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# Influence of modulation of the actin cytoskeleton on NF-κB activation and interleukin-6 signaling

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*Abstract:*

Transcription factor NF-κB and its target gene *IL6* are involved in many key processes like immune defense, inflammatory, cell proliferation and apoptosis. At the same time actin cytoskeleton plays a crucial role not only in the cell motility, but also in the proliferation and cell survival, as well as in the intracellular signal transduction.

In the current work we have investigated the influence of actin cytoskeleton reorganization on the signaling pathways of NF-κB and cytokine interleukin 6 (IL-6) as well as on the proteolytic production of soluble IL-6 receptors (sIL-6R). Manipulation of the actin cytoskeleton was achieved with the help of actin-modulating substances like jasplakinolide and cytochalasins B and D.

Our results showed that actin-modulating compounds do not have an effect on the phosphorylation state of NF-κB subunits p65 and RelB in the cell lines Hep3B and HCT116. Hence, perturbation of actin cytoskeleton does not lead to activation of canonical and non-canonical pathways of NF-κB.

By contrast, manipulation of actin cytoskeleton with cytochalasins and jasplakinolide led to reduction in the activation of STAT3 after stimulation with IL-6 in Hep3B cells. Furthermore, production of sIL-6R by metalloprotease ADAM10 as well as synthesis of IL-6 itself was investigated. Neither the IL-6R proteolysis nor the IL-6 synthesis was affected from the used substances. In conclusion, modulation of actin cytoskeleton shows a minimal influence on the investigated processes.

*Key words*:

IL-6, NF-κB, intracellular signaling, actin, actin cytoskeleton reorganization, actin-modulating compounds, soluble IL-6 receptors

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**Abbreviations**

| | |
|---|---|
| **ABP** | Actin binding protein |
| **ADAM** | A disintegrin and metalloproteinase domain-containing protein |
| **ADF** | Actin depolymerization factor |
| **ADP** | Adenosine diphosphate |
| **AP-1** | Activator protein 1 |
| **APS** | Ammonium Persulfate |
| **Arid5a** | AT-rich interactive domain containing protein 5a |
| **Arp2/3 complex** | Actin Related Protein 2/3 complex |
| **ATO** | Arsenic trioxide |
| **ATP** | Adenosine triphosphate |
| **BAFF** | B-cell activating factor |
| **Bax** | Bcl-2-associated X protein |
| **BBC3** | BCL2 binding component 3 |
| **BCDF** | B cell differentiation factor |
| **Bcl3** | B-cell lymphoma 3-encoded protein |
| **BCL2L1** | BCL2 like 1 |
| **BIRC** | Baculoviral IAP repeat containing |
| **BSF-2** | B cell stimulatory factor 2 |
| **CAC** | Colitis-associated cancer |
| **CB** | cytochalasin B |
| **CBP** | CREB-binding protein |
| **CD** | cytochalasin D |
| **CD40** | Cluster of differentiation 40 |
| **c-IAPs** | Cellular inhibitors of apoptosis |
| **CK2** | Casein kinase-II |
| **CLC** | Cardiotrophin-like cytokine |
| **CNTF** | Ciliary neurotrophic factor |
| **CRC** | Colorectal carcinoma |
| **CRP** | C reactive protein |
| **CRS** | Cytokine release syndrome |
| **CT-1** | Cardiotrophin-1 |
| **DAMPs** | Damage-associated molecular patterns |
| **DCs** | Dentritic cells |
| **DHMEQ** | Dehydroxymethylepoxyquinomicin |
| **DNA** | Deoxyribonucleic acid |
| **DMARDs** | Disease-modifying antirheumatic drugs |
| **DMEM** | Dulbecco's Modification of Eagle's Medium |
| **DMSO** | Dimethyl sulfoxide |
| **dsRNA** | Double-stranded ribonucleic acid |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EMSA** | Electrophoretic mobility-shift assay |
| **F-actin** | Filamentous actin |
| **FASLG** | Fas ligand |
| **FBS** | Fetal Bovine Serum |

**Abbreviations**

| | |
|---|---|
| **G-actin** | Globular actin |
| **GCA** | Giant cell arteritis |
| **G-CSF** | Granulocyte-colony stimulating factor |
| **GLP-1** | Glucagon-like-peptide-1 |
| **gp130** | Glycoprotein 130 |
| **GPCRs** | G-protein coupled receptors |
| **HCC** | Hepatocellular carcinoma |
| **HER2** | Human epidermal growth factor receptor 2 |
| **HGF** | Hepatocyte growth factor |
| **IAP** | Inhibitor of apoptosis proteins |
| **IBD** | Inflammatory bowel disease |
| **IER3** | Immediate early response 3 |
| **CBP/p300** | CREB-binding protein |
| **ICAM** | Intercellular Adhesion Molecule 1 |
| **IFN-β2** | Interferon β2 |
| **IκB** | Inhibitor of nuclear factor κB |
| **IκBαSR** | IκBα super suppressor |
| **IκBNS** | Inhibitor of nuclear factor κB NS |
| **IKK** | IκB complex |
| **IKKβDN** | Dominant negative mutant IKKβ constructs |
| **IL-6** | Interleukin-6 |
| **IL-6R** | Interleukin-6 receptor |
| **IPSIs** | Immunoproteasome-specific inhibitors |
| **IRF-1** | Interferon regulatory factor1 |
| **JAK1** | Janus kinase 1 |
| **JP** | jasplakinolide |
| **kDa** | Kilodalton |
| **LIF** | Leukemia inhibitory factor |
| **LPS** | Lipopolysaccharide |
| **LTβ** | Lymphotoxin β |
| **M-CSF** | Macrophage colony-stimulating factor |
| **MAPK** | Mitogen-activated protein kinase |
| **MCL** | Mantle cell lymphoma |
| **MEKK3** | Mitogen-activated protein kinase kinase kinase3 |
| **MHC** | Major histocompatibility complex |
| **MnSOD** | Manganese-dependent superoxide dismutase |
| **mRNA** | Messenger RNA |
| **miRNA** | MicroRNA |
| **mTOR** | Mechanistic target of rapamycin |
| **MTT** | Thiazolyl blue tetrazolium bromide |
| **NBD-peptides** | NEMO-binding domain peptides |
| **NEMO** | NF-κB essential modulator |
| **NGF** | Nerve growth factor |
| **NF-IL6** | Nuclear factor IL-6 |
| **NF-κB** | Nuclear factor-κB |
| **NIK** | NF-κB-inducing kinase |

**Abbreviations**

| | |
|---|---|
| **NLS** | Nuclear localization site |
| **NNT-1/BSF-3** | Novel neurotrophin 1/B cell stimulating factor 3 |
| **ns** | not significant |
| **NSAIDs** | Nonsteroidal anti-inflammatory drugs |
| **OSM** | Oncostatin M |
| **PAMPs** | Pathogen-associated molecular patterns |
| **PBS** | Phosphate-buffered saline |
| **PIs** | Proteasome inhibitors |
| **PI3 kinase** | Phosphoinositide kinase |
| **pJIA** | Polyarticular juvenile idiopathic arthritis |
| **PH** | Partial hepatectomy |
| **PMA** | Phorbol-12-myristate 13-acatate |
| **PMR** | Polymyalgia rheumatica |
| **PPAR** | Peroxisome proliferator-activated receptors |
| **RA** | Rheumatoid arthritis |
| **RANKL** | Receptor of nuclear factor kappa-B ligand |
| **Regnase-1** | Regulatory RNase-1 |
| **RHD** | Rel homology domain |
| **RIP1** | Receptor–interacting protein kinase 1 |
| **RNAi** | Ribonucleic acid interference |
| **RT-PCR** | Reverse transcription polymerase chain reaction |
| **SAA** | Serum amyloid A |
| **SDS-PAGE** | Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis |
| **sIL-6R** | Soluble Interleukin-6 receptor |
| **siRNA** | Small interfering RNA |
| **sJIA** | Systemic juvenile idiopathic arthritis |
| **SOCS** | suppressor of cytokine signaling |
| **SP-1** | Specifity protein 1 |
| **STAT** | Signal transducer and activator of transcription |
| **SSc** | Systemic sclerosis |
| **TAD** | Transactivation domain |
| **TAK1** | TGF-β-activated kinase 1 |
| **TCZ** | Tocilizumab |
| **TEMED** | Tetramethylethylenediamine |
| **TGFβ** | Transforming growth factor β |
| **Th17** | T helper 17 cells |
| **TLR** | Toll-like receptor |
| **TNFα** | Tumor necrosis factor α |
| **TNFRSF10B** | TNF receptor superfamily member 10 b |
| **TNF-R1** | Tumor necrosis factor receptor 1 |
| **TRAF** | TNF receptor-associated factor |
| **Treg** | Regulatory T cells |
| **USP7** | Ubiquitin-specific-processing protease 7 |
| **UV** | Ultra-Violet |
| **VEGF** | Vascular endothelial growth factor |
| **XIAP** | X-linked inhibitor of apoptosis protein |

# 1. Introduction

## 1.1. NF-κB

### 1.1.1. The NF-κB family of proteins

NF-κB has been identified as a regulator of κB light chain expression in mature B and plasma cells (Sen & Baltimore 1986a). Induction and activation of this transcription factor in response to exogenous stimuli was demonstrated in various cell types (Sen & Baltimore 1986a, Sen & Baltimore 1986b). After years of intensive research it has been shown that NF-κB is expressed in almost all cell types and tissues and the specific NF-κB binding sites are present in the promoters/ enhancers of a large number of genes (Baldwin 1996).

NF-κB is a transcription factor that consists of homo- or heterodimers formed by members of the Rel family of proteins. The mammalian NF-κB family includes five cellular DNA-binding subunits namely NF-κB1 (p50), NF-κB2 (p52), c-Rel (Rel), p65 (RelA) and RelB (Ghosh et al. 1998). All of these members share an N-terminal Rel homology domain (RHD), which is responsible for DNA binding, dimerization, nuclear translocation and interaction with inhibitory IκB proteins (Baldwin 1996). The p50/p65 heterodimer represents the most frequently formed NF-κB complex in the cells (Ghosh et al. 1998). p65, RelB and c-Rel possess a C-terminal transactivation domain (TAD), which is necessary for their transcriptional activity. Among these proteins, p65 mediates the strongest gene activation due to its two potent TADs (Schmitz & Baeuerle 1991). By contrast, p50 and p52 each lack a TAD and as such do not possess any intrinsic transcriptional activity (Zhong et al. 2002). Generation of p50 and p52 from their respective precursor proteins p105 and p100 occurs through a process called processing (Baldwin 1996). In unstimulated cells, p100 undergoes constitutive processing to produce p52 (Xiao et al. 2004, Qing & Xiao 2005). Its phosphorylation via IKKα and NIK (Senftleben et al. 2001, Xiao et al. 2001, Xiao et al. 2004) leads to either degradation or processing to p52 (Fong & Sun 2002, Amir et al. 2004, Liang et al. 2006). The other precursor protein p105 undergoes a constitutive proteasomal processing to p50 (Fan & Maniatis 1991, Palombella et al. 1994, Lin et al. 1998). This limited proteolysis is mediated by the 20S proteasome (Moorthy et al. 2006) and may occur both cotranslationally (Lin et al. 1998) and post-translationally (Fan & Maniatis 1991, Moorthy et al. 2006).

## 1 Introduction

Transcriptional activity of NF-κB is controlled by inhibitors of κB molecules (IκB). Common for all members of this family is that they possess a number of protein/protein interaction domains called ankyrin repeats. These inhibitors form a complex with NF-κB subunits and mask their nuclear localization site, thus they retain the inactive NF-κB dimers in the cytoplasm (Arenzana-Seisdedos et al. 1997). The family of inhibitors of κB molecules includes the typical members IκBα, IκBβ, IκBγ, IκBε, the atypical IκBζ, Bcl-3 and IκBNS, as well as the precursor forms p105 and p100 (Arenzana-Seisdedos et al. 1997, Yamamoto & Takeda 2008).

Because of its ability to influence expression of numerous genes, activity of NF-κB is highly regulated at multiple levels. The primary mechanism for NF-κB regulation is through inhibitory IκB proteins and the IKK complex, a kinase that phosphorylates them. This high-molecular-complex consists of three tightly associated IKK polypeptides, namely the two catalytic subunits IKK1 (IKKα) and IKK2 (IKKβ) and the regulatory subunit called NEMO (IKKγ, IKKAP-1) (Mercurio et al. 1997, Mercurio et al. 1999, Delhase et al. 1999, Woronicz et al. 1997, Zandi et al. 1997). Furthermore, IκB can terminate NF-κB activation also at the transcriptional level via a negative feedback loop. Increased levels of IκB proteins induced by activated NF-κB block NF-κB-induced gene expression (Arenzana-Seisdedos et al. 1997). Newly synthetized IκB is able to enter the nucleus, to remove NF-κB from DNA and thus to relocalize it to the cytosol (Hayden & Ghosh 2004). A number of post-translational modifications such as phosphorylation and acetylation also modulate the activity of IκB and IKK proteins as well as NF-κB molecules themselves (Ghosh & Karin 2002).

Activation of NF-κB can be triggered by a diversity of external stimuli such as the inflammatory cytokines TNFα and IL-1, microbial products like LPS and dsRNA, or the activation of antigen receptors. Moreover, physical (UV- or γ-irradiation), physiological (ischemia and hyperosmotic shock) or oxidative stress can also result in NF-κB induction (Baeuerle & Henkel 1994, Hayden et al. 2006). In addition, there are some reports indicating that NF-κB can be activated by diverse viral oncoproteins and by oncogenic forms of Ras (Finco et al. 1997, Cahir McFarland et al. 1999, Norris & Baldwin 1999, Rayet & Gélinas 1999, Sun & Ballard 1999) and that these oncoproteins require its activity to induce cellular transformation.

### 1.1.2. Functions of NF-κB

As a transcription factor, NF-κB is responsible for expression of numerous genes, including those involved in immune and inflammatory responses, cell proliferation and apoptosis (Ghosh et al. 1998). More than 200 NF-κB target genes have been identified in the recent years (Aggarwal 2004).

Many of the NF-κB target genes are central components of the immune response and inflammatory processes. Parts of them are acute phase proteins, components of the complement cascade, immune receptor subunits, MHC molecules, cell adhesion molecules, chemokines and cytokines like TNFα and IL-6. NF-κB regulators IκBα, p105 or A20 are themselves NF-κB-inducible, which results in auto-regulatory feedback loops (Chen & Manning 1995, Wissink et al. 1997). Furthermore, NF-κB controls some genes responsible for apoptosis. It induces not only anti-apoptotic (*BIRC2, BIRC3, TRAF1, TRAF2, BCL2L1 , XIAP, MnSOD* and *IER3*) (Karin & Greten 2005), but also pro-apoptotic genes (e.g. *TNFRSF10B, FASLG, BBC3* and *Bax)* (Singh et al. 2007, Shou et al. 2002, Wang et al. 2009). NF-κB plays also an important role in cell growth, proliferation and survival (Chen & Manning 1995, Wissink et al. 1997), as it triggers the expression of cyclins such as c-Myc and Cyclin D (Karin & Greten 2005) and growth factors like G-CSF and M-CSF. Furthermore, NF-κB participates in embryonic development and in the physiology of the bones, the skin and the central nervous system (Hayden & Ghosh 2004).

Due to its pleiotropic functions, inappropriate regulation of NF-κB pathway results in severe human disorders including a variety of cancers such as HCC and CAC (Karin 2009), pancreatic (El-Rayes et al. 2006), lung (Tang et al. 2006), breast (Huber et al. 2004), cervical (Li et al. 2009) and prostate (Huang et al. 2001b) cancer as well as neurodegenerative diseases (Grilli & Memo 1999). Moreover, NF-κB has been involved in the pathogenesis of several inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis, systemic lupus erythematosus, type I diabetes, ulcerative colitis and Crohn's disease (Herrington et al. 2016).

### 1.1.3. NF-κB signaling

Two main pathways that lead to NF-κB activation can be differentiated, namely the canonical (classical) and the non-canonical pathways (Hayden & Ghosh 2004). Furthermore, a DNA damage-induced pathway and other atypical pathways have been identified (Janssens & Tschopp

2006). In addition, it has been shown that p65 posttranscriptional modifications also regulate the activity of this transcription factor (Luedde & Trautwein 2006).

The canonical pathway (Figure 1A) is the best described and probably the most important mediator of NF-κB activation. It is the major pathway in most cell types (Hayden & Ghosh 2004) and can be activated by numerous pro-inflammatory agents such as TNFα and IL-1β or bacterial LPS (Karin & Ben-Neriah 2000, Ghosh & Karin 2002).

Induction of NF-κB pathway by TNFα (Figure 1A) is the most studied one and represents the typical canonical NF-κB activation. TNFα binds to TNFR1 and thus recruits IKK to the TNFR1 signaling complex through TRAF2 and RIP1. IKK is then activated through a RIP-mediated phosphorylation that involves MEKK3 or TAK1 (Devin et al. 2000, Yang et al. 2001). The activated catalytic subunit IKKβ afterwards phosphorylates the serine residues at positions 32 and 36 in IκBα. This results in IκBα polyubiquitination and its degradation by the 26S proteasome. These steps lead to NF-κB release from its inhibitory IκB-binding partner and allow the NF-κB dimer to translocate into the nucleus and therefore to promote transcription of NF-κB-dependent target genes (Karin 1999, Yamamoto & Gaynor 2004).

By contrast, the non-canonical pathway (Figure 1C) is IκB-independent and involves the IKK subunit IKKα. It is induced by members of the TNF super family like BAFF, LTβ or CD40 ligand. This stimulation results in NF-κB-inducing kinase (NIK)-mediated activation of IKKα and thus in release of p52 by processing of the p100 precursor protein (Senftleben et al. 2001). The released p52 forms a functional complex with RelB and translocates to the nucleus, where it promotes the expression of NF-κB target genes (Hayden & Ghosh 2004).

Beside canonical and non-canonical pathways, other mechanisms to NF-κB activation have been identified (Figure 1B). Genotoxic stimuli such as ionizing radiation and chemotherapeutic drugs lead to NEMO-dependent IKKβ activation. Activated IKKβ subunit triggers IκBβ degradation, which results in activation of the canonical NF-κB pathway (Wu et al. 2006). Other atypical IKK-independent pathways of NF-κB activation have also been described. Stimuli as hypoxia and reoxygenation, hydrogen-peroxide stimulation and treatment of cells with NGF or the tyrosine-phosphatase inhibitor pervanadate lead to phosphorylation of IκBα and thus also to its degradation and dissociation from NF-κB (Perkins 2006). Treatment with ultraviolet light or

expression of the *HER2* oncogene in breast cancer cells can result in IκBα phosphorylation by CK2 (Perkins 2006).

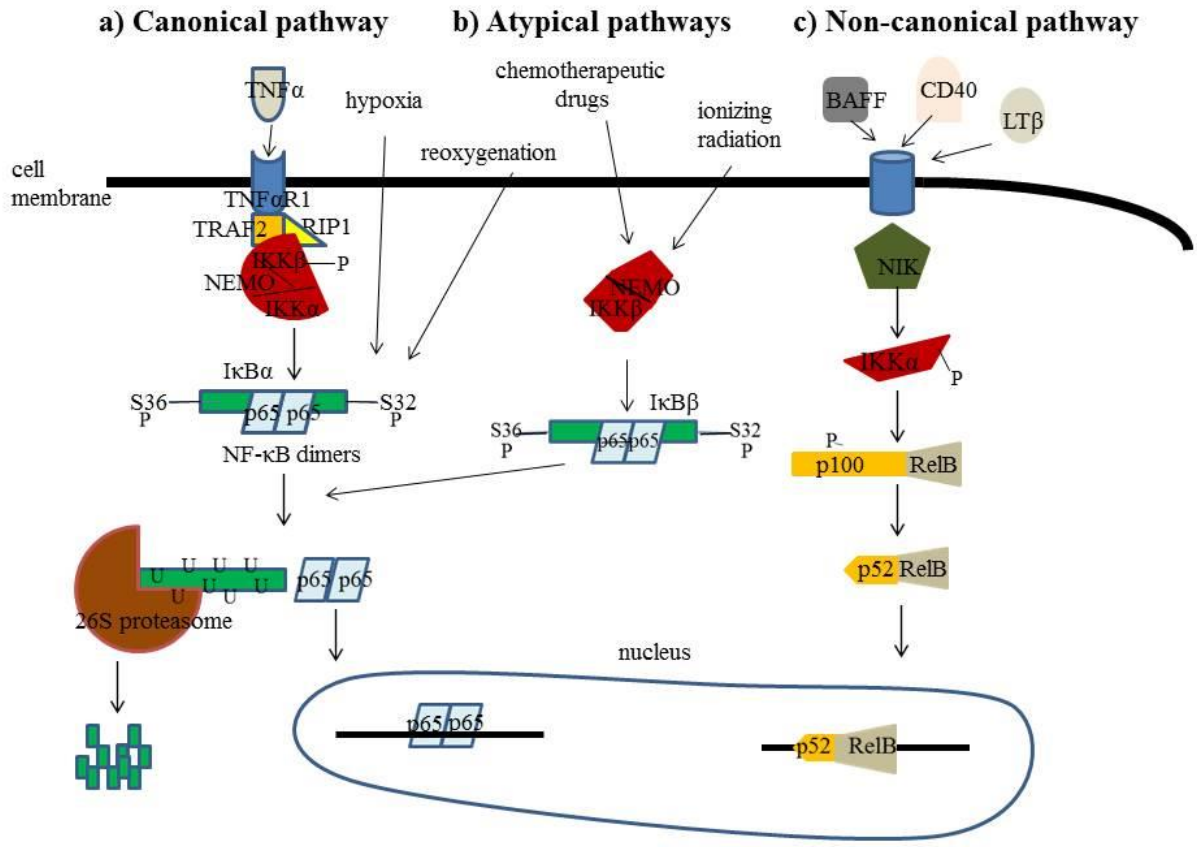


**Figure 1: NF-κB signaling. (A) Canonical pathway:** TNFα binds to TNFR1, which triggers the recruitment of IKK to the TNFR1 signaling complex through TRAF2 and RIP1. Activated catalytic subunit IKKβ phosphorylates the serine residues at positions 32 and 36 in IκBα. This results in IκBα polyubiquitination and its degradation by the 26S proteasome. NF-κB releases from IκBα so that the p65/p65 formed dimer is able to translocate into the nucleus and there promotes transcription of the NF-κB-dependent target genes. **(B) Atypical pathways**: Stimuli such as hypoxia and reoxygenation lead to phosphorylation of IκBα and thus also to its degradation and to the dissociation of NF-κB subunits. Genotoxic stimuli such as ionizing radiation and chemotherapeutic drugs lead to NEMO-dependent IKKβ activation. Activated IKKβ subunit triggers IκBβ degradation, which also results in activation of NF-κB. **(C) Non-canonical pathway:** BAFF, LTβ or CD40 ligand bind to their respective receptor. This stimulation results in NIK-mediated activation of IKKα and thus in release of p52 by processing of the p100 precursor protein. p52 forms a dimer with RelB, which translocates to the nucleus and there promotes the expression of NF-κB target genes. Figure is adapted from (Lin et al. 2010).

