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PATHOGENETICS MECHANISMS OF CELIAC DISEASE

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I wish to dedicate this work to my parents and all my family which always trusted in me.

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1. Summary

Celiac disease (CD) is an enteropathy caused by permanent intolerance to gluten/gliadin, in genetically susceptible individuals (Sollid 2000; Shan *et al.*, 2002). This pathology is characterized by the presence of anti-tissue transglutaminase antibodies in the serum and by damage at the level of the small intestine with villous atrophy, intraepithelial lymphocyte infiltration, chronic inflammation and activation of lamina propria T cells. Nevertheless, patients go into remission when they are put on a gluten-free diet (Sollid, 2002) The mechanisms by which gluten/gliadin damages the intestinal mucosa of coeliac patients have not been elucidated. Different hypotheses have been proposed to explain the origin of the mucosal damage. Some reports include a direct toxic effect of gliadin on intestinal mucosa (Auricchio *et al.*, 1990; Maiuri *et al.*, 1996). Most observations favour a dysregulated immune response to gluten-derived peptides in coeliac patients. Toxic gluten peptides are absorbed across the epithelium, presented in associated with HLA class-II molecules by macrophages in the lamina propria and recognised by gliadin antigen specific $CD4^+$ T cells (Marsh, 1992; Sollid, 2000). As a result, secreted mediators, including interferon-γ (IFN-γ), may cause activation of macrophages which, in turn, produce pro-inflammatory cytokines contributing to the damage of the mucosal matrix (Przemioslo *et al.*, 1994; Kontakou *et al.*, 1995; Nilsen *et al.*, 1995; Pender *et al.*, 1996). It has been reported that nitric oxide (NO) and prostaglandins (PGs) may play an important role in the mucosal lesion (Beckett *et al.*, 1999; Lavo *et al.*, 1990). High levels of nitric oxide products (nitrate/nitrite) in the urine of children with active CD have been correlated with the expression of inducible nitric oxide synthase (iNOS) in the small intestine (Holmegren Peterson *et al.*,1998; van Straaten *et al.*, 1999; ter Steege *et al.*, 1997). Increased

amounts of prostaglandin E_2 (PGE_2) in homogenized small bowel biopsies from patients with active CD have been detected (Lavo *et al.*, 1990). Recently, it has been reported that lamina propria cells from coeliac patients produce high levels of cyclooxygenase-2 (COX-2) (Kainulainen *et al.*, 2002). It has been shown that in CD both enterocytes and cells with macrophage-like morphology in the lamina propria are responsible for increased iNOS expression (ter Steege *et al.*, 1997). Recent report has shown that gluten or gliadin and their proteolytic fragments enhanced NO production and iNOS mRNA level in mouse peritoneal macrophages stimulated with IFN-γ (Tuckovà *et al.*, 2000). A common paradigm for the pathogenesis of CD is that several genes whose expression is induced in the inflamed mucosa, such as those encoding for iNOS and COX-2, contain κB sites for Nuclear Factor-κB (NF-κB) (Xie *et al.*, 1994; Yamamoto *et al.*, 1995). NF-κB has been identified in epithelial cells and lamina propria macrophages from biopsy specimens or cultured cells of patients with Crohn's disease, ulcerative colitis and unspecific colitis (Schmid and Adler, 2000). Recently it has been assessed IRF-1 and STAT-1α expression in the mucosa of active CD (Mazzarella *et al.*, 2000). The promoter region of the iNOS gene has been characterized in different species, including human, rat and mouse (Xie *et al.*, 1993; Chu *et al.*, 1995; Eberhardt *et al.*, 1996). Sequence analysis of promoter revealed the presence of consensus motifs for binding of transcription factors, such as NF-κB, interferon regulatory factor-1 (IRF-1) and signal transducer and activator of transcription-1α (STAT-1α), which are essential for the iNOS induction by IFN-γ (Kamijo *et al.*, 1994; Martin *et al.*, 1994; Weisz *et al.*, 1994; Gao *et al.*, 1997; Kim *et al.*, 1997). The aim of this present doctoral thesis was to investigate the molecular mechanisms involved in the pathogenesis of celiac disease. In

the first phase of the study, I have investigated *ex vivo* NF-κB activation in intestinal biopsy specimens from patients with active CD. In the second phase of the study, I have investigated *in vitro* the effect of gluten-derived peptides on iNOS gene expression in RAW 264.7 macrophages stimulated with IFN-γ, a murine cell line. In the third phase of the study, I have further investigated *in vitro* the synergistic induction of iNOS gene expression by gliadin plus IFN-γ focusing on the differential kinetics of activation and expression of NF-κB, IRF-1 and STAT-1α.

2. Celiac disease

2.1 Introduction

Celiac disease (CD) is a gluten sensitive entheropathy in which ingested wheat gluten or related proteins from rye and barley are not tolerated (Trier, 1991). CD has a chronic nature where particular HLA alleles are overrepresented among the patients (Thorsby, 1997). Usually these disorders are multifactorial, due to HLA genes wrong expression and environmental factors. Moreover the expression of CD is strictly dependent on dietary exposure to gluten and similar cereal protein (Trier, 1991). Patients go into complete remission when they are put on a gluten-free diet and relapse when gluten is reintroduced into the diet. From this point of view CD is the unique, among the chronic inflammatory HLA-associated diseases, in that a critical environmental factor has been identified. CD is mostly recognized among Europeans and presents in early childhood with classic symptoms including chronic diarrhoea, abdominal distension and failure to thrive (Schmitz, 1992). Anyway the disorder may also present later in life with symptoms that tend to be more vague.

CD patients have increased levels of serum anti-bodies to a variety of antigens, including gluten and the auto-antigen tissue transglutaminase (tTG) (Dieterich *et al.*, 1997). Testing of serum antibodies to gluten and tissue tTG is utilized to predict CD. The final diagnosis rests on the demonstration of typical mucosal damage by histhological examination of small intestinal biopsies. Current treatment of CD is a lifelong exclusion of gluten from the diet. Patients who do not observe this diet may have complications including anemia, infertility, osteoporosis and intestinal lymphoma. Moreover untreated CD is associated with increased mortality (Maki and Collin, 1997).

The lesion in CD is localized in the proximal part of the small intestine and is characterized by villous atrophy, crypt cell hyperplasia, lymphocytic infiltration of the

epithelium and increased density of various leukocytes in the lamina propria (Figure 1). These alterations have been classified into three stages:

- infiltrative lesion, chraracterized by infiltration of small nonmitotic lymphocytes in the villous epithelium
- hyperplastic lesion, similar to infiltrative but in addition has hypertrophic crypts whose epithelium may be infiltrated by lymphocytes
- destructive lesion, in which villi appear totally atrophic.

Oral challenge with gluten have demonstrated that these stages are dynamically related.

Many populations participate at mucosal lesion: enterocytes, intraepithelial lymphocytes, lamina propria leukocytes and the extracellular matrix (ECM).

In CD there is a loss of epithelial cells and increased proliferation of epithelial cells in the crypts. Both these factors have been used to explain the villous atrophy found in CD (Booth, 1970).

The two phenomena probably reflect increased apoptosis of enterocytes whereas the increased enterocyte proliferation in the crypts may be due to an increased production of keratinocyte growth factor (KGF) by stromal cells (Bajaj-Elliot *et al.*, 1998).

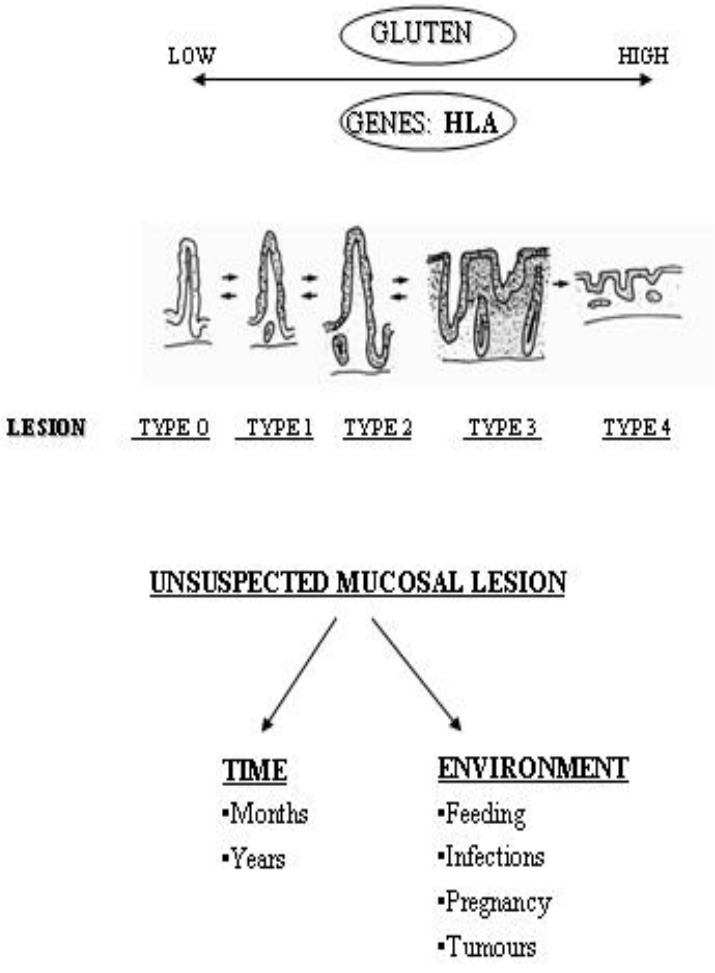

Figure 1. Mucosal lesion phases in patients with celiac disease

This population returns to normal when gluten is removed from the diet while the in the normal human intestine occur three major lineages of intraepithelial lymphocytes (IELs); the most prominent as the TCRαβ+ $CD8^+$ $CD4^-$. TCRγδ+ remains at elevated levels. However both the lineages express the Ki67 proliferation marker, suggesting intraepithelial proliferation of both populations in CD (Halstensen and Brandtzaeg, 1993a). Activated human IELs are able to produce a number of cytokines including IFN-γ, IL-2, IL-8 and TNF-α which are known to have a lytic potential (Lundqvist *et al.*, 1996). A marked infiltration of TCRαβ+ T cells appears in the lamina propria in the active lesion. These cells are mostly $CD4^+$ and carry a memory phenotype. Moreover, there seems to be a particular increase in cells producing IFN-γ since mRNA for IFN-γ has been found to be increased more than 1000 fold in untreated disease (Nilsen *et al.*, 1998). A characteristic of CD lesion is an accumulation of IgA, IgM and IgG producing plasma-cells. In the normal mucosa a high number of macrophage/dendritic cells which express abnormally HLA genes. Furthermore, increased ECM degradation has been suggested to play a role in the villous atrophy. Infact this is supported by the demonstration of a decreased ratio of cells expressing collagen I and tissue inhibitor of metalloproteinase-1 (MMP-1) and -3 mRNA in untreated CD (Daum *et al.*, 1999). Expression of MMP-1 and MMP-3 mRNA is mainly localized to subepithelial fibroblasts and macrophages. It is likely that the increased expression of metalloproteinases is related to activation of mucosal T cells.

2.2 Molecular mechanisms

2.2.1 Genetic aspects

A high prevalence rate among first degree relatives of CD patients indicates a strong genetic influence on susceptibility to develop CD (Ellis, 1981). This strong influence is further supported by a high concordance rate in monozygotic twins (Polanco *et al.*, 1981).

CD was first found to be associated with the HLA class I molecule B8. Later stronger association were found to the HLA class II molecules DR3 and DQ2, both containing the B8-DR3-DQ2 or the B18-DR3-DQ2 extended haplotypes (Alper *et al.*, 1987; Congia *et al.*, 1992). CD is also associated with DR7. In many studies patients have been shown to carry either the DR3-DQ2 haplotypes or are DR5-DQ7/DR7-DQ2 heterozygous. Probably CD patients with these DR-DQ combinations share the genetic information conferring CD susceptibility (Sollid and Thorsby, 1993). It can be argued that susceptibility for CD depends on an interaction between at least two genes on the DR3-DQ2 haplotype that are reunited in DR5-DQ7/DR7-DQ2 heterozygous individuals (Figure 2). Theoretically this gene interaction could involve any HLA-linked genes in the DQ region. However, complete sequencing of an 86-kb genomic fragment spanning the DQ subregion of the DR3-DQ2 haplotype failed to identify genes other than DQA1 and DQB1 in this region (Ellis, 1997). Furthermore, the DQA1 and DQB1 are very good candidates because their products interact by forming a class II heterodimer and because they are situated close to the putative recombination site. This evolutionary consideration together with the fact that most CD patients share a particular pair of DQA1 and DQB1 genes located either in *cis* or in *trans* are strong arguments that these alleles jointly confer susceptibility to CD.

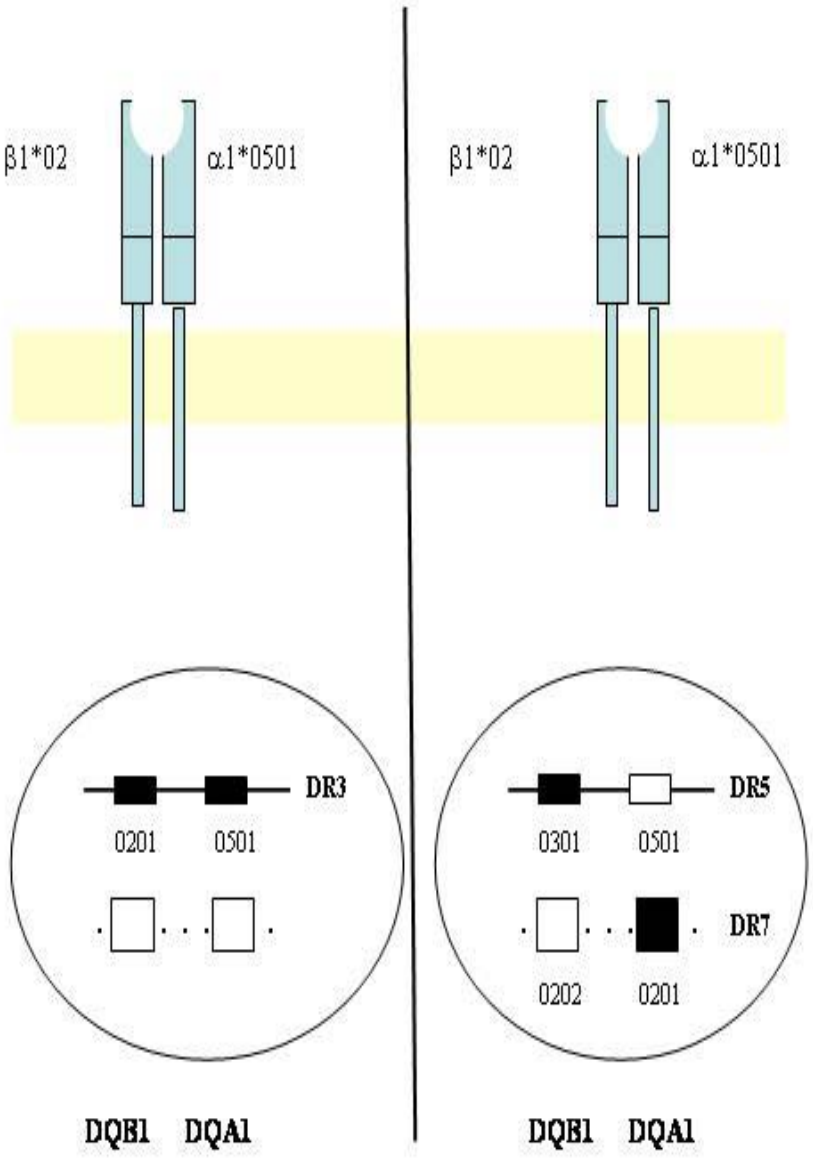

Figure 2. Differences in CD susceptibility genetic information

From Molecular basis of Celiac Disease, Sollid LM, Annu Rev Immunol (2000) 18:53-81

Genes located in the HLA gene complex other than DQ might also contribute to CD susceptibility. Association to particular DP alleles have been reported in different population, but many of these association can be explained by linkage disequilibrium between the involved DP and the DQA1 and DQB1 alleles (Sollid and Thorsby, 1993). Studies of Irish CD patients have indicated an additional predisposing role of TNF genes, an association independent of DQ2 that has been demonstrated using a microsatellite polymorphism situated near rhe TNF genes (McManus *et al.*, 1996). Moreover, a polymorphism of the TNF-α gene promoter has been demonstrated to be a component of the DR3-DQ2 haplotype. Other studies failed to demonstrate the finding of a DQ2-indipendent association of the TNF microsatellites but there could be a discrepancy related to population differences. Thus available data suggest that susceptibility to develop CD is primarily associated to two conventional peptide-presenting DQ molecules DQ2 and to a lesser extent DQ8. An issue to be clarified is whether there are additional molecules encoded by unidentified genes in the HLA gene complex that also contribute to the genetic predisposition for CD. However any effect of these additional genes is likely to be moderate. A key question for the understanding of the molecular basis for CD is therefore to define the functional role of the DQ2 and DQ8 molecules. Peptides binding to DQ2 have anchor residues in the relative positions P1, P4, P6, P7 and P9 (Johansen *et al.*, 1996; van de Wal *et al.*, 1997). The binding motif of DQ8 is different from that of DQ2, but DQ8 also dispays a preference for binding negatively charged residues at several positions (Figure 3). Anyway both the DQ2 and DQ8 molecules share a preference for negatively charged residues at some of their anchor positions. The DQ2 and DQ8 molecules could confer susceptibility to CD by presenting disease-related peptides in the target organ or alternatively by shaping the

T cell repertoire during T cell development in the thymus. This issue has been addressed by studies of T cells derived from the celiac lesion. Stimulation of small intestinal biopsy specimens with a peptic/tryptic digest of gluten induces rapid activation of the T cells in the lamina propria of CD patients, but not of non-CD control-subjects (Halstensen *et al.*, 1993b). Gluten-reactive T cells can be isolated and propagated from intestinal biopsies of CD patients but not from non-CD controls (Molberg *et al.*, 1997). These T cells are $CD4^+$ and use the αβ TCR. Importantly, T cells isolated from biopsy specimens of patients carryiing the DR3-DQ2 haplotype typically recognize gluten fragments presented by the DQ2 molecule rather than the other HLA molecules carryed by the patients (Lundin *et al.*, 1994). Both DR3-DQ2-positive and DR5-DQ7/DR7-DQ2-positive antigen-presenting cells are able to present the gluten antigen to these T cells. Interestingly, gluten-specific T cells can also be found in the peripheral blood. These T cells are restricted either by DR, DP or DQ molecules and they do not therefore display the predominant DR, DP or DQ restriction observed for gluten-specific T cells from the intestinal mucosa (Gjertsen *et al.*, 1994).

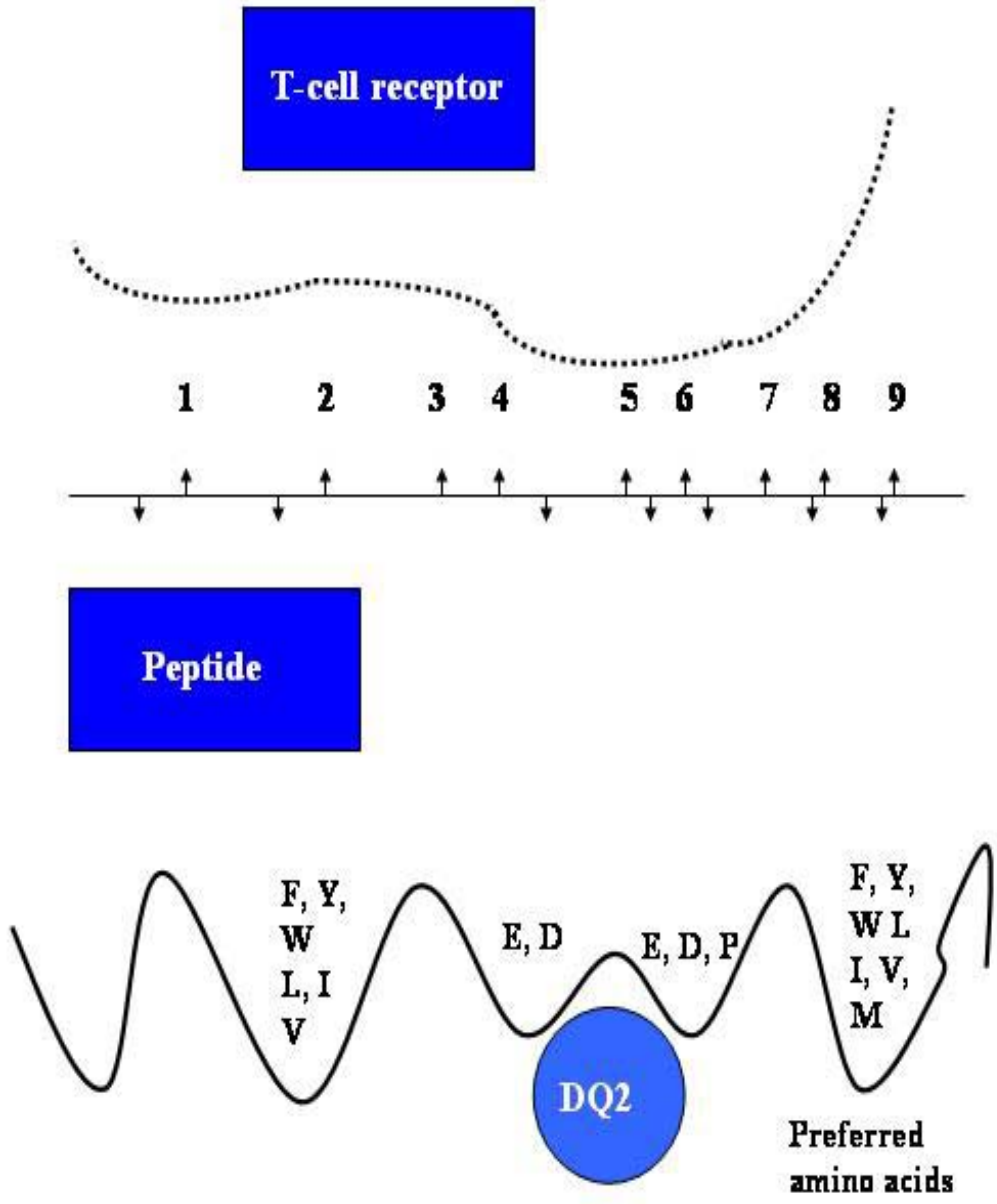

Figure 3. Schematic depiction of the peptide binding groove of HLA-DQ2

From Molecular basis of Celiac Disease, Sollid LM, Annu Rev Immunol (2000) 18:53-81

One explanation for this could be that the majority of gluten-specific T cells of peripheral blood recognize epitopes different from those recognized by T cells of the small intestine. Studies of lamina propria T cells *in situ* have indicated that gluten-reactive T cells have a cytokine profile dominated by IFN-γ. This notion is sustained by the characterization of gut-derived DQ2 and DQ8-restricted gluten-specific T cell clones. These T cells uniformly secrete IFN-γ at high concentrations and some produce IL-4, IL-5, IL-6, IL-10, TNF-α and TGF-β (Nilsen *et al.*, 1995).

2.2.2 T cells activation by gluten peptides

Wheat gluten is a mixture of numerous proteins grouped into the gliadin and glutenin fractions. These proteins serve as a source of nitrogen and carbon for the growing seedling during germination. A vast sequence heterogeneity among gliadin and glutenin probably reflects that these proteins have been subjected to few structural constraints during evolution. Generally, gluten proteins contain a large percentage of proline and glutamine residues, while many other amino acids, including aspartic and glutamic acid, are unusually scarce. Feeding experiments have demonstrated that the gliadin fraction can precipitate CD (van de Kamer *et al.*, 1953), whereas the role of glutenin is still inconclusive. Proteins of the gliadin fraction can be subdivided according to their sequence into the α-, γ- and ω-gliadins (Shewry *et al.*, 1992) (Table 1). A large number of different gliadins exist within each of these gliadin families. Estimates suggest that as many as 50 to 150 different α-gliadin genes may be present in a single wheat cultivar (Anderson *et al.*, 1997). For the work of identifying peptide fragments recognized by T cells, the complexity of this antigen presents a big challenge.

Table I. Unique epitopes recognized by gut T cells

Epitope	Derived from protein	Presentation element	Recognized by patients
DQ2-γ-I-gliadin	γ-gliadin	DQ2	Infrequently
DQ2-α-I-gliadin	α-gliadin	DQ2	Frequently
DQ2-α-II-gliadin	α-gliadin	DQ2	Frequently
DQ8-α-I-gliadin	α-gliadin	DQ8	Frequently?
DQ8-I-glutenin	Glutenin	DQ8	Not known

From Molecular basis of Celiac Disease, Sollid LM, Annu Rev Immunol (2000) 18:53-81

The stimulatory capacity of gliadin preparations for gliadin-specific intestinal T cells is significantly enhanced following treatments at high temperatures and low pH (Lundin *et al.*, 1997). These conditions are known to cause nonspecific deamidation of glutamines to glutamic acid and many thus convert gliadin from a protein with very few peptides with the potential to bind DQ2/DQ8 into one with many such. Five unique epitopes of gluten that are recognized by gut T cells have been identified: three restricted by DQ2 (Sjostrom *et al.*, 1998) and two restricted by DQ8 (van de Wal *et al.*, 1999). The first ones fail to stimulate T cells in their native form but are potent antigens when a single glutamine residue is exchanged with glutamic acid in certain positions. The second ones recognition is augmented by introduction of negatively charged residues (van de Wal *et al.*, 1998). These data demonstrate that most gluten-specific intestinal T cells from CD patients recognize gluten proteins only after they have undergone deamidation. Moreover, the results with the gluten epitope demonstrate that intestinal T cells can recognize gluten proteins other than gliadins (van de Waal *et al.*, 1999). The deamidation of gliadin *in vivo* may take place in the acidic environment in the stomach (Sjostrom *et al.*, 1998) or, altenatively, it can be mediated by the enzyme tissue tansglutaminase (tTG) which is expressed in many different tissues and organs (Molberg *et al.*, 1998). In the small intestine it is expressed just beneath the epithelium in the gut wall (Figure 4). This enzyme is present both intracellularly and extracellularly and in the extracellular environment plays a role in the extracellular matrix assembly, cell adhesion and wound healing (Bruce *et al.*, 1985).

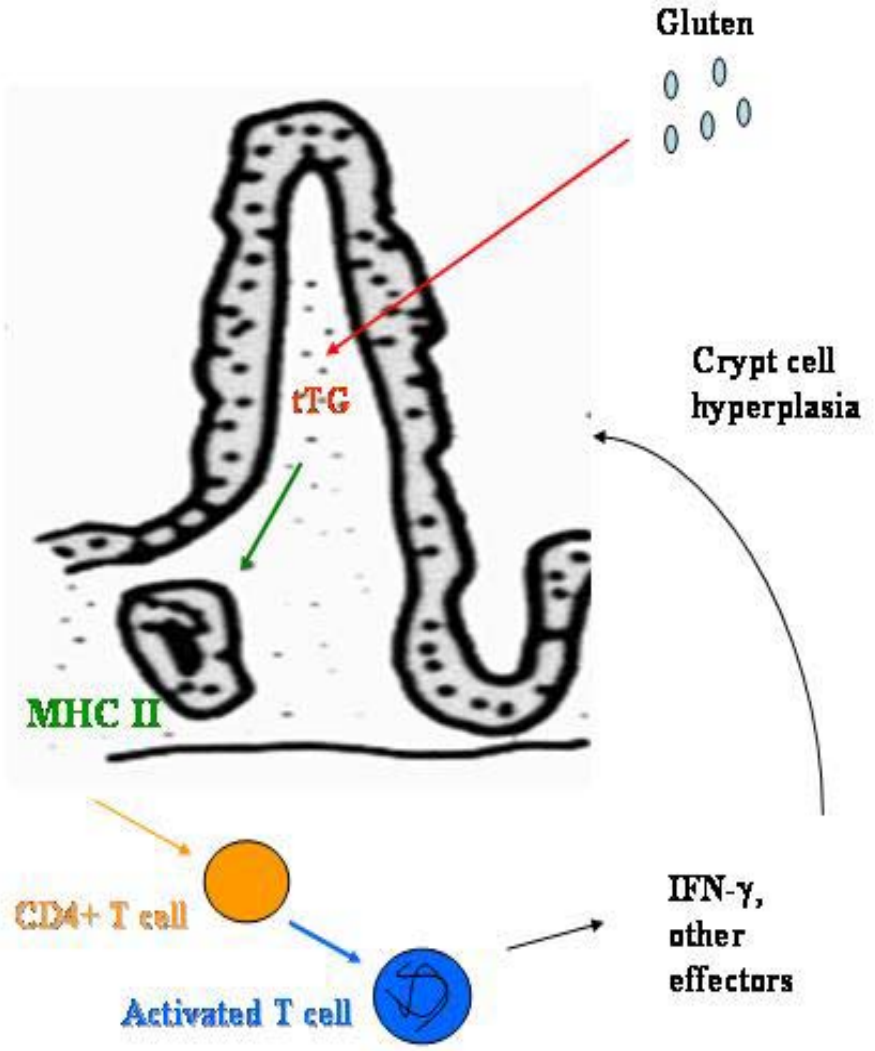

Figure 4. Hypotetical scheme for interaction between tTG and the specifical immune system in celiac disease

Modified from Mowat A, Celiac disease, a meeting point for genetics, immunology and protein chemistry. Lancet (2003) 361:1290-1292

The calcium-dependent transglutaminase activity of tTG catalyzes selective cross-linking or deamidation of protein-bound glutamine residues (Folk, 1983) and appears to carry out an ordered deamidation of some few specific glutamines (Molberg *et al.*, 1998).

