TNFalpha expression in monocytes/macrophages

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Abstract

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Dexamethasone, a synthetic glucocorticoid, inhibits bacteria-induced TNFα production in mouse macrophages. Effects on TNFα expression when cells are pretreated with dexamethasone are a decrease in gene transcription as well as posttranscriptional inhibition. Okadaic acid, a protein phosphatase inhibitor, seems to overcome the posttranscriptional inhibition exerted by dexamethasone when TNFα expression is induced.

The posttranscriptional regulation of TNFα was further studied in human monocytic THP-1 cells. THP-1 cells express TNFα mRNA when unstimulated, but produce no TNFα protein. Induction of TNFα production can be achieved by activation of the classical MAP kinase or p38 MAP kinase pathways without any significant change in gene transcription.

TNFα mRNA expression is not detectable in primary mouse macrophages but when challenged with LPS, TNFα mRNA and subsequent protein production increases. TNFα production is regulated both at the level of gene transcription and translation of the transcripts. The kinase Mnk1, activated by ERK and/or p38, phosphorylates the translation initiation factor eIF4E and this correlates with TNFα production. A proposed signalling pathway to translational regulation of TNFα in mouse macrophages is presented.

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TNFα expression in monocytes/macrophages

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To My Family

CONTENTS

ABSTRACT

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LIST OF PAPERS

This thesis is based on the following papers and referred to in the text by their Roman numeral.

I. Gewert K, Svensson U, Andersson K, Holst E, Sundler R (1999) Dexamethasone differentially regulates cytokine transcription and translation in macrophages responding to bacteria or okadaic acid *Cell signal* 11:665-70

II. Andersson K and Sundler R (2000) Signalling to translational activation of tumour necrosis factor-α expression in human THP-1 cells *Cytokine* 12:1784-87

III. Andersson K and Sundler R Posttranscriptional regulation of TNFα expression via eukaryotic initiation factor 4E (eIF4E) phosphorylation in mouse macrophages (manuscript submitted for publication)

ABBREVIATIONS

4E-BP	eIF4E binding protein
A	adenylate
AIDS	acquired immunodefiency syndrome
AP-1	activator protein 1
ARE	AU-rich elements
ATF	activating transcription factor
cAMP	cyclic adenosine monophosphate
CD	cluster designation. Nomenclature for cell surface markers
cGMP	cyclic guanosine monophosphate
c-jun	cellular proto-oncogene product, homologous to virus-Jun avian sarcoma virus 17 oncogene
CREB	cAMP-response element binding
Dex	dexamethasone
DNA	deoxyribonucleic acid
Egr	early growth response gene
eIF	eukaryotic initiation factor
Elk	Ets-like
ERK	extracellular signal regulated kinase
Ets	E26 avian erythroblastosis virus oncogene-E twenty-six
FRAP/mTOR	FKBP12-rapamycin associated protein/mammalian target of rapamycin
GTP	guanosine triphosphate
HuR	human protein R
IκB	inhibitory κB
IKK	IκB kinase
IL	interleukin
IP_3	inositol 1,4,5-triphosphate
IRAK	IL-1 receptor associated kinase
JNK	c-jun N-terminal kinase
kDa	kilo Dalton
LITAF	lipopolysaccharide-induced TNF-alpha factor
LPS	lipopolysaccharide
Mal	MyD88-adaptor-like
MAP	mitogen activated protein , additional K means kinase
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony stimulating factor

MEK	mitogen activated protein kinase kinase
MIP	macrophage inflammatory protein
MK	MAPK activated kinase
Mnk	MAPK-interacting kinase
mRNA	messenger ribonucleic acid
Msk	mitogen- and stress-activated kinase
MyD	myeloid differentiation marker
NEMO	NFκB essential modifier
NFκB	nuclear factor κB
NF-AT	nuclear factor of activated T cells
NF-IL-6	nuclear factor interleukin 6
NO	nitric oxide
PABP	poly (A)-binding proteins
PIP_2	phosphatidylinositol 4,5 biphosphate
PKC	protein kinase C
PMA	4β-phorbol 12-myristate 13-acetate
Rsk	ribosomal S6kinase
Sp	specificity protein
TAK	transforming growth factor-β-activated kinase
TGF	transforming growth factor
TIA	T-cell intracytoplasmic (internal) antigen
TIAR	TIA-1-related-protein
TIR	Toll/Interleukin 1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFR	TNF receptor
Tpl	tumour progression locus
TRAF	tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-containing adapter molecule
U	uridylate
UTR	untranslated region

