Regulation of IL-18 Binding Protein by IFN-γ

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meinen Eltern

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I
Introduction

1.1 Cytokines

The term cytokine is derived from the Greek words κυτοζ (cell) and κινειν (kinesis) meaning as far as moving between cells. Cytokines are small proteins often glycoproteins with molecular weights ranging from 8 to 40 kD. They serve as biochemical messengers between cells and are involved in processes such as regulation of the immune response (host response to disease or infection), stress responses, cell growth/differentiation, and finally tissue repair and remodeling, respectively.

The nomenclature of cytokines was often based on their cellular sources. Cytokines produced by mononuclear phagocytes were sometimes called monokines, and those produced by lymphocytes were commonly called lymphokines, a term first introduced in 1969. But with the advent of molecular cloning it became evident, that several distinct cell lineages could produce the same mediator. Historically, the nomenclature for individual soluble factors was also based on their biological activity, and names were often abbreviated to acronyms that reflect the studied activity, e.g. tumor necrosis factor-α (TNF-α). TNF-α previously has been shown to induce necrosis within tumors. Problems arose immediately when it became clear that more than one protein could share the same biological activity or one cytokine exerted numerous and varied biological activities. TNF-α, for instance, induces prostaglandins, IL-1 and NO. Therefore the generic term cytokines has become the preferred name of this class of mediators. Because many cytokines are made by leukocytes and act on other leukocytes most of these are also called interleukins (IL). The term interleukin with a numerical identifier was first introduced in 1979 (e.g. IL-1). It is imperfect since some of these mediators also serve to communicate signals between immune and nonimmune cells and even between non-immune cells [Dinarello and Moldawer, 2000; Abbas *et al.*, 2000].

Because of their structural characteristics, cytokines can be divided into four groups: α-helical cytokines, long-chain-β-sheet cytokines, α/β-cytokines, and finally mosaic cytokines. These different cytokine classes show only limited homology among each other. Moreover, the maximal homology between selected cytokines in one of these classes is not more than 20 % to 30 %. Although cytokines are in principle structurally diverse, they share several properties. Cytokine secretion is a brief, self-limited event. Their production is usually

initiated by transient *de novo* gene transcription as a result of cellular activation by immune signals or stress [Loppnow, 2001]. Furthermore the mRNAs encoding most cytokines are rather unstable. Additionally, the production of some cytokines may be controlled by RNA processing and posttranscriptional mechanisms, such as proteolytic release of an active product from an inactive precursor (e.g. pro-IL-1β or pro-IL-18). The actions of cytokines are often pleiotropic and redundant. Pleiotropism refers to the ability of one cytokine to act on different cell types mediating diverse biologic effects. This property limits their therapeutic use. On the other hand multiple cytokines have the same functional effects, so that the use of antagonist against a single cytokine may not have functional consequences. Another key property of cytokines is to influence the synthesis and actions of other cytokines. Two cytokines may interact to antagonize each other's action or to produce additive or synergistic effects. Furthermore, the ability of one cytokine to induce the synthesis of others leads to cascades, in which a second or third cytokine may mediate the biologic effects of the upstream mediator. For instance, IL-18 induces TNF-α that itself mediates expression of matrix metalloproteinase-9 (MMP-9). In general cytokine actions are local and under certain conditions systemic. However, most of them act close to where they are produced in a autocrine or paracrine manner.

Cytokines initiate their function by binding to specific membrane receptors on target cells. These receptors often bind their ligands with very high affinities ($K_d = 10^{-10}$ to 10^{-12} M). As a consequence only small quantities of a cytokine are sufficient to elicit maximal biologic effects. There are different classes of cytokine receptors namely class I-IV-cytokine-receptors, the receptor-kinase-family, and the chemokine-receptors. Stimulation of T or B lymphocytes by antigens leads to increased expression of cytokine receptors. Receptor expression is also regulated by cytokines themselves permitting positive or negativ feedback regulation. In general cellular response to most cytokines mediates changes in gene expression, resulting in new cellular functions that may be accompanied by proliferation or differentiation. Two exceptions to this rule are chemokines, which elicit rapid cell migration without new gene expression, and tumor necrosis factor (TNF-α), which can induce cell death often without requiring new protein synthesis.

1.2 Cytokines and inflammation

Some cytokines initiate and aggravate inflammatory reactions in some cases making disease worse (proinflammatory cytokines) whereas others serve to reduce inflammation and promote healing (antiinflammatory cytokines). The balance of pro- and antiinflammatory cytokines determines kinetic and outcome of an inflammatory response, it is dynamic and ever-shifting. Cytokines also act in concert with specific cytokine inhibitors

(e.g. IL-1Ra) and soluble cytokine receptors (e.g. sIL-1R) to regulate the human immune response. Taken together, the net effect of any cytokine is dependent on the timing of cytokine release, the local milieu in which it acts, the presence of competing or synergistic elements, cytokine receptor density, and tissue responsiveness to each cytokine. The most prominent immunoregulatory cytokines are listed in the following figure.

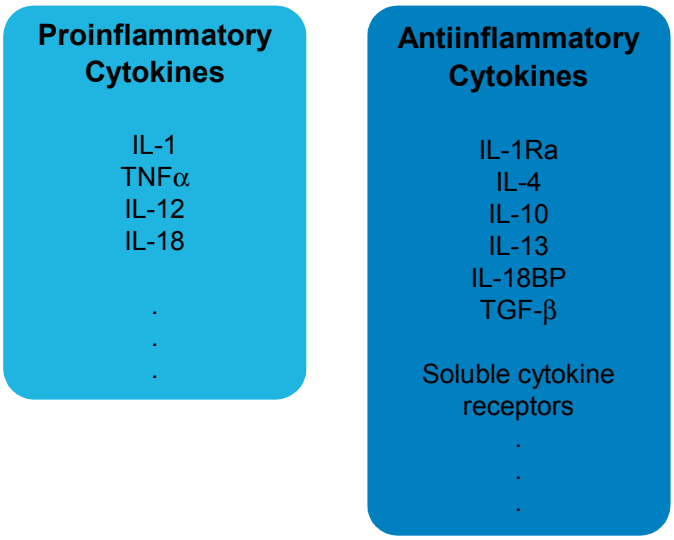


Figure A.

Important cytokines with pro- and antiinflammatory properties

Proinflammatory cytokines initiate and aggravate inflammatory reactions. They are important for host defense against infections. In contrast, antiinflammatory cytokines serve to reduce inflammation and promote healing. These cytokines control the proinflammatory cytokine response by virtue of their ability to suppress genes encoding for proinflammatory cytokines.

1.2.1 Proinflammatory cytokines

Proinflammatory cytokines induce a cascade of gene products usually not produced in healthy persons. Examples for such genes are the type IIA phospolipase (PL) A_2, cyclooxygenase (COX)-2, inducible NO-synthase (iNOS), adhesion molecules, and chemokines. They are coding for enzymes responsible for increased synthesis of platelet-activating factor (PAF), prostaglandins, leukotrienes, NO, O_2^-, and proteases, etc.. These mediators of inflammation finally mediate the classical cardinal symptoms of inflammation: heat, redness, swelling, pain, and loss of function which finally may end in sclerosis (figure B) [Dinarello, 2000]. IL-1, TNFα and IL-18 have the potential to initiate this cascade of inflammatory mediators. These cytokines are induced by infection, tissue damage or ischemia. They themselves induce secondary cytokines e.g. IFNγ and are known to direct and maintain T cell development along a T_H1 pathway. IL-1 and TNF-α are prototype proinflammatory cytokines. When administered to humans, they produce fever. Excessive production leads to inflammation, tissue destruction, and, in some cases shock and death as seen in septic shock patients. Proinflammatory cytokines activate important regulators of gene transcription (e.g. NF-κB, AP-1, NF-IL-6). Their overproduction is a frequent characteristic of human autoimmune diseases such as: Crohn's disease, lupus erythematosus, and rheumatoid arthritis.

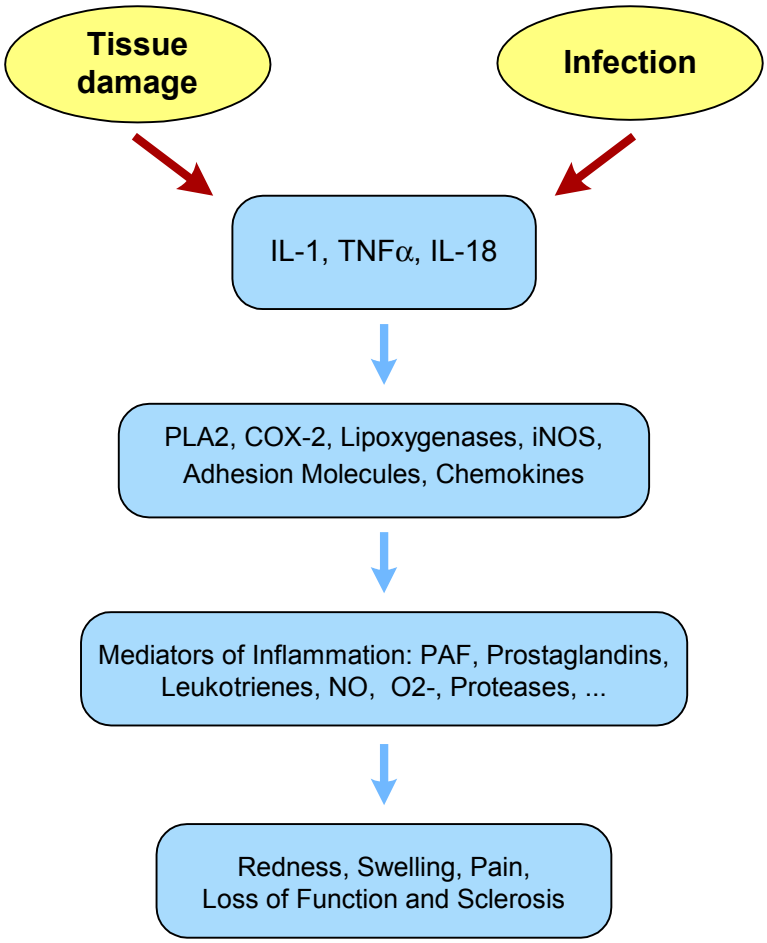


Figure B. The inflammatory cascade.

1.2.1.1 Interleukin-12

IL-12 is a principal mediator of the early innate immune response to intracellular microbes and is a key inducer of cell-mediated immunity, the adaptive immune response to these microbes. This secreted cytokine stimulates the differentiation of $CD4^+$ helper T lymphocytes into IFN-γ-producing T_H1 cells. The stimuli for IL-12 production are LPS, infection by intracellular bacteria, such as Listeria and mycobacteria, and virus infections. In addition, antigen-stimulated helper T-cells induce the production of IL-12 by macrophages and dendritic cells (CD40/CD40-ligand interactions). Originally this cytokine was identified as inducer of NK cell mediated cytotoxicity, but its most important action is to stimulate IFN-γ production by T cells and NK cells. Vice versa IFN-γ produced by NK cells or T cells also stimulates IL-12 production. This induction of IFN-γ by IL12 requires IL-18. It is likely that the ability of IL-12 to induce IFN-γ includes up-regulation of receptors for IL-18

[Yoshimoto *et al.*, 1998] as well as activation of ICE (Caspase-1), a protease that is involved in the processing of the inactive precursor of IL-18 (pro-IL-18). Furthermore, IL-12 is highly inflammatory because it induces TNF-α, which appears to be involved in mediating the effects of IL-12 on NK cells. Finally, IL-12-induced IFN-γ activates macrophages to kill phagocytosed microbes which underlines the importance of IL-12 in cell-mediated immune defense. IL-12 enhances not only the cytolytic functions of activated NK cells but also of $CD8^+$ cytolytic T lymphocytes. The heterodimeric glycoprotein IL-12 consists of the two subunits p40 and p35. The p40 subunit of murine IL-12 has been shown to specifically antagonize the effects of the IL-12 heterodimer in different assay systems thereby function as an endogenous specific inhibitor for the IL-12 heterodimer [Mattner *et. al.*, 1993]. This classical T_H1 cytokine is an important link between innate and adaptive immunity, being produced by macrophages and dendritic cells during early innate immune reactions against intracellular microbes. Moreover, IL-12 stimulates adaptive immune responses that protect the host against these microbes through specific T cell differentiation.

Large amounts of IL-12 are produced in severe gram-negative sepsis, resulting in excessive production of IFNγ, which synergizes with LPS to stimulate macrophage production of TNF-α, one principal mediator of septic shock. IL-12-deficient mice are impaired but not completely lack the ability to produce IFN-γ following endotoxin administration and to mount a T_H1 T-helper cell response *in vivo*. IL-12 has been shown to inhibit the growth of a variety of experimental tumors *in vivo* most likely via induction of IFN-γ and inhibition of angiogenesis.

1.2.1.2 Interleukin-18

IL-18 has been introduced as a novel member of the IL-1 family of cytokines due to its structural properties. It participates in both innate and acquired immunity. As opposed to IL-1, IL-18 appears to be a pivotal mediator of the T_H1 cytokine response [Dinarello *et al.*, 1998; Dinarello, 1996; Okamura *et al.*, 1995]. However, both cytokines also share a variety of functions like induction of inflammatory cytokines [Puren *et al.*, 1998]. IL-18 was first described in 1989 as IFN-γ inducing factor (IGIF) [Nakamura *et al.*, 1989.]. It is produced by macrophages in response to LPS and other microbial products and in concert with IL-12, IL-2, or antigenic stimulation IL-18 induces IFN-γ production by T-cells [Okamura *et al*, 1995] and natural killer cells [Tsutsui *et al.*, 1996]. A variety of immune and non-immune cells express IL-18, including monocytes and macrophages, Kupffer cells, T-cells and B-cells, dendritic cells, osteoblasts, epidermal keratinocytes, intestinal epithelial

cells, corneal epithelial cells, glucocorticoid-secreting adrenal cortex cells, astrocytes, and microglia [Dinarello, 2000]. IL-18 lacks a classical signal sequence necessary for secretion via the Golgi apparatus [Okamura *et al.*, 1998; Ushio *et al.*, 1996]. Like IL-1β, IL-18 is synthesized as an inactive precursor (pro-IL-18) that has to be cleaved by caspase-1 (ICE) to generate its biological active form mature-IL-18. But also other proteases like proteinase 3 (Myeloblastin) [Sugawara *et al.*, 2001] certain MMPs, mast cell chymase as well as cathepsin D may provide activation pathways for pro-IL-18. IL-18 primarily seems to function as a costimulant for T_H1 cytokine production. It has been found to inhibit production of the T_H2 response promoting cytokine IL-10 in concanavalin A-stimulated human PBMCs. Recent data imply that IL-18 in the absence of IL-12, may also facilitate the development of T_H2 responses [Nakanishi *et al.*, 2001] IL-18 can directly induce TNF-α, FasL, as well as chemokines, e.g. IL-8 [Puren *et al.*,1998]. The bioactivities of IL-18 are modulated by the secreted IL-18 binding protein a (IL-18BPa).

IL-18 bioactivity is connected with the pathogenesis of different inflammatory diseases, for example, septic shock, colitis, Crohn's disease, myasthenia gravis, multiple sclerosis, rheumatoid arthritis, atherosclerosis and organ transplant rejection. By stimulating immune cells IL-18 exhibits a strong antitumoral activity, protecting experimental animals against repeated challenges with tumor cells [Micallef *et al.*, 1997]. Transgenic knockout mice lacking expression of IL-18 have been generated by Wei *et al.*, [1999]. These mice are viable and fertile, they do not show any evident histopathologic abnormalities. However, they produce significantly lower levels of IFN-γ following infection by the protozoan parasite *Leishmania major*. IL-18-deficient mice were also employed to investigate the role of IL-18 in LPS-induced liver injury and endotoxic shock in mice primed with *Propionibacterium acnes* [Sakao *et al.*, 1999]. These mice showed resistance to LPS-induced liver injury, but were highly susceptible to LPS-induced endotoxin shock. The latter effect was due to remarkably high levels of TNF-α, which were produced in IL-18 knockout mice compared to wild-type mice after LPS challenge [Akira, 2000]. The following figure summarizes the main functions of the T_H1 response prime mediator IL-18.

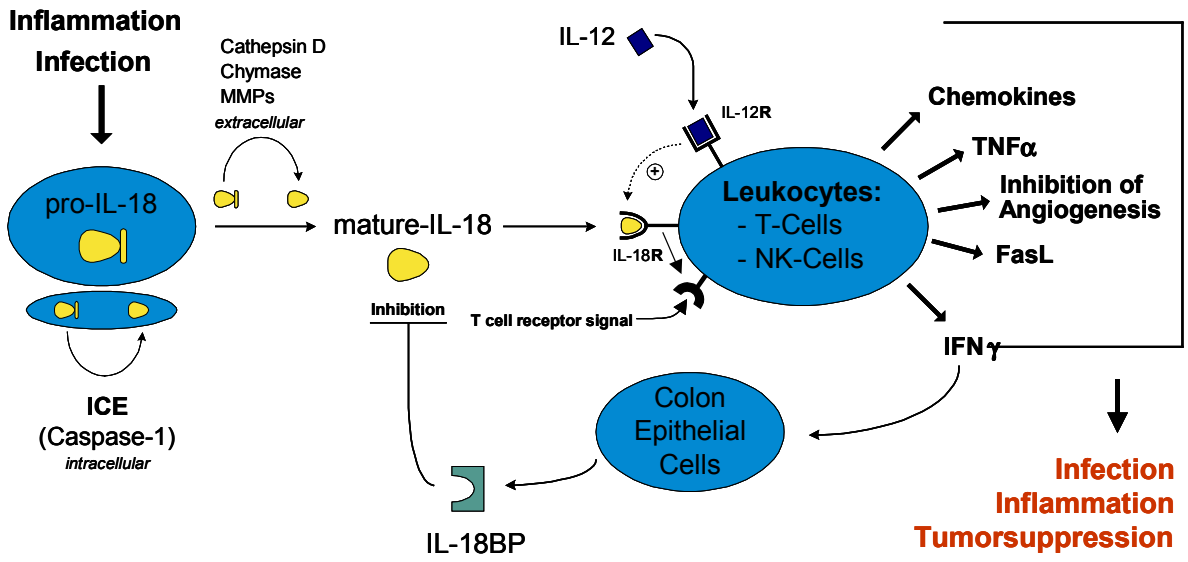


Figure C. Overview about IL-18 action and its naturally occuring counterpart IL-18BP.

1.2.2 Antiinflammatory cytokines

Antiinflammatory cytokines are involved in controlling the proinflammatory cytokine response by virtue of their ability to suppress genes for proinflammatory cytokines such as IL-1, TNF, and chemokines [Opal and DePalo, 2000]. In the presence of antiinflammatory cytokines like IL4, IL-6, and IL-10 activated naive T cells develop along a T_H2 pathway.

1.2.2.1 Cytokine inhibitors

Not only cytokine receptor antagonists like the IL-1Ra, but also membrane-bound or soluble decoy receptors are important tools to counterregulate an inflammatory immune response. These are providing potential targets for the treatment of chronic or excessive inflammatory responses. At high molar ratios, some soluble receptors inhibit their corresponding cytokines and have been used as immunosuppressors in patients, e.g. soluble TNF-αR for the treatment of rheumatoid arthritis (RA). In contrast, at low concentrations these soluble receptors may function as stabilizers of cytokines and enhance their activity [Novick *et al.*, 1999]. Interestingly microbial pathogens may actually use components of the cytokine network for their own advantage. Viral-encoded inhibitory cytokines appear to assist the virus in the promotion of viral replication and evasion of host-derived clearance mechanisms. Furthermore, several bacterial pathogens and viruses have the capacity to alter host cell cytokine synthesis, degrade proinflammatory cytokines, or use cytokine receptors as portals of entry for cellular invasion, e.g. *Malaria falciparum* [Pogo *et al.*, 2001], HIV-I (CCR5, CXCR4) [Dragic, 2001].

1.2.2.1.1 Interleukin-18 binding protein (IL-18BP)

IL-18BP is a secreted protein that functions as a natural antiinflammatory and immunosuppressive molecule by neutralizing the effects of IL-18 during inflammation. This highly glycosylated protein suppresses the production of IL-18-induced IFN-γ resulting in a reduced T_H1 immune response. IL-18BP was first isolated from 500 l of human urine by affinity chromatography. IL-18BP does not have transmembrane domain and hence is not anchored to the cell membrane [Novick *et al.*, 1999]. There are at least four human (hIL-18BPa/b/c/d) and two mouse (mIL-18BPc/d) isoforms resulting from mRNA splicing. These were found in various cDNA libraries with hIL-18BPa cDNA as the most abundant clone. The human isoforms IL-18BPa and IL-18BPc exhibited the greatest affinity for IL18 with dissociation constants K_d of 399 pM and K_d of 2.94 nM, respectively. A large mixed electrostatic and hydrophobic binding site in the immunoglobulin domain of IL-18BP has been found to be responsible for its high affinity binding to the ligand. On contrary, the other two isoform IL-18BPb and IL-18BPd only possess an incomplete immunoglobulin-domain. By virtue of this structural difference these variants are unable to neutralise IL-18 [Kim *et al.*, 2000].

IL-18BPa is the major form constitutively expressed in human spleen (Novick *et al.*, 1999). It has been identified as IFN-γ-inducible gene in a variety of human cell types such as: colon carcinoma epithelial cells, renal mesangial cells, immortalized and primary keratinocytes, endothelial cells, endometrical epithelia cells, and monocytes. Levels of IL-18BP are upregulated in patients with septic shock [Novick *et al.*, 2001] and Crohn's disease [Corbaz *et al.*, 2002]. In mice, injection of human IL-18BPa inhibits LPS-induced circulating IFN-γ by > 90% [Novick *et al.*, 1999]. Furthermore it has been shown that therapeutically employed IFN-α induces IL-18BP in chronic hepatitis C patients [Kaser *et al.*, 2002].

IL-18BP is a member of a novel family of soluble proteins, which also includes Poxvirus-encoded decoy receptors for many cytokines. These receptors are instrumental in viral evasion of immune responses, as demonstrated by reduced virulence following their deletion (Spriggs *et al.*, 1996; Ploegh *et al.*, 1998). For instance, *M contagiosum* viral proteins MC53 and MC 54 share a significant homology to mammalian IL-18BP [Novick *et al.*, 1999]. These proteins possess the ability to bind and neutralise human IL-18 in a fashion similar to that of IL-18BP [Xiang *et al.*, 1999]. It has been hypothesized that IL-18BP evolved from a primordial cell-surface protein which lost its membrane anchoring domain [Novick *et al.*, 1999].

1.2.2.1.2 IL-1 receptor antagonist (IL-1Ra) and antagonistic IL-1 receptors

Two different membrane receptors for IL-1 (IL-1R) have been characterized. Both are members of the Ig superfamily. Binding of IL-1 to the type I receptor (IL-1RI) forms a signal transducing complex. IL-1RAcP (IL-1R Accessory Protein) participates in the signaling by this complex. In contrast binding of IL-1 to the cell-bound type II receptor (IL-1RII) does not lead to signal transduction, because this receptor lacks such an intracellular signaling domain. During inflammatory conditions soluble forms of IL-1 receptors (particularly the type II receptor) can be detected in human sera and likely control excessive IL-1 bioactivity associated with inflammatory diseases. Soluble IL-1RII is a decoy receptor that can capture IL-1 thereby preventing its binding to the type I receptor and thus suppresses IL-1 bioactivity. Mononuclear phagocytes produce a natural competitive inhibitor of IL-1 called IL-1R antagonist (IL-1Ra) that is structurally homologous to IL-1. IL-1Ra binds to the same receptor but does not translate into receptor activation, since IL-1Ra does not recruit the IL-1RAcP to form the heterocomplex required to trigger a signal [Sims *et al.*, 2002]. The type I receptor is expressed on almost all cell types and is seen as the major receptor for IL-1-mediated responses. In contrast the type II receptor is constitutively expressed on B cells but may be induced in other cell types, e.g. by antiinflammatory glucocorticoids [Re *et al.*, 1994]. The cytoplasmatic portion of the type I receptor is homologous to that of a *Drosophila* cell surface receptor called Toll that is involved in defense against microbial infections.

1.3 Infection and inflammation

1.3.1 Innate immunity

Defense against microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity. The components of innate immunity recognize structures that are characteristic of microbial pathogens and not present on mammalian cells. Inhibiting or eliminating the mechanisms of innate immunity markedly increases susceptibility to severe infection, even when the adaptive immune system is intact and functional. The innate immune system is unable to recognize nonmicrobial chemical substances or macromolecules, in contrast to the adaptive immune system that recognizes foreign antigens produced by microbes or other sources, including synthetic antigens. It relies primarily on cell surface receptors, called Toll-like receptors (TLR), and secreted proteins to recognize carbohydrate, lipid, protein, and DNA structures associated with microbial infection. Probably the most well-described cell surface receptor system for

recognition by the innate immune system is the lipopolysaccharide (LPS) recognition complex composed of CD14 (Cluster of Differentiation) and the TLR family, which also recognize other microbial products apart from LPS [Wright *et al.*, 1990]. Whereas LPS (in Gram-negative bacteria) appears to be the principle ligand for TLR4, lipoteichoic acids and peptidoglycans from yeast and Gram-positive bacterial pathogens are potential ligands for TLR2 [Brightbill *et al.*, 1999; Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999]. The microbial components recognized by the innate immune system are often essential for survival of the microbes. This host adaption ensures that the targets of innate immunity cannot be discarded by microbes in an effort to evade recognition by the host. The receptors of the innate immune system are encoded in the germline without recombination as seen for receptors of the adaptive immune system, which limits the repertoire of specificities. Therefore they only can distinguish classes of microbes.

The principal effector cells of innate immunity are neutrophils, mononuclear phagocytes, and natural killer (NK) cells. Macrophages and NK cells secrete cytokines that activate phagocytes and stimulate inflammation associated with innate immunity. Inflammation consists of recruitment of leukocytes into a site of infection and their activation to eliminate the infectious agent. The process of leukocytes migration is mediated by leukocyte homing receptors and endothelial ligands for these receptors. TNF-α and IL-1 secreted by macrophages stimulates endothelial cells to sequentially express adhesion molecules (E-selectin, ICAM-1, and VCAM-1), that mediate the preferential attachment of different types of leukocytes to the endothelium. Selectins mediate the initial loose attachment of neutrophils and other leukocytes to venules at sites of inflammation. In the presence of flowing blood, the loosely adherent leukocytes are propelled to roll along the endothelial surface (“multistep model”). In the next step, TNF-α mediates expression of vascular cell adhesion molecule-1 (VCAM-1, the ligand for VLA-4 integrin) and ICAM-1 (or CD 54; the ligand for LFA-1 and Mac-1 integrins) on the inflamed endothelium. Integrins on the leukocytes then can mediate the stable attachment of leukocytes to the endothelium via interaction with VCAM-1 and ICAM-1. Stable adhesion is followed by their migration through the interendothelial spaces into extravascular tissue. Chemokines are of pivotal importance for leukocyte recruitment to sites of infection. They work to promote leukocyte binding to endothelium and stimulate the chemotaxis of leukocytes within the tissues. Chemokines increase the affinity of leukocyte integrins for their ligands, which stabilizes their attachment to the endothelium and promotes subsequent extravasation.

Cytokine-mediated leukocyte recruitment and activation are responsible for the injury to normal tissues that often accompanies innate immune reactions to infection. These primarily macrophage-derived cytokines, especially TNF-α, IL-1, IL-12 and IL-18 are also responsible for the systemic manifestations of infection.

1.3.1.1 Epithelial barriers

Epithelial barriers represent a component of the innate immune system. Intact epithelial surfaces form physical barriers between microbes in the external environment and host tissue. The three main interfaces are the skin, and the mucosal surfaces of the gastrointestinal and respiratory tracts. They all are protected by continuous epithelia that serve to prevent the entry of microbes. Epithelia not only secrete several cytokines that function in innate immunity but also produce peptides that have a natural antibiotic function. The best known of these peptides are defensins present in the skin of many organisms. Defensins are broad-spectrum antibiotics. Their synthesis is increased in response to inflammatory cytokines (IL-1 and TNF). Barrier epithelia and serosal cavities contain intraepithelial T lymphocytes and the B-1 subset of B cells, respectively. These cells may recognize and respond to commonly encountered microbes. The third population of cells present within many epithelia are mast cells, which respond to microbes and various mediators by release of inflammatory substances.

1.3.1.2 Phagocytes: neutrophils and macrophages

The function of ingesting and destroying microbes is mediated by phagocytes, which include neutrophils early in the innate immune response (hours after infection) and macrophages later in the response (1 or 2 days after infection). The latter typical respond to microbes nearly as rapidly as neutrophils do but persist much longer at sites of inflammation, because macrophages are more long lived than neutrophils (circulate in the blood for only 6 hours), additionally macrophages can undergo cell division at an inflammatory site. Monocytes are the circulating precursors of macrophages. After entering into tissue, monocytes differentiate into tissue macrophages. Neutrophils and macrophages recognize microbes by several receptors that function to stimulate migration of the cells to the site of infection, promote phagocytosis of microbes, and stimulate the production of microbicidal substances to kill the microbes. These receptors are: seven α-helical transmembrane receptors, Toll-like receptors, and phagocytic receptors. Ligands for seven α-helical transmembrane receptors, which function mainly to stimulate leukocyte migration into sites of infection, are *N*-formylmethionyl peptides, chemokines, and lipid mediators. Toll-like receptors recognize e.g. the LPS/LPS-binding protein complex in cooperation with CD14. Finally phagocytic receptors Fc are necessary for microbe recognition by neutrophils and macrophages, examples are the mannose receptor and receptors for Fc portions of IgG antibodies. The latter are responsible for microbe coating and additionally promote phagocytosis. The mannose receptor consists of a lectin that binds terminal mannose and

fucose residues of glycoproteins and glycolipids, sugars typically found in microbial cell walls. Neutrophils and macrophages ingest microbes into vesicles, in order to kill them by fusion of lysosomes with phagosomes to so-called phagolysosomes. The lysosomes contribute proteolytic enzymes that come in contact with and destroy phagocytosed microbes. Neutrophil lysosomes are particularly rich in elastase. The primary free radical-generating system is the phagocyte oxidase system. The phagocyte oxidases located in the plasma membrane of activated phagocytes and in the phagolysosomal membrane reduce molecular oxygen and produce reactive oxygen intermediates (ROIs) such as superoxide radicals. A second free radical-generating system is the inducible nitric oxide synthase (iNOS) system. This enzyme is absent in resting macrophages but can be induced in response to LPS in combination with IFN-γ. Subsequently, it catalyzes the conversion of arginine to citrulline, and freely diffusible nitric oxide gas is released. These radicals mediate killing of ingested microbes (ROI, NO, and their reaction product peroxynitrite).

1.3.1.3 Natural killer (NK) cells

NK cells are a subset of bone marrow-derived lymphocytes, distinct from B or T cells. The principal physiologic role of NK cells is in defense against infections by viruses and some other intracellular microbes. NK cells proliferate, show increased cytolytic activity and IFN-γ production in response to IL-15 and IL-12, which are mainly produced by macrophages. IL-18 potently augments most likely also actions of IL-12. The effector function of NK cells are to lyse virus-infected cells and most likely also tumor cells. NK cells have lytic granules that contain the protein called perforin, which can create pores in target cell membranes. Moreover, NK cells release a protein called granzyme, which is a serine protease. Granzymes enter target cells through perforin pores and induces apoptosis in these cells through activation of intracellular caspases. NK cells also produce an antibiotic peptide called granulysin, which can pass through the mentioned pores to directly kill intracellular microbes [Boman, 1995]. Some tumors are targets of NK cells (especially those of hematopoietic origin), likely because the tumor cells do not express normal levels of class I MHC molecules. Lack of MHC-expression appears to label cancer cells for the attack by NK cells.