Interestingly, the deamidation of glutamines that are not targetedby tTG can be deleterious for T cell recognition (Quarsten *et al.*, 1999). It is intriguing to hypothesize that tTG plays a central role in the selection of gliadin T cells epitopes. This idea is supported by the observation that the intestinal T cell response to α-gliadin in adults is focused on a single deamidated glutamine that is targeted by tTG (Arentz-Hansen *et al.*, 1999). The IgG and IgA serum antibodies to tTG (anti-endomysial antibodies) are a hallmark of CD and detection of serum IgA tTG-antibodies is utilized to predict the disease (Sulkanen *et al.*, 1998). An important physiological role of tTG is the catalysis of isopeptide bond formation between glutamine and lysine residues. *In vitro* treatment of gliadin fragments with tTG leads to some gliadin fragments becoming covalently attached to tTG by autocatalysis (Molberg *et al.*, 1998). tTG specific B cells may selectively bind and internalize gliadin-tTG complexes via specific surface immunoglobulis. The gliadin fragment may finally be processed and presented by DQ2 or DQ8 to the gliadin-specific T cells, thereby providing cognate help for B cell maturation, isotype switching and antibody secretion. This observation could explain why tTG antibody-levels in CD are dependent on the presence of gliadin in the diet because its removal is able to abolish the T cell help needed for antibody production.

It is demonstrated that $CD4^{+}TCR\alpha\beta^{+}$ T cells in the lamina propria are central for controlling the immune response to gluten that produces the immunopathology of CD. The knowledge of the events downstream of T cell activation is, however, still

incomplete. Anyway, the characterization of mechanisms operating in the model of human fetal gut explant cultures, where activation of T cells induces villous atrophy and hyperplasia of the crypts, has provided interesting clues and indicated some major pathways (Pender *et al.*, 1997). Recent *in vitro* organ cultures studies have indicated that gluten exerts additional immune relevant effects independent of T cell activation (Maiuri *et al.*, 1996; Maiuri *et al.*, 1998), some of which have rapid kinetics and conceivably the direct effects of gluten may facilitate subsequent T cell responses.

Cytokines produced by lamina propria $CD4^+$ T cells may be involved in the increased crypt cell proliferation and the increased loss of epithelial cells. Moreover, IFN-γ induces macrophages to produce TNF-α which, in turn, activates stromal cells to produce KGF which causes epithelial proliferation and crypt cell hyperplasia (Bajaj-Elliot *et al.*, 1998) (Figure 5). IFN-γ and TNF-α can jointly have a direct cytotoxic effect on intestinal epithelial T cells. Alterations of the ECM can also distort the epithelial arrangement, as the ECM provides the scaffold on which the epithelium lies. Infact enterocytes adhere to basement membrane through ECM so that modifications or loss of the basement membrane can result in enterocyte shedding. The increase of ECM degeneration might be important for the mucosal transformation found in CD (Daum *et al.*, 1999). Also the increased production of MMPs by subepithelial fibroblasts and macrophages is likely to be directly or not induced by cytokines that are released from activated T cells.

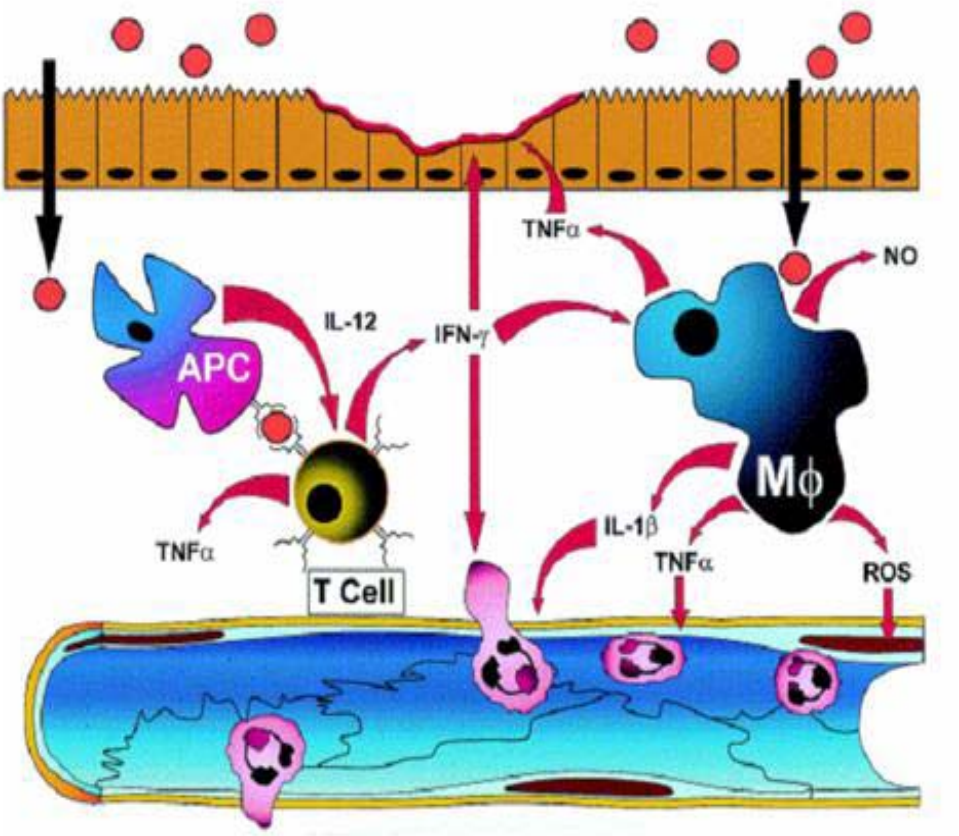

Figure 5. Illustration of activation of immunal response to enteric antigens

From Grisham M et al., Nitric oxide and chronic gut inflammation: controversies in inflammatory bowel disease. J Invest Med 50:1-12

Instead very few is known about the autoantibodies: the endomysial antibodies can be involved in the disease development by blocking interactions between mesenchymal cells and epithelial cells during the migration of epithelial cells and fibroblasts from the crypts to the tips of the villi.

tTG is necessary for activation of TGF-β (Nunes *et al.*, 1997) which is known to affect the differentiation of the intestinal epithelium (Halttunen and Maki, 1999), to stimulate the ECM (Bonewald, 1999) and to regulate the function of many immune competent cells within the gut microenvironment (Letterio and Roberts, 1998). Moreover it has been demonstrated that tTG is involved in attachment of fibroblasts to the ECM (Verderio *et al.*, 1998) thereby suggesting that the autoantibodies could also be involved in lesion formation by perturbing important contacts between fibroblasts and ECM components. In addition tTG autoantibodies may modulate the deamidating activity of tTG in either an inhibiting or a promoting fashion (Sollid and Scott, 1998).

2.2.3 A new mechanism

In the early 1960s it was suggested that gliadins, or peptic-tryptic digests, had a directly toxic effect on the mucosa of patients with active CD. To explain this research some scientists proposed "the lectin hypotesis" which suggested that gliadins interacted directly with glycoproteins in the epithelial cell membrane. Moreover, this hypothesis is supported by the presence of specific antigliadin antibodies and disease specific auto-antibodies against connective tissue components such as endomysium and reticulin, along with the presence of activated lymphocytes in the mucosa of untreated patients.

However, the most compelling evidence for an immune-mediated pathogenesis is the extremely strong association of the disease with HLA class II molecules (Sollid and

Thorsby, 1993). Gliadin-derived peptides may be processed for presentation by HLA class II molecules to helper T cells. Once activated T cells might orchestrate the changes leading to entheropathy (Gjertsen *et al.*, 1994). In a recent study mucosal biopsy specimens were taken from the duodenum of patients or controls and cultured for approximately 24 hours with digest of gliadin or control prolamins from maize, which are known not to be toxic. The results showed not only the expected changes accompanying immune activation but also an early, immediate effect of gliadins on the tissue. Infact it was demonstrated that activated helper $CD4^+$ T cells and macrophages migrated to the subepithelial compartment. HLA-DQ expression was up-regulated on mononuclear cells and the epithelium was invaded by $CD8^+$ T cells. Furthermore, also disease specific antiendomysial antibodies were produced (Picarelli *et al.*, 1996; Godkin and Jewell, 1998). Moreover, another recent study has shown that gliadin can directly interact with the extracellular protein transglutaminase (Dieterich *et al.*, 1997). This enzyme catalyzes the transfer of an acil group from a specific Gln donor to a non specific Lys acceptor, resulting in protein cross-linking. This means that the antiendomysial antibody is directed to the transglutaminase itself, suggesting that T-cell epitopes in the gliadin provide help in driving the antibody response to both the gliadin and transglutaminase. Alternatively, the interaction between transglutaminase and gliadin may result in the expression of novel epitopes. Although the endomysial antibody may or may not have a direct role in the pathogenesis disease this study confirms that gliadin directly interacts with components of mucosa (Godkin and Jewell, 1998).

3. Nitric oxide (NO)

NO is one of the smallest biologically active messenger molecules. It is also one of the first gaseous biological messenger which has a wide range of physiological and patophysiological actions in mammalian cells. NO is synthesized by the enzyme nitric oxide synthase (NOS) *via* the 5-e oxidation of one of the chemically equivalent guanidino nitrogens associated with L-arginine (Figure 6). NO is involved in the control of cardiovascular, central and peripheral nervous and immune systems (Figure 7). *In vivo* NO reacts with oxyhemoglobin, resulting in the formation of its stable metabolite nitrite (Butler and Williams, 1993) but can also react with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), a relatively stable product. Moreover, NO can react with thiols to form S-nitrothiols, such as S-nitrosocysteine and S-nitrosoglutathione (Figure 8). Many of the biological actions of NO are mediated through the guanylate-cyclase-cGMP system. NO diffuses to adjacent cells, activates soluble guanylate cyclase by binding to the iron on its heme component and moves the iron out of the plane of the porphyrin ring. This mediates some of the biological effects of NO such as the relaxation of vascular and nonvascular smooth muscle, inhibition of platelet adhesion and aggregation, inhibition of the chemotaxis of polymorphonuclear cells and the signal transduction pathways in the central and peripheral nervous systems (Murad *et al.*, 1987; Schmidt *et al*., 1993). It is now well estabilished that NO has also a number of cGMP-independent actions. The cytotoxic effects of NO involve the inhibition of tumor cell NADH ubiquitinone reductase, NADH-succinate oxidoreductase, cis-aconitase and all iron-sulfur prosthetic groups of the enzymes that contain them (Nathan, 1992).

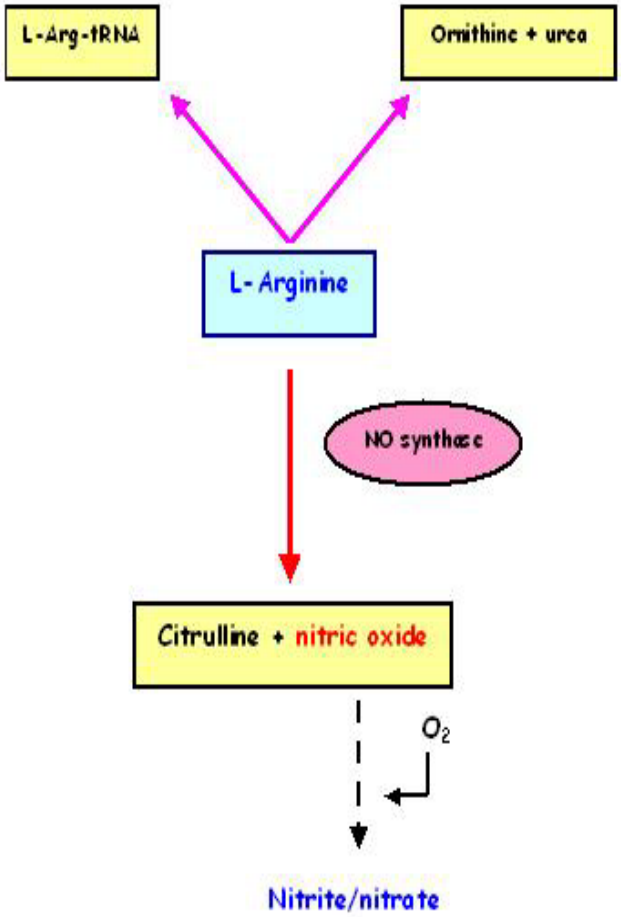

Figure 6. Biosynthesis of nitric oxide (NO)

Figure 7. Nitric oxide activities

cGMP-independent effects of NO on other enzymes, such as cyclooxygenase (COX) have also been described in macrophages and islet cells (Salvemini *et al.*, 1993). These effects may be related to the reaction of NO with the iron heme center in the active site of the enzyme. Thus NO, at high concentrations, inhibits COX activity (Stadler *et al.*, 1993) as well as the activity of NO synthase itself (Assreuy *et al.*, 1993). NO can also inhibit the cativity of cytochrome p-450 enzymes *in vivo* and *in vitro*. In macrophages NO also inhibts vacuolar-type proton-ATPase with a subsequent impairment in phagocytosis, protein synthesis and antimicrobial activity (Swallow *et al.*, 1991). Cytotoxicity caused by high local levels of NO can also be due to NO-mediated DNA damage, which is due to inhibition of ribonuclease reductase (Lepoivre *et al.*, 1990; Kwon *et al.*, 1991); it can also cause mutations by nitrosative deamination (Nguyen *et al.*, 1992). Accordingly, NOS inhibitors inhibit the promotion of neoplastic transformation.

Some of the oxidant/cytotoxic effects of NO are due to reaction of NO with O_2^- to form peroxynitrite $ONOO^-$, with subsequent formation of NO_2^- and hydroxyl radical[1] (Beckman *et al.*, 1990). It has been demonstrated that the majority of NO produced by activated macrophages is converted to $ONOO^-$, through this reaction (Zhu *et al.*, 1992).

3.1 Nitric oxide synthases isoforms and nomenclature

The nitric oxide synthases constitute a family with at least three distinct isoforms. In order of their molecular characterization, these include the neuronal (nNOS or NOS1)

[1] Peroxynitrite has a half-life of 1.9 seconds at pH 7.4 and exists in equilibrium with peroxynitrous acid, as shown in the equation $O_2^{-\cdot} + NO^{\cdot} \longleftrightarrow ONOO^- + H^{+\cdot} \longleftrightarrow$ ONOOH

(Bredt and Snyder, 1991; Nakane *et al.*, 1993), inducible (iNOS or NOS2) (Xie *et al.*, 1992; Lowenstein *et al.*, 1992) and endothelial constitutive (ecNOS, NOS3) (Lamas *et al.*, 1992; Janssens *et al.*, 1992) NOSs. Their apparent molecular masses are 160, 130 and 135 kDA, respectively (Table 2). Originally purified from neurons, cytokine-induced macrophages and vascular endothelium (hence the designations nNOS, iNOS and ecNOS), the three isoforms are now appreciated to distribute across a wide spectrum of cell types and tissues. Furthermore, recent findings indicate that a cell may express more than one isoform of NOS (Radomski *et al.*, 1990; Mehta *et al.*, 1995) thereby complicating the interpretation of NO derived from any given cell.

3.2 Biochemical functions

The NOSs are well characterized as cytochrome P-450-like hemeproteins (Bredt and Snyder, 1991; White and Marletta, 1992). They can be broadly divided into a reductase domain at the COOH terminus and an oxidative domain at the NH_2 terminus. The primary amino acid sequences of NOS isoforms share common consesus sequences binding site for calmodulin, NADPH, flavin-adenin dinucleotide (FAD) and flavin mononucleotide (FMN) (Bredt and Snyder, 1991; Lowenstein *et al.*, 1992; Xie *et al.*, 1992). Each enzyme function has a dimeric protein in catalyzing the NADPH-dependent five-electron oxidation of L-arginine to generate NO. L-Citrulline is a by-product (Abu and Stuehr, 1993). Electrons are supplied by NADPH, transferred along the flavins and calmodulin and presented to the catalytic heme center (Stuehr and Ikeda, 1992). The NOS apoenzyme requires tetrahydrobiopterin, prosthetic heme (ferroprotoporphyrin IX), calmodulin, FMN and FAD as cofactors for monomer assembly and/or catalytic activity (Abu and Stuehr, 1993).

Table 2. Isoforms of nitric oxide synthase

Type	Alternative name	Subcelhular location	Regulation	MW (Da)	Localization
I	nNOS	Cytosol	Ca/CAM	160.000	Brain, neurons skeletal muscle, kidney, pancreatic islet cells
II	iNOS	Cytosol	Ca	130.000	Macrophages, PMNs hepatocytes, mesangial cells endothelial cells
II	eNOS	Membrane bound	Ca/CAM	135.000	Colon intestinal cells endothelial cells

From Grisham M et al., Nitric oxide and chronic gut inflammation: controversies in inflammatory bowel disease. J Invest Med (2003) 50:1-12

The nNOS and ecNOS isoforms are constituvely expressed, but enzyme activation requires stimulation of the calcium/calmodulin signalling pathway (Zhang and Vogel, 1994). The synthesis and release of NO by constitutive NOS isoforms are rapid and do not depend on new protein synthesis. Calmodulin, in the presence of an elevated level of intracellular free calcium, activates constitutive NOS activity. In contrast the expression of iNOS is stimulated by cytokines or lipopolysaccharide (LPS) over a period of many hours. This process is dependent on new mRNA and protein synthesis (Vodovotz *et al.*, 1994). Once it has been induced, this enzyme produces large amounts of NO and its activity is independent of intracellular calcium levels. Despite this difference in their dependence on calcium, all three NOS isoforms appear to be related.

3.3 Genes encoding the three isoforms

NOS cDNA from many species have been cloned and functionally expressed. These include rat (Bredt and Snyder, 1991), mouse (Ogura *et al.*, 1993) and human (Nakane *et al.*, 1993) nNOS; mouse (Xie *et al.*, 1992; Lowenstein *et al.*, 1992), rat (Wood *et al.*, 1993) and human (Geller *et al.*, 1993a) iNOS and bovine (Nishida *et al.*, 1992) and human (Janssen *et al.*, 1992) ecNOS. Three distinct genes encoding the family of human NOS proteins have been identified and characterized. Analysis of these complex loci reveals that the mechanisms implicated in controlling mRNA expression and structure are unique for the different NOS isoforms. Human nNOS, iNOS and ecNOS are present on chromosomes 12, 17 and 7, respectively. Detailed analysis of genomic organization and exon/intron structure indicates a remarkably high degree of relatedness among the three genes (Hall *et al.*, 1994). The only related gene in the human genome is cytochrome P-450 reductase.

3.4 iNOS gene: transcriptional and posttranscriptional regulation

iNOS is, under physiological conditions, absent from mammalian cells and induced by proinflammatory stimuli such as bacterial LPS or cytokines or combinantions.

Evidences suggest that induction of iNOS in a number of patophysiological conditions is part of an uncontrolled and deleterious immune activation, for inhibition of NOS exerts protective effects in these conditions (Nussler and Billiar, 1993).

The human iNOS gene localizes to the 17q11.2-q12 region of chromosome 17 (Xu *et al.*, 1994). This human locus has been a region of interest in cardiovascular disease, expecially hypertension.

The iNOS gene consists of 26 exons and 25 introns spanning 37 kb of human genomic DNA (Chartrain *et al.*, 1994). The full-lenght open reading frame is 3459 bp, encoding a protein of 1153 amino acids., a palindromic TNF-RE-like site and a liver-specific transcription factor consesus sequence, AABS (Chartrain *et al.*, 1994; Nunokawa *et al.*, 1994). Sequence analysis of promoter revealed the presence of consensus motifs for binding of transcription factors, such as NF-κB, IRF-1, STAT-1α and nuclear factor interleukin-6 (NF-IL-6) which are essential for iNOS induction by IFN-γ (Kamijo *et al.*, 1994; Gao *et al.*, 1994; Kim *et al.*, 1997). Binding of IFN-γ to its receptor induces phosphorylation of STAT-1α via activation of the Janus family tyrosine kinases, JAK1 and JAK2. The phosphorylated STAT-1 α assembles to form a homodimer, which then translocates into the nucleus and binds to a specific DNA sequence motif, termed the IFN-γ activation site (GAS). The activated STAT-1 α also binds to the GAS site of the IRF-1 promoter and induces IRF-1 (Seidel *et al.*, 1995; Decker *et al.*, 1991). Furthermore, the IRF-1 gene promoter has been described as containing a composite

GAS/κB element (Sims *et al.*, 1993; Harada *et al.*, 1994), and cooperative regulation of transcription by IRF-1 and NF-κB has been previously described (Garoufalis *et al.*, 1994; Neish *et al.*, 1995).

iNOS can be induced in almost every tissue and nucleated cell type. These include macrophages (Xie *et al.*, 1992; Lowenstein *et al.*, 1992), lymphocytes (Kirk *et al.*, 1990), hepatocytes (Adachi *et al.*, 1993), chondrocytes (Palmer *et al.*, 1993), glia and neurons (Koprowski *et al.*, 1993), tumor cells (Sherman *et al.*, 1993), pancreatic islets (Yamada *et al.*, 1993), vascular smooth muscle cells (Perrella *et al.*, 1994), platelets (Mehta *et al.*, 1995), mesangial cells (Kunz *et al.*, 1994) and renal tubular epithelium (Markewitz *et al.*, 1993) (Table 3). The predominant mechanism underlying the induction of iNOS in these varied cell types is transcriptional regulation. Low basal rates of transcription are markedly enhanced by the treatment of cells with cytokines or LPS and maximal promoter activation occurs with synergistic combinations of proinflammatory cytokines such as TNF-α, IL-1β, IFN-γ and LPS. Functional characterization of the murine promoter of the 5'-flanking regions of the iNOS gene participate in the transcriptional response to the different exogenus stimuli (Xie *et al.*,1993; Xie *et al.*, 1994; Lowenstein *et al.*, 1993) (Figure 9).

A proximal region of the promoter interacts with the NF-κB trans-acting factor. This DNA-binding complex resides in the cytosol of quiescent cells, bound to an inhibitory complex, IκB. Following the activation of cells by various agents, IκB dissociates and degrades and the DNA-binding complex migrate to the nucleus where it binds to cis-regulatory regions of the iNOS promoter (Grilli *et al.*, 1993; Xie *et al.*, 1994).

Table 3. Cells and tissues that express human inducible nitric oxide synthase (iNOS)

Astrocytes	**Mast cells**
Chondrocytes	**Neurons**
Cardiac myocytes	**Neutrophils**
Eosinophils	**Osteoblasts**
Endometrium	**Osteoclasts**
Fibroblasts	**Placental trophoblasts**
Hepatocytes	**Platelets**
Islet cells	**Retinal epithelium**
Keratinocytes	**Sertoli cells**
Kupffer cells	**Skeletal muscle**
Lipocytes	**Vascular endothelial cells**
Lung epithelium	**Vascular smooth muscle cells**
Monocytes/macrophages	

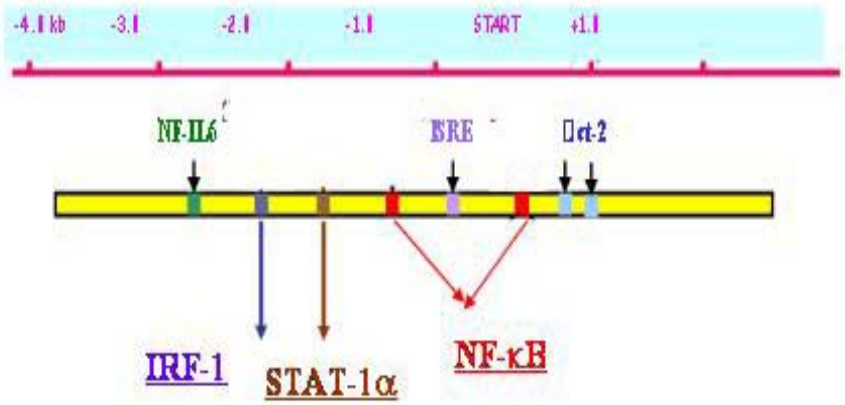

Figure 9. Nitric oxide synthase promoter region

p50/cRel and p50/RelA heterodimers represent at least a component of the trans-acting NF-κB complex that participates in transcriptional regulation (Xie *et al.*,1994). The NF-κB binding site in the proximal region of the promoter has been shown to be critical for induction of the iNOS gene by LPS (Xie *et al.*,1993; Xie *et al.*, 1994; Lowenstein *et al.*, 1993). Analysis of promoter/reporter constructs indicates that more distal regions of the promoter are involved in IFN-γ-stimulated changes in iNOS mRNA expression (Lowenstein *et al.*, 1993; Xie *et al.*, 1993). An IRF-1 cis-regulatory DNA region is implicated in this effect. Infact homozygous mice IRF-1 (-/-) do not exhibit an iNOS response following infection with cytokines or viruses (Kamijo *et al.*, 1994).

Thus, IFN-γ and LPS also exert effects on posttranscriptional regulation of the iNOS by increasing iNOS mRNA stability (Vodovotz *et al.*, 1993; Weisz *et al.*, 1994) which is stabilized also by cAMP elevating agents, such as dibutyryl cAMP and forskolin (Kunz *et al.*, 1994). In constrat TGF-β destabilizes iNOS mRNA in various cell types (Perrella *et al.*, 1994; Vodovotz *et al.*, 1993), decreases the translation of iNOS and interferes with protein stability (Vodovotz *et al.*, 1993).

3.5 Role of NO in bowel disease

A consistent finding in experimental and human bowel disease is the upregulation of iNOS and overproduction of NO (Rachmilewitz *et al.*, 1995; Singer *et al.*, 1996). Despite the role of iNOS-derived NO in bowel disease remains unclear, it is a particular interesting mediator that possesses both anti- and pro-inflammatory properties. The first ones are probably attributable to its ability to attenuate adhesion and recruitment of leukocytes in postcapillary venules exposed to different acute inflammatory stimuli. To

explaine antiadhesive properties of NO it has been proposed NO acts as a physiologic scavenger of reactive oxygen metabolites (Grisham *et al.*, 1998) (Figure 10).

The second ones are attributable to neovascularization by NO, which is known to be associated with bowel disease (Montrucchio *et al.*, 1997). Infact the concentrations of citrulline, the co-product of NO synthase, were found to be higher in rectal biopsy specimens from patients with ulcerative colitis than in those from patients with quiescent disease or normal histology; incubation with inhibitors of NO synthases significantly reduced the concentration of citrulline in colonic biopsies, suggesting a role for iNOS (Middleton *et al.*, 1993). Moreover it has been demonstrated that iNOS-derived NO seems to be critical in mice colitis development. Genetic absence or pharmacological blockade of iNOS significantly attenuates the severity of colonic injury and inflammation in a model miming ulcerative colitis (Krieglstein *et al.*, 2001). Finally it has been proposed that the high output production of NO from iNOS causes injury, perhaps through the generation of potent radicals such as peroxynitrite (Pryor and Squadrito, 1995).

Although there is much evidence that peroxynitrite in buffer system can be extremely harmful to most cells, *in vivo* this evidence is forthcoming. It has been demonstrated that children with active CD have a gluten-induced NO production in small intestine reflected by urine levels of nitrate/nitrite and iNOS expression in the intestine (Holmgren Peterson *et al.*, 1998). Moreover, recent studies reported that NO is produced in biopsy specimens cultured *in vitro* and that plays a role in the regulation of cytokine production, such as IL-1β (Beckett *et al.*, 1999). Furthermore it has been shown that lamina propria cells of patients with active CD are positive to iNOS (Beckett *et al.*, 1998). Finally, other studies *ex vivo* have correlated IRF-1 and STAT-1α

activation in small intestinal mucosa of celiac patients with the enhancement of iNOS gene expression (Mazzarella *et al.*, 2003; Salvati *et al.*, 2003).

