Study of crosstalk between G-protein coupled receptor-mediated signals and the Nuclear Factor-κB signal transduction cascade

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Summary

Nuclear Factor-κB (NF-κB) is an ubiquitously expressed transcription factor that is activated in response to a broad spectrum of inflammatory stimuli, including the proinflammatory cytokine Tumour Necrosis Factor-α (TNF-α). Whereas NF-κB is pivotal for coordination of the immune/inflammatory response, its excessive activation is associated with the onset and propagation of multiple disease processes. NF-κB activity is mostly studied in cells subjected to proinflammatory stimuli, but in "real life" cells are simultaneously exposed to a plethora of signalling molecules that can modulate NF-κB activity. It has been known for many decades that sympathetic stress modulates immunity and inflammation, yet the molecular bases are not completely understood. Therefore, in this thesis, we focused on the activity of the β_2-adrenergic receptor (β_2-AR), one of the key mediators of the stress response, as a modulator of NF-κB function.

In line with other reports describing the anti-inflammatory action of β_2-AR agonists (β-agonists), we observed that cotreatment of human astrocytes with TNF-α and a β-agonist, inhibited the expression of several NF-κB-driven genes. However, we found that at the same time it potently enhanced the expression of other prototypical NF-κB target genes, including the proinflammatory cytokine Interleukin-6 (IL-6). We found that the IL-6 synergy, depended on the formation of an enhanceosome structure, and hypothesized that the IL-6 promoter acted as a "coincidence" detector, which requires input from multiple signalling cascades for maximal activation. Our previous research was limited to the study of β_2-AR/NF-κB crosstalk in the central nervous system, using astrocytes as a cellular model system. In this thesis, we have extended our previous research to skeletal muscle cells. In addition, we have attempted to further unravel the molecular details of the very strong transcriptional synergy apparent at the IL-6 gene using a proteomics approach.

Firstly, we have investigated signalling in response to TNF-α/β-agonist cotreatment in C2C12 cells, a murine skeletal muscle model, representing a physiologically relevant cell type to study β_2-AR/NF-κB crosstalk. We observed many similarities in the outcome of β_2-AR/NF-κB crosstalk in skeletal muscle cells as compared to astrocytes, although cell-type specific differences in the signalling cascades induced by β-agonists/TNF-α were also apparent. In particular, the very potent synergy at the IL-6

promoter was also detected in skeletal muscle cells. In addition, we found that the expression of several chemokines, influencing the migration potential of undifferentiated skeletal muscle cells, was upregulated upon TNF-α/β-agonist costimulation At the molecular level, we demonstrated that β-agonist-induced potentiation of NF-κB-dependent transcription of the IL-6 gene was associated with histone modifications, chromatin relaxation and formation of an enhanceosome structure.

Secondly, using an unbiased proteomics approach, combining DNA-affinity purification and mass spectrometric analysis, we identified Transcription Enhancer Factor 1 (TEF-1) as a novel interactor of the IL-6 promoter. We found that TEF-1 recruitment to the IL-6 promoter was induced upon TNF-α/β-agonist costimulation and that it acted as a transcriptional repressor. Our results furthermore indicate that TEF-1 modulates the transcriptional activity of CREB, but not NF-κB, and that this is associated with altered accessibility of the IL-6 promoter to transcriptional regulators. Importantly, TEF-1 modulated NF-κB-dependent transcription in a gene selective manner. As the effects of β-agonists appear to be highly gene-selective, further elucidation of its molecular basis might lead to the identification of novel targets for the development of selective NF-κB inhibitors.

In conclusion, these findings indicate that β_2-AR/NF-κB crosstalk promotes potent transcriptional synergy for a subset of NF-κB target genes, including IL-6 and several chemokines. This synergy is apparent in multiple relevant cell types, suggesting it might have general significance. As IL-6 has been attributed with devastating properties in inflammatory disease, and as β-agonists are mainstream therapy for respiratory disease, our data warrant further investigation into the outcome of β_2-AR/NF-κB crosstalk *in vivo*.

Samenvatting

Nuclear Factor-κB (NF-κB) is een alomtegenwoordig geëxpresseerde transcriptiefactor die geactiveerd wordt als antwoord op een breed spectrum aan inflammatoire stimuli, waaronder het inflammatoire cytokine Tumour Necrosis Factor-α (TNF-α). Alhoewel NF-κB onmisbaar is voor de coördinatie van de immuun/inflammatoire respons, is excessieve activering geassocieerd met de aanvang en voortzetting van verschillende ziekteprocessen. NF-κB activiteit wordt meestal bestudeerd in cellen die onderworpen zijn aan pro-inflammatoire stimuli, maar in werkelijkheid worden cellen simultaan blootgesteld aan een heel gamma van signaalmoleculen die NF-κB activiteit kunnen moduleren. Alhoewel het reeds verschillende decennia geweten is dat sympatische stress immuniteit en inflammatie moduleert, is de moleculaire basis van dit proces niet volledig ontrafeld. Daarom hebben we in deze thesis gefocust op de activiteit van de β_2-adrenerge receptor (β_2-AR), één van de sleutelmediatoren van de stress respons, als modulator van NF-κB werking.

In overeenstemming met andere rapporten die de ontstekingsremmende werking van β_2-AR agonisten (β-agonisten) beschrijven, observeerden we dat een gecombineerde behandeling van humane astrocyten met TNF-α en een β-agonist de expressie van verschillende NF-κB-gedreven genen inhibeerde. Daarentegen vonden we dat deze gecombineerde behandeling terzelfdertijd heel sterk de expressie van andere prototypische NF-κB doelwitgenen, waaronder het pro-inflammatoire cytokine Interleukine-6 (IL-6), opreguleerde. We observeerden dat de IL-6 synergie afhankelijk is van de totstandkoming van een enhanceosoom structuur, en veronderstellen dat de IL-6 promotor werkt als een detector die input van verschillende signaalcascades integreert. Ons eerder onderzoek was beperkt tot het bestuderen van β_2-AR/NF-κB crosstalk in het centraal zenuwstelsel, waarbij astrocyten als cellulair modelsysteem gebruikt werden. In deze thesis hebben we ons vorig onderzoek uitgebreid naar skeletspiercellen. Daarbovenop hebben we geprobeerd om de moleculaire details van de sterke transcriptionele synergie die werkzaam is aan het IL-6 gen verder te ontrafelen met behulp van een proteomics aanpak.

Samenvatting

Eerst hebben we signaaloverdracht als antwoord op TNF-α/β-agonist gecombineerde behandeling onderzocht in C2C12 cellen, een muis skeletspiermodel dat een fysiologisch relevant celtype representeert om β_2-AR/NF-κB crosstalk te bestuderen. We observeerden vele overeenkomsten in de uitkomst van β_2-AR/NF-κB crosstalk in skeletspiercellen vergeleken met astrocyten, alhoewel celtype specifieke verschillen in signaalbanen geïnduceerd door β-agonists/TNF-α ook merkbaar waren. De heel sterke synergie aan de IL-6 promotor werd, in bijzonder, ook gedetecteerd in skeletspiercellen. Daarenboven vonden we dat de expressie van verschillende chemokines die het migratie potentieel van niet-gedifferentieerde skeletspiercellen beïnvloeden, opgereguleerd was na TNF-α/β-agonist costimulatie. Op moleculair niveau toonden we aan dat β-agonist geïnduceerde potentiatie van NF-κB afhankelijke transcriptie van het IL-6 gen geassocieerd was met histon modificaties, chromatine relaxatie en vorming van een enhanceosoom structuur.

Vervolgens identificeerden we Transcription Enhancer Factor 1 (TEF-1) als een nieuwe interactor van de IL-6 promotor door gebruik te maken van een proteomics aanpak die DNA-affiniteitszuivering en massa spectrometrische analyse combineert. We vonden dat TEF-1 rekrutering naar de IL-6 promotor geïnduceerd werd na TNF-α/β-agonist costimulatie en dat TEF-1 functioneert als transcriptionele repressor. Onze resultaten toonden voorts aan dat TEF-1 de transcriptionele activiteit van CREB, maar niet van NF-κB, moduleert en dat dit geassocieerd is met veranderde toegankelijkheid van de IL-6 promotor voor transcriptionele regulatoren. Belangrijk is dat TEF-1 NF-κB afhankelijke transcriptie moduleert op een genselectieve manier. Aangezien de effecten van β-agonisten heel genselectief blijken te zijn, kan verdere opheldering van hun moleculaire basis leiden tot identificatie van nieuwe doelwitten voor de ontwikkeling van selectieve NF-κB inhibitoren.

Samengevat tonen deze bevindingen aan dat β_2-AR/NF-κB crosstalk sterke transcriptionele synergie promoot voor een subset van NF-κB doelwitgenen, inclusief IL-6 en verschillende chemokines. Deze synergie is duidelijk in verschillende relevante celtypes, wat suggereert dat het een algemeen mechanisme kan betreffen. Aangezien aan IL-6 verschillende verwoestende eigenschappen in ontstekingsziekten worden toegekend en β-agonisten mainstream therapie zijn voor ademhalingsziekten, motiveren onze data verder onderzoek naar de uitkomst van β_2-AR/NF-κB crosstalk *in vivo*.

Résumé

Le facteur de transcription NF-κB (Nuclear Facteur-κB) est exprimé de manière ubiquitaire et est activé en réponse à un large spectre de stimuli inflammatoires, y compris la cytokine pro-inflammatoire TNF-α (Tumour Necrosis Factor-α). Tandis que NF-κB est essentiel pour la coordination de la réponse immunitaire/inflammatoire, son activation excessive est associée à l'apparition et à la propagation de multiples processus pathologiques. L'activité de NF-κB a surtout été étudié dans des cellules soumises à des stimuli pro-inflammatoires, mais dans les « conditions réelles » les cellules sont simultanément exposées à une multitude de molécules capables de moduler l'activité de NF-κB. Il est connu depuis de nombreuses décennies que la stimulation sympathique module l'immunité et l'inflammation, cependant les bases moléculaires ne sont pas entièrement comprises. Pour cette raison, le travail de cette thèse se concentre sur l'activité du récepteur β_2-adrénergique (β_2-AR), l'un des médiateurs clés de la réponse au stress, comme modulateur de la fonction de NF-κB.

De façon cohérente avec d'autres études décrivant l'action anti-inflammatoire des agonistes β_2-AR (β-agonistes), nous avons observé que la stimulation simultanée des astrocytes humains avec le TNF-α et l'β-agoniste inhibe l'expression de plusieurs gènes cibles de NF-κB. Néanmoins, nous avons observé que ce même traitement augmente fortement l'expression d'autres gènes cibles prototypiques de NF-κB, y compris la cytokine pro-inflammatoire Interleukine-6 (IL-6). Nous avons constaté que la synergie au niveau du promoteur de l'IL-6 dépendait de la formation d'une structure d'un enhanceosome. Par conséquent, nous avons émis l'hypothèse que le promoteur de l'IL-6 agit comme un détecteur de "coïncidences", qui requiert la contribution de plusieurs cascades de signalisation pour une activation maximale. Nos précédents travaux se limitaient à l'étude du crosstalk entre β_2-AR et NF-κB dans le système nerveux central, en utilisant des astrocytes comme modèle cellulaire. Dans cette thèse, nous avons étendu nos recherches précédentes aux cellules des muscles squelettiques. De plus, nous avons essayé d'élucider les détails moléculaires de la très forte synergie transcriptionnelle visible au niveau du gène de l'IL- 6 en utilisant une approche protéomique.

Résumé

Dans un premier temps, nous avons étudié la signalisation en réponse au co-traitement TNF-α/β-agoniste des cellules murines de muscle squelettique C2C12, représentant un type cellulaire physiologiquement pertinent pour étudier le crosstalk entre β_2-AR et NF-κB. Nous avons observé de nombreuses similitudes dans les résultats (de crosstalk β_2-AR/NF-κB dans les cellules des muscles squelettiques) par rapport aux astrocytes, bien que des différences dans les cascades de signalisation induites par β-agonistes/TNF-α sont également apparues dues aux différents types cellulaires. En particulier, la synergie très puissante au niveau du promoteur de l'IL-6 a également été détectée dans les cellules des muscles squelettiques. De plus, nous avons constaté que l'expression de plusieurs chimiokines, qui influencent le potentiel de la migration des cellules du muscle squelettique indifférenciées, a été augmentée lors de la co-stimulation TNF-α/β-agoniste. Au niveau moléculaire, nous avons démontré que la potentialisation de la transcription du gène de l'IL-6 dépendante de NF-κB induite par le β-agoniste est associée à des modifications d'histones, la relaxation de la chromatine et la formation d'une structure d'enhanceosome.

Dans un deuxième temps, en utilisant une approche protéomique, combinant la purification liée à l'affinité d'ADN et l'analyse par spectrométrie de masse, nous avons identifié TEF-1 (Transcription Enhancer Factor 1) comme un nouvel interacteur du promoteur IL-6. Nous avons constaté que le recrutement de TEF-1 sur le promoteur IL-6 est induit par la co-stimulation TNF-α/β-agoniste et qu'il agi comme un répresseur transcriptionnel. Nos résultats indiquent également que TEF-1 module l'activité transcriptionnelle de CREB, mais pas de NF-κB, et que cela est associé à la modification de l'accessibilité des régulateurs de transcritpion au promoteur de l'IL-6. De façon importante, TEF-1 module la transcription dépendante du NF-κB de manière sélective en fonction des gènes impliqués. Comme les effets des β-agonistes semblent être hautement dépendant des gènes, l'élucidation du mécanisme moléculaire permettra l'identification de nouvelles cibles pour le développement d'inhibiteurs sélectifs de NF-κB.

En conclusion, les résultats présentés ici indiquent que le crosstalk entre β_2-AR et NF-κB favorise une forte synergie transcriptionnelle d'un sous-ensemble de gènes cibles de NF-κB, y compris l'IL-6 et plusieurs chimiokines. Cette synergie existe dans plusieurs types cellulaires exprimant des récepteurs β-adrénergiques, suggèrant qu'il pourrait y avoir une portée générale. Comme il a été attribué des propriétés néfastes importantes à l'IL-6 dans le développement des pathologies inflammatoires,

fréquentes et importantes pour la santé publique, et que les β-agonistes font partie du traitement de certaines maladies respiratoires courantes, nos données justifient une enquête plus approfondie sur la nature et les conséquences du crosstalk entre β2-ARet NF-κB *in vivo*.

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List of abbreviations

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AC	adenylate cyclase
ACF	apobec-1 complementation factor
ADAM	disintegrin and metalloproteinase domain-containing protein
Akt	protein kinase Akt/protein kinase B
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AP	activator protein
APEX-1	DNA-(apurinic or apyrimidinic site) lyase-1
AR	adrenergic receptor
ARE	AU rich elements
Arid5a	AT-rich interactive domain-containing protein 5a
ASK-1	apoptosis signal-regulating kinase-1
ASP	acylation-stimulating protein
ATF	activating transcription factor
ATM	ataxia telangiectasia-mutated kinase
ATP	adenosine triphosphate
AUF-1	ARE/poly(U) binding degradation factor-1
BACH1	BTB and CNC homolog 1
BCR	B-cell receptor
BRF	butyrate response factor
bZIP	basic leucine zipper domain
C/EBP	CCAAT/enhancer-binding protein
C3	complement component 3
CAMKII	Ca2+/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CCL	xhemokine (C-C motif) ligand
CD	cluster of differentiation/cluster of designation/classification determinant
cIAP	cellular inhibitor of apoptosis
CK2	casein kinase 2
COX	cyclooxygenase
CRE site	cAMP response element
CREB	cAMP response element binding protein
CREM	cAMP response element modulator
CRTC	CREB regulated transcription coactivator
CTKD	C-terminal kinase domain
CUL	cullin
CXCL	chemokine (C-X-C motif) ligand
DAMP	damage associated molecular pattern
DD	death domain
DDB	DNA damage-binding protein

List of abbreviations

DR	death receptor
Epac	exchange protein directly activated by cAMP
ERKs	extracellular signal-regulated kinase
FADD	Fas-associated death domain
G-CSF	granulocyte colony-stimulating factor
GCN	histone acetyltransferase GCN5
GDP	guanosine diphosphate
GM-CSF	granulocyte/macrophage-colony-stimulating factor
GNAT	GCN-related N-acetyltransferases
Gp	glycoprotein
GPCR	G protein-coupled receptor
Grb2	growth-factor-receptor-bound Protein 2
GTP	guanosine triphosphate
Gαi	Gα subunit of the G inhibitory protein
Gαs	Gα subunit of the G stimulatory protein
Gβγ	Gβγ subunit of the G protein
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
HMG	high mobile group protein
HPA	hypothalamic-pituitary-adrenal
ICAM	intercellular adhesion molecule
IFN	interferon
IKK	IκB kinase
IL	interleukin
IL-6R	IL-6 receptor
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
IκB	inhibitor of NF-κB
JAK	Janus kinase
JNKs	c-Jun N-terminal kinase
KID	kinase inducible domain
KIX	KID interaction domain
KSRP	Kaposi sarcoma-associated herpes virus open reading frame
L-DOPA	L-3,4-di-hydroxy-phenylalanine
LPS	lipopolysaccharide
LUBAC	linear ubiquitin chain assembly complex
M-CSF	macrophage-colony-stimulating factor
MAP2Ks, MAPKKs, MKKs, MEK	MAPK kinase
MAP3Ks, MAPKKKs, MKKKs, MEKKs	MAPK kinase kinase
MAPK	mitogen-activated protein kinase
MAPKAPs	MAPK-activated proteins
MK	MAPK Activated Protein Kinase
MMP	matrix metaloprotease
MNK	MAPK-interacting kinase

MnSOD	mitochondrial superoxide dismutase
MSK	mitogen and stress-activated kinase
MuRF	murine ring finger
MYST	histone acetyltransferase MYST
NADPH	nicotinamide adenine dinucleotide phosphate
NCD	non communicable disease
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-κB
NGF	nerve growth factor
NIK	NF-κB-inducting kinase
NLS	nuclear localization sequence
NTKD	N-terminal kinase domain
p38 MAPKs	p38 mitogen-activated protein kinase
p300	histone acetyltransferase p300
PAMP	pathogen associated molecular pattern
PARP-1	poly [ADP-ribose] polymerase 1
PCAF	p300/CBP-associated factor
PI3K	phosphoinositide 3-kinase
PIDD	p53-inducible death domain-containing protein
PKA	protein kinase A
PKAc	protein kinase A catalytic subunit
PKAr	protein kinase A regulatory subunit
PKC	protein kinase C
PP	protein phosphatase
PRR	pattern recogintion receptor
Q	glutamine rich domain
R6K	ribosomal S6 kinase
RHD	Rel-homology domain
RIP	receptor-interacting protein
ROS	reactive oxigen species
SAPK	stress-activated protein kinase
Sgp	soluble glycoprotein
sIL-6R	soluble IL-6 receptor
SMRT	silencing mediator fot retinoic acid receptor and thyroid hormone receptor
SNS	sympathetic nervous system
SOCS-3	suppressor of cytokine signaling-3
SODD	silencer of the death domain
SOS	son of sevenless
SRC	steroid receptor coactivator
STAT	signal transducer and activator of transcription
sTNF-α	soluble tumour necrosis factor-α
SWI/SNF	switch/sucrose nonfermentable
TAB	TGF-β-activated kinase binding protein
TACE	TNF-α converting enzyme
TAD	transactivation domain

TAK	TGF-β-activated kinase
TBP	TATA-binding protein
TCR	T-cell receptor
TDIID	transcription factor II D
TEAD	TEA domain family member
TEF	transcription enhancer factor
TGF-β	transforming growth factor-β
TIM	TRAF interacting motif
TLR	toll-like receptor
tmTNF-α	transmembrane tumour necrosis factor-α
TNF-R	TNF-α receptor
TNF-α	tumour necrosis factor-α
TORC	transducer of regulated CREB
TRADD	TNF-R-associated death domain
TRAF	TNF-R-associated factor
TTP	tristetraproline
UTR	untranslated region
VCAM	vascular adhesion molecule
WHO	World Health Organization
β-agonist	β-adrenoreceptor agonist
β-antagonist	β-adrenoreceptor antagonist
κB site	NF-κB response element
CRF	corticotropin-releasing factor
POMC	proopiomelanocortin
ACTH	adrenocorticitropic hormone
α-MSH	α-melanocyte stimulating hormone

Introduction

Chapter 1

Inflammation and stress

1. Inflammation, old victories and new concerns in medicine

During the past century, remarkable progress has been made in medicine to counteract infectious diseases that in the middle of 20th century still decimated thousands of human lives worldwide. This virtual victory on the "medical battlefield" was overshadowed by a plague of non-communicable diseases (NCDs). NCDs are tightly associated with the development of modern and aging societies, however, along with the popularisation of Western lifestyle, they also became common in low- and middle-income countries. The most prevalent types of NCDs are cardiovascular diseases, cancer, chronic respiratory diseases and diabetes. Overall, these disorders account currently for approximately 63% of premature deaths and constitute the major healthcare concern according to estimations by the World Health Organization (WHO). From the medical point of view, NCDs are a heterogeneous group of disorders with very diverse clinical manifestations. Nevertheless, most of them are characterized by a chronic inflammatory state that might be triggered by genetic, social, lifestyle and environmental factors (Global status on non-communicable diseases 2011, WHO).

Inflammation (from the Latin *inflammare*: to set afire) is a protective tissue reaction to injury, irritation or infection that aims at neutralizing the extracellular insult and/or repair intracellular damage, ultimately restoring tissue homeostasis. The cardinal symptoms of inflammation are redness (*rubor*), swelling (*tumor*), heat (*calor*) and pain (*dolor*). At the molecular level, inflammation involves the orchestrated action of signals instructing components of the immune system how to defend and reinstate the internal balance in the organism. Nowadays, it is well accepted that inflammation involves many different cell types, including immune cells but also non-immune cells, such as glia cells, fibroblasts, and endothelial cells. In general, inflammation can be classified as either acute or chronic, each with its own collection of cellular and humoral components. Whereas the acute state is characterized by a prompt, temporary and localized immune response at the "inflamed site", the chronic inflammatory

response, originating from the persistent presence of the insult, may acquire systemic characteristics and have devastating consequences. Chronic inflammation is associated with a shift in the type of cells present at the site of inflammation, disturbances in the circulating level of immunomodulatory mediators as well as changes in the morphology and function of the "inflamed tissue". Finally, it can be considered as an abnormal type of immune response eliciting deleterious effects at the site of inflammation as well as on whole body homeostasis (Cone 2001; Lawrence and Gilroy 2007).

Inflammation is orchestrated by the timely action of a variety of immunomodulatory factors, including diverse cytokines, chemokines, growth factors, receptors, adhesion molecules, enzymes, inhibitory proteins, peptides, lipid-derived mediators, etc., which act as "checkpoints" to control the course and magnitude of the inflammatory response. Deregulation of production and/or secretion of these mediators predisposes to the development of an inadequate immune response, which can trigger the transition of acute to chronic inflammation and the development of disease (Nathan 2002). Although multiple classes of drugs are available in the clinic for treatment of inflammatory disorders (e.g. steroidal and non-steroidal anti-inflammatory drugs) a constant search for new therapeutics with improved specificity and reduced side-effects is ongoing. To identify novel targets for combatting chronic inflammation, intensive research efforts are directed towards a better understanding of the molecular basis of inflammation.

2. Tumour Necrosis Factor-α

One of the key mediators of inflammation is Tumour Necrosis Factor-α (TNF-α).

TNF-α was discovered as a macrophage-derived factor inducing necrosis of tumour cells (Carswell et al. 1975). In spite of this spectacular property, the anti-tumour action of TNF-α has not been exploited clinically because of its strong systemic toxicity (K. J. Tracey et al. 1987). Today, TNF-α is known as a pleiotropic cytokine, which plays an important role in the regulation of cell proliferation, differentiation, survival and apoptosis. It also substantially contributes to the regulation of the immune response and the maintenance of homeostasis (Wajant et al. 2003). Ample evidence indicates that deregulation of TNF-α production and signalling leads to the development of various chronic inflammatory disorders, suggesting the importance of the TNF-α axis as a drug target (Bradley 2008; Van Hauwermeiren et al. 2011).

2.1. Regulation of TNF-α expression

TNF-α is expressed at very low or undetectable levels in healthy individuals, while elevated levels are found during inflammatory and infectious conditions. The primary sources of TNF-α are activated immune cells, mainly macrophages and T-cells at the site of inflammation, but multiple other cell types like for instance skeletal muscle, astrocytes or adipocytes can express this cytokine during various (patho)physiological circumstances (Bradley 2008; Cawthorn and Sethi 2008; Y. P. Li and Reid 2001; Montgomery and Bowers 2012; Van Hauwermeiren et al. 2011).

The stimuli triggering TNF-α production are very diverse and include anything that causes an inflammatory response. Among these factors, there are viral and bacterial components collectively known as pathogen associated molecular patterns (PAMPs), molecules associated with host cell damage, collectively known as damage-associated molecular patterns (DAMPs), various cytokines, including TNF-α itself, complement factors, immune complexes and many others (Spriggs et al. 1992).

The human TNF-α gene is approximately 3000 base pairs long, consists of four exons and localizes to chromosome 6p21. Expression of this cytokine is stringently controlled by the activity of several transcriptional regulators, primarily Activator Protein (AP)-1, AP-2, Nuclear Factor-κB (NF-κB), but also cAMP responsive element binding protein (CREB) (Spriggs et al. 1992). In addition, rapid production of TNF-α is made possible via tightly regulated post-transcriptional mechanisms. In homeostatic conditions, the TNF-α transcript is rapidly degraded due to the presence of AU rich elements (ARE) in its 3' untranslated region (UTR). However, upon encountering inflammatory stimuli, the mRNA of TNF-α is stabilized via binding of tristetraproline (TTP) to the ARE, leading in turn to rapid upregulation of TNF-α protein levels (Clement et al. 2011; Deleault et al. 2008). Structurally, TNF-α exists as a homotrimer with a protein structure resembling a cone shape. A single monomer is composed of two anti-parallel β-pleated sheets that are formed by eight anti-parallel β-strands arranged in a β-jellyroll topology, typical for the TNF ligand family (Eck and Sprang 1989).

Human TNF-α is synthesized as a 26 kDa transmembrane protein (tmTNF-α) that can be processed into a 17 kDa soluble form (sTNF-α) via the activity of an extracellular metalloprotease, the TNF Alpha Converting Enzyme (TACE also known as ADAM-17) (R. A. Black et al. 1997). The sTNF-α and tmTNF-α show different biological activities, but in general sTNF-α is less active than tmTNF-α (Wajant et al. 2003).

TNF-α transmits its biological activity via binding to two different receptors: either TNF-R1 (p55 or CD120a), which is activated by both sTNF-α and tmTNF-α, or TNF-R2 (p75 or CD120b), which is activated almost exclusively by tmTNF-α (Smith et al. 1994). Whereas TNF-R1 is constitutively expressed in all nucleated cells, inducibly produced TNF-R2 is chiefly found on endothelial and hematopoietic cells (Carpentier et al. 2004). Each receptor displays a unique panel of activities but examples of redundancy, cooperation and crosstalk between the TNF-Rs exist (Naude et al. 2011). Interestingly, TACE can also "clip" the TNF-Rs generating soluble forms of each one (J. Wang et al. 2003). Thus, TACE may be recognized as a modulator of inflammation bearing pro- or anti-inflammatory properties depending on whether it acts on effector or target cells, releasing respectively ligand or receptor (Xanthoulea et al. 2004). TNF-α signalling mechanisms are summarized in Figure 1.

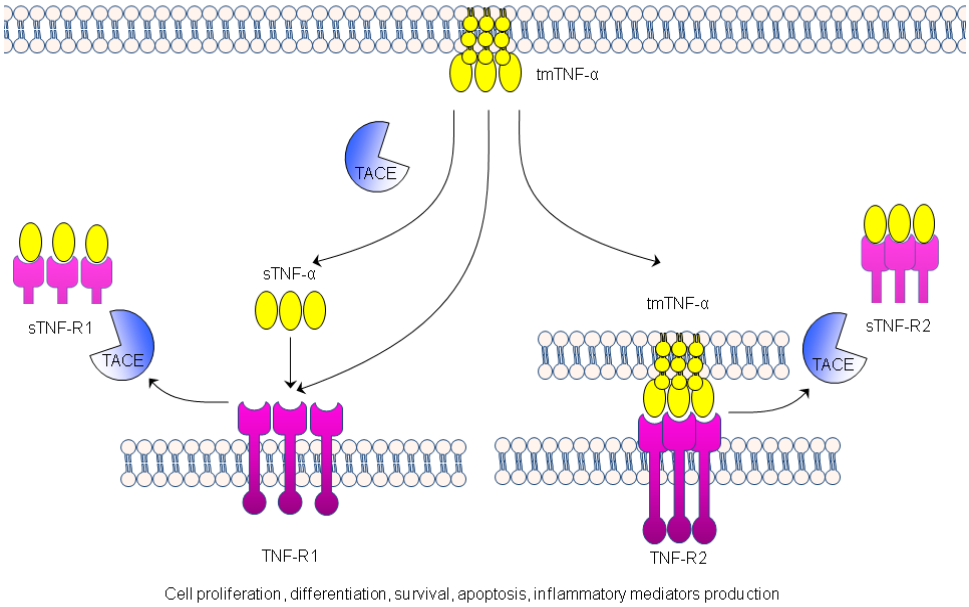

Figure. 1. TNF-α signalling mechanisms. TNF-α is expressed as a transmembrane protein that can activate both TNF-R1 and TNF-R2. Processing by TACE generates sTNF-α, which mainly activates TNF-R1. Signalling through both receptors can result in a variety of cellular responses, depending on the cell type and biological context. TACE is able to cleave both TNF-Rs to generate sTNF-Rs, which can still bind and inhibit TNF-α.

2.2. TNF-α signalling pathways

Trimeric occupation of TNF-Rs by TNF-α induces receptor trimerization and consequently a cascade of receptor proximal events leading to the recruitment of signalling proteins (Dempsey et al. 2003).

TNF-R1 belongs to a group of death receptors (DR) containing a death domain (DD) responsible for interaction with adaptor proteins upon ligand triggering of the receptor (Dempsey et al. 2003). In the resting state, the receptors are associated with a cytoplasmic silencer of the death domain (SODD) that prevents signal transduction (Jiang et al. 1999). Ligand binding to TNF-R1 leads to receptor aggregation and SODD dissociation from its DD, which can then interact with the DD of the adaptor protein TNF Receptor Associated Death Domain (TRADD). Subsequently, TRADD recruits Receptor-Interacting Protein 1 (RIP1), TNF Receptor-Associated Factor 2 (TRAF2) and Fas-Associated Death Domain (FADD) eliciting formation of the Signalling Complexes. Briefly, the Signalling Complex I, composed of TRADD/RIP1/TRAF2, triggers among others Mitogen-Activated Protein Kinases (MAPKs) and transcription factor NF-κB (these signalling events are summarized in Figure 2 and are discussed more extensively later), while the Signalling Complex II, composed of TRADD/RIP1/TRAF2/FADD, recruits procaspase-8/10 resulting in apoptosis (H. Li and Lin 2008; Naude et al. 2011; Schneider-Brachert et al. 2004).

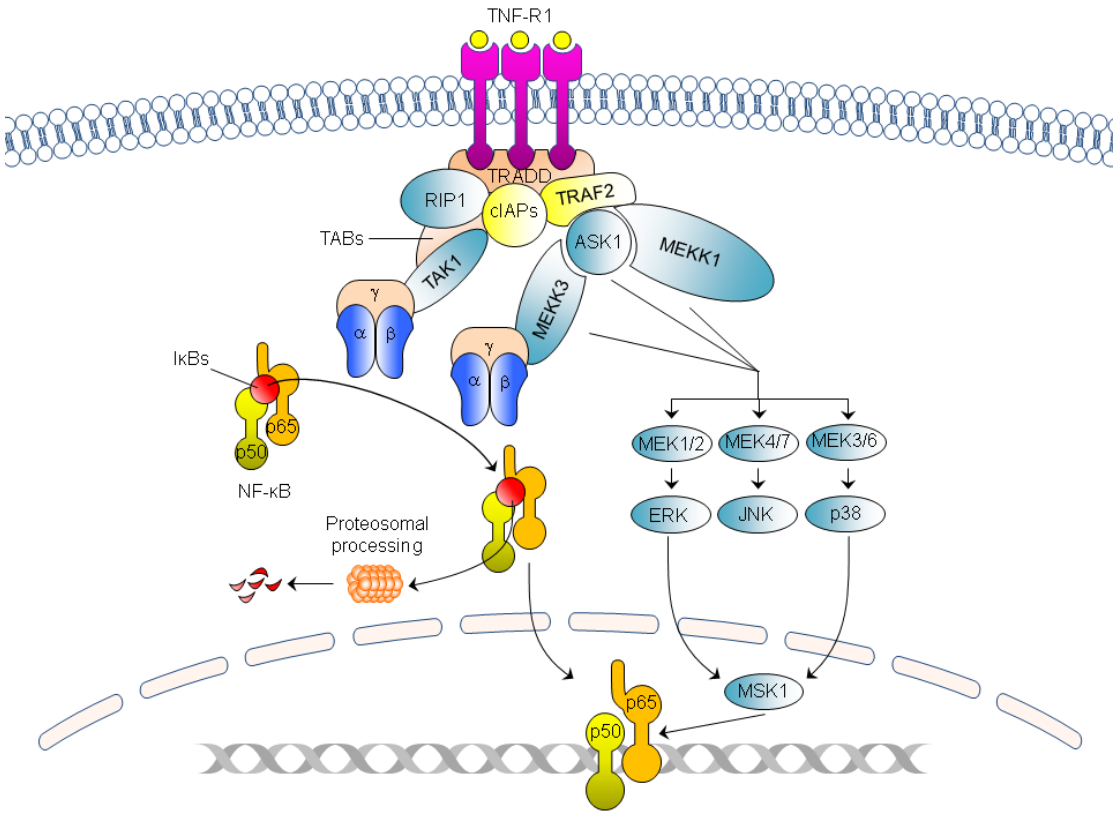

Figure 2. The signalling pathways activated downstream of TNF-R1. Activation of TNF-R1 leads to the recruitment of adaptor proteins, which in turn activate multiple kinases that ultimately launch the NF-κB signalling pathway and various MAPK signalling cascades. MAPK can modulate the activity of NF-κB.

TNF-R2, on the other hand, belongs to a group of receptors bereft of a DD but containing a TRAF-interacting motif (TIM). Consequently, binding of tmTNF-α to TNF-R2 elicits TRAF2 recruitment (Dempsey et al. 2003). TRAF2 associates with cIAPs, which prevents caspase activation via an intrinsic ubiquitin-ligase activity. Moreover, TNF-R2 stimulation in several cell lines was shown to launch NF-κB (Naude et al. 2011). Some research groups also reported that TNF-R2 utilizes TRAF2 to interact with TRADD and RIP1, which are typical mediators of TNF-R1 signalling, and in that way contributes to NF-κB activation (Faustman and Davis 2010). In comparison with TNF-R1 signalling, which is the predominant and strong activator of NF-κB, TNF-R2 activates the NF-κB-dependent transcriptional response poorly and slowly but for a longer time (McFarlane et al. 2002).

Thus, TNF-α signalling is a very complex process. The main TNF-α signalling cascades are depicted in Figure 3 and culminate in activation of multiple protein kinases and transcription factors or triggering of apoptosis. The character and ultimate effect of the TNF-α signalling pathway depends on the adaptor proteins involved in formation of the signalling complex, the molecules transmitting the signals as well as crosstalk between activated pathways. Lastly, branches of TNF-α signalling can antagonise each other's action, for example NF-κB activation antagonizes caspase-8-induced apoptosis or p38-mediated signals that promote growth and differentiation are in opposition with caspase-8-mediated apoptosis (Dempsey et al. 2003; MacEwan 2002a, 2002b).

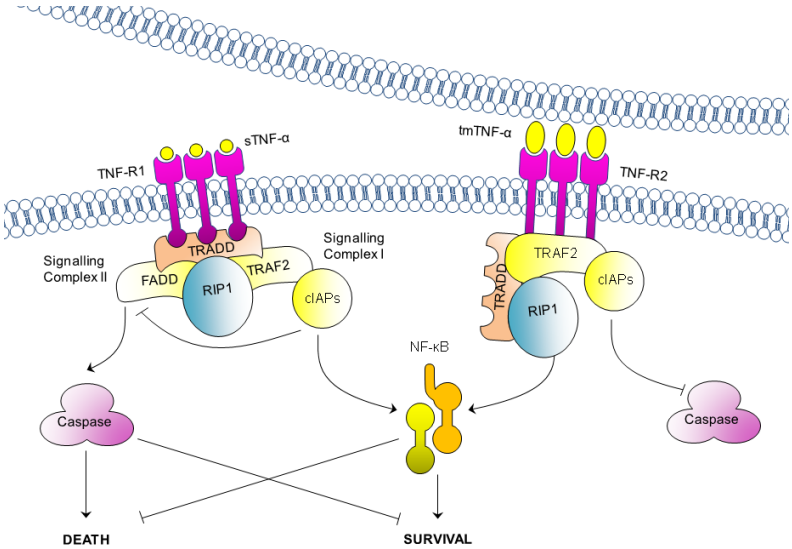

Figure 3. Overview of the TNF-α signalling pathway upon activation of TNF-R1 and TNF-R2 as well as their main intracellular responses. Binding of sTNF-α to the TNF-R1 initiates either activation of the NF-κB signalling cascade or apoptosis via activity of the Signalling complex I (composed of TRADD/TRAF2/RIP1/cIAPs) or Signalling complex II (composed of TRADD/TRAF2/RIP1/FADD), respectively. The Signalling complex I has antagonistic activity towards Signalling complex II. Binding of tmTNF-α to the TNF-R2 launches the NF-κB signalling cascade and inhibits apoptosis.

2.2.1. Mitogen-activated protein kinases

Within the signalling network in response to TNF-α, activation of the mitogen-activated protein kinase (MAPKs) signalling pathway enables efficient orchestration of a wide range of cellular processes, including gene transcription, mRNA metabolism, protein biosynthesis, proliferation, differentiation, survival and apoptosis. Three families of MAPKs have been identified: the Extracellular Signal-Regulated Kinases (ERKs), c-Jun N-terminal Kinases (JNKs), and p38 MAPKs (Sabio and Davis 2014).

All of these MAPKs are activated as a result of dual phosphorylation of threonine and tyrosine in the three-step signalling cascade. The signalling cascade is composed of: MAP3Ks (MAPKKKs, MKKKs, MEKKs; activated by a receptor protein complex), MAP2Ks (MAPKKs, MKKs, MEKs; activated by an upstream component – a MAPK3K) and culminate on a MAPK protein. This multistep mode of activation enables amplification of the initial signal that is generated upon receptor triggering. Activated MAPKs in turn phosphorylate diverse proteins on serine or threonine residues. The determinant of the kinase specificity is the presence of a specific docking site for this kinase and vicinity of proline residue to the phosphorylated motif.

In the context of inflammatory gene expression, MAPKs target transcriptional and translational (co)regulators, diverse enzymes and structural proteins in the nucleus, which directly or indirectly regulate transcription, the stability and transport of mRNA, and protein biosynthesis. Furthermore, MAPKs catalyse the phosphorylation of MAPK-activated protein kinases (MK), which represent the most downstream protein kinases in this signalling cascade. The MK family is composed of the Ribosomal S6 Kinases (RSKs), the Mitogen- and Stress-activated Kinases (MSKs), the MAPK-interacting kinases (MNKs), and the MAPK-activated protein kinases 2, 3 and 5 (MK2, MK3 and MK5) (Kyriakis and Avruch 2012).

Studies on MAPKs knockout animals demonstrated that individual MAPKs mediate processes essential for normal development and survival. Furthermore, they are required for a tightly tailored inflammatory response. However, it is impossible to assign specific functions to individual family members because of great functional redundancy (Aouadi et al. 2006). As overactivation of MAPKs has been reported in numerous chronic inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel disease, neurodegenerative pathologies, this group of enzymes constitutes an

attractive target for the development of anti-inflammatory therapies (Boldt and Kolch 2004; Gaestel and Kracht 2009; Kaminska 2005).

2.2.1.1. ERK MAPKs

ERK MAPKs are ubiquitously expressed protein kinases involved in the regulation of proliferation, differentiation and survival. There are five ERK family members, which are activated in response to mitogens, such as growth factors, and to a lower extent by cytokines (Kyriakis and Avruch 2012; Sabio and Davis 2014). In resting cells, ERKs localize in the cytoplasm. However, upon activation, which prototypically involves a cascade composed of Ras/Raf and MEK1/2, they rapidly translocate to the nucleus (Pouyssegur et al. 2002). In the nucleus, ERKs target multiple nuclear proteins, including NF-κB, hence, affecting cellular gene expression programs (Vanden Berghe et al. 1998). Alternatively, these protein kinases can transduce signalling by activation of several MKs, including MSK-1 (Yoon and Seger 2006).

Whereas knockout of ERK2 causes embryonic death, animals with a targeted deletion of ERK1 are viable and fertile indicating functional compensation by another family member. Interestingly, these animals display an impaired development of T cells beyond the CD4+CD8+ stage (Aouadi et al. 2006). Several inhibitors targeting components of the ERK pathway enable to study the cellular response evoked by the ERKs family members. Among them, PD98059 and U0126, which inhibit MEK1/2 are commonly used *in vitro*.

2.2.1.2. p38 MAPKs

The family of p38 MAPKs consists of four isoforms: α, β, γ and δ, which are activated in response to various environmental stressors. While p38 α and β are ubiquitously expressed, p38 γ and δ are synthesized only in selected tissues, such as skeletal muscle. Activation of this kinase is preceded by MEK3/4/6 activation. Whereas both MEK3 and MEK6 exclusively mediate p38 phosphorylation, MEK4 is more promiscuous as it induces both the JNK and p38 MAPK signalling cascades and displays only limited selectivity towards p38. p38 MAPK targets multiple transcription factors, including NF-κB, c-Jun (a component of the AP-1 transcription factor) and Activating Transcription Factor-1 (ATF-1), and furthermore phosphorylates several MKs, such as MSK-1/2 (Kyriakis and Avruch 2012; Sabio and Davis 2014). Ample evidence indicates the importance of p38 MAPK in the

regulation of immunity at the transcriptional level. Specifically, p38 MAPK orchestrates NF-κB, histone H3 and TATA-binding protein (TBP) phosphorylation via an intermediate kinase, called MSK-1 (Carter et al. 1999; Saccani et al. 2002; Vanden Berghe et al. 1998).

Targeted disruption of the p38 α isoform is lethal, while isolated p38 β, γ and δ or double p38 γ and δ knockouts do not display a lethal phenotype. The p38 α knockout mice die due to defective placenta development. Interestingly, when the placental defect was rescued, p38 α deficient embryos developed normally, indicating that p38 α is not crucial for other aspects of embryonic development. Conversely, lack of the p38 β, γ and δ isoforms can be sufficiently compensated by p38α (Aouadi et al. 2006). The p38 MAPK inhibitor SB203580 enables selective cassation of kinase activity.

2.2.1.3. JNKs

JNKs are the family of stress-activated protein kinases (SAPK) responding to a panel of cellular stressors as well as serum and growth factors. Three genes encode the JNK family members. Among them JNK 1 and 2 are ubiquitously expressed, while JNK3 is primarily present in the brain. Furthermore, all JNKs exist in a number of spliced forms. JNKs are activated upon phosphorylation of multiple upstream kinases including MEK4/7. The main cellular targets of the JNKs are the transcription factor AP-1 family members, including c-Jun, JunB, JunD, c-Fos, ATF-2. In comparison to p38 and ERKs, JNKs do not target any MKs (Johnson and Nakamura 2007; Kyriakis and Avruch 2012; Sabio and Davis 2014).

Knockout of individual JNK family members does not significantly affect the animal phenotype. These mice are viable, fertile and display normal development. However, alterations in T cell differentiation were observed in animals bearing targeted disruption of either JNK1 or JNK2. Interestingly, JNK1 deficient animals displayed T cell hyperproliferation and exhibited decreased apoptosis. Furthermore, these T cells preferentially differentiated into Th2 cells, which induce humoral response that is mediated by activated B cells. T cells from JNK2 knockout mice also had impaired differentiation into the Th1 effector cells, mediators of cellular response, due to defective interferon-γ (IFN-γ) production at the early stages of differentiation. Furthermore, targeted deletion of both JNK1 and JNK2 family members leads to death at the embryonic stage, while JNK1/3 and JNK2/3 knockout animals are

relatively normal (Aouadi et al. 2006). In cellular studies, JNK activity is commonly suppressed by SP600125.

2.2.1.4. MSK-1

MSK-1 is a member of the MK family and a downstream substrate of ERKs and p38 MAPK (Deak et al. 1998; New et al. 1999; Pierrat et al. 1998; Vermeulen et al. 2003), which activity is controlled by multiple phosphorylation sites (McCoy et al. 2005). It shares over 60% homology with its sibling isoform, MSK-2. Both kinases derive from separate genes and display nuclear localization(Arthur 2008). The highest expression of MSK-1 has been reported in the brain, skeletal muscle and placenta (Deak et al. 1998).

The architecture of the MSK-1 polypeptide is quite unique as it is composed of two kinase domains combined by a linker. The N-terminal kinase domain (NTKD) resembles those belonging to the AGC family of protein kinases (which includes among others Protein kinase A), while the C-terminal kinase domain (CTHD) shares homology with the calmodulin-activated protein kinases. In addition, the C-terminal motif contains a nuclear localization signal (NLS) and a MAPK-docking site. From the functional point of view, the CTKD mediates kinase autophosphorylation and the NTKD targets diverse nuclear proteins (Manning et al. 2002). MSK-1 is involved in the regulation of gene expression. It was shown that MSK-1 mediates phosphorylation of diverse transcription factors, such as CREB and NF-κB (Deak et al. 1998; Vermeulen et al. 2003), as well as various structural proteins, like for instance histone H3 or high mobility group protein-14 (HMG-14) (Soloaga et al. 2003; Thomson et al. 1999b). Furthermore, activated MSK-1 can interact with the transcriptional cofactor CREB-binding protein (CBP) and its paralog p300 (Janknecht 2003).

Single and double MSK knockout animals are viable and fertile. However, they express elevated levels of proinflammatory cytokines in response to Toll-Like Receptor (TLR) triggering due to diminished production of the anti-inflammatory Interleukin-10 (IL-10) by macrophages. This implicates that MSKs serve as negative regulators of TLR-induced inflammation by phosphorylation of CREB and histone H3 at the IL-10 promoter and activation of its gene transcription. Additionally, studies on MSK1/2 knockout revealed that these kinases fulfil an important role in regulation

of a subset of rapidly transcribed genes in a variety of cell types (Arthur and Elcombe 2012).

2.2.2. Nuclear Factor-κB

One of the key transcription factors activated in response to TNF-α is Nuclear Factor-κB (NF-κB). NF-κB is a ubiquitously expressed transcriptional regulator for genes involved in inflammation and immunity as well as other biological processes. The activity of this transcription factor can be also induced by a wide array of other inflammatory and environmental stimuli (Bonizzi and Karin 2004; Hayden and Ghosh 2011). Importantly, deregulation of NF-κB signalling is associated with a long list of inflammatory disorders (Baker et al. 2011; DiDonato et al. 2012). Hence, it is not surprising that NF-κB activation must be precisely coordinated. The activation of NF-κB can be grossly divided into two phases: cytoplasmic and nuclear.

The first phase involves cytoplasmic events culminating in the activation of the Inhibitor of NF-κB Kinase (IKK) signalosome that in turn phosphorylates the IκB inhibitory molecules, hence targeting them for ubiquitin-dependent degradation via the proteasome. The liberated NF-κB complexes enter the nucleus, ending the first phase. Fine-tuning of cytoplasmic NF-κB activation is made possible by the existence of several family members of IKK, IκB and NF-κB.

The second phase occurs in the nucleus and involves the following regulatory mechanisms: an interaction between NF-κB dimers and DNA response elements, epigenetic events at the promoters of NF-κB target genes, interaction of NF-κB with other transcriptional (co)regulators in the enhanceosomes structure, an array of post-translational modifications of NF-κB family members and negative feedback loops (Smale 2011).

Another layer of complexity is imposed via the omnipresent regulatory molecules, called microRNA (miRNA). miRNAs are short RNA of 20-25 nucleotides in length that negatively regulate gene expression by targeting 3'UTR in transcripts. Several miRNAs participate in the degradation of mRNAs that encode upstream regulators and effector as well as key components of the NF-κB signalling cascade. Thus, these powerful molecules orchestrate signalling events by affecting key molecules participating in positive and negative feedback loops. Interestingly, their production can depend on the transcriptional activity of NF-κB as well as other transcription

factors. Finally, deregulation of miRNA production can lead to development of various disorders (Boldin and Baltimore 2012; Ma et al. 2011).

Finally, both cytoplasmic and nuclear events can be shaped by crosstalk with other signalling cascades, which can amplify or attenuate the NF-κB signalling cascade (Chapter II).

All of these regulatory mechanisms are interrelated and determine the strength as well as duration of the NF-κB-dependent transcriptional responses (Smale 2011).

2.2.2.1. Members of the NF-κB signalling cascade

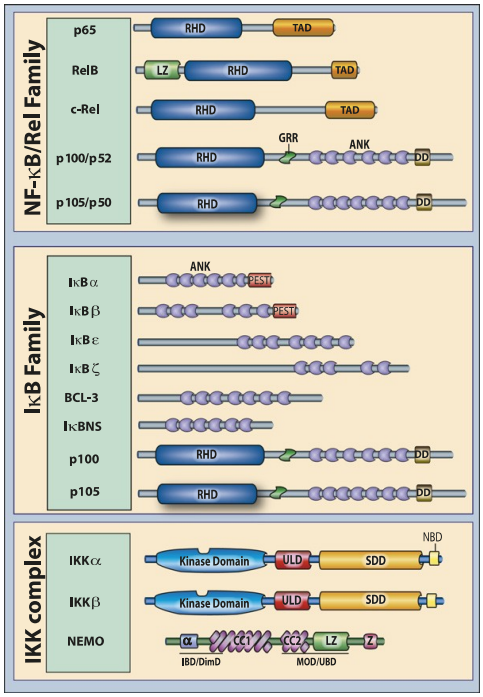

Figure 5. Members of the NF-κB, IκB and IKK families are depicted. RHD, Rel hompology domain; TAD, transactivation domain; LZ, leucine zipper domain; ANK – anykine domains; GRR, glycine-rich region; HLH, helix-loop-helix domain; Z, zinc finger domain; CC1/2, coiled-coil domains; NBD, NEMO-binding domain; MOD/ UBD, minimal dimerization domain and ubiquitin-binding domain; and DD, death domain (Hayden and Ghosh 2012).

NF-κB is actually a generic term for a family of proteins that share a similar structural and functional architecture. Mammals have five NF-κB family members: RelA/p65, RelB, c-Rel, NF-κB1 (p50 deriving from a precursor protein p105) and NF-κB2 (p52 deriving from a precursor protein p100). All of them are characterized by an N-terminal Rel-homology domain (RHD), which is responsible for sequence specific DNA-binding, dimerization, interaction with inhibitory proteins (the IκB family members) and nuclear translocation. Only RelA, RelB and c-Rel possess a C-terminal transactivation domain (TAD) that enables interaction with transcriptional cofactors. NF-κB1 and NF-κB2 contain multiple copies of ankyrin repeats that serve to inhibit their function. These regions are required for proteosomal processing, yielding respectively p50 or p52 that are able to modulate target gene expression (Hayden and Ghosh 2012).

NF-κB regulates transcription as homo- or heterodimers. In the context of inflammatory gene expression, the activity of the p65-p50 NF-κB heterodimer has been the most

extensively explored. However, it is necessary to emphasize that there are fifteen possible NF-κB dimer combinations that regulate a broad spectrum of genes. In general, it is considered that dimers berefted of a TAD acts as transcriptional suppressors, while dimers containing a TAD domain stimulate transcription (Hoffmann et al. 2006).

The transcriptional activity of NF-κB dimers is halted by the IκB inhibitory molecules. The IκB family consists of six members: IκBα, IκBβ, IκBγ, IκBε, IκBζ and Bcl-3 (Hayden and Ghosh 2012). Furthermore, as mentioned before, the precursor molecules NF-κB1 and NF-κB2 can also act as NF-κB inhibitors. All of these proteins contain ankyrin repeats, which cover the nuclear localization sequence (NLS) within the RHD of NF-κB subunits, and hence sequester NF-κB dimers in the cytoplasm. Furthermore, IκBα can complex and "pull out" NF-κB dimers from the nucleus because they contain a nuclear export signal (Arenzana-Seisdedos et al. 1995; Arenzana-Seisdedos et al. 1997). Finally, in the resting state, the NF-κB dimers actually shuttle between the nucleus and the cytoplasm because the NLS is only partially masked by the ankyrin repeats. Of note, the inhibitory proteins Bcl-3 and IκBζ display a rather non-canonical function as they can mediate transcriptional activation upon complexing with p52 and p50 homodimers, hence, acting as transcriptional coactivators (Gilmore 2006; Hayden and Ghosh 2012).

Activation of NF-κB dimers is made possible by the Inhibitor of NF-κB Kinase (IKK) complex, which is essentially composed of two catalytic subunits (IKKα and IKKβ) possessing kinase activity, and a regulatory subunit (IKKγ or NF-κB Essential Modulator (NEMO)). There are two NF-κB signalling pathways, which are distinguished based on the IKK subunits involved: the canonical (IKKβ and NEMO) and the non-canonical (IKKα) cascade. Their triggering is primarily dependent on the stimulus and the cell type (Gilmore 2006; Hayden and Ghosh 2012).

A graphical summary of the domain structures of NF-κB, IκB and IKK families are displayed in Figure 5.

2.2.2.2. NF-κB signalling cascades

As mentioned before, NF-κB activation is achieved via the classical (canonical) or alternative (non-canonical) signalling cascade. In response to TNF-α stimulation, the classical NF-κB signalling cascade is activated.

Activation of the classical or canonical signalling pathway occurs also in response to various other proinflammatory cytokines, ligation of pattern recognition receptors (PRR) or the engagement of T/B cell receptors (TCR/BCR). Subsequently, a cascade of adaptor proteins acting in the proximity of the receptor leads to activation of NEMO and IKKβ, which phosphorylates IκBα inhibitory molecules. This in turn engenders proteasome-dependent degradation of IκBα. Liberated NF-κB dimers, chiefly p65-p50, can then enter the nucleus and drive transcription of various inflammatory mediators (Hayden and Ghosh 2008).

In response to a distinct set of stimuli, such as CD40 or lymphotoxin β, NF-κB activation occurs via the alternative or non-canonical signalling cascade. In that case, the signalling events include IKKα-mediated activation of NF-κB inducing kinase-1 (NIK-1), which induces proteasomal processing of NF-κB1/p100 to p52. p52 forms complexes with RelB and operates at a different pool of NF-κB-dependent promoters, transcribing genes involved in multiple non-inflammatory responses, such as lymphoid development and B cell maturation (Hayden and Ghosh 2008).

Interestingly, it was also described that triggers of the canonical NF-κB pathway, such as TNF-α, lead to IKKα activation. In that context, IKKα phosphorylates a diverse pool of intracellular targets, such as histone H3, promoting expression of TNF-α-responsive genes that are expressed as a result of the classical NF-κB pathway (Anest et al. 2003). Furthermore, IKKα was shown to interact with the transcriptional cofactor CBP enhancing histone H3 acetylation and as consequence NF-κB-dependent transcription (Yamamoto et al. 2003).

Apart from the above-described signalling cascades, various atypical mechanisms exist. For instance, UV exposure launches the p38-dependent activation of casein kinase 2 (CK2), which in turn phosphorylates IκBα molecules leading to the ubiquitinylation and proteasomal degradation of inhibitory molecules and NF-κB activation (Kato et al. 2003; Neumann and Naumann 2007).

Figure 6 presents a scheme illustrating the classical, alternative and atypical NF-κB signalling pathway.