GENERAL INTRODUCTION

Background

Everyday we encounter infectious agents, such as viruses and bacteria, and when they are allowed to multiply unrestricted they cause pathological damage. One defence we have against microbes is the physical barriers of which the skin might be the most important since most infectious agents can not penetrate intact skin. Other epithelial linings such as the mucosal surfaces protect the inside of the body from harmful agents. Mucosal membranes are protective but in contrast to the dry skin, where most bacteria do not grow, the moist environment is ideal for microbial growth. Apart from the mucus itself that protects epithelial cells from infectious agents, some epithelial surfaces are covered with bacteria (referred to as the normal flora) that prevent colonization by pathogenic bacteria.

The inside of the body is protected by additional mechanisms, for instance cilia in the trachea that removes particles by its mechanical movement. In the gastrointestinal system, acid in the stomach, defensins (microbicidal peptides) in the small intestine and bacteria in the large intestine help to protect against infectious agents. The urogenital system is protected by the washing effect and acidity of urine as it flows out of the urethra and the vagina is protected by mucus, in which bacteria producing lactic acid reside, and the acidic environment thus created further improves protection. When our defensive barrier is broken the immune system is activated.

Our immune system is composed of two parts: the innate and adaptive immune systems. Innate immunity is the first line of defence against infectious agents. Phagocytes and natural killer cells are part of the innate immune system and lymphocytes account for the adaptive immune system. Inflammation is the response to an insult such as an infection. The cardinal signs of inflammation are redness, pain, swelling, heat and sometimes loss of function and are caused by increased blood supply to the infected area, a change in blood capillaries that result in oedema and migration of leukocytes into the surrounding tissues. Leukocytes are white blood cells and somewhat simplified they are classified into phagocytes and lymphocytes. Leukocytes originate from pluripotent stem cells which give rise to two lineages, one for myeloid cells and the other for lymphoid cells. The myeloid precursor cell differentiates into monocytes/macrophages, dendritic cells, granulocytes and mast cells. The granulocytes are further divided into neutrophils, basophils and eosinophils.

Circulating monocytes, tissue macrophages and neutrophils are referred to as "professional phagocytes" and once at the site of inflammation phagocytes recognize infectious agents by receptors on their cell surface. After recognition the phagocytes can engulf particles by a process called phagocytosis. Phagocytosis is mediated by activation of the phagocytes through cell surface receptors. The cell then extends around the infectious agent and the formed phagosome is fused with lysosomes containing degradative enzymes. Phagocytes also process the engulfed particle and present fragments of it on their surface allowing recognition by lymphocytes.

The adaptive or acquired immune system consists of T– and B-lymphocytes. B-lymphocytes can transform into antibody producing cells and T-lymphocytes are mediating the cellular immune response. T-cells recognize processed material from infectious agents presented by antigen presenting cells, for instance dendritic cells and phagocytes.

Macrophages

Macrophages are part of the innate immune system. They originate from the bone marrow and are released into the bloodstream as monocytes. The monocytes either reside in the blood or migrate through the vessel endothelium into the surrounding tissues. Once in the tissues they differentiate into specific macrophages. Macrophages can be found in large numbers in the lung, spleen, liver (Kupffer cells), brain (microglial cells) and body cavities such as the peritoneum. The migration of monocytes from the blood is enhanced at the site of inflammation. Monocytes/macrophages are professional phagocytes [1] and one of their main functions is phagocytosis, or ingestion of particles such as infectious agents. Macrophages are also important in clearance of damaged or dying cells. Macrophages play an essential role in inflammation (Figure 1); as they produce mediators that initiate and propagate inflammation, are antigen presenting cells and have microbicidal activity.

