range of 450 to 721) as can be seen in figure 4.2, 4.3 and 4.4 respectively. At

24 hours there still was no statistical significance between unstimulated cells (interquartile range of 234 to 677) and cells stimulated with both PT Gliadin (interquartile range of 372 to 605) and BSA (interquartile range of 263 to 994). At 24 hours in coeliac blood monocytes a significant increase in surface HLA-DR was found upon stimulation with IFN- γ with a median fold increase of 5(interquartile fold range of 1599 to 2042).

Using a Mann Whitney rank-sum test to evaluate statistical differences between control and coeliac subjects, no statistical differences between the control and coeliac group was found.

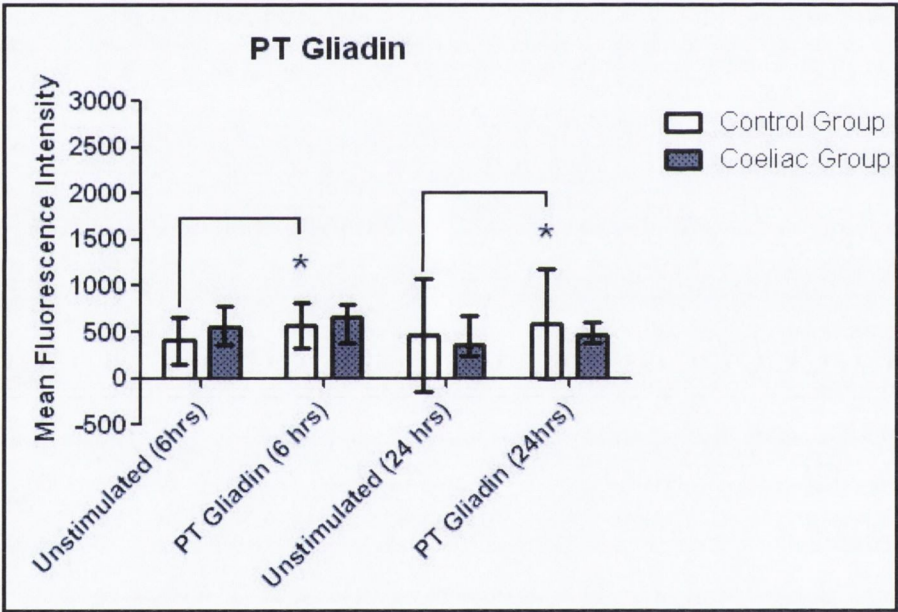


Figure 4.2: HLA-DR surface expression on control and coeliac monocytes over time in response to PT Gliadin: each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects.

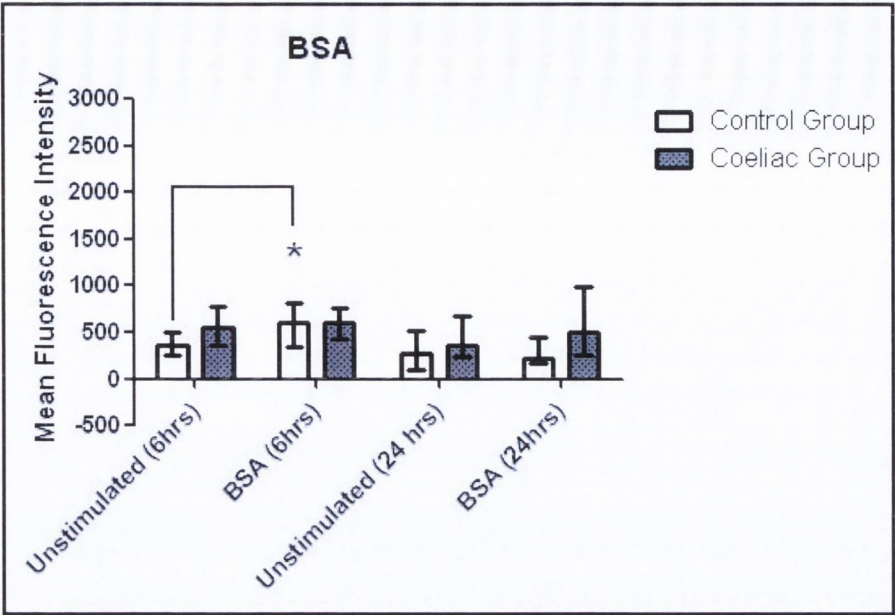


Figure 4.3: HLA-DR surface expression on control and coeliac monocytes over time in response to BSA: each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects

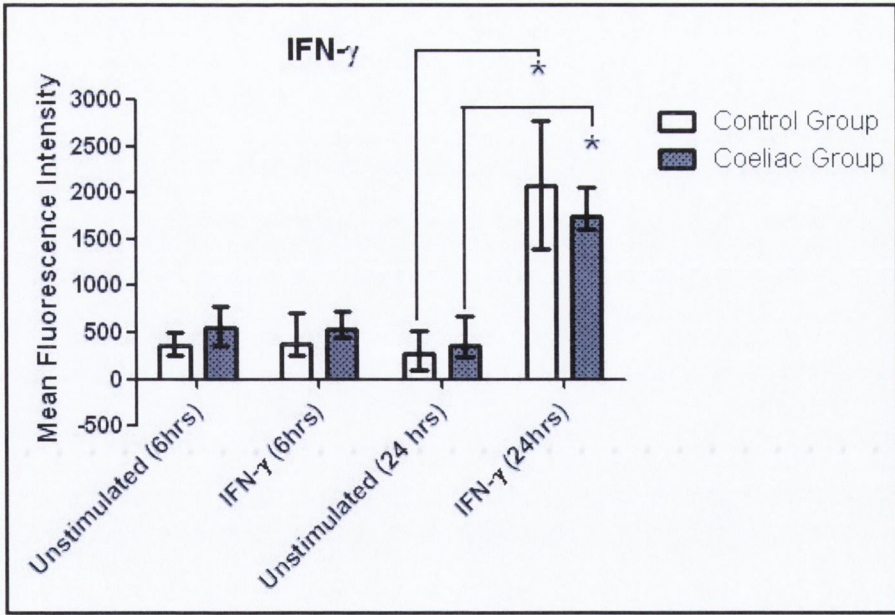


Figure 4.4: HLA-DR surface expression on control and coeliac monocytes over time in response to IFN- γ : each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects

4.3.2 Effect of stimulation on surface IL-15 expression on blood monocytes

Control Group

No statistical significance was found at 6hrs between unstimulated cells (interquartile range of -.06 to 2.1) and cells stimulated with PT Gliadin (interquartile range of -0.39 to 1.76), BSA (interquartile range of -0.05 to 1.2) or IFN- γ (interquartile range of 0.18 to 2.1). At 24 hours PT gliadin (interquartile range of 0.64 to 2.15) failed to cause a statistical significant change in surface IL-15 expression compared to unstimulated cells as seen in figure 4.5. Despite a statistical significance seen in cells stimulated with the control peptide (BSA) at 24 hours (interquartile range of 0.53 to 2.55) the median fold increase of 1.3 was very small compared to unstimulated as seen in figure 4.6. IFN- γ stimulated cells also showed a statistical significant increase in surface IL-15 compared to controls (interquartile range of 2.97 to 4.68) with a 2.5 fold increase in IL-15 compared to unstimulated cells. The median and interquartile fold ranges for can be seen in figure 4.7.

Coeliac Group

At 6 hours no statistical significance was found in PT Gliadin stimulated cells (interquartile range of 0.35 to 1.83) or cells stimulated with BSA (interquartile range of 0.36 to 2.06) or cells stimulated with IFN- γ (interquartile range of 0.68 to 2.05) compared to unstimulated cells (interquartile range of 0.33 to 1.25). No significance was also noted at the later time point of 24 hours with all three stimuli (PT Gliadin interquartile range of 0.16 to 1.8; BSA interquartile range of 0.9 to 1.9 and IFN- γ interquartile range of 0.94 to 4.95) compared to unstimulated cells (interquartile range of -0.08 to 1.28). The median and interquartile ranges can be seen in figure 4.5, 4.6 and 4.7. In summary no statistical significant difference was found between coeliac monocytes stimulated using all three stimuli over the time course investigated.