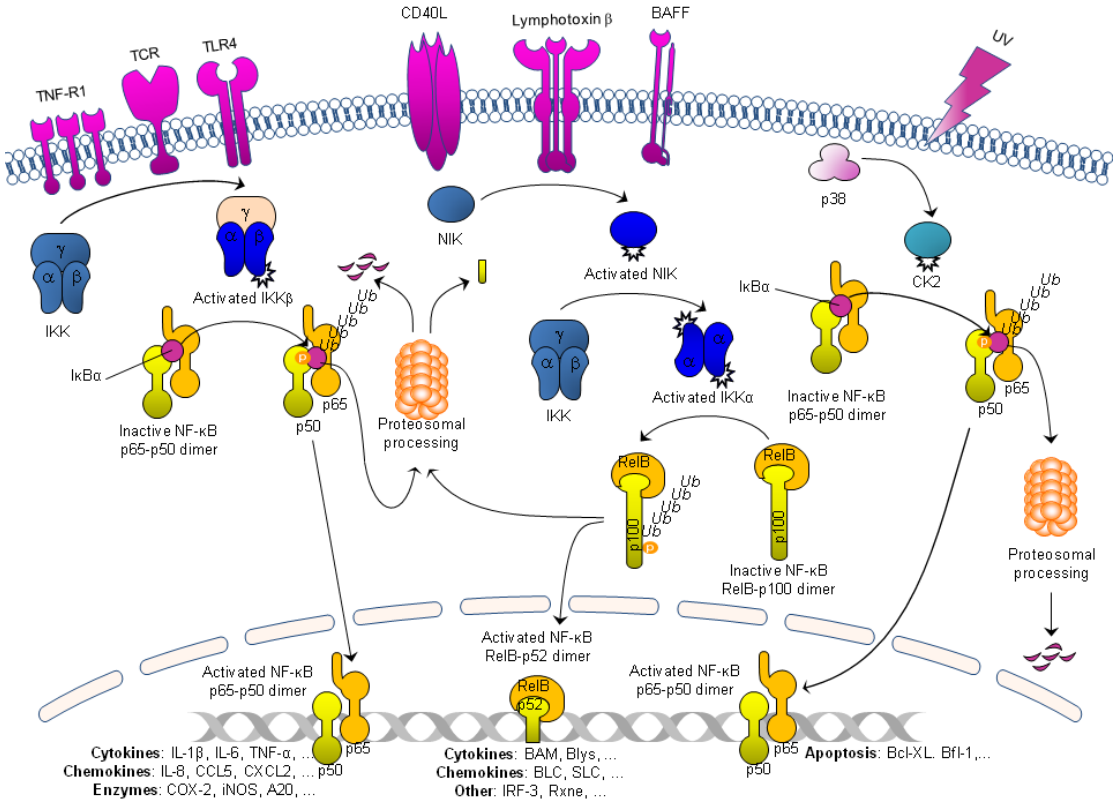

Figure 6. Figure summarizes the main signalling events occurring during the activation of the classical, alternative or atypical NF-κB. (Left) The canonical (classical) NF-κB pathway depends on the site specific phosphorylation of IκBs by activated IKK complex. Phosphorylated IκBs are degraded in proteasome, allowing for nuclear entry of p65-p50 NF-κB dimers. (Middle) The non-canonical (alternative) NF-κB pathway is dependent on NIK-1, which phosphorylates IKKα. Subsequent phosphorylation of p100 by IKKα leads to the proteasome processing of this NF-κB subunit. RelB-p52 NF-κB dimers can then translocate to the nucleus. (Right) In response to cellular stressors, such as UV, NF-κB is activated in atypical manner. In response to UV, p38 becomes activated and triggers CK2 activation which in turn phosphorylates IκBs.

2.2.2.3. Interaction of NF-κB dimers with DNA response elements

A fundamental feature of NF-κB-driven transcription is the selective induction of genes bearing a DNA motif, called a κB element, in their promoter/enhancer(s). Most of these transcription factor binding sites are 10 bp long with the following consensus sequence 5'-GGGRNWYYCC-3', where N – denominates any base; R – purine, W – adenine or thymine; Y – pyrimidine. Furthermore, this regulatory motif can be divided into two functional half-sites depending on the interacting NF-κB subunit. Whereas the first half-site is recognized by p50 and p52 subunits, the second one is targeted by p65, RelB or c-Rel (Hoffmann et al. 2006; Natoli 2009). Despite the transparency and simplicity of this model, recent studies pointed out that NF-κB

dimers interact with a panel of non-canonical DNA binding motif as well as shorter sequences than the classical consensus. For instance, the nonameric CD28 reponse element in the IL-2 enhancer binds the c-Rel NF-κB homodimers with high efficiency. Yet, another example constitutes κB site (AGGAAAGTAC) in the urokinase plasminogen activator gene promoter, which is recognized by the p65-c-Rel NF-κB heterodimers (Natoli et al. 2005). More recently, three distinct groups of NF-κB dimers, which display diverse DNA binding preferences, were described in literature. First group encompasesses p50 and p52 homodimers, which display high affinity for 11-12 bp motifs, second cluster heterodimer that interact with 10 bp motifs, while third contains p65 and c-Rel homodimers that preferentially interacts with 9 bp motifs Recognized consensus for each group is depicted in Figure 7. Furthermore, it has been suggested that transactivation of binding sites for RelA and c-Rel homodimers as well as heterodimers requires interaction with coregulators, while the repression of binding sites recognized by p50 and p52 homodimers is achieved via completion with other transcriptional regulator or recruitment of repressory complex (Siggers et al. 2012; Smale 2012).

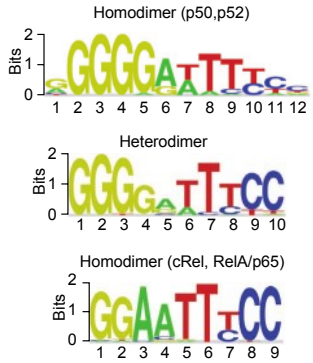

Figure 7. Representative DNA-binding site motifs for each NF-κB dimer class (Siggers et al. 2012).

The interaction between an NF-κB dimer and its binding site is highly specific as targeted deletion of Rel family members cannot be compensated for other subunits to provide comparable level of a gene transcript (Weih et al. 1995). Interestingly, κB elements can function as gene selective allosteric regulators, which are able to induce different conformations in the NF-κB subunits modulating their activity (Chen-Park et al. 2002). These conformational changes, furthermore, can affect binding of additional transcriptional coregulators (Leung et al. 2004). Whereas recruitment of a selected NF-κB dimer to its respective recognition motifs is highly dependent on the stimulus as well as on the promoter context (Hoffmann et al. 2003), some genes recruit all NF-κB proteins with no apparent specificity (Saccani et al. 2003). Finally, the association between NF-κB dimer and DNA sequence is a very dynamic process (Bosisio et al. 2006).

2.2.2.4. Epigenetic events at NF-κB-dependent promoters

In the cell, DNA is organized in chromatin. The fundamental unit of chromatin is the

nucleosome, which consists of a DNA fragment wrapped around an octamer of the four core histones (H2A, H2B, H3 and H4). Binding of nucleosome to a linker histone H1 forms the chromatosome structure. Positioning of these nucleosomes determines the condensation state of chromatin and consequently its transcriptional activity. Whereas condensed chromatin (euchromatin) is regarded as transcriptionally inactive due to steric hindrance for the interaction between transcription factors and their respective DNA motifs, relaxed chromatin (heterochromatin) is highly accessible for transcription factors and concomitantly more prone for the initiation of transcription. Alterations in chromatin structure can be achieved by two coupled mechanisms: the post-translational modifications of histone tails and nucleosome repositioning.

Histones are a family of small, positively charged globular proteins with unstructured N-terminal "tails". The serine, threonine, arginine, lysine, arginine, glutamic acid and proline residues within the histone tails undergo a panel of post-translational modifications by various histone-modifying enzymes. There are at least eight different types of histone modifications, of which acetylation and phosphorylation of lysine and serine/threonine residues have been most intensively studied. These histone modifications define chromatin accessibility via a so-called "histone code". Consequently, there are specific proteins able to read this code. Another layer of complexity is imposed by crosstalk between several types of modifications simultaneously deposited on the histones' tail contributing to the fine-tuning of transcriptional response (Kouzarides 2007).

2.2.2.4.1. Chromatin remodelling complexes

NF-κB-dependent genes can be grossly divided into two groups depending on their requirement for chromatin remodelling complexes to enable gene expression: the constitutive/immediate accessible genes and the regulated/late accessible genes. Whereas the first group does not depend on chromatin remodelling events, the transcription of the second group is preceded by the recruitment of the chromatin remodelling machinery (Natoli 2009; Smale 2010; Vanden Berghe et al. 2006). Changes in the chromatin environment are executed by chromatin remodelling complexes, such as Switch/Sucrose Non-Fermentable (SWI/SNF), that mediate a shift in nucleosomes position using energy deriving from ATP hydrolysis (Tang et al. 2010). In line, silencing of the SWI/SNF subunits, namely Brg1 and Brm1, resulted in a drop of the transcription rate of all late NF-κB-dependent genes, such as IL-6 or

chemokine (C-C motif) ligand-5 (CCL-5), while expression of immediate early genes, such as chemokine (C-X-C motif) ligand-2 (CXCL-2) or TNF-α, remained unaffected. Hence, it was proposed that the SWI/SNF complex induces nucleosome repositioning to generate a relaxed chromatin environment easily accessible for NF-κB culminating in a potent transcriptional response (Ramirez-Carrozzi et al. 2006).

2.2.2.4.2. Histone phosphorylation

Nowadays, a clear connection has been established between histone phosphorylation and positive regulation of gene expression (Kouzarides 2007). It is postulated that histone phosphorylation mediates changes in nucleosome and chromatin structure by altering the histone-DNA interface, thereby facilitating access for transcription factors to the underlying DNA sequence. One of the histone marks with a crucial role in transcriptional regulation is phosphorylation of histone H3 at serine 10. In yeast for example, this modification stimulates transcription by promoting subsequent acetylation (Lo et al. 2000; Lo et al. 2001). Similarly, in higher eukaryotes, phosphorylation of histone H3 induces other covalent modifications of the histone H3 tail, such as acetylation of lysine 14 (S. J. Nowak and Corces 2004). Relatively few interactors of this modification have been identified. In mammals, the histone H3 serine 10 mark is recognized by a domain within the 14-3-3 protein, which is a sequence-dependent phosphoserine/phosphothreonine motif-binding protein (Macdonald et al. 2005; Walter et al. 2008; Winter et al. 2008b; Winter et al. 2008a). Interaction of this protein with phosphorylated histone H3 engenders enrichment of SWI/SNF chromatin-remodelling complexes at the gene promoter (Drobic et al. 2010), followed by enhanced recruitment of RNA polymerase II (Vicent et al. 2006).
Several kinases, including Protein kinase A (PKA), IKKα and MSK-1, can phosphorylate histone H3 serine 10. Cessation of their activity by means of pharmacological inhibitors or gene silencing was shown in multiple studies to disrupt histone phosphorylation and concomitantly suppress or substantially affect the profile of gene expression (Anest et al. 2003; DeManno et al. 1999; Drobic et al. 2004; Drobic et al. 2010; G. Y. Park et al. 2006; Salvador et al. 2001; Soloaga et al. 2003; Thomson et al. 1999a; Thomson et al. 1999b; Yamamoto et al. 2003). Finally, p38 MAPK-mediated phosphorylation of histone H3 at serine 10 on a subset of NF-κB-dependent promoters, such as IL-8 and CCL2, augments the accessibility of NF-κB transcription factor binding sites. Oppositely, histone H3 at the IκBα promoter

undergoes rapid phosphorylation that is independent of p38 MAPK, suggesting the existence of alternative mechanisms to efficiently "fine-tune" gene expression at other genes populations (Saccani et al. 2002).

2.2.2.4.3. Histone acetylation

Prototypically histone acetylation is almost exclusively associated with transcriptional activation, while deacetylation correlates chiefly with gene repression. Deposition or removal of acetyl groups is mediated via distinct families of enzymes, termed respectively histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs can be grouped into three main families: GNAT, MYST and CBP/p300. In general, they are promiscuous regarding the targeted residue but in some cases limited specificity has been described. HDACs are also divided into three distinct families: the class I HDACs, the class II HDACs and the class III NAD-dependent enzymes of the Sir family. In general, these enzymes are present in various repressive complexes. Finally, histone acetylation is recognized via proteins possessing a bromodomain, which in turn can recruit chromatin-remodelling complexes (Kouzarides 2007).

Some of the above-described factors were shown to be inducibly recruited to NF-κB-regulated promoters and mediate chromatin opening/shutting. For instance, CBP, p300 and p300/CBP-associated factor (PCAF) enhance transcriptional rate by acetylation of histone H3 and H4 at the E-selectine, Vascular Cell Adhesion Molecule-1 (VCAM-1), TNF-α and cyclooxygenase-2 (COX-2) promoters upon TNF-α stimulation (Edelstein et al. 2005; Lee et al. 2006; Miao et al. 2004). On the other hand, HDAC1 and HDAC2 inhibit IL-8 gene expression by the removal of acetylmoieties from histone tails within surrounding chromatin (Ashburner et al. 2001; Zhong et al. 2002). In the context of NF-κB-dependent gene expression, acetylation of histone H3 on lysine 9 and lysine 14 has been reported in literature (Anest et al. 2003; Ashburner et al. 2001; Saccani et al. 2002; Yamamoto et al. 2003).

2.2.2.4.4. Histones phosphoacetylation

The transcriptional process can be effectively controlled and highly fine-tuned via deposition of several covalent modifications on a single histone tail, which can be "read" by various proteins in the frame of the "histone code". In line, signal-induced histone phosphoacetylation has been reported in a number of cellular systems using diverse stimuli (Clayton and Mahadevan 2003). Although the precise function of this

dual histone modification is unknown, it probably serves as a highly specific mark for the recruitment of chromatin remodelling complexes that are necessary for the removal of transcriptional barriers and repressors (Mateescu et al. 2004) as well as recruitment of coactivators that are required for concomitant transcriptional activation (Winter et al. 2008b; Winter et al. 2008a). It was also demonstrated that the synergistic coupling of histone phosphorylation and acetylation at promoters occurs in a highly coordinated fashion to ensure high rates of transcription. For instance, histone H3 phosphorylation can affect the efficiency of a subsequent acetylation reaction as the GCN5 HAT exhibits 10-fold higher preference for phosphorylated over nonphosphorylated histone H3 (Cheung et al. 2000). However, not always the prior presence of phosphorylation at a histone tail marks it for subsequent acetylation (Clayton and Mahadevan 2003).

2.2.2.5. NF-κB-dependent enhanceosomes

The association of multiple proteins with gene promoters and enhancers tightly regulates transcription of genetic information. Whereas the promoter localizes in the close proximity of a gene's transcription starting site, enhancers are usually embedded more distal from the transcription starting site. These two regulatory elements are usually separated in the genomic sequence by many thousands of base pairs. However, promoter and enhancer can be brought into proximity via looping. This physical vicinity between regulatory regions in a native nuclear environment contributes to potent transcriptional activation (Nolis et al. 2009). Both promoter and enhancer contain a collection of short DNA motifs that are recognized by sequence specific transcription factors. These factors are instrumental for the recruitment of additional cofactors, either coactivators or corepressors, as well as components of the basal transcriptional machinery. By contrast to transcription factors, accessory proteins do not directly interact with DNA sequence but have other functions that are indispensable for efficient and precisely regulated transcription (Smale 2011).

Bazett-Jones et al. (Bazett-Jones et al. 1994) were the first to propose that efficient gene expression requires the assembly of an "enhanceosome". This three dimensional and highly dynamic structure is created by cooperative binding of transcription factors to the control regions in the promoter/enhancer regions of a given gene. Together, these transcriptional regulators via direct interaction with the DNA sequence form a platform for the recruitment of additional cofactors, chromatin remodelling

complexes and general transcription factors, that is essential for a stringent and tightly tailored transcriptional response driven by RNA polymerase II (Merika and Thanos 2001).

One of the best-characterized enhanceosomes is the one formed at the regulatory regions of the virus-triggered IFN-β. Expression of the IFN-β gene requires concomitant activation and cooperative binding of several transcription factors, including ATF-2, c-Jun, isoforms of Interferon Regulatory Factor-3 (IRF-3) and IRF-7 as well as the NF-κB p65-p50 heterodimer, to the respective DNA-response elements within a 55 bp nucleosome-free region of the IFN-β promoter (Agalioti et al. 2000; Maniatis et al. 1998). The architectural protein HMG-I/Y is also important for enhanceosome assembly. It facilitates binding of transcriptional factors to unbended DNA sequence by mimicking the fully assembled complex. HMG-I/Y is, however, displaced from the final enhanceosome complex by other transcriptional regulators due to steric hindrance (Falvo et al. 1995; Thanos et al. 1993). The nucleosome-free region of the IFN-β promoter is flanked by two nucleosomes that bind respectively in the vicinity of the TATA box and the transcription starting site. Enhanceosome formation is associated with the recruitment of transcriptional coregulators CBP/p300 and GCN5, which interact with each of the predeposited transcription factors through distinct protein-protein interactions. Whereas CBP/p300 mediates histone acetylation, GCN5 promotes displacement of the nucleosome masking the TATA box via recruitment of the SWI/SNF chromatin-remodelling complex. These events enable access by the transcription factor II D (TFIID) complex that contains the TATA-binding protein (TBP), along with other general transcription factors and the RNA polymerase II to the IFN-β enhancer (Agalioti et al. 2000; Merika and Thanos 2001). Subsequent crystallography studies provided significant insight into DNA-protein and protein-protein interactions occurring within the IFN-β enhanceosome (Figure 8). These studies clearly demonstrated that virtually every nucleotide matters for the IFN-β enhancer activity by creating a composite surface for the eight transcriptional regulators: ATF-2, c-Jun, IRF-3A, IRF-7B, IRF-3C, IRF-7D, p50 and p65 (Panne 2008).

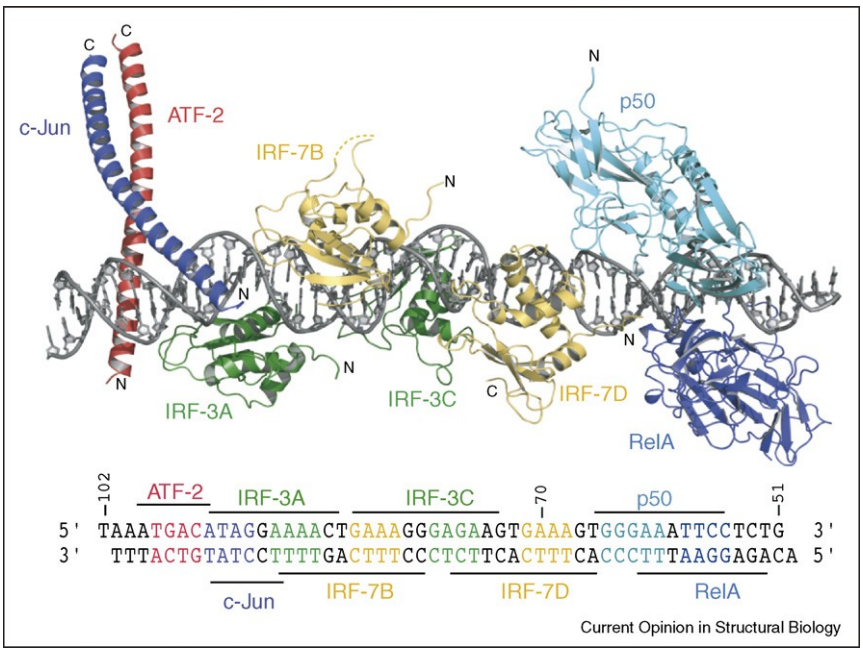

Figure 8. The model of INF-β enhanceosome (Panne et al. 2007). The p50 is in light blue and RelA in dark blue. IRF-7B and IRF-7D are in yellow and IRF-3A and IRF-3C are in green. ATF-2 is in red and c-Jun in blue. The DNA sequence is shown with the core-binding sites coloured accordingly.

A similar enhanceosome architecture has been proposed for various other NF-κB-dependent gene promoters, such as IL-6 (Vanden Berghe et al. 1999), CXCL-1 (Amiri and Richmond 2003; Amiri et al. 2006; Ueda et al. 2007), IL-8 (Amiri and Richmond 2003) (Figure 9).

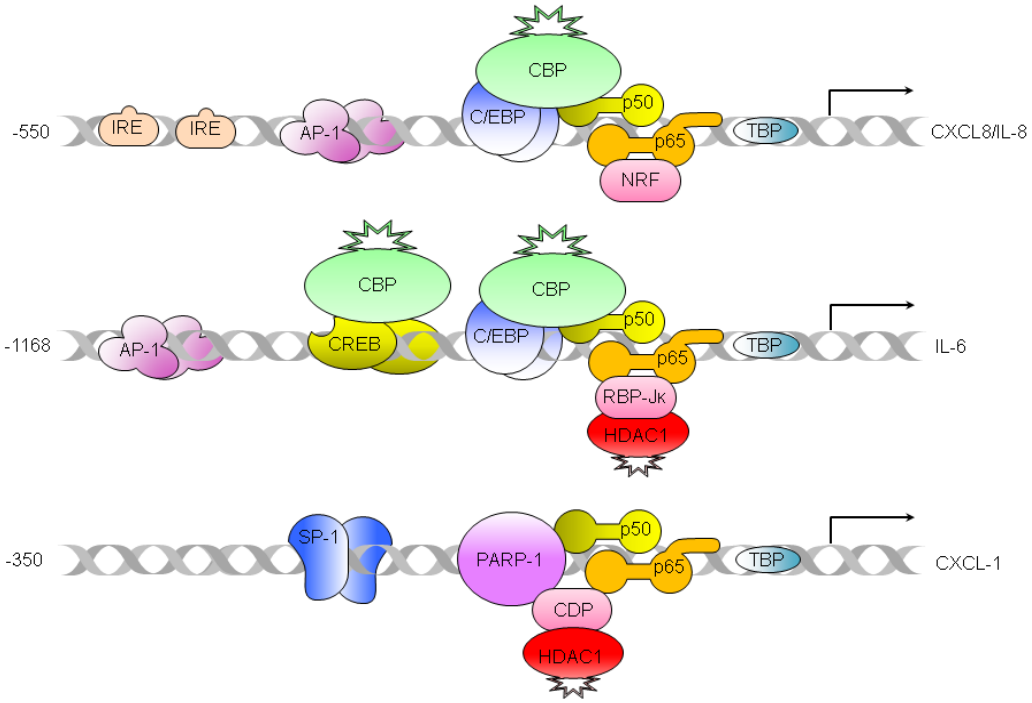

Figure 9. Schemes of promoter architecture of prototypical NF-κB target genes for which the enhanceosome structure is well characterized or being extensively explored.

The central role in all of these complexes is played by NF-κB, which enables transcriptional activation via the recruitment of the transcription cofactor CBP/p300. CBP/p300 functions among others as a scaffold protein that brings together diverse groups of transcriptional factors, cofactors, and components of the basal transcriptional machinery and recruits chromatin-remodelling complexes increasing their relative concentration in the transcription area. Furthermore, CBP/p300 takes advantage of either its intrinsic HAT activity or other HATs assembled in multiprotein complexes to modulate the strength of these protein-protein and/or protein-DNA interactions via deposition of acetyl moieties on enhanceosome components. Both bridging and acetyltransferase activity substantially contribute to upregulation of target gene expression (Chan and La Thangue 2001; McManus and Hendzel 2001). Another feature of these NF-κB-dependent enhanceosomes is transcriptional cooperation of NF-κB with other transcription factors, such as HMG, CCAAT/enhancer-binding protein (C/EBP), ATF, CREB, IRF, Specificity Protein-1 (SP-1) and/or AP-1, to ensure potent gene expression (Amiri and Richmond 2003; J. M. Park et al. 2005; Spooren et al. 2010; Ueda et al. 2007).

2.2.2.6. Post-translational modifications of NF-κB

Activity of all NF-κB subunits is fine-tuned by a broad spectrum of post-translational modifications (Perkins 2006). Here, we will focus on the modifications important in TNF-α signalling cascade. In that context, the role of phosphorylation and acetylation in the modulation of p65 transcriptional activity has been the most extensively explored. The NF-κB p65 subunit possesses a number of amino acid residues, localized within the RHD and TAD, which are targets for post-translational modification (Figure 10). These reversible modifications regulate the intracellular localization, dimerization, DNA binding ability, transcriptional potential and protein-protein interactions of NF-κB subunits (Calao et al. 2008; Vermeulen et al. 2006).

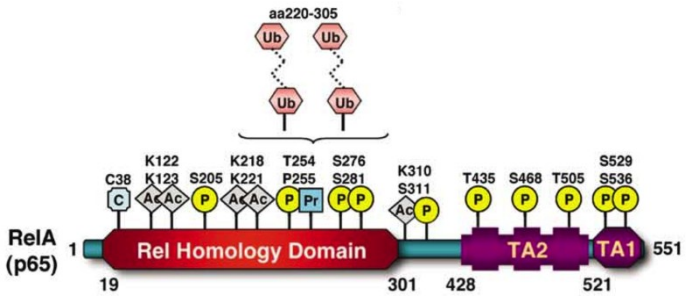

Figure 10. Scheme presenting different described post-translational modifications of the p65 NF-κB subunit (Perkins 2006).

2.2.2.6.1. Phosphorylation of p65 at serine 276

Phosphorylation of serine 276, a residue embedded within the RHD of p65, is instrumental for the efficient transcription of a specific pool of NF-κB target genes, which do not constitutively associate with RNA polymerase II (D. E. Nowak et al. 2008). Initially, this post-translational modification was described to be deposited by the lipopolysaccharide (LPS)-activated PKAc (Zhong et al. 1997) but further studies demonstrate that it is also imposed by MSK-1 upon activation of upstream p38 and ERK1/2 MAPK in TNF-α stimulated cells (Vermeulen et al. 2003).

Overall, serine 276 phosphorylation of p65 augments the NF-κB transcriptional activity by disrupting the intramolecular association of the N- and C-terminal ends (Zhong et al. 1997), producing in turn conformational changes that promote association with CBP/p300. Together, p65 and CBP/p300 ensure potent induction of NF-κB-dependent genes (Zhong et al. 1998). Subsequently, another study demonstrated that serine 276 phosphorylation of p65 promotes displacement of the repressive complex, composed of p50-p50 NF-κB homodimers and HDAC1, from the gene promoters (Zhong et al. 2002).

Significant insight into the importance of p65 serine 276 phosphorylation was provided by two *in vivo* studies using knock-in mice. In one study, Dong et al. (Dong et al. 2008) demonstrated that constitutive inactivation of p65 by serine to alanine mutation leads to suppression of a subset of NF-κB target genes due to recruitment of HDAC3. Interestingly, the result of the HDAC3 recruitment was epigenetic suppression of genes positioned in the vicinity of NF-κB-binding sites. These molecular changes manifested themselves in embryonic death due to variegated developmental abnormalities. Opposite study performed by the same group (Dong et al. 2010) demonstrated that constitutive activation of p65 by serine to aspartic acid mutation of serine 276 leads to enhanced expression of NF-κB-dependent inflammatory mediators, including several cyto-/chemokines. These mice eventually died because of amplification of the inflammatory state. Strikingly, the inflammatory phenotype could be partially reversed by crossing the p65 knock-ins with the TNF-R1 deficient mice indicating the destructive potential of the TNF-α/NF-κB axis. Nevertheless, some effects are independent of TNF-α as deleterious effects of NF-κB overactivity become again apparent upon aging of these rescued mice, which suffer from chronic keratitis accompanied by increased corneal expression of several inflammatory mediators.

2.2.2.6.2. Phosphorylation of p65 at serine 536

Phosphorylation of p65 at serine 536, which localizes within the TAD, is another modification that modulates the NF-κB transcriptional responsiveness. It is mediated by multiple kinases IKKα, IKKβ or TANK binding kinase-1 (TBK-1) upon TNF-α treatment (Sakurai et al. 1999a; Sakurai et al. 1999b).

It was shown that p65 phosphorylation on serine 536 is pivotal for NF-κB transcriptional potential. Similarly to serine 276 phosphorylation, phosphorylation at serine 536 induces conformational changes in the p65 NF-κB subunit, modulating its interaction with transcriptional cofactors (L. F. Chen et al. 2005b). For instance, phosphorylation of serine 536 has been reported to promote p65 association with the transcriptional coactivator CBP/p300, while hampering binding with the Silencing Mediator for Retinoic acid receptor and Thyroid hormone receptor (SMRT) transcriptional corepressor (Buss et al. 2004; L. F. Chen et al. 2005b; Hoberg et al. 2006; Sasaki et al. 2005). Finally, this modification inhibits the reassociation of IκBα with DNA-bound NF-κB, hence, hampering its nuclear export and consequently regulating the duration of the NF-κB-dependent transcriptional response (Adli and Baldwin 2006; Sasaki et al. 2005).

Finally, it is not well understood whether phosphorylation of p65 at serine 536 by IKKβ is part of the activation mechanism that is required for the nuclear entry of NF-κB dimers (Perkins 2006).

2.2.2.6.3. p65 Acetylation

The p65 subunit of NF-κB is acetylated at multiple lysine residues, including lysine 122, 123, 218, 221, 310, 314 and 315, which are spread across the protein chain. The majority of these lysines are targeted by CBP/p300, but some can be modified by PCAF or members of the p160 family of steroid-receptor coactivators (SRC-1) in response to various stimuli, including TNF-α (Calao et al. 2008).

Acetylation of p65 regulates distinct nuclear properties of NF-κB. CBP/p300-mediated acetylation of p65 at lysine 218 and 221 specifically enhances its DNA binding ability and impairs assembly with IκBα (with a pivotal role played by lysine 221), while modification of lysine 310 is required for full transcriptional activity (L. F. Chen et al. 2002). Conversely, HDAC3-mediated deacetylation of these residues is involved in termination of the NF-κB-dependent transcriptional responses by the reassociation with IκBα and subsequent nuclear export of newly formed complexes

(L. Chen et al. 2001; L. F. Chen et al. 2002). Furthermore, TNF-α-induced phosphorylation of p65 at either serine 276 or 536 promotes its subsequent association with CBP/p300 that in turns acetylates p65 at lysine 310. Combination of these two modifications (phosphoacetylation) at a single transcription factor is required to achieve full transcriptional activation. These specific marks deposited on the p65 subunit of NF-κB correspond to a "transcription factor code" that governs the recruitment or displacement of selected transcriptional cofactors that contribute to the transcriptional activity (L. F. Chen et al. 2005b; Hoberg et al. 2006). Oppositely, acetylation of p65 at lysine 122 and 123 by CBP/p300 or PCAF diminishes p65 DNA binding ability and facilitates its interaction with IκBα (Kiernan et al. 2003). Finally, acetylation of p65 at lysine 314 and 315 was observed to have no effect on interaction with DNA and intracellular localization (Buerki et al. 2008). It is noteworthy that in response to TNF-α, p300 acetylates various p65 residues (Perkins 2006), which in tune differentially regulate the expression pattern of NF-κB target genes (Buerki et al. 2008; Kiernan et al. 2003).

2.2.2.7. Negative feedback mechanisms

Cessation of NF-κB transcriptional activity occurs via various negative feedback loops.

The most common mechanism involves synthesis of negative regulators. The prototypical example is IκBα, an NF-κB target gene and powerful inhibitor of its signalling cascade. The protein, upon synthesis, enters the nucleus, associates with DNA-bound NF-κB complexes and drives them to the cytoplasm, terminating in that way the transcriptional response. This process is also tightly linked with the removal of post-translational modifications, such as phosphorylation of p65 serine 536 (Adli and Baldwin 2006; Sasaki et al. 2005) and acetylation of p65 lysine 221 (L. Chen et al. 2001; L. F. Chen et al. 2002), which impede association with the inhibitory molecules.

Another important protein, which turns off the NF-κB signalling cascade, is TNF-α-induced protein 3 (A20). Its expression is also upregulated via NF-κB-dependent mechanisms. The protein by itself is a ubiquitin-editing enzyme that possesses a deubiquitinase and an E3 ligase domain. A20 interacts with RIP-1 and using the enzymatic activity removes the K63-ubiquitin chain and subsequently attaches a K48-ubiquitin chain, targeting RIP-1 for proteosomal degradation (Wertz et al. 2004).

More recently, A20 was demonstrated to interact with other receptor adaptor proteins, such as TRAF-2, as well as other downstream components of the NF-κB signalling pathway, like for instance NEMO. Finally, it acts downstream multiple receptors able to activate NF-κB signalling cascade (Heyninck and Beyaert 2005).
Alternatively termination of NF-κB-dependent gene expression occurs via proteasome-dependent degradation of NF-κB dimers occupying promoters of target genes (Saccani et al. 2004) or exchange of NF-κB dimers interacting with promoters of target genes (Saccani et al. 2003).

2.2.2.8. Physiological and pathological role of NF-κB

NF-κB is a pivotal transcription factor regulating expression of genes involved in inflammation and immunity. Activation of this powerful transcription factor upregulates expression of various effector molecules, such as cytokines, chemokines, enzymes, signalling molecules, receptors, receptor ligands, adhesion molecules and many others. Several of these gene products directly activate the NF-κB signalling cascade creating a positive feedback loop that promotes amplification of inflammatory response and prolongs its duration. In line, lack of NF-κB transcriptional activity leads to higher susceptibility to infections, augmented severity of disease processes and the inability to resolve inflammation and restore homeostasis. Furthermore, NF-κB is known to govern several other cellular processes, such as proliferation, differentiation and apoptosis by driving expression of genes involved in cell cycle and cell death (Hayden and Ghosh 2011; Lawrence 2009). An extract from the long list of NF-κB targets can be found in Table 1.
Given the broad spectrum of cellular processes under the control of NF-κB, it is not surprising that deregulation of this powerful transcription factor is tightly associated with a variety of disease processes, such as autoimmune disorders, chronic inflammatory diseases and cancer. Indeed, whereas coordinated activation of NF-κB is beneficial in terms of whole body homeostasis, its aberrant activation has deleterious effects (Courtois and Gilmore 2006; Karin 2006; Sarkar et al. 2008).
With respect to autoimmune and chronic inflammatory disorders, NF-κB-dependent cyto-/chemokines attract and activate various immune cells at the site of inflammation augmenting the severity of the inflammatory process. Concomitantly, the same products further amplify NF-κB activity via a positive feedback loop (Karin et al. 2006; Tak and Firestein 2001). Recently, it has become evident that tumour

development is tightly associated with the generation of an inflammatory microenvironment. In that context, NF-κB was shown to drive expression of genes stimulating cell growth (e.g. IL-6, TNF-α) and division (e.g. cyclin D1, Ephrin) as well as tumour malignancy (e.g. MMP-2, MMP-9) and angiogenesis (e.g. VEGF). In addition, NF-κB governs expression of pro-survival genes (e.g. cIAP1, Bcl-XL) resulting in augmented therapy resistance of malignant cells (DiDonato et al. 2012).

Cytokines	IL-1α, IL-1β, IL-1ra IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, IL-23, IL-27, TNF-α, INF-β,
Chemokines	CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-3, CXCL-5, IL-8/CXCL-8, CXCL-10, eotaxin, G-CSF, M-CSF, GM-CSF,
Enzymes	iNOS, COX-2, MnSOD, MMP-2, MMP-9, elastase, MuRF1
Receptors and signalling factors	CD40, CD86, c-IAP1, c-IAP-2, TRAF-1, TRAF-2, IκBα
Adhesion molecules	CD44, ICAM-1, VCAM-1, P-selectin, Fibronectin,
Cell growth and division	Cyclin D1, Cyclin D2, Cyclin D3, Ephrin
Cell death	Bax, Bcl-2, Bcl-xL, Bim, caspase-11, Fas, FasL, c-Flip

Table 1. Examples of prototypical NF-κB target genes grouped in functional categories. For more extended list, it is advised to refer to an online resource available at http://www.bu.edu/nf-kb/gene-resources/target-genes.

Deregulation of NF-κB activity might occur at all levels of the signalling cascade. Principally, it involves augmented activation of the IKK signalosome, diminished level of IκB inhibitory molecules and elevated transcriptional activity of NF-κB. Furthermore, it couples with the ameliorated stimulation of positive feedback loops or diminished activity of negative feedback mechanisms. Perturbations of these processes have been observed in a number of inflammatory disease processes (Hayden and Ghosh 2011; Lawrence 2009). In this thesis, we have studied the molecular mechanism associated with NF-κB activity in skeletal muscle and brain. The pathological role of NF-κB will be illustrated on these organs. For instance, aberrant activation of NF-κB signalling pathway from immune and muscle cells has been detected and implicated in the pathogenesis of numerous muscular dystrophies and myopathies (Jackman et al. 2013; Mourkioti and Rosenthal 2008; Peterson and

Guttridge 2008). Interestingly, enhanced NF-κB activity has also been detected in muscle biopsies obtained after an acute boost of exercises (Kramer and Goodyear 2007). In the central nervous system (CNS), deleterious effects of Alzheimer's disease, Parkinson disease, and multiple sclerosis are associated with aberrant activation of the NF-κB signalling axis and deregulation of its transcriptional program (Camandola and Mattson 2007; Kaltschmidt and Kaltschmidt 2009; Memet 2006). Enhanced activation of NF-κB is also tightly linked to development of various metabolic disorders, such as atherosclerosis, diabetes and obesity (Baker et al. 2011).

2.3. Role of the TNF-α/TNF-Rs axis in immunity

TNF-α-induced signals launch several downstream transcription factors, which in turn drive the expression of various genes involved in inflammation. Examples include inflammatory cytokines (e.g. IL-6, IL-1β, TNF-α), chemokines (e.g IL-8, CCL-5, CXCL-5), receptors (e.g. TNF-R1, TNF-R2), adhesion molecules (e.g. VCAM-1, P-selectin), enzymes (e.g. COX-2, inducible NO synthase (iNOS)), acute phase proteins (e.g. C reactive protein (CRP)) and matrix metalloproteases (e.g. MMP-2, MMP-9). Stringently controlled expression of these immunomodulatory mediators is critical for resolution of infection and restoration of homeostasis (Feldmann and Steinman 2005). Studies on TNF-R-deficient mice have shed light on the role of TNF-α in the immune system. TNF-R1 signalling is crucial for the development and maintenance of lymphoid organs (Hehlgans and Pfeffer 2005). Furthermore, cessation of TNF-R1 signalling sensitizes animals to infection with several pathogens, including bacteria, yeast and certain viruses (Ehlers et al. 2000; Everest et al. 1998; Flynn et al. 1995; Laichalk et al. 1996; Pasparakis et al. 1996; Pavic et al. 1993; Rothe et al. 1993). The TNF-α/TNF-R1 axis is required for the activation of various immune cells, such as lymphocytes and neutrophils. It also stimulates the formation and maintenance of granulomas, in which macrophages limit pathogen growth, at the site of inflammation. TNF-α induces expression of chemokines and cell adhesion molecules, which enable navigation of leukocytes and their migration through endothelium to the "inflamed tissue" (Kneilling et al. 2009). Genetic deletion of TNF-R1 precludes formation of splenic primary B cell follicles as well as the organization of follicular dendritic cell networks and germinal centres (Pasparakis et al. 1996). Moreover, TNF-α regulates proliferation and differentiation of various immune cell-types, such as T-cells into Th-cells (Ehlers 2003) and drives the humoral immune response to T-cell-

dependent antigens (Pasparakis et al. 1997; Rothe et al. 1993). The biological activity of TNF-α is manifested during the inflammatory process through a number of clinical symptoms, such as vasodilatation, oedema, blood coagulation and fever (Zelova and Hosek 2013). Altogether, these findings indicate that TNF-α shapes the outcome of the innate and adaptive immune response.

Although TNF-R2 signalling has not received as much attention as the one downstream of TNF-R1, recent evidence indicates that the tmTNF-α/TNF-R2 axis also plays an important role in the regulation of immunity. For instance, activation of T cells (Aspalter et al. 2003; E. Y. Kim and Teh 2004) as well as proper proliferation and function of T regulatory cells (X. Chen et al. 2007) are dependent on signalling downstream of TNF-R2. Moreover, tmTNF-α plays a non-redundant role in the dendritic cells-mediated activation of natural killer (NK) cells (Xu et al. 2007).

Finally, the above-mentioned reports point out some important and non-redundant immunomodulatory functions of the TNF-α/TNF-R axis that cannot be compensated for other proinflammatory factors.

2.4. Pathophysiological role of TNF-α

Although TNF-α plays a crucial role in the normal host immune response, uncontrollable TNF-α expression and signalling contributes to the pathogenesis of several chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, inflammatory bowel disease, inflammatory myopathies and various neurodegenerative disorders. In all of these conditions, TNF-α mediates various deleterious effects promoting disease development. Furthermore, elevated levels of the cytokine in serum correlate with pathological changes in whole body metabolism, such as insulin resistance and type II diabetes (Bradley 2008; Clark 2007; De Paepe et al. 2012; Montgomery and Bowers 2012; Van Hauwermeiren et al. 2011).

As the TNF-α/TNF-R axis is disrupted in a number of pathological conditions, it is an excellent target for development of immunomodulatory therapies. Currently, four monoclonal TNF-α antibodies (infliximab, adalimumab, certolizumab and golimumab) and one TNF-R Fc fusion protein (etanercept) are on the pharmaceutical market. All of these inhibitors are among the most successful protein-based drugs, showing a remarkable clinical efficacy against various chronic inflammatory disorders, such as arthritis, psoriasis, Crohn's disease and ankylosing spondylitis. (Croft et al. 2013; Kontermann et al. 2009). Although TNF-α antagonistic approaches

are becoming a part of standard therapy against arthritis and inflammatory bowel disease, several obstacles push researchers towards further improvement of these therapeutic strategies. An important subpopulation of patients exists that is refractive to anti-TNF-α treatment. Furthermore, long-term administration of antagonists promotes side effects, such as a greater predisposition to infection, congestive heart failure, neurologic changes or development of autoimmune disorders and tumours. Thus, it was proposed that selective targeting of the proinflammatory sTNF-α/TNF-R1 axis, while keeping the immunomodulatory tmTNF-α/TNF-R2 axis intact, could be much more beneficial in the treatment of inflammatory disorders with elevated levels of TNF-α. Finally, the widespread clinical use of anti-TNF-α monoclonals is hampered by their complicated route of administration (injection) and relatively high cost (D. Tracey et al. 2008).

3. Adrenergic signalling

Norepinephrine and epinephrine are mediators of the "fight or flight response". The principle aim of this evolutionary conserved reaction is to prepare the "stressed subject" to escape or surmount the threat by eliciting immediate and fairly brief changes in the functioning of individual organs, tissues and cell types. With a few exceptions, the two hormones exert the same effects. The action of epinephrine is manifested in a stimulation of metabolic activities, bronchial dilatation, and increased blood flow to skeletal muscles and heart, while norepinephrine has the greater influence on peripheral vasoconstriction and blood pressure. Overall, the ultimate aim of these changes is to deliver maximum energy and oxygen to muscles for combat or escape.

The release of epinephrine and norepinephrine in response to various psychosocial or environmental stressors is a tightly regulated physiological process. Perception of "stress" by the high cortical centres of the brain initiates a series of impulses, which are relayed to the limbic system to release various neuromodulators, such as norepinephrine. These neuromodulators activate the hypothalamus to secrete corticotropin-releasing factor (CRF), which is the primary coordinator of the stress response as it activates the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). CRF stimulates the pituitary gland to produce proopiomelanocortin (POMC), a polyprotein that is subsequently cleaved to form adrenocorticitropic hormone (ACTH), β-endorphin, and α-melanocyte stimulating

hormone (α-MSH). Concomitantly, CRF also triggers the locus coeruleus, a dense collection of neuron cells in the brain, to release norepinephrine from the sympathetic nerves axonal terminals. The action of norepinephrine and ACTH converges at the level of the chromaffin cells in the adrenal medulla, which are responsible for production of epinephrine and small amounts of norepinephrine (P. H. Black 2002).

At the cellular level, the production of norepinephrine and epinephrine is also precise controlled. These neuroendocrine factors belong to a group of monocatecholamines. Both of them are synthesized from the amino acid tyrosine in a multistep biochemical pathway. Briefly, tyrosine is converted by tyrosine hydroxylase into L-3,4-di-hydroxy-phenylalanine (L-DOPA). L-DOPA undergoes decarboxylation via activity of aromatic L-amino acid decarboxylase to produce dopamine. Hydroxylation of dopamine via dopamine β-hydroxylase yields in turn norepinephrine. Subsequent methylation of the amine group in norepinephrine by phenylethanolamine N-methyltransferase generates the homologous catecholamine epinephrine. The fate of norepinephrine does not finish upon release from the axonal terminal as this catecholamine may undergo rapid uptake by sympathetic nerve terminal, hence resulting in the termination of the response. “Recycled” norepinephrine can be metabolized by monoamine oxidase in neuronal cells or catechol-O-methyltransferase in non-neuronal cells, rendering its inactive forms (Kvetnansky et al. 2009).

It was also postulated since the earliest days of medicine that the “mind” can affect the severity and course of physical illness. Today, this classical paradigm is supported by multiple lines of evidence showing that the brain and immune system are indeed interconnected through the HPA axis and the SNS. Each route involves the secretion of various hormones, including epinephrine and norepinephrine, which in turn modulate, mostly suppress, multiple aspects of both innate and adaptive immune response, by acting on a wide variety of immune organs and cell types (Elenkov et al. 2000; Ley et al. 2010; Nance and Sanders 2007; Padro and Sanders 2014; Powell et al. 2013; Sanders 2012). Furthermore, diverse non-immune cell types, such as glial cells display immune-like behaviour, are also targets of norepinephrine and epinephrine (Marino and Cosentino 2013). Finally, the communication between the neuroendocrine and immune system is bidirectional, meaning that the immune system is able to instruct the brain how to shape the course of immune response. This “instruction” constitutes of a wide panel of immunomodulatory factors, which are produced by the immunogen-activated immune cells. Importantly, some

immunomodulatory factors are able to traverse the blood-brain barrier (Galea et al. 2007). Thus, a reciprocal communication between neuroendocrine and immune system guards homeostasis in the organism as depicted in Figure 11 (Eskandari and Sternberg 2002).

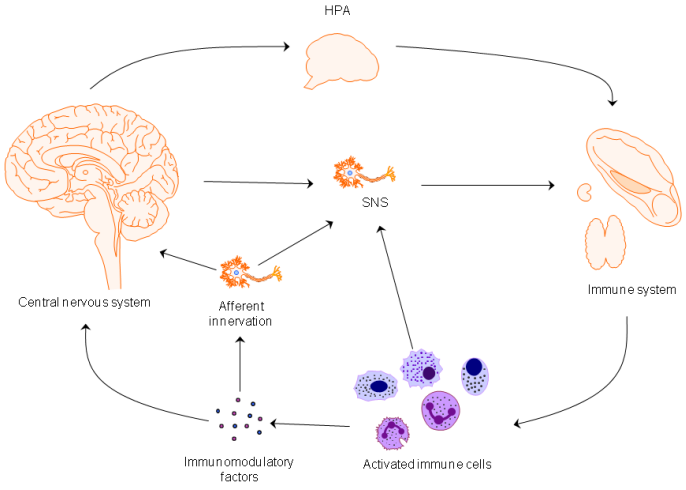

Figure 11. Bidirectional communication between the nervous and immune system.

3.1. Adrenergic receptors

Norepinephrine and epinephrine specifically target adrenergic receptors (AR). There are two major families of adrenergic receptors: the α-ARs and β-ARs and molecular cloning led to identification of nine AR subtypes: α_1A, α_1B, α_1D, α_2A, α_2B, α_2C, β_1, β_2 and β_3. All of these receptors belong to the family of G-protein coupled receptors (GPCRs) (Philipp and Hein 2004).

GPCRs, also known as seven transmembrane receptors, constitute the largest, the most ubiquitous and the most versatile class of membrane receptors. They sense a variety of extracellular stimuli and transduce them to diverse intracellular responses (Pierce et al. 2002). ARs, like all members of the GPCR family, interact with heterotrimeric G proteins that are composed of an α, β and γ subunit. Ligand triggering of the receptor induces its guanine nucleotide exchange factor activity, which mediates the substitution of GDP for GTP in the Gα subunits of the G protein and allows for its detachment from the Gβγ subunits (Neves et al. 2002). As a consequence, these subunits stimulate different effector molecules, thereby activating or inhibiting the production of a wide variety of second messengers which in turn initiate downstream signal transduction. Alternatively, GPCRs can induce cellular responses that are independent of the heterotrimeric G protein and instead involve members of the β-arrestin protein family (Shenoy and Lefkowitz 2011).

One of the ARs that attracted a lot of attention in the field of inflammation and drug discovery is the β_2-AR (Kobilka 2011). Drugs activating the β_2-AR (β_2-agonists) are widely used in clinical practice to treat airway disorders, such as asthma and chronic obstructive pulmonary disease, as they provoke relaxation of airway smooth muscle resulting in bronchodilatation (Cazzola et al. 2011; Theron et al. 2013). On the other hand, nonselective β-antagonists (drugs inhibiting the activity of β_1-AR and β_2-AR), in addition to selective β_1-antagonists, are employed in management of cardiovascular disease as they lower the heart rate and reduce blood pressure (Barrese and Taglialatela 2013). Recently, administration of β_2-AR antagonist has been also suggested to have a therapeutic potential in the menagment of asthma (Thanawala et al. 2014). Since we have studied β_2-AR-mediated effects in this thesis, the relevance of this receptor subtype and its downstream signalling in (patho)physiological conditions will be highlighted.

In humans, the β_2-AR is expressed in several organs, such as lungs, skeletal muscle and brain (Perez et al. 2014). Furthermore, lymphoid organs (for instance: spleen and thymus) and various immune cell types (for instance: lymphocytes and macrophages) display a high number of functional receptors (Ley et al. 2010; Nance and Sanders 2007; Padro and Sanders 2014; Sanders 2012). Studies on knockout mice showed that the β_2-AR is not an essential gene for prenatal and postnatal development and reproductive function. In fact, adult animals appear grossly normal under basal conditions, while β_2-AR deficiency results in alteration of vascular tone and energy metabolism, promoting greater resistance to the stress of exercise (Chruscinski et al. 1999). Since the report by Chruscinski and colleagues, the function of β_2-AR in the regulation of immunity and other physiological processes has been intensively studied. Here, we will discuss only those studies in which the link between immunity and stress has been investigated. One of the earliest studies showed that β_2-AR deficient mice have a normal adaptive immune response and possibly non-adrenergic mechanisms developed early in life can contribute to compensatory effects *in vivo* (Sanders et al. 2003). Furthermore, cold restrain as a psychological and physiological stressor does not influence the course of the innate immune response upon Listeria challenge in animals lacking β_2-ARs. However, changes have been observed in case of adaptive immune response. Precisely, these animals displayed a predominance of the humoral over the cellular immune response that correlated with substantially higher production of antibodies (Emeny et al. 2007). Another report demonstrated

sexual dimorphism for murine leukocyte migration. Female mice recruit four times more leukocytes than male mice upon LPS exposure but this difference is not apparent in β_2-AR-deficient animals (de Coupade et al. 2007). β_2-AR knockout was shown to prevent recruitment of inflammatory cells to the lungs during asthma development (Nguyen et al. 2009). Finally, a recent study by Vida *et al.* (Vida et al. 2011) has shown that the presence of β_2-ARs on splenic regulatory lymphocytes is crucial to control the inflammatory response as transfer of this lymphocyte population from wild type to β_2-ARs knockout mice could reestablish the anti-inflammatory potential in response to β_2-agonist administration. In line, treatment with β_2-agonists also suppressed cytokine production in an *in vitro* culture of wild type but not β_2-ARs deficient splenocytes and prevented from systemic inflammation, organ damage and lethal endotoxic shock upon LPS challenge in wild type but not β_2-ARs knockout animals.

Using these full β_2-AR knockout animals, the repertoire of β_2-AR functions was also extensively studied in other tissues. For instance, it was shown that the β_2-AR in skeletal muscle promotes clenbuterol-induced tissue hypertrophy and concomitantly protects from atrophy (Hinkle et al. 2002). In addition to these anabolic properties, β_2-ARs are modulators of the skeletal muscle capacity for endurance exercise (Davis et al. 2008). In the brain, β_2-ARs deficiency has a neuroprotective effect that results from the downregulation of NF-κB signalling components as well as its effector genes. Suppression of NF-κB signalling and its target gene expression prevents the inflammatory and apoptotic phenotype (White et al. 2012).

3.2. β_2-adrenoreceptor signalling

Signalling from the β_2-AR, is prototypically initiated by either a G protein or β-arrestin (Neves et al. 2002; Shenoy and Lefkowitz 2011).

3.2.1. Signalling pathways dependent on the G protein

According to the classical paradigm, β_2-AR triggering activates coupling of the receptor to Gs, resulting in activation of adenylyl cyclase (AC), which converts ATP to cAMP (McKnight 1991). Cyclic AMP acts as a second messenger, launching either the cAMP-dependent protein kinase A (PKA) (Walsh et al. 1968) or the guanine exchange proteins directly activated by cAMP (EPACs) (de Rooij et al. 1998). EPACs (EPAC-1 and EPAC-2) further transduce signals though the Rap family of

small Ras-like GTPases (Bos 2003; Gloerich and Bos 2010). PKA is a tetrameric holoenzyme, composed of two regulatory (PKAr) and two catalytic subunits (PKAc), which in the inactive form reside in the cytoplasm. Binding of cAMP to the PKAr induces conformational changes resulting in release of the PKAc. Subsequently, PKAc targets multiple cytoplasmic and nuclear proteins bearing a consensus motif RRXS/T where X represents any amino acid. Some variations with regard to spacing and basic residue are permissible. Vicinity of phenylalanine to the phosphorylated motif hampers PKA-mediated phosphorylation of serine/threonine residues in the target protein because of steric hindrance (Ubersax and Ferrell 2007). Subsequently, PKA phosphorylates cytoplasmic kinases, including c-Src kinase (Schmitt and Stork 2002) and p38 MAPK (Zheng et al. 2000) or translocates to the nucleus to phosphorylate the cAMP response element-binding protein (CREB transcription factor, which activity will be discussed later (Mayr and Montminy 2001). More recently, it was demonstrated that in cardiomyocytes and airway smooth muscle cells, the β_2-AR can also couple to the Gi protein (McGraw et al. 2007; Xiao et al. 1995). The molecular mechanism underlying this phenomenon was investigated in Hek293T cells and is based on PKA-mediated phosphorylation of β_2-ARs. This event serves as a molecular switch, changing receptor coupling from Gs to Gi, which leads to release of Gβγ from activated Gi and launches the c-Src/ERK1/2 signalling cascade (Daaka et al. 1997). Furthermore, the Gβγ subunit deriving from the Gαi/o protein was demonstrated to activate phosphoinositide-3-kinase (PI3K), which in turn stimulates Akt in cardiomyocytes (Yano et al. 2007). The most important β_2-AR-dependent, G protein-mediated, signalling pathways are summarized in Figure 13.

3.2.2. Signalling pathways dependent on β-arrestin

Alternatively, β_2-ARs can induce cellular responses that involve the members of the arrestin protein family. The latter comprises four members; of these, β-arrestin-1 (also called arrestin-2) and β-arrestin-2 (also called arrestin-3) are ubiquitously expressed, while arrestin-1 and arrestin-4 are exclusively present in the retina. β-arrestins were initially demonstrated to play an important role in attenuation of β_2-AR signalling and receptor internalization, while recent reports point to their role as signal transducers, connecting ARs to multiple signalling pathways, such as p38 and ERK MAPKs and NF-κB. Moreover, it was also suggested that β-arrestins have a nuclear function and participate in the regulation of transcription (Ma and Pei 2007; Shenoy and Lefkowitz

2011). The most important β_2-AR-dependent, β-arrestin-mediated signalling pathways are summarized in Figure 12.

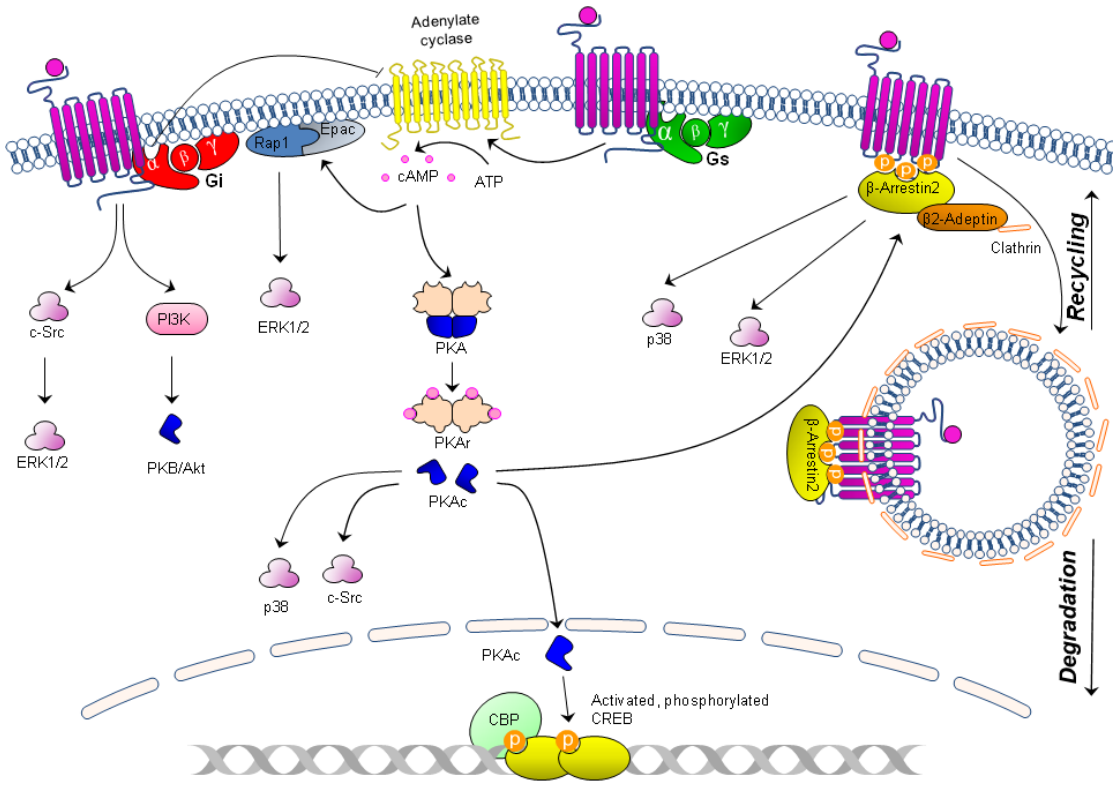

Figure 12. The main signalling cascades initiated by ligation of β_2-ARs depend on interaction of the receptor with either a heterotrimeric G protein or β-arrestin. Coupling of the β_2-AR to Gs or Gi proteins respectively activates or inhibits the adenylate cyclase, hence modulating intracellular cAMP levels and the activity of cAMP-dependent effector proteins, including PKA and EPAC. Coupling of the β_2-AR to β-arrestin or the Gβγ subunit of Gi triggers activation of ERK and p38 MAPKs. Finally, β-arrestin coupling promotes receptor internalization and subsequent recycling or degradation.