NF-κB transcription activity can be further regulated by phosphorylation and acetylation of the p65 subunit. These processes affect DNA binding of NF-κB or its interaction with transcriptional

co-activators such as CBP/p300 (Hayden & Ghosh 2004). In addition, there are several observations indicating that the redox status of the cells also participates in the modulating of NF-κB activation (Kustermans et al. 2005).

### 1.1.4. Therapy

Due to the general involvement of NF-κB in cell survival and proliferation and the observations that it is commonly activated in the cancer cells, it is expected that its blockade may be a promising strategy in anticancer therapy. Numerous chemical compounds, monoclonal antibodies and viral vectors that inhibit or modulate the NF-κB pathway have been developed or are under development (Herrington et al. 2016, Ramadass et al. 2020).

It has been shown that many of the commonly used anti-inflammatory agents are able to modulate NF-κB transcription activity as a part of their mechanism of action and thus can be simultaneously described as unspecific inhibitors of NF-κB. The group of these drugs includes glucocorticoids (Scheinman et al. 1995, Auphan et al. 1995), NSAIDs such as aspirin and sodium salicylate (Kopp & Ghosh 1994, Yin et al. 1998) and the DMARD agents sulfasalazine (Wahl et al. 1998) and mesalamine (Egan et al. 1999). Furthermore, certain immunosuppressive agents like anakinra (IL-1R antagonist), infliximab and adalimumab (both anti-TNFα monoclonal antibodies) also mediate their effect through modulation of NF-κB activity (Müller-Ladner et al. 2007).

Presently, there are also several drugs used in anticancer therapy, which have been described to inhibit NF-κB. For instance, bortezomib is the first approved NF-κB blocking drug for the treatment of multiple myeloma and represents a reversible 26S proteasome inhibitor (Dingli & Rajkumar 2009). It is currently in clinical use as a therapy in combination with other drugs for the treatment of multiple myeloma and mantle cell lymphoma (MCL) (Manasanch & Orlowski 2017). The second-generation proteasome inhibitor, carfilzomib, has been approved as a single agent as well in combination with other agents for the treatment of patients with relapsed or refractory multiple myeloma. It is an epoxyketone compound, acting as an irreversible proteasome inhibitor (Manasanch & Orlowski 2017). Ixazomib is the first reversible oral proteasome inhibitor, which has been approved in combination with lenalidomide and dexamethasone for the treatment of patients with relapsed or refractory multiple myeloma (Raedler 2016). Thalidomide represents another agent in clinical use that inhibits NF-κB

activation. This occurs via inhibiting of IκB kinase activity (Keifer et al. 2001). It has also been approved for the treatment of multiple myeloma (Raab et al. 2009). Arsenic trioxide (ATO) is non-specific inhibitor of IKKβ and thus of NF-κB (Kapahi et al. 2000) and has been approved as a treatment of the acute promyelocytic leukemia (Lo-Coco et al. 2013). Another drug in clinical use, able to inhibit NF-κB, is dexamethason (Yamamoto & Gaynor 2001). It has been suggested that it induces the synthesis of IκBα and thus the inhibition of NF-κB activity (Scheinman et al. 1995). Another mechanism of NF-κB inhibition such as direct protein-protein interactions between the activated glucocorticoid receptor and NF-κB has been also proposed (Bosscher et al. 1997, Scheinman et al. 1995). Dexamethasone is in clinical use as a combination therapy in the treatment of multiple myeloma (Raab et al. 2009).

The central role of NF-κB in a wide range of human malignant and non-malignant pathological states has led to intensive research in this area with the purpose a specific NF-κB or IKK inhibitor to be developed (Begalli et al. 2017). The list with the identified and tested agents has been increased rapidly in the past years, but none of them has been clinically approved, due to the dose-limiting toxicities associated with the global suppression of NF-κB (Begalli et al. 2017, Ramadass et al. 2020).

The agents developed as specific NF-κB inhibitors have diverse mechanisms of action and target different steps in the NF-κB pathways (Begalli et al. 2017) They have been divided in different groups on the basis of the level, where they act on the NF-kB signaling. For instance, upstream of IKK (e.g. at a receptor or adaptor level), directly at the IKK complex or IκB phosphorylation, NIK, ubiquitination or proteasomal degradation of IκB, nuclear translocation of NF-κB, NF-κB DNA binding and NF-κB-directed gene transactivation (Gilmore & Herscovitch 2006, Ramadass et al. 2020). These compounds include antioxidants, peptides, small RNA/DNA, microbial and viral proteins, small molecules, and engineered dominant-negative or constitutively active polypeptides (Gilmore & Herscovitch 2006). In the recent years many small molecules, able to inhibit the post-translational modifications, have been approved in the therapy of various pathological states (Ramadass et al. 2020).

## 1.2. IL-6

### 1.2.1. Basic biology of IL-6

Interleukin-6 is a small glycoprotein (21-26 kDa) known under different names. It was first cloned in 1986 (Hirano et al. 1985). Initially, this cytokine was identified as an antigen nonspecific B cell differentiation factor, which induces B cells to produce immunoglobulins (Muraguchi et al. 1981, Yoshizaki et al. 1982). In the following years, different working groups have described other molecules with the same characteristics. Later it turned out that this protein is the same molecule as B cell stimulatory factor 2 (Hirano et al. 1985), hepatocyte stimulating factor (Andus et al. 1987, Gauldie et al. 1987, Castell et al. 1988), IFN-β2 (Zilberstein et al. 1986, Sehgal et al. 1987) and hybridoma/ plasmacytoma growth factor (van Damme et al. 1987, Nordan et al. 1987, Uyttenhove et al. 1988, van Snick et al. 1988). Finally, this molecule was named IL-6. IL-6 is part of the IL-6 family, which consists of IL-6, IL-11, IL-27, IL-31, ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), oncostatin M (OSM), leukemia inhibitory factor (LIF) and cardiotrophin-like cytokine (CLC) (Rose-John 2018). Common for these family members is that each of them transmits its signals via the signal transducing receptor glycoprotein 130 (gp130) as a part of their receptor complex (Taga & Kishimoto 1997).

IL-6 can be secreted from diversity of cells in response to wide range of stimuli. During infection stimulation of Toll-like receptors (TLRs) with distinct pathogen-associated molecular patterns (PAMPs) triggers IL-6 production in monocytes and macrophages. In non-infectious inflammation like burns or traumatic injuries, activation of TLRs with damage-associated molecular patterns (DAMPs) from damaged or dying cells also induces its production (Akira et al. 1993, Kishimoto 1989). In the liver, stimulation of Kupffer cells with TNFα results in IL-6 secretion (Michalopoulos & DeFrances 1997, Taub 2004). The list of cells able to produce IL-6 further includes dendritic cells (DC), T and B cells, neutrophils, mast cells, fibroblasts, synovial cells, endothelial cells, stromal cells, mesangial cells, glial cells, neurons, chondrocytes, keratinocytes, osteoblasts, smooth muscle cells, adipocytes and some tumor cells (Akira et al. 1993, Kishimoto 1989).

A diversity of transcriptional and posttranscriptional mechanisms regulates the process of IL-6 synthesis (Tanaka et al. 2014). It has been shown that a number of transcription factors such as NF-κB, NF-IL6, AP-1, SP-1 and IRF-1 induce *IL6* gene expression (Libermann & Baltimore

1990, Akira & Kishimoto 1992, Matsusaka et al. 1993). At the same time, it is believed that activated NF-κB is the most important inducer of IL-6 transcription (Shimizu et al. 1990, Libermann & Baltimore 1990). It has also been demonstrated that some transcription factors such as PPARs are able to repress IL-6 expression (Delerive et al. 1999). Retinoblastoma protein, p53 (Santhanam et al. 1991), activated estrogen (Jilka et al. 1992) as well as corticoid receptors (Ray & Prefontaine 1994) were also identified as suppressors of IL-6 transcription. In addition, some microRNAs are also considered as repressors of IL-6 expression (He et al. 2009, Chen et al. 2012, Zilahi et al. 2012). In the past few years, novel regulators of posttranslational modifications of IL-6 mRNA have been discovered. One of them is a RNA binding protein called Arid5a, which leads to selective stabilization of IL-6 mRNA (Masuda et al. 2013). Another one is a nuclease known as Regnase-1 that participates in destabilization of IL-6 mRNA (Matsushita et al. 2009).

### 1.2.2. Functions of IL-6

IL-6 is a pleiotropic cytokine involved in multiple pivotal biological processes. It has been demonstrated, that it affects a wide range of cell types, especially the immune cells and hepatocytes due to their ability to express IL-6 receptors (IL-6R) (Taga 1992) .

In the immunity, there are a lot of cells, whose proliferation and differentiation are IL-6-dependant. For instance, it triggers differentiation of B cells into immunoglobulin-producing cells (Muraguchi et al. 1981, Yoshizaki et al. 1982) and this of $CD8^+$ T cells into cytotoxic T cells (Okada et al. 1988). In addition, IL-6 switches differentiation of monocytes from DCs to macrophages (Chomarat et al. 2000). The cooperation with TGF-β leads to differentiation of Th17cells from naive $CD4^+$ T cells and thus to IL-17 production (Korn et al. 2009). Recently, it has been demonstrated that the serum amyloid A proteins (SAAs) together with IL-6 lead directly to development of pathogenic pro-inflammatory Th17 cells from naive $CD4^+$ T cells, which results in the initiation of pro-inflammatory diseases (Lee et al. 2020). At the same time, IL-6 inhibits the TGF-β-mediated Treg cells differentiation (Bettelli et al. 2006). Furthermore, IL-6 is responsible for T-follicular helper cells differentiation and synthesis of IL-21 (Ma et al. 2012). As a regulator of chemokines and chemokine receptors expression IL-6 affects recruitment of neutrophils from blood and bone marrow as well as their survival (Scheller et al. 2011, Fielding et al. 2008, Asensi et al. 2004).

## 1 Introduction

In the liver IL-6 initiates the acute phase response as it stimulates hepatocytes to produce acute phase proteins such as CRP, fibrinogen, $\alpha_1$-antitrypsin and SSA (Gauldie et al. 1987, Castell et al. 1988). Therefore it is considered to play a central role in defense against microbial pathogens like bacteria and in restoring of the physiological homeostasis (Heinrich et al. 2003). Simultaneously, IL-6 suppresses production of albumin, fibronectin and transferrin (Andus et al. 1987). It has been also shown that IL-6 takes a part in the liver regeneration by promoting hepatocellular proliferation (Peters et al. 2000). Moreover, IL-6 provides protection from apoptosis induced by TGF-β as it blocks the activation of caspase-3 (Chen et al. 1999). In addition, IL-6 is involved in the metabolic functions of the liver. It participates in production of GLP-1 (Ellingsgaard et al. 2008, Ellingsgaard et al. 2011) and thus achieves the control of insulin sensitivity and glucose tolerance (Wunderlich et al. 2010, Matthews et al. 2010).

Besides immune cells and hepatocytes, IL-6 affects a broad spectrum of other cells. It acts as a growth factor for renal mesangial cells (Horii et al. 1989) and epidermal keratinocytes (Grossman et al. 1989, Yoshizaki 1990). It promotes megakaryocyte maturation in the bone marrow, which results in the release of platelets (Ishibashi et al. 1989). Moreover, IL-6 induces osteoclasts differentiation and activation via triggering RANKL synthesis by synovial fibroblasts (Hashizume et al. 2008). Another important function of IL-6 is the regulation of vascular permeability, which occurs trough induction of the VEGF production (Nakahara et al. 2003, Hashizume et al. 2009). Additionally, it has been shown that it increases collagen production by dermal fibroblasts (Duncan & Berman 1991) and is also involved in the neural activities (März et al. 1998, März et al. 1999) and protection of the cardiac myocytes from oxidative stress and apoptosis (Yamauchi-Takihara & Kishimoto 2000, Terrell et al. 2006, Wollert & Drexler 2001). Last but not least, IL-6 stimulates the growth of various types of tumor cells, e.g. plasmacytoma (Nordan et al. 1987), multiple myeloma (Kawano et al. 1988), renal cell carcinoma (Miki et al. 1989) and Kaposi's sarcoma (Streetz et al. 2000).

Aside from its physiological functions, it has been demonstrated that IL-6 is involved in multiple pathological states. In the past few years numerous working groups and their studies have been reported that dysregulation of IL-6 expression leads to development of different kind of pathologies including autoimmune and inflammatory diseases and some malignancies.

Elevated IL-6 levels are associated with RA (Houssiau et al. 1988), juvenile rheumatoid arthritis (Robak et al. 1998), vasculitis (e.g. giant cells arteritis) (Dasgupta & Panayi 1990, Roche et al. 1993, Weyand et al. 1994), polymyalgia rheumatica (Tanaka & Kishimoto 2014), Castlemann's disease (Yoshizaki et al. 1989) as well as Chron's disease (Kishimoto 2010). Furthermore, it has been shown that IL-6 is responsible for hypoferremia or anemia associated with chronic inflammation (Nemeth et al. 2004) and for bone resorption and osteoporosis (Hashizume et al. 2008, Kotake et al. 1996, Poli et al. 1994). It has been also reported that prolonged elevation of SAA levels leads to development of amyloid A amyloidosis, which is considered as a serious complication of the chronic inflammatory diseases (Gillmore et al. 2001). In addition, IL-6-induced production of collagen by dermal fibroblasts and their differentiation into myofibroblasts may also play a role in the pathology of fibrosing disorders such as systemic sclerosis (Duncan & Berman 1991).

Several studies have described an IL-6 elevation in distinct malignances such as colon and liver cancers (Bromberg & Wang 2009). Persistent activation of the IL-6 signaling pathway has been proposed as an underlying cause of liver cancer (Schmidt-Arras & Rose-John 2016). Recently, studies have demonstrated a correlation between elevated IL-6 serum levels and the increased risk to develop HCC (Aleksandrova et al. 2014). Moreover, it was found that IL-6 and sIL-6R serum levels in patients suffering from HCC are elevated (Soresi et al. 2006). Over the past few years, several studies using animal models have confirmed the role of IL-6 signaling in development of liver cancer (Maeda et al. 2005, Hatting et al. 2015, Pikarsky et al. 2004, Mair et al. 2010). In addition, it has been shown that persistent activation of IL-6 trans-signaling pathway induces formation of hepatocellular adenoma (Maione et al. 1998).

### 1.2.3. IL-6 signaling

IL-6 signaling is initiated by the binding of IL-6 to the non-signaling IL-6R. Association of IL-6/IL-6R complex with another receptor, namely gp130, results in dimerization of the second receptor. This step leads consequently to autophosphorylation and activation of the tyrosine kinase JAK1 that is bound to the cytoplasmic part of gp130 (Kishimoto 2005). Activated JAK1 phosphorylates tyrosine residues within the cytoplasmic part of gp130 and thus activates several intracellular pathways such as MAP kinase, $PI_3$ kinase and STAT pathways (Schaper & Rose-John 2015). Among them the JAK-STAT (Figure 2B) pathway is the most studied one.

Phosphorylated and thereby activated STAT3 dimerizes and then translocates into the nucleus, where it regulates transcription of its target genes (Heinrich et al. 2003).

With the exception of granulocytes, gp130 is expressed ubiquitously (Wilkinson et al. 2018). In contrast, expression of the IL-6R is limited to a few cell types such as hepatocytes, megakaryocytes and some immune cells like macrophages and monocytes (Scheller & Rose-John 2006). Hence, only cells expressing IL-6R on their surface are capable to respond directly to IL-6 (Kishimoto 2005). However, three IL-6 signaling pathways can be differentiated. The first one is the above described pathway in which cells directly respond to IL-6 and is called classic signaling (Figure 2B). The second one is called *trans*-signaling (Figure 2C) and expands the spectrum of cells that can be activated by IL-6. Here, the complex consisting of IL-6/sIL-6R binds to a gp130 homodimer and as a consequence triggers the intracellular IL-6 signaling. By a process called ectodomain shedding (Figure 2A) the membrane-bound IL-6R is cleaved by the metalloproteases ADAM10 and ADAM17 and thus the soluble IL-6 receptor (sIL-6R) is produced (Müllberg et al. 1993, Matthews et al. 2003). Distinct stimuli, which induce both metalloproteases, have been identified. The most potent and used in several *in vitro* studies is the phorbol ester PMA, which activates ADAM17 (Müllberg et al. 1993). Cholesterol depletion (Matthews et al. 2003), apoptosis (Chalaris et al. 2007) and CRP (Jones et al. 1999, Zhao et al. 2011) have been described as *in vivo* acting ADAM17 stimuli. $Ca^{2+}$-influx induced by ionomycin is used as an activator of ADAM10 in the *in vitro* (Maretzky et al. 2005), whereas cholesterol depletion (Matthews et al. 2003), activation of the P2X7R by ATP (Garbers et al. 2011) and mTOR activation (Garbers et al. 2013) have been discovered as *in vivo* stimuli of ADAM10. The other mechanism responsible for sIL-6R production is the alternative splicing of mRNA. In contrast to proteolysis, alternative splicing provides ~15% of the total amount of sIL-6R (Chalaris et al. 2011, Müller-newen et al. 1996, Riethmueller et al. 2017, Lust et al. 1992) .

Not long ago a third mode of IL-6 signaling, called *trans*-presentation has been discovered in mice. Here, IL-6 is *trans*-presented from DCs via their membrane-bound IL-6R to T cells (Heink et al. 2017). The response of T cells to the DC-derived IL-6-IL-6R complex occurs through their own gp130, which results in phosphorylation of STAT3 in T cells. All that leads to initiation of a pathogenic effector program, namely the differentiation of the T cell to a highly tissue-destructive phenotype (Heink et al. 2017).

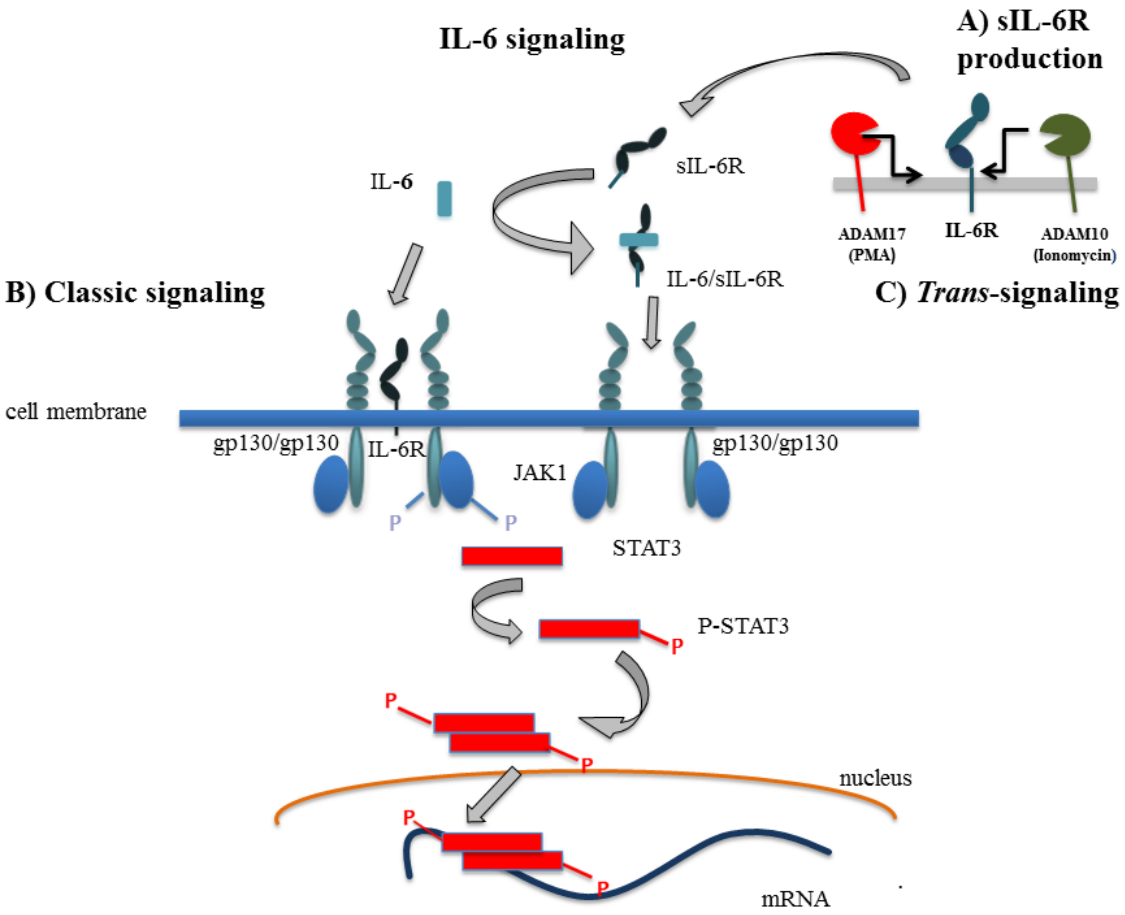


**Figure 2: IL-6 signaling. (A) sIL-6R production**: The membrane-bound IL-6R is cleaved by the metalloproteases ADAM10 and ADAM17 in a process called ectodomain shedding. Diverse stimuli such as PMA and ionomycin induce the metalloproteases. **(B) Classic signaling**: Binding of IL-6 to IL-6R results in dimerization of gp130, its autophosphorylation and the activation of JAK1. So activated JAK1 phosphorylates STAT3, which dimerizes and translocates into the nucleus, where it regulates the transcription of IL-6 target genes**. (C) *Trans*-signaling:** A complex consisting of IL-6/sIL-6R binds to a gp130 homodimer. As a result the intracellular IL-6 signaling is initiated. Figure is adapted from (Lokau et al. 2017).

Classic and *trans*-signaling differ from each other due to their functions. As an illustration, activation of the classic pathway leads to induction of anti-inflammatory processes such as the host defense against microbial pathogens (Mauer et al. 2015, Hoge et al. 2013). It is responsible for induction of the acute phase response via the production of acute phase proteins in the liver (Gauldie et al. 1987, Schmidt-Arras & Rose-John 2016). Furthermore, it has been shown that classic signaling has a crucial role in preventing development of insulin resistance (Matthews et al. 2010).

By contrast, activated *trans*-signaling pathway triggers the pro-inflammatory responses trough induction of pathogenic adaptive immune cells. It is considered that dysregulated IL-6 can contribute to the tissue damage in the autoimmunity and in the chronic inflammation (Calabrese & Rose-John 2014). It has been proposed that the negative metabolic effects of IL-6 are caused by trans-signaling (Kraakman et al. 2013, Kraakman et al. 2015). For example, increased IL-6 serum levels have been observed in patients with obesity (Bastard et al. 2000) and diabetes mellitus type 2 (Pradhan et al. 2001). Moreover, in the liver *trans*-signaling is the important trigger of the liver regeneration (Cressman et al. 1996, Peters et al. 1997, Schirmacher et al. 1998, Taub 2004, Fazel Modares et al. 2019, Yeoh et al. 2007).

