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Negative Feedback Loops in Inflammation

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Der Zweifel ist der Beginn der Wissenschaft. Wer nichts anzweifelt, prüft nichts. Wer nichts prüft, entdeckt nichts. Wer nichts entdeckt, ist blind und bleibt blind.

Teilhard de Chardin

Danksagung:

Mein Dank gilt Prof. Dr. Rainer de Martin, der es mir ermöglicht hat in seinem Labor an meinem Diplomprojekt zu arbeiten.

Ebenfalls möchte ich mich bei meinen Kollegen Uli, Yvonne und Renate bedanken, die mir die Arbeit durch praktische Tipps und oft auch durch ihre moralische Unterstützung erleichtert haben.

Besonderer Dank gebührt meiner Familie, die mich während meines gesamten Studiums in jeder erdenklichen Hinsicht unterstützt hat.
Auch zu erwähnen sei meine Studienkollegin Maria Hölzl die mir vor allem in administrativen Belangen zur Seite gestanden ist.

Table of Contents:

1. Summary:

Inflammation is a beneficial response of the organism to harmful stimuli, but it can have detrimental effects if not resolved in time or occurs exaggerated. Chronic inflammatory conditions play a role in the pathogenesis of various severe diseases. Thus, mechanisms that cause shutting down of inflammation are important targets of studies. Endothelial cells lining the vasculature are actively contributing to inflammatory reactions. Upon pro-inflammatory stimulation they undergo phenotypic changes that enable the passage of immune cells through the endothelium to the side of injury. On the molecular level these processes are mainly regulated by inducible transcription factors like NF-κB, AP-1 and NFAT. Amongst their target genes are not only pro-inflammatory genes, but also genes encoding proteins that cause the resolution of the reaction at later stages. These gene products represent “negative feedback loops”. For the identification of novel negative feedback loops, some genes were selected that were found previously to be inducible in human vascular endothelial cells (HUVEC) by the pro-inflammatory cytokine IL-1, and are supposed to have repressive potential. These genes of interest were cloned into mammalian expression vectors and used to perform different reporter gene assays.

One of the examined genes, GG2-1, encodes a member of the FLICE-inhibitory family of anti-apoptotic proteins that possesses oncogenic potential. In AP-1 and NF-κB reporter gene assays, the protein exhibited significant inhibitory effects on the transcription factors when their activation was achieved by co-transfection of MEKK1. Accordingly, the activity of the IL-8 promoter was suppressed by GG2-1. Western blotting revealed that the decrease of MEKK1 on the protein level correlated with increasing concentrations of GG2-1. This effect seemed to be specific for MEKK1, since protein levels of other MAP3K were not influenced by GG2-1 overexpression. Co-immunoprecipitation excluded direct protein interaction between the two proteins.

Another target of studies was JAG1 that encodes one of the ligands of Notch1, Jagged-1. Notch signalling is involved in cell-cell communication and developmental processes. Mutations of JAG1 cause the Alagille syndrom (AGS), an autosomal dominant disorder affecting various systems of the body. In endothelial cells Jagged-1 plays a role in contact-inhibition by regulating the Retinoblastoma (Rb) protein, in other cell types Jagged-1 exhibits oncogenic properties. In reporter gene assays examining pro-inflammatory transcription factors, Jagged-1 did not show considerable inhibitory effects, whereas the activity of the tumor-suppressor p53 was significantly suppressed.

2. Introduction

2.1. Inflammation as a beneficial response

Inflammation is a complex response of the organism to harmful stimuli like toxic, infectious, traumatic or autoimmune injury. [1] In the course of an inflammatory reaction these stimuli are removed and the healing process is initiated. At best the original structure and tissue function is reconstituted. If the injury is too extensive and the initial state can not be recreated, structural integrity is provided by mechanisms like scarring, fibrosis or sclerosis. [2]

A classical acute inflammation manifests itself with the symptoms *rubor, dolor, calor, tumor* and *laesa* (redness, pain, heat, swelling, loss/disturbance of function). [1] Most of these symptoms are caused by changes in the vasculature. In the surrounding of the injury small blood vessels widen and thereby cause a local increase of blood supply. As a consequence, the tissue shows redness and development of heat. Simultaneously, the blood vessels enhance their permeability and fluid containing immune cells accumulates in the surrounding tissue which leads to swelling. [3] The pains that typically go along with an inflammation are caused by mediators that act on C-type sensory nerve fibers. [4] All these symptoms are reversible, only the loss or disturbance of function can persist if the severity of the injury necessitates replacement of the original structures by fibrotic material. [2]

An inflammation is based on the interplay of many different cells types. Communication between theses cells is mainly mediated by inflammatory mediators, including chemokines, cytokines and lipid mediators. These are soluble molecules that bind to specific receptors on inflammatory cells and thereby stimulate their targets.

The first step of an inflammatory reaction is the detection of the injury. Not only cells of innate immunity, but also most somatic cells can sense the presence of intruders or damages of the tissue. In response they initiate an inflammatory reaction by liberation of pro-inflammatory signal transducers. [2] The formerly mentioned vasodilation and vascular permeability is caused by histamines and prostagladins. These molecules are released from the granulae of mast cells located in peripheral tissues and circulating basophiles in response to injury. As consequence, phagocytes recruited by chemotactical signals can accumulate in the tissue. [3] The first immune cells arriving in the lesion are neutrophiles. They carry various receptors for sensing stress signals sent by somatic cells and pathogen-associated molecules. Additionally they express adhesion molecules for interactions with activated endothelial cells.

These properties allow them to invade the side of injury very fast and remove bacteria and particles by phagocytosis. [5] Second, monocytes accumulate. Monocytes are long living cells. During the course of the inflammatory reaction they differentiate into macrophages and dentritic cells. The former ones do not only remove invaders and cell debris, but also apoptotic inflammatory cells, like the short-lived neutrophiles. Dendritic cells are professional "antigen-presenting" cells. Upon uptake and processing of antigenic material they migrate from the side of injury and activate T cells. By this process, activation of the adaptive immune system is induced and provides antigen-specific reactions and immunological memory mediated by T- and B-lymphocytes [5].

In summary, inflammation plays an essential role in the maintenance of tissue homeostasis by restoration of original tissue structure and function. It is an important defense mechanism against infections and induces both, innate and adaptive immune response [2].

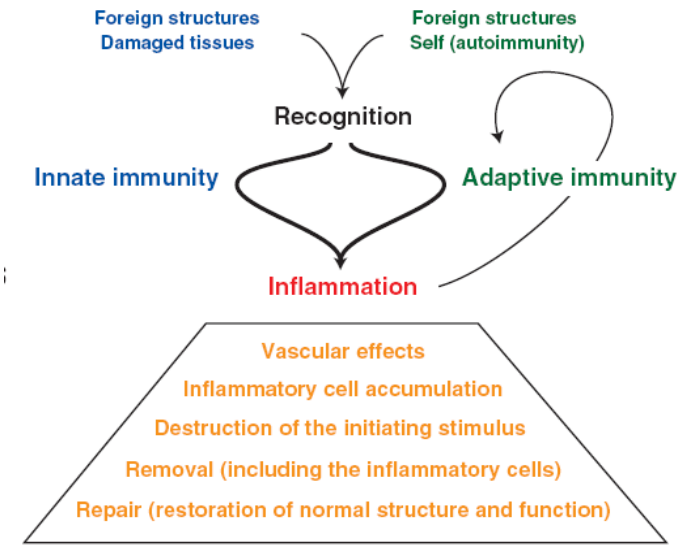


Fig.2.1.: **Inflammation and its effects**
Graphic: "Dampening Inflammation" [2]

2.2. Termination of inflammatory reactions

As described in the previous chapter, inflammation is originally an advantageous reaction of the organism. However, if it occurs exaggerated or is not terminated in time, it can have detrimental effects on the organism.[1,6] Chronic inflammation is a factor in the pathogenesis of diseases like cancer, asthma, Alzheimer, Crohn´s disease, multiple sclerosis, artheriosclerosis, rheumatoid arthritis, juvenile diabetes, eczema, and psoriasis. [1, 6-10] For this reason the resolution of the inflammatory reaction is an important topic of studies. There are mainly three prerequisites for the shutting down of the reaction:

1) An essential factor is the complete elimination of the harmful stimulus. Chronic inflammations are often caused the continuous confrontation with the initial trigger. Thus, autoimmune reactions that are caused by recognition of the tissue itself are predestinated to cause persistent inflammations. [2]

2) To avoid the chemoattraction of new inflammatory cells to the side of injury, a switch has to occur from pro-inflammatory to anti-inflammatoy mediators. Whereas in the initial phase prostaglandines and leukotrienes are produced, in the resolution phase lipoxines, resolvins and protectins are enriched.[1,11] This switch is, amongst others, achieved by changes in signalling pathways that initially lead to transcription of genes encoding enzymes that direct the synthesis of pro-inflammatory mediators belonging to the eicosanoids (e.g. E_2, D_2). In the course of the inflammatory reaction they induce the generation of enzymes that catalyze the synthesis of anti-inflammatory classes of these lipid mediators. The formerly mentioned lipoxines do not only actively prevent the invasion of new neutrophiles, but also mediate a decrease of the vascular permeability and stimulation of macrophages. Additionally, they seem to play an active role in the initiation of wound healing. [11]

3) Last but not least, apoptotic immune cells and debris of somatic cells have to be removed from the lesion. Especially the short-lived neutrophiles must be elimiminated after they have undergone apotosis. The removal is achieved by “efferocytosis”, a phagoytic process performed mainly by macrophages, but also by dentritic and somatic cells. As a side effect, the recognition of apoptotic cells leads to an increase of anti-apoptotic mediators and growth or maintenance factors for tissue cells (e.g. TGF-β). This helps to restore tissue structure and function. [2]

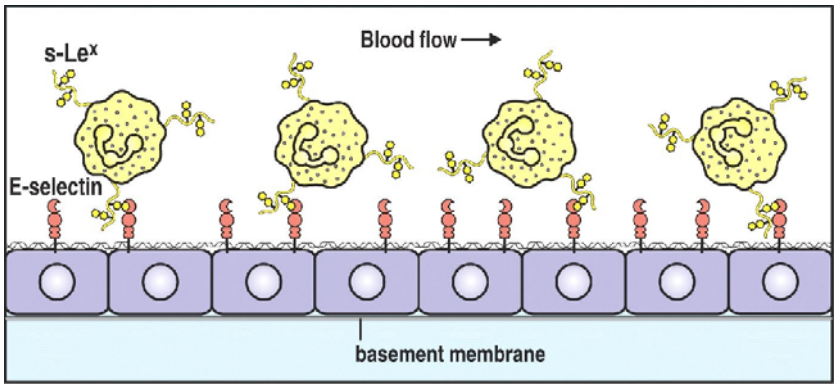
The last step of an inflammatory reaction is the exit of surviving monocytes through the lymphatic system. [1]

2.3. Endothelial cells in inflammation:

Endothelial cells represent the inner surface of blood as well as lymphatic vessels all over the circulatory system. This layer it builds an anticoagulant, nonadhesive barrier between the blood stream and the underlying tissue. [3]

The endothelium contributes actively to inflammatory reactions. In response to pro-inflammatory stimuli (IL-1, TNF-α, LPS) it undergoes changes in permeability, enables adhesion of leucocytes and develops thrombogenic properties. [6] The basis for these alterations is the induction of a complex gene expression program. Up-regulated genes include pro-inflammatory cytokines, chemokines, growth factors (e.g. IL-1, IL-6, IL-8, M-CSF, G-CSF, GM-CSF) and cell-adhesion molecules (E-selectin, VCAM-1, ICAM-1). [12]

By chemoattraction, circulating leucocytes are recruited to the vascular wall. There they are “tethered” by low affinity bindings to selectins on the surface of activated EC. By forming and breaking these bindings the leucocytes “roll” along the vessel wall and become activated. This activation is supported by chemokines released by macrophages and causes a switch of the surface receptors from low to high affinity state. As consequence the rolling leucocytes are “arrested” on the endothelium and polarization occurs. Subsequently they can migrate through the venular wall by “diapedesis”. This process requires transient opening of endothelial cell junctions and degradation of the basement membrane. Alterations in the function of cell junction proteins and contractile forces in the EC are induced by interaction of up-regulated adhesion molecules (e.g. PECAM-1) with their ligands on the activated leucocytes. The penetration of the basement membrane is enabled by secretion of leucocytic proteases. After entering the interstitial fluid the leucocytes move to the side of injury guided by chemoattractants. [5]



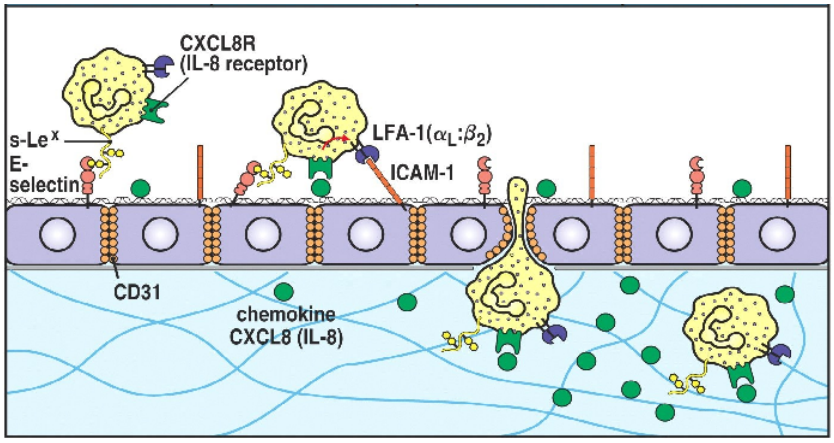


Fig.2.2.:
Tethering and **Rolling** of leucocytes on the surface of activated endothelium
Fig. 2.3.:
Arrest and **diapedesis**
Both Graphics from the Homepage of "UCL Division of Medicine, Royal Free & University College Medical School, Centre for Rheumatology and connective Tissue Disease"

2.4. Regulation of Inflammation in Endothelial Cells

In response to pro-inflammatory stimulation, endothelial cells change their phenotype. The molecular basis for these processes is a mostly a complex gene expression program that requires tightly controlled regulatory mechanisms. These seem to be provided mainly by transcriptional regulation mediated by different inducible transcription factors. [6]

2.4.1. Activation of NF-κB

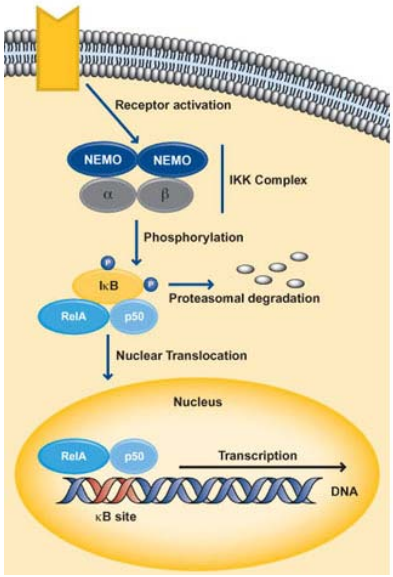
NF-κB is probably the most prominent and best characterized example for a primary transcription factor. It is present in the cell in an inactive state, upon induction it gets activated. Thus, *de novo* synthesis is not necessary and the transcription factor is able to react very fast to stimulation of the cell.

In humans there are five members of the NF-κB family: p50, p52, RelA, RelB and c-Rel. All of them carry a Rel homology domain (RHD) on their N-terminus. This 300 amino acid spanning domain enables the formation of homo- and heterodimers and binding to κB sides in the promoter and enhancer sequences of target genes. Via transcriptional activation domains (TADs) on their C-terminus RelA, RelB and c-Rel can activate the expression of target genes. p50 and p52 lack this domain and therefore act either as transcriptional repressors when forming homodimers, or as activators when heterodimerizing with proteins carrying a TAD. In contrast to the other members of the family, p50 and p52 are derived from precursors (p105, p100, respectively). [13]

NF-κB is activated by two different signaling pathways. The canonical or classical pathway is initiated by pro-inflammatory inducers like TNFα, IL-1 or LPS. These ligands bind to their specific receptors (TNFRs, IL-1 receptor, TLRs) on the surface of the endothelial cell and thereby cause the recruitment of various cytoplasmatic adaptor molecules (e.g. RIP, TRAF-1, TRAF -2, TRAF-6, MyD88). The signals are transmitted to different mitogen activated protein (MAP) kinases (e.g. MEKK1, MEKK2, MEKK3, TAK1) that activate various signaling cascades and converge at the IKK signaling complex. This consists of IKKα, IKKβ and a noncatalytic subunit (NEMO or IKKγ). Upon activation, the IKKs phosphorylate the inhibitors of NF-κB (IκBα) on their serine residues e.g., 32 and 36 in IκBa. These specific phosphorylations enable ubiquitination of IκBα by an E3 ubiquitin ligase complex followed by proteasomal degradation via the 26S proteasome. As a consequence the complex between NF-κB and its inhibitors is dissociated, its nuclear localization signal is exposed and the transcription factor translocates to the nucleus where it directs the expression of its target

genes. [1,14] Among them are chemokines and cytokines (e.g. IL-1, IL-2, IL-6, IL-8, TNFα, TNFβ), adhesion molecules (VCAM-1, ICAM-1, E-selectin, P-selectin), growth factors (G-CSF, M-CSF, GM-CSF) and stress response genes. [15]

The alternative or non-canonical pathway is induced e.g. by activation of the lymphotoxin-β receptor or TNFR1 and results in the activation of NIK (NF-κB-inducing kinase). NIK phosphorylates and thereby activates an IKK complex consisting of two IKKα subunits. The complex phosphorylates the C-terminal domain of p100. Consequently p52/RelB is liberated and can translocate to the nucleus.



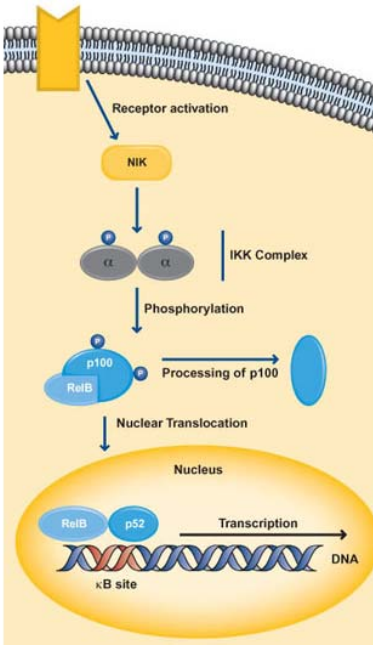


Fig.2.4., (left):
The **canonical/classical pathway of NF-κB activation**
Various signalling pathways lead to activation of the IKK complex. As consequence, IκBα is phosphorylated, ubiquitinated and degraded.
Fig.2.5., (right):
The **non-canonical/alternative pathway of NF-κB activation**
Upon receptor stimulation NIK is activated and phosphorylates the IKK complex consisting of two IKKα subunits. Consequently the IkB domain of p100 is phosphorylated and p52/RelB can translocate to the nucleus.
Both graphics: Overview of NF-kB signalling;
www.abcam.com [13]

2.4.2. Activation of AP-1

Another important transcription factor contributing to inflammatory reactions is the activator protein 1. AP-1 is a heteromeric protein consisting of proteins that belong to the c-Jun, c-Fos, ATF and JDP families. Activation is caused by a variety of different stimuli like cytokines, growth factors and bacterial and viral infections. Its composition and target genes are dependent on the type of signal and the consequently activated signaling pathway. [16] Binding of ligands to TNF-R1 leads to recruitment of TRADD to the cytoplasmatic “death domain” of the receptor, followed by binding of RIP-1 and TRAF-2. As a consequence, the SAP kinase cascade is induced and leads to activation of JNK. This kinase phosphorylates c-Jun and thereby enables the formation of a heterodimer with e.g. c-Fos. Expression of c-Fos is mainly achieved via the ERK pathway. In this composition AP-1 affects the expression

of genes required for inflammatory processes like cytokines, chemokines and adhesion molecules. In other variations AP-1 is involved in the control of various cellular processes including differentiation, proliferation and apoptosis. [17]

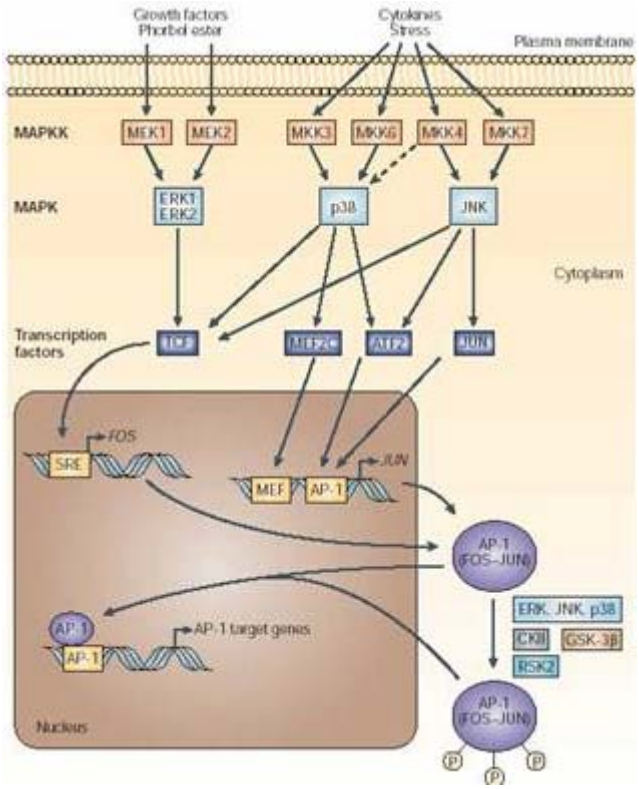


Fig.2.6.:
Activation of AP-1
In response to pro-inflammatory stimulation the ERK-pathway is activated and leads to expression of c-Fos.
Induction of the SAPK cascade leads to activation of JNK. Consequently c-Jun is phosphorylated and is able form a heterodimer with c-Fos.
Graphic from „Regulation der Stabilität der proangiogenen Transkriptionsfaktoren c-Jun, Id1 und Id3 durch das COP9-Signalosom“; [18]