3.2.3. cAMP response element binding protein

The transcription factor cAMP response element binding protein (CREB) was the first identified transcription factor regulated by phosphorylation and this was crucial for expression of the somatostatin gene in PC12 pheochromocytoma cells. Since then CREB was shown to occupy the promoters of multiple genes bearing a cAMP response element (CRE). CREs typically appear as palindromic (TGACGTCA) or half-site (either TGACG or CGTCA) sequences (B. Mayr and Montminy 2001), although a small number of atypical variants have also been described in the literature. For instance, the IL-8 promoter possesses the CRE-like site (TGACATAA) with two changes from a canonical CRE consensus that are well tolerated by CREB

(Iourgenko et al. 2003). Another noncanonical CRE site (TTACGTAA) was described in the glucose-6-phosphatase gene and its sequence has been reported to alter CREB binding (Hornbuckle et al. 2004). It is noteworthy that half site CRE motifs are less active than the full CRE palindrome for CREB binding (B. Mayr and Montminy 2001). Furthermore, most of CRE sites are unable to bind CREB due to disruptive cytosine methylation within CREB-binding motif (Iguchi-Ariga and Schaffner 1989; Zhang et al. 2005). The ability of CREB to drive transcription further depends on the relative vicinity of CRE site to the TATA box (B. Mayr and Montminy 2001).

CREB belongs to the family of transcription factors that encompasses also cAMP-Response Element Modulator (CREM) and Activating Transcription Factor 1 (ATF-1). All family members share a similar modular organisation. They all contain a transactivation domain (TAD) and a basic leucin zipper domain (bZIP). The bZIP domain is responsible for DNA binding and dimerization. The TAD contains the kinase-inducible domain (KID) that enables interaction with transcriptional cofactors. The KID is flanked by two glutamine-rich domains: the Q1 and Q2 domain, which are necessary to elicit the maximal transcriptional activity of CREB. Whereas Q2 interacts with the components of the basal transcriptional machinery, Q1 interactors have not been identified (B. Mayr and Montminy 2001). Scheme illustrating domains organization in CREB is shown in Figure 13.

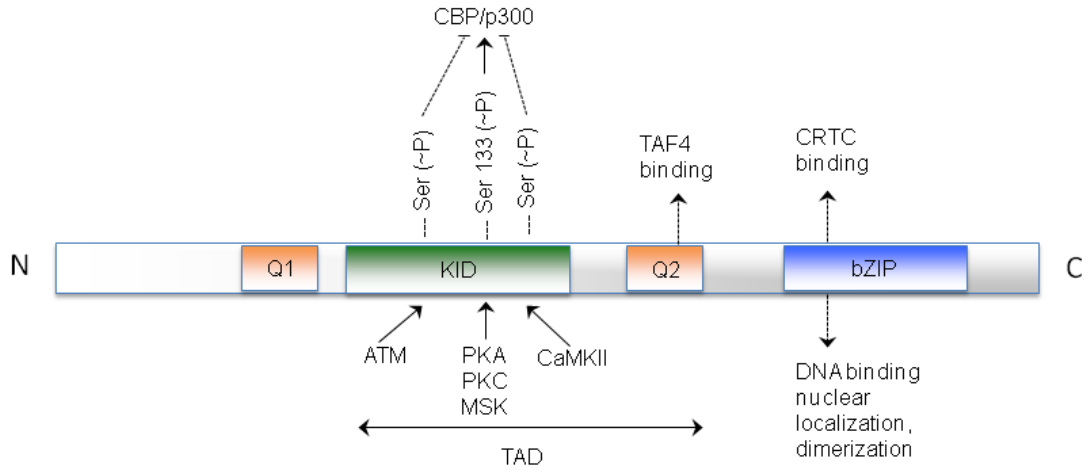

Figure 13. Structure of CREB. CREB: various phosphorylation sites are marked. Q1 and Q2 are glutamine-rich domains; the TAD (transactivation domain) is responsible for interaction with transcriptional cofactors, which occurs predominantly via the kinase-inducible domain (KID)*, the bZIP domain is responsible for DNA binding, nuclear localization, dimerization and interaction with transcriptional cofactors.*

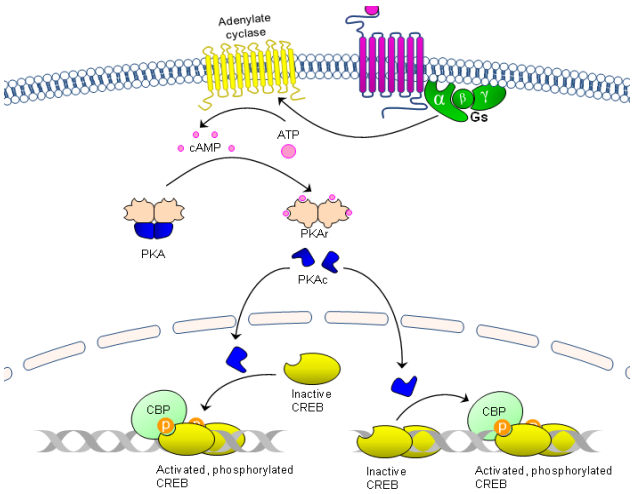

Figure 14. The canonical signalling pathway activated upon β_2-AR triggering. For detailed description, we refer to the above-section and section dedicated to cAMP-response element binding protein.

CREB is activated in response to the intracellular accumulation of cAMP via AC activity. Nuclear PKAc leads to phosphorylation of CREB at serine 133 (Figure 14). This event is pivotal for the recruitment of the transcriptional cofactor paralogues CBP and p300 (Chrivia et al. 1993; Lundblad et al. 1995). Apart from cAMP, other intracellular signals, such as protein kinase C (PKC) or MSK-1, are known to modulate the transcriptional potential of CREB via serine 133 phosphorylation in response to a diverse panel of environmental stimuli (Shaywitz and Greenberg 1999). Finally, calmodulin-dependent kinase II (CaMKII) and ATM phosphorylate different serine residue within the TAD of CREB as shown in Figure 13, hampering its interaction with CBP/p300 (Shi et al. 2004; P. Sun et al. 1994).

3.2.3.1. Regulation of CREB-dependent gene expression

Expression of CREB-dependent genes is regulated at multiple levels. First of all, most of the CREs in the human genome are repressed via cytosine methylation, which has a disruptive effect on CREB binding (Iguchi-Ariga and Schaffner 1989; Zhang et al. 2005). By contrast, transcriptionally active sites are primarily localized in the proximal promoter region, which is usually within 250 base pairs upstream to the transcription start site and in the close vicinity of the TATA box (Impey et al. 2004; Zhang et al. 2005). Furthermore, transcription from CREB-dependent promoters is regulated by as yet unknown epigenetic mechanisms because only a limited pool of CREB-occupied promoters, which contain a TATA-box, is transcribed in response to elevation of cAMP (Altarejos and Montminy 2011). The pattern of CREB phosphorylation also plays a pivotal role in the recruitment of transcriptional cofactors, which affects the transcriptional activity (Altarejos and Montminy 2011; B. M. Mayr et al. 2001). Finally, whereas it is commonly accepted that CREB is

constitutively bound to its target gene promoters and transcriptional activation requires its phosphorylation, several reports have demonstrated that at selected gene promoters, CREB is recruited by certain stimuli (Figure 12). For instance, it has been demonstrated that CREB is enriched at the c-fos promoter in neurons treated with brain-derived nuclear factor (Riccio et al. 2006), at the glucagon-responsive gene promoter in Hek293T treated with forskolin (Y. Wang et al. 2010) or at the IL-6 promoter upon combinational treatment with TNF-α and isoproterenol (Spooren et al. 2010). Finally, activation of CREB-dependent genes is terminated by dephosphorylation of CREB, a process regulated by the serine/threonine phosphatases: protein phosphatase 1 (PP1) and PP2A (B. Mayr and Montminy 2001).
Multiple lines of evidence indicate that association of CREB with its transcriptional coregulators, such as CBP/p300 or cAMP-regulated transcriptional coactivators (CRTCs, previously referred to as transducers of regulated CREB activity (TORCs)), is a crucial step for its transcriptional activity (Altarejos and Montminy 2011; Conkright et al. 2003b; B. Mayr and Montminy 2001). It is now a well-established fact that phosphorylation of CREB at serine 133, which is localized within the KID, is crucial for interaction with the KID interaction domain (KIX) of CBP/p300 (Parker et al. 1998). Remarkably, although CBP and p300 bind CREB to a similar extent and can compensate for each other *in vitro*, their role *in vivo* was shown not to be completely interchangeable (Vo and Goodman 2001). Furthermore, simultaneous knockout of both CBP and p300 genes in mouse embryonic fibroblasts disrupts the expression of certain CREB target genes, such as *areg* or *rgs2*. Nevertheless, a subset of genes with multiple CREs, including *crem*, is still transcribed by CREB, probably due to recruitment of another group of transcriptional coregulators, namely CRTCs/TORCs, to those CREB-dependent promoters (Kasper et al. 2010). CRTCs/TORCs are a group of cofactors, which are kept in the cytoplasm through phosphorylation-dependent interaction with the 14-3-3 protein. Elevation of the intracellular levels of cAMP and calcium, but not other signalling messengers, promotes calcineurin-mediated dephosphorylation and nuclear entry of CRTC, which in turn interacts with the bZIP domain of CREB at certain promoters independently of serine 133 phosphorylation (Conkright et al. 2003a). Importantly, it seems that CBP/p300 and CRTCs/TORCs may provide a cooperative mode to induce transcription from CREB occupying promoters (Rarnskjaer et al. 2007).

3.2.3.2. Physiological and pathological role of CREB

As evident from genome-wide studies, CREB promotes the expression of over 5000 genes or grossly one-quarter of the mammalian genome. CRE motifs are frequently present in cAMP-regulated genes, with a function linked to cellular proliferation and differentiation, survival and regulation of metabolism and immunity (Impey et al. 2004; Zhang et al. 2005).

Indeed, CREB was shown to play an important role in proper functioning of highly metabolic tissues, such as skeletal muscle, liver, pancreas, adipose tissue and the brain. For instance, CREB is an essential factor for differentiation of embryonic skeletal muscle progenitors and survival of adult skeletal muscle. In particular, expression of various myogenic factors, such as Pax3, MyoD and Myf5, which regulate myotome formation, is dependent on the cAMP/PKA/CREB-dependent pathway as shown using CREB knockouts (A. E. Chen et al. 2005a). Furthermore, stimulation of β_2-AR in skeletal muscle augments CREB-dependent expression of salt-inducible kinase, which in turn phosphorylates HDAC5 leading to its nuclear exclusion and activation of myogenic program driven by the myocyte enhancer factor-2 (Berdeaux et al. 2007). Activation of a CREB-dependent transcriptional program in both myogenic precursor cells and newly regenerating myofibers contributes to skeletal muscle regeneration upon acute injury (Stewart et al. 2011).

CREB also plays an instrumental role in CNS homeostasis. It plays an important role in long-term memory, regulation of behaviour, food intake and drug addiction (Altarejos and Montminy 2011; Carlezon et al. 2005). Furthermore, CREB regulates neuronal activity and survival (Riccio et al. 1999). In addition, this transcription factor governs various processes in glial cells. In that context, CREB was shown to regulate the expression of neurotrophins, such as nerve growth factor (NGF), as well as cytokines, like for instance IL-6, which are crucial for communication between the nervous and immune system (Otten et al. 2000).

As CREB is a sensor of hormonal and metabolic signals in various tissues, deregulation of its activity may underlie a number of pathological changes such as insulin resistance, hyperglycaemia, hyperinsulinaemia, and obesity (Altarejos and Montminy 2011).

Finally, inflammatory stimuli can launch the p38/MSK-1 axis that promotes CREB activation, which in turn contributes to the induction of several inflammatory mediators, including various cyto-/chemokines and cell adhesion molecules (Kang et

al. 2008; C. Kim et al. 2008). In this context, CREB-dependent expression of these inflammatory mediators appears to be independent of the recruitment of the CBP/p300 cofactor as shown using embryonic fibroblasts from the knock-in mice bearing the CREB serine 133 to alanine mutation (Naqvi et al. 2014).

3.2.4. Influence of the adrenergic and cAMP-dependent pathway on the Interferon-β enhanceosome

The IFN-β enhanceosome is composed of four positive regulatory domains (PRD), I through IV, which are recognized by several transcription factors. More specifically, the PRDI and PRDIII are bound by various isoforms of the IRF transcription factor family, while PRDII and PRDIV are recognized respectively via the ATF-2/c-JUN and the p65-p50 NF-κB heterodimer. The highly cooperative binding of these transcriptional regulators enables synergistic activation of the intact IFN-β enhancer in response to viral infection. Interestingly, the cAMP-dependent signalling has no effect on the activity of the full-length IFN-β enhancer or its isolated IRF-binding site (Thanos and Maniatis 1995).

Noteworthy, adrenergic signals via the PKA-dependent pathway impair TLR3-induced IFN-β expression in both lymphoid and myeloid dendritic cells by interfering with transactivation of IRF binding factors. In addition to IFN-β, norepinephrine also supresses INF-α transcription (Collado-Hidalgo et al. 2006). In line, stimulation of β_2-AR inhibits IFN-γ production in a cAMP-dependent manner in Th1 cells (Cole et al. 1998, Borger et al. 1998). These data corroborate the suppressive effects of adrenergic activation on immune response during infection (Sloan et al. 2008).

There are also some reports showing opposite effects of adrenergic signalling on IFN-γ expression. For instance, exposure in vitro to norepinephrine induces naive T cells to differentiate into Th1 cells that produced an augmented level of IFN-γ upon restimulation (Swanson et al. 2001). In vivo study performed in mice infected with Listeria monocytogenes or Mycobacterium tuberculosis showed that norepinephrine ensured immunoprotection by increasing the level of IFN-γ produced by CD4+ T-cells (Alaniz et al. 1999). However, the precise molecular mechanisms remain to be elucidated.

Literature lacks mechanistic reports that investigated the influence of adrenergic signals with cytoplasmic or nuclear events leading to IRF activation upon stimulation with INFs.

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Chapter 2

Mechanisms of NF-κB modulation by β_2-AR-dependent signals

Adapted from manuscript entitled:
"β_2-adrenergic receptors in immunity and inflammation: stressing NF-κB"
by Krzysztof Kolmus, Jan Tavernier, Sarah Gerlo
submitted to "Brain, Behavior, and Immunity"

In both physiological and pathological circumstances, cells are exposed to a wide variety of environmental stimuli, which intersect intracellularly and are integrated to ensure appropriate context-dependent gene expression.
In this chapter, we will summarize the progress that has been made in understanding the molecular mechanisms of β_2-AR/NF-κB crosstalk. We will show that the NF-κB signalling cascade provides multiple targets for negative and positive regulation by adrenergic signals. This chapter is organized according to the different steps in NF-κB activation, starting at the cell membrane and ending in the cell nucleus, at the promoters/enhancers of NF-κB target genes.

1. Interactions at the level of plasma membrane receptors

Wang et al. (Wang et al. 2009) showed that fenoterol, a selective β_2-AR agonist, elicits translocation of β-arrestin-2 to the cell membrane in THP-1 monocytes. β-arrestin-2, via a non-defined mechanism, subsequently mediates redistribution of TLR4/CD14 complexes in the cell membrane and downregulates plasma membrane

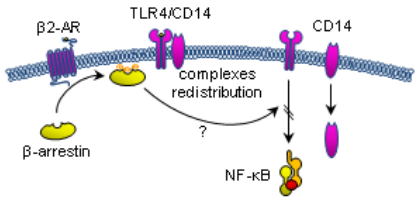

Figure 1. Negative β_2-AR/NF-κB crosstalk at the level of cell membrane.

display of CD14 costimulatory molecules, hampering NF-κB-dependent gene expression (Fig. 1). These findings suggest β_2-AR activation can disrupt NF-κB activity already at an early step in the TLR4 signalling cascade.

2. Cytosolic interactions

2.1. Effects of β_2-AR modulators on IKK activation and cellular IκB levels

2.1.1. Inhibitory effects

Phosphorylation of IκB by the IKKβ kinase, leading to IκB ubiquitinylation and proteasome-dependent degradation, is the key regulatory event in canonical NF-κB activation. A large body of evidence suggests β_2-AR triggering attenuates NF-κB via upregulation of cellular IκBα levels. The inhibitory effects can be grossly divided in those engaging cAMP-regulated effector proteins and those involving members of the β-arrestin family (Gerlo et al. 2011; Ma and Pei 2007).

2.1.1.1. cAMP-mediated

The influence of β_2-AR triggering on the upregulation of basal IκBα levels (Madrigal et al. 2006) or on the repression of stimulus-induced IκBα degradation (Gavrilyuk et al. 2001; Loop et al. 2004; Mortaz et al. 2008; Strell et al. 2009; Yang et al. 2010) has been investigated in a number of studies, using a variety of cell types and different stimuli to activate both NF-κB and β-AR-dependent signalling cascades (For details refer to Table 1) but the precise mechanism(s) explaining the observed effects have not been elucidated in most of these studies.

Farmer and Pugin (Farmer and Pugin 2000) showed that in monocytic cells inhibition of LPS-induced NF-κB by isoproterenol is the result of elevation of intracellular IκBα levels. Isoproterenol had no influence on basal IκBα levels and also did not affect immediate LPS-induced IκBα degradation, while it significantly increased IκBα protein levels upon prolonged cotreatment with LPS (Fig. 2A). In line with these findings, norepinephrine was reported to upregulate astroglial IκBα expression in a dose-dependent manner at the mRNA and protein levels, hence elevating the threshold required for NF-κB activation (Gavrilyuk et al. 2002). Studies investigating molecular mechanism underlying NF-κB suppression by β_2-agonists reported

enhanced IκBα gene transcription upon β_2-AR triggering (Farmer and Pugin 2000; Gavrilyuk et al. 2002). The IκBα promoter contains a transcription factor binding site for the CREB transcription factor and it was postulated that CREB could mediate β-agonist induced IκBα transcription (Gavrilyuk et al. 2002), yet this hypothesis was not supported by experimental evidence (Fig. 2B). Interestingly, whereas these publications indicated that the inhibitory effects of β_2-agonists on IκBα degradation are mediated via the cAMP/PKA-dependent cascade (Farmer and Pugin 2000; Gavrilyuk et al. 2002), Dello Russo et al. (Dello Russo et al. 2004) proposed that suppressive signals could be also mediated via other cAMP-responsive proteins. Indeed, inhibition of the PKA pathway did not attenuate the effect of norepinephrine on NF-κB activity in primary microglia, leading to the speculation that an alternative signalling cascade is implicated. In line with this, Oldenburger et al. (Oldenburger et al. 2012) recently demonstrated that in airway smooth muscle cells, fenoterol, a selective β_2-AR agonist, hampers IκBα degradation via a cAMP-dependent mechanism depending on the Epac proteins (Fig. 2C).

Although upregulation of IκBα as a result of β_2-AR-induced, cAMP-dependent, signalling appears to be a common mechanism controlling NF-κB activity, the molecular events behind this phenomenon remain poorly understood. It was postulated that in monocytes augmentation of the IκBα levels results from stabilization of IκBα protein (Farmer and Pugin 2000) (Fig. 2A). Nevertheless, to date no detailed study evaluating IκBα protein phosphorylation and ubiquitinylation was performed to determine where β_2-AR-mediated signals interfere with the IκBα degradation cascade. In astrocytes, the elevated level of IκBα mRNA is independent of post-transcriptional events, such as mRNA stabilization, indicating transcriptional upregulation of IκBα levels. Intriguingly, elevated protein levels of IκBα were observed not only in the cytoplasm but also in the nucleus upon norepinephrine treatment. This observation implies that IκBα, besides inhibiting NF-κB nuclear translocation, might stimulate prompter nuclear export of NF-κB dimers and/or inhibit inflammatory gene expression via interaction with promoter-bound NF-κB (Gavrilyuk et al. 2002) (Fig. 2B).

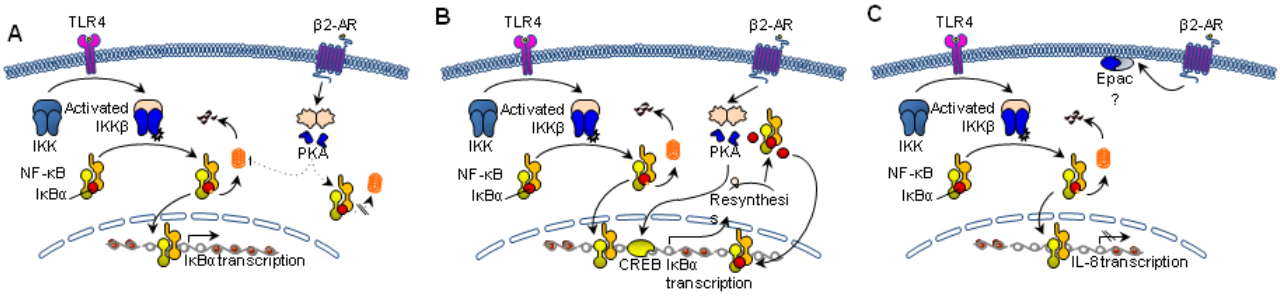

Figure 2. Negative β_2-AR/NF-κB crosstalk at the level of cytosol via the cAMP-dependent pathway. Fig. 2B compiles mechanisms described in two different reports.

Table 1.

Summary of reported effects of β_2-AR modulators on NF-κB activity.

Cell type or tissue	*β_2-AR modulator*	*NF-κB stimulus*	*Overall effect on NF-κB activity*	*Reference*
Lymphocytes				
Human T cells	Clenbuterol	PMA	↓	(Loop et al. 2004)
	Isoproterenol	PMA	↓	(Loza et al. 2006)
	Norepinephrine *	CD3+CD28+	↓	(Strell et al. 2009)
	Carvedilol	PMA + Ionomycin, H2O2	↓	(Yang et al. 2003)
Mouse B cells	Terbutaline	$F(ab')_2$ anti-IgM	↑	(Kohm et al. 2002)
Monocytes/Macrophages				
THP-1	Isoproterenol Albuterol Fenoterol Epinephrine Norepinephrine	LPS	↓	(Farmer and Pugin 2000)
	Norepineprine *	None	↑	(Bierhaus et al. 2003)
	Norepineprine *	None	↑	(Djuric et al. 2012)
Neutrophils				
Human Neutrophils	Salmeterol	Cigarette smoke extract	↓	(Mortaz et al. 2008)
Mast cells				
HMC-1	Epinephrine	IL-1β	↑	(Chi et al. 2004)
Dendritic Cells				
Mouse Dendritic Cells	Salbutamol	LPS	↓	(Herve et al. 2013)
Neurons				
Rat Primary Neurons	Norepinephrine *	None	↓	(Madrigal et al. 2006)
Glial cells				
C6/ Rat Primary Astrocytes	Norepinephrine *	LPS/IFN-γ	↓	(Gavrilyuk et al. 2001)
	Norepinephrine *	None	↓	(Gavrilyuk et al. 2002)
Rat Primary Glial Cells	Norepinephrine *	LPS	↓	(O'Sullivan et al. 2009)
Rat Primary Microglia	Norepinephrine	LPS	↓	(Dello Russo et al. 2004)
N9/ Primary Microglia	Norepinephrine Isoproterenol	None	↑	(Kong et al. 2010)

132.1N1	Isoproterenol	TNF-α	=	(Spooren et al. 2010b)
	Isoproterenol	IL-1β, TNF-α	=	(Spooren et al. 2011)
Airway Smooth Muscle cells				
Human ASM	Isoproterenol Formoterol Salmeterol Albuterol	TNF-α	↑	(Ammit et al. 2002)
	Salmeterol	TNF-α	↓	(Nie et al. 2005)
	Salbutamol	IL-1β	↓	(Kaur et al. 2008)
hTERT-ASM/ Primary ASM	Fenoterol	Cigarette smoke extract	↓	(Oldenburger et al. 2012)
Fibroblasts				
Bronco5 MyoICIG7	Salmeterol	TNF-α	↓	(Baouz et al. 2005)
Mouse Neonatal Cardiac Fibroblasts	Isoproterenol	None	↑	(Yin et al. 2006)
NIH-3T3 And other cell lines: A549, Human Bronchial Smooth Muscle, HEK293	Salbutamol	TNF-α	↓	(Yang et al. 2010)
Epithelial cells				
ACHN	Terbutaline	Shiga Toxin 2	↓	(Nakamura et al. 2001)
Endothelial cells				
Mouse Cardiac-Derived Endothelial Cells	Isoproterenol	None	↑	(Chandrasekar et al. 2004)
Mouse Aortic Endothelial Cells	Isoproterenol	None	↑	(Ciccarelli et al. 2011)
Skeletal muscle cells				
C2C12 Myoblasts	Epinephrine	LPS	=	(Frost et al. 2004)
Parotid gland				
Rat Parotid Gland	Isoproterenol	None	↑	(Yeh et al. 2012)

* Involvement of β_2-AR not confirmed
↑ enhanced activity upon β_2-AR (co)stimulation
↓ decreased activity upon β_2-AR (co)stimulation
= no change in activity upon β_2-AR costimulation

2.1.1.2. β-arrestin-mediated

Members of the β-arrestin family, acting downstream of the β_2-AR, have emerged as important modulators of NF-κB. Both β-arrestin-1 and β-arrestin-2 bind IκBα directly, preventing its phosphorylation-induced proteasomal degradation (Gao et al. 2004; Luan et al. 2005; Witherow et al. 2004). Whereas stimulation of β_2-ARs in

multiple cellular backgrounds induces a stable physical association of β-arrestin-2 with IκBα, the strength of the interaction between β-arrestin-1 and IκBα appears to be cell type-specific and in co-immunoprecipitation experiments β-arrestin-1 and IκBα interacted with different affinities in Hek293, HeLa and COS-7 cells. The basis of this cell type-specificity, however, remains to be established. In line with these findings, pretreatment of cells with β_2-AR antagonists entirely abrogates β-arrestin-IκBα complex formation (Gao et al. 2004; Luan et al. 2005). Using deletion mutants, it was shown that the N-terminal amino acid sequence of β-arrestin-2 is essential for the interaction with the C-terminal region of IκBα. These data suggest that β-arrestin-2 may impair the IκBα degradation pathway, which requires phosphorylation of four C-terminal serine residues within the PEST domain (Gao et al. 2004). In addition, it was shown that in response to UV exposure, CK2 phosphorylates IκBα and other target proteins, including β-arrestin-2, while stimulation of β_2-ARs promotes β-arrestin dephosphorylation. Unphosphorylated β-arrestin binds IκBα and masks its CK2 target sites, hence preventing its degradation and activation of NF-κB (Luan et al. 2005) (Fig. 3A). Yet another study showed that a non-selective β-blocker, carvedilol, inhibited IKKβ kinase activity, thereby preventing phorbol 12-myristate 13-acetate (PMA)/ionomycin-induced IκBα degradation in T cells (Yang et al. 2003) (Fig. 3B). The molecular events associated with suppression of IKK activity remain to be established but they probably rely on the induction of β-arrestin by this biased agonist for β-AR In conclusion, β-arrestin appears to act as a negative regulator of NF-κB.

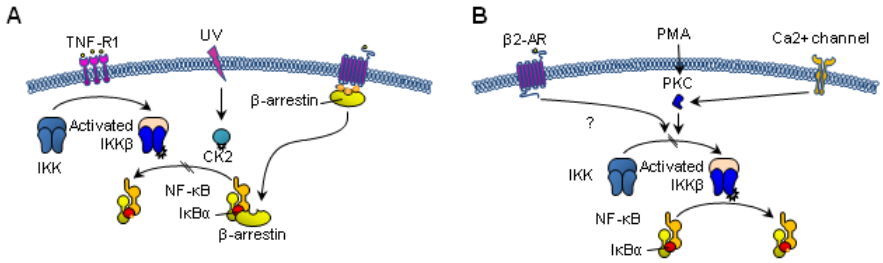

Figure 3. Negative β_2-AR/NF-κB crosstalk at the level of cytosol via β-arresting. Fig. 3A represent the action of β_2-AR agonists, while Fig. 3B depicts the action of an antagonist.

2.1.2. Stimulatory effects.

Few reports describe activation of canonical NF-κB signalling by β_2-AR stimulation. For instance, Chandrasekar et al. (Chandrasekar et al. 2004) showed that β_2-AR triggering activates NF-κB in cardiac endothelial cells. At the molecular level,

signalling was initiated by the Gβγ subunit of the Gi/o protein and involved activation of the PI3K/Akt pathway, which in turn activated the IKKβ kinase. The latter resulted in NF-κB-dependent expression of IL-18 (Fig. 4A). Stimulation of NF-κB activity by β-agonists was also reported in N9 microglia cells, where β_2-AR-dependent activation of the p38 MAPK, promoted degradation of IκBα and consequently activated NF-κB-driven transcription of the mouse formyl peptide receptor (mFPR2) gene (Kong et al. 2010) (Fig. 4B).

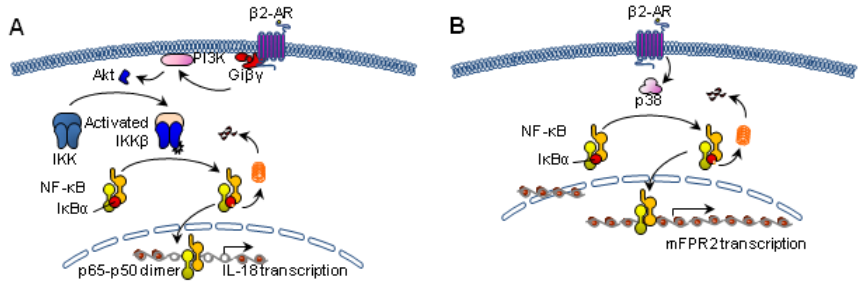

Figure 4. Positive β_2-AR/NF-κB crosstalk in the cytosol.

2.2. Effects of β_2-AR modulators on the function of NF-κB subunits

In addition to the above-described reports of β_2-AR-mediated regulation of NF-κB at the level of the IκB inhibitor protein, several reports showed that β_2-AR stimulation mitigates NF-κB nuclear translocation, DNA binding and transcriptional activity, in both immune (Farmer and Pugin 2000; Herve et al. 2013; Loop et al. 2004; Loza et al. 2006; Mortaz et al. 2008; Strell et al. 2009; Yang et al. 2003) and non-immune cell types (Baouz et al. 2005) (For details refer to Table 1). In many cases NF-κB activity was measured via electrophoretic mobility shift assay (EMSA), which assesses DNA binding, or using microscopy to image NF-κB localization in the cell. However, in most of these studies, the exact level of the β_2-AR/NF-κB crosstalk was not established. Nevertheless, some reports indicate that regulatory events occur downstream of the release of NF-κB from its inhibitor.

In renal tubular epithelial cells, treated with Shiga toxin, β_2-AR triggering modulates NF-κB activity via cooperation of the cAMP/PKA pathway, which inhibits the DNA binding ability of the p65 subunit, and an unidentified cAMP/PKA-independent pathway that represses p50 DNA binding (Nakamura et al. 2001) (Fig. 5A). Whereas β_2-AR stimulation is prototypically associated with cAMP-dependent activation of PKA or Epac, Yin et al. (Yin et al. 2006) demonstrated that in cardiac fibroblasts, the isoproterenol-induced increase in cAMP, via an unidentified cAMP effector, leads to

the activation of the p38 MAPK, which in turn enhances NF-κB DNA binding and IL-6 expression (Fig. 5B). Also, in THP-1 monocytic cells, norepinephrine treatment promotes activation of the PI3K, the Ras/Raf, ERK1/2 and the p38/JNK pathway and inhibition of these kinases reduces NF-κB DNA-binding and IL-6 transcription (Bierhaus et al. 2003) (Fig. 5C). Another study, also in THP-1 cells, showed that norepinephrine simultaneously activates p38 MAPK, protein kinase C (PKC) and PI3K, which promote nuclear entry and enrichment of, respectively, p65, c-Rel and p50 NF-κB complexes, at target gene promoters. Via chromatin immunoprecipitation (ChIP), it was furthermore demonstrated that this control of individual NF-κB subunits by diverse signalling proteins is crucial for selective regulation of inflammatory gene expression, such as intracellular adhesive molecule 1 (ICAM-1), tissue factor (TF) and manganese superoxide dismutase (MnSOD). How the different kinases modulate the activity of the different NF-κB family members was however not established (Djuric et al. 2012) (Fig. 5D).

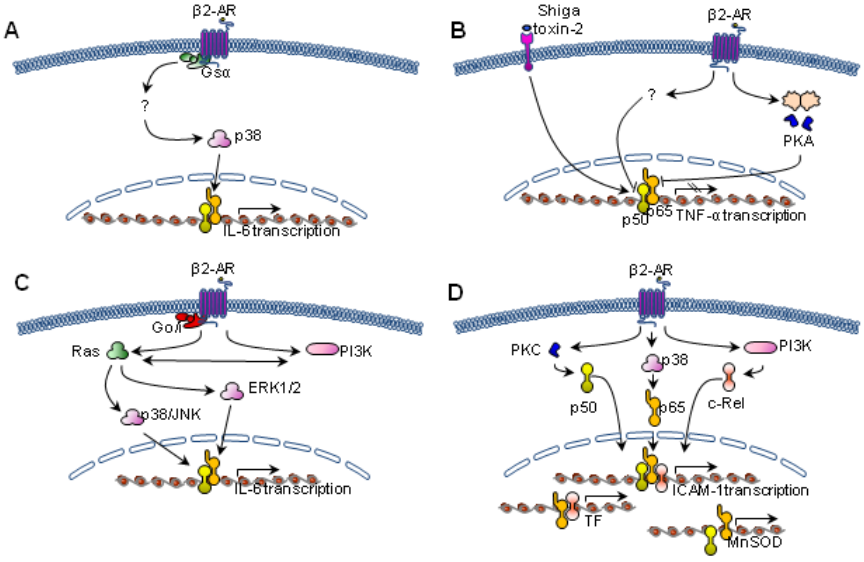

Figure 5. Influence of β_2-AR signals on the activity of NF-κB subunits.

3. Nuclear interactions

3.1. Effects of β_2-AR modulators on enhanceosome formation

The efficient transcription of many genes requires formation of an enhanceosome structure, composed of multiple transcription factors, cofactors and the basal transcriptional machinery, which is essential for fine-tuning and selectivity of gene expression (Kim and Maniatis 1997). The formation of enhanceosome-like structures

has also been described at prototypical NF-κB-dependent target genes such as the pro-inflammatory cytokine Interleukin-6 (IL-6) (Vanden Berghe et al. 1999) and several chemokines (Richmond 2002). Ample evidence suggests that β-agonists modulate the composition of the IL-6 enhanceosome. Using IL-6 promoter mutants, it was demonstrated that in airway smooth muscle cells β-agonists potentiate TNF-α-induced IL-6 transcription via an additive effect between κB and CRE response elements (Ammit et al. 2002). In accordance, we found that in astrocytes and skeletal muscle cells β_2-AR/TNF-R1 coactivation results in enhanced accumulation of NF-κB and CREB transcription factors at the IL-6 promoter, and that this leads to cooperative recruitment of the transcriptional coactivator CBP and components of the basic transcriptional machinery, hence promoting transcriptional synergy (Spooren et al. 2010b) (Fig. 6). These findings are in contrast with previous studies demonstrating competition between NF-κB and CREB for a limiting amount of CREB-binding protein (CBP) (Parry and Mackman 1997). Importantly, the synergy observed at the IL-6 promoter did not occur at other NF-κB target genes, such as IL-8, ICAM-1 or VCAM-1, indicating it is a gene-selective event unique for a subset of NF-κB-dependent target gene promoters (Spooren et al. 2010b).

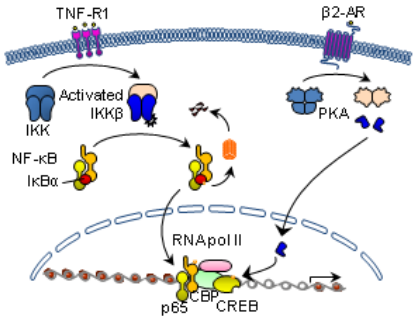

Figure 6. β_2-AR/NF-κB crosstalk in the nucleus.

CBP is an essential component of NF-κB enhanceosomes. Interestingly, upon TLR4 triggering, PKA, a key mediator of β_2-AR signalling, was shown to phosphorylate the p65 subunit at serine 276 and this was essential for recruitment of CBP and NF-κB-induced transcriptional activation (Zhong et al. 1997; Zhong et al. 1998). Although in the original report of Zhong et al. (Zhong et al. 1997), PKA phosphorylated p65 in an unusual, cAMP-independent manner, several more recent studies have reported p65 phosphorylation at serine 276 in cells treated with cAMP-elevating agents (Gao et al. 2010; Moon et al. 2011; Yoon et al. 2008). As in many studies the role of p65 serine 276 phosphorylation is solely supported by the use of phosphospecific antibodies, the specificity of which has however been questioned, whether the cAMP/PKA cascade

promotes serine 276 phosphorylation of p65 is still controversial (Herkenham et al. 2011; Spooren et al. 2010a). Also, whether β_2-agonist-activated PKA can phosphorylate p65 at serine 276 remains to be elucidated.

3.2. Epigenetic effects induced by β_2-AR modulators

A wealth of evidence is available on epigenetic regulation of NF-κB-dependent gene transcription (Bhatt and Ghosh 2014; Vanden Berghe et al. 2006) and recent studies indicate that signals transduced via the β_2-AR can also modulate NF-κB-dependent gene transcription at the chromatin level. Kohm et al. (Kohm et al. 2002) showed that β_2-AR/BCR coactivation of B-cells launches multiple intracellular kinases, including PKA, PKC, p38, and an unidentified protein tyrosine kinase (PTK), that promote cooperative binding of NF-κB, SP1 and Transcription Factor II D (TFIID) associated with the recruitment of chromatin remodelling complexes, promoting B7-2 (CD86) gene expression (Fig. 7A). Frost et al. (Frost et al. 2004) found that cotreatment of C2C12 skeletal muscle cells with LPS/epinephrine promoted IL-6 transcriptional synergy that unexpectedly was associated with histone deacetylation. They furthermore showed that the JNK MAPK, acting downstream of both TLR4 and β_2-AR, was required for the transcriptional response (Fig. 7B). Finally, Nie et al. (Nie et al. 2005) showed that salmeterol prevents TNF-α-induced histone H4 acetylation at the promoter region of the chemokine eotaxin. This, in turn, hampers p65 subunit recruitment to the promoter, reducing eotaxin gene expression. Interestingly, these effects appear to be gene selective, as β_2-AR costimulation did not interfere with chromatin loosening and p65 recruitment to the IL-8 promoter (Fig. 7C).

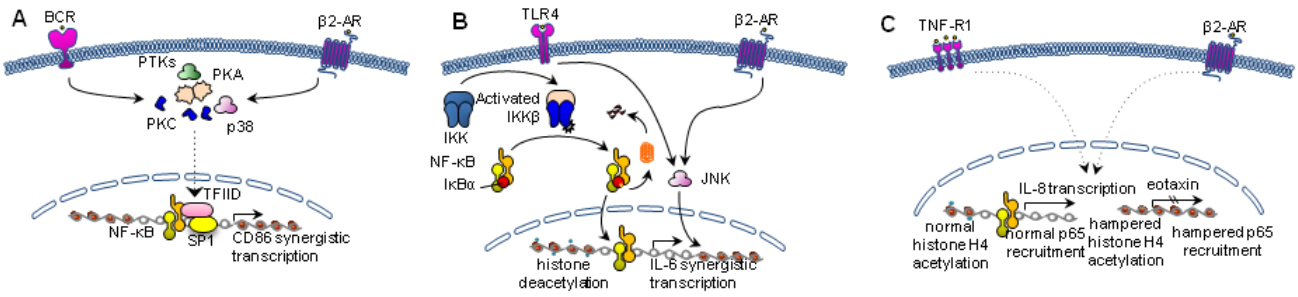

Figure 7. Nuclear β_2-AR/NF-κB crosstalk involving epigenetic events.

3.3. Effect of β_2-AR modulators on NF-κB-dependent gene expression

In Table 2, we have summarized the reported effects of (nor)epinephrine and pharmacological ligands of β_2-ARs on the expression of selected NF-κB target genes.

Effects have been reported in different immune cell types, but also in non-immune cells, such as glia, fibroblasts, epithelial and endothelial cells and smooth and skeletal muscle cells. As evident from the Table, the transcription of several prototypical NF-κB target genes, such as IL-2, IL-8 and TNF-α, appears to be consistently inhibited by β_2-agonists, while the effects on other targets, like for instance IL-6, IL-10 and IL-13, are less uniform. Interestingly, IL-6, IL-8, IL-13, CXCL-2, CXCL-3, B7-2 and COX-2 genes undergo synergistic expression upon co-activation of β_2-ARs and the NF-κB pathway in various cell types.

Table 2.

Summary of reported effects of β_2-AR agonists on NF-κB target gene expression.

Effect on target gene expression	*Cell type*	*NF-κB stimulus*	*β_2-AR ligand*	*Reference:*
Cytokines, chemokines and complement components				
C3				
-	132.1N1, Rat Primary Astrocytes, Rat Brain Tissue	TNF-α	Clenbuterol	(Laureys et al. 2014)
CCL-5 / RANTES				
-	Rat Cortex	LPS	Clenbuterol	(McNamee et al. 2010)
	132.1N1, Rat Primary Astrocytes, Rat Brain Tissue	TNF-α	Clenbuterol	(Laureys et al. 2014)
CXCL-2 / MIP-2α				
++	132.1N1, Rat Primary Astrocytes, Rat Brain Tissue	TNF-α	Clenbuterol	(Laureys et al. 2014)
CXCL-3 / MIP-2β				
++	132.1N1, Rat Primary Astrocytes, Rat Brain Tissue	TNF-α	Clenbuterol	(Laureys et al. 2014)
CXCL-10 / IP-10				
-	Rat Cortex	LPS	Clenbuterol	(McNamee et al. 2010)
Neutrophil elastase				
=	Human Neutrophils	Cigarette smoke extract	Salmeterol	(Mortaz et al. 2008)
Eotaxin				
-	Human Airway Smooth Muscle	TNF-α	Salmeterol	(Nie et al. 2005)
IL-1β				
+	Rat Cortex	None	Clenbuterol	(McNamee et al. 2010)
-		LPS	Clenbuterol	(McNamee et al.

				2010)
-	Rat Primary Glial Cells	LPS	Norepinephrine	(O'Sullivan et al. 2009)
IL-1ra				
+	Rat Cortex	None	Clenbuterol	(McNamee et al. 2010)
		LPS	Clenbuterol	(McNamee et al. 2010)
IL-1RII				
+	Rat Cortex	None	Clenbuterol	(McNamee et al. 2010)
		LPS	Clenbuterol	(McNamee et al. 2010)
IL-2				
-	Human T-cells	Phytohemagglutinin Concanavalin A α-CD3/α-CD28 mAb	Carvedilol	(Yang et al. 2003)
		PMA+Calcimycin α-CD3/α-CD28 mAb	Isoproterenol	(Loza et al. 2006)
		α-CD3/α-CD28 mAb	Norepinephrine	(Strell et al. 2009)
	Mouse Dendritic Cells	LPS	Salbutamol	(Herve et al. 2013)
IL-4				
-	Human T-cells	Phytohemagglutinin Concanavalin A α-CD3/α-CD28 mAb	Carvedilol	(Yang et al. 2003)
IL-6				
+++	HMC-1	IL-1β	Epinephrine	(Chi et al. 2004)
	C2C12 Myoblasts	LPS	Epinephrine	(Frost et al. 2004)
	132.1N1	TNF-α	Isoproterenol	(Spooren et al. 2010b)
	132.1N1	IL-1β, TNF-α	Isoproterenol	(Spooren et al. 2011)
	132.1N1, Rat Primary Astrocytes, Rat Brain Tissue	TNF-α	Clenbuterol	(Laureys et al. 2014)
++	Human Airway Smooth Muscle	TNF-α	Isoproterenol Formoterol Salmeterol Albuterol	(Ammit et al. 2002)
+	THP-1	None	Norepineprine	(Bierhaus et al. 2003)
	Mouse Neonatal Cardiac Fibroblasts	None	Isoproterenol	(Yin et al. 2006)
-	Bronco5 MyoICIG7	TNF-α	Salmeterol	(Baouz et al. 2005)
	Rat Cortex	LPS	Clenbuterol	(McNamee et al. 2010)
=	Human Airway Smooth Muscle	IL-1β	Salbutamol	(Kaur et al. 2008)
IL-8 / CXCL-8				
+++	HMC-1	IL-1β	Epinephrine	(Chi et al. 2004)
=	Human Airway Smooth Muscle	TNF-α	Salmeterol	(Nie et al. 2005)
	Human Airway	IL-1β	Salbutamol	(Kaur et al.

	Smooth Muscle			2008)
-	THP-1	LPS	Isoproterenol	(Farmer and Pugin 2000)
	Human T-cells	PMA	Clenbuterol	(Loop et al. 2004)
	Human Neutrophiles	Cigarette smoke extract	Salmeterol	(Mortaz et al. 2008)
	A549, Human Bronchial Smooth Muscle	TNF-α	Salbutamol	(Yang et al. 2010)
	132.1N1	TNF-α	Isoproterenol	(Spooren et al. 2010b)
	hTERT-Airway Smooth Muscle/ Primary Airway Smooth Muscle	Cigarette smoke extract	Fenoterol	(Oldenburger et al. 2012)
IL-10				
+	Mouse Dendritic Cells	LPS	Salbutamol	(Herve et al. 2013)
-	Human T cells	PMA	Clenbuterol	(Loop et al. 2004)
		Phytohemagglutinin Concanavalin A α-CD3/α-CD28 mAb	Carvedilol	(Yang et al. 2003)
IL-12				
-	Mouse Dendritic Cells	LPS	Salbutamol	(Herve et al. 2013)
IL-13				
+++	HMC-1	IL-1β	Epinephrine	(Chi et al. 2004)
+	Human T- cells	PMA	Isoproterenol (low doses)	(Loza et al. 2006)
-		PMA	Isoproterenol (high doses)	(Loza et al. 2006)
IL-18				
+	Mouse Cardiac-Derived Endothelial Cells	None	Isoproterenol	(Chandrasekar et al. 2004)
INF-γ				
-	Human T- cells	PMA	Isoproterenol	(Loza et al. 2006)
		Phytohemagglutinin Concanavalin A α-CD3/α-CD28 mAb	Carvedilol	(Yang et al. 2003)
TNF-α				
-	THP-1	LPS	Isoproterenol	(Farmer and Pugin 2000)
	ACHN	Shiga Toxin 2	Terbutaline	(Nakamura et al. 2001)
	Rat Primary Glial Cells	LPS	Norepinephrine	(O'Sullivan et al. 2009)
	Rat Cortex	LPS	Clenbuterol	(McNamee et al. 2010)
	Rat Cortex, Hippocampus	LPS	Clenbuterol	(Ryan et al. 2013)
=	C2C12 Myoblasts	LPS	Norepinephrine	(Frost et al. 2004)
Growth factors				
GM-CSF				

-	Human Airway Smooth Muscle	IL-1β	Salbutamol	(Kaur et al. 2008)
VEGF				
+	Primary Endothelial Cells	None	β_2-AR deficiency	(Ciccarelli et al. 2011)
Adhesion molecules				
ICAM-1				
+	THP-1	None	Norepineprine	(Djuric et al. 2012)
-	Human Airway Smooth Muscle	IL-1β	Salbutamol	(Kaur et al. 2008)
	132.1N1	TNF-α	Isoproterenol	(Spooren et al. 2010b)
	Rat Cortex	LPS	Clenbuterol	(McNamee et al. 2010)
	Rat Cortex, Hippocampus	LPS	Clenbuterol	(Ryan et al. 2013)
	132.1N1, Rat Primary Astrocytes, Rat Brain Tissue	TNF-α	Clenbuterol	(Laureys et al. 2014)
VCAM-1				
-	132.1N1	TNF-α	Isoproterenol	(Spooren et al. 2010b)
-	132.1N1, Rat Primary Astrocytes, Rat Brain Tissue	TNF-α	Clenbuterol	(Laureys et al. 2014)
Immunoreceptors				
CD40				
-	Rat Primary Glial Cells	LPS	Norepinephrine	(O'Sullivan et al. 2009)
-	Rat Cortex	LPS	Clenbuterol	(McNamee et al. 2010)
CD86/ B7-2				
+++	Mouse B-cells	$F(ab')_2$ anti-IgM	Terbutaline	(Kohm et al. 2002)
FPR2				
+	N9/ Mouse Primary Microglia	None	Norepinephrine Isoproterenol	(Kong et al. 2010)
TF/ CD142/ Tromboplastin				
+	THP-1	None	Norepineprine	(Djuric et al. 2012)
Enzymes				
COX-2				
+++	132.1N1	TNF-α	Isoproterenol	(Spooren et al. 2010b)
iNOS/ NOS2				
-	Rat Primary Microglia	LPS	Norepinephrine	(Dello Russo et al. 2004)
-	Rat Primary Glial Cells	LPS	Norepinephrine	(O'Sullivan et al. 2009)
MMP-2				
=	Human Neutrophils	Cigarette smoke extract	Salmeterol	(Mortaz et al. 2008)
MMP-9				
=	Human	Cigarette smoke extract	Salmeterol	(Mortaz et al.

	Neutrophils			2008)
MnSOD				
+	THP-1	None	Norepineprine	(Djuric et al. 2012)
Modulators of NF-κB signalling pathway components				
A20				
=	132.1N1, Rat Primary Astrocytes, Rat Brain Tissue	TNF-α	Clenbuterol	(Laureys et al. 2014)
IκBα				
+	C6/ Rat Primary Astrocytes	None	Norepineprine	(Gavrilyuk et al. 2002)
	Rat Primary Microglia	LPS	Norepinephrine	(Dello Russo et al. 2004)
	Rat Primary Neurons	None	Norepinephrine	(Madrigal et al. 2006)
	Rat Cortex	None	Clenbuterol	(McNamee et al. 2010)
	Rat Cortex, Hippocampus	None	Clenbuterol	(Ryan et al. 2013)
=	Rat Primary Astrocytes	LPS+IFN-γ	Norepinephrine	(Gavrilyuk et al. 2001)
	C2C12 Myoblasts	LPS	Epinephrine	(Frost et al. 2004)
-	C6	LPS+IFN-γ	Norepinephrine	(Gavrilyuk et al. 2001)

+ upregulation upon β_2-AR (co)stimulation
++ additive effect upon β_2-AR costimulation
+++ synergistic effect upon β_2-AR costimulation
- inhibition upon β_2-AR costimulation
= no change upon β_2-AR costimulation

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Chapter 3

Molecular biology of IL-6

In the present thesis, we have used the IL-6 gene as a model system to explore the nuclear crosstalk events associated with the coactivation of the β_2-AR/NF-κB signalling cascades.

1. Interleukin-6

IL-6 was originally discovered as a lymphocyte-derived factor that stimulates the final maturation of B-cells to immunoglobulin-secreting plasma cells (Muraguchi et al. 1981). Since then, the cytokine has been linked to a dizzying array of immunomodulatory functions (Scheller et al. 2011). Its multifunctionality is reflected in a wide number of alternative names (interferon-β2, B-cell stimulatory factor 2, hepatocyte stimulatory factor, myeloma/plasmocytoma growth factor, macrophage-granulocyte-inducing factor and T-cell-replacing factor) that had been assigned to this cytokine by different investigators before the research community realised that it is one and the same factor (Wolvekamp and Marquet 1990). Today, IL-6 is known to regulate various aspects of the immune response and deregulation of its expression was observed in several disease processes (Tanaka and Kishimoto 2012; Yao et al. 2014). Naugler and Karin (Naugler and Karin 2008) compare IL-6 to "the wolf in sheep's clothing", where IL-6 is "the sheep" in a physiological immune response but becomes "the wolf" when its expression is persistently elevated as in chronic inflammatory conditions. Furthermore, mounting evidence indicates that IL-6 is an important player in the regulation of whole body metabolism (Hoene and Weigert 2008; Pedersen and Febbraio 2005; Pedersen and Febbraio 2008).

2. Regulation of IL-6 expression

In homeostatic conditions, IL-6 levels are low, while during inflammation, disease processes, psychosocial stress and upon exercise, the amounts of the cytokine are rapidly and substantially elevated. Multiple cell types have been shown to produce and secrete IL-6. Whereas monocytes and macrophages are the primary source of this cytokine at the site of acute inflammation, IL-6 in chronic inflammatory conditions

additionally derives from T-cells (Naugler and Karin 2008). Furthermore, in the resting state, adipocytes are the chief producers of systemic IL-6 (Kern et al. 2001; Mohamed-Ali et al. 1997), while upon exercise skeletal muscle produces substantial amounts of IL-6 (Steensberg et al. 2002). Finally, in the CNS, both glial cells and neurons constitute a source of IL-6 (Spooren et al. 2011).

The stimuli that trigger IL-6 production, just like in the case of TNF-α, include multiple cytokines, growth factors, PAMPs and DAMPs. Furthermore, various hormones, such as epinephrine, and neurotransmitters, like for instance norepinephrine, substantially elevate IL-6 production (Naugler and Karin 2008; Tanaka et al. 2014; Vanden Berghe et al. 2000).

The human IL-6 gene is over 6000 base pairs long and has been mapped to chromosome 7p21. It consists of six exons and gives rise to ten alternative transcripts. IL-6 expression is regulated at the transcriptional and posttranscriptional levels, and separate sections will be devoted to these events. Human IL-6 is produced as a 20 kDa protein which includes a short signal peptide marking the protein for secretion. Before secretion, the cytokine undergoes glycosylation that accounts for the 21-26 kDa size of the native IL-6 protein. Mature IL-6 has four-α helix structure connected by three loops (Kishimoto 1989; Tanaka et al. 2014).

IL-6 transduces its biological activity via the classical or alternative signalling pathway. In the classical cascade, the cytokine binds to its membrane-bound receptor (IL-6Rα), while in the alternative pathway, signalling is initiated via a soluble form of this receptor (sIL-6Rα) present in serum. IL-6Rα (also known as gp80 or CD126) by itself is unable to transmit signals. Moreover, in contrast to other soluble forms of receptors, such as those for IL-1β and TNF-α, sIL-6Rα exhibits agonistic but not antagonistic properties (Heinrich et al. 2003). How sIL-6Rα is generated is not entirely clear, however, it was postulated that it involves alternative splicing (Lust et al. 1992) or proteolytic cleavage of the receptor expressed on the cell surface by ADAM10 and ADAM17 proteases (Chalaris et al. 2010; Matthews et al. 2003). Both soluble and membrane-anchored IL-6Rα interact with gp130, which functions as a signal transducer protein. An additional level of complexity is added by the existence of a soluble form of gp130 (sgp130), which was proposed to act as a negative regulator of IL-6 signalling. Specifically, sgp130 binds preformed IL-6/sIL-6Rα complexes in the serum and prevents their interaction with membrane bound gp130,

which is a key component to initiate intracellular signalling (Heinrich et al. 2003; Scheller et al. 2011). IL-6 signalling mechanisms are summarized in Figure 1.

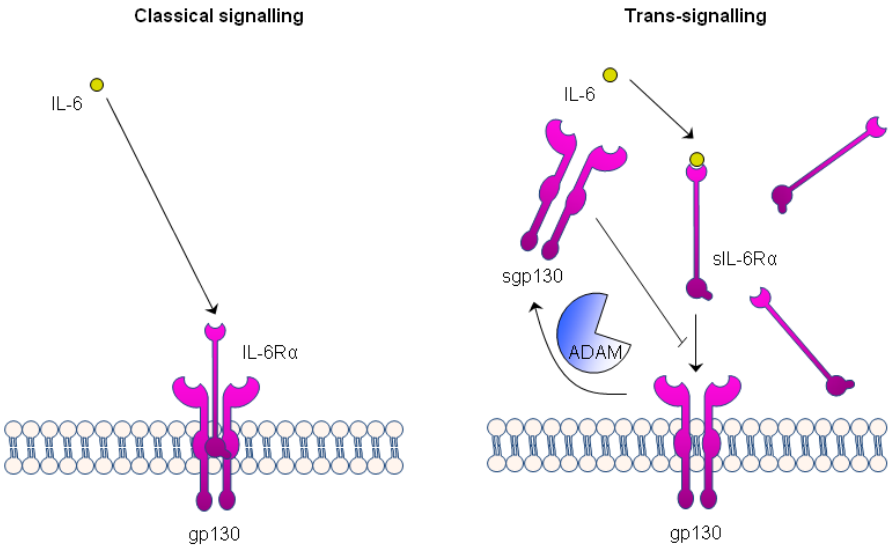

Figure 1. IL-6 signalling mechanisms. Secreted IL-6 can initiate the classical or alternative signalling pathway. In the classical signalling cascade, the cytokine binds to the membrane anchored IL-6Rα/gp130 complexes to induce intracellular events. Alternatively, IL-6 is "caught" by the sIL-6Rα to form complexes that together trigger the membrane-bound gp130 to initiate intracellular signalling. Additionally, the alternative IL-6 signalling cascade can be fine-tuned by a soluble form of gp130, which displays antagonist properties.

3. IL-6 signalling pathways

At the cellular level, binding of IL-6 to its receptor induces homodimerization of the gp130 signal transducer protein. Dimerization of gp130 engenders recruitment of Janus kinases (JAK1 and JAK2), which in turn phosphorylate specific tyrosine residues in the intracellular portion of gp130 forming a platform for recruitment of the Signal Transducer and Activator of Transcription (STAT) transcription factor. Subsequently, JAKs phosphorylate STATs. Phosphorylated STAT dimerizes and translocates from the cytoplasm to the nucleus, where it controls a transcriptional program of genes containing STAT-responsive elements (Heinrich et al. 2003). In the context of immunity, the STAT-3 family member is responsible for the vast majority of the IL-6-induced responses. STAT-3 promotes expression of genes involved in proliferation, differentiation and apoptosis. Furthermore, STAT-3 drives expression of negative regulators of IL-6 signalling, such as suppressor of cytokine signalling 3 (SOCS-3) (Aggarwal et al. 2009).