### 1.2.4. Therapy

In the last years several studies have demonstrated pathological roles of IL-6 and IL-6 signaling in the development of several diseases. As a cytokine with a wide range of biological functions, IL-6 is considered as a potential target for the therapy of different pathologies including inflammatory, autoimmune and malignant diseases.

All these studies and observations have led to development of the first humanized anti IL-6R antibody tocilizumab (TCZ). TCZ blocks IL-6-mediated signal transduction by inhibiting IL-6 binding to both membrane-bound and soluble IL-6R and thus inhibits both classic and *trans*-signaling (Nishimoto et al. 2005).This blockade of both anti- and pro-inflammatory processes results in several side effects such as infections, significant reduction in the number of peripheral neutrophils (Berti et al. 2013) and increase in the body weight and triglyceride levels (Nishimoto et al. 2005, Richez et al. 2012). It has been shown that bacterial infections are the major side effect of anti-IL-6-therapy. It is considered that the compromised acute phase response due to the blockade of the IL-6 receptors on hepatocytes may be the reason (Lang et al. 2012). TCZ is the first approved IL-6-targeted therapy indicated for the treatment of RA (Kneepkens et al. 2017, Narazaki et al. 2017), juvenile idiopathic arthritis (JIA) (Tanaka et al. 2014), giant cell arteritis (GCA) (Villiger et al. 2016, Stone et al. 2017), Castleman disease (Tanaka et al. 2012, Kang et al. 2015) and as well as for cytokine release syndrome (CRS) (Garbers et al. 2018). In addition, sarilumab is another anti-IL-6R antibody approved for the treatment of RA (Narazaki et al. 2017). Ni-1201 and vobarilizumab are other specific anti-IL-6R antibodies that are still under investigation (Garbers et al. 2018).

Currently several other drugs, which inhibit different steps of the IL-6 signaling are being tested. For instance, sirukumab, olokizumab, clazakizumab and siltuximab represent a group of IL-6 specific monoclonal antibodies, which bind to different binding sites of IL-6 (Garbers et al. 2018). Among them siltuximab has been approved for the treatment of Castleman disease (van Rhee et al. 2014, Kang et al. 2015). The rest of them are still in different phases of clinical trials (Garbers et al. 2018).

Olamkicept, which is still under clinical testing, presents the only substance able to block selectively the *trans*-signaling (Garbers et al. 2018). It is a soluble gp130Fc fusion protein of the extracellular part of gp130 with Fc portion of an IgG antibody that targets sIL-6/IL-6R complex (Calabrese & Rose-John 2014, Hunter & Jones 2015).

There is a group of small-molecule kinase inhibitors with different selectivity, which block JAKs. For instance, the non-selective tofacitinib (O'Shea et al. 2013, Winthrop 2017) and baricitinib (Dougados et al. 2017) have been approved for the therapy of RA and ruxolitinib for the treatment of myelofibrosis (Meyer & Levine 2014). Upadacitinib, filgotinib (Genovese et al. 2016) and PF-04965842 (Garbers et al. 2018) represent selective JAK inhibitors, which are still under different phases of clinical trial.

Targeting of the transcription factor STAT3 represents another alternative for the therapeutic blockade of IL-6 signaling. STA-21 is a small chemical compound, which is still tested in different clinical trials (Miklossy et al. 2013). It inhibits both the STAT3 dimerization and the DNA binding (Song et al. 2005). The compound stattic selectively inhibits activation, dimerization, and nuclear translocation of STAT3 (Schust et al. 2006) and has been shown to induce the apoptosis in breast cancer cell lines (Schust et al. 2006) and different nasopharyngeal carcinoma cell lines (Pan et al. 2013). The small interfering RNA (siRNA) represents another mechanism of STAT3 targeting, which is planned to be tested in clinical trials in patients with diffuse large B cell lymphoma (Kortylewski et al. 2009, Kortylewski & Moreira 2017).

## 1.3. Actin

### 1.3.1. Actin cytoskeleton

The actin cytoskeleton is an extremely dynamic and complex system composed of diverse actin superstructures organized by numerous actin binding proteins. Together with other components

of the cytoskeleton such as microtubules and intermediate filaments, the actin cytoskeleton plays a pivotal role in many cellular processes (dos Remedios et al. 2003).

Actin is a major component of the cytoskeleton and is the most abundant protein in eukaryotic cells (Carpenter 2000). It is a globular 43 kDa protein, which consists of three main isotypes. While α-actin can be found in muscle cells, β- and γ-actin are typical for non-muscle cells (Schoenenberger et al. 1999). It is known that actin can exist both as a monomer and as a multimer. The globular or G-actin is able to polymerize into the other form, which is called filamentous or F-actin. As polar structures, actin filaments possess two ends, at which the polymerization occurs at different rates. As a result, a fast growing "plus" or barbed end and a slow-growing "minus" or pointed end can be differentiated. While the "plus" end is composed from high concentrated ATP-actin monomers and is oriented towards the cell membrane, the orientation of the ADP-actin consisting "minus" end is towards the cytoplasm (Begg et al. 1978, Stossel 1984). It has been shown that polymerization of actin monomers leads to the formation of a helical structure (Holmes et al. 1990). F-actin takes a part in the building of diverse complex structures. For instance, actin filaments form bundles called stress fibers, major mediators of cell contraction (Pellegrin & Mellor 2007), or build fine and complex networks underneath the plasma membrane. Another structure with important role is the contractile ring, which participates in the separation of the cell during cytokinesis (Stossel 1984).

The actin cytoskeleton permanently interacts with various proteins. These are responsible not only for polymerization, but also for the stability and length of the actin filaments (Pollard & Cooper 2009). These accessory proteins regulate the assembly and turnover of the filaments and provide the necessary pool of actin monomers. Besides, they mediate the cross-linking of the filaments into networks and bundles (Pollard & Cooper 2009).

These accessory proteins, called actin binding proteins (ABPs), have been divided in groups on the base of their functions. The sequestering proteins profilin and thymosin-β4 form one of these groups. They bind to the actin monomers and sequester them into the cytoplasm. This action prevents G-actin to participate in the processes of nucleation and elongation (Pollard 2016). Capping proteins are another group of the ABPs. They bind to the fast growing end of the filaments and thus slow or inhibit their elongation (Blanchoin et al. 2014). In cooperation with profilin they are able to maintain the required pool of actin monomers, to limit the number of

barbed ends available for the growth and to stabilize them (Pollard 2016). Another class of regulatory proteins, called nucleation proteins, initiates the actin polymerization. For instance, Arp2/3 complex induces filaments branching, whereas formin triggers the formation of unbranched filaments (Pollard 2016). Furthermore, other proteins like fimbrin and filamin have been described as regulators of the actin network's architecture. These so-called cross-linking proteins (Matsudaira 1994) lead to formation of higher-order assemblies, which can be found as parallel bundles in the filopodia and microvilli, as contractile bundles in the lamella or as complicated networks in the cortex (Gardel et al. 2010, Parsons et al. 2010, Schwarz & Gardel 2012). Cofilin and gelsolin families are typical members of the actin filament-severing proteins, which represent another group of the ABPs. These proteins sever actin filaments into short fragments and therefore accelerate their turn over (Pollard 2016).

### 1.3.2. Actin dynamics

Actin filaments are highly dynamic structures and the polymerization of G-actin into F-actin represents a multi-level process. Generally, actin polymerization occurs over three phases: nucleation, elongation and steady state phase (Pollard 2016).

During the nucleation phase (Figure 3B), an actin nucleus, usually composed of three actin monomers, is formed. Spontaneous nucleation of actin filaments from monomers is aggravated by the relative instability of actin trimers and by G-actin-binding and sequestering proteins, such as profilin and thymosin-β4 (Pantaloni & Carlier 1993). In the elongation phase (Figure 3C), when the actin nucleus is formed, actin monomers are rapidly added to the barbed end of the growing filament under ATP hydrolysis until almost actin monomers are part of the filament. This step is often facilitated by additional elongation factors such as formin and Arp2/3 (Pollard & Borisy 2003). In the steady state phase (Figure 3D), filaments dynamics enter a state of equilibrium, where monomer disassembly from "minus" end and the polymerization at the "plus" end are balanced and have maintained the critical concentration of actin monomers in the cytoplasm. This steady stay is also known as treadmilling (Carlier & Pantaloni 1997).

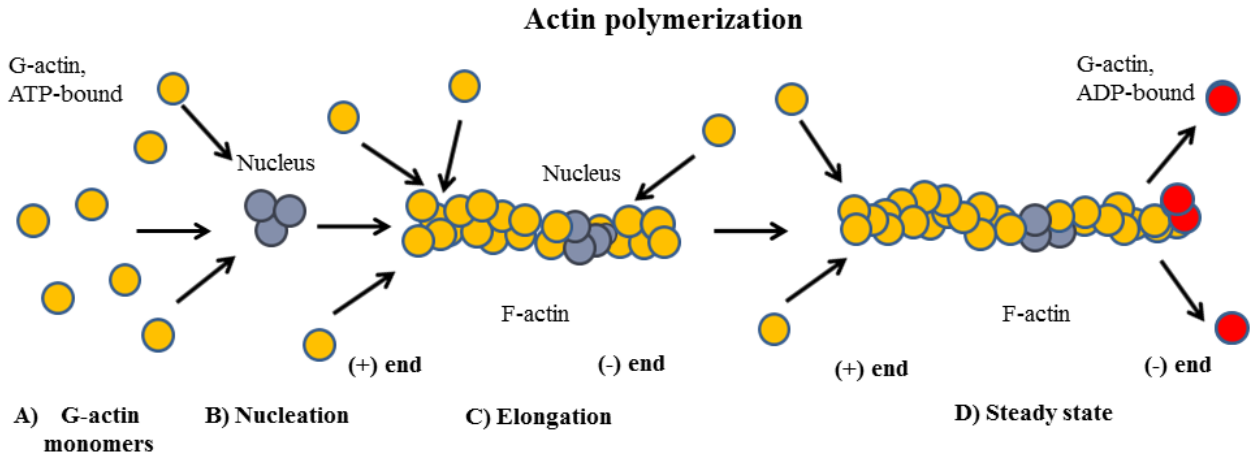


**Figure 3: Actin polymerization. (A) ATP-bound G-actin monomers**. **(B) Nucleation phase**: formation of the nucleus. **(C) Elongation phase:** activated G-actin monomers are rapidly added to the fast growing (+) end. **(D) Steady state = treadmilling:** monomers assembly at (+) end and their disassembly at (-) end are balanced. Figure is adapted from (Lodish HF 2003).

Depolarization of actin filaments occurs at pointed ends and is driven by the ADF/cofilin family proteins. Actin monomers intrinsically disassociate from the barbed end at a faster rate than they do from the pointed end (Pollard 1986). Cofilin severs filaments on the pointed ends into short fragments. This destabilizes the filaments and promotes the release of ADP-actin monomers. When the dissociation rate of ADP-actin exceeds the rate of ATP-actin association, the filament shrinks. Actin treadmilling occurs, when the rate of ATP-actin association and the ADP-actin loss are balanced (Carlier et al. 1997).

### 1.3.3. Functions of actin

The actin cytoskeleton is a dynamic system that participates in many pivotal cellular processes including cell survival and proliferation, apoptosis and motility (Papakonstanti & Stournaras 2008). Due to actin dynamics, cells can change their shape and move via formation of structures like filopodia and lamellipodia. In muscle cells, actin filaments participate in the muscle contraction, whereas in non-muscle cells, they form a system for intracellular transport of vesicles and organelles (Carpenter 2000). Furthermore, actin participates in processes like secretion, endocytosis and phagocytosis. Moreover, actin supports cell division during cytokinesis. In addition, it provides connections to other cells and extracellular matrix through

formation of cell junctions (Carpenter 2000). Finally, actin participates in cell signaling and thus in the activation of transcription factors and control of gene expression (Ben-Ze'ev 1991, Frixione et al. 2001).

In recent years, several signal transduction pathways have been described as able to regulate actin polymerization and contractility. These pathways allow cells to respond to extracellular signals and thus trigger a variety of actin-depending responses. GTP-binding proteins, phosphoinositide kinases, protein phosphatases and protein kinases have been described as mediators of this signaling (Carpenter 2000). Furthermore, several stimuli known to activate NF-κB such as TNFα (Peppelenbosch et al. 1999), IL-1β (Singh et al. 1999), PMA (Downey et al. 1992) and LPS (Chakravortty et al. 2000) have been identified as inducer of actin cytoskeleton reorganization.

### 1.3.4. Actin modulating compounds

Several small molecule drugs have been used in order to examine actin dynamics. These are naturally occurring toxins like phaloidins, cytochalasins, lantrunculin A and jasplakinolide that bind to actin and alter its dynamics (Holzinger & Blaas 2016).

Phalloidins stabilize actin filament and prevent its depolymerization by specifically binding at the interface between F-actin subunits and thus locking adjacent actin subunits together (Cooper 1987). In addition, jasplakinolide also stabilizes actin and therefore enhances the filament nucleation and polymerization (Bubb et al. 2000). By contrast, cytochalasins bind to the barbed ends of actin filaments and thus prevent the addition of monomers during the elongation. As a result polymerization and elongation of actin filaments are inhibited (Cooper 1987). Lantrunculin A binds to G-actin monomers and therefore prevents their polymerization. Furthermore, this compound may also inhibit the nucleated exchange on the actin subunits (Yarmola et al. 2000, Morton et al. 2000).

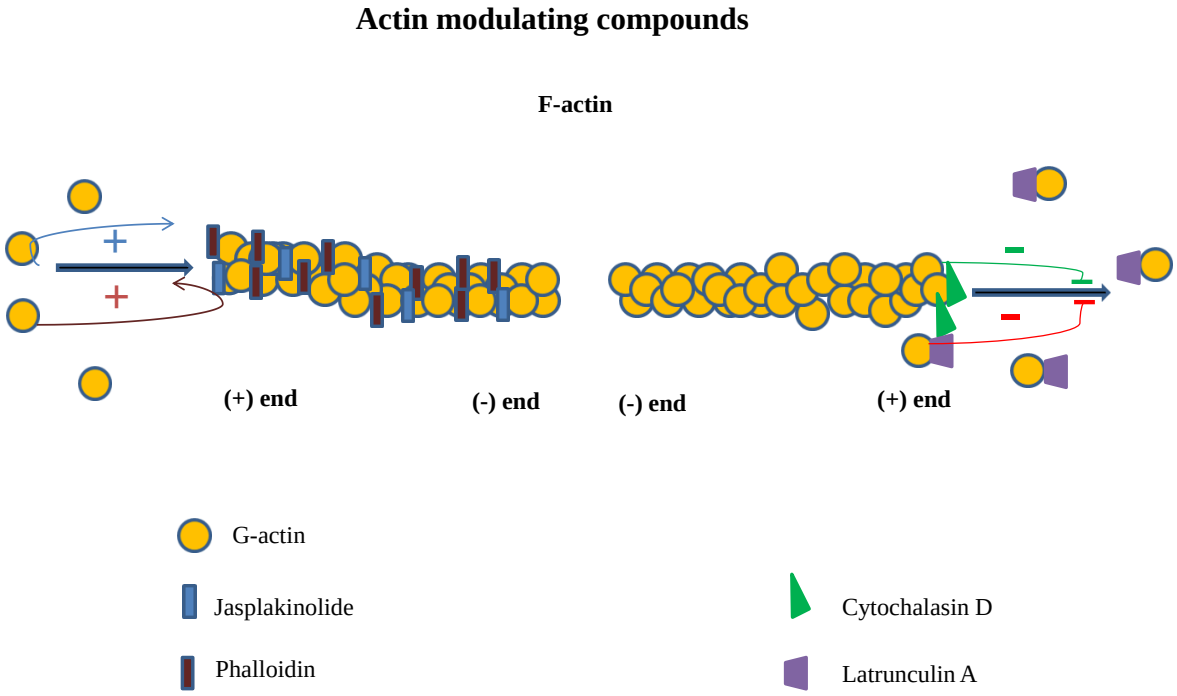


**Figure 4: Actin modulating compounds.** Jasplakinolide enhances polymerization by inducing nucleation. Phalloidin stabilizes actin. Both Jasplakinolide and Phalloidin compete for the same binding site. Cytochalasin D binds F-actin at barbed ends. Latrunculin A binds to G-actin monomers. Both cytochalasin D and Latrunculin A inhibit actin polymerization. Figure is adapted from (https://www.mechanobio.info/cytoskeleton-dynamics/what-is-the-cytoskeleton/what-are-actin-filaments/what-factors-influence-actin-filament-length-and-treadmilling/).

### 1.4. Aims of the current work and hypothesis

In the past years many working groups have shown connection between persistent activated NF-κB and development of different pathological states like inflammatory and malignant diseases. Due to its control over the expression of genes involved in inflammation, cell proliferation and apoptosis and the hypothesis that tumor formation is favored in an inflammatory environment, it is not surprising that NF-κB is a potential therapeutic target for anti-cancer therapy.

*IL6* as NF-κB target gene and due to its pleiotropic functions and involvement in different malignant and nonmalignant pathologies also arouses the interest of the scientists. Therefore it was no wander that it has led to intensive research in the area of the anti-cancer and anti-inflammatory therapy.

# 1 Introduction

The actin cytoskeleton supports a wide range of important cellular processes and mediates cell responses to external stimuli like TNFα. It was found that stabilization of actin filaments prevents the nuclear translocation of NF-κB and its binding activity (Papakonstanti & Stournaras 2004). Furthermore, it has been demonstrated that diverse alterations in the actin dynamics lead to different cell responses (Papakonstanti & Stournaras 2008).

Manipulation of actin dynamics led to activation of NF-κB, which was demonstrated for myelomonocytic (Kustermans et al. 2005) and for intestinal epithelial cells (Németh et al. 2004). The aim of the current work was to investigate whether manipulation of the actin cytoskeleton with actin modulating compounds results in activation of NF-κB in hepatocytes. Furthermore, we analyzed whether IL-6 signaling and generation of sIL-6R would be altered in cells, which were pre-treated with actin modulating compounds.

# 2. Materials and methods

## 2.1. Materials

### 2.1.1. Cell culture media and reagents

**Table 1: List of cell culture media and reagents.**

| Media/Reagent | Abreviation | Manufacturer (Content) |
|---|---|---|
| Dimethyl sulfoxide | DMSO | Life Technologies, USA |
| Dulbecco's Modification of Eagle's Medium | DMEM | Gibco, USA (1g/L D-Glucose, L-Glutamin, 110 mg/L Sodium Pyruvate) |
| Fetal Bovine Serum | FBS | Gibco, USA |
| Freeze media | | DMEM + 10% FBS + 5% DMSO |
| Penicillin, Streptomycin | Pen Strep | Gibco, USA (10 000U/ml Penicillin, 10 000 µg/ml Streptomycin) |
| Phosphate-buffered saline 10 x | PBS | Gibco, USA (pH 7.4) |
| RPMI 1640 | | PAN Biotech, Germany |
| Trypan blue stain 0.4% | | Invitrogen, USA |
| Trypsin/EDTA | | PAN Biotech, Germany (0.25%/ 0.02% in PBS) |

### 2.1.2. Stimulants

**Table 2: List of cell stimulants.**

| Stimulant | Manufacturer |
|---|---|
| Cytochalasin B | ENZO Life Sciences, USA |
| Cytochalasin D | Sigma Aldrich, USA |
| Human IL-6 | Self-produced, full recipe is described in J Immunol, 1992 (Mackiewicz et al. 1992) |
| Ionomycin | Thermo Fischer Scientific, USA |
| Jasplakinolide | EMD Millipore, Germany |
| Recombinant Human Lymphotoxin α1/β2 | R&D Systems, USA |

| | |
|---|---|
| Recombinant human TNFα | PeproTech, USA |

### 2.1.3. Buffers and solutions

**Table 3: List of buffers and solutions.**

| Buffer/Solution | Recipe/ Manufacturer |
|---|---|
| TBST (washing buffer) | 100 ml TBS 10x; 900 ml Aqua Dest; 1 ml Tween 20 |
| TBS (Tris-Buffered Saline) 1x | 100 ml TBS 10x; 900 ml Aqua Dest |
| TBS (Tris-Buffered Saline) 10x | 9 g Tris-Base; 68.5 g Tris-HCl; 87.8 g NaCl (pH 7.4-7.6) |
| Restore TM PLUS Western Blot Stripping Buffer | Thermo Fisher Scientific, USA |
| Rotiphorese 10x SDS-Page | Carl Roth GmbH+Co.KG, Germany |
| Ponceau S solution | Sigma-Aldrich, USA : 0.1% Ponceau S (w/v) in 5% (v/v) acetic acid |
| Promocell Lysis buffer | 1 ml Tris HCL 1M pH 7.5; 820 mg NaCl; 1ml Triton x 100; 1 ml 0.5 M EDTA (pH 8.0) |
| Laemmli buffer, 2x | Sigma-Aldrich, USA: 0,063 M Tris HCl; 10 % Glycerol; 2 % SDS; 5 % Mercaptoethanol; Bromophenol blue |
| Immobilon Western HRP substrate | EMD Millipore, Germany |
| Blocking buffer | 10 g BSA; 50 ml TBS 10x; 450 ml Aqua Dest; 1 ml NP 40 |
| 1x Transfer buffer (Blotting buffer) | 100 ml 10x Transfer buffer; 200 ml Methanol; 700ml Aqua Dest |
| 10x Transfer buffer (Blotting buffer) | 32,8 g Tris; 144 g Glycin |
| Resolving gel buffer | 1.875 M Tris/Base (22.7 g/ 100 ml), 1 % SDS (1g) pH 8.8 |
| Stacking gel buffer | 1.875 M Tris/Base (22.7 g/100 ml), 0.5 % SDS (0.5g) pH 6.8 |

**Table 4: Recipes of gels.**

| reagents | resolving gel 10% | resolving gel 12% | stacking gel |
|---|---|---|---|
| Aqua Dest | 4,67 ml | 3,93 ml | 6, 33 ml |
| Acrylamide | 3,33 ml | 3,93 ml | 1,65 ml |
| Buffer | 2 ml | 2 ml | 2 ml |
| APS 10% | 75 µl | 75 µl | 75 µl |
| TEMED | 8 µl | 8 µl | 8 µl |