2.4.3. Activation of NFAT

Nuclear factor of activated T-cells (NFAT) is known to be required for the expression of pro-inflammatory cytokines like IL-2, IL-4 and TNF-α. Usually it acts in association with other transcription factors, e.g. AP-1. In mammals the NFAT family consists of four members: NFAT1-NFAT4. NFAT is regulated by calcium signaling. In unstimulated cells, NFAT is present in an inactive, serine-phosphorylated state. In response to increasing calcium levels the calcium sensor protein calmodulin (CaM) activates the serin/threonine phosphatase calcineurin (CN) and thereby leads to dephosphorylation of NFAT. As consequence its nuclear localization signal is exposed, NFAT can translocate to the nucleus and modulate the expression of its target genes. [19]

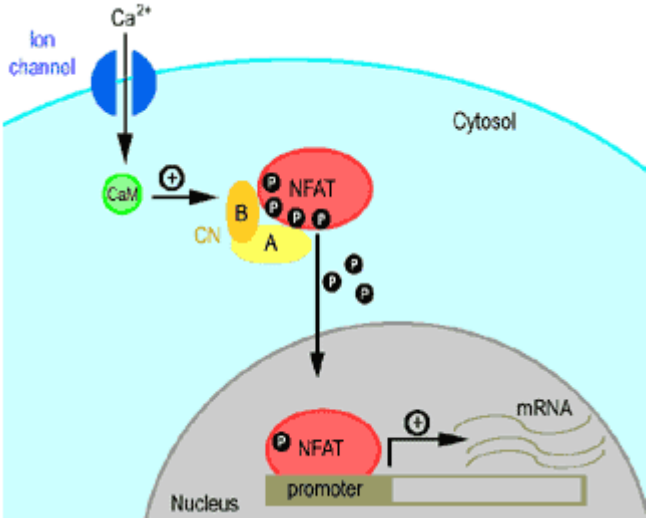


Fig. 2.7.:
Activation of NFAT
In response to increasing calcium levels Calmodulin (CaM) activates calineurin (CN). Consequently NFAT is dephoshorylated and thereby activated.
Graphic:www.angiobodies.com; "Universidad Autonoma de Madrid"

2.4.4. Activation of CHOP

CHOP is a member of the C/EBP (CCAAT/enhancer binding protein) family of transcription factors. It acts as central mediator of ER-stress induced apoptosis and is therefore activated by two signaling pathways involved in the UPR (unfolded protein response), the PERK and the ATF6 pathway. CHOP is also target of the p38 MAPK pathway, another signaling cascade induced by cellular stress like stimulation by cytokines, irradiation, heat or osmotic shock. Target genes of activated CHOP are pro-apoptotic factors like DR5, TRB3, BIM and GADD34. [20,21] The mechanism by which CHOP mediates apoptosis is not known, but because the ER-stress pathway stands in close context to the initiation of inflammatory reactions CHOP is a target of these studies.

2.4.5. Activation of p53

The transcription factor p53 plays an important role as tumor suppressor and "guardian of the genome" (Lane, 1992). It is massively involved in the regulation of the cell cycle and is able to avoid the amplification of damaged DNA. p53 is activated by stress signals including DNA damage by post-transcriptional modifications. Upon activation it induces growth arrest and the expression of a variety of genes involved e.g. in the DNA repair. If the damage is too extensive, p53 can send the cell into growth arrest or apoptosis.
The regulation of p53 is very complex, several negative and positive feedback loops are known, and the pathway stands in close context to many other signaling pathways of the cell. [22] Also, some members of the MAP kinases (JNK, p38, ERK) are able to transmit stress

signals to p53 by phosphorylation of its transcriptional activation domain. [23] The inducibility of p53 by MAP kinases involved in pro-inflammatory signaling and the observation that it can be inactivated by a pro-inflammatory cytokine (MIF) [24] makes it to a target of studies in the context of inflammation, although it is not directly involved in this reaction.

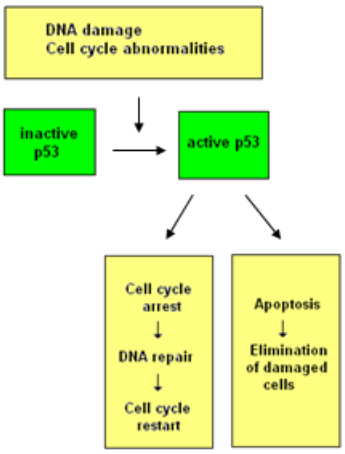


Fig. 2.8.:
Function of p53
In response to stress signals tp53 is activated and causes cell cycle arrest allowing DNA repair or apoptosis
Graphic: „the tp53 webside“

2.5. Negative Feedback Loops

The termination of an inflammatory reaction occurs on various levels. On the level of gene expression it is partly achieved by “negative feedback loops”. This term describes the phenomenon, that pro-inflammatory stimuli do not only induce the expression of pro-inflammatory genes, but also of those that cause the resolution of the reaction at later time points. Thus, the stage for termination of the reaction is set very early in the activation phase.[1]

A prominent example for a “negative feedback loop” is the NF-κB-dependent synthesis of IκBα. Among a variety of other target genes, NF-κB induces also the expression of its inhibitor IκBα. The newly synthesized protein translocates to the nucleus where it targets NF-κB for export to the cytoplasm and thereby inactivates it. [25, 26]

Another NF-κB-inducible gene is A20. A20 inhibits the TNF-induced activation of NF-κB by two different functions. As mentioned above, an essential step in TNFR signaling is the recruitment of cytoplasmatic adaptor proteins to the activated receptor complex. One such adaptor is RIP1, which is ubiquitiniated by TRAF-2 and can consequently transmit the signal towards the IKK complex. A20 inhibits RIP-1 signalling in a process involving two steps: First, it removes activating Lys63-linked ubiquitin chains via its N-terminal OTU domain. Second, it attaches Lys48-linked ubiquitin chains by its C-terminal zinc finger (Ub ligase) domain. This ubiquitination targets RIP-1 for proteosomal degradation and thereby leads to the inhibition of TNF-R signalling. [27]

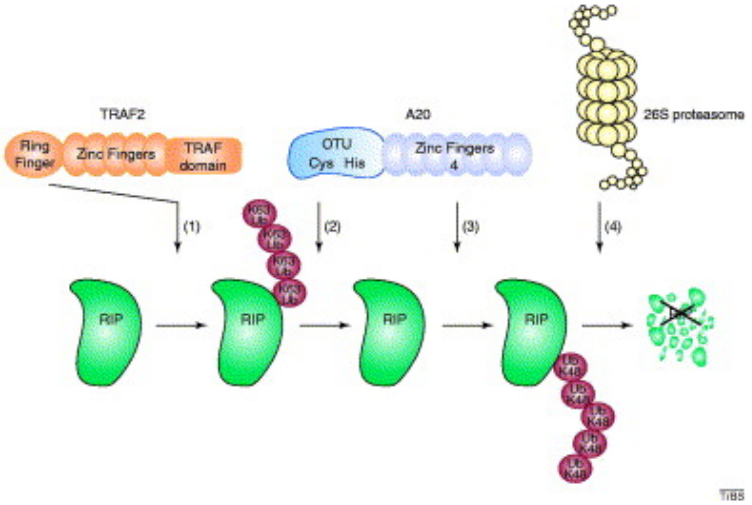


Fig. 2.9.:
Inhibition of TNFR signaling by dual activity of NF-κB induced A20
(1) TRAF2 ubiquitinates and thereby activates RIP
(2) A20 removes activating ubiquitin chains from RIP via its OUT domain
(3) A20 attaches ubiquitin chaines at RIP via its zinc-finer domain
(4) This ubiquitination targets RIP for proteosomal degradation resulting in inhibition of TNFR signalling
Graphic: “A20 inhibits NF-kB activation by dual ubiquitin editing function” [28]

2.6. Background of the project

The foundations of the project were laid by a micro array experiment performed by Mayer, de Martin et al. [6] The aim of this experiment was to get an overview of the immediate-early to early gene expression in stimulated HUVECs.

The cells were stimulated with the pro-inflammatory cytokine IL-1 for various periods of time (0.5h, 1h, 2.5h and 6h) or remained unstimulated as control. Expression profiles were analysed using Affimetrix U133 micro arrays. 137 genes were shown to be regulated more than four-fold, among these genes 18 were known to encode transcription factors. In order to identify novel negative regulatory mechanism of inflammation, genes were selected that were known or suspected to have repressive potential in a different context.

Gene	Function
IκB	inhibitor of NF-κB
A20	inhibitor of NF-κB, inhibitor of apoptosis
Zfp36/TTP	destabilization of specific ARE-containing mRNAs
DSCR1	inhibitor of NF-AT
C8FW	homologue of SINK and SHIK (involved in NF-κB p65 transactivation)
Etr101	inhibitor of several signaling pathways
DUSP1	dual-specificity phosphatase, inhibits MAPKs
DUSP5	dual-specificity phosphatase, inhibits MAPKs
SOCS1	suppressor of cytokine signaling (JAK-STAT, TLR pathways)
RGS7	regulator of G-protein signaling
ARTS-1	aminopeptidase regulator, promotes TNFR1 ectodomain shedding
EHD1	endosomal sorting, recycling of GF receptors (?)
TNFRSF11B	osteoprotegerin, soluble decoy receptor for RANKL
OATP-C	Na-independent transport of prostaglandin E2, thromboxane B2, leukotriene C3, leukotriene E4 (clearence of proinflammatory mediators?)
TCF8	transcriptional repressor (for IL-2 in T cells)
GG2–1	negative regulator of T cell signaling
TBX3	transcriptional repressor (in development)
ADAMTS1	disintegrin, metalloproteinase, anti-angiogeneic properties
TNFSF15	inhibits EC proliferation
Jag1	activates notch signaling, thereby represses EC proliferation via Rb

Tab. 2.1.: **Micro array experiment**
IL-1 induced genes with inhibitory potential
Table from “Resolution of inflammation” [1]

During the “Vertiefungsübungen” the results of the micro array were verified for a number of selected genes using real time PCR. At first the original cDNA analyzed in the micro array was tested. I was largely able to confirm the expression patterns, also when the magnitude of expression was in some cases different from the results of the micro array. The four genes that showed the highest inducibility were chosen to be targets of further studies: GG2-1, JAG1, TNFRSF11B and TNFSF15.

To substantiate the results for these genes, an independent source of mRNA was analyzed. HUVECs were stimulated with IL-1 for two different durations (2h, 4h) or remained unstimulated. Also in this approach the expression patterns could be reproduced by real time PCR (data not shown).

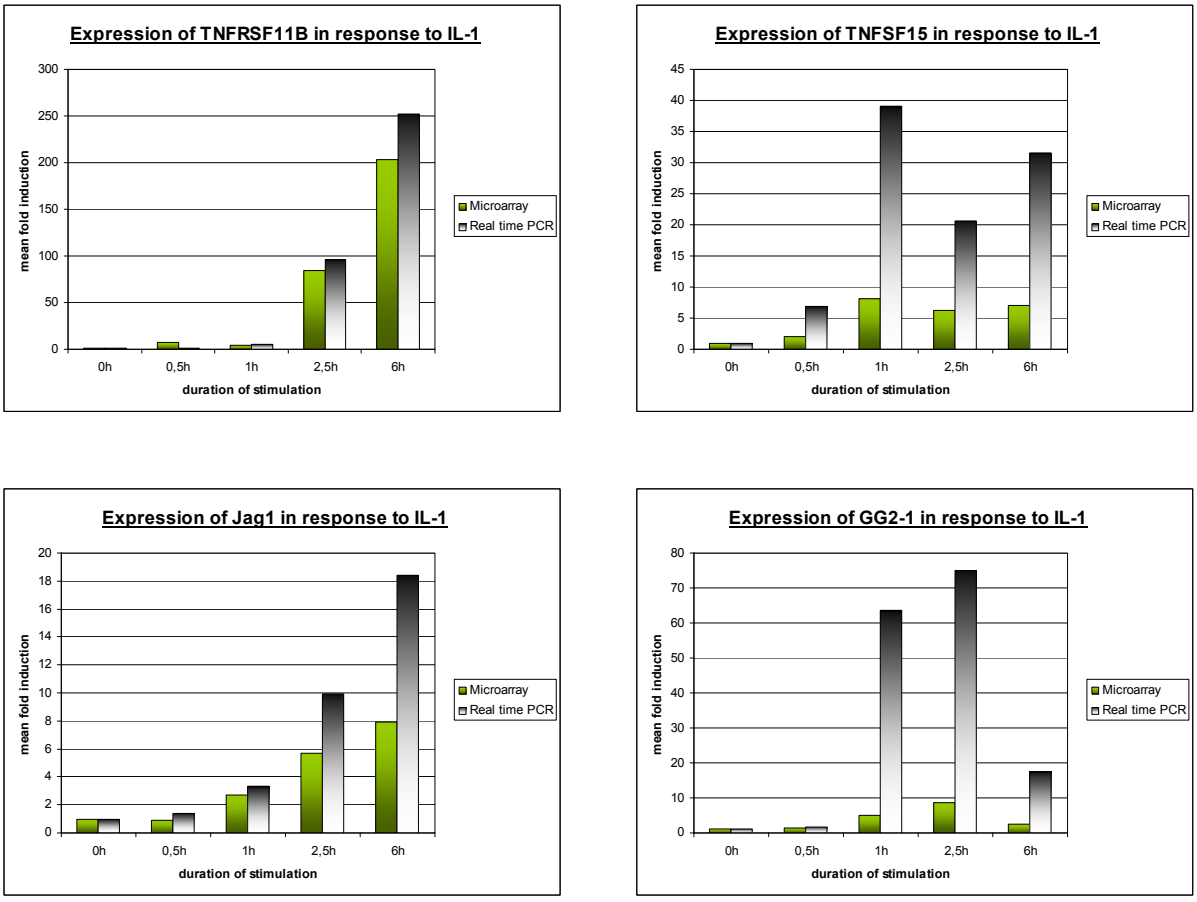


Fig.2.10.: Fold-induction of selected genes in response to IL-1 stimulation of HUVECs
Comparison micro array – real time PCR

2.7. GG2-1

GG2-1 encodes a cytosolic protein of ~21kDa and is induced by TNF-α via activation of NF-κB in various human cancer cells, rheumotoid arthritis synovial fibroblasts and vascular endothelial cells.[29]

G22-1 was described the first time in a publication of Patel, Wang et al. in 1997. In order to identify genes involved in tumor progression and/or metastasis, they established cell lines of primary and metastatic human head and neck squamos cell carcinomas (HNSCC) from the same patient. The expression profiles were compared and beside six other genes, GG2-1 was found to be differentially expressed. The fact that the expression level of GG2-1 was significantly higher in the metastatic cell line led to the suggestion that it encodes a protein involved in tumorigenesis. [30]

Further studies using bioinformatical methods revealed that GG2-1 contains a death effector domain (DED) on its amino-terminal end. DEDs are important features of proteins involved in apoptosis. The death receptors TNFR1 and Fas carry a cytoplasmic domain that is bound by TRADD (TNFR-associated death domain). TRADD serves as adaptor molecule and can recruit FADD. Via death effector domains FADD interacts with caspase-8 resulting in the receptor-associated death inducing signaling complex (DISC). This is the beginning of a caspase cascade leading to apoptosis. Not in every cell type apoptosis is induced by TNFR1 or Fas activation. In these cases the signaling pathway is inhibited by members of the FLICE inhibitory family of anti-apoptotic proteins (FLIP, CASH, I-FLICE, FLAME-1). These proteins contain sequences with a significant homology to the death effector domains of TRADD and caspase-8, but no catalytic caspase domain. Via the death effector domain they can bind caspase-8 and thereby inhibit its capacity to activate down stream caspases by proteolytic cleavage.

The presence of a DED and the observed ability of GG2-1 to inhibit TNF-induced apoptosis, led to the conclusion that GG2-1 belongs to this protein family. [31] This suggestion could be supported by the observation that GG2-1 inhibits the activity of caspase-8, whereby it is not known how it is acting on the signal pathway. In contrast to other members of its protein family (FLIP) it does not block the processing of caspase-8. Thus, it might act downstream from the DISC formation.

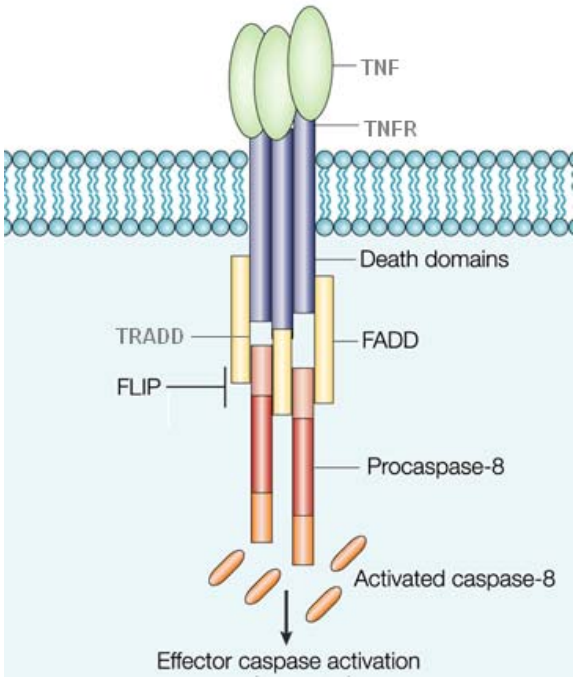


Fig.2.11.:
TNFR signaling pathway leading to apoptosis
The activated TNFR recruits the adapter molecule TRADD which activates FADD. Interactions beween the death effector domains of procaspase-8 and FADD lead to the formation of the death inducing signaling complex (DISC). Activated caspase-8 initiates a caspase cascade leading to apoptosis.
FLIP can prevents the processing of caspase-8 and thereby inhibit the signalling pathway.
Modified Graphic: "How melanoma cells evade trail-induced apoptosis" [32]

The oncogenic function of GG2-1 could be confirmed by various studies. Human breast cancer cell lines show enhanced DNA synthesis, proliferation and tumor growth rate when it is over-expressed. Additionally it is up-regulated in various human breast and renal carcinomas. *In vivo* experiments showed that GG2-1 over-expression in human breast cancer cells enhances their potential to perform pulmonary colonization. Vice versa, the inhibition of enogenous GG2-1 using antisense nucleotides causes decreased metastasis. Eventually these effects are caused by downregulation of VEGFR-2. This receptor tyrosine kinase is expressed in vascular endothelial cells and many types of tumor cells. As major receptor of VEGF-A it plays an essential role in endothelial cell proliferation and in autocrine and paracrine mechanisms of tumor growth and metastasis. It has been shown that reduced GG2-1 expression correlates with decreased VEGFR-2 expression, whereby VEGFR-1 and -3 expression is not affected. Also members of another protein family known to be involved in metastasis are suppressed when GG2-1 is down-regulated. Matrix degrading metalloproteases (MMPs) act by degrading components of the ECM and thereby facilitate invasion and extravasion of tumor cells. The expression of MMP-1 and MMP-9 correlates with GG2-1 expression in human cancer cells, whereas MMP-2 expression does not. [29]
GG2-1 seems also to act as inhibitor in another context. By performing a large-scale functional genetic screen for inhibitors of TCR signaling, GG2-1 was among the identified genes. The mechanism causing GG2-1 mediated TCR inhibition remains to be elucidated [33]. The function of GG2-1 in endothelial cells is unknown.

2.8. JAG1

JAG1 encodes the jagged 1 protein that functions as a ligand of Notch1. The Notch signaling pathway plays an important role in cell-cell communication and developmental processes. During embryogenesis it regulates cell fate decisions in tissues derived from all three primary germ layers. Thus, it is obvious that Notch signaling is involved in various independent processes. Among others it plays an important role in hematopoiesis and lymphocyte development, somitogenesis, neurogenesis and vasculogenesis.

Four different Notch receptors (Notch1-4) are expressed in mammals. All of them are synthesized as single polypeptides, but upon cleavage and transport to the cell surface, they act as heterodimers. Notch receptors are membrane spanning molecules. In their extracellular domains they contain variable numbers of tandem EGF (epidermal growth factor)-like repeats that enable interactions with the respective ligands. Upon ligand binding their intracellular domain (N^{IC}) is set free by proteolytic cleavage and translocates to the nucleus. There it binds CSL, a transcriptional regulator. In the absence of N^{IC} CSL blocks gene expression by binding RTGGGAA elements and recruiting various co-repressors. Cleaved N^{IC} replaces these inhibitory elements, recruits transcriptional co-activators and consequently induces expression of its target genes. Target genes are members of the HES (hairy/enhancer split) and HRT (hairy-related transcription factor) family of proteins.