IL-6 also induces other intracellular responses. For instance, the cytokine engenders the JAK-mediated binding of SHP2, a protein-tyrosine phosphatase, which in this case forms a bridge to Growth-Factor-Receptor-Bound Protein 2/Son of Sevenless (Grb2/SOS) that in turns promotes the signalling via the Ras/Raf/ERK1/2 signalling axis culminating in the activation of the transcription factor C/EBPβ. Alternatively, IL-6 stimulates the PI3K/Akt signalling pathway. Activated Akt can launch the classical NF-κB signalling cascade (Heinrich et al. 2003). Intracellular events downstream of IL-6R are depicted in Figure 2.

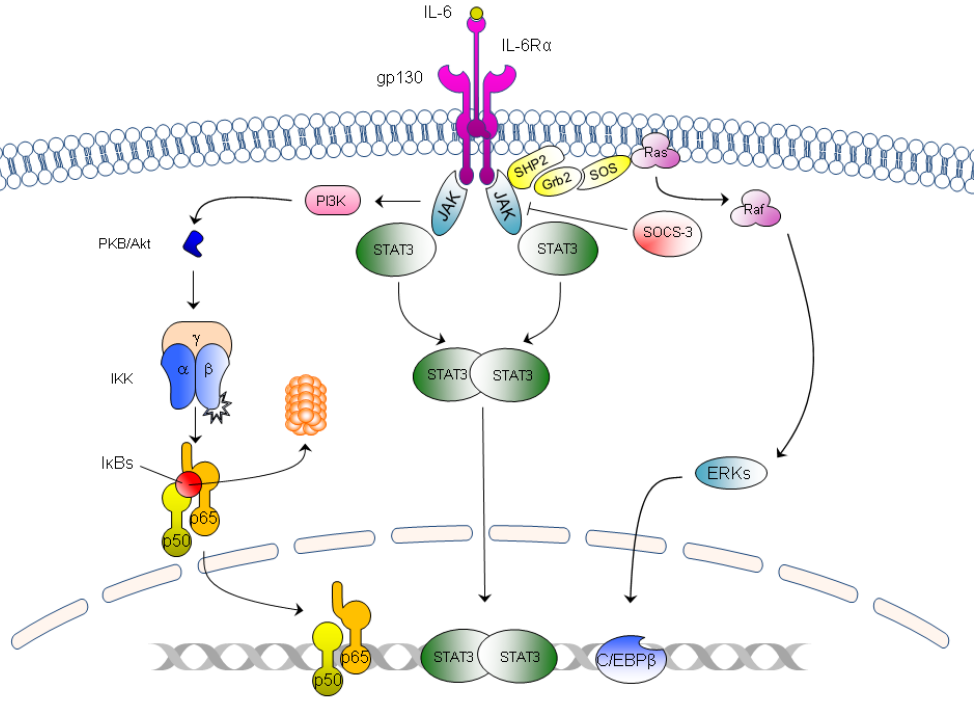

Figure 2. Intracellular events upon formation of the IL-6/(s)IL-6Rα/gp130 complexes. Assembly of complexes is followed by the recruitment of JAK, series of phosphorylation events, dimerization of STATs and its nuclear entry. Alternative signalling pathways promote activation of NF-κB or C/EBPβ transcription factors.

4. Physiological role of IL-6

Substantial insight into the role of IL-6 in immunity has been provided by studies on IL-6 knockout mice. Although, animals with genetic deletion of IL-6 develop normally, they display a markedly impaired response to infection due to abolishment of the acute-phase response mediated by the liver and a severe defect in T-cell-dependent antibody production (Kopf et al. 1994). Another study using IL-6

knockouts demonstrated that IL-6 is a crucial mediator of the localized inflammatory response and lack of IL-6 can be compensated to a certain extent by TNF-α (Fattori et al. 1994). In line with the above, the IL-6 knockout animals are resistant to the development of B lineage neoplasm indicating an essential role of the cytokine in the development of these tumours *in vivo* (Hilbert et al. 1995).

Furthermore, IL-6 is a crucial factor mediating the switch from the initiatory innate immune response to the more focused adaptive immune response. In that context, the cytokine orchestrates the shift of leukocyte populations from predominantly neutrophilic to mainly monocytic via upregulation of CXC and CC motif chemokine expression (Hurst et al. 2001). Accumulating evidence shows that IL-6 is also important for regulating T-cell differentiation. For instance, IL-6 induces differentiation of CD8+ T cells into cytotoxic T cells (Okada et al. 1988). Furthermore, the cytokine together with TGF-β promotes development of Th17 lymphocytes from naive T cells, while in the absence of IL-6, cell differentiation towards the suppressive T regulatory population dominates (Bettelli et al. 2006). Whereas T regulatory lymphocytes contribute to progressive quenching of the immune response, via the production of among others IL-10 and TGF-β, Th17 lymphocytes have a proinflammatory action via secretion of substantial amounts of IL-17 (Park et al. 2005). Deregulation of the balance between these populations underlies the development of various pathological conditions (Bettelli et al. 2006). Finally, IL-6 governs differentiation of follicular T helper cells as well as the production of IL-21, which regulates antibody production (Ma et al. 2012). Hence, IL-6 modulates the immune response at multiple levels acting at diverse cell populations.

In addition to its role in immunity, IL-6 is an important regulator of other physiological processes. In this thesis, we have studied regulation of IL-6 expression in skeletal muscle and brain. Thus, the role of this cytokine will be exemplified on these organs. IL-6 is synthesized and secreted from skeletal muscle in response to exercise and this muscle-derived cytokine was show to modulate several metabolic processes within skeletal muscle as well as other organs acting in a hormone-like manner. Whereas locally acting IL-6 increases muscles glucose uptake and fat oxidation, upon release into the circulation, it potentiates hepatic glucose production or lipolysis in adipose tissue (Pedersen and Febbraio 2008). Mounting evidence also shows the importance of IL-6 in the process of hypertrophic muscle growth and

myogenesis (Munoz-Canoves et al. 2013). In the CNS, IL-6 also displays variegated functions. This cytokine is one of the regulators of appetite, energy expenditure and body composition (K. Wallenius et al. 2002a). Furthermore, IL-6 promotes proliferation, differentiation and regeneration of neurons, influences neuronal activity, secretion of neurotransmitters to synaptic cleft and finally affects the cellular fate of neuronal stem, progenitor and differentiated cells (Spooren et al. 2011).

5. Pathophysiological role of IL-6

Accumulating evidence indicates that elevated levels of IL-6 are associated with the development of multiple chronic inflammatory and autoimmune diseases, such as rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, asthma, various neurodegenerative disorders as well as conditions leading to skeletal muscle wasting (Carson and Baltgalvis 2010; Naugler and Karin 2008; Spooren et al. 2011). In support of these observations, studies on IL-6 knockout mice indicate that blockage of IL-6 signalling could have therapeutic potential for the treatment of multiple sclerosis and rheumatoid arthritis (Ohshima et al. 1998; Samoilova et al. 1998). Furthermore, elevated serum levels of IL-6 have been measured in patients with obesity and insulin resistance (Kern et al. 2001). On the other hand, IL-6 deficiency was also reported to have a substantial impact on the development of mature-onset obesity due to increased expression of muscle and adipocyte-derived factors that participate in the formation of acylation-stimulating protein (ASP), a cleavage product of the C3 complement component. ASP stimulates synthesis and uptake of triacylglycerol by adipocytes, hence contributing to development of obesity in IL-6 knockout mice (V. Wallenius et al. 2002b; Wernstedt et al. 2006). Finally, a progressive increase in IL-6 expression is observed during aging (Maggio et al. 2006; Sarkar and Fisher 2006).

The primary initiator of the abovementioned conditions remains obscure, however, a milieu rich in inflammatory mediators, including IL-6, is present in all. Furthermore, these disease processes are often associated with elevated levels of TNF-α and enhanced NF-κB activity. Both factors are well-known upstream regulators of IL-6 expression (Naugler and Karin 2008). Thus, expression of IL-6 and the IL-6/(s)IL-6Rα signalling axis appears to be a promising targets for therapeutic intervention.

Administration of corticosteroids and non-steroidal anti-inflammatory drugs is routinely used for the treatment of chronic inflammatory and autoimmune diseases with elevated levels of IL-6 (Ataie-Kachoie et al. 2013; Yao et al. 2014). Although

these drugs effectively interfere with inflammatory process via suppression of multiple inflammatory mediators, more selective anti-IL-6 therapies are available or are under development. Tocilizumab is the only monoclonal antibody-based IL-6Rα inhibitor currently available on the pharmaceutical market and this monoclonal antibody is used for treatment of rheumatoid arthritis. Clinical responsiveness to this drug was also demonstrated for other chronic inflammatory disorders, such as Castleman's disease and systemic juvenile idiopathic arthritis. Furthermore, it is expected to be applicable for various other autoimmune and chronic inflammatory disorders (Tanaka and Kishimoto 2012). At the molecular level, it neutralizes IL-6-mediated activities by blocking the binding site in both sIL-6Rα and membrane-bound IL-6Rα (Mihara et al. 2005). Additionally, several therapeutic interventions based on monoclonal antibodies targeting IL-6 or IL-6Rα are in clinical trials. Examples include Sarilumab, which is also designed to interfere with the signalling originating from IL-6Rα, or Olokizumab, Sirukumab, Siltuximab and Clazakizumab that specifically target the IL-6 cytokine. As these protein inhibitors show clinical efficacy and are well tolerated, they constitute a promising alternative for strategies aimed at blocking the TNF-α/TNF-R axis. Finally, all of these drugs are developed primarily against rheumatic disease, while there is a gap in the testing of IL-6 blockage strategies against other chronic/autoimmune disorders in which IL-6 levels are elevated (Neurath and Finotto 2011; Woodrick and Ruderman 2012).

6. Molecular biology of IL-6 expression

6.1. Transcriptional regulation of IL-6

The IL-6 promoter has a unique architecture with multiple motifs that are recognized by various transcriptional regulators, suggesting it could serve as a platform for crosstalk between multiple signalling pathways. Indeed, IL-6 expression is induced by a wide array of environmental stimuli (Sehgal 1992), which in turn activate a panel of transcription factors converging their activity at the IL-6 promoter. These factors include among others: Nuclear Factor-κB (NF-κB), Specificity Protein-1 (SP-1), CCAAT/enhancer-binding protein (C/EBP), cAMP response element binding protein (CREB) and Activator Protein 1 (AP-1) (Dendorfer et al. 1994; Grassl et al. 1999; Vanden Berghe et al. 1998) (Figure 3). Among these transcription factors, NF-κB is

indispensable for efficient IL-6 expression (Libermann and Baltimore 1990). The contribution of other transcriptional regulators differs between cell types and stimuli.

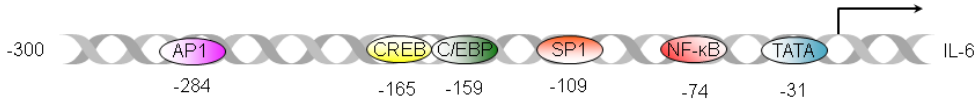

Figure 3. Schematic representation of the human IL-6 promoter.

Previous studies showed that TNF-α triggering leads to assembly of an enhanceosome structure composed of NF-κB, CREB, C/EBP, AP-1 that are spatially organized by CBP at the IL-6 promoter (Vanden Berghe et al. 1999). Recently, Spooren *et al.* (Spooren et al. 2010) have reported that concurrent treatment with TNF-α and isoproterenol results in the assembly of enhanceosome at the IL-6 promoter to ensure synergistic gene expression in the human 1321N1 astrocytes. The foundations of the latter structure consist of NF-κB p65 subunit and CREB, which cooperatively recruit CBP and RNA polymerase II to ensure high rate of transcription.

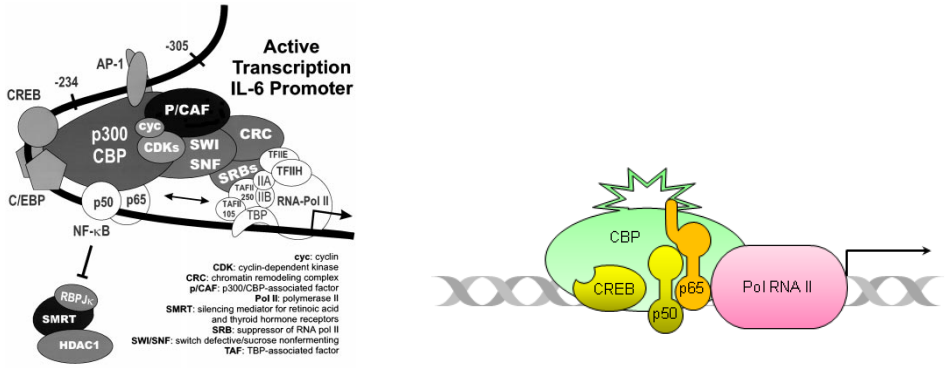

Figure 4. Hypothetical models of the IL-6 enhanceosomes upon TNF-α stimulation (left) (scheme reproduced from (Vanden Berghe et al. 1999)) and combined treatment with TNF-α/isoproterenol (right).

Epigenetic regulation of IL-6 expression has also attracted a lot of attention. IL-6 is prototypically classified as a "late gene" because its transcription requires histone modifications and reorganization of chromatin structure within its promoter sequence. In that context, phosphorylation of histone H3 at serine 10 has been recurrently reported to promote IL-6 transcription (Anest et al. 2003; Pathak et al. 2006; Saccani et al. 2002; Vanden Berghe et al. 2006; Yang et al. 2008). Furthermore, histone acetylation has been both positively (Kim et al. 2012; Lee et al. 2013; Vanden Berghe

et al. 1999; Wada et al. 2014) and negatively (Frost et al. 2004; Mishra et al. 2003) associated with expression of IL-6, suggesting a context-dependent role of this modification in the regulation of gene transcription.

6.2. Post-transcriptional regulation of IL-6

At post-transcriptional level, the fate (stabilization/degradation) of IL-6 mRNA is controlled through both the 5' and 3' UTR (Anderson 2008). Whereas the 5'UTR is responsible for initiation of translation, the AREs localized within the 3'UTR govern the stability of IL-6 mRNA (Chen and Shyu 1995). Multiple RNA-binding proteins and microRNAs control IL-6 mRNA decay via interaction with regulatory motifs in the UTRs and recruitment of accessory proteins, which mediate mRNA cleavage or stabilization (Jing et al. 2005; Khabar 2010; Schott and Stoecklin 2010; Stoecklin et al. 2006).

Several RNA-binding proteins, such as TTP, butyrate response factor-1 (BRF-1) and BRF-2, ARE/poly(U) binding degradation factor-1 (AUF-1), Kaposi sarcoma-associated herpes virus open reading frame (KSRP) are implicated in regulation of IL-6 mRNA decay (Paschoud et al. 2006; Tanaka et al. 2014; Winzen et al. 2007). Furthermore, various microRNAs, such as microRNA-26, microRNA-365, microRNA-608 and let-7, promote IL-6 mRNA degradation (Iliopoulos et al. 2009; Jones et al. 2009). Finally, the regulatory RNA nuclease Regnase-1 has been recently appointed as a new regulator of IL-6 mRNA fate and its IKK-induced degradation inversely correlates with IL-6 mRNA stability (Iwasaki et al. 2011).

Oppositely, AT-rich interactive domain-containing protein 5a (Arid5a) was shown to selectively stabilize the IL-6 transcript in response to stimulation with inflammatory triggers, such as LPS, IL-1β and IL-6 itself. In addition, Arid5a counteracts the Regnase-1 activity on IL-6 mRNA (Masuda et al. 2013). Interestingly, HuR and apobec-1 complementation factor (ACF) were also reported to protect IL-6 mRNA from degradation (Blanc et al. 2010; Nabors et al. 2001; Zhou et al. 2007). Finally, the influence of KSRP on the IL-6 transcript appears to be context dependent as this protein was demonstrated to compete with miRNA-608 to IL-6 mRNA, thereby leading to its stabilization (Tanaka et al. 2014).

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Scope of thesis

Scope of thesis

NF-κB is the generic name of a family of transcription factors that regulates the expression of genes involved in immunity, inflammation but also other biological processes. In research settings, NF-κB activation is classically triggered by typical proinflammatory stimuli, such as LPS or TNF-α (Hayden and Ghosh 2012). Whereas NF-κB activation is instrumental for successful coordination of the immune/inflammatory response, excessive NF-κB activation is associated with the onset and propagation of chronic inflammatory and auto-immune diseases (Baker et al. 2011; DiDonato et al. 2012). In this respect, developing strategies that block NF-κB signalling have become a major focus for research. However, the same functions that make NF-κB attractive for developing inhibitors for treating disease also play a role in homeostasis, and disruption of the NF-κB pathway during development or in adults has unfavourable consequences. It is this balance, between treating human disease and unwanted side-effects, that remains a big challenge in NF-κB drug discovery. Importantly, various environmental stimuli can activate/modulate NF-κB, and the kinetics and gene expression spectrum associated with NF-κB activation is highly context-dependent. Designing efficient, yet safe, NF-κB inhibitors will therefore require an in depth understanding of signalling crosstalk with the NF-κB pathway in physiological and pathological conditions (Karin 2005).

There is overwhelming physiological evidence for immunomodulatory, mostly immunosuppressive, effects associated with sympathetic stress (Padro and Sanders 2014; Powell et al. 2013, Chapter 2). The molecular basis of these effects and their impact on NF-κB function are however still poorly understood. As a paradigm to study how stress signals affect NF-κB activity, we investigated how coactivation of the β_2-AR, a prototypical GPCR that is activated by catecholamine stress hormones/neurotransmitters, modulates NF-κB function. Previous work from our group showed that in human astrocytoma cells, β_2-agonists modulate NF-κB activity in a gene-selective manner, via nuclear crosstalk mechanisms involving cooperation of NF-κB and CREB transcription factors. Importantly, we observed that the expression of selected NF-κB target genes, including IL-6, was synergistically enhanced upon β-agonist/TNF-α cotreatment, which seems counterintuitive in view of

previous reports mainly showing anti-inflammatory effects of adrenergic stress (Spooren et al. 2010).

The aims of the present study were to extend these findings and 1) investigate whether the unexpected regulation we observed in astrocytes also applies to other cell types responsive to adrenergic stimulation, 2) further elucidate the molecular basis of the nuclear crosstalk between β_2-AR and NF-κB.

Therefore, in a first part of this thesis, we investigated β_2-AR/TNF-α crosstalk in C2C12 cells, a murine model for skeletal muscle. Using this model system, we performed an in depth biochemical analysis of β_2-AR/TNF-α crosstalk and the result of this crosstalk at the chromatin level. In a second part of the thesis we used a proteomics approach to identify nuclear factors involved in the synergistic IL-6 transcription apparent upon TNF-α/β-agonist cotreatment.

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Results

1. β-agonists selectively modulate proinflammatory gene expression in skeletal muscle cells via non-canonical nuclear crosstalk mechanisms

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In this part of the thesis, we have investigated the nuclear crosstalk events upon β_2-AR and TNF-R coactivation in C2C12 cells, a murine model for skeletal muscle.

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Contribution:
Conceived and designed the experiments: KK SG JT GH MVT CA.
Performed the experiments: KK SG KVW MVT.
Analyzed the data: KK SG MVT.
Contributed reagents/materials/analysis tools: SG KK JT GH MVT CA.
Wrote the paper: KK SG JT GH MVT CA.
Designed figures and tables: KK SG MVT

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β-Agonists Selectively Modulate Proinflammatory Gene Expression in Skeletal Muscle Cells via Non-Canonical Nuclear Crosstalk Mechanisms

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Abstract

The proinflammatory cytokine Tumour Necrosis Factor (TNF)-α is implicated in a variety of skeletal muscle pathologies. Here, we have investigated how *in vitro* cotreatment of skeletal muscle C2C12 cells with β-agonists modulates the TNF-α-induced inflammatory program. We observed that C2C12 myotubes express functional TNF receptor 1 (TNF-R1) and β2-adrenoreceptors (β2-ARs). TNF-α activated the canonical Nuclear Factor-κB (NF-κB) pathway and Mitogen-Activated Protein Kinases (MAPKs), culminating in potent induction of NF-κB-dependent proinflammatory genes. Cotreatment with the β-agonist isoproterenol potentiated the expression of inflammatory mediators, including Interleukin-6 (IL-6) and several chemokines. The enhanced production of chemotactic factors upon TNF-α/isoproterenol cotreatment was also suggested by the results from migrational analysis. Whereas we could not explain our observations by cytoplasmic crosstalk, we found that TNF-R1-and β2-AR-induced signalling cascades cooperate in the nucleus. Using the IL-6 promoter as a model, we demonstrated that TNF-α/isoproterenol cotreatment provoked phosphorylation of histone H3 at serine 10, concomitant with enhanced promoter accessibility and recruitment of the NF-κB p65 subunit, cAMP-response element-binding protein (CREB), CREB-binding protein (CBP) and RNA polymerase II. In summary, we show that β-agonists potentiate TNF-α action, via nuclear crosstalk, that promotes chromatin relaxation at selected gene promoters. Our data warrant further study into the mode of action of β-agonists and urge for caution in their use as therapeutic agents for muscular disorders.

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Introduction

Skeletal muscle atrophy is a devastating consequence of a large number of diseases, including cancer and myopathies, but is also apparent in physiological processes, such as aging or disuse. Several lines of evidence indicate that inflammatory factors contribute to the loss of skeletal muscle mass and function [1]. One of the cytokines that has been especially associated with the development of skeletal muscle abnormalities is Tumour Necrosis Factor (TNF)-α and elevated levels of TNF-α are apparent in skeletal muscle wasting disorders [2]. TNF-α transduces its activity via two different types of membrane-bound receptors, namely TNF-receptor 1 (TNF-R1) and TNF-receptor 2 (TNF-R2), which stimulate different cellular processes. TNF-R ligation leads to the recruitment of receptor-specific adaptor proteins, which in turn activate a cascade of protein kinases and several downstream transcription factors, including the Nuclear Factor (NF)- κB [3].

NF-κB is the generic term for members of a family of ubiquitously expressed transcription factors, that act as homo- or heterodimers to regulate genes involved in immunity and inflammation [4]. In the context of inflammatory gene expression, the p65-p50 NF-κB heterodimer has been most intensively studied. TNF-α induces the canonical NF-κB signalling pathway, marked by activation of the IκB kinase β (IKKβ) complex, which phosphorylates the IκBα inhibitor proteins that, in resting cells, sequester NF-κB in the cytoplasm. Phosphorylated IκBα is ubiquitinylated and targeted for proteasomal degradation, allowing NF-κB to migrate from the cytoplasm to the nucleus, where it drives transcription of genes containing NF-κB-responsive elements [4]. Whereas NF-κB function has been mainly studied in immune cells, recent reports have demonstrated a role for NF-κB in a variety of other cell types, including skeletal muscle. For instance, it was shown that interference with NF-κB activity, via overexpression of IκB supersuppressor or p65 knock-out, reduces inflammation and improves the regeneration process in different skeletal muscle disease models [1,5].

The adrenergic receptors belong to the family of G-protein coupled receptors (GPCRs) and skeletal muscle cells express mainly the β_2-adrenoreceptor (β_2-AR) subtype [6]. β_2-AR agonists (β-agonists) are well known for their anabolic properties and several *in vivo* studies support the therapeutic potential of β-agonists in skeletal muscle wasting disorders [7-9]. Interestingly, the existence of extensive crosstalk between β_2-AR and TNF-R-

mediated signalling cascades was documented in different cell types and it was postulated that β-agonists have anti-inflammatory effects, that can be, at least in part, explained by inhibition of NF-κB activity [10–12]. Arguing against the anti-inflammatory effects of β_2-AR stimulation is the repeated observation, in different model systems, that β-agonists potentiate the expression of the prototypical inflammatory cytokine IL-6, a phenomenon that was also reported in skeletal muscle cells *in vivo* and *in vitro* [13,14].

Recent genome-wide expression studies have yielded detailed information on the individual effects of TNF-α or β-agonists on the skeletal muscle transcriptome [15,16]. However, the effect of controlled cotreatment of skeletal muscle cells with TNF-α and β-agonists has, to our knowledge, never been investigated. In addition, the molecular basis for the reported selective effect of β-agonists on NF-κB dependent gene expression remains to be elucidated. Therefore, we have investigated how β-agonists modulate TNF-α-induced signalling cascades, focusing on the NF-κB pathway and its target genes in C2C12 myotubes. As opposed to previous reports in other cell types, we found no direct inhibitory effects of β-agonists on the NF-κB cascade in C2C12 skeletal muscle cells. Instead, we found that the β-agonist isoproterenol potently enhanced TNF-α-induced expression of selected NF-κB target genes, such as interleukin-6 (IL-6) and chemokine (C-X-C motif) ligand 5 (CXCL5). In addition, we report that this selective potentiation is not mediated by cytoplasmic modulation of NF-κB function, but instead relies on atypical epigenetic events. Finally, in support of the physiological relevance of our findings, the co-activation of β_2-AR/TNF-Rs in myotubes promoted the migration of undifferentiated myoblasts.

Materials and Methods

Reagents and Antibodies

Isoproterenol and Trichostatin A (TSA) were purchased from Sigma Aldrich (St. Louis, MO, USA) and used at 10 μM and 100 nM, respectively. Murine TNF-α was a gift from the VIB Department for Molecular Biomedical Research of Ghent University (VIB-UGent, Gent, Belgium) and was used at 2000 IU/ml. Insulin-like growth factor-1 (IGF-1) was from ImmunoTools (Friesoythe, Germany) and was used at 10 ng/ml. Anti-β_2-AR, anti-TNF-R1, anti-myogenin, anti-PARP, anti-P-H3-Ser10, anti-CBP, anti-RNA polymerase II, anti-p65, anti-IκBα, anti-P-CREB-Ser133 and anti-PKAc were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-P-p65-Ser536, anti-P-ERK-Thr202/Tyr204, anti-P-JNK-Thr183/Tyr185, anti-P-p38-Thr180/Tyr182, anti-P-MSK-1-Thr581, anti-Lamin A/C and anti-CREB were from Cell Signaling Technology (Danvers, MA, USA). Anti-Ac-H3-Lys27 and GAPDH were from AbCam (Cambridge, UK). Anti-α-tubulin and anti-α-actin were from Sigma-Aldrich. In figures, the expression "antibody anti-" was substituted by the Greek letter "α-". AatII and HincII restriction enzymes were obtained from New England BioLabs (Ipswich, MA, USA).

Cell culture

Murine C2C12 cells (European Collection of Cell Cultures, Salisbury, UK) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco by Life Technologies) supplemented with 10% foetal bovine serum (Gibco by Invitrogen, Paisley, UK), 100 IU/ml penicillin and streptomycin (Gibco by Life Technologies, Grand Island, NY, USA) (referred to herein after as growth medium or GM). Cells were cultured in an incubator at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged using 0.05% Trypsin+EDTA (Gibco by Life Technologies). In experiments using myoblasts, cells were seeded at 200000 cells/well in a 6-well format and GM was substituted by starvation medium (DMEM containing 1% foetal bovine serum, 100 IU/mL penicillin and streptomycin, referred as SM) for 24 hours. In experiments using myotubes, cells were seeded at 300000 cells/well in a 6-well format and GM was substituted by differentiation medium to induce differentiation (DMEM containing 2% horse serum, 100 U/ml penicillin and streptomycin, referred as differentiation medium or DM). To obtain myotubes, cells were cultured for 5 days in DM.

RNA isolation, cDNA synthesis and Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Reverse transcription was performed on 0.5 μg of total mRNA using the PrimeScript RT reagent kit from Takara Bio Inc. (Shiga, Japan). For real time cDNA amplification we used the Roche SYBR Green Mastermix (Roche Applied Science, Penzberg, Germany) and primers listed in Table S1. Fluorescence was monitored using the Light Cycler 480II (Roche). A serial dilution of a representative cDNA sample was used to generate a standard curve and determine the efficiency of the PCR reaction for all primer sets which was used in the subsequent calculation of relative mRNA inputs. Expression of each gene was normalized to expression of the HPRT house-keeping gene and results are presented as fold induction compared to untreated cells. For clarity the Y-axis is interrupted in some cases.

Luciferase reporter assay

The NF-κB-luciferase reporter plasmid and the β-galactosidase control constructs have been described elsewhere [17]. The CRE-luciferase reporter plasmid was from Stratagene (LaJolla, CA). Transient transfection was performed using the Neon Transfection system (Invitrogen), according to the manufacturer's instructions for the murine C2C12 cell line. Briefly, cells were transfected with 1 μg of luciferase reporter constructs harbouring a consensus NF-κB site or CREB binding site and seeded to semi-confluence in 24-well plates. Cells were cotransfected with a β-galactosidase reporter construct (200 ng) containing the constitutively active pPGK promoter to correct for transfection efficiency. Following transfection, cells were differentiated for 5 days. Subsequently, cells were induced with isoproterenol and/or TNF-α for 6 hours. Total lysate was then incubated with luciferase substrate and luminescence was measured on a TopCount luminometer (PerkinElmer Life Sciences, Canberra-Packard, Waverley, UK). Luciferase activity was expressed as fold induction (treated/untreated) upon normalisation for transfection efficiency.

Isolation of cellular proteins

For preparation of total protein lysates, cells were washed with ice-cold Phosphate Buffered Saline (PBS) and subsequently lysed in RIPA lysis buffer (200 mM NaCl, 50 mM Tris HCl pH 8.0, 0.05% SDS, 2 mM EDTA, 1% NP40, 0.5% Na-Deoxycholate, 2 mM Na_2MoO_4 and 10 mM NaF, Complete Protease Inhibitor Cocktail (Roche)) or in SDS sample buffer (10% glycerol, 62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.5% β-mercaptoethanol and Bromophenol Blue).

For isolation of nuclear proteins, cells were washed with PBS, PBS with 2 mM Na_2MoO_4 and 10 mM NaF and buffer HB (20 mM Hepes pH 7.5, 10 mM NaF, 2 mM Na_2MoO_4, 0.2 mM EDTA pH 7.5). After aspiration of the washing buffer, lysis buffer (20 mM Hepes pH 7.5, 10 mM NaF, 2 mM Na_2MoO_4, 0.2 mM EDTA pH 7.5, 0.05% NP40, Complete Protease Inhibitor

Cocktail (Roche)) was added. Subsequently, cells were scraped and collected. Samples were centrifuged at 17900 g for 1 minute in 4°C. The pellet was recovered and resuspended in Resuspension Buffer (20 mM Hepes pH 7.5, 0.1 mM EDTA pH 7.5, 5 mM NaF, 1 mM Na_2MoO_4, 20% glycerol) supplemented with the Complete Protease Inhibitor Cocktail (Roche). Next, equal volume of salt buffer (20 mM Hepes pH 7.5, 1.6 M NaCl, 0.1 mM EDTA pH 7.5, 5 mM NaF, 1 mM Na_2MoO_4, 20% glycerol, Complete Protease Inhibitor Cocktail (Roche)) was added. Samples were incubated for 30 minutes in the shaker at 4°C and subsequently centrifuged at 17900 g for 10 minutes at 4°C. Samples were next diluted to equal protein concentration (determined via BioRad protein assay) and 5x concentrated SDS sample buffer was added. The extracts were used subsequently for Western blotting analysis.

Protein contents of RIPA lysates and nuclear extracts were determined using the Bio-Rad protein assay according to manufacturer's instructions. Samples were next diluted to equal protein concentration and 5x concentrated SDS sample buffer was added. The extracts were used subsequently for Western blotting analysis.

Western blotting

10 µg of nuclear proteins, 25 or 50 µg RIPA protein lysates or 25 µl of SDS sample buffer protein lysates were resolved using SDS-PAGE on 10% or 12% polyacrylamide gels, transferred to nitrocellulose membranes (Amersham, Dubendorf, Switzerland) and analysed by Western blotting. Briefly, membranes were incubated with Blocking Buffer (LICOR Biosciences, Lincoln, NE, USA) diluted with PBS in a 1:1 ratio. Subsequently, membranes were probed with primary antibody diluted 1:1000 in Blocking Buffer diluted 1:1 with PBS containing 0.1% Tween (PBS-T). After washing in PBS-T, DyLight secondary antibody (Pierce, Rockford, IL, USA) was applied diluted 1:10000 in Blocking Buffer/PBS-T (1:1). The membranes were then washed in PBS-T and detection was performed using the Odyssey Imaging System (Licor). Subsequently, the membranes were reprobed with Actin, Tubulin, GAPDH, Lamin A/C or PARP antibodies to verify equal loading. Densitometric analysis averaging the results from independent Western blotting experiments was performed using ImageJ.

Chromatin accessibility assay via Real-Time PCR (CHART-PCR)

The nuclease accessibility technique was performed as follows: cells were washed in PBS, scraped and collected by centrifugation at 453 g for 5 minutes at 4°C. Next, cells were resuspended in buffer A (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.3 M sucrose) and incubated for 10 minutes at 4°C. Next, equal volume of lysis buffer was added (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.3 M sucrose, 0.4% NP40 and 2 mM Na-butyrate) and cells were incubated for 10 minutes at 4°C. After centrifugation at 240 g for 5 minutes at 4°C, chromatin was resuspended in buffer R (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM Na-butyrate) digested for 30 min at 37°C with restriction enzymes in the buffer recommended by the manufacturer (New England BioLabs). Reactions were terminated by the addition of 2× Proteinase K buffer (100 mM Tris, pH 7.5, 200 mM NaCl, 2 mM EDTA, 1% SDS). Following proteinase K (Qiagen) and RNase A (Qiagen) treatment, genomic DNA (gDNA) was extracted using the phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) method and subsequently resuspended in sterile water after ethanol precipitation. Purified gDNA (10 ng) was used for RT-PCR. Primers were designed to amplify sequences within the murine IL-6 promoter (Table S1). A serial dilution of a representative gDNA sample was used to generate a standard curve and determine the efficiency of the PCR reaction for all primer sets and to calculate the relative gDNA concentration ([gDNA]) of the samples. Data are presented as chromatin opening which was defined as the ratio of [gDNA] of samples digested with restriction enzymes over [gDNA] of undigested samples: Chromatin opening = [gDNA]digested/[gDNA]undigested.

Chromatin immunoprecipitation (ChIP)

For ChIP experiments, protein/DNA complexes were cross-linked *in cellulo* by adding formaldehyde directly to the culture medium to a final concentration of 1%. After 10 minutes, glycine was added to a final concentration of 125 mM for another 10 minutes. Cells were washed, scraped and collected. Pellets were lysed in FA lysis buffer (0.1% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0) supplemented with Complete Protease Inhibitor Cocktail (Roche). Cells were sonicated using the Diagenode Bioruptor (Liège, Belgium) at high settings (8 min, 30 s on/30 s off, samples cooled on ice and repeat of the 8 minute cycle). Sonicated lysates were mixed in 1:5 ratio with incubation buffer (0.15% SDS, 1% Triton X-100, 150 mM, 1 mM EDTA, 20 mM HEPES-KOH pH 7.5) followed by immunoprecipitation with 5 µg of anti-p65, anti-polymerase RNA II, anti-P-H3 Serine 10, anti-Ac-H3 Lys27, anti-CBP or anti-CREB using Protein A Sepharose 4 Fast Flow beads (Amersham). Samples were decrosslinked overnight at 65°C. All samples were treated with 50 µg/ml of RNase A and 100 µg/ml of Proteinase K. Immunoprecipitated genomic DNA (gDNA) was purified with the QiaQuick PCR purification kit (Qiagen) and subsequently quantified by Real Time PCR using the Roche SYBR Green Mastermix (Roche). Primers used for amplification of the IL-6 promoter and GAPDH control region are listed in Table S1. Quantitative PCR was performed on the Light Cycler 480II (Roche). Determination of [gDNA] in the input and immunoprecipitated samples (IPs) was performed as for CHART-PCR. Data are presented as the percentage (%) of [gDNA] in the IPs as compared to the [gDNA] in the corresponding input sample: %IP = ([gDNA]IP/[gDNA]input) ×100.

Medium swap and migration assay

To investigate the influence of myotube-secreted factors on myoblast migration, a medium swap, followed by a migration assay, was performed. Conditioned media were prepared from untreated myotubes or myotubes treated with isoproterenol and/or TNF-α for 60 minutes. Subsequently, cells were washed twice with DMEM to remove the remaining inducers and incubated with serum-free DMEM for 24 hours to collect conditioned media with secreted factors. Conditioned media were filtered on 0.22 µm pore membranes (MILLEX GS, MF-Millipore MCE Membrane) and applied to myoblasts. As a positive control, IGF-1, dissolved in serum free DMEM, was used. Myoblasts were seeded in GM (20000 cells/well) on a collagen coating (25 µg/mL rat tail monomeric collagen type I; BD Biosciences, Two Oak Park, Bedford, MA, USA) in wells of a 96-well plate. A cell-free area was generated in the middle of the well using stoppers, according to the ORIS cell migration protocol (ORIS; Platypus Technologies). After 5 hours of cell adhesion, stoppers were removed, GM was aspirated, 100 µl of conditioned media was applied and cells were allowed to migrate for 24 hours into the cell-free central zone. Phase-contrast time-lapse movies were recorded for 24 hours with an interval of 10 minutes using a 10x UPlanFL objective (N.A. 0.30) on a CellM system with a IX81 microscope (Olympus). Four

to eleven replicates were tested per condition and three independent experiments were performed. The area covered by the cell layer (that increases due to migration towards the central zone) was determined in time using customized software (CELLMIA, unpublished data). Cell migration velocity (mean of n replicates as indicated) is determined based on the slope of linear fit on the area over time data. The relative migration efficiency is the normalized cell velocity over different independent experiments.

Statistical analysis

Statistical analysis was performed using Student's t-test or one-way ANOVA followed by Bonferroni's multiple comparison test with Graphpad Prism 4 software (Graphpad Software Inc.). Migration data are presented as mean ± standard error and statistical analysis was performed using Wilcoxon pairwise comparison with Bonferroni correction for multiple testing in R version 2.15.2 (The R Foundation for Statistical Computing). Results were considered significant when p-value <0.05.

Results

C2C12 myotubes express TNF-R1 and β2-ARs

The murine C2C12 cell line is a well-established in vitro model for the study of molecular interactions occurring in skeletal muscle cells at different steps of myogenesis [18,19]. We first investigated the expression levels of β2-ARs and TNF-R1/2 in undifferentiated myoblasts versus differentiated myotubes. Progression in myogenesis was confirmed by measuring myogenin expression (Figure S1A). We observed that myogenic differentiation was associated with augmented mRNA levels of TNF-R1 as well as β2-AR, as shown in Figure 1A and B, respectively. At the protein level, we did, however, not detect more TNF-R1 in myotubes as compared to myoblasts. Also, we did not detect an increase in the immunoreactive signal representing the monomeric β2-AR, which has a molecular weight of approximately 50 kDa. We did however observe that multiple high-molecular weight immunoreactive bands appear upon myogenic differentiation of C2C12 cells (Figure 1B). To corroborate that the high-molecular weight immunoreactivities indeed represent β2-ARs, we overexpressed a hemagglutinin (HA)-tagged β2-AR (β2-AR-HA) in HEK-293T cells, which do not express endogenous β2-AR. Like in C2C12 cells, we found higher molecular weight bands that react with the anti-β2-AR antibody. In addition, we found that these bands overlap with immunoreactivities detected with anti-HA, indicating they indeed derive from β2-AR species, and probably represent post-translationally modified or oligomeric forms of the β2-AR (Figure S1B) [20,21]. In conclusion, these data indicate C2C12 myotubes express both TNF-R1 and β2-AR protein and indicate the expression of variant β2-AR species is upregulated in the course of myogenesis. Given that myotubes have been shown to secrete a wide variety of mediators when subjected to pro-inflammatory stimuli [22], and as C2C12 myotubes express both TNF-R1 and β2-AR2, we have used C2C12 myotubes for further experiments into β2-AR2/TNF-R1 crosstalk.

Effect of β-agonist cotreatment on TNF-α-induced, NF-κB-dependent, inflammatory gene expression in skeletal muscle cells

In physiological and pathological circumstances, such as during an acute boost of exercise or in chronic inflammatory disease, skeletal muscle cells express a number of NF-κB-dependent cytokines and other inflammatory markers [1,23]. Here, we performed a RT-qPCR analysis to assess how β-agonist cotreatment modulates the expression of well-known NF-κB target genes with a previously demonstrated role in skeletal muscle homeostasis [15,23,24]. The presence of NF-κB binding sites in the promoters of the selected genes was confirmed by a bioinformatics analysis using P-Scan (Table S2). For each gene, the promoter region of 500 bp upstream to the Transcription Starting Site was investigated with the Transcription Factor Binding Sites matrices available in the TRANSFAC databases.

We found that in C2C12 myotubes, TNF-α significantly induced the expression of intercellular adhesion molecule 1 (ICAM-1), NF-κB inhibitor alpha (IκBα), chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-C motif) ligand 5 (CCL5), whereas it had modest yet insignificant effects on the expression of interleukin-6 (IL-6) and chemokine (C-X-C motif) ligand 5 (CXCL5) (Figure 2). We found no clear effects of TNF-α on the expression of interleukin-7 (IL-7), interleukin-15 (IL-15) and Brain derived neurotrophic factor (BDNF) (Figure S2). Isoproterenol by itself did not significantly affect the expression of any of the investigated genes, but had a mild positive effect on the expression of CXCL5 and IL-6. Interestingly, we found that isoproterenol cotreatment did not significantly modulate TNF-α-induced CCL5 and IκBα transcription (Figure 2D, F), but enhanced the effect of TNF-α on the expression of CCL2 and ICAM-1 (Figure 2C, E) and induced a pronounced synergy for IL-6 and CXCL5 (Figure 2A, B, Table 1). Overall, the effect of TNF-α on the expression of the selected genes was rapid and transient, peaking after 2 hours of treatment and then rapidly declining, and these kinetics were not modulated by isoproterenol cotreatment. Only CCL5 mRNA levels peaked at 5 hours, both upon TNF-α and TNF-α and isoproterenol cotreatment, and were maintained even after 16 hours induction (Figure 2 D). We concluded that in C2C12 myotubes a subset of NF-κB-dependent genes exists with different responses to TNF-α or costimulation with isoproterenol.

Cytoplasmic signalling crosstalk upon β_2-AR/TNF-R co-activation in C2C12 myotubes

Because isoproterenol enhanced the expression of several typical NF-κB target genes, we explored what could be the molecular basis of these effects. Upon binding to its receptor(s), TNF-α triggers the canonical NF-κB cascade, initiated by IKKβ activation, IκBα degradation, phosphorylation of the NF-κB p65 subunit on serine 536 and its subsequent translocation from the cytoplasm to the nucleus [4]. As evaluated by Western blotting, we detected canonical NF-κB activation in C2C12 myotubes treated with TNF-α. We observed rapid phosphorylation of p65 on serine 536, degradation of IκBα (Figure 3 A), and nuclear translocation of p65 (Figure 3B). In line with this, TNF-α also induced an NF-κB reporter gene transiently transfected in C2C12 cells (Figure 3C). Isoproterenol treatment by itself had no effect on any of these aspects of canonical NF-κB activation and did not modulate the effects of TNF-α (Figure 3A-C).

In addition to the NF-κB pathway, TNF-α triggers activation of Mitogen Activated Protein Kinases (MAPKs) and Mitogen and Stress-activated Kinase 1 (MSK-1) which cooperate with NF-κB in driving inflammation [17,25]. We found that in C2C12 myotubes, TNF-α activated JNK (Jun N-terminal kinase), ERK (Extracellular signal-regulated kinase) and p38 MAPKs and MSK-1 (Figure 3D-G). Isoproterenol by itself did not have significant effects on the phosphorylation of any of the MAPKs or MSK-1. Isoproterenol slightly inhibited TNF-α-induced phosphorylation of ERK, JNK and MSK-1, whereas it did not significantly affect TNF-α-induced p38 MAPK activation (Figure 3D–G). Collectively, this suggests that the potentiation of TNF-α-induced gene expression by

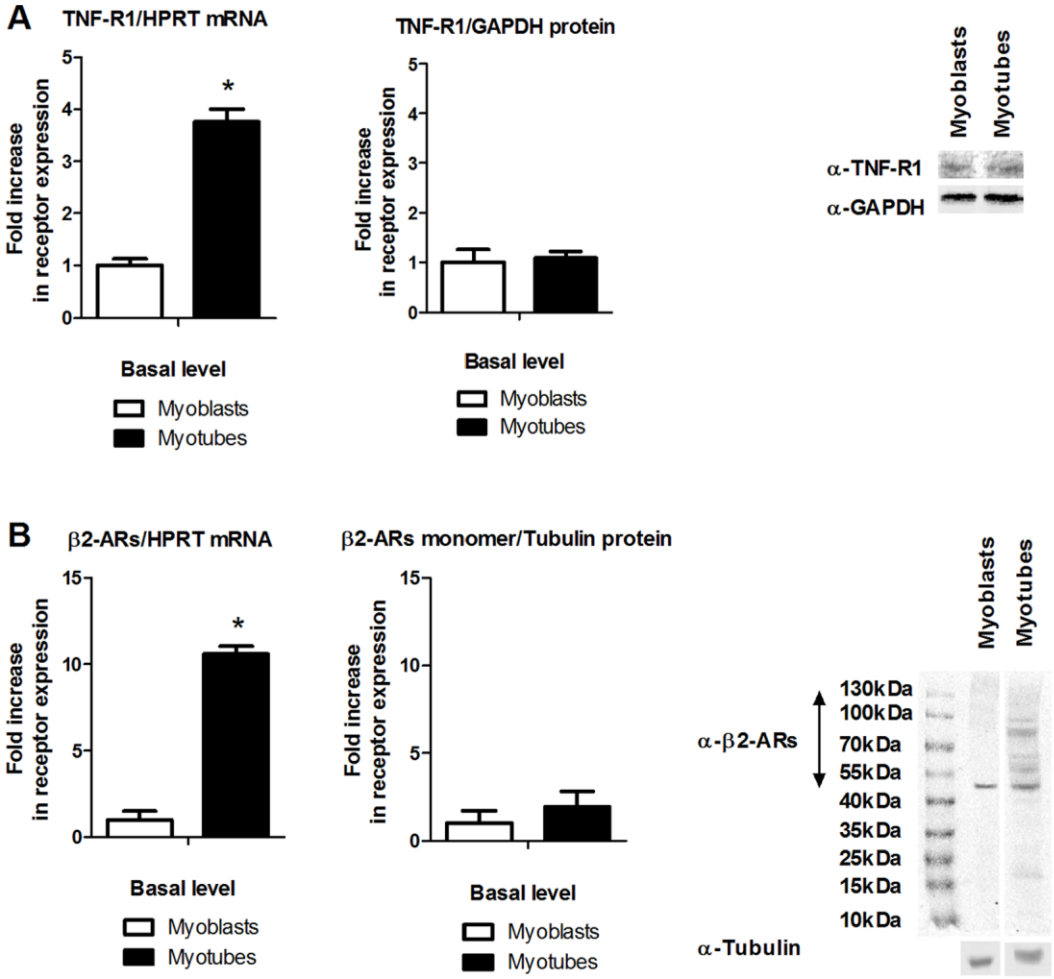

Figure 1. Expression of TNF-R1 and β_2-AR in C2C12 myoblasts and myotubes. The basal expression of TNF-R1 (A) and β_2-AR (B) mRNA and protein was compared in C2C12 myoblasts versus myotubes using RT-qPCR and Western blotting. RIPA lysates were prepared and equal amounts of protein were loaded on gel. Bar charts represent average ± SD of densitometric analysis of three independent experiments. (For the β_2-AR Western blot only the monomeric form of the receptor was densitometrically quantified.) A representative blot is shown. (*) significantly different from myoblasts using student's t-test.
doi:10.1371/journal.pone.0090649.g001

isoproterenol is unlikely to be mediated by cytoplasmic modulation of NF-κB activity (Figure 2).

The canonical signalling pathway that is induced by β_2-AR triggering is the protein kinase A (PKA)/ cAMP-response element-binding protein (CREB) cascade. We detected rapid phosphorylation of CREB on serine 133 in cells treated with isoproterenol as well as TNF-α. Combined treatment did not further potentiate CREB phosphorylation (Figure 4A). Nuclear translocation of the PKA catalytic subunit (PKAc) was only detected in cells treated with isoproterenol and not in TNF-α-treated cells (Figure 4B). Whereas both TNF-α and isoproterenol induced CREB phosphorylation, only isoproterenol-activated CREB was able to induce a CRE reporter gene in C2C12 cells (Figure 4C). TNF-α cotreatment did not modulate isoproterenol-induced CRE activity.

β_2-AR/TNF-R cotreatment induces chromatin modifications required for efficient recruitment of NF-κB

Because we did not detect any cytoplasmic β_2-AR/TNF-Rs crosstalk events that could explain the observed synergy, we next explored nuclear crosstalk. We used the IL-6 gene, a prototypical NF-κB target gene, with known function in skeletal muscle, and the most potently affected gene in our RT-qPCR analysis, as a model system. The IL-6 gene has a complex promoter architecture and, among others, contains functional transcription factor binding sites for NF-κB and CREB [17,26].

The accessibility of a gene's promoter to transcription factors is reflected in its susceptibility to nuclease digestion. In CHART-PCR, this feature is exploited and the accessibility of a selected DNA sequence is determined by digesting chromatin using nucleases and then quantifying the amount of remaining uncut gDNA in the digested chromatin sample via qPCR. Here, we digested the proximal IL-6 gene promoter using two restriction enzymes (AatII, HincII) that cut in the proximal IL-6 promoter, in the vicinity of CREB and NF-κB binding sites respectively. Then, we amplified the sequence of interest via qPCR, using primers that recognise the sequence flanking the restriction enzyme recognition sites (Figure 5A). Results were normalised as explained in the Materials and Methods section and expressed as chromatin opening. We found that the IL-6 promoter is susceptible to digestion with the AatII and HincII restriction enzymes, only upon cotreatment with TNF-α and isoproterenol (Figure 5B). As

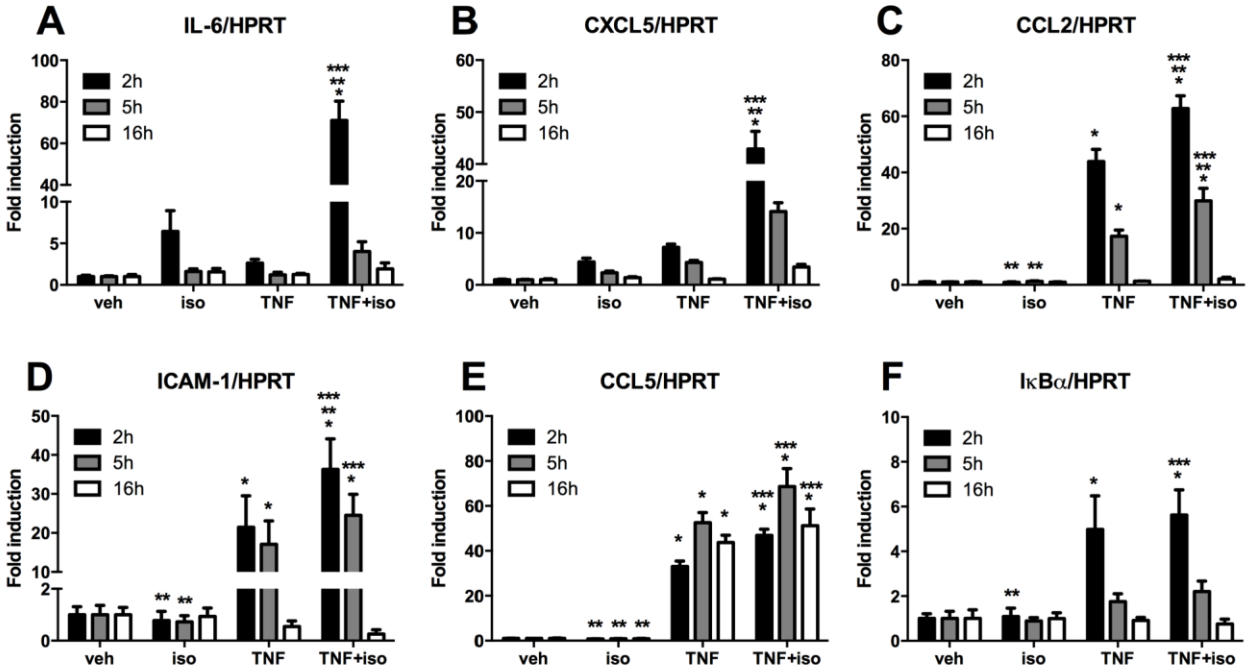

Figure 2. Effect of isoproterenol/TNF-α cotreatment on NF-κB-dependent gene expression in C2C12 myotubes. Expression of inflammatory markers was measured by RT-qPCR after 2, 5 and 16-hours induction with vehicle (veh), isoproterenol (iso) and/or TNF-α (TNF) in C2C12 myotubes. Fold induction for each gene was calculated versus veh control at the corresponding time point. Results represent average ± SD of three independent experiments. Statistical significance was determined via ANOVA followed by Bonferroni's multiple comparison test. (*) Significantly different from veh. (**) Significantly different from TNF. (***) Significantly different from iso.
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expected, no chromatin opening was apparent, at an irrelevant region (not containing restriction sites for AatII and HincII) of the IL-6 gene (Figure 5B).

Covalent modification of histone tails is a crucial event in the regulation of chromatin dynamics. In particular enhanced acetylation of histone H3 has been detected at transcriptionally active chromatin [27]. To investigate whether histone acetylation is implicated in IL-6 transcription induced by TNF-α/isoproterenol cotreatment, we first explored how the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) affects IL-6 transcription. Remarkably, we found that TSA, at subcytotoxic dose (100 nM), almost completely abrogated IL-6 transcription triggered by isoproterenol, TNF-α or the combination of both, indicating IL-6 transcription is inhibited, rather than enhanced by histone acetylation (Figure 5C). To assess histone H3 acetylation at the IL-6 promoter, we performed ChIP using an antibody recognising histone H3 acetylated at the Lys 27 residue, a modification for which the association with transcriptional activation is well established [28-30]. We found that, at the IL-6 promoter, histone H3 carried a constitutive acetylation mark at Lys 27, that was not significantly enhanced upon isoproterenol and/or TNF-α treatment (Figure S3A). We also failed to detect enhanced histone H3 acetylation using a pan acetyl-histone-H3 antibody (data not shown). The efficiency of the dose of TSA that we used to promote histone acetylation was confirmed by the detection of histone H3 Lys 27 acetylation in TSA-treated C2C12 cells by confocal microscopy (Figure S3B).

We found that isoproterenol stimulated PKA nuclear entry in C2C12 cells, while TNF-α promoted phosphorylation of MSK-1. Intriguingly, both PKA and MSK-1 have been shown to trigger phosphorylation of histone H3 on serine 10, a modification associated with chromatin relaxation and transcriptional activity [31,32]. Via ChIP analysis, we detected very modest histone H3 phosphorylation at the IL-6 promoter upon isoproterenol stimulation that was significantly potentiated upon cotreatment of C2C12 cells with TNF-α (Figure 5D). Finally, we observed that isoproterenol promoted recruitment of CREB, CREB-binding protein (CBP) and RNA polymerase II to the IL-6 promoter, whereas TNF-α induced recruitment of NF-κB p65. Combined treatment with TNF-α and isoproterenol moreover potentiated the recruitment of p65, CBP and RNA polymerase II to the IL-6 promoter (Figure 5E-H). Isoproterenol-induced CREB recruitment was not modulated by TNF-α cotreatment. The specificity of

Table 1. Synergy factors upon combined isoproterenol/TNF-α treatment in C2C12 skeletal muscle.