No statistical difference was found between the control and coeliac subjects as assessed by the Mann Whitney rank sum test.

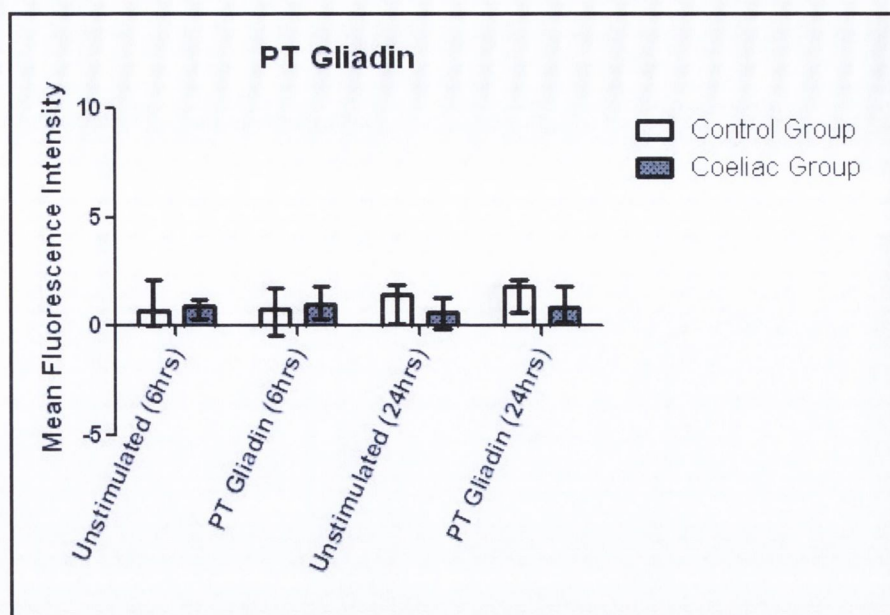


Figure 4.5: IL-15 surface expression on control and coeliac monocytes over time in response to PT Gliadin: each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects

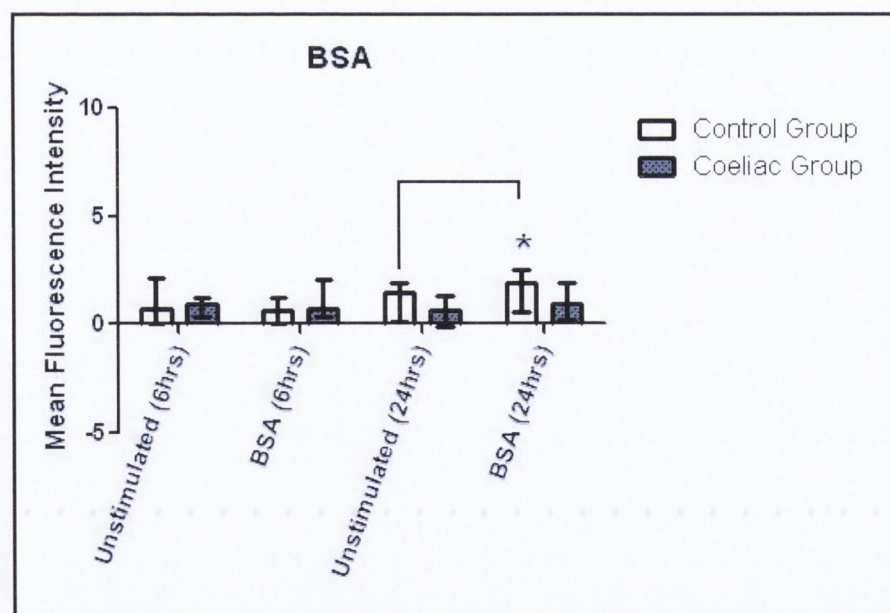


Figure 4.6: IL-15 surface expression on control and coeliac monocytes over time in response to BSA: each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects

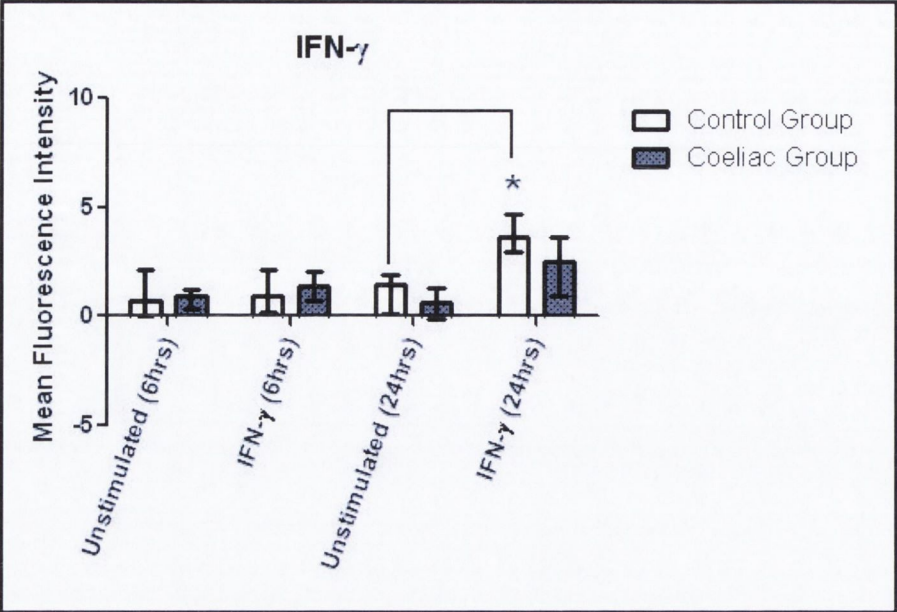


Figure 4.7: IL-15 surface expression on control and coeliac monocytes over time in response to IFN- γ : each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects

4.3.3 Effect of stimulation on intracellular IL-15 in blood monocytes

Control Group

At 6 hours no statistical significance fold change was found between unstimulated cells (interquartile range of 6.1 to 15.4) and cells stimulated with PT Gliadin (interquartile range of 8.2 to 16), BSA (interquartile range of 7.3 to 15.8) or IFN- γ (interquartile range of 7.3 to 16).

At 24 hours no statistical significance was found in PT Gliadin stimulated cells (interquartile range of 5.9 to 11) compared to unstimulated cells (interquartile range of 4.6 to 10.3) as seen in figure 4.8. Whilst the control peptide showed a statistical significance using the Wilcoxon signed rank test at 24 hours (interquartile range of 5.3 to 13) as seen in figure 4.9 this only represents a very marginal median fold increase of 1.2 compared to unstimulated cells. Statistical analysis of IFN- γ stimulated cells (interquartile range of 8.3 to 14) found a significant increase in intracellular IL-15 levels at 24 hours compared to unstimulated cells. On closer examination this increase was marginal compared to unstimulated cells with a median fold increase of 1.3 as seen in figure 4.10. In summary despite marginal increases in intracellular IL-15 induced by both BSA and IFN- γ stimulated cells at 24 hours, these fold increases were very small and no major changes could be seen in control monocytes upon stimulation with all 3 stimuli.

Coeliac Group

At 6 hours no statistical significant fold change was noted between unstimulated cells (interquartile range of 6.1 to 9.2) and cells stimulated with PT Gliadin (interquartile range of 5 to 10), BSA (interquartile range of 5.5 to 9) or IFN- γ (interquartile range of 4.1 to 10.3). No significant changes in intracellular IL-15 expression were seen at 24 hours in either PT Gliadin (interquartile range of 2.6 to 12.8), BSA (interquartile range of 4.1 to 11.8) or IFN- γ stimulated cells (interquartile range of 3.7 to 17.3) compared to unstimulated cells. In summary no significant difference in coeliac monocyte intracellular IL-15 levels could be seen at both time points in response to all stimuli tested as seen in figures 4.8, 4.9 and 4.10.