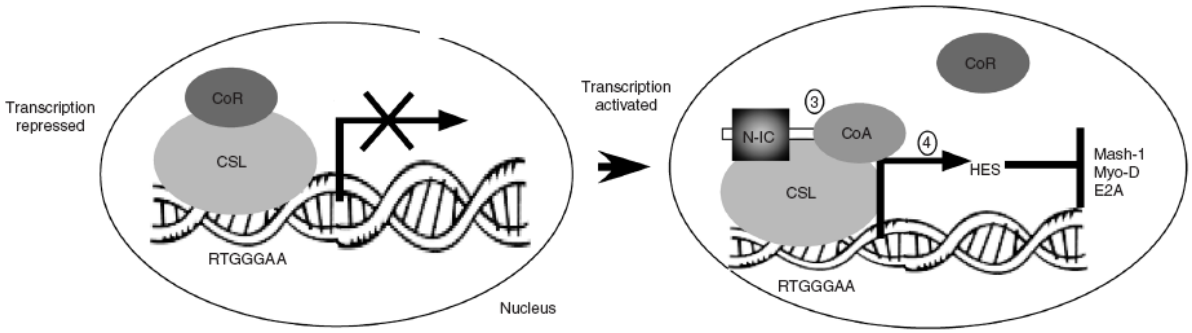


Fig. 2.12.:
Gene expression induced by Notch signaling
In unstimulated cells gene expression is inhibited by binding of the transcriptional regulator CSL and co-repressors to specific elements.
Upon activation of the Notch receptor its intracellular domain (N-IC) is released, translocates to the nuleus and interacts with CSL and co-activators leading to expression of target genes
Graphic: “Notch signaling in development and disease” [34]

Five different ligands are known to exist in humans: Three delta-like ligands (Dll1-3), and two Serrate-like ligand (Jagged-1 and Jagged-2). All of them are transmembrane proteins possessing tandem EGF-like repeats and a single DSL-binding domain in their N-terminus for interactions with the Notch receptors.

There are two different possibilities for the activation of the Notch signaling pathway: The term “inductive signaling” describes the interactions between two different cell types expressing either the receptor or the ligand. In this case only in the cell possessing the receptor the pathway is initiated. “Lateral signaling” occurs between two cells of the same type expressing both, the receptor and the ligand. As consequence Notch signaling is induced in both cells. [34]

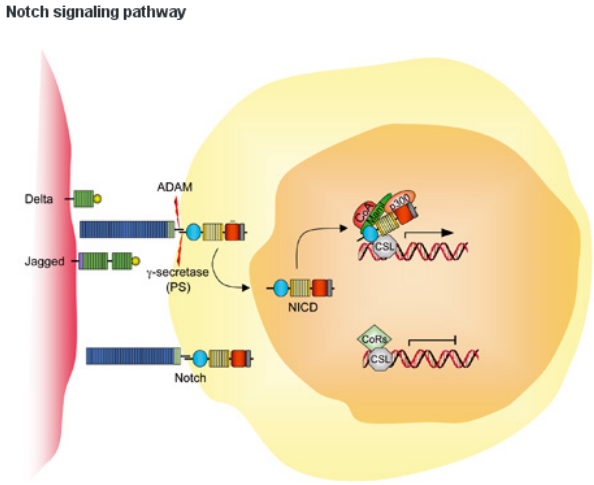


Fig.2.13.:
„Inductive“ Notch signaling
Two different cell types interact via Notch receptor and ligand.
Graphic: “Molecular mechanisms controlling homeostasis of self-renewing organs and their dysregulation during tumorigenesis” [35]

Mutations of JAG1 cause the Alagille syndrom (AGS). This autosomal dominant hereditary disease is a developmental disorder affecting various systems of the body. Symptoms are a reduced number of bile ducts and abnormalities concerning the heart, eyes, skeleton, face, kidneys, pancreas and vasculature. [36] 94% of patients suffering from AGS have been shown to carry mutations of JAG1, whereby these mutations are very versatile. They comprise total gene deletions, frameshifts, nonsense and missense mutations. [37] The fact that a small number of patients show deletions of the entire gene indicates that AGS is caused by haploinsufficiency. This suggestion is confirmed by the observation that these patients show a similar phenotype to patients carrying intragenic mutations. [36, 37]

JAG1 expression has been proven to be essential for vascular remodeling. JAG1 knockout mice die between embryonic day 10.5 and 11.5 due to abnormalities in the yolk sac and vasculature. [38]

In endothelial cells Jagged-1 seems to play a role in contact inhibition by preventing the phosphorylation of the retinoblastoma protein (Rb). Rb is a tumor suppressor that inhibits cell cycle progression between G1- and S-phase. In the hypophosphorylated state it is active and inhibits transcription factors of the E2F family that cause entry into the S-phase. Rb is phosphorylated and thereby inactivated by cyclinD/CDK4 and cyclinE/CDK2 complexes. Through Jagged 1 activated Notch signaling, phosphorylation of Rb is inhibited and the cell cycle is arrested. These effects seem to be partly achieved by changes in the expression of p21. [39]

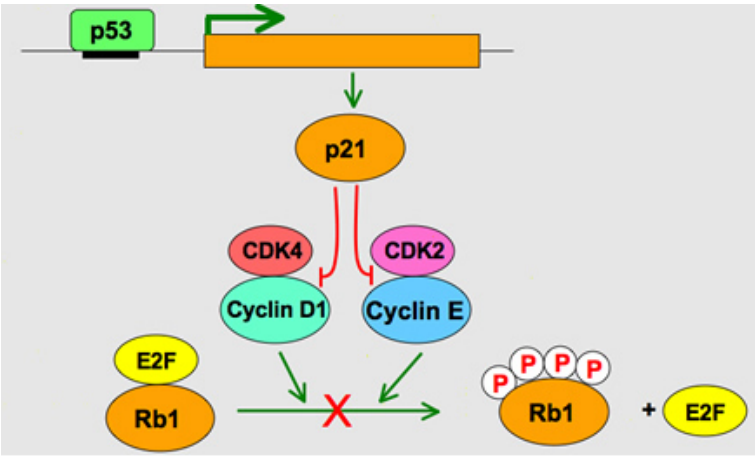


Fig.2.14.:
Regulation of the Rb protein
Active Rb inhibits E2F and thereby inhibits cell cycle progression
By cyclin/CdK complexes it is phosphorylated, the elongation factor is released and the cell can enter the S-phase.
Graphic:"The tp53 web side"

Additionally, endothelial expression of JAG1 plays an important role in smooth muscle development. [38]

Jagged 1 also has oncogenic properties. It has been found to be overexpressed in patients suffering from acute myeloid leukemia (AML). [40] In breast cancer patients elevated levels of JAG1 mRNA is associated with poor diagnosis. [41]. Together, although Jag-1 has been described in endothelial cells, its role in the inflammatory process remains to be established.

2.9. TNFRSF11B

TNFRSF11B encodes osteoprotegerin (OPG), a protein essential for bone homeostasis. Bone is constantly remodelled by osteoclasts that have degrading functions and osteoblasts synthesizing new material. This balance between resorption and bone formation is partly regulated by the RANK/RANKL/OPG system. The cytokine RANKL (receptor activator of nuclear factor NF-κB ligand) belongs to the family of tumor necrosis factors (TNFs) and is expressed on osteoblasts and bone marrow cells. Binding of RANKL to its transmembrane receptor RANK stimulates bone resorption by two different mechanisms: On the one hand it induces the differentiation of precursor cells to osteoclasts, on the other it activates mature osteoclasts. The soluble protein osteoprotegerin is the second receptor for RANKL. Binding of RANKL to OPG prevents RANK/RANKL interactions and therefore inhibits bone resorption. Thus, OPG acts as decoy receptor [42].

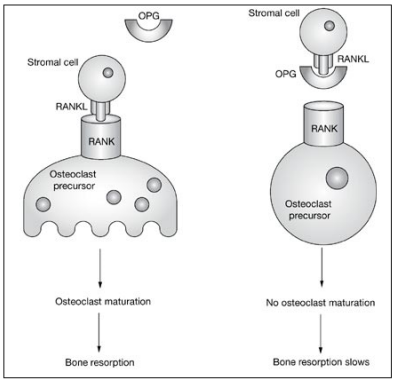


Fig.2.15:
The RANK/RANKL/OPG system
OPG acts as decoy receptor by preventing RANK/RANKL interactions
Grafic: "The osteoprotegerin/RANK/RANKL axis of bone formation regulation."

An imbalance of the RANK/RANKL/OPG system is involved in the pathogenesis of various diseases concerning the skeleton like postmenopausal or glucocorticoid-induced osteoporosis and rheumatoid arthritis. Further, the system plays a role in oncologic processes like the progression of multiple myelomas. This plasma cell cancer is associated with bone loss caused by enhanced osteoclastogenesis. Other cancer types (e.g. breast and prostate carcinoms) do not develop in the bone, but have a strong affinity to develop skeletal metastases. As prerequisite for colonization of the bone, osteolysis must occur. Beside to the capacity of tumor cells to develop an osteoclast phenotype, degradation of the bone is caused by changes in the RANK/RANKL/OPG system of osteoclasts.
Juvenile Paget´s disease (JPD) is an autosomal recessive disorder that can be caused by mutations in the TNFRSF11B gene. The phenotypic effects are versatile and include skeletal

and dental abnormalities, fractures and hearing deficits. JPD is caused by different kinds of mutation, whereby all of them affect the ligand-binding properties of OPG. [43]

The RANK/RANKL/OPG system seems also to play a role in vascular calcification associated with arteriosclerosis and osteoporosis. The observation that $OPG^{-/-}$ mice develop arterial calcification and aneurysms in the large arteries support the hypothesis that it is involved in the vascular pathophysiology.

RANK is expressed in endothelial cells of the vasculature and is activated by RANKL expressed in smooth muscle cells in a paracrine manner. These interactions cause activation of the PI3´-kinase/ Akt pathway and provide endothelial cell integrity by regulating cell survival. Upon TNF-α stimulation OPG is up-regulated and inhibits RANK/RANKL signalling and consequently the survival pathway. Thus, OPG is eventually contributing to the TNF-α induced endothelial damage [59].

2.10. TNFSF15

TNFSF15 encodes a member of the TNF family, vascular endothelial growth factor (VEGI) and shows 20-30% homology to other members of the superfamily. VEGI is expressed in many different tissues including lung, kidney, placenta, small intestine and colon, spleen, pancreas and prostate. The highest levels of TNFSF15 expression have been observed in vascular endothelial cells. In the endothelium VEGI specifically acts as strong growth inhibitor, whereby its effect is dependent on the cell stage. In G_0-G_1 endothelial cells VEGI induces growth arrest, in proliferating cells it causes apoptosis. As a result, it has been shown to act as a potent inhibitor of angiogenesis. The vascular homeostasis provided by VEGI is essential for the maintenance of vascular integrity (hairy/enhancer split) and HRT (hairy-related transcription factor) [45, 46].

The previously described VEGI protein is one of three different splicing forms of VEGI called VEGI-174. This 174 amino acids containing gene product is a type II membrane bound protein. Two other splicing variants are known: VEGI-192 and VEGI-251. The soluble VEGI-251 protein is also termed TL1A and is involved in the reaction of the organism to inflammation. It acts as ligand for DR3, a receptor up-regulated during T-cell activation. Upon binding activating and proliferative signals are induced, mainly by activation of NF-κB. Because the secretion of INF-γ is induced by TL1A, it is thought that it directs the immune answer towards a T_H1-mediated response [47]. Recent data revealed that DR3 is also expressed on NK cells, macrophages and endothelial cells, whereby the effect of receptor activation is not known in theses cell types [48]. The interactions between TL1A and DR3 can be inhibited by when TL1A binds the decoy receptor DcR3. Imbalances in this system might be involved in the pathogenesis of rheumatoid arthritis [49].

The capacity of TL1A to act as T_H1-polarizing cytokine is also the background of its role in the pathogenesis of the inflammatory bowel disease (IBD). TNFRSF15 expression is significantly up-regulated in macrophages and lymphocytes of patients suffering from the major forms Crohn´s disease (CD) and ulcerative colitis (UC) [50].

2.11. Principle of Reporter gene assays

Generally, a reporter gene encodes a protein that can be easily identified and quantified. Fused to appropriate regulatory elements it can be used to analyze the activity of transcription factors or promoter regions of certain genes.

Two different systems are available for these purposes: The *cis*-system consists of one plasmid carrying a luciferase gene under the control of a minimal promoter and a variable enhancer element. When the transcription factor of interest is activated, it binds to its specific enhancer element and activates the expression of luciferase. In the case of the NF-κB reporter system the luciferase gene is under the control of an artificial promoter containing five κB binding sides. For the examination of the promoter region of e.g. IL-8, a more natural promoter is used. This element is – like *in vivo* - activated by a combination of different transcription factors (NF-κB and AP-1).

The *trans*-system is composed of two plasmids. The transactivator plasmid expresses a fusion protein consisting of the DNA binding domain of GAL4 and the transactivation domain of a transcription factor of interest. The reporter plasmid carries the reporter gene under the control of a minimal promoter and a multimerized GAL4 binding side. If the transactivator protein is activated e.g. by phosphorylation, the fusion protein can induce the expression of luciferase. This system allows the observation of specific signalling pathways.

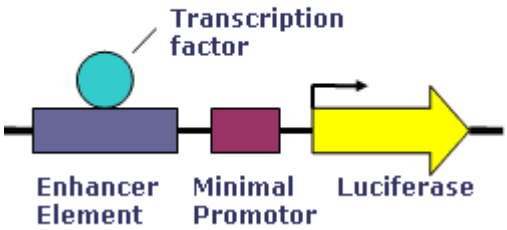


Fig.2.15.:
Scheme of Reporter constructs, *cis-system*
The luciferase gene is under the control of a minimal promoter and an enhancer element specific for a particular transcription factor

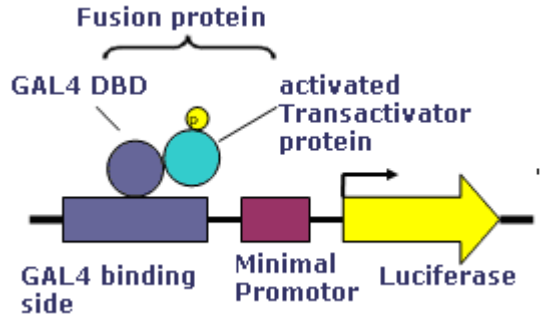


Fig.2.16.:
Scheme of Reporter constructs, *trans-system*
The luciferase gene is under the control of a minimal promoter and fused to a GAL4 binding side.
The fusion protein expressed on another plasmid binds the GAL4 binding side via its GAL4 DNA binding domain. Prerequisite for binding and luciferase expression is the activation of the transactivator protein

In both cases the expression of the reporter gene is dependent on the transcription factors of interest. Thus, proportional to its increasing/decreasing activity the measurable expression of luciferase increases/decreases.

To eliminate experimental variabilities e.g. variations in transfection efficiency, a second reporter gene (β-galactosidase or *Renilla* luciferase) is co-transfected as internal control and used for normalization. To keep the total amount of transfected DNA constant when combining different plasmids, an empty vector is used.

3. Material and Methods

3.1. DNA Methods:

3.1.1. Restriction Digest:

For restriction analysis of DNA the “Roche Buffer System” was used according to the manufacturer`s instructions. Control digests of plasmids to confirm their integrity were done in a final volume of 20µl. For the cloning of the constructs higher amounts of DNA were digested, therefore the reaction was performed in 50µl. All approaches were incubated at least for 1 hour at 37°C. For double digestions, enzymes were chosen that work in the same buffer as far as possible. When this was impractical the DNA was digested by the first enzyme for 1 hour at 37°C in the half of the final volume. Subsequently the second half of the digestion mix containing the second enzyme and its appropriate buffer was added and the incubation repeated. The first digest was always performed using the enzyme with the lower salt requirement. The samples were analyzed on agarose gels of different concentrations according to the size of the expected fragments. As control undigested/single digested samples were applied.

Single digest / double digest with enzymes working in the same buffer:

DNA (1µg/µl)	1µl / 3µl
Buffer	2µl / 5µl buffer (10% of final volume)
Enzyme (each)	0,5µl
dd$H_2$0	to 20µl / 50µl

Double digest with enzymes working in different buffers:

DNA (1µg/µl)	1µl / 3µl
Buffer 1	1µl / 2,5µl buffer
1st Enzyme	0,5µl
dd$H_2$0	to 10µl / 25µl
Buffer 2	1µl / 2,5µl buffer
2nd Enzyme	0,5µl
dd$H_2$0 to	10µl / 25µl

3.1.2. Re-Transformation:

Chemical competent cells were thawed on ice and 150µl of the suspension united with 0.5µg plasmid DNA. After 20min incubation on ice the cells were exposed to a “heat shock” (1min; 42°C), subsequently 1ml LB media was added and the cells were shaken (37°C; 30min; 250rpm). By centrifugation (5min; 3.500rpm, RT) the cells were pelleted and the supernatant was discarded to a large extent. The cells were resuspended in the remaining ~100µl and plated on selective agar plates.

3.1.3. Miniprep:

Plasmids were isolated using the “Qiagen buffer system” and a modified protocol.
Depending on the resistance provided by the respective plasmid, a single colony was inoculated in 3ml LB + Ampicillin (100 µg/ml) or Kanamycin (50 µg/ml) and shaken o.n. at 37°C and 250rpm. 1.5ml of the culture were transferred in Eppendorf tubes and centrifuged (5min; 8000rpm; RT). The supernatant was discarded and the pelleted bacterias were resuspended in 250µl buffer P1 by vortexing. Lysis of the cells was achieved by addition of 250µl buffer P2, inversion of the tubes and 5min incubation at RT. The lysate was neutralized by addition of 250µl buffer P3, followed by shaking of the tube. Debris was removed by a centrifugation step (10min; 13.000rpm; RT) and the supernatant transferred into a new Eppendorf tube. Precipitation of plasmid DNA was achieved by addition of 500µl Isopropanol followed by vortexing and incubation for 5min at RT. The DNA was harvested by centrifugation (20min; 13.000rpm; RT). After washing the pellet in 0.2ml 70% ethanol (centrifugation: 5min; 13.000rpm; RT) it was air-dried and resuspended in 50µl TE buffer.

Buffer P1 (resuspension buffer)	50 mM TrisCl, pH 8.0
	10 mM EDTA
	100 µg/ml RNAse A
Buffer P2 (lysis buffer)	200 mM
	1% SDS (w/v)
Buffer P3 (neutralization buffer)	3.0 M potassium acetate, pH 5.5

3.1.4. Maxiprep:

For the isolation of higher amounts of DNA the “Qiagen buffer” system and a modified protocol was used.
A single colony was inoculated in 10ml LB+Ampicillin(100 µg/ml) or Kanamycin (50 µg/ml) and incubated for ~8 hours at 37°C and 250rpm. The suspension was transferred into 240ml of the same media and cultivated o.n. under the same conditions. The cells were harvested by centrifugation (15min; 6000rpm; 4°C [Sorvall centrifuge; rotor: SLA-1500]) and resuspended in 10ml of buffer P1 by vortexing. Cell lysis was achived by addition of 10ml buffer P2, inversion of the tube and an incubation of 5min at RT. The lysate was neutralized by addition of 10ml buffer P3 and vigorous shaking. Debris was removed by centrifugation (10min; 4.000rpm; 4°C [Sorvall centrifuge; rotor: SLA-1500 or HB-4]) and the supernatant purified by filtering. Endotoxin removal was achived by addition of 3ml (10vol%) ER buffer and 30min incubation on ice. During this time the Quiagen Tip was equilibrated with 10ml buffer QBT; afterwards the endotoxin-cleared supernatant was loaded onto the column and could enter the resin by gravity flow. After washing the column twice with 10ml buffer QC, the DNA was eluated with 15ml buffer QF into a corex tube. Precipitation was achived by addition of 10,5ml (70vol%) Isopropanol and vigorous shaking of the sealed tube. The DNA was harvested by centrifugation (30min; 9000rpm; 4°C [Sorvall centrifuge; rotor: SLA-1500 or HB-4]). After discarding the supernatant the pellet was air-dried and resuspended in 400µl TE buffer. Subsequently an EtOH precipitation was performed: 40µl (10vol%) NaAc and 1ml (250vol%) EtOH abs. were added and the suspension was vortexed. After an incubation of for ~1hour at -20°C the DNA was pellet by centrifugation (30min; 13.000rpm; 4°C), washed with 0,5ml 70% EtOH twice (5min; 13.000rpm;RT) and resuspended in 200µl sterile TE-buffer.

Buffer QBT (equilibration buffer)	750mM NaCl
	50mM MOPS, pH 7.0
	15% Isopropanol (v/v)
	0,15% Triton X-100 (v/v)
Buffer QC (wash buffer)	1.0 M NaCl
	50mM MOPS, pH 7.0
	15% Isopropanol (v/v)

Buffer QF (elution buffer)	1.25mM NaCl
	50mM TrisCl, pH 8.5
	15% Isopropanol (v/v)

3.1.5. Determination of DNA concentrations:

For the determination of RNA or DNA concentrations, the samples were diluted 1:100 with H_20 and their optical density measured in the spectrophotometer at 260nm. As reference H_20 was used. The concentration was calculated using the following formula:

$c = (OD_{260} \times F \times D) /1000$

c: concentration in µg/µl
OD_{260}: optical density at 260nm
F: specific factor; DNA: 50; RNA: 40
D: dilution factor

The ration 260nm/280nm is a measure for the purity of a solution. When purifying nucleic acids a ration of 1.8-2.0 is desired. If the ration is less than 1.7 the solution is probably contaminated. (e.g. with proteins).