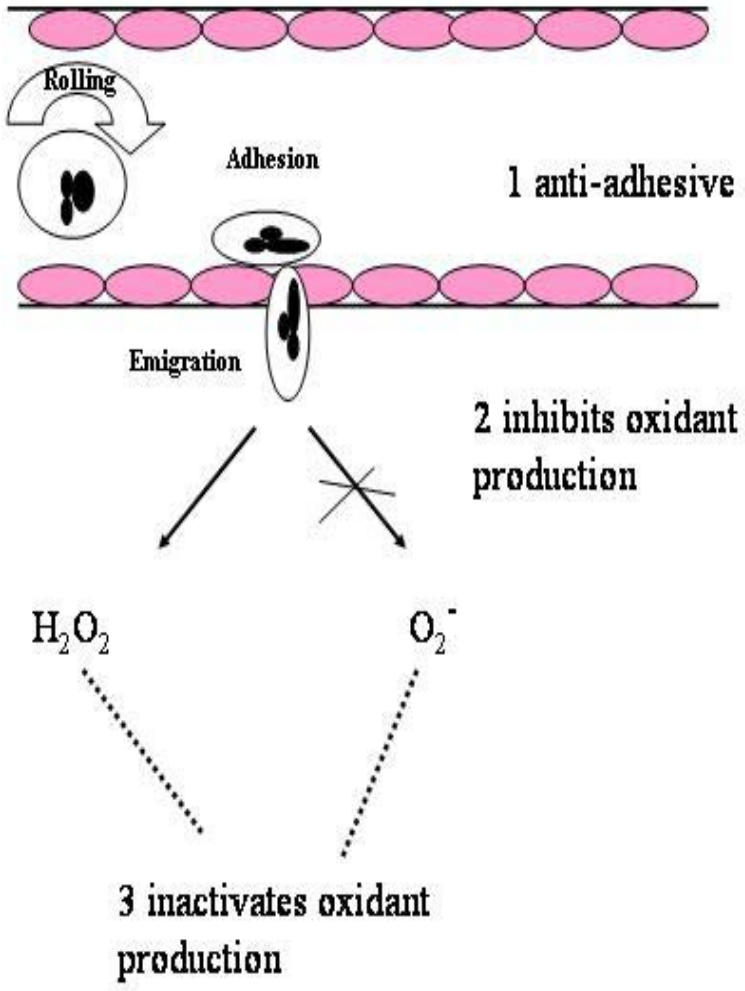

Figure 10. Antioxidant potential of nitric oxide.

From Kubes and McCafferty, Nitric oxide and intestinal inflammation. Am J Mol Med (2000) 150-158

4. Nuclear factor-κB (NF-κB)

NF-κB is a transcription factor initially discovered and characterised by Sen and Baltimore in the kappa light chain of immunoglobulins in B cells (Sen and Baltimore, 1986). Research over the last few years has revealed that NF-κB is an inducible and ubiquitously expressed transcription factor which plays an evolutionarily conserved and critical role in the triggering and coordination of inflammatory processes and in both innate and adaptive immune responses (Ghosh *et al.*, 1998; Silverman and Maniatis, 2001). NF-κB can be activated by a variety of pathogenic stimuli, including bacterial products and viral proteins, cytokines, growth factors, radiation, ischemia/reperfusion and oxidative stress. NF-κB activated, in turn, regulates the expression of wide variety of genes included those encoding cytokines, chemokines, adhesion molecules, acute phase proteins and inducible enzymes (Pahl, 1999). In addition, some of the chemokines and cytokines produced in response to NF-κB activation can stimulate the migration and maturation of lymphocytes. Furthermore, NF-κB is central for the overall immune response through its ability to activate genes coding for regulators of apoptosis and cell proliferation, that are critical for apoptotic processes (Karin and Lin, 2002).

4.1 Structure and activation of nuclear factor-κB

NF-κB exists in the cytoplasm of the majority of cells as homo or heterodimers of a family of structurally related proteins. Five proteins of the NF-κB family has been identified in mammalian cells: Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (Ghosh *et al.*, 1998) (Figure 11). The first three of these are produced as transcriptionally active proteins; the latter

are synthesised as large precursors, p105 and p100, respectively, that require proteolytic processing to produce the mature p50 and p52 proteins. The mechanisms by which the p105 and p100 precursor proteins are processed are not fully understood. What seems to be clear is that while p105 processing is constitutive, the processing of p100 is regulated (Karin and Ben-Neriah, 2000; Xiao *et al.*, 2001). Each member of NF-κB/Rel family contains conserved a 300 amino acid long N-terminal region called *Rel*-homology domain (RHD) responsible for DNA-binding, dimerization and association with the IκB inhibitory proteins through the nuclear localisation signal (NLS) (Baldwin, 1996; Ghosh *et al.*, 1998).

Activation of NF-κB is regulated by its cytoplasmic inhibitor, IκB. IκB binds NF-κB and masks its NLS, thus retaining it in the cytoplasm (Baldwin, 1996). Like NF-κB, IκB is a member of a larger family of inhibitory molecules, including IκBα, IκBβ, IκBε, IκBγ and Bcl-3 in mammals (Figure 12) and the recently described inducible IκBζ (Yamazaki *et al.*, 2001). The IκBs are characterized by the presence of multiple regions of homology known as the ankyrin-repeat motifs. The ankyrin repeats are regions of protein/protein interaction, and the specific interaction between ankyrin repeats and *Rel*-homology domains appears to be a crucial, evolutionary conserved feature of the regulation of NF-κB protein. Each IκB differs in the number of ankyrin repeats, and this number appears to influence the specificity by which IκB pairs with *Rel* dimers (Ghosh and Karin, 2002).

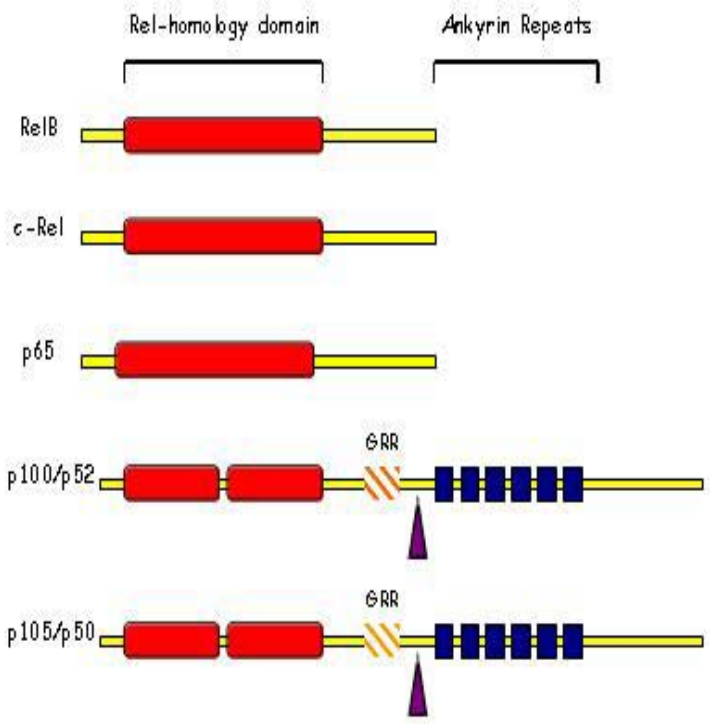

Figure 11. Members of NF-κB/Rel family.

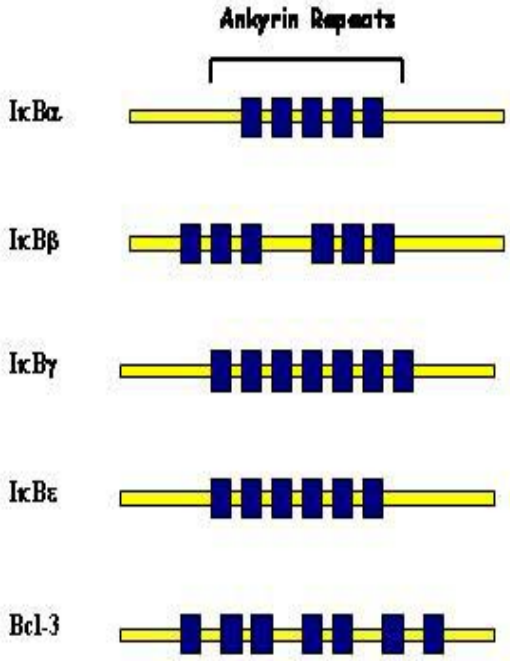

Figure 12. Members of IκB/Rel family.

The C-terminal region of p100 and p105 also contain similar ankyrin repeats and, prior to proteolytic processing, can function as IκB-like proteins, retaining NF-κB complex in the cytoplasm (Palombella *et al.*, 1994; Baldwin, 1996).

The best characterised IκBs is IκBα, mainly because it was the first member of this family to be cloned (Davis *et al.*, 1991; Haskill *et al.*, 1991). IκBα is a 37 kDa protein that has a tripartite organization also seen in IκBβ: an N-terminal domain that is phosphorylated in response to signal, a central ankyrin repeat domain and a C-terminal PEST domain that is involved in the basal turnover of the protein (Verma *et al.*, 1995). Physiologically the defining characteristic of IκBα is its ability to regulate rapid but transient induction of NF-κB activity, owing to the participation of IκBα in an auto-regulatory feedback loop; that is, the activation of NF-κB causes up-regulation of the transcription of IκBα, which serves to shut off the signal (Brown *et al.*, 1993; Scott *et al.*, 1993). This up-regulation occurs owing to the presence of κB sites in the IκBα promoter (de Martin *et al.*, 1993; Le Bail *et al.*, 1993). Thus IκBα is thought to maintain the transient effect of inducing agents on the transcription of NF-κB responsive genes. The continuing presence of certain inducing agents (for example, LPS) however causes NF-κB to be maintained in the nucleus despite the up-regulation of IκBα mRNA synthesis, and this persistent activation of NF-κB is regulated by IκBβ (Thompson *et al.*, 1995). IκBβ transcription is not regulated by NF-κB; therefore, the protein is slowly degraded following IL-1β and LPS stimulation and leads to persistent activation of NF-κB (Weil *et al.*, 1997; Johnson *et al.*, 1996). The mechanism of persistent activation may involve a chaperone-like role of unphosphorylated or newly synthesized IκBβ that allows transport of NF-κB to the nucleus without being trapped by IκBα (Suyang *et al.*,

1996; Ghosh *et al.*, 1998). This pool of variably responding IκB molecules, with different kinetics of degradation, synthesis and affinity, may allow cells to activate NF-κB differentially and, consequently, to regulate down-stream genes differentially in response to the wide array of stimulating agents (Ghosh *et al.*, 1998).

The activity of NF-κB is controlled by shuttling from the cytoplasm to the nucleus in response to cell stimulation. A large number of extracellular signals can trigger distinct signal transduction pathways, each of which culminates in the degradation of IκB proteins. These signal transduction pathways lead to the activation of the IκB kinase (IKK) and the subsequent phosphorylation of serine residues on the N-terminal region of IκB proteins (i.e. Ser_{32} and Ser_{36} for IκBα and Ser_{32} and Ser_{36} for IκBβ) (Karin and Ben-Neriah, 2000; Ben-Neriah, 2002). Phosphorylation determines the immediate recognition of IκB by the recently identified $E3RS^{IκB/β\text{-}TrCP}$, an SKp1-Cullin-F-box (SCF)-type 3 ubiquitin ligase (Yaron *et al.*, 1998; Laney and Hochstrasser, 1999; Maniatis, 1999), leading to its polyubiquitination (i.e. Lys_{21} and Lys_{22} for IκBα) and consequently rapid degradation by the ATP-dependent 26S proteasome (Ben-Neriah, 2002; Karin and Ben-Neriah, 2000). Neither phosphorylation, nor ubiquitination alone is sufficient to dissociate the NF-κB-IκB complex, and hence free NF-κB is only released after degradation of IκB. The degradation of IκB unmasks the NLS of the NF-κB leading to its nuclear translocation and binding to enhances or promoters of target genes (Figure 13).

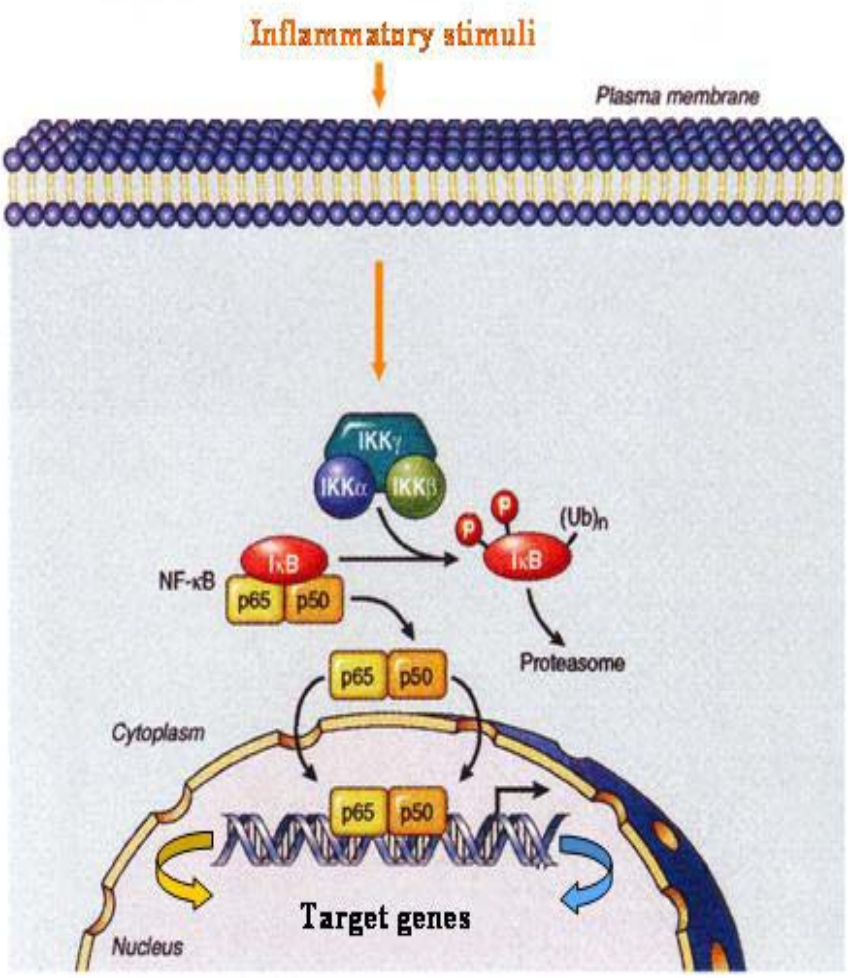

Figure 13. NF-κB activation pathway
Modified from Lee and Collins, Nuclear factor-kappaB and cell survival: IAPs call for support. Circ Res (2001) 88:262-264.

IKK is a complex composed of three subunits: IKKα (IKK1), IKKβ (IKK2) and IKKγ (also referred as NF-κB essential modulator, NEMO or IKK associated protein1, IKKAP1). IKKα and IKKβ are 85 and 87kDa proteins, respectively (Di Donato *et al.*, 1997; Mercurio *et al.*, 1997; Zandi *et al.*, 1997; Mégrier *et al.*, 1997; Woronicz *et al.*, 1997). IKKα and IKKβ are higly homologous proteins (50% sequence identity; >70% sequence similarity) and contain N-terminal protein kinase domains as well as leucine zipper (LZ) and helix-loop-helix (HLH) motifs. IKKγ is 48kDa protein (Rotwarf *et al.*, 1998; Yamaoka *et al.*, 1998; Mercurio *et al.*, 1999). Secondary-structure prediction algorithm indicates that IKKγ is predominantly helical with large stretcher of coiled-coil structure, including an LZ motif near the C terminus (Rotwarf *et al.*, 1998) (Figure 14). IKKα and IKKβ are the catalytic subunits of the complex. IKKγ has regulatory role and is required for activation of the complex by upstream signalling pathways (Ghosh and Karin, 2002). While IKKβ is mostly required for the canonical NF-κB pathway, triggered by TNF-α, IL-1 or by products of bacterial and viral infections (i.e. LPS) (Li Q *et al.*, 1999; Delhase *et al.*, 1999; Li Z *et al.*, 1999), IKKα is involved in a non-canonical NF-κB pathway, stimulated by only a few members of the TNF family, such as lymphotoxin β (LT β), that regulates the RelB/p52 dimer (Solan *et al.*, 2002; Senftleben *et al.*, 2001a) (Figure 15). Whereas the canonical NF-κB pathway, which is based on IκB degradation, is essential for innate immunity (Senftleben *et al.*, 2001b), the second pathway, which is based on NF-κB2 processing, is mostly involved in lymphoid organ development and adaptive immunity (Senftleben *et al.*, 2001a).

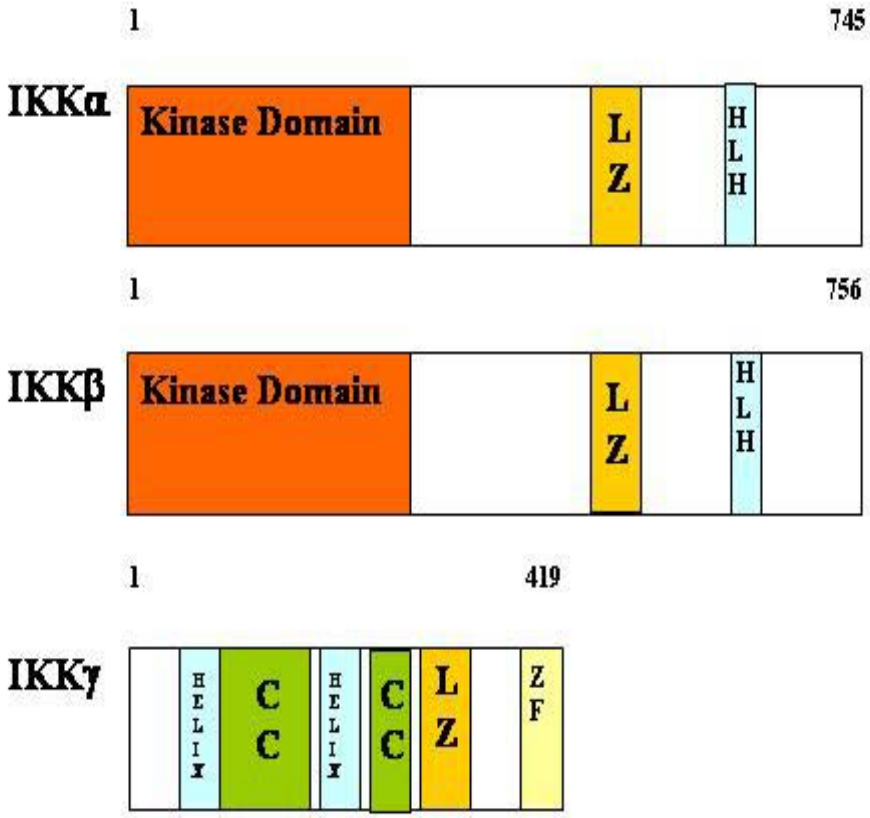

Figure 14. Structure of IKKα, IKKβ and IKKγ.

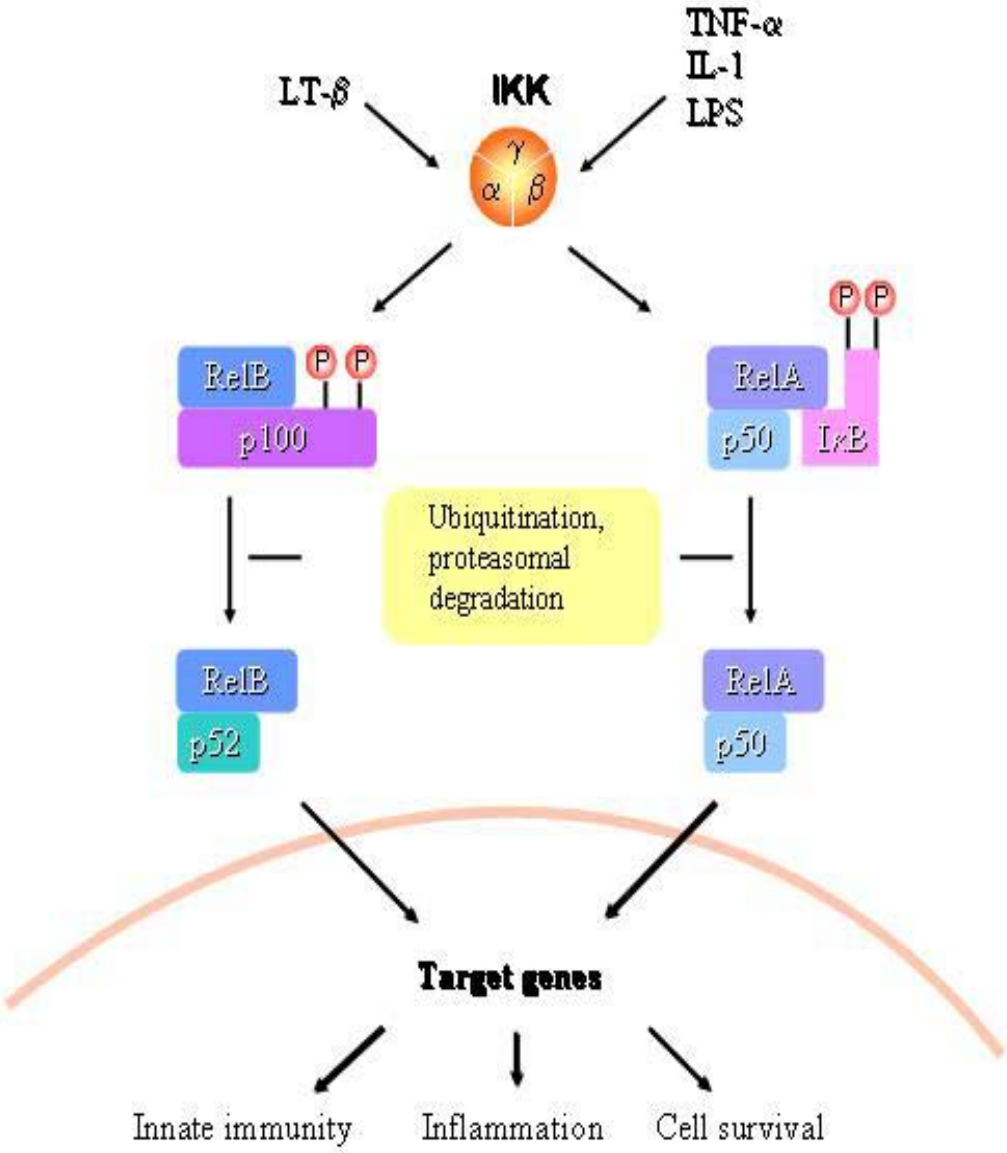

Figure 15. Two distinct IKK signaling pathways that lead to NF-κB activation.
Modified from Karin and Lin, NF-κB at the crossroads of life and death. Nat Immunol (2002) 3:221-227

Once in the nucleus, NF-κB dimers are subject to further regulation mainly through phosphorylation of the Rel proteins, which is required for full induction of NF-κB target genes (Zhong *et al.*, 1997; Sizemore *et al.*, 1999). Several signalling pathways, which induce the phosphatidyl inositol 3-kinase (PI 3-K)/Akt pathway, are thought to be involved in this process (Madrid *et al.*, 2001).

4.2 NF-κB and inflammation

NF-κB plays a crucial role in many inflammatory diseases, such as rheumatoid arthritis (RA), asthma and inflammatory bowel disease. It has also been implicated in other diseases such as atherosclerosis and Alzheimer's disease (Baldwin, 2001). In cells associated with these diseases, NF-κB has been found with aberrant, constitutively nuclear localization and enhanced transcriptional activity. It is probable that the role of NF-κB in inflammatory diseases results from defect in the regulatory mechanisms controlling its activation. Experimental evidence suggests a pivotal role for NF-κB both at the stage of initiation and perpetuation of chronic inflammation, because an important aspect of the significance of NF-κB in these processes stems from it both being activated by and inducing the expression of inflammatory mediators.

$CD4^+$ T cells are considered a trigger of immune inflammation. They become activated when the T-cell receptor (TCR) recognizes fragments of auto-antigens presented by the MHC class II molecules of APCs. Another signal required for T-cell activation is provided by the interaction of CD28 with its ligands, the co-stimulatory molecules CD80 and CD86 that are expressed by APCs. The engagement of TCR and CD28 activates NF-κB in T cells, which is required for the induction of NF-κB-dependent

genes controlling activation and proliferation of T cells, such as interleukin-2 (IL-2), IL-2 receptor (IL-2R) and IFN-γ (Ghosh *et al.*, 1998; Schulze-Osthoff *et al.*, 1997; Sheppard *et al.*, 1999; Gerondakis *et al.*, 1998).

T cells that are found in inflammatory lesions express on their surface CD40 ligand (CD40L) (Grewal and Flavell, 1998; Stout and Suttles, 1996) and TRANCE (TNF-related activation-induced cytokine also known as RANKL, receptor activator of NF-κB ligand) (Anderson *et al.*, 1997; Wong *et al.*, 1999), the molecules of the TNF superfamily. Interactions of CD40L and TRANCE/RANKL with the APC receptors CD40 and RANK induce NF-κB activation, promote survival of APC cells and augment their ability to stimulate T-cell proliferation and activation, possibly by upregulating the expression of NF-κB-dependent molecules MHC class II, CD80 and CD86 (Li J *et al.*, 1999; Verhasselt *et al.*, 1999). Thus, NF-κB appears to be an important mediator of antigen-induced T cell activation.

Activated $CD4^+$ T helper (Th) cells can differentiate into the subsets whose cytokine profile is restricted to Th1 (IL-2 and IFN-γ-dominant) or Th2 (IL-4-, IL-5-dominant) patterns. Th1 cells, that mediate cellular immunity and activate macrophages, are considered pro-inflammatory, whereas Th2 cells, that potentiate antiparasite and humoral immunity and down-regulate macrophage activation, are considered anti-inflammatory. Suppression of NF-κB pathway in T cells promotes Th2 development (Aronica *et al.*, 1999), whereas NF-κB activation promotes type 1 responses, presumably through the induction of NF-κB-dependent Th1 cytokines IL-2, IFN-γ and IL-12 (Li B *et al.*, 2000).

Secreted products of activated T cells and direct cell-cell contacts cause activation of macrophages, fibroblast and endothelial cells. NF-κB controls the expression of the cytokines IL-1β and TNF-α, which are essential mediators of chronic inflammation and implicated in leukocytosis, hyperplasia and tissue breakdown (Hiscott *et al.*, 1993; Shakhov *et al.*, 1990). Because both IL-1β and TNF-α are also potent inducers of NF-κB activation, an interdependence of persistent NF-κB activation and sustained levels of IL-1β and TNF-α is indicated (Miagkov *et al.*, 1998; Tomita *et al.*, 1999). Elevated levels of other NF-κB-dependent gene products, including IL-6 and IL-15, the adhesion molecules E-selectin, vascular cellular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), and the chemokines IL-8, macrophage chemotactic protein-1 (MCP-1) and MIP-1α, promote recruitment of inflammatory cells and their activation (Libermann and Baltimore, 1990; Azimi *et al.*, 1998; Iademarco *et al.*, 1992; van de Stolpe *et al.*, 1994; Kunsch and Rosen, 1993; Ueda *et al.*, 1994; Grove and Plumb, 1993; Pahl, 1999).

Activation of non-immune cells, particularly fibroblasts, promotes tissue remodelling in inflammatory lesions. In RA, activated fibroblast-like synoviocytes secrete large amounts of matrix proteinases, including NF-κB-dependent metalloproteinases (MMPs) MMP-1, MMP-3 and MMP-9 (Bond *et al.*, 1998; Bond *et al.*, 1999). Another mechanism of tissue remodelling/destruction is mediated by the induction of NF-κB-dependent genes encoding inducible forms of cyclooxygenase (COX-2) and iNOS, enzymes catalyzing the synthesis of pro-inflammatory prostaglandins, NO and nitric oxide metabolites, respectively (Yamamoto *et al.*, 1995; Xie *et al.*, 1996). Activation of

NF-κB in fibroblasts also promotes neovascularization of inflammatory lesions through induction of vascular endothelial growth factor (VEGF) (Chilov *et al.*, 1997).