No statistical significance was found between the control and coeliac group.

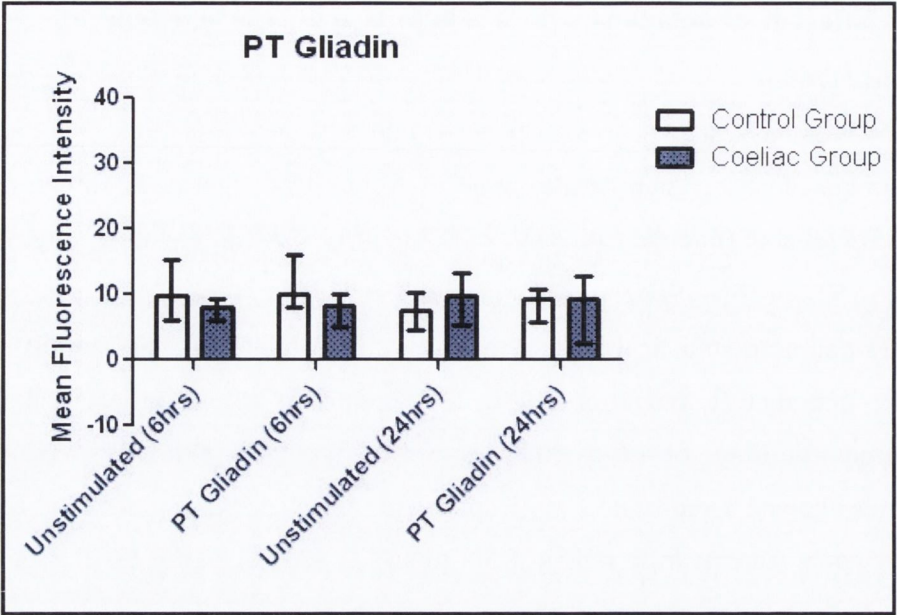


Figure 4.8: IL-15 intra-cellular expression in control and coeliac monocytes over time in response to PT Gliadin: each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects

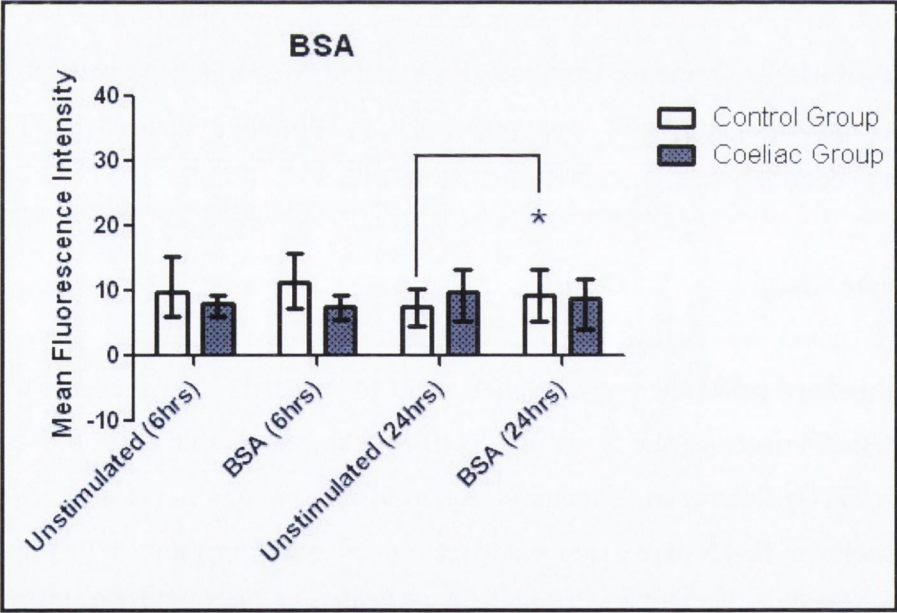


Figure 4.9: IL-15 intra-cellular expression in control and coeliac monocytes over time in response to BSA: each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects

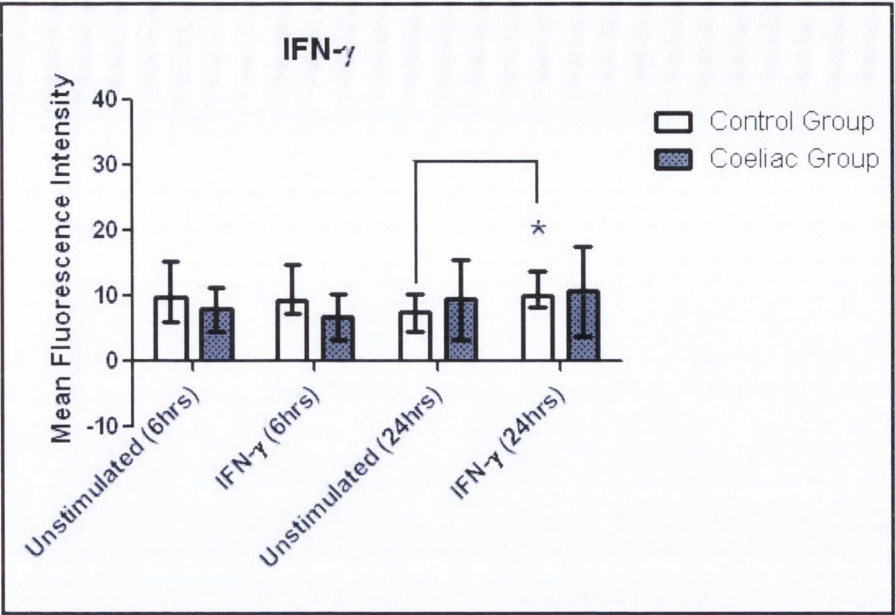


Figure 4.10: IL-15 intracellular expression in control and coeliac monocytes over time in response to IFN- γ . each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects

4.3.4 Effect of stimulation on nitrite production in peripheral blood mononuclear cells

Control Group

Nitrite levels were assessed at 24 hours in response to PT Gliadin and IFN- γ . As can be seen in figure 4.11, PT Gliadin showed a statistical significant increase in nitrite (interquartile range of 0.11 to 10.35) compared to unstimulated PBMCs (-0.3 to 6) with a median fold increase of 3.08 ($p < 0.05$). In contrast IFN- γ (interquartile range of -2.6 to 4) showed a decrease in nitrite upon stimulation which just barely reached significance ($p = 0.049$). However there is a wide variability in results as depicted in figure 4.11 with interquartile ranges.

Coeliac Group

PT gliadin (interquartile range of -1.96 to 4.6) caused an increase in nitrite production of PBMCs compared to unstimulated cells (interquartile range of 2.87 to 4.60) though this did not reach statistical significance as can be seen in figure 4.11. No statistical significance could be seen in cells stimulated with IFN- γ (interquartile range of -5 to 6.7).