3.1.6. Agarose gel electrophoresis:

To separate DNA molecules by their size, gel electrophoresis was performed. The concentration of the gel was chosen due to the length of the expected fragments:

Gel Concentration (%)	DNA Size (kb)
0.3	5-50
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

The desired quantity of agarose was weighed in and 30ml (small gel) or 100ml (big gel) 1xTAE buffer were added. The agarose solution was heated in the microwave until all

particles were completely dissolved. After cooling down to ~60°C ethidium bromide (500ng/ml) was added and the gel poured into the appropriate apparature.
Before loading the DNA samples onto the hardened gel, they were united with a sixth of the final volume of 6xloading dye (Fermentas™). Depending on the length of the expected fragments appropriate DNA size markers (100bp DNA ladder plus/ 1kb DNA ladder plus; Fermentas™) were used.
Electrophoresis was performed at 80V for small or 100V for big gels. The bands were made visible using a 300nm transilluminator.

3.1.7. Extraction of DNA from Agarose Gel

DNA was extracted from agarose gels using the "GFX PCR DNA and Gel Band Purification Kit" (GE Healthcare) and an optimized protocol.
The required bands were excised with a sterile scalpel and transferred into 1.5ml Eppendorf tubes. The weights of the gel pieces were determined; per 1mg agarose gel 1µl capture buffer was added. The tube was incubated at 60°C until the agarose was completely dissolved and the solution turned clear, then it was loaded onto a GFX MicroSpin column. After an incubation of 3min at RT, the column was centrifuged (1min; 6000rpm; RT) and the flow-through discarded. A wash step was performed by application of 0.5ml washing buffer and centrifugation (1min; 6000rpm; RT). Afterwards DNA was eluted by applying 25µl elution buffer, 3min incubation at RT and centrifugation (1min; 6000rpm; RT). To maximize the yield the elution was performed twice.

3.1.8. Sequencing of DNA:

To ensure that the IMAGE clones or the constructed expression vectors did not carry mutations, they were sent to MWG to be sequenced. As prerequisite 1µg DNA per sequencing reaction had to be precipitated. The required quantity of DNA solution was brought to a final volume of 100µl with TE buffer. 10µl (10vol%) NaAc and 70µl (70vol%) Isopropanol were added, the solution was vortexted and incubated for 10 min at 4°C. The DNA was pelleted by centrifugation (20min; 13.200rpm), washed with 200µl 70% EtOH and air-dried.
The appropriate primers were chosen either by studying the vector map or the homepage of Image consortium. When internal primers were necessary (in one reaction only ~1kb can be

sequenced) they were designed using the program Primer3 and ordered at MOLBIOL or Invitrogen.
The sequences were analyzed and compared with the reference sequence using the program DSgene.

3.1.9. Cloning into expression plasmids:

For further usage in reporter gene assays and other experiments, the genes of choice had to be cloned into mammalian expression vectors. Also genes that had been delivered in expression vectors were re-cloned, because no specific antibodies for the encoded proteins were available. To enable their detection by western blotting they were cloned into vectors carrying a flag tag.

3.1.9.1. Design of expression primers:

For the design of expression primers the program DSgene was used. The reference sequences of the genes were downloaded from the NCBI data base. The aim was to design primers that enable cloning of the complete CDS into the expression vector of choice in the right orientation and in frame. Using the maps of the expression vectors restriction sides were chosen that cut the respective vector only once in its multiple cloning side. To ensure that the corresponding enzyme would not cut the gene to be cloned, a virtual restriction analysis was performed. Because the insert had to ligate in a certain orientation, two different enzymes were chosen. GG2-1 and JAG1 were cloned into pcDNA3. The recognition sites of the forward primers were set directly before the start codon (ATG). In the cases were the reading frame was changed by the restriction, one or two bases had to be inserted, whereby it was important to ensure that the encoded amino acid would be as neutral as possible. The recognition site of the second restriction enzyme was set directly after the stop codon. TNFRSF11B and TNFSF15 carry a signal peptide on their 5´-terminus that could interfere with the tag of pcDNA3. Thus, these two genes were cloned into another expression construct carrying a tag localized on the 3´-terminus of the insert, pcDNA3.1/myc.HIS A. To ensure the expression of the inserts, a Kozak sequence cut by a restriction enzyme had to be incorporated into the forward primer. The reverse primer was designed like described before, whereby the stop-codon was left out.

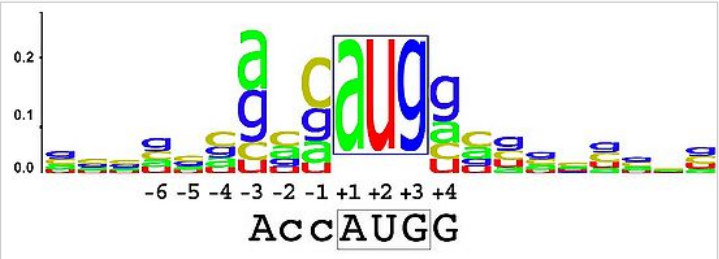


Fig. 3.1.:
Kozak sequence
Graphik: "Wikipedia"

Ideally both primers should have an annealing temperature of 52°C. This could be calculated using the following formula (only the annealing bases of the primers were counted):

Annealing temperature = [A+T] x 2°C+ [G+C] x 4°C

Primer	Sequence	Enzyme	Annealing Tm
exGG2-1fwd	5´- **AAGCTT**AATGCTCTCCGAAGCGG-3´	HindIII	52°C
exGG2-1rev	5´-**GGATCC**TCATATGTTCTCTTCATCC-3´	BamHI	52°C
exJAG1fwd	5´-**AAGCTT**AATGCGGTCCCCACGG-3´	HindIII	52°C
exJag1rev	5´-**GGATCC**CTATACGATGTATTCCATC-3´	BamHI	52°C
exTNFRSF11Bfw	5`-**GGTACC**ATGAACAAGTGGCTGTGC-3`	KpnI	54°C
exTNFRSF11Brev	5`-**TTCGAA**TAAGCAGCTTATTTTCACG-3`	SfuI	54°C
exTNFSF15fw	5`-**GGTACC**ATGGCAGAGGAGCTGG-3`	KpnI	54°C
exTNFSF15rev	5`-**TTCGAA**TAGCAAGAAAGCTCCAAAG-3`	Sfu	5$4°C

Tab.3.1.: **Sequences of expression primers** designed for cloning into expression constructs
Bold letters represent bases forming recognition sites of restriction enzymes, the underlining marks bases that anneal to the template

3.1.9.2. TOPO Cloning

As intermediate step the genes of interest were cloned into a TOPO vector (pVP22). This technique is very efficient and provides the possibility to cut out the insert from the vector by various combinations of enzymes if required for other application at a later date. TOPO vectors are supplied in a linearized form, carrying a specific sequence [(C/T)CCTT-3´] and single 3´ thymidine overhangs on both ends. DNA Topoisomerase I is covalently attached to this recognition sequence. When PCR products with a single 3´deoxyadenosine on their ends are present, the enzyme is able to link the vector and the insert independently from the sequence of the PCR product.

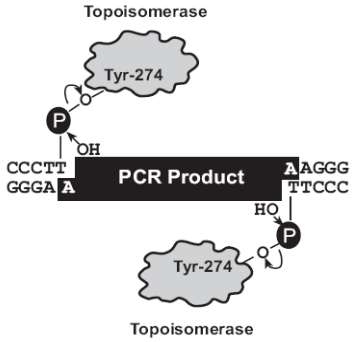


Fig. 3.2.:
Principle of TOPO cloning
PCR products with 3´deoxyadenosin ends are ligated with the 3´thymidine ends of the linearized TOPO vector. This reaction is catalyzed by Topoisomerase I.

4µl extracted PCR product, 0.5µl pVP22 vector and 1µl salt solution were mixed, incubated 5min at RT and placed on ice. As control the same reaction mix was prepared using H_2O instead of PCR product. After the ligation a transformation was performed: 100µl competent DH5α were added and the suspension incubated on ice for 30min, afterwards the competent bacterias were exposed to a "heat shock" (42°C, 1min). After addition of 1ml LB-media the cells were shaken (1 hour; 37°C; 250rpm) and pelleted by centrifugation (5min, 1100rcf; RT). The supernatant was discarded, the cells resuspended in ~100µl of the remaining media and plated on selective agarose plates. After an incubation of about 12 hours at 37°C various colonies were picked and inoculated in 3ml LB+Amp o.n. To check if a certain colony was carrying a TOPO vector ligated with the insert, minipreps were performed, the DNA digested and analyzed by electrophoresis.

3.1.9.3. Polymerase chain reaction (PCR):

For the amplification of the genes of interest from plasmids the Expand High Fidelity ^{Plus} PCR System (Roche) and a modified protocol was used.

Component	Volume	Final Concentration
5x Expand Hifi^{Plus} Reaction Buffer	10µl	1.5mM $MgCl_2$
PCR Grade Nucleotide Mix	1µl	200µM
Upstream primer	1.5µl	300mM
Downstream primer	1.5µl	300mM
Template DNA	1.5µl	15ng
Expand Hifi^{Plus} Enzyme Blend	0.75µl	3.75U
H_2O	33.75µl	
Final Volume	**50µl**	

	Temperature	Time	Cycles
Initial Denaturation	94°C	2min	1x
Denaturation	94°C	0.25min	
Annealing	52°C	0.5min	25x
Elongation	68°C (> 3kb)	2.5min	
	72°C (< 3kb)		
Final elongation	68°C (> 3kb)	7min	1x
	72°C (< 3kb)		

The PCR products were applied onto agarose gels and the fragments required for further usage extracted from the gels.

3.1.9.4. DNA Ligation

After restricting vector and insert, both was loaded onto an agarose gel and the appropriate bands were extracted. To achieve a final volume of 20µl, 2µl 10xBuffer for T4 DNA Ligase (New England BioLabs) was diluted with 12µl H_2O. In this solution 1µl vector was united with the 4-fold amount of insert and 1µl T4 DNA Ligase (New England BioLabs). The reaction mixture was incubated at 16°C o.n and 3µl used for a transformation in DH5α cells. The success of the procedure was checked by digesting the isolated plasmid DNA and analyzing the fragments on a gel.

3.2. Bacteriological techniques:

3.2.1. Chemical competent cells:

A single DH5α colony was inoculated in 5ml LB-media and incubated o.n at 37°C. 2ml of the culture was transferred into 200ml of the same media and grown until the OD_{600} had reached a value between 0.4 and 0.5. The cell suspension was centrifuged (15min; 4000rpm; 4°C) and the pellet resuspended in 20ml TSS buffer. After addition of 8,7ml 50% glycerol and an incubation of 10min on ice the cells were aliquoted (150µl) into pre-cooled tubes. After flash freezing the cells in liquid nitrogen they were stored at -80°C.

3.2.2. Preparation of LB medium and agarose plates:

For the preparation of LB medium 2.5vol% Broth Base was weighed in, dissolved in Aqua bidest. and autoclaved. For agar plates 1.5vol% agar was added. After the media had reached a temperature of 56°C the required antibiotic was added (Ampicillin:100 µg/ml; Kanamycin 50 µg/ml) and the plates were poured under sterile conditions. The plates were stored at 4°C.

25g LB Broth Base (Invitrogen)
15g Agar
H_20 to 1l

3.2.3. Cultivation of Image Clones:

The vectors containing the desired genes were delivered in bacterial hosts growing on agar. Because all of the vectors express an Ampicillin resistance gene, the colonies were plated on Amp-plates. After 24h of incubation one single colony from every plate was picked, inoculated in LB+Amp and shaken at 37°C and 250rpm o.n.

3.3. Cell culture

3.3.1 Cultivation of HEK293

HEK293 were grown under sterile conditions in DMEM (Dulbeccos Modified Eagle Medium; Gibco Invitrogen) supplemented with fetal calf serum, penicillin/streptomycin and glutamin in culture flasks (TC Flask 25CM2; Nunc). In the incubator a humidified atmosphere of 5% CO_2 was provided. When cells reached 80% - 90% confluence they were splitted. Usually this was done every 2-3 days 1:5 or 1:6. Before passaging the cells, the layer was washed with 3ml sterile PBS, then the cells were trypsinised (1.5ml Trypsin) and observed under the microscope. When most of the cells were detached, the reaction was stopped by addition of 10ml DMEM (FCS inhibits Trypsin).The required amount of cell suspension was transferred into a new culture flask and DMEM was added for a final volume of 15ml.

DMEM: HyClone; DMEM/High Glucose; Cat.No. SH30022.0; Thermo Scientific
10% (v/v) FCS, heat inactivated; Gibco
Penicillin / Streptomycin (Pen: 50U/ml; Strep: 50µg/ml); BioWhittaker
1mM Glutamin; BioWhittaker0

The components were united, steril filtered (0.2µm) and the medium stored on 4°C.

3.3.2. Freezing of HEK293

The required number of cryo-tubes was placed in a freezing pot filled with isopropanol and precooled at 4°C. Freezing media (90% DMEM+10% DMSO [containing 10% FCS, Pen/Strep and Glutamin]) was stored on ice. The cells were washed with 2.5ml sterile PBS and trypinised (1.5ml trypsin). The single cells were resuspended in 10ml media and centrifuged (3min; 1000rpm; RT), the pellet was resuspended in 3ml freezing media. 1ml aliquots were transferred into the cryo-tubes and stored at -80°C o.n. Subsequently the cells were stored in liquid nitrogen.

3.3.3. Thawing of HEK293 cells

The frozen cell suspension was placed in a 37°C water bath. When the media was almost completely thawed it was transferred into a 15ml falcon tube containing 10ml DMEM and centrifuged (5min; 1000rpm; RT). The supernatant was discarded carefully, the pellet was

resuspended in 5ml DMEM and the solution transferred into a T80 culture flask already containing 10ml DMEM. Before the cells were used for experiments (e.g. reporter gene assays) they were passaged at least twice.

3.3.4. DAPI staining

To test the cells for mycoplasm and other intracellular infections, DAPI staining was performed. DAPI stains DNA, so in an infected culture not only the chromosomal DNA of the cells but also the DNA of the bacterias gets visible. Four chamber slides (Lab-Tek II Chamber Slide; Nalge Nunc International) were coated with 0.5ml gelatine for at least 15min. Afterwards different quantities of cells suspension (100µl, 200µl, 300µ, 400µl) were given into four chambers, brought to a final volume of 400µl with pre-warmed DMEM and incubated o.n at 37°C. As preparation for the staining the cells layers were washed with 100µl PBS complete (2x) and fixed with 150µl 4% paraformaldehyd for at least 15min at 37°C. After two additional wash steps with 100µl PBS complete, the cells were incubated with 150µl DAPI solution (1:500 with PBS complete) for at least 15min and washed again (2x). The chamber was removed from the slide; rests of glue were removed using a sterile scalpel. One drop of mounting fluid (Geltol Mounting Medium; Immunon™ Thermo Shandon) was applied per chamber and a cover glass put onto the slide. The cells were analyzed using fluorescence microscopy.

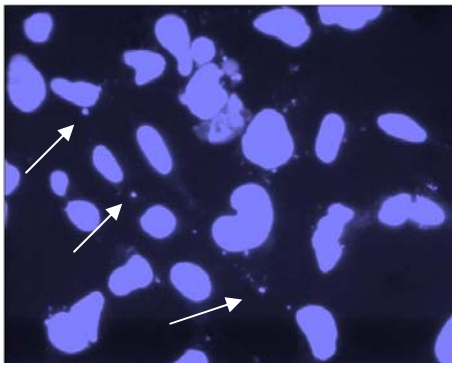


Fig.3.3.:
DAPI staining of an mycoplasma infected HEK293 culture
Arrows indicate mycoplasma DNA

3.3.5 Transient transfection and Reporter gene assay

3.3.5.1 Calcium-Phosphat Transfection of HEK293:

For introducing foreign DNA into HEK293, cells were transiently transfected using the calcium-phosphat method. This technique is based on the fact, that $CaCl_2$ and HeBS (HEPES-buffered saline) form a fine precipitate when united under advantageous conditions. The positively charged calcium and the negatively charged phosphate form complexes that are able to bind the DNA to be transfected on their surface. When this suspension is added to the cells the bound DNA is able to pass the plasma membrane mediated by a process that is not completely understood.
In preparation HEK293 were seeded in gelatine-coated multi-well dishes ($1x10^6$ cells per 6-well or $0.25x10^6$ per 24–well) approximately 16 hours before the experiment was done. When the transfection was performed the cells were supposed to be 50-60% confluent. For the transfection of one 6-well a total of 4µg DNA was united in a 1.5ml Eppendorf tube. A dilution of 9µl $CaCl_2$ in 58 µl H_2O was added. 71µl HeBS were added dropwise while bubbling the DNA solution with a Pasteur pipette on a pipette boy. After an incubation of 1 min the whole mixture (142µl) was applied onto the 6-well. When 24-well plates were transfected, 1/4 of the suspension (35,5µl) was applied per well.
After incubation of optimally 16, but at least 4 hours, the medium was replaced. Cells were harvested 48 hours after transfection.

2xHeBS: 280mM NaCl
1.5mM $Na_2HPO_4 \cdot 7H_2O$
50mM HeBS (Sigma)
In A. bidest. ; pH 7.05

$CaCl_2$: 2M $CaCl_2 \cdot 2H_2O$

Both solutions were sterile filtered (0.2µm), aliquoted and stored at -20°C.

3.2.5.2. Reporter gene assay:

To observe the effects of the genes of interest in HEK293, cells were transfected with a constant quantity of reporter constructs and increasing concentrations of the genes. In parallel the same experiment was performed, co-transfecting the cells with a constant amount of an inducer of the respective transcription factor. All experiments were performed in triplicates.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Gene of interest	0	0.5	1.0	1.5	0	0.5	1.0	1.5
Reporter plasmid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Normalizing Reporter	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Empty Vector	3.0	2.5	2.0	1.5	2.5	2.0	1.5	1.0
Pathway specific inducer	0	0	0	0	0.5	0.5	0.5	0.5
Total	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**

Fig. 3.4.: **Pipetting scheme** of a typical reporter gene assay performed in a 24-well plate

After 48 hours the cells were harvested and frozen at -20°C for ~1 hour to facilate the subsequent lysis. Lysates were produced by adding 50µl 1xPassive Lysis Buffer (Promega) per well and shaking for 1 hour at RT.

For the luciferase assay 10µl of the lysates were transferred into appropriate tubes (5ml, 75x12mm, PS; Sarstedt). Because the injection mix was added automatically (100µl per sample), the total prepared volume was set in the reservoir of the luminometer (Lumat LB 9501; Berthold). To avoid contaminations from former experiments, a single wash cycle using this mix was performed. To each sample 350µl reaction mix was added manually right before measuring. In the presence of ATP and Mg^{+2}, Luciferase catalyzes the oxidation of Luciferin to Oxyluciferin, resulting in bioluminescence. This light output was usually measured for 10 sec.

For the β-galactosidase assay 10µl of the lysates were transferred into 96-well plates and incubated with 90µl CPRG reagent until a clear colour change was observable. β-

Galactosidase catalyzes the cleavage of CPRG. The resulting product was quantified by spectrophotometry in an Anthos 2 plate reader at 580nm.
For the normalization of the results, the RLUs (relative light units) from the Luciferase assay were divided by the β-Galactosidase values for each triplicate. Then the average value was calculated for each sample. To illustrate the fold-induction/inhibition, the values of the samples transfected with the gene of interest, were referred to the control-sample.

Reaction mix:	25mM Glycine-Glycine, pH 7.8
	1M $MgSO_4$
	20mM Adenosin-5´- triphosphat (ATP)
Injection mix:	25mM Glycin-Glycin
	1mM Luciferine (Roche)
CPRG:	0.05% (w/v) CPRG (Roche) in PBS with 0.1% (w/v) BSA

3.4. Protein methods

3.4.1. Preparation of lysates for western blotting

To avoid degradation of the proteins all steps were performed on ice. 50µl 1 x Passive lysis buffer were applied onto the cells. After removing the required quantities for the reporter gene assay, the triplicates were pooled, united with a fifth of the final volume of 5xLämmli buffer and boiled at 95°C for 10min.
For testing on the expression of the genes of interest 6–wells were transfected. In these cases the cell layer was washed with 1ml PBS and the cells flushed from the surface and resupended in the same volume. The cells were pelleted (5min; 4000rpm; 4°C), resuspended in 100µl Laemmli buffer and boiled at 95°C. After three freeze and thaw cycles (5min, 95°C; freezing in liquid nitrogen) the samples were applied onto the gene.

3.4.2. Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE):

For separating proteins according to their size SDS-PAGE was performed. The concentration of the separating gel was chosen due to the size of the protein to be detected (see table). If this was unknown it was calculated using following formula:

1kb DNA = 333aa coding capacity = $3.7x10^4$ (= 37 kDa)

Gel Concentration (%)	Proteine Size (kDa)
8%	40-200kDa
10%	21-100kDa
12%	10-40kDa

The components of the separating gel were united, poured into the appropriate apparature and allowed to polymerize. To ensure a flat surface and avoid air bubbles, the gel was overlaid with butanol, which was removed later by H_20 and Whatman paper. To create the slots, a comb was inserted and the stacking gel poured. After complete polymerization the gel was transferred into a chamber containing 1x Running buffer. The samples were applied using a “Hamilton needle”. 5µl of a defined protein mixture served as size marker. While the samples were passing the stacking gel electrophoresis was carried out at 80V, afterwards the gel was allowed to run at 120V.