4.2.1 NF-κB in inflammatory bowel disease

Recent studies have shown increased production of pro-inflammatory cytokines in inflammatory bowel disease (MacDonald *et al.*, 1990). NF-κB is thought to be an important factor in pathophysiology of intestinal inflammation in inflammatory bowel disease. However the pivotal elements in the regulation of the increased inflammatory activity remain unclear. An important candidate involved in the regulation of inflammation gene transcription is NF-κB. Infact many of the inflammatory cytokine genes include NF-κB binding sites, and most have been shown to be regulated by this factor (Baldwin, 1996). Most interestingly NF-κB dimers containing p65 appear to have profound proinflammatory activity, whereas the p50 homodimer is either inactive or may be even involved in blocking NF-κB sites in some inflammation gene promoters against binding of p65 dimers (Ledebur and Parks, 1995). Recent studies suggest that activation of NF-κB involving the p65 subunit may be a key event in trinitrobenzenesulphonic acid (TNBS)/ethanol colitis (Neurath *et al.*, 1996). Although not shown *in vivo*, a role for NF-κB/p65 has been suggested in Chron's disease. Infact nuclear extracts from lamina propria of these patients contained substantially higher levels of NF-κB/p65 (MacDonald *et al.*, 1990) and biopsy specimens as well as isolated lamina propria mononuclear cells from patients with active inflammatory bowel disease show enhanced activation of NF-κB. Therefore when activation of NF-κB involves the

p65 subunit, may contribute to the enhanced expression of inflammation genes observed in inflammatory bowel disease (Schreiber *et al.*, 1998).

5. Signal Transducer and Activator of Transcription 1-α (STAT-1α)

5.1 Structure and activation of Signal Transducers and Activators of Transcription (STATs)

Cytokines regulate numerous aspects of hematopoiesis and immune response. They mediate their responses through activation of the Jak/STAT signaling pathway. STATs comprise a family of seven structurally and functionally related proteins: STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b and STAT-6. Janus kinases (Jak) represent a family of four non-receptor tyrosine kinases, Jak1, Jak2, Jak3 and Tyk2. These kinases selectively phosphorylate STATs, leading to their activation. Once activated, STAT then play a critical role in regulating innate and acquired host immune responses. Dysregulation of at least two STAT signaling cascades is associated with cellular transformation. STATs transduce signals for the large hematopoietin subfamily of cytokines and the conserved family of the receptors they bind. This includes the IFN family (IFN-α,β and γ; IL-10, IL19, IL-20, IL-22), the gp130 family (IL-6, IL-11, GCSF, Leptin), the γC family (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) and the single chain family (Epo, GH, PRL, Tpo) of the receptors (Schindler and Strehlow, 2000) (Figure 16). STATs can also be activated by receptor tyrosine kinases; also several members of the G-protein-coupled receptors, a primitive family, have also been shown to signal through STATs (Leonard and O'Shea, 1998). Signaling through the Jak/STAT pathway is initiated when cytokine binds to its corresponding receptor; this leads to a conformational change in the cytoplasmic portion of the receptor, initiating activation of receptor associated members of the Jak family of kinases.

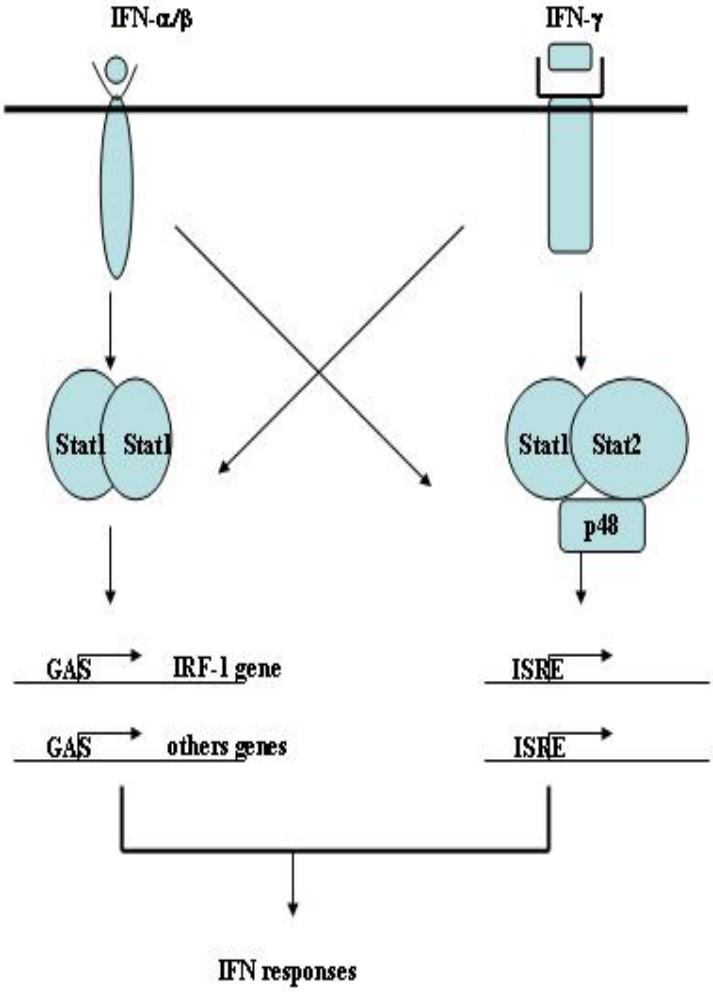

Figure 16. Activation of STATs after IFNs challenge.

Modified from Harada et al., The role of interferon regulatory factors in the IFN system and cell growth. Biochimie (1998) 80:641-650

The Jaks, in turn, mediate phosphorylation at the specific receptor tyrosine residues, which then serve as docking sites for STATs and other signaling molecules. Once recruited to the receptor, STATs also become phosphorylated by Jaks, on a single tyrosine residue. Activated STATs dissociate from the receptor, dimerize and translocate to the nucleus binding to members of the gamma activated site (GAS) family of enhancers.

Upon ligand stimulation, cytokines receptors undergo the conformational changes that bring Jaks, which are constituvely associated with a proline-rich membrane proximal domain of these receptors, into proximity of each other, to mediate signal transduction. The four members of Jak family (Jak1, Jak2, Jak3 and Tyk2) are over 1000 amino acids in lenght, ranging in molecular weight from 120 to 130 kDa. Jak1, Jak2 and Tyk2 are expressed ubiquitously, whereas the expression of Jak3 is restricted to cells of the myeloid and lymphoid lineages (Leonard and O'Shea, 1998). Comparison of Jak sequences reveals seven regions of high homology, JH1-JH7. Altough the biological activity of each of these regions has not been fully elucidated, several domains are well characterized (Ihle, 2001). JH1 has been shown to encode the kinase while JH2 represents a pseudokinase domain, which appears to be required for JH1 catalytic activity (Yeh, 2000). The amino-terminal JAK homology domains, JH3-JH7, have been implicated in receptor association altough the role of JH7 region in this interaction has not been confirmed.

The seven STAT proteins identified in mammals range in size from 750 and 850 amino acids. Both, the cromosomal distribution of these STATs, as well as the identification of STATs in more primitive eukaryotes, suggest that this family arose from a single primordial gene. Duplications of this locus appear to reflect an increasing need for cell-

to-cell communication as eukaryotes became more complex. Consistent with this evolutinary pattern, STATs share structurally and functionally conserved domains. This includes the amino-terminal domain, the coiled-coil domain, the DNA binding domain, the linker domain and the SH2/tyrosine activation domain. In contrast the carboxy-terminus domain is quite divergent and contributes to STAT specificity. Despite the fact that over 50 members of the hematopoietin family transduce signals through a more limited number of STATs, significant specificity is achieved. This is largely because the receptors that transduce signals for these cytokines can be placed into five structurally and functionally related subfamilies, each of which tend to transduce signals through a single STAT. Further specificity can be attributed both to tissue specific patterns of expression and the activation of additional signaling pathways by these receptors.

Structural studies of several hematopoietin receptors from the cytokine family indicate that ligand binding promotes the dimerization of receptors into an active conformation (Wells, 1996). In each case of activation is believed to lead to close approximation of cytoplasmic receptor tails, enabling the transphosphorylation of the receptor-associated Jaks. Activated Jaks then phosphorylate specific tyrosine motifs present in the receptor endodomains, which in turn mediate the recruitment of STATs to their appropriate receptor. This entails the ability of a STAT domain to recognize a phosphotyrosine residue and 4-5 carboxy-proximal amino acids, known as the receptor STAT recruitment motif. In unstimulated cells STATs predominantly localize to the cytoplasm and upon stimulation rapidly translocate to the nucleus and induce gene expression. After termination of the signal, STATs translocate back to the cytoplasm (Figure 17).

This regulated mobilization of STATs is an essential step for signaling and is mediated by the nuclear pore complex (NPC) (Doye and Hurt, 1997). Like all molecules larger

than 60 kDa STATs are transported across the NPC in an active bidirectional process that is energy and activation dependent.

5.2 IFN-γ receptor

IFN-γ, like IFN-α plays a key role in innate and acquired host immune responses (Bach, 1997). It can inhibit viral and cellular proliferation and regulate apoptosis. Unlike IFN-α, IFN-γ function is required for host defenses against several intracellular pathogens. IFN-γ is predominantly produced by activated T lymphocytes and natural killer (NK) cells. However its receptor is expressed on most cells. Cellular responses following IFN-γ stimulation are mediated through the recruitment of STAT-1 to a single STAT-1 binding site and its subsequent activation by Jak1/Jak2 (Greenlund, 1994). Both IFN-γ receptors chains participate in signal transduction as demonstrated by targeted disruption of the two chains (Lu, 1998). Investigations of IFN responses has identified two different transcriptional enhancers within the promoter elements of STAT target genes (Schindler and Brutsaert, 1999). IFN-γ, through STAT-1 homodimers, binds to a distinct response element, known as GAS (Decker, 1997). This palindromic element has a consensus sequence of TTTCCNGGAAA.

Similar responsive elements have been identified in the promoters of other STAT-induced genes. Biochemical studies have determined that $TTCN_{2-4}GAA$ consensus defines the optimal binding site for all STATs, with an exception of STAT-2, which appears to be defective in DNA binding. This provides an opportunity for most STAT homodimers to exhibit unique DNA binding preferences. In part this is determined by spacing between palindromic half sites (Ehret, 2001).

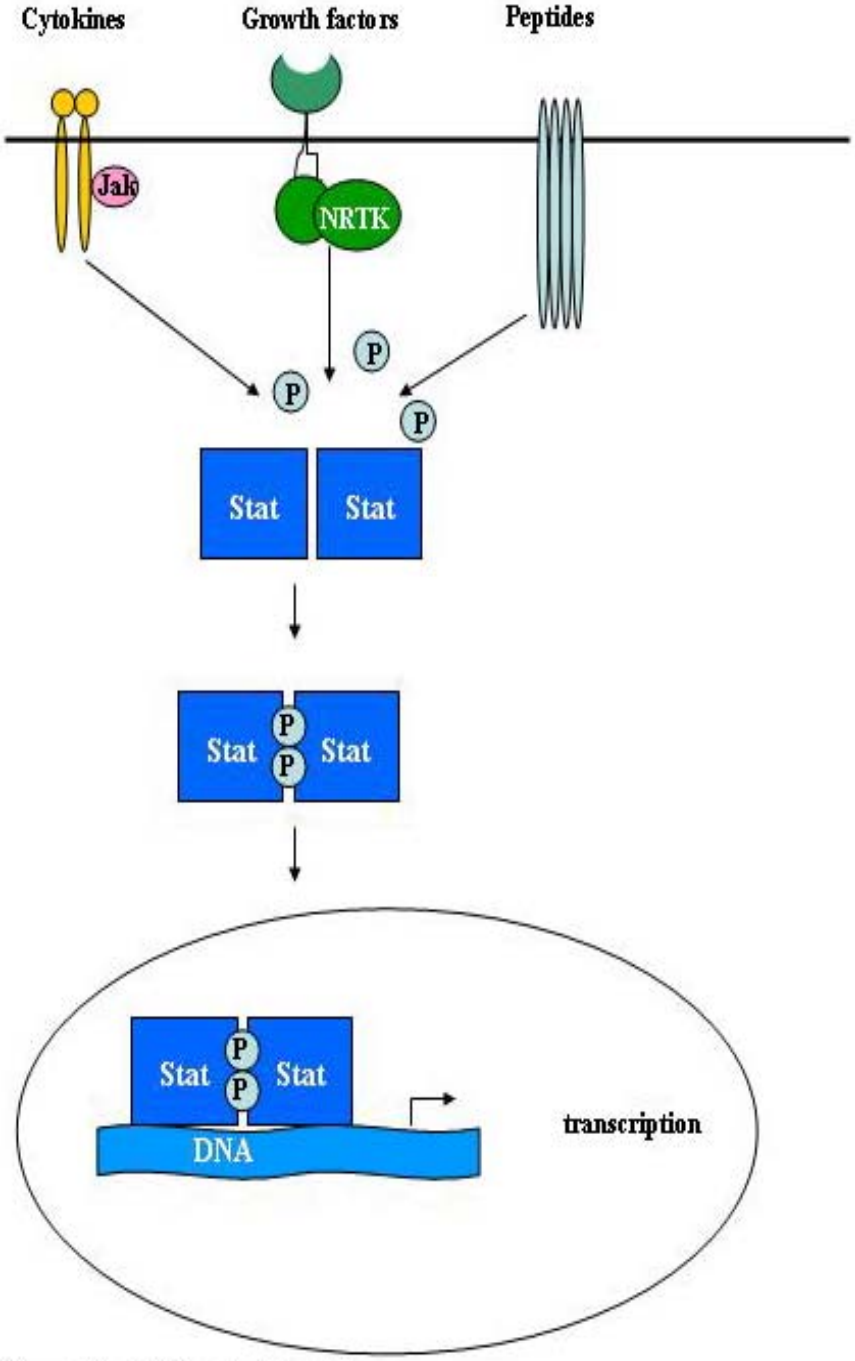

Figure 17. STATs activation pathway.

Modified from Levy and Darnell. Stats: transcriptional control and biological impact. Nat Rev (2002) 3:651-662

Recent studies indicate the ability of STATs to bind cooperatively to tandem GAS elements also contributes to DNA binding specificity. Moreover these tandem sites often include nonconsesus GAS elements (Ehret, 2001)

5.3 Genetics and functions of STATs family

Gene-targeting studies indicate that STATs largely evolved to mediate host response to stress in mammals (Ihle, 2001). Moreover, these studies highlighted a remarkable level of specificity in STAT function: for example STAT-1 and STAT-2 predominately transduce signals for IFNs, likewise STAT-4 and STAT-6 play an important role in the polarization of native T cells into Th1 and Th2 cells, respectively. However STAT-3 and STAT-5, which are activated by more cytokines, are more pleiotropic in their function so they appear to be more closely realted to the STATs found in lower eukaryotes.

STATs are well conserved during eukaryotic evolution and may encode the primordial SH2 domain. Studies in *Drosophila* demonstrate how the Jak/STAT pathway is important for many functions, like hematopoiesis, sexual identity and embryo segmentation (Zeidler, 2000).

In mammals the seven STATs are segregated to three clusters each of which represents a tandem duplication. STAT-1 and STAT-4 map to chromosome 1, STAT-2 and STAT-6 cosegregate on chromosome 10 while STAT-3 and STAT-5 are located on chromosome 11 although the STAT-5 has undergone an additional duplication more recently in evolutionary history (Copeland, 1995). According to this, analysis of STAT-3 and STAT-5 gene structure suggests that these two STATs may have been the first

mammalian STATs (Miyoshi, 2001). This may also account for the relative functional pleiotropy of these two STATs.

5.4 STAT-1

Investigations into IFNs signalling pathways led to the discovery of STAT-1. IFN-γ activates STAT-1 homodimers, which initiate transcription of GAS-driven genes. This has been confirmed by the generation of STAT-1 knockout mouse (Durbin, 1996). Infact these mice were defective in IFN dependent immune response to both viral and microbial agents because they failed to induce transcription of STAT-1 target genes. Over the last several years STAT-1 null mice have been exploited to demonstrate the wide role IFN-γ plays in regulating innate and acquired immunity (Schindler and Strehlow, 2000). Recent studies underline the role IFN-γ plays in protecting mice from chemically induced and spontaneous tumors. STAT-1 and IFN-γ deficient animals develop these tumours more rapidly and frequently than wild-type mice. These observations provide compelling evidence for an IFN-γ/STAT-1 dependent tumor immune surveillance system in immune competent host (Shankaran, 2001). Moreover, studies in STAT-1 null mice have also provided evidence for non-immune role for STAT-1. For example many studies have suggested that STAT-1 affects apoptosis through the regulation of caspase 1, 2 and 3 (Chin, 1997; Huang *et al.,* 2000) and that inactive STAT-1 prevents cell apoptosis (Kumar, 1997). The identification of low levels of STAT-1 in the nucleus of unstimulated cells suggests that STAT-1 might cooperate with other transcriptional factors to regulate the basal expression of genes that regulate apoptosis (Schindler and Strehlow, 2000).

5.5 Regulation of the Jak/STAT signalling pathway

Numerous regulatory layers exist to modulate this signalling pathway. The effects of these regulatory processes, which can be both negative and positive, determine the rate at which STAT signals are transduced.

Recent studies determined that STAT signals are downregulated at several points (Figure 18). A common mechanism of downregulation involves receptors and entails receptor endocytosis (Dittrich, 1996). Also ubiquitin-proteasome dependent degradation may play a role in lowering cytokine signalling: proteasome inhibitors have been found to impede the degradation of both STAT-1 and IFN-γ receptors (Kim and Maniatis, 1996) and to prolong Jak activity (Yu and Burakoff, 1997).

Since Jak activation depends on tyrosine phosphorylation phosphatases have been found to negatively regulate its activity. STAT activation is directly antagonized by the suppressor of cytokine signalling (SOCS), a family of STAT target genes that estabilishes an important and classic feedback loop (Kile *et al.*, 2001). STAT activity can also be negatively regulated through direct modifications, for example dephosphorylation and generation of carboxy-terminally truncated isoforms (Mowen *et al.*, 2001). Reporter gene studies have determined that serine phosphorylation enhances transcriptional activity of STATs, except STAT-5 and STAT-6 (Kovarik, 2001; Visconti, 2000; Yamashita, 1998). Numerous studies have identified a substantial interaction between STATs and other transcription factors. Analysis of STAT dependent promoters provided functional and biochemical evidence for the interaction with other transcription factors: STAT-1 was found to interact with NF-κB, Sp1, USF-1 and the glucocorticoid receptor (Look, 1995; Ohmori, 1997; Aittomaki, 2000).

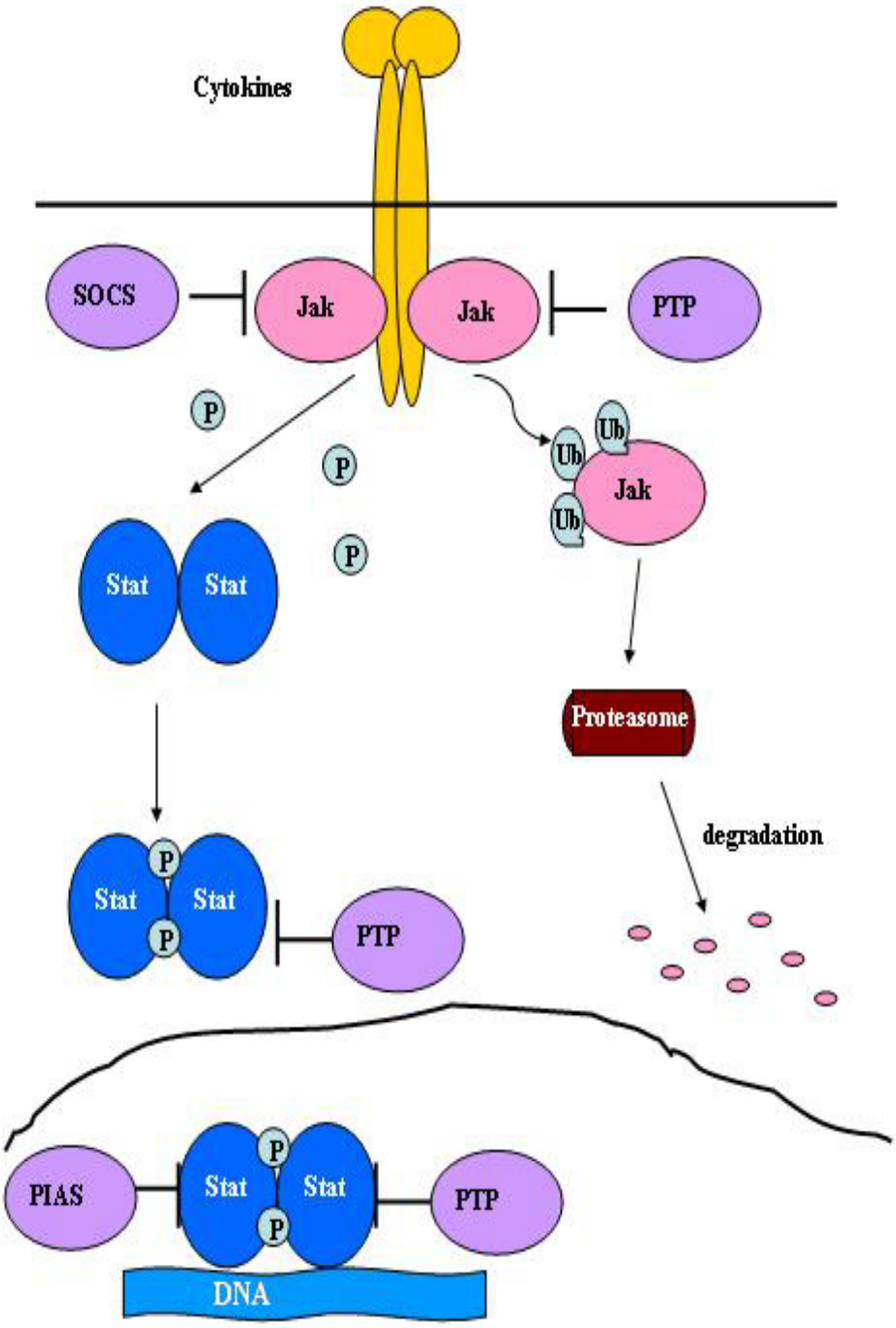

Figure 18. Negative regulation of the Jak/STAT activation pathway. Modified from Shuai and Liu Regulation of the Jak/Stat signalling in the immune system. Nat Rev (2003) 3:900-911

Other studies have demonstrated the association between STATs and proteins that facilitate transcription through chromatin modification (Horvath and Mehta, 2000). Finally yeast two hybrid screens have identified non-nuclear proteins that associate with STATs (Collum, 2000).

6. Interferon Regulatory Factors (IRF)

6.1 Structure and activation of Interferon Regulatory Factors (IRFs)

IFNs are a heterogeneous family of multifunctional cytokines, which were originally identified as proteins responsible for the induction of cellular resistance to viral infections. Subsequently, much evidence has accumulated with their regard to their roles in cell growth, differentiation and immunomodulation (Vilcek, 1996). Studies of IFN-α and IFN-β gene regulation have identified a factor termed IRF-1 as a protein that binds to the virus-inducible elements of the human IFN-β gene (Miyamoto, 1988). Subsequently it has been identified also IRF-2 by cross-hybridization with cDNA (Harada, 1989). Mutational analysis of IRF-1 and IRF-2 revealed that DNA-binding activity resides in the amino-terminal region, with some highly conserved amino acids, particularly five tryptophan repeats. After the cloning of IRF-1 and IRF-2, several factors which share a significant homology with IRF-1 and IRF-2 in their amino-terminal region have been identified. Notably these factors conserved the five trypthophan repeats. These factors now constitute a family of transcription factors, which includes IRF-3, IRF-4 (Pip, LSIRS, ICSAT, MUM1), IRF-5, IRF-6, IRF-7, IRF-8 (ICSBP) and IRF-9 (p48, ISGF3γ) (Taniguchi, 2001). These members are ubiquitously expressed, except for IRF-4 and IRF-8 whose expression is restricted to hematopietic cells. The consesus IRF-1/IRF-2 binding motif, termed IRF Element (IRF-E) was determine to be G(A)AAA$^{C}/_{T}$G$^{G}/_{T}$AAA$^{G}/_{T}$C$^{C}/_{C}$ by the random oligosection method (Tanaka, 1993). As the DNA binding domain of these factors is highly conserved, IRF members recognize similar DNA sequences (Taniguchi, 2001). In contrast recent gene targeting for IRF members clearly demonstrate their specialized role in the regulation of target gene expression and host defense.

6.2 Expression of IRF-1

IRF-1 was isolated by virtue of its affinity to specific DNA sequences in the IFN-β promoter that mediates virus responsiveness (Fujyta, 1988; Miyamoto, 1988). With the exception of early embrional cells, IRF-1 mRNA is expressed at a low basal level in all cell types examined. IRF-1 mRNA levels accumulate in response to IFNs, double-stranded RNA (dsRNA), cytokines and some hormones. IFN-γ is the strongest IRF-1 inducer known, altough some combinations, such as IFN-γ and tumor necrosis factor alpha (TNF-α), induce even higher levels of IRF-1 mRNA (Ohmori *et al.*, 1997). On the other hand, other cytokines can antagonize IRF-1 induction; for example, interleukin 4 (IL-4) reduces IFN-γ-stimulated IRF-1 transcription (Coccia *et al.*, 2000). As the IRF-1 protein has a short half life of 30 min, IRF-1 mRNA levels most probably correlate with IRF-1 protein abundance (Watanabe *et al.*, 1991). IRF-1 mRNA levels are cell-cycle regulated (Harada *et al.*, 1993). The levels are elevated in serum-starved cells in G_0 and successively lowered after serum-induced growth. IRF-1 mRNA levels increase before and during S phase. This is indicative of a short half-life of IRF-1 mRNA that permits such rapid changes. Altough it is not known what mechanism control cell cycle-dependent accunulation of IRF-1 mRNA levels, most inducers lead to activation of IRF-1 gene transcription. Key promoter elements are IFN-γ-activated sequences (GAS) and binding sites for NF-κB. These sites mediate transcription induction on binding STAT-1 and NF-κB respectively (Harada, 1994; Durbin, 1996) (Figure 19). Interestingly, IRF-1 can also activate IRF-2 promoter. IRF-2 inhibits the transcription of IRF-1-activated genes. Because IRF-2 accumulation is delayed relative to IRF-1 due to a much longer

half-life, IRF-2 acts as a negative feed-back mechanism to limit postinduction IRF-1 activity (Harada *et al.*, 1994).

6.3 Structure and activities of IRF-1

The most crucial activity of IRF-1 is its ability to activate transcription from specific promoters. Inactivation of its DNA-binding capacity or deletion of the amino acid sequences required for transcriptional activation eliminates all phenotypic response to IRF-1 overexpression (Kirchhoff, 1993). IRF-1 is a typical modular protein (Schaper, 1998). Distinct protein domains have been characterized that mediate transcriptional activation, DNA binding, nuclear translocation and heterodimerization with IFN consesus sequence binding protein (ICSBP). The N-terminal 125 amino acids encoding the DNA-binding domain (DBD) are structurally and functionally conserved among the IRF family. DBD is characterized by a winged type helix-loop-helix motif with five tryptophan repeats (Uegaki, 1995). IRF-1 binding sites contain a direct hexameric nucleotide repeat; IRF-1 may bind DNA either as a monomer or as a dimer. The dimer could interact with opposing sites of the DNA double helix. IRF-1 is constituvely localized in the nucleus (Pine *et al.*, 1990). The transcription factor contains two consesus signal sequences (NLS). It is a weak transcriptional activator: two activator fragments located between amino acids 185 and 256 function in an additive manner (Schaper, 1998).