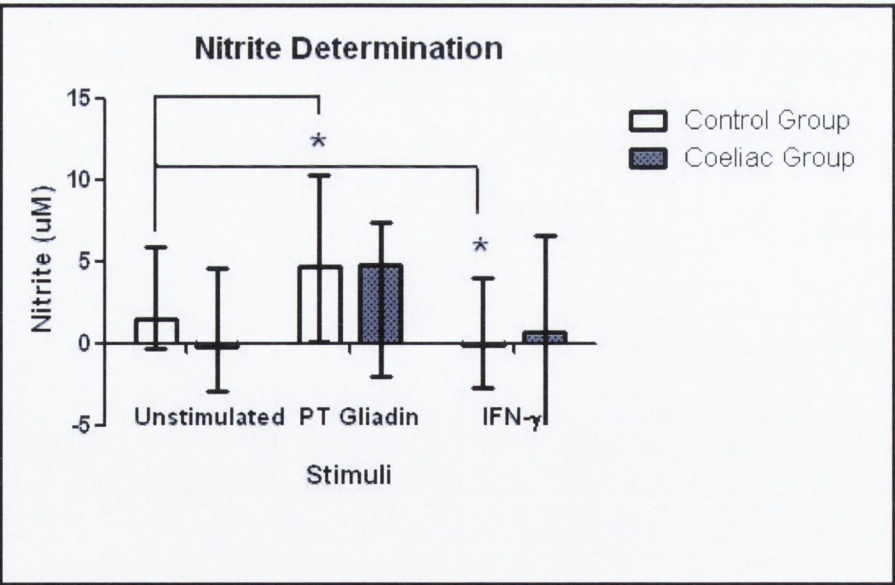


Figure 4.11: Nitrite production in control and coeliac peripheral blood mononuclear cells over time in response to PT Gliadin and IFN- γ . Each column represents the median $\pm$ the interquartile range of 10 control and 10 coeliac subjects.

4.3.5 Effect of stimulation on HLA expression in the Caco-2 cell line

Surface HLA-DR and HLA-ABC was assessed using the Caco-2 cell line in response to PT Gliadin and IFN- γ . A cell enzyme linked immunosorbent assay (CELISA) technique was used as adherent cells are quite cumbersome using flow cytometry which requires that cells are in a single cell suspension. Trypsinisation of samples to facilitate their detachment for flow cytometry may also interfere with markers on the cell surface. For this reason a CELISA was performed to assess HLA expression in response to stimulation using the Caco-2 cell line. As can be seen in figure 4.12 Caco-2 cell HLA-DR expression does not respond to PT gliadin or IFN- γ stimulation. Subsequently changes in HLA-ABC were assessed as this has previously been shown by other groups to increase with stimulation (Imanishi, Kamigaki et al. 2006). As can be seen in figure 4.13, whilst IFN- γ showed a significant increase in HLA-ABC expression compared to controls this was not seen in PT Gliadin stimulated cells. However co-efficient of variation values for unstimulated and PT Gliadin stimulated samples were > 100%. Poor sensitivity along with high background interference in the absence of antibody and wide inter-assay variability found this assay unsuitable for detecting surface markers in response to stimulation.

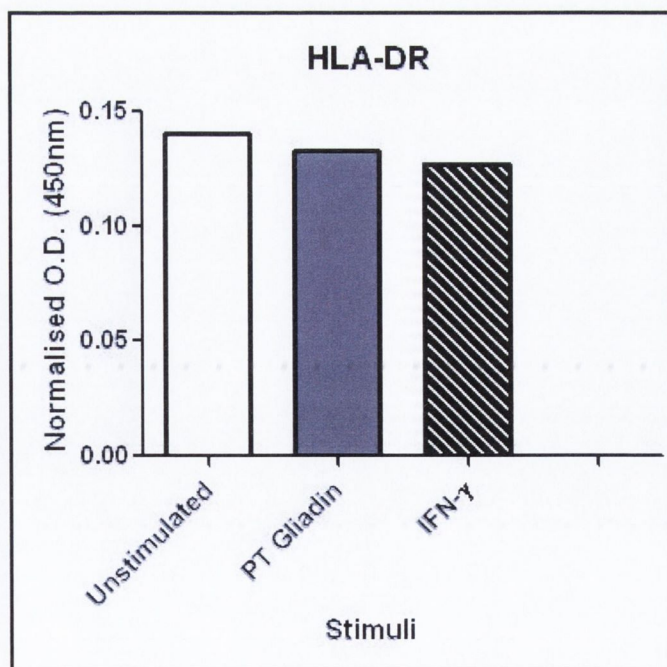


Figure 4.12: HLA-DR surface expression on the Caco-2 cell line in response to PT Gliadin and IFN- γ stimulation.

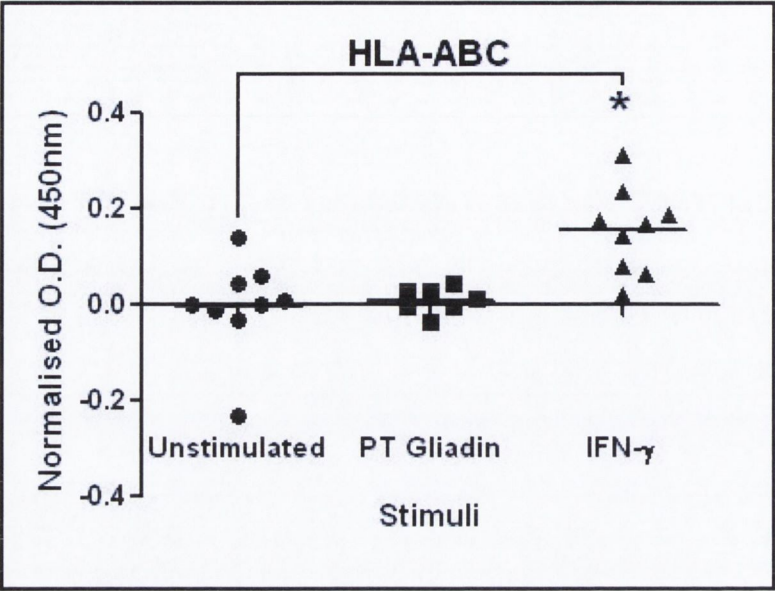


Figure 4.13: HLA-ABC surface expression on the Caco-2 cell line in response to PT Gliadin and IFN- γ stimulation.

4.4 Discussion:

Increasing evidence supports a direct effect of gliadin on cells of the innate immune system both in the small intestinal lesion and in circulation in coeliac subjects and in controls. In cells of the monocytic lineage this has been demonstrated using *in vitro* organ culture, cell lines and cells isolated from the intestine and in peripheral blood of human and murine origin. These studies show an up-regulation of antigen presenting cell capacity along with increases in pro-inflammatory cytokine secretion and nitrite production (Maiuri, Picarelli et al. 1996; Tuckova, Flegelova et al. 2000; Tuckova, Novotna et al. 2002; Maiuri, Ciacci et al. 2003; Jelinkova, Tuckova et al. 2004; Nikulina, Habich et al. 2004; Palova-Jelinkova, Rozkova et al. 2005; De Stefano, Maiuri et al. 2006; Thomas, Sapone et al. 2006; Cinova, Palova-Jelinkova et al. 2007). This study was performed to further investigate these findings by studying blood monocytes isolated from control and coeliac cohorts and testing their response to gliadin challenge. This was assessed by measuring alterations in HLA-DR and IL-15 expression and nitrite production.

In the study PT Gliadin caused a slight increase in monocyte HLA-DR expression in the control subjects when compared to unstimulated cells. This is not seen in the treated coeliac group. However, due to a similar effect with the control peptide (BSA), this response has not been found to be specific to gliadin fragments. PT Gliadin failed to elicit a direct effect on both surface and intra-cellular IL-15 levels in both study groups. Assessment of nitrite production in response to gliadin stimulation found a significant increase in the control group. In contrast, IFN- γ causes a significant increase in HLA-DR expression in both the control and coeliac group and caused a significant increase in both surface and intracellular IL-15 levels in the control group. IFN- γ showed a significant reduction in nitrite levels compared to unstimulated cells in the control group. To further investigate the roles of innate cells in antigen presenting function the enterocyte Caco-2 cell line was employed. Both gliadin and IFN- γ fails to elicit an effect on HLA-DR expression. IFN- γ but not PT Gliadin causes a significant increase in HLA-ABC expression.