### 2.1.4. Chemicals

**Table 5: List of chemicals.**

| Chemical | Abreviation | Manufacturer |
|---|---|---|
| Albumin Fraction V | BSA | Carl Roth GmbH + Co. KG, Germany |
| Ammonium Persulfate 0.1 g/1ml (10%) | APS | Sigma Aldrich, USA |
| BM Blue POD Substrate, soluble | | Roche Diagnostics, Germany |
| Ethanol 96% | $C_2H_5OH$ | Otto Fischer GmbH & Co. KG, Germany |
| Ethylenediaminetetraacetic acid | EDTA | Sigma-Aldrich, Germany |
| Glycin | | Carl Roth GmbH + Co. KG, Germany |
| Isopropyl alcohol | $C_3H_8O$ | Carl Roth GmbH + Co. KG, Germany |
| Methanol | $CH_3OH$ | Carl Roth GmbH + Co.KG, Germany |
| Sodium chloride | NaCl | Carl Roth GmbH + Co.KG, Germany |

| | | |
|---|---|---|
| Nonidet® P40 | NP 40 | Appli Chem GmbH, Germany |
| Octylphenol-polyethylene glycol ether | Triton® X-100 | Serva Electrophoresis GmbH, Germany |
| PBS-Tween® Tablets | | Calbiochem, Germany |
| Powdered milk (blotting grade, powdered, low fat) | | Carl Roth GmbH + Co. KG, Germany |
| Phosphatase Inhibitor Cocktail 3(P0044) | | Sigma-Aldrich, USA |
| Precision Plus Protein™ Western C™ Standards | | Bio-Rad Laboratories, USA |
| Protease Inhibitor Cocktail (P8340) | | Sigma-Aldrich, USA |
| Rotiphorese® Gel 30 (37.5>1) - 30% Acrylamidstammlösung mit 0.8% Bisacrylamid im verhältnis 37.5:1 | AA | Carl Roth GmbH + Co. KG, Germany |
| Sodium dodecyl sulfate | SDS | Carl Roth GmbH + Co. KG, Germany |
| Sulfuric acid | $H_2SO_4$ | Carl Roth GmbH + Co. KG, Germany |
| Tetramethylethylenediamine | TEMED | Carl Roth GmbH + Co. KG, Germany |
| Thiazolyl blue tetrazolium bromide | MTT | Sigma Aldrich, USA |
| TRIS | | Carl Roth GmbH + Co. KG, Germany |
| TRIS-hydrochloride | | Carl Roth GmbH + Co. KG, Germany |
| Trypan blue stain 0.4% | | Invitrogen, USA |
| Tween 20 | | Carl Roth GmbH + Co. KG, Germany |

### 2.1.5. Antibodies

**Table 6: List of antibodies.**

| Antibody | Catalogue № | Manufacturer | Dilution |
|---|---|---|---|
| IκBα | #4812S | Cell Signaling Technology, USA | 1:5,000 |
| Phospho-IkBα (Ser32) | #2859T | Cell Signaling Technology, USA | 1:1,000 |
| NF-κB p65 | #8242 | Cell Signaling Technology, USA | 1:1,000 |
| Phospho-NF-κB p65 (Ser536) | #3031S | Cell Signaling Technology, USA | 1:1,000 |
| RelB | #4922 | Cell Signaling Technology, USA | 1:1,000 |
| Phospho-RelB (Ser552) | #4999 | Cell Signaling Technology, USA | 1:1,000 |
| Stat3 | #9139 | Cell Signaling Technology, USA | 1:1,000 |
| Phospho-Stat3 (Tyr705) | #9145 | Cell Signaling Technology, USA | 1:1,000 |
| GAPDH | #2118S | Cell Signaling Technology, USA | 1:1,000 |
| Anti-rabbit Ig G HRP-linked Antibody | #7074 | Cell Signaling Technology, USA | 1:3,000 |
| Anti-mouse Ig G HRP-linked Antibody | #7076S | Cell Signaling Technology, USA | 1:3,000 |
| Precision Protein™ Strep Tactin-HRP Conjugate | #1610380 | Bio-Rad laboratories, USA | 1:2,000 |

### 2.1.6. Kits

**Table 7: List of kits.**

| Kit | Manufacturer |
|---|---|
| Bio-Rad DC™ Protein Assay | Bio-Rad laboratories, USA |
| Duo Set® Human sIL-6Rα | R&D systems, USA |
| Human IL-6 Duo Set ELISA | R&D systems, USA |

### 2.1.7. Consumables

The list of all used consumables can be found as an appendix 1 (table 9).

### 2.1.8. Equipment

The list of all used equipment can be found as an appendix 2 (table 10).

## 2.2. Methods

### 2.2.1. Cell cultures and cell culturing

In this study two cell lines, which have been originally isolated from human hepatocellular carcinoma, were used. Hep3B cells were obtained from the Department of Pathology of the Otto von Guericke University Magdeburg and HepG2 cells from the Department of Pathology of the Medical University Graz (Austria). Both cell lines are adherent and were cultivated in DMEM medium supplemented with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 µg/ml). Furthermore, the cell line HCT116 was used. It is an adherent colorectal carcinoma cell line and was cultivated in RPMI 1640 medium, supplemented with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 µg/ml). HCT116 cells were also obtained from the Department of Pathology of Otto-von-Guericke-University Magdeburg. All cells were grown in a humidified incubator at 37°C under 5% $CO_2$. Growth media were changed every two days.

When reaching a confluence of 80% cells were passaged. Culture media was removed, cells were washed with PBS and then trypsinized with 0.25% trypsin/EDTA for 5 minutes. Cells were then resuspended in prewarmed media and centrifuged at 700 x g at room temperature for 5 minutes. Supernatant was discarded and cells were resuspended in prewarmed culture media. Cells were counted using a Countess II cell counter and an appropriate cell number was transferred to a 75 $cm^2$ cell culture flask with fresh prewarmed media.

### 2.2.2. Cell thawing and freezing

Cells were thawed in a water bath at 37° C, transferred in a tube containing 10 ml prewarmed culture medium and then centrifuged at 700 x g at room temperature for 5 minutes. Supernatant was discarded and cells were resuspended in prewarmed culture media and transferred to a 75 $cm^2$ cell culture flask. Growth medium was changed on the following day.

Cells were cryopreserved after reaching 80% confluence. Cells were trypsinized as described above, centrifuged at 700 x g at room temperature for 5 minutes and resuspended in freezing medium (DMEM + 10% FBS + 5% DMSO). A desirable amount of cells was transferred into

cryovials, which were placed in a freezing box containing isopropyl for a night at -80°C. On the following day cryovials were transferred into a -150°C liquid nitrogen freezer for definitive storage.

### 2.2.3. Cellular protein extraction and analysis

#### 2.2.3.1. Total cell lysate preparation

Cells were lysed using Promocell lysis buffer. Before usage, 10 µl protease inhibitor cocktail (for use with mammalian cell and tissue extracts) and 10 µl phosphatase inhibitor cocktail (for Serine/Threonine Protein Phosphatase and L-Isozymes of Alkaline Phosphatase) were added to 1 ml lysis buffer. All of the following steps were performed on ice.

Cell culture medium was discorded and an appropriate volume of the lysis buffer was added to the culture plate (e.g. 80 µl/ well for 12-well plate and 100 µl/ well for a 6-well plate) and left on the cells for 20 minutes. Then cells were scraped from the bottom of the plate with the help of a cell scraper. Cell suspensions were transferred to a 1.5 ml Eppendorf tube and then centrifuged for 10 minutes at 14000 x g and 4° C. Supernatants were transferred to a new tube and stored at -80° C.

#### 2.2.3.2. Determination of protein concentrations

Protein concentrations were determined with the help of the Bio-Rad DC™ Protein Assay, which is a calorimetric assay. Its principle of action is similar to the Lowry protein assay, where a reaction between proteins and divalent cooper under alkaline conditions leads to reduction of the copper to a monovalent ion. The Folin Reagent (Reagent B) forms with $Cu^{+}$ a complex with blue colour with maximum absorbance at 750 nm and minimum absorbance at 450 nm.

A protein standard was prepared using a stock solution of 10 mg/ml BSA at following concentrations: 0, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0 and 3.0 µg BSA/ml Aqua Dest. 5 µl from the standards as well as from the samples in duplicate were mixed with 25 µl from the reagents mixture (20 µl Reagent S solved in 1 ml Reagent A and 200 µl from Reagent B) in a 96-well plate. The plate was left for 15 min at room temperature on a thermomixer for incubation. Finally, absorption was measured using the detection system GLOMAX Multi. Determination of the protein concentrations in the samples was based on the creation of a standard curve, using the absorption values of the standards.

#### 2.2.3.3. Discontinuous SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analyzed with the help of SDS-polyacrylamide gel electrophoresis. For this, a gel consisting of a large-pore stacking gel on the top of a small-pore resolving gel was used. The first one was used to concentrate the proteins and the second one to separate them by their molecular weight.

Protein samples were mixed with 2x Laemmli buffer and then heated at 95°C for 5 min to denature proteins and to reduce the disulfide bonds. Mixtures with volumes of 40 to 60 µl containing 30 to 35 µg proteins were loaded into the wells of the stacking gel alongside Precision Plus Protein™ Western C™ Standards molecular weight marker. An electrophoresis system from Bio-Rad Laboratories was used and gels were run at 100 V for approximately two hours. The recipes for gels and buffers are listed in section 2.2.3.

#### 2.2.3.4. Western blotting

After separation proteins were transferred from the gel onto a nitrocellulose membrane using a Trans-Blot® Turbo™ Transfer System. Western blotting is a technique by which an electric field is built perpendicular to the gel. This field leads to migration of negatively charged proteins from the gel to positively charged anode and in this way onto the nitrocellulose membrane.

The prewetted with transfer buffer membrane and the gel were placed between three sheets of buffer-soaked filter papers on each side. A blott roller was gently used to remove possible air bubbles between the gel and membrane. So assembled transfer pack was laid between two iron cassettes and the blotting system was run at 25 V for 40 minutes. After that membrane was stained with Ponceau S solution to verify the blotting. Membrane was washed then with Aqua Dest and blocked for 1 hour at room temperature with blocking buffer (5% BSA in TBST and 1% NP 40), then washed 3 times for 5 min with washing buffer (TBST). Finally, specific primary antibodies were added and membrane was incubated at 4°C overnight. All of the used antibodies were diluted in 5% BSA in TBST following the instructions of the manufacturer. The recipes for the buffers and antibodies dilutions are listed in sections 2.2.3 and 2.2.5.

#### 2.2.3.5. Immunological detection of proteins

On the following day the membrane was washed 3 times for 5 min with washing buffer. HRP-conjugated secondary antibodies, diluted in 5% milk in TBST following the instructions of the manufacturer, were added and incubated for 1 hour at room temperature. Thereafter, the

membrane was washed 3 times with washing buffer for 5 minutes and then 3 minutes incubated in Immobilon Western HRP Substrate. Detection of chemiluminescent blots was achieved with the help of a western blot imaging system (Fluor Chem E System). Protein bands were visualized and quantified using Image Studio version 5.2.

In cases when a second protein should be detected, the membrane was washed again 3 times for 5 min with washing buffer and then stripped for 30 min at room temperature with Restore PLUS Western Blot Stripping buffer. After that, the membrane was washed again 3 times for 5 min with washing buffer and then blocked for 1 hour with blocking buffer. The membrane was incubated with specific primary antibodies at 4°C overnight. The above described procedure was repeated as often as required to detect all proteins of interest.

### 2.2.4. Manipulation of actin cytoskeleton and stimulation of cells

Cells were seeded at appropriate density and then stimulated with different substances. Hep3B and HepG2 cells were seeded at a density of 4.0 x $10^5$ cells/ well and 7.5 x $10^5$ cells/ well in a 6-well plate, whereas Hep3B and HCT116 respectively at density of 6.5 x $10^4$ cells/ well and 1.2 x $10^5$ cells/ well in a 12-well plate. Cell culture plates were left under standard conditions in a humidified incubator overnight. On the following day cells were washed with serum free DMEM, then fresh serum free DMEM was added and cells were stimulated with actin-modulating compounds for 15 min or 2 hours. Depending on the experiment cells were treated afterwards with IL-6 or ionomycin for 15 minutes or 1 hour, respectively. Total cell lysate was prepared as described in section 2.3.3.1. Collected supernatants were centrifuged for 10 minutes at 1, 000 x g and 4° C and then transferred into new tubes, which were stored at -20° C.

### 2.2.5. MTT assay

In order to test the cytotoxic effect of different substances on cells a MTT assay was performed. It is a calorimetric test that measures cells viability. Its principle of action is the reduction of the yellow, water soluble compound tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple-coloured insoluble formazan. The entire process of reduction depends on cellular reductase enzymes in living cells, which means that conversion of the substrate is proportional to living cells. An increased signal and intensive purple colour indicates that cells possess high metabolic activity, they are alive and proliferating. A decrease in

the signal and a weak purple shade means low metabolic rate and reduced proliferation. Reduced values in the MTT Assay can be an indication for a cytotoxic effect of the tested compounds.

A cell suspension containing 1.0 x $10^5$ cells/ ml medium was prepared and 100 µl of it were pipetted in the wells of a 96-well culture plate. Plate was left for 24 h under standard conditions in an incubator to allow adherence of the cells. On the following day cells were stimulated with increasing concentrations of the substances to be tested in quadruplicates for different time periods. A solution of 5 mg MTT tetrazolium salt and 1 ml PBS was mixed and then filtrated through a sterile filter. 10 µl of the MTT solution were added to the cells and then they were incubated for 2 h at 37°C. After that supernatants were discarded and 100 µl DMSO were pipetted in each well. Following that, the plate was incubated for 15 min at room temperature on a rotor. Absorbance was read using 562 nm as the primary wavelength and 620 nm as a reference wavelength with the help of the detection system GLOMAX Multi. The percentage of living cells was formed from the quotient between absorption values of stimulated cells and the values of unstimulated controls.

### 2.2.6. ELISA ( Enzyme-linked immunosorbent assay)

For the quantification of sIL-6R and IL-6 in culture medium the Duo Set® Human sIL-6Rα and Human IL-6 Duo Set ELISA kits from R&D Systems, USA were used. The principle of ELISA technique is the attachment of antigens from the sample to a specific capture antibody, which is immobilized on a surface. Detection antibodies are applied afterwards in order to bind to the antigens. These antibodies are covalently linked with enzymes, which are able to convert a substrate, which can be measured, e.g. a change in colour.

A Nunc Maxi Sorp™ 96-well ELISA plate was coated with 50 µl capture antibodies, diluted in PBS according to manufacturer's instructions (see Table 8) and incubated overnight at room temperature. On the following day non-bound capture antibodies were aspirated and wells were washed twice with 300 µl PBST (1 tablet PBS-Tween® Tablets solved in 1 l Aqua Dest). The plate was blocked with 1 % BSA in PBS at room temperature for 1 hour. A standard stock solution was used to prepare a two-fold dilution series at the following concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 pg/ml (sIL-6R ELISA) and 600, 300, 150, 75, 37.5, 18.75, 9.38 and 0 pg/ ml (IL-6 ELISA). Afterwards, the aspiration/ wash step was repeated, 50 µl respectively from standards and samples were added in each well in triplicate and incubated for 2

hours at room temperature. Wells were washed afterwards twice and 50 µl detection antibodies, diluted in 1 % BSA in PBS, were added per well and incubated for 1 h. Following this, the wash step was repeated and 60 µl streptavidin-HRP, diluted in 1 % BSA in PBS, were pipetted into each well. The plate was incubated for 20 min, avoiding direct light. After washing twice 60 µl BM blue POD substrate were added into each well and incubated for 10 min at room temperature, again avoiding direct light. The reaction was stopped by adding 60 µl 2N $H_2SO_4$ per well. The optical density was measured using a CLARIO star® Plus microplate reader, set to 450 nm wavelengtht.

**Table 8: Dilutions of reagents of ELISA kits.**

| reagent | sIL-6R - dilution | IL-6 - dilution |
|---|---|---|
| **capture antibodies** | 1:180 | 1:120 |
| **detection antibodies** | 1:180 | 1:60 |
| **standard** | 1:110 | 1:300 |
| **streptavidin-HRP** | 1:200 | 1:40 |

### 2.2.7. Statistical analysis

Quantitative data are expressed as the means ± SD. For all western blots one representative example is shown. Statistical significance of the intergroup differences was determined using GraphPad Prism with one-way analysis of variance (ANOVA) or two-tailed Student's unpaired t-test as described in the figure legends.

# 3. Results

## 3.1. Effect of TNFα on NF-κB in Hep3B cells

In order to demonstrate that stimulation with TNFα leads to phosphorylation of the p65 subunit of NF-κB and thus to activation of the NF-κB signaling in Hep3B cells, the following experiment was performed. Hep3B cells were seeded and stimulated with TNFα (100 U/ml) for 30 min 24 h, 48 h and 72 h after seeding. Total cell lysates were analyzed with the help of western blot technique. In addition, it was intended to analyze whether cell confluence influences the response of Hep3B cells to TNFα stimulation.

Figure 5A is a representative western blot image and presents a comparison between TNFα stimulated cells and the unstimulated ones. Apart from that a comparison between all investigated time points was also made. Figure 5B presents the quantification of P-p65 expression and like Figure 5A demonstrates that cell stimulation with TNFα results in phosphorylation of the p65 subunit at all investigated time points in comparison to the untreated cells. The maximal response is reached at first 24 h after seeding followed by a slight, but not significant decrease with increasing confluence of the cells.

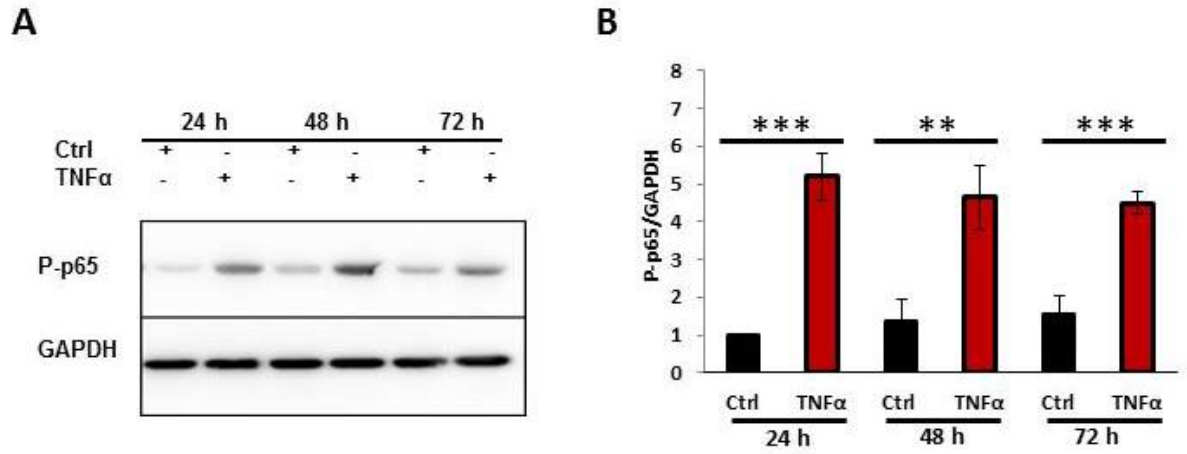


**Figure 5: Stimulation of Hep3B cells with TNFα (100 U/ml) for 30 min 24 h, 48 h and 72 h after seeding. (A)** Representative immunoblot of Phospho (P)-p65 expression. GAPDH was visualized to verify equal loading. **(B)** Data present the quantification of P-p65 expression normalized to GAPDH. Data are shown as means ± SD of three independent experiments. Statistical significance was determined using two-tailed unpaired t tests (**: $p < 0.01$; ***: $p < 0.001$).

In conclusion, stimulation of Hep3B cells with TNFα resulted in phosphorylation of p65 and thus in activation of NF-κB. Moreover, confluence state of cells did not significantly influence the activation of the transcription factor.

### 3.2. Effect of actin-modulating compounds on the morphology of Hep3B and HepG2

With the aim of documenting the morphological changes, which have occurred in both used hepatocellular cell lines as a result of stimulation with actin-modulating compounds, the following experiment was made. Hep3B and HepG2 cells were seeded and on the following day stimulated with the actin polymerization inhibitors cytochalasin B (15 µM) and cytochalasin D (1 µg) and the actin polymerization inducer jasplakinolide (0.25 µM). Morphological changes, which have occurred after 1 h and 2 h, as a consequence of the treatment, were documented with images, taken by light microscopy.

Figure 6 shows microscopic images of Hep3B and HepG2 cells and demonstrates that all of the used compounds lead to morphological changes such as cell shrinking and development of long branches in the periphery of the cells 2 h after treatment in both Hep3B and HepG2 cells in comparison to the untreated controls. Furthermore, with the increasing concentrations a formation of apoptotic bodies was also observed. Likewise, the above described morphological changes could be detected even 1 h after treatments begin in both hepatocellular carcinoma cell lines. All of the used compounds showed an impact in comparison to the untreated controls. At the same time, the effect of the substances at this time point was weaker as that after 2 h incubation.

To conclude, usage of actin-modulating compounds on Hep3B and HepG2 resulted in morphological changes in the sense of branching and cell shrinking. So produced effect became stronger and more obvious with the increasing time points and concentrations of the used substances.

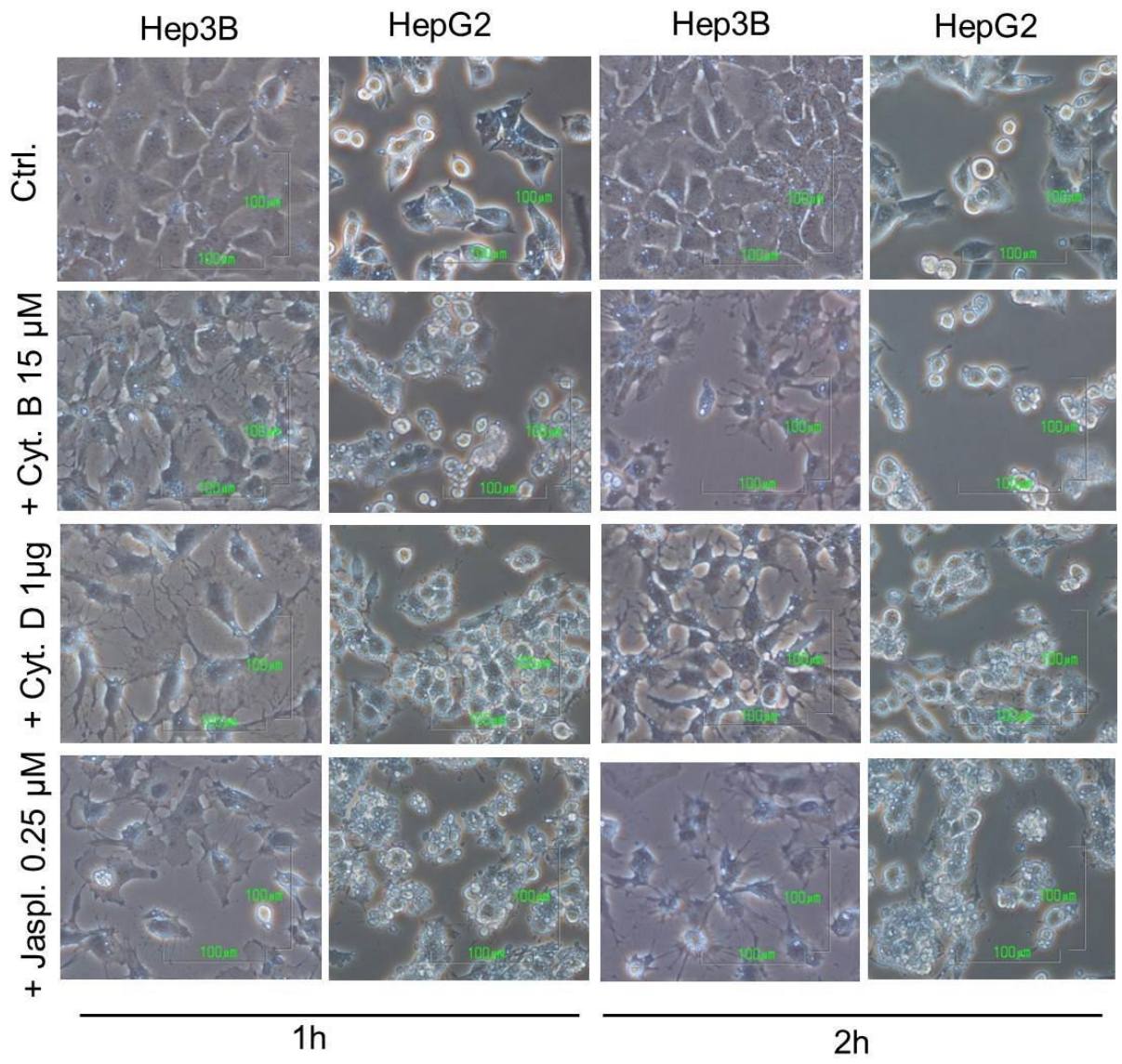


**Figure 6: Microscopic images of Hep3B and HepG2.** Cells are treated with cytochalasin B (15 µM), cytochalasin D (1 µg) and jasplakinolide (0.25 µM) for 1h and 2 h. Morphological changes such as branch formation and cell shrinking can be observed.