	Synergy Factor					
	2 h		5 h		16 h	
	Mean	St Dev	Mean	St Dev	Mean	St Dev
IL-6	8.80 (*)	3.13	1.48	0.74	0.68	0.31
CXCL5	3.98 (*)	1.20	2.07	0.51	1.38	0.37
CCL2	1.48	0.38	1.59	0.39	0.92	0.21
CCL5	1.52	0.44	1.22	0.28	1.14	0.30
ICAM-1	1.66	1.06	1.35	0.80	0.18	0.10
IκBα	0.93	0.46	0.77	0.29	0.40	0.16

The strength of the synergies was calculated by dividing the relative mRNA levels upon TNF-α/isoproterenol induction (Z) by the sum of the relative mRNA levels after individual TNF-α (X) and isoproterenol (Y) induction (i.e. Z/(X+Y)). (*) Significant synergy meaning Z/(X+Y) significantly larger than X+Y.
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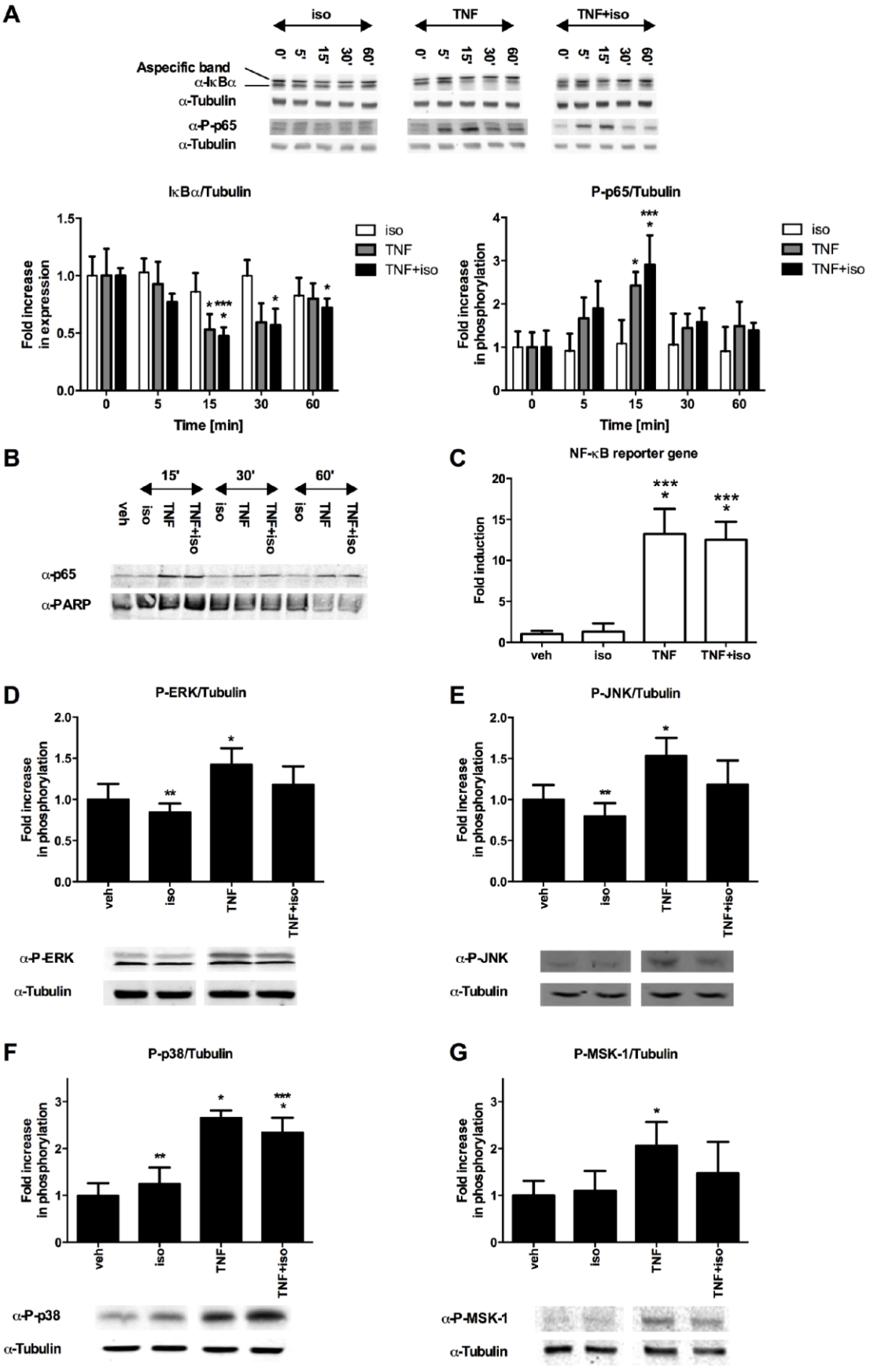
A
iso
TNF
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0'
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α-IκBα
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α-Tubulin
IκBα/Tubulin
Fold increase in expression
Time [min]
iso
TNF
TNF+iso
P-p65/Tubulin
Fold increase in phosphorylation
Time [min]
B
15'
30'
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veh
iso
TNF
TNF+iso
α-p65
α-PARP
C
NF-κB reporter gene
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E
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Fold increase in phosphorylation
α-P-JNK
α-Tubulin
F
P-p38/Tubulin
Fold increase in phosphorylation
α-P-p38
α-Tubulin
G
P-MSK-1/Tubulin
Fold increase in phosphorylation
α-P-MSK-1
α-Tubulin

Figure 3. Effect of isoproterenol on TNF-α-induced NF-κB and MAPKs activation in C2C12 myotubes. (A) Kinetics of IκBα degradation and increase in phosphorylation of p65 at serine 536. After veh, iso and/or TNF treatment cells were lysed in SDS sample buffer and analysed via Western blotting. Bar charts represent average ± SD of densitometric analysis of three independent experiments. A representative blot is shown. (B) Kinetics of p65 nuclear translocation. After veh, iso and/or TNF treatment, nuclear extracts were prepared and analysed via Western blotting. A representative blot from two independent experiments is shown. (C) Induction of NF-κB reporter gene activity. C2C12 myotubes, transfected with the κB-luciferase reporter plasmid, were treated with veh, iso and/or TNF for 6 hours, before analysis of luciferase production. Fold induction was calculated versus veh control. Results represent average ± SD of three independent experiments. (D–G) Increase in phosphorylation of ERK (D), JNK (E), p38 (F) and MSK-1 (G). After induction of C2C12 myotubes for 15 minutes with veh, iso and/or TNF, cells were lysed in SDS sample buffer and analysed via Western blotting. Bar charts represent average ± SD of densitometric analysis of three independent experiments. A representative blot is shown. Statistical significance was determined via ANOVA followed by Bonferroni's multiple comparison test (*) Significantly different from veh. (***) Significantly different from iso.
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the observed responses is indicated by the fact that we did not detect any of the observed responses at an irrelevant housekeeping gene (GAPDH) or using aspecific antibodies or beads only for the immunoprecipitation (Figure S3A, C-H).

These results show that only combined stimulation with TNF-α and isoproterenol leads to chromatin remodelling at the IL-6 promoter.

Myotube-derived cytokines induce migration of myoblasts

Recent literature shows that, upon contraction or inflammation, skeletal muscle cells express several cytokines with chemotactic properties [24,33]. Because we observed that cotreatment of C2C12 myotubes with isoproterenol enhanced transcription of several TNF-α-induced cyto-/chemokines that are known to modulate the migratory properties of cells, we investigated whether these factors induced migration of undifferentiated C2C12 myoblasts. It was reported that C2C12 myoblasts exhibit spontaneous mobility, which can be further stimulated by IGF-1 [33]. To analyse whether TNF-α/isoproterenol cotreatment induces the production of chemotactic factors by myotubes, we performed migration assays using myoblasts treated with myotube-conditioned medium. IGF-1 stimulation was used as a positive control. As shown in Figure 6 and Figure S4, myoblast cells treated with conditioned medium from myotubes stimulated with only TNF-α or isoproterenol displayed a modest, albeit significant increase in myoblast migration efficiency compared to the control condition. Importantly, myoblasts treated with conditioned medium derived from myotubes, which were stimulated with the combination of TNF-α and isoproterenol, migrated significantly faster than those treated with conditioned medium from myotubes treated with only TNF-α or isoproterenol (Figure 6C, Figure S4). This indicates that the conditioned medium of the cotreated myotubes has a stronger chemotactic potential on undifferentiated myoblasts.

Discussion

The devastating role of TNF-α and NF-κB in skeletal muscle inflammation and atrophy is well documented [2,5]. Here, we investigated whether and how β-agonists, that are known for their anabolic and anti-inflammatory properties, modulate the effects of TNF-α on the expression of inflammatory factors in skeletal muscle cells.

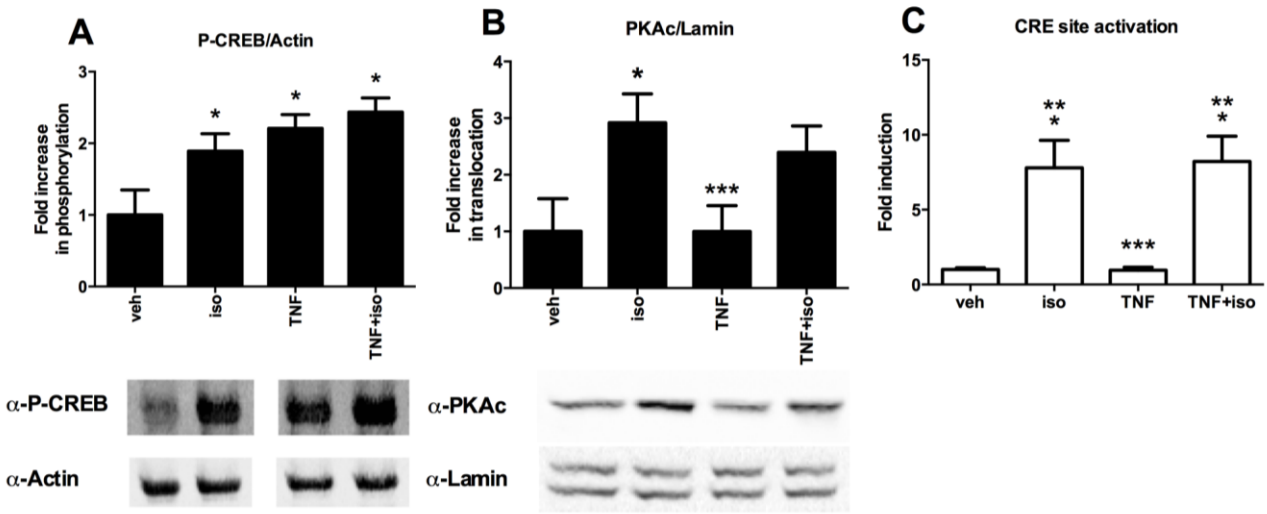

Figure 4. Effect of isoproterenol/TNF-α cotreatment on activation of the PKA/CREB cascade. (A) Increase in phosphorylation of CREB at serine 133. After induction of C2C12 myotubes for 15 minutes with veh, iso and/or TNF, cells were lysed in SDS sample buffer and analysed via Western blotting. Bar charts represent average ± SD of densitometric analysis of three independent experiments. A representative blot is shown. (B) Effect on nuclear translocation of PKAc. After veh, iso and/or TNF treatment for 15 min, nuclear extracts were prepared and analysed via Western blotting. Bar charts represent average ± SD of densitometric analysis of three independent experiments. A representative blot is shown. (C) Effect on the activation of CREB. C2C12 myotubes, transfected with the CRE-luciferase reporter plasmid, were treated with veh, TNF and/or iso for 6 hours, before analysis of luciferase production. Fold induction was calculated versus veh control. Results represent average ± SD of three independent experiments. Statistical significance was determined via ANOVA followed by Bonferroni's multiple comparison test (*) Significantly different from veh. (***) Significantly different from iso.
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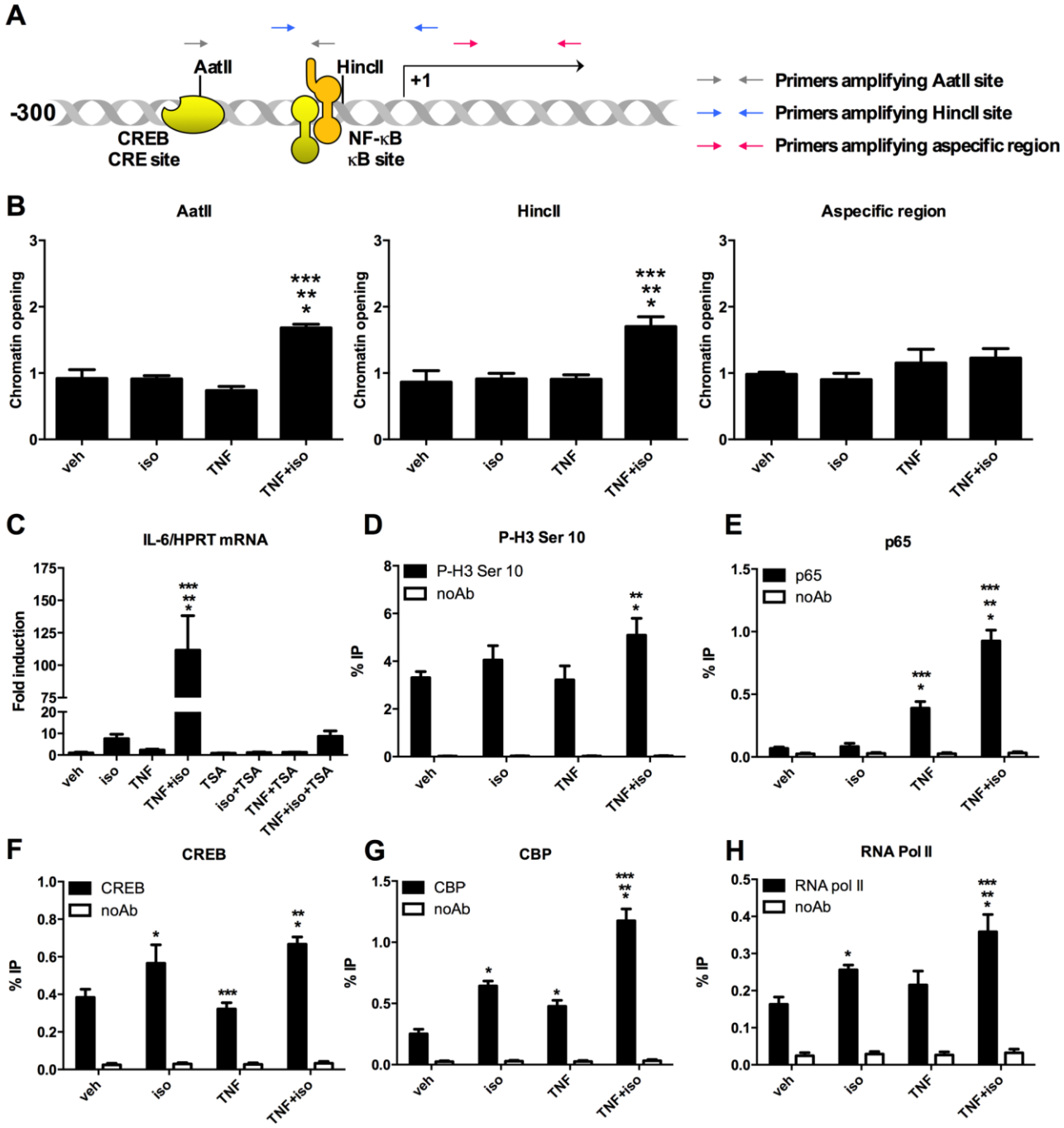

Figure 5. TNF-α/isoproterenol cotreatment induces chromatin remodelling and histone H3 modifications at the IL-6 promoter in C2C12 myotubes. (A) Schematic representation of the localisation of CREB- and NF-κB-responsive elements in the IL-6 promoter. Relative position of the recognition sites for AatII and HincII and primers used in the restriction enzyme accessibility assay via Real Time PCR (CHART-PCR) are indicated. (B) TNF/iso cotreatment promotes accessibility of the IL-6 promoter. C2C12 cells were treated for 2 hours with veh, iso and/or TNF. Chromatin opening of the promoter region was determined by CHART-PCR as detailed in Materials and Methods. Results represent average ± SD of three independent experiments. (C) Effect of TSA, a histone deacetylase inhibitor, on IL-6 transcription. C2C12 cells were treated for 2 hours with combinations of veh, TSA, iso and/or TNF. mRNA levels of IL-6 were determined via RT-qPCR. Results represent average ± SD of three independent experiments. (D) TNF/iso cotreatment enhances phosphorylation of histone H3 at the IL-6 promoter. C2C12 cells were treated with veh, iso and/or TNF for 2 hours. Phosphorylation of histone H3 at serine 10 was determined via ChIP. Results represent average ± SD of three independent experiments. (E-H) Effect of TNF and/or iso treatment on the recruitment of NF-κB, CREB, CBP and RNA polymerase II to the IL-6 promoter. C2C12 cells were treated with veh, iso and/or TNF for 2 hours. Recruitment of NF-κB p65, CREB, CBP and RNA polymerase II was measured via ChIP. Results represent average ± SD of three independent experiments. (*) Significantly different from veh. (**) Significantly different from TNF. (***) Significantly different from iso.

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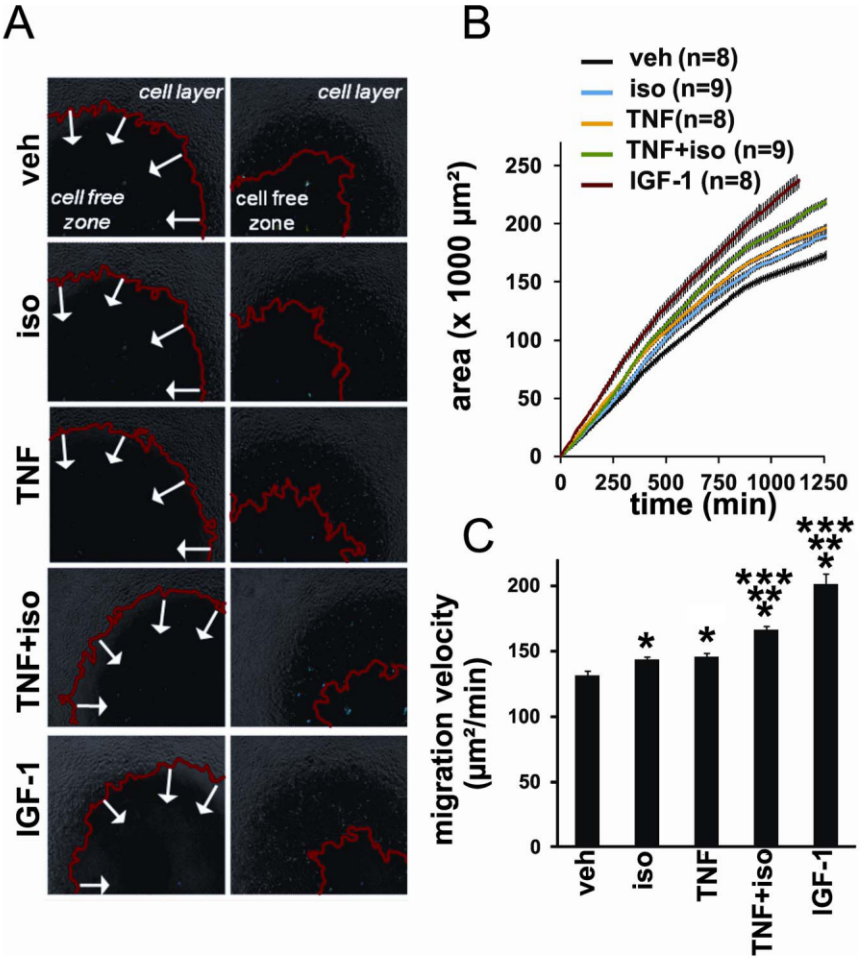

Figure 6. C2C12 myotubes secrete factors that promote the migration of C2C12 myoblasts. C2C12 myotubes were treated for 24 hours with veh, iso and/or TNF. Conditioned medium was prepared as described in Materials and Methods and applied to C2C12 myoblasts. Recombinant IGF-1 was used as a positive control. Cell migration was monitored for 24 hours. (A) Selected start and end phase contrast images for the different conditions from a representative experiment. The red line delineates the confluent cell layer that migrates inwards in time; arrows show migration direction into the cell-free zone. (B) Cell-covered area over time plot from a representative experiment. The lines represent the mean area for technical replicates (n indicated in the Figure); error bars are SEM. (C) Mean migration velocity (n replicates, see data in B) for the tested conditions in a representative experiment. (Relative migration efficiencies based on the cumulated data of three independent biological experiments are shown in Figure S4). Error bars are SEM. Statistical analysis was performed using Wilcoxon pairwise comparison with Bonferroni correction for multiple testing. (*) Significantly different from veh. (**) Significantly different from TNF. (***) Significantly different from iso.
doi:10.1371/journal.pone.0090649.g006

We used the murine C2C12 cell line as *in vitro* model system and found that the expression of β_2-ARs was much more prominent, both at the mRNA and protein level, in differentiated myotubes as compared to undifferentiated myoblasts, indicating myotubes would be more susceptible to regulation by β_2-AR-agonists. In agreement with this finding, elevated expression of the β_2-AR was reported in regenerating myofibers after injury [7]. Whereas we also observed increased TNF-R1 expression at the mRNA level, this was not reflected at the protein level. Enhanced TNF-R1 mRNA levels were previously reported in regenerating myofibers, but the level of protein in the latter study was not investigated [34]. In another study decreased activation of NF-κB upon TNF-α treatment was observed upon myogenesis [35], but in this study TNF-R1 expression levels were not investigated and the diminished response could also be due to post-receptor regulatory mechanisms [36]. Our data suggest that in C2C12 cells TNF-R1 protein levels are not regulated upon myogenic differentiation, despite clear differences in mRNA levels. The reason for this discrepancy remains to be elucidated, but probably depends on post-transcriptional regulation of TNF-R1 expression.

Several proinflammatory mediators are upregulated in myotubes upon TNF-α challenge [15,24] and in muscle wasting disorders [24,37]. In line with this, we found a significant increase in the expression of selected NF-κB-dependent target genes in C2C12 cells treated with TNF-α. Interestingly, we observed that isoproterenol treatment by itself did not significantly affect the expression of these genes, but potently enhanced TNF-α-induced expression of a subset of inflammatory factors, such as IL-6 and CXCL5, and to a lesser extent that of CCL2 and ICAM-1, whereas that of others (CCL5 and IκBα) remained unaffected. The regulation of IL-6 expression was investigated previously in C2C12 cells stimulated with lipopolysaccharide (LPS) and epinephrine [13]. Similar to what we observe here using TNF-α as a proinflammatory stimulus, the authors reported synergistic IL-6 expression upon LPS/epinephrine cotreatment, indicating the synergy depends on a signalling protein that is common to TNF-R and Toll-like receptor (TLR) pathways. As opposed to previous

reports of anti-inflammatory effects of β-agonists [10–12], we did not find any evidence for that in C2C12 cells. The anti-inflammatory effects of β-agonists have been mostly explained by β_2-AR-mediated upregulation of IκBα levels, either via induction of its transcription, or by promoting its stability through interaction with the GPCR adaptor β-arrestin [10,12,38]. In C2C12 cells, we and others [13] did not detect any modulation of IκBα mRNA or protein levels, indicating β_2-AR-dependent regulation of IκBα levels is a cell type-specific process. The lack of effects on IκBα expression was confirmed by our observation that isoproterenol cotreatment did not hamper nuclear translocation of NF-κB p65. In addition, we did not find potentiation of TNF-α-induced MAPKs or MSK-1 activation by isoproterenol, rather we found mild inhibition of MSK-1, ERK and JNK – indicating the effect of isoproterenol cannot be explained by cytoplasmic crosstalk with canonical TNF-α-induced signalling cascades. Whereas Frost *et al.* [13] suggested that epinephrine promotes activation of ERK, p38 and JNK MAPKs, we did not find any evidence for that using isoproterenol as a stimulus. Activation of these MAPKs by epinephrine in the publication by Frost *et al.* was however solely supported by effects of pharmacological inhibitors on IL-6 expression.

The most important novel finding in this paper is the observation that, in addition to IL-6, the expression of several chemokine genes is potentiated upon TNF-α/isoproterenol cotreatment of skeletal muscle cells. The genes we investigated in this study all contain NF-κB responsive elements in their proximal promoters. As is evident from Table S2, there is significant variation in the sequences of these elements. Although the investigated selection of genes is too small to make any reliable predictions, it is noteworthy that the NF-κB consensus sequences in the most responsive IL-6 (GGGATTTTCC) and CXCL5 (GGGAATTTCC) promoters bear the strongest similarity among the investigated genes. Recently, Siggers *et al.* [39] performed a comprehensive *in vitro* analysis of DNA binding by NF-κB dimers. Using Siggers' open source dataset, we evaluated whether the IL-6 and CXCL5 NF-κB sequences showed a particular preference for selected NF-κB dimers, as compared to genes that were not prone to TNF-α/isoproterenol coregulation, but could not find evidence for that. In fact, the binding preference of the NF-κB sequence in the IL-6 promoter resembled most that of the κB sequence in the IκBα promoter, which was not susceptible to synergistic regulation by TNF-α/isoproterenol. Evaluating whether the sequence of the NF-κB responsive element determining for the response to isoproterenol will however require more extensive, genome-wide, analysis. In addition to specifying NF-κB dimer binding, the sequence of the κB site also dictates which co-activators will bind to a selected promoter, and accumulating evidence suggests NF-κB dimers can adopt different conformations when bound to different DNA sequences [40]. In line with this, even a single nucleotide change within a κB sequence was shown to affect cofactor specificity of NF-κB dimers [41]. Cofactor specificity is also determined by post-translational modifications of NF-κB family members. Interestingly, both PKA and MSK-1 have been shown to phosphorylate p65 on its serine 276 residue, which promotes its association with the CBP coactivator and selective NF-κB dependent gene induction [42,43]. Because of the lack of specific antibodies recognizing p65 phosphorylated at the serine 276 residue, we were unable to investigate whether this phosphorylation is triggered in C2C12 cells upon activation of PKA and/or MSK-1 [44]. We recently also reported synergistic IL-6 expression in human 1321N1 astrocytes cotreated with TNF-α/isoproterenol [26] indicating the TNF-R1/β_2-AR synergy is not a phenomenon restricted to C2C12 cells. Nevertheless, in astrocytes we also detected inhibitory effects of isoproterenol (for instance at the ICAM-1 gene), whereas in the current study isoproterenol did not block the expression of any of the investigated genes (including ICAM-1). Our results indicate that the effects of isoproterenol are not only gene-selective, with only a selection of NF-κB dependent genes being susceptible to potentiation by isoproterenol, but that there is also an important cell type-specific component determining the outcome of TNF-R1/β_2-AR co-activation. This cell-type specificity is supported by other reports describing different effects of β-agonists on the expression of selected inflammatory mediators, depending on the cell type investigated [11,45,46].

Expression of NF-κB target genes is regulated at multiple levels and selectivity is accomplished, among others, by co-operation of multiple transcription factors and cofactors in so-called enhanceosome structures as well as by epigenetic mechanisms [47]. We showed via chromatin accessibility assays that β_2-AR/TNF-R co-activation induces chromatin remodelling at the IL-6 promoter. Both MSK-1 and PKA can directly phosphorylate histone H3 and recruit chromatin-remodelling complexes leading to increased promoter accessibility [31,32,48]. In agreement with this, we observed that in C2C12 cells concurrent activation of MSK-1 and PKA is associated with enhanced histone H3 phosphorylation and chromatin relaxation at the IL-6 promoter. Whereas we cannot exclude that there are also effects of the individual triggers (as is indicated by detectable recruitment of p65, CREB, CBP and RNA polymerase II also upon stimulation of cells with only isoproterenol or TNF-α) that are below the detection limit of our assays, it is clear that β_2-AR/TNF-R co-activation promotes transcriptional synergy.

Intriguingly, it was reported that at the c-fos promoter (*in vitro*) CREB binding and phosphorylation are required to recruit MSK-1, which then phosphorylates histone H3 [49]. Here, we observed that, although both TNF-α and isoproterenol stimulate phosphorylation of CREB on serine 133, CREB activation is only promoted in the presence of isoproterenol. These results are in agreement with previous reports demonstrating that both MSK-1 and PKC phosphorylate CREB on serine 133 to a similar extent as PKA, but that only PKA-mediated phosphorylation resulted in CREB-dependent transcription [50,51]. According to the generally accepted model for CREB-dependent transcriptional activation, CREB is constitutively bound to its target gene promoters and transcriptional activation requires phosphorylation of CREB. We have attempted to immunoprecipitate phosphorylated CREB from the IL-6 promoter using two antibodies that recognise CREB phosphorylated at serine 133, but failed to recover it (data not shown). Instead, we found that CREB was actively recruited to the IL-6 promoter upon isoproterenol, but not upon TNF-α treatment. We previously reported CREB recruitment at the IL-6 promoter upon TNF/isoproterenol cotreatment in human astrocytes [26]. These findings are moreover in line with other reports demonstrating active CREB recruitment at selected gene promoters. For instance, it has been shown that CREB recruitment occurs at glucagon-responsive gene promoters in hepatocytes treated with forskolin [52], at the c-fos promoter in neurons treated with BDNF [53] and at the ICER promoter in forskolin-treated PC12 cells [54].

We found that TNF-α induced CREB phosphorylation, but did not promote activation of a CREB-dependent reporter gene and did not induce CREB recruitment to the IL-6 promoter. It is, however, possible that TNF-α-induced CREB phosphorylation plays a role in the transcriptional activation of other genes, perhaps requiring different CREB co-activators. Evaluating the contribution of TNF-α induced MSK-1 versus isoproterenol-

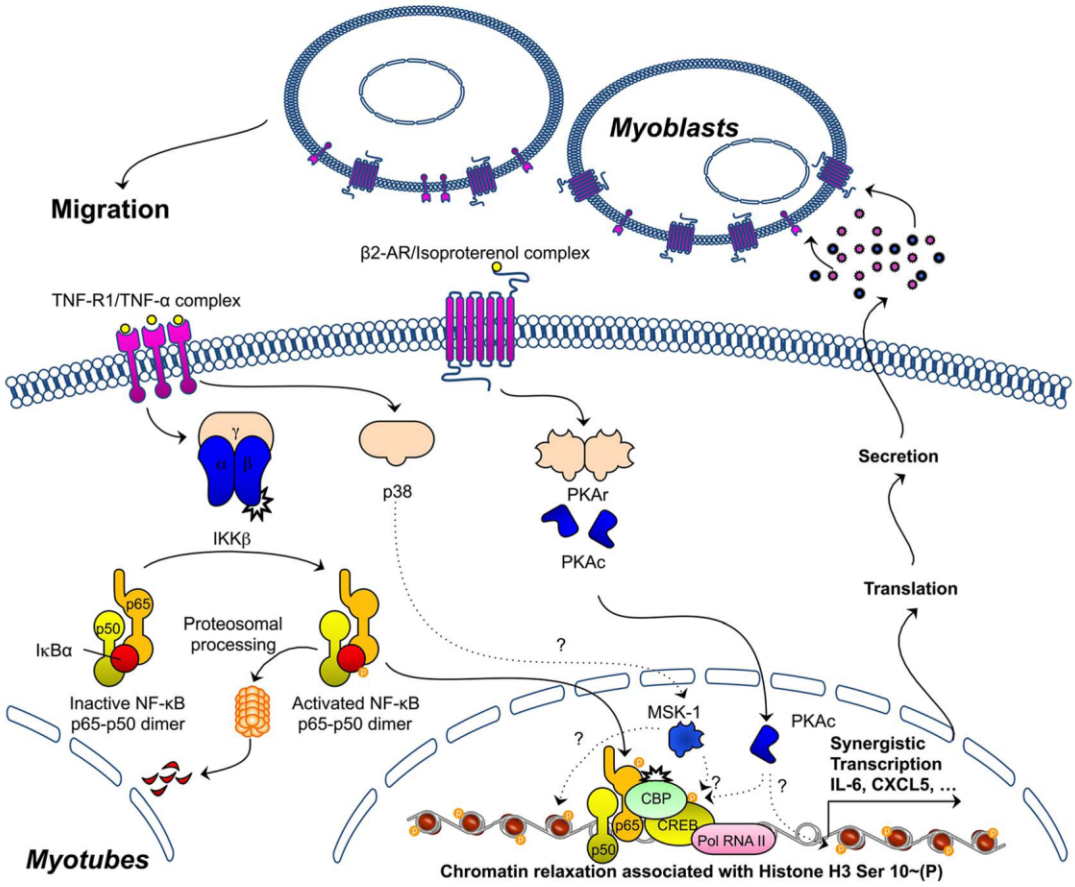

Figure 7. Model describing signalling events that are associated with β2-AR/TNFR coactivation in C2C12 cells, ultimately leading to transcriptional synergy at selected NF-κB-dependent promoters. For a detailed description we refer to the text in the discussion. Dotted lines indicate connections that are based on correlations and hence should be interpreted as assumptions rather than proven facts.
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induced PKA in CREB-mediated gene transcription will, however, require further study.

In addition to phosphorylation, acetylation of histones plays a crucial role in gene expression [27,28]. Moreover, combinations of multiple histone modifications can reinforce the robustness of the chromatin state and as a consequence modulate gene expression [55,56]. We previously proposed that the selective synergy at the IL-6 promoter in astrocytes was the result of co-operative recruitment of the transcriptional coactivator CBP by CREB and NF-κB, that are positioned in close proximity at the IL-6 promoter [26]. Interestingly, also the CXCL5 promoter contains a CREB and NF-κB element in its proximal promoter, indicating this could be a signature of genes susceptible to TNF-R1/β_2-AR co-activation. CBP acts as a transcriptional coactivator by either acetylating histone tails via its intrinsic acetyl-transferase activity or by recruiting additional histone acetyl-transferases (HATs), hence promoting chromatin accessibility [57,58]. Surprisingly, whereas CBP is efficiently recruited to the IL-6 promoter upon TNF-α/isoproterenol cotreatment in the present study, we did not find evidence for a positive role of histone acetylation in promoting chromatin relaxation. This observation is in line with a previous report showing reduced IL-6 expression in C2C12 cells treated with TSA [13] and suggests the existence of cell-type specific epigenetic regulatory mechanisms acting at the IL-6 promoter. Importantly, whereas previous studies show coupling of histone H3 phosphorylation and acetylation as prerequisite for transcriptional activation [59,60], we could not find evidence for that in our model system. It could be that, at the IL-6 promoter, CBP has a function independent of its HAT activity. It is indeed well known that CBP can acetylate non-histone proteins and in this way affect transcriptional activity. For instance, it has been shown that CBP acetylates CREB [61] and NF-κB p65 [62,63], and that these modifications are instrumental for efficient transcriptional activation. Further experiments will however be required to investigate whether this indeed occurs at the IL-6 promoter.

In conclusion, the most striking observation of this study is that co-activation of the TNF-R1 and β_2-AR potently enhances the expression of a subset of inflammatory mediators in C2C12 skeletal muscle cells. Recent reports demonstrated that IL-6, as well as C-C and C-X-C chemokines, regulate various stages of skeletal muscle regeneration [33,64,65]. For example, IL-6, CXCL5, CCL2 and CCL5 were shown to induce leukocyte influx to remove damaged myofibers [24,65] and stimulate myoblast migration to fill in damaged areas [33,64,66]. Here, we show that cotreatment of myotubes with TNF-α and isoproterenol synergistically promotes the secretion of factors that induce the migration of undifferentiated myoblasts *in vitro*. In future studies, it would be interesting to investigate how this affects the recruitment of satellite cells and leukocytes *in vivo*, and in particular in models of TNF-α-dependent muscle disease.

Based on our findings, we propose the following model (Figure 7): in C2C12 myotubes (I) TNF-R activation promotes

activation of the canonical NF-κB signalling cascade and MAPKs, including the downstream p38 MAPK target MSK-1, which can phosphorylate CREB (II) β_2-AR activation is associated with activation of PKA, which is the canonical CREB kinase (III) β_2-AR activation does not interfere with early events in TNF-triggered pro-inflammatory NF-κB and MAPK activation (IV) β_2-AR/TNF-R coactivation is associated with enhanced phosphorylation of histone H3 and chromatin relaxation at selected gene promoters (i.e. IL-6) and this is paralleled by recruitment of CREB, NF-κB p65 and CBP transcriptional coactivators as well as RNA polymerase II (V) TNF-R/β_2-AR coactivation leads to synergistic gene expression of selected pro-inflammatory mediators and the secretion of factors that promote the migration of undifferentiated myoblasts.

Finally, as we did not detect anti-inflammatory effects of β-agonists in C2C12 skeletal muscle cells, but instead potentiation of TNF-α action, we suggest caution in the proposed use of β-agonists as therapeutic agents for inflammatory myopathies and urge for further study.

Supporting Information

Figure S1 Myogenesis correlates with the expression of myogenin (Myog) in C2C12 skeletal muscle cells. The basal expression patterns of myogenin (Myog) mRNA (A) and protein (B) were compared in C2C12 myoblasts versus myotubes using RT-qPCR and Western blotting. For Western blotting, cells were lysed in RIPA buffer. A representative blot from two independent experiments is shown. (C) Confirmation of the β_2-AR antibody specificity. Increasing amounts of an expression plasmid encoding a haemagglutinin-tagged β2-AR-HA (β2-AR-HA) was transiently transfected in HEK 293T cells. Cells were lysed in SDS sample buffer and analysed via Western blotting using α-β_2-AR and/or α-HA. A representative blot from two independent experiments is shown.
(TIF)

Figure S2 Effect of β-agonist cotreatment on NF-κB-dependent gene expression in C2C12 myotubes. Expression of muscle-derived cytokines was measured by RT-qPCR after 2, 5 and 16-hours induction with veh, iso and/or TNF in C2C12 myotubes. Fold induction for each gene was calculated versus veh control at the corresponding time point. Results represent average ± SD of three independent experiments.
(TIF)

Figure S3 Nuclear events associated with iso/TNF cotreatment. (A) ChIP analysis of histone H3 acetylation. C2C12 cells were treated with vehicle or iso/TNF for up to 2 hours. Kinetics of histone H3 acetylation were determined via ChIP using an antibody recognizing histone H3 acetylated at Lys 27. Results represent average ± SD of three independent experiments. (B) TSA treatment promotes global histone H3 acetylation at Lys 27 in C2C12 cells. The efficiency of TSA in promoting histone acetylation was checked via confocal microscopy using anti-acetyl Lys 27 histone H3. The scale bar in the image equals 5 μM. The experiment was performed three times and a representative image is shown. (C–G) Control of ChIP assay gene specificity. Control ChIP experiments showing specificity of the observed responses for the IL-6 promoter. ChIP samples from the experiments shown in Figure 5 were re-analyzed using primers amplifying the GAPDH housekeeping gene promoter. Results represent average ± SD of three independent experiments. Statistical signifance was determined via ANOVA followed by Bonferroni's multiple comparison test. (*) Significantly different from veh. (**) Significantly different from TNF. (***) Significantly different from iso. (H) ChIP aspecific antibody background control. Control ChIP experiments comparing signals obtained using protein A beads only or aspecific control antibodies (normal rabbit IgG or normal goat IgG) versus signals obtained using selected specific antibodies (CREB and p65). Cells were treated for 2 hrs with iso+TNF, which is the optimal time point for detection of CREB and p65 recruitment. Results represent average ± SD of three independent experiments.
(TIF)

Figure S4 C2C12 myotubes secrete factors that promote migration of C2C12 myoblasts. C2C12 myotubes secrete factors that promote the migration of C2C12 myoblasts. Relative migration efficiency based on the cumulated data of three independent biological experiments (total number of technical replicates, n = 22,24,23,25,25 for veh, iso, TNF, TNF/iso and IGF-1 respectively). Migration efficiency is a measure of the ratio of the obtained velocity versus the one in 'veh' condition. Statistical analysis was performed using Wilcoxon pairwise comparison with Bonferroni correction for multiple testing. (*) Significantly different from veh. (**) Significantly different from TNF. (***) Significantly different from iso. The table shows p-values indicating the result of pairwise comparison of the indicated conditions. Only 'TNF' and 'iso' do not significantly differ in migration efficiency ($p>0.05$).
(TIF)

Table S1 Summary of primer sequences used in the present study.
(DOCX)

Table S2 Summary of the position and sequence of NF-κB binding sites in the proximal promoters of selected genes.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SG KK JT GH MVT CA. Performed the experiments: KK SG KVW MVT. Analyzed the data: KK SG MVT. Contributed reagents/materials/analysis tools: SG KK JT GH MVT CA. Wrote the paper: SG KK JT GH MVT CA.

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Supplementary information

Supplementary data for the paper entitled: "β-agonists selectively modulate proinflammatory gene expression in skeletal muscle cells via non-canonical nuclear crosstalk mechanisms" can be found in Supplementary data section at the end of present thesis.

2. A proteomics strategy identifies TEF-1 as a regulator of IL-6 transcription in astrocytes

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Manuscript in preparation

In this part of the thesis, we have used a proteomics approach to identify nuclear factors involved in the synergistic IL-6 transcription apparent upon TNF-α/β-agonist cotreatment in the human 1321N1 astrocytoma cell line.

Contribution:

Conceived and designed the experiments: KK SG AT PR SE KG JT.

Performed the experiments: KK AT MD ER WC.

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Wrote the paper: KK SG JT KG PR.

Designed figures and tables: KK SG.

Abstract

Uncontrolled expression of Interleukin (IL)-6 in the central nervous system is a hallmark of neuroinflammation and is associated with the development of several neurodegenerative diseases. IL-6 transcription is initiated by proinflammatory stimuli, including the proinflammatory cytokine Tumour Necrosis Factor (TNF)-α, and depends on the formation of a cell-type and context-dependent enhanceosome structure at the IL-6 promoter. We and others previously reported that β-agonists potentiate IL-6 transcription in astrocytes. The molecular basis of the transcriptional synergy that is promoted by β-agonists is however not fully understood. We used a proteomics approach to identify novel components of the IL-6 enhanceosome that might play a role in the potentiation of IL-6 transcription by β-agonists. Using DNA affinity purification followed by mass spectrometry, we identified Transcription Enhancer Factor (TEF)-1 as a novel interactor of the IL-6 promoter. We confirmed the interaction in the native IL-6 promoter environment and found that TEF-1 functions as a transcriptional repressor. The inhibitory effects of TEF-1 on IL-6 expression probably involve modulation of chromatin remodelling and cAMP response element binding protein (CREB), but not Nuclear Factor (NF)-κB transcriptional activity. We also demonstrated that TEF-1 is a target of the cAMP/Protein Kinase A pathway. Finally, we observed that the action of TEF-1 as a repressor is gene-selective, acting only at selected pro-inflammatory gene promoters.

2.1. Introduction

During the past decade, evidence has accumulated indicating that deregulated interactions between glia cells and neurons are involved in the pathogenesis of neuroinflammation and neurodegenerative disease. The proinflammatory cytokine Tumour Necrosis Factor (TNF)-α is a pivotal factor driving neuroinflammation (Montgomery and Bowers 2012). One of the key transcription factors activated in response to TNF-α is Nuclear Factor (NF)-κB. NF-κB is a generic term for a family of transcription factors that act as dimers and share a similar structure and function (Hayden and Ghosh 2012). Whereas most studies have focused on the role of microglia in neuroinflammation, accumulating evidence also points to astrocytes as important effectors of inflammatory responses. Indeed, recent reports have shown the involvement of astrocytic NF-κB in neuroinflammatory disorders *in vivo* (Brambilla et al. 2005; van Loo et al. 2006).

NF-κB dimers, upon binding to specific motifs in target gene promoters, drive expression of a panel of inflammatory mediators (Hayden and Ghosh 2012). The nuclear activity of NF-κB is fine-tuned by interaction with other proteins in enhanceosome structures and via diverse epigenetic mechanisms (Bhatt and Ghosh 2014; Vanden Berghe et al. 2006). NF-κB function is also modulated by crosstalk with other signalling pathways, which interfere with NF-κB activity at different levels of the signalling cascade (Oeckinghaus et al. 2011). The G-protein-coupled receptor (GPCR) family is the largest class of transmembrane receptors in the human genome and their ligands are important modulators of NF-κB function (Fraser 2008). One of the GPCRs that has drawn a lot of attention in the field of inflammatory research is the β_2-adrenoreceptor (β_2-AR) (Kobilka 2011). In the central nervous system (CNS), this receptor is targeted by the neurotransmitter norepinephrine, which is released by noradrenergic neurons in the locus coeruleus. Noradrenergic neurons project to different brain areas involved in arousal and attention. Along with epinephrine, norepinephrine also coordinates the "fight-or-flight response". Interestingly, it has been demonstrated that locus coeruleus destruction in rodent models of Alzheimer's and Parkinson's disease promotes neuroinflammation (Marien et al. 2004), indicating it has an anti-inflammatory action in the brain. This is supported by several *in vitro* studies in which norepinephrine suppressed the expression of intracellular cell adhesion molecule-1 (ICAM-1) (Ballestas and Benveniste 1997), inducible nitric oxide synthase (iNOS) (Feinstein 1998) and TNF-α (Nakamura et al. 1998).

As opposed to these studies, we observed that β_2-AR triggering engenders bidirectional effects on NF-κB-dependent gene expression *in vitro* in astrocytes (Spooren et al. 2010) and *in vivo* in the CNS (Laureys et al. 2014). Whereas transcription of certain NF-κB targets was inhibited, expression of others, and in particular Interleukin (IL)-6, was synergistically potentiated. IL-6 plays an important role in neuroinflammation (Spooren et al. 2011). To identify enhanceosome components that might explain the very potent transcriptional synergy at the IL-6 gene that is apparent upon combined triggering of β_2-AR and TNF-Receptors (TNF-R), we used a proteomics approach based on DNA affinity purification (AP) and identified and further validated Transcriptional Enhancer Factor (TEF)-1 as a repressor of IL-6 transcription, whose activity is regulated by β_2-AR-dependent signals.

2.2. Materials and Methods

2.2.1. Reagents and Antibodies

Isoproterenol and forskolin were purchased from Sigma Aldrich (St. Louis, MO, USA) and used at 10 μM. Murine TNF-α was obtained from the VIB Department for Molecular Biomedical Research of Ghent University (VIB-UGent, Ghent, Belgium) and was used at 2000 IU/ml. Anti-p65 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Lamin A/C and anti-phospho-PKA substrate were from Cell Signaling Technology (Danvers, MA, USA). Anti-TEF-1 was from BD Biosciences (San Jose, CA, USA). Anti-Flag M2 was from Sigma-Aldrich. XbaI restriction enzyme and MNase nuclease were obtained from New England BioLabs (Ipswich, MA, USA).

2.2.2. Cell culture

The human astrocytoma cell line 1321N1 was a gift from Prof. Dr. Müller (University of Bonn). Human HEK293T cells were obtained from ATCC. 1321N1 and HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco by Life Technologies, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (Gibco, Paisley, UK), 100 IU/ml penicillin and streptomycin (Gibco by Life Technologies). Cells were cultured in an incubator at 37°C in a humidified atmosphere containing 5.5% CO_2. Cells were passaged using 0.05% Trypsin-EDTA (Gibco by Life Technologies). Prior to all experiments, cells were starved in DMEM containing 1% foetal bovine serum.

2.2.3. Nuclear Extract Preparation

Nuclear extracts for AP-mass spectrometry (MS) were prepared from $40x10^6$ cells of untreated and TNF-α/isoproterenol treated cells for 60 minutes. Cells were washed first with 20 ml of PBS, then with 20 ml of PBS with 2 mM Na_2MoO_4 and 10 mM NaF and finally with 20 ml of hypotonic buffer HB (20 mM Hepes pH 7.5, 10 mM NaF, 2 mM Na_2MoO_4, 0.2 mM EDTA pH 7.5). After aspiration of the final washing buffer, 1.2 ml of lysis buffer (20 mM Hepes pH 7.5, 10 mM NaF, 2 mM Na_2MoO_4, 0.2 mM EDTA pH 7.5, 0.05% NP40, Complete Protease Inhibitor Cocktail without EDTA (Roche Applied Science, Penzberg, Germany)) was added. Subsequently, cells were scraped and collected. Samples were centrifuged at 17900 g for 1 minute at 4°C. The pellet was recovered and resuspended in 200 μl Resuspension Buffer (20 mM

Hepes pH 7.5, 0.1 mM EDTA pH 7.5, 5 mM NaF, 1 mM Na_2MoO_4, 20% glycerol) supplemented with the Complete Protease Inhibitor Cocktail without EDTA (Roche). Next, an equal volume of Salt Buffer (20 mM Hepes pH 7.5, 1.6 M NaCl, 0.1 mM EDTA pH 7.5, 5 mM NaF, 1 mM Na_2MoO_4, 20% glycerol, Complete Protease Inhibitor Cocktail without EDTA (Roche)) was added. Samples were incubated for 60 minutes by shaking at 4°C and subsequently centrifuged at 17900 g for 10 minutes at 4°C. Protein contents of the supernatant obtained after the last centrifugation were determined using the Bio-Rad protein assay (Hercules, CA) according to the manufacturer's instructions. Samples were next diluted to equal protein concentrations (1 mg/ml). The extracts were used subsequently for DNA-affinity purification (see below).

2.2.4. Plasmids

The wild type 1168-base pair (bp) human IL-6 promoter construct coupled to luciferase, the NF-κB-luciferase reporter plasmid and the β-galactosidase control constructs were described elsewhere (Vanden Berghe et al. 1998). The CRE-luciferase reporter plasmid was from Stratagene (LaJolla, CA). The Flag-TEF-1 expression vector was generated via Gateway cloning (Invitrogen by Life Technologies) by the transfer of the TEF-1 gene from a Gateway entry clone (Internal ID: 56266) of the human ORFeome v8.1 collection (http://horfdb.dfci.harvard.edu) to a Gateway destination vector for expression of N-terminally Flag tagged proteins under control of the SR-α promoter.

2.2.5. Site-directed mutagenesis

The Flag-TEF-1-S87A mutant construct was generated by site directed mutagenesis. Briefly, the codon corresponding to serine 87 in the Flag-TEF-1 construct was mutated according to the manufacturer's instructions using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (LaJolla, CA, USA) with primers listed in Supplementary Table 1.

2.2.6. DNA affinity purification followed by mass spectrometry

The DNA affinity approach using a desthiobiotin-tagged DNA bait followed by mass spectrometry (MS) was performed as described earlier (Tacheny et al. 2012). This method is referred to throughout the manuscript as “Method A”. The bait in Method

A consisted of a 329-bp-long desthiobiotinylated double stranded oligonucleotide encompassing 333 base pairs upstream of the transcription start site of the wild type IL-6 promoter, produced by polymerase chain reaction (PCR) using the 1168-bp human IL-6 promoter construct as template and primers listed in Supplementary Table 1. Upon amplification via PCR, the DNA bait was resolved on agarose gel and purified using NucleoSpin (Macherey-Nagel MN GmbH, Duren, Germany). Using this technology, two independent biological replicates for each experimental condition (untreated and TNF-α/isoproterenol treated cells) were performed.

The DNA affinity purification approach for DNA-binding proteins using a TEG-biotinylated DNA bait followed by MS analysis was developed based on the method by Tacheny (2012) (Tacheny et al. 2012) and adapted according to Mittler (2009) (Mittler et al. 2009). This method is referred to throughout the manuscript as "Method B". In this method the same IL-6 promoter fragment as in Method A was used as bait, but an XbaI restriction enzyme site and a 6-bp spacer sequence were added to allow release of the DNA from the streptavidin beads using restriction enzyme digest instead of excess biotin competition in Method A. Primers used for PCR amplification of the bait are listed in Supplementary Table 1. Upon amplification, the DNA bait was resolved by agarose gel electrophoresis and purified using NucleoSpin. Next, 20 pmol of DNA bait in a final volume of 200 µl of low saline phosphate buffer (PBS_{50}) (10 mM NaH_2PO_4 pH 7.4, 50 mM NaCl) was incubated for 1 hour at room temperature on a rotary wheel with 1 mg of streptavidin-coupled beads (Dynabeads MyOne Streptavidin C1, Invitrogen by Life Technologies) that had been washed six times with 200 µl of PBS_{50}. Excess unbound DNA bait was removed by three washes with PBS_{50}. One mg of nuclear extract was pre-incubated on ice with 1.5x volume of binding buffer (4 mM Hepes pH 7.4, 120 mM KCl, 8% glycerol, 2 µM DTT (Sigma), 0.166 mg/ml salmon sperm DNA (Sigma) and 0.166 mg/ml PolydIdC (Sigma or Roche)) and subsequently with the immobilized DNA bait for 1 hour at room temperature. DNA-protein complexes immobilized on magnetic beads were then washed with 500 µl of binding buffer, three times with 1 ml of PBS_{50} + 0.1% Tween-20 and twice with 1 ml of 50 mM NH_4HCO_3. The DNA-protein complexes were eluted from the beads upon incubation for 2 hours (room temperature) in 30 µl of NH_4HCO_3 supplemented with 20 IU of XbaI (New England BioLabs (Ipswich, MA, USA)) and NEBuffer#4 (20 mM Tris-acetate, 10 mM magnesium acetate, 50 potassium acetate, 1 mM DTT, pH 7.9) in a shaker with vigorous agitation. The

supernatant containing the DNA-protein complexes was collected and the beads were discarded. Subsequently, isolated proteins were reduced for 30 minutes at 50°C with 5 mM DTT, alkylated for 30 minutes at room temperature in darkness using 15 mM iodoacetamide and then digested overnight at 37°C with 1 µg of trypsin (1 mg/ml in 50 mM NH_4HCO_3, 1 mM $CaCl_2$; Trypsin Gold, Mass Spectrometry Grade; Promega). After digestion, the peptides were acidified by adding acetic acid to a final concentration of 5%, incubated at 37°C for 45 minutes and possible debris was removed by centrifugation for 10 minutes at 17900 g (4°C).

Peptides were separated on a reverse phase column (made in-house, 100µm I.D. x 20mm, 5µm beads C18 Reprosil-HD, Dr. Maisch) using the Ultimate 3000 RSLC nano system (Dionex, Amsterdam, The Netherlands) in-line connected to an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) for identification. The sample was loaded in buffer A (0.1% trifluoroacetic acid, 2% acetonitrile), and separated using a linear gradient from 2% buffer A (0.1% formic acid) to 50% buffer B (0.1% formic acid and 80% acetonitrile) at a flow rate of 300 nl/minute followed by a wash reaching 100% buffer B. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS/MS acquisition for the ten most abundant peaks in a given MS spectrum. In the LTQ-Orbitrap Velos, full scan MS spectra were acquired in the Orbitrap at a target value of 1E6 with a resolution of 60 000. The ten most intense ions were then isolated for fragmentation in the linear ion trap, with a dynamic exclusion of 30 seconds. Peptides were fragmented after filling the ion trap at a target value of 1E4 ion counts. Mascot Generic Files were created from the MS/MS data using Distiller software (version 2.4.3.3, Matrix Science, www.matrixscience.com/Distiller). While generating these peak lists, grouping of spectra was allowed in distiller with max intermediate retention time of 30 seconds and maximum intermediate scan count of 5 was used where possible. Grouping is done with 0.005 precursor tolerance. A peak list is only generated when the MS/MS spectrum contains more than 10 peaks. There was no de-isotoping and the relative signal to noise limit was set at 2. Using this technology three independent experiments for each experimental condition (untreated and TNF-α/isoproterenol treated cells) were performed. A scheme of the two DNA AP-MS methods is presented in Figure 1.

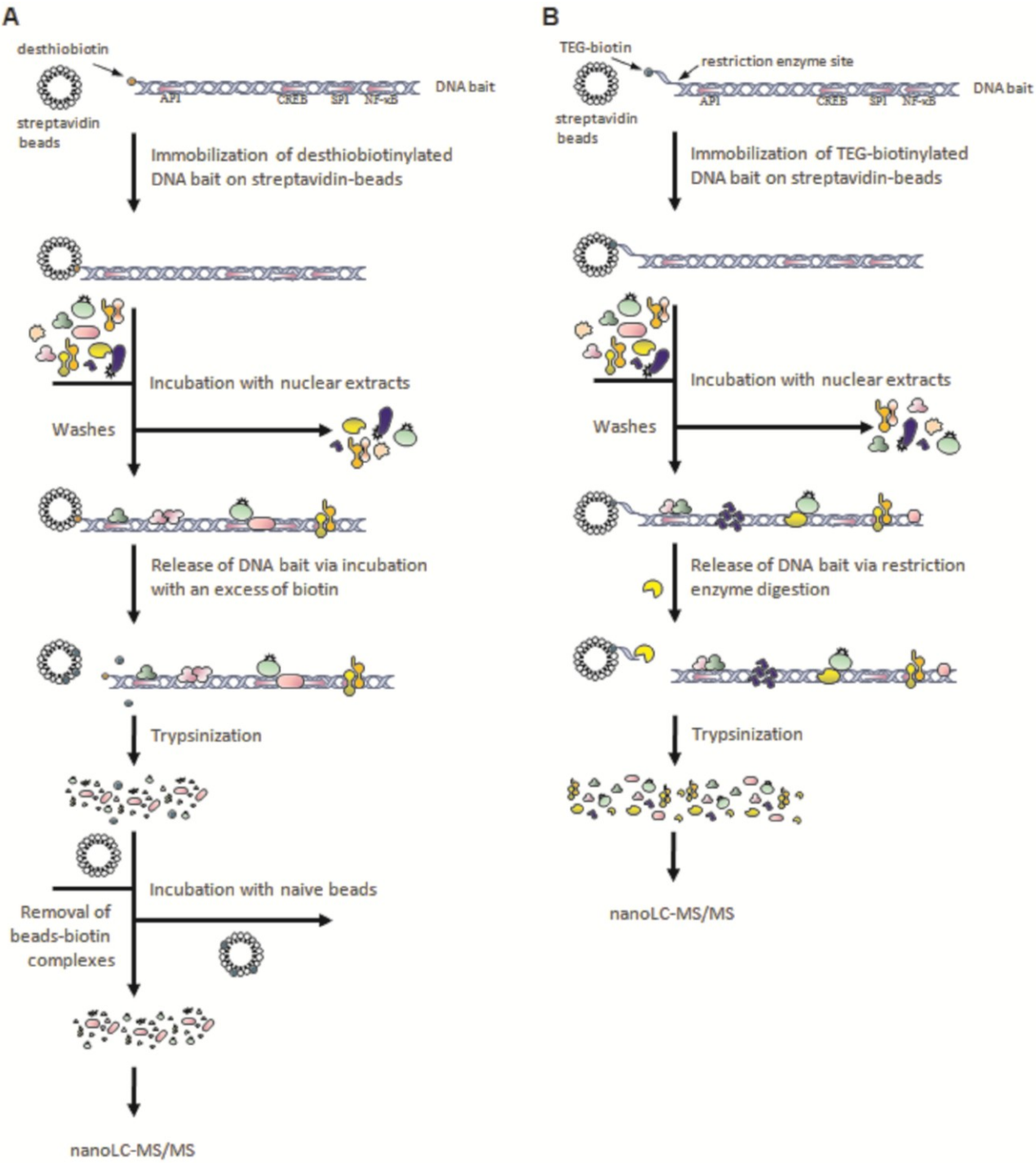

Figure 1. Schematic representation of the mass spectrometry-coupled DNA affinity purification (AP) approaches. *(A) Method using desthiobiotinylated DNA bait and competition between desthiobiotin and biotin to liberate DNA-protein complexes as described by Tacheny (2012) (Tacheny et al. 2012) (B) Method using biotinylated DNA bait and restriction enzyme digestion to liberate DNA-protein complexes adapted from Mittler (2009) (Mittler et al. 2009).*

2.2.7. Mass spectrometry data analysis

MS/MS spectra were searched with SearchGui (Vaudel et al. 2011), a bioinformatics tool that employs two search engines, OMSSA (Geer et al. 2004) and X!TANDEM

(Craig and Beavis 2003), to obtain peptide-to-spectrum matches. Corresponding proteins were searched using Peptide-Shaker (Barsnes et al. 2011) by confronting peptide-to-spectrum matches with the human subsection of the UniProt database. The search settings were as follows: (1) trypsin as protease with a maximum of two missed cleavage sites, (2) MS and MS/MS tolerance of 10 ppm and 0.5 Da, (3) cysteine S-carbamidomethylation as fixed modification, (4) pyroglutamic acid and methionine oxidation as variable modifications, (5) a decoy database generated automatically from the human subsection of the UniProt database was used and the major criterion to consider proteins was a False Discovery Rate (FDR) < 1%. Proteins recovered with at least two unique peptides yielded in both DNA AP approaches were considered for further validation. To achieve relative quantification of the different proteins bound to the IL-6 promoter bait, the total number of peptides and the exponentially modified protein abundance index (emPAI) (Ishihama et al. 2005) were calculated per protein.