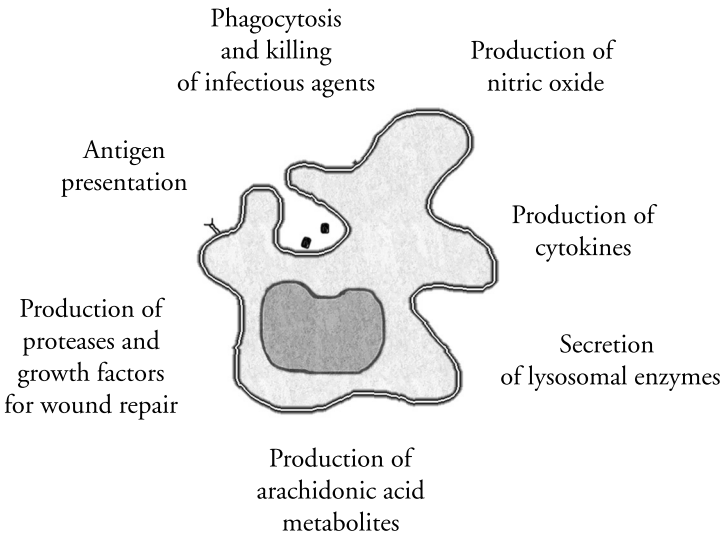

Figure 1. Schematic overview of macrophage functions in inflammation.

Bacteria that have crossed the defensive barrier encounter cells that are capable to mount an inflammatory response and macrophages are most likely to be activated by such an insult. Macrophages produce mediators essential for the inflammatory response and some of the products are listed in Table 1. The inflammatory response starts with activated macrophages producing cytokines to activate other macrophages, attract more phagocytes and initiate the process where phagocytes are allowed to adhere to the endothelium. The first cells recruited to the site of inflammation are the neutrophils and shortly thereafter the monocytes enter the tissues and differentiate into macrophages.

The clearance of apoptotic cells occurs by phagocytosis but it does not provoke an inflammatory response. In macrophages the phagocytosis of apoptotic cells invokes an anti-inflammatory response [12] including generation of IL-10 and PGE_2. This could explain the differences between phagocytosis of the apoptotic cell and the necrotic cell, as the latter induces proinflammatory mediators when engulfed.

Resident macrophages become activated (the classical pathway [13]) when exposed to two signals, where one is the priming of the cells with interferon gamma and the other is an inducer of tumour necrosis factor α (TNFα). Induction of TNFα is usually a result of an encounter with microbes or microbial products such as lipopolysaccharide (LPS). LPS is a potent stimulus of macrophages and as presented in Figure 2, LPS induces extensive spreading of the cells.

Another response elicited by macrophages after an exposure to microbes/microbial constituents, cytokines or growth factors is the production of nitric oxide (NO). NO is a short lived free radical, formed from arginine. NO synthases, enzymes responsible for the conversion of arginine to NO, are found in three isoforms. Two of these are constitutively expressed in endothelial and neural cells, whereas the inducible form is found in macrophages [9]. Chronic toxoplasma infection in macrophages induces cytotoxic effects towards fibroblasts and the effect is due to the inducible synthesis of NO [14].

Much of the tissue injury associated with the inflammatory response elicited by macrophages comes from the degradative enzymes which are released by lysosomal secretion. Secretion of lysosomal contents can be a regulated process. For instance the yeast cell wall preparation, zymosan, induces massive exocytosis of lysosomal contents in mouse peritoneal macrophages [15].

Another group of mediators produced by macrophages are the arachidonic acid metabolites, eicosanoids (prostaglandins, tromboxanes and leukotrienes). These lipid mediators are important in the inflammatory responses and e.g. prostaglandins increase vascular permeability, induce immunosuppressive activity in leukocytes and stimulate bone resorption. The synthesis of eicosanoids starts with formation of arachidonate, which is formed by hydrolysis of membrane phospholipids. The cytosolic phospholipase A_2 is the main enzyme responsible for the generation of arachidonate in mouse macrophages [16]. The formation of eicosanoids from arachidonate is catalyzed by different enzymes, cyclooxygenase 1-3 and lipoxygenase. The nonsteroid anti-inflammatory drugs inhibit the production of arachidonate lipid metabolites [17]. For instance aspirin inhibits cyclooxygenase 1 activity and paracetamol inhibits cyclooxygenase 3 [18].