Component (ml)	5.0%	7.5%	10%	12.5%	15%	20%
H_2O	5.75	4.95	4.1	3.3	2.45	0.8
Solution A	2.5	2.5	2.5	2.5	2.5	2.5
Solution B	1.7	2.5	3.4	4.2	5.0	6.7
10% APS (µl)	50	50	50	50	50	50
Temed (µl)	10	10	10	10	10	10

Tab.3.2: **Components of separating gels of different concentration**

Component (ml)	4%
H_2O	3.1
Solution Ast	1.25
Solution B	0.65
10% APS (µl)	50
Temed (µl)	10

Tab.3.2:
Components of the stacking gel

Solution A: 1.5M Tris
0.4% SDS
in A.dest.
pH 8.8

Solution B: 30% Acrylamid / bis-Acrylamid (37.5:1; Serva)

Solution Ast: 0.5M Tris
0.4% SDS
in A.dest.
pH 6.8

Temed: N, N, N`,N`- Tetramethylen-ethylendiamine (Fluka)

APS: 10% (v/v) Ammoniumperoxydisulfate (Sigma) in A. dest.

3.4.3. Western blotting:

To detect the proteins of interest, western blots were performed. This technique is based on the principle of antigen-antibody binding. To expose the proteins for detection they are transferred from the polyacrylamide gel to a nitrocellulose membrane by electroblotting. The

first antibody binds specifically to an epitope on the protein of interest and – as long it is used in appropriate concentrations- not to any other target on the membrane. The secondary antibody must be chosen due to the origin of the first one (e.g. mouse, goat) because it binds the Fc-region of the primary antibody, which is variable between different species. This secondary antibody is conjugated with an enzyme like HRP (horseradish peroxidase) that catalzyses a visible reaction and therefore enables the detection of the antigen-antibody complex.

For the transfer a “blotting sandwich” was prepared: The membrane was incubated in Towbin buffer for at least 5min and placed onto three pieces of Whatman paper that had been soaked with the same buffer. The polyacrylamid gel was carefully taken out from the apparature, the stacking gel was removed and the separating gel laid onto the membrane. Finally, the “blotting sandwich” was covered with three pieces of Whatman paper soaked with Towbin Buffer and air bubbles were removed by rolling a glas pipette. Blotting was carried out at 350mA.

To confirm that the transfer had been successful, the membrane was washed with tab water and stained with a Ponceau S solution. To facilitate the later analysis of the blot, the membrane was scanned or copied and destained with tab water.

For avoiding non-specific bindings the membrane was blocked in 5% milk for 30min. After washing the membrane three times with PBS Tween for 5min, it was incubated o.n at 4°C with the first antibody (diluted 1:2000 in 5% milk). To remove unbound primary antibodies the washing step was repeated (3 x 5min in PBS Tween) and the membrane incubated with the second antibody (diluted 1:5000 in 1% milk) for 1 hour at RT. For developing the blot, the membrane was incubated for 5min with a substrate containing luminol (SuperSignal West Pico Chemiluminiscent Substrate; Thermo Scientific) between two transparent foils and put into a film cassette. The HRP conjugated to the secondary antibody catalyzes the oxidation of luminol and thereby produces a chemoluminiscent signal. X-ray films (Super RX, Fuji) were laid onto the membrane and exposed for different durations (30sec-30min). The films were developed in a Curix 60 developing machine (Agfa).

10 x Running Buffer:	250mM Tris-base
	2M Glycin
	1% SDS
	in A. dest.

10 x Towbin Buffer:	250mM Tris-base
	1.92M Glycin
	20% (v/v) Methanol
	In A. bidest., pH 8.6
Milk:	5% skim milk powder (Merck) in A. bidest.
	0.02% NaN_3
PBS Tween:	136.89mM NaCl
	2.68mM KCl
	10mM $Na_2HPO_4 \cdot 7H_2O$
	1.76mM KH_2PO_4
	In A. bidest. ; pH 7.4
	0.1% (v/v) Tween

Primary Antibodies	Species	Dilution	Secondary Antibodies	Dilution
α-flag antibody	mouse	1:2000	α-mouse antibody	1:5000
α-HA antibody	rabbit	1:2000	α-rabbit antibody	1:5000
α-TAK antibody	rabbit	1:2000	α-rabbit antibody	1:5000

3.4.4. Co-Immunoprecipitation:

For the examination of protein interactions, co-immunoprecipitations were performed. For this purpose, HEK293 cells were transfected with an empty vector only or increasing concentrations of one of the genes encoding the proteins of interest. In parallel the same experiment was performed, co-transfecting the cells with constant amounts of a second gene encoding the eventually interacting product. To keep the total quantity of DNA constant, an empty vector was used.

	1	2	3	4	5	6
Gene 1 (µg)	0	1	2	0	1	2
Gene 2 (µg)	0	0	0	1	1	1
EV(µg)	4	3	2	3	2	1

Fig.3.5.:
Pipetting scheme of a typical co-immunoprecipitation performed in a 6-well plate

After 48 hours the medium was removed and the plate placed on ice. Cells were resuspended in 1ml cold PBS, transferred into pre-cooled Eppendorf tubes and harvested by centrifugation (700g; 5min; 4°C). Cells were washed in 1ml PBS (700g; 5min; 4°C) and lysates produced by resuspending them in 400µl Co-IP Buffer. After 30min incubation on ice debris was removed by centrifugation (13000rpm; 15min; 4°C). To ensure the over-expression of both proteins, 40µl of the supernatant were separated and boiled with 10µl 5xLaemmli at 95° for 5min (Input). For Co-Immunoprecipitation, 1µg antibody specific for one of the over-expressed proteins was added to the remaining supernatant. The antigen-antibody mixture was rotated at 4°C for 30min. Before adding 30µl Agarose beads, they were equilibrated by washing them in Co-IP Buffer. The beads are associated with proteins that bind the Fc region of immunglobulins and can therefore pull out antibody-associated protein complexes from the solution. The choice of the appropriate bead-associated protein is dependent on the origin of the antibody. For the precipitation of rabbit antibodies, protein A beads were used. After rotation at 4°C for at least 2 hours, the beads were pelleted by centrifugation (5000g; 2min; 4°C) and washed three times with Co-IP Buffer (5000g; 2min; 4°C). 20µl 2xLaemmli was added and the protein solution boiled on 95°C for 5min. For detecting the proteins, Western blotting was performed with the samples of the input and the Co-IP. If the two overexpressed proteins are interacting, both of them have to be detectable in the samples of the Co-immunoprecipitation, because the whole complex is pulled out of the solution. Two ensure that both proteins are overexpressed, both of them have to be detected in the samples from the input.

Co-IP Buffer: PBS + 0.5% (v/v) Triton X
0.3% (v/v) NP40

4. Aim and Strategy

Aim of the project was the identification of genes involved in negative regulatory mechanisms of inflammation.
“Negative feedback loops” are genes with inhibitory function that are expressed in response to pro-inflammatory stimulation. It has been shown by several examples (IκBα, A20) that proteins involved in the termination of an inflammatory reaction are expressed very early in the beginning of an inflammatory response. Thus, it could be supposed that among the 137 IL-1 induced genes identified in the microarray one or more could be involved in negative regulatory mechanisms. After selection of the targets of studies the strategy was as follows:

- Image Clones of the genes of interest were obtained and their identity pre-tested by restriction digestion. To exclude mutations of the plasmids the DNA was sequenced and compared to the reference sequence.
- By PCR the inserts were amplified and cloned into a TOPO vector as intermediate step. To exclude mutations the construct was sequenced.
- The inserts were subcloned into a mammalian expression vector carrying a flag epitope.
- The expression of the genes of interest was confirmed by transfection of HEK293 followed by western blotting using anti-flag antibodies.
- To study the influences of the genes of interest on different signaling pathways, a number of different reporter gene assays was performed (NF-κB, AP1-luc, NFAT-luc).

5. Results

5.1. Testing of IMAGE clones:

The IMAGE clones were cultivated and the plasmids isolated. For testing the identity and integrity of the plasmids, restriction analysis was performed. Restriction enzymes that cut sites flanking the genes in a most minimal distance were chosen, so the expected fragments were corresponding to their length.

Gene Symbol	**GG2-1**	**JAG1**	**TNFSF15**	**TNFRSF11B**
I.M.A.G.E. ID	3965693	6834418	30740802	6506550
Geneservice Co-ordinate	AV21-C8	AV131-G11	AV151-b8	AV105-H5
MGC ID	11714	76404	0	59365
Species	Mouse	Mouse	Mouse	Mouse
Vector	pCMV-SPORT6	pYX-Asc	pCMV-SPORT6	pCMV-SPORT6.1
Collection	IRAV	IRAV	IRAV	IRAV
GenBank Accession Number	BC009090	BC058675	CF905061	BC049782
Unigene Cluster	27740	22398	BC096494	15383
Genbank Definition	TNFα-induced protein 8	Jagged-1	Tumor necrosis factor (ligand) superfamily	Tumor necrosis factor receptor 11B

Table 5.1: **IMAGE clones**

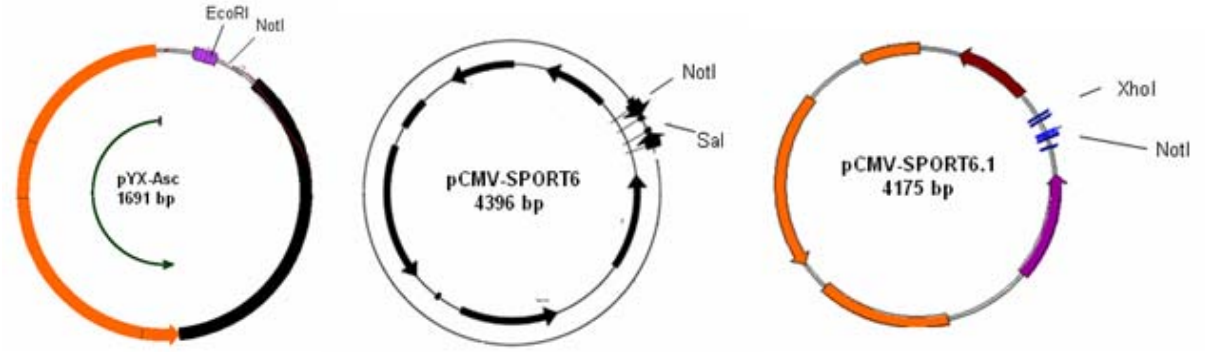


Fig. 5.1: **Vector maps of Image Clones** and recognition sites used for restriction digest

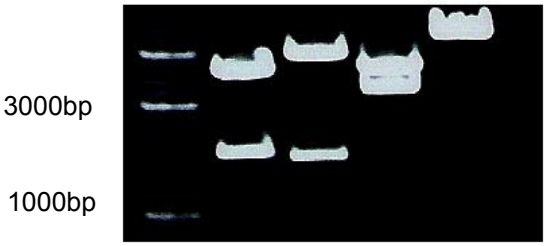


Fig. 5.2:
Restriction analysis of IMAGE clones

Lane 1: GG2-1 (1.8kb) excised from SPORT6 (4.4kb) using NotI and SalI
Lane 2: JAG1 (5.4kb) excised from pYX-Asc (1.7kb) using EcoRI and NotI
Lane 3: TNFSF15 (3.4kb) excised from SPORT6 (4.4kb) using NotI and SalI
Lane 4: TNFRSF11B, uncut by XhoI and NotI

The restriction analysis let suggest that the desired IMAGE clones had been delivered in the cases of JAG1, GG2-1 and TNFSF15. TNFRSF11B was not restricted as expected. To verify these results and exclude mutations in the genes of interest, the plasmids were sequenced. The coding regions of GG2-1, JAG1 and TNFSF15 were identical with the reference sequence. The sequence of TNFRSF11B was different. BLAST analysis showed that the gene was not mutated, but that the original IMAGE clone was distinct from the reference sequence. Thus, working on this gene was discontinued.

5.2. TOPO Cloning

The genes of interest were amplified by PCR from the plasmids isolated from the IMAGE clones. Since the expression primers had been designed to anneal at the ends of the coding regions the expected products were corresponding to their lengths.

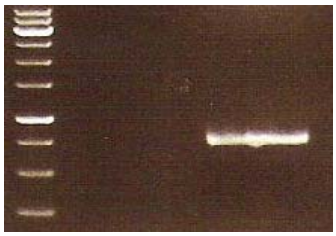


Fig. 5.3.:
PCR products of TNFSF15
Coding region: 0.76kb
1kb ladder

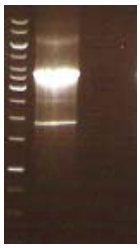


Fig. 5.4.:
PCR products of JAG1
Coding region: 3.6kb
1kb ladder

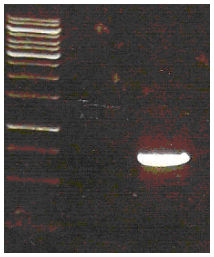


Fig. 5.5:
PCR products of GG2-1
Coding region: 0.6kb
1kb ladder

The bands representing the coding regions were excised, the DNA extracted and the PCR products ligated into pVP22 vectors. After transformation of competent DH5α, colonies carrying the vector were identified by cultivation on a selective plate. To exclude that the colonies were carrying a re-ligated vector only, several of them were grown up. The plasmids were isolated and restriction analysis performed. For restriction, the enzymes corresponding to the recognition sequences within the primers were used, so the expected fragments were almost identical with the lengths of the coding regions.

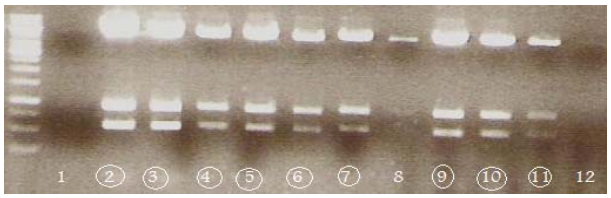


Fig. 5.6.:
Restriction analysis of TOPO vectors containing GG2-1
The coding region (0.6kb) was excised using BamHI and HindIII
1kb ladder

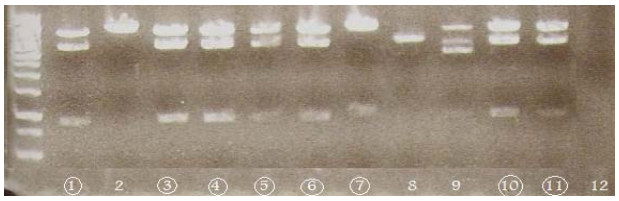


Fig. 5.7.:
Restriction analysis of TOPO vectors containing JAG1
The coding region (3.5kb) was excised using BamHI and HindIII
1kb ladder

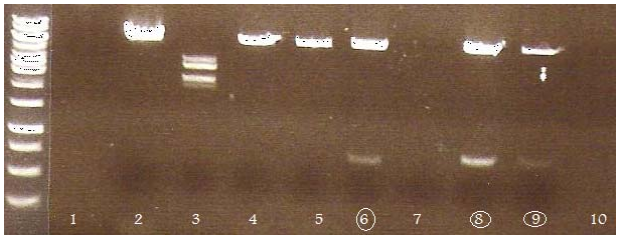


Fig. 5.8.:
Restriction analysis of TOPO vectors containing TNFSF15
The coding region (0.76kb) was excised using KpnI and SfuI
1kb ladder

5.3. Cloning into pcDNA3

Plasmid DNA of colonies carrying the pPV22 vector with the desired insert was isolated by performing a maxi-prep. The genes of interest were cut out of the TOPO vector using the restriction sites incorporated in the primers. The expression vectors pcDNA3 or pcDNA3.1-His A were digested with the same enzymes.

Fig. 5.9.:
Restriction of pcDNA3 and GG2-1 for cloning
Left: pcDNA3 (5.4kb) linearized by BamHI and HindIII
Right: coding region of GG2-1 (0.6kb) and pVP22 backbone
1kb DNA ladder

Fig. 5.10.:
Restriction of pcDNA3 and JAG1 for cloning
Left: pcDNA3 (5.4kb) linearized by BamHI and HindIII
Right: coding region of JAG1 (3.5kb) and pVP22 backbone
1kb DNA ladder

Fig. 5.11:
Restriction of pcDNA3.1 His A and TNFSF15 for cloning
Left: pcDNA3.1 HisA (5.5kb) linearized by SfuI and KpnI
Right: coding region of TNFSF15 (0.76kb) ad pVP22 backbone
1kb DNA ladder

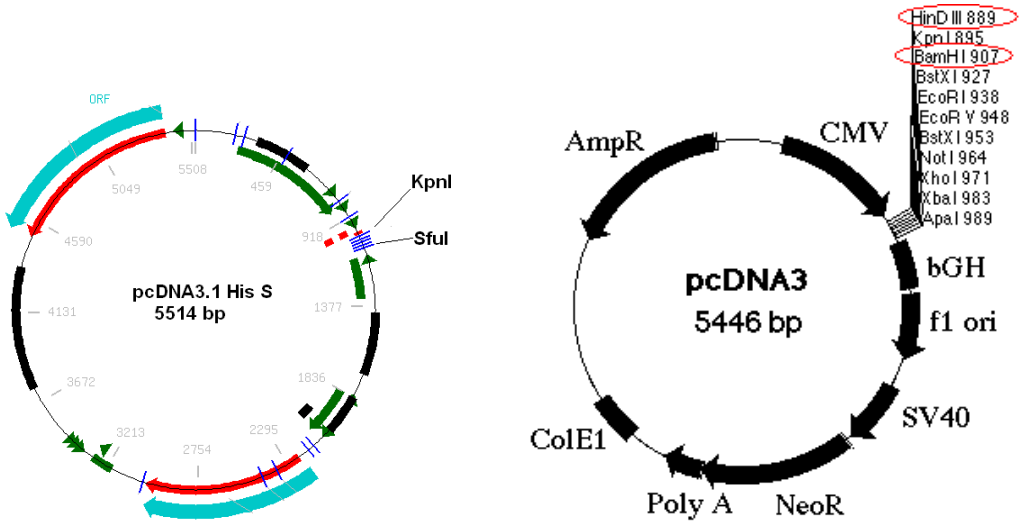


Fig. 5.12:
Gene maps of mammalian expression vectors pcDNA3 / pcDNA3.1-HisA
with restriction sites used for incorporating the genes of interest

The bands were excised from the gel, the DNA extracted and vector and insert ligated. Positive colonies were identified with the same strategy pursued after TOPO cloning.

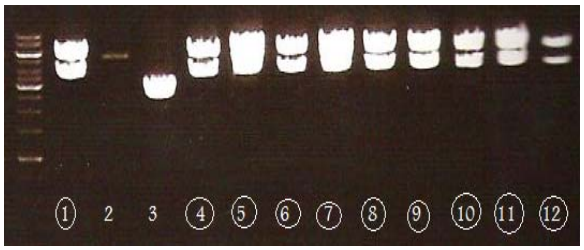


Fig. 5.13:
Restriction analysis of pcDNA3 ligated with JAG1 (3.5kb)
The insert was excised using BamHI and HindIII

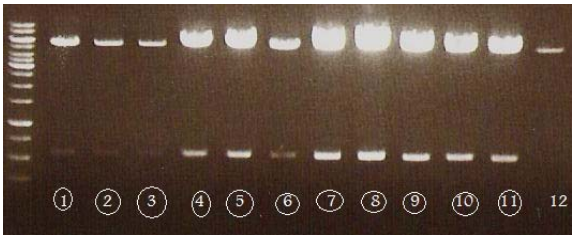


Fig. 5.14:
Restriction analysis of pcDNA3 ligated with GG2-1 (0.6kb)
The insert was excised using BamHI and HindIII

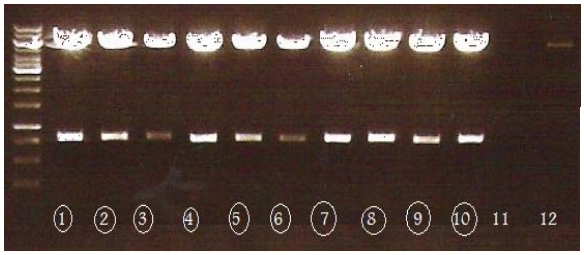


Fig. 5.15:
Restriction analysis of pcDNA3.1-His A ligated with TNFSF15 (0.76bp)
The insert was excised using SfuI and KpnI

5.4. Test on expression of constructs

The constructs were transfected into HEK293 cells in different concentrations. Expression of the genes was demonstrated by production of cell lysates, performance of SDS PAGE and detection of the proteins by western blotting. Expression of GG2-1 and JAG1 could be confirmed. The protein encoded by TNFSF15 was not detectable. Re-cloning of the construct gave the same result. The reasons for the lack of expression for this protein could not be clarified, therefore this gene was not further examined.