2.2.8. siRNA silencing

Transient silencing of TEF-1 in 1321N1 cells was accomplished by transfecting 40 nM siGENOME SMART pool specifically targeting TEF-1 (Dharmacon RNAi Technology, Lafayette, CO, USA). Cells transfected with an equal amount of Renilla luciferase siRNA (Dharmacon) were used to assess for unspecific effects. $3x10^5$ cells in 6-well plate format were transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen by Life Technologies) according to the manufacturer's instructions. 48 hours after transfection and after overnight starvation, cells were left untreated or induced for the indicated time.

2.2.9. Luciferase reporter assay

Transient transfection with reporter gene constructs and siRNA was done using the Lipofectamine 2000 Transfection Reagent (Invitrogen by Life Technologies) according to the manufacturer's instructions. Briefly, $3x10^5$ cells were seeded in a 6-well plate and cotransfected with 1 µg of luciferase reporter constructs (the IL-6 promoter construct, NF-κB- or CRE-reporter gene constructs), 200 ng of a β-galactosidase-coupled housekeeping reporter construct and either TEF-1 or control siRNA. Total lysates were incubated with luciferase substrate and luminescence was measured on a TopCount luminometer (PerkinElmer Life Sciences, Canberra-

Packard, Waverley, UK). Luciferase activity was expressed as fold induction (treated/untreated) upon normalisation for transfection efficiency.

2.2.10. RNA isolation, cDNA synthesis and Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Reverse transcription was performed on 0.5 µg of total mRNA using the PrimeScript RT reagent kit from Takara Bio Inc. (Shiga, Japan). For real time cDNA amplification we used the Roche SYBR Green Mastermix (Roche Applied Science, Penzberg, Germany) and primers listed in Supplementary Table 1. Fluorescence was monitored using the Light Cycler 480II (Roche). A serial dilution of a representative cDNA sample was used to generate a standard curve and determine the efficiency of the PCR reaction for all primer sets, which was used in the subsequent calculation of relative mRNA inputs. Expression of each gene was normalized to expression of the HPRT or GAPDH housekeeping genes and results are presented as fold induction compared to untreated cells. For clarity the Y-axis is interrupted in some cases.

Chromatin accessibility assay via Real-Time PCR (CHART-qPCR)

Cells ($1x10^6$) were washed in PBS, scraped and collected by centrifugation at 453 g for 5 minutes at 4°C. Next, cells were re-suspended in 600 µl buffer A (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.3 M sucrose) and incubated for 10 minutes at 4°C. Subsequently, an equal volume of lysis buffer was added (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.3 M sucrose, 0.4% NP40 and 2 mM Na-butyrate) and cells were incubated for 10 minutes at 4°C. After centrifugation at 240 g for 5 minutes at 4°C, the chromatin pellet was resuspended in buffer R (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$) digested for 10 minutes at 37˚C with 3.3 IU/ml MNase and MNase Buffer (New England BioLabs). Reactions were terminated by adding PBS to obtain a final volume of 200 µl. Following proteinase K (Qiagen) and RNase A (Qiagen) treatment, genomic DNA (gDNA) was isolated using the QIAamp DNA purification kit (Qiagen). Purified gDNA (10 ng/reaction) was quantified by qPCR using the Roche SYBR Green Mastermix (Roche). Primers were designed to amplify sequences within the human IL-6 promoter (Supplementary Table 1). A serial dilution of a representative gDNA sample was used to generate a standard curve and to determine the efficiency of the PCR reaction for all primer sets and to calculate the relative gDNA concentration ([gDNA]) of the samples. Data are

presented as chromatin opening which was defined as the ratio of [gDNA] of samples digested with restriction enzymes over [gDNA] of undigested samples (Chromatin opening = [gDNA]digested/[gDNA]undigested).

2.2.11. Chromatin immunoprecipitation (ChIP)

For ChIP experiments, protein/DNA complexes were crosslinked *in cellulo* by adding formaldehyde directly to the culture medium to a final concentration of 1%. After 10 minutes, glycine was added to a final concentration of 125 mM and cells were incubated for 5 minutes, then washed with 30 ml PBS, scraped and collected.

Pellets were lysed in FA lysis buffer (50 mM HEPES pH 7,5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.01% SDS and 0.1% sodium deoxycholate) supplemented with Complete Protease Inhibitor Cocktail without EDTA (Roche)). Cells were sonicated at high settings (twice for 8 minutes using 30 s on/ 30 s off cycles with an intermediate cooling on ice) using the Diagenode Bioruptor (Liège, Belgium). Sonicated lysates were pre-cleared by centrifugation at 17900 g for 10 minutes at 4°C, followed by immunoprecipitation with 5 µg of anti-p65 or anti-TEF-1 using Protein A Sepharose 4 Fast Flow beads (Amersham). Samples were decrosslinked overnight at 65°C. All samples were treated with 50 µg/ml of RNase A and 100 µg/ml of Proteinase K. Immunoprecipitated genomic DNA (gDNA) was purified with the QiaQuick PCR purification kit (Qiagen) and subsequently quantified by qPCR using the Roche SYBR Green Mastermix (Roche). Primers used for amplification of the IL-6 promoter and β-actin (control) are listed in Supplementary Table 1. qPCR was performed on the Light Cycler 480II (Roche). Determination of [gDNA] in the input and immunoprecipitated samples (IPs) was performed as for CHART-PCR. Data are presented as the percentage (%) of [gDNA] in the IPs as compared to the [gDNA] in the corresponding input sample (%IP = ([gDNA]IP/[gDNA]input) ×100).

2.2.12. Overexpression experiments

For overexpression of TEF-1, HEK293T cells were transfected with either the Flag-TEF-1 or the Flag-TEF-1 S87A expression constructs. Briefly, $1x10^6$ cells were seeded in a 10 cm plate and transfected with 8 µg of DNA using the calcium phosphate method. 48 h after transfection and after overnight starvation, cells were treated with different inducers for the indicated time periods. Subsequently, cells were subjected to immunoprecipitation.

2.2.13. Immunoprecipitation (IP)

For IP experiment, cells were lysed in 500 µl of Lysis Buffer (50 mM Tris-HCl pH 7.5, 20 mM NaCl, 1% NP40, 1 mM Na_3VO_4, 10 mM NaF and Complete Protease Inhibitor Cocktail without EDTA (Roche)). After 20 minutes of incubation at 4°C, the lysates were cleared by centrifugation at 17900 g for 10 minutes at 4°C. Subsequently, supernatants were incubated overnight with 20 µl monoclonal anti-flag M2 agarose beads (Sigma) at 4°C to precipitate the Flag-TEF-1 protein. The protein/bead complexes were washed four times with 1 ml of lysis buffer. Proteins were released from the beads by incubation with 50 µl of SDS-PAGE sample buffer. After centrifugation of the beads, phosphorylation of immunoprecipitated TEF-1 as well as the level of TEF-1 expression were evaluated by Western blotting.

2.2.14. Western blotting

25 µl of SDS sample buffer protein lysates was resolved using SDS-PAGE on 12% polyacrylamide gels, proteins were then transferred to a nitrocellulose membrane (Amersham, Dubendorf, Switzerland) and analysed by Western blotting. Briefly, membranes were incubated with a 1:1 dilution of Blocking Buffer (LICOR Biosciences, Lincoln, NE, USA) in PBS. Subsequently, membranes were probed with primary antibodies, targeting either phosphorylated PKA substrate (1:1000) or anti-Flag (1:5000), diluted in Blocking Buffer (1:1 in PBS containing 0.1% Tween (PBS-T)). After three washes in PBS-T, DyLight secondary antibody (Pierce, Rockford, IL, USA) diluted 1:10000 in Blocking Buffer/PBS-T (1:1) was applied. The membranes were then washed three times in PBS-T and detection was performed using the Odyssey Imaging System (Licor).

2.2.15. Statistical analysis

Statistical significance was determined using Student's t-test or one-way ANOVA followed by Bonferroni's multiple comparison test. Results are considered significant when $p\text{-value} < 0.05$.

2.3. Results

2.3.1. Identification of proteins binding to the IL-6 promoter

As a model for this study we used the human 1321N1 astrocytoma cell line, in which we previously observed synergistic IL-6 expression upon cotreatment with TNF-α and

the β-agonist isoproterenol (Spooren et al. 2010). To identify mediators of this synergistic IL-6 transcription, we here used a proteomics approach based on DNA AP. As a DNA bait, we used the minimal IL-6 promoter (Vanden Berghe et al. 1998) and fished for interactors in nuclear extracts of untreated cells or cells co-treated for 60 minutes with TNF-α and isoproterenol to enrich for transcriptional (co)regulators. We used two independent approaches for the MS-coupled DNA AP and a scheme presenting the two approaches is depicted in Figure 1. A list of all proteins identified in the different experiments is shown in Supplementary Table 2. Combining data from all experiments and considering only proteins identified with at least two unique peptides, whilst excluding common contaminants, we recovered a total of 142 putative IL-6 promoter interactors (Supplementary Table 3).

Although we expected to identify differential recruitment of proteins to the IL-6 promoter upon TNF-α and isoproterenol treatment, our approach did not reveal this. A few proteins were identified only when using lysates from either untreated cells (e.g. DPY30, HMGA1) or cells treated with TNF-α/isoproterenol (e.g. JDP2, NFKB2), however, they were not reproducibly identified (Supplementary Table 3). Therefore, we further analysed our dataset irrespective of the treatment cells underwent.

To prioritize proteins for further analysis, we focused on 17 proteins that were identified in both independent AP-MS approaches. Next, we compared the abundance of the recovered proteins using the emPAI abundance index values, which enable comparison of protein abundance (Ishihama et al. 2005). Putative IL-6 promoter interactors were ranked according to their mean emPAI value per sample (Tables 1 and Supplementary Table 3). The validity of this prioritization becomes clear by the fact that this list contains eight transcription factor (JUNB, C/EBPB, FOSL2, JUND, FOSL1, JUN, NFKB1, RELA) that were previously shown to drive IL-6 expression (Dendorfer et al. 1994; Grassl et al. 1999; Szabo-Fresnais et al. 2008; Vanden Berghe et al. 1998) (Table 1, marked in bold). As expected we also identified proteins linked to the regulation of the DNA damage response, such as Poly (ADP-ribose) polymerase 1 (PARP1), DNA-(apurinic or apyrimidinic site) lyase 1 (APEX1), members of the DNA damage-binding (DDB) protein and cullin (CUL) protein families. According to the CRAPome database (Mellacheruvu et al. 2013), these proteins are also frequently identified in AP-MS control samples (Supplementary Table 4). Although, according to CRAPome, DDB2 was identified only in a limited number of negative control samples, we assumed that

Table 1. Shortlist of putative interactors of the IL-6 promoter identified via AP-MS.

Accession	Protein Name	Sum # Validated Peptides	Av emPAI	Nuclear localization	Function	Reference	Known interaction with the IL-6 promoter	Reference
Q16531	DNA damage-binding protein 1 (DDB1)	435	2,46	X	DNA-binding protein	(Iovine et al. 2011)		
Q92466	DNA damage-binding protein 2 (DDB2)	230	2,24	X	DNA-binding protein	(Stoyanova et al. 2009)		
P17275	**Transcription factor jun-B (JUNB)**	99	1,08	X	Transcription	(Shaulian and Karin 2002)	X	(Grassl et al. 1999)
P17676	**CCAAT/enhancer-binding protein beta (CEBPB)**	79	0,87	X	Transcription	(Tsukada et al. 2011)	X	(Grassl et al. 1999)
P15408	**Fos-related antigen 2 (FOSL2)**	59	0,52	X	Transcription	(Shaulian and Karin 2002)	X	(Szabo-Fresnais et al. 2008)
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	202	0,52	X	Transcription	(Thomas and Tulin 2013)		
Q13619	Cullin-4A (CUL4A)	136	0,38	X	DNA-binding protein	(Jackson and Xiong 2009)		
P17535	**Transcription factor jun-D (JUND)**	37	0,37	X	Transcription	(Shaulian and Karin 2002)	X	(Grassl et al. 1999)
P15407	**Fos-related antigen 1 (FOSL1)**	27	0,29	X	Transcription	(Shaulian and Karin 2002)	X	(Grassl et al. 1999)
Q13620	Cullin-4B (CUL4B)	83	0,20	X	DNA-binding protein	(Jackson and Xiong 2009)		

						(Anbanandam et al. 2006)		
P28347	*Transcriptional enhancer factor TEF-1 (TEAD1)*	30	0,18	X	Transcription	(Pobbati and Hong 2013)		
P27695	DNA-(apurinic or apyrimidinic site) lyase (APEX1)	22	0,14	X	Transcription	(Tell et al. 2009)		
P05412	**Transcription factor AP-1 (JUN)**	14	0,13	X	Transcription	(Shaulian and Karin 2002)	X	(Grassl et al. 1999)
O14867	*Transcription regulator protein BACH1 (BACH1)*	30	0,10	X	Transcription	(Motohashi et al. 2002)		
						(Hayden and Ghosh 2012)		
Q04206	**Transcription factor p65 (RELA)**	12	0,07	X	Transcription	(Bhatt and Ghosh 2014)	X	(Grassl et al. 1999)
Q14938	*Nuclear factor 1 X-type (NFIX)*	11	0,06	X	Transcription	(Gronostajski 2000)		
						(Hayden and Ghosh 2012)		
P19838	**Nuclear factor-kappa-B p105 subunit (NFKB1)**	14	0,04	X	Transcription	(Bhatt and Ghosh 2014)	X	(Grassl et al. 1999)

this protein is also non-specifically captured in our study just like its sibling family member, DDB1. Hence, we considered PARP1, APEX1, DDB1, DDB2, CUL1A, CUL1B proteins as likely contaminants and excluded them from further analysis. It must, however, be emphasized that the CRAPome database is primarily built using AP-MS data with proteins and not DNA as baits. Thus, we finally limited our initial list to 3 putative novel interactors of the IL-6 promoter: TEF-1, BTB and CNC homolog 1 (BACH1) and Nuclear factor 1 X-type (NF1X) (Table 1, *marked in italics*). Each of these proteins has a known function linked to regulation of gene transcription (Anbanandam et al. 2006; Gronostajski 2000; Motohashi et al. 2002). We further focused on TEF-1 as this transcriptional regulator is a direct target of protein kinase A (PKA) (Gupta et al. 2000), which we have previously identified as an indispensable mediator of IL-6 transcriptional synergy upon concurrent β_2-AR/TNF-R triggering (Spooren et al. 2010). Interestingly, phosphorylation of TEF-1 by PKA can affect its DNA binding ability (Gupta et al. 2000).

2.3.2. Ablation of TEF-1 expression leads to increased IL-6 transcription

To establish whether TEF-1 plays a role in the regulation of IL-6 gene expression upon treatment with TNF-α and/or isoproterenol, we silenced TEF-1 using siRNA targeting selectively this family member (siTEF-1). In agreement with our previous data, in control siRNA-transfected cells (siCtrl), TNF-α/isoproterenol co-treatment synergistically induced IL-6 promoter activity in 1321N1 human astrocytoma cells. As we were unable to detect endogenous TEF-1 at the protein level in 1321N1 cell lysates using an anti-TEF-1 antibody, that according to the supplier should enable detection of TEF-1 expression via Western blotting, we checked the level of TEF-1 silencing only at the mRNA level, via RT-qPCR. Using siRNA targeting TEF-1, we achieved approximately 50% downregulation of the TEF-1 mRNA level (Figure S1 A). As evident from Figure 2 A, TEF-1 silencing modestly promoted IL-6 promoter activity in all treatment settings, yet the most pronounced effects were apparent in cells treated with isoproterenol alone or in combination with TNF-α. Subsequently, we investigated the effect of TEF-1 silencing on endogenous IL-6 transcription (Figure 2 B). In this set of experiments, we also observed only 50% reduction of TEF-1 mRNA level (Figure S1 B). IL-6 mRNA levels were undetectable both in control siRNA or TEF-1 siRNA-treated cells. TEF-1 silencing upregulated IL-6 transcript

levels in all treatments, and the most pronounced effects were again seen in cells treated with isoproterenol alone or in combination with TNF-α.

Next, we explored whether TEF-1 physically interacts with the endogenous IL-6 promoter by means of ChIP. However, we could not recover TEF-1 interaction with the IL-6 promoter upon concurrent TNF-R/β_2-AR triggering or in untreated cells (Figure 2 C). It is still possible that interaction is indirect, too weak or too transient to be detected using ChIP. The specificity of the observed responses is indicated by the fact that we did not detect any of the observed responses at an irrelevant housekeeping gene (β-actin) or using beads only for the immunoprecipitation (Figure S1 C). Furthermore, in our hands the TEF-1 antibody did not produce consistent results in ChIP assays and we were able to immunoprecipitate TEF-1 with the IL-6 promoter in two out of four experiments. Similar problems using anti-TEF antibodies were previously reported by others (Cuddapah et al. 2008; Ribas et al. 2011).

These results indicate that TEF-1 might indirecty interact with the IL-6 promoter and probably acts as a repressor of IL-6 transcription.

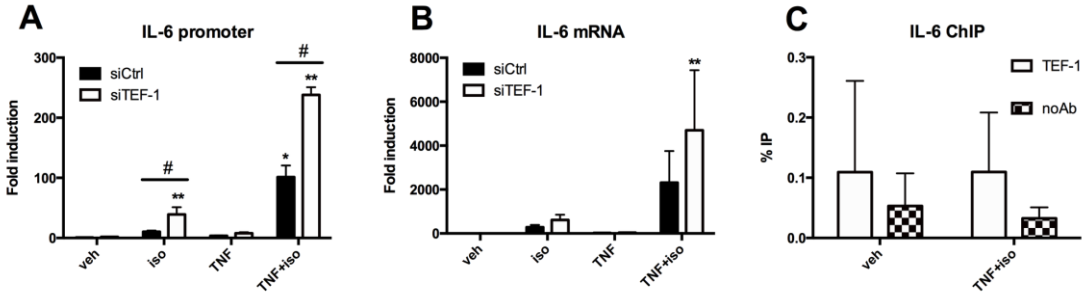

Figure 2. Role of TEF-1 as a regulator of IL-6 expression. *(A) Influence of TEF-1 silencing on IL-6 promoter activity in 1321N1 cells. 1321N1 cells, cotransfected with an siRNA targeting TEF-1 and IL-6-luciferase reporter plasmid, were treated for 6 hours with vehicle (veh), isoproterenol (iso) and/or TNF-α (TNF), before analysis of luciferase production. The specificity of observed responses was evaluated by comparison to cells transfected with control siRNA. Fold induction values are normalized to that of untreated siCtrl-transfected cells for which fold induction was set as 1. (B) Influence of TEF-1 silencing on IL-6 mRNA level in 1321N1 cells. 1321N1 cells, transfected with an siRNA targeting TEF-1, were treated for 2 hours with veh, iso and/or TNF, before analysis of IL-6 mRNA via RT-qPCR. The specificity of the observed responses was evaluated by comparison to cells transfected with control siRNA. Fold induction values are normalized to that of untreated siCtrl-transfected cells for which fold induction was set as 1. (C) Effect of TNF/iso cotreatment on the recruitment of TEF-1 and NF-κB p65 to the IL-6 promoter. 1321N1 cells were treated with veh or TNF/iso for 1 hour. Recruitment of TEF-1 and NF-κB p65 was measured via ChIP. The experiment was performed four times. All data presented*

in Figure 2 are displayed as mean ± SD of three independent experiments. (*) Statistically different from untrated, siCtrl transfected cells. (**) Statistically different from untreated, siTEF-1 transfected cells. (#) Statistically different between siCtrl and siTEF-1 condition.

2.3.3. TEF-1 regulates the accessibility of the IL-6 promoter for transcription factors

Since silencing of TEF-1 enhanced the expression of the IL-6 gene and as we expected association of this factor with the IL-6 promoter, we explored the molecular mechanism of these effects. An *in silico* analysis of the proximal IL-6 promoter using the JASPAR database did not predict a consensus binding site for TEF-1, indicating that the interaction of TEF-1 with the IL-6 promoter is most likely indirect (data not shown). The TEF-1 transcription factor was previously reported to function as a transcriptional repressor, downregulating target gene expression via interaction with other transcriptional (co)regulators (Liu et al. 2014).

The IL-6 gene has a complex promoter architecture and, among others, contains functional transcription factor binding sites for NF-κB and CREB, binding of which is pivotal for synergistic transcription upon TNF-α/isoproterenol co-treatment (Spooren et al. 2010; Vanden Berghe et al. 1998). To investigate whether TEF-1 modulates the transcriptional activity of NF-κB and CREB, we first tested the effect of TEF-1 silencing using synthetic NF-κB- and CREB-dependent luciferase constructs. As we previously reported (Spooren et al. 2010), TNF-α activated NF-κB-dependent luciferase activity, whereas isoproterenol activated CREB-dependent luciferase activity in control siRNA-treated cells. Co-treatment did not lead to synergistic activation of the isolated CREB or NF-κB response elements. Silencing of TEF-1 (Figure S1 D and Figure S1 E) did not affect the activity of the NF-κB reporter, whereas a modest stimulation of the CRE reporter gene was apparent (Figure 3 A and Figure 3 B). The accessibility of a gene promoter to transcription factors is reflected in its susceptibility to nuclease digestion. In CHART-PCR, this feature is exploited and the accessibility of a selected DNA sequence is determined by digesting chromatin using nucleases and then quantifying the amount of remaining uncut gDNA in the digested chromatin sample via qPCR. Here, we digested the proximal IL-6 gene promoter using MNase that non-selectively cuts DNA. Then, we amplified the sequence of interest via qPCR, using primers that recognise the sequence flanking the DNA motifs for NF-κB and CREB transcription factors (Figure 3 C). Results were

normalised as explained in the Materials and Methods section and expressed as chromatin opening. We found that the IL-6 promoter is susceptible to digestion with MNase in the vicinity of CREB and NF-κB binding sites, only upon co-treatment with TNF-α and isoproterenol (Figure 3 D and Figure 3 E). Interestingly, silencing of TEF-1 promoted further chromatin loosening at the IL-6 promoter in the proximity of the transcription factor-binding site for NF-κB and CREB (Figure 3 G and Figure 3 H). As expected, neither TNF-α/isoproterenol treatment nor TEF-1 silencing affected chromatin opening at the β-actin promoter, which we considered as an irrelevant region (Figure 3 F and Figure 3 I). The efficiency of silencing TEF-1 in CHART-PCR experiments is represented in Figure S1 F.

These results show that TEF-1 acts as a repressor at the IL-6 promoter, interferes with transcriptional activity of CREB and regulates the accessibility of IL-6 promoter for transcription factors.

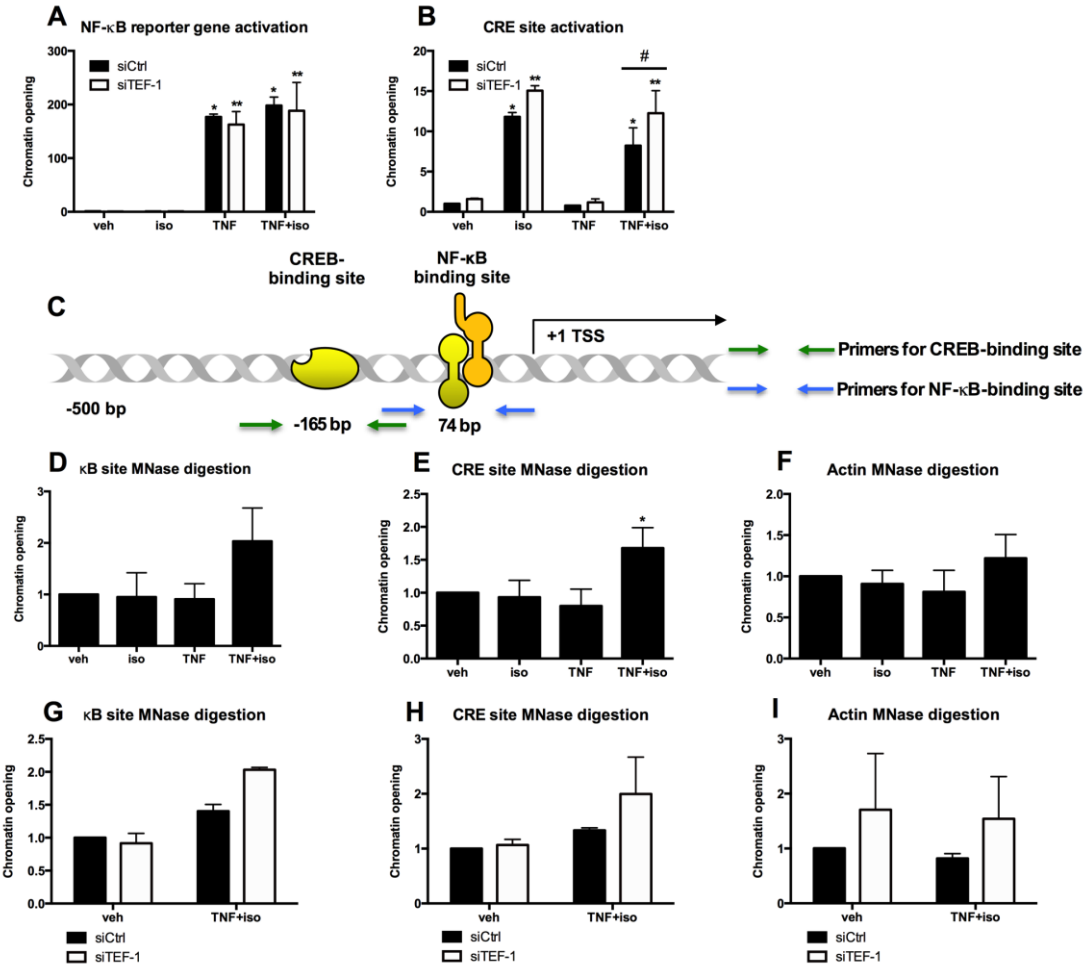

Figure 3. Effect of TEF-1 on nuclear events associated with synergistic IL-6 expression. *(A) Effect of TEF-1 on the activation of NF-κB. 1321N1 cells, cotransfected with an siRNA targeting TEF-1 and the*

NF-κB-luciferase reporter plasmid, were treated with veh, iso and/or TNF for 6 hours, before analysis of luciferase production. The specificity of the observed responses was evaluated by comparison to cells transfected with control siRNA. Fold induction values are normalized to that of untreated siCtrl-transfected cells for which fold induction was set as 1. (B) Effect of TEF-1 on the activation of CREB. 1321N1 cells, cotransfected with an siRNA targeting TEF-1 and the CRE-luciferase reporter plasmid, were treated with veh, iso and/or TNF for 6 hours, before analysis of luciferase production. The specificity of observed responses was evaluated by comparison to cells transfected with control siRNA. Fold induction values are normalized to that of untreated siCtrl-transfected cells for which fold induction was set as 1. (C) Schematic representation of the localisation of CREB- and NF-κB-responsive elements and the transcription start site (TSS) in the IL-6 promoter. Relative positions of primers used in the chromatin accessibility assay via Real Time PCR (CHART-PCR) are indicated. (D, E, F) Influence of TNF/iso cotreatment on the accessibility of the IL-6 promoter and irrelevant region. 1321N1 cells were treated for 1 hour with veh, iso and/or TNF. Chromatin opening of the promoter region was determined by CHART-PCR as detailed in Materials and Methods. (G, H, I) Role of TEF-1 in IL-6 promoter relaxation upon TNF/iso cotreatment. 1321N1 cells, transfected with an siRNA targeting TEF-1, were treated for 1 hour with veh, iso and/or TNF. The specificity of observed responses was evaluated by comparison to cells transfected with control siRNA. Chromatin opening of the promoter region was determined by CHART-PCR as detailed in Materials and Methods. The experiment was performed 2 times. Data present in Figure 3 are displayed as mean ± SD of independent experiments. If not mentioned otherwise experiments presented in Figure were performed three times. (*) Statistically different from untrated, siCtrl transfected cells. (**) Statistically different from untreated, siTEF-1 transfected cells. (#) Statistically different between siCtrl and siTEF-1 condition.

2.3.4. Gene selectivity of TEF-1 repressor action

To assess the gene selectivity of TEF-1 as a transcriptional coregulator, we performed RT-qPCR analysis to investigate how TEF-1 silencing modulates the effects of β-agonist co-treatment on the expression of well-known NF-κB target genes with a previously demonstrated role in neuroinflammation, such as cyclooxygenase-2 (COX-2), chemokine (C-X-C motif) ligand 2 (CXCL-2), ICAM-1 and IL-8, which we previously reported to be affected by TNF/isoproterenol co-treatment in astrocytes (Laureys et al. 2014; Spooren et al. 2010).

In line with our previous reports, we found bidirectional effects of β_2-AR co-treatment on TNF-α-induced NF-κB-dependent transcription (Figure 4). Specifically, we found that in 1321N1 cells transfected with control siRNA, TNF-α potently induced expression of IL-8 and ICAM-1, and TNF-α-induced expression of these genes was inhibited by isoproterenol co-treatment. Silencing of TEF-1 did not modulate the

effect of TNF-α and isoproterenol on IL-8 and ICAM-1 transcription (Figure 4 C and Figure 4 D). COX-2 and CXCL-2 mRNA levels expression were induced both with TNF-α and isoproterenol and co-treatment potentiated mRNA expression of these genes. Whereas silencing of TEF-1 showed trend towards upregulation of COX-2 transcription in both untreated cells and in cells treated with TNF-α and/or isoproterenol, CXCL-2 mRNA levels remained unchanged in all treatment settings upon TEF-1 knockdown. Interestingly, knockdown of TEF-1 doubled basal expression of COX-2, while it did not have an effect on CXCL-2 expression (Figure 4 A and Figure 4 B).

We thus conclude that in 1321N1 astrocytes a subset of NF-κB-dependent genes exists that is under transcriptional control of TEF-1.

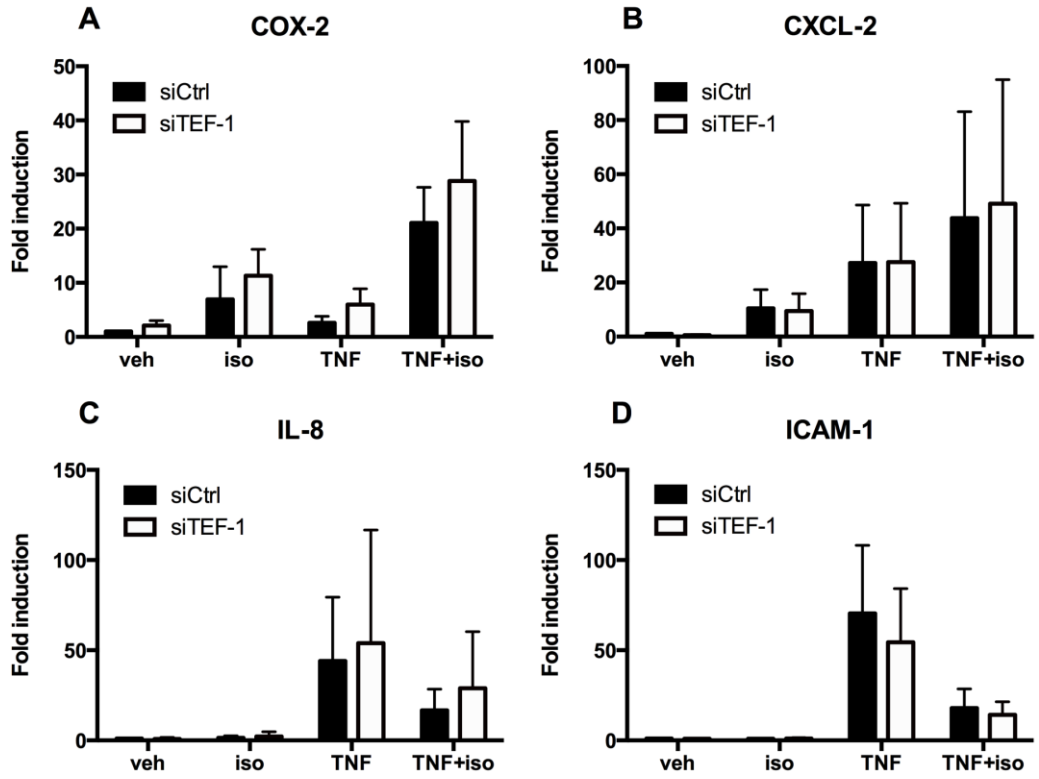

Figure 4. Influence of TEF-1 on NF-κB-dependent gene expression in 1321N1 cells upon TNF-α/isoproterenol cotreatment. *Expression of inflammatory markers was measured via RT-qPCR after 2 hours induction with veh, iso and/or TNF in 1321N1 cells transfected either with siRNA targeting TEF-1 or control siRNA. All data present in Figure 4 are displayed as mean ± SD of three independent experiments. (*) Statistically different from untrated, siCtrl transfected cells. (**) Statistically different from untreated, siTEF-1 transfected cells. (#) Statistically different between siCtrl and siTEF-1 condition.*

2.3.5. TEF-1 is a substrate of the PKA signalling cascade

The canonical signalling pathway activated upon β_2-AR triggering involves activation of adenylyl cyclase, which catalyses the production of cAMP that in turn activates PKA. PKA enters the nucleus and phosphorylates multiple proteins bearing the RRXS/T consensus sequence (Ubersax and Ferrell 2007). The TEF-1 serine 102 residue is part of a consensus recognition site for PKA (Gupta et al. 2000). Since specific antibodies recognising the phosphorylated form of TEF-1 are not available, we transfected HEK293T cells with an expression plasmid encoding either Flag-TEF-1 wild type or Flag-TEF-1 serine 87 to alanine mutant (S87A). The TEF-1 isoform that we obtained from the ORFeome collection displayed only 79 % identity to the amino acid sequence deposited in UniProt, lacking 15 amino acids at the N-terminus and 58 amino acids in the transactivation domain, whilst harbouring a 4 amino acid insertion in position 110 (Figure 5 A). Thus, the previously reported phosphorylated serine 102 in the TEF-1 protein (Gupta et al. 2000) is on position 87 in the ORFeome isoform we cloned. Importantly, we observed that the PKA-phosphorylated motif was not altered.

To investigate whether TEF-1 is a substrate of the cAMP/PKA signalling cascade, HEK293T cells, transfected with either wild type Flag-TEF-1 wild type or mutant Flag-TEF-1, were left untreated or induced with forskolin (Fsk), an adenylyl cyclase activator raising intracellular cAMP levels, TNF-α or a combination of both. Next, the TEF-1 proteins were immunoprecipitated, probed in Western blotting with an antibody detecting motifs phosphorylated by PKA and re-probed with anti-Flag antibody. As expected in the Fsk-treated cells, we observed multiple immunoreactive bands corresponding to various cellular targets of PKA. Upon immunoprecipitation, fewer bands were detected, one of which had a molecular weight of approximately 43 kDa, corresponding to the expected weight of the Flag-tagged TEF-1 protein. Probing of the blot using an anti-Flag antibody confirmed that the 43 kDa band indeed represented Flag-TEF-1. Furthermore, we found that in HEK293T cells, Fsk induced phosphorylation of TEF-1 (Figure 5 B). TNF-α by itself did not have any significant effect on TEF-1 phosphorylation and it did not affect Fsk-induced phosphorylation of TEF-1. As expected, the S87A mutation in the TEF-1 protein abrogated phosphorylation by PKA (Figure 5 C).

Collectively, these data demonstrate that TEF-1 is phosphorylated by PKA at serine 87.

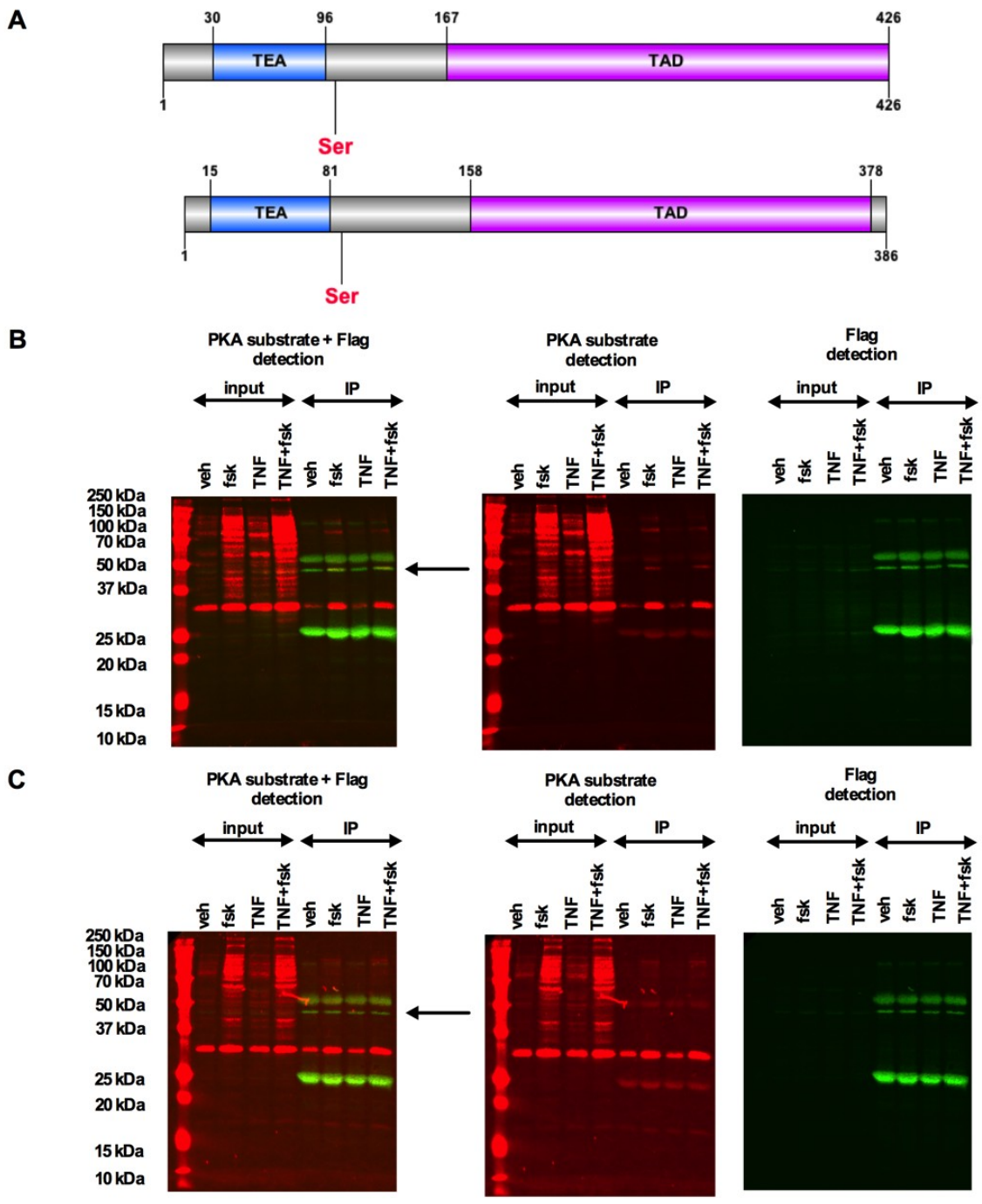

Figure 5. TEF-1 is a target of cAMP/PKA-dependent pathway. *(A) Schematic representation of the domain structure of TEF-1. The upper panel depicts the domain structure of the protein sequence deposited in the UniProt database. The lower panel depicts the domain structure of the isoform recovered from the v8.1 ORFeome. (B-C) TEF-1 phosphorylation was evaluated in HEK293T cells overexpressing either Flag-TEF-1 or Flag-TEF-1 S87A. TEF-1 was immunoprecipitated from cells stimulated with veh, forskolin (fsk) and/or TNF. Blots were probed with antibody targeting PKA substrates (red channel) and Flag (green channel) motif. Merge of green and red signals gives a yellow signal. A representative blot from three independent experiments for Flag-TEF-1 and two independent experiments for Flag-TEF-1 S87A is shown.*

Discussion

The ambiguous role and transcriptional regulation of IL-6 in health and disease are not fully understood (Spooren et al. 2011). We recently reported that concurrent treatment of human 1321N1 astrocytes with TNF-α and isoproterenol, a β-adrenoreceptor agonist, results in recruitment of NF-κB, CREB and CREB-binding protein (CBP) to the IL-6 promoter and that these factors cooperate in an enhanceosome structure to ensure synergistic gene expression (Spooren et al. 2010). Here, we used a proteomics approach to identify unknown elements of the IL-6 enhanceosome.

We used two independent methods that are adaptations of previously reported technologies (Mittler et al. 2009; Tacheny et al. 2012), to capture and identify proteins interacting with the minimal IL-6 promoter. The validity of our method is supported by the recovery of several known IL-6 promoter interactors belonging to the NF-κB, AP-1 and C/EBP transcription factor families (Dendorfer et al. 1994; Grassl et al. 1999; Szabo-Fresnais et al. 2008; Vanden Berghe et al. 1998). However, we did not recover all proteins previously reported to interact with the IL-6 promoter. For instance, via ChIP analysis we also detected CBP at the IL-6 promoter in TNF-α/isoproterenol co-treated 1321N1 cells (Spooren et al. 2010) and this protein was not among the hits of our AP-MS study. Possibly, the synthetic DNA bait lacks certain features of the native IL-6 promoter environment, which are required for interaction with some nuclear proteins. Another potential explanation could be that the binding buffer that we have used in our DNA AP assays might not provide the optimal conditions to capture the interaction of very diverse groups of transcriptional (co)regulators with the synthetic DNA bait. In addition, we cannot exclude that certain transcriptional (co)regulators of low abundance are lost before subjecting samples to MS sequencing (Tacheny et al. 2013). Nevertheless, we identified 3 putative novel IL-6 promoter interactors: TEF-1, BACH1, and NFIX. All of these proteins possess a nuclear function linked to gene expression (Anbanandam et al. 2006; Gronostajski 2000; Motohashi et al. 2002) but have never been shown to interact with the IL-6 promoter. An *in silico* analysis of the 500 bp IL-6 promoter fragment upstream of the transcriptions starting site using JASPAR database of transcription factor binding sites revealed the presence of putative DNA responsive motifs for TEF-1 and BACH1. Interestingly, our bioinformatics approach also identified a binding site for NFIC, a transcription factor closely related to the NFIX

transcription factor here identified (data not shown).

We decided to focus on TEF-1 as a novel interactor of the IL-6 promoter. TEF-1, also called TEAD-1, is a member of a transcription factor family that consists of four members (TEF-1, TEF-3, TEF-4 and TEF-5). TEAD genes are ubiquitously expressed in embryonic and adult tissues, and display a distinct but overlapping expression pattern. All TEAD proteins share a TEA domain that enables binding of specific DNA elements and a transactivation domain responsible for interaction with coactivators (Pobbati and Hong 2013).

Our results suggest a repressive role of TEF-1 at the IL-6 promoter. We observed that silencing of TEF-1 upregulated IL-6 transcription as measured via reporter gene assay or RT-qPCR. Although the effect of TEF-1 silencing that we observed was rather modest, this could be explained by the fact that we only obtained partial knock-down using our siRNA-based approach (Figure S1). In addition, although these did not pass our selection filter, we also identified two other TEAD family members, TEF-3 and TEF-5. As all TEAD family members recognize the same consensus motif, it is possible that there is redundancy between TEFs in the regulation of IL-6 expression, which could also contribute to the modest effect of TEF-1 silencing.

TEF-1 can act both as a transcription factor and cofactor, interacting respectively with DNA or modulating the activity of other transcription regulators, such as serum response factor (SRF), and its action appears to be cell type and gene specific (Anbanandam et al. 2006; Liu et al. 2014; Pobbati and Hong 2013). Furthermore, the transcriptional activity of TEF-1 is controlled by its interaction with several coregulators belonging to three main categories: YAP/TAZ, Vgl1 and p160 nuclear coactivator proteins (Pobbati and Hong 2013). However, none of these proteins got identified in our AP-MS study.

Since the IL-6 promoter probably does not contain a binding site for TEF-1, the action of TEF-1 as a repressor could result from modulation of the activity of other transcriptional (co)factor(s) or component(s) of the basal transcriptional machinery that are essential for IL-6 transcription. Here, we observed that silencing of TEF-1 potentiates transactivation of isolated CREB but not NF-κB response elements, suggesting that TEF-1 regulates the transcriptional activity of CREB. In line with our data, Kessler (2008) (Kessler et al. 2008) showed that overexpression of TEF-1 in uterine decidual cells has a dominant negative effect on the activity of the prolactin promoter probably via interference with an unidentified transcription factor. As

expression of decidual prolactin is primarily driven by CREB (Gerlo et al. 2006), one might speculate that this unidentified transcription factor inhibited by TEF-1 at the prolactin promoter is CREB. TEF-1 also is a general repressor of muscle-specific genes, interfering with the binding of SRF and myocardin to particular smooth muscle gene promoters (Liu et al. 2014). As the IL-6 promoter contains a binding site for SRF at the position -158 upstream of the transcription starting site, it is possible that this factor mediates the interaction of TEF-1 with the IL-6 promoter (Ray et al. 1989). Interestingly, the SRF-binding site is also recognised by the C/EBP transcription factor, that we fished out in our AP/MS approach (Grassl et al. 1999; Tsukada et al. 2011). We also observed that silencing of TEF-1 promotes chromatin loosening at the IL-6 promoter, suggesting that TEF-1 could alter the recruitment of a chromatin remodelling complex to the IL-6 promoter. Previously, TEF-1 was shown to be required for SWI/SNF-like BAF complex-mediated chromatin remodelling necessary for transcription of the interferon inducible transmembrane (IFITM) gene (Cuddapah et al. 2008). Alternatively, TEF-1 can inhibit target gene expression via binding of TATA binding protein (TBP), a component of the basal transcriptional machinery, hence inhibiting its interaction with the TATA box and disrupting the formation of the pre-initiation complex (Jiang and Eberhardt 1996). Finally, it is possible that several mechanisms operate at the same time to precisely control and fine-tune IL-6 transcription.

Intriguingly, in addition to IL-6, silencing of TEF-1 alters expression of a selected pool of NF-κB-dependent targets in 1321N1 astrocytes. Previously, we found that combined triggering with TNF-α/isoproterenol elicits bidirectional effects on the expression of NF-κB-dependent genes (Spooren et al. 2010). Here, we additionally showed that TEF-1 selectively modulates expression of synergistically induced NF-κB targets, such as IL-6 and COX-2, but not those that are inhibited by β-AR co-stimulation, like IL-8 or ICAM-1. These data indicate that TEF-1 could be a unique coregulator affecting only a selected pool of NF-κB-dependent promoters that are susceptible for synergistic upregulation by β-adrenergic signals. TEF-1 is a prototypical transcriptional regulator of muscle specific genes (Liu et al. 2014; Ribas et al. 2011) and it has never been linked to modulation of TNF-α-induced gene expression. Nevertheless, TEF-1 was reported to modulate the interferon-α-induced transcriptional program, further strengthening the hypothesis that the pool of genes regulated by TEF-1 is larger than was previously assumed (Cuddapah et al. 2008).

We observed that cAMP-dependent signals stimulate phosphorylation of TEF-1 by PKA. Although we did not investigate how this phosphorylation affects the interaction of TEF-1 with the IL-6 promoter and other transcriptional (co)regulators in 1321N1 cells, it was previously shown that PKA-mediated phosphorylation of TEF-1 at serine 102 represses its DNA binding without affecting its interaction with the Max transcription factor in cardiac myocytes (Gupta et al. 2000). Interestingly, there is also evidence linking elevated levels of cAMP to inhibition of TEF-1-dependent transcription (Thompson et al. 2003). Whereas TEF-1 activity was shown to be regulated by signals downstream of the α_1-AR (McLean et al. 2003; Ueyama et al. 2000), our findings suggest that TEF-1 phosphorylation can also be regulated by β-adrenergic signals.

In summary, we here used a proteomics approach to identify novel regulators of IL-6 transcription in human astrocytes. We focused on TEF-1 and demonstrated that it represses the endogenous IL-6 promoter, probably via indirectly interaction that we could not reproducibly capture using ChIP asssay. The action of TEF-1 as a repressor of IL-6 transcription involves modulation of CREB activity and unidentified chromatin remodelling complexes. Our data indicate that TEF-1 is phosphorylated by PKA, acting downstream of the β_2-AR. Finally, we show that the inhibitory effect of TEF-1 on the expression of inflammatory mediators is gene selective. In view of the crucial role of IL-6 as a driver of neuroinflammation, it would be interesting to further explore how TEF-1 and the other TEAD family members modulate inflammatory processes in the central nervous system.

Acknowledgements

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Supplementary information

Supplementary data for the paper entitled: “A proteomics strategy identifies TEF-1 as a regulator of IL-6 transcription in astrocytes” can be found in Supplementary data section at the end of present thesis.

General discussion

&

Perspectives

General discussion & Perspectives

Ample evidence indicates that adrenergic stress and β_2-AR ligands can modulate various aspects of inflammation and immune function during health and disease (Padro and Sanders 2014; Powell et al. 2013). Furthermore, as evident from the literature summarized in Chapter 2, β_2-adrenergic signals interact with the NF-κB signalling cascade at multiple levels. Interestingly, whereas the immunosuppressive effects of adrenergic stress and β_2-agonists are widely accepted and often associated with inhibition of NF-κB-driven transcriptional programs, we have shown before that stimulation of astrocytic β_2-ARs elicits bimodal effects (potentiation vs. inhibition) on the expression of NF-κB-dependent genes (Spooren et al. 2010). Importantly, these dichotomic effects were observed both *in vitro* and *in vivo* indicating their (patho)physiological relevance (Laureys et al. 2014). Here, we further explored whether potentiation of selected NF-κB-dependent gene expression by β-agonists is a general phenomenon occurring in different cell types. In addition, we zoomed in on the molecular basis of this crosstalk.

1. β_2-AR/NF-κB crosstalk might be a general phenomenon

In the first part of the thesis, we have investigated whether the upregulation of selected NF-κB-dependent genes by β_2-AR-mediated signals, which we observed in astrocytes, also occurs in other cell types responsive to adrenergic stimulation. As a model for our research, we chose skeletal muscle cells since they display a high level of β_2-ARs and several studies suggested a therapeutic potential of β_2-agonists in skeletal muscle wasting disorders (Lynch and Ryall 2008). We and others observed that combination of a proinflammatory stimulus and a β_2-AR agonist results in strong synergistic IL-6 expression in skeletal muscle (Frost et al. 2004; Kolmus et al. 2014). Although there are many reports of cytoplasmic crosstalk mechanisms, targeting IKK activity and IκB degradation (Chapter 2), that lead to modulation of NF-κB activity, we did not find any proof for that in the C2C12 skeletal muscle cell line we used as a model. We observed that TNF-α launched the classical NF-κB signalling cascade and the p38 MAPK/MSK-1 axis, while activation of the β_2-AR induced the PKA/CREB pathway. Concurrent receptor triggering did not produce cytoplasmic crosstalk, but converged in the nucleus, at the promoters of NF-κB target genes. This nuclear

crosstalk involved chromatin remodelling and the formation of enhanceosome structures, which were associated with activation of transcription. Specifically, we found that combined β_2-AR and TNF-R triggering promoted phosphorylation of histone H3 and chromatin relaxation at the IL-6 promoter. This was paralleled by enhanced recruitment of NF-κB and CREB, which cooperatively recruited the CBP transcriptional cofactor that integrated synergistic transcription driven by RNA polymerase II (Kolmus et al. 2014). Additionally, we have abundant evidence that β_2-AR-mediated effects in skeletal muscle and astrocytes are dependent on the cAMP pathway and almost solely originate from this receptor subtype. In line with this, we also found the IL-6 synergy in macrophages upon combined stimulation with LPS (acting as an NF-κB-activating stimulus) and an cAMP analogues. As β_2-AR triggering potentiated, via the cAMP-dependent pathway, NF-κB-dependent gene expression in skeletal muscle and astrocytes, a similar mode of regulation might also occur in other cell types expressing a high level of β_2-AR, such as airway smooth muscle cells or hepatocytes but also in immune cells, such as macrophages. This hypothesis requires, however, further experimental verification.

2. Subtle differences in intracellular signalling might account for the robustness of nuclear crosstalk

Although the overall outcome of β-agonist/TNF-α cotreatment was very similar when skeletal muscle cells were compared to astrocytes, we observed some interesting cell-type specific differences in signalling. One of the most striking differences was the activation of the p38/MSK-1 axis upon TNF-R triggering, which was apparent in C2C12 cells, but not in astrocytes. We found that MSK-1 potently phosphorylated CREB at serine 133 in C2C12 cells but this was not associated with augmented recruitment of neither CREB nor CBP to the IL-6 promoter. In line, Naqvi *et al.* (Naqvi et al. 2014) showed that phosphorylation of CREB on serine 133 by MSK-1 does not promote strong recruitment of the CBP/p300 cofactor in response to phorbol myristate acetate (PMA), another inflammatory trigger. Nevertheless, CREB serine 133 phosphorylation was indispensable for the induction of CREB-dependent genes, including IL-6. Overall, these findings suggest that although MSK-1 and PKA both phosphorylate CREB at serine 133, they promote the use of different cofactors. In C2C12 cells, we observed that TNF-R/β_2-AR coactivation promotes potent recruitment of CREB at the IL-6 promoter that is paralleled by enrichment for CBP.

An outstanding question is whether other unidentified cofactors interacting with MSK-1-phosphorylated CREB are also recruited to the IL-6 promoter. In addition to CREB, MSK-1 also phosphorylates the NF-κB p65 subunit at serine 276, and this accounts for potent interaction with CBP and selective gene expression (Vermeulen et al. 2003; Zhong et al. 1998). Interestingly both p65 and CREB can be phosphorylated by PKA and MSK-1 at the same residues, serine 276 for p65 and serine 133 for CREB. It would be interesting to identify additional substrates of both PKA and MSK-1 that could perhaps explain differences in gene expression profiles activated by both kinases.

3. Gene selectivity of β_2-AR/NF-κB crosstalk

Overall, our findings in C2C12 skeletal muscle cells closely parallel what we previously reported in astrocytes *in vitro* (Laureys et al. 2014; Spooren et al. 2010) and in the CNS *in vivo* (Laureys et al. 2014) and indicate β-agonists potentiate IL-6 expression in different cell types subjected to proinflammatory stimuli, supporting the general relevance of this phenomenon. Although we focused on IL-6 as a model gene to study the β_2-AR/TNF-R crosstalk, we found that in C2C12 cells β-agonists not only promote IL-6 expression but also that of other NF-κB target genes that are important in skeletal muscle (patho)physiology. Positive crosstalk was apparent for CXCL-5, CCL-2 and ICAM-1 transcription. Other genes, such as CCL-5 and IκBα, were not affected by β_2-AR cotreatment. Although β_2-AR triggering also inhibited the expression of a subset of NF-κB target genes in astrocytes (eg. ICAM-1, VCAM-1 and IL-8), none of the NF-κB target genes we investigated in C2C12 cells were inhibited by β-agonists. These data further indicate that potentiation of TNF-α-induced, NF-κB-dependent target gene expression by β_2-adrenergic signals is a general phenomenon occurring in different cell types at selected pool of promoters. Moreover, our data suggest that there is probably a universal mechanism underlying synergistic gene expression during β_2-AR/NF-κB crosstalk as we detected activation of the same signalling pathways in astrocytes and skeletal muscle cells. Furthermore, our data indicate that the "skeleton" of the IL-6 enhanceosome is identical in skeletal muscle and astrocytes as we found that the same principal components (the NF-κB p65 subunit, CREB, CBP and RNA polymerase II) were recruited to the promoter upon β_2-AR/TNF-R coactivation (Kolmus et al. 2014; Spooren et al. 2010). In line, the variegated outcome of β_2-AR/NF-κB crosstalk in the context of inflammatory

gene expression is probably due to differences in the enhanceosome composition that are generated at promoters of inflammatory mediators. A model summarizing the intracellular events in response to combined β_2-AR and TNF-R triggering in skeletal muscle cells is depicted in Figure 1.