1.3.2 Roles of T cell cytokines in adaptive immunity

Adaptive immunity is a highly evolved defense mechanism stimulated by exposure to infectious agents. It increases in magnitude and defensive capabilities with each successive exposure to a particular microbe. This form of immunity develops as a response to infection and adapts to the infection. In response to the protein antigens of microbes, $CD4^+$ helper T cells may differentiate into special subsets of helper T cells called T_H1 and T_H2 cells. These T cell populations are distinguished most clearly by the cytokines they produce. These not only determine the T cell effector function, but also participate in the development and expansion of the respective subsets. For instance, IFN-γ secreted by T_H1 cells promotes further T_H1 differentiation and inhibits the proliferation of T_H2 cells in parallel. Conversely, IL-4 produced by T_H2 cells promotes T_H2 differentiation. IL-10, also produced by T_H2 cells inhibits activation of T_H1 cells. Thus, each subset amplifies itself and cross-inhibits the reciprocal subset. For this reason once an immune response develops along one pathway, it becomes increasingly polarized in that direction. Chronic immune reactions are often dominated by either T_H1 or T_H2 populations. The pattern of T cell differentiation is determined by stimuli present early during immune responses. The most important differentiation-inducing stimuli are cytokines, with IFN-γ, IL-12, and IL-18 being the major inducer of T_H1 cells (for cell-mediated immune response) and IL-4, IL-6, and IL-10 of T_H2 cells (for humoral response). The induction of IFN-γ and IL-12 is dominated by IL-1, TNF-α, and IL-18, produced by accessory or antigen presenting cells such as macrophages or dendritic cells, thereby linking the acquired and innate immune responses. The T_H1 differentiation pathway is stimulated by many intracellular bacteria, such as *Listeria* and mycobacteria, and by some parasites, such as *Leishmania*, all of which infect macrophages. It is also stimulated by viruses. Many organ-specific autoimmune diseases and inflammatory reactions such as granulomas are due to excessive activation of T_H1 (e.g. rheumathoid arthritis and Crohn's disease). In contrast, T_H2 differentiation occurs in response to helminths and allergens, which cause chronic T cell stimulation, often with little macrophage activation. The principal effector function of T_H2 cells is in IgE and eosinophil/mast cell-mediated immune reactions. T_H2 cells are responsible for defense against helminthic and arthropod infections and for allergic reactions as well.

1.3.3 Interferon-γ

Originally identified 30 years ago as an agent with antiviral activity, IFN-γ has since been characterized as a homodimeric glycoprotein with pleiotropic immunologic functions in cell-mediated immunity against intracellular microbes. It is primarly secreted by activated T

cells and NK cells. IFN-γ can promote the microbicidal function of macrophages by stimulating the synthesis of ROI and NO. These effects are mediated by activating transcription and/or assembly of the enzymes phagocyte oxidase and iNOS. These reactive molecules are produced within lysosomes to destroy microbes contained within phagolysosomes. IFN-γ enhances MHC-associated antigen presentation and amplifies the recognition phase of immune responses by increasing expression of ligands necessary for T cell interaction with APC (antigen presenting cells). This cytokine also activates vascular endothelial cells and potentiates many of the actions of TNF on endothelial cells promoting T lymphocyte adhesion and extravasation to sites of infection. Furthermore, it has been found that IFN-γ promotes the differentiation of naive $CD4^+$ T cells to the T_H1 subset and inhibits the proliferation of T_H2 cells in parallel. This T_H1 inducing effect is mediated indirectly by activating mononuclear phagocytes to produce IL-12, a typical T_H1 cytokine. In mice IFN-γ also enhances expression of the signaling chain of the IL-12 receptor. The combination of IL-12 and IL-18 induces IFN-γ expression in human PBMCs as performed in our coculture experiments. It induces antibody responses that also participate in phagocyte-mediated elimination of microbes, in concert with its described macrophage-activating effects. The B-cell IgG subclasses induced by IFN-γ bind to Fcγ receptors on phagocytes and activate complement. Both these mechanisms promote the phagocytosis of opsonized microbes. Furthermore, IFN-γ activates neutrophils and enhances the cytotoxicity of NK cells.

IFN-γ specifically induces the transcription of a number of genes (e.g. IL18BPa, IP-10, and iNOS), using special IFN-γ responsive sites such as γ-IRE (γ-interferon responsive element), and GAS (gamma-activated site) for direct activation, and ISRE (IFN-stimulated response element) for indirect activation mediated by IRF-1 (Interferon regulating factor-1). Interferon-γ employes the JAK/Stat (JAK1/2; Stat1) pathway to transduce its signaling events [Tau and Rothman, 1999]. The net effect of all these activities is to promote macrophage-rich inflammatory reactions while inhibiting IgE-dependent eosinophil-rich reactions. Knockout mice lacking IFN-γ or the IFN-γ receptor are susceptible to infections with intracellular microbes, such as mycobacteria, because of defective macrophage activation. Moreover, patients with inherited disorders of IFN-γ-mediated immunity also appear to be specifically vulnerable to mycobacterial infections [Dupuis *et al.*, 2000].

1.4 Effects of IFN-γ, IL-18 and NO in carcinogenesis

The response of the body to cancer has many parallels with inflammation. There is evidence that inflammatory cells and cytokines found in tumors are more likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective host antitumor response. Deletion or inhibition of inflammatory cytokines inhibits development of experimental cancer. Inflammatory bowel diseases demonstrate that inflammation can be a cofactor in carcinogenesis leading to colorectal cancer.

Nevertheless, IFN-γ exerts potent antitumor effects. IFN-γ production promotes immune recognition of tumor cells by enhancing the expression of MHC molecules and stimulates cytotoxicity of NK cells, T lymphocytes as well as macrophages [Billiau *et al.*, 1996]. It also may have an anti-proliferative effect on tumor cells [Xu *et al.*, 1998]. In part IFN-γ is responsible for a T_H1 polarization of the immune response that has been shown to be a factor of good prognosis in certain types of cancer [Tartour *et al.*, 1998; Fridman *et al.*, 1998]. Contrary, T_H2 responses are ineffective against tumors and viruses. IL-18 is a prime inducer of a T_H1 response. IL-18 production in human colon may play an important role in homeostasis and tumor immune surveillance, by enhancing IFN-γ production and Fas-L-dependent cytotoxicity of immune cells. In colon cancer, the synthesis of the active form of IL-18 may be decreased or abolished. Inhibition of tumor angiogenesis appears to be a major mechanism of IL-18 antitumor activity [Coughlin *et al.*, 1998]. Many lines of evidence support the hypothesis that tumor growth and metastasis are angiogenesis dependent [Kim *et al.*, 1993]. It has been shown that the anti-angiogenic activity of IL-18 in part appears to be specifically mediated by IFN-γ [Coughlin *et al.*, 1998], which induces interferon-inducible protein 10 (IP-10) [Angiolillo *et al.*, 1995] and monokine induced by γ-IFN (MIG) [Sgadari *et al.*, 1997]. Both anti-angiogenic chemokines can initiate tumor necrosis. But IFN-γ also has other activities that may promote tumor regression [Boehm *et al.*, 1997], such as inhibition of cell proliferation, and proapoptotic effects, particularly in combination with TNF-α. Furthermore, the tumor suppressive pathway of IL-18 may not be completely mediated by IFN-γ signaling, but also through FAS-L mediated killing. Additional IL-18 antitumor effects are mediated by $CD4^+$ T cells and NK cells, but in an most likely IFN-γ and IL-12 independent manner [Osaki *et al.*, 1998].

NO plays an ambivalent role in tumorigenesis depending on NOS activity and resulting NO concentrations. Although there are some reports showing induction of tumor cell apoptosis and cytostasis by high levels of NO [Xie and Fidler, 1998], an increasing number of reports shows a positive correlation between NO and tumor progression [Thompsen and Miles, 1998]. The induction of inducible NO-synthase (iNOS or NOS II) is an important part of macrophage cytotoxicity against tumor cells. iNOS is generally inducible by pro-

inflammatory stimuli and mediates a high-output long-lasting release of NO. It is frequently expressed constitutively in human malignancies including colorectal cancer [Ambs *et al.*, 1998/1999]. If NO concentrations do not reach a cytotoxic level, NO favors tumor growth, neovascularization as well as invasiveness by induction of p53 tumor suppressor gene mutations and upregulation of angiogenic factors such as vascular endothelial growth factor (VEGF) and IL-8. Contrary high levels of NO trigger p53 accumulation, of which wild-type form is known to be an inhibitor of angiogenesis [Dameron *et al.*, 1994]. High concentrations of NO are known to be antiproliferative leading to apoptosis.

1.5 Aim of this study

I intended to investigate the regulation of IL-18BPa expression and release induced by IFNγ in different human cell types that are associated with the ethiology of colon carcinogenesis and autoimmune diseases such as Crohn's disease (CD) and rheumatoid arthritis (RA).

II

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acrylamide/bisacrylamide-solutions	Roth, Karlsruhe
Actinomycin D	Sigma Biochemicals, Deisenhofen
Agar	Gibco Life Technologies, Eggenstein
Agarose	Biozym, Oldendorf
Ammoniumpersulfate	Sigma Biochemicals, Deisenhofen
Ampicillin	Sigma Biochemicals, Deisenhofen
Aprotinin	Roche Biochemicals, Mannheim
[γ-^{32}P]ATP	Amersham Pharmacia, Braunschweig
Bovine serum albumin	Sigma Biochemicals, Deisenhofen;
Brefeldin A	Sigma Biochemicals, Deisenhofen
5-Bromo-4-chloro-3-indolyl-β-D-galactoside	Roth, Karlsruhe
Cell culture media	Gibco Life Technologies, Eggenstein
Complete (protease inhibitor)	Roche Biochemicals, Mannheim
Coomassie	Sigma Biochemicals, Deisenhofen
DETA-NONOate	Alexis, Grünberg
Diethylpyrocarbonate	Sigma Biochemicals, Deisenhofen
Dithiothreitol	Sigma Biochemicals, Deisenhofen
Ethidium bromide	Sigma Biochemicals, Deisenhofen
Ficoll	Sigma Biochemicals, Deisenhofen
FuGENE 6 Transfection Reagent	Roche Biochemicals, Mannheim
Glutathione	Sigma Biochemicals, Deisenhofen
Isopropylthiogalactoside	Roth, Karlsruhe
Leupeptin	Roche Biochemicals, Mannheim
Lipopolysaccharide	Sigma Biochemicals, Deisenhofen
β-Mercaptoethanol	Sigma Biochemicals, Deisenhofen
Molecular weight markers (DNA) 1 kb/100 bp	MBI Fermentas, St. Leon-Rot
Molecular weight markers (protein)	Amersham Pharmacia, Braunschweig; Roth, Karlsruhe
N-naphtylethylendiamine	Sigma Biochemicals, Deisenhofen
Nucleotide triphosphates	Applied Biosystems Applera, Weiterstadt

Okadaic acid	Calbiochem-Novabiochem, Bad Soden
Oligonucleotides	MWG Biotech, Ebersberg; Santa Cruz Biotechnologies, Heidelberg
Pepstatin	Roche Biochemicals, Mannheim
Peptone	Gibco Life Technologies, Eggenstein
Phenol/Chloroform	Roth, Karlsruhe
Ponceau S	Sigma Biochemicals, Deisenhofen
Roti Load1	Roth, Karlsruhe
RNasin	Promega, Mannheim
Sodium butyrate	Sigma Biochemicals, Deisenhofen
Skim milk (non fat)	Fluka, Deisenhofen
Sulfanilamide	Sigma Biochemicals, Deisenhofen
Triton X-100	Sigma Biochemicals, Deisenhofen
tRNA, RNase free	Roche Biochemicals, Mannheim
Trypan blue	Gibco Life Technologies, Eggenstein
Tween 20	Sigma Biochemicals, Deisenhofen
[α-^{32}P]UTP	Amersham Pharmacia, Braunschweig
Yeast extract	Gibco Life Technologies, Eggenstein

Acetone, chloroform, ethanol, ether, methanol, isopropanol, acids and lyes were provided by the central store of the university hospital Frankfurt. All other, not listed chemicals were supplied from Merck (Darmstadt), Roth (Karlsruhe) or Sigma Biochemicals (Deisenhofen).

2.1.2 Other materials and kits

DNAzol	Molecular Research Center, Cincinnati
ECL Detection Kit/Films	Amersham Pharmacia, Freiburg
Endofree Plasmid Maxi Kit (Qiagen-tip 500)	Qiagen, Hilden
LDH Assay	Roche Biochemicals, Mannheim
Luciferase Assay System	Promega, Mannheim
Nanoquant	Roth, Karlsruhe
Nick Columns	Amersham Pharmacia, Braunschweig
NucleoBond PC 100 Kit (AX 100)	Macherey & Nagel, Düren
NucleoSpin Extract 2 in 1	Macherey & Nagel, Düren
OptEIA Elisa Kits for IFN-γ, TNF-α and, IL-8 detection	BD PharMingen, Heidelberg
Polystyrene plates	Greiner, Frickenhausen
TGFβ1 ELISA	R&D Systems, Wiesbaden
TOPO TA Cloning	Invitrogen, Groningen (Netherlands)
Transwell-Clear inserts 6-well polystyrene-plates	Costar, Bodenheim
Trizol	Sigma Biochemicals, Deisenhofen

2.1.3 Buffers and solutions: Immunoblot-analysis

4x Laemmli-buffer	125 mM Tris/HCl pH 6.8 10% (w/v) SDS 50 mM Dithiothreitol 30% (v/v) Glycerol 0.01% (w/v) Bromphenol blue
10x PAGE	250 mM Tris 1% (w/v) SDS 520 mM Glycin
50x TBST	0.1 M Tris pH 8.0 1.5 M NaCl 5% (v/v) Tween 20
Transfer buffer	25 mM Tris 192 mM Glycine pH 8.3 20% (v/v) Methanol
Triton-X100 lysis-buffer	300 mM NaCl 50 mM TrisHCl pH 7.6 0.5% Triton X-100 (supplemented with protease inhibitor cocktail Complete)
Protein molecular weight marker	1 µl molecular weight marker (Roth) 5 µl 4x Loading-Dye (Roth) 14 µl H_2O

2.1.4 Buffers and solutions: β-gal assay

100x Mg^{2+}-buffer	100 mM $MgCl_2$ 5 mM 2-mercaptoethanol
0.1 M $NaP0_4$-buffer	100 ml of 0.1 M Na_2HPO_4 adjusted to pH 7.3 at 37°C using 0.1 M NaH_2P0_4
ONPG-solution	4 mg/ml in OJM $NaP0_4$-buffer
Stop-solution	1 M Na_2CO_3 solution

2.1.5 Buffers and solutions: Mini preparation

CTAB/NaCl	5% (w/v) Cetyltrimethyl-ammonium bromide 0.2 M NaCl
STET	8% (w/v) Saccharose 50 mM Tris/HCl pH 8.0 50 mM EDTA pH 8.0 0.1% (w/v) Triton X-100

2.1.6 Buffers and solutions: EMSA and nuclear extracts

Deionizised formamide (EMSA)	500 ml Formamide +50 g Mixed-bed resin (Bio-Rad 501-X8) incubation at 4°C for 30 min, afterwards filtered, stored at –20°C
3x Hybridisation buffer (EMSA)	12% (w/v) Ficoll 60mM Hepes ph7,9 150mM KCl 3mM EDTA 3mM DTT 3mM PMSF 0.75mg/ml BSA stored at –20°C
Puffer A (cytosolic extract)	10mM Hepes (pH 7,5) 10 mM KCl 0.1 mM EDTA 0.1mM EGTA Complete protease inhibitor (freshly added)
Puffer B (nuclear extract)	20mM Hepes (pH 7.5) 0.4M NaCl 1mM EDTA 1mM EGTA 1mM DTT Complete protease inhibitor (freshly added)

2.1.7 Buffers, media and sera for cell culture

Dulbecco´s modified Eagles medium	Gibco, Berlin
Fetal calf serum	Gibco, Berlin
Human AB serum	Sigma, Deisenhofen
Phosphate buffered saline	Gibco, Berlin
Penicillin/Streptomycin	Gibco, Berlin
RPMI 1640	Gibco, Berlin
Trypsin/EDTA	Gibco, Berlin

2.1.8 Buffers and media for bacteria culture

Medium components	**LB medium*** (sterilized)	**SOB medium*** (sterilized)	**SOC medium*** (sterilized)
Bacto-tryptone	1.0% (w/v)	2.0% (w/v)	2.0% (w/v)
Bacto-yeast extract	0.5% (w/v)	0.5% (w/v)	0.5% (w/v)
NaCl	1.0% (w/v)	10 mM	10 mM
KCl	---	2.5 mM	2.5 mM
$MgCl_2$	---	10 mM	10 mM
$MgSO_4$	---	10 mM	10 mM
Glucose	---	---	20 mM

**For solid agar plates, the different media were supplemented with 1.5% (w/v) Agar!*

TB – Buffer (pH 6.7)	10mM Pipes, 15mM $CaCl_2$,
for competent bacteria:	250mM KCl, 55mM $MnCl_2$

2.1.9 Additional buffers and solutions

10x PBS (pH 7.4)	1.3 M NaCl 30 mM NaH_2PO_4 70 mM Na_2HPO_4
50x TAE	2 M Tris 1 M Acetic acid 50 mM EDTA
10x TBE	0.45 M Tris 0.45 M Boric acid 10 M EDTA

2.1.10 DEPC-treatment

Solutions for RNA based methods were treated with diethyl pyrocarbonate (DEPC) to inactivate RNases. Note that DEPC reacts with amines such as Tris. Hence, Tris and similar buffers were made from DEPC treated water, but not treated with DEPC directly. DEPC treated water was obtained by adding 1 ml DEPC per liter of Aq. dest. After mixing in an overnight step at RT, the solution was autoclaved.

2.1.11 Laboratory equipment

ABI-Prism 310 Genetic Analyser	Applied Biosystems Applera, Weiterstadt
Gel dryer 583	Bio-Rad, München
GeneAmp 2400/9600 Thermocycler	Applied Biosystems Applera, Weiterstadt
Gene Quant II	Amersham Pharmacia, Braunschweig
Herasafe clean bench	Heraeus, Hanau
Hyperprocessor	Amersham Pharmacia, Braunschweig
Incubator Heraeus BBD 6220	Heraeus, Hanau
AutoLumat LB953	Berthold, Pforzheim
Microplate reader Benchmark	Bio-Rad, München
PhosphoImager BAS 1500	Raytest, Straubenhardt
TRI-CARB 2100 TR β-counter	Canberra-Packard, Dreieich

2.1.12 Enzymes

Alkaline shrimps phosphatase	Roche Biochemicals, Mannheim
DNA polymerase T4	MBI-Fermentas, St. Leon-Rot
Turbo Pfu/Pfu-DNA Polymerase	Stratagene, Heidelberg
Proteinase K	Roche Biochemicals, Mannheim
Restriction Enzymes	NEB, Frankfurt a.M.; MBI-Fermentas, St. Leon-Rot
Reverse Transcriptase	Applied Biosystems Applera, Weiterstadt
T3 RNA Polymerase	Roche Biochemicals, Mannheim
T7 RNA Polymerase	Roche Biochemicals, Mannheim
RNase A	Roche Biochemicals, Mannheim
RNase T_1	Roche Biochemicals, Mannheim
T4-DNA Ligase	Gibco Life Technologies, Eggenstein
Taq-DNA polymerase	Applied Biosystems Applera, Weiterstadt
Taq-Gold-DNA polymerase (Hot Start)	Applied Biosystems Applera, Weiterstadt

2.1.12.1 Pretreatment of enzymes

Proteinase K

The lyophilized enzyme was dissolved in $Aqua_{dest}$ (10 mg/ml), incubated for 30 min at 37°C and aliquoted. The aliquots were stored at –20°C until use.

RNase A

RNase A was dissolved to a final concentration of 10 mg/ml in RNase-buffer [Tris/HCl (10 mM, pH 7.5), NaCl (15 mM)]. The enzyme solution was incubated for 30 min at 95°C and cooled to room temperature over night. Aliquots were stored at –20°C until use.

2.1.13 Antibodies and antisera

anti-human IL18 (rabbit, polyclonal)	PeproTech, Frankfurt
anti-human iNOS (rabbit, polyclonal)	Santa Cruz Biotechnologies, Heidelberg
anti-mouse IgG (horseradish-peroxidase coupled)	Bio-Rad, München
anti-rabbit IgG (horseradish-peroxidase coupled)	Bio-Rad, München
hIL18BP 147A (rabbit, polyclonal)	Eurogentech, Seraing (Belgium)
IFNγ neutralizing antibody monoclonal + control AB	PharMingen, NALE Quality, Hamburg
Stat1 9H2 (mouse, monoclonal)	Cell Signaling, distributed by NEB, Frankfurt a.M.

2.1.14 Recombinant and purified proteins

IFN-γ (human)	PeproTech, Frankfurt
IL-12	R&D Systems, Wiesbaden
IL-18	PeproTech, Frankfurt
IL-1β	Cell Concepts, Umkirch
TGF-β1	Roche Biochemicals, Mannheim

2.1.15 Plasmids

2.1.15.1 Vectors

pBluescript II KS (+)	Stratagene, Heidelberg
pCR 2.1 TOPO	Invitrogen, Groningen (Netherlands)
pCR II TOPO	Invitrogen, Groningen (Netherlands)
pCR 4 TOPO	Invitrogen, Groningen (Netherlands)
pGL3-Basic Vector	Promega, Mannheim
β-Gal (pcmv)	Stratagene, Heidelberg

2.1.15.2 Recombinant plasmids

	Reference	Purpose
pBKS(+)*GAPDH* (human, nucleotides 148-302)	Tokunaga *et al.* 1987	RPA
pBKS(+)*IL-18* (human, nucleotides 335-627)	Ushio *et al.* 1996	RPA
pBKS(+)*IL-18BP* (human, nucleotides 457-644)	Novick *et al.* 1999	RPA
pBKS(+)*iNOS* (human)	kindly provided by Dr. H. Kleinert, Mainz	RPA
pBKS(+)*TGF-β* (murine, nucleotides 1735-1974)	EMBL Acc. No. NM011577	RPA
pBKS(+)*VEGF* (human, nucleotides 339-498)	Weindel *et al.* 1992	RPA
pORF *IL18BPa*	InvivoGen, San Diego (USA)	Overexpression ⇒ TCA-Precipitation

2.1.15.3 Recombinant IL18BP promoter-constructs

IL-18BP promoter luciferase vectors	Genomic position of cloned cDNA (Chromosome 11q13) 5' ⇒ 3'
pGL3 + IL18BProm Frag4	nucleotides 2405990 ⇒ 2407104
pGL3 + IL18BProm Frag5	nucleotides 2406459 ⇒ 2407104

Pub Med human genome: URL

http://www.ncbi.nlm.nih.gov:80/cgi-bin/Entrez/map_search.cgi?chr=hum_chr.inf&advsrch=off&query=il18bp

2.1.16 Bacterial strains

		Genotype
***E.coli* DH5α**	Gibco Life Technologies, Eggenstein	*supE44 Δlac U169 (∅80lacZΔM15) hsd R17 recA endA1 gyrA96 thi-1 relA1*
***E.coli* XL-1 blue**	Stratagene, Heidelberg	*supE44 hsdR17 recA1 endA1 gyrA46thi relA1 lac⁻ F`[proAB⁺ lacI^q lacZΔM15 Tn10(tet^r)]*
TOP10	Invitrogen, Groningen (Netherlands)	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC)Φ80lacZ ΔM15 ΔlacX74 recA1 deoR araD139 Δ(araleu)7697 galU galK rpsL (Str^R) endA nupG*

2.1.17 Eukaryotic cell lines

Caco-2	Colon carcinoma cell line (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany)
DLD-1	Colon carcinoma cell line (Centre for Applied Microbiology & Research, Salisbury, United Kingdom)
HaCaT	provided by Dr. N.E. Fusenig [Boukamp *et al*., 1988]
HCT116	Colon carcinoma cell line (American type tissue collection, Manassas, VA, USA)
LoVo	Colon carcinoma cell line (American type tissue collection)

2.1.18 Oligonucleotides

2.1.18.1 Cloning of IL18BP Promoter-Fragments

Fragment Number	**Forward Primer (MluI)**	**Reverse Primer (BglII)**
Frag4 IL18BProm	5'IL18BProm forB MluI	3'IL18BProm BglII rev
Frag5 IL18BProm	5'IL18BProm FragB MluI	3'IL18BProm BglII rev

Forward Primer (MluI *ACG CGT*)*	**Sequence 5' ⇒ 3'**
5'IL18BProm forB MluI	CTG TCA CC***A CGC GT***T GCA CCC TCC
5'IL18BProm FragB MluI	CCT AGA GGG ***ACG CGT*** CTG GAA AGG
Reverse Primer (BglII *AGA TCT*)*	
3'IL18BProm BglII rev	TTC AGC ACC ***AGA TCT*** GCT TCT GGG

** Mutated sequences for generating artificial restriction sites are shown in italic characters!*

2.1.18.2 Semiquantitative PCR (human specific)

	Fragment size (bp)	**Sequence 5' ⇒ 3'**
GAPDH for	452	ACC ACA GTC CAT GCC ATC AC
GAPDH rev		TCC ACC ACC CTG TTG CTG TA
IL18 for	293	ACC AAG TTC TCT TCA TTG ACC
IL18 rev		TTG CAT CTT ATT ATC ATG TCC
IL18BPa for	295	CCT CTA CTG GCT GGG CAA TGG
IL18BPa rev		TTA ACC CTG CTG CTG TGG AC
iNOS for	258	GGT GCT GTA TTT CCT TAC GAG GCG AAG AA
iNOS rev		GGT GCT ACT TGT TAG GAG GTC AAG TAA AGG
IP-10 for	289	AGT GGC ATT CAA GGA GTA CC
IP-10 rev		ATC CTT GGA AGC ACT GCA TC

2.1.18.3 Sequencing primer

	Sequence 5' ⇒ 3'
IL18BProm Seq1	GTT GAG CCA GTC CGC CTC TTC
IL18BProm Seq2	GTA TGG CAT TGA GCC TGA AGT GGT CC
IL18BProm Seq3	GGT AGA TTA GAG ATC CCA GTC TGG
M13 for	CAG GAA ACA GCT ATG AC
M13 rev	GTA AAA CGA CGG CCA G
pGL3 Basic for	CTA GCA AAA TAG GCT GTC CC
pGL3 Basic rev	CTT TAT GTT TTT GGC GTC TTC CA

2.1.18.4 IL18BP promoter GAS sites (EMSA)

Stat1 binding sites	**Sequence 5' ⇒ 3'**
EMSA GAS1 for ss	CAG TGC TTT CCC AGA AGG ATT GC
EMSA GAS1 rev ss	GCA ATC CTT CTG GGA AAG CAC TG

Stat1 p84/p91 ***Mutant*** Oligonucleotide (Santa Cruz Biotechnologies, Heidelberg)	CAT GTT ATG CAT ***ATT GGA GTA AG***T G **3' --** GTA CAA TAC GTA ***TAA CCT CAT TC***A G **-- 5'**
Stat1 p84/p91 ***Consensus*** Oligonucleotide (Santa Cruz Biotechnologies, Heidelberg)	CAT GTT ATG CAT ***ATT CCT GTA AG***T G **3' --** GTA CAA TAC GTA ***TAA GGA CAT TC***A C **-- 5'**

2.1.19 Computer software

DNA/Protein homology search	BLAST search (National Center of Biotechnology, USA; URL: *http://www.ncbi.nlm.nih.gov);*
DNA analysis software	Analyze
Graphic processing	Corel Draw 8.0/10.0
Presentations	Powerpoint 2000
Statistical analysis	Sigma Plot 4.0 (Students T-Test)
Text processing	Microsoft Word 2000

Programs belonging to special devices are mentioned separately in the appropriate sections.

2.2 Methods

2.2.1 Microbiologic methods

2.2.1.1 Bacterial culture

The *E. coli* strains DH5α (Gibco Life Technologies) and XL1-blue (Stratagene) were used for amplification of plasmid DNA. Both strains were either grown in liquid LB (**L**auria-**B**ertani) or SOC medium (2.1.8). For selection, the media contained ampicillin (50 µg/ml). Agarplates were generated with LB-ampicillin medium supplemented with agar (15 g/l). For long-term preservation of transformed bacteria, cells were mixed with sterile glycerol [30% (v/v)] and stored at –80°C.

2.2.1.2 Competent bacteria for transformation

To yield high transformation efficiencies from plasmid DNA in bacteria, cells have to be pretreated chemically. To this end, 250 ml SOB-Medium (2.1.8) were inoculated with 200 µl of an overnight bacterial culture and grown at 18°C until the suspension reaches an optical density of 0.5 ($OD_{600\ nm}$). The bacterial growth was stopped by storing the suspension for

10min on ice. Bacterial cells were concentrated by centrifugation (15 min, 2500g, 4°C; Heraeus Megafuge 1.0, rotor 7570F). The cellular pellet was subsequently resuspended in 80 ml solution TB (2.1.8), mixed and incubated on ice for 10 min. The bacterial suspension was centrifuged again (15 min, 2500 g, 4°C), the pellet was gently resuspended in 20 ml of solution TB + 7% DMSO, and incubated on ice for additional 10 min. Thereafter, aliquots of competent bacteria were snap frozen in liquid nitrogen and stored at –80°C.

2.2.1.3 Transformation

100 µl of a competent bacteria suspension was thawed on ice and 5 µl of the ligation reaction (2.2.3.11) was added. The bacteria/DNA mixture remained on ice for additional 30 min followed by an incubation at 42°C for 1 min. The bacteria were chilled on ice again for 2 min, before 300 µl of SOC-medium were added. For initial expression of the plasmid encoded ampicillin resistance, bacteria were incubated for 1 h at 37°C on a shaker. Subsequently, 20 - 200 µl of this transformation solution was plated on ampicillin containing agar plates depending on the efficiency of transformation. To enable a blue/white screening for recombinant clones (pBSII(KS)+/pCR TOPO), the agar plate was supplemented with 50 µl X-Gal (2% in DMSO) and 50 µl IPTG (0.1 M in $Aqua_{dest}$). The plates were incubated overnight at 37°C.

2.2.2 Cellbiologic methods

2.2.2.1 Cultivation of DLD-1, Caco-2, LoVo, HCT116, and HaCaT cells

Human DLD-1 (Centre for Applied Microbiology & Research, Salisbury, United Kingdom), HCT116 (American type tissue collection, Manassas, VA, USA), Caco-2 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) colon carcinoma cells, and HaCaT keratinocytes (provided by Dr. N.E. Fusenig) were grown in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FCS (GIBCO-BRL, Eggenstein, Germany). LoVo (American type tissue collection) colon carcinoma cells were maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FCS (GIBCO-BRL).
All these cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 (Heraeus BBD 6220 incubator). For subcultivation, cells were washed twice in phosphate buffered saline (PBS), subsequently trypsinized (Trypsin, EDTA) and diluted dependent on their growth properties in a 1:10 and 1:20 ratio.