### 3.3. Effect of actin-modulating compounds on the viability of Hep3B cells

In order to investigate whether the used actin-modulating compounds are cytotoxic for cells, a MTT assay was performed. Hep3B cells were seeded and on the following day treated with the inducer of actin depolymerization cytochalasin B and the inducer of actin polymerization jasplakinolide at increasing concentrations for 15 min, 120 min and with cytochalasin B also for 24 h. For cytochalasin B were chosen the following concentrations: 1 µM, 2 µM, 5 µM, 10 µM

and 15 µM and for jasplakinolide the following ones: 0.025 µM, 0.05 µM, 0.10 µM, 0.15 µM and 0.25 µM. Untreated cells and cells treated with 0.1 % DMSO served as controls. Optical density was measured with the help of a microplate reader at wavelength of 560 nm.

Figure 7 shows the quantification of Hep3B proliferation and viability after treatment with actin-modulating compounds. The figure demonstrates that stimulation with cytochalasin B does not produce a cytotoxic effect on Hep3B cells after 15 min (A) and 120 min (B) incubation, while 24 h (C) treatment results in cytotoxicity in comparison to the controls. As it can be seen, the more concentrations increase, the more severe and stronger is the produced effect. Despite this, the toxic effect became statistically significant not until 24 h. In the same time, treatment with jasplakinolide does not influence the viability of Hep3B after 15 min (D) despite the increasing concentration. After 120 min (E) incubation time jasplakinolide again does not demonstrate an effect on the viability of Hep3B cells. Regardless of the variation in the used concentrations, experiment did not result in differences in the impact on the cells.

To conclude, usage of cytochalasin B did not produce a significant cytotoxic effect on Hep3B after 15 min and 120 min, but did so after 24 h. On the other hand, treatment with jasplakinolide did not affect the viability of the cells at any of the analyzed time points.

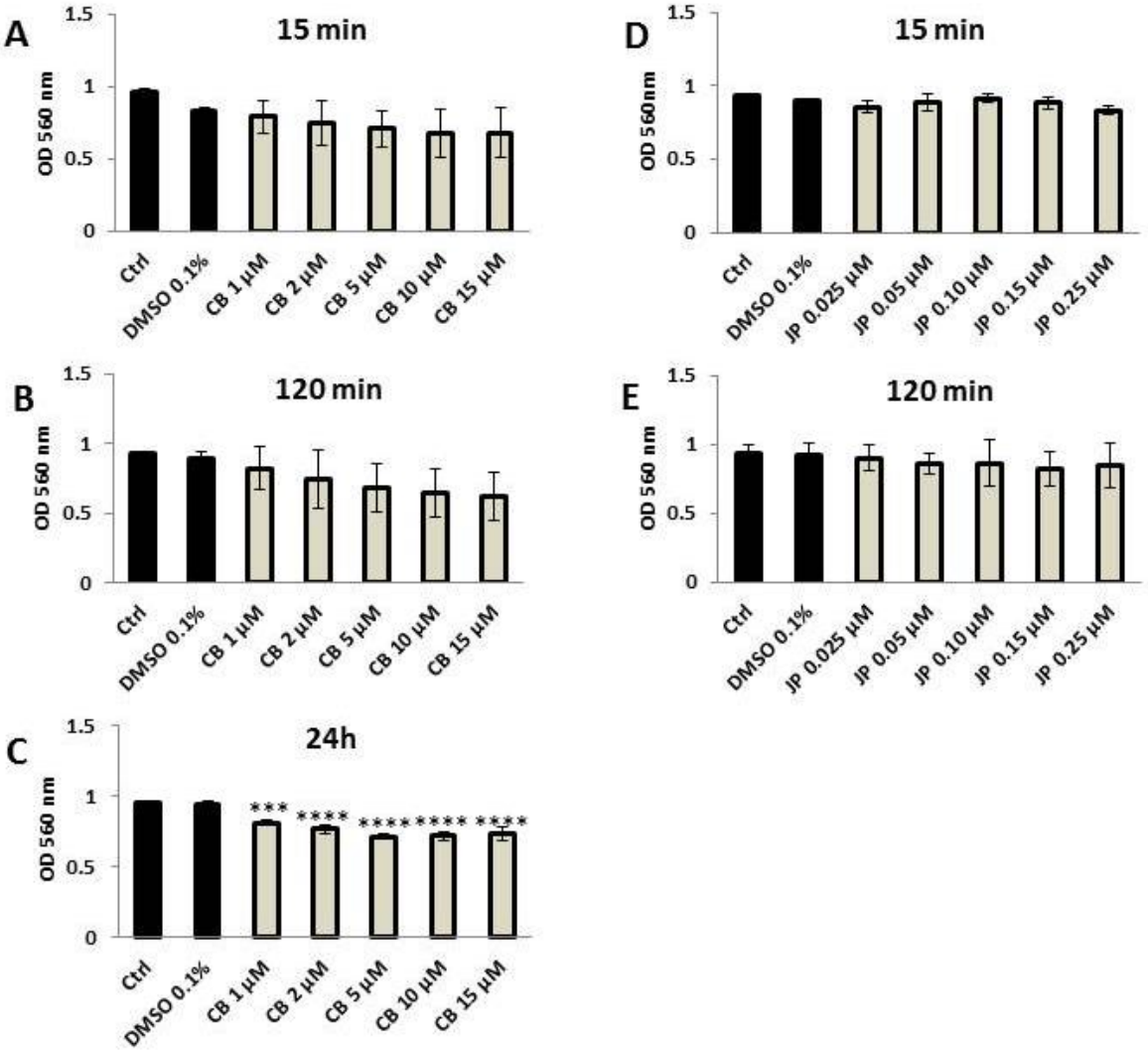


**Figure 7: MTT assay of Hep3B cells.** Cells were stimulated with cytochalasin B and jasplakinolide at increasing concentrations and the optical density was read after 15 min, 120 min and 24 h. Cytochalasin B does not produce a significant cytotoxic effect in comparison to the controls on Hep3B after **(A)** 15 min and **(B)** 120min, but does it after **(C)** 24 h. Jasplakinolide does lead to cytotoxicity in contrast to the controls neither after **(D)** 15 min nor after **(E)** 120 min incubation. Data are shown as means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons test (all treated samples were compared to the DMSO-treated cells; ***: $p < 0.001$; ****: $p < 0.001$; all other samples were not significantly different). CB: cytochalasin B; CD: cytochalasin D; JP: jasplakinolide.

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### 3.4. Effect of actin modulating compounds on the NF-κB signaling in Hep3B and HCT116 cell lines

The purpose of the following experiments was to support the hypothesis that reorganization of actin cytoskeleton, caused by usage of actin-modulating compounds, leads to activation of NF-κB transcription factor in Hep3B and HCT116 cells.

For this aim Hep3B and HCT116 were seeded and on the following day stimulated with actin-modulating compounds at increasing concentrations for 15 min or 120 min. The chosen concentrations were the following ones for the inducer of actin depolymerization cytochalasin B: 1 µM, 2 µM, 5 µM, 10 µM and 15 µM and the following ones for the inducer of actin polymerization jasplakinolide: 0.025 µM, 0.05 µM, 0.10 µM, 0.15 µM and 0.25 µM. As controls were used untreated cells and cells, stimulated with 0.1 % DMSO. As positive control cells were treated for 30 min with TNFα (100 U/ml). Phosphorylated and unphosphorylated components of the NF-κB system such as IκBα, p65 and RelB were quantified using western blot of total cell lysates. Interestingly, the signal of Phospho (P)-IκB was detectable neither after 15 min nor after 120 min stimulation, consequently, signals of Phospho (P)-p65 and IκBα were normalized to those of p65 and GAPDH respectively. Additionally to IκBα and p65, the expression of RelB was also quantified by HCT116 cell line. Similarly to P-IκBα, detection of Phospho (P)-RelB was not successful. Therefore RelB was normalized to GAPDH.

Figure 8 presents the effect of 15 min stimulation with cytochalasin B and jasplakinolide on activation of NF-κB system in Hep3B cells. Figure 8A is a representative western blot image of experiments made with cytochalasin B and compares the effect of the used compound with different controls. From the figure can be obtained, that only stimulation with TNFα leads to the expected increase in phosphorylation of p65 and decrease in amount of IκBα. Figures 8B and 8C present the quantification of expression of the NF-κB system components P-p65 and IκBα in the same experiments. The analyzed data showed that stimulation of cells with cytochalasin B does not lead to phosphorylation of p65 and to decrease in amount of IκBα in contrast to the positive control TNFα. Even the increase of cytochalasin B concentrations did not influence the activation rate of NF-κB.

In Figure 8D, which is a representative western blot image of jasplakinolide experiments, the expected increase in phosphorylation of p65 can be seen again only by the positive control TNFα. In comparison, stimulation with jasplakinolide does not have an effect on the activation of p65. Figures 8E and 8F present the quantified expression of P-p65 and IκBα. From this data can be obtained that not only jasplakinolide but also TNFα does not lead to the expected decrease in IκBα amount. As well as by the cytochalasin experiments, the increasing of jasplakinolide concentration does not show an impact on the activation of NF-κB.

To conclude, both of the used actin-modulating compounds did not lead to phosphorylation of p65 and to decrease of IκBα amount in Hep3B after 15 min stimulation. Even the increasing concentrations did not produce an effect.

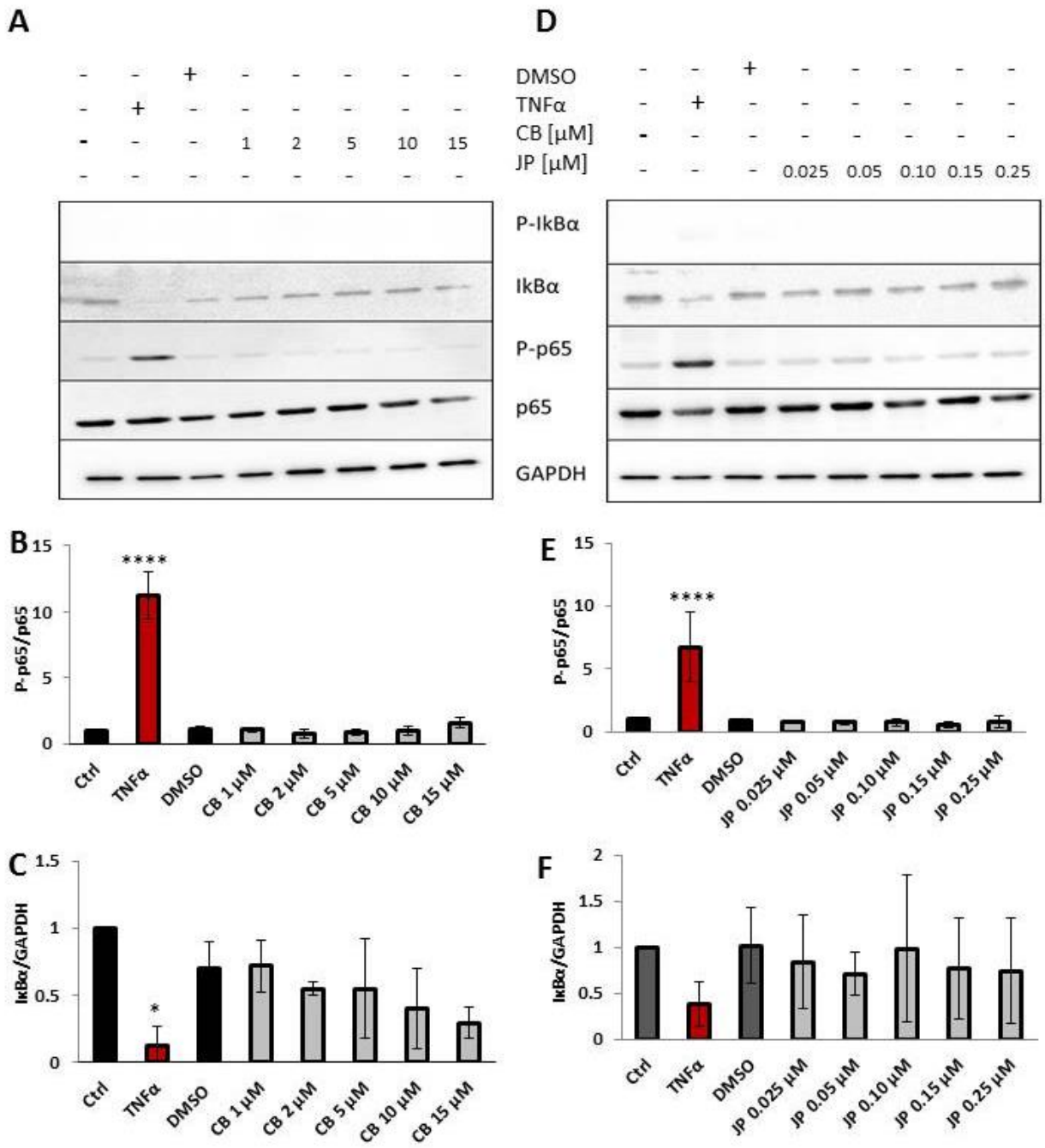


**Figure 8: Short-term perturbation of the actin cytoskeleton does not activate NF-κB in Hep3B.** Stimulation of Hep3B with TNFα and cytochalasin B at increasing concentrations for 15 min. **(A)** Representative immunoblots for P-IκBα, IκBα P-p65, p65 and GAPDH. Quantification of expression of **(B)** P-p65/p65 and **(C)** IκBα/GAPDH is shown. Data are shown as means ± SD of three independent experiments. **(D- F)** The experiments were performed as described for panels A-C, but cells were pre-incubated with different amounts of jasplakinolide instead of CB. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons test (all treated samples were compared to the DMSO-treated cells; *: $p < 0.05$; ****: $p < 0.001$; all other samples were not significantly different). CB: cytochalasin B; JP: jasplakinolide.

Figure 9 presents the results of experiments, where Hep3B cells were stimulated with actin-modulating compounds cytochalasin B and jasplakinolide for 120 min. Figure 9A is a representative western blot image and compares the impact of cell stimulation with cytochalasin B at increasing concentration with different controls. Figures 9B and 9C present the analyzed data of the conducted experiments. Here can be obtained that only stimulation with TNFα causes the expected phosphorylation of p65, whereas treatment with the inducer of actin depolymerization does not influence the activation of NF-κB. Moreover, increasing concentrations also do not have an impact. Interestingly, both cytochalasin B and TNFα do not lead to decrease in IκBα amounts.

In Figure 9D, which is a representative western blot image of Hep3B stimulation with jasplakinolide at increasing concentrations, can be seen that treatment with TNFα activates p65 in contrast to the treatment with the compound. Similarly to the above described experiments, the increasing concentrations of the substance do not influence the phosphorylation state of p65. As expected, stimulation of the cells with TNFα leads to decrease in IκBα expression. Interestingly, a significant reduction of IκBα expression can also be detected by treatment of the cells with 0.25 µM jasplakinolide, although the effect was still much smaller compared to TNFα. Despite this sporadic result, it could be concluded that manipulation of the actin cytoskeleton do not affect the activation of the transcription factor. Figures 9E and 9F present the expression of P-p65 and IκBα and support the in western blot images made observations.

To sum up, treatment of Hep3B cells with both used substances for 120 min did not induce the phosphorylation of p65 and the decrease of IκBα in comparison to the positive control. Despite increasing concentrations, by both compounds activation rate of NF-κB stayed unaffected and equal to that of the untreated and with DMSO treated cells.

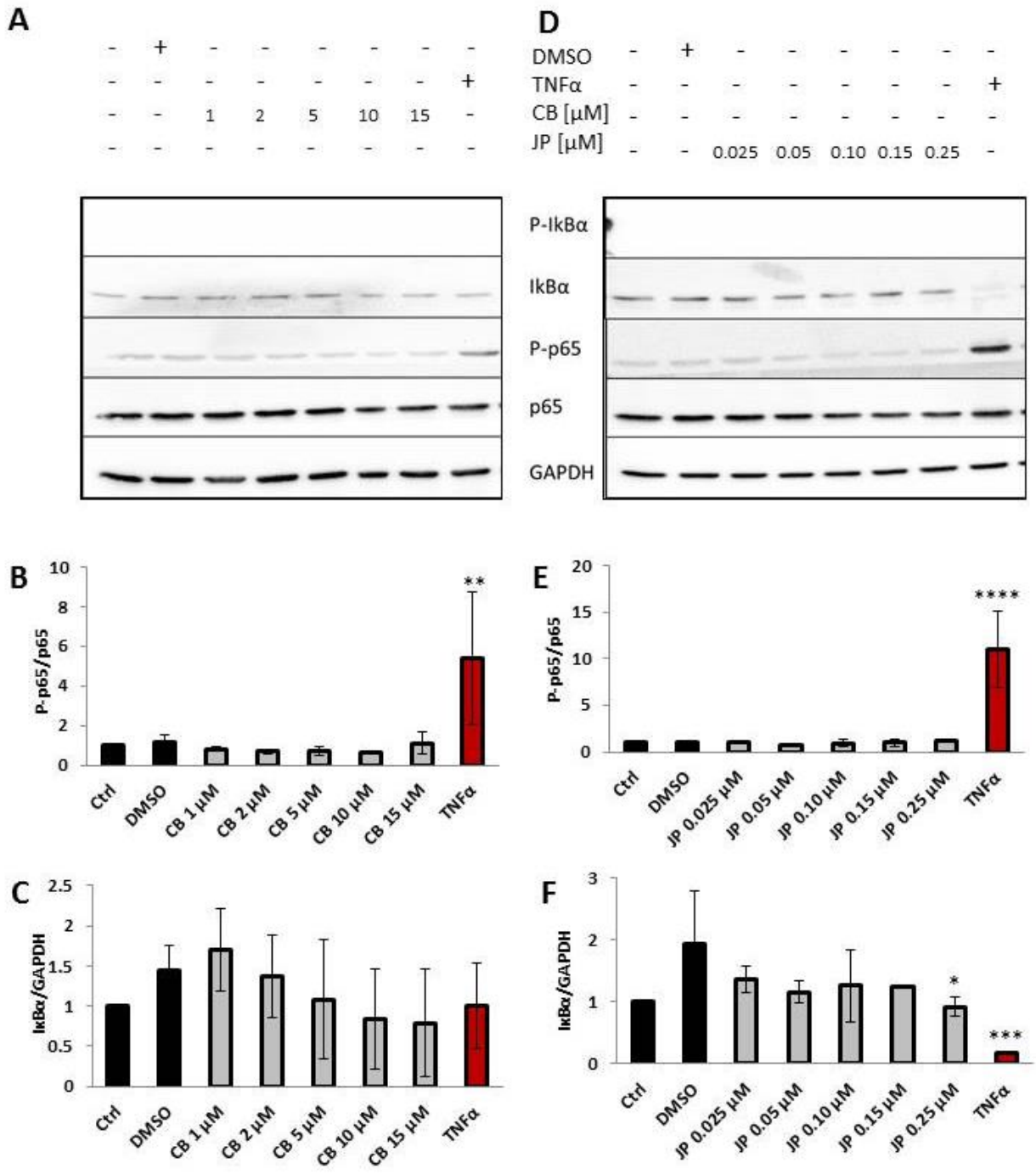


**Figure 9: Long-term perturbation of the actin cytoskeleton does not activate NF-κB in Hep3B.** Stimulation of Hep3B with TNFα and cytochalasin B at increasing concentrations for 120 min. **(A)** Representative immunoblots for P-IκBα, IκBα P-p65, p65 and GAPDH. Quantification of expression of **(B)** P-p65/p65 and **(C)** IκBα/GAPDH is shown. Data are shown as means ± SD of three independent experiments. **(D- F)** The experiments were performed as described for panels A-C, but cells were pre-incubated with different amounts of jasplakinolide instead of CB. Data are shown as means ± SD of two independent experiments. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons test (all treated samples were compared to the DMSO-treated cells; **: $p < 0.01$;

***: $p < 0.001$; ****: $p < 0.001$; all other samples were not significantly different). CB: cytochalasin B; JP: jasplakinolide.

Figure 10 illustrates results of HCT116 stimulation with cytochalasin B for 15 min and 120 min. Figure 10A is a representative western blot image, which compares the effect of the 15 min cytochalasin B stimulation on HCT116 cells with different controls. From this figure can be obtained that only TNFα control leads to phosphorylation of p65, whereas, the usage of inhibitor of actin polymerization does not influence the activation rate of NF-κB. Figure 10B shows that treatment with the actin-modulating compound does not affect the activation of RelB in both controls and cells, treated with the substance. Figures 10C and 10D, which present the expression of P-p65 and IκBα, demonstrate once again that only TNFα control leads to alteration in the phosphorylation of p65, whereas the usage of the compound does not have an influence. Even the increase of cytochalasin B concentrations does not produce an effect. On the other hand, stimulation with TNFα does not result in drastically reduction of IκBα as expected.

A representative western blot image of HCT116 stimulation with cytochalasin B for 120 min is shown in Figure 10D. From it can be obtained that in comparison to the positive control TNFα, the inhibitor of actin polymerization does not activate p65 and thus NF-κB transcription factor. Figure 10E demonstrates that the expression of RelB stays unaffected by both TNFα and cytochalasin B. Figure 10G and 10H also illustrate that HCT116 treatment with increasing concentrations of the actin-modulating compound does not influence the expression of P-p65 and IκBα. Similarly to 15 min experiments, here also cannot be seen the expected decrease in IκBα amount by stimulation with TNFα.