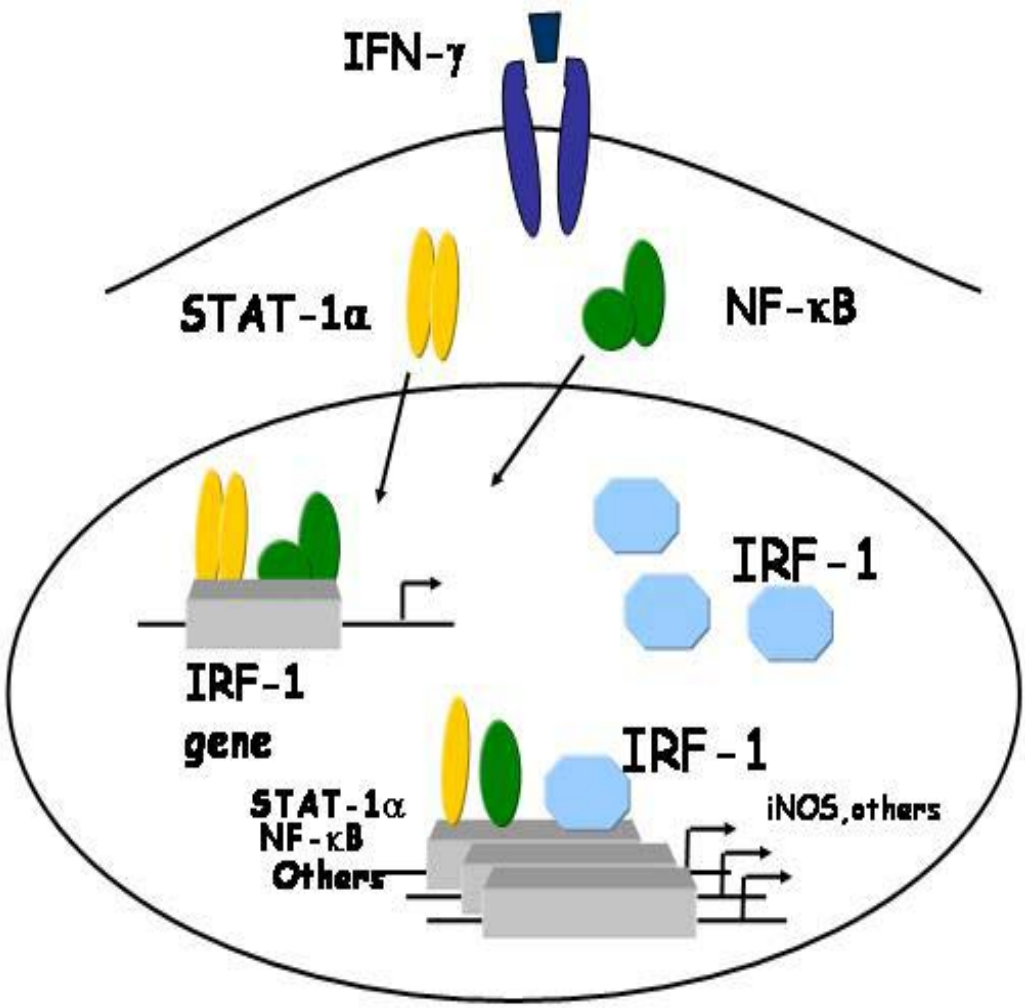

Figure 19. IRF-1 activation pathway.

A C-terminal fragment of IRF-1, which has no activator function by itself, acts as a strong enhancer of these activator sequences. A novel type of repression domain in the N-terminal 60 amino acids of IRF-1 that strongly inhibits its transcriptional activity and is conserved in some but not all IRF family members was identified (Kirchhoff *et al.*, 2000). These antagonistic acting domains may contribute to a balanced overall IRF-1 activity. This is regulated by different means: primarily it is regulated at the transcriptional level; since IRF-1 is serine/threonine phosphorylated at multiple sites the role of this phosphorylation was examined but this kind of regulation can neither be verified nor excluded (Lin, 1999). IRF-2 and IRF-8 are functional antagonists of IRF-1. The first one represses the IRF-1 transactivation of certain promoters. This is thought to be due to competition of both factors for the same DNA-binding sites (Yamamoto *et al.*, 1994). The second one has been shown to repress IFN and IRF-1 mediated activation of IFN-stimulated response element (ISRE)-driven genes (Weisz *et al.*, 1994).

IRF-1 interacts also with other transcription factors. The interaction with NF-κB is crucial for the induction of several promoters (Thanos and Maniatis, 1992). One such example is the iNOS promoter. Induction of iNOS is relevant for production of NO as defense against bacterial infections and for elimination of tumor cells. Bacterial compounds, such as lipopolysaccharides (LPS), induce NF-κB and hypoxia in tumor activates hypoxia-induced factor-1 (HIF-1) (Tendler *et al.* 2001). A combined action of IFN-γ with either of the coinducers leads to iNOS promoter activation. IRF-1 also cooperates with the tumor suppressor p53 (Tanaka *et al.*, 1996).

In a wide variety of unrelated cell types from different mammalian species, such as human and mouse, ectopic overexpression of IRF-1 leads to growth inhibition (Coccia *et al.* 1999). A first indication of these antiproliferative effects was the low number of

stable transfectants that were obtained when cells were transfected with DNA encoding IRF-1 and none of these transfectants efficiently expressed IRF-1 (Kirchhoff *et al.*, 1993). To investigate the relationship of IRF-1 activities and cell growth, recombinant IRF-1 was expressed from a tetracycline-regulated promoter. Alternatively, a fusion protein of IRF-1 with the hormone-binding domain of the human estrogen receptor was expressed, such that the DNA-binding specificity of IRF-1 was preserved and the transcriptional activator function of the fusion protein was dependent on estrogen. The antiproliferative effects of these constructs and the inactivity of DBD or DNA activation domain mutants showed that the growth inhibition is due to the potential of IRF-1 to stimulate transcription of antiproliferative acting agents (Kirchhoff *et al.*, 1993; Nguyen *et al.*, 1997). Physiologically, IRF-1 is transiently induced. Infact the growth inhibitory effect of IRF-1 extends significantly beyond the IRF-1 activation period. Activation of the IRF-1-estrogen-receptor fusion protein for a day or less is sufficient to mantain a reduced growth rate for several days in the absence of cell death. However, the cells recover and resume normal growth subsequently, demonstrating that IRF-1-induced growth arrest is reversible (Geserick *et al.*, 2000). IRF-1 might cause cell growth inhibition by inducing IFN-β gene expression or by other secreted factors.

The role of IRF-1 as tumor suppressor was first revealed by its ability to reverse IRF-2-induced transformation of NIH-3T3 cells and tumor formation in nude mice (Harada *et al.*, 1993). IRF-1 can prevent anchorage-independent growth of cells transformed by various oncogenes, such as c-myc and c-fos (Tanaka *et al.*, 1994). In addition, IRF-1 expression reverses the transformed phenotype of tumor cell lines and of cells transformed by one or more oncogenes, indicating that IRF-1 can act as general suppressor of transformation (Tanaka *et al.*, 1994). It has been shown that inactivation

of IRF-1 is implicated in the development and progression of human cancer, such as medulloblastoma and glioblastoma (Park *et al.*, 1998). The mechanisms by which IRF-1 exerts its tumor suppressive activities are not well understood. Several IRF-1-induced genes may be involved whose induction leads to proliferation inhibition, induction of apoptosis, stimulation of the immune response and reversion of transformed phenotype.

In general IRF-1 does not promote cell death. Under certain conditions, however, it promotes apoptosis. DNA damage-induced apoptosis of mitogen-activated T lymphocytes is dependent on IRF-1 (Tamura *et al.*, 1995). It has been shown that the induction of caspase-1 by IRF-1 is an essential component of apoptosis induction. Furthermore, IRF-1 can act as a mediator of cytokine-induced apoptosis. One underlying mechanism seems to be caspase-3 activation by IFN-γ. Caspase inhibition cannot prevent cell death but directs cells to undergo necrosis instead of apoptosis. Consequently, IRF-1 induction appears to be an important signaling event in initiation of apoptosis by IFN-γ.

Besides its function as apoptosis promoter, a role as apoptosis suppressor has been ascribed to IRF-1 (Chapman *et al.*, 2000). Moreover IRF-1 regulates the expression of a number of genes whose products play a central role in both innate and adaptative immunity, suggesting that IRF-1 may provide a link between the two systems.

IRF-1 induces the transcription of many genes involved in first reaction to viral invasion, such as PKR (Kirchhoff *et al.*, 1995). IRF-1 regulates the expression of iNOS in macrophages (Kamijo *et al.*, 1994). iNOS catalyzes the reaction of short-lived volatile gas NO, which is one of the principal mechanisms of macrophage cytotoxiciyìty against tumor cells, bacteria, protozoa, helmints and fungi (Nathan, 1991). iNOS gene transcription is induced by both IFN-γ and LPS (Ding *et al.*, 1990). IRF-1 binding sites

were identified in the promoter of iNOS gene and IRF-1 is essential for iNOS induction[2], demonstrating that IRF-1 is involved in antibacterial response. IRF-1 overexpression induces the transcription of IFN-α and IFN-β. Besides the involvement in antiviral effects, IFN-α and IFN-β have a number of other immune stimulatory effects on macrophages and T cells (Bogdan *et al.*, 2000). Also IRF-1 influences development and function of natural killer (NK) cells (Taki *et al.*, 1997). Finally, overexpression of IRF-1 by tumor cells leads to increased MHC class II and IFN-β gene expresssion (Kroger *et al.*, 2001). IRF-1 overexpression suppresses tumor formation in syngenic mice and subsequently protects mice against rechallenge with related tumor cells that are not overexpressing IRF-1. This indicates that IRF-1 plays a role not only in antagonism of tumor cell growth but also in stimulating an immune respone against tumor cells.

[2] IRF-1 binding site on iNOS promoter is showed in Figure 9 and descripted in chapter 3.4 at page 17

7. Materials and methods

7.1 Gliadin and Zein digest

Pure bread wheat (*Triticum Aestivum*, var. San Pastore) was kindly supplied by the "Istituto Sperimentale per la Cerealicoltura", Rome, Italy. Peptic-tryptic digest of gliadin (Pt-G), purified prolamin fraction, were prepared following a two-step procedure as previously described (De Ritis *et al.*, 1979). Gliadin (100 g) pool was digested in 1 liter of HCl 0.2 M (pH 1.8) with 2 g of purified pepsin at 37 °C for 2 h. The resultant peptic digest was further digested by addition of 2 g of purified trypsin after pH adjustment to 8.0 with NaOH 2 M. The reaction mixture was vigorously stirred at 37 °C for 4 h at pH 8.0. During the entire digestion procedure, the pH was checked periodically and when needed adjusted with HCl or NaOH. At the end of the whole digestion period, the digest was submitted to gel filtration and the peptide fractions eluted after cytocrome c were collected, freeze-dried and stored at –20 °C. Zein (Z) from maize (Sigma, Milan, Italy), was extracted from commercial preparations with 70 % (v/v) ethanol and freeze-dried. Z (1 g) was dissolved in 10 ml of HCl 0.1M. Pepsin (20 mg) was added and after digestion for 4 h at 37 °C, pH was adjusted to 8.0 by NaOH 5.0M. The zein was then further digested with 20 mg trypsin at 37°C for 4 h. Inactivation of trypsin was achieved by heating at 90 °C for 5 min. Insoluble material was removed by centrifugation at 10,000 g for 30 min. The peptic-tryptic digest of zein (Pt-Z) was finally freeze-dried and stored at -20 °C.

7.2 Patients

Biopsy specimens from the distal duodenum were obtained by upper gastrointestinal endoscopy from 6 children with coeliac disease on a normal gluten containing diet

(untreated) and 7 with celiac disease following gluten-free diet from almost 3 years (treated). Histological examination was performed on half specimen, while half sample-tissue was immediately frozen in liquid nitrogen and then tested. Diagnosis of coeliac disease was performed in all patients for anti-endomisial antibodies positivity (EMA) and typical mucosal lesions with crypt hyperplasia, villous atrophy, increased number of intraepithelial lymphocytes. Control pediatric patients (n = 5) underwent upper gastrointestinal endoscopy for gastrointestinal symptoms, but were EMA negative and their duodenal histology was normal. This study received ethical approval from local committee (University Federico II, Naples, Italy).

7.2.1 Organ culture

The mucosal specimens from other untreated children (n = 4) were cultured as previously described (Picarelli *et al.*, 1996) were incubated with medium alone at different time points (0, 2, 4, 6, 12 and 24 h). After 24 h incubation with medium alone the specimens were incubated for 2, 4 and 6 h with peptic-tryptic digest of gliadin (Pt-G; 1 mg/ml). Pt-G, purified prolamin fraction, was prepared as previously described (De Ritis *et al.*, 1979). The dishes were placed in a tight container with 95% O_2/ 5% CO_2 at 37 °C, at 1 bar. Biopsies were snap-frozen and stored at –80 °C until used.

7.2.2 Cytosolic and nuclear extracts from biopsy specimens

Cytosolic and nuclear extracts of biopsy specimens were prepared as previously described with some modification (D'Acquisto *et al.*, 2001). Briefly, each biopsy specimen was frozen in liquid nitrogen, immediately suspended in 150 μl of ice-cold hypotonic lysis buffer (10 mM Hepes, 10 mM KCl, 0.5 mM

phenylmethylsulphonyfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and homogenized using a glass homogenizer and a teflon pestle. The homogenates were chilled on ice for 15 min and then vigorously shaken for another 15 min in the presence of 20 μl of 10% Nonidet P-40. The nuclear fraction was precipitated by centrifugation at 1500 g for 5 min, the supernatant containing the cytosolic fraction was removed and stored at –80 °C. The nuclear pellet was resuspended in 100 μl of high salt extraction buffer (20 mM Hepes pH 7.9, 10 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulphonyfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000 g and supernatant was aliquoted and stored at –80 °C. Protein concentration was determined by Bio-Rad (Milan, Italy) protein assay kit.

7.2.3 Electrophoretic mobility shift assay (EMSA)

Double stranded oligonucleotides containing the NF-κB, IRF-1 (5'-GGAAGCGAAAATGAAATTGACT-3') and STAT-1α (5'-CATGTTATGCATATTCCTGTAAGTG-3') recognition sequences were endlabelled with ^{32}P-γ-ATP. Nuclear extracts containing 5 μg protein were incubated for 15 min with radiolabeled oligonucleotides (2.5-5.0 x 10^4 cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol. The specificity of the DNA/protein binding was determined by competition reaction in which a 50-fold molar excess of unlabeled wild-

type, mutant or Sp-1 oligonucleotide was added to the binding reaction 15 min before addition of radiolabeled probe. In supershift assay, antibodies reactive to p50 or p65, IRF-1 or STAT-1α proteins were added to the reaction mixture 15 min before the addition of radiolabeled probe. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1x TBE buffer at 150 V for 2 h at 4 °C. The gel was dried and autoradiographed with intensifying screen at –80 °C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst; IBM).

7.2.4 Western blot analysis

Immunoblotting analysis of anti-p50, anti-p65, anti-IRF-1, anti-STAT-1α, anti-IκBα, anti-IκBβ, anti-iNOS, anti-COX-2, anti-histone and anti-β-actin was performed on biopsy. Cytosolic and nuclear fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml of bromophenol) in a ratio of 1:1, boiled for 3 min and centrifuged at 10,000 g for 5 min. Protein concentration was determined and equivalent amounts (50 μg) of each sample were electrophoresed in a 12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitro-cellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4 °C overnight with 10% non-fat dry milk in PBS and then incubated with (1:1000) anti-IRF-1, anti-STAT-1α, anti-IκBα, anti-IκBβ, anti-iNOS, anti-COX-2 for 1 h at room temperature. The membranes were washed three times with 0.05% Triton 100x in PBS and then

incubated with anti-rabbit or anti-goat immunoglobulins coupled to peroxidase (1:1000). The immunocomplexes were visualized by the ECL chemiluminescence method (Amersham, Milan, Italy). The membranes were stripped and re-probed with β-actin or histone-1 antibody to verify equal loading of proteins. Subsequently, the relative bands of p50 and p65, IRF-1 and STAT-1α in nuclear fraction as well as iNOS and COX-2 in cytosolic fraction were quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

7.2.5 Immunofluorescence of p65 subunit

Immunological sections of small intestinal biopsy specimens were prepared by cutting paraffinated tissues and subsequently prepared for NF-κB immunofluorescence. The slides were gently washed with sterile phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The slides were incubated with primary antibody (anti-p65) for 1 hr or overnight at room temperature, and then washed 3 times in 0.1% Tween 20 in PBS, followed by 30 min incubation with fluorescein isothiocyanate (FITC-conjugated secondary antibody, Clontech, Inc., Palo Alto, CA). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Coverslips were mounted and images were acquired on Nikon TE 300 inverted microscope equipped with Leica DC-100 colour digital camera.

7.3 Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 was cultured at 37 °C in humidified 5 % CO_2/95% air in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes and 5 mM sodium pyruvate. The cells were plated in 24 culture wells at a density of 2.5 x 10^5 cells/ml per well or 10 cm diameter culture dishes at a density of 3 x 10^6 cells/ml per dish and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated with IFN-γ (25 U/ml). Pt-G (50, 100, 200, 400 and 800 μg/ml), G (50, 100, 200, 400 and 800 μg/ml), Bovine Serum Albumin (BSA; 50, 100, 200, 400 and 800 μg/ml), Pt-Z (50, 100, 200, 400 and 800 μg/ml), Z (50, 100, 200, 400 and 800 μg/ml), Pyrrolidine dithiocarbamate (PDTC; 0.1, 1 and 10 μM) or N-α-para-tosyl-L-lysine chloromethyl ketone (TLCK; 1, 10 and 100 μM) were added to the cells 5 min after IFN-γ challenge, alone or in combination. The cell viability was determined by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay (D'Acquisto *et al.*, 2001). Briefly, 100 μl MTT (5 mg/ml in complete DMEM) was added and the cells were incubated for an additional 3 h. After this time point the cells were lysed and the dark blue crystals solubilized with 500 μl of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCCC/340) equipped with a 620 nm filter. The cell viability in response to treatment with test compounds was calculated as % dead cells = 100 – (OD treated / OD control) x 100.

7.3.1 Nitrite determination

NO was measured as nitrite (NO_2^-, nmol/10^6 cells) accumulated in the incubation medium after 24, 48 and 72 h. A spectrophotometric assay based on the Griess reaction was used (D'Acquisto *et al.*, 2001). Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in phosphoric acid) was added to an equal volume of cell culture supernatant and the absorbance at 550 nm was measured after 10 min. The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

7.3.2 Cytosolic and nuclear extracts from cultured cells

Cytosolic and nuclear extracts of macrophages stimulated for 1, 3, 6**,** 24, 48 and 72 h with IFN-γ (25 U/ml) in presence or absence of Pt-G (50, 100, 200, 400 and 800 μg/ml), G (50, 100, 200, 400 and 800 μg/ml), PDTC (0.1, 1 and 10 μM) or TLCK (1, 10 and 100 μM) were prepared as previously described with some modification (D'Acquisto *et al.*, 2001). Briefly, harvested cells (3 x 10^6) were washed two times with ice-cold PBS and centrifuged at 180 g for 10 min at 4 °C. The cell pellet was resuspended in 100 μl of ice-cold hypotonic lysis buffer (10 mM Hepes, 10 mM KCl, 0.5 mM phenylmethylsulphonyfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle for five or six times and the cytoplasmic fraction was then obtained by centrifugation for 1 min at 13,000 g. The supernatant containing the cytosolic fraction was removed and stored at –80°C. The nuclear pellet was resuspended in 60 μl of high salt extraction buffer (20 mM Hepes pH 7.9, 10 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM

phenylmethylsulphonyfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000 g and supernatant was aliquoted and stored at –80 °C. Protein concentration was determined by Bio-Rad (Milan, Italy) protein assay kit.

7.3.3 Real time quantitative polymerase chain reaction (RT-PCR)

RAW 264.7 cells (1.0×10^6) were seeded onto 10 cm culture plates in 10 ml of DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. IFN-γ (25 U/ml), G (800 μg/ml), PDTC (10 μM), Gen (25 μM), TB42 (50 μM) were added to the cells alone or in combination at 1, 6 and 24 h. Total RNA was prepared from cells by using the Rneasy Mini Kit (Qiagen) and subjected to cDNA synthesis with random hexanucleotide primers and MultiScribe Reverse Transcriptase (Invitrogen) at 48°C for 1 h. The cDNA was then amplified in a iCycler Iq Real-Time PCR Detection System (Biorad) using iQ™SYBR Green Supermix in triplicate in 25 μl reaction volumes. Relative quantification of gene expression was performed using the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001). Changes in mRNA expression levels were calculated following normalization to c-Abl transcripts. The ratios obtained following normalization are expressed as fold increase. The primer sequences were as follows:

c-Abl-F: 5'GGTATGAAGGGAGGGTGTACCA-3'

c-Abl-R: 5'GTGAACTAACTCAGCCAGAGTGTTGA-3'

NF-κB-F: 5'GGGGATGTGAAGATGTTG-3'

NF-κB-R: 5'CCAAGTGCAGAGGTGTCTGA-3'

STAT-1α–F: 5'GTTGCAGCACAACATACGGAA-3'

STAT-1α-R: 5'GATCATGGACATCTGTACGGGA-3'

IRF-1-F: 5'CAATCACTGGAATGCGGATGA-3'

IRF-1-R: 5'TTGTTGATGTCCCAGCCGTG-3'

iNOS-F: 5'GAGCATCCCAAGTACGAGTGGT-3'

iNOS-R: 5'GGCCACGGCAGGCAG-3'

7.3.4 Reporter vectors and transfection experiments

RAW 264.7 macrophages were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml L-glutamine. Cells (3×10^6) were transfected in triplicate with Lipofectamine™ Reagent (Life Technologies, Invitrogen). Each transfection, according to the manufacturer's instructions, was performed with 10 μl of Lipofectamine™ Reagent and 2 μg of piNOS CAT1 plasmid (Weisz *et al.*,1996) plus 0.2 μg of CMV-Luc vector, to correct for variations in DNA uptake and transfection efficiency. After 24 h of transfection the cells were treated with IFN-γ (25 U/ml), G (800 μg/ml), PDTC (10μM), Gen (25 μM) and TB42 (50 μM) alone or in combination for 1, 6 and 24 h. Luciferase activities were then measured in a microplate luminometer (EG&G Berthold) as previously described (Bevilacqua *et al.*, 1997). CAT gene expression was assessed by measuring the CAT protein levels as Reverse transcriptase-polymerase chain reaction (RT-PCR) relative activity, by ELISA. (Roche Molecular Biochemicals, Mannheim, Germany). The values reported in the figures are the mean of three independent transfection experiments.

7.4 Reagents

^{32}P-γ-ATP was from Amersham (Milan, Italy). Poly dI-dC and T4 polynucleotide kinase were from Boehringer-Mannheim (Milan, Italy). Anti-p50, anti-p65, anti-iNOS, anti-COX-2, anti-IκBα, anti-IκBβ and β-actin antibodies were from Santa Cruz (Milan, Italy). Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim, Genova, Italy). Non-fat dry milk was from Bio-Rad (Milan, Italy). DL-dithiothreitol, pepstatin A, leupeptin, benzamidine, phenylmethylsulfonilfluoride were from Applichem (Darmstadt, Germany). Pt-G from pure bread wheat (*Triticum aestivum*, var. San Pastore) was kindly supplied by the "Istituto Sperimentale per la Cerealicoltura" (Rome, Italy). All other reagents were from Sigma (Milan, Italy). Recombinant mouse interferon-γ was from Vinci-Biochem (Florence, Italy). ^{32}P- γ -ATP was from Amersham (Milan, Italy). Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). Anti-p50, anti-p65, anti-IRF-1, anti-STAT-1α, anti-iNOS, anti-IκBα, anti-β-actin and anti-histone1 antibodies was from Santa Cruz (Milan, Italy). Phosphate buffer saline was from Celbio (Milan, Italy). Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim, Genova, Italy). Non-fat dry milk was from Bio-Rad (Milan, Italy). CAT ELISA was from Molecular Biochemicals (Roche Mannheim, Germany). Lipofectamine™ and Reagent iQ™SYBR Green Supermix were from Life Technologies (Invitrogen, Milan, Italy).

DL-dithiothreitol, pepstatin A, leupeptin, benzamidine, phenylmethylsulfonilfluoride were from Applichem (Darmstadt, Germany). Gliadin, BSA, MTT, PDTC and TLCK as well as other reagents were from Sigma (Milan, Italy).

7.5 Statistics

Results are expressed as the means ± S.E.M. of *n* experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected P-value for multiple comparison test. The level of statistically significant difference was defined as $P<0.05$. Linear associations between variables were assessed by the use of standard-least-square linear regression. Correlation coefficient (r) was presented as measure of linear association for regression relationship.

Nuclear factor κB is activated in small intestinal mucosa

of celiac patients

8.1 Results

8.1.1 NF-κB activity is increased in intestinal mucosa of celiac patients

To detect NF-κB/DNA binding activity, nuclear extracts from biopsy specimens of untreated patients, treated patients and normal controls were analyzed by EMSA. As shown in Figure 20 A and B, a low basal level of NF-κB/DNA binding activity was detected in nuclear extracts from biopsy specimens of controls. The NF-κB/DNA binding activity markedly increased in nuclear extracts obtained from biopsy specimens of untreated patients, while it significantly decreased in nuclear extracts from biopsy specimens of patients treated. The composition of the NF-κB complex was determined by competition and supershift experiments in nuclear extracts from untreated patients. The specificity of NF-κB/DNA binding complex was demonstrated by the complete displacement of NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabeled NF-κB probe (W.T., 50x) in the competition reaction. In contrast, a 50-fold molar excess of unlabeled mutated NF-κB probe (Mut., 50x) or Sp-1 oligonucleotide (Sp-1, 50x) had no effect on this DNA-binding activity. The subunit composition of the NF-κB complexes was determined by incubating nuclear extracts with specific antibodies against p50 or p65 subunits and observing the effects on the electrophoretic mobility of NF-κB DNA complexes. Addition of anti-p65 to the binding reaction caused the appearance of low mobility complex whereas addition of anti-p50 caused the appearance of the faster migrating complex. Concomitant addition of anti-p50 + anti-p65 to the binding reaction resulted in a marked reduction of the levels of NF-κB complexes, suggesting that NF-κB consisted primarily of p50 and p65 dimers (Figure 20 C).

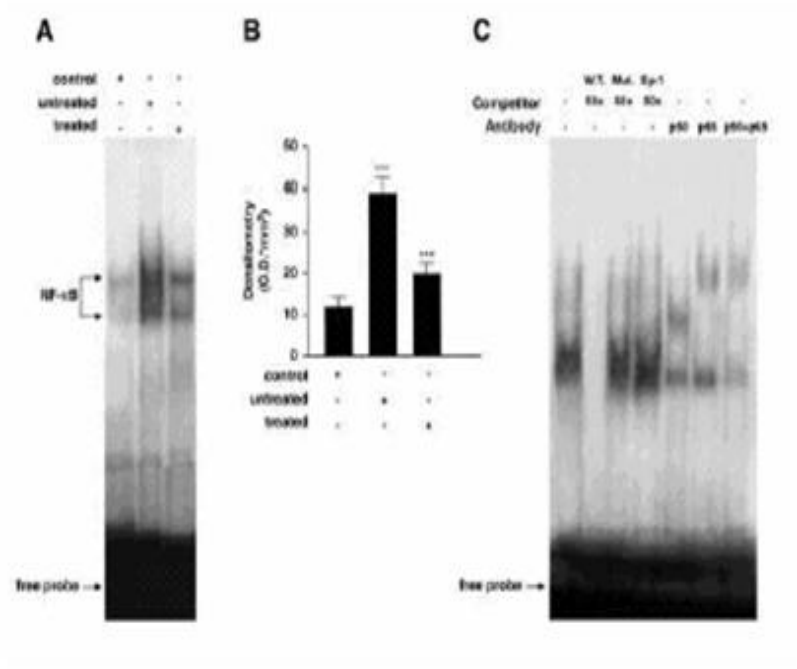

Figure 20. NF-κB activation and characterization of NF-κB complex (A, B). Representative electrophoretic mobility shift assay (A) and densitometric analysis (B) shows NF-κB/DNA binding activity in nuclear extracts from biopsy specimens of control, untreated and treated patients. Nuclear extracts were prepared as described in the text and incubated with ^{32}P-labeled NF-κB probe. (A) Data are from a single experiment. (B) Mean ± SEM of fourteen experiments. °°° $P < 0.0001$ *vs.* control; *** $P < 0.0001$ *vs.* untreated. (C) Characterization of NF-κB complex. In competition reaction nuclear extracts from biopsy specimens of untreated patients were incubated with radiolabeled NF-κB probe in absence or presence of identical but unlabeled oligonucleotides (W.T., 50x), mutated nonfunctional κB probe (Mut., 50x) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50x). In supershift experiments nuclear extracts were incubated with antibodies against p50, p65 or p50+ p65 15 min before incubation with radiolabeled NF-κB probe. Data are from a single experiment and representative of six experiments.

The NF-κB activation was confirmed by immunofluorescence analysis performed on these specimens and as well as a larger number of patients and controls. An increased nuclear p65 expression was detectable in crypt epithelial cells as well as in lamina propria mononuclear cells from untreated patients as compared to treated and controls (Figure 21).

8.1.2 Nuclear level of p50 and p65 subunits

The level of p50 and p65 in nuclear extracts from biopsy specimens was examined by Western blot analysis. Biopsy specimens from controls expressed a basal level of p50 and p65, whereas from untreated patients the levels of p50 and p65 were markedly increased as compared with treated patients (Figure 22).