For long-term storage, the cells were treated as subsequently described: after trypsinization, cells were concentrated (5 min at 1100 rpm, Heraeus Megafuge 1.0, rotor 75750F), diluted in freezing medium (growth medium supplemented with 10% DMSO) and stored in cryotubes (Nunc). The cryotubes were cooled down slowly overnight to –70°C and finally stored in liquid nitrogen.
For TCA precipitation of cell culture supernatants, cells on polystyrene plates (Greiner, Frickenhausen) were washed twice with PBS and incubated in the aforementioned DMEM medium without FCS.

2.2.2.2 Cultures of colonic intestinal biopsy specimens

The study protocol and consent documents were approved by the 'Ethik Kommission' of the Klinikum der Johann Wolfgang Goethe-Universitat, Frankfurt am Main. Informed consent was obtained from the donors. All patients required a colonoscopy for medical reasons. For the experiments a macroscopically non-diseased biopsy site was chosen. Cultivation was performed as recently described [Reimund *et al.*, 1996]. Within a maximal lag of 0.5 hours after biopsy, specimens were washed carefully in PBS (GIBCO-BRL). Thereafter, tissues were placed in 24-well tissue culture plates (Greiner) and maintained in DMEM without phenol red (GIBCO-BRL, 1 ml/well) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO-BRL) and 1% (v/v) heat-inactivated human AB serum (Sigma). Stimuli were added and after the indicated incubation periods cells were harvested for RNA isolation.

2.2.2.3 Isolation of PBMC

The study protocol and consent documents were approved by the *´Ethik Kommission´* of the Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main. Healthy volunteers abstained from using any drugs during 2 weeks before the study.
Blood was drawn into heparinized syringes (10 U/ml final concentration) and subsequently PBMC were isolated by centrifugation through Ficoll (Histopaque-1077, Sigma Biochemicals) for 15 min at 1900 rpm without brake (Heraeus Megafuge 1.0, rotor 75750F). PBMC were washed three times with PBS and resuspended in RPMI 1640 supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO-BRL) and 1% (v/v) heat-inactivated human AB serum (Sigma, Deisenhofen, Germany) and seeded at 2 x 10^6 cells/ml in round-bottom polypropylene tubes.

2.2.2.4 Cocultures of PBMC/DLD-1

For indirect (transwell-assay) cocultures, PBMC (5 x 10^6 cells per insert) were seeded into Transwell-Clear inserts (0.4 μm pore size, Costar, Bodenheim). Inserts were placed onto confluent DLD-1 cells, grown in 6-well polystyrene-plates (Costar). Incubations were performed in RPMI 1640 supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO-BRL) and 1% (v/v) heat-inactivated human AB serum (Sigma, Deisenhofen, Germany). In some of these experiments, DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 1% (v/v) heat-inactivated human serum was used instead of the RPMI medium specified above. This change of medium was necessary due to the high content of nitrate in the RPMI medium which interferes with determination of nitrate production by activated cells. The choice of medium did not affect iNOS induction in DLD-1 cells of cocultures.
After 45h inserts with PBMC and the supernatants were carefully removed. Thereafter, adherent DLD-1 cells in the polystyrene wells were washed twice with PBS followed by cell-lysis for RNA-isolation or immunoblot analysis (2.2.3.6/2.2.4.4).

2.2.2.5 IFNγ production by IL-12/IL-18-stimulated PBMC cultivated in DLD-1 cell-derived conditioned media

To investigate whether conditioned medium from IFNγ-stimulated DLD-1 cells may contain IL-18BPa activity, the following experimental protocol was performed: DLD-1 cells were kept as unstimulated control, or were stimulated with IFNγ (20 ng/ml), with sodium butyrate alone (5 mM), or with IFNγ plus sodium butyrate for 14 h using DMEM medium (containing 10 % FCS) in order to induce expression of IL-18BPa. Thereafter, cultures were thoroughly washed three times with PBS. After an additional 48 h incubation period in control DMEM without FCS, cells were lysed for isolation of total RNA. Cell-free culture supernatants were either TCA-precipitated (for SDS-PAGE) or concentrated 5-fold using Ultrafree-4 Biomax 10K centrifugal filters (Millipore, Bedford, MA). Concentrated conditioned media were preincubated for 30 min without stimuli or with IL-12 (20 ng/ml)/IL-18 (20 ng/ml) at 37°C. PBMC were then resuspended in these conditioned media and after an additional 24h of incubation, IFNγ production was assessed by ELISA (Pharmingen, Hamburg).

2.2.2.6 Reporter gene assays

The regulation of gene expression can be examined using a reporter-gene-assay. For this purpose DNA sequences carrying putative regulatory elements were cloned in front of a reporter gene, in this case the gene encoded luciferase enzyme [Alam and Cook, 1990]. By liposomal transfection newly generated constructs were transferred into the eukaryotic target cells. Thereafter transfected cells were stimulated with IFN-γ. After transfection and stimulation, cells were harvested to measure the amount of synthesized reporter gene products. To assess the influence of a certain sequence on IL-18BP gene regulation, reporter gene expression in IFN-γ-stimulated cells and unstimulated cells were compared.

2.2.2.6.1 Transient transfection of DLD-1 cells with luciferase constructs

Confluent grown DLD-1 cells were trypsinized and cultured in fresh medium. The cells should have reached a confluency of about 50 - 60%. The cells were seeded in 2 ml medium per well onto 6-well-culture-plates and incubated for 18 - 24 h under standard conditions. IL-18BP promoter luciferase expression contructs (2 µg DNA/35 mm well) were transfected into DLD-1 cells using Fugen 6 Transfection Reagent (Roche Biochemicals) according to the manufacturer's instruction. The transfection conditions for this cell line had to be optimized. A final volume of 4.5 µl of Fugene 6 Transfection Reagent was used per well of a 6-well plate. These 4.5 µl were diluted 90 µl using serum-free DMEM and incubated for 5 min. Meanwhile 2 µg plasmid DNA and 500 ng of pCMV-β galactosidase DNA (2.1.15.1), for controlling the transfection efficiency, were brought to a final volume of 10 µl also employing serum-free medium. Subsequently both solutions were combined and mixed gently. In the following 15 min the formation of DNA-liposome complexes took place at room temperature.

DLD-1 target cells were rinsed two times with PBS and maintained in 2 ml of fresh serum-containing medium. Cells were transfected with 100 µl DNA-liposome complexes and incubated for 42 h without replacing the transfection mixture. After the 42 h transfection period, cells were washed twice with PBS. Subsequently DLD-1 cells were either kept as unstimulated control or stimulated with IFNγ (20 ng/ml). 6 h later, cells were harvested to determine luciferase (Promega Luciferase Assay System) and β-Gal activity (2.2.2.6.3), respectively.

2.2.2.6.2 Luciferase assay

The luciferase of the Firefly (*Photinus pyralis*) catalyses the oxidation of luciferin. During this reaction photons are released at a wavelength of 562 nm. By employing a luminometer (AutoLumat LB953, Berthold) a quantitative measurement of the emitted light was performed. Cells were transiently transfected with a luciferase-construct, stimulated and harvested in 1x Reporter-Lysisbuffer (Promega) as described by the manufacturer. The solutions needed for measurement were prewarmed at RT. 20 µl of cell-extract were pipetted in a polystyrene-tube. Subsequently, the reaction was started in the Luminometer by injection of 100 µl Luciferase-Assay-Reagent (Promega) (20 mM Tricin, 1.07 mM $(MgCO_3)Mg(OH)_2$ x 5 H_20, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM Coenzym A, 470 µM Luciferin, 530 µM ATP pH 7.8). The emitted light was measured for 10 s with a Photomultiplier and expressed in Relative Light Units short (RLUs) by the luminometer.

2.2.2.6.3 ß-Galactosidase assay

The ß-galactosidase is an bacterial enzyme which catalyses the breakdown of lactate. It is encoded by the bacterial lacZ gen in the lac-Operon. Besides lactate also X-Gal is substrate for this decomposition process, which product leads to yellow colour. Therefore, the transfection efficiacy can be determined by transferring the lacZ gene into a cell under control of a constitutive promoter. Using this method the amount of ß-galactosidase was measured in lysates of cells transfected with β-Gal and luciferase genes. Activation of the luciferase gene in cells was normalized using the β-Gal counts.
For this experimental set up the following reagents (2.1.4) were combined: 100 µl of cell extract (extraction as previously described in 2.2.2.6.2), 3 µl of 100x Mg-buffer, 66 µl ONPG solution and $NaP0_4$-buffer were added to a final volume of 300 µl. The reaction was incubated from 30 min up to several hours at 37°C until a yellow color became visible. Finally, the reaction was terminated by adding 0.5 ml of stop solution. The color reaction was quantified photometrically at 410 nm and 595 nm for reference.

2.2.2.7 Measurement of cell parameters

2.2.2.7.1 Cell viability: LDH assay

Cell viability was also determined by a lactate dehydrogenase (LDH) activity assay according to the manufacturer's instructions (Roche Molecular Biochemicals). LDH is usually located intracellularly in viable cells. During cell death this enzyme is released into the cell culture supernatant where its activity can be detected.

2.2.2.7.2 Nitric oxide synthase activity: Griess assay

Nitrite (NO_2^-) is a stable NO oxidation product, that has been used as a direct readout for nitric oxide synthase activity using the Griess-assay as recently described (Mühl *et al*., 1997). Detection limit for nitrite in this assay was 1.5 µM.

Cell culture supernatants were cleared by centrifugation (5 min at 400 g). 200 µl of the cleared lysates or supernatants were mixed with an equal volume of ready-to-use Griess Reagent (Roche, Mannheim). After 5 min at room temperature, the absorbance was measured at 540 nm with a reference wavelength at 690 nm.

For detection of total nitrite/nitrate a colorimetric assay kit was used according to the manufacturer's instructions (Alexis Biochemicals). Detection limit for nitrite/nitrate was 3 µM in this assay.

2.2.3 Molecular biology methods

2.2.3.1 Reverse transcriptase reaction (RT)

The enzyme reverse transcriptase, originally discovered in RNA tumor viruses, synthesizes a complementary DNA strand using RNA as a template. This enzymatic activity provides access to the generation of cDNA. Random hexamers (50 µM) were used as internal enzyme start sites.

Reverse transcriptase reaction:		
	X µl (*max. 3 µl)*	RNA (1 µg)
	4 µl	$MgCl_2$ (25 mM)
	2 µl	10x PCR-Buffer
	2 µl	d**A**TP (each 10 mM) d**C**TP d**G**TP d**T**TP
	1 µl	RNase Inhibitor (20U/µl)
	1 µl	Random Hexameric Primers (50 µM)
	1 µl	Reverse Transcriptase (50U/µl; Muloney Virus) Applied Biosystems Applera
	X µl	DEPC H_2O
	20 µl	**Total Volume**

1 µg of total RNA was added to the reagents as listed in the table. The reaction was performed for 30 min at 42°C. Subsequently the mix was terminated by incubation for 5 min at 99°C and cooled to 4°C. Aliquots of the cDNAs were stored at 4°C.

2.2.3.2 Polymerase chain reaction (PCR)

This method enables the *in vitro* amplification of DNA fragments without time consuming cloning and identification steps [Mullis & Foloona, 1987]. The method is based on the availability of heat-stable DNA polymerases which allow multiple denaturing of template DNA, annealing of driver sequences (primer) and synthesis of DNA by amplification steps within one tube.

Polymerase chain reaction:

2 µl	RT-product (2.2.3.1) or
X µl	Plasmid-DNA Template (5-10 ng) or
X µl	genomic-DNA Template (250 ng)
5 µl	10x PCR-Buffer
3 µl	$MgCl_2$ (25 mM)
1 µl	d**A**TP (each 10 mM) d**C**TP d**G**TP d**T**TP
5 µl	Forward/Reverse Primer-Mix (5 µM)
0.4 µl	*Taq-Gold* Polymerase (5U/µl)
X µl	DEPC H_2O
50 µl	**Total Volume**

The reaction was performed in a thermocycler (GeneAmp 2400 or 9600, *PE* Biosystems). Conditions varied dependently on the gene of interest, the type of polymerase used for amplification and the experimental set up. For **semi-quantitative gene analysis** *taq-gold* polymerase has been used. This enzyme has to be activated by a single incubation for 10 min at 94°C (hot start!).

In general, 25-30 cycles of the following steps have been carried out: 1 min 94°C, 1 min e.g. 60°C (primer-annealing) and 2 min 72°C (endelongation). The amplification was completed by a final 7 min incubation step at 72°C. Depending on special conditions of template, primers and type of cell the protocol was adapted individually (see table below). The samples were stored at 4°C and analyzed by gel electrophoresis.

Semi-quantitative gene analysis for	Annealing temperature	Cycle number
GAPDH	60°C	23-25
hiNOS	62°C	30-32
IL8	60°C	28
IL18	58°C	30-32
IL18BP	58°C	31
IP10	60°C	30

For **amplification of genomic DNA** *taq* and *(turbo)-pfu*-polymerase have been used, since genomic DNA is damaged by long term heatment needed for *taq-gold* polymerase activation. In contrast to *taq* polymerase the *(turbo)-pfu* polymerase provides proofreading activity, but at a reduced rate of synthesis. 30–35 cycles were sufficient to amplificate genomic IL18BP promoter fragments by using to following PCR program: 1 min 94°C, 1 min 65°C and 4 min 72°C. Finally the amplification was completed by a 7 min incubation step at 72°C.

2.2.3.3 Real-time quantitative PCR: analysis of IL-18BPa expression

Real-time PCR analysis of cDNA is based on the direct detection of amplicons by fluorescence. Changes in fluorescence are caused by the *Taq*-polymerase degrading the probe that contains a fluorescent dye (FAM for IL-18BPa, VIC for GAPDH) and a quencher (TAMRA). Primers and probe for IL-18BPa were designed using the Primer Express software from Applied Biosystems according to the published sequence (XM 035063.1): forward 5´-acc tcc cag gcc gac tg-3´; reverse 5´-cct tgc aca gct gcg tac c-3´; probe 5´-cac cag ccg gga acg tgg ga-3´. The possibility to amplify contaminating genomic DNA was eliminated by selecting an amplicon which crosses an exon/intron boundary. For GAPDH, we used a pre-developed assay reagent including primers and probe (Applied Biosystems). Specificity of PCR products was tested by classic PCR using the aforementioned primers. 1 µg of total RNA was transcribed in RT reaction using random hexameric primers and Muloney virus RT (Applied Biosystems Applera) according to the manufacturer´s instructions. Real-time PCR was performed on the AbiPrism 7700 Sequence Detector (Applied Biosystems Applera) as follows: One initial step at 50°C for 2 minutes and 95°C for 10 minutes was followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Detection of the dequenched probe, calculation of threshold cycles (Ct values), and further analysis of these data were performed by the Sequence Detector software. All results for IL-18BPa were normalized to GAPDH.

2.2.3.4 Cloning of PCR products in cloning vectors (pBSII(+)KS/TOPO) and luciferase vector (pGL3)

After amplification, PCR products were cloned into the pBluescript II (+) KS vector (Stratagene), into the TOPO-Cloning Vectors (Invitrogen), or directly ligated to the target vector pGL3 (Promega). PCR inserts in pBSII/TOPO were restricted by type II endonucleases (MluI/BglII) and separated from these cloning vectors by gel electrophoresis and subsequent gel elution using a column gel extracting kit (NucleoSpin

Extract 2 in 1 by Macherey & Nagel), followed by ligation into the prepared luciferase-vector pGL3. These luciferase constructs were used to transfect DLD-1 cells.

2.2.3.5 Preparation of plasmid DNA: CTAB Miniprep

Isolation of plasmid DNA was performed as described by Del Sal et al (1989) with slight modifications. 1.5 ml cells of an overnight culture were collected at 15.000 g (1 min, 4°C). The bacterial pellet was resuspended in 200 µl Lysis-STET-buffer (2.1.5; supplemented with a final concentration of 1 mg/ml lysozyme) and incubated for 10 min at room temperature. After a boiling step (1 min at 95°C), the mixture was centrifuged for 10 min at 15,000 g. The pellet containing cell debris, protein, and genomic DNA, was removed using a tip. 10 µl 5% (w/v) cetyltrimethylammonium bromide (CTAB) / 0.2 M NaCl (2.1.5) was added for 3 min to precipitate RNA and plasmid DNA. After centrifugation (15 min, 15,000 g), the pellet was resuspended in 300 µl 1.2 M NaCl containing 5 µg/ml RNase A, incubated for 15 min at 37°C (or 0.5 - 1 h at room temperature, to ensure a complete degradation of RNA), and subsequently precipitated with 750 µl ethanol for 1 h at –20°C. The precipitated plasmid DNA (15 min, 15,000 g) was dissolved in 20 µl $Aqua_{dest}$.

Midiprep: Higher amounts of plasmid DNA (expected yields: 75 - 100 µg) were obtained using the NucleoBond PC 100 Kit (AX 100) as described by the manufacturer.

Maxiprep: To obtain endotoxinfree plasmid DNA for transfection experiments we employed Endofree Plasmid Maxi Kit (Qiagen-tip 500) according to the manufacturer's instruction.

2.2.3.6 RNA isolation from cultured cells

RNA isolation was performed with TRI-Reagent according to the protocol from the manufactor (Sigma Biochemicals). Cells were grown and stimulated as described above (2.2.2.1). Last traces of media were removed by a pipette tip attached to a vacuum line. Subsequently, cells were lysed with 1 ml of TRI-Reagent per well using 6-well-plate. The lysate was transferred into an Eppendorf tube. After addition of 200 µl chloroform, the samples were inverted for 30 s. The inverted tubes were stored RT for 10 min and subsequently centrifuged (15,000 g, 15 min at 4°C). Afterwards the aqueous upper phase was transferred into a fresh tube. RNA was precipitated using 500 µl of isopropanol and stored again for 10 min at RT. Finally a single centrifugation step (15,000 g) was carried out for 15 min at 4°C to pellet the precipitated RNA. Then the RNA pellet was washed with cold 70% ethanol (with DEPC-treated water), followed by centrifugation (15,000 g, 10 min

at 4°C). The final RNA pellet was resuspended in 20 – 25 µl DEPC-treated water. After a 10 min incubation at 55°C, the amount of isolated RNA was quantified photometrically (2.2.3.7). 3 µg of the isolated RNA was controlled for integrity by agarose gel electrophoresis (1%, 1x TBE buffer).

2.2.3.7 Quantification of nucleic acid concentrations

Concentrations of nucleic acids were determined photometrically using a wavelength of 260 nm (Gene Quant II, Amersham Pharmacia). An optical density (OD) of 1 corresponds to approximately 50 µg/ml double-stranded DNA or 40 µg/ml for single stranded DNA and RNA [Sambrook *et al*. 1989]. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimation of the purity of the nucleic acid preparation. Highly pure DNA or RNA are characterized by ratios between 1.8 and 2.0. Concentrations were calculated according to the following equation:

c [µg/ml] = OD_{260} x V x F	V = dilution factor
	F = multiplication factor (dsDNA = 50; RNA = 40)

Low amounts of DNA were estimated by agarose gel electrophoresis (2.2.3.8) in comparison with a known standard.

2.2.3.8 Agarose gel electrophoresis of nucleic acids

Nucleic acids were separated by gel electrophoresis using agarose gels. The agarose concentration was dependent on the molecular weight of the analyzed nucleic acids. For separation of DNA molecules from 0.5 to 2 kbp usually 1% agarose gels (w/v) were employed. Smaller DNA fragmentes (100 bp – 500 bp) were separated in high density gels (1.5 - 2% agarose gels) [Sambrook et al., 1989]. Agarose (Roth/Gibco Life Siences) was dissolved in 1x TBE gel electrophoresis buffer. Ethidium bromide was added to a final concentration of 500 ng/µl. Ethidium bromide binds to DNA or RNA by intercalation between the bases and, thus enables an ultraviolet fluorescence illumination of nucleic acids. The DNA/RNA samples were diluted with loading buffer [6x loading buffer: 30% glycerol (v/v), 0.25% bromophenol blue (w/v), 0.25% xylenecyanole (w/v), 60% 10x TBE buffer (v/v)] and transferred into the appropriate gel wells. Electrophoresis was performed in 1x TBE buffer with a voltage of 10 V/cm electrode distance. DNA fragment sizes were estimated using molecular weight markers (MBI Fermentas).

2.2.3.9 DNA isolation from agarose gels

The use of the NucleoSpin Extract 2 in 1 Kit (Macherey & Nagel) enables a pure extraction of DNA fragments directly from agarose gels. The system is based on a silica membrane, which binds single and double stranded DNA. The DNA fragments of interest were cut from the gel with a razor blade and further processed according to the instructions of the manufacturer.

2.2.3.10 Restriction

Type II endonucleases isolated from bacteria specifically bind palindromic sequences with a subsequent cleavage of the DNA molecule at their recognition site. This process generates either blunt-end fragments or overhanging cohesive ends, which allow the generation of recombinant DNA by enzymatic ligation. The standard approach for DNA digestion is subsequently listed. After incubation at the appropriate temperature, DNA cleavage was checked by agarose gel electrophoresis (2.2.3.8*)*.

DNA digestion:		
	X µl	DNA (3 µg) target-vector
	2.0 µl	10x buffer
	1.8 µl	restriction enzyme (10U/µl)
	X µl	H_2O
	50 µl	**Total volume**

2.2.3.11 Ligation

Generation of covalent phosphodiester bonds between the 5´-phosphate and the 3´-OH of DNA fragments is catalyzed by T4-DNA ligase. The ligation reaction was performed with restricted or PCR amplified DNA. The DNA was separated in a agarose gel. Subsequently the DNA fragment of interest was isolated using a column gel extracting kit by Macherey & Nagel. The gel extracted fragment was added to the ligation reaction. The mixture was incubated overnight at 16°C. Afterwards, an aliquot of this reaction was transformed into competent bacteria as described in section 2.2.1.3 .

DNA ligation:		
	X µl	target vector DNA (70 ng)
	X µl	gel-extracted DNA fragment (130 ng)
	2 µl	5x ligase buffer
	0.5 µl	T4-DNA ligase (1U/µl)
	X µl	H_2O
	10 µl	**total volume**

2.2.3.12 DNA sequencing

DNA sequencing was performed using the ABI-Prism 310 Genetic Analyser (Applied Biosystems Applera) based on the dideoxynucleotide chain termination method [Sanger *et al.* 1977]. In the termination labeling mix, the four dideoxy terminators (ddNTPs) were tagged with different fluorescent dyes. This technique allows the simultaneous sequencing of all four reactions (A, C, G, T) in one reaction tube. The probes were separated electrophoretically using a micro capillary. As each dye terminator emits light at a different wavelength when excited by laser light, all four colors corresponding to the four nucleotides can be detected and distinguished within a single run. Raw data were evaluated by the Abi Prism sequencing analysis software on a Power G3 Macintosh computer. The sequencing reaction, as listed in the table, was performed in a thermocycler (GeneAmp 2400, Applied Biosystems Applera) with 25 cycles of the following temperature steps: 96°C for 10 sec, 55°C for 5 sec, 60°C for 2 min. For the detection process, probes were prepared as described by the manufacturer.

DNA sequencing:		
	X µl	DNA (250 ng plasmid DNA or 50 ng PCR derived DNA)
	2 µl	sequencing premix
	0.5 µl	primer (200 µM)
	X µl	H_2O
	6 µl	**total volume**

2.2.3.13 RNase protection assay

This method was used to quantify the amounts of specific RNA transcripts from total cellular RNA. Compared to Northern blot analysis, this technique possesses increased sensitivity and specificity. The assay is based on the principle, that double stranded RNA hybrids are protected from cleavage by RNases A and T1.

2.2.3.13.1 Preparation of a radiolabeled antisense probe

The desired probes (150-450 nt in length) were cloned into the transcription vector pBluescript II KS (+) as described in section 2.2.3.4. The recombinant plasmids (see 2.1.15.2) were linearized with restriction enzymes, phenol/chloroform extracted, precipitated and dissolved in $Aqua_{dest}$ to a final concentration of 1 µg/µl. A pure single stranded, [α-^{32}P]UTP radiolabeled antisense transcript was synthesized using T3 or T7 RNA polymerase dependent on insert direction. The reaction mix was prepared as listed in the following table and incubated for 1 h at 37°C:

***In vitro* transcription:**		
	1.1 µl	DEPC H_2O
	1.0 µl	nucleotides (ATP, CTP, GTP; each 5 mM)
	2.0 µl	transcription buffer (5x)
	0.4 µl	RNasin (40 U/µl)
	0.5 µl	T3 or T7 RNA polymerase, depending on insert direction
	3.5 µl	[α-^{32}P]UTP (800 Ci/mM)
	0.5 µl	linearized template (1 µg/µl)

Subsequently 90 µl DEPC-H_2O were added. The reaction was extracted with an equal volume of phenol/chloroform and centrifuged (15,000 g, 2 min). The radiolabeled RNA was precipitated from the aqueous phase using 40 µl of 7.5 M ammoniumacetate, 1.5 µl of tRNA (10 µg/µl) and 350 µl of ethanol for 15 min at –20°C. Following a 15 min centrifugation step at 4°C, the radiolabeled pellet was dissolved in 20 µl FLB 80 and purified using an acrylamide/urea gel (5% acrylamide/bisacrylamide, 29:1; 8 M urea; 1x TBE). Electrophoresis (1x TBE buffer, 300 V) was terminated after 90 min. The radiolabeled probe was located by autoradiography, cut out and eluted into 300 µl elution buffer (0.1x TBE, 0.2% SDS). The incorporated activity of the freshly prepared radiolabeled probe was determined using a β-counting device (TRI-CARB 2100 TR, Canberra-Packard).

Buffer components	**FLB 80 buffer**	**FAB buffer**	**RNase buffer**
Formamide (deionized)	80% (v/v)	80% (v/v)	---
EDTA (pH 8.0)	1 mM	1 mM	5 mM
PIPES (pH 6.4)	---	40 mM	---
NaCl	---	400 mM	---
NaOAc (pH 7.0)	---	---	300 mM
Tris/HCl (pH 7.5)	---	---	10 mM
TBE (1x)	0.01% (w/v)	---	---
Bromophenol blue	0.05% (w/v)	---	---
Xylenecyanole	0.05% (w/v)	---	---

2.2.3.13.2 Hybridization and cleavage

20 µg of total RNA from cell culture (see 2.2.3.6) were used for the experiments. Total RNA and 100,000 cpm of the radiolabeled probe were co-precipitated using a 2.5-fold excess of ethanol/0.1 M NaOAc. The samples were incubated at –20°C for 15 min and subsequently

centrifuged (15,000 g, 10 min). The supernatants were removed and the RNA/antisense probe pellet was resuspended carefully in 30 µl FAB hybridization buffer. Samples were subsequently denatured for 10 min at 85°C and hybridized overnight (42°C in a water bath). Following hybridization, samples were treated with 300 µl RNase T1/A-mix (RNase buffer supplemented with 10 µg of RNase A and 200 units of RNase T1) for 1 h at 30°C [Melton *et al*. 1984]. Under these conditions, every single mismatch was recognized by the RNases. RNases were inactivated by an addition of 6.6 µl SDS (10%) and 4.4 µl proteinase K (10 µg/ml) for 15 min at 42°C. The samples were extracted with phenol/chloroform and centrifuged (15,000 g, 2 min). Protected double-stranded RNA hybrids were precipitated using 880 µl of ethanol and 1.5 µl of tRNA (10 µg/µl). After centrifugation, (15,000 g, 15 min), the pellet was resuspended in 25 µl FLB 80 buffer, heated for 5 min at 95°C and loaded onto the gel.

2.2.3.13.3 Analytical gel electrophoresis and signal detection

The protected RNA fragments were separated on acrylamide/urea gels (5% acrylamide/bisacrylamide, 29:1; 8 M urea; 1x TBE). 1000 cpm of the radiolabeled antisense probe served as a size marker. Electrophoresis (300 V, 1x TBE buffer) was stopped after 60 min. The gel was fixed on Whatman 3MM paper and subsequently dried on a gel drying system (Bio Rad). The radiolabeled gel was exposed to a phosphoimager plate (BAS-MP 2040S, Fuji) and analyzed using a Fuji PhosphoImager BAS-1500.

2.2.3.14 Electrophoretic mobility shift assay (EMSA)

EMSA is used to investigate protein/dsDNA interaction using nuclear extracts. It is based on the binding of a particular protein providing a special 3D structure (e.g. transcription factor) to a specific radiolabeled dsDNA sequence (see GAS1 2.1.18.4). This labeled protein/dsDNA complex can be separated from non protein-bound dsDNA on a native acrylamide gel.

An additional proof for modified gel mobility of the dsDNA bound to protein is the socalled Super-Shift, which identificates the protein being involved in dsDNA binding. In this experimental set up an antibody is added to the reactionmix that specifically binds to the putative dsDNA bound protein. Does the shifting antibody successfully detects the protein/dsDNA complex a Super-Shift becomes visible which is characterized by a higher molecular weight compared to the single shift.

2.2.3.14.1 Nuclear extracts

Subconfluent cells on 10 cm dishes were stimulated for 0.5 – 2 h washed twice with cold PBS and scraped in ice cold 1.5 ml PBS/EDTA (0.1mM/pH8.0). All steps were performed strictly in cold enviroment at 4°C. The cells were pelleted by centrifugation in a pre-cooled centrifuge at 13000 rpm for 5 min. Afterwards the pellet was resuspended in 300 µl cold buffer A + 0.6% NP40 and incubated for 15 min by vigerous shaking at 4°C. Subsequently, nuclei and cell fragments were pelleted by centrifugation (13000 rpm, for 15 min at 4°C). The supernatant consists of cytosolic extract which can be investigated by western-blot analysis. In contrast the DNA-binding proteins are located in their active form in the nucleus. Therefore the pellet has to be resuspended in 70 µl ice cold buffer B containing protease inhibitor and incubated for additional 20 min by vigerous shaking at 4°C. Nuclei- and cellfragments were pelleted by centrifugation (13000 rpm, for 5 min 4°C). Finally, the supernatant contains the nuclear extract. Protein content was determined using the Nanoquant (Roth) protein assay. The protein solutions were aliquoted, shock frozen in liquid nitrogen and stored at –80°C until use.

2.2.3.14.2 Labeling of oligonucleotides

The identified GAS site (GAS1) in the active IL-18BP promoter fragments have been synthesized by MWG Biotech. Control consensus oligonucleotides for Stat1 p84/p91 sites (wildtype/mutated control) used in the binding reactions as additional competitors were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Sequences of all these double-stranded oligonucleotides are listed in 2.1.18.4. Complementary oligonucleotides were end labeled by T4 polynucleotide kinase using [γ-^{32}P]ATP (3000 Ci/mmol).

Oligo labeling:	2 µl	dsGAS1oligonucleotides (pgml/µl) **or** ds control consensus oligonucleotides
	2 µl	10x Polynucleotid Kinase Buffer
	1 µl	T4 Polynucleotidkinase
	5 µl	[γ-^{32}P] ATP
	10 µl	H_2O

The reaction mix was incubated for 1 h at 37°C. Afterwards the enzyme was heat-inactivated at 75°C for 10 min and slowly cooled down overnight. The labeled probe was cleaned up using Nick Columns (Amersham, Pharmacia) according to the manufacturer's protocol. The incorporated radioactivity of the freshly prepared radiolabeled probe was determined using a β-counting device (TRI-CARB 2100 TR, Canberra-Packard).