To conclude, stimulation of HCT116 cells with the inhibitor of actin polymerization cytochalasin B did not lead to phosphorylation of p65 in contrast to the positive control TNFα at both investigated time points. Moreover, decrease in amount of IκBα and RelB could be observed neither after 15 min nor after 120 min. Despite the increasing concentrations of the substance, the activation of NF-κB stayed unaffected.

In summary, manipulation of actin cytoskeleton with cytochalasin B and jasplakinolide did not lead to activation of NF-κB system in contrast to TNFα in both Hep3B and HCT116. Even increasing concentrations did not activate NF-κB after 15 min or after 120 min.

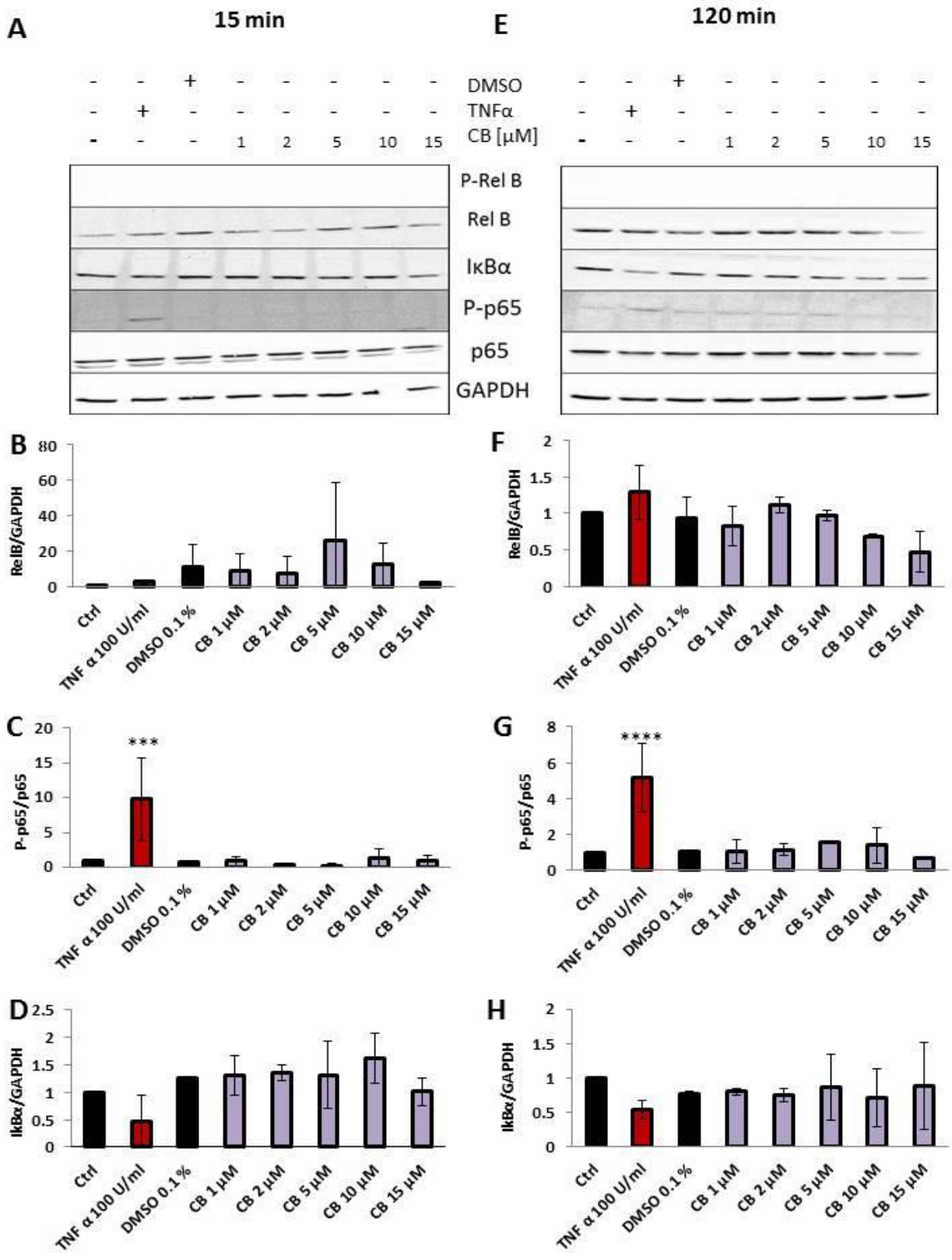


**Figure 10: Short- and long-term perturbation of actin cytoskeleton does not activate NF-κB in HCT116 cells.** Stimulation of HCT116 cells with TNFα and cytochalasin B at increasing concentrations

for **(A-D)** 15 min and **(E-H)** 120 min**. (A, E)** Representative immunoblots for P-Rel, RelB, P-IκBα, IκBα P-p65, p65 and GAPDH. Quantification of expression of **(B, F)** RelB/GAPDH, **(C, G)** P-p65/p65 and **(D, H)** IκBα/GAPDH is shown. Data are shown as means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons test (all treated samples were compared to the DMSO-treated cells; ***: $p < 0.001$; ****: $p < 0.001$; all other samples were not significantly different). CB: cytochalasin B.

## 3.5. Effect of actin-modulating compounds on the IL-6 signaling in Hep3B and HepG2 cell lines

### 3.5.1. Effect of actin-modulating compounds on sIL-6R production

The aim of the following experiment was to investigate whether manipulation of actin cytoskeleton with inducers of actin depolymerization leads to changes in production of sIL-6R in both used cell lines.

Hep3B and HepG2 cells were both seeded in 6-well plates and on the following day cells were washed with serum free medium and serum free DMEM was added. After that cells were pretreated with the inhibitor of actin polymerization cytochalasin B (15 µM) in duplicate for 120 min. 60 minutes after stimulation begin half of the cell samples were stimulated with the ADAM10 inducer ionomycin (1 µM) and the rest of them with DMSO (0.1%) as a control. After 120 min, supernatants were collected and later analyzed by ELISA.

Figure 11A presents the results of experiments done with Hep3B. The quantification of sIL-6R amount in the supernatant demonstrates that stimulation of the cells with ionomycin does lead to increase in the production of sIL-6R in Hep3B. Nevertheless, statistically significant differences in the sIL-6R amounts cannot be observed between the cells treated with cytochalasin B and those with DMSO. From Figure 11B, which shows the results of HepG2 stimulation with the inducers of actin depolymerization, can be obtained that treatment with ionomycin also results in the expected increase of sIL-6R amount in the supernatant in contrast to untreated cells. On the other hand, usage of cytochalasin B does not lead to change in production of sIL-6R in HepG2 in comparison to DMSO control.

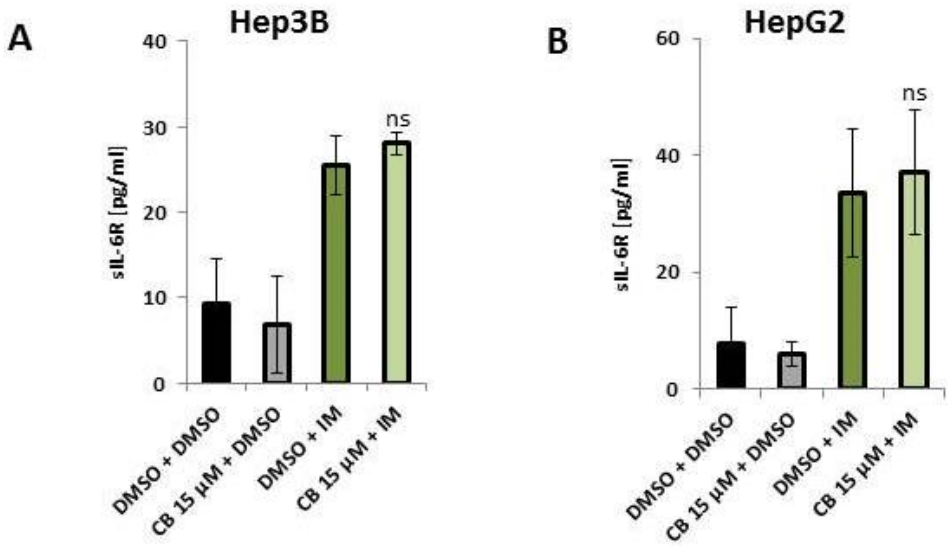


**Figure 11: Perturbation of the actin cytoskeleton with cytochalasin B does not influence IL-6R proteolysis by ADAM10**. **(A, B)** ELISA quantification of sIL-6R in culture supernatant after pretreatment of **(A)** Hep3B and **(B)** HepG2 cells with cytochalasin B (15 µM) for 120 min. Proteolysis by ADAM10 was activated via stimulation with ionomycin (1 µM) for 60 min. Data are shown as means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA with Sidaks's multiple comparisons test (cells pre-treated with actin-modulating compounds and stimulated with ionomycin were compared to ionomycin-stimulated cells only); ns = no significant difference; CB: cytochalasin B; IM: ionomycin.

In order to compare the influence of compounds with opposite effect on actin cytoskeleton on production of sIL-6R, the above described experiment was repeated with other substances. Here were compared the inhibitor of actin polymerization cytochalasin D (1 µg) and the inducer of actin polymerization jasplakinolide (0.25 µM).

Figure 12A introduces the results of Hep3B experiments and makes a comparison between the effect caused from the both used compounds and the control. Here once again is demonstrated that stimulation with ionomycin induces the proteolysis of IL-6R by ADAM10. Nonetheless, usage of cytochalasin D and jasplakinolide does not influence the production of sIL-6R. The quantification of sIL-6R by HepG2 is shown in Figure 12B. As expected, treatment with ionomycin leads to increase in the amount of sIL-6R in the supernatant. Likewise to the Hep3B experiments, here also cannot be seen a statistically significant difference in the impact of both substances on the synthesis of sIL-6R.

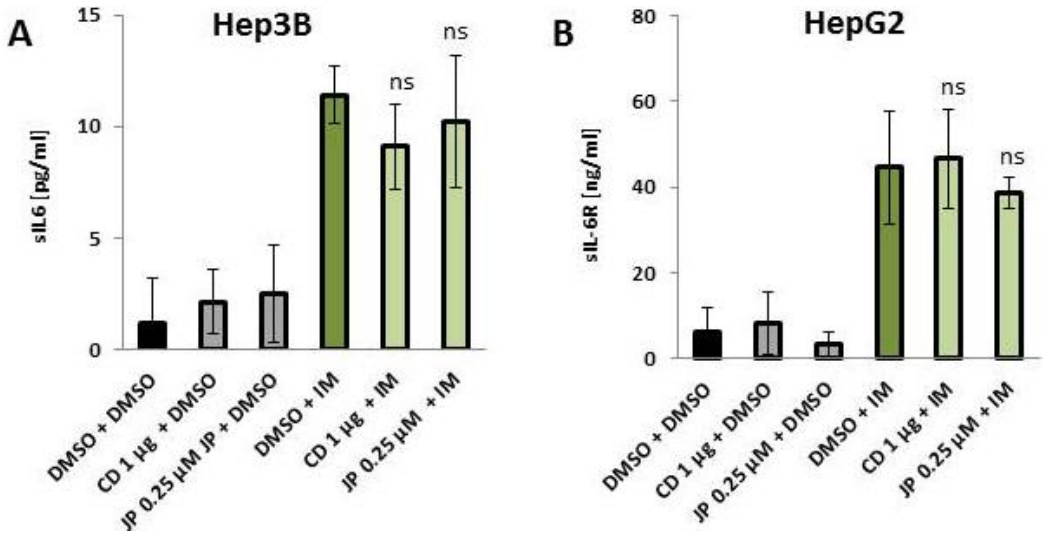


**Figure 12: Perturbation of the actin cytoskeleton with actin-modulating compounds does not influence IL-6R proteolysis by ADAM10**. **(A, B)** ELISA quantification of sIL-6R in culture supernatant after pretreatment of **(A)** Hep3B and **(B)** HepG2 cells with cytochalasin D (1 µg) and jasplakinolide (0.25µM) for 120 min. Proteolysis by ADAM10 was activated via stimulation with ionomycin (1 µM) for 60 min. Data are shown as means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA with Sidaks's multiple comparisons test (cells pre-treated with actin-modulating compounds and stimulated with ionomycin were compared to ionomycin-stimulated cells only); ns = no significant difference; CD: cytochalasin D; JP: jasplakinolide; IM: ionomycin.

To sum up, reorganization of actin cytoskeleton, caused by actin-modulating compounds such as cytochalasins and jasplakinolide, did not influence in any way the proteolysis of IL-6R by ADAM10 in both Hep3B and HepG2.

### 3.5.2. Effect of actin-modulating compounds on the activation of STAT3 by IL-6

Aim of the following experiment was to investigate, whether manipulation of actin cytoskeleton with inhibitors of actin polymerization would lead to changes in activation of IL-6 signaling, namely phosphorylation of STAT3. The amounts of STAT3 and its phosphorylated form were quantified and the ratio between them was calculated.

Hep3B and HepG2 cells were seeded in 6-well plates and on the following day washed with serum free DMEM and as well fresh serum free medium was added. Afterwards cells were stimulated for 120 minutes with DMSO (0.1 %) or cytochalasin B (15 µM). All substances were added in duplicates. Plate with treated cells was left in the incubator and 15 min before cell

harvesting IL-6 (10 ng/ml) was added in only three of plate wells. Cells, which were left untreated with IL-6, served as controls. Total cell lysates were analyzed with western blotting.

As we can see in the western blot image in Figure 13A, stimulation of Hep3B cells with IL-6 leads to phosphorylation of STAT3 in comparison to the unstimulated controls. Figure 13B presents the quantification of STAT3 activation. Interestingly, Hep3B pretreatment with cytochalasin B results in a reduction of STAT3 phosphorylation in comparison to the DMSO control.

Figure 13C is a representative western blot image and shows the results of HepG2 stimulation with the inducer of actin depolymerization. This figure demonstrates that treatment of HepG2 cells with IL-6 results in phosphorylation and thus to activation of STAT3. On the other hand, as we can obtain from Figure 13D, which shows the analyzed data of HepG2 experiments, manipulation of actin cytoskeleton with cytochalasin B does not have an effect on the activation of STAT3 after 120 min incubation.

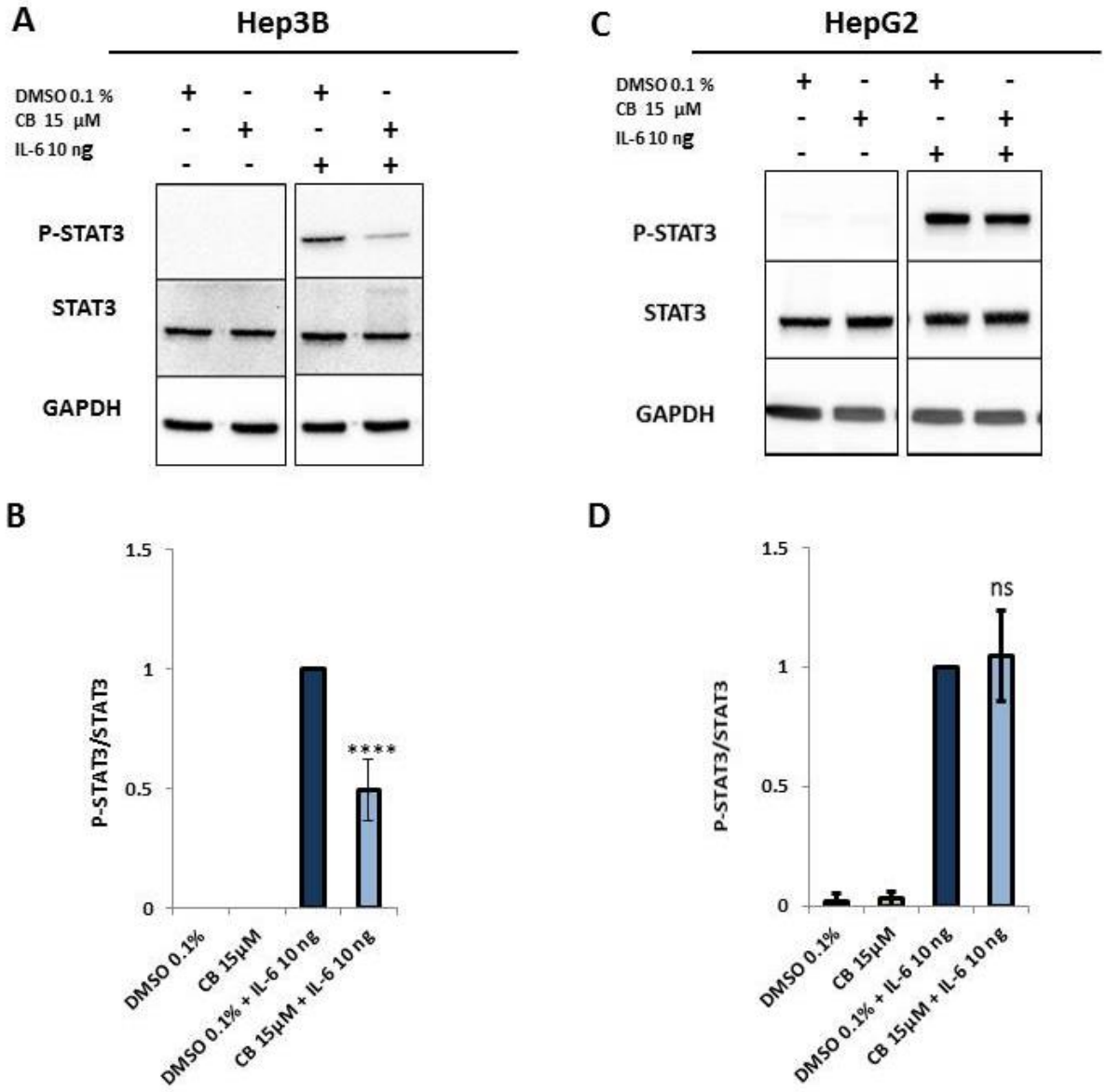


**Figure 13: Perturbation of the actin cytoskeleton with cytochalasin B interferes with STAT3 signaling in Hep3B cells, but not in HepG2 cells. (A, B)** Hep3B and **(C, D)** HepG2 cells were pretreated with cytochalasin B (15µM) or DMSO (0. 1 %) as a control for 120 min. IL-6 (10 ng/ml) was added for 15 min in order to activate STAT3 signaling. **(A, C)** Representative western blot images from one experiment are shown. **(B, D)** The quantitative data are shown as means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA with Sidaks's multiple comparisons test (cells pre-treated with actin-modulating compounds and stimulated with IL-6 were compared to IL-6-stimulated cells only); ****: $p < 0.001$; ns = no significant difference; CB: cytochalasin B**.**

With the aim to compare the effect of compounds with opposite impact on the actin cytoskeleton, the above described experiment was performed with other substances. Therefore next to the 0.1% DMSO control here were used 1 µg cytochalasin D as inhibitor of actin polymerization and 0.25 µM jasplakinolide as inducer of actin polymerization. Again IL-6 (10 ng/ml) was used to activate STAT3 and added 15 min before cell lysis.

Figure 14A, which is a representative western blot image of experiments done with Hep3B, and Figure 14B, which presents the quantification of the STAT3 activation in the same experiments, demonstrates that treatment with IL-6 results in phosphorylation of STAT3 in the stimulated cells in comparison to the untreated controls. Furthermore, it can be seen that the usage of actin-modulating substances for 120 min in Hep3B leads to a half reduction in activation rate of STAT3 in contrast to DMSO control. In addition, the effect produced from jasplakinolide is stronger than that of cytochalasin D.

In the meantime, Figures 14C and 14D, which are representative western blot image of HepG2 experiments and a graph with analyzed data from the same ones, illustrate the lack of impact of both used substances on the phosphorylation of STAT3 after 120 min incubation period. As expected, the activation of STAT3 after IL-6 treatment can be observed as well.

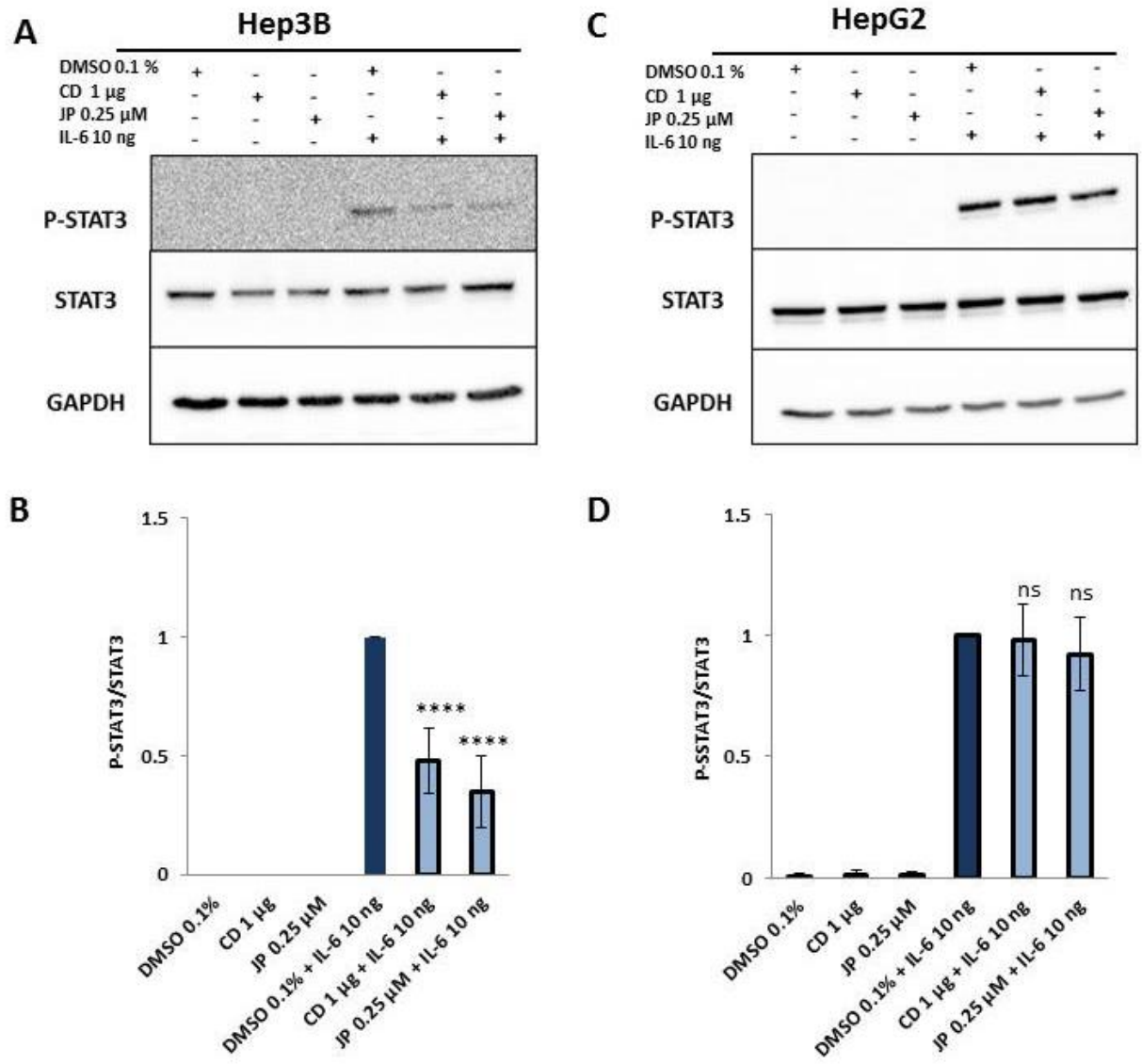


**Figure 14: Perturbation of the actin cytoskeleton with actin-modulating compounds interferes with STAT3 signaling in Hep3B cells, but not in HepG2 cells. (A, B)** Hep3B and **(C, D)** HepG2 cells were pretreated with cytochalasin D (1µg), jasplakinolide (0.025 µM) or DMSO (0.1%) as a control for 120 min. IL-6 (10 ng/ml) was added for 15min in order to activate STAT3 signaling. **(A, C)** Representative western blot images from one experiment are shown**. (B, D)** The quantitative data are shown as means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA with Sidaks's multiple comparisons test (cells pre-treated with actin-modulating compounds and stimulated with IL-6 were compared to IL-6-stimulated cells only; ****: $p < 0.001$; ns = no significant difference; CD: cytochalasin D; JP: jasplakinolide.