HLA-DR

Lamina propria mononuclear expression of HLA-DR has been found to be up-regulated in treated coeliac biopsies cultured in the presence of gliadin using *in vitro* organ culture. This has found to be specific for the coeliac disease group (Maiuri, Picarelli et al. 1996; Maiuri, Ciacci et al. 2003). A previous study by another group using blood monocytes from a healthy donor population failed to show a significant increase in HLA-DR in response to gliadin or IFN- γ challenge (Cinova, Palova-Jelinkova et al. 2007). In contrast in this current study, PT Gliadin showed a marginal 1.5 median fold increase compared to unstimulated cells in HLA-DR expression within a healthy population that reached statistical significance. However as reactivity was also demonstrated with BSA, this effect was not specific to gliadin peptides. One possible explanation for increases in HLA-DR in peptide stimulated samples may be that this represents an immunosurveillance function whereby in the presence of potential antigenic stimuli, monocytes increase surface HLA-DR to sample antigenic material. No significant difference was seen in treated coeliac monocytes between unstimulated and PT Gliadin stimulated cells. Baseline levels of HLA-DR were higher on coeliac monocytes compared to control monocytes though this did not reach significance. Thus in the coeliac cohort, monocytes seem to be in a more activated state and HLA-DR does not increase significantly upon peptide challenge.

In contrast this study demonstrated a significant increase in HLA-DR expression in both the control and coeliac group in response to IFN- γ . IFN- γ is up-regulated within the coeliac lesion of patients with coeliac disease and is secreted by gliadin reactive T cells (Brandtzaeg 2006). This cytokine has previously been shown to increase HLA-DR in healthy donor blood monocytes and this study supports these findings (Littman, Dastvan et al. 1989; Varis, Deneys et al. 2001). This study demonstrated an increase in HLA-DR surface expression on blood monocytes of both control and treated coeliac disease patients upon IFN- γ challenge. As IFN- γ is not present in the circulation of healthy individuals in the absence of inflammation this HLA-DR induction would not be expected to occur (Kerr, Barah et al. 2001). However in coeliac patients in whom circulating IFN- γ is elevated, this may

stimulate coeliac monocytes to express significant amounts of HLA-DR (Hansson, Dannaeus et al. 1999; Ben-Horin, Green et al. 2006). As has been demonstrated previously, gliadin can induce maturation of monocytic derived dendritic cells with induction of antigen presentation associated markers such as HLA-DR (Palova-Jelinkova, Rozkova et al. 2005). In the presence of elevated levels of IFN- γ , circulating coeliac monocytes may increase the expression of HLA molecules also. Upon antigen uptake these cells through chemotaxis could home to the gut and increase antigen presentation and cytokine secretion in the small intestine. This would result in potentiating the existing inflammatory response. Such a hypothesis would suggest that circulating monocytes respond indirectly to gliadin through T cell derived IFN- γ produced during coeliac disease and that this is the principal inducer of HLA-DR on circulating monocytes. However a direct induction by PT Gliadin may also occur as shown in this study.

IL-15

Previous findings by other groups have shown PT Gliadin to increase IL-8 and TNF- α directly in blood monocytes from both control and coeliac patients though this does not reach statistical significance in the control group. (Cinova, Palova-Jelinkova et al. 2007). The number of IL-15 positive cells has shown to increase in coeliac disease biopsies challenged with gliadin using *in vitro* organ culture (Maiuri, Ciacci et al. 2003). In addition, increased IL-15 mRNA has been shown in response to gliadin challenge using murine macrophages (Thomas, Sapone et al. 2006). To further these findings on the role of gliadin in induction of pro-inflammatory cytokines, this study investigated the direct effect of gliadin on surface and intracellular levels of IL-15. Unlike a number of cytokines which become secreted, IL-15 is biologically active in a membrane bound form and both surface and intracellular stores of IL-15 have been shown in monocytes (Musso, Calosso et al. 1999; Neely, Robbins et al. 2001). The experiments described in this study did not support a direct role for PT gliadin in increasing surface bound or intra-cellular levels of IL-15 in both the control and treated coeliac disease group. In contrast, an increase in surface and intracellular IL-15 was noted in response to the control peptide (BSA). Although this reached statistical significance, the median fold increase was minimal compared to

unstimulated cells (median fold increase surface IL-15 of 1.3 and intracellular of 1.2) and was only seen in the control monocytes IFN- γ caused a significant increase of surface IL-15 in control monocytes with a median fold increase of 2.5 at 24 hours and whereas coeliac monocytes showed an increasing trend in surface IL-15 (mean fold increase of 3.8) this did not reach significance due to a wide range of variability between samples. IFN- γ also induced a minimal 1.3 fold increase in intra-cellular levels of IL-15. The findings here support a role for IFN- γ as a main inducer of IL-15 in control monocytes which concurs with previous findings of IFN- γ up-regulation of IL-15 in healthy donors (Musso, Calosso et al. 1999).

Nitrite production

Previously, using murine macrophage cell lines, gliadin in conjunction with IFN- γ has been shown to significantly increase nitric oxide production (Tuckova, Flegelova et al. 2000; Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003; De Stefano, Maiuri et al. 2006). In this current study the direct effect of gliadin on peripheral blood mononuclear cells in inducing nitrite using a normal and coeliac cohort was investigated. In this study PT Gliadin was found to induce a significant increase in nitrite, a marker of nitric oxide production in the control group. Similarly in treated coeliac disease in peripheral blood mononuclear cells, an increase was noted in response to PT Gliadin but did not reach significance over the time course studied. As a positive control was not incorporated within these experiments, definitive conclusions on the role of gliadin on iNOS activity cannot be made. In contrast IFN- γ showed a significant decrease in nitrite production in the control group. Though not significant, there also was a decline in nitrite production in coeliac PBMCs in response to IFN- γ stimulation. Discrepancies between these and previously reported results in response to IFN- γ may be due to the origin of the monocytic cells which represent the major producers of iNOS within PBMC preparations. IFN- γ represents a potent inducer of rodent monocytes but is less potent in *in vitro* activation of human monocytes (Martin and Edwards 1993; Martin and Edwards 1994; Sharara, Perkins et al. 1997). IFN- γ has previously been shown by other groups to require co-incubation with IL-4 to induce nitrite production (Kolb, Paul-

Eugene et al. 1994). As nitrite levels were assessed in a peripheral blood mononuclear population of cells it is reasonable to assume that these cells could produce factors in response to IFN- γ which may cause suppression of iNOS. Previously it has been shown that IL-4 and IL-13 can cause inhibition IFN- γ induced iNOS within lung epithelial cells (Berkman, Robichaud et al. 1996). A similar inhibition by cells distinct from blood monocytes within the PBMC preparation may be responsible for the decrease in iNOS activity. In the presence of oxyhemoproteins found in plasma, nitrite may become oxidized to nitrate and this would not be detected in the assay used in this study because high constitutive nitrate levels within the culture medium made it impossible to determine induced nitrate levels. Thus, nitrite levels alone may not indicate the true level of nitric oxide activity (Ignarro, Fukuto et al. 1993). However, in this study cells were washed free of plasma prior to incubation and thus the level of oxyhemoproteins present should have been negligible. In contrast to previous findings, this study supports a direct role of PT gliadin in induction of nitric oxide production within blood monocytes (Maiuri, De Stefano et al. 2003). The mechanisms involving PT Gliadin induction of nitrite, however, are unclear.

Enterocyte HLA expression

In this current study, investigations of enterocyte HLA-DR expression in response to gliadin and IFN- γ showed no significant difference between both stimulated and unstimulated cells. Caco-2 cells have previously been shown by other groups not to express HLA-DR on resting cells and that IFN- γ stimulation does not induce HLA-DR (Rodriguez-Juan, Perez-Blas et al. 2001). To further investigate the role of IFN- γ and PT gliadin on the expression of surface markers on enterocytes, alterations in surface expression of HLA ABC was assessed. Despite up-regulation of HLA-ABC by IFN- γ , PT Gliadin failed to elicit any direct effect. Thus using this cell system, gliadin did not appear to regulate either HLA-DR or HLA-ABC directly. IFN- γ up-regulation of HLA-ABC on Caco-2 cells has previously been described (Siavoshian, Blottiere et al. 1996; Imanishi, Kamigaki et al. 2006).