2.2.3.14.3 Binding reaction, analytical gel electrophoresis and signal detection

Binding reactions were performed for 40 min on ice with 10 μg of protein in 20 μl of binding buffer containing 4% Ficoll, 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.25 mg/ml BSA, 2 μg of poly(dI-dC), and 10,000 - 15,000 cpm of ^{32}P-labeled ds oligonucleotide. For Stat1 Super-Shift analysis nuclear proteins were preincubated for 40 minutes at room temperature with the monoclonal AB Stat1 9H2 (mouse; Cell Signaling, distributed by NEB, Frankfurt a.M.). DNA-protein complexes were separated from unbound oligonucleotide by electrophoresis through a 4% polyacrylamid gels using 0.5x TBE buffer.

Solution components	Component volumes (ml) per 36 ml gel		
	4%	6%	8%
H_2O	28.3	27	24.1
Acrylamide mix (30%/0.8%)	5.4	7.2	9.6
10 x TBE	1.8	1.8	1.8
APS (10%)	0.4	0.4	0.4
TEMED	0.3	0.3	0.3

Thereafter, gels were fixed and analyzed by PhosphoImager analysis (Fuji). Competition experiments were performed by coincubation with a 100-fold excess (20 pmol) of unlabeled double-stranded oligonucleotides (GAS1 and wildtype/mutated control oligonucleotides by Santa Cruz) in the DNA-protein binding reaction.

2.2.3.14.4 Cloning of IL-18BP promoter fragments

Synthesis of the different IL-18BP promoter fragments was carried out by PCR (2.2.3.2) using genomic DNA template isolated from DLD-1 cells (DNAzol, MRC). In order to generate artificial restriction sites for directed ligation to the target vector pGL3, mutated oligonucleotides (2.1.18.1) were constructed. Forward primer were supplied with a MluI site, whereas reverse primer were modified by generating a BglII site. The maximal amplificated IL-18BP promoter fragment (pGL3 + IL-18BProm Frag4) contains a genomic region of 1114 bp (nucleotides 2405990 ⇒ 2407104, see 2.1.15.3).

2.2.4 Biochemical Methods

2.2.4.1 Preparation of cell lysates

Cells were grown and stimulated as described above (2.2.2.1). For harvesting, cells were washed twice with ice-cold PBS. Last traces of PBS were removed by a pipette tip attached to a vacuum line. DLD-1 cells were treated with Triton X-100 lysis-buffer (300 mM NaCl, 50 mM TrisHCl, pH 7.6, 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Roche Biochemicals). To remove cellular debris, probes were centrifuged (15,000 g, 20 min at 4°C). Finally the supernatants were stored at –80°C until use. Protein content was determined using the Roth Nanoquant Protein Assay.

2.2.4.2 Trichloroacetic acid (TCA) precipitation

This method was used to concentrate proteins from a defined volume of cell culture supernatant (without serum!) for Western blot analysis. Cell culture supernatants were cleared from cellular contaminations using a single centrifugation step (1000 rpm, 5 min at 4°C; Heraeus Megafuge 1.0, rotor 7570F). Subsequently, 0.1 volume of 70% trichloroacetic acid (TCA) was added to 1 ml of conditioned cell culture supernatant, mixed and incubated for 30 min on ice. TCA-precipitated proteins were concentrated by centrifugation (15,000 g, 30 min, 4°C). The protein pellet was washed with 200 µl of ice-cold acetone, again centrifuged for 5 min at 15,000 g and finally resuspended in 1:4 Roti Load1 (Roth). After neutralization (1 µl of 1 M Tris/HCl, pH 8.5), the samples were ready to use for Western blot analysis. Their protein content was normalized by Ponceau S staining.

2.2.4.3 Determination of protein concentration

The amount of protein in cellular lysates was determined using the Roth Nanoquant Protein Assay (Bradford method). 50 µl of the samples (1:5 - 1:200 prediluted in Triton X-100 lysis-buffer) were pipetted in duplicate into appropriate wells of a 96-well ELISA plate. Different BSA concentrations (20-100 µg/ml) were used as a standard. 200 µl of Roti Nanoquant (Roth, 1:5 diluted in $Aqua_{dest}$) were added to each well. After 10 min of incubation, the optical density was measured at a wavelength of 595 nm and reference wavelength 450 nm using a microplate reader (Bio Rad). The absorption values were calculated using the Microplate Manager 4.0 software (Bio Rad).

2.2.4.4 Western blot analysis

The Western blot technique represents a sensitive method to detect specific polypeptides within a complex mixture of proteins. Proteins are separated electrophoretically and transferred to a membrane, which is subsequently incubated with antibodies specific for the protein of interest. Finally, the bound antibody is recognized by a second anti-immunoglobulin that is coupled to horseradish peroxidase or alkaline phosphatase. The detection limit of this method ranges between 1-5 ng of an average-sized protein using the ECL-Detection Kit by Amersham Pharmacia (Freiburg).

Buffer components	Laemmli buffer (4x)	Electrophoresis buffer (1x)	Transfer buffer (1x)	TBST buffer (10x)
Tris/HCl	125 mM (pH 6.8)	25 mM	25 mM	100 mM (pH 8.0)
SDS	10% (w/v)	0.1% (w/v)	---	---
Glycine	---	250 mM	192 mM	---
Dithiothreitol	50 mM	---	---	---
Bromophenol blue	0.01% (w/v)	---	---	---
Glycerol	30% (v/v)	---	---	---
Methanol	---	---	20% (v/v)	---
Tween 20	---	---	---	5% (v/v)
NaCl	---	---	---	1.5 M

2.2.4.4.1 SDS gel electrophoresis

Electrophoretic separation of proteins was carried out in the discontinuous buffer system for SDS polyacrylamide gels as originally described by Laemmli [1970]. For detection of intracellular proteins 20 - 100 µg of total protein were dissolved in 4x Roti Load1 (Roth). However, for detection of TCA precipitated proteins the whole supernatants were used. After heating for 5 min at 95°C, samples were loaded onto a mini gel. Subsequently, it was run at a current of 20 mA for a period of 1 - 2 hours.

Resolving gel:	Component volumes (ml) per 20 ml gel		
	6% (> 120 kDa)	**10%** (60-120kDa)	**12%** (< 60 kDa)
H_2O	10.6	7.9	6.6
Acrylamide mix (30%)	4.0	6.7	8.0
Tris/HCl (1.5 M, pH 8.8)	5.0	5.0	5.0
SDS (10%)	0.2	0.2	0.2
APS (10%)	0.2	0.2	0.2
TEMED	0.016	0.008	0.008

Stacking gel :	Component volumes (ml) per gel volume of		
	2 ml	**5 ml**	**10 ml**
H_2O	1.4	3.4	6.8
Acrylamide mix (30%)	0.33	0.83	1.7
Tris/HCl (1.5 M, pH 6.8)	0.25	0.63	1.25
SDS (10%)	0.02	0.05	0.1
Ammonium persulfate (10%)	0.02	0.05	0.1
TEMED	0.002	0.005	0.01

2.2.4.4.2 Transfer to PVDF membrane

After gel electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting (Hoefer SemiPhor, Amersham Pharmacia). Prior to use, the PVDF membrane was activated in isopropanol for 15 s and subsequently rinsed in deionized water for additional 2 min. Three pieces Blotting PAPER (Sigma) were soaked in transfer buffer and positioned on the anode side of the transfer apparatus. The PVDF membrane was placed directly on the stack of blotting paper. The SDS gel containing the separated proteins was taken off the glasplates, rinsed shortly in transfer buffer, and placed on the top of the PVDF membrane. Finally, the gel was covered with three additional, transfer buffer-soaked Blotting PAPER. Air bubbles were squeezed out by a roller apparatus. The upper electrode (cathode) was positioned on the top of the stack and a current of 0.8 mA/cm^2 (nearly 60 mA per mini gel) was applied. Transfer of proteins was carried out at room temperature and terminated after 75 min. After blotting, the membrane was checked by Ponceau S staining for correct electrophoretic transfer and equal loading. The successful protein transfer can also controlled by staining the blotted SDS gel with Coomassie-Brilliant-Blue (2.5 mg/ml Coomassie-Brilliant-Blue G250, 45% Methanol, 45% H_2O and 10% acetic acid).

2.2.4.4.3 Immunodetection

After blotting the PVDF membrane was directly shaked in a TBST-buffered non-fat skim milk solution (10%) for 1 h at room temperature (or overnight at 4°C) to block non-specific binding sites. The membrane was subsequently exposed to primary antibodies (diluted 1:500 - 1:2000 in 1x TBST buffer) specific for the protein of interest and incubated overnight at 4°C. The blot was washed four times for 10 min in 1x TBST. Specific binding of primary antibody was detected by incubation of the membrane with a secondary antibody

coupled to horseradish peroxidase (diluted 1:10,000 in 1x TBST) for 2 h at room temperature. For detection of the corresponding bands, we used the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia) according to the instructions of the manufacturer. The membrane was exposed to a special ECL film (Amersham Pharmacia) and developed (Hyperprocessor, Amersham Pharmacia). Developed films were scanned (GS 700 Imaging Densitometer, Bio Rad) and analyzed using the Molecular Analyst software from Bio Rad.

2.2.4.5 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) technique is the most sensitive method to specifically determine protein concentrations from different sources. Sensitivity is approximately 100-fold increased compared to the Western blot technique. The ELISA was performed according to the instructions of the manufacturers' (R&D Systems and PharMingen). Briefly, microtiter plates were coated with a monoclonal antibody or a specific receptor. Equal volumes of samples were pipetted into the wells of the microtiter plate and incubated for 2 h. Subsequently, wells were washed. An enzyme-linked antibody specific for the target protein was added for additional 2 h. The wells were subsequently washed for three times and afterwards incubated with substrate solution. The color reaction develops in direct proportion to the amount of bound antigen. The optical densities were calculated using the Microplate Manager 4.0 software from Bio Rad.

Used ELISAs:	• human IFNγ (PharMingen)
	• human IL-8 (PharMingen)
	• human TGFβ1 (R&D Systems)
	• human TNFα (PharMingen)

III
Results

3.1 Expression and release of IL-18BPa in response to IFN-γ

Colon epithelial cells have been identified as a potential source of IL-18. Its expression is upregulated not only during the course of *Morbus Crohn* [Takeuchi *et al.*, 1999; Pizarro *et al.*, 1999], but also in other inflammatory diseases such as *rheumatoid arthritis* [Dinarello *et al.*, 1998; Gracie *et al.*, 1999] and *psoriasis* [Ohta *et al.*, 2001]. Furthermore, IL-18 appears to be modulated in colorectal cancer [Pages *et al.*, 1999]. We therefore intended to investigate the expression of its natural occuring inhibitor IL-18BPa in different colon carcinoma cell lines and organ cultures of colonic intestinal biopsy specimens. Moreover, HaCaT keratinocytes as well as synovial fibroblasts isolated from rheumatoid arthritis patients were investigated.

3.1.1 IFNγ induces expression and release of IL-18BPa in the colon carcinoma cell lines DLD-1 and Caco-2, as well as in HaCaT keratinocytes

We recently reported on induction of IL-18BPa gene expression by IFN-γ in human nonleukocytic cells [Mühl *et al.*, 2000]. In the present study we focussed on the colon carcinoma/epithelial cell line DLD-1 and sought to extend the abovementioned observations by including the level of IL-18BPa protein release and function. IFN-γ-induced IL-18BPa mRNA (Fig.1A, lower panel) was paralleled by secretion of the corresponding protein as detected by immunoblotting analysis of TCA-precipitated cell culture supernatants (Fig. 1A and B). IL-18BPa appeared as IFN-γ-inducible immunoreactivity in the expected molecular weight range between 40 kD and 50 kD [Novick *et al.*, 1999; Kim *et al.*, 2000]. This heterogeneity in the molecular weight agrees with a high degree of glycosylation as has been reported (Novick *et al.*, 1999). Strong immunoreactivity was observed when human IL-18BPa was transiently expressed in DLD-1 cells (Fig. 2A). In contrast, control transfection with empty vector did not result in any immunoreactivity (data not shown). IL-18BPa immunoreactivity induced by IFN-γ consisted of two major bands. This was particularly evident when lower amounts of proteins were separated on a 12% SDS-PAGE (Fig. 1A, Fig. 2B: lane 2). The doublet was also observed when lower amounts of IL-18BPa transiently expressed in DLD-1 cells were subjected to SDS-PAGE (data not

shown). Addition of immunizing peptide to the antiserum impaired immunodetection of IL-18BPa (Fig. 2C). No immunoreactivity was detectable when preimmune serum was used (data not shown). To exclude that effects of IFNγ were due to endotoxin which is heat stable, IFNγ was heat-inactivated by boiling (99 ^{o}C, 30 minutes) before use. Immunoreactivity disappeared after heat-inactivation of IFNγ (Fig. 2D). In addition, LPS (10 μg/ml) did not induce IL-18BPa in DLD-1 cells (data not shown). IL-18BP is synthesized with a 28 residue signal peptide and is supposed to be secreted via the endoplasmatic reticulum/Golgi pathway [Novick *et al.*, 1999]. Accordingly, Brefeldin A abrogated release of IL-18BPa (Fig. 2E). However, despite readily detectable immunoreactivity of IL-18BPa in supernatants of IFN-γ-treated cells, we could not demonstrate intracellular IL-18BPa by immunoblotting when whole cell lysates were analyzed from IFNγ-treated cells (Fig. 2F) or from cells treated with IFN-γ/Bref A (data not shown). This discrepancy between cell lysates and TCA-precipitated supernatants is likely to be due to the huge enrichment factor immanent to the method of TCA precipitation.

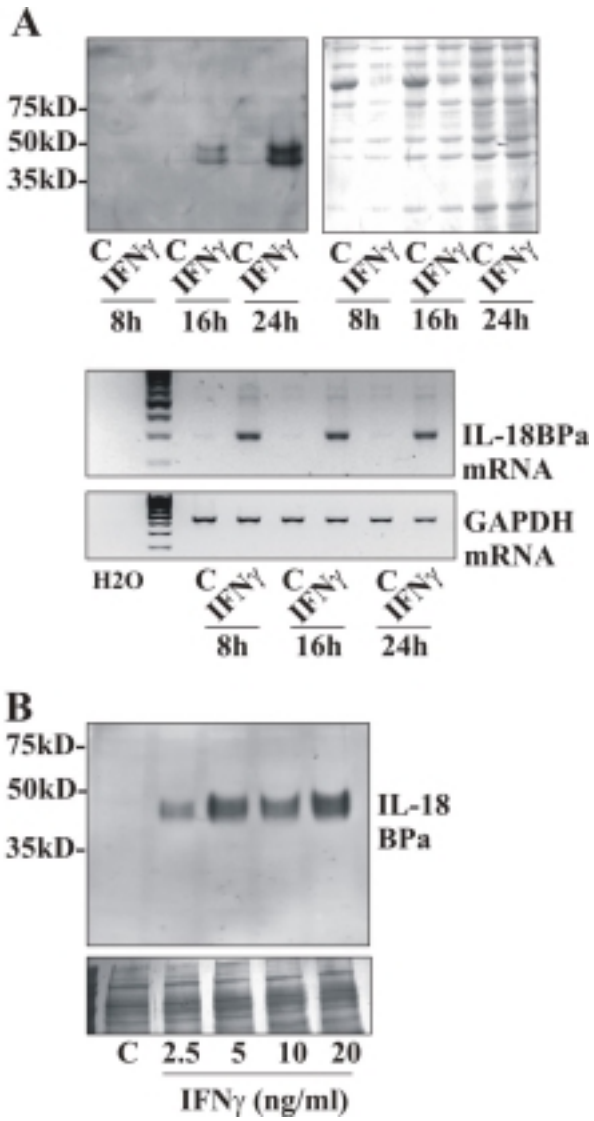


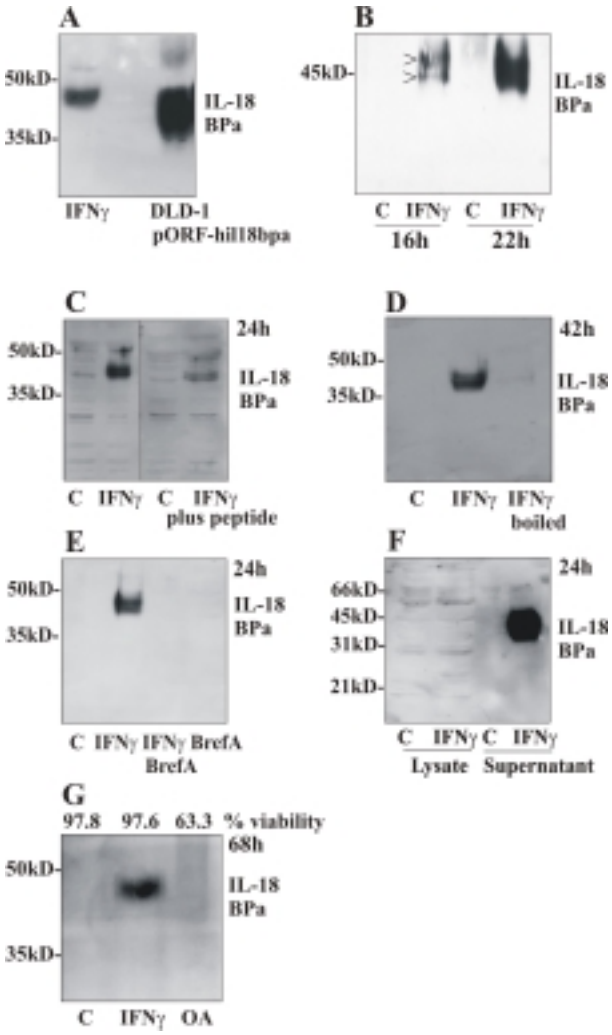
Figure 1. **Time course and dose-response analysis of IFN-γ-induced IL-18BPa.**

(A) DLD-1 cells were kept as controls or stimulated with IFN-γ (20 ng/ml) for the indicated time periods. TCA-precipitated supernatants were analyzed by immunoblotting. Lower panel: IL-18BPa mRNA was evaluated by RT-PCR in these same experiments. One representative of four independently performed experiments is shown.

(B) DLD-1 cells were kept as controls or stimulated for 24h with the indicated concentrations of IFN-γ. TCA-precipitated supernatants were analyzed by immunoblotting. Membranes were stained by Ponceau S (A&B).

Figure 2. IL-18BPa immunoreactivity as detected using a polyclonal antiserum.

(A) DLD-1 cells were transiently transfected with pORF-hil18bpa. TCA-precipitated supernatants of IFN-γ-stimulated (20 ng/ml) cells were run in parallel.
(B) DLD-1 cells were stimulated with IFNγ (50 ng/ml) for the indicated times periods. TCA-precipitated supernatants were analyzed by immunoblotting. Arrows indicate IL-18BPa immunoreactivity (second lane).
(C) DLD-1 cells were kept as controls or stimulated with IFN-γ (20 ng/ml) for 24h. TCA-precipitated supernatants were analyzed by immunoblotting. One half of the blot was stained with IL-18BPa antiserum, the other with IL-18BPa antiserum plus immunizing peptide (2.5 μg/ml).
(D) DLD-1 cells were kept as controls, stimulated with IFN-γ (20 ng/ml), or with IFN-γ (20 ng/ml) which had been inactivated by boiling. After 42h, TCA-precipitated supernatants were analyzed by immunoblotting.
(E) DLD-1 cells were kept as controls, stimulated with IFN-γ (20 ng/ml) alone, with Bref A (1 μg/ml), or with IFN-γ (20 ng/ml)/Bref A (1 μg/ml). After 24h, TCA-precipitated supernatants were analyzed by immunoblotting. Similar results were obtained using Bref A at 0.25 μg/ml (data not shown).
(F) DLD-1 cells were kept as controls or stimulated with IFN-γ (20 ng/ml) for 24h. TCA-precipitated supernatant proteins and whole cell lysates were prepared and analyzed by immunoblotting.
(G) DLD-1 cells were kept as controls, stimulated with IFN-γ (20 ng/ml), or exposed to okadaic acid (OA) (50 nM) for 68h. TCA-precipitated supernatants were analyzed by immunoblotting. Cell viability was determined in these same experiments by LDH activity analysis.



There was no induction of cytotoxicity in IFN-γ-treated cultures as examined by determination of LDH activity in culture supernatants: viability was 98.3 ± 0.4% versus 97.5 ± 0.4% for control versus IFNγ-treated cells (20 ng/ml, 42h, n = 3). Ponceau S staining of membranes after blotting revealed no differences in the total amounts of proteins loaded onto the gels (Fig. 1A and B). Moreover, induction of cell death by okadaic acid did not result in release of immunoreactivity, despite a 36.7% loss of cell viability (Fig. 2G). Altogether, these observations argue against IFN-γ-induced cytotoxicity with passive release of cellular proteins as the driving force behind IL-18BPa immunoreactivity in TCA-precipitated supernatants.

To investigate whether IFNγ-induced secretion of IL-18BPa is restricted to DLD-1 cells or is of more general relevance, experiments were performed using additional colon carcinoma/epithelial cell lines. As shown in Fig. 3, IFN-γ-induced IL-18BPa mRNA accumulation (A and B, left panel) was associated with secreted IL-18BPa immunoreactivity (A and B, right panel) in the respective culture supernatants of LoVo (A)

and Caco-2 (B) colon carcinoma cells. Similar data were obtained using HCT116 colon carcinoma cells (data not shown). We also investigated the keratinocyte cell line HaCaT (Fig. 3C). Induction of IL-18BPa mRNA (left panel) was paralleled by appearance of IL-18BPa in IFN-γ-conditioned culture supernatants (right panel).

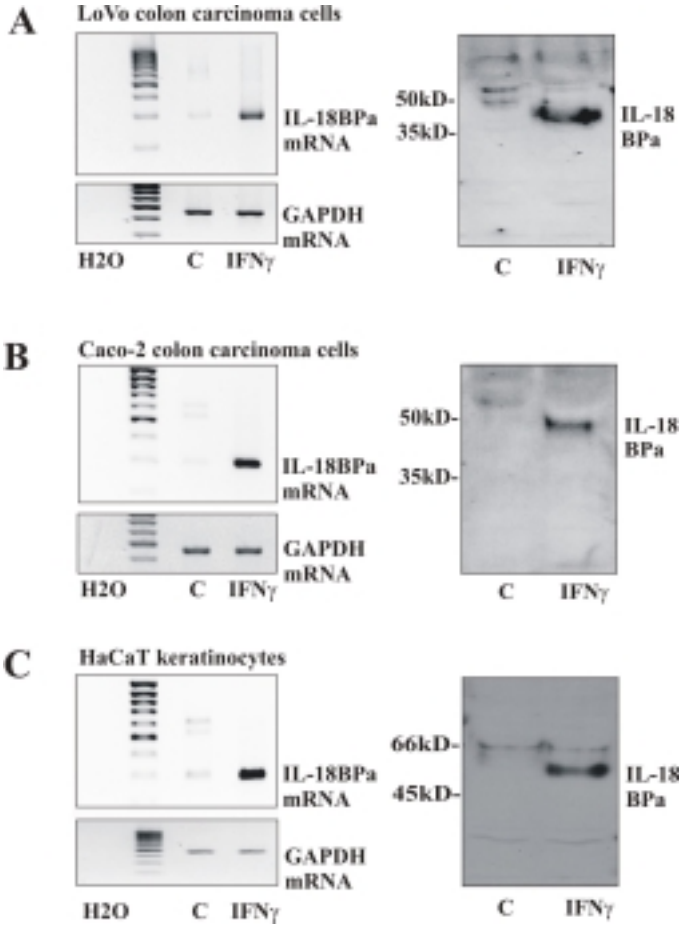


Figure 3. IFN-γ-induced IL-18BPa in LoVo and Caco-2 colon carcinoma cells, as well as in HaCaT keratinocytes.

LoVo cells **(A)**, Caco-2 cells **(B)**, or HaCaT keratinocytes **(C)** were kept as unstimulated controls or incubated with IFN-γ (20 ng/ml). After 24h, IL-18BPa mRNA was evaluated by RT-PCR (left panel) and TCA-precipitated supernatants were analyzed by immunoblotting (right panel). One representative of two independently performed experiments is shown for each cell line.

3.1.2 Sodium butyrate inhibits IFN-γ-induced IL-18BPa in colon carcinoma cell lines cell lines

Butyrate is a short-chain fatty acid that is produced by intestinal bacteria and is supposed to be an important regulator of colonic epithelial cell biology [Wächtersheimer and Stein, 2000]. Sodium butyrate (B) at 5 mM efficiently suppressed IFN-γ-induced IL-18BPa protein release as well as mRNA induction (Fig. 4A and B). It is important to take into account that peak concentrations of butyrate in the colon can reach 20 mM [Wächtersheimer and Stein, 2000]. Sodium butyrate alone did not change background expression of IL-18BPa. Biological activity of IL-18 is supposed to be determined by local concentrations of IL-18 versus IL-18BP. Therefore, we determined the effect of sodium butyrate on expression of IL-18 in these same experiments. Notably, sodium butyrate (5 mM) did not change IL-18

expression in DLD-1 cells exposed to IFN-γ (Fig. 4C). Sodium butyrate is supposed to trigger apoptosis in colon carcinoma cells [Wächtersheimer and Stein, 2000]. Thus, cell viability was determined in these experiments. Sodium butyrate at 5 mM alone or in combination with IFN-γ did not modulate cell viability in DLD-1 cells during a 24h incubation period (Fig. 4D). In contrast, cell death was detectable when DLD-1 cells were incubated for 48h with sodium butyrate at 25 mM. Again, cell death was not associated with appearence of IL-18BPa immunoreactivity in these supernatants. Inhibition of IFN-γ-induced IL-18BPa expression by sodium butyrate was also observed in the colon carcinoma cell lines HCT116 (Fig. 4E), LoVo, and Caco-2 (data not shown).

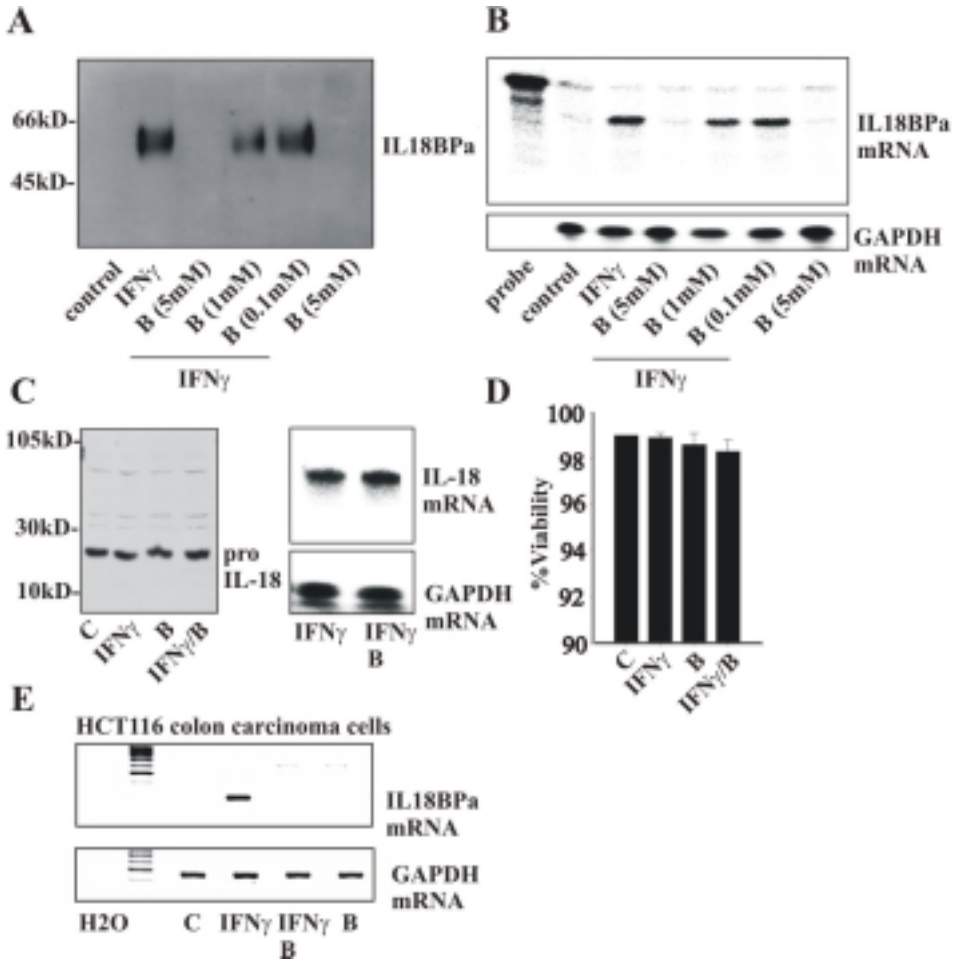


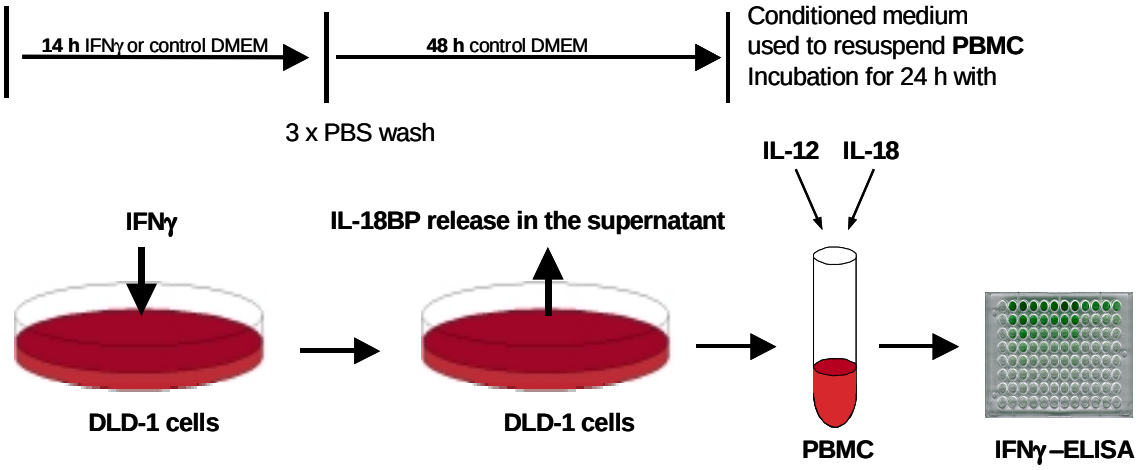
Figure 4. Sodium butyrate suppresses IFN-γ-induced IL-18BPa expression, but not expression of IL-18.

DLD-1 cells were kept as controls, stimulated with sodium butyrate (B) (5 mM), with IFN-γ (20 ng/ml), or with IFN-γ (20 ng/ml) plus the indicated concentrations of sodium butyrate. After 24h, TCA-precipitated supernatants were analyzed by immunoblotting **(A)** and IL-18BPa mRNA was evaluated by RNase protection assay **(B)**. One representative of three independently performed experiments is shown. **(C)** DLD-1 cells were kept as control, stimulated with IFN-γ (20 ng/ml), with sodium butyrate (5 mM), or with IFNγ (20 ng/ml)/sodium butyrate (5 mM). After 24h, cell-lysates were analyzed for IL-18 by immunoblotting. IL-18 mRNA expression was evaluated by RNase protection assay. One representative of three independently performed experiments is shown. **(D)** In these experiments viability was determined by LDH activity analysis. % viability ± SD is shown (n = 3). **(E)** HCT116 colon carcinoma cells were kept as unstimulated control, or stimulated with IFN-γ (20 ng/ml), with sodium butyrate (5 mM), or with the combination IFN-γ plus sodium butyrate. After 24h, IL-18BPa mRNA expression was evaluated by RT-PCR. One representative of two independently performed experiments is shown.