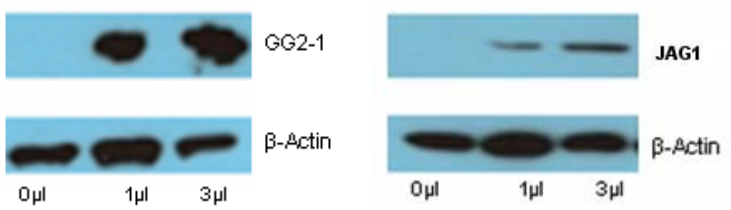


Fig 5.16: **Western Blots** detecting GG2-1 (left) and JAG1 (right)

5.5. GG2-1: Reporter gene assays

5.5.1. Effects of GG2-1 on NF-κB activity

TNFα is a ligand for TNFR1 and TNFR2. TNFR1 is expressed in almost all tissues and activates NF-κB mediated by the classical and the non-canonical pathway. Upon ligand binding it recruits TRADD and TRAF2. TRAF2 activates RIP by ubiquitination. Consequently the TAK1/TAB1/TAB2 complex is activated leading to phosphorylation of the IKK complex. [51, 52]
The TNFR further transmits signals to MAP3K like MEKK1, MEKK3 and ASK1. Also MEKK1 and MEKK3 have been shown to phosphorylate the IKK complex by direct interactions. [53]

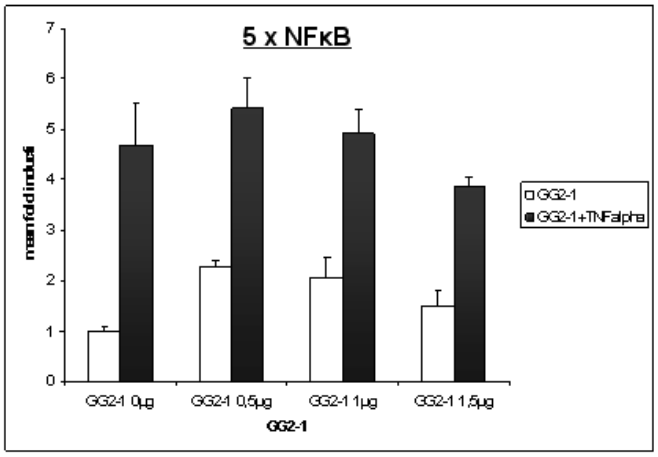


Fig. 5.17.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV only or increasing amounts of GG2-1 Cells remained unstimulated or were stimulated with a constant quantity of TNF-α

GG2-1 over-expression did not show inhibitory influence on NF-κB activity in HEK293 cells stimulated with TNF-α. The protein rather seemed to have activating effects on the transcription factor. However, the effects of GG2-1 overexpression on TNF-α stimulated reporter gene expression were not extensive.
Since the downstream components of the TNFR signalling pathways are also activated by distinct signalling cascades, the assay was repeated co-transfecting HEK293 with some of these proteins.

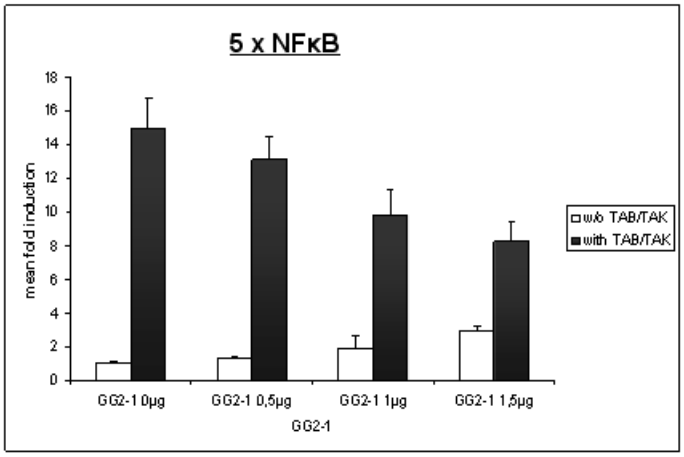


Fig. 5.18.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV only or increasing amounts of GG2-1 NF-κB induction is achieved by co-transfection of constant quantities of TAB1 and TAK1

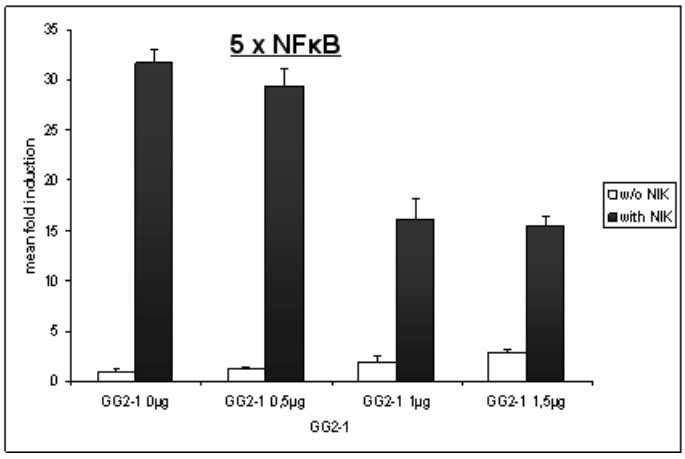


Fig. 5.19.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV or increasing amounts of GG2-1 NF-κB induction is achieved by co-transfection of a constant quantity of NIK

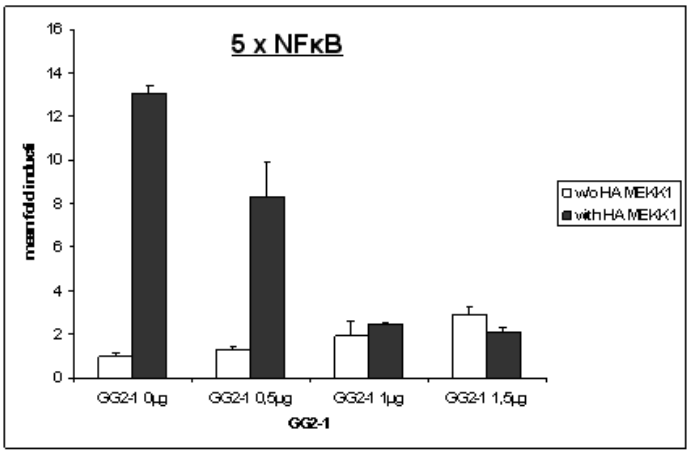


Fig. 5.20.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV or increasing amounts of GG2-1 NF-κB induction is achieved by co-transfection of a constant quantity of MEKK1

In cells co-stimulated by TAK1/TAB1 a reduction of NFκB activity was observed with increasing concentrations of GG2-1. Similar results were achieved by co-stimulation with NIK, an inducer of the non-classical pathway of NFκB activation. However, in both cases the inhibition of luciferase activity was not more that 50% in response to the highest transfected

amount of GG2-1. The only significant inhibition could be observed in the cells over-expressing MEKK1. In these assays a mean inhibition of NFκB activity to ~20% was achieved by smaller concentrations of GG2-1.

To see if GG2-1 acts similarly down-stream from the MAP3K, cells were co-transfected with IKK2, a catalytic subunit of the IKK complex. The NFκB activity was not influenced by GG2-1 over-expression in this approach.

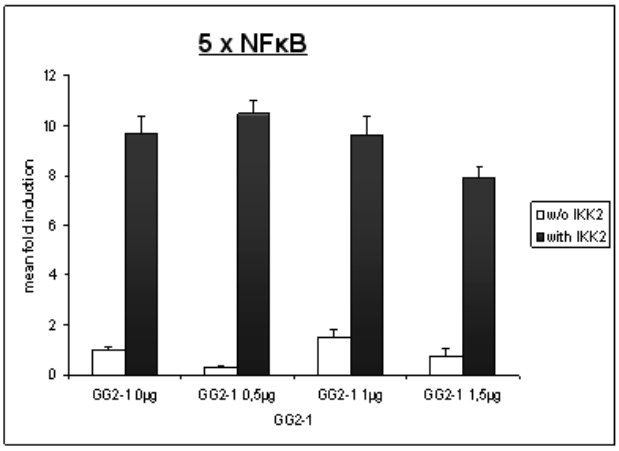


Fig. 5.21.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV or increasing amounts of GG2-1
NF-κB induction is achieved by co-transfection of a constant quantity of IKK2.

5.5.2. Effects of GG2-1 on AP-1 activity

Like mentioned above MAP/ERK kinase kinase 1 (MEKK1) is activated via TNFR signaling. Consequently it can activate the MAPK kinase 7 (MKK7) that leads to activation of the MAP kinase JNK (c-Jun terminal kinase). JNK phosphorylates c-Jun and thereby enables the formation of heterodimers with other proteins.

MEKK1 also induces another kinase cascade. Via activation of the MAPK kinases MEK1/2 it leads to activation of ERK1/2 and enables subsequent phosphorylation of ELK1. In the phosphorylated form this transcription factor induces the expression of its target genes including c-Fos, another component of AP-1. [54,55]

Because MEKK1 activates both, the ERK and the JNK pathway, it is a very strong inducer of AP-1. HEK293 cells were transfected with a constant quantity of a plasmid expressing the catalytic domain of MEKK1 and increasing concentrations of GG2-1. A concentration-dependent decrease of AP-1-dependent reporter gene expression could be observed. 1µg GG2-1 caused a mean inhibition of more than 50%, with higher concentration a reduction to 15% compared to the control sample was achieved.

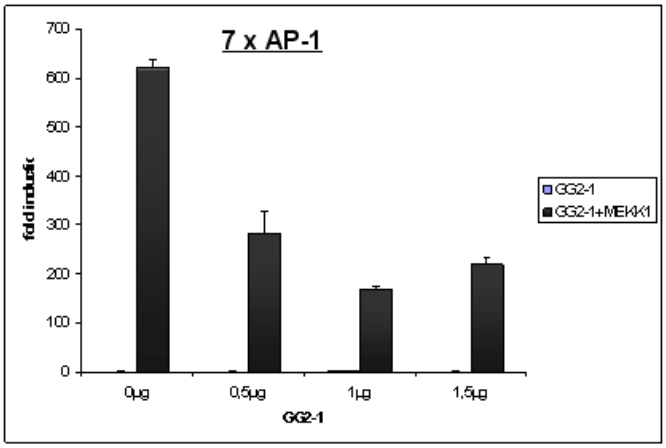


Fig. 5.22.:
Reporter gene assays in HEK293
AP-1 promoter activity in cells transfected with EV or increasing amounts of GG2-1
AP-1 induction is achieved by co-transfection of a constant quantity of MEKK1

In order to detect the stimulating protein by western blotting, the experiment was repeated with a construct encoding the full length form of MEKK1 fused to a HA-tag. The results were similar. However, the inhibition of luciferase activity by increasing concentrations of GG2-1 was significantly higher. Transfection of 0.5µg GG2-1 caused a decrease of reporter gene expression to 25% compared to the control sample. Higher concentrations lead to a reduction of 95%.

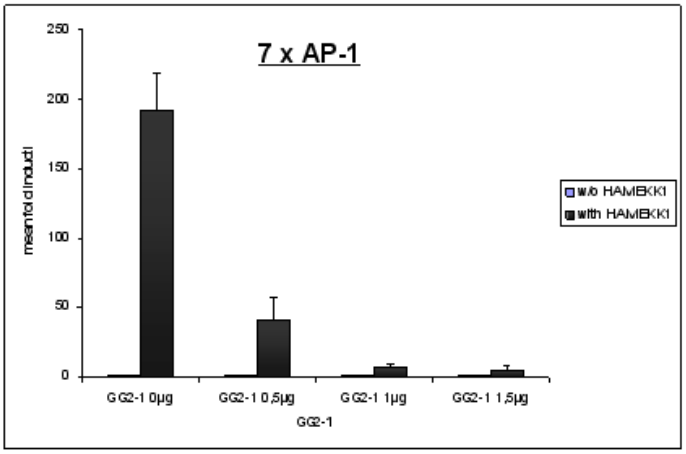


Fig. 5.23.:
Reporter gene assays in HEK293
AP-1 promoter activity in cells transfected with EV or increasing amounts of GG2-1
AP-1 induction is achieved by co-transfection of a constant quantity of HA-tagged MEKK1

To test if GG2-1 has an effect on other MAP3K, cells were transfected with TAK1/TAB1 and MEKK3. TAK1 is activated by TNFR and IL1/TLR signaling. RIP1 or TRAF6 enable the formation of a complex of TAK1 with the adapter proteins TAB1 and TAB2. Consequently MEKK7 is phosphorylated and leads to activation of JNK. [57]
GG2-1 over-expression caused a decrease of AP-1 activity in HEK293 cells transfected with a constant quantity of TAK1 and TAB1. This suppression was not extensive. In average the lowest transfected quantity of GG2-1 caused a reduction of reporter gene activity of 30%. Higher concentrations did not enhance this decrease significantly.

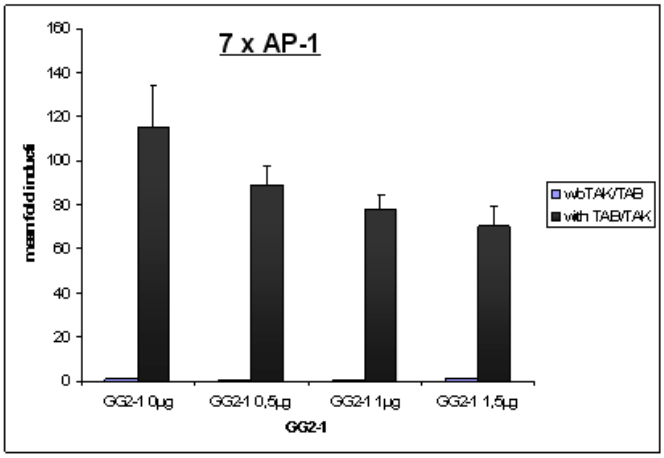


Fig. 5.24.:
Reporter gene assays in HEK293
AP-1 promoter activity in cells transfected with EV or increasing amounts of GG2-1
AP-1 induction is achieved by co-transfection of constant quantities of TAB1 and TAK1

MEKK3 also induces the JNK pathway via activation of MKK7. Amongst others, it is activated via TNFR signaling. In cells co-transfected with MEKK3 the results of the reporter gene assay were completely different from the approaches with the other MAP3K. The activity was not decreasing, but rather showing the opposite tendencies.

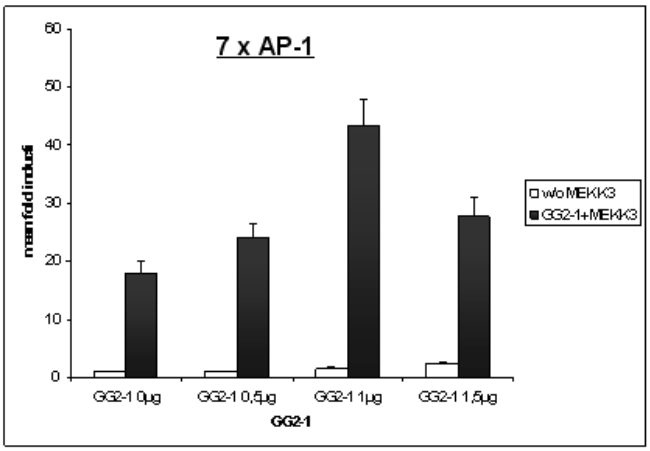


Fig. 5.25.:
Reporter gene assays in HEK293
AP-1 promoter activity in cells transfected with EV or increasing amounts of GG2-1
AP-1 induction is achieved by co-transfection of a constant quantity of MEKK3

To study the effects of GG2-1 over-expression on the ERK pathway, the cells were stimulated with phorbol 12-myristate 19-acetate. PMA activates the protein kinase C (PKC) that activates Raf. Consequently the signaling cascade consisting of MEK1/2, ERK1/2 and ELK leads to expression of c-Fos [57]. In HEK293 cells, stimulated with a constant amount of PMA these pathway was not affected by GG2-1.

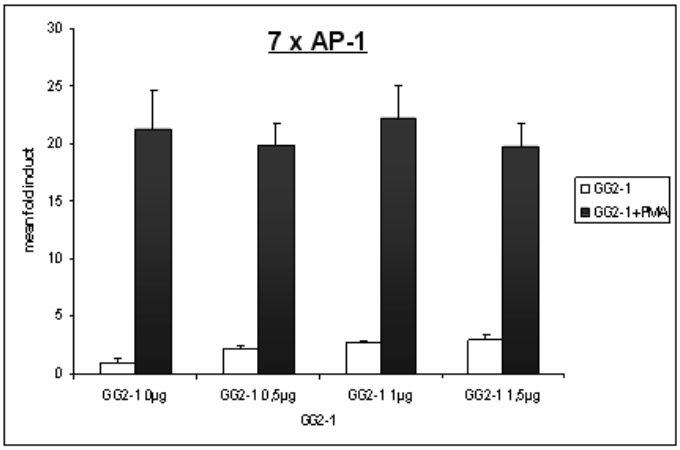


Fig. 5.26.:
Reporter gene assays in HEK293
AP-1 promoter activity in cells transfected with EV or increasing amounts of GG2-1
Cells remained unstimulated or were stimulated with a constant quantity of PMA.

5.5.3. Effects of GG2-1 on NFAT activity

Activation of NFAT is achieved by various signaling pathways that cause an increase of the intracellular calcium levels. For instance the activation of phospholipase C (PLK) can lead to the release of intracellular calcium stores. The enzyme cleaves phosphatidylinositol-4,5-biphosphate (PIP_2) to diacyl glycerol (DAG) and inositol-1,4,5,-triphosphate (IP_3). The former one is bound to the membrane and is involved in the activation of PKC leading to induction of the ERK pathway as decribed in the former chapter. The latter one translocates to its specific receptors on the endoplasmatic reticulum (ER) resulting in the release of Ca^+. The sensor protein calmodulin (CaM) activates the phosphatase calcineurin (CN) leading to dephosphorylation of NFAT. [58, 59]

The activity of NFAT in response to increasing concentrations of GG2-1 was examined. Induction of NFAT-dependent luciferase expression was achieved by co-transfection of a constant quantity of NFAT-GFP. GG2-1 reduced the reporter gene expression in a concentration-dependent manner. Compared to the mock sample transfection of 0.5µg, 1µg or 1.5µg led to a decrease of 75%, 50% or 25%.

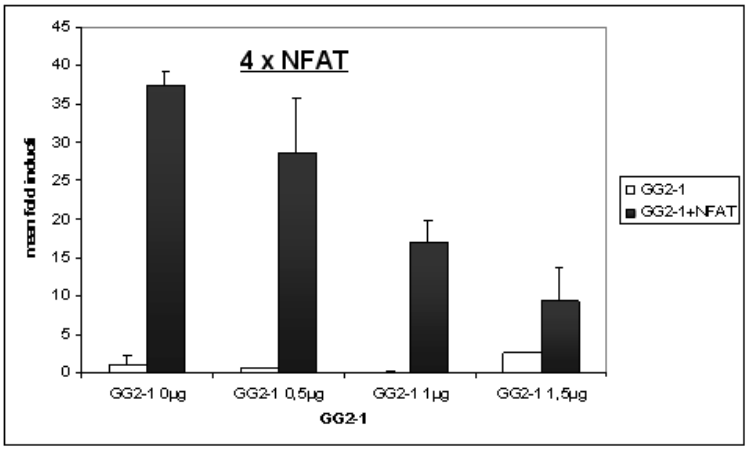


Fig. 5.27.:
Reporter gene assays in HEK293
NFAT promoter activity in cells transfected with EV or increasing amounts of GG2-1
NFAT induction is achieved by co-transfection of a constant quantity of NFAT-GFP

5.5.4. Effects of GG2-1 on CHOP activity

Another transcription factor mediating the transmission of stress signals is CHOP. It is induced by phosphorylation by the active MAPK p38 and acts on genes involved in cellular growth, differentation and inflammation. The activation of p38 is induced by environmental stress or pro-inflammatory cytokines. TNFR signaling activates p38 via activation of ASK resulting in activation of MEKK3/6. These kinases are also activated by TAB1/TAB1/TAB2 resulting in the phosphorylation of p38. Stress signals causing p38 activation are transmitted by small GTPases of the Rho family (Rho,Rac, Cdc42). [60]

In HEK293 cells co-transfected with p38α increasing amounts of GG2-1 had no inhibitory effects on the activity of CHOP. Rather it caused enhanced reporter gene expression, whereby this effect was not extensive. The highest transfected quantities of GG2-1 caused a 2-fold induction compared to the control sample.

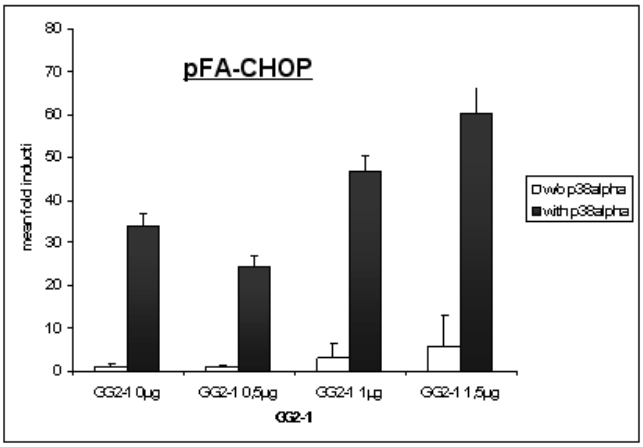


Fig. 5.28.:
Reporter gene assays in HEK293
CHOP activity in cells transfected with EV or increasing amounts of GG2-1
CHOP induction is achieved by co-transfection of a constant quantity of p38α

5.5.5. Effects of GG2-1 on p53 activity

HEK293 cells co-transfected with constructs encoding the tumor suppressor p53 showed a concentration-dependent decrease of p53-dependent reporter gene activity. The highest transfected quantity of GG2-1 caused a mean suppression of reporter gene activity to 50% compared to the control sample.