Table 1. Abridged list of monocyte/macrophage derived products

Pro-inflammatory cytokines	TNFα, IL-12 [2], IL-1 [3]
Anti-inflammatory cytokines	IL-10 [4]
Chemokines	MCP-1,MIP-1α,MIP1β [5]
Arachidonic acid metabolites	Prostaglandins [6]
Complement system proteins	C1,C2 [7]
Coagulation factors	Prothrombin [8]
Plasma proteins	ApoE [7]
Free radicals	Nitric oxide [9]
Growth factors	Colony stimulating factors [10]
Mikrobicidals	Lysozyme [11]

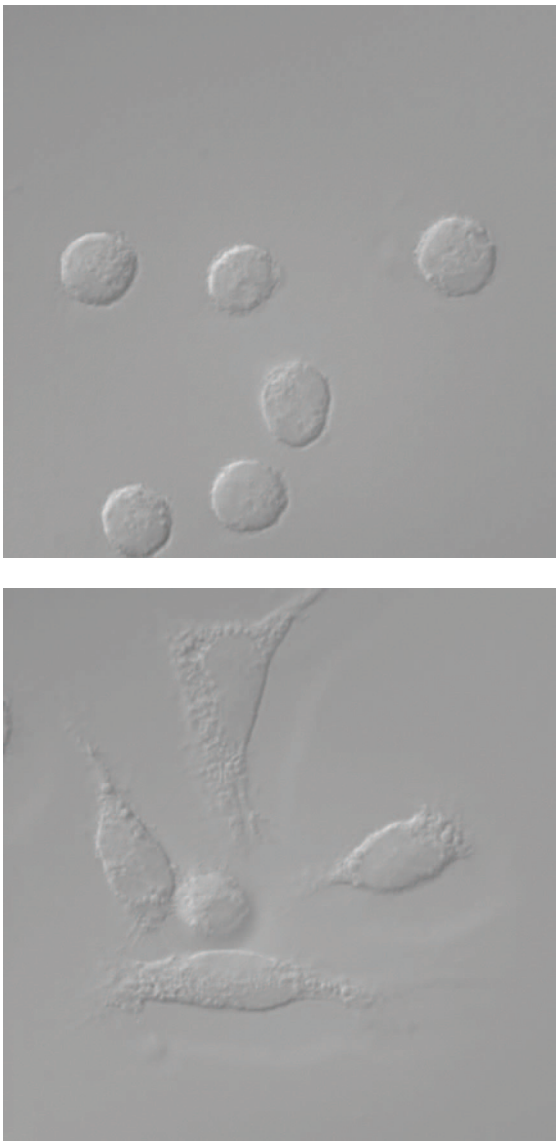

Figure 2. Resident mouse peritoneal macrophages.

Macrophages cultured overnight and thereafter left untreated (upper image) or stimulated for four hours with 10 ng/ml LPS (lower image). Images were recorded on an inverted microscope at x100 magnification.

Cytokines

Cytokines are a group of low-molecular weight proteins produced by different types of cells. They are essential mediators of immunity, inflammation, cell growth and differentiation. They mediate responses in an autocrine and/or paracrine fashion, although endocrine signalling also occurs. Excessive production of cytokines contributes to diseases related to inflammation and autoimmunity. Cytokine expressions are under strict control. Members of the cytokine group include interleukins, growth factors, interferons, tumour necrosis factors, chemokines and colony stimulating factors. Cytokines bind to high affinity receptors and have high biological activities, as they can induce responses at low molar concentrations. Cytokines induce or suppress their own synthesis, regulate expression of cytokine receptors and can antagonize or synergize with each other. The interaction between cytokines is a complex network which can be rearranged dependent on stimuli.

The name interleukin is misleading as the term means "between leukocytes" and interleukins (IL) not only affect leukocytes. They are designated IL-1 to 29 (to date 29). Interleukins are produced mainly by leukocytes and their biological effects are e.g. production of acute phase proteins from the liver, stimulation of leukocytes, antiviral responses, induction and repression of inflammation, proliferation and differentiation of cells. The anti-inflammatory cytokine IL-10 exerts its effects on monocytes partly by inhibiting the production of TNFα [19]. Interleukin 1 will be discussed in more detail below.