To conclude, the manipulation of actin cytoskeleton with actin-modulating compounds resulted in reduction of STAT3 phosphorylation in Hep3B after 120 min stimulation. Both Cytochalasins

and Jasplakinolide did not influence the activation of IL-6 signaling in HepG2 cell line after 120 min incubation.

### 3.5.3. Effect of actin-modulating compounds on production of IL-6

With the purpose to discover whether manipulation of actin cytoskeleton with inhibitors of actin polymerization would influence in some way the synthesis of IL-6 in the both used hepatocellular carcinoma cell lines, the following experiment was performed.

Hep3B and HepG2 cells were seeded in 6-well plates and on the following day washed with serum free medium and after that serum free DMEM was added to them. Then cells were stimulated with cytochalasin B (15 µM) or DMSO (0.1%) as a control. After 120 min incubation supernatants were collected and then IL-6 amounts in them were quantified with ELISA.

The results of performed experiments with Hep3B are shown in Figure 15A and those of HepG2 in Figure 15B. The analysis of the IL-6 amounts in the supernatants demonstrated that treatment with cytochalasins B does not influence the production of IL-6 in both Hep3B and HepG2 cell lines. After 120 min incubation period detection of IL-6 was impossible not only in DMSO control but also in the cytochalasin samples.

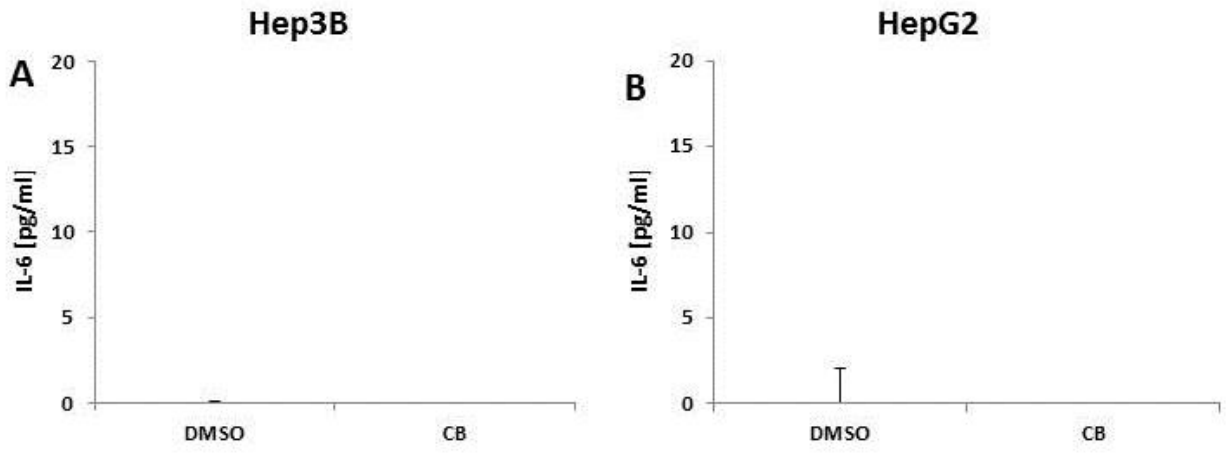


**Figure 15: Perturbation of actin cytoskeleton with cytochalasin B does not affect the production of IL-6.** ELISA quantification of IL-6 amount in culture supernatant after pretreatment of (**A)** Hep3B and **(B)** HepG2cells with cytochalasin B (15 µM) for 120 min. Data are shown as means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons test and revealed no differences. CB: cytochalasin B.

Accordingly, an experiment, which compares the effect of actin polymerization inhibitor and inducer, was performed. Likewise, cells were stimulated with cytochalasin D (1µg), jasplakinolide (0.25 µM) and DMSO (0.1%) as a control and after 120 min supernatants were collected.

Figure 16A presents the quantification of IL-6 amount in Hep3B experiments and Figure 16B that in HepG2 ones. Similarly to results of above described experiment, manipulation of actin cytoskeleton with both cytochalasin D and jasplakinolide did not lead to change in synthesis of IL-6 in both cell lines. Here detection of the cytokine was again not possible in both control and actin-modulating compounds samples.

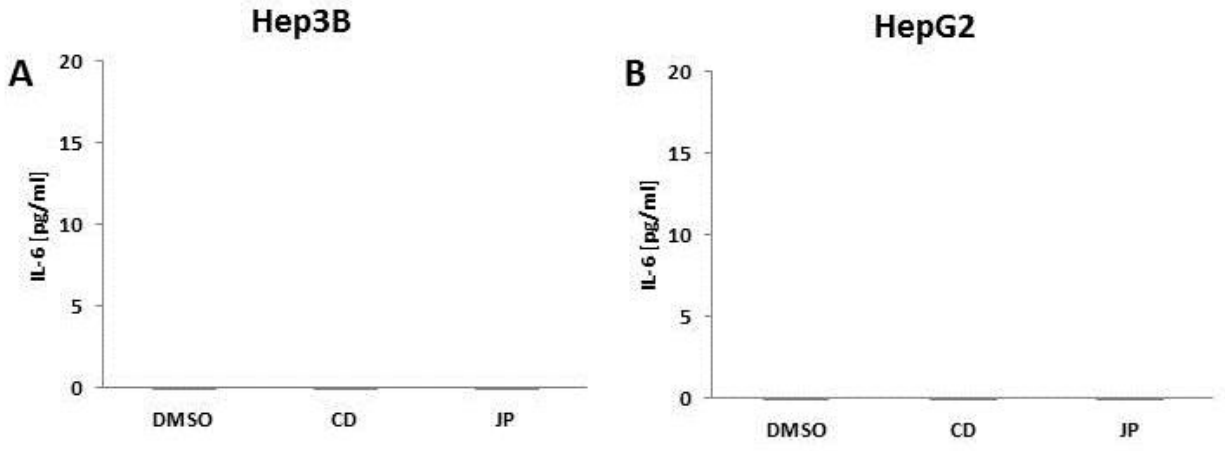


**Figure 16: Perturbation of actin cytoskeleton with actin-modulating compounds does not affect the production of IL-6.** ELISA quantification of IL-6 amount in culture supernatant after pretreatment of **(A)** Hep3B and **(B)** HepG2 cells with cytochalasin D (1 µg) and jasplakinolide (0.25 µM) for 120 min. Data present means ± SD of three independent experiments. Data are shown as means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons test and revealed no differences. CD: cytochalasin D; JP: jasplakinolide.

In summary, reorganization of actin cytoskeleton, induced from actin-modulating compounds such as cytochalasin B and D and jasplakinolide, did not influence in any way the synthesis of IL-6 in Hep3B and HepG2 cells after 120 min treatment. Furthermore, the mechanism of action of the used substances appeared to not have an impact on the process of IL-6 production.

# 4. Discussion

## 4.1. NF-κB, IL-6 and the actin cytoskeleton

Are and colleagues demonstrated that p65 and actin colocalize and that p65 physically interacts with actin-containing structures in the cytoplasm of normal rat fibroblasts (Are et al. 2000). Moreover, they found that p65 concentrates in the places of the focal contacts and along the length of stress fibers (Are et al. 2000). In addition, the group of Babakov showed that p65 colocalizes with α-actinin isoforms in the human epidermoid carcinoma cell lines A431 (Babakov et al. 2008). Rosette and Karin suggested that actin reorganization mediates its effect by a change in the cell shape (Rosette & Karin 1995). At the same time this tandem reported that changes in the cell shape also lead to activation of NF-kB, possibly through multimerization and clustering of membrane receptors (Rosette & Karin 1995). Furthermore, many working groups have already demonstrated that NF-kB activation and expression of pro-inflammatory cytokines accompany many physiological processes such as phagocytosis (Friedland et al. 2001), cellular adhesion (Reyes-Reyes et al. 2002) and chemotaxis (Huang et al. 2001a).

Based on these reports, we decided to investigate possible connections between actin dynamics and NF-κB signaling in hepatocytes to identify possible intervening points for clinical treatment of diseases. For this aim, we manipulated the actin cytoskeleton with different actin-modulating compounds and then analyzed their influence on the activation of the above mentioned system. Our hypothesis that manipulation of actin cytoskeleton leads to activation of the transcription factor NF-κB was based on previous studies (Németh et al. 2004, Kustermans et al. 2005). In addition, we also analyzed the influence of actin cytoskeleton reorganization on IL-6 signaling, which is closely linked to NF-κB, and the proteolysis of the IL-6R.

## 4.2. Effect of TNFα on NF-κB in Hep3B cells

In the current work stimulation of Hep3B cells with TNFα led to phosphorylation of p65 subunit and thus to activation of NF-κB. The confluence affected neither the cell response to TNFα nor the activity of the transcription factor significantly.

A study from Hellweg and colleagues demonstrated that stimulation of HEK-293 cells with TNFα leads to activation of NF-κB. Culture conditions like growth duration before treatment and cell density were shown to influence the response of the cells to TNFα. The increasing cell

confluence corresponded with the decreasing activation potential of NF-κB (Hellweg et al. 2006).

A decrease in activation of the transcription factor could be observed in our work too. The maximum of the NF-κB activation was reached by stimulation with TNFα 24 h after seeding, followed by a decrease in the activation rate at the other time points (48 h, 72 h). By contrast to the study of Hellweg and colleagues (Hellweg et al. 2006), influence of the culture conditions on the NF-κB activation was not significant. A possible explanation could be the usage of different methods for determination of the NF-κB activity. Whereas for the quantifying of NF-κB activation we applied the technique of western blotting, the group of Hellweg used flow cytometry. In addition, experiments were performed with different cell types. Here, we used the human hepatocellular carcinoma cells Hep3B, while the other group used the human embryonic kidney cells HEK-293 (Hellweg et al. 2006).

A possible mechanism for decreasing activity of NF-κB could be the reorganization of actin cytoskeleton (e.g. formation of gap junctions) or the production of soluble factors, induced by the increasing confluence, which both could diminish the cellular response to TNFα and even directly inhibit NF-κB activation.

In addition to this experiment, we could have investigated the impact of actin-modulating compounds on the TNFα effect on NF-κB. It would be interesting to examine whether TNFα effect could be influenced in hepatocytes via actin cytoskeleton reorganization, knowing the fact that TNFα itself leads to actin cytoskeleton reorganization (Peppelenbosch et al. 1999).

### 4.3. Effect of actin-modulating compounds on the morphology of Hep3B and HepG2

Treatment of Hep3B and HepG2 cells with actin-modulating compounds led to morphological changes in sense of branch formation and cell shrinking. When higher concentrations were used and the cells were longer incubated with the substances, the morphological changes that occurred in both cell lines were stronger and more obvious.

Sanger and Holtzer used cytochalasin B to manipulate actin cytoskeleton in myoblasts, fibroblasts and stellate chondroblasts (Sanger et al. 1971). As a result of the treatment, cells were displayed with a markedly branched or arborized morphology, which was documented with the help of phase-contrast microscopy. In another study, BSC-1 cells (African green monkey kidney cells) were treated with cytochalasin D (Schliwa 1982). A diversity of morphological changes

like inhibition of ruffling, formation of phase-dense aggregates or “foci”, dissolution of stress fibers, cell arborization, rounding up of the perinuclear area were observed (Schliwa 1982). Here, light microscopy and high-voltage electron microscopy were used (Schliwa 1982). Nemeth and colleagues confirmed the actin cytoskeleton disruption trough cytochalasin D via Alexa Fluor 546 phalloidin staining (Németh et al. 2004). Stimulation of porcine ERM (epithelial cell rests of Malassez) cells with jasplakinolide led to shrinking and irregular cytoplasm, as well as to fragmentation of nuclei (Aida et al. 2016). These morphological changes were investigated again by staining with Alexa Fluor 568 phalloidin and observed under a fluorescence microscope.

The observed morphological changes documented in this study correspond well with already published data. Formation of branches and cell shrinking in both hepatocyte cell lines could be due to the reorganization of actin cytoskeleton induced by the applied actin-modulating compounds. For documentation of the morphological changes we used light microscopy, which could be considered as a limitation of the work. Usage of electron microscopy or fluorescence microscopy would have allowed us to document not only the morphological changes, but also to demonstrate the reorganization of the actin cytoskeleton in hepatocytes.

### 4.4. Effect of actin-modulating compounds on the viability of Hep3B cells

Treatment of Hep3B cells with increasing concentrations of the actin-modulating compounds cytochalasin B and jasplakinolide for 15 min and 120 min did not result in cytotoxicity and did not affect the viability of the cells. At the same time, stimulation of Hep3B cells with cytochalasin B for 24 h led to a significant cytotoxic effect.

Stimulation of the human breast cancer cell lines MDA-MB-231 and ZR-75-1 (Chang et al. 2016) and HeLa cells (Hwang et al. 2013) with cytochalasin B for 24 h or 48 h led to significant dose- and time-dependent inhibition of the cell proliferation and a reduction of the cell viability. The examined parameters were determined respectively with WST-1 and WST-8 assays, which represent ready-to-use calorimetric assays for measuring the cell viability, the proliferation and the cytotoxicity in the mammalian cells (Lutter et al. 2017). Another study has examined the effect of cytochalasin D and B on the viability of human intestinal epithelial cells after 18 h treatment. While cytochalasin D caused a slight decrease in MTT levels (5–15%) at all tested concentrations, the usage of cytochalasin B stayed without effect (Németh et al. 2004). The cytotoxic effect of jasplakinolide was investigated on the human leukemia Jurkat T cell line 24 h

or 48 h after treatment. As a consequence, a dose- and time dependent inhibition of cell proliferation and a reduction of the cell viability were observed. Here, the cell proliferation and the cell viability were determined with the help of [$^3$H] thymidine uptake rate and the trypan blue dye exclusion assay, respectively (Odaka et al. 2000).

Results of the MTT assays in the current work correspond with the results of other published studies. By contrast to this work, none of the above mentioned studies has examined the cytotoxic effect of the actin-modulating compounds at the earlier time points after treatment. Our studies showed that the used compounds are not cytotoxic by shorter incubation periods in comparison to the longer ones. These experiments allowed us to determine the appropriate incubation time and the working concentrations for the further investigations so that the used drugs do not have a negative impact on the results, e.g. due to induction of apoptosis or other forms of cell death.

### 4.5. Effect of actin modulating compounds on the NF-κB signaling in Hep3B and HCT116 cell lines

Treatment of Hep3B and HCT116 cells with increasing concentrations of actin-modulating compounds cytochalasin B and jasplakinolide for 15 min and 120 min did not result in phosphorylation of the subunit p65 and thus in activation of NF-κB. Furthermore, neither a decrease in RelB amounts, nor a phosphorylation of RelB in HCT116 cells after stimulation with cytochalasin B could be observed. Even an increase of the concentrations did not affect the activation of the transcription factor.

It was previously reported that disruption of the actin cytoskeleton with cytochalasin D in human intestinal epithelial cell lines (HT-29 and Caco-2) leads to activation of NF-κB and therefore to expression of inflammatory genes like *IL8* (Németh et al. 2004). Furthermore, it was shown that NF-κB activation is IκBα-dependent (Németh et al. 2004), which corresponds to activation of the canonical pathway. Another research demonstrated that actin cytoskeleton reorganization induced by actin-depolymerizing agents such as cytochalasin D and latrunculin B and the actin-polymerizing compound jasplakinolide results in dose-dependent NF-κB activation in myelomonocytic cell lines as well as in human monocytes (Kustermans et al. 2005). Here the IKK-dependency of NF-κB activation was also demonstrated. Stimulation with cytochalasin D led to the cytoplasmic IκBα degradation that began after 30 min and was maximal after 2 h

(Kustermans et al. 2005). By contrast, no significant modification of DNA-binding activities of NF-κB was observed in nuclear extracts of several other cell lines, such as human epithelial cervix carcinoma cells (HeLa), murine fibroblasts (L929), human T lymphocytes (CEM) and human embryonic kidney cells (HEK-293) (Kustermans et al. 2005). Moreover, analysis of nuclear extracts and immunoblotting indicated accumulation of p65, p50 and α-actinin-4 in nuclear extracts of A431 cells 30 min after cell stimulation with cytochalasin D (Babakov et al. 2008). Pretreatment of the cells with cytochalasin D prior to TNF-α-stimulation further increased the nuclear accumulation of p65 (Babakov et al. 2008). On the other hand, Eswarppa and colleagues demonstrated that treatment of murine macrophage-like cells (RAW 264.7) with cytochalasin D or lantrunculin B alone for 1 h does not results in a significant change in the transcriptional activity of NF-κB in comparison to the positive controls (Eswarappa et al. 2008). A study by Fazal and colleagues followed up with actin cytoskeleton-dependent and -independent pathways for p65 nuclear translocation in human umbilical vein endothelial cells (HUVEC) (Fazal et al. 2007). They observed that modulation of the actin cytoskeleton with cytochalasin D and jasplakinolide does not lead to alterations in the NF-κB activity, in the expression of ICAM-1, RelA/p65 DNA binding, IκBα-degradation or the RelA nuclear translocation in comparison to treatment with TNFα (Fazal et al. 2007).

Our results correspond to the majority of the studies already published on this subject (Kustermans et al. 2005, Fazal et al. 2007, Eswarappa et al. 2008). Kustermans et al. 2005 reported mixed results depending on the used cell line whether modulation of the actin cytoskeleton resulted in NF-κB activation, while the study of Németh et al. 2004 does not correspond to our data. This discrepancy could be explained with the usage of different cell lines and methods applied for determination of NF-κB activation. Moreover, variations in the drug concentrations and duration of the treatments could also influence the results.

Activation of NF-κB induced by actin cytoskeleton reorganization was investigated in several cell lines. Interestingly, only a few cell types such as human intestinal epithelial cells (Németh et al. 2004), myelomonocytic cells (Kustermans et al. 2005) and human epidermoid carcinoma cells (Babakov et al. 2008) showed this activation. At the same time, there are a lot of cell lines such as human epithelial cervix carcinoma cells (HeLa), murine fibroblasts (L929), human T lymphocytes (CEM), human embryonic kidney cells (HEK-293) (Kustermans et al. 2005), murine macrophage-like cells (RAW 264.7) (Eswarappa et al. 2008) and human umbilical vein

endothelial cells (HUVEC) (Fazal et al. 2007), in which disruption of the actin cytoskeleton did not lead to activation of NF-κB, comparable to our own results. Thus, it could be speculated that Hep3B and HCT116 belong to a group of cell types in which reorganization of actin cytoskeleton does not affect the transcription activity of NF-κB.

### 4.6. Differentiation of canonical and non-canonical pathway and further considerations

In order to investigate the activation of NF-κB via the canonical pathway, we analyzed the phosphorylation of the p65 subunit and the degradation of IκBα. Moreover, to determine a possible participation of the non-canonical NF-κB signaling pathway, the phosphorylation of the subunit RelB in HCT116 cells was also examined. In previous studies, a luciferase assay and EMSA were used to determine NF-κB activation (Németh et al. 2004, Kustermans et al. 2005, Fazal et al. 2007, Eswarappa et al. 2008). Participating NF-κB subunits were investigated with the help of the supershift assay (Németh et al. 2004, Kustermans et al. 2005) and the degradation of IκBα with immunoblotting (Németh et al. 2004, Fazal et al. 2007, Eswarappa et al. 2008, Babakov et al. 2008). The expression of NF-κB target genes was measured via RT-PCR and ELISA (Németh et al. 2004, Fazal et al. 2007). Hence, it could be speculated that in comparison to the other studies, our methods for investigation of NF-κB activation were limited und could have been complemented with one of the other methods mentioned above.

The lack of phosphorylation of p65 and RelB suggests that neither of the both signaling pathways can be activated after actin cytoskeleton manipulation in Hep3B and HCT116 cells. Possible explanation for the absent signal of phosphorylated IκBα even by the positive control TNFα could be its rapid cytoplasmic degradation. A previous study showed that IκBα degradation in HT-29 cells can be masked by its rapid resynthesis (Jobin et al. 1997). Similar mechanism could hide behind some of our results showing lack of IκBα decrease.

Whereas we defined two fixed time points (15 min and 120 min) for all our experiments, treatment duration chosen by the other groups varied method-dependent. For luciferase assay all of the groups expect one (Eswarappa et al. 2008) stimulated their cells with actin-modulating compounds for 6 h. For EMSA and supershift assay the treatment took between 60 min and 150 min (Németh et al. 2004, Kustermans et al. 2005). IκBα-degradation was analyzed after 60 min (Fazal et al. 2007, Eswarappa et al. 2008) or 120 min (Németh et al. 2004) stimulation.

Expression of NF-κB target genes was measured after 3 h (Németh et al. 2004) or 18 h (Fazal et al. 2007) treatment. Consequently, it could be possible that the stimulation times we chose might not be long enough to induce the process of NF-κB activation.

In the already published studies 0.1 µM - 1 µM were chosen as working concentrations for jasplakinolide (Kustermans et al. 2005, Fazal et al. 2007). Cytochalasin D was applied at a broader spectrum of concentrations (1 µM - 100 µM) (Fazal et al. 2007, Kustermans et al. 2005). Whereas all above mentioned studies used one fixed concentration of the consequent substance, only one group used increasing concentrations like we did (Kustermans et al. 2005). Furthermore, the higher concentrations of the actin-modulating drugs were used, the more obvious and stronger the caused effect was. Cytochalasin D and jasplakinolide had an impact by concentrations higher than 20 µM and 0.25 µM respectively (Kustermans et al. 2005). Therefore, it might be possible that performing our experiments with higher concentrations could have led to different results.