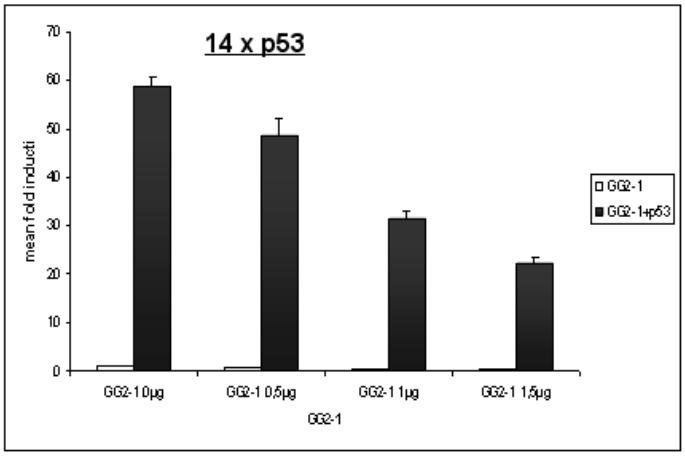


Fig. 5.29.:
Reporter gene assays in HEK293
tp53 activity in cells transfected with EV or increasing amounts of GG2-1
tp53-dependent reporter gene expression induction is achieved by co-transfection of a constant quantity of p53

5.5.6. Effects of GG2-1 on the IL-8 promoter

The pro-inflammatory cytokine IL-8 contains binding sides for NF-κB and AP-1 in its promoter.

An assay using a reporter construct carrying a luciferase gene under the control of a -600 bp IL-8 promoter was performed. In former experiments cells co-transfected with MEKK1 showed a decrease of NFκB and AP-1 activity in response to GG2-1. In line with these observations, the IL-8 promoter-dependent reporter gene expression was strongly inhibited by GG2-1 in cells co-transfected with the full length form of MEKK1.

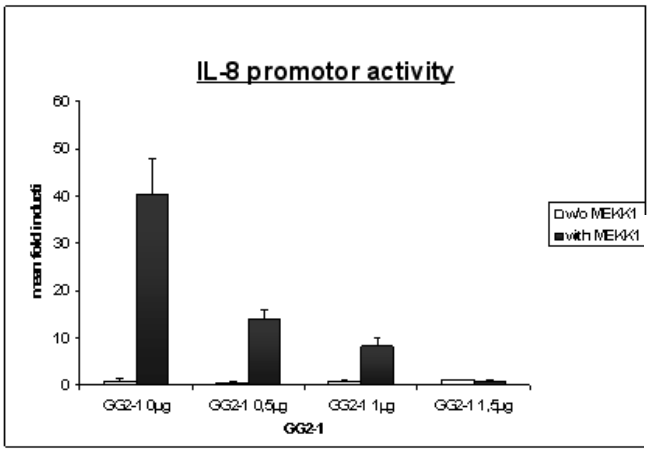


Fig. 5.30.:
Reporter gene assays in HEK293
IL-8 promoter activity in cells transfected with EV or increasing amounts of GG2-1
IL-8 promoter activity is induced by co-transfection of a constant quantity of MEKK1

The same approach was performed by co-transfecting MEKK3. In the AP-1 reporter gene assay GG2-1 showed an inductive effect in MEKK3 stimulated HEK293 cells. A similar influence was observed in the IL-8 reporter assay. On average GG2-1 caused 5-fold induction of reporter gene expression compared to the control sample. This effect was widely concentration-independent.

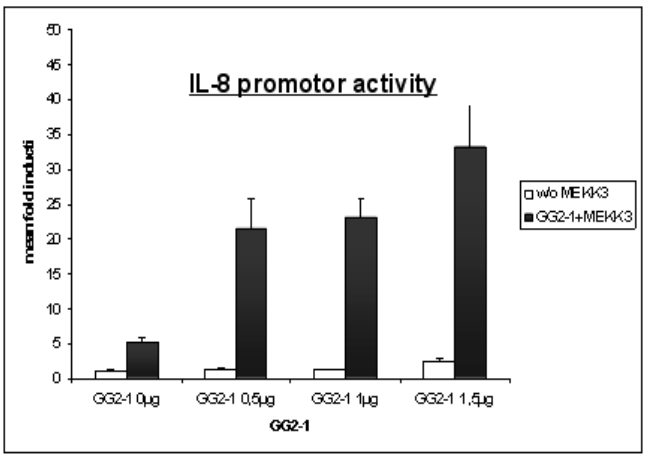


Fig. 5.31.:
Reporter gene assays in HEK293
IL-8 promoter activity in cells transfected with EV or increasing amounts of GG2-1
IL-8 promoter activity is induced by co-transfection of a constant quantity of MEKK3

TNF-α stimulation of the cells led to a similar result like on the NF-κB-reporter. No inhibitory effects could be observed. In contrast, GG2-1 over-expression seemed to have an enhancing effect on reporter genes expression. However, this effect was not very pronounced, the maximal induction was 2-fold in average.

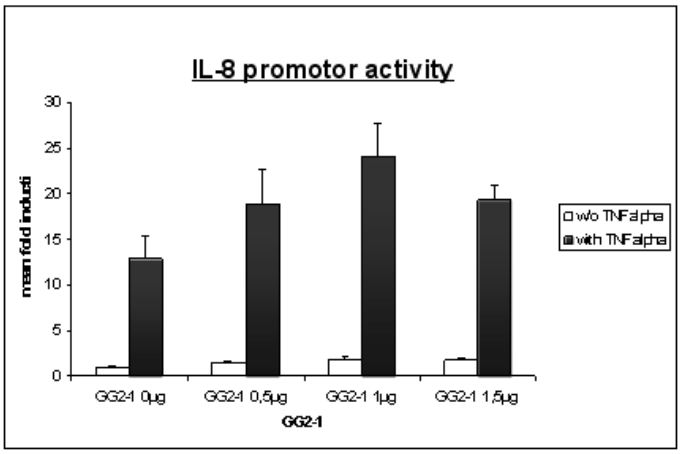


Fig. 5.32.:
Reporter gene assays in HEK293
IL-8 promotor activity in cells transfected with EV or increasing amounts of GG2-1
Cells remained unstimulated or were stimulated with a constant quantity of TNFα.

In summary, the reporter gene assays showed, that GG2-1 has strong inhibitory effects on the activity of the NF-κB, AP-1 and IL-8 promoters in cells co-transfected with MEKK1. Also TAB/TAK and NIK induced signaling pathways were moderately suppressed by GG2-1 overexpression as could be observed in NF-κB and/or AP-1 reporter gene assays. Pathways induced by components downstream from the MAP3K were hardly affected by GG2-1, as was shown by stimulation of the cells with PMA or co-transfection of IKK2. Beside to the influence of GG2-1 on promoters containing κB or AP-1 binding sides, it also exhibited inhibitory effects on NFAT and p53 promoter activity, but not on CHOP.

5.6. GG2-1: Western Blots

To prove that the observed effects were caused by over-expression of the transfected genes, Western blots were performed detecting the encoded proteins.

An inhibiting influence of GG2-1 on NF-κB, AP-1 and IL-1 promoter activity had been observed in cells co-transfected with MEKK1. The lysates of NF-κB and AP-1 reporter gene assays were analyzed by Western blotting. Detection of MEKK1 via its HA-tag revealed that the inhibitory effect resulted from decreasing concentrations of the stimulating protein. The more GG2-1 was transfected into the cells, the less MEKK1 was detectable in the lysates.

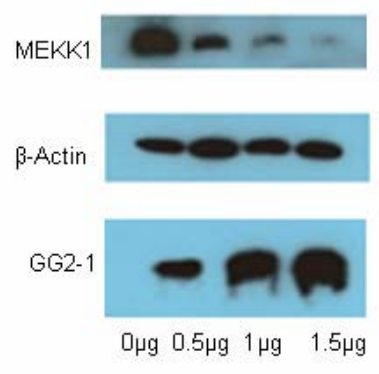


Fig. 5.33.:
Western Blot
Detection of MEKK1 (anti HA-AB), GG2-1 (anti-flag-AB) and β-Actin (anti-β-Actin-AB) as loading control in cell lysates from an AP-1reporter gene assay
The 196kDa form of MEKK1 is decreasing with increasing GG2-1 levels.

In contrast, the protein levels of other MAP3K like MEKK1 or TAK1 seemed to be independent from GG2-1 over-expression

The lysates of cells from AP-1 or NF-κB reporter gene assay were analysed. In both cases the protein levels of MEKK3 or TAK1 were not altered in the presence of GG2-1.

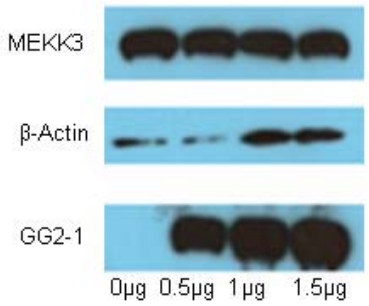


Fig. 5.34.:
Western Blot
Detection of MEKK3 (anti-flag-AB), GG2-1 (anti-flag-AB) and β-Actin (anti-β-Actin-AB) as loading control in lysates from an AP-1 reporter gene assay
The concentration of MEKK3 is independent on the presence and concentration of GG2-1

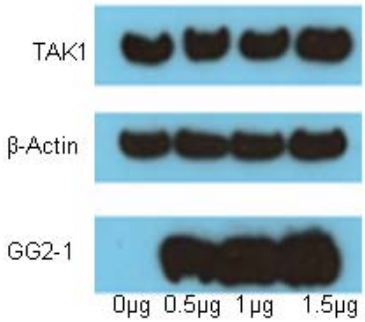


Fig. 5.35.:
Western Blot
Detection of TAK1 (anti-TAK1-AB), GG2-1 (anti-flag-AB) and β-Actin (anti-β-Actin-AB) as loading control in lysates from an AP-1 reporter gene assay
The concentration of TAK1 is independent from the presence or concentration of GG2-1

5.7.: GG2-1: Co-Immunoprecipiation

In order to examine if the degrading effect of GG2-1 on MEKK1 is caused by direct interactions between the two proteins, co-immunoprecipitation was performed. For this purpose, cells were transfected without or with increasing amounts of MEKK1 in the presence or absence of GG2-1.
In this experiment no direct interaction of the two proteins could be observed.

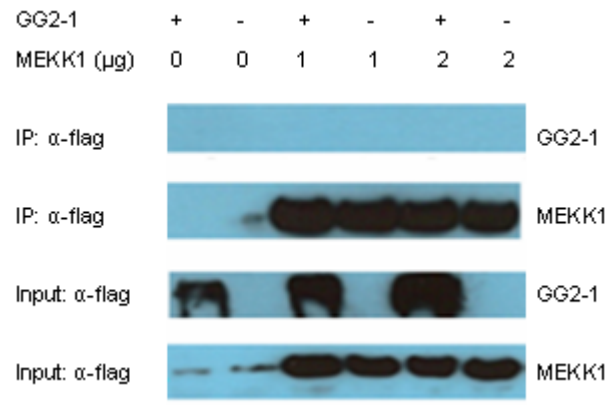


Fig. 5.36.:
Co-Immunoprecipitation
GG2-1 (anti-flag-AB) and MEKK1 (anti-HA-AB) were detected by Western blotting
GG2-1 was detectable in the input, but not in the co-immunoprecipitation
As control, MEKK1 was detected in both, the input and the co- immunoprecipitation

5.8. JAG1: Reporter gene assays

5.8.1. Effects of JAG1 on NF-κB activity

Over-expression of JAG1 caused a decrease of NF-κB-dependent reporter gene activity in TNF-α-stimulated HEK293 cells. Transfection of lower amounts of JAG1 caused reductions of luciferase expression to 63% or 55% compared to the control sample. A significant effect was observable in the cells transfected with the highest concentration of JAG1. Reporter gene activity was reduced to 11%.

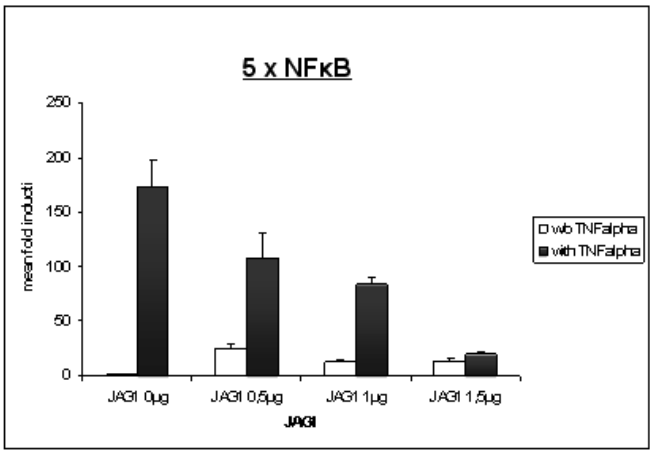


Fig 5.37 .:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV only or increasing concentrations of JAG1
Cells remained unstimulated or were stimulated with a constant quantity of TNF-α.

To get further information, cells were co-transfected with components of pathways downstream from TNFR. In cells co-transfected with TAK1/TAB1 no effect of JAG1 on NF-κB activity was observed. Only the highest transfected concentration of JAG1 caused a decrease of reporter gene expression. However, the first two samples show the opposite tendencies and therefore it is probable that the reduction is caused by an “over-loading” of the system.

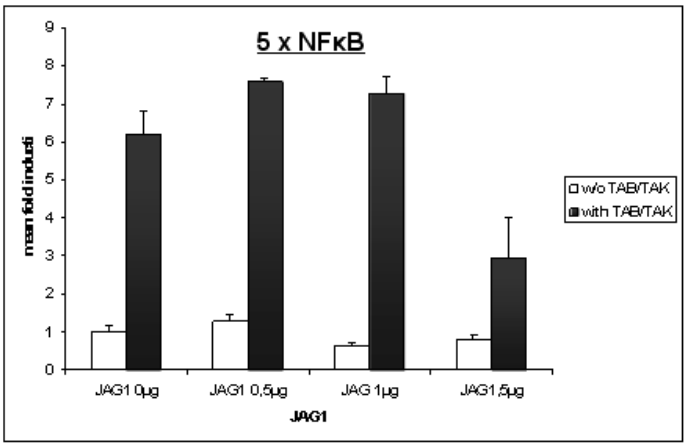


Fig 5.38.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV only or increasing amounts of JAG1
NF-κB induction is achieved by co-transfection of constant quantities of TAB1 and TAK1

Similarly to the TNF-α stimulated cell, HEK293 co-transfected with NIK1 showed a concentration-dependent decrease of reporter gene expression. The lower transfected concentrations caused a decrease of NF-κB activity of 62% or 47% compared to the control-sample. Higher amounts of JAG1 caused a reduction to 12%. Almost identical results were achieved by co-transfection of MEKK1. Compared to the control-sample the reporter gene expression was reduced proportional to increasing concentrations of JAG1 to 60%, 52% or 13%. Transfection of the full length form of MEKK1 showed similar results. (data not shown)

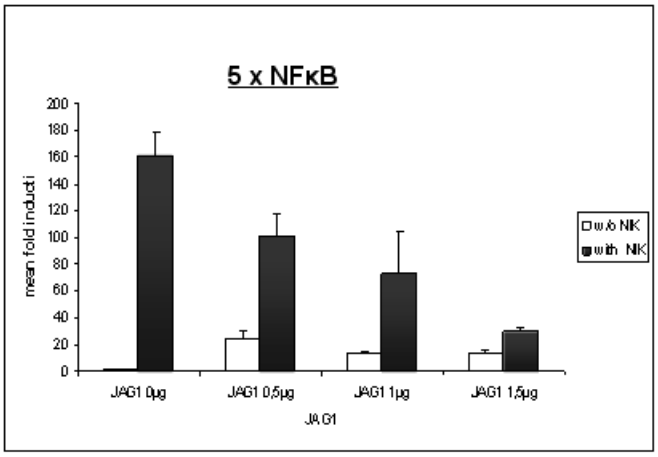


Fig.5.39.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV only or increasing amounts of JAG1 NF-κB induction is achieved by co-transfection of a constant quantity of NIK1

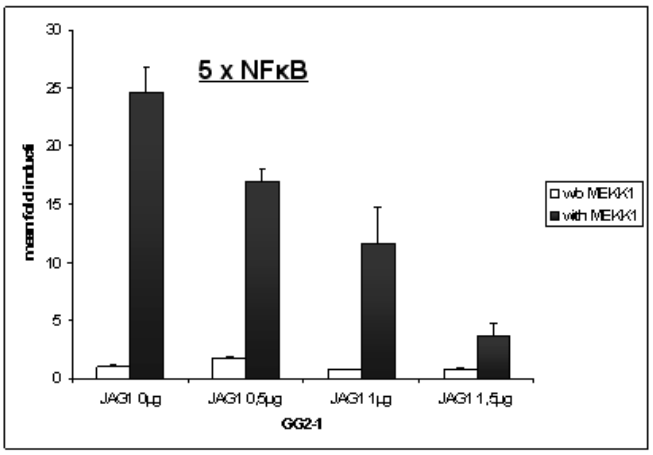


Fig.5.40.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV only or increasing amounts of JAG1 NF-κB induction is achieved by co-transfection of a constant quantity of MEKK1

To get an impression of the effect of JAG1 downstream from the MAP3K, cells were co-transfected with IKK2. In these approaches JAG1 did not show effects on NF-κB-dependent reporter gene expression.

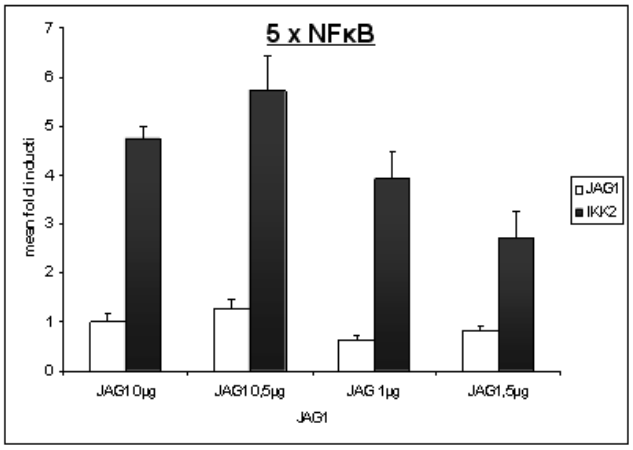


Fig.5.41.:
Reporter gene assays in HEK293
NF-κB promoter activity in cells transfected with EV only or increasing amounts of JAG1
NF-κB induction is achieved by co-transfection of a constant quantity of IKK2

5.8.2. Effects of JAG1 on AP-1 activity

HEK293 cells co-transfected with MEKK1 showed a decrease of AP-1 dependent reporter gene activity correlating with increasing amounts of JAG1. However, the effect was not extensive. The lowest transfected quantity of JAG1 did hardly inhibit luciferase expression, higher amounts led to a reduction to 33% compared to the control-sample.

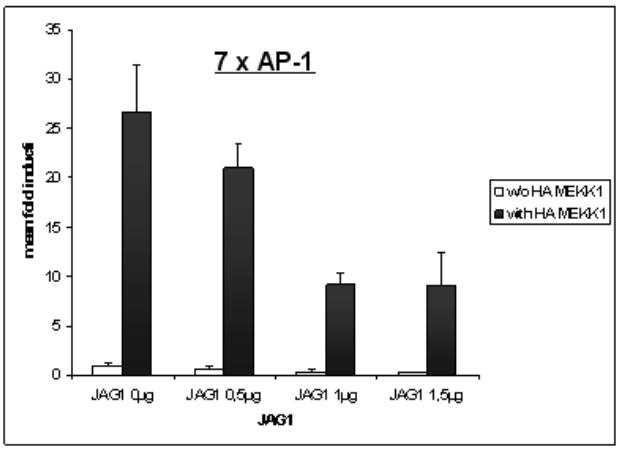


Fig.5.42.:
Reporter gene assays in HEK293
AP-1 promoter activity in cells transfected with EV only or increasing amounts of JAG1
AP-1 induction is achieved by co-transfection of a constant quantity of HA-tagged MEKK1

Similar results were achieved by co-transfection of TAK1/TAB1. AP-1 activity decreased with increasing levels of JAG1, whereby with the highest transfected quantity of JAG1 led only to a reduction of 50% compared with the control-sample.

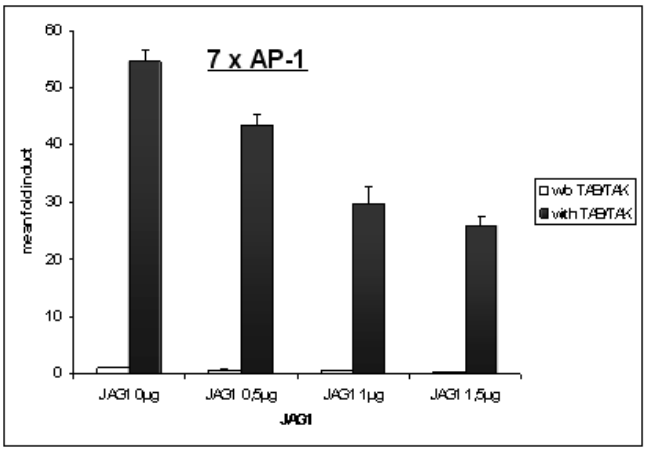


Fig.5.43.:
Reporter gene assays in HEK293
AP-1 promoter activity in cells transfected with EV only or increasing amounts of JAG1
AP-1 induction is achieved by co-transfection of constant quantities of TAB1 and TAK1

5.8.3. Effects of JAG1 on NFAT activity

Over-expression of JAG1 did not show significant effects on NFAT activity. NFAT-dependent reporter gene expression was reduced to ~60% compared to the control-sample, whereby this effect seemed to be widely concentration-independent.