Growth factors are proteins binding to receptors on cell surfaces inducing cell proliferation and differentiation. Many growths factors are quite versatile as they induce cell responses in many different cell types, still there are others specific to one cell type. Transforming growth factor β (TGFβ) is involved in different activities such as proliferation of lymphocytes, differentiation of cells of the myeloid lineage and regulating immunoglobulin production [20]. TGFβ is a negative growth factor for epithelial cells [21], and many human tumours do not have the growth inhibition otherwise induced by TGFβ. Platelet derived growth factor (PDGF) was one of the first growth factors described. PDGF was first characterized as a product of platelets which stimulate proliferation of mesenchymal cells [22]. Excessive stimulation of PDGF receptor signalling leads to transformation of cells, and PDGF receptor antagonists have been suggested as cancer drugs [23].

Chemokines are chemotactic cytokines, and they attract immune cells to the site of inflammation. The induction of chemokines can either be through irritants or endogenous ligands. Chemokines are essential in the immune response, as the recruitment of circulating monocytes is an important step in the inflammatory response. Macrophages produce chemokines to attract T-helper cells, T-cytotoxic cells and natural killer cells [24].

The macrophage colony stimulating factor (M-CSF) is produced by endothelial cells, fibroblasts and monocytes/macrophages. M-CSF acts exclusively on cells of the monocytic lineage. The effects on monocytes/macrophages are enhancement of phagocytosis, chemotaxis, cytotoxicity and superoxide production [10].

Tumour necrosis factor (TNF) superfamily [25] not only includes the α and β forms of TNF but also FAS ligands and herpes virus entry mediators. TNFβ, also called lymphotoxin, is mainly produced by lymphocytes. Signalling through TNF superfamily receptors induces apoptosis, proliferation, differentiation, and modified gene expression. TNFα will be presented separately.

Interleukin 1

Interleukin 1 (IL-1) is a classical proinflammatory cytokine with diverse biological activities such as activation of the acquired immune system, fibroblast proliferation, and induction of fever. IL-1 production is increased in chronic inflammatory diseases [26] and diseases related to autoimmunity [27].

Interleukin 1 (IL-1) is produced in a variety of cells such as monocytes/macrophages, endothelial, cells and epidermal cells. The production of IL-1 is induced in cultured cells by e.g. LPS [3], phorbol esters [28], other cytokines [29], influenza virus [30] and bacteria/bacterial products [31, 32]. There are two transcripts of IL-1, designated α and β, which encode different products with molecular weight of 31 kDa. The products of IL-1 gene transcripts are both produced as cytoplasmic precursor proteins which are proteolytically cleaved yielding 17 kDa extracellular proteins.

The promoter region of IL-1 includes binding sites for AP-1 [33], NFκB, NF-IL-6 and CREB/ATF [34].

The predominant IL-1 mRNA, induced by LPS is the β transcript [29]. The exact event following the production of proIL-1 is not clear, as to way most of the expressed protein is in the precursor state and not processed and secreted. The IL-1β transcript product lacks a secretory signal peptide and one suggestion is that it is stored and released from lysosomes [35]. A fraction of the proIL-1β cell content is localized to lysosomes and stored together with its proteolytically active enzyme caspase-1. When a signal leading to exocytosis is transduced, IL-1β is secreted.

Tumour necrosis factor α

In the beginning, before TNFα was cloned and sequenced, two factors with different biological activities were discovered. One induced cachexia in chronic disease, termed cachectin, and the other induced necrosis in tumours as a result of bacterial infection. Later studies revealed that the factors were in fact the same, now designated TNFα [36].

Tumour necrosis factor α is a pleiotropic cytokine with diverse biological activities and are associated with diseases related to inflammation and autoimmunity. A summary of TNFα associated diseases are presented in Table 2. TNFα is produced mainly by monocytes and macrophages as a 26 kDa membrane bound precursor that is proteolytically cleaved yielding a 17 kDa mature soluble form. Both the cell associated and the secreted forms are biologically active in trimers. The half-life of TNFα in serum is short, ranging from 6 to 20 minutes [37], and clearance of the cytokine is via the kidneys. Initially TNFα was considered a potential antitumour agent, as it induced necrosis of tumour cells *in vitro*. Further studies revealed that the cytotoxic effect seen with TNFα was not restricted to tumours but also occurred in normal cells. However, TNFα is a potent antitumour agent when administered locally together with vasoactive drugs in isolated limb perfusion, providing a cytotoxic effect rather than systemic toxic effects [38]. On the other hand, TNFα also causes progression of cancer as it is considered a growth factor for some tumours [39].