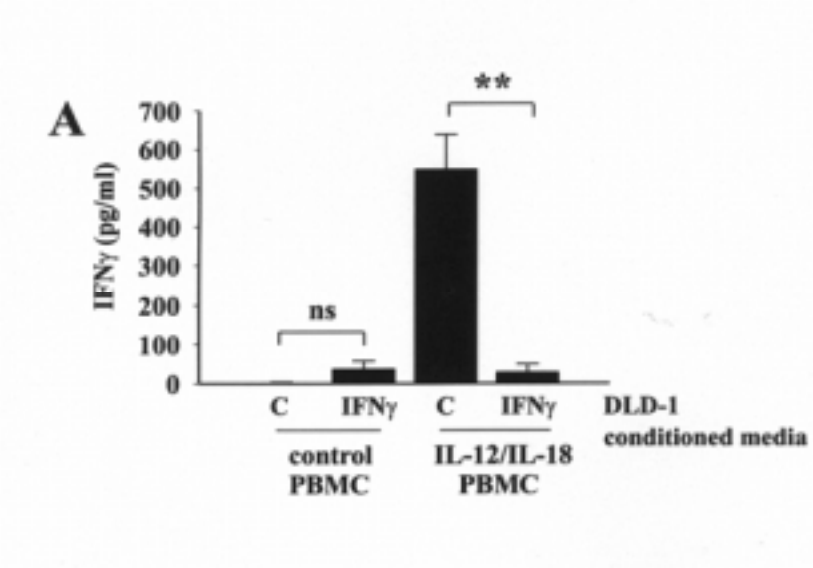
3.1.3 IFNγ-stimulated DLD-1 cells release an activity which impairs IFN-γ production by IL-12/IL-18-stimulated PBMC

As shown in Fig. 5A2, compared to conditioned media obtained from unstimulated cells, conditioned media from IFN-γ-stimulated DLD-1 cells impaired production of IFN-γ in PBMC exposed to IL-12/IL-18. To confirm that IL-18BPa is actually detectable in these conditioned media, immunoblot analysis was performed. As shown in Fig. 5B, a 14 h stimulation with IFN-γ was sufficient to trigger detectable release of IL-18BPa during an additional 48 h incubation period in control medium. Notably, in these DLD-1 cells augmented levels of IL-18BPa mRNA were still detectable after this 48 h incubation in control medium. This is consistant with a long half-life (> 8 h) of IFN-γ-induced IL-18BPa mRNA in DLD-1 cells as detected in actinomycin D experiments (data not shown). We also investigated effects of sodium butyrate using this experimental protocol. However, conditioned media from DLD-1 cells stimulated with sodium butyrate alone significantly reduced later IFN-γ production of IL-12/IL-18-activated PBMC by 60.1 ± 12.4 % (n = 3, $p < 0.05$). This indirect inhibitory effect of sodium butyrate interfered with efficient recovery of IL-12/IL-18-induced IFN-γ in PBMC by use of conditioned media from DLD-1 cells exposed to the combination IFN-γ plus sodium butyrate (data not shown).

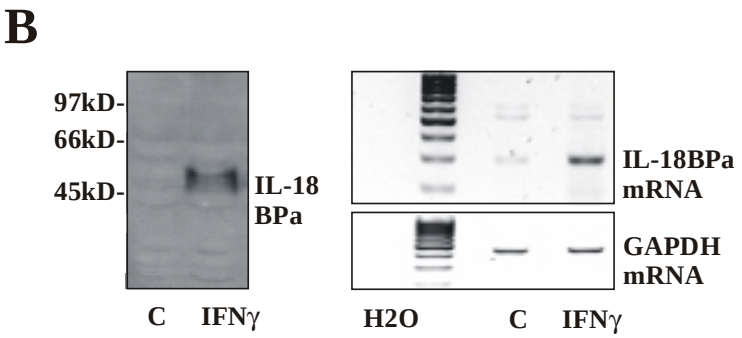
Figure 5. **IFN-γ-stimulated DLD-1 cells release an activity which impairs IFN-γ production by IL-12/IL-18-stimulated PBMC.**



(A1) Scheme of experimental design: Control conditioned and IFN-γ-conditioned media from DLD-1 cell cultures (generated as outlined in the *materials and methods* section) were used to resuspend control PBMC and IL-12 (20 ng/ml)/IL-18 (20 ng/ml)-stimulated PBMC, respectively. After a 24 h incubation period, IFN-γ production was determined by ELISA.



(A2) Data are mean ± SEM (n = 3); **p < 0.01 versus use of conditioned media from IFN-γ-treated DLD-1 cells.



(B) Control conditioned and IFN-γ-conditioned media from DLD-1 cell cultures (generated as outlined in the *materials and methods* section) were TCA-precipitated and analyzed for IL-18BPa expression by immunoblotting. IL-18BPa mRNA of this same experiment was evaluated by RT-PCR analysis.

3.1.4 IFN-γ mediates gene expression of IL-18BPa in organ cultures of colonic intestinal biopsy specimens

Colonic intestinal biopsy specimens obtained from five different donors were cultivated as unstimulated control, or exposed to either IFN-γ alone, or the combination IFN-γ/sodium butyrate. Upregulation of IL-18BPa mRNA by IFN-γ was observed after an 8 h incubation period (Fig. 6). Transcripts remained elevated for at least another 15 h of stimulation (data not shown). In accord with data obtained using colon carcinoma cell lines, sodium butyrate suppressed IFN-γ-induced IL-18BPa expression in all organ culture experiments performed (n = 5). In contrast, expression of IL-18 mRNA in these same IFN-γ-treated organ cultures was not modulated by coincubation with sodium butyrate (Fig. 6).

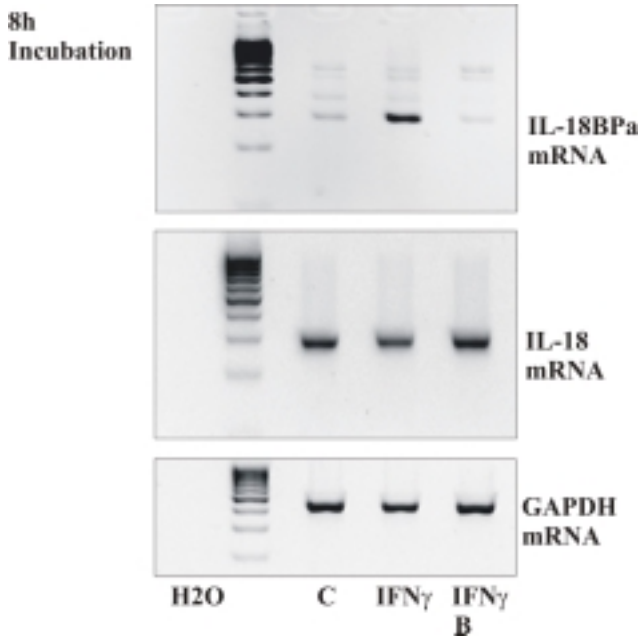


Figure 6. Induction of IL-18BPa by IFN-γ in cultures of colonic biopsy specimens: modulation by sodium butyrate.

Cultures of colonic biopsy specimens from five different donors were kept as unstimulated control, or stimulated with IFN-γ (40 ng/ml) alone, or in combination with sodium butyrate (B) (10 mM) for different time periods (8h, 16h, 22h). Thereafter, IL-18BPa and IL-18 mRNA expression was evaluated by RT-PCR. No differences dependent on the duration of stimulation (8h, 16h, 22h incubation) were observed. One representative experiment is shown (8h stimulation).

3.1.5 IFN-γ induces expression of IL-18 binding protein in fibroblast-like synoviocytes

3.1.5.1 Detection of IL-18BPa by immunoblotting

IFN-γ induces IL-18BPa mRNA expression in rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS). This induction was paralleled by secretion of the corresponding protein as detected by immunoblotting analysis of TCA-precipitated cell culture supernatants (Fig. 7A). Immunoreactivity appeared with a molecular mass of about 45 kDa, which is in keeping with previous reports (Kim *et al.*, 2000).

3.1.5.2 IFN-γ production by IL12/IL-18-stimulated PBMC cultivated in RA-FLS-derived conditioned medium

To investigate whether conditioned media from IFN-γ-stimulated RA-FLS may contain IL-18BPa activity, RA-FLS were kept as control or stimulated with IFN-γ (20 ng/ml, 16 h). These conditioned media were used to resuspend control PBMC and IL-12/IL-18 stimulated PBMC, respectivley. After a 24 h incubation period, IFN-γ production was determined by ELISA. Compared to conditioned media obtained from unstimulated cells conditioned media from IFN-γ-stimulated RA-FLS showed reduced production of IFN-γ in PBMC exposed to IL-12/IL-18 (Fig. 7B).

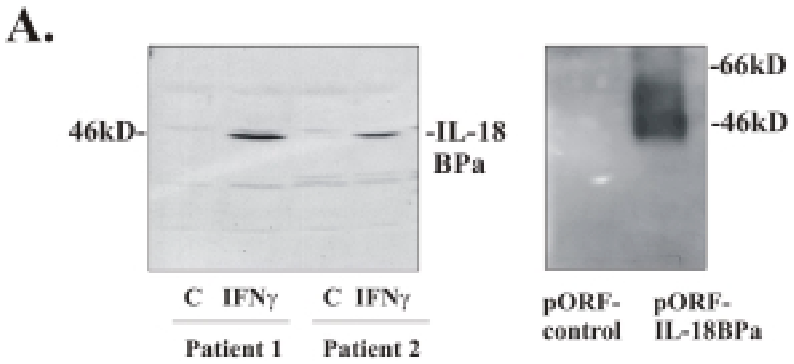
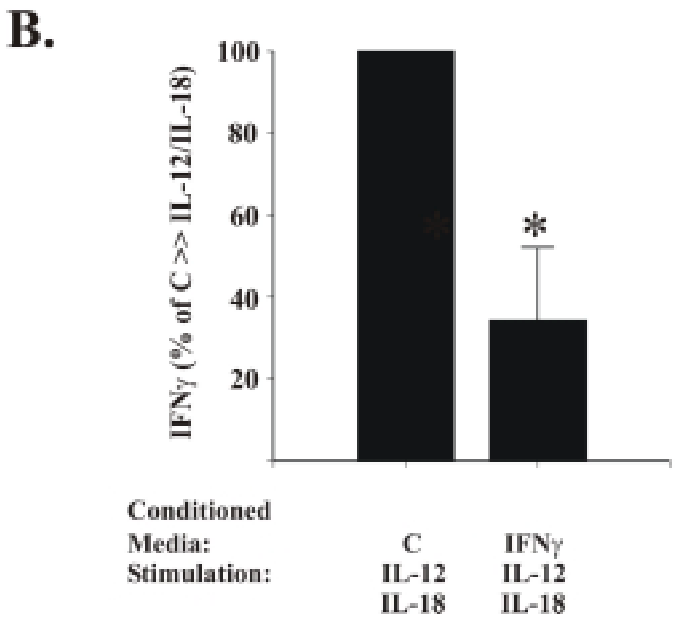


Figure 7. IFN-γ mediates release of IL-18BPa from FLS.

(A) RA-FLS cultures from RA patients were kept as unstimulated controls or stimulated with IFN-γ (5 ng/ml). After 48 h, secreted IL-18BPa was detected by immunoblotting.
Representative results from RA-FLS cultures of two patients are shown. The right figure shows over-expressed and TCA precipitated IL-18BPa as positiv detection control.



(B) Control- and IFN-γ-conditioned media from RA-FLS were used to resuspend control or IL-12/IL-18-PBMC cultures (compare method section 2.2.2.5). IFN-γ production was determined after 24 h by ELISA. Data are expressed (as % of IFN-γ production ± SEM) by IL12/IL-18-stimulated PBMC re-suspended in control conditioned RA-FLS medium (n=4).

3.2 Cell-to-cell crosscommunication during T_H1 responses mediates coordinated expression of modulatory IL-18 binding protein and inducible NO synthase in adjacent local resident cells

IL-18, IL-18 binding protein (IL-18BP), and inducible nitric oxide synthase (iNOS) are parameters that mediate important functions in the context of inflammation, infection, and cancer. Here we performed coculture studies in order to simulate a T_H1 response. In this experimental set up we investigated IL-18BP and iNOS gene induction in human DLD-1 colon carcinoma/epithelial cells cocultured with IL-12/IL-18-activated PBMC (Fig. 8).

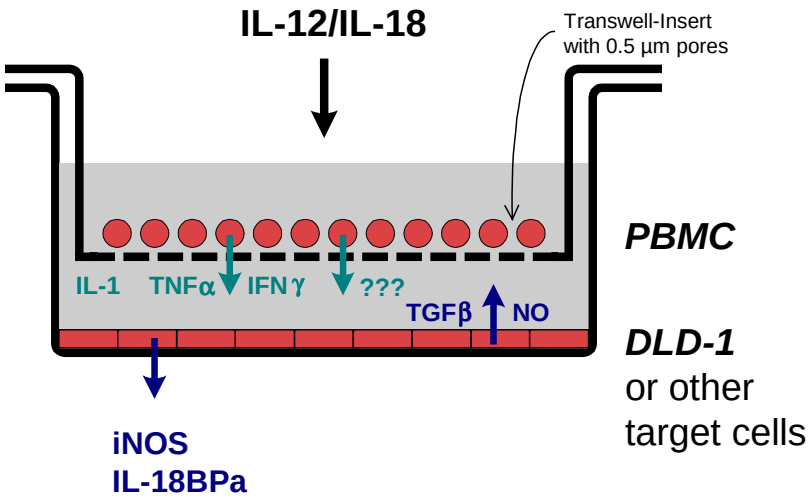


Figure 8. Schematic representation of the indirect transwell-coculture system.

3.2.1 Induction of IL-18BPa in DLD-1 cells cocultured with PBMC

Recently, we reported that stimulation of DLD-1 cells with exogenous IFN-γ mediates expression and release of IL-18BPa. Induction of IL-18BPa correlated with release of an activity from DLD-1 cells that impaired IL-12/IL-18-induced release of IFN-γ from PBMC [Mühl *et al.*, 2000; Paulukat *et al.*, 2001]. That observation appears to be of general relevance as this regulatory pathway has been observed in various different cell types. In the present study, we investigated whether IL-12/IL-18 can induce IL-18BPa in DLD-1 cells in our transwell-coculture system. In contrast to IFN-γ, IL-12/IL-18 were unable to stimulate expression of IL-18BPa in DLD-1 cells directly (Fig. 9A). However, in parallel to iNOS and production of IFN-γ, IL-18BPa was induced by IL-12/IL-18 in DLD-1 cells cocultured with PBMC. Again in accord with data on iNOS, use of neutralizing antibodies revealed that production of IFN-γ by PBMC was a prerequisite for expression of IL-18BPa (Fig. 9B). IL-18BPa mRNA accumulation in DLD-1 cells was associated with appearance of IFN-γ-inducible IL-18BPa immunoreactivity as detected by immunoblot analysis of TCA-precipitated coculture supernatants (Fig. 9C). We also compared inducibility of IL-18BPa expression by IFNγ in PBMC and DLD-1 cells using quantitative real-time PCR. As shown in Fig. 10, IL-18BPa mRNA was inducible by IFN-γ in PBMC. However, induction was far more pronounced in DLD-1 cells compared to PBMC (21 ± 5.5 versus 3.3 ± 1.1 fold induction relative to GAPDH and compared to unstimulated control). A 21-fold induction in DLD-1 cells is in keeping with previous data on IFN-γ-induced IL-18BPa in these cells as detected by RNAse protection assay [Mühl *et al.*, 2000].

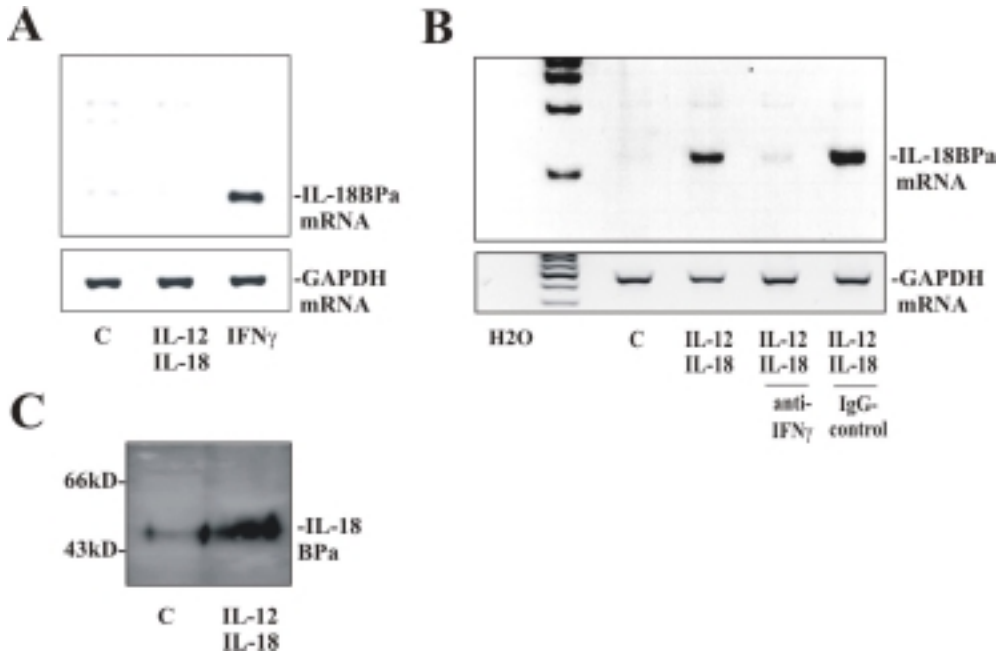


Figure 9. IL-12/IL-18 mediate IFN-γ-dependent induction of IL-18BPa in DLD-1 cells cocultured with PBMC. (A) DLD-1 cells were stimulated with IL-12 (20 ng/ml)/IL-18(50 ng/ml) or with IFN-γ (20 ng/ml) for 24h. Thereafter, mRNA expression of IL-18BPa was investigated by RT-PCR. One representative of three independently performed experiments is shown. **(B)** Indirect cocultures (transwell-assay) were kept as unstimulated controls or stimulated with IL-12 (25 ng/ml)/IL-18 (80 ng/ml) in the presence or absence of anti-IFN-γ antibody (20 μg/ml) or control IgG (20 μg/ml). After 45h, DLD-1 cells were lysed and iNOS mRNA expression was analyzed by RT-PCR. One representative RT-PCR of two independently performed experiments is shown. **(C)** Indirect cocultures (transwell-assay) were kept as unstimulated controls or stimulated with IL-12 (20 ng/ml)/IL-18 (100 ng/ml) for 60h. Thereafter, TCA-precipitated supernatants were evaluated for IL-18BPa content by immunoblotting analysis. One representative of three independently performed blots is shown.

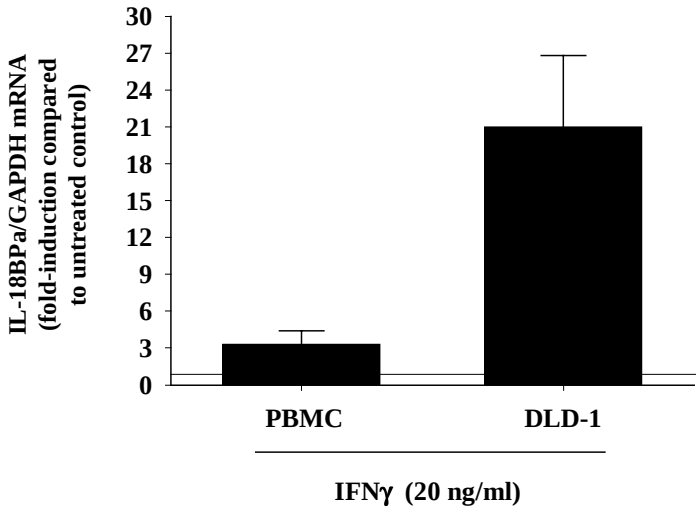


Figure 10. IFN-γ-induced IL-18BPa in PBMC and DLD-1 cells as detected by quantitative real-time PCR.

PBMC or DLD-1 cells were kept as unstimulated control or stimulated with IFN-γ (20 ng/ml). After 21 h cells were harvested and total RNA was isolated. Expression of IL-18BPa transcripts was assessed by quantitativ real-time PCR. Data are expressed relative to GAPDH and as fold-induction ± SEM compared to unstimulated control (n = 3).

3.2.2 Induction of iNOS in DLD-1 cells cocultured with PBMC

In accord with previous data [Salzman *et al.*, 1996], we confirm that activated DLD-1 cells express iNOS. Immunoblotting analysis (Fig. 11A) and RT-PCR (Fig. 11B) analysis revealed that IFN-γ alone is sufficient to trigger iNOS induction. Coincubation with IL-1β/IFN-γ resulted in a marked synergistic effect (Fig. 11A). We next sought to investigate a possible role for IL-18 in iNOS expression in DLD-1 cells. As IL-12 usually enhances actions of IL-18 [Dinarello *et al.*, 1998; Nakanishi *et al.*, 2001], we chose to incubate DLD-1 cells with the combination of both cytokines. IL-12/IL-18 did not result in upregulation of iNOS, neither on the level of mRNA (Fig. 11B), nor on the level of protein (Fig. 11C and Fig. 12A). As expected, exposure to IL-12/IL-18 did not induce secretion of IFN-γ from DLD-1 cells (data not shown).

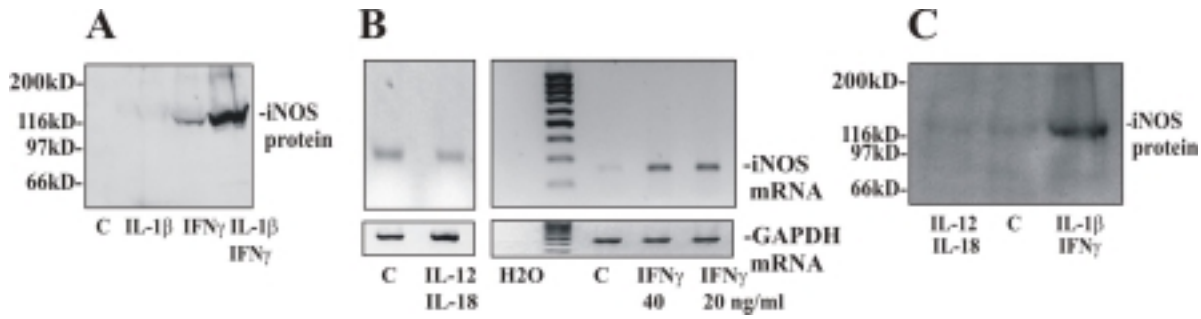


Figure 11. IFN-γ, but not IL-12/IL-18 mediates induction of iNOS in DLD-1 cells. (A) Confluent DLD-1 cells were stimulated for 48h with IL-1β (50 ng/ml), IFNγ (20 ng/ml), with IL-1β (50 ng/ml) plus IFN-γ (20 ng/ml), or as unstimulated control. Thereafter, iNOS protein expression was investigated by immunoblot-analysis. One representative of two independently performed experiments is shown. **(B)** Confluent DLD-1 cells were stimulated for 24h with IL-12 (25 ng/ml) plus IL-18 (80 ng/ml), with the indicated concentrations of IFN-γ, or kept as unstimulated control. Thereafter, iNOS mRNA expression was evaluated by RT-PCR. **(C)** Confluent DLD-1 cells were stimulated with IL-12 (25 ng/ml) plus IL-18 (80 ng/ml), with IL-1β (50 ng/ml) plus IFN-γ (20 ng /ml), or as unstimulated control. After 48h, iNOS protein expression was investigated by immunoblot-analysis. One representative of three independently performed experiments are shown.

We first performed *direct cocultures* of PBMC and DLD-1 cells (Fig. 12A). Absence of iNOS in untreated controls revealed that cell-cell contact between PBMC and DLD-1 colon carcinoma cells was not sufficient to trigger induction of iNOS. Consistent with induction of IFN-γ in IL-12/IL-18-activated PBMC, stimulation of cocultures with IL-12/IL-18 resulted in release of IFN-γ from PBMC in all direct coculture experiments performed (n = 3). In all cases, this induction of IFN-γ was accompanied with expression of iNOS in the adherent cells (Fig. 12A). Although DLD-1 cells were carefully washed twice with PBS before cell lysis was performed, we could not rule out the possibility that adherent mononuclear cells might have contributed to iNOS expression under these conditions. Therefore, we

performed *indirect cocultures* with transwell inserts (Costar) (Fig. 8). Using this method iNOS gene induction was analyzed specifically in DLD-1 cells exposed to secretory products of PBMC. In all donors/cocultures (transwell-assay) investigated (n = 5), addition of IL-12/IL-18 again resulted in release of IFN-γ. This was always paralleled by mRNA (Fig. 12B) and protein expression (Fig. 12C) of iNOS in DLD-1 cells (n = 5). To investigate the role of endogenous IFN-γ in this process, a neutralizing antibody for IFN-γ was used. As shown in Fig. 12B and Fig. 12C, coincubation of IL-12/IL-18 with this antibody markedly reduced induction of iNOS mRNA and protein.

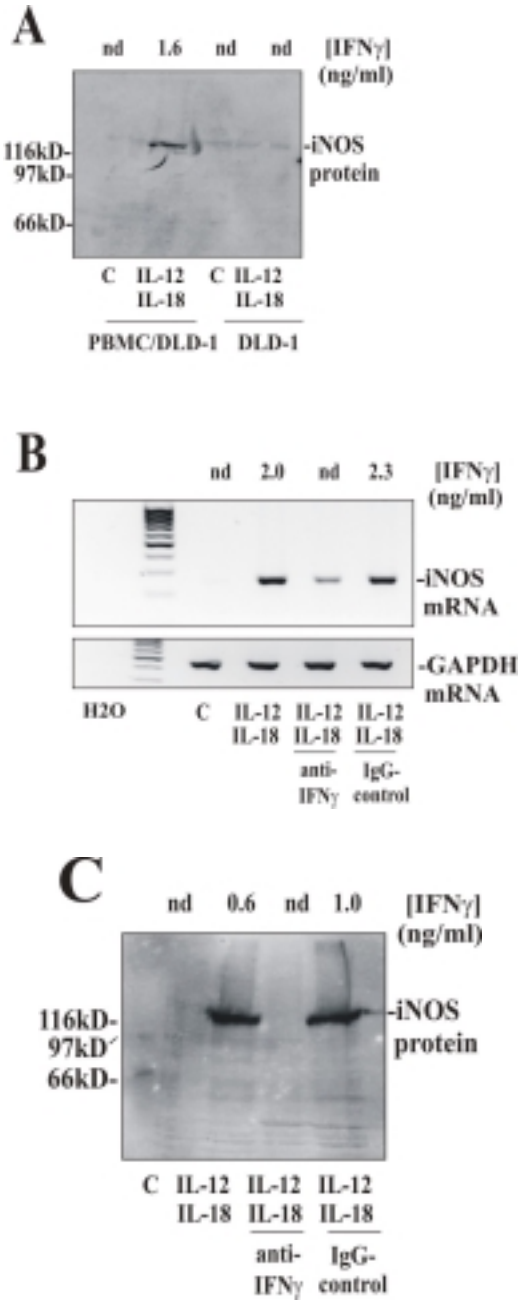


Figure 12. IL-12/IL-18 mediate IFN-γ-dependent induction of iNOS in DLD-1 cells cocultured with PBMC.

(A) ***Direct cocultures*** were stimulated with IL-12 (25 ng/ml)/IL-18 (80 ng/ml) or kept as unstimulated controls. In these same experiments DLD-1 cells were also stimulated in the absence of PBMC with IL-12 (25 ng/ml)/IL-18 (80 ng/ml) or kept as unstimulated control. After 45h, adherent cells were lysed for immunoblot analysis. One representative of three independently performed experiments is shown. Cell-free supernatants were assayed for IFN-γ content by ELISA. IFN-γ levels which correspond to the immunoblot shown are given as absolute concentrations.
(B) ***Indirect cocultures*** (transwell-assay) were kept as unstimulated controls or stimulated with IL-12 (25 ng/ml)/IL-18 (80 ng/ml) in the presence or absence of anti-IFN-γ antibody (20 μg/ml) or control IgG (20 μg/ml). After 45h, DLD-1 cells were lysed and iNOS mRNA expression was analyzed by RT-PCR. Five independent coculture experiments were performed that all demonstrate strong IL-12/IL-18-mediated iNOS mRNA induction. One representative RT-PCR of two independently performed experiments using neutralizing antibodies is shown. IFN-γ levels in cell-free culture supernatants corresponding to the RT-PCR experiment displayed are given as absolute concentrations.
(C) ***Indirect cocultures*** (transwell-assay) were kept as unstimulated controls or stimulated with IL-12 (10 ng/ml)/IL-18 (20 ng/ml) in the presence or absence of anti-IFN-γ antibody (20 μg/ml) or control IgG (20 μg/ml). After 45h, iNOS expression in DLD-1 cells was analyzed by immunoblot-analysis. Five independent coculture experiments were performed that demonstrate strong IL-12/IL-18-induced iNOS protein induction. One representative immunoblot of two independently performed experiments using neutralizing antibodies is shown. IFN-γ levels in cell-free culture supernatants corresponding to the immunoblot displayed are given as absolute concentrations; **nd, denotes non detectable** (IFN-γ < 32.5 pg/ml).

3.3 IL-18 binding protein promoter function

IFN-γ has been described as potent inducer of IL-18BPa expression on protein and mRNA level in different colon carcinoma cell lines, organ cultures of colonic intestinal biopsy specimens, HaCaT keratinocytes as well as rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) [Mühl *et al.*, 2000; Paulukat *et al.*, 2001; Möller *et al.*, 2003]. We therefore decided to investigate the IL-18BP promoter function. A recent report defined the transcription start site of IL-18BPa [Hurgin *et al.*, 2002]. In the upstream area of this determined transcriptional starting point we found different putative sites which might be responsible for IFN-γ mediated IL-18BPa induction as shown in figure 13.

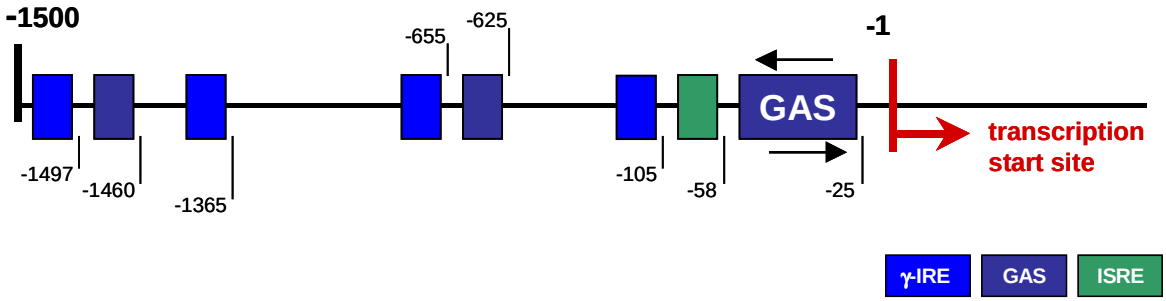


Figure 13. Putative IFN-γ responsive sites responsible for IL-18BPa induction. Different IFN-γ responsive sites upstream of the transcription start site are shown. **γ-IRE:** γ-Interferon responsive element **ISRE:** IFN-stimulated response element **GAS:** Gamma-activated site. The transcription start has been defined to position 785 of the genomic IL-18BP DNA sequence (Gen Bank accession no. AF110798).