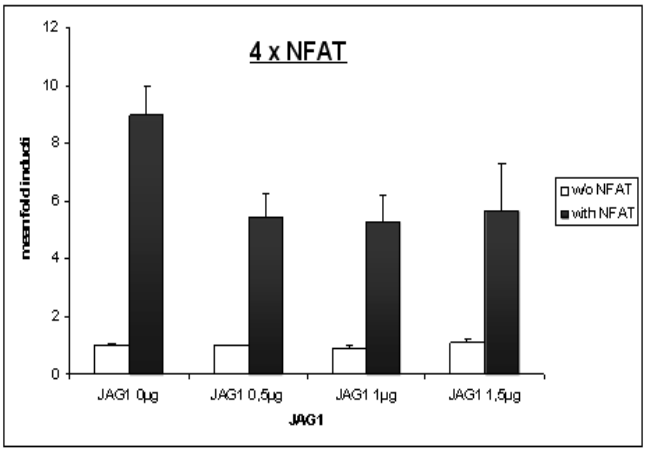


Fig.5.44.:
Reporter gene assays in HEK293
AP-1 promoter activity in cells transfected with EV only or increasing amounts of JAG1
NFAT induction is achieved by co-transfection of a constant quantity of NFAT-GFP

5.8.4. Effects of JAG1 on p53 activity

Reporter gene assay examining the activity of p53 revealed a significant inhibitory effect of JAG1 on this transcription factor. Also when low concentrations of JAG1 did not show a relevant influence on reporter gene expression, higher amounts led to a reduction to 11% or 2% compared to the control sample.

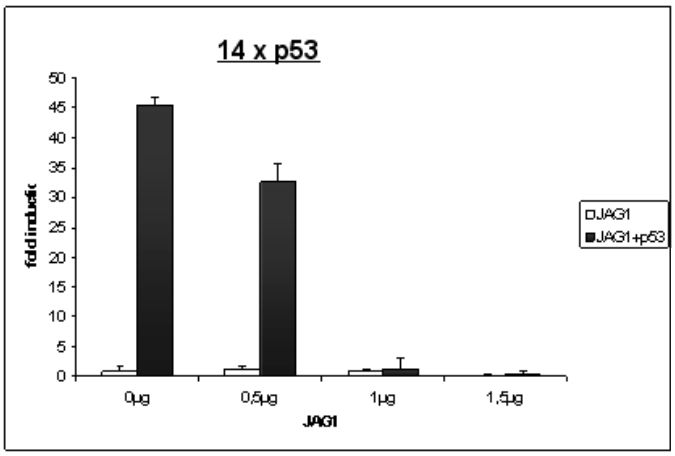


Fig.5.45.:
Reporter gene assays in HEK293
AP-1 promotor activity in cells transfected with EV only or increasing amounts of JAG1
tp53-dependent reporter gene expression induction is achieved by co-transfection of a constant quantity of p53

5.8.5. Effects of JAG1 on the IL-8 promoter

The results of the NF-κB and AP-1 reporter gene assays were confirmed examining the IL-8 promoter. Accordingly to the former experiments, JAG1 exhibited a moderate inhibitory effect on IL-8 promoter activity. Over-expression of JAG1 caused a decrease of reporter gene expression to 55%. This effect was widely concentration-independent.

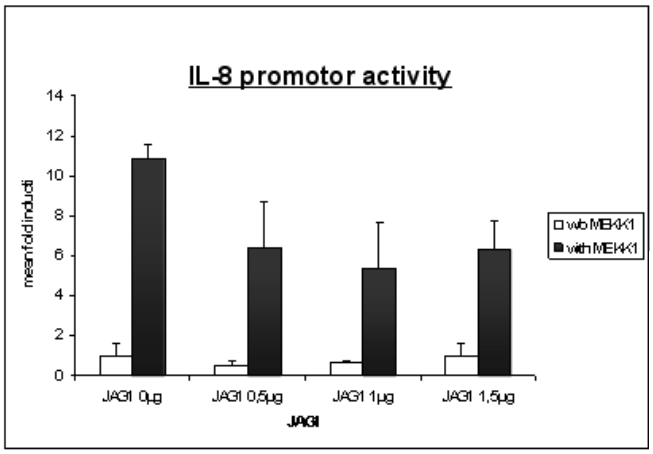


Fig.5.46 .:
Reporter gene assays in HEK293
IL-8 promotor activity in cells transfected with EV only or increasing concentrations of JAG1
IL-8 promotor activity is induced by co-transfection of a constant quantity of MEKK1

In summary, the reporter gene assays let suggest that Jagged-1 has inhibitory effects on some of the NF-κB inducing pathways. In cells stimulated by TNF-α or co-transfected with NIK or MEKK1, reporter gene expression was reduced. In contrast, when NF-κB was activated by co-transfection of TAB/TAK or IKK-β, JAG1 over-expression did not seem to have inhibitory influences. Beside to the suppressive effect of Jagged-1 on NF-κB it also seemed to have inhibitory influences on AP-1, NFAT and the IL-8 promoter. However, these effects were not extensive. The by far strongest influence of Jagged-1 was observed in the p53-reporter gene assays.

5.9. JAG1: Western Blots

Western blotting revealed that - in contrast to GG2-1 - JAG1 had no influence on the protein level of MEKK1.

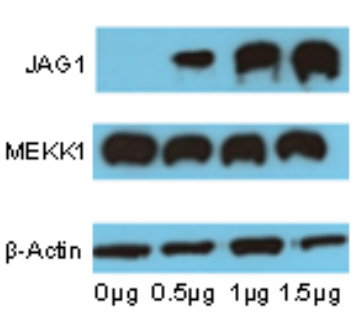


Fig. 5.47.:
Western Blot
Detection of MEKK1 (anti-HA-AB), JAG1 (anti-flag-AB) and β-Actin (anti-β-Actin-AB) as loading control in lysates from an AP-1reporter gene assay
The concentration of MEKK1 is independent from the presence or concentration of JAG1

Also the levels of other MAP3K like TAK1 were constant in all samples.

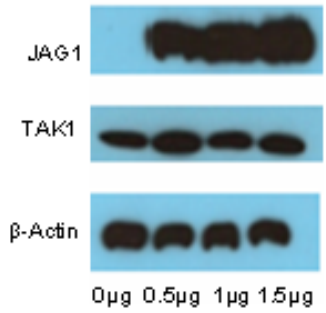


Fig. 5.48.:
Western Blot
Detection of TAK1 (anti-TAK-AB), JAG1 (anti-flag-AB) and β-Actin (anti-β-Actin-AB) as loading control in lysates from an AP-1reporter gene assay
The concentration of TAK1 is independent from the presence or concentration of JAG1

6. Discussion

The performed reporter gene assays revealed a significant inhibitory effect of GG2-1 on both, AP-1 and NF-κB when their induction was achieved by MEKK1. Accordingly, reporter gene expression regulated by the -600 bp IL-8 promoter was decreased in cells co-transfected with MEKK1. Western blotting revealed that this effect is caused by a decrease of the 196kDa form of MEKK1 proportionally to increasing GG2-1 levels. Further it was demonstrated that this influence of GG2-1 was restricted to this respective MAP3K. The protein levels of TAK1 and MEKK3 were constant in all samples, independently from the presence or concentration of GG2-1.

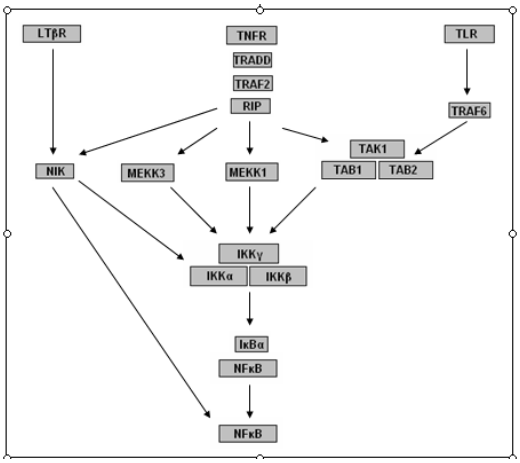
The 196kDa form of MEKK1 activates the ERK and the JNK pathway and thereby induces AP-1. By direct phosphorylation of the IKK complex it activates NF-κB. Besides these functions MEKK1 can also play a role in apotosis. Stress signals activate caspase-3 which cleaves MEKK1. The resulting 91kDa kinase domain fragment translocates to the cytoplasma and activates further caspases. Unfortunately, it was not possible to examine if the observed effect of GG2-1 on MEKK1 leads to formation of this respective fragment. Upon cleavage of MEKK1 the N-terminal domain is released. Since the HA-tag of the used construct was placed on the C-terminus and no 91kDa-fragment-specific antibody was available, detection of the pro-apoptotic form was not possible.

In other cell lines (e.g. human fibrosarcoma cell line HT1080) GG2-1 has been shown to have anti-apoptotic properties and inhibit caspase-8. Although GG2-1 does not prevent the proteolytic cleavage activating caspase-8, it suppresses downstream events like the activation of caspaspe-3 and the cleavage of Bid. [61] MEKK1 is cleaved to its pro-apototic 91kDa form by caspase-3 [62]. Neglecting the possibility that GG2-1 shows contrary functions in HEK293 cells, it is therefore rather probable that it does no induce the cleavage of MEKK1 to a pro-apoptotic form. During the course of the experiments there was also no increase of apoptotic cells correlating with increasing GG2-1 levels observed. Eventually the decreasing 196kDa MEKK1 protein levels are not caused by cleavage to a specific fragment, but GG2-1 may have a degrading effect on MEKK1. The hypothesis that GG2-1 and MEKK1 could directly interact was excluded by Co-immunopreciptation. However, there still remains the possibility that GG2-1 causes the decreasing MEKK1 concentrations on the protein level by indirect mechanisms. Another explanation would be that GG2-1 has an influence on the stability of MEKK1 mRNA. The results of the reporter gene assays showed remarkable differences between HEK293 cells stimulated with full length form and the truncated form of MEKK1. Since the truncated form contains the catalytic subunit only, it can be suggested that

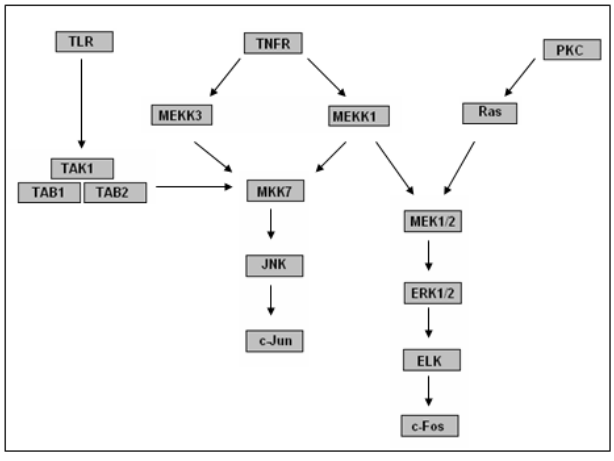
additional domains of the protein are involved in the decrease of the full length form of MEKK1.

Apart from the significant reduction of activity in MEKK1 stimulated cells, moderate inhibitory effects of GG2-1 on NF-κB could also be observed in other reporter gene assays. In the cells stimulated with TAK1/TAB1 reporter gene activity was reduced to ~60%. In contrast to MEKK1 the protein levels of TAK1 were not affected by co-transfection of GG2-1. In two independent experiments it was observed that co-transfection of TAK1 only did not induce AP-1 activity (data not shown). This allows the suggestion that complex formation of at least TAK1 and TAB1 is essential for NF-κB induction in HEK293. Maybe the decreasing effects on NF-κB activity are caused by suppression of this dimerization by GG2-1 over-expression. This speculation was supported by AP-1 reporter gene assays that show very similar results in TAK1/TAB1 stimulated cells. Also when TAK1/TAB1 causes different degrees of induction in the AP-1 and the NF-κB reporter genes assays, the decrease of activity related to the control samples were almost identical and the TAK1 protein level constant. Because the inhibitory effect was in both cases not very strong, it is doubtful if it is specific and plays are role *in vivo*.

Also HEK293 cells stimulated with NIK, another MAP3K showed a moderate decrease of NF-κB activity. Inhibiting functions of GG2-1 downstream from the IKK complex could be excluded. Thus, GG2-1 overexpression in HEK293 cells seems to have inhibiting influences on NF-κB activity on the level of the MAP3K.



Pic.6.1:
Schematic illustration of the examined
NF-κB activating pathways



Pic.6.2:
Schematic illustration of the examined
AP-1 activating pathways

AP-1 reporter gene assays supported the impression that GG2-1 acts very far upstream in the signalling cascades. In these approaches the cells were stimulated with PMA that activates PKC. Consequently the Ras signalling cascade leads to induction of the ERK pathway. Since c-Fos induction was not affected by GG2-1 over-expression it can be suggested that GG2-1 does not exhibit inhibitory effects on the ERK pathway downstream from the MAP3K level. Activation of c-Jun, the second component of AP-1 was induced by another MAP3K, MEKK3. In these experiments no decrease of AP-1 regulated reporter gene activity was observed. In contrast, GG2-1 showed an inducing effect on reporter gene expression, whereby these effects were not extensive. In summery the results of the AP-1 reporter gene assays implicate that GG2-1 inhibits the activation of this transcription factor via inhibition of the MEKK1 mediated pathway.

The results of the NF-κB and AP-1 reporter gene assays were confirmed by experiments examining the IL-8 promoter. Probably due to the presence of binding sides for AP-1 and for NF-κB, over-expression of GG2-1 in cells co-transfected with MEKK1 caused a significant decrease of reporter gene expression. Similarly to the results of the AP-1 reporter gene assay, GG2-1 exhibited an enhancing effect on IL-8 promoter activity in MEKK3 stimulated cells. IL-8 regulated reporter gene expression in TNF-α stimulated cells was not significantly effected by GG2-1. MEKK1 and MEKK3 are involved in signalling cascades downstream from the TNFR. The opposite effects of GG2-1 on pathways downstream from the TNFR possibly explain these results.

GG2-1 also suppressed the activity of NFAT and CHOP, whereas it did not have effects on p53-regulated promoters.

In summary the results of the reporter gene assays lead to the conclusion that GG2-1 may have anti-inflammatory properties. The activity of the most important inflammation initiating transcription factors AP-1, NF-κB and NFAT are reduced by over-expression. Additionally, transcription regulated by the natural IL-8 promoter is suppressed. This chemokine is one of the major mediators of inflammatory reactions. Amongst others it is expressed on endothelial cells and it induces chemotaxis in target cells like neurophiles.

Because GG2-1 is induced by NF-κB and acts as negative regulator of pro-inflammatory molecules it can be regarded as a negative feedback loop.

The results of the reporter gene assays implicate that Jagged-1 has inhibitory functions on NF-κB activation induced by TNFR signalling. Cells stimulated with TNF-α showed a Jagged-1 concentration-dependent decrease of reporter gene activity. Examination of downstream components of TNFR-induced pathways gave more information about the background of this

effect. In both, cells co-transfected with NIK and MEKK1, similar effects were observed like in TNF-α stimulated cells. Thus, it can be suggested that Jagged-1 has inhibitory functions on the classical and the non-classical pathway of NF-κB activation. The inhibitory function of Jagged-1 does not affect all pathways induced by MAP3K. In cells stimulated with TAK1/TAB1 over-expression of JAG1 did not show suppressive effects on NF-κB activity. Also in HEK293 cells co-transfected with IKKβ reporter gene expression was not inhibited by JAG1 over-expression. These results allow the suggestion that Jagged-1 affects NF-κB activation upstream from the IKK complex.

Jagged-1 exhibited also inhibitory effects on other transcription factors. However, these effects were less significant. AP-1 activity was only remarkably inhibited by high concentrations of Jagged-1 in all approaches. In contrast to the NF-κB reporter gene assays, Jagged-1 showed an inhibiting effect on luciferase expression in TAK1/TAB1 stimulated cells. This observation may suggest that the signalling pathway leading to MKK7 activation is moderately inhibited by Jagged-1, whereas IKK phosphorylation is not. NFAT activity was in average reduced to 65% and the suppression did not rise with increasing concentrations of Jagged-1. Examination of the influence of Jagged-1 on the natural IL-8 promoter confirmed the observation that it has inhibitory effects on pro-inflammatory processes. However, also in these experiments the suppression of luciferase expression was not very pronounced. In summary, it remains doubtful if JAG1 plays a relevant role in the resolution of the inflammation *in vivo.*

A significant inhibitory effect of Jagged-1 was observed in p53-reporter gene assays. These results support the hypothesis that this protein has oncogenic properties. By suppression of p53, cell cycle check points are abrogated which can lead to amplification of damaged DNA and loss of repair mechanisms. Noseda, Chang et al. postulated in 2004 that Jagged-1 plays a role in contact inhibition of endothelial cells by inhibiting the phosphorylation of the retinoblastoma protein (Rb). Notch activated by Jagged-1 suppresses the up-regulation of p21. Consequently cyclinD-cdK4-kinase activity is both, delayed and reduced and the release of E2F is inhibited.[39] Since p21 is a target gene of p53, the observation that p53 activity is inhibited by Jagged-1 overexpression is according to this hypothesis.

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Zusammenfassung:

Entzündung ist im Prinzip eine schützende Reaktion des Organismus auf verschiedenste schädigende Reize. Kommt es aber zu einer übertriebenen oder ständigen Entzündungsreaktion, so werden durch den ursprünglich nutzbringenden Abwehrmechanismus Schädigungen verursacht. Chronische Entzündungen sind in die Pathogenese von vielen unterschiedlichen Erkrankungen involviert. Die Aufklärung von Mechanismen, die die adäquate Beendigung dieser Reaktion ermöglichen ist daher ein wichtiges Ziel der Entzündungsforschung.

Neben verschiedenen Immunzellen tragen auch endotheliale Zellen aktiv zu einer Entzündung bei. Das Endothelium kleidet die Vaskulatur aus und bildet unter normalen Bedingungen eine anti-koagulante Oberfläche und eine Barriere zwischen dem Blutstrom und dem umliegenden Gewebe. Als Reaktion auf pro-inflammatorische Stimulation kommt es zu phenotypischen Veränderungen der endothelialen Zellen, die es den Immunzellen erlauben diese Schranke zu passieren und ins Gewebe einzuwandern. Auf molekularer Ebene sind diese Prozesse hauptsächlich durch verschiedene induzierbare Transkriptionsfaktoren (e.g. NF-κB, AP-1, NFAT) reguliert. Ihre Aktivierung führt nicht nur zur Expression einer Vielzahl von pro-inflammatorischen Genen, sondern auch von solchen, die in einem späteren Stadium einer Entzündungsreaktion deren Ende induzieren. Die Proteine, die von solchen Genen codiert werden, formen so genannte „negative feedback loops“. Um neue „negative feedback loops“ zu identifizieren wurden Gene selektiert, die in Endothelzellen aus der humane Nabelschnur (HUVECs) durch das pro-inflammatorische Cytokin IL-1 induziert werden und inhibitorisches Potential aufweisen. Im Rahmen dieser Diplomarbeit wurden einige diese Kandidatengene in passende Expressionsvektoren kloniert und ihr Einfluss auf Transkriptionsfaktoren und Signaltransduktionswege die bei der Entzündung eine wichtige Rolle spielen, mittels Reportergen Assays analysiert.

Eines der untersuchten Gene, GG2-1, kodiert ein FLICE–inhibierendes, anti-apoptotisches Protein mit onkogenem Potential. Reportergen Assays in HEK293 zeigten, dass Überexpression von GG2-1 einen signifikanten inhibitorischen Effekt auf die Aktivität von NF-κB und AP-1 hat, wenn deren Aktivierung durch Transfektion der MAP3 Kinase MEKK1 erreicht wurde. Damit übereinstimmend wurde beobachtet, dass die MEKK1-induzierte Aktivität des IL-8-Promoters ebenfalls proportional zu steigenden Mengen von GG2-1 sinkt. Detektierung des Proteins mittels „Western Blotting“ zeigte, dass die Inhibierung der Reportergen Expression mit abnehmenden Proteinmengen von MEKK1 assoziiert ist. Die

Konzentrationen von anderen MAP3 Kinasen wurde durch die Anwesenheit von GG2-1 nicht beeinflusst, was die Vermutung erlaubt, dass der beobachtete Effekt spezifisch für MEKK1 ist. Durch Co-Immunopräzipitaton wurde eine direkte Interaktion zwischen den beiden Proteinen ausgeschlossen.

JAG1 kodiert Jagged-1, einen der vier Liganden von Notch 1. Aktives Notch setzt Signalkaskaden in Gang, die essentiell für die Zell-Zell Kommunikation sowie diverse Entwicklungsprozesse sind. Mutationen in JAG1 verursachen das Alagille Syndrom (AGS), eine autosomal dominate Erbkrankheit, die Auswirkungen auf verschiedenste Bereiche des Organismus hat. In endothelialen Zellen ist Jagged-1 in die Kontaktinhibierung involviert, wahrscheinlich durch Regulierung des Retinoblastoma Proteins (Rb). In anderen Zelltypen zeigt Jagged-1 onkogene Eigenschaften. Die durchgeführten Reportergen Assays zeigten keinen signifikanten inhibitorischen Effekt von Jagged-1 auf pro-inflammatorische wirkende Transkriptionsfaktoren. Im Gegensatz dazu wurde die Aktivität des Tumorsuppressors p53 stark unterdrückt, was vermuten lässt, dass Jagged-1 auch in endothelialen Zellen einen Einfluss auf onkogene Prozesse haben könnte.

8. Curriculum Vitae

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