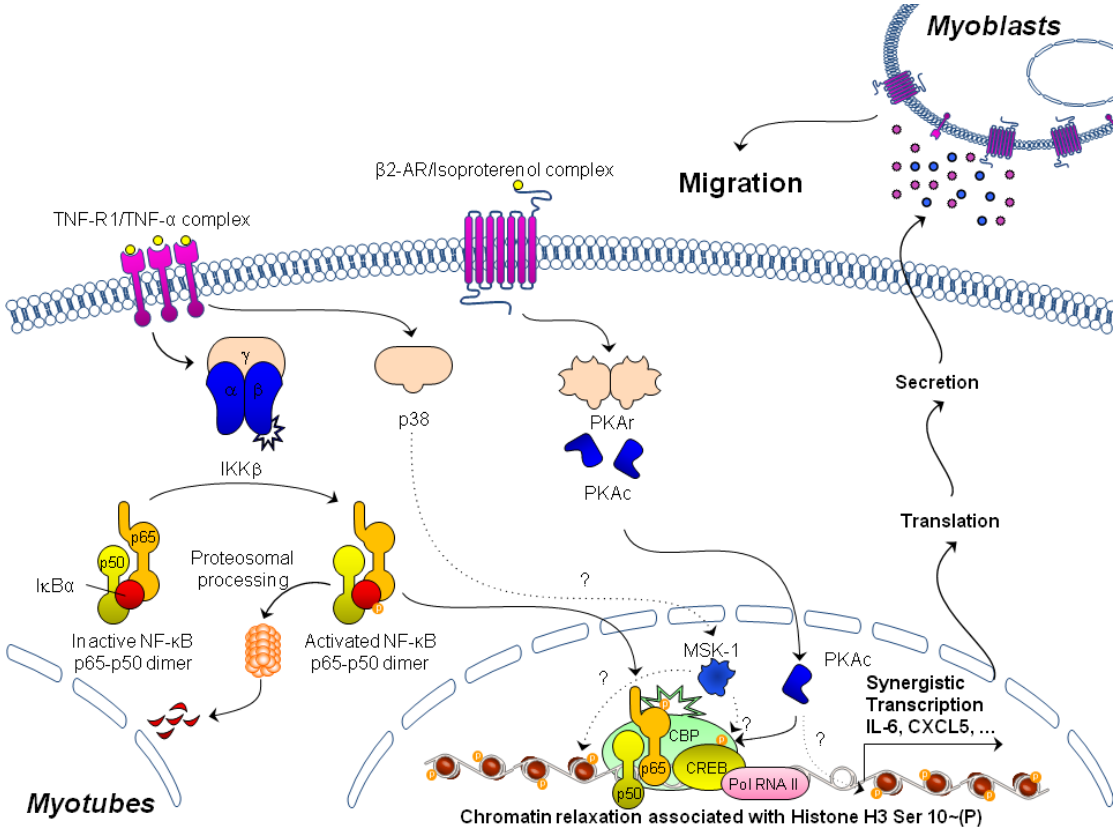

Figure 1. Model summarizing the intracellular events in response to combined β_2-AR and TNF-R triggering in skeletal muscle cells.

4. TEF-1 as a novel regulator of inflammatory genes

In the second part of this thesis, we employed MS-coupled DNA affinity purification to capture and identify novel components of the IL-6 enhanceosome involved in synergistic transcription upon concurrent TNF-R/β_2-AR triggering. Using this strategy, we recovered several known and three novel IL-6 promoter interactors: TEF-1, BACH1 and NF1X. Interestingly, TEF-1, BACH1 and NF1X are known transcriptional regulators, but have never been connected to transcriptional regulation of the IL-6 gene (Kolmus et al. in preparation). Because it is a known substrate of PKA (Gupta et al. 2000), we further focused on TEF-1 and demonstrated that it interacts with the endogenous IL-6 promoter and acts as a transcriptional repressor. The action of TEF-1 as a repressor of IL-6 transcription involved modulation of CREB but not NF-κB activity (Kolmus et al. 2014 in preparation). Although the

molecular mechanism underlying TEF-1-dependent inhibition of CREB remains to be established, we hypothesize that TEF-1 inhibits CREB transcriptional activity via blockage of its KID domain, which is responsible for contact with CBP. It is generally accepted that CBP mediates transcriptional activation by its intrinsic acetyltransferase activity or by recruiting additional acetyltransferases, hence promoting chromatin loosening (Vo and Goodman 2001). Thus, one could speculate that TEF-1 represses chromatin remodelling via precluding cooperative CBP recruitment by CREB and NF-κB upon blockage of the KID domain in CREB (Kolmus et al. in preparation). A model summarizing our current understanding of the enhanceosome generated at the IL-6 promoter in astrocytes upon combined β2-AR and TNF-R triggering is depicted in Figure 2.

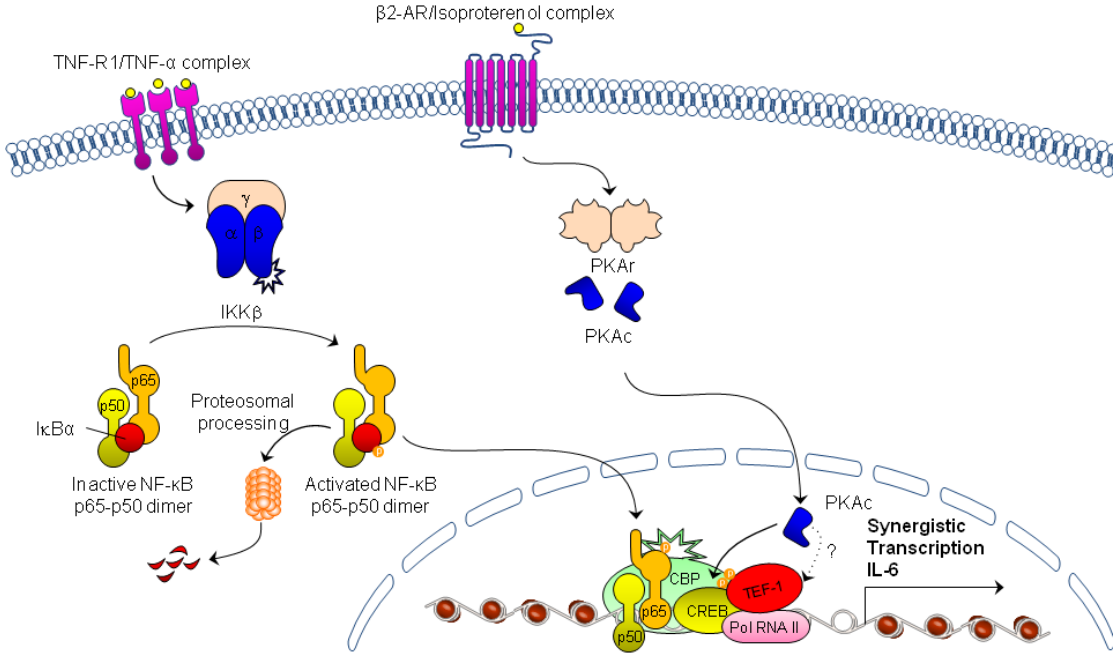

Figure 2. Model summarizing the composition of the IL-6 enhanceosome upon β_2-AR/TNF-R triggering in astrocytes.

We also observed that the TEF-1 represses only a subset of NF-κB-dependent genes. Interestingly, we found that TEF-1 acts as a repressor of both IL-6 and COX-2, both of which display synergistic upregulation upon β-agonist/TNF-α cotreatment. Intriguingly, IL-6 and COX-2 are important mediators of inflammation. It will be interesting to further elucidate the role of TEF-1 in fine-tuning the inflammatory response and to investigate whether it could be a target for selective inhibition of NF-κB-dependent gene expression (Kolmus et al. in preparation).

5. Role of β_2-adrenergic signals in the modulation of TEF-1 nuclear activity

As we found that TEF-1 is phosphorylated by PKA acting downstream of the β_2-AR, these data further strengthen the importance of modulatory input originating from β_2-AR. Previous findings showed that PKA-phosphorylated TEF-1 displays a diminished ability to interact with DNA, whilst this post-translational modification did not modify the ability of TEF-1 to interact with the Max transcription factors (Gupta et al. 2000). Here, we would like to propose two possible roles of PKA-mediated phosphorylation of TEF-1 in the regulation of IL-6 expression. As TEF-1 can act both as a transcription factor and cofactor, phosphorylation of TEF-1 could serve as a switch button "turning on" cofactor ability and "turning off" the transcriptional activity by altering its interaction with DNA. In that context, PKA-phosphorylated TEF-1 would not interact directly with the IL-6 promoter but instead binds CREB, precluding recruitment of cofactors and chromatin remodelling complexes. Alternatively, phosphorylation could induce dissociation of TEF-1 from the IL-6 promoter or mark the protein for deposition of other post-translational modification(s) that evokes its degradation. In that context, phosphorylation would contribute to abolishment of TEF-1 repressor activity. Clearly, further studies will be required to resolve the exact mechanism via which TEF-1 represses IL-6 transcription (Kolmus et al. in preparation).

6. TEF-1 as a therapeutic target in inflammatory disorders

TEF-1 is an imperative transcription factor for expression of developmental genes and its role in inflammatory/immune processes is not really well understood. Studies using TEF-1 knockout mice demonstrated that lack of TEF-1 leads to embryonic lethality due to cardiac defects (Chen et al. 1994). Subsequent *in vitro* and *in vivo* reports showed that this protein also regulates the transcriptional programs in skeletal and smooth muscle. Intriguingly, in some of cases TEF-1 acted as a transcriptional coactivator while in others as a corepressor (Yoshida 2008). Overall, it appears that TEF-1 is a mediator in a broad spectrum of nuclear processes via a variegated panel of activities.

To our best knowledge, we are the second group showing the importance of TEF-1 in the regulation of inflammatory mediators (Cuddapah et al. 2008). Thus, it is difficult to immediately answer the question whether TEF-1 could be a good therapeutic target in inflammatory disorders. Nevertheless, as TEF-1 acts in a gene selective manner to

regulate expression of two important mediators in inflammatory process, namely IL-6 and COX-2, it is possible that enhancement of TEF-1 binding to promoters of inflammatory genes could be a novel strategy to deliver a therapeutic gain. However, more in-depth characterization of the molecular mechanisms governing TEF-1 activity and TEF-1-mediated coregulation during gene expression is required.

7. Effect of β_2-AR modulators on NF-κB-dependent gene expression

Currently, it is clear that (nor)epinephrine and pharmacological ligands of β_2-ARs differentially affect the expression of selected NF-κB target genes. These effects have been reported in different immune cell types, but also in non-immune cells, such as glia, fibroblasts, epithelial and endothelial cells and smooth and skeletal muscle cells (Chapter 2). As evident from Table 2 in Chapter 2 and the findings summarized in the present thesis (Kolmus et al. 2014), the transcription of several prototypical NF-κB target genes, such as IL-2, IL-8 and TNF-α, appears to be consistently inhibited by β_2-agonists, while the effects on other targets, like for instance IL-6, IL-10 and IL-13, are less uniform. Interestingly, IL-6, IL-8, IL-13, CXCL-2, CXCL-3, CXCL-5, B7-2 and COX-2 genes undergo synergistic expression upon co-activation of β_2-ARs and the NF-κB pathway in various cell types.

We previously speculated that promoter context may play an important role in determining the effect of β_2-AR stimulation on NF-κB-dependent gene expression (Spooren et al. 2010). Using the Regulatory Sequence Analysis Tool (http://rsat.ulb.ac.be), a bioinformatics tool that is designed for detection of regulatory elements in non-coding sequences, we performed a search for genes that have NF-κB and CREB binding sites in close vicinity (<300 bp) in their proximal promoters (500 bp upstream to the transcription starting site). We searched for CRE and κB motifs identical to the ones present in the IL-6 promoter and this analysis recovered only the IL-6 gene. When more flexible settings were applied, searching for consensus κB and CRE sites or, in the latter case, also functional half-sites, we retrieved a total of 991 genes. However, except IL-6, there were no other genes in the hit list for which β_2-AR/NF-κB synergy was previously reported. This indicates that coexistence of binding motifs for NF-κB and CREB cannot explain all synergistic effects described in literature and probably gene-selective mechanisms are at play.

A major limitation of our study is the focus on a limited and biased selection of NF-κB target genes. It would be interesting to use an unbiased RNAseq analysis to verify these *in silico* predictions and reveal how β_2-AR/TNF-R coactivation globally affects gene expression patterns.

8. Association of enhanceosome architecture and global changes in gene expression patterns during β_2-AR/NF-κB crosstalk

Most studies investigating β_2-AR/NF-κB crosstalk published to date focused on a limited pool of NF-κB target genes (Chapter 2) (Kolmus et al. 2014). In addition, most experiments were performed in highly controlled *ex vivo* or *in vitro* conditions and their translation to *in vivo* situations remains to be tested. Therefore, we believe a more translational approach, applying state-of-the art genome-wide technologies (e.g. RNAseq, ChIP-seq) to relevant animal models of disease or primary human samples of patients undergoing β-(ant)agonist treatment would yield highly useful information, that could then be validated *in vitro*. For instance, a recent microarray study demonstrated that psychological stress, associated with cancer patient caregiving, mitigates glucocorticoid receptor gene transcription but promotes that driven by NF-κB (Miller et al. 2008). In line, a transcriptomics analysis of β_2-AR knockout mice brains after ischemia showed lower expression of NF-κB signalling components as well as its target genes when compared to wild type mice (White et al. 2012).

Furthermore, combining transcriptomics analysis with ChIP-seq to profile NF-κB, CREB, CBP and TEF-1 recruitment to regulatory regions could yield valuable information on how these transcriptional coregulators interact to regulate transcription. More specifically, it might provide an answer to the question whether "synergy susceptible genes" have a particular promoter signature. This strategy could perhaps lead to the identification of novel targets for the development of highly selective anti-inflammatory drugs, with a nuclear action at selected gene promoters.

9. Explaining the heterogeneous outcome of studies on β_2-AR/NF-κB crosstalk

A large number of reports showed that the outcome of β_2-ARs/NF-κB in the context of inflammatory gene expression can be bidirectional, either positive or negative. The dichotomy in the reported effects probably reflects the use of different model systems

and illustrates the importance of cell type- and stimulus-specific factors in determining the outcome of β_2-AR/NF-κB crosstalk. Interestingly, the variegated impact of β_2-agonists on NF-κB function also reflect a dose-dependent switch in signalling originating from the β_2-AR localized in different microdomains of the plasma membrane (Bruzzone et al. 2014). This fascinating area of regulation has only recently gained more attention. Overall, β_2-adrenergic signals indeed regulate NF-κB activity by various mechanisms, operating at the level of the plasma membrane, in the cytoplasm and in the nucleus, engaging diverse molecular switches, scaffolding proteins, signal transducers and transcriptional cofactors.

10. Universal patterns in β_2-AR/NF-κB crosstalk and their therapeutic relevance

Although no systematic analysis of β_2-AR/NF-κB crosstalk has been performed, based on the reports published to date several universal patterns can be identified (Table 1 and Table 2 in Chapter 2).

Firstly, β_2-AR triggering was recurrently observed to stimulate NF-κB activity in the absence of an inflammatory trigger in cell populations belonging to the immune and cardiovascular system. In line with these findings, several clinical reports showed that administration of β-antagonists to patients suffering from burn trauma could hold a therapeutic promise as elevated levels of stress catecholamines in these patients were associated with enhanced production of inflammatory mediators and detrimental effects on the cardiovascular system (Friese et al. 2008; Jeschke et al. 2011; Jeschke et al. 2007).

Secondly, all studies performed to date show that β_2-AR triggering inhibits NF-κB-driven expression of IL-2 in activated T cells. β_2-AR activation also represses the LPS-induced NF-κB-dependent pro-inflammatory program (IL-1β, IL-8 and TNF-α) and augments expression of anti-inflammatory factors (IL-1ra, IL-10 and IκBα) in cells belonging to the immune and central nervous system. Based on those observations, one can propose that β_2-AR stimulation could offer a therapeutic intervention against sepsis, a condition in which severe infection cause production of NF-κB-dependent inflammatory mediators that in turn activate a positive feedback loop (de Montmollin et al. 2009). Concomitantly, β_2-agonists also counteract expression of pro-inflammatory mediators suggesting their utility in the management of neurodegenerative disorders (McNamee et al. 2010; Ryan et al. 2013). On the other

hand, as there are also reports showing potentiation of inflammatory factors, such as IL-6, upon β_2-AR triggering (Laureys et al. 2014), it is necessary to carefully test administration of β_2-agonists in various animal models of inflammatory disease prior to considering their evaluation in patients.

Despite the fact that our observations (Kolmus et al. 2014), and those of others (Table 2 in Chapter 2), clearly indicate β-agonists trigger the release of selected proinflammatory mediators, β-agonists are mainstream therapy for respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD). If enhanced production of proinflammatory mediators would result from β-agonist treatment and have deleterious effects, one would expect that there would be reports of that. Epidemiological evidence indeed indicates that the use of long-acting beta-agonists (LABAs) as monotherapy for asthma is associated with increased risk for exacerbations and morbidity, and current asthma guidelines recommend the use of LABAs only in combination with glucocorticoids. A detailed analysis of inflammatory parameters in the lungs of patients experiencing deleterious effects of LABA treatment has to our knowledge, however, not been performed. Nevertheless, it was demonstrated in mice that chronic β_2-agonist treatment exacerbates lung inflammation (Lin et al. 2012). Also, an *in vitro* study on human airway epithelial cells indicated that β_2-agonists promote IL-1β-induced expression of IL-6 and glucocorticoid cotreatment inhibited this effect (Holden et al. 2010). In line, a recent study exploring the influence of air pollution on the expression of IL-6 showed that particular matter air pollution potentiates sympathetic tone, which via the cAMP/CREB-dependent pathway enhances NF-κB-mediated transcription of IL-6 in human and murine alveolar macrophages, resulting in a prothrombotic state and accelerated arterial thrombosis. Importantly, *in vitro* administration of β_2-agonists mimicked the effects of stress catecholamines on the particular matter-induced IL-6 expression (Chiarella et al. 2014).

Finally, we (Kolmus et al. 2014) and others (Table 2 in Chapter 2) observed that not only IL-6 but also several chemokines can be synergistically or additively upregulated in different cell types in response to combined situation with a proinflammatory stimulus and β_2-agonist. As these factors are known to affect cell migration, the synergistic upregulation of a selected pool of NF-κB-dependent cyto-/chemokines by β_2-agonists may have substantial physiological consequences. For instance, a recent

study demonstrated that intracerebroventricular coadministration of a β_2-agonist and TNF-α was associated with synergistic expression a subset of NF-κB target genes and modulated leukocyte infiltration in the brain, with a skewing of the T-cell population towards a double-negative phenotype, whilst promoting neutrophilic predominance in the myeloid subset (Laureys et al. 2014). Based on these observations, future studies should elucidate whether production of chemoattractants *in vivo* have relevance for the recruitment of immunocompetent cells and cells contributing to tissue regeneration in relevant models of inflammatory disorders or upon exercise. Importantly, recent evidence has also linked excessive adrenergic signalling to tumour-related biological processes, such as metastasis, and β_2-antagonists were proposed to hold therapeutic potential against tumour malignancies (Cole and Sood 2012; Powell et al. 2013). In this context, our finding that β_2-AR/TNF-R coactivation leads to potent upregulation of IL-6 as well as C-C and C-X-C motif chemokines, which are typical malignancy-promoting components of the tumour microenvironment, deserves further attention.

11. Selective modulation of NF-κB-dependent gene expression by means of biased agonists

Recently, the crystal structure of the human β_2-AR was solved and has provided molecular insights that will be key to the design of selective modulators of receptor signalling (Cherezov et al. 2007; Rosenbaum et al. 2007). In particular, biased ligands or nanobodies could become important therapeutic tools and could also provide opportunities for selective manipulation of NF-κB activity. For instance, as β_2-AR-dependent activation of β-arrestin represses NF-κB activity (Gao et al. 2004; Luan et al. 2005), one could envisage that selective stimulation of the β-arrestin pathway using biased ligands for the β_2-AR could deliver an anti-inflammatory effect by inhibiting NF-κB (Yang et al. 2003), without affecting G-protein dependent signalling, the outcome of which is currently less clear (Chandrasekar et al. 2004; Farmer and Pugin 2000). In line with this, the β_2-AR agonists S-albuterol and R,R-formoterol were shown to have differential effects on the concentration of inflammatory mediators in the bronchoalveolar lavage fluid in a mouse model of acute lung injury (Bosmann et al. 2012). Although no molecular explanation for the discrepant effects of both agonists was provided, one could envisage that they trigger

different activation states of the β_2-AR. Recently, a systematic screen was published in which an attempt was made to quantify ligand bias (towards cAMP generation, ERK MAPK activation, calcium signalling and receptor endocytosis) for 19 clinically relevant β-adrenergic ligands (van der Westhuizen et al. 2014). It would be highly relevant to extend this study and investigate also ligand bias towards effects on NF-κB activation.

12. Modulation of NF-κB-dependent gene expression at inflammatory gene promoters

Whilst biased ligands and nanobodies might hold promise for treating inflammation, they interfere with the initial steps of cytoplasmic NF-κB signalling and will probably trigger global inhibition of NF-κB, which has been associated with defects in immunity and an enhanced risk for infection (Powell et al. 2013). Enhanced selectivity could be accomplished if one could inhibit NF-κB at selected gene promoters, suppressing transcription of only a subset of NF-κB target genes, preferentially those with the most deleterious properties. Compounds that interfere with the interaction of transcription factors with their cofactors are already known. For instance, a small molecule, called chetomin, disrupts interaction of hypoxia-inducible factor with p300 offering a therapeutic window against tumours (Kung et al. 2004). As we and others have demonstrated that only selected NF-κB target genes, including IL-6 and several chemokines, are susceptible to β_2-AR-induced transcriptional synergy, a better understanding of the molecular basis of this synergy might identify novel, more selective, therapeutic targets.

13. Novel strategies to investigate β_2-AR/NF-κB crosstalk

In addition, several interesting technologies, such as enChIP (engineered DNA-binding molecule-mediated chromatin immunoprecipitation) coupled to mass spectrometry (Fujita and Fujii 2013) or FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) (Giresi et al. 2007), have recently emerged and could be used to gain a better insight into transcription factor recruitment and chromatin remodelling events associated with β_2-AR/TNF-R co-activation.

14. Summary of outstanding questions for further research

- What is the outcome of systemic administration of β_2-(ant)agonists in various models of inflammatory disorders and tumour?
- What is the signature of synergy susceptible promoters as compared to those that are repressed?
- What are the global changes in inflammatory gene expression profile upon β_2-AR coactivation?
- Does targeting of TEF-1 offer a novel selective approach to combat inflammatory disorders with uncontrolled IL-6 expression?
- Does β_2-AR coactivation enhancer expression of chemotactic factors *in vivo* to modulate cell migration in various disease models and upon exercise?

15. Overall conclusion

Taken together, we believe that our findings warrant caution in the use of β_2-agonists in any therapeutic disorder and urge for further study into their immunomodulatory properties.

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Supplementary information

Supplementary data for the paper entitled:

"β-agonists selectively modulate proinflammatory gene expression in skeletal muscle cells via non-canonical nuclear crosstalk mechanisms".

Figure S1.

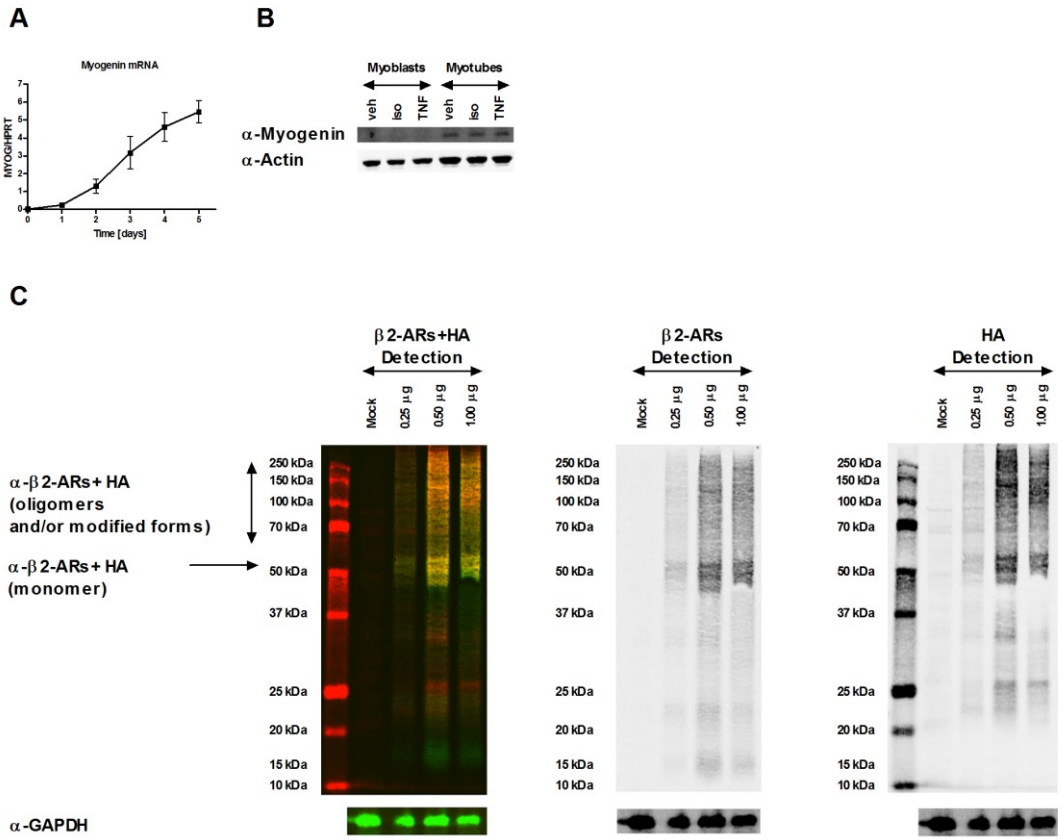


Figure S2.

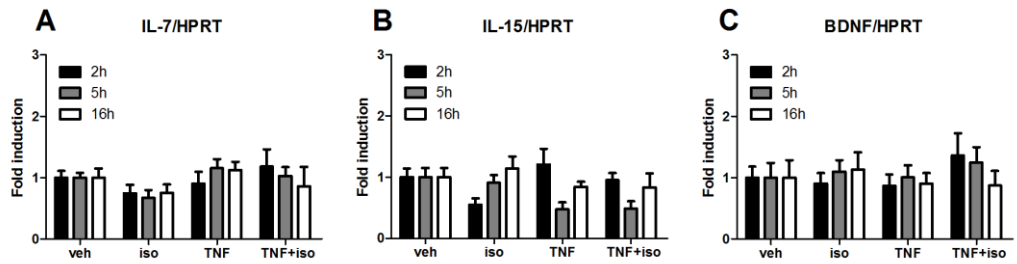
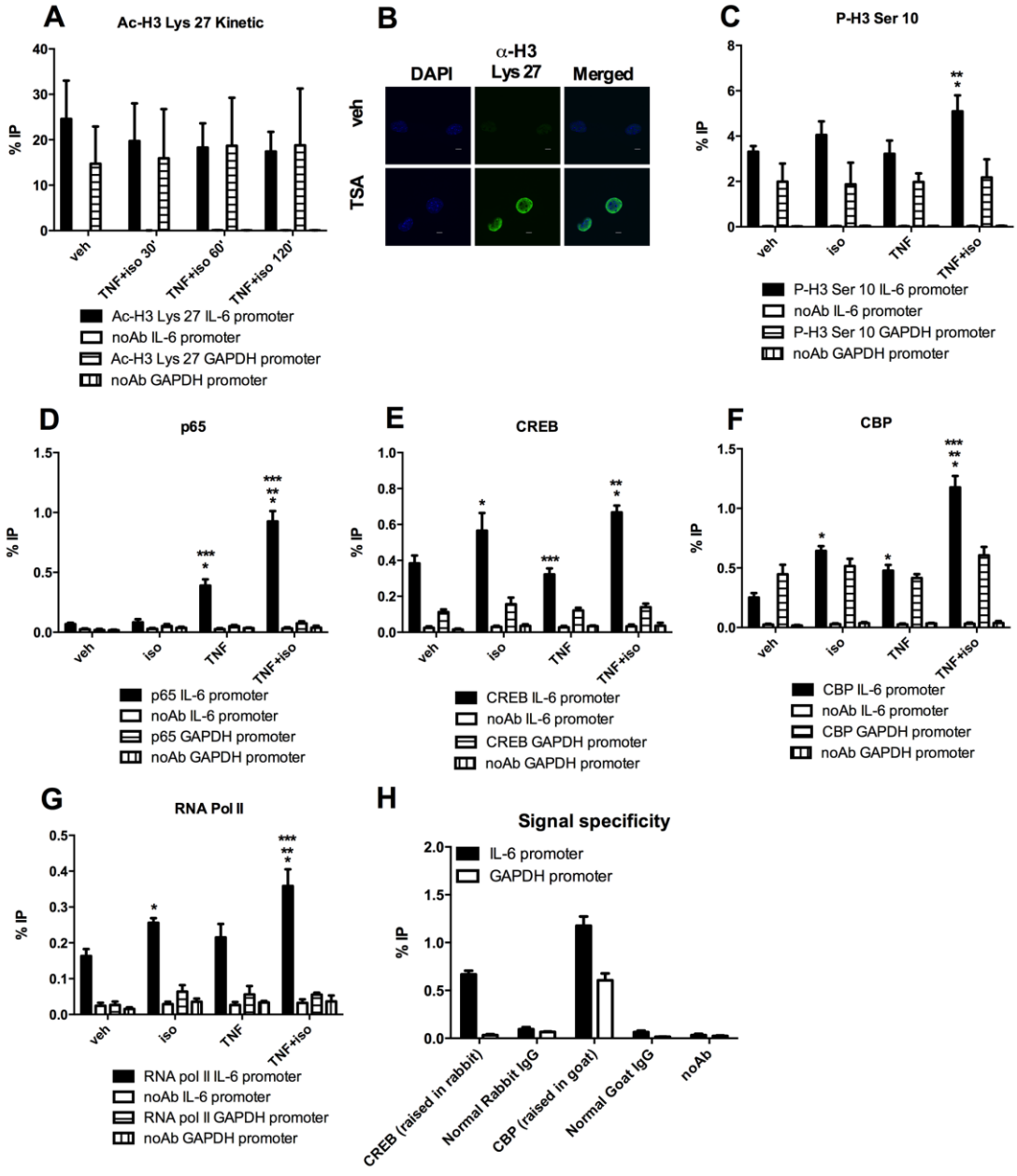


Figure S3.


A
Ac-H3 Lys 27 Kinetic
% IP
veh
TNF+iso 30'
TNF+iso 60'
TNF+iso 120'
Ac-H3 Lys 27 IL-6 promoter
noAb IL-6 promoter
Ac-H3 Lys 27 GAPDH promoter
noAb GAPDH promoter
B
α-H3 Lys 27
DAPI
Merged
veh
TSA
C
P-H3 Ser 10
% IP
veh
iso
TNF
TNF+iso
P-H3 Ser 10 IL-6 promoter
noAb IL-6 promoter
P-H3 Ser 10 GAPDH promoter
noAb GAPDH promoter
D
p65
% IP
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noAb IL-6 promoter
RNA pol II GAPDH promoter
noAb GAPDH promoter
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Signal specificity
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GAPDH promoter
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Normal Goat IgG
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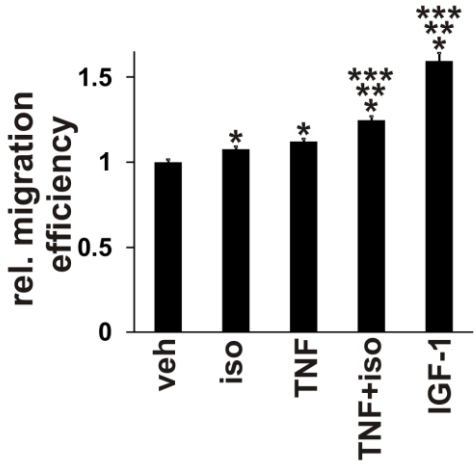
Figure S4.



p-value	IGF	iso	TNF	TNF_iso
iso	6.70E-13	-	-	-
TNF	9.70E-12	6.85E-02	-	-
TNF_iso	2.50E-07	5.60E-07	6.10E-05	-
veh	6.70E-13	5.20E-03	1.70E-05	1.20E-09

Table S1. Summary of primer sequences used in the present study.

Name			Sequence	
Hypoxanthine guanine phosphoribosyl transferase (HPRT)	fw	5'	CCTAAGATGAGCGCAAGTTGAA	3'
	rv	5'	CCACAGGACTAGAACACCTGCTAA	3'
Interleukin-6 (IL-6)	fw	5'	AGTCCTTCCTACCCCAATTTCC	3'
	rv	5'	TTGGTCCTTAGCCACTCCTTC	3'
Interleukin-7 (IL-7)	fw	5'	GTGCTGCTCGCAAGTTGAAG	3'
	rv	5'	AGTTCACCAGTGTTTGTGTGC	3'
Interleukin-15 (IL-15)	fw	5'	CATCCATCTCGTGCTACTTGTG	3'
	rv	5'	GCCTCTGTTTTAGGGAGACCT	3'
Brain-derived neurotrophic factor (BDNF)	fw	5'	TCATACTTCGGTTGCATGAAGG	3'
	rv	5'	AGACCTCTCGAACCTGCCC	3'
Chemokine (C-C motif) ligand 2 (CCL2)	fw	5'	TTAAAAACCTGGATCGGAACCAA	3'
	rv	5'	GCATTAGCTTCAGATTTACGGGT	3'
Chemokine (C-C motif) ligand 5 (CCL5)	fw	5'	TTTGCCTACCTCTCCCTCG	3'
	rv	5'	CGACTGCAAGATTGGAGCACT	3'
Chemokine (C-X-C motif) ligand 5 (CXCL5)	fw	5'	GTGTTTGCTTAACCGTAACTCCA	3'
	rv	5'	CTTCCACCGTAGGGCACTG	3'
Intercellular adhesion molecule1 (ICAM-1)	fw	5'	CCGCAGGTCCAATTCACACT	3'
	rv	5'	TCCAGCCGAGGACCATACAG	3'
Nuclear factor of kappa B inhibitor α (IκBα)	fw	5'	TGAAGGACGAGGAGTACGAGC	3'
	rv	5'	TTCGTGGATGATTGCCAAGTG	3'
Myogenin (MYOG)	fw	5'	GGGCAATGCACTGGAGTTCG	3'
	rv	5'	CAGATTGTGGGCGTCTGTAG	3'
IL-6 NheI, CHART-PCR	fw	5'	CGTGCATGACTTCAGCTTTAC	3'
	rv	5'	TGCAGCTTAGGTCGTCATTG	3'

IL-6 AatII, CHART-PCR, ChIP	fw	5'	GCCTCAAGGATGACTTAAGC	3'
	rv	5'	TGTGACGTCGTTTAGCATCG	3'
IL-6 aspecific region, CHART-PCR	fw	5'	ACCGCTATGAAGTTCCTCTC	3'
	rv	5'	AACCCACAATGCTGGCTCTC	3'
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ChIP	fw	5'	GATGCAGGGATGATGTTC	3'
	rv	5'	TGCACCACCAACTGCTTAG	3'

Table S2. Summary of the position and sequence of NF-κB binding sites in the proximal promoters of selected genes.

Name	Position	Sequence		
Interleukin-6 (IL-6)	-91	5'	GGGATTTTCC	3'
Interleukin-7 (IL-7)	-24	5'	TCAGATCCCC	3'
Interleukin-15 (IL-15)	-243	5'	CGCCTTGTTT	3'
Brain-derived neurotrophic factor (BDNF)	-117	5'	AGAAGTTTCC	3'
Chemokine (C-C motif) ligand 2 (CCL2)	-152	5'	GGAAACACCCG	3'
Chemokine (C-C motif) ligand 5 (CCL5)	-90	5'	GGAAACTCCC	3'
Chemokine (C-X-C motif) ligand 5 (CXCL5)	-29	5'	GGGAATTTCC	3'
Intercellular adhesion molecule1 (ICAM-1)	-174	5'	TGGAAATTCC	3'
Nuclear factor of kappa B inhibitor α (IκBα)	-29	5'	GGAAATTCCC	3'

Bioinformatics analysis of promoters was performed using P-Scan. A region of 500 bp upstream to the transcription starting site of the selected promoters was investigated with the Transcription Factor Binding Sites matrices from TRANSFAC databases.

Supplementary data for the paper entitled:

"A proteomics strategy identifies TEF-1 as a regulator of IL-6 transcription in astrocytes"

Supplementary Figure 1.

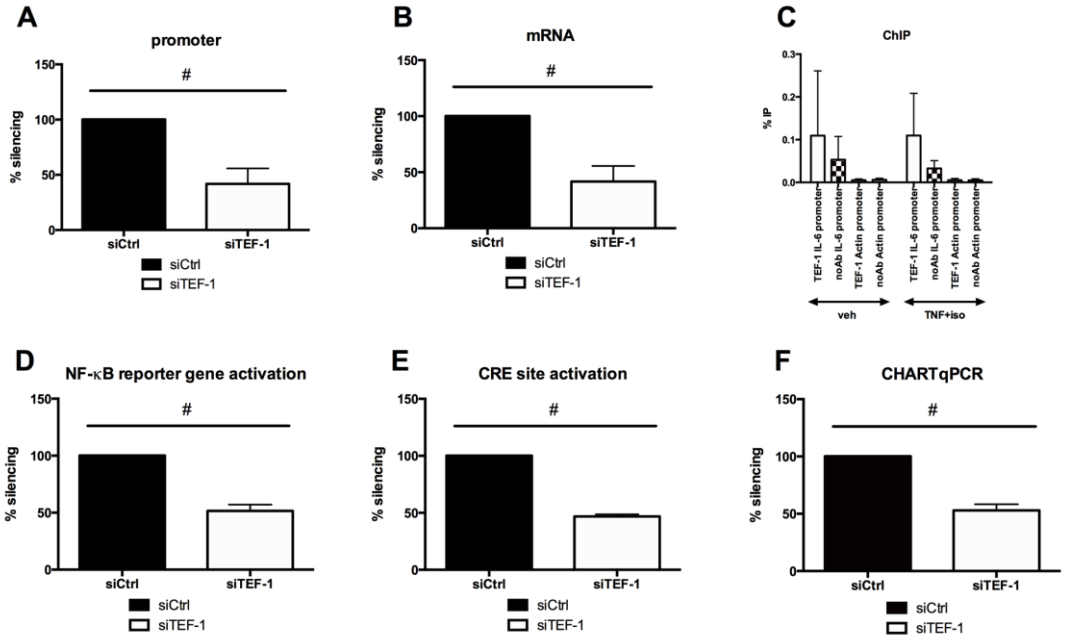


Figure S1. Control of siRNA-mediated TEF-1 silencing. (A, B, D, E, F) Level of TEF-1 mRNA levels was measured via RT-qPCR in untreated 1321N1 cells transfected either with siRNA targeting TEF-1 or control siRNA. Data are presented as average ± SD of independent experiments. (C) Control of ChIP assay gene specificity. Control ChIP experiments showing specificity of the observed responses for the IL-6 promoter. ChIP samples from the experiment shown in Figure 2C were reanalysed using primers amplifying the β-actin gene promoter. Data are presented as average of technical replicates ± SD of three independent experiments. (#) Statistically different between siCtrl and siTEF-1 condition.

Supplementary Table 1. Supplementary Table 1. Summary of primer sequences used in the present study.

Name	Sequence			
Hypoxanthine guanine phosphoribosyl transferase (HPRT)	fw	5'	TGACACTGGCAAAACAATGCA	3'
	rv	5'	GGTCCTTTTCACCAGCAAGCT	3'
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	fw	5'	TGCACCACCAACTGCTTAGC	3'
	rv	5'	GGCATGGACTGTGGTCATGAG	3'
Interleukin-6 (IL-7)	fw	5'	GACAGCCACTCACCTCTTCA	3'
	rv	5'	AGTGCCTCTTTGCTGCTTTC	3'
Interleukin-8 (IL-8)	fw	5'	GCTCTCTTGGCAGCCTTCCTGA	3'
	rv	5'	ACAATAATTTCTGTGGCGC	3'
Chemokine (C-X-C motif) ligand 2 (CXCL2)	fw	5'	CCCATGGTTAAGAAAATCATCG	3'
	rv	5'	CTTCAGGAACAGCCACCAAT	3'
Cyclooxygenase 2 (COX-2)	fw	5'	GCCCTTCCTCCTGTGCC	3'
	rv	5'	AATCAGGAAGCTGCTTTTTACCTTT	3'
Intercellular adhesion molecule 1 (ICAM-1)	fw	5'	GCAGACAGTGACCATCTACAGCTT	3'
	rv	5'	CTTCTGAGACCTCTGGCTTCGT	3'
Desthiobiotin IL-6 DNA bait	fw	5'	TCGTGCATGACTTCAG	3'
	rv	5'	CTTCGTGCATGACTTCAG	3'
TEG-biotin IL-6 DNA bait	fw	5'	cccggttctagaGCATGACTTCAGCTTTAC	3'
	rv	5'	CTTCGTGCATGACTTCAG	3'
IL-6 ChIP, CHART-PCR κB site	fw	5'	ACCCTCACCCTCCAACAAAG	3'
	rv	5'	CAGAATGAGCCTCAGACATC	3'
IL-6 ChIP, CHART-PCR CRE site	fw	5'	GGGCTGATTGGAAACC	3'
	rv	5'	CACCGGGAACGAAAGAGAAG	3'
β-actin (Actin) ChIP	fw	5'	TCCACCTTCCAGCAGATGTG	3'
	rv	5'	GCAACTAAGTCATAGTCCGCCTAGA	3'
Transcription Enhancer Factor-1 (TEF-1)	fw	5'	GCTAAAGGATCAGACTGCAAAGG	3'
	rv	5'	TTATGAATGGCAGTGGCCGA	3'
Transcription Enhancer Factor-1 (TEF-1) mutagenesis primers	fw	5'	GCTTGGAATGAAAATCTCG AGCTTTCCTTCTGGCAAGAACC	3'
	rv	5'	GGTTCTTGCCAGAAGGAAA GCTCGAGATTTTCATTCCAAGC	3'

Supplementary Table 2. List of all proteins identified in the different AP-MS experiments.

Exp 1	TNF+iso	Method A	
Accession	Description	#Validated Peptides	emPAI
Q16531	DNA damage-binding protein 1 (DDB1)	68	4,28937
Q13619	Cullin-4A (CUL4A)	25	0,73020
Q13620	Cullin-4B (CUL4B)	30	0,78690
Q92466	DNA damage-binding protein 2 (DDB2)	25	2,16228
P04264	Keratin, type II cytoskeletal 1 (K2C1)	9	0,37550
P35908	Keratin, type II cytoskeletal 2 epidermal (K22E)	6	0,23682
P02538	Keratin, type II cytoskeletal 6A (K2C6A)	7	0,27662
P04259	Keratin, type II cytoskeletal 6B (K2C6B)	7	0,27662
P17275	Transcription factor jun-B (JUNB)	7	0,56475
P13645	Keratin, type I cytoskeletal 10 (K1C10)	6	0,34171
P08779	Keratin, type I cytoskeletal 16 (K1C16)	7	0,39905
P08651	Nuclear factor 1 C-type (NFIC)	3	0,13921
P28347	Transcriptional enhancer factor TEF-1 (TEAD1)	2	0,10294
Q14938	Nuclear factor 1 X-type (NFIX)	3	0,15478
P02533	Keratin, type I cytoskeletal 14 (K1C14)	5	0,23764
P02768	Serum albumin (ALBU)	2	0,05777
P15407	Fos-related antigen 1 (FOSL1)	4	0,40653
P27695	DNA-(apurinic or apyrimidinic site) lyase (APEX1)	2	0,11034
P17535	Transcription factor jun-D (JUND)	2	0,16016
O14867	Transcription regulator protein BACH1 (BACH1)	5	0,15478
P15408	Fos-related antigen 2 (FOSL2)	3	0,20526
P05412	Transcription factor AP-1 (JUN)	2	0,16016
P17676	CCAAT/enhancer-binding protein beta (CEBPB)	3	0,21153
P35527	Keratin, type I cytoskeletal 9 (K1C9)	2	0,09648
Q99594	Transcriptional enhancer factor TEF-5 (TEAD3)	1	0,04914
Q15699	ALX homeobox protein 1 (ALX1)	3	0,18850
Q04206	Transcription factor p65 (RELA)	2	0,10294
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	2	0,03056
P02545	Prelamin-A/C (LMNA)	1	0,02239
P21802	Fibroblast growth factor receptor 2 (FGFR2)	1	0,02883
P19838	Nuclear factor NF-kappa-B p105 subunit (NFKB1)	2	0,05311
Q00653	Nuclear factor NF-kappa-B p100 subunit (NFKB2)	1	0,02682
O15525	Transcription factor MafG (MAFG)	1	0,07711
P02656	Apolipoprotein C-III (APOC3)	1	0,29155
Q7L2Z9	Centromere protein Q (CENPQ)	1	0,05133
O75531	Barrier-to-autointegration factor (BAF)	1	0,19378
O95497	Pantetheinase (VNN1)	1	0,07007
Q99958	Forkhead box protein C2 (FOXC2)	1	0,05777

Exp 1	veh	Method A	
Accession	Description	#Validated Peptides	emPAI
Q16531	DNA damage-binding protein 1 (DDB1)	65	3,91461
Q92466	DNA damage-binding protein 2 (DDB2)	26	2,31131
Q13619	Cullin-4A (CUL4A)	23	0,65595
Q13620	Cullin-4B (CUL4B)	21	0,50131
P17275	Transcription factor jun-B (JUNB)	6	0,46780

P04264	Keratin, type II cytoskeletal 1 (K2C1)	6	0,23682
P13645	Keratin, type I cytoskeletal 10 (K1C10)	4	0,21648
P15408	Fos-related antigen 2 (FOSL2)	4	0,28265
P02768	Serum albumin (ALBU)	4	0,11887
O14867	Transcription regulator protein BACH1 (BACH1)	1	0,02920
P15407	Fos-related antigen 1 (FOSL1)	2	0,18597

Exp 2	TNF+iso	Method A	
Accession	Description	#Validated Peptides	emPAI
Q16531	DNA damage-binding protein 1 (DDB1)	5	0,13029
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	9	0,14505
Q92466	DNA damage-binding protein 2 (DDB2)	4	0,20226
P04264	Keratin, type II cytoskeletal 1 (K2C1)	4	0,15223
P35908	Keratin, type II cytoskeletal 2 epidermal (K22E)	3	0,11213
P13645	Keratin, type I cytoskeletal 10 (K1C10)	3	0,15832
P02768	Serum albumin (ALBU)	2	0,05777

Exp 2	veh	Method A	
Accession	Description	#Validated Peptides	emPAI
Q16531	DNA damage-binding protein 1 (DDB1)	4	0,10294
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	3	0,04618
Q92466	DNA damage-binding protein 2 (DDB2)	2	0,09648
P04264	Keratin, type II cytoskeletal 1 (K2C1)	5	0,19378
P35527	Keratin, type I cytoskeletal 9 (K1C9)	1	0,04713
P13645	Keratin, type I cytoskeletal 10 (K1C10)	1	0,05021
Q13619	Cullin-4A (CUL4A)	1	0,02217
P17275	Transcription factor jun-B (JUNB)	2	0,13646
P35908	Keratin, type II cytoskeletal 2 epidermal (K22E)	1	0,03606
P02768	Serum albumin (ALBU)	2	0,05777
P05412	Transcription factor AP-1 (JUN)	1	0,07711
P04259	Keratin, type II cytoskeletal 6B (K2C6B)	1	0,03550

Exp 1	TNF+iso	Method B	
Accession	Description	#Validated Peptides	emPAI
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	73	2,00002
Q16531	DNA damage-binding protein 1 (DDB1)	47	2,16228
P12956	X-ray repair cross-complementing protein 6 (XRCC6)	42	2,07873
P13010	X-ray repair cross-complementing protein 5 (XRCC5)	42	1,96422
P02545	Prelamin-A/C (LMNA)	27	0,81809
P02751	Fibronectin (FINC)	23	0,34652
P39880	Homeobox protein cut-like 1 (CUX1)	20	0,31324
Q92466	DNA damage-binding protein 2 (DDB2)	21	1,63027
P08670	Vimentin (VIME)	20	1,10175
P46063	ATP-dependent DNA helicase Q1 (RECQ1)	22	0,89881
P49916	DNA ligase 3 (DNLI3)	15	0,26689
Q5T5X7	BEN domain-containing protein 3 (BEND3)	17	0,46234
P17275	Transcription factor jun-B (JUNB)	16	1,78256
Q13619	Cullin-4A (CUL4A)	14	0,35936
Q14764	Major vault protein (MVP)	15	0,39390
P61978	Heterogeneous nuclear ribonucleoprotein K (HNRPK)	15	0,91875

P18887	DNA repair protein XRCC1 (XRCC1)	13	0,49857
Q00059	Transcription factor A, mitochondrial (TFAM)	11	0,67683
P04406	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	10	0,93070
Q04206	Transcription factor p65 (RELA)	10	0,63217
P17676	CCAAT/enhancer-binding protein beta (CEBPB)	10	0,89574
P20700	Lamin-B1 (LMNB1)	9	0,26896
Q8IVF2	Protein AHNAK2 (AHNK2)	7	0,02248
P02768	Serum albumin (ALBU)	9	0,28753
P27695	DNA-(apurinic or apyrimidinic site) lyase (APEX1)	9	0,60157
P15408	Fos-related antigen 2 (FOSL2)	8	0,64519
P68104	Elongation factor 1-alpha 1 (EF1A1)	9	0,42083
Q09666	Neuroblast differentiation-associated protein AHNAK (AHNK)	6	0,01616
P11142	Heat shock cognate 71 kDa protein (HSP7C)	8	0,25535
P19838	Nuclear factor NF-kappa-B p105 subunit (NFKB1)	8	0,22995
P09429	High mobility group protein B1 (HMGB1)	8	0,47983
P09651	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	8	0,58489
P28347	Transcriptional enhancer factor TEF-1 (TEAD1)	8	0,47983
O15527	N-glycosylase/DNA lyase (OGG1)	6	0,45265
P60709	Actin, cytoplasmic 1 (ACTB)	8	0,66810
Q13620	Cullin-4B (CUL4B)	6	0,12310
P02452	Collagen alpha-1(I) chain (CO1A1)	5	0,10069
Q14204	Cytoplasmic dynein 1 heavy chain 1 (DYHC1)	5	0,02059
P17535	Transcription factor jun-D (JUND)	5	0,44974
P04075	Fructose-bisphosphate aldolase A (ALDOA)	5	0,35388
P14866	Heterogeneous nuclear ribonucleoprotein L (HNRPL)	6	0,25418
Q96AE4	Far upstream element-binding protein 1 (FUBP1)	5	0,24262
P05204	Non-histone chromosomal protein HMG-17 (HMGN2)	5	0,68761
P15407	Fos-related antigen 1 (FOSL1)	5	0,53174
P16220	Cyclic AMP-responsive element-binding protein 1 (CREB1)	5	0,46780
Q14938	Nuclear factor 1 X-type (NFIX)	4	0,21153
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	5	0,31537
Q00653	Nuclear factor NF-kappa-B p100 subunit (NFKB2)	5	0,14149
P26583	High mobility group protein B2 (HMGB2)	4	0,22713
P35579	Myosin-9 (MYH9)	4	0,02866
P07355	Annexin A2 (ANXA2)	3	0,14207
O15525	Transcription factor MafG (MAFG)	4	0,34596
P22415	Upstream stimulatory factor 1 (USF1)	3	0,25893
P26599	Polypyrimidine tract-binding protein 1 (PTBP1)	3	0,15478
Q06330	Recombining binding protein suppressor of hairless (SUH)	3	0,13921
Q15853	Upstream stimulatory factor 2 (USF2)	3	0,25893
Q9Y2S6	Translation machinery-associated protein 7 (TMA7)	2	0,27427
O14867	Transcription regulator protein BACH1 (BACH1)	3	0,09018
P05412	Transcription factor AP-1 (JUN)	2	0,16016
P08123	Collagen alpha-2(I) chain (CO1A2)	1	0,02040
P09382	Galectin-1 (LEG1)	3	0,77828
P17544	Cyclic AMP-dependent transcription factor ATF-7 (ATF7)	3	0,15832
P21333	Filamin-A (FLNA)	1	0,00964
P31943	Heterogeneous nuclear ribonucleoprotein H (HNRH1)	3	0,16999
Q14103	Heterogeneous nuclear ribonucleoprotein D0 (HNRPD)	3	0,16591
P53567	CCAAT/enhancer-binding protein gamma (CEBPG)	2	0,22168
P62987	Ubiquitin-60S ribosomal protein L40 (RL40)	2	0,20226
P06733	Alpha-enolase (ENOA)	2	0,08902

P17096	High mobility group protein HMG-I/HMG-Y (HMGA1)	2	0,23285
P35527	Keratin, type I cytoskeletal 9 (K1C9)	2	0,09648
P35637	RNA-binding protein FUS (FUS)	2	0,09854
P51991	Heterogeneous nuclear ribonucleoprotein A3 (ROA3)	2	0,10530
P52926	High mobility group protein HMGI-C (HMGA2)	2	0,27427
P61956	Small ubiquitin-related modifier 2 (SUMO2)	2	0,46780
P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	1	0,12202
Q13409	Cytoplasmic dynein 1 intermediate chain 2 (DC1I2)	2	0,07115
Q15365	Poly(rC)-binding protein 1 (PCBP1)	2	0,17210
Q92945	Far upstream element-binding protein 2 (FUBP2)	2	0,07978
Q96T60	Bifunctional polynucleotide phosphatase/kinase (PNKP)	1	0,03912
P05114	Non-histone chromosomal protein HMG-14 (HMGN1)	1	0,10069
P06748	Nucleophosmin (NPM)	1	0,06082
P16949	Stathmin (STMN1)	1	0,07461
P17844	Probable ATP-dependent RNA helicase DDX5 (DDX5)	1	0,02813
P23246	Splicing factor, proline- and glutamine-rich (SFPQ)	1	0,02746
P23284	Peptidyl-prolyl cis-trans isomerase B (PPIB)	1	0,07227
P55145	Mesencephalic astrocyte-derived neurotrophic factor (MANF)	1	0,07461
P60903	Protein S100-A10 (S10AA)	1	0,19378
P63167	Dynein light chain 1, cytoplasmic (DYL1)	1	0,21153
P68363	Tubulin alpha-1B chain (TBA1B)	1	0,06082
Q15233	Non-POU domain-containing octamer-binding protein (NONO)	1	0,03393
Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3 (SH3L3)	1	0,25893
Q9P016	Thymocyte nuclear protein 1 (THYN1)	1	0,06605
P19338	Nucleolin (NUCL)	2	0,04160
P63167	Dynein light chain 1, cytoplasmic (DYL1)	3	0,77828

Exp 1	veh	Method B	
Accession	Description	#Validated Peptides	emPAI
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	81	2,38386
Q16531	DNA damage-binding protein 1 (DDB1)	60	3,34808
P13010	X-ray repair cross-complementing protein 5 (XRCC5)	57	3,36968
P12956	X-ray repair cross-complementing protein 6 (XRCC6)	48	2,61526
P08670	Vimentin (VIME)	40	3,41734
P21333	Filamin-A (FLNA)	31	0,34638
P02545	Prelamin-A/C (LMNA)	30	0,94295
Q92466	DNA damage-binding protein 2 (DDB2)	29	2,80189
P02751	Fibronectin (FINC)	25	0,38181
P39880	Homeobox protein cut-like 1 (CUX1)	24	0,38679
P35579	Myosin-9 (MYH9)	21	0,15989
Q13619	Cullin-4A (CUL4A)	17	0,45179
P49916	DNA ligase 3 (DNLI3)	17	0,30749
P17275	Transcription factor jun-B (JUNB)	15	1,61016
P04075	Fructose-bisphosphate aldolase A (ALDOA)	15	1,48163
P46063	ATP-dependent DNA helicase Q1 (RECQ1)	17	0,64131
P60709	Actin, cytoplasmic 1 (ACTB)	15	1,61016
Q00059	Transcription factor A, mitochondrial (TFAM)	13	0,84207
P04406	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	15	1,68270
P06733	Alpha-enolase (ENOA)	12	0,66810
P61978	Heterogeneous nuclear ribonucleoprotein K (HNRPK)	14	0,83718
P02768	Serum albumin (ALBU)	13	0,44058