8.1.3 Cytoplasmic and nuclear level of IκB proteins

Since NF-κB activation is controlled by inhibitory IκB proteins, we examined the presence of IκBα and IκBβ proteins in cytosolic and nuclear extracts from biopsy specimens of untreated, treated patients and controls in an attempt to underlying mechanisms to sustained activation of NF-κB. In biopsy specimens from untreated patients IκBα and even more IκBβ disappeared from the cytosolic fraction whereas high levels of IκBα and lower levels of IκBβ were detectable in specimens from treated patients and controls. Significant amounts of IκBα and IκBβ were observed in the nuclear extracts from biopsy specimens of untreated patients, while lower amounts of nuclear IκBα and IκBβ were observed in specimens from treated patients. Basal levels of IκBα and IκBβ were present in the nuclear extracts from specimens of controls (Figure 23).

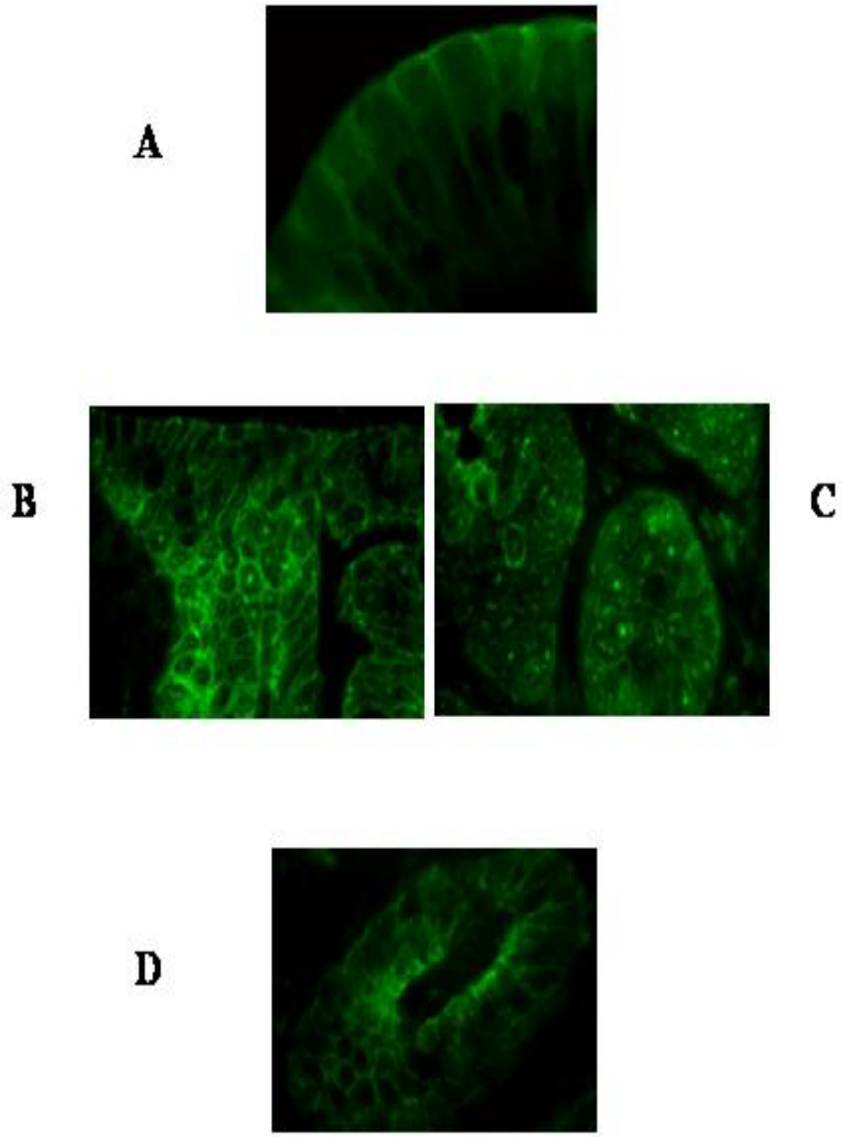

Figure 21. Representative immunofluorescence of p65 subunit confirms NF-κB activation. Crypt epithelial and lamina propria mononuclear cells are positive for p65 subunit. (A, C) Data are from a single experiment and representative of five experiments.

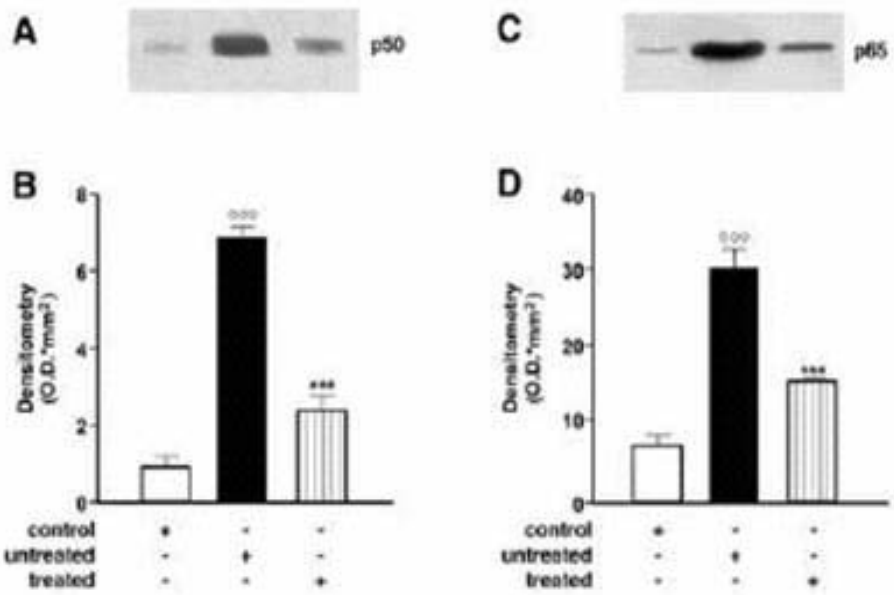

Figure 22. Nuclear level of p50 and p65 subunits. Representative western blots of p50 (A) and p65 (C) and densitometric analysis (B, D) show the nuclear level in biopsy specimens from control, untreated and treated patients. (A, C) Data are from a single experiment. (B, D) Mean ± SEM of five experiments. °°° $P < 0.0001$ *vs.* control; *** $P < 0.0001$ *vs.* untreated.

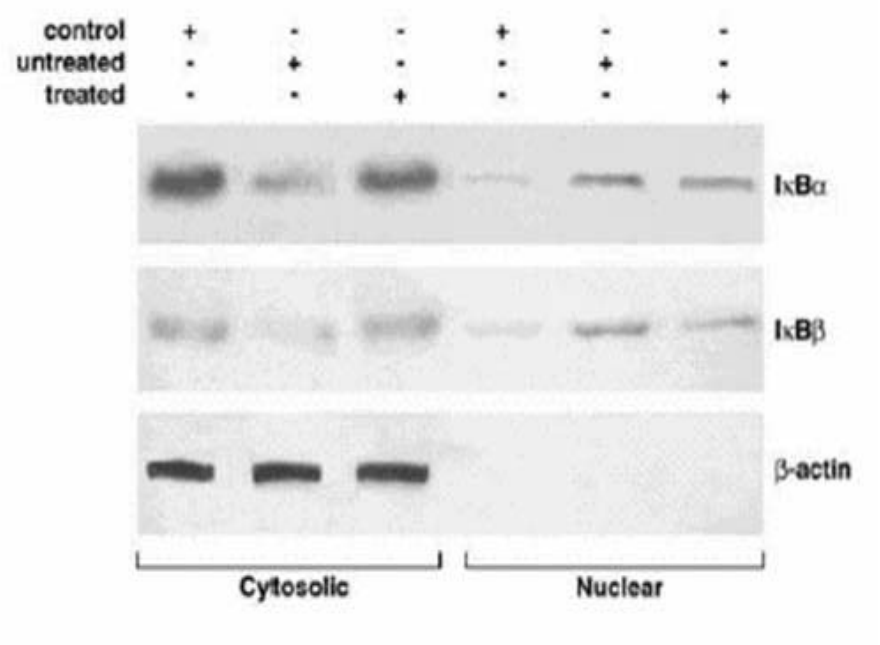

Figure 23. Cytosolic and nuclear level of IκB subunits. Representative western blots shows the cytosolic and nuclear level of IκBα and IκBβ in biopsy specimens from control, untreated and treated patients. β-actin is shown as a control. Data are from a single experiment and representative of five experiments.

8.1.4 iNOS and COX-2 protein expression

iNOS and COX-2 protein level in cytosolic extracts from biopsy specimens were determined by Western blot analysis. As shown in Figure 24 a significantly high level of either iNOS and COX-2 protein expression was detected in biopsy specimens from untreated patients when compared with controls. In biopsy specimens from treated patients the level of either iNOS or COX-2 protein expression was significantly reduced as compared with untreated patients.

8.1.5 Kinetic analysis of NF-κB activation in cultured biopsy specimens from untreated patients

To determine whether NF-κB activity was also sustained *ex vivo*, we used an in vitro model of mucosal biopsies. Biopsy specimens from untreated patients were cultured with medium alone for 0, 2, 4, 6, 12 and 24 h before assessment of NF-κB/DNA binding activity. The results in Figure 25 A show that in nuclear extracts from specimens cultured for 0, 2, 4 and 6 h NF-κB activity was maintained at high levels, while in those cultured for 12 and 24 h NF-κB activity was decreased. When NF-κB activity was sustained at high levels, both iNOS and COX-2 protein expression was also maintained at high levels. Conversely, reduced NF-κB activity was accompanied by a decrease in both iNOS and COX-2 protein expression (Figure 25 B and C, respectively). NF-κB/DNA binding activity and either iNOS and COX-2 protein expression were correlated ($r = 0.99$, $^{***}P<0.0001$ and $r = 0.98$, $^{***}P<0.0001$, respectively).

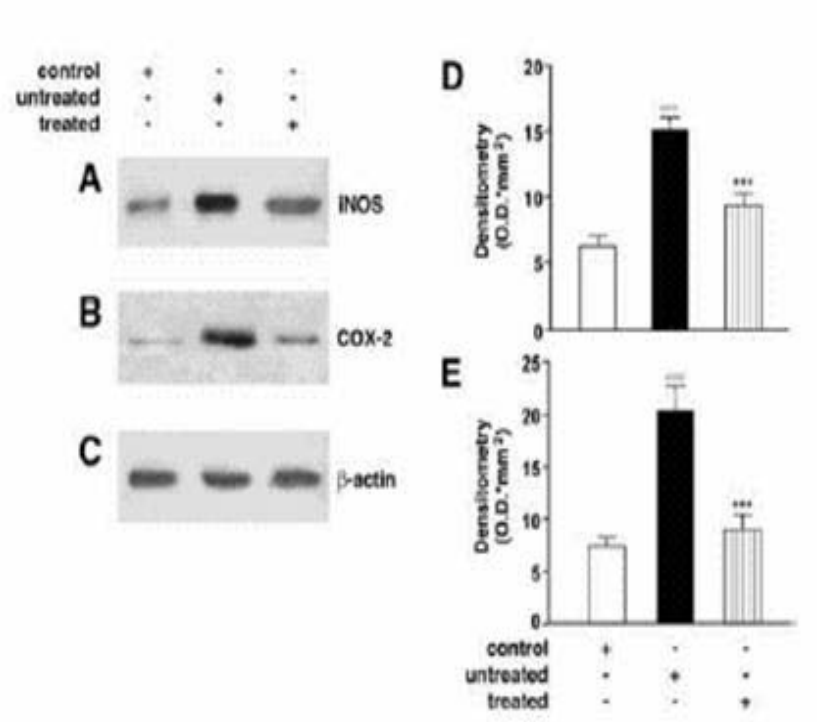

Figure 24. Expression of iNOS and COX-2. Representative western blots of iNOS (A) and COX-2 (B) and densitometric analysis (D, E) show the protein expression in cytosolic extracts from biopsy specimens from control, untreated and treated patients. (C) β-actin expression is shown as a control. (A-C) Data are from a single experiment. (D, E) Mean ± SEM of five experiments. °°°P< 0.0001 *vs.* control; ***P< 0.0001 *vs.* untreated.

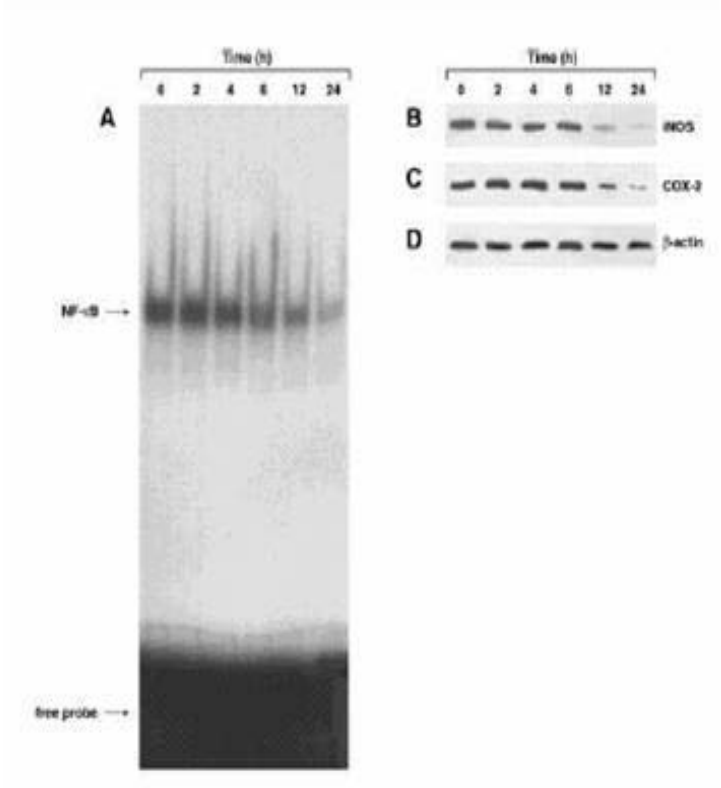

Figure 25. Kinetic analysis of NF-κB activation in cultured biopsy specimens from untreated patients (A). Representative electrophoretic mobility shift assay shows NF-κB/DNA binding activity in nuclear extracts from biopsy specimens cultured with medium alone for 0, 2, 4, 6, 12 and 24 h. (B, C) Representative western blots of iNOS (B) and COX-2 (C) show the protein expression in cytosolic extracts from biopsy specimens cultured with medium alone for 0, 2, 4, 6, 12 and 24 h. (D) β-actin expression is shown as a control. Correlation coefficients between the intensity of NF-κB/DNA binding activity and both iNOS and COX-2 protein expression bands, determined by densitometric analysis, were 0.99 ($P< 0.0001$) and 0.98 ($P< 0.0001$), respectively. (A) Data are from a single experiment and representative of seven experiments. (B, D) Data are from a single experiment and representative of four experiments.

In addition, to evaluate whether NF-κB activity decreased at 24 h recovered, the specimens were incubated with Pt-G (1 mg/ml) for 2, 4 and 6 h. As shown in Figure 26 A, NF-κB activity increased 6h after addition of Pt-G whereas the levels of either iNOS and COX-2 protein expression were at the limit of detection (Figure 26 B and C, respectively). These results show that NF-κB activity is sustained in intestinal mucosa of patients with untreated CD even 6 h after removal from the causative environment, decreases at 12 and 24 h and increases 6 h after the addition of Pt-G.

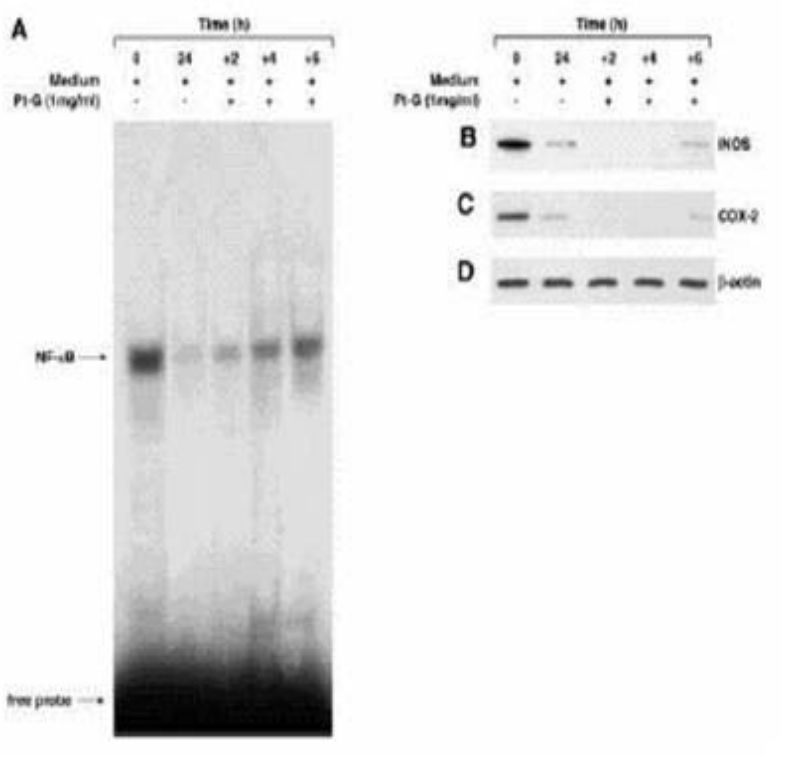

Figure 26. Kinetic analysis of NF-κB activation in cultured biopsy specimens from untreated patients in the presence or absence of Pt-G (A). Representative electrophoretic mobility shift assay shows NF-κB/DNA binding activity in nuclear extracts from biopsy specimens cultured with medium alone for 0 and 24 h and then incubated with Pt-G (1 mg/ml) for 2, 4 and 6 h. (B, C) Representative western blots of iNOS (B) and COX-2 (C) show the protein expression in cytosolic extracts from biopsy specimens cultured with medium alone for 0 and 24 h and the incubated with Pt-G (1 mg/ml) for 2, 4 and 6 h . (D) β-actin expression is shown as a control. (A) Data are from a single experiment and representative of three experiments. (B, D) Data are from a single experiment and representative of two experiments.

8.2 Discussion

NF-κB is a transcriptional regulator that mediates key immune and inflammatory response (Ghosh *et al.*, 1998). In this report we present evidence for the first time that NF-κB is constitutively activated in intestinal mucosa of patients with untreated CD. We found that NF-κB/DNA binding activity is significantly greater in biopsy specimens from untreated patients than in those from treated patients, indicating that NF-κB activation occurs in this mucosal compartment and declines on removal of gluten from diet. Levels of p50 and p65 subunits were higher in nuclear extracts from biopsy specimens of untreated patients than in those from treated patients. IκBα and IκBβ were degraded in the cytosol and present in the nucleus, suggesting that IκBβ plays a role in maintaining NF-κB/DNA binding activity in inflamed mucosa of patients with untreated CD. It has been reported that agents promote persistent NF-κB activity induce IκBα and IκBβ degradation (Bourke *et al.*, 2000; Johnson *et al.*, 1996). IκBα is implicated in regulating the persistent NF-κB activation in inflammatory chronic diseases (De Luca *et al.*, 1999; Thompson *et al.*, 1995). It has been shown that following degradation of the initial pool of IκBβ, newly unphosphorylated synthesized IκBβ, can act as a chaperone of NF-κB blocking the inhibitory effect of IκBα in the nucleus and therefore maintain NF-κB activity even after IκBα resynthesis (Suyang *et al.*, 1996). Furthermore, other studies have demonstrated that the dynamic state of degradation and resynthesis of IκBβ may result in the continuous production of hypophosphorylated IκBβ form that is unable to mask the nuclear localization signal of RelA, permitting NF-κB/ IκBβ complexes to enter into the nucleus and bind DNA (Ghosh *et al.*, 1998; McKinsey *et al.*, 1997).

In this study we demonstrate that IκBβ is present in the nuclear fraction. At present we were unable to determinate whether IκBβ is as part of NF-κB/DNA complex and in hypoor unphosphorylated state. Nevertheless, we have found that NF-κB activation persists for up 6 h in cultured biopsy specimens from untreated patients and is lower at 12 and 24 h. In addition, NF-κB activation was correlated with either iNOS and COX-2 protein expression. Previous studies have shown that iNOS is expressed more in enterocytes and COX-2 in the cells of lamina propria (ter Steege *et al.*, 1997; Kainulainen *et al.*, 2002). These enzymes catalyzing the synthesis of nitric oxide and proinflammatory prostaglandins, respectively, have been shown to be involved in disease induction and maintenance (Beckett *et al.*, 1999; Lavo *et al.*, 1990; ter Steege *et al.*, 1997; van Straaten *et al.*, 1999). Our finding that both iNOS and COX-2 expression is increased in biopsy specimens from untreated patients is in agreement with previous observations, although in other studies iNOS and COX-2 appear to play a protective role in intestinal injury (Kainulainen *et al.*, 2002; McCafferty *et al.*, 1997; Grisham *et al.*, 2002; Newberry *et al.*, 1999; Morteau, 1999). Interestingly, our findings show that removal of the inflamed mucosa from the causative environment reduces the expression of both iNOS and COX-2, two molecular events downstream of NF-κB activation, and suggest that NF-κB activation is diminished in patients with a strict gluten-free diet. Moreover, we observed that NF-κB activity is decreased at 24 h and increased in cultured biopsy specimens 6 h after the addition of Pt-G.

Taken together our results show that NF-κB is indeed activated in intestinal mucosa of untreated CD patients and suggest a role for IκBβ in regulating the persistent activation of NF-κB in this disease. Therefore NF-κB might play a pivotal role in the perpetuation

of inflammatory process in CD and even at early stage. NF-κB appears to be an important mediator of antigen-induced T cell activation and promotes Th1 subset development through the induction of NF-κB-dependent cytokines such as IFN-γ (Aronica *et al.*, 1999).

Secreted products of activated T cells are capable of maintaining the activation of nonimmune cells within the lesion, thereby perpetuating the chronic inflammatory process (Makarov *et al.*, 2000). Gluten induces activation of mucosal Th1 cells in patients with susceptibility of CD, thereby leading to local secretion of high levels of interferon-γ, which alone or together with other mediators activates macrophages and directly or indirectly damages enterocytes or alter their maturation (Nilsen *et al.*, 1995). Activated macrophages secrete cytokines, adhesion molecules, and enzymes whose gene expression is known to be transcriptionally regulated by NF-κB (Makarov *et al.*, 2000). These mediators may contribute to a perpetuation of the inflammatory reaction (Sollid, 2000; Beckett *et al.*, 1999). Thus our findings may be of clinical relevance because the sustained activation of NF-κB in intestinal mucosa of CD patients leads to prolonged induction of inflammatory gene expression and thereby perpetuates the chronic inflammatory process. In conclusion, the presence of activated NF-κB in human mucosal lesion in CD may yield new insights into the understanding of the pathogenesis of this disorder.

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Gliadin increases iNOS protein expression in interferon-γ RAW 264.7 cells through a mechanism involving NF-κB

9.1 Results

9.1.1 Effect of Pt-G and G on nitrite production by RAW 264.7 cells stimulated with IFN-γ for 24 h

The nitrite production by unstimulated cells was undetectable. The stimulation of cells with IFN-γ (25 U/ml) for 24 h resulted in an accumulation of nitrite in the medium. Addition of Pt-G (50, 100, 200, 400 and 800 μg/ml) or G (50, 100, 200, 400 and 800 μg/ml) to the cells increased significantly and in a concentration-dependent manner the nitrite production as compared with IFN-γ alone (Figure 27). Enhancement of nitrite production by G was greater than that exhibited by Pt-G. In contrast, Pt-G or G added to the cells alone, at the same concentrations, did not elicit the nitrite production (data not shown). BSA, Pt-Z and Z (50, 100, 200, 400 and 800 μg/ml), used as other food antigens, alone or in combination whit IFN-γ had no effect (Table 4). All compounds did not affect cell viability (>95%).

9.1.2 Effect of PDTC and TLCK on increase of nitrite production and iNOS protein expression by Pt-G and G in RAW 264.7 macrophages stimulated with IFN-γ for 24 h

Pt-G (800 μg/ml) or G (800 μg/ml), added to the cells together with IFN-γ (25 U/ml), caused an increase of nitrite production (55.4 ± 2.7 and 86.4 ± 3.4, respectively; $n = 3$) as compared with IFN-γ alone (36.0 ± 0.9; $n = 3$).

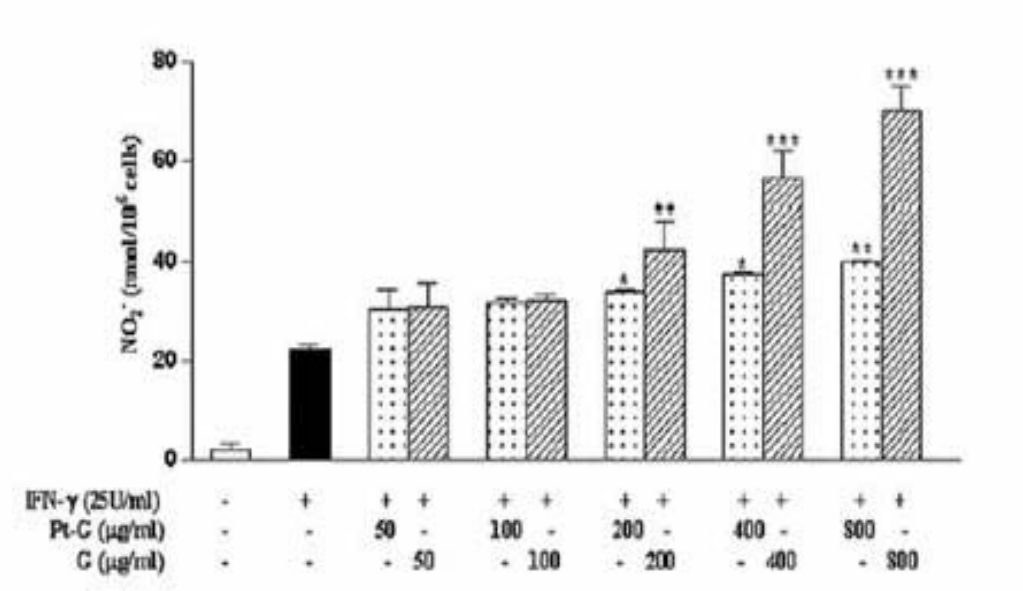

Figure 27. Concentration-dependent increase of nitrite production by Pt-G and G in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. Data are expressed as mean ± SEM of five experiments in triplicate. *P<0.05, **P<0.001, ***P<0.0001 *vs.* IFN-γ alone.

Table 4 Effect of food antigens on nitrite production in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. Data are expressed as mean ± SEM of five experiments in triplicate. BSA = Bovine serum albumin; Z= Zein; Pt-Z= Peptic-tryptic digest of zein

Treatment	Control cells	BSA (800 μg/ml)	Z (800 μg/ml)	Pt-Z (800 μg/ml)	
None	2.1 ± 1.2	2.3 ± 0.9	2.2 ± 0.6	2.4 ± 0.8	nmol/10^6 cells
IFN-γ (25 U/ml)	22.2 ± 1.2	23.1 ± 1.5	20.3 ± 0.9	21.4 ± 1.2	nmol/10^6 cells

PDTC or TLCK inhibited significantly the increase of nitrite production induced by Pt-G and G (Figure 28 A and B). PDTC (0.1, 1 and 10 μM) or TLCK (1, 10 and 100 μM) caused a reduction of nitrite production induced by IFN-γ alone (by 31.9 ± 2.1%, 37.5 ± 1.4%, 66.8 ± 1.8% and 40.0 ± 2.35%, 49.0 ± 3.0%, 64.0 ± 2.1%, respectively; $n = 3$).

Upon stimulation with IFN-γ (25 U/ml) for 24 h, cells expressed high level of iNOS protein expression as compared with untreated cells. Pt-G (800 μg/ml) or G (800 μg/ml), added to the cells together with IFN-γ (25 U/ml), increased iNOS protein expression (by 1.8 and 3.0 fold, respectively). PDTC (10 μM) or TLCK (100 μM) reduced significantly the increase of iNOS protein expression induced by Pt-G (by 80.0 ± 0.1% and 90.0 ± 0.2%, respectively) or by G (by 55.1 ± 0.1% and 65.0 ± 0.2%, respectively) (Figure 29). Pt-G and G alone failed to augment the iNOS protein expression level (data not shown).