Summary

Based on the findings of the current study, IFN- γ is the principal inducer of HLA-DR, surface and intracellular IL-15 on blood monocytes. Gliadin does not appear to play a specific role in up-regulation of these molecules as similar effects were noted in response to the negative peptide. IFN- γ affects were more pronounced in the healthy control subjects. This study shows a direct role for PT Gliadin in nitrite induction particularly in control subject peripheral blood mononuclear cells with an increase also noted in the coeliac group.

GENERAL DISCUSSION

5.0 General Discussion

5.1 Background to study

Coeliac disease is defined by a well recognised, coeliac specific adaptive immune response to gliadin. These T cell mediated events include the infiltration of intraepithelial lymphocytes into the small intestinal mucosa which results in inflammation that is central to gliadin specific T cell responses and the modification of gliadin by deamidation for efficient adaptive activation. However, in recent times the direct role of gliadin as a triggering event of innate immunity in coeliac disease and its ability to influence subsequent effective activation of this specific adaptive response has been the focus of much research.

Initially the participation of innate immunity in coeliac disease pathogenesis appeared to be disease specific with early events triggered by unique peptides not found to trigger the T cell mediated responses, in coeliac patients. These initial innate events did not appear to occur in non-coeliac individuals suggesting that these direct gliadin-triggered events were also specific to coeliac disease patients (Maiuri, Picarelli et al. 1996; Maiuri, Ciacci et al. 2003). However, numerous studies with cell lines of both the enterocytic and monocytic lineage have shown gliadin induced effects in non-coeliac associated cells implying that the role of gliadin in innate modification occurs in the absence of disease susceptibility. These gliadin induced modifications include the maturation of dendritic cells, apoptosis induction in enterocyte cell lines and the secretion of cytokines and nitric oxide by monocytic cells not of coeliac origin (Giovannini, Luchetti et al. 1997; Elli, Dolfini et al. 2003; Giovannini, Matarrese et al. 2003; Maiuri, De Stefano et al. 2003; Jelinkova, Tuckova et al. 2004; Palova-Jelinkova, Rozkova et al. 2005). Through elucidation of the initial mechanisms that govern disease progression in genetically predisposed individuals, this would provide the basis of novel methods of disease intervention and therapy. This would help to determine why individuals with the disease susceptibility HLA genes proceed to develop coeliac disease despite the knowledge that 30% of the

general population with disease susceptibility genes remain unaffected (Dieterich, Esslinger et al. 2003).

The purpose of this study was to investigate the direct effects of gliadin on cells of the innate immune system to study its effect on tight junction integrity and to assess alterations in the expression of surface and intracellular molecules associated with the disease following gliadin exposure. As gliadin has been found to induce a number of biological modifications through the cytokine IL-15, the induction of this cytokine in both control and coeliac monocytes and the potential regulatory role of IL-15 on enterocyte enzyme gene expression and activity were assessed, along with the direct effect of IL-15 on epithelial barrier function. The regulation of the pro-inflammatory enzymes iNOS and COX-2, whose activity is up-regulated during coeliac disease, was investigated along with the effect of nitric oxide, a key iNOS metabolite on tight junction integrity. Cytokines associated with the adaptive branch of the immune response were also investigated to assess the potential role of innate versus adaptive responses in inducing the modifications under investigation.

5.2 Modifications in tight junction integrity are not gliadin induced

This study does not support a direct role for gliadin in alteration of epithelial barrier function despite previous findings of gliadin induced modifications (Clemente, De Virgiliis et al. 2003; Sander, Cummins et al. 2005; Drago, El Asmar et al. 2006). Gliadin failed to exert any effect on monolayer permeability over a wide range of concentrations tested using both T 84 and Caco-2 cells: the latter cell line was derived from two different laboratory sources to account for known heterogeneity of this cell line. Immunofluorescent staining for key tight junctional proteins failed to show any changes in tight junctional protein localisation in response to gliadin exposure. Previous findings of gliadin induced disruption of tight junctions have been attributed to zonulin, a mediator of tight junction permeability which is up-regulated in coeliac disease. In previous studies, disruption of barrier integrity has been shown in Caco-2 cells which were found to be zonulin responsive but not in T 84 cells which do not appear to have the necessary zonulin receptors for zonulin binding and activation (Drago, El

Asmar et al. 2006). However this study does not support a direct role for gliadin in tight junction disruption by a zonulin dependent or independent mechanism.

5.3 Novel role of IL-15 in disruption of barrier function

This study shows a novel role for IL-15 in disruption of tight junction integrity. Whilst significant decreases in TEER was not shown in both cell lines at all time points, discrete disruption of tight junctional proteins in both cell lines were noted. A speculative decrease in TEER may be expected with further disruption of tight junctional proteins with time. Administration of exogenous IL-15 to T 84 cell lines has previously been shown to take three to four days before any appreciable change in TEER (depicted by an increase in TEER) was shown (Nishiyama, Sakaguchi et al. 2001). As the present study was performed for 24 hours, this may have been too short a time frame to observe significant changes in TEER. However as both cell lines have previously been shown to express high levels of endogenous IL-15, this may also explain the non-responsiveness of both cell lines to exogenously administered IL-15 on TEER (Hue, Mention et al. 2004). Previous reports have shown positive regulation of IL-15 on TEER in T 84 cells. The T-84 cell line which lacks the RIL-15 β , upon transfection with RIL-15 β shows an increase in TEER compared to the parental T 84 cell line lacking the β chain. in the absence of exogenous IL-15 Administering exogenous IL-15 did not significantly increase TEER suggesting that over-expression of the RIL-15 β is the cause of increasing TEER in response to endogenous IL-15 and not due to the exogenous IL-15 (Nishiyama, Sakaguchi et al. 2001).

Subtle changes in tight junction patterns were found to occur in both cell lines in response to IL-15 in this study. Alterations in T84 cell lines in response to exogenous IL-15 have previously been reported by signalling via RIL-15 β dependent and independent mechanisms (Nishiyama, Sakaguchi et al. 2001) Incubation with IL-15 for 24 hours resulted in discrete areas of cell separation at ZO-1 contact points and these were seen in both T 84 and Caco-2 cell lines. The cell separation at ZO-1 contact points has previously been demonstrated in airway epithelial cells in response to IFN- γ and IL-4 (Relova, Shahana et al. 2005). The disruption of barrier function described in

this study caused by IL-15 may represent another feature of coeliac disease pathogenesis attributed to this cytokine. IL-15 has been shown to induce key features of innate immunity in coeliac disease including up-regulation of antigen presenting molecules and COX-2 on lamina propria mononuclear cells, induction of stress molecules such as MICA on enterocytes and the proliferation of intraepithelial cells and their subsequent secretion of IFN- γ in coeliac disease tissue. Some of these effects have also been demonstrated in healthy controls though not with the same magnitude of response (Maiuri, Ciacci et al. 2003; Hue, Mention et al. 2004; Di Sabatino, Ciccocioppo et al. 2006). Recently it has been shown that IL-15 induction in response to gliadin is not coeliac disease specific and occurs in healthy controls also (Bernardo, Garrote et al. 2007). Thus IL-15 production in response to gliadin exposure could result in disruption to epithelial barrier function as an early event in coeliac disease development. The cell separation demonstrated in this study would enable peptide fragments to cross paracellularly through the epithelium into the underlying mucosa. This could occur in both healthy and coeliac-susceptible individuals. However, IL-15 production in response to gliadin would be expected to be greater in disease susceptible individuals as coeliac disease patients have been shown to produce increased IL-15 compared to healthy controls (Di Sabatino, Ciccocioppo et al. 2006). Interaction of gliadin with tissue transglutaminase and activation of gliadin specific T cells may be the determining factor in disease development.