P68104	Elongation factor 1-alpha 1 (EF1A1)	10	0,47738
P07355	Annexin A2 (ANXA2)	10	0,55707
P18887	DNA repair protein XRCC1 (XRCC1)	12	0,45265
Q14764	Major vault protein (MVP)	12	0,30432
P15408	Fos-related antigen 2 (FOSL2)	9	0,75083
P62736	Actin, aortic smooth muscle (ACTA)	10	0,89574
P09651	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	10	0,77828
P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	10	2,16228
P11142	Heat shock cognate 71 kDa protein (HSP7C)	9	0,29155
Q09666	Neuroblast differentiation-associated protein AHNAK (AHNK)	8	0,02160
P17676	CCAAT/enhancer-binding protein beta (CEBPB)	9	0,77828
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	7	0,46780
Q5T5X7	BEN domain-containing protein 3 (BEND3)	8	0,19583
P27695	DNA-(apurinic or apyrimidinic site) lyase (APEX1)	7	0,44242
P20700	Lamin-B1 (LMNB1)	8	0,23582
P26038	Moesin (MOES)	6	0,14658
Q13620	Cullin-4B (CUL4B)	6	0,12310
Q14103	Heterogeneous nuclear ribonucleoprotein D0 (HNRPD)	7	0,43072
Q16658	Fascin (FSCN1)	7	0,32035
Q96AE4	Far upstream element-binding protein 1 (FUBP1)	6	0,29780
Q99594	Transcriptional enhancer factor TEF-5 (TEAD3)	6	0,33352
P17535	Transcription factor jun-D (JUND)	4	0,34596
Q8IVF2	Protein AHNAK2 (AHNK2)	4	0,01278
P09382	Galectin-1 (LEG1)	6	2,16228
P05204	Non-histone chromosomal protein HMG-17 (HMGN2)	6	0,87382
P16220	Cyclic AMP-responsive element-binding protein 1 (CREB1)	6	0,58489
P28347	Transcriptional enhancer factor TEF-1 (TEAD1)	6	0,34171
O15527	N-glycosylase/DNA lyase (OGG1)	5	0,36501
P07900	Heat shock protein HSP 90-alpha (HS90A)	5	0,11249
P09429	High mobility group protein B1 (HMGB1)	6	0,34171
P13639	Elongation factor 2 (EF2)	6	0,14207
P15407	Fos-related antigen 1 (FOSL1)	4	0,40653
P16949	Stathmin (STMN1)	5	0,43301
P02452	Collagen alpha-1(I) chain (CO1A1)	4	0,07978
P11021	78 kDa glucose-regulated protein (GRP78)	4	0,11304
P14866	Heterogeneous nuclear ribonucleoprotein L (HNRPL)	5	0,20772
P62826	GTP-binding nuclear protein Ran (RAN)	4	0,42510
Q13263	Transcription intermediary factor 1-beta (TIF1B)	4	0,12365
Q14204	Cytoplasmic dynein 1 heavy chain 1 (DYHC1)	2	0,00818
P60174	Triosephosphate isomerase (TPIS)	3	0,25893
Q14938	Nuclear factor 1 X-type (NFIX)	4	0,21153
O14867	Transcription regulator protein BACH1 (BACH1)	4	0,12202
O15525	Transcription factor MafG (MAFG)	3	0,24961
P04264	Keratin, type II cytoskeletal 1 (K2C1)	3	0,11213
P08107	Heat shock 70 kDa protein 1A/1B (HSP71)	4	0,12202
P08238	Heat shock protein HSP 90-beta (HS90B)	3	0,06800
P14618	Pyruvate kinase PKM (KPYM)	4	0,16298
P18846	Cyclic AMP-dependent transcription factor ATF-1 (ATF1)	2	0,17877
P19338	Nucleolin (NUCL)	3	0,06304
P20810	Calpastatin (ICAL)	4	0,09750
P22415	Upstream stimulatory factor 1 (USF1)	3	0,25893
P26599	Polypyrimidine tract-binding protein 1 (PTBP1)	3	0,15478

P31943	Heterogeneous nuclear ribonucleoprotein H (HNRH1)	4	0,23285
P60660	Myosin light polypeptide 6 (MYL6)	4	0,93070
P78527	DNA-dependent protein kinase catalytic subunit (PRKDC)	1	0,00482
Q15233	Non-POU domain-containing octamer-binding protein (NONO)	4	0,14280
Q15853	Upstream stimulatory factor 2 (USF2)	3	0,25893
Q92945	Far upstream element-binding protein 2 (FUBP2)	2	0,07978
P31946	14-3-3 protein beta/alpha (1433B)	3	0,25893
P04083	Annexin A1 (ANXA1)	3	0,15140
P07437	Tubulin beta chain (TBB5)	3	0,21153
P15311	Ezrin (EZRI)	3	0,07227
P17096	High mobility group protein HMG-I/HMG-Y (HMGA1)	3	0,36887
P17544	Cyclic AMP-dependent transcription factor ATF-7 (ATF7)	3	0,15832
P23284	Peptidyl-prolyl cis-trans isomerase B (PPIB)	3	0,23285
P26583	High mobility group protein B2 (HMGB2)	2	0,10776
P27797	Calreticulin (CALR)	2	0,11034
P40926	Malate dehydrogenase, mitochondrial (MDHM)	3	0,23285
P63167	Dynein light chain 1, cytoplasmic (DYL1)	3	0,77828
P63241	Eukaryotic translation initiation factor 5A-1 (IF5A1)	3	0,53993
P68363	Tubulin alpha-1B chain (TBA1B)	3	0,19378
P68363	Tubulin alpha-1B chain (TBA1B)	3	0,19378
P80723	Brain acid soluble protein 1 (BASP1)	3	0,26896
Q13409	Cytoplasmic dynein 1 intermediate chain 2 (DC1I2)	3	0,10860
Q15365	Poly(rC)-binding protein 1 (PCBP1)	3	0,26896
Q15366	Poly(rC)-binding protein 2 (PCBP2)	3	0,27980
P53567	CCAAT/enhancer-binding protein gamma (CEBPG)	2	0,22168
P62987	Ubiquitin-60S ribosomal protein L40 (RL40)	2	0,20226
Q07666	KH domain-containing, RNA-binding, signal transduction-associated protein 1 (KHDR1)	1	0,04197
P00558	Phosphoglycerate kinase 1 (PGK1)	2	0,09450
P23246	Splicing factor, proline- and glutamine-rich (SFPQ)	2	0,05567
P23528	Cofilin-1 (COF1)	2	0,17877
P27816	Microtubule-associated protein 4 (MAP4)	1	0,02022
P30050	60S ribosomal protein L12 (RL12)	1	0,09648
P36578	60S ribosomal protein L4 (RL4)	1	0,02591
P50454	Serpin H1 (SERPH)	2	0,09260
P52926	High mobility group protein HMGI-C (HMGA2)	2	0,27427
P61313	60S ribosomal protein L15 (RL15)	1	0,05372
P61956	Small ubiquitin-related modifier 2 (SUMO2)	2	0,46780
P62917	60S ribosomal protein L8 (RL8)	2	0,09450
Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3 (SH3L3)	2	0,58489
Q9Y2S6	Translation machinery-associated protein 7 (TMA7)	2	0,27427
P63313	Thymosin beta-10 (TYB10)	1	0,33352
O00148	ATP-dependent RNA helicase DDX39A (DX39A)	1	0,04618
P38159	RNA-binding motif protein, X chromosome (RBMX)	1	0,03444
P00338	L-lactate dehydrogenase A chain (LDHA)	1	0,06082
P02795	Metallothionein-2 (MT2)	1	0,33352
P05114	Non-histone chromosomal protein HMG-14 (HMGN1)	1	0,10069
P05386	60S acidic ribosomal protein P1 (RLA1)	1	0,38950
P06703	Protein S100-A6 (S10A6)	1	0,21153
P06748	Nucleophosmin (NPM)	1	0,06082
P08123	Collagen alpha-2(I) chain (CO1A2)	1	0,02040
P10412	Histone H1,4 (H14)	1	0,04197

P10599	Thioredoxin (THIO)	1	0,23285
P10809	60 kDa heat shock protein, mitochondrial (CH60)	1	0,03250
P18858	DNA ligase 1 (DNLI1)	1	0,01954
P29034	Protein S100-A2 (S10A2)	1	0,29155
P35527	Keratin, type I cytoskeletal 9 (K1C9)	1	0,04713
P35637	RNA-binding protein FUS (FUS)	1	0,04811
P37802	Transgelin-2 (TAGL2)	1	0,11034
P51858	Hepatoma-derived growth factor (HDGF)	1	0,07007
P51991	Heterogeneous nuclear ribonucleoprotein A3 (ROA3)	1	0,05133
P55145	Mesencephalic astrocyte-derived neurotrophic factor (MANF)	1	0,07461
P59998	Actin-related protein 2/3 complex subunit 4 (ARPC4)	1	0,11034
P60903	Protein S100-A10 (S10AA)	1	0,19378
P62328	Thymosin beta-4 (TYB4)	1	0,33352
P62906	60S ribosomal protein L10a (RL10A)	1	0,05925
P83731	60S ribosomal protein L24 (RL24)	1	0,05777
Q06330	Recombining binding protein suppressor of hairless (SUH)	1	0,04440
Q13148	TAR DNA-binding protein 43 (TADBP)	1	0,06082
Q8NC51	Plasminogen activator inhibitor 1 RNA-binding protein (PAIRB)	1	0,03606
Q92841	Probable ATP-dependent RNA helicase DDX17 (DDX17)	1	0,02714
Q96T60	Bifunctional polynucleotide phosphatase/kinase (PNKP)	1	0,03912
Q9BQ61	Uncharacterized protein C19orf43 (CS043)	1	0,08902
Q9P2V4	Leucine-rich repeat, immunoglobulin-like domain and transmembrane domain-containing protein 1 (LRIT1)	1	0,03663
P07737	Profilin-1 (PROF1)	1	0,16591
Q9UKF2	Disintegrin and metalloproteinase domain-containing protein 30 (ADA30)	1	0,02591
P21802	Fibroblast growth factor receptor 2 (FGFR2)	1	0,02883
Q09028	Histone-binding protein RBBP4 (RBBP4)	1	0,07711
P14316	Interferon regulatory factor 2 (IRF2)	1	0,05925

Exp 2	TNF+iso	Method B	
Accession	Description	#Validated Peptides	emPAI
Q16531	DNA damage-binding protein 1 (DDB1)	68	4,28937
Q13619	Cullin-4A (CUL4A)	29	0,88882
Q92466	DNA damage-binding protein 2 (DDB2)	36	4,24807
Q13620	Cullin-4B (CUL4B)	16	0,36287
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	15	0,25325
P15408	Fos-related antigen 2 (FOSL2)	17	1,88044
P17676	CCAAT/enhancer-binding protein beta (CEBPB)	18	2,16228
P17275	Transcription factor jun-B (JUNB)	17	1,96635
O14867	Transcription regulator protein BACH1 (BACH1)	13	0,45378
P04406	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	10	0,93070
P02768	Serum albumin (ALBU)	11	0,36190
P28347	Transcriptional enhancer factor TEF-1 (TEAD1)	10	0,63217
Q99594	Transcriptional enhancer factor TEF-5 (TEAD3)	9	0,53993
P02545	Prelamin-A/C (LMNA)	10	0,24783
Q15561	Transcriptional enhancer factor TEF-3 (TEAD4)	7	0,45476
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	6	0,38950
P15407	Fos-related antigen 1 (FOSL1)	7	0,81660
P17535	Transcription factor jun-D (JUND)	7	0,68192
P05412	Transcription factor AP-1 (JUN)	6	0,56152
P17544	Cyclic AMP-dependent transcription factor ATF-7 (ATF7)	6	0,34171

O15525	Transcription factor MafG (MAFG)	4	0,34596
O60675	Transcription factor MafK (MAFK)	3	0,26896
P19838	Nuclear factor NF-kappa-B p105 subunit (NFKB1)	4	0,10903
P53539	Protein fosB (FOSB)	2	0,20226
P53567	CCAAT/enhancer-binding protein gamma (CEBPG)	1	0,10530
P23246	Splicing factor, proline- and glutamine-rich (SFPQ)	3	0,08466
P16220	Cyclic AMP-responsive element-binding protein 1 (CREB1)	2	0,16591
P62877	E3 ubiquitin-protein ligase RBX1 (RBX1)	1	0,23285
P15336	Cyclic AMP-dependent transcription factor ATF-2 (ATF2)	2	0,10294
Q15233	Non-POU domain-containing octamer-binding protein (NONO)	1	0,03393
Q8WYK2	Jun dimerization protein 2 (JDP2)	2	0,17877
Q96AE4	Far upstream element-binding protein 1 (FUBP1)	1	0,04440
Q9ULX9	Transcription factor MafF (MAFF)	1	0,08264
P09651	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	1	0,05925
P62273	40S ribosomal protein S29 (RS29)	2	0,51991
Q9UKF2	Disintegrin and metalloproteinase domain-containing protein 30 (ADA30)	1	0,02591
O00178	GTP-binding protein 1 (GTPB1)	1	0,02996
P02751	Fibronectin (FINC)	1	0,01302
P12956	X-ray repair cross-complementing protein 6 (XRCC6)	1	0,02714
P22415	Upstream stimulatory factor 1 (USF1)	1	0,07978
P29372	DNA-3-methyladenine glycosylase (3MG)	1	0,06247
P51991	Heterogeneous nuclear ribonucleoprotein A3 (ROA3)	1	0,05133
Q00839	Heterogeneous nuclear ribonucleoprotein U (HNRPU)	1	0,02135
Q14938	Nuclear factor 1 X-type (NFIX)	1	0,04914
Q8N108	Mesoderm induction early response protein 1 (MIER1)	1	0,04440
P20700	Lamin-B1 (LMNB1)	2	0,05436
P14866	Heterogeneous nuclear ribonucleoprotein L (HNRPL)	2	0,07842
Q92945	Far upstream element-binding protein 2 (FUBP2)	2	0,07978
P61978	Heterogeneous nuclear ribonucleoprotein K (HNRPK)	1	0,04440
Q9BW61	DET1- and DDB1-associated protein 1 (DDA1)	1	0,15478

Exp 2	veh	Method B	
Accession	Description	#Validated Peptides	emPAI
Q16531	DNA damage-binding protein 1 (DDB1)	28	0,98551
Q92466	DNA damage-binding protein 2 (DDB2)	26	2,31131
P17676	CCAAT/enhancer-binding protein beta (CEBPB)	22	3,08424
P17275	Transcription factor jun-B (JUNB)	21	2,83119
P17535	Transcription factor jun-D (JUND)	12	1,43835
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	10	0,73020
P15408	Fos-related antigen 2 (FOSL2)	11	0,98288
P02768	Serum albumin (ALBU)	9	0,28753
P02545	Prelamin-A/C (LMNA)	8	0,19378
P04406	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	5	0,38950
Q13619	Cullin-4A (CUL4A)	8	0,19176
O14867	Transcription regulator protein BACH1 (BACH1)	5	0,15478
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	5	0,07815
P05412	Transcription factor AP-1 (JUN)	6	0,56152
P28347	Transcriptional enhancer factor TEF-1 (TEAD1)	4	0,21648
P53567	CCAAT/enhancer-binding protein gamma (CEBPG)	4	0,49250
P15407	Fos-related antigen 1 (FOSL1)	5	0,53174

O15525	Transcription factor MafG (MAFG)	4	0,34596
Q9ULX9	Transcription factor MafF (MAFF)	3	0,26896
P17544	Cyclic AMP-dependent transcription factor ATF-7 (ATF7)	3	0,15832
P16220	Cyclic AMP-responsive element-binding protein 1 (CREB1)	4	0,35936
P23246	Splicing factor, proline- and glutamine-rich (SFPQ)	2	0,05567
P20700	Lamin-B1 (LMNB1)	2	0,05436
Q15233	Non-POU domain-containing octamer-binding protein (NONO)	2	0,06902
Q13620	Cullin-4B (CUL4B)	4	0,08047
O60675	Transcription factor MafK (MAFK)	2	0,17210
P22415	Upstream stimulatory factor 1 (USF1)	2	0,16591
Q15853	Upstream stimulatory factor 2 (USF2)	2	0,16591
P15336	Cyclic AMP-dependent transcription factor ATF-2 (ATF2)	1	0,05021
P09651	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	1	0,05925
P02751	Fibronectin (FINC)	1	0,01302
Q14938	Nuclear factor 1 X-type (NFIX)	1	0,04914
O60282	Kinesin heavy chain isoform 5C (KIF5C)	1	0,01601
P29372	DNA-3-methyladenine glycosylase (3MG)	1	0,06247
P39880	Homeobox protein cut-like 1 (CUX1)	1	0,01372
P51991	Heterogeneous nuclear ribonucleoprotein A3 (ROA3)	1	0,05133
P62273	40S ribosomal protein S29 (RS29)	1	0,23285
Q00839	Heterogeneous nuclear ribonucleoprotein U (HNRPU)	1	0,02135
Q92945	Far upstream element-binding protein 2 (FUBP2)	1	0,03912
Q9UKF2	Disintegrin and metalloproteinase domain-containing protein 30 (ADA30)	1	0,02591

Exp 3	TNF+iso	Method B	
Accession	Description	#Validated Peptides	emPAI
Q16531	DNA damage-binding protein 1 (DDB1)	70	4,55496
Q92466	DNA damage-binding protein 2 (DDB2)	40	5,30957
Q13619	Cullin-4A (CUL4A)	14	0,35936
P02768	Serum albumin (ALBU)	14	0,48160
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	6	0,09450
P02751	Fibronectin (FINC)	7	0,09478
P04264	Keratin, type II cytoskeletal 1 (K2C1)	5	0,19378
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	4	0,24520
P04406	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	6	0,48398
P13645	Keratin, type I cytoskeletal 10 (K1C10)	4	0,21648
P17676	CCAAT/enhancer-binding protein beta (CEBPB)	4	0,29155
P15408	Fos-related antigen 2 (FOSL2)	2	0,13254
P28347	Transcriptional enhancer factor TEF-1 (TEAD1)	1	0,05021
P17275	Transcription factor jun-B (JUNB)	2	0,13646
P17535	Transcription factor jun-D (JUND)	2	0,16016
P29372	DNA-3-methyladenine glycosylase (3MG)	1	0,06247
P35908	Keratin, type II cytoskeletal 2 epidermal (K22E)	2	0,07342
P62273	40S ribosomal protein S29 (RS29)	2	0,51991
Q13951	Core-binding factor subunit beta (PEBB)	1	0,07978
P04908	Histone H2A type 1-B/E (H2A1B)	1	0,09648
P16220	Cyclic AMP-responsive element-binding protein 1 (CREB1)	1	0,07978
P35527	Keratin, type I cytoskeletal 9 (K1C9)	1	0,04713
P53567	CCAAT/enhancer-binding protein gamma (CEBPG)	1	0,10530
Q8N108	Mesoderm induction early response protein 1 (MIER1)	1	0,04440

Q9NZB2	Constitutive coactivator of PPAR-gamma-like protein 1 (F120A)	1	0,02059
Q9H251	Cadherin-23 (CAD23)	1	0,01011

Exp 3	veh	Method B	
Accession	Description	#Validated Peptides	emPAI
P02545	Prelamin-A/C (LMNA)	41	1,47874
P23246	Splicing factor, proline- and glutamine-rich (SFPQ)	25	0,96842
Q92466	DNA damage-binding protein 2 (DDB2)	23	1,88403
P02751	Fibronectin (FINC)	22	0,32922
Q16531	DNA damage-binding protein 1 (DDB1)	18	0,55414
Q09666	Neuroblast differentiation-associated protein AHNAK (AHNK)	17	0,04646
Q15233	Non-POU domain-containing octamer-binding protein (NONO)	18	0,82335
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	17	1,53958
P08670	Vimentin (VIME)	16	0,81161
P17275	Transcription factor jun-B (JUNB)	13	1,29674
P17676	CCAAT/enhancer-binding protein beta (CEBPB)	12	1,15443
P21333	Filamin-A (FLNA)	13	0,13284
P09651	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	6	0,41254
P61978	Heterogeneous nuclear ribonucleoprotein K (HNRPK)	12	0,68428
Q96AE4	Far upstream element-binding protein 1 (FUBP1)	13	0,75907
P04406	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	10	0,93070
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	8	0,12794
P51991	Heterogeneous nuclear ribonucleoprotein A3 (ROA3)	6	0,35031
P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	9	1,81838
Q14103	Heterogeneous nuclear ribonucleoprotein D0 (HNRPD)	8	0,50584
P02768	Serum albumin (ALBU)	9	0,28753
P04075	Fructose-bisphosphate aldolase A (ALDOA)	8	0,62378
P14866	Heterogeneous nuclear ribonucleoprotein L (HNRPL)	8	0,35253
P20700	Lamin-B1 (LMNB1)	6	0,17210
P11387	DNA topoisomerase 1 (TOP1)	3	0,04122
Q99729	Heterogeneous nuclear ribonucleoprotein A/B (ROAA)	7	0,45476
P10412	Histone H1,4 (H14)	7	0,33352
P16401	Histone H1,5 (H15)	7	0,32035
Q92841	Probable ATP-dependent RNA helicase DDX17 (DDX17)	6	0,17427
P35579	Myosin-9 (MYH9)	6	0,04329
O14979	Heterogeneous nuclear ribonucleoprotein D-like (HNRDL)	5	0,21957
P12956	X-ray repair cross-complementing protein 6 (XRCC6)	5	0,14325
Q92945	Far upstream element-binding protein 2 (FUBP2)	5	0,21153
P17535	Transcription factor jun-D (JUND)	5	0,44974
Q8IVF2	Protein AHNAK2 (AHNK2)	3	0,00957
P15408	Fos-related antigen 2 (FOSL2)	5	0,36501
P06748	Nucleophosmin (NPM)	5	0,34340
P0C0S8	Histone H2A type 1 (H2A1)	6	0,73780
P13010	X-ray repair cross-complementing protein 5 (XRCC5)	4	0,10903
Q13619	Cullin-4A (CUL4A)	5	0,11588
P07355	Annexin A2 (ANXA2)	3	0,14207
P27695	DNA-(apurinic or apyrimidinic site) lyase (APEX1)	4	0,23285
P13639	Elongation factor 2 (EF2)	4	0,09260
Q07666	KH domain-containing, RNA-binding, signal transduction-associated protein 1 (KHDR1)	1	0,04197

Q92804	TATA-binding protein-associated factor 2N (RBP56)	3	0,11034
P06733	Alpha-enolase (ENOA)	2	0,08902
P15311	Ezrin (EZRI)	3	0,07227
P60660	Myosin light polypeptide 6 (MYL6)	3	0,63789
P98179	Putative RNA-binding protein 3 (RBM3)	4	0,58489
Q13263	Transcription intermediary factor 1-beta (TIF1B)	4	0,12365
O15525	Transcription factor MafG (MAFG)	3	0,24961
P18754	Regulator of chromosome condensation (RCC1)	2	0,11304
P50454	Serpin H1 (SERPH)	3	0,14207
P52943	Cysteine-rich protein 2 (CRIP2)	2	0,19378
P62273	40S ribosomal protein S29 (RS29)	3	0,87382
P62826	GTP-binding nuclear protein Ran (RAN)	2	0,19378
P63241	Eukaryotic translation initiation factor 5A-1 (IF5A1)	3	0,53993
P53567	CCAAT/enhancer-binding protein gamma (CEBPG)	2	0,22168
Q9C005	Protein dpy-30 homolog (DPY30)	2	0,58489
Q9P258	Protein RCC2 (RCC2)	1	0,03663
P09429	High mobility group protein B1 (HMGB1)	1	0,05021
O60814	Histone H2B type 1-K (H2B1K)	2	0,18597
P07910	Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRPC)	2	0,10294
P10809	60 kDa heat shock protein, mitochondrial (CH60)	1	0,03250
P16220	Cyclic AMP-responsive element-binding protein 1 (CREB1)	2	0,16591
P24534	Elongation factor 1-beta (EF1B)	1	0,09648
P25398	40S ribosomal protein S12 (RS12)	2	0,27427
P29401	Transketolase (TKT)	1	0,03550
P31949	Protein S100-A11 (S10AB)	2	0,42510
P33240	Cleavage stimulation factor subunit 2 (CSTF2)	1	0,04528
P62805	Histone H4 (H4)	1	0,10069
P68104	Elongation factor 1-alpha 1 (EF1A1)	1	0,03980
P82979	SAP domain-containing ribonucleoprotein (SARNP)	2	0,12534
Q13151	Heterogeneous nuclear ribonucleoprotein A0 (ROA0)	2	0,14976
Q13409	Cytoplasmic dynein 1 intermediate chain 2 (DC1I2)	1	0,03496
Q8WXF1	Paraspeckle component 1 (PSPC1)	2	0,06701
Q9NP97	Dynein light chain roadblock-type 1 (DLRB1)	2	0,46780
Q9ULX9	Transcription factor MafF (MAFF)	2	0,17210
P05204	Non-histone chromosomal protein HMG-17 (HMGN2)	1	0,11034
P27797	Calreticulin (CALR)	1	0,05372
A5A3E0	POTE ankyrin domain family member F (POTEF)	1	0,01695
O95785	Protein Wiz (WIZ)	1	0,01388
P00558	Phosphoglycerate kinase 1 (PGK1)	1	0,04618
P05386	60S acidic ribosomal protein P1 (RLA1)	1	0,38950
P11021	78 kDa glucose-regulated protein (GRP78)	1	0,02714
P16949	Stathmin (STMN1)	1	0,07461
P17096	High mobility group protein HMG-I/HMG-Y (HMGA1)	1	0,11034
P22415	Upstream stimulatory factor 1 (USF1)	1	0,07978
P30050	60S ribosomal protein L12 (RL12)	1	0,09648
P35637	RNA-binding protein FUS (FUS)	1	0,04811
P51858	Hepatoma-derived growth factor (HDGF)	1	0,07007
Q15365	Poly(rC)-binding protein 1 (PCBP1)	1	0,08264
P60174	Triosephosphate isomerase (TPIS)	1	0,07978
P61158	Actin-related protein 3 (ARP3)	1	0,05372
Q00839	Heterogeneous nuclear ribonucleoprotein U (HNRPU)	1	0,02135
Q12888	Tumor suppressor p53-binding protein 1 (TP53B)	1	0,01332
Q12905	Interleukin enhancer-binding factor 2 (ILF2)	1	0,06421

Q13765	Nascent polypeptide-associated complex subunit alpha (NACA)	1	0,11034
Q15651	High mobility group nucleosome-binding domain-containing protein 3 (HMGN3)	1	0,11034
Q16630	Cleavage and polyadenylation specificity factor subunit 6 (CPSF6)	1	0,03847
Q92499	ATP-dependent RNA helicase DDX1 (DDX1)	1	0,02591
Q99832	T-complex protein 1 subunit eta (TCPH)	1	0,03550
Q9Y224	UPF0568 protein C14orf166 (CN166)	1	0,07007
Q9Y3I0	tRNA-splicing ligase RtcB homolog (RTCB)	1	0,04122
Q9Y6D9	Mitotic spindle assembly checkpoint protein MAD1 (MD1L1)	1	0,02059
P68431	Histone H3,1 (H31)	2	0,17210
P26038	Moesin (MOES)	1	0,02306
P55145	Mesencephalic astrocyte-derived neurotrophic factor (MANF)	1	0,07461
Q9UKF2	Disintegrin and metalloproteinase domain-containing protein 30 (ADA30)	1	0,02591
Q9BQ61	Uncharacterized protein C19orf43 (CS043)	1	0,08902
Q9H910	Hematological and neurological expressed 1-like protein (HN1L)	1	0,11588
P02452	Collagen alpha-1(I) chain (CO1A1)	1	0,01937
Q14847	LIM and SH3 domain protein 1 (LASP1)	1	0,07461
Q9BRJ9	Mesoderm posterior protein 1 (MESP1)	1	0,07978
P16949	Stathmin (STMN1)	2	0,15478
Q9BVC5	Ashwin (ASHWN)	1	0,07007
P63313	Thymosin beta-10 (TYB10)	1	0,33352
P19338	Nucleolin (NUCL)	1	0,02059
Q15853	Upstream stimulatory factor 2 (USF2)	1	0,07978
Q9ULV4	Coronin-1C (COR1C)	1	0,03980

Supplementary Table 3. Overview of proteins identified in the different AP-MS experiments after elimination of technical contaminants and proteins identified with only 1 unique peptide. Blank boxes indicate that the protein was not identified in a given treatment/experiment.

				Exp 1 Method A				Exp 2 Method A				Exp 1 Method B				Exp 2 Method B				Exp 3 Method B				
				TNF/iso		veh		TNF/iso		veh		TNF/iso		veh		TNF/iso		veh		TNF/iso		veh		
Accession	Description	Localization	Function	#Validated Peptides	emPAI	#Validated Peptides	emPAI	#Validated Peptides	emPAI	#Validated Peptides	emPAI	#Validated Peptides	emPAI	#Validated Peptides	emPAI	#Validated Peptides	emPAI	#Validated Peptides	emPAI	#Validated Peptides	emPAI	#Validated Peptides	emPAI	Sum # Validated Peptides	Av emPAI
Q16531	DNA damage-binding protein 1 (DDB1)	N	DNA nucleic acid-associated protein	68	4,28937374	65	3,91461341	5	0,13029417	4	0,10294331	47	2,16227766	60	3,34808346	68	4,28937374	28	0,98551119	72	4,83387939	18	0,55413722	435	2,46104873
Q92466	DNA damage-binding protein 2 (DDB2)	N	DNA nucleic acid-associated protein	25	2,16227766	26	2,31131121	4	0,20226443	2	0,09647819	21	1,63026799	29	2,80189396	36	4,24807460	26	2,31131121	38	4,75439937	23	1,88403150	230	2,24023102
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	N	Transcription related protein	2	0,03055671			9	0,14504757	3	0,04618344	73	2,00002022	81	2,38385515	15	0,25325433	5	0,07815136	6	0,09449979	8	0,12794411	202	0,51595127

Q13619	Cullin-4A (CUL4A)	N	DNA nucleic acid-associated protein	25	0,73019573	23	0,65595152					14	0,35935639	17	0,45179321	29	0,88881958	8	0,19176458	15	0,38949549	5	0,11588399	136	0,37832605
P02545	Prelamin-A/C (LMNA)	N	Transcription related protein									27	0,81809002	30	0,94294961	10	0,24782547	8	0,19377664			41	1,47873882	116	0,36813806
P13010	X-ray repair cross-complementing protein 5 (XRCC5)	N	Transcription related protein									42	1,96421781	57	3,36967803							4	0,10903134	103	0,54429272
P17275	Transcription factor jun-B (JUNB)	N	Transcription related protein	7	0,56474814	6	0,46779926			2	0,13646366	16	1,78255940	15	1,61015721	17	1,96634883	21	2,83118685	2	0,13646366	13	1,29673617	99	1,07924632
P12956	X-ray repair cross-complementing protein 6 (XRCC6)	N	Transcription related protein									42	2,0787334	48	2,6152599							5	0,1432455	95	0,4837239
Q13620	Cullin-4B (CUL4B)	N	DNA nucleic acid-associated protein	30	0,78690243	21	0,50131072					6	0,12310450	6	0,12310450	16	0,36286795	4	0,08047182					83	0,19777619

P17676	CCAAT/enhancer-binding protein beta (CEBPB)	N	Transcription related protein	3	0,21152765							10	0,89573565	9	0,77827941	18	2,16227766	22	3,08423865	5	0,37685716	12	1,15443469	79	0,86633509
P02751	Fibronectin (FINC)	O	Not described nuclear function									23	0,34652209	25	0,38181352					7	0,09477744	22	0,32921583	77	0,11523289
P08670	Vimentin (VIME)	O	Not described nuclear function									20	1,10174801	40	3,41734470							16	0,81160919	76	0,53307019
P15408	Fos-related antigen 2 (FOSL2)	N	Transcription related protein	3	0,20526093	4	0,28264983					8	0,64519058	9	0,75082703	17	1,88044415	11	0,982883949	2	0,13254131	5	0,36500780	59	0,52448056
P04406	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	N	Transcription related protein									10	0,93069772	15	1,68269579	10	0,93069772	5	0,38949549	6	0,48398178	10	0,93069772	56	0,53482663
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	N	RNA nucleic acid-associated protein									5	0,31536905	7	0,46779926	6	0,38949549	10	0,73019573	4	0,24519708	17	1,53958003	49	0,36876367

P21333	Filamin-A (FLNA)	O	Not described nuclear function						31 0,34637694				13 0,13283502	44 0,04792120
P39880	Homeobox protein cut-like 1 (CUX1)	N	Transcription related protein					20 0,31323725	24 0,38679361					44 0,07000309
P61978	Heterogeneous nuclear ribonucleoprotein K (HNRPK)	N	Transcription related protein					15 0,91875209	14 0,83717676				12 0,68428311	41 0,24402120
P46063	ATP-dependent DNA helicase Q1 (RECQ1)	N	DNA nucleic acid-associated protein					22 0,89880782	17 0,64130719					39 0,15401150
P17535	Transcription factor jun-D (JUND)	N	Transcription related protein	2 0,16015530				5 0,44974067	4 0,34596032	7 0,68192432	12 1,43835409	2 0,16015530	5 0,44974067	37 0,36860307
P23246	Splicing factor, proline- and glutamine-rich (SFPQ)	N	Transcription related protein						2 0,05567299	3 0,08466123	2 0,05567299		25 0,96841944	32 0,11644267

P49916	DNA ligase 3 (DNLI3)	N	DNA nucleic acid-associated protein					15 0,266892386	17 0,30748994					32 0,05743823
P35579	Myosin-9 (MYH9)	O	Not described nuclear function					4 0,02865546	21 0,15989099				6 0,04328967	31 0,02318361
Q09666	Neuroblast differentiation-associated protein AHNAK (AHNK)	N	Not described nuclear function					6 0,0161564	8 0,02159966				17 0,04645745	31 0,00842135
O14867	Transcription regulator protein BACH1 (BACH1)	N	Transcription related protein	5 0,15478198				4 0,12201845	3 0,09018449	5 0,15478198	13 0,45378438			30 0,09755513
P28347	Transcriptional enhancer factor TEF-1 (TEAD1)	N	Transcription related protein	2 0,10294331				8 0,47983319	6 0,34171283	10 0,63217212	4 0,21648394			30 0,17731454
P04075	Fructose-bisphosphate aldolase A (ALDOA)	O	Not described nuclear function					5 0,35387618	15 1,48162892				8 0,62377673	28 0,24592818

P15407	Fos-related antigen 1 (FOSL1)	N	Transcription related protein	4 0,40652724	2 0,18597101			5 0,53174046	4 0,40652724	7 0,81659978	5 0,53174046			27 0,28791062
P20700	Lamin-B1 (LMNB1)	N	Transcription related protein					9 0,26896100	8 0,23581659	2 0,05435899	2 0,05435899		6 0,17210229	27 0,07855979
Q14764	Major vault protein (MVP)	N	RNA nucleic acid-associated protein					15 0,39389727	12 0,30432138					27 0,06982187
P18887	DNA repair protein XRCC1 (XRCC1)	N	DNA nucleic acid-associated protein					13 0,49856531	12 0,45265392					25 0,09512192
Q5T5X7	BEN domain-containing protein 3 (BEND3)	N	Transcription related protein					17 0,46234062	8 0,19583126					25 0,06581719
P09651	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	N	RNA nucleic acid-associated protein					8 0,58489319	10 0,77827941				6 0,41253754	24 0,17757101

Q00059	Transcription factor A, mitochondrial (TFAM)	O	Not described nuclear function					11 0,67683293	13 0,84206996					24 0,15189029
Q15233	Non-POU domain-containing octamer-binding protein (NONO)	N	Transcription related protein						4 0,14280206		2 0,06901920		18 0,82334800	24 0,10351693
Q96AE4	Far upstream element-binding protein 1 (FUBP1)	N	Transcription related protein					5 0,24262367	6 0,29779933				13 0,75906958	24 0,12994926
P60709	Actin, cytoplasmic 1 (ACTB)	O	Not described nuclear function					8 0,66810053	15 1,61015721					23 0,22782578
P27695	DNA-(apurinic or apyrimidinic site) lyase (APEX1)	N	Transcription related protein	2 0,11033631				9 0,60156826	7 0,44241725				4 0,23284673	22 0,13871686
P14866	Heterogeneous nuclear ribonucleoprotein L (HNRPL)	N	Transcription related protein					6 0,25418226	5 0,20772265	2 0,07841739			8 0,35253197	21 0,08928543

P16220	Cyclic AMP-responsive element-binding protein 1 (CREB1)	N	Transcription related protein					5 0,46779926	6 0,58489319	2 0,16591440	4 0,35935639		2 0,16591440	19 0,17438777
P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	O	Not described nuclear function						10 2,16227766				9 1,81838293	19 0,39806606
P68104	Elongation factor 1-alpha 1 (EF1A1)	O	RNA nucleic acid-associated protein					9 0,42083083	10 0,47737765					19 0,08982085
O15525	Transcription factor MafG (MAFG)	N	Transcription related protein					4 0,34596032	3 0,24960914	4 0,34596032	4 0,34596032		3 0,24960914	18 0,15370993
Q14103	Heterogeneous nuclear ribonucleoprotein D0 (HNRPD)	N	Transcription related protein					3 0,16591440	7 0,43072298				8 0,50583635	18 0,11024737
P11142	Heat shock cognate 71 kDa protein (HSP7C)	N	Transcription related protein					8 0,25535174	9 0,29154966					17 0,05469014

P07355	Annexin A2 (ANXA2)	O	Not described nuclear function					3 0,14206890	10 0,55706840				3 0,14206890	16 0,08412062
P06733	Alpha-enolase (ENOA)	N	Transcription related protein					2 0,08902296	12 0,66810053				2 0,08902296	16 0,08461465
P17544	Cyclic AMP-dependent transcription factor ATF-7 (ATF7)	N	Transcription related protein					3 0,15832328	3 0,15832328	6 0,34171283	3 0,15832328			15 0,08166827
Q99594	Transcriptional enhancer factor TEF-5 (TEAD3)	N	Transcription related protein						6 0,33352143	9 0,53992652				15 0,08734480
P05412	Transcription factor AP-1 (JUN)	N	Transcription related protein	2 0,16015530						6 0,56152300	6 0,56152300			14 0,12832013
P09429	High mobility group protein B1 (HMGB1)	N	Transcription related protein					8 0,47983319	6 0,34171283					14 0,08215460

P19838	Nuclear factor NF-kappa-B p105 subunit (NFKB1)	N	Transcription related protein	2	0,05310557									8	0,22995052			4	0,10903134							14	0,03920874
Q8IVF2	Protein AHNAK2 (AHNK2)	N	Not described nuclear function									7	0,02248082	4	0,01278495							3	0,00957347	14	0,00448393
Q04206	Transcription factor p65 (RELA)	N	Transcription related protein	2	0,10294331							10	0,63217212											12	0,07351154
O15527	N-glycosylase/DNA lyase (OGG1)	N	DNA nucleic acid-associated protein									6	0,45265392	5	0,36500780									11	0,08176617
P05204	Non-histone chromosomal protein HMG-17 (HMGN2)	N	Transcription related protein									5	0,68761247	6	0,87381742									11	0,15614299
Q14938	Nuclear factor 1 X-type (NFIX)	N	Transcription related protein	3	0,15478198							4	0,21152765	4	0,21152765									11	0,05778373

Q92945	Far upstream element-binding protein 2 (FUBP2)	N	Transcription related protein					2 0,07977516	2 0,07977516	2 0,07977516			5 0,21152765	11 0,04508531
P13639	Elongation factor 2 (EF2)	O	Not described nuclear function						6 0,14206890				4 0,09260086	10 0,02346698
P53567	CCAAT/enhancer-binding protein gamma (CEBPG)	N	Transcription related protein					2 0,22167734	2 0,22167734		4 0,49249554		2 0,22167734	10 0,11575276
P62736	Actin, aortic smooth muscle (ACTA)	O	Not described nuclear function						10 0,89573565					10 0,08957357
P09382	Galectin-1 (LEG1)	O	Not described nuclear function					3 0,7782794	6 2,1622776					9 0,2940557
P22415	Upstream stimulatory factor 1 (USF1)	N	Transcription related protein					3 0,25892541	3 0,25892541		2 0,16591440			8 0,06837652

P51991	Heterogeneous nuclear ribonucleoprotein A3 (ROA3)	N	RNA nucleic acid-associated protein					2 0,10529514					6 0,35031403	8 0,04556092
Q13263	Transcription intermediary factor 1-beta (TIF1B)	N	Transcription related protein						4 0,123654				4 0,123654	8 0,024730
Q15853	Upstream stimulatory factor 2 (USF2)	N	Transcription related protein					3 0,25892541	3 0,25892541		2 0,16591440			8 0,06837652
P10412	Histone H1,4 (H14)	N	Transcription related protein										7 0,33352143	7 0,03335214
P16401	Histone H1,5 (H15)	N	Transcription related protein										7 0,3203517	7 0,0320351
P16949	Stathmin (STMN1)	O	Not described nuclear function						5 0,43301257				2 0,15478198	7 0,05877946

P31943	Heterogeneous nuclear ribonucleoprotein H (HNRH1)	N	RNA nucleic acid-associated protein					3 0,16998910	4 0,23284673					7 0,04028358
P60660	Myosin light polypeptide 6 (MYL6)	O	Not described nuclear function						4 0,93069772				3 0,63789370	7 0,15685914
P62273	40S ribosomal protein S29 (RS29)	O	RNA nucleic acid-associated protein							2 0,51991108		2 0,51991108	3 0,87381742	7 0,19136396
Q14204	Cytoplasmic dynein 1 heavy chain 1 (DYHC1)	O	Not described nuclear function					5 0,02058588	2 0,00818405					7 0,00287699
Q15561	Transcriptional enhancer factor TEF-3 (TEAD4)	N	Transcription related protein							7 0,45475781				7 0,04547578
Q16658	Fascin (FSCN1)	O	Not described nuclear function						7 0,3203517					7 0,0320351

Q99729	Heterogeneous nuclear ribonucleoprotein A/B (ROAA)	N	RNA nucleic acid-associated protein										7 0,45475781	7 0,04547578
P0C0S8	Histone H2A type 1 (H2A1)	N	Transcription related protein										6 0,73780082	6 0,07378008
P15311	Ezrin (EZRI)	O	Not described nuclear function						3 0,07226722				3 0,07226722	6 0,01445344
P26038	Moesin (MOES)	O	Not described nuclear function						6 0,14658416					6 0,01465842
P26583	High mobility group protein B2 (HMGB2)	N	Transcription related protein					4 0,22712524	2 0,10775685					6 0,03348821
P26599	Polypyrimidine tract-binding protein 1 (PTBP1)	N	RNA nucleic acid-associated protein					3 0,15478198	3 0,15478198					6 0,03095640

P62826	GTP-binding nuclear protein Ran (RAN)	N	Transcription related protein						4 0,42510267				2 0,19377664	6 0,06188793
P63167	Dynein light chain 1, cytoplasmic (DYL1)	O	Not described nuclear function					3 0,7782794	3 0,7782794					6 0,1556558
P63241	Eukaryotic translation initiation factor 5A-1 (IF5A1)	O	RNA nucleic acid-associated protein						3 0,53992652				3 0,53992652	6 0,10798531
Q92841	Probable ATP-dependent RNA helicase DDX17 (DDX17)	N	Transcription related protein										6 0,17426858	6 0,01742686
O14979	Heterogeneous nuclear ribonucleoprotein D-like (HNRDL)	N	Transcription related protein										5 0,2195704	5 0,0219570
O60675	Transcription factor MafK (MAFK)	N	Transcription related protein							3 0,26896100	2 0,17210229			5 0,04410633

P06748	Nucleophosmin (NPM)	N	Transcription related protein										5 0,34339933	5 0,03433993
P07900	Heat shock protein HSP 90-alpha (HS90A)	O	Not described nuclear function						5 0,11249046					5 0,01124905
P19338	Nucleolin (NUCL)	N	Transcription related protein					2 0,04159554	3 0,06303771					5 0,01046333
P50454	Serpin H1 (SERPH)	O	Not described nuclear function						2 0,09260086				3 0,14206890	5 0,02346698
Q00653	Nuclear factor NF-kappa-B p100 subunit (NFKB2)	N	Transcription related protein					5 0,14148777						5 0,01414878
Q13409	Cytoplasmic dynein 1 intermediate chain 2 (DC1I2)	O	Not described nuclear function					2 0,07115112	3 0,10860317					5 0,01797543

Q15365	Poly(rC)-binding protein 1 (PCBP1)	N	Transcription related protein					2 0,17210229	3 0,26896100					5 0,04410633
Q9ULX9	Transcription factor MafF (MAFF)	N	Transcription related protein								3 0,26896100		2 0,17210229	5 0,04410633
P08107	Heat shock 70 kDa protein 1A/1B (HSP71)	O	Not described nuclear function						4 0,12201845					4 0,01220185
P11021	78 kDa glucose-regulated protein (GRP78)	O	Not described nuclear function						4 0,11304219					4 0,01130422
P14618	Pyruvate kinase PKM (KPYM)	O	Not described nuclear function						4 0,16298408					4 0,01629841
P20810	Calpastatin (ICAL)	O	Not described nuclear function						4 0,09749876					4 0,00974988

P52926	High mobility group protein HMGI-C (HMGA2)	N	Transcription related protein					2 0,27427498	2 0,27427498					4 0,05485500
P61956	Small ubiquitin-related modifier 2 (SUMO2)	N	Transcription related protein					2 0,46779926	2 0,46779926					4 0,09355985
P62987	Ubiquitin-60S ribosomal protein L40 (RL40)	N	RNA nucleic acid-associated protein					2 0,20226443	2 0,20226443					4 0,04045289
P98179	Putative RNA-binding protein 3 (RBM3)	N	RNA nucleic acid-associated protein										4 0,58489319	4 0,05848932
Q9Y2S6	Translation machinery-associated protein 7 (TMA7)	O	Not described nuclear function					2 0,27427498	2 0,27427498					4 0,05485500
P04083	Annexin A1 (ANXA1)	N	Not described nuclear function					3 0,15139539						3 0,01513954

P07437	Tubulin beta chain (TBB5)	O	Not described nuclear function						3 0,21152765					3 0,02115277
P08238	Heat shock protein HSP 90-beta (HS90B)	O	Not described nuclear function						3 0,06800043					3 0,00680004
P08651	Nuclear factor 1 C-type (NFIC)	N	Transcription related protein	3 0,13920996										3 0,01392100
P11387	DNA topoisomerase 1 (TOP1)	N	DNA nucleic acid-associated protein										3 0,04122325	3 0,00412233
P17096	High mobility group protein HMG-I/HMG-Y (HMGA1)	N	Transcription related protein						3 0,3688745					3 0,0368874
P23284	Peptidyl-prolyl cis-trans isomerase B (PPIB)	O	Not described nuclear function						3 0,23284673					3 0,02328467

P31946	14-3-3 protein beta/alpha (1433B)	N	Transcription related protein						3 0,25892541					3 0,02589254
P40926	Malate dehydrogenase, mitochondrial (MDHM)	O	Not described nuclear function						3 0,23284673					3 0,02328467
P60174	Triosephosphate isomerase (TPIS)	O	Not described nuclear function						3 0,25892541					3 0,02589254
P68363	Tubulin alpha-1B chain (TBA1B)	O	Not described nuclear function						3 0,19377664					3 0,01937766
P80723	Brain acid soluble protein 1 (BASP1)	N	Transcription related protein						3 0,26896100					3 0,02689610
Q06330	Recombining binding protein suppressor of hairless (SUH)	N	Transcription related protein					3 0,13920996						3 0,01392100

Q15366	Poly(rC)-binding protein 2 (PCBP2)	N	Transcription related protein						3 0,27980221					3 0,02798022
Q15699	ALX homeobox protein 1 (ALX1)	N	Transcription related protein	3 0,18850222										3 0,01885022
Q92804	TATA-binding protein-associated factor 2N (RBP56)	N	Transcription related protein										3 0,11033631	3 0,01103363
O60814	Histone H2B type 1-K (H2B1K)	N	Transcription related protein										2 0,18597101	2 0,01859710
P00558	Phosphoglycerate kinase 1 (PGK1)	N	DNA nucleic acid-associated protein						2 0,09449979					2 0,00944998
P07910	Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRPC)	N	RNA nucleic acid-associated protein										2 0,10294331	2 0,01029433

P15336	Cyclic AMP-dependent transcription factor ATF-2 (ATF2)	N	Transcription related protein							2 0,10294331				2 0,01029433
P18754	Regulator of chromosome condensation (RCC1)	N	DNA nucleic acid-associated protein										2 0,11304219	2 0,01130422
P18846	Cyclic AMP-dependent transcription factor ATF-1 (ATF1)	N	Transcription related protein						2 0,17876863					2 0,01787686
P23528	Cofilin-1 (COF1)	N	Not described nuclear function						2 0,17876863					2 0,01787686
P25398	40S ribosomal protein S12 (RS12)	O	RNA nucleic acid-associated protein										2 0,27427498	2 0,02742750
P27797	Calreticulin (CALR)	N	Transcription related protein						2 0,11033631					2 0,01103363

P31949	Protein S100-A11 (S10AB)	N	DNA nucleic acid-associated protein										2 0,4251026	2 0,0425102
P35637	RNA-binding protein FUS (FUS)	N	RNA nucleic acid-associated protein					2 0,09854114						2 0,00985411
P52943	Cysteine-rich protein 2 (CRIP2)	N	Transcription related protein										2 0,19377664	2 0,01937766
P53539	Protein fosB (FOSB)	N	Transcription related protein							2 0,20226443				2 0,02022644
P62917	60S ribosomal protein L8 (RL8)	O	RNA nucleic acid-associated protein						2 0,09449979					2 0,00944998
P68431	Histone H3,1 (H31)	N	Transcription related protein										2 0,17210229	2 0,01721023

P82979	SAP domain-containing ribonucleoprotein (SARNP)	N	RNA nucleic acid-associated protein										2 0,12533558	2 0,01253356
Q13151	Heterogeneous nuclear ribonucleoprotein A0 (ROA0)	N	RNA nucleic acid-associated protein										2 0,14975699	2 0,01497570
Q8WXF1	Paraspeckle component 1 (PSPC1)	N	Transcription related protein										2 0,06701128	2 0,00670113
Q8WYK2	Jun dimerization protein 2 (JDP2)	N	Transcription related protein							2 0,17876863				2 0,01787686
Q9C005	Protein dpy-30 homolog (DPY30)	N	Transcription related protein										2 0,58489319	2 0,05848932
Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3 (SH3L3)	O	Not described nuclear function						2 0,58489319					2 0,05848932

Q9NP97	Dynein light chain roadblock-type 1 (DLRB1)	O	Not described nuclear function										2	0,46779926	2	0,04677993

Legend

N	Nucleus
O	Other

Supplementary Table 4. Results of CRAPome analysis of 17 common IL-6 promoter interactors detected using both AP-MS methodologies.

Accession	Protein Name	User Input	Mapped Gene Symbol	Num of Expt. (found/total)	Ave SC	Max SC
Q16531	DNA damage-binding protein 1 (DDB1)	DDB1	DDB1	103 / 411	4.8	52
Q92466	DNA damage-binding protein 2 (DDB2)	DDB2	DDB2	2 / 411	1.5	2
P17275	Transcription factor jun-B (JUNB)	JUNB	JUNB	9 / 411	1.4	3
P17676	CCAAT/enhancer-binding protein beta (CEBPB)	CEBPB	CEBPB	4 / 411	1.3	2
P15408	Fos-related antigen 2 (FOSL2)	FOSL2	not found			
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	PARP1	PARP1	182 / 411	11.8	151
Q13619	Cullin-4A (CUL4A)	CUL4A	CUL4A	46 / 411	3.5	25
P17535	Transcription factor jun-D (JUND)	JUND	JUND	2 / 411	1.5	2
P15407	Fos-related antigen 1 (FOSL1)	FOSL1	not found			
Q13620	Cullin-4B (CUL4B)	CUL4B	CUL4B	58 / 411	3	16
P28347	Transcriptional enhancer factor TEF-1 (TEAD1)	TEAD1	TEAD1	3 / 411	1.3	2
P27695	DNA-(apurinic or apyrimidinic site) lyase (APEX1)	APEX1	APEX1	54 / 411	2.7	8
P05412	Transcription factor AP-1 (JUN)	JUN	JUN	6 / 411	1.5	3
O14867	Transcription regulator protein BACH1 (BACH1)	BACH1	not found			
Q04206	Transcription factor p65 (RELA)	RELA	RELA	9 / 411	1.2	2
Q14938	Nuclear factor 1 X-type (NFIX)	NFIX	NFIX	10 / 411	1.4	3
P19838	Nuclear factor NF-kappa-B p105 subunit (NFKB1)	NFKB1	NFKB1	9 / 411	1.5	4

SC Spectral Counts

Curriculum Vitae

Curriculum Vitae

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Research topic: "*Study of crosstalk between GPCR-mediated signals and NF-κB signal transduction cascade*"
Promoter: Prof. Dr. Sarah Gerlo

July 2008 – October 2008
Scientific Researcher
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Research topic: "*Probing Hax-1 RNA-binding properties in respect to its domain structure*"
Promoter: Dr. Ewa Grzybowska

February 2007 – July 2007
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University of Wageningen and Research Center, The Netherlands
Minor thesis: "*Expression of Thyroid Hormone Receptor in EDS treated rat testis*"
Promoter: Dr. Katja Teerds

October 2003 – June 2008
MSc. Biotechnology
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MSc thesis: "*Analysis of the interaction of Hax-1 protein with 3'UTR of DNA polymerase β transcript in rat*", Graduation with the highest distinction
Promoter: Dr. Ewa Grzybowska

List of publications:

A proteomics strategy identifies TEF-1 as a regulator of IL-6 transcription in astrocytes. (2014) Kolmus K, Tacheny A, Eyckerman A, Dieu M, Ruyssinck E, Claeys W, Gevaert K, Renard P, Tavernier J, Gerlo S. (in preparation)

β_2-adrenergic receptors in immunity and inflammation: stressing NF-κB (2014) Kolmus K, Tavernier J, Gerlo S. Brain, Behaviour and Immunity (submitted)

β-Agonists Selectively Modulate Proinflammatory Gene Expression in Skeletal Muscle Cells via Non-Canonical Nuclear Crosstalk Mechanisms (2014) Kolmus K, Van Troys M, Van Wesemael K, Ampe C, Haegeman G, Tavernier J, Gerlo S. *PLoS One.*

Cyclic AMP: a selective modulator of NF-κB action (2011) Gerlo S, Kooijman R, Beck IM, Kolmus K, Spooren A, Haegeman G. *Cell Mol Life Sci.*

IL-1β potently stabilizes IL-6 mRNA in human astrocytes (2011) Spooren A, Mestdagh P, Rondou P, Kolmus K, Haegeman G, Gerlo S. *Biochem Pharmacol.*

Interleukin-6, a mental cytokine (2011) Spooren A, Kolmus K, Laureys G, Clinckers R, De Keyser J, Haegeman G, Gerlo *S. Brain Res Rev.*

Hunting for serine 276-phosphorylated p65. (2010) Spooren A, Kolmus K, Vermeulen L, Van Wesemael K, Haegeman G, Gerlo S. *J Biomed Biotechnol.*

Conferences:

β-agonists selectively modulate the TNF-α-induced inflammatory program in skeletal muscle cells. Kolmus K, Van Troys M, Van Wesemael K, Ampe C, Haegeman G, Tavernier J, Gerlo S. *UPAI meeting.* Brussels, Belgium, 2013

Exploring the IL-6 promoter interactome upon β_2-adrenergic/TNF-α receptor co-activation via proteomic approaches. Kolmus K, Tacheny A, Dieu M, Eyckerman S, Gevaert K, Renard P, Tavernier J, Gerlo S. *Wellcome Trust Advanced Course: Proteomics Bioinformatics*. Cambridge, United Kingdome, 2013

β-agonists potentiate TNF-α-induced expression of proinflammatory mediators in skeletal muscle cells. Kolmus K, Van Troys M, Van Wesemael K, Haegeman G, Tavernier J, Gerlo S. *Scientific day of the Faculty of Medicine and Health Sciences, UGent.* Ghent, Belgium, 2013.

Gel-free proteomic analysis of enhanceosome composition at the IL-6 promoter upon β_2-adrenergic/TNF-α receptor co-activation. Kolmus K, Tacheny A, Dieu M, Eyckerman S, De Bock P-J, Gevaert K, Renard P, Tavernier J, Gerlo S. *206th Proteomic-Interactomic Meeting of the Belgian Society of Biochemistry and Molecular Biology.* Ghent, Belgium, 2012.

Proinflammatory stimuli and β_2-adrenoreceptor agonists synergistically induce IL-6 expression in myotubes. Kolmus K, Spooren A, Van Wesemael K, Haegeman G, Gerlo S. *3rd BENELUX Nuclear Receptor Meeting.* Ghent, Belgium, 2010.

Exploring TNF-α and cAMP-induced signaling pathways regulating IL-6 expression in skeletal muscle cells. Kolmus K, Haegeman G and Gerlo S. Signal Transduction and Disease, Trinational Fall Meeting 2009 of the Society of Biochemistry and Molecular Biology of Belgium, Germany and the Netherlands, Aachen, Germany, 2009.

Awards:

First prize for the poster entitled: "*Gel-free proteomic analysis of enhanceosome composition at the IL-6 promoter upon β_2-adrenergic/TNF-α receptor co-activation*" at the 206th Proteomic-Interactomic Meeting of the Belgian Society of Biochemistry and Molecular Biology, Ghent, Belgium.

Trainings:

Novermber 2013
Wellcome Trust Advanced Course: Proteomics Bioinformatics. Cambridge, United Kingdome

April 2013
Training in R programming organized by FLAMES (Flanders Training Network for Methodology and Statistics). Ghent, Belgium

September 2012
Participant of Prime-XS program, exchange scientist involved in LOPIT (Localisation of Organelle Proteins by Isotope Tagging) technology transfer between the University of Cambridge and Ghent University. Cambridge, United Kingdom.

August 2012
VRTC – Tech Transfer and Science-based entrepreneurship – Course 2012 organized by VIB. Ghent, Belgium.

June 2012
Training in Mass spectrometry data processing organized by VIB Bioinformatics Training & Services (BITS). Ghent, Belgium

November 2011
Training in Basic bioinformatics concepts, databases and tools organized by VIB Bioinformatics Training & Services (BITS). Ghent, Belgium

Skills:

Microsoft Office Packages: Word, Excel, PowerPoint
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GraphPad Prism
Advanced Academic English: Writing Skills (Life Sciences and Medicine)
Scientific training and guidance of MSc and BCs students
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