9.1.3 Effect of Pt-G and G on NF-κB/DNA binding activity in RAW 264.7 macrophages stimulated with IFN-γ for 24 h

The effects of Pt-G (800 μg/ml) or G (800 μg/ml) on NF-κB/DNA binding activity in RAW 264.7 macrophages stimulated with IFN-γ (25 U/ml) for 24 h were evaluated by EMSA. A low basal level of NF-κB/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected following stimulation with IFN-γ. Pt-G and G caused an increase of NF-κB/DNA binding activity (by 1.3 and 1.6 fold, respectively) which was reduced by

PDTC (10 μM; 90.0 ± 4.5% and 90.0 ± 3.8%, respectively) or TLCK (100 μM; 73.0 ± 2.0% and 83.5 ± 1.5%, respectively) (Figure 30 A).

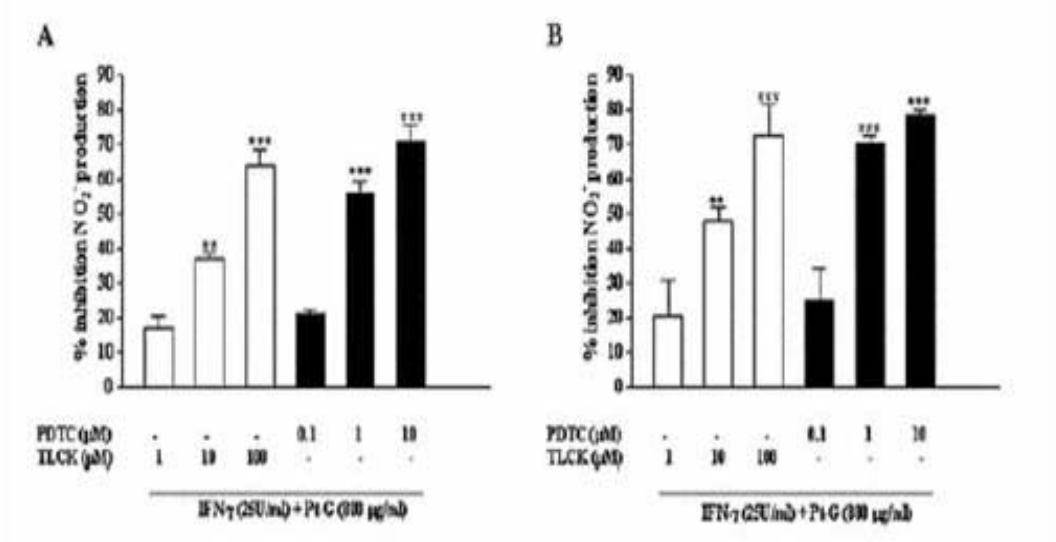

Figure 28. RAW 264.7 macrophages stimulated with IFN-γ for 24 h in the presence of (A) Pt-G or (B) G were treated with pyrrolidine dithiocarbamate (PDTC) and N-α-para-tosyl-L-lysine chloromethyl ketone (TLCK). Data are expressed as mean ± SEM of three experiments in triplicate. (A) ***P*<0.001, *** *P*<0.0001 *vs.* IFN-γ plus Pt-G; (B) ***P*<0.001, ****P*<0.0001 *vs.*IFN-γ plus G.

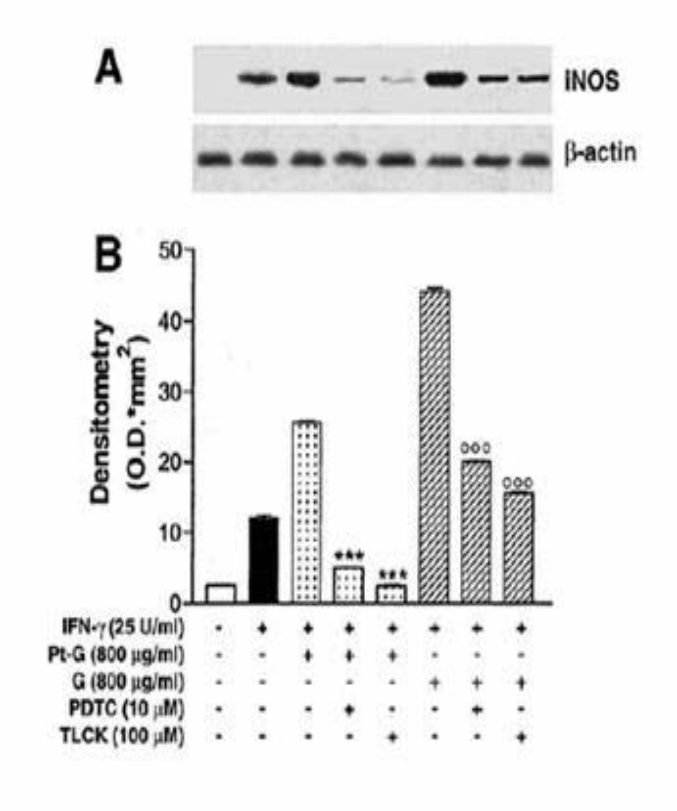

Figure 29. (A) Representative western blots of iNOS protein as well as (B) densitometric analysis shows the effect of PDTC and TLCK on enhancing iNOS protein expression by Pt-G and G in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. (A) Data are from a single experiment and representative of five experiments. β-actin expression is shown as a control. (B) Data are expressed as mean ± SEM of five experiments. °°° $P < 0.0001$ *vs.* IFN-γ plus Pt-G; °°° $P < 0.0001$ *vs.* IFN-γ plus G.

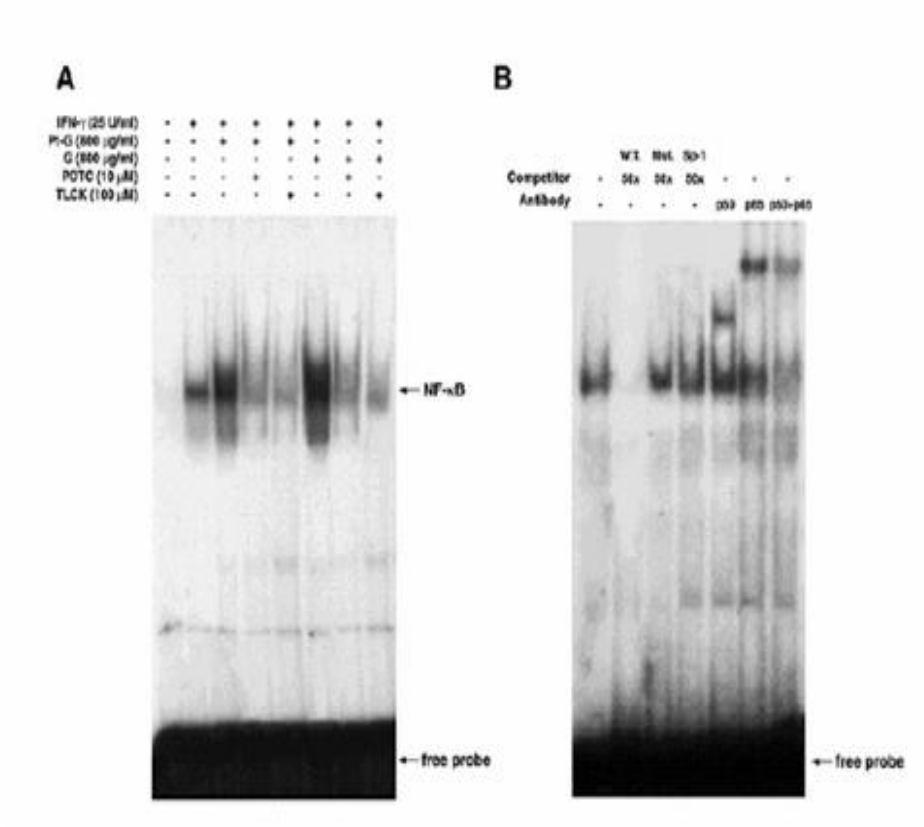

Figure 30. (A) Representative EMSA shows the effect of Pt-G and G in presence of PDTC and TLCK on NF-κB/DNA binding activity in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. Data are from a single experiment and representative of six experiments. (B) Characterization of NF-κB/DNA complex. In competition reaction nuclear extracts were incubated with ^{32}P-labeled NF-κB probe in absence or presence of identical but unlabeled oligonucleotides (W.T., 50x), mutated nonfunctional κB probe (Mut., 50x) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50x). In supershift experiments nuclear extracts were incubated with antibodies against p50, p65 or p50 + p65 15 min before incubation with radiolabeled NF-κB probe. Data are from a single experiment and representative of three experiments.

The composition of the NF-κB complex was determined by competition and supershift experiments. The specificity of NF-κB/DNA binding complex was evident by the complete displacement of NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabelled NF-κB probe in the competition reaction. In contrast a 50-fold molar excess of unlabeled mutated NF-κB probe or Sp-1 oligonucleotide had no effect on DNA-binding activity. The anti-p50 and anti-p65 antibodies clearly gave rise to a characteristic supershift of the retarded complex. Addition of anti-p50 + anti-p65 to the binding reaction resulted in a marked reduction of the intensity of NF-κB specific bands, suggesting that the NF-κB complex contained p50 and p65 dimers (Figure 30 B).

9.1.4 Effect of Pt-G and G on degradation of IκBα and nuclear translocation of NF-κB subunits in RAW 264.7 macrophages stimulated with IFN-γ for 24 h

The presence of IκBα in the cytosolic fraction or p50 and p65 subunits in nuclear fraction was examined by immunoblotting analysis. Unstimulated cells as well as cells treated with Pt-G (800 μg/ml) or G (800 μg/ml) alone expressed high levels of IκBα in the cytosolic fraction and basal levels of p50 and p65 in the nuclear fraction. Stimulation of the cells with IFN-γ (25 U/ml) caused a reduction of IκBα band intensity which almost disappeared in presence of Pt-G (800 μg/ml) or G (800 μg/ml). PDTC (10 μM) or TLCK (100 μM) prevented IκBα degradation (Figure 31 A).Upon the stimulation with IFN-γ, cells exhibited p50 and p65 high nuclear levels which were increased by Pt-G or G. PDTC or TLCK prevented p50 and p65 nuclear translocation (Figure 31 B).

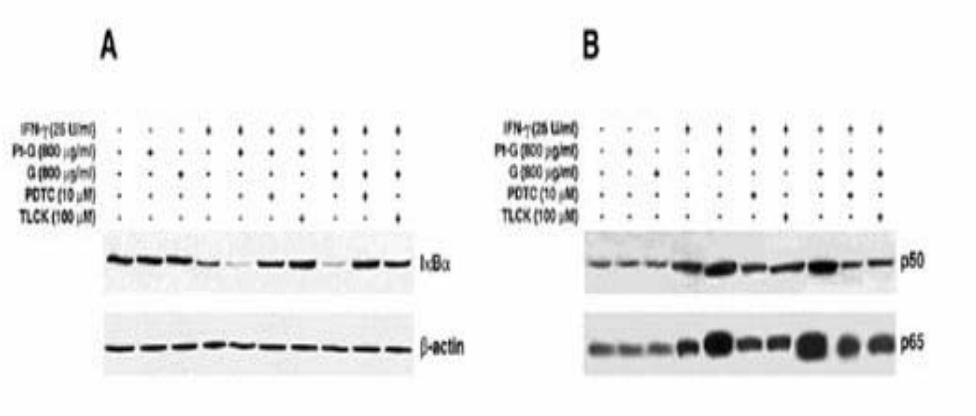

Figure 31. Representative Western blots show the effect of Pt-G and G in the presence or absence of PDTC and TLCK on (A) cytosolic degradation of IκBα and (B) nuclear translocation of p50 and p65 in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. β-actin expression is shown as a control. (A) Data are from a single experiment and representative of four experiments.

9.1.5 Effect of Pt-G and G on nitrite production, iNOS protein expression and NF-κB/DNA binding activity in RAW 264.7 macrophages stimulated with IFN-γ for 48 and 72 h

The cells stimulated with IFN-γ (25 U/ml) for 48 and 72 h caused nitrite accumulation which was increased in a significant and concentration-dependent manner by Pt-G (50, 100, 200, 400 and 800 μg/ml) or G (50, 100, 200, 400 and 800 μg/ml) (Figure 32 A and B). Interestingly, Pt-G and G at lower concentrations (50 and 100 μg/ml) enhanced significantly ($P<0.0001$) the nitrite production. In contrast, Pt-G or G added to the cells alone, at the same concentrations, did not elicit the nitrite production (data not shown). Pt-G (800 μg/ml) or G (800 μg/ml) in combination with IFN-γ also increased both iNOS protein expression (by 1.7 and 2.6 fold, at 48 h; by 1.3 and 1.7 fold, at 72 h, respectively) and NF-κB/DNA binding activity (by 1.4 and 1.8 fold, at 48 h; by 1.3 and 1.6 fold, at 72 h, respectively) as compared with IFN-γ alone (Figure 33 A and B). The increase of iNOS protein expression was correlated with NF-κB/DNA binding activity ($r = 0.99$,$^{*}P<0.05$, Pt-G; $r = 0.99$,$^{*}P<0.05$, G) at 24, 48 and 72 h.

9.1.6 Effect of Pt-G and G on IRF-1 and STAT-1α DNA/binding activity in RAW 264.7 macrophages stimulated with IFN-γ: comparison with kinetic analysis of NF-κB DNA/binding activity

We investigated the enhancing effect of Pt-G and G on IFN-γ-induced IRF-1 or STAT-1α DNA binding activity.

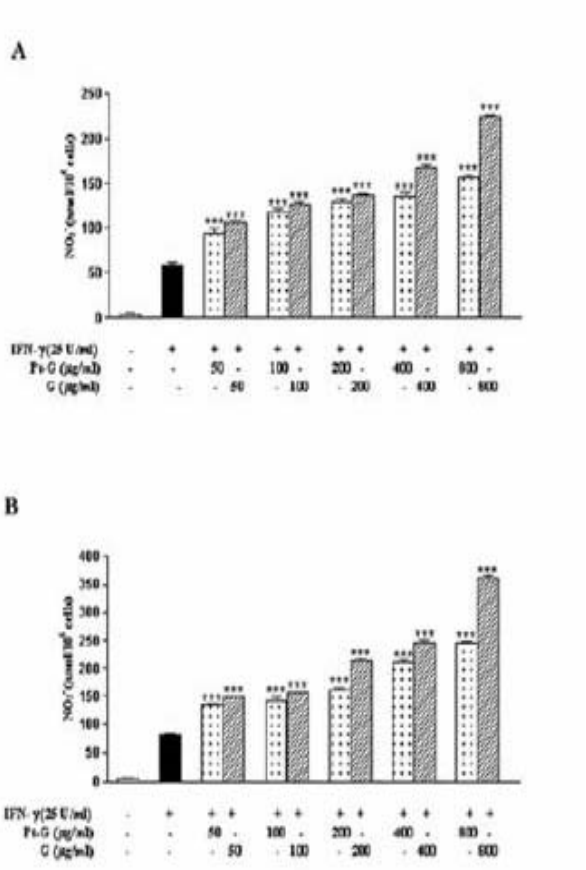

Figure 32. Concentration-dependent increase of nitrite production by Pt-G and G in RAW 264.7 macrophages stimulated with IFN-γ for (A) 48 and (B) 72 h. Data are expressed as mean ± SEM of five experiments in triplicate. ***P<0.0001 *vs.* IFN-γ alone.

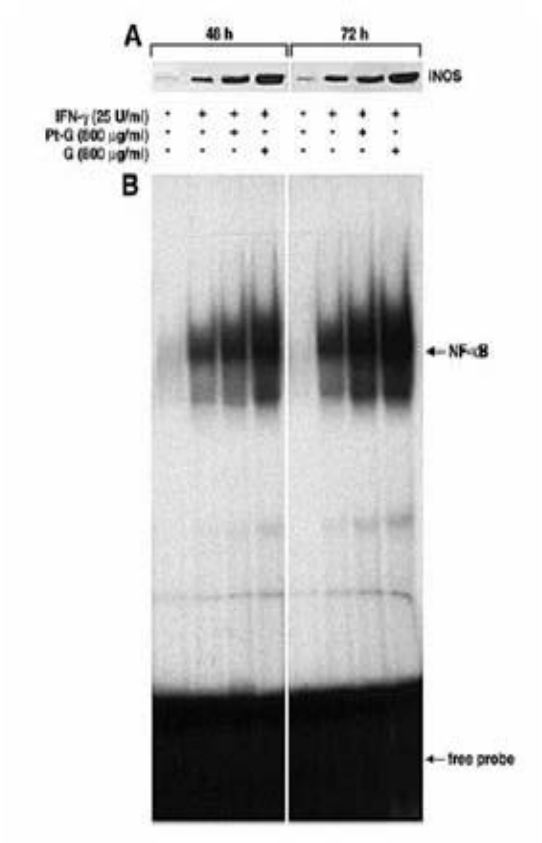

Figure 33. Representative Western blot and EMSA show the effect of Pt-G and G on (A) iNOS protein expression and on (B) NF-κB/DNA binding activity in IFN-γ-stimulated RAW 264.7 macrophages at 48 and 72 h. (A) Data are from a single experiment and representative of four experiments. (B) Data are from a single experiment and representative of five experiments

Nuclear extracts from RAW 264.7 cells treated for 1, 3, 6, 24, 48 and 72 h with IFN-γ (25 U/ml) or both IFN-γ and Pt-G (800 μg/ml) or G (800 μg/ml) were subjected to EMSA using the specific binding elements for each transcription factor. As shown in Figure 34, a low basal level of IRF-1 and STAT-1α /DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected at 1 h after stimulation with IFN-γ. The addition of Pt-G and G to the cells caused an increase of band intensity up to 24 h thereafter decreased at 48 h and 72 h. In contrast, NF-κB/DNA binding activity was evident at 3 h and increaseḍ up to 72 h. Pt-G and G alone did not change the binding activity of IRF-1, STAT-1α and NF-κB at time points considered. These results indicate that the enhancing effect of Pt-G and G on IFN-γ-induced iNOS gene expression is mainly mediated through the NF-κB activation.

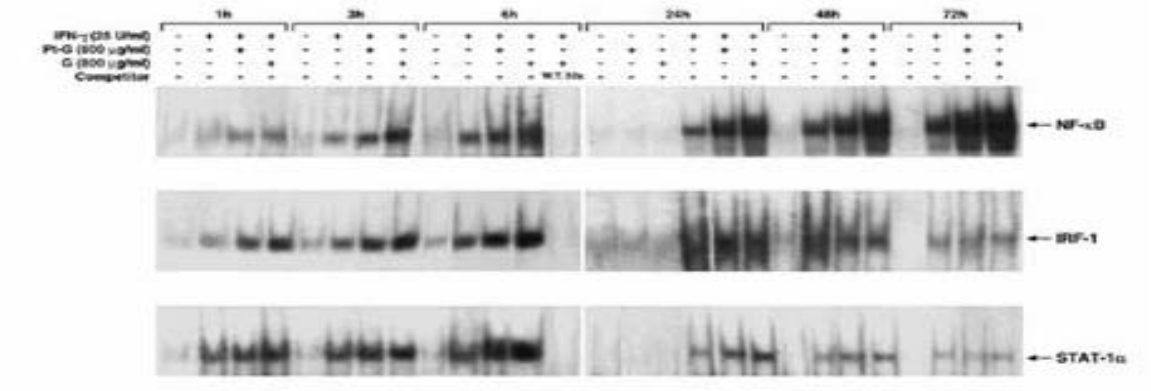

Figure 34. Kinetic analysis shows the effect of Pt-G and G on IFN-γ-induced NF-κB, IRF-1 and STAT-1α/DNA binding activity in RAW 264.7 macrophages. In competition reaction nuclear extracts were incubated with 50-fold molar excess of each unlabeled probe (W.T., 50x). Data are from a single experiment and representative of three to six experiments.

9.2 Discussion

There is increasing evidence that NO plays important role in the pathogenesis of the histologic changes seen in CD (ter Steege *et al.*, 1997; Beckett *et al.*, 1998; Holmegren Peterson *et al.*, 1998; Beckett *et al.*, 1999; van Straaten *et al.*, 1999). Recently, it has been shown that gluten or gliadin and their proteolytic fragments enhance iNOS mRNA level and NO production by mouse peritoneal macrophages stimulated with IFN-γ (Tuckovà *et al.*, 2000, 2002). The molecular mechanisms by which gluten/gliadin induces iNOS expression and NO production by intestinal mucosa cells in CD and cultured activated macrophages have not been investigated. Here, we report that Pt-G and G were able to increase significantly and in a concentration-dependent manner NO production by RAW 264.7 macrophages stimulated with IFN-γ for 24 h. The increase of NO production by Pt-G and G was associated with an increased expression of iNOS protein compared with IFN-γ alone. In addition, we found that Pt-G and G increased NF-κB/DNA binding activity, IκBα cytosolic degradation and nuclear translocation of p50 and p65 subunits. It is particularly interesting to observe that NF-κB/DNA binding activity as well as iNOS protein expression and NO production were ulteriorly increased by Pt-G and G at 48 and 72 h. In addition, Pt-G and G also at lower concentrations were able to increase significantly NO production. G exhibited greater effects in comparison with Pt-G. These results show that the type and concentration as well as time exposure of gluten derived-peptides induce IFN-γ-stimulated RAW 264.7 cells to produce high levels of NO and suggest a direct toxic action of gliadin in CD. However, Pt-G and G increased iNOS protein expression and NF-κB/DNA binding

activity compared with IFN-γ alone. Moreover, the NF-κB activation inhibitors, PDTC and TLCK, reduced significantly these effects, thereby indicating that Pt-G and G may modulate iNOS protein expression as co-signal with IFN-γ in RAW 264.7 cells through NF-κB activation. Besides NF-κB, we also investigated the involvement of IRF-1 and STAT-1α in the increase of iNOS expression induced by Pt-G and G in IFN-γ-stimulated RAW 264.7 cells. Interestingly, the kinetics of IRF-1 and STAT-1α activation are rapid and decrease compared with NF-κB activation, which is persistent. It has been reported that STAT-1α and IRF-1 can cooperate with NF-κB to promote synergistically transcriptional activity (Drew *et al.*, 1995; Ohmori *et al.*, 1997; Teng *et al.*, 2002). Although the effect of Pt-G and G on IFN-γ-induced iNOS protein expression seems to depend on the enhanced activity of all three transcription factors, a major role for NF-κB cannot be ruled out. Further studies will be addressed to investigate whether this effect is mediated at the molecular level by a cooperation between transcription factors. Taken together our results show that Pt-G and G cause a time and concentration-dependent increase of IFN-γ-induced NF-κB/DNA binding as well as molecular events downstream of NF-κB activation and suggest a role for gluten/gliadin in maintaining activated NF-κB in infiltrating lamina propria cells of inflamed mucosa of coeliac patients. In CD, it is widely accepted that specific cellular and humoral factors are implicated in morphological and functional changes following gluten challenge in intestinal mucosa (Auricchio *et al.*, 1990). Nevertheless, some reports shown the involvement of non specific immune reaction, caused by direct interaction of gluten of innate immune system (Auricchio et al. 1990; Maiuri et al. 1996; Tuckovà et al. 2000). Gliadin is a lectin that is able to bind glycosylated residues,

called “lectinic binding sites” and expressed on various cells, by a non-covalent bound (Kolberg and Sollid, 1985; Damjanov, 1987; Amore *et al.*, 1994). Other studies have shown that gliadin is able to associate with many proteins, mainly by hydrophobic interactions (Farré Castany *et al.*, 1995; Pittschieler *et al.*, 1994; Tuckovà *et al.*, 2000). It has been reported that different cell types in response to pro-inflammatory stimuli, including IFN-γ, induce the expression of cell surface molecules capable of acting as a potent triggers of intracellular activation signals (Marzio *et al.*, 1997). Since Pt-G and G alone had no effect, it could be hypothesised that IFN-γ induces the expression of macrophage surface molecules able of interacting with Pt-G or G and triggering signal transduction pathway. Moreover, gliadin and other lectin fractions of gluten have been shown to be potent modulators of leukocyte function, enhancing chemotaxis and generation of reactive oxygen species (Roccatello *et al.*, 1990; Amore *et al.*, 1994; Rivabene *et al.*, 1999). Several evidences suggest that NF-κB activation may also be under the control of oxidant/antioxidant balance (Flohè *et al.*, 1997). The antioxidant PDTC is thought to inhibit NF-κB activation by depleting the cells of oxygen radicals, whereas TLCK acts by inhibiting proteasome function and hence IκBα degradation (Sherman *et al.*, 1993; Kim *et al.*, 1995; Epinat and Gilmore 1999). We found that Pt-G and G enhanced IκBα degradation, which was prevented by either PDTC or TLCK suggesting that these compounds inhibit NF-κB/DNA binding activity by stabilising IκBα in IFN-γ-stimulated RAW 264.7 macrophages. Thus, it is conceivable that Pt-G and G in combination with IFN-γ increase NF-κB activation through an oxidant mechanism.

Moreover, our results show that PDTC and TLCK are able to reduce the increased iNOS protein expression and NO production by activated macrophages suggesting that NF-κB is responsible for the synergistic effect of Pt-G and G together IFN-γ on NO production. High levels of NO are present in serum and urine of children with CD and correlated with an increased iNOS expression in the small intestine (ter Steege *et al.*, 1997; Beckett *et al.*, 1998; Holmegren Peterson *et al.*, 1998; Beckett *et al.*, 1999; van Straaten *et al.*, 1999). However, the molecular mechanisms by which NO induces, directly or indirectly, injury of the small-intestine in coeliac patients are not clear. Excessively produced NO is known to act as a free radical and cause tissue damage (Liu and Hotchkiss, 1995). In conclusion, our study provides evidence that the effect of Pt-G and G on iNOS protein expression in IFN-γ-treated RAW 264.7 cells is mediated through NF-κB and suggests that blockage of NF-κB activation reduces enhancing effect of gluten on NO production in inflamed mucosa of celiac patients.

This work has been published in N-S Arch Pharmacol, vol 368 pages 63-71, 2003. Maiuri MC, De Stefano D, Mele G, Iovine B, Bevilacqua MA, Greco L, Auricchio S and Carnuccio R. Gliadin increases iNOS gene expression in interferon-γ-stimulated RAW 264.7 cells through a mechanism involving NF-κB.

Role of NF-κB, IRF-1 and STAT-1α transcription factors in the iNOS gene induction by gliadin and IFN-γ in RAW 264.7 macrophages

10.1 Results

10.1.1 Effects of PDTC, Gen and TB42 on G-induced increase of nitrite production and iNOS protein expression in RAW 264.7 stimulated with IFN-γ for 24 h

The nitrite production by unstimulated cells was undetectable. The stimulation of cells with IFN-γ (25U/ml) for 24 h resulted in an accumulation of nitrite (27.8 ± 4.7 nmol/10^6 cells; n = 5) in the medium. G (800 μg/ml) added to the cells together with IFN-γ caused a significant increase of the nitrite production (85.9 ± 3.9 nmol/10^6 cells; n = 5) as compared with IFN-γ alone. Treatment of cells with PDTC (0.1, 1 and 10 μM), Gen (12.5, 25 and 50 μM) and TB42 (25, 50 and 100 μM) inhibited significantly and in a concentration-manner the increase of nitrite production induced by G plus IFN-γ (by 25.0 ± 1.1%; 70.0 ± 2.2% and 78.2 ± 1.5%; 35.8 ± 3.8%; 59.6 ± 0.9% and 86.9 ± 0.5%; 2.7 ± 1.4%; 35.2 ± 2.5% and 65.7 ± 1.7%, respectively; n = 3) (Figure 35). Upon stimulation with IFN-γ (25 U/ml) for 24 h, cells expressed high level of iNOS protein expression as compared with untreated cells. G (800 μg/ml), added to the cells together

with IFN-γ (25 U/ml), increased iNOS protein expression compared to IFN-γ alone. PDTC (10 μM), Gen (25 μM) and TB42 (50 μM) inhibited significantly the increase of iNOS protein expression induced by G plus IFN-γ (by 80.3 ± 0.08%; 35.1 ± 0.75%; 47.4 ± 1.7%; ***$P < 0.0001$ respectively; $n = 5$) (Figure 36). G alone failed to augment the iNOS protein expression level. Both EtOH and DMSO vehicles did not affect cell viability (data not shown).