3.3.1 Induction of IL-18BP promoter luciferase constructs by IFN-γ

To further characterize the promoter function in DLD-1 cells, we constructed a luciferase reporter vector containing up to 1104 bp (Fig. 14A) corresponding to the DNA sequence upstream of base -1. In addition, we generated another luciferase reporter vector that has been deleted in the upstream area by 469 bp (Fig. 14B). Subsequently the mechanism of IL-18BP gene induction by IFN-γ has been investigated using the described luciferase constructs (Fig. 14). After transfection of human DLD-1 cells with fragment 4 (pGL3 + IL-18BProm Frag4; Fig. 14A), IFN-γ stimulation for 6 h increased the luciferase activity by 15.8 - fold over the unstimulated control (15.8 ± 6.4, Fig. 15). This demonstrates the presence of IFN-γ responsive sites in the investigated fragment, which might be necessary for IL-18BP promoter induction. Furthermore we measured the second generated luciferase reporter vector fragment 5 (pGL3 + IL-18BProm Frag5; Fig. 14B), which was inducible after 6 h IFN-γ stimulation 23.2-fold over the unstimulated control (23.2 ± 5.8, Fig.

15). Fragment 5 shows higher luciferase activities compared to fragment 4 (Fig. 15) This observation might be due to one or more silencer elements located upstream of fragment 5 which are being part of fragment 4.

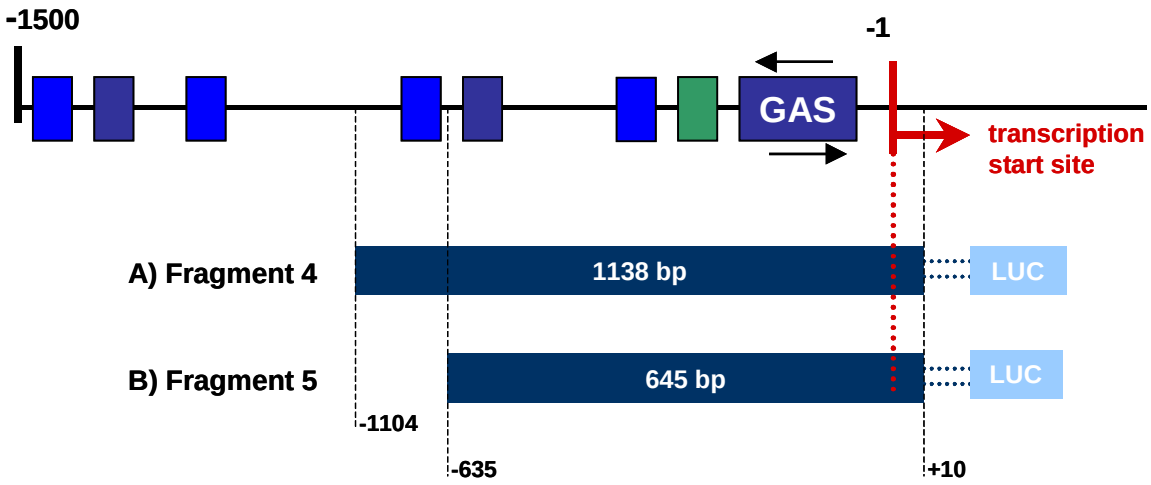


Figure 14. IL-18BP luciferase reporter constructs generated for promoter analysis. (A) Fragment 4 (pGL3 + IL-18BProm Frag4) consists of 1138 bp. Upstream of the transcription start site (TSS) this construct contains -1104 bp. **(B)** The smaller fragment 5 consists of 645 bp. Its region upstream of the TSS contains -635 bp. Both fragments include IFN-γ responsive sites. Compared to fragment 4, fragment 5 lacks an upstream γ-IRE site.

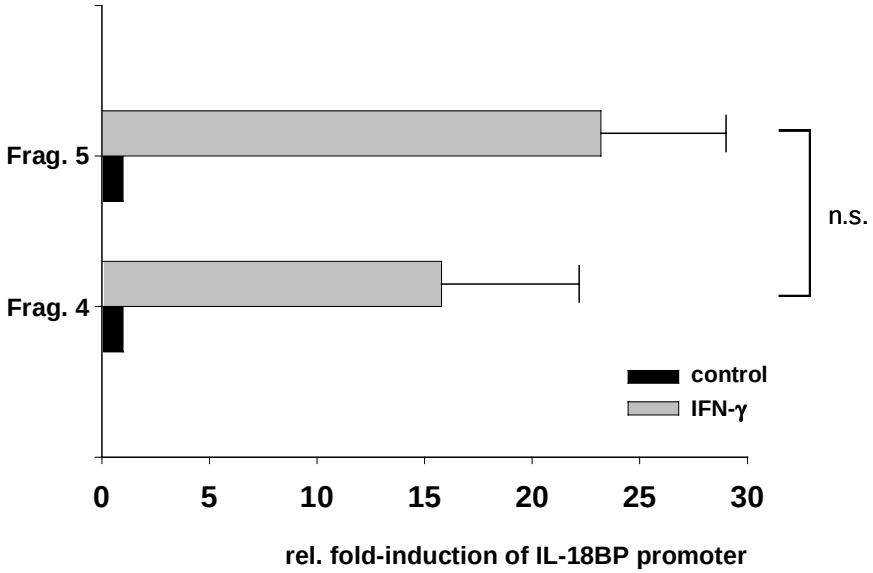


Figure 15. Luciferase activity of IL-18BP promoter reporter vectors. After transfection of DLD-1 cells and an additional 42 h incubation period, the cells were stimulated with IFN-γ (20 ng/ml) for 6 h or kept as unstimulated controls. For internal transfection control ß-Gal plasmids were co-transfected. IFN-γ-induced IL-18BP promoter luciferase activity is shown normalized to β-Gal counts with control conditions set to 1 (Frag4 n=12, Frag5 n=9; data are expressed as fold induction ± SD).

3.3.2 Mutagenesis of putative IFN-γ-inducible GAS site

In order to define sites responsible for IFN-γ-mediated induction of IL-18BP promoter we mutated the GAS site closely located to the transcription start site. Two different mutations (mutGAS 5'/mutGAS 3') have been synthesized by PCR with two complementary mutated primers using fragment 4 (pGL3 + IL18BProm Frag4) and fragment 5 (pGL3 + IL18BProm Frag5) as templates. Subsequently, the bacteria-derived methylated templates were decomposed by DpnI, whereas the newly synthesized PCR products have been transformed into competent *E.coli* strain DH5α. Finally, endotoxinfree preparations of pGL3 + mutGAS 5'/3' Frag4 and pGL3 + mutGAS 5'/3' Frag5 respectively were transfected into DLD-1 cells to investigate responsiveness of the mutated GAS sites toward IFN-γ-stimulation by performing luciferase assays.

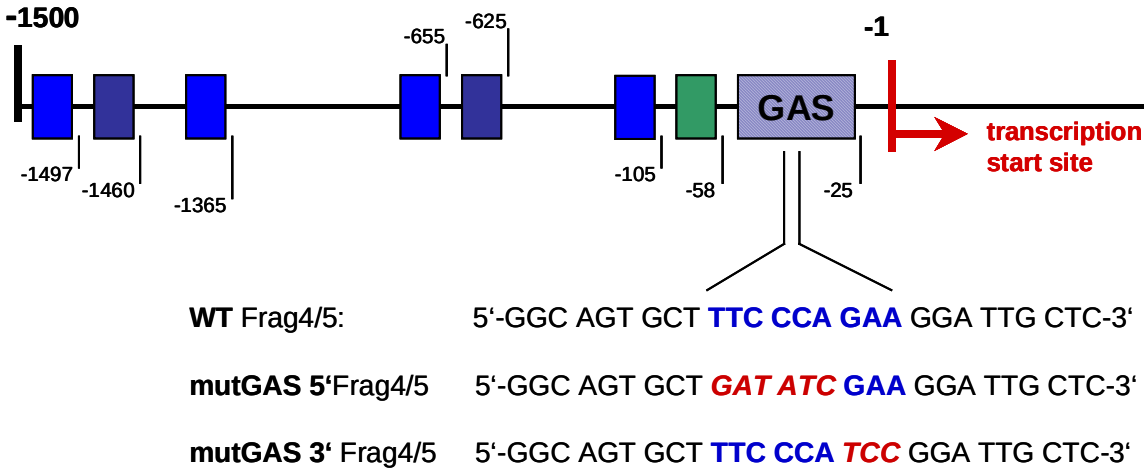


Figure 16. 5'/3'-mutagenesis of GAS site proximal to transcription start site. The two different GAS-mutations have been synthesized by PCR using mutated complementary primers and pGL3+IL18BProm Frag4/Frag5 (wildtype controls) as template. These modified IL18BP promoter constructs were used for transfection and subsequent luciferase assay.

After 6 h IFN-γ stimulation promoter activity of GAS-mutated fragment 4 (mutGAS 5'/3') and fragment 5 (mutGAS 5'/3') was decreased compared to non-mutated control. Whereas the mutated constructs of fragment 4 (mutGAS 5': 6.1 ± 1.7 /mutGAS 3': 6.9 ± 2.6) only show half of its wildtype activity (WT Frag4: 13.9 ± 5.8) in luciferase assay, mutated fragment 5-derived (mutGAS 5': 7.2 ± 1.6 /mutGAS 3': 7.2 ± 0.9) promoter activity was decreased by 71.9 % compared to fragment 5 control (WT Frag5: 25.6 ± 4.5).

According to our observations this mutated GAS site appears to be relevant for IFN-γ-mediated induction of the IL-18BP promoter as investigated by use of luciferase constructs including fragments 4 and 5.

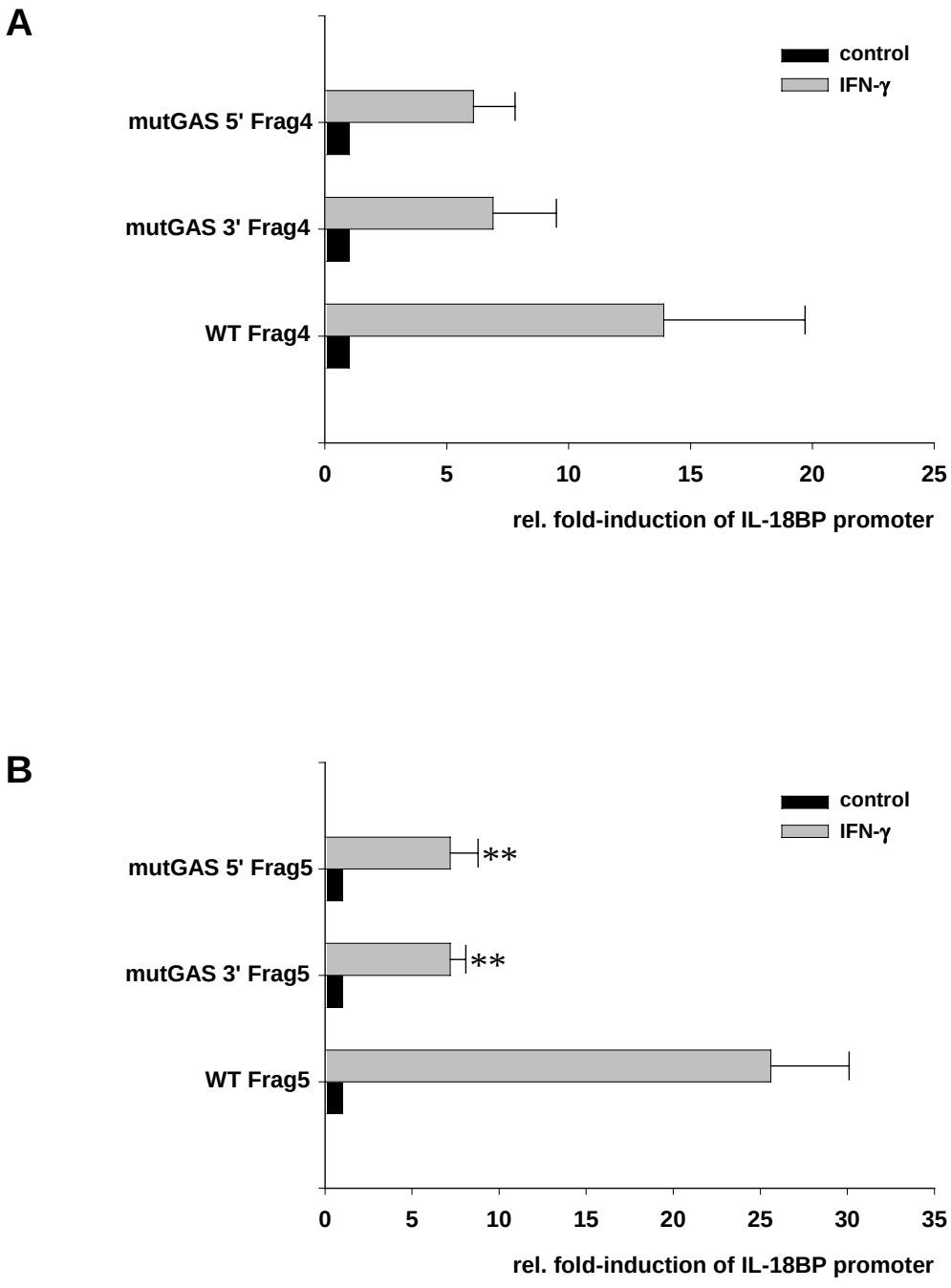


Figure 17. Luciferase activity of 5'/3'-GAS-mutated IL-18BP promoter fragment 4 (A) and fragment 5 (B). After transfection of DLD-1 cells and an additional 42 h incubation period, the cells were stimulated with IFN-γ (20 ng/ml) for 6 h or kept as unstimulated controls. For internal transfection control ß-Gal plasmids were co-transfected. IFN-γ-induced IL-18BP promoter luciferase activity is shown normalized to β-Gal counts with control conditions set to 1 (mutGAS 5'/3' Frag4/5 n=3; data are expressed as fold induction ± SD).

3.3.3 STAT-1 binding to GAS site following IFN-γ stimulation

Next we used EMSA to identify protein/ds-DNA interaction at the GAS site proximal to the transcription start site within the IL-18BP promoter. Labeled double-stranded DNA probes (oligo GAS1) corresponding to bases -18 to -41 (containing GAS) were allowed to bind with nuclear extracts of control and IFN-γ-treated DLD-1 cells. A complex of the GAS-containing probe and nuclear protein(s) was apparent after incubation of cells for 0.5 h or 2 h with IFN-γ (Fig. 18A lane 2 and 18B lane 8). In contrast, no such complex was obtained with extracts of untreated control cells (Fig. 18A lane 1 and 18B lane 7). A 10-fold excess of cold oligo GAS1 competed with the labeled dsDNA-probe. Thus, no shift was detectable (Fig. 18A lane 3 and 18B lane 8). The binding of nuclear proteins to the radioactive ds-oligonucleotide GAS1 was also abolished by using 10-fold excess of an unlabeled wildtype dsDNA probe purchased from Santa Cruz that is known to be specific for GAS (Fig. 18A lane 5 and 18B lane 11). In contrast, the mutated version of this Santa Cruz WT dsDNA probe, namely Mut Santa Cruz, did not affect nuclear protein binding to the labeled GAS containing dsDNA-probe (Fig. 18A lane 4 and 18B lane 10). Finally, addition of a monoclonal antibody against STAT-1 caused a “supershift”, which is due to antibody binding to the complex of labeled dsDNA probe interacting with STAT-1, that had been induced by IFN-γ (Fig. 18A lane 6 and 18B lane 12).

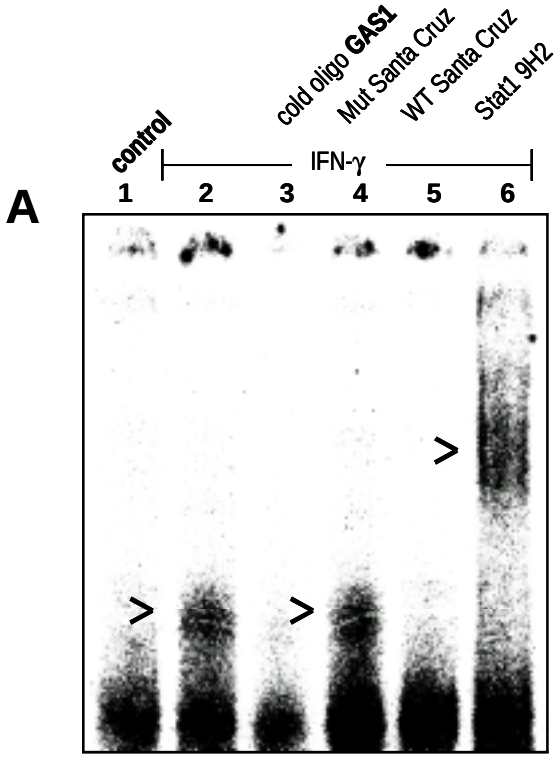
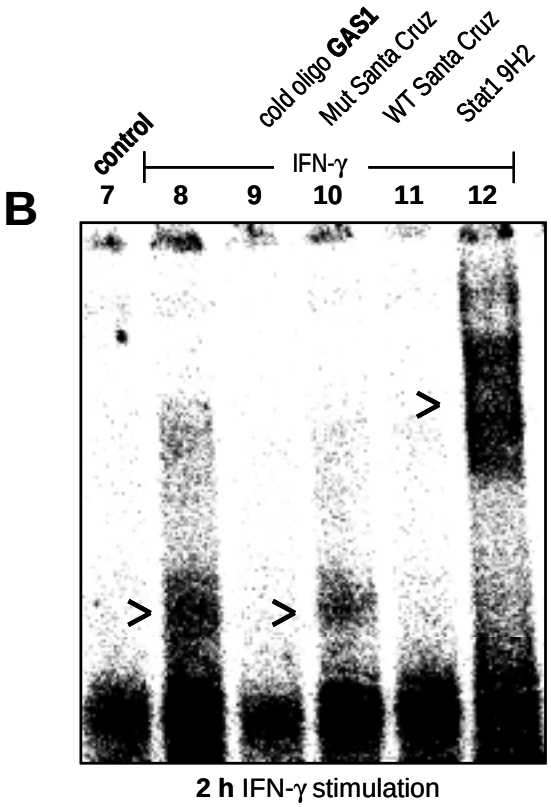


Figure 18. EMSA and ´supershift´ analysis after IFN-γ stimulation of DLD-1 cells.

(A) EMSA following 0.5 h IFN-γ stimulation. DLD-1 cells were stimluted with IFN-γ (20 ng/ml) for 0.5 h or kept as unstimulated control. Where indicated, the nuclear extracts were shifted with the given ds DNA probe (lane 2 and 4) respectively supershifted with the specified antibody (lane 6). Additionally, in lane 3 and 5 cold ds oligonucleotides have been used in 10-fold excess to compete for nuclear extract protein binding. Arrows indicate shifts and supershifts, respectively.



B) EMSA following 2 h IFN-γ stimulation. DLD-1 cells were stimluted with IFN-γ (20 ng/ml) for 2 h or kept as unstimulated control. Where indicated, the nuclear extracts were shifted with the given ds DNA probe (lane 8 and 10) respectively supershifted with the specified antibody (lane 12). Additionally, in lane 9 and 11 cold ds oligonucleotides have been used in 10-fold excess to compete for nuclear extract protein binding. Arrows indicate shifts and supershifts, respectively.

IV
Discussion

4.1 Expression and release of IL-18BPa in response to IFN-γ

IL-18 is a pivotal mediator of a T_H1 cytokine response [Nakamura *et al.* 1989] and its naturally occuring inhibitor IL-18 binding protein (IL-18BP) [Novick *et al.* 1999] are two newly described opponents in the cytokine network. Local concentrations of these two players may determine biological functions of IL-18 in the context of inflammation, infection, and cancer. IL-18 bioactivity is connected with the pathogenesis of several inflammatory diseases, for example, septic shock, colitis, Crohn's disease, myasthenia gravis, multiple sclerosis, rheumatoid arthritis, and atherosclerosis. Furthermore, IL-18-promoted T_H1 responses are associated with several autoimmune diseases as well as organ transplant rejection. IL-18 action is modulated by IL-18BPa which is the most abundant IL-18BP splice variant found in human cDNA libraries. This isoform exhibits greatest affinity for IL-18. In the first part of this study we raised the question how IL-18BP expression is regulated on mRNA and protein level particularly in different colon carcinoma cells but also in other cell types.

4.1.1 IFN-γ induces expression and release of IL-18BPa in different colon carcinoma cell lines, organ cultures of colonic intestinal biopsy specimens, and keratinocytes

In this study we demonstrate that IL-18BPa is released by IFN-γ-activated DLD-1 colon carcinoma/epithelial cells. IFN-γ induction of IL-18BPa was not only restricted to DLD-1 cells but was also confirmed in other colon carcinoma cells using LoVo, Caco-2, and HCT116 cell lines. Additionally, we detected IL-18BPa release in IFN-γ-stimulated HaCaT keratinocytes indicating that IFN-γ-induced IL-18BPa expression is a more general phenomenom not being restricted only to colonic epithelial cells. In each cell line the secretion of IL-18BPa immunoreactivity coincided with induction of IL-18BPa expression on mRNA level. Expression and secretion of IL-18BPa in response to IFN-γ was accompanied by appearance of an extracellular activity that was able to impair IL-12/IL-18-induced IFN-γ in PBMC. Gene induction of IL-18BPa by IFN-γ was also observed in organ cultures of colonic biopsy specimens. These latter results demonstrate upregulation of IL-18BPa gene

expression in an *ex vivo* setting and underscore the potential significance of the data obtained using colon carcinoma/epithelial cell lines. The present data are consistent with work by Fantuzzi et al. demonstrating reduced expression of IL-18BP in interferon regulatory factor-1 (IRF-1) deficient mice [Fantuzzi *et al.*, 2001]. These observations may have important implications for the action of IFN-γ and IL-18 under pathophysiological conditions. By inducing IL-18BP, IFN-γ appears to trigger a negative feedback loop which limits IFN-γ-dependent and -independent actions of IL-18. Accordingly, overproduction of IFN-γ has been observed in IFN-γ receptor-deficient mice used in models of hapten-induced colitis [Camoglio *et al.*, 2000] and collagen-induced arthritis [Matthys *et al.*, 1998].

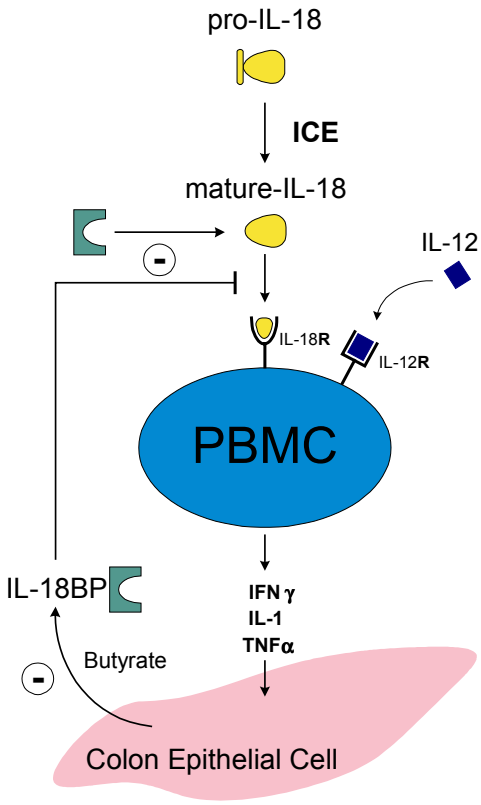


Figure 19.

Negative feedback regulation of IL-18 action by IFN-γ-induced IL-18BPa.

IL-18 induces IFN-γ expression in synergism with IL-12. IFN-γ itself induces IL-18BP expression in colon epithelial and likely also in other local cells thereby providing a negative regulator for IL-18 action. Sodium butyrate suppresses IFN-γ-induced IL-18BPa expression thus promoting IL-18 action.

The present data are compatible with data on IL-18BP expression in graft versus host disease, where production of IL-18BP increases in parallel with IFN-γ [Nagler *et al.*, 2000]. Moreover, in adult Still's disease serum levels of IL-18 correlate with the presence of an inhibitory activity that impairs binding of IL-18 to its membrane receptor [Kawashima *et al.*, 2001]. Upregulation of IL-18BP might also be involved in the diminished capability of IFN-γ production in *ex vivo* whole blood cultures obtained from patients with septic shock [Oberholzer *et al.*, 2000]. In fact, Novick et al. recently demonstrate augmented serum levels of IL-18BP in septic shock patients [Novick *et al.*, 2001]. Therapeutic use of IL-18BPa may restore a hypothetically disturbed IL-18/IL-18BP balance in diseases that are

associated with augmented production of T_H1 cytokines, such as Crohn's disease. In addition, induction of IL-18BP may contribute to protective functions of IFN-γ as seen in murine models for rheumatoid and septic arthritis [Vermeire *et al.*, 1997; Puliti *et al.*, 2000], and in rheumatoid arthritis patients [German Lymphokine Study Group, 1992].

4.1.2 Influence of short chain fatty acid (SCFA) sodium butyrate on IFN-γ-induced IL-18BPa expression in colon carcinoma cells

IFN-γ induces IL-18BPa expression in different colon carcinoma cell lines as well as in cultures of colonic intestinal biopsy specimens. With sodium butyrate we determined an inhibitor of IFN-γ induced IL-18BP expression in these cells. This short chain fatty acid is formed in the gastrointestinal tract of mammals as a result of anaerobic bacterial fermentation of undigested dietary components and is avidly absorbed by the colonic epithelium [Velázquez *et al.*, 1996].

In the colon butyrate has been shown to be the dominant energy source for epithelial cells. Furthermore it affects cellular proliferation and differentiation by yet unknown mechanisms. Recent data suggest that the luminal provision of butyrate may be an appropiate means to ameliorate symptoms of inflammatory bowel diseases [Wächtersheimer and Stein, 2000]. Interestingly, previous reports also demonstrate that butyrate can protect against the development of colon cancer [Wächtersheimer and Stein, 2000]. According to our observations we suggest that intestinal butyrate may have the capability to strengthen the bioactivity of IL-18 at the colonic microenvironment by modulating IFN-γ-induced IL-18BPa expression. There is ample evidence that IL-18 is an inhibitor of tumor growth. In this context, suppression of a proposed IL-18BP activity should be beneficial. IL-18 acts as inhibitor of angiogenesis [Cao *et al.*, 1999] and augments Fas/Fas-ligand dependent $CD4^+$ T cell- and NK cell-cytotoxicity [Dao *et al.*, 1996; Tsutsui *et al.*, 1996; Osaki *et al.*, 1998]. Taking into account these antitumor functions of IL-18, the capability of sodium butyrate to strengthen the bioactivity of IL-18 through modulation of IL-18BP expression is in accord with its tumor-suppressive potential. Actually, sodium butyrate augments the sensitivity of colon carcinoma cells towards Fas-mediated apoptosis [Bonnotte *et al.*, 1998] and Fas-related $CD4^+$ T cell- and NK cell-cytotoxicity is characteristically enhanced by IL-18 [Dao *et al.*, 1996; Tsutsui *et al.*, 1996; Osaki *et al.*, 1998]. Although IFNγ should contribute to tumor suppressive actions of IL-18 *in vivo*, antitumor functions of IL-18 have been observed in an IFN-γ-independent manner [Cao *et al.*, 1999; Dao *et al.*, 1996, Osaki *et al.*, 1998]. It is noteworthy that under defined conditions IFN-γ in fact appears to be capable of enhancing growth of colon carcinomas [Ramani *et al.*, 1987]. Induction of IL-18BP by IFNγ might

contribute to this observation. Altogether, sodium butyrate appears to shift the IL-18/IL-18BP balance in colon carcinoma cells in favour of IL-18, which may contribute to local tumor protection by this compound, likely via IFN-γ-independent tumor-suppressive actions of IL-18. However, IL-18 may also augment expression of genes which have been associated with cancer progression. For example, IL-18 can upregulate hepatic melanoma metastasis via induction of vascular cell adhesion molecule-1 expression [Vidal-Vanaclocha *et al.*, 2000]. In addition, IL-18 can mediate production of nitric oxide [Zhang *et al.*, 1997; Olee *et al.*, 1999], which has been shown to facilitate growth of certain tumors among them melanoma (Ekmekcioglu *et al.*, 2000) and colorectal cancer (Ambs *et al.*, 1998).

4.1.3 IFN-γ induces expression of IL-18BPa in rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS)

IL-18BPa is not only induced by IFN-γ in cells of colonic lineage. This induction seems to be a more general phenomenom since this regulatory pathway is observed in several cell types. In this study we also observed IFN-γ induced IL-18BPa expression and secretion in resident rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS). Furthermore, release was accompanied with appearance of an activity associated with IFN-γ-treated RA-FLS which was able to impair the production of IFN-γ in PBMC exposed to IL12/IL-18. Induction of IL-18BP in RA-FLS might be an important regulative, since IL-18 seems to play an important role in the pathogenesis of rheumatoid arthritis (RA) [Dinarello *et al.*, 1998; Gracie *et al.*, 1999]. Yet FLS might contribute to the complex network of immunoregulation which ensures control of primarily macrophage-derived IL-18 during synovitis. IL-18 is able to activate both, T cells and macrophages in the microenviroment of RA synovitis. IFN-γ, IL-1β, and TNF-α are supposed to be induced in RA joints [Dinarello *et al.*, 1998; Gracie *et al.*, 1999; Klimiuk *et al.*, 1997] and successful anti-cytokine therapies in RA reflect the paramount relevance of the proinflammatory cytokines IL-1β and TNF-α [Feldmann *et al.*, 1998; Jiang *et al.*, 2000] in this context. IL-18 was first identified as IFN-γ-inducing factor [Dinarello *et al.*, 1998], and is capable of directly inducing expression of the TNF-α [Dinarello *et al.*, 1998; Gracie *et al.*, 1999]. Although IFN-γ levels are often low, it is detectable in all histological variants of rheumatoid arthritis synovitis [Klimiuk *et al.*, 1997]. But the role of IFN-γ in RA synovitis is somewhat contradictory. Addition of IFN-γ to T cell/monocyte co-cultures enhances TNF-α production, which should aggravate the disease [Sebbag *et al.*, 1997]. Furthermore, a murine model of septic antigen-induced arthritis illustrates that IFN-γ can display proinflammatory properties when given in the

inflammation initiation phase. Here the detrimental functions of IFN-γ may include a more effective antigen presentation at the onset of disease. On the other hand, IFN-γ can downregulate parameters of joint destruction such as proinflammatory IL-1, matrix-metalloproteases, and FLS proliferation [Ghezzi *et al.*, 1988; Alvaro-Gracia *et al.*, 1990]. Another report provide evidence that IFN-γ ameliorates joint inflammation when given later in the inflammatory process. In this state of disease inhibition of IL-18 through IFN-γ-induced IL-18BPa could represent a negativ feedback mechanism upon established inflammation comparable to our proposed model for colonic cells in connection with inflammatory bowel diseases (Figure 19). Such a negative feedback principle concurs with reported overexpression of IFN-γ in IFN-γ receptor deficient mice evaluated in collagen induced arthritis [Matthys *et al.*, 1998]. Additionally, in patients with adult onset Still's disease [Kawashima *et al.*, 2001], in Crohn's disease [Corbaz *et al.*, 2002], as well as in patients with sepsis [Novick *et al.*, 2001], levels of IFN-γ-inducing IL18 activity appear to correlate with expression of IL-18BP. All these observations suggest a close association of IL-18 with expression of its decoy receptor in inflammatory diseases. Induction of IL-18BPa by IFN-γ could prove to be a crucial endogenous corrective that counterregulates IL-18-mediated leukocyte activation in rheumatoid arthritis. In spite of this functional feedback loop in RA materials, there is still IL-18 bioactivity detectable in RA synovial fluids, even though an IL-18 inhibitory activity is present [Yamamura *et al.*, 2001]. Therefore, therapeutic administration of IL-18BPa in patients may modulate the cytokine balance in order to ameliorate established arthritis. This concept has been confirmed recently in a murine rheumatoid arthritis animal model [Plater-Zyberk *et al.*, 2001].