5.4 NO modification of barrier function

This study also demonstrated a role in nitric oxide disruption of barrier function. An increase in intestinal permeability was shown in both cell lines at 24 hours in response to nitric oxide though this only reached statistical significance in the Caco-2 cell line. Marked decreases were shown in TEER in two of the Caco-2 cell line experiments. Cell separation at ZO-1 contact points could be seen in T 84 cell experiments along with some unusual tight junctional protein patterns. In two of the Caco-2 experiments complete cell separation at ZO-1 contact points was seen in areas of the cell monolayer with internalisation of occludin and claudin-4. This is in agreement with findings using the Caco-2 clone BBe in which nitric oxide donors induced

increased permeability and widened tight junctions (Salzman, Menconi et al. 1995). Nitric oxide metabolites are reported to be increased in active coeliac disease in both the plasma and urine of patients and increased activity of iNOS, the nitric oxide producing enzyme has been found within the small intestine (ter Steege, Buurman et al. 1997; Laurin, Falth-Magnusson et al. 2003; Spencer, Daniels et al. 2004). No definitive roles have been demonstrated for this increased iNOS activity and this study supports a role for NO in regulation of intestinal permeability during inflammation. iNOS has been found to be up-regulated by IFN- γ in macrophages and this study, along with others have shown the up-regulation of enterocyte iNOS in response to IFN- γ (Chavez, Morin et al. 1999). NO upregulation may also occur as an early innate event through induction of macrophage iNOS by IL-15, as has previously been shown (Liu, Zhai et al. 2004). Thus during inflammation, nitric oxide generated as part of the inflammatory response to gliadin may disrupt barrier function resulting in uncontrolled entry of gliadin into the mucosa and thereby potentiate the inflammatory response. . However due to low sample number and morphological changes occurring in only some of the NO treated cells, it is difficult to interpret the NO effect. It would appear NO causes subtle changes in tight junctional proteins compared to untreated cells but that this effect is not always reproducible.

5.5 Gliadin and IL-15 do not directly stimulate enterocyte iNOS

Both gliadin and IL-15 in this study failed to directly regulate gene expression, protein production and activity of iNOS in Caco-2 cells. Gliadin has been shown to directly induce cytokines such as IL-8 and TNF- α in monocyte cell lines and increase iNOS activity in conjunction with IFN- γ in murine macrophages (Maiuri, De Stefano et al. 2003; Jelinkova, Tuckova et al. 2004). IL-15 has previously been found to induce iNOS activity within macrophages and gingival epithelial cells (Yanagita, Shimabukuro et al. 2002; Liu, Zhai et al. 2004). Studies in tumour bearing muscle cells have shown IL-15 down-regulation of iNOS protein production (Figueras, Busquets et al. 2004). In the present study both PT gliadin and IL-15 failed to have an effect on iNOS within the Caco-2 cell line.

5.6 COX-2 activity in enterocytes is not influenced by coeliac triggering factors

The investigation of a direct role for gliadin, IL-15 and IFN- γ on COX-2 gene expression and activity in enterocytes failed to show any regulatory role for all of the tested stimuli. COX-2 activity has been shown to be up-regulated in coeliac disease (Lavo, Knutson et al. 1990). Its induction upon gliadin exposure on coeliac cultured biopsies revealed a direct role for IL-15 in its induction within lamina propria mononuclear cells (Maiuri, Ciacchi et al. 2003). This study demonstrates that this is not the case within the enterocyte population as represented by the Caco-2 cell line.

5.7 Modifications in HLA-DR and IL-15 in blood monocytes are not disease specific

In this study, HLA-DR up-regulation was shown to occur in response to IFN- γ in both control and treated coeliac disease blood monocytes following 24 hours of stimulation. This is in contrast to previous findings which showed that IFN- γ incubation both in the absence of gliadin and in conjunction with gliadin failed to show an up-regulation in HLA-DR in healthy blood monocytes (Cinova, Palova-Jelinkova et al. 2007). However up-regulation of HLA-DR in healthy donors in response to IFN- γ has been previously described by other groups (Littman, Dastvan et al. 1989; Varis, Deneys et al. 2001). It can be reasonably assumed, however, in the absence of inflammation, no appreciable level of circulating IFN- γ would be present to induce these responses (Kerr, Barah et al. 2001). In contrast, coeliac disease patients show an increase in circulating IFN- γ during active disease and thus circulating blood monocytes in the presence of IFN- γ would demonstrate increased HLA-DR expression levels (Cataldo, Lio et al. 2003). Expression of HLA-DR on monocytes is indicative of cell activation and increased antigen presenting function: thus, in the presence of IFN- γ , coeliac blood monocytes would be in a heightened activation state capable of T cell activation (Kanakoudi-Tsakalidou, Debonera et al. 2001). IFN- γ was also found to induce both intracellular and surface IL-15 on control monocytes

but not on coeliac monocytes. IL-15 up-regulation by IFN- γ has previously been demonstrated in blood monocytes (Musso, Calosso et al. 1999).

PT gliadin caused a minimal increase in HLA-DR on control blood monocytes; however as this was also seen in BSA stimulated cells, this increase was not peptide specific. In contrast increased HLA-DR in coeliac patients was not found in response to PT gliadin stimulation. Baseline levels of HLA-DR were higher in coeliac monocytes compared to controls and gliadin stimulation did not cause a significant increase. PT gliadin did not induce IL-15 in either the control or coeliac study group contrary to findings of increased IL-15 expression on lamina propria mononuclear cells in coeliac biopsies cultured with gliadin (Maiuri, Ciacci et al. 2003). Thus IFN- γ appears to regulate HLA-DR and IL-15 but PT gliadin does not play a major role in induction of these molecules.

5.8 iNOS activity in response to PT gliadin and IFN- γ

This study showed a significant increase in iNOS activity in control blood peripheral blood mononuclear cells in response to gliadin exposure. Previous reports of murine macrophages have shown gliadin in the presence of IFN- γ to increase iNOS activity; however no direct induction by gliadin alone was found (Tuckova, Novotna et al. 2002; Maiuri, De Stefano et al. 2003). This current study demonstrates increased activity of iNOS in direct response to gliadin in the absence of IFN- γ in control peripheral blood mononuclear cells.. An increase in nitrite was also found in coeliac blood PBMCs but this failed to reach significance with the small sample number tested. However further studies are necessary to speculate on these preliminary results with inclusion of both negative and positive controls within the experiment IFN- γ exposure in this study was found to cause a decrease in iNOS activity in PBMCs from both control and coeliac but only reaching significance in the former. Discrepancies in results may be explained by species specific differences. Murine macrophages, which were used in previous experiments of gliadin co-administered with IFN- γ , have been shown to produce higher levels of nitric oxide in response to IFN- γ compared to human macrophages

and monocytes (Denis 1994; Sharara, Perkins et al. 1997). IFN- γ has been shown to require co-stimulation with IL-4 in human blood monocytes to induce iNOS activity (Kolb, Paul-Eugene et al. 1994).

5.9 Role of the innate and adaptive responses in coeliac disease

In this study the role of innate participation via direct induction by gliadin the innate cytokine IL-15 and NO was assessed in contrast to the adaptive immune response in which IFN- γ is the predominant cytokine. These effects were assessed in relation to tight junction integrity, expression of iNOS and COX-2 enzymes on enterocytes and the expression of monocytes surface and intracellular molecules. Direct effects were shown with IL-15 and nitric oxide by subtle disruption of tight junction integrity. The direct induction (though minimal) of HLA-DR by gliadin was also demonstrated along with gliadin induction of nitrite in peripheral blood mononuclear cells. In contrast, IFN- γ was shown to have pleiotrophic effects as shown by alteration of barrier function, induction of iNOS gene expression in enterocytes and up-regulation of HLA-DR and IL-15 in blood monocytes. Thus whilst this study supports the direct role of innate immunity on influencing key changes noted in coeliac disease, it also supports the fundamental role of cytokines up-regulated in the adaptive phase of coeliac disease namely IFN- γ , in successful disease development.