### 4.7. Effect of actin-modulating compounds on sIL-6R production in Hep3B and HepG2

Our data show that manipulation of actin cytoskeleton with cytochalasins and jasplakinolide did not affect the production of sIL-6R by ADAM10-mediated proteolysis in both hepatocellular carcinoma cell lines.

Behind our idea to investigate the impact of actin reorganization on the production of sIL-6R stands the suggestion that the activation of proteases that cleave the IL-6R could be influenced by changes in the cell shape induced by actin dynamics.

It was reported that L-selectin, a leukocyte adhesion molecule, which mediates the process of neutrophils infiltration in to sites of inflammation (Kansas et al. 1993, Pavalko et al. 1995), interacts directly with cytoplasmic actin-binding protein α-actinin (Pavalko et al. 1995). Furthermore, diverse groups have demonstrated that L-selectin is shed mainly by ADAM17 (Li et al. 2006, Wang et al. 2010, Tang et al. 2011, Ager 2012), one of the main sheddases that is responsible for production of sIL-6R. A study of Le Gall and colleagues has demonstrated that ADAM10, another sheddase able to cleave IL-6R, is also responsible for the shedding of L-selectin, but not in the same extent like ADAM17 (Le Gall et al. 2009).

We concluded from the results of our experiments that reorganization of the actin cytoskeleton does not influence the production of sIL-6R in Hep3B and HepG2. Therefore, it appears that there is no connection between actin cytoskeleton reorganization and sIL-6R generation by ADAM10 in these cell lines.

In order to trigger production of sIL-6R we used an inducer of ADAM10, but not an inducer of ADAM17. It would be interesting to investigate, whether performing the experiments with PMA, an inducer of ADAM17, would lead to the same results or to alterations in the amount of produced sIL-6R.

Faure and colleges have demonstrated that mechanical stretch regulates, in frequency dependent manner, the vascular endothelial growth factor-A (VEGF-A) alternative splicing in osteoblastic cells through actin polymerization. (Faure et al. 2008). The study showed that jasplakinolide-induced stress fibers result in an increase of matrix-bound VEGF protein amounts, but not with an effect on soluble VEGF production (Faure et al. 2008). A recent study with different cell lines (Hela, Flp-In™ T-REx™ 293 and U2OS) has linked actin to several steps of transcription, to RNA processing, as well as to the RNA splicing (Viita et al. 2019).

The alternative splicing represents the other possibility to produce sIL-6R. In the current work we did not investigate whether manipulation of actin cytoskeleton would have some impact on this process, which could be considered as a limitation. Further investigation in this direction would also be a subject of interest.

Moreover, cell lines other than hepatocytes could be used in order to see whether this aspect also could have an influence on the outcomes.

### 4.8. Effect of actin-modulating compounds on the activation of STAT3 by IL-6

In the present work, treatment of Hep3B cells with cytochalasins and jasplakinolide for 120 min led to reduction of STAT3 phosphorylation and thus to a decrease in its activation when cells were stimulated with recombinant IL-6. By contrast, stimulation of HepG2 cells with the same compounds did not influence the activation of STAT3.

A possible explanation could be the different origin and characteristics of the used cell lines. It was already demonstrated that there are differences in the intracellular signal cascades, in the

responses to stimuli, in the gene expression and in the drug effects on both cell lines (Qiu et al. 2015). Therefore, it is not surprising that in response to the same drug treatment under the same experimental conditions it could come to different or even opposite outcomes (Qiu et al. 2015). To illustrate, a study from Chiao and colleagues showed that by contrast to HepG2, by Hep3B the transcription factor RelA/NF-κB is constitutively activated (Chiao et al. 2002). Moreover, both cell lines express differently SOCS (suppressor of cytokine signaling), which are inducible STAT inhibitors (Qiu et al. 2015). Difference in the levels of these inhibitors might be the explanation for the different chemoresistance of HepG2 and Hep3B to drug treatments (Kusaba et al. 2007, Fuke et al. 2007). In order to investigate whether the observed alliterations in the IL-6-mediated signaling could be demonstrated in other cell types, experiments with other cell lines able to respond directly to IL-6 like T cells, B cells or monocytes could be performed.

In the current study we have not explored the exact mechanism how actin modulation leads to inhibition of STAT3 phosphorylation. A study from Guo and colleges demonstrated the physically association between actin filaments, STAT3 and JAK2 in A549 cells (Guo et al. 2018). Furthermore, it was shown that this interaction regulates phosphorylation of these proteins via two signaling complexes, namely the IL-6 receptor complex and the focal adhesion complex (Guo et al. 2018). Therefore it could be speculated that pretreatment of the cells with actin-modulating compounds possibly leads to disruption of this interaction and thus to alliterations in the phosphorylation of STAT3.

In this work we have investigated the effect of actin modulation only on STAT3 knowing the fact that it is the most important protein in the downstream cascade of IL-6 signaling. Exploring possible alliterations in other downstream proteins such as JAK, MAPK or PI3 kinase would be also a subject of interest.

Our study demonstrated that both stabilizing and depolymerizing substances result in reduction of STAT3 phosphorylation, which uncovers a previously unknown role of actin dynamics in the IL-6-mediated signaling.

### 4.9.Effect of actin-modulating compounds on production of IL-6

In the present work actin cytoskeleton manipulation with inducers of actin depolymerization cytochalasins B and D and with inducer of actin polymerization jasplakinolide did not affect the production of IL-6 neither by Hep3B nor by HepG2 cells.

## 4 Discussion

Most of the published works have investigated the connection between actin cytoskeleton reorganization and *IL8*, also a NF-κB target gene. By contrast, little is known about the influence of actin cytoskeleton dynamics on IL-6 expression. Nemeth and colleagues demonstrated that disruption of actin cytoskeleton with cytochalasin D results in increased IL-8 production in human intestinal epithelial cells (Németh et al. 2004). A study from Kustermans and colleagues showed an increase of the IL-8 mRNA level and stimulation of its secretion after incubation with 5 µM cytochalasin D in human promyelocytes (HL-60 cells) (Kustermans et al. 2005).

In comparison to the studies about IL-8, our examinations showed that manipulation of the actin cytoskeleton does not influence the IL-6 production in human hepatocellular carcinoma cells. Interestingly, hepatocytes are a cell type for which IL-6 production is not a typical process. It was demonstrated that under certain conditions such as stimulation with LPS, HGF (hepatocyte growth factor) or bacterial hepatotoxin, hepatocytes are capable to produce IL-6 (Norris et al. 2014). Therefore, the lack of IL-6 production after stimulation with actin-modulating compounds was not a surprising result. Due to the fact that IL-6 is produced mainly by immune cells (Norris et al. 2014), a repetition of the experiments with other cell lines, would be meaningful. For instance, Kustermans and colleagues have used for their research monocytes, which represent the major source of IL-8 in blood and are also known to produce IL-6 (Kustermans et al. 2005).

In addition, it could be suggested that conditions affect production of the both cytokines IL-6 and IL-8 differentially. To illustrate, a study from van der Berg demonstrated that disruption of actin cytoskeleton in airway epithelial cells has a different effect on both degradation and translation of IL-6 and IL-8 mRNA (van den Berg et al. 2006). Whereas disrupted actin and microtubule system of the cells led to reduced IL-8 mRNA degradation and increased IL-8 production in response to TNFα, the same process resulted in suppression of the IL-6 production. At the same time IL-6 mRNA was stabilized to the same extent as the IL-8 mRNA (van den Berg et al. 2006).

Usage of different cell lines could also influence the results from experiments and could be possible explanation for the discrepancy between our and already published data. Whereas other groups used human intestinal epithelial cells (Németh et al. 2004) and monocytes (Kustermans et al. 2008), we worked with human hepatocellular carcinoma cells.

Duration of the drug stimulation and diversity of the concentrations could be another possible explanation for the different results. The other groups stimulated their cells longer then us and

then measured IL-8 with the help of ELISA. To illustrate, Nemeth and colleagues stimulated their cells with cytochalasin D for 18 h (Németh et al. 2004), while the Kustermans group for 8 h and 24 h (Kustermans et al. 2008). By contrast, we stimulated our hepatocytes notably shorter – only for 2 h. It could be suggested that these 2 h were not long enough to induce the process of IL-6 production. In the previous studies IL-8 mRNA was analyzed via RT-PCR additionally to ELISA. Duration of the stimulation varied from 30 min to 240 min (Németh et al. 2004, Kustermans et al. 2008). Moreover, we used a lower concentration of cytochalasin D (1 µg) in comparison to the Kustermans group (5 µM) (Kustermans et al. 2008). Experiments with higher concentration of the substances could be done in addition to the already performed ones in order to examine whether the used compounds could have a dose dependent effect.

Last but not least, the tight link between IL-6 and NF-κB should also be considered by the analysis of the current results. Our previous experiments showed that reorganization of actin cytoskeleton via diverse actin-modulating compounds did not lead to activation of NF-κB in Hep3B. Knowing the fact that *IL6* is NF-κB target gene, it could be expected that by lack of NF-κB activation in the earlier experiments the synthesis of IL-6 would stay unaffected in the current ones too. Therefore, the absent NF-κB activation seems to be the most reasonable and meaningful explanation for the unaffected IL-6 production in our experiments.

### 4.10. Outlook

Several studies showed that disruption of the microtubule system results in NF-κB activation (Rosette & Karin 1995, Bourgarel-Rey et al. 2001). Over the past few years, induction of the transcription factor activity after actin cytoskeleton reorganization was also demonstrated (Németh et al. 2004, Kustermans et al. 2005). In the current work, we could not support the hypothesis that reorganization of actin cytoskeleton activates NF-κB and IL-6 in human hepatocellular carcinoma cells. Therefore, it is possible that NF-κB and IL-6 behave differentially depending on the used cell type and stimulus. The exact mechanism of NF-κB activation after actin cytoskeleton disruption is still unclear and further examinations with other cell lines are required.

In the last years, several reports have proven the connection between Rho family of small GTPases, which are critical regulators of actin cytoskeleton reorganization, and NF-κB (Perona et al. 1997, Montaner et al. 1998, Montaner et al. 1999). It was demonstrated that the family

members RhoA, Cdc42 and Rac-1 induce transcriptional activity of NF-κB by phosphorylation of IκBα and translocation of dimers to the nucleus (Perona et al. 1997). Furthermore, it was shown that the Rho GTPases can play a positive or a negative regulatory role in the activation of NF-κB depending on the context (Tong & Tergaonkar 2014). For instance, in hepatocellular carcinoma cells (HepG2 cells) the family members RhoA and Rac activate NF-κB via phosphorylation of p65, via association of p65 with NIK or through nuclear p52 processing (Choudhary et al. 2007). On the other hand, in fibroblasts (NIH3T3 cells) RohB represses NF-κB signaling due to IκBα targeting (Fritz & Kaina 2001). Moreover, it was demonstrated that NF-κB activation through RhoA is involved in the LPS-induced IL-8 production in human cervical stromal cells (Shimizu et al. 2007). Interestingly, there are some publications which showed the relation between NF-κB, inflammation, cancer and the small GTPases (Benitah et al. 2004). The simultaneous overexpression of GTPases and NF-κB was demonstrated by many human tumor types such as melanoma (Hodge et al. 2003), gastric (Benitah et al. 2003), colorectal (Benitah et al. 2003) and prostate cancer (Hodge et al. 2003). Studies have shown that the small GTPases modulate not only the activity of NF-κB, but also this of STATs (Aznar & Lacal 2001). It was reported that Rho GTPases regulate STAT3 through phosphorylation of tyrosine and serine residues (Turkson et al. 1999, Aznar & Lacal 2001). Furthermore, activation of STAT3 by autocrine IL-6 mediated through Rac1 was also demonstrated. Interestingly, expression of IL-6 and IL-6R was initiated by NF-κB (Faruqi et al. 2001).

Taking all these reports into consideration, investigation of a possible link between actin cytoskeleton reorganization, small GTPases, NF-κB and IL-6 seems to be a promising area for future research. Do dysregulations in the above mentioned signaling pathways and proteins lead to development of malignancies? Are there other human tumors where NF-κB and the small GTPases are simultaneously overexpressed? Does actin reorganization lead to alterations in the activity of GTPases and NF-kB and therefore to tumorigenesis and development of diseases? Are there some intervening points for future therapies of diseases and malignancies? Could some of the proteins, participating in these cascades, be used in the diagnosis of diseases? These and many other questions arise and represent an area with a lot of potential for following investigations and studies.

## Summary

Both the transcription factor NF-κB and the cytokine IL-6 participate in many pivotal processes like immune responses, inflammation, cell proliferation and apoptosis. At the same time the actin cytoskeleton plays an important role not only in cell motility, but also in cell proliferation, cell survival and even in the intracellular signal transduction. In the last years, it was shown that dysregulation of the signaling pathways of NF-κB and IL-6 is linked to many pathological states like chronic inflammatory and malignant diseases.

In the current work, we have investigated the influence of cytoskeleton reorganization on the signaling pathways of NF-κB and IL-6 in different cell lines. Actin modulating compounds such as cytochalasin B and D and jasplakinolide were used to manipulate the actin cytoskeleton. Cells were pretreated with actin-modulating compounds and analyzed afterwards with the help of different techniques like western blotting and ELISA.

Stimulation of Hep3B and HCT116 cells with cytochalasin B and jasplakinolide at increasing concentration did not lead to phosphorylation of p65 and thus to activation of NF-κB. Moreover, treatment of HCT116 cells with the both substances did not affect the phosphorylation state of RelB. Taken together, these results demonstrate that reorganization of the actin cytoskeleton did not induce the activation of the canonical and the non-canonical NF-κB pathway in the analyzed cell lines.

Treatment of Hep3B and HepG2 cells with cytochalasins and jasplakinolide did also not show an effect on the amount of sIL-6R produced by ADAM10-mediated cleavage. In contrast, manipulation of the actin cytoskeleton with these substances led to significant reduction of STAT3 phosphorylation in Hep3B cells by stimulation with recombinant IL-6. Furthermore, perturbation of the actin cytoskeleton with the applied compounds did not result in IL-6 production in both Hep3B and HepG2 cell lines.

In conclusion, this work demonstrated that NF-κB signaling, IL-6 synthesis and ADAM10-induced production of sIL-6R stay unaffected by actin dynamics. By contrast, perturbation of actin cytoskeleton resulted in reduced IL-6-mediated activation of the transcription factor STAT3 in Hep3B cells.

**Zusammenfassung**

Der Transkriptionsfaktor NF-κB und das Zytokin IL-6 sind an Schlüsselprozessen wie Immunabwehr, Immunantwort, Entzündung, Zellproliferation und Apoptose beteiligt. Gleichzeitig spielt das Aktinzytoskelett eine entscheidende Rolle, nicht nur in der Zellmotilität, sondern auch in Proliferation und Überleben der Zelle, sowie in der intrazellulären Signalübertragung. Eine Dysregulation in den Signalwegen von NF-κB und IL-6 ist mit vielen pathologischen Zuständen wie chronisch entzündlichen oder malignen Erkrankungen verbunden.

In der vorliegenden Arbeit wurde der Einfluss der Reorganisation des Aktinzytoskeletts auf die Signalwege von NF-κB und IL-6 untersucht. Zum Zweck der Manipulation des Aktinzytoskeletts wurden Aktin-modulierende Substanzen wie Cytochalasin B und D und Jasplakinolid benutzt. Nach Vorbehandlung der Zellen mit diesen Substanzen wurden die gesammelten Proben mit Hilfe von verschiedenen Methoden wie Western Blot und ELISA untersucht.

Die Stimulation der Hep3B und HCT116 Zellen mit Cytochalasin B und Jasplakinolid führte nicht zur erwarteten Phosphorylierung von p65 und dadurch nicht zur Aktivierung von NF-κB. Darüber hinaus zeigte die Stimulation der HCT116 Zellen mit den Substanzen keinen Effekt auf die Phosphorylierung von RelB. Zusammenfassend führte die Reorganisation des Aktinzytoskeletts bei keiner der beiden untersuchten Zelllinien zu einer Aktivierung des kanonischen und des nicht-kanonischen NF-κB Signalwegs.

Die Behandlung von Hep3B und HepG2 Zellen mit Cytochalasinen und Jasplakinolid übte keinen Effekt auf die Menge der ADAM10-vermittelten Produktion von sIL-6R aus. Im Gegensatz dazu führte jedoch die Manipulation des Aktinzytoskeletts zum signifikanten Rückgang in der Phosphorylierung von STAT3 nach Stimulation mit rekombinantem IL-6 in Hep3B-Zellen. Daneben löste die Manipulation des Aktinskeletts mit den verwendeten Substanzen keine Produktion von IL-6 in beiden Zelllinien aus.

Zusammenfassend zeigte die vorliegende Arbeit, dass die NF-κB-Signalübertragung, die Synthese von IL-6 und die ADAM10-vermittelte Produktion von sIL-6R unabhängig von der Dynamik des Aktinzytoskeletts sind. Dagegen führte die Reorganisation des Aktinskeletts zur Reduktion der IL-6 induzierten Aktivierung des Transkriptionsfaktors STAT3 in Hep3B Zellen.

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# Ehrenerklärung

Ich erkläre, dass ich die in der Medizinischen Fakultät der Otto-von-Guerike-Universität Magdeburg zur Promotion eingerichtete Dissertation mit dem Titel:

**Influence of modulation of the actin cytoskeleton on NF-κB activation and interleukin-6 signaling**

am Institut für Pathologie an der Medizinischen Fakultät der Otto-von-Guerike-Universität Magdeburg mit Unterstützung durch Herrn Prof. Dr. rer. nat. Christoph Garbers und Herrn Prof. Dr. med. univ. Dr. sc. nat. Prof. h.c. Johannes Haybäck ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine andere als dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 07.10.2021

Elizabeta Georgieva

# Erklärung zur strafrechtlichen Verurteilung

Ich erkläre hiermit, nicht wegen einer Straftat verurteilt worden zu sein, die Wissenschaftsbezug hat.

Magdeburg, den 07.10.2021

Elizabeta Georgieva

# Curriculum vitae

**Der Lebenslauf ist in der Version aus Datenschutzgründen nicht enthalten.**

**Curriculum vitae**

**Der Lebenslauf ist in der Version aus Datenschutzgründen nicht enthalten.**

## Appendix 1

**Table 9: List of consumables.**

| Material | Type | Manufacturer |
|---|---|---|
| cell counting chambers slides | | Invitrogen, USA |
| cell culture flasks | CELLSTAR® - 25 und 75 cm2, PS | Greiner Bio One, Austria |
| cell culture multiwell plate | CELLSTAR® - 6-, 12- and 96-well with lid | Greiner Bio One, Austria |
| cell scrapers | 25 cm | Sarstedt AG & Co. KG, Germany |
| coated plates | Nunc MaxiSorp™ 96 well ELISA plate | Thermo Scientific, USA |
| cryovials | T310-2A, 2 ml | Simport, Canada |
| filter tips | ep T.I.P.S.® Standard Bulk 0,1-10 µl, 0,5-20 µl, 50 -1000 µl, 100-5000 µl, 1-10 ml | Eppendorf, Germany |
| filter tips | 200 µl, 300 µl | Sarstedt AG & Co. KG, Germany |
| glass plates | Mini PROTEAN® System Glass plates, Short Plates 1.5 mm 10 x 8.2 cm, Short Plates 10 x 7,3 cm | Bio-Rad Laboratories, USA |
| nitrocellulose membrane | 30 cm x 3,5 mm, 1 roll | Bio-Rad Laboratories, USA |
| Pasteur pipettes | 230 mm | Carl Roth GmbH+Co.KG, Germany |
| pipette filler | Pipetus® | Hirschmann Laborgeräte GmbH & Co. KG Laboratories, Germany |
| pipettes | Research Plus 2,5 µl, 20 µl, 200 µl, 1000 µl, 5 ml | Eppendorf, Germany |
| reaction tubes | Safe Seal tube 0,5 ml | Sarstedt AG & Co. KG, Germany |
| reaction tubes | 1,5 ml | Greiner Bio One, Austria |

**Appendix**

| | | |
|---|---|---|
| reaction tubes | 2,0 ml, 5,0 ml | Sarstedt AG & Co. KG, Germany |
| reaction tubes | 15ml, 50 ml | Greiner Bio One, Austria |
| serological pipettes | 2 ml, 5 ml, 10 ml, 25 ml | TPP Techno Plastic Products AG, Switzerland |
| Western Blotting filter paper | 8 x 10,5 cm | Thermo Scientific, USA |

**Appendix 2**

**Table 10: List of equipment.**

| Equipment | Type | Manufacturers |
|---|---|---|
| incubator | C200 | Labotect, Germany |
| biological safety cabinet | Class II A/B3 NU-440-401E | NuAire, USA |
| microscope | AE21 | Motic, USA |
| microscope | Nikon Eclipse TE 300 | Nikon, Japan |
| cell counter | Countess II | Invitrogen, USA |
| water bath | W6 | Störk Tronic, Germany |
| centrifuge | Multiguge 3 S-R Heraeus | Thermo Fisher Scientific, USA |
| centrifuge | Centrifuge 5417R | Eppendorf, Germany |
| mini centrifuge | Mini Star silver line | VWR international, USA |
| thermomixer | Thermomixer comfort | Eppendorf, Germany |
| rotator | Fröbel Rocky GRS | Fröbel, Germany |
| rotator | Polymax1040 | Heidolph Instruments, Germany |
| vortexer | IKA® MS 3 basic | IKA-Werke GmbH & Co.KG, Germany |

**Appendix**

| | | |
|---|---|---|
| hotplate stirrer | CB162 | Stuart, UK |
| hotplate stirrer | MR 3001 K | Heidolph Instruments, Germany |
| electrophoresis system | Mini-PROTEAN Tetra Cell | Bio-Rad Laboratories, USA |
| transfer system | Trans-Blot® Turbo™ Transfer System | Bio-Rad Laboratories, USA |
| transfer system | Mini Trans-Blot® cell | Bio-Rad Laboratories, USA |
| power supply | Power NPAC 1000 | Bio-Rad Laboratories, USA |
| power supply | Power NPAC 200 | Bio-Rad Laboratories, USA |
| Western Blot Imaging System | Fluoro Chem E System | Protein Simple, USA |
| detection System | GLOMAX Multi | Promega, USA |
| plate reader | CLARIO star® Plus | BMG Labtech, Germany |
| electronic balance | BL 1200 | Sartorius, Germany |
| electronic balance | BP 121S | Sartorius, Germany |
| freezer (-20°C) | Premium No Frost | Liebherr, Germany |
| ultra-low freezer (-86°C) | Hera freeze | Thermo Electron Corporation, USA |
| cryogenic ultra-low freezer (-152°C) | MDF-1156 | Sanyo Electric Co. , Japan |