4.1.4 Antiinflammatory properties of IFN-γ apart from IL-18BPa induction

IL-18BPa might play an important role in the counterregulation of proinflammatory IL-18 action. But apart from IL-18BPa induction, IFN-γ also mediates other antiinflammtory effects. For instance, IFN-γ induces so-called SOCS proteins (**S**uppressor **o**f **c**ytokine **s**ignaling), that are known to be negative regulators of cytokine signaling [Krebs and Hilton, 2001]. Also known as STAT-induced STAT inhibitors (SSI-1) or JAK (JAB) 2-binding proteins, they may act in part in a negative feedback loop. IFN-γ signaling takes place using the JAK/STAT-pathway. Once phosphorylated, two STAT-1 proteins homodimerize. Subsequently these homodimers are able to induce transcription of SOCS1 and SOCS3. Precisely SOCS1 interacts directly with active JAKs in a phosphorylation dependent manner and inhibits its catalytic activity whereas SOCS3 binds to JAK-proximal sites and inhibits JAK activity. In conclusion, by inducing SOCS proteins IFN-γ-dependent IL-18

action might be inhibited by interference with the intracellular signal transduction machinery responsible for STAT-activation. IFN-γ also shows antiinflammatory properties by modulating the bioactivity of pro-inflammatory IL-1. Interestingly, IFN-γ has been reported to suppress IL-1-induced transcription of IL-1. This IL-1-mediated induction of its own expression is supposed to be part of a self-amplification loop in a variety of inflammatory responses. Endogenous regulation of IL-1 production should contribute to the outcome of inflammatory responses [Schindler *et al.*, 1990]. IFN-γ-treatment also modulates IL-1 action by induction of circulating IL-1 receptor antagonist (IL-1Ra), which is known to be a specific inhibitor of IL-1. These regulatory loops may contribute to antiinflammatory effects of IFN-γ [Tilg *et al.*, 1993].

4.1.5 Proapoptotic properties of IFN-γ

IFN-γ does not only exert antiinflammatory properties but also exhibit proapoptotic functions. It has been shown that IFN-γ sensitized colonic epithelial cell lines to diverse inducers of apoptosis of physiologic or therapeutic relevance to the colon [O'Connell *et al.*, 2000]. These apoptosis inducers included Fas (CD95/APO-1) ligand (FasL), short- chain fatty acids like sodium butyrate, as well as chemotherapeutic drugs. IFN-γ-mediated sensitization has been correlated to upregulation of the proapoptotic protease caspase-1 (ICE) [O'Connell *et al.*, 2000]. In many cell types, including various epithelial cells, apoptosis in response to diverse stimuli proceeds via activation of caspase-1 [Boudreau et al., 1995; Enari et al., 1995/1996; Kondo et al., 1995; Henkart, 1996; Tamura et al., 1996; Los et al., 1997]. Activated caspases induce proteolysis of specific substrates, which contributes to apoptotic processes including mitochondrial permeability transitions, DNA fragmentation, and phosphatidylserine externalization. By catalyzing IL-18 maturation, caspase-1 induced by IFN-γ contributes to its tumorsuppressive properties.

4.2 Cell-to-cell crosscommunication during T_H1 responses mediates coordinated expression of modulatory iNOS and IL-18BP in adjacent local resident cells

So far we reported that IFN-γ induces IL-18BPa expression in different cell types of colonic lineage, in keratinocytes as well as in primary cultures of fibroblast-like synoviocytes. We therefore concluded that IFN-γ induced IL-18BPa expression seems to be a more general phenomenom. All these findings have been achieved performing exposure to exogenous recombinant IFN-γ. To be more close to the *in vivo* situation, we intended to carry out

coculture experiments. This experimental set up allowed us to investigate cell-to-cell crosscommunication during T_H1 responses. In the present study we report on coordinated induction of IL-18BPa and iNOS in adjacent local resident DLD-1 during T_H1 responses initiated by PBMC exposed to IL-12/IL-18. Both parameters mediate important functions in the context of inflammation, infection, and cancer. The induction of IL-18BPa respectively iNOS was mediated by endogenously synthesized IFN-γ.

4.2.1 Induction of iNOS in DLD-1 cells cocultured with PBMC

Inducible nitric oxide synthase (iNOS) has been recognized as a marker of human diseases associated with inflammation and immunoactivation, among them rheumatoid arthritis [St. Clair, *et al.*, 1996], glomerulonephritis [Furusu *et al.*, 1998], and inflammatory bowel disease [Singer *et al.*, 1996]. iNOS has also been detected in a broad range of human malignancies including melanoma and colorectal cancer [Ekmekcioglu *et al.*, 2000; Ambs *et al.*, 1998; Ambs *et al.*, 1999]. In these tumors, iNOS is not only expressed in invading mononuclear cells, but also in the cancer cells [Ambs *et al.*, 1999]. Accordingly, several studies imply a role for NO in carcinogenesis [Ambs *et al.*, 1998; Ambs *et al.*, 1999; Jenkins *et al.*, 1995]. Despite its unambiguous presence in inflammatory diseases and cancer, current knowledge of endogenous pathways which establish early expression of human iNOS is still incomplete. *In vitro,* iNOS is inducible by exogenous interferon-γ (IFN-γ) in a variety of different cell types e.g. human epithelial-like colon carcinoma cells such as DLD-1 cells [Kleinert *et al.*, 1998]. However, little is known about the role of cytokines that intervene more upstream in the cytokine cascade.

Although data on immunoregulatory roles of NO are not entirely uniform [Bogdan, 2001], analysis of iNOS knockout mice suggests that high-output production of NO likely represents a pathway that inhibits production of IFN-γ [Wei *et al.*, 1995; Shi *et al.*, 2001]. In accord with this assumption, it has been reported that endogenous production of NO can reduce release of IFN-γ from human natural killer cells [Salvucci *et al.*, 1998]. Different mechanisms may account for impaired production of IFN-γ associated with NO: i) NO is supposed to interfere with maturation of pro-IL-18 by inhibiting caspase-1 (ICE) activity (Figure 20). That mechanism has been documented by use of iNOS knockout mice [Kim *et al.*, 1998]. However, mechanisms of pro-IL-18 processing and release have not been fully characterized for the human system. Particularly the role of caspase-1 remains elusive. A recent study demonstrates that lipopolysaccharide (LPS) alone does not mediate secretion of IL-18 from human monocytes. Instead, release of mature IL-18 was induced by the combination LPS/ATP. Processing and secretion was, however, independent of caspase-1

[Mehta *et al.*, 2001]. Constitutive release of mature IL-18 has been observed in *ex vivo* cultures of human whole blood taken from septic patients. Notably, secretion is not modulated by inhibition of caspase-1 activity. In contrast, release of mature IL-1β is suppressed in these same experiments [Oberholzer *et al.*, 2000]. ii) NO is capable of inhibiting proliferation of T-lymphocytes, possibly via disruption of the Jak3/STAT5 signaling pathway [Bingisser *et al.*, 1998; Koblish *et al.*, 1998] (Figure 20). iii) NO is able to impair production of IL-12 by macrophages [Huang *et al.*, 1998]. In conclusion iNOS derived NO might provide an inhibitory feedback loop that modulate IFN-γ production at different stages. This negative feedback loop is bound up with a postulated second inhibitory loop consisting of IFN-γ-induced IL-18BPa [Paulukat *et al.*, 2001] as discussed in the following section.

4.2.2 Induction of IL-18BPa in DLD-1 cells cocultured with PBMC

Induction of IL-18BPa by IFN-γ represents a further mode of feedback inhibition that should serve to control IL-18 biological activity under pathophysiological conditions (Figure 16). Present and previous data from our group suggests that local resident cells may constitute an important source of inducible IL-18BP production. Here we present first data that IL-18BPa is also inducible by IFNγ in PBMC. Although induction was modest compared to DLD-1 cells, we cannot entirely exclude that PBMC contributed to secretion of IL-18BP protein as detected by immunoblotting in our coculture system. The proposed IFN-γ-driven feedback modulation agrees with amplified production of IFN-γ in IFN-γ receptor knockout mice, evaluated in models of inflammatory diseases [Camoglio *et al.*, 2000; Matthys *et al.*, 1998]. In the present context it is noteworthy that an association between IL-18 and expression of its decoy receptor has been observed in patients with sepsis [Novick *et al.*, 2001], Still's disease [Kawashima *et al.*, 2001], and psoriasis [Ohta *et al.*, 2001]. Altogether, induction of both, IL-18BPa and iNOS, may serve to control the strength and duration of local IL-18/IFN-γ action and should affect the pathogenesis of inflammatory diseases such as Crohn's disease and autoimmune glomerulonephritis.

4.2.3 Synchronized induction of IL-18BPa and iNOS in carcinogenesis

In the context of tumor biology, coordinated induction of IL-18BP and iNOS in cancer cells is of particular significance. In several murine models IL-18 exerts either a direct or an IFNγ-dependent tumor-suppressive function that is associated with FasL-mediated killing and reduction of angiogenesis in the tumor microenviroment [Coughlin *et al.*, 1998; Osaki

et al., 1998; Cao *et al.*, 1999]. Upregulation of iNOS on the other hand has been observed in human colorectal cancer [Ambs *et al.*, 1998; Ambs *et al.*, 1999], breast cancer [Thomsen *et al.*, 1995], in prostate cancer [Klotz *et al.*, 1998], in lung cancer [Liu *et al.*, 1998], as well as in melanomas [Ekmekcioglu *et al.*, 2000], and head and neck cancer [Gallo *et al.*, 1998]. Besides suppression of IFNγ production (Figure 20), NO may facilitate tumor growth by several additional mechanisms: iNOS in human colon cancer tissues is positively correlated with the frequency of mutations in the p53 tumor suppressor gene. NO may thus provide a selection pressure for nonfunctional p53, a mechanism which should ultimately promote tumor growth [Ambs *et al.*, 1999]. Interestingly, NO has been recognized as an inhibitor of apoptotic cell death under certain conditions [Liu and Stamler 1999]. Therefore, autocrine NO may increase cancer cell survival. It has also been reported that overexpression of iNOS in DLD-1 cells results in enhanced tumor growth in xenografted nude mice. Tumors derived from these iNOS overexpressing DLD-1 cells appear markedly more vascularized [Jenkins *et al.*, 1995]. Similar observations have been reported using Calu-6 lung carcinoma cells [Ambs *et al.*, 1998]. Notably, DLD-1 cells [Kagawa *et al.*, 1997] as well as Calu-6 [Ambs *et al.*, 1998] cells do not express wild-type p53. NO-mediated promotion of tumor growth agrees as well with a report on head and neck cancer which identifies iNOS as a marker of progression that closely correlates with angiogenesis and tumor vascularization [Gallo *et al.*, 1998]. Furthermore, iNOS-derived NO should mediate vasodilatation at the tumor site. The resulting increase in blood supply will likely favour tumor growth [Andrade *et al.*, 1992]. Finally, NO not only appears to be a marker but also a mediator of inflammation. Inflammatory actions of NO include activation of the transcription factor NF-κB [Lander *et al.*, 1993], as well as augmented production of TNFα and of chemokines [Mühl and Dinarello, 1997; Lander *et al.*, 1993; Mühl *et al.*, 2000; Guo *et al.*, 2001; Walpen *et al.*, 2001], which can mediate leukocyte infiltration into tumor tissues. As colonic inflammation and macrophage infiltration are supposed to support progression of colorectal cancer [Etoh *et al.*, 2000], these inflammatory actions of NO are compatible with NO-mediated tumor promotion.

Lack of IFNγ is associated with distant metastasis and poor prognosis in colorectal cancer [Pages *et al.*, 1999]. By counterregulating the IL-18- and IFNγ-dependent arm of immune defenses against tumors, both IL-18BP and iNOS, have the potential to promote carcinogenesis by mechanisms that likely include tumor-mediated immunosuppression and angiogenesis.

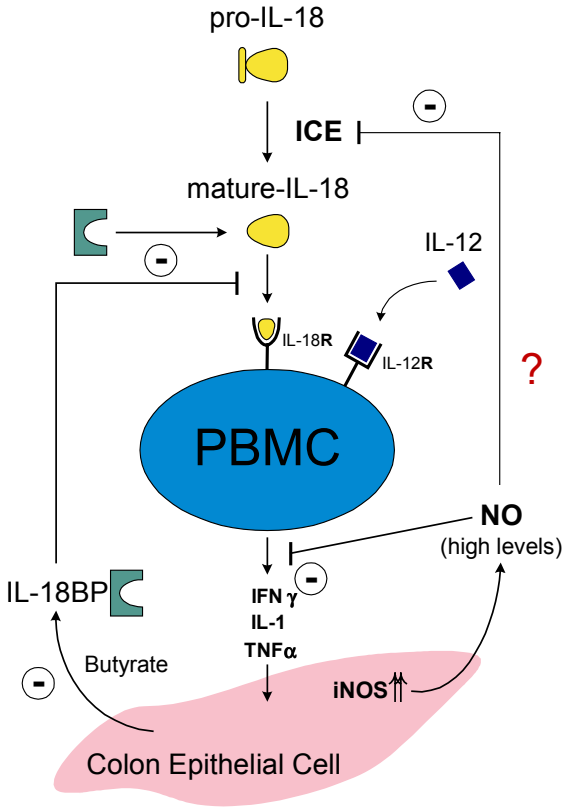


Figure 20.

Negative feedback regulation of IL-18 action by iNOS-derived NO.

IL-18 induces IFN-γ expression in synergism with IL-12. IFN-γ in combination with costimulant IL-1 induces iNOS expression in colon epithelial cells. iNOS derived NO negatively modulates IFN-γ production at different stages. IFN-γ also induces IL-18BPa expression as second negative feedback loop for IL-18 action. Both proteins counterregulate IL-18- and IFN-γ-mediated action. This might be of particular significance, since IL-18 exerts either a direct or an IFN-γ-dependent tumor-suppressive function. On the other hand upregulation of iNOS has been observed in different human cancer.

4.3 IL-18 binding protein promoter function

We postulate that IFN-γ-induced IL-18BPa expression seems to be a general phenomenom as shown in this study in cells of colonic lineage, keratinocytes and rheumatoid arthritis fibroblast-like synoviocytes. There are further reports that IFN-γ-induced IL-18BPa expression also take place in renal mesangial cells [Mühl *et al.*, 2000], hepatocytes [Rubinstein *et al.*, 2001], PBMC and monocytes [Corbaz *et al.*, 2002] as well as retinal pigment epithelial cells (data not shown), and endothelial cells (Huvec) [Corbaz *et al.*, 2002]. Therefore, we were interested in the mechanism of IFN-γ-mediated IL-18BPa induction on promoter level.

4.3.1 Induction of IL-18BP promoter by IFN-γ

We cloned two IL-18BP promoter luciferase reporter vectors which were highly inducible by IFN-γ in luciferase assays. Both fragments habored diverse IFN-γ-responsiv elements (γ-IRE: γ-Interferon responsive element; GAS: Gamma-activated site; ISRE: IFN-stimulated response element [Pearse *et al.*, 1991; Decker *et al.*, 1997; Taniguchi *et al.*, 2001] that are known to transduce IFN-γ signaling and therefore might be responsible for IL-18BPa expression (Figure 21).

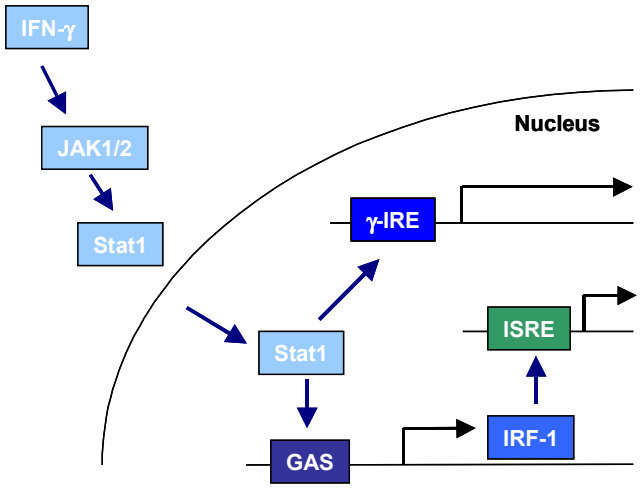


Figure 21. IFN-γ responsive sites

IFN-γ signaling is via the Jak /Stat pathway (Janus family of protein tyrosin kinases/**S**ignal **T**ransducers and **A**ctivators of **T**ranscription family). Stat1 is able to bind directly to γ-IRE as well as GAS. Furthermore, GAS itself is part of the IRF-1 (**I**nterferon **R**egulatory **F**actor-1) promoter. Expressed IRF-1 subsequently can mediate IFN-γ signaling by binding to ISRE sites.

The 5' 469 bp shortened fragment 5 showed higher luciferase activities compared to fragment 4. This observation might be due to one or more silencer elements located upstream of base -635 not yet defined. A recent report confirmed our data suggesting that a silencer element resides within bases –656 to –1107 [Hurgin *et al.*, 2002]. To identify sites responsible for IFN-γ-mediated induction of the IL-18BP promoter we mutated the GAS site closely located to the transcription start site. Our mutagenesis data reveal that this site is likely of relevance for IFN-γ-mediated induction of the IL-18BP promoter fragments 4 and 5. Whereas the mutated constructs of fragment 4 (pGL3 + mutGAS 5'/3') only showed about half of its wildtype activity in luciferase assay, mutated fragment 5 vectors (pGL3 + mutGAS 5'/3') promoter activity were decreased by 71.9 %.

In the present work we focussed on the GAS site (**G**amma **A**ctivated **S**ite) closely located to the IL-18BP gene transcription start site and performed electrophoretic mobility shift assays. Our data revealed STAT-1 binding to this GAS element following IFN-γ stimulation. Previously it was shown that GAS binds an IFN-γ-induced STAT1 dimer [Shuai *et al.*, 1994]. This observation is also in accord with other reports, indicating that IFN-γ ligand binding leads to receptor oligomerization, with two IFN-γR_1 (α) chains bound to one IFN-γ homodimer, and the subsequent recruitment of two IFN-γR_2 (β) chains to the complex. IFN-γ-mediated receptor aggregation brings the inactive JAKs associated with the cytoplasmatic tails of the α and β chains into close proximity. Once clustered, the JAKs are reciprocally activated through sequential auto- and transphosphorylation events. Activated JAKs themselves phosphorylate a specific c-terminal located tyrosine residue of each IFN-γR_1 [Tau and Rothman, 1999]. This phosphorylated tyrosine residue pair is embedded within a recognition sequence to which STAT-1 binds through its SH_2 (src homology 2) domain. By

docking of STAT-1 molecules to this sequence these become phosphorylated by the receptor associated JAKs. After phosphorylation two STAT-1 proteins homodimerize and finally translocate to the nucleus, where they bind to the consensus sequence TTCN2-4GAA, known as GAS element [Leonard and O'Shea, 1998; Decker *et al.*, 1997]. A recently published report is contradictory to our data. Hurgin *et al.* (2002) did not see any STAT-1 binding to GAS following IFN-γ stimulation in EMSA studies. They suggest that IRF-1 also induced by IFN-γ forms a physical complex with C/EBPβ (CCAAT/enhancer binding protein β), which subsequently binds not only to the ISRE but also to the GAS-containing proximal DNA, proclaiming a fundamental role of such a complex in regulating further IFN-γ-responsive genes. There is a partial homology between GAS and the C/EBP-E (C/EBPβ response element) that might provide the basis for the observed binding. However, our divergent observations might be due to the employment of another cell system. Whereas Hurgin *et al.* used the hepatocytic cell line Hep G2, we stimulated the colon carcinoma cell line DLD-1. GAS also controls expression of IRF-1 (**I**nterferon **R**egulatory **F**actor-1) [Kumatori *et al.*, 2002]. Accordingly, IFN-γ-induced expression of IRF-1 is suppressed in STAT-1 KO mice [Meraz, 1996]. By binding of STAT-1 to this GAS element IRF-1 expression is induced. The latter might then bind to an ISRE site also present in the IL-18BP promoter (see results figure 14). Besides the GAS element under investigation, we cannot exclude that other IFN-γ responsive sites might contribute to IFN-γ-induced IL-18BPa expression.

4.4 Therapeutic use of IL-18BPa

IL-18 is examined as a prime mediator of T_H1 responses [Nakamura *et al.*, 1989] that are known to be associated with several autoimmune diseases as well as organ transplant rejection. Its opponent IL-18 binding protein (IL-18BP) [Novick *et al.,* 1999] is likely therapeutic useful in the treatment of diseases such as septic shock, inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, toxic liver damage, lupus erythematosus, Still's disease, psoriasis, graft versus host, as well as transplant rejection [Dinarello, 2000]. The most interesting target in counteracting IL-18 action seems to be the IL-18BP splice variant a. This naturally occuring inhibitor exhibited the greatest affinity for IL-18 with a K_d of 399 pM [Kim *et al.*, 2000]. All four IL-18BP isoforms (IL-18BPa/b/c/d) found in humans vary significantly in their ability to neutralize the biological activity of IL-18. Therefore the T_H1 response will be affected in individuals expressing one isoform preferentially compared with another isoform. Perhaps changes in IL-18BP splicing events of hnRNA may provide a regulative tool of IL-18 action. In humans, IL-18BP is constitutively expressed in the spleen

and circulates at plasma concentrations of about 2.5 ng/ml [Novick *et al.*, 2001]. Serum IL-18BP is significantly elevated during sepsis and able to reduce free serum IL-18, indicating its role in regulating immune responses *in vivo* [Novick *et al.*, 2001]. The importance of IL-18BP is further underscored by the observation that Pox virus encode proteins sharing a significant homology to mammalian IL-18BP. Thus infected cells secret a soluble Pox virus-derived vIL-18BP that may modulate host antiviral responses [Vincent *et al.*, 2000]. As shown in previous reports as well as in this study IL-18BPa is induced by IFN-γ in various cells suggesting that it serves as a negative feedback inhibitor of the IL-18-mediated immune response [Mühl *et al.*, 2000; Paulukat *et al.*, 2001]. This induction is quite specific, as it was not triggered by other proinflammatory cytokines. In Hep G2 cells TNF-α and IL-6 enhanced IFN-γ-induced IL-18BPa [Hurgin *et al.*, 2002].

The therapeutic use of IL-18BPa depends on its respective function in disease. For instance, by stimulating immune cells IL-18 exhibits a strong antitumoral activity, protecting experimental animals against repeated challenges with tumor cells [Micallef *et al.*, 1997]. A upregulated IL-18BPa expression would therefore probably suppress anti-tumoral activity. As reported in the present study IFN-γ induces IL-18BPa expression in different colon carcinoma cell lines as well as in cultures of colonic intestinal biopsy specimens. With sodium butyrate we found an inhibitor of IFN-γ-induced IL-18BP expression in these cells. Its capability to strengthen the bioactivity of IL-18 at the colonic microenviroment might explain previous reports demonstrating a protective role of butyrate in colon carcinogenesis [Wächtersheimer and Stein, 2000].

In Crohn's disease (CD), a chronic inflammatory bowel disease, the situation is different. Here IL-18BPa might be an appropiate means to ameliorate disease symptoms. CD is characterized by a marked accumulation of activated T_H1 cells and macrophages in the inflamed intestinal mucosa. There is increasing evidence that an imbalance of T_H1/ T_H2 polarization in favor of T_H1 cell subsets may be a key pathogenic mechanism in a variety of chronic inflammatory disorders and organ-specific autoimmune diseases. Upregulation of bioactive IL-18, as prime mediator of a T_H1 response, has been reported in CD mucosa [Pizarro *et al.*, 1999; Monteleone *et al.*, 1999]. Furthermore, it has been demonstrated that the serum level of IL-18 and the expression of IL-18 by macrophages in inflamed intestinal mucosa are significantly higher in patients with CD. Additionally, there was significant correlation between serum IL-18 concentration and disease severity [Kanai *et al.*, 2000]. Different findings suggest that macrophage-derived IL-18 acting synergistically with IL-12 serves as a potent regulatory factor for intestinal mucosal lymphocytes and contributes to chronic intestinal inflammation in CD. Administration of neutralizing anti-IL-18 antibodies to

the TNBS-treated (2,4,6,-trinitro-benzenesulfonic acid) mice led to a striking improvement in both clinical and histopathological aspects of the disease and frequently completely abrogated the established colitis [Kanai *et al.*, 2001]. Thus macrophage-derived IL-18 system seems to play an important role in the pathogenesis of CD and inhibitory IL-18BP should be therapeutically useful in the treatment of CD patients.

IL-18 mRNA and protein are also present in rheumatoid synovial macrophages [Yamamura *et al.*, 2001], suggesting an important role of this proinflammatory cytokine in the pathogenesis of rheumatoid arthritis. The proinflammatory effects of IL-18 include the direct stimulation of nitric oxide, GM-CSF, TNF-α, IL-1, as well as IL-6 production by macrophages [Gracie *et al.*, 1999; Yamamura *et al.*, 2001; Netea *et al.*, 2000]. The combination of IL-12, IL-15, and IL-18 potently induce IFN-γ production in synovial tissues. Moreover, T_H1 development is further enhanced through IL-1- and TNF-α-stimulated IL-18 production in cultured rheumatoid synovial fibroblasts. In the present study we report IFN-γ-induced IL-18BPa expression in fibroblast-like synoviocytes (FLS) isolated from rheumatoid arthritis patients. Therefore we postulate that FLS might contribute to the complex network of immunoregulation which would ensure control of primarily macrophage-derived IL-18 during synovitis. Administration of IL-18BP ameliorates collagen-induced arthritis (CIA) in mice. Yet the pharmaceutical industry investigates IL-18BPa as therapeutic agent for the treatment of rheumatoid arthritis and clinical phase I studies have been completed.

4.5 Summarizing discussion

IL-18 plays an important role during T_H1 responses in the context of inflammation, infection, and cancer. Accordingly, IL-18 has been associated with human autoimmune diseases and organ transplant rejection. IL-18BPa is a naturally occuring inhibitor that counteracts IL-18 bioactivity. So far IFN-γ is the only known single stimulus for IL-18BPa expression in different human cell lines [Mühl *et al.*, 2000]. In HepG2 cells it has been shown that TNF-α and IL-6 synergistically enhance IFN-γ-induced IL-18BPa [Hurgin *et al.*, 2002]. In the present study we report IFN-γ-mediated induction of IL-18BPa in different colon carcinoma cell lines, in organ cultures of colonic intestinal biopsy specimens, keratinocytes and in fibroblast-like synoviocytes isolated from rheumatoid arthritis patients (RA-FLS). Therefore, we conclude that IFN-γ-induced IL-18BPa expression likely is a more general phenomenom, common to various cell types. By inducing IL-18BPa, IFN-γ triggers a negative feedback loop which limits IFN-γ-dependent and –independent actions of IL-18. The proposed IFN-γ-driven feedback regulatory pathway agrees with amplified production of IFN-γ in IFN-γ receptor knockout mice, evaluated in models of inflammatory diseases

[Camoglio *et al.*, 2000; Matthys *et al.*, 1998]. Therefore, IL-18BPa has the potential to restore a hypothetically disturbed IL-18/IL-18BP balance in autoimmune diseases such as rheumatoid arthritis and Crohn's disease. The association between IL-18 and expression of its decoy receptor has been observed in patients with sepsis [Novick *et al.*, 2001], Still's disease [Kawashima *et al.*, 2001], and psoriasis [Ohta *et al.*, 2001]. On the contrary, in the case of carcinogenesis IFN-γ-induced IL-18BPa expression might be disadvantageous. In particular, when coinduced together with IFN-γ-dependent iNOS, IL-18BPa may facilitate tumorgenesis.

Protective functions of IFN-γ have been shown in murine models for rheumatoid and septic arthritis (Vermeire *et al.*, 1997; Puliti *et al.*, 2000), and in rheumatoid arthritis patients (German Lymphokine Study Group, 1992). These effects may be due to IL-18BPa expression. IFN-γ-mediated induction of IL-18BPa in RA-FLS could be an important regulative, since proinflammatory IL-18 appears to play an important role in the pathogenesis of rheumatoid arthritis. IL-18 is able to activate T cells and macrophages in the microenviroment of RA synovitis. Located upstream in the cascade of proinflammatory cytokines, IL-18 can be seen as pivotal inducer of subsequent cardinal inflammatory cytokines (e.g. IL-1 and TNF-α) which contribute to the pathogenesis of RA. By producing IL-18BPa, FLS may contribute to the complex network of immunoregulation which ensures control of IL-18 during synovitis. A recent report provides evidence that IFN-γ ameliorates joint inflammation when given later in the inflammatory process. In this state of disease inhibition of IL-18 through IFN-γ-induced IL-18BPa should represent a negative feedback mechanism upon established inflammation. Indeed, the pharmaceutical industry successfully used IL-18BP as therapeutic agent in a murine model of RA [Plater-Zyberk *et al.*, 2001] and in phase I clinical trials [http://www.serono.com/index.jsp]. Another autoimmune disease being connected with pathologic IL-18 action is Crohn's disease (CD). CD is characterized by a marked accumulation of activated T_H1 type $CD4^+$ T cells and macrophages in inflamed intestinal mucosa. Upregulation of bioactive IL-18 has been reported in CD mucosa [Pizarro *et al.*, 1999; Monteleone *et al.*, 1999]. Furthermore, there was significant correlation between serum IL-18 concentration and disease severity [Kanai *et al.*, 2000]. Therefore, therapeutic use of IL-18BPa may restore a hypothetically disturbed IL-18/IL-18BP balance in Crohn's disease thereby counteracting a T_H1-promoted immune response.

In contrast, IL-18BPa expression might be disadvantageous, too. There is evidence that IL-18 is an inhibitor of tumor growth. IL-18 acts as inhibitor of angiogenesis [Cao *et al.*, 1999] and augments Fas/Fas-ligand dependent $CD4^+$ T cell- and NK cell-cytotoxicity [Dao *et al.*, 1996; Tsutsui *et al.*, 1996; Osaki *et al.*, 1998]. Previous reports demonstrate that sodium

butyrate can be protective against development of colon cancer by augmenting the sensitivity of colon carcinoma cells towards Fas-mediated apoptosis [Bonnotte *et al.*, 1998]. In our experimental set up butyrate inhibited IFN-γ-induced IL-18BPa expression. Intestinal butyrate may therefore have the capability to strengthen the bioactivity of IL-18 at the colonic environment, which should be beneficial. IFN-γ itself shows tumor suppressive potential, but under defined conditions IFN-γ also appears to be capable of enhancing growth of colon carcinomas [Ramani *et al.*, 1987]. This observation might be due to IFN-γ-mediated induction of IL-18BPa expression. Altogether, sodium butyrate appears to shift the IL-18/IL-18BP balance in colon carcinoma cells in favor of IL-18, which may contribute to local tumor protection by this compound, likely via IFN-γ-independent tumor-suppressive actions of IL-18. To complete the picture it also noteworthy, that IL-18 may also augment expression of certain genes which have been associated with cancer progression, e.g. adhesion molecules. Accordingly, IL-18 increased metastasis in murine melanoma model [Wang *et al.*, 2001].