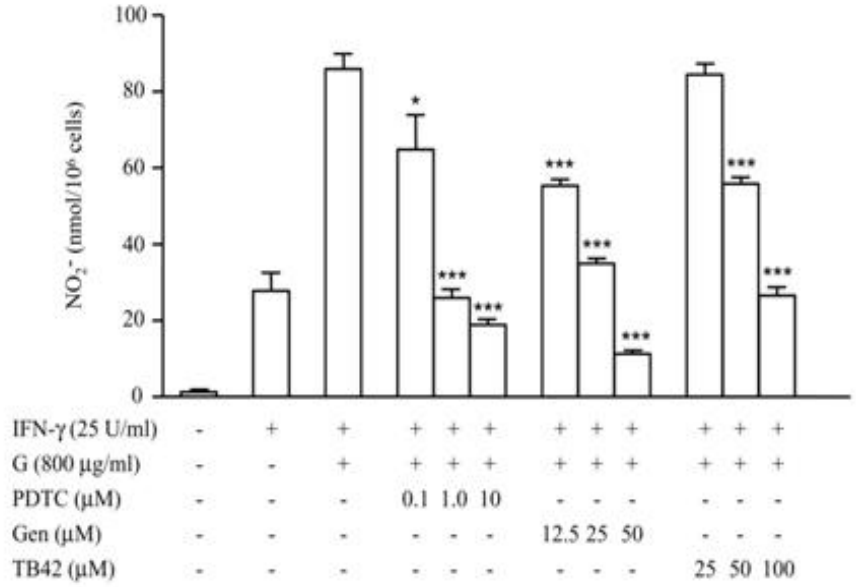

Figure 35. Effects of PDTC, Gen and TB42 on G-induced increase of nitrite production by RAW 264.7 stimulated with IFN-γ for 24 h. Data are expressed as mean ± S.E.M. of five experiments in triplicate; $*P < 0.05$, $***P < 0.0001$ *vs.* IFN-γ plus G.

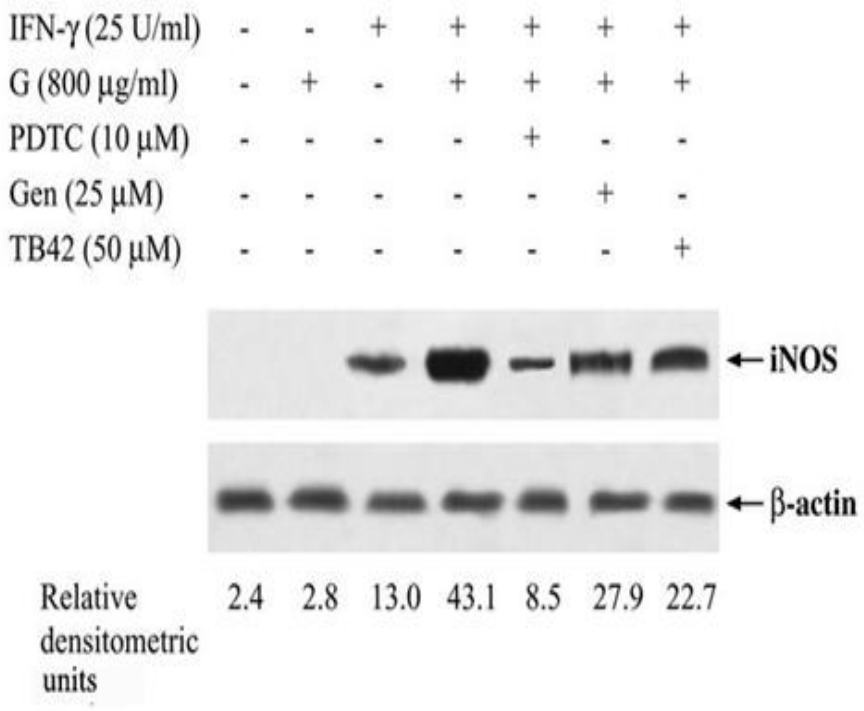

Figure 36. Representative Western blot as well as relative densitometric analysis shows theeffects of PDTC, Gen and TB42 on enhancing iNOS protein expression induced by G in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. β-actin expression is shown as a control. Western blot data are from a single experiment and are representative of five separate experiments. Densitometric data are expressed as units of Optical Density mm^2 and reported under "Results" as percentage of the mean ± S.E.M. of five experiments.

10.1.2 Effects of PDTC, Gen and TB42 on NF-κB, IRF-1 and STAT-1α activation induced by G in RAW 264.7 stimulated with IFN-γ for 24 h

We have previously described the activation of NF-κB, IRF-1 and STAT-1α by G and IFN-γ combination treatment (Maiuri *et al.*, 2003)**,** here we tested the effects of PDTC (10 μM), Gen (25 μM), and TB42 (50 μM) on NF-κB, IRF-1 and STAT-1α/DNA binding activity induced by G (800 μg/ml) in RAW 264.7 macrophages stimulated with IFN-γ (25 U/ml) for 24 h by EMSA studies using the specific binding elements for each transcription factor. A low basal level of NF-κB as well as IRF-1 and STAT-1α/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected following stimulation with IFN-γ, the intensity of which was markedly increased in extracts from cells exposed to G plus IFN-γ. Treatment of cells with PDTC or Gen and TB42 caused a significant reduction of specific protein-DNA complexes induced by G plus IFN-γ (by 90.3 ± 3.8%; 41.9 ± 3.7%; 46.5 ± 2.2%; ***$P < 0.0001$ respectively; $n = 6$) (Figure 37 A, B and C).

The composition of protein-DNA binding complexes of respective transcription factors was determined by competition. In the reaction of competition the specificity of NF-κB/DNA binding complexes was evident by the complete displacement of protein-DNA binding in the presence of a 50-fold molar excess of unlabeled NF-κB probe. In contrast a 50-fold molar excess of unlabeled mutated NF-κB probe or Sp-1 oligonucleotide had no effect on DNA binding activity.

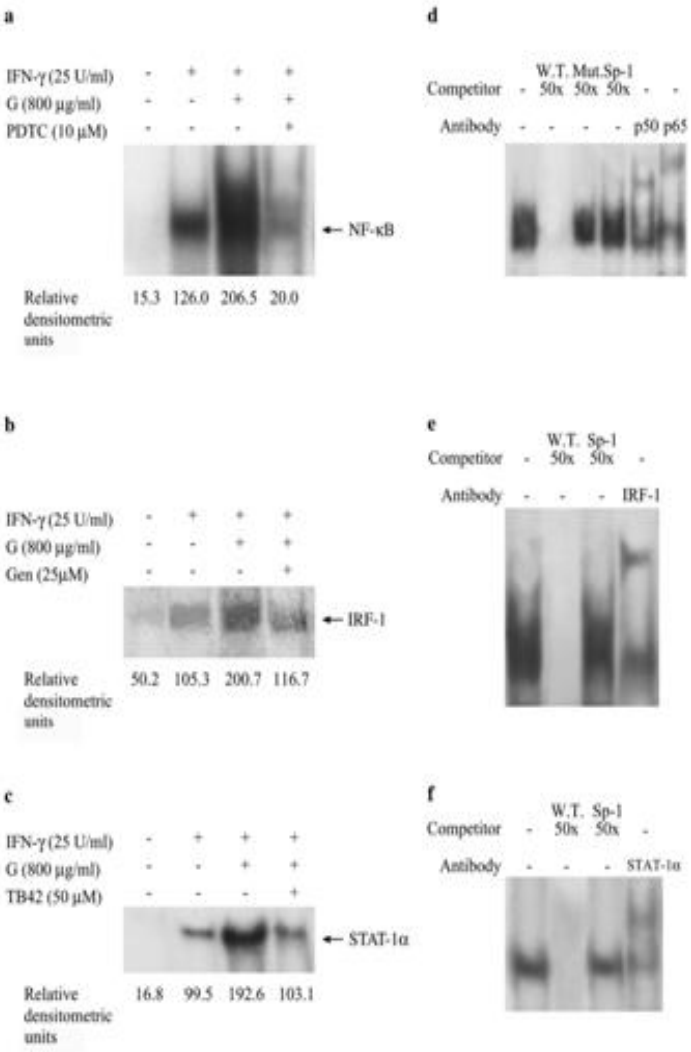

Figure 37. Representative EMSA as well as relative densitometric analysis shows the effects of PDTC, Gen and TB42 on NF-κB, IRF-1 and STAT-1α/DNA binding activity induced by G in RAW 264.7 stimulated with IFN-γ for 24 h (A), (B), (C). EMSA data are from a single experiment and are representative of six separate experiments. Densitometric data are expressed as units of Optical Density mm^2 and reported under "Results" as percentage of the mean ± S.E.M. of six separate experiments. (D) In competition reaction nuclear extracts were incubated with radiolabeled NF-B probe in absence or presence of: identical but unlabeled oligonucleotides (W.T., 50x), mutated non-functional NF-B probe (Mut.,50x) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50x). (E) In competition reaction nuclear extracts were incubated with radiolabeled IRF-1 probe in absence or presence of: identical but unlabeled oligonucleotides (W.T., 50x) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50x). (F) In competition reaction nuclear extracts were incubated with radiolabeled STAT-1 probe in absence or presence of: identical but unlabeled oligonucleotides (W.T., 50x) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50x).

Addition of either anti-p50 and anti-p65 antibodies to the binding reaction clearly gave rise to a characteristic supershift of the retarded complex, suggesting that the NF-κB complex contained p50 and p65 dimers (Figure 37 D). Also the specificity of IRF-1 or STAT-1α/DNA binding complexes was evident by the complete displacement of protein-DNA binding in the presence of a 50-fold molar excess either of unlabeled IRF-1 and STAT-1α probe, respectively. In contrast a 50-fold molar excess either of unlabeled mutated IRF-1 and STAT-1α probe (data not shown) or Sp-1 oligonucleotide had no effect on DNA/binding activity. Addition of anti-STAT-1α or anti-IRF-1 antibody to the binding reaction clearly gave rise to a characteristic supershift of the retarded complex, respectively (Figure 37 E and F). Moreover, the presence of p50 and p65 as well as IRF-1 and STAT1-α subunits in nuclear fraction was examined by immunoblotting analysis. Unstimulated cells expressed basal levels of p50 and p65, IRF-1 and STAT1-α. Upon the stimulation with IFN-γ, cells exhibited p50 and p65, IRF-1 and STAT-1α high nuclear levels which were increased by G. PDTC prevented the nuclear translocation of subunits p50 and p65 (by 50.8 ± 0.2% and 63.5 ± 2.8%, ***$P < 0.0001$, *vs.* IFN-γ plus G , $n = 5$, respectively) (Figure 38 A).

Similarly Gen as well as TB42 prevented the nuclear translocation of IRF-1 and STAT-1-α respectively (by 61.9 ± 0.2% and 44.7 ± 1.6%, ***$P < 0.0001$ *vs.* IFN-γ plus G; $n = 5$) (Figure 38 B and C). These results show that the PDTC as well as the Gen and TB42 induce a decrease of NF-κB, IRF-1 and STAT-1α binding to its own specific DNA target sequence.

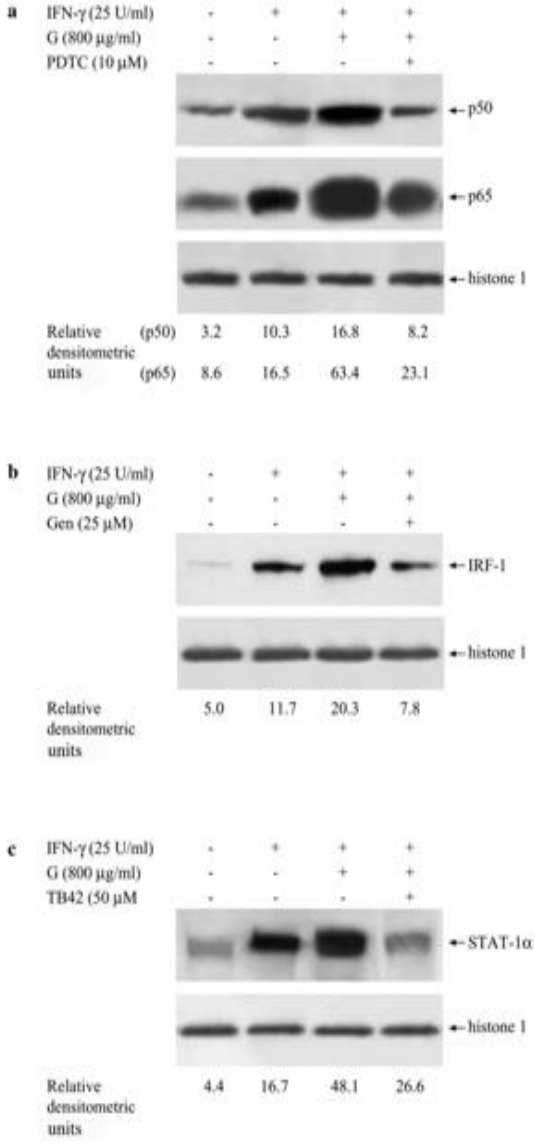

Figure 38. Representative Western blot shows the effects of PDTC, Gen and TB42 on p50 and p65 (A), IRF-1 (B), STAT-1α (C) protein expression induced by G in RAW 264.7 macrophages stimulated with IFN-γ for 24 h. Histone 1 expression is shown as a control. Western blot data are from a single experiment and are representative of five separate experiments. Densitometric data are expressed as units of Optical Density mm^2 and reported under "Results" as percentage of the mean ± S.E.M. of five separate experiments.

10.1.3 Effects PTDC, Gen and TB42 on G-induced increase of iNOS gene expression in RAW 264.7 stimulated with IFN-γ for 1, 6 and 24 h

To explore whether the reduced iNOS protein content observed in cells treated with PTDC, Gen and TB42 could be attributed to a reduced gene transcription, first we analyzed the expression of iNOS mRNA by RT-PCR analysis. The IFN-γ and G combination treatment led to a higher increase of iNOS mRNA levels at 1, 6 and 24 h compared to IFN- γ alone. Gen and TB42 treatment reduced iNOS mRNA levels mainly at 1 h, whereas PDTC treatment was able to inhibit iNOS mRNA levels at 6 and 24 h (Figure 39). To test if iNOS expression was regulated at transcriptional level, we have measured the promoter activity of the iNOS gene in cells treated with G (800 ∝g/ml), IFN- γ (25 U/ml), PTDC (10 μM), Gen (25 μM), TB42 (50 μM) and their combinations for 1, 6 and 24 h. RAW 264.7 cells were transiently transfected with piNOS CAT plasmid (Weisz *et al.*, 1996). The results normalized by using CMV-Luc as an internal control are reported in Figure 40 and obtained by quantifying CAT activity in cellextracts. IFN-γ was efficient in enhancing basal transcription of iNOS promoter whereas G alone had no effect (data not shown). The G plus IFN-γ combination treatment led to a higher augmentation of promoter activity compared to IFN-γ alone. The synergistic induction of iNOS promoter activity was inhibited by Gen and TB42 at 1 h, whereas it was inhibited by PDTC at 6 and 24 h. These observations indicate that the G and IFN-γ combination treatment increases iNOS gene expression through a transcriptional mechanism mediated by all three transcription factors at different time points.

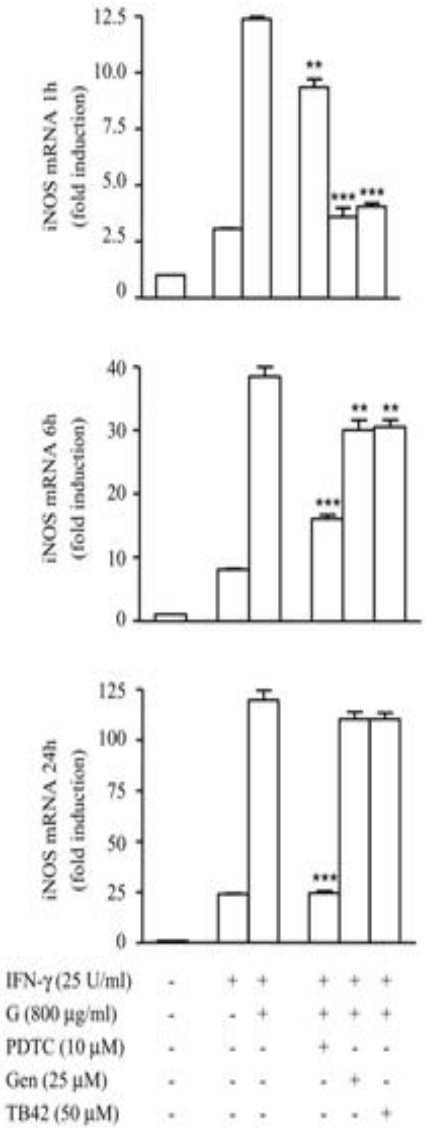

Figure 39. Effects of PDTC, Gen and TB42 on G-induced increase of iNOS mRNA expression in RAW 264.7 cells stimulated with IFN-γ for 1, 6 and 24 h. mRNA levels were measured by RT-PCR as described under "Materials and Methods". The same total mRNAs analyzed in the experiments were used to calculate the changes in the concentration of c-Abl transcripts as control. The data reported are expressed as mean ± S.E.M. of three separate experiments in triplicate; $^{**}P < 0.001$, $^{***}P < 0.0001$ *vs.* IFN-γ plus G.

Although a functional analysis of iNOS promoter was not performed, nevertheless the inhibitory effects exerted by PDTC, Gen and TB42 on iNOS gene transcription appear primarily to involve IRF-1 and STAT-1α at early time points while NF-κB at late time points.

10.1.4 Kinetics of the induction of NF-κB/p65, IRF-1 and STAT-1α mRNA accumulation by G in RAW 264.7 stimulated with IFN-γ

Our demonstration that PDTC, Gen and TB42 inhibited the synergistic induction iNOS gene transcription by G plus IFN-γ at different time points (Figure 38 and 39), prompted us to further investigate the induction of NF-κB/p65, IRF-1 and STAT-1α mRNA expression. As shown in Figure 41**,** the IFN-γ and G combination treatment led to a higher increase of NF-κB/p65, IRF-1 and STAT-1α mRNA levels compared to IFN-γ alone, but with different kinetics of accumulation. NF-κB/p65 mRNA was induced after 1 h in an amount which progressively increased up to 24 h (Figure 41 A). In contrast, IRF-1 and STAT-1α mRNA levels were highest after 1h, and then declined time dependently (Figure 41 B and C). These findings show that the IFN-γ and G combination treatment induces a different amount of NF-κB/p65, IRF-1 and STAT-1α mRNA level.

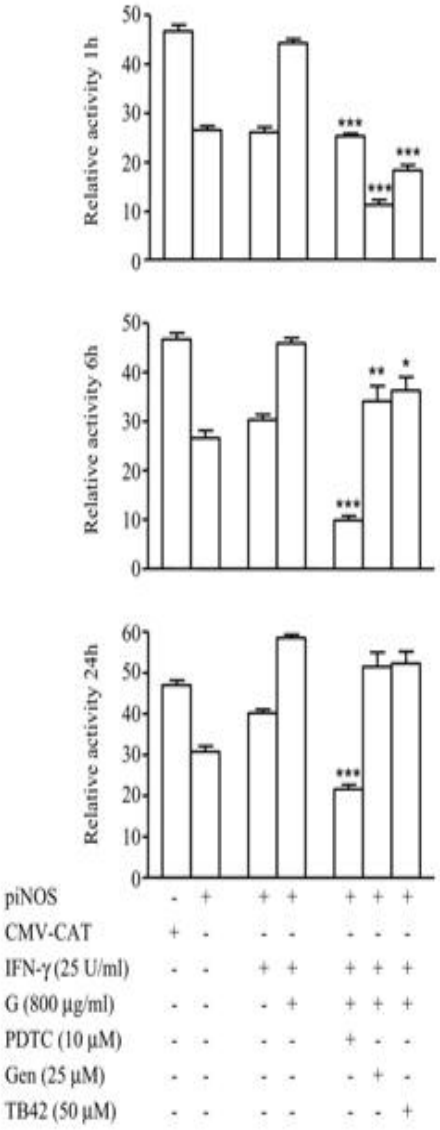

Figure 40. Effects of PDTC, Gen and TB42 on transcription efficiency of the piNOS CAT1 vector in RAW 264.7 cells stimulated with IFN-γ alone or with IFN-γ plus G combination for 1, 6 and 24 h. The efficiency of the CMV-CAT control vector is reported as percentage of the mean value of transcription of this vector in transfected RAW 264.7 cells and treated similarly at the indicated time points. The data reported are expressed as mean ± S.E.M. of three transfection experiments. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ *vs*. IFN-γ plus G.

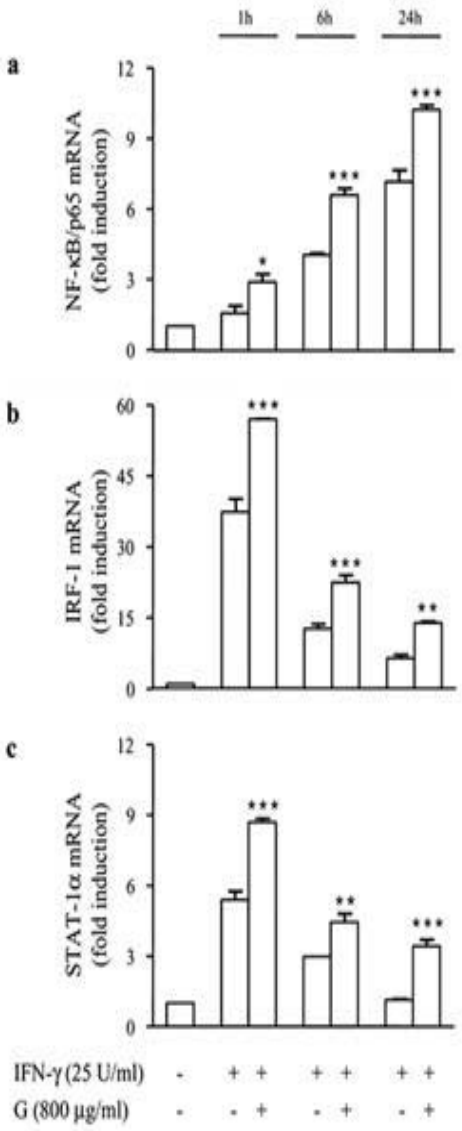

Figure 41. Kinetics of NF-κB/p65, IRF-1 and STAT-1α mRNA accumulation induced by G in RAW 264.7 stimulated with IFN-γ for 1, 6 and 24 h. mRNA levels were measured by RT-PCR as described under "Materials and Methods". The same total mRNAs analyzed in the experiments were used to calculate the changes in the concentration of c-Abl transcripts as control. The data reported are expressed as mean ± S.E.M. of three separate experiments intriplicate; $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$ *vs.* IFN-γ alone.

10.2 Discussion

The molecular mechanisms by which gluten/gliadin induces iNOS expression and NO production by intestinal mucosa cells in celiac disease are unclear. It has been shown that gluten or gliadin and their proteolytic fragments enhance iNOS mRNA level and NO production by mouse peritoneal macrophages stimulated with IFN-γ (Tuckovà *et al.*, 2000; Tuckovà *et al.*, 2002).

We have previously reported that G increases iNOS gene expression in IFN-γ-stimulated RAW 264.7 cells through a mechanism involving NF-κB (Maiuri, *et al.*, 2003). In the present study, besides NF-κB we have further examined the involvement of IRF-1 and STAT-1α on the synergistic induction of iNOS gene expression by G in association with IFN-γ in RAW 264.7 macrophages. The results provided evidence that PDTC as well as Gen and TB42, inhibitors of NF-κB, IRF-1 and STAT-1α activation respectively, were able to reduce significantly the increase of NO production and iNOS protein expression induced by G in RAW 264.7 cells stimulated with IFN-γ for 24 h. The well-characterized transcription factors involved in IFN-γ signaling, NF-κB, IRF-1 and STAT-1α, have been previously demonstrated to play a role in iNOS gene regulation (Kamijo *et al.*, 1994; Weisz *et al.*, 1994; Gao *et al.*, 1997 ; Kim *et al.*, 1997 ; Saura *et al.*, 1999 ; Xie *et al.*, 1994). Previously, we demonstrated that the G plus IFN-γ treatment combination increased the NF-κB, IRF-1 and STAT-1α/DNA binding activity in RAW 264.7 cells compared with IFN-γ alone (Maiuri *et al.*, 2003). In the present study, EMSA experiments using antibodies against p50 and p65 as well as IRF-1 and STAT-1α allowed us to confirm that the formation of specific protein-DNA complexes induced by G and IFN-γ involved NF-κB, IRF-1 and STAT-1α. When cells were

treated with PDTC, Gen and TB42, the NF-κB, IRF-1 and STAT-1α/DNA binding activity as well as respective nuclear subunit level was drastically reduced. Moreover, the G plus IFN-γ combination treatment also led to a higher augmentation of iNOS promoter activity compared with IFN-γ alone. The synergistic induction of iNOS promoter activity was mainly reduced by treatment of cells with Gen and TB42 at 1 h and with PDTC at 6 and 24 h. These observations indicate that a synergistic effect of G plus IFN-γ on iNOS expression occurs at the transcriptional level and is mediated by NF-κB, IRF-1 and STAT-1α. In addition, G plus IFN-γ were found to induce time-dependently a significant increase of iNOS mRNA accumulation compared with IFN-γ alone.

Gen and TB42 were mainly able to reduce the iNOS mRNA accumulation at 1 h, whereas PDTC reduced it at 6 and 24 h. These findings suggest that G may modulate iNOS gene expression as co-signal with IFN-γ in RAW 264.7 cells through IRF-1 and STAT-1α in the early phase of the induction and NF-κB in the late phase. Interestingly, the kinetics of inhibition of iNOS gene expression by PDTC, Gen and TB42 correlated with the induction of NF-κB/p65, IRF-1 and STAT-1α mRNA. NF-κB/p65 mRNA accumulation was induced after 1 h in an amount which progressively increased up to 24 h. In contrast, IRF-1 and STAT-1α mRNA levels were highest after 1h, and then declined time dependently confirming the contribution of each transcription factor in the regulation of iNOS gene expression at different time points. These observations are supported by finding that phosphorylated STAT-1α and IRF-1 are degraded by the ubiquitin-proteasome pathway decreasing to low levels between 1 and 2 h (Kim and Maniatis, 1996). Taken together our results show that PDTC, Gen and TB42 are able to

reduce the increased iNOS gene expression by activated macrophages indicating NF-κB, IRF-1 and STAT-1α as responsible for the synergistic effect of G together with IFN-γ on iNOS gene induction. Moreover, the evidence that maximal accumulation either of iNOS and transcription factors mRNA occurs when cells are stimulated with a combination of G plus IFN-γ suggests a role for G together with IFN-γ as direct activator of macrophages in celiac disease (Jelinkova *et al.*, 2004; De Stefano *et al.*, 2005). The molecular mechanisms that regulate the increase of NF-κB, IRF-1 and STAT-1α /DNA binding activity induced in response to G plus IFN-γ remain to be determined. Further study of functional analysis of deletion mutants of piNOS and/or specific inhibitors of transcription factors will better clarify the cooperative mechanism of these transcription factors and even synergistic effect of G plus IFN-γ on iNOS gene expression. High levels of NO are present in serum and urine of children with celiac disease and correlated with an increased iNOS expression in the small intestine (Beckett *et al.*, 1998; ter Steege *et al.*, 1997). We have previously demonstrated that NF-κB is activated in small intestinal mucosa of celiac patients and NF-κB/DNA binding was correlated with iNOS protein expression (Maiuri *et al.*, 2003).

However, the molecular mechanisms by which NO induces, directly or indirectly, injury of the small-intestine in celiac patients are not clear. Excessively produced NO is known to act as a free radical and cause tissue damage (Liu and Hotchkiss, 1995).

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11. Conclusions and future perspectives

Celiac Disease is a complex coordinated pathology. It involves the activation of immune system as well as the genesis of a inflammatory pathway and interactions of many cellular populations. I have described experimental evidence both *ex vivo* and *in vitro* showing that some of the events correlated with CD could be regulated by NF-κB, IRF-1 and STAT-1α signalling pathway. The results of these investigations report, for the first time, NF-κB activation in biopsy specimens from patients with active CD and *in vitro* emphasize iNOS gene induction and subsequent NO production by gliadin plus IFN-γ. These events seem to be related to IRF-1 and STAT-1α activation in the early phase and to NF-κB activation in the late phase. These findings may contribute to lead to new insights into the molecular mechanism governing the inflammatory process in CD. It remains to be elucidated the precise molecular mechanisms by which gluten/gliadin interacts with activated macrophages and induces NF-κB, IRF-1 and STAT-1α activation even in intestinal mucosa cells in CD. Futhermore, it would also be interesting to analyse the signals that lead to cooperation between these transcription factors. Therefore, there is an exciting perspective that using agents capable of selectively inhibiting these transcription factors or downstream iNOS induction could represent a new therapy for CD.

In conclusion, these findings may provide a better understanding of pathogenic mechanisms associated with CD.

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