5.10 Novel findings of thesis and future work

This study has demonstrated a number of novel findings which require further characterisation and study. The direct role of IL-15 in tight junction integrity has not been reported before and represents a potential key role for IL-15 in augmenting disease development by facilitating the entry of gliadin through the epithelium where it can interact with the underlying T cell rich mucosa. Further studies would involve the isolation of coeliac tissue and assessment of IL-15 exposure on tight junction integrity and comparing these findings to control, healthy tissue. Tracer flux experiments and time-course confocal imaging of gliadin peptide transport across the epithelium would also provide invaluable information on the mechanism of gliadin entry This

finding supports the already strongly held viewpoint regarding the potential use of anti-IL-15 therapy as a method of coeliac disease intervention. Paracellular tracer flux experiments would also demonstrate whether altered permeability occurs in response to IL-15 stimulation.

Another novel finding was the direct positive regulation of iNOS activity in blood monocytes by gliadin. Understanding the source of iNOS and its role in coeliac disease would give a better understanding of why nitric oxide products are elevated during disease. This study has also suggested a potential role for nitric oxide in disruption of barrier function.

The source of COX-2 and its regulation was not determined in this study. However, data relating to COX-2 activity and regulation in coeliac disease would address the potential use of COX-2 inhibitors within disease and would help to define whether prostaglandins up-regulated during active disease play a pro-inflammatory or anti-inflammatory role. Cell culture experiments using enterocytes isolated from coeliac tissue along with analysis of other potential sources of COX-2 e.g T lymphocytes and the subsequent regulation of COX-2 may provide key information on the role of this enzyme in disease.

This study supports the viewpoint that the innate immune response in coeliac disease is not disease specific and can be shown in non-coeliac cells. This indicates that whilst innate immunity may influence the effective activation of the adaptive response, it does not appear to be the determining factor in the development of coeliac disease in genetically pre-disposed individuals.

IFN- γ has been shown in this study to regulate a number of effects. It is important to note however that the source of IFN- γ may not be the specific CD4⁺ adaptive cells but also could be derived from intraepithelial lymphocytes which form an important part of innate immunity. Intraepithelial cells have been found to be as equally an important source of IFN- γ during disease (Brandtzaeg 2006). Recently it has been shown that IL-15 can induce significant IFN- γ secretion in both healthy and coeliac disease intraepithelial

cells (Di Sabatino, Ciccocioppo et al. 2006). Thus even the IFN- γ specific effects described in this study may be mediated in the absence of T cell activation by the direct role of gliadin induced IL-15. This demonstrates the increasing importance of studies with IL-15 and determination of its relationship to key aspects of disease induction, including its effect on breachment of the epithelium which is a necessary pre-requisite for entry of gliadin to trigger disease.

In summary

This study supports important roles for IL-15 in tight junction integrity and iNOS activity along with a potential function of nitric oxide in coeliac disease pathogenesis through disruption of tight junctions. This study demonstrates the importance of IFN- γ in its regulation of both monocytes and enterocyte function. Whilst some direct effects for PT gliadin in iNOS activity have been shown, gliadin was not the major inducer of monocyte or enterocyte activity.

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APPENDICES

APPENDIX I: REAGENTS

10X Phosphate buffered saline (PBS)

80g NaCl

2g KCl

11.5g Na₂HPO₄

2g KH₂PO₄

Make up to 1 litre with distilled water

Store at 4°C

Adjust pH to 7.6.

1 X PBS (working solution)

10 X PBS was diluted 1:10 with distilled water

50 X Tris acetate EDTA (TAE)

242 g Tris base

57.1ml Acetic acid

18.6 g EDTA

Make up to 1 litre with distilled water

Store at room temperature

Adjust to pH 7.6

1 X TAE (working solution)

50 X TAE was diluted 1:50 with distilled water

4% Paraformaldehyde (Cellular ELISA fixative)

800mg paraformaldehyde dissolved in 20 mls 1 x PBS

Heat to 56°C to dissolve

Store at 4°C

1% Sodium dodecyl sulphate (SDS) (Cellular ELISA)

500mg SDS dissolved in 50mls 1 x PBS

Store at room temperature

Flow Cytometry buffer (for intracellular staining)

5% foetal calf serum, 1mM EDTA, 0.05% saponin

50mg saponin

5mls foetal calf serum

37.22gms EDTA

Make up to 100mls with 1 x PBS

Blocking buffer (Immunofluorescent staining)

5% Normal goat serum in 1 x PBS

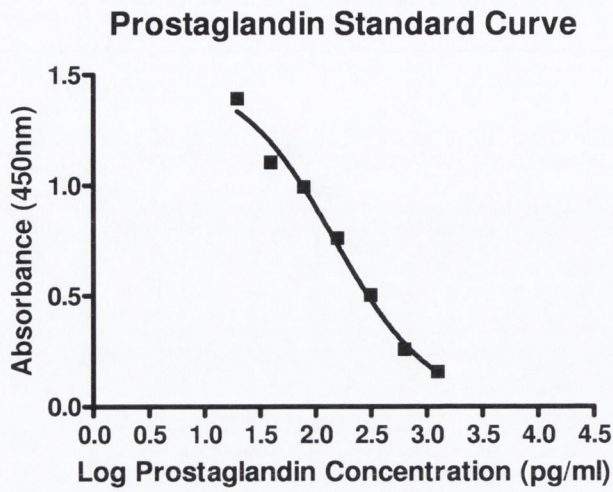
2mls normal goat serum

38mls 1 x PBS

APPENDIX II: mRNA AND cDNA QUANTIFICATION FOR REAL TIME PCR

Stimulus	Expt. No.	Incubation time (hours)	mRNA conc. (ng/ul)	cDNA conc. (pg/ul)
Unstimulated	1	4	166.1	809.92
PT Gliadin	1	4	125	883.85
IL-15	1	4	111.5	993.75
IFN	1	4	98.38	856.88
Unstimulated	1	30	167.7	893.84
PT Gliadin	1	30	209.2	942.8
IL-15	1	30	118.8	768
IFN	1	30	134.4	941.8
Unstimulated	2	4	57.33	915.85
PT Gliadin	2	4	81.73	664.43
IL-15	2	4	98.23	1078.27
IFN	2	4	38.19	579.89
Unstimulated	2	30	152.4	711.16
PT Gliadin	2	30	134.41	973.7
IL-15	2	30	139.36	617.71
IFN	2	30	168.97	786.8
Unstimulated	3	4	108.48	764.55
PT Gliadin	3	4	111.32	822.4
IL-15	3	4	116.39	1015.97
IFN	3	4	108.25	1053.79
Unstimulated	3	30	224.51	1062.64
PT Gliadin	3	30	195.49	802.38
IL-15	3	30	192.47	862.45
IFN	3	30	181.98	855.78
Unstimulated	4	4	100.07	1401.5
PT Gliadin	4	4	51.43	1373.04
IL-15	4	4	130.09	1215.68
IFN	4	4	55.45	1198.94
Unstimulated	4	30	110.27	1660.98
PT Gliadin	4	30	82.18	1383.09
IL-15	4	30	52.39	1212.33
IFN	4	30	71.84	1085.11

APPENDIX III: COX-2 Activity



Standards (pg/ml)	Absorbance
4.9	1.999
9.8	1.952
19.6	1.893
39	1.8275
78	1.54

Standard Curve Raw Data for Prostaglandin Determination

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	4hrs	30hrs	4hrs	30hrs	4hrs	30hrs	4hrs	30hrs
Unstimulated	2.126	2.155	2.063	1.798	1.989	1.826	1.985	1.679
PT Gliadin	2.113	2.089	1.978	1.921	1.926	1.856	1.879	1.587
IL-15	2.014	1.912	1.737	1.946	1.930	2.083	1.915	1.888
IFN-g	2.069	2.014	1.857	2.003	1.991	2.179	1.919	2.065

Raw data of Prostaglandin E₂ ELISA: Values represent absorbance values determined at a wavelength of 450nm. As can be seen from standard curve all sample values fall outside of the detectable range of the assay.