The suggested negative feedback model of IL-18 action (figure 17) shown in this study can also be observed in the cocultur system, that we employed to simulate an inflammatory T_H1 response. By counterregulating the IL-18 arm of immune defenses against tumors, IL-18BP may have the potential to promote carcinogenesis. In several murine models IL-18 exerts either a direct or an IFNγ-dependent tumor-suppressive function. These are associated with enhanced NK dependent cell killing or with reduction of angiogenesis in the tumor microenviroment, respectively [Coughlin *et al.*, 1998; Osaki *et al.*, 1998; Cao *et al.*, 1999]. In addition, induction of iNOS may provide a second inhibitory way for tumor suppressive effects of IL-18. Upregulation of iNOS has been shown in different types of cancer. Although data on immunoregulatory roles of NO are not entirely uniform [Bogdan, 2001], analysis of iNOS knockout mice suggests that high-output production of NO likely represents a pathway that inhibits production of IFN-γ [Wei *et al.*, 1995; Shi *et al.*, 2001]. Different mechanisms for impaired IFN-γ production associated with NO can be envisioned (figure 17). NO is supposed to interfere with maturation of pro-IL-18 by inhibiting caspase-1 (ICE) activity [Kim *et al.*, 1998], but there are also reports of caspase-1 independent IL-18 processing and secretion [Mehta *et al.*, 2001]. Furthermore, NO is capable of reducing proliferation of T-lymphocytes possibly via disruption of the Jak3/STAT5 signaling pathway [Bingisser *et al.*, 1998; Koblish *et al.*, 1998]. NO also has been shown to impair IL-12 production by macrophages [Huang *et al.*, 1998], which usually acts in synergism with IL-18 to induce IFN-γ. Besides suppression of IFN-γ production, NO may facilitate tumor growth by several additional mechanisms. For instance, iNOS in human colon cancer tissues is positively correlated with the frequency of mutations in the p53 tumor suppressor

gene. Furthermore, NO has been recognized as an inhibitor of apoptotic cell death under certain conditions [Liu and Stamler, 1999]. Tumors derived from iNOS overexpressing DLD-1 cells are markedly more vascularized [Jenkins *et al.*, 1995]. Moreover, iNOS-derived NO should mediate vasodilatation at the tumor site leading to increased blood supply, which will likely favor tumor growth [Andrade *et al.*, 1992]. Finally, NO appears to be a mediator of inflammation, leading to leukocyte infiltration into tumor tissues. Colonic inflammation and macrophages infiltration are supposed to support progression of colorectal cancer [Etoh *et al.*, 2000]. In conclusion, lack of IFN-γ is associated with distant metatasis and poor prognosis in colorectal cancer [Pages *et al.*, 1999]. By counterregulating the IL-18- and IFNγ-dependent arm of immune defenses against tumors, both IL-18BP and iNOS, have the potential to promote carcinogenesis by mechanisms that likely include tumor-mediated immunosuppression and angiogenesis.

As mentioned earlier, IFN-γ is the only known single stimulus for IL-18BPa induction so far [Mühl *et al.*, 2000]. In HepG2 cells it has been reported that TNF-α and IL-6 synergize with IFN-γ for IL-18BPa expression [Hurgin *et al.*, 2002]. IFN-γ uses the Jak/STAT-pathway for signal transduction. Phosphorylated STAT-1 proteins homodimerize and finally translocate to the nucleus, where they bind to a consensus sequence called GAS (Gamma-activated-site). In the present work we identified a GAS element proximal to the start of transcription. EMSA analysis revealed STAT-1 binding to this element. Furthermore, two IL-18BP promoter fragments containing this GAS element were highly inducible following IFN-γ-stimulation in luciferase assays. Mutagenesis of this GAS site led to strong reduction of the fragments IFN-γ-inducibility. We also found other IFN-γ responsive sites in our promoter fragments, namely γ-IRE (γ-Interferon responsive element) and ISRE (IFN-stimulated response element). STAT-1 might also directly bind to two γ-IRE sites present in the cloned IL-18BP promoter luciferase fragments. Putative induction via ISRE may be mediated by STAT-1 induced IRF-1 (Interferon Regulatory Factor-1), since a GAS element controls IRF-1 expression as well.

V
Summary

In this study we investigated the regulation of IL-18BPa by IFN-γ in the context of colon cancer and human autoimmune diseases. IL-18BPa is a naturally occuring inhibitor that counteracts IL-18 bioactivity. By enhancing IFN-γ production IL-18 has been introduced as pivotal mediator of T_H1 immune responses. Indeed, many IL-18 effects are mediated by IFN-γ. IL-18 bioactivity is connected with the pathogenesis of different inflammatory diseases, for instance, septic shock, colitis, Crohn's disease, myasthenia gravis, multiple sclerosis, rheumatoid arthritis, atherosclerosis, and organ transplant rejection. In addition, IL-18 has tumor-suppressive properties. IFN-γ induced IL-18BPa expression was shown on protein and mRNA level in different colon carcinoma cell lines, organ cultures of colonic intestinal biopsy specimens, HaCaT keratinocytes as well as rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS). The IFN-γ-mediated induction of IL-18BPa appears to be a more general phenomenom. The capability of IFN-γ to induce IL-18BPa also has been confirmed on the promoter level by performing luciferase reporter gene studies with two IL-18BP promoter fragments. A GAS-site proximal to the transcription start site has been identified to be relevant for IFN-γ-mediated induction of these two IL18BP promoter fragments. The induction of IL-18BPa is most likely mediated by STAT-1 in DLD-1 colon carcinoma cells. Sodium butyrate inhibited IFN-γ-induced IL-18BPa expression in these cells. On the basis of our observations, we postulate a negative feedback mechanism, by which IFN-γ-dependent and –independent IL-18 action might be counterregulated. In this model sodium butyrate is an additional player, that may interrupt the postulated negative feedback loop. A coculture system was performed to simulate an inflammatory T_H1 response. This model which is more close to the *in vivo* situation, confirmed upregulation of IL-18BPa by endogenously produced IFN-γ.

The role of IL-18BPa is manifold and depends on IL-18 function in each particular case. In autoimmune diseases, for instance, which are often characterized by a T_H1 polarized immune response, IL-18BPa might counterregulate IL-18 and/or IL-18-induced IFN-γ bioactivity. Important examples are Crohn's disease and rheumatoid arthritis. In CD therapeutic use of IL-18BPa may therefore restore a hypothetically disturbed IL-18/IL-18BP balance. Concerning RA, IL-18BPa expression might contribute to protective functions of IFN-γ, observed in different murine models for arthritis and in rheumatoid arthritis patients.

Moreover, IL-18BPa might inhibit IL-18-mediated induction of subsequent cardinal inflammatory cytokines responsible for the pathogenesis of these diseases. Indeed, the pharmaceutical industry successfully used IL-18BP as therapeutic agent in a murine model of RA and in phase I clinical trials. On the contrary, in the context of carcinogenesis IFN-γ-mediated IL-18BPa expression might be disadvantageous. By counterregulating the IL-18 arm of immune defenses against tumors, IL-18BP may have the potential to promote carcinogenesis. Our hypothesis is underlined by the observation that sodium butyrate, known to be protective in colon cancer, inhibited IFN-γ-induced IL-18BPa expression. In parallel, IL-18-induced IFN-γ is also responsible for iNOS induction. iNOS-derived NO provides a second possible way for inhibition of IFN-γ-dependent and –independent tumor suppressive effects of IL-18. Finally, IFN-γ-induced IL-18BPa expression was confirmed on the promoter level. This induction on the promoter level was associated with STAT-1 binding to the GAS element proximal to the start of transcription.

It is tempting to speculate that blockage of the cytokine cascade upstream of IL-1 and TNF-α on the level of IL-18 may be of therapeutic benefit. Our data reflect the relationship between inflammation and cancer, in that inflammatory cells and cytokines found in tumors are likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective host antitumour response.

VI
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VII
Appendix

7.1 Abbreviations

B	sodium butyrate
BSA	bovine serum albumin
Bref A	Brefeldin A
CD	Crohn's disease, cluster of differentiation
cDNA	copy or complementary DNA
cpm	counts per minute
C	Control
d	Day
DEPC	diethyl pyrocarbonate
dest	Distilled
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	*et alter*
FCS	fetal calf serum
fig	Figure
FLS	fibroblast-like synoviocytes
γ-IRE	γ-interferon responsive element
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAS	gamma-activated site
h	hour(s), human
HEPES	N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)
ICE	interleukin-1β converting enzyme
IFN	Interferon
Ig	Immunoglobulin

IGIF	interferon-γ inducing factor
IL	Interleukin
IL-18BP	interleukin-18 binding protein
iNOS	inducible nitric oxide synthase
IPTG	isopropylthiogalactoside
ISRE	IFN-stimulated response element
kDa	kilo Dalton
LB	Lauria-Bertani
LPS	lipopolysaccharide
LUC	Luciferase
m	mature, molar
min	minute(s)
MMP	matrix metalloproteinase
mRNA	messenger RNA
nd	not detecable
NO/NOS	nitric oxide/nitric oxide synthase
OA	ocadaic acid
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	potentia hydrogenii
PVDF	polyvinylidene fluoride
RA	rheumatoid arhtritis
RNA	ribonucleic acid
RNase	Ribonuclease
ROS	reactive oxygen species
rpm	rounds per minute
RT	reverse transcription, room temperature
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	second(s)
SEM	standard error of the mean
TCA	trichloroacetic acid
TEMED	N,N,N´,N´-tetramethylethylenediamine
Th	T helper cell
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
U	unit(s)

UV	Ultraviolet
URL	uniform resource locator
UTP	uridine 5´-triphosphate
v/v	volume per volume
VEGF	vascular endothelial growth factor
w/v	weight per volume
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

7.2 Journal publications

Muhl H, Chang JH, Huwiler A, Bosmann M, **Paulukat J**, Ninic R, Nold M, Hellmuth M, and Pfeilschifter J (2000) Nitric oxide augments release of chemokines from monocytic U937 cells: modulation by anti-inflammatory pathways. Free Radic Biol Med 29: 969-980.

Kämpfer H, **Paulukat J**, Muhl H, Wetzler C, Pfeilschifter J, Frank S (2000) Lack of interferon-gamma production despite the presence of interleukin-18 during cutaneous wound healing. Mol Med 6: 1016-1027.

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Moeller B, **Paulukat J**, Kokoc-Zivojnov N, Pfeilschifter J, and Mühl H (2003) Interferon-γ induces expression of interleukin-18 binding protein in fibroblast-like synoviocytes. Rheumatology 42: 442-445.

7.3 Congress contributions

7.3.1 Poster presentation

Paulukat J, Bosmann Markus, Pfeilschifter J, and Mühl H (2001) Interferon-γ mediates release of interleukin-18 binding protein from colon carcinoma/epithelial cells. Scand J Immunol 54, Suppl. 1. *11th International Congress of Immunology, Stockholm (Sweden), July 2001.*

7.3.2 Talk

Paulukat J, Nold M, Ninic R, Bosmann M, Pfeilschifter J, and Mühl H (2002) IL-12 and IL-18 activated PBMC mediate expression of IL-18 binding protein and iNOS in adjacent DLD-1 colon carcinoma/epithelial cells. N-S ARCH PHARMACOL 365: 279 Suppl. 1. *43rd Spring meeting of DGPT, März 2002.*

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7.5 Curriculum vitae

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04/1998 – 10/1999	Diplomarbeit am Botanischen Institut der Technischen Universität Carolo-Wilhemina zu Braunschweig bei Prof. Dr. R. R. Mendel mit dem Titel: „Charakterisierung der Domänen und Splicevarianten des Gephyrins hinsichtlich ihrer Beteiligung an der Molybdäncofaktor Biosynthese“.
11/1999 – 11/2002	Experimentelle Arbeiten der Dissertation am Institut für Allgemeine Pharmakologie und Toxikologie (Direktor: Prof. Dr. J. Pfeilschifter) des Klinikums der Johann Wolfgang Goethe-Universität Frankfurt am Main unter Betreuung von Dr. H. Mühl. Thema der Dissertation: „Regulation of IL-18 binding protein by IFN-γ“.

7.6 Deutsche Zusammenfassung

Zytokine fungieren als biochemische Botenstoffe zwischen Zellen. Sie sind involviert in Prozesse wie der Regulation der Immunantwort, Streß, Zellwachstum und -differenzierung, sowie Gewebsregeneration. Die Balance zwischen pro- und antientzündlichen Zytokinen bestimmt den Ausgang einer durch Entzündung bedingten Immunantwort. Zytokine interagieren mit spezifischen Zytokininhibitoren sowie löslichen Zytokinrezeptoren. Solche Mechanismen ermöglichen eine genau abgestimmte Regulation der Immunantwort. Der Effekt eines jeden Zytokins ist abhängig von der zeitlichen Zytokin-Freisetzung, dem lokalen Milieu in welchem es agiert, der Präsenz von verdrängenden oder synergistischen Elementen, der Dichte von Zytokinrezeptoren sowie der gewebeabhängigen Antwort auf das jeweilige Zytokin.
Interleukin 18 (IL-18) als Hauptmediator einer T_H1 Immunantwort, sowie sein natürlich vorkommender Inhibitor IL-18 Bindeprotein (IL-18BP) sind zwei neu beschriebene Kontrahenten im Zytokinnetzwerk. IL-18 verstärkt die IFN-γ Produktion, welche letztendlich viele Effekte dieses Zytokins vermittelt. Lokale Konzentrationen beider Gegenspieler, IL-18 und IL-18BP, bestimmen die biologische Funktion von IL-18 im Zusammenhang mit Entzündung, Infektion und Krebs. Die jeweilige IL-18 Bioaktivität steht dabei in Verbindung mit der Pathogenese diverser entzündlicher Krankheiten wie z.B. Morbus Crohn, Multiple Sklerose, rheumatoide Arthritis (RA) und Atheriosklerose. Durch IL-18 ausgelöste T_H1 Immunantworten sind von Bedeutung bei verschiedenen Autoimmunerkrankungen sowie der Abstoßung von transplantierten Organen. Die Bioaktivität von IL-18 wird besonders durch IL-18BPa moduliert. Diese Splice-Variante des Bindeproteins wurde als Hauptform in verschiedenen humanen cDNA Bibliotheken entdeckt und zeigt höchste Affinität für IL-18. IFN-γ scheint der vorherrschende Stimulus für IL-18BPa Expression in verschiedensten humanen Zelltypen zu sein [Mühl *et al.*, 2000].

Mit diesem Hintergrund haben wir die IFN-γ-vermittelte Regulation der Expression von IL-18BPa sowie dessen Freisetzung untersucht. Die Experimente wurden in verschiedenen humanen Zelltypen durchgeführt, welche in Verbindung stehen mit der Etiology von Kolon Karzinogenese und Autoimmunerkrankungen, wie z.B. Morbus Crohn und rheumatoide Arthritis. In der vorliegenden Arbeit konnten wir die IFN-γ-vermittelte Induktion von IL-18BPa in verschiedenen Kolon Karzinom Zellinien (DLD-1, LoVo, Caco-2 und HCT116), in Kulturen von Dickdarm Biopsien, in Fibroblasten-ähnlichen Synoviozyten isoliert von rheumatoide Arthritis - Patienten sowie in HaCat Keratinozyten zeigen. Wir schlossen daraus, daß IFN-γ-vermittelte IL-18BPa Expression ein generelles Phänomen ist, das verschiedensten Zelltypen eigen zu sein scheint. Durch die Induktion von IL-18BPa durch

IFN-γ wird ein negativer Rückkopplungsmechanismus eingeschaltet, der IFN-γ-abhängige und -unabhängige IL-18 Aktivität limitiert. Dieser durch IFN-γ-ausgelöste Rückkopplungsmechanismus zeigt seine Berechtigung in der beobachteten vermehrten Produktion von IFN-γ in IFN-γ-Rezeptor „Knockout" Mäusen [Camoglio *et al.*, 2000; Matthys *et al.*, 1998]. Auf diese Weise hat IL-18BPa das Potential, eine gestörte T_H1/T_H2-Balance bei Autoimmunerkrankungen wie rheumatoide Arthritis und Morbus Crohn, wiederherzustellen. Eine Verbindung zwischen IL-18 sowie der Expression seines inhibitorischen Gegenspielers IL-18BPa ist in Patienten mit Sepsis [Novick *et al.*, 2001], Still's Krankheit [Kawashima *et al.*, 2001] und Schuppenflechte [Ohta *et al.*, 2001] beobachtet worden. An dieser Stelle sei aber auch erwähnt, daß IL-18BPa eine komplexe vielfältige Rolle spielt, die von der IL-18 Funktion im jeweiligen speziellen Fall abhängig ist. So könnte sich die IFN-γ-induzierte IL-18BPa Expression im Zusammenhang mit Karzinogenese nachteilig auswirken und das Tumorwachstum verstärken.

Protektive Funktionen von IFN-γ sind gezeigt worden in murinen Modellen für rheumatoide und septische Arthritis (Vermeire *et al.*, 1997; Puliti *et al.*, 2000) sowie in RA-Patienten (German Lymphokine Study Group, 1992). Diese Effekte könnten auf die Induktion des IL-18BPa zurückgeführt werden. IFN-γ-vermittelte Expression von IL-18BPa in RA-fibroblasten-ähnlichen Synoviozyten wäre somit ein wichtiges Regulativ für IL-18 Aktivität, welche eine wichtige Rolle in der Pathogenese von rheumatoider Arthritis zu spielen scheint [Dayer, 1999]. IL-18 ist in der Lage, T Zellen und Makrophagen in der Mikroumgebung der rheumatoide Arthritis Synovitis zu aktivieren. In der Kaskade von proentzündlichen Zytokinen proximal angesiedelt, kann IL-18 als ursprünglicher Auslöser von nachfolgenden kardinalen inflammatorischen Zytokinen angesehen werden. Dazu gehören IL-1 und TNF-α, welche entscheidend zur Pathogenese von rheumatoider Arthritis beitragen. Durch die Synthese von IL-18BPa könnten Fibroblasten-ähnlichen Synoviozyten zum komplexen immunoregulatorischen Netzwerk beitragen, das die Kontrolle von IL-18 Aktivität während Synovitis sicherstellt. Aktuelle Daten aus Tiermodellen belegen, daß IFN-γ die Symptomatik einer Gelenkentzündung verbessern kann, wenn es spät im entzündlichen Prozeß verabreicht wird. In diesem Stadium der Krankheit könnte die Hemmung von IL-18 durch IFN-γ-induziertes IL-18BPa einen negativen Rückkopplungsmechanismus gegenüber fortwährender Entzündung repräsentieren. In der Tat wird IL-18BPa zur Zeit in klinischen Phase I [http://www.serono.com/index.jsp] Studien zur späteren Behandlung der rheumatoiden Arthritis getestet.

Eine weitere Autoimmunerkrankung, die in enger Verbindung steht mit pathologischer IL-18 Aktivität, ist Morbus Crohn. Diese ist charakterisiert durch eine auffällige Akkumulation von aktivierten $CD4^+$ T Zellen vom T_H1-Typ und Makrophagen in der entzündeten intestinalen Mucosa. Eine vermehrte Produktion von bioaktiven IL-18 ist in der Mucosa von Morbus

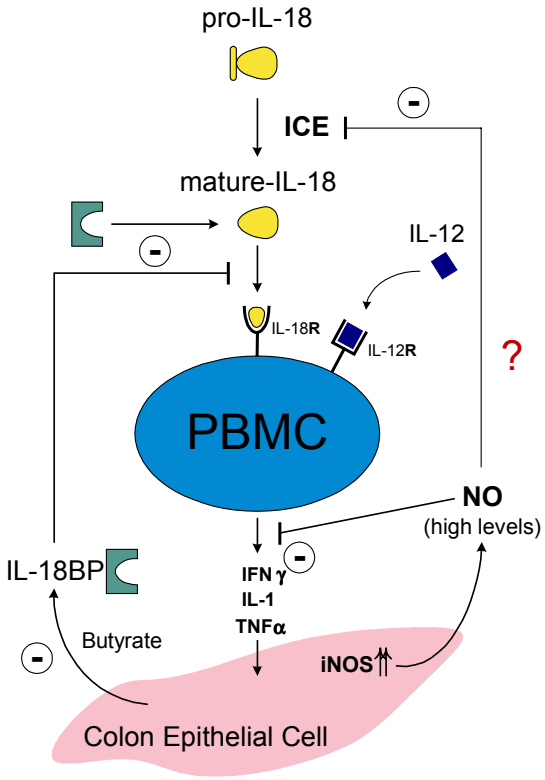
Crohn Patienten gezeigt worden [Pizarro *et al*., 1999; Monteleone *et al.*, 1999]. Weiterhin war eine signifikante Korrelation zwischen Serum IL-18 Konzentrationen und der jeweiligen Schwere der Erkrankung auffällig [Kanai *et al.*, 2000]. IL-18BPa könnte aus diesen angeführten Gründen von therapeutischem Nutzen sein, indem es eine hypothetisch gestörte IL-18/IL-18BPa Balance bei Morbus Crohn wiederherstellt und somit einer vorangetriebenen T_H1-Immunantwort entgegenwirkt.

Eine erhöhte IL-18BPa Expression könnte sich im Rahmen der Kolon Karzinogenese nachteilig auswirken. So gibt es Hinweise, daß IL-18 als Inhibitor von Tumor Wachstum fungiert, indem es als Angiogenese Hemmer [Cao *et al.*, 1999] arbeitet und die Fas/Fas-Ligand abhängige $CD4^+$ T Zell- und NK Zell-Zytotoxicität verstärkt [Dao *et al.*1996; Tsutsui *et al.*, 1996; Osaki *et al.*, 1998]. Da gezeigt worden ist, daß Butyrat in punkto Entwicklung von Kolon Krebs potentiell protektiv ist, haben wir uns für den Einfluß von Butyrat auf die IL-18BPa Expression interessiert. Wir konnten zeigen, daß Butyrat die IFN-γ-induzierte IL-18BPa Expression und Freisetzung aus DLD-1 Zellen hemmt. Intestinales Butyrat könnte daher die Fähigkeit haben, die Bioaktivität von IL-18 in der Umgebung des Dickdarms zu verstärken. Dies wäre ein durch IL-18 vermittelter tumor-suppressiver Effekt des Butyrat, welcher übereinstimmt mit der Beobachtung, daß diese kurzkettige Fettsäure die Sensitivität von Kolon Karzinom Zellen gegenüber Fas-vermittelter Apoptose erhöht [Bonnotte *et al.*, 1998]. Interessanterweise scheint IFN-γ unter definierten Bedingungen in der Lage zu sein, das Wachstum von Kolon Karzinomzellen zu verstärken [Ramani *et al.*, 1987]. Diese Beobachtung könnte sich erklären aus IFN-γ-vermittelter Induktion von IL-18BPa Expression. Zusammengefaßt läßt sich festhalten, daß Butyrat die IL-18/IL-18BPa Balance in Kolon Karzinomzellen in Richtung von IL-18 verschiebt, was zur lokalen Tumorprotektion durch diese Substanz beitragen könnte.

Bisher haben wir berichtet, daß IFN-γ IL-18BPa Expression in verschiedenen Kolon Zelltypen, Keratinozyten, sowie Primärkulturen von fibroblasten-ähnlichen Synoviozyten induziert. Unsere Daten stammen aus Stimulationsexperimenten mit exogen hinzugegebenen rekombinanten IFN-γ. Um eher einer *in vivo* Situation zu entsprechen, entschieden wir uns für Kokultur Experimente, welche uns die Simulation einer T_H1-Immunantwort erlaubten. In diesem experimentellen Aufbau untersuchten wir die IL-18BP- sowie induzierbare NO-Synthase (iNOS) - Geninduktion in humanen DLD-1 Kolon Karzinom/Epithelzellen, die mit IL-12/IL-18 aktivierten PBMC in einem „Transwell"-System kokultiviert wurden. Ebenso wie IL-18 vermittelt die iNOS wichtige Funktionen im Zusammenhang mit Entzündung, Infektion und Krebs. In verschiedenen Typen von Krebs ist eine Hochregulation der iNOS tatsächlich beschrieben worden. In unseren Kokulturen

wurden iNOS und IL-18BPa in DLD-1 Zellen nur in Gegenwart von IL-12/IL-18-stimulierten PBMC exprimiert. Diese Induktion von iNOS und IL-18BPa war abhängig von endogen produziertem IFN-γ, das von IL-12/IL-18-aktivierten T und NK Zellen freigesetzt wurde.

Auch innerhalb dieses Kokultursystems konnte also der von uns vorgeschlagene negative Rückkopplungsmechanismus (IL-18 => IFN-γ => IL-18BPa) beobachtet werden. IL-18BPa hat hierbei das Potential, Karzinogenese zu fördern, indem es den IL-18 Arm der Immunabwehr gegenüber Tumoren schwächt. Die Induktion der iNOS parallel zu IL-18BPa stellt einen weiteren Weg dar, der das Tumorwachstum begünstigen könnte. Obwohl die Datenlage für die immunregulatorische Rolle von NO nicht einheitlich ist [Bogdan, 2001], zeigt die Analyse von iNOS Knockout Mäusen, daß die verstärkte Produktion von NO wahrscheinlich einen inhibitorischen Weg für IFN-γ Produktion repräsentiert [Wei *et al.*, 1995; Shi *et al.*, 2001]. Dabei sind verschiedene Mechanismen vorstellbar: Einerseits wird diskutiert, daß NO mit der Reifung von pro-IL-18 interferiert durch Hemmung der Caspase-1 (ICE) Aktivität [Kim *et al.*, 1998]. Andererseits gibt es auch Berichte über Caspase-1 unabhängiges IL-18 „Processing“ im humanen System [Mehta *et al.*, 2001]. Insofern steht der Nachweis der Relevanz für das humane System in Bezug auf die Hemmung der Caspase-1 durch NO noch aus. Daneben kann NO die Proliferation von T-Lymphozyten einschränken. Dies geschieht wahrscheinlich durch die Unterbrechung des Jak3/STAT5 Signaltransduktionsweges [Bingisser *et al.*, 1998; Koblish *et al.*, 1998]. Es konnte auch gezeigt werden, daß NO die IL-12 Produktion von Makrophagen beeinträchtigt, welches häufig mit IL-18 synergistisch zur Induktion von IFN-γ beiträgt. Neben der Unterdrückung der IFN-γ Produktion, kann NO Tumorwachstum durch einige weitere Mechanismen erleichtern. Beispielsweise korreliert die iNOS in humanen Kolonkrebs Geweben positiv mit der Frequenz von Mutationen im p53 Tumor Suppressor Gen. Ein weiterer Beleg für die Tumorwachstum fördernde Wirkung von NO ist die Beobachtung, daß iNOS-überexprimierende Tumore deutlich stärker vaskularisiert sind [Jenkins *et al.*, 1995]. Darüberhinaus kann durch iNOS gebildetes NO zur Vasodilatation im Tumor führen. Dies hat eine gesteigerte Blutzufuhr und Energieversorgung im Tumor zur Folge und würde zwangsläufig das Tumorwachstum begünstigen. Weiterhin scheint NO ein Entzündungsmediator zu sein, der eine verstärkte Infiltration von Leukozyten ins Tumorgewebe nach sich zieht. Dies ist relevant, weil gastrointestinale Entzündungen als Präkanzerosen die Progression von Kolontumoren begünstigen. Es konnte gezeigt werden, daß das Fehlen von IFN-γ verbunden ist mit der Bildung von entfernten Metastasen und schlechter Prognose in punkto Dickdarm Krebs [Pages *et al.*, 1999]. Durch Gegenregulation des IL-18- und IFN-γ-abhängigen Armes der Immunabwehr gegenüber Tumoren haben IL-18BP und iNOS das Potential, eine Karzinogenese zu fördern.



Negativer Rückkopplungsmechanismus reguliert die Bioaktivität von IL-18 und die Produktion von IFN-γ.

Zwei verschiedene negative Rückkopplungsmechanismen sind denkbar, welche die Bioaktivität von IL-18 und die Produktion von IFN-γ inhibieren können. Dabei induziert zunächst IL-18 die Expression von IFN-γ in Synergismus mit IL-12.

1) IFN-γ vermittelt die Expression der iNOS in Kolon Epithelzellen. Hohe Konzentrationen des durch die iNOS gebildeten NOs modulieren die IFN-γ Produktion an verschiedenen Stellen.

2) IFN-γ induziert die Expression von IL-18BPa in Kolon Epithelzellen.
Butyrat unterdrückt die IFN-γ-induzierte IL-18BPa Expression und fördert damit die IL-18 Bioaktivität.

Wir stellten bereits fest, daß die IFN-γ-induzierte IL-18BPa Expression ein generelles Phänomen zu sein scheint, welches nicht nur auf Epithelzellen im Dickdarm beschränkt ist. Weitere Publikationen berichten über die IFN-γ-induzierte IL-18BPa Expression auch in renalen Mesangiumzellen [Mühl *et al.*, 2000], Hepatozyten [Rubinstein *et al.*, 2001] PBMCs und Monozyten [Corbaz *et al.*, 2002], sowie endotheliale HUVEC („human umbilical vein endothelial cells“) Zellen [Corbaz *et al.*, 2002]. Daher entschlossen wir uns dazu, den Mechanismus der IFN-γ-vermittelten IL-18BPa Induktion näher auf Ebene des Promoters zu untersuchen. Wie bereits erwähnt ist IFN-γ der Hauptstimulus einer IL-18BPa Induktion. In der vorliegenden Arbeit wurde von uns ein GAS („Gamma activated site“) Element proximal zum Transkriptionstart identifiziert. Mit Hilfe einer EMSA Analyse konnte die Bindung des Transkriptionsfaktors STAT-1 an dieses Element nachgewiesen werden. Weiterhin wurden zwei IL-18BP Promoter Fragmente kloniert, bei denen das beschriebene GAS Element im 3'-Bereich lokalisiert ist. Diese Konstrukte waren nach IFN-γ Stimulation stark induzierbar im Luciferase-Assay. Mutagenese des GAS Elements hingegen führte zur starken Reduktion der Induzierbarkeit beider Fragmente durch IFN-γ. Neben GAS wurden von uns noch weitere Elemente im IL-18BP Promoter entdeckt, die auf IFN-γ reagieren können, wie z.B. γ-IRE („γ-Interferon responsive element“) und ISRE („IFN-stimulated response element“). STAT-1 könnte daher ebenfalls direkt an zwei γ-IRE Regionen binden,

welche Bestandteil der von uns klonierten IL-18BP Promoter Luciferase Fragmente sind. Ebenso denkbar wäre eine putative Induktion über ISRE vermittelt durch STAT-1 induziertes IRF-1 („Interferon Regulatory Factor-1“), da ein GAS Element auch die Expression von IRF-1 kontrolliert. Nachfolgende Arbeiten werden diese Fragestellung im einzelnen behandeln.

Aufgrund der Datenlage ist man versucht zu schließen, daß eine Blockade der Zytokine Kaskade oberhalb von IL-1 und TNF-α auf der Ebene von IL-18 einen therapeutischen Nutzen bringen könnte. Die endogene Produktion von IL-18BPa nach IFN-γ stellt einen negativen Rückkopplungsmechanismus dar, der einerseits Entzündungsreaktionen limitiert, andererseits aber auch tumorsuppressive Eigenschaften von IL-18 einschränkt und so im Rahmen der Tumorabwehr kontraproduktiv sein kann.