TNFα is a key player in acute and chronic inflammation, antitumour responses and infection. The effects on acute inflammation mediated by TNF are numerous and include the activation of leukocytes and enhancement of adherence of neutrophils and monocytes to the endothelium, promotion of migration of inflammatory cells to the intercellular matrix, stimulation of proliferation of fibroblasts and induction of production of other cytokines and chemokines. In chronic inflammation, as rheumatoid arthritis and Crohn's disease, the treatment of the diseases is

a combination of anti-TNF therapy and conventional drugs [40, 41]. Anti-TNF therapy [42] is the treatment of patients with either monoclonal antibodies against TNFα or soluble TNF receptors. The two different approaches of the therapeutic agents have the same goal; to decrease TNF activity in chronic diseases. There are many other cytokines involved in the progression of the diseases, but TNFα is a central cytokine that induces the expression of other mediators [37]. The anti-TNF therapy was at first an attempt to treat sepsis as TNFα had been shown to be an important mediator in sepsis induced by Gram-negative bacteria [36].

The biosynthesis of TNFα in cellular systems is often studied by the use of endotoxin, LPS, derived from the outer membrane of Gram-negative bacteria. LPS is one of the most potent inducers of macrophage derived TNFα. A first challenge with LPS renders macrophages unresponsive to a second challenge with LPS; this is often referred to as tolerized cells. The exact mechanism by which cells become tolerized is controversial, but seems to involve decreased activation of the MAP kinases [47] ERK, p38 and JNK as well as decreased phosphorylation of IκB [48] and subsequent release of NFκB. The hyporesponsiveness induced by preexposure to LPS is not a general deactivation process as the production of nitric oxide is enhanced [49].

Table 2. Biological activities of TNFα. Example of diseases where TNFα plays a permissive role.

Septic chock [43]
Graft-versus-host disease [44]
Cachexia accompanying other diseases [36]
Rheumatoid arthritis [42]
Crohn's disease [45]
Tissue injury [46]
Insulin resistance [27]

The secretion of TNFα induced by LPS in macrophages [50] is preceded by an increased rate of transcription of the gene, augmented mRNA content and protein production. Regulation of biosynthesis occurs at the level of gene transcription as well as posttranscriptionally. Induction of TNFα gene transcription is cell type and stimulus specific. The TNFα gene promoter contains binding sites for numerous transcription factors, including NFκB, Ets, Elk-1, Sp1, NF-AT, Egr-1, ATF-2, c-jun, LITAF, CREB [51-56] and the combination of different enhancers binding to the promoter is dependent upon stimuli (virus, LPS, bacteria) and cell type (monocytes/macrophages, T cells, B cells). Posttranscriptional regulation occurs at the level of translation of mRNA (discussed below) and, as mentioned above, cleavage of membrane associated TNFα by specific proteases [57].

TNFα transduces responses through two structurally distinct receptors: type I (TNFR1) and type II (TNFR2) and these are expressed on all nucleated cells. The outcome of receptor ligation [58] is not clear as TNFR1 can induce both activation of gene transcription as well as apoptosis by the activation of caspases. The closely related TNFβ (30 % sequence identity) also signals via these receptors and induces the same responses. Macrophages are not the main source of TNFβ; instead it is produced by lymphocytes and was earlier called lymphotoxin.

SIGNAL TRANSDUCTION

Introduction

Signal transduction at the cellular level refers to signals from the outside being transmitted to the inside to induce responses in the target cell. Extracellular signalling molecules bind to cell surface receptors or intracellular receptors. The intracellular event following ligand binding to the receptor involves production of second messengers, activation of protein kinases with subsequent phosphorylation of substrates and activation of phosphatases. Second messengers can activate different classes of protein kinases. The responses to ligand binding vary between cell types and also the responsiveness of a cell to a particular ligand can be modulated.

Cell surface receptors are classified into groups depending on the signalling cascade they induce in the cytoplasm. Some receptors induce protein tyrosine phosphorylation, either the receptor has intrinsic kinase activity (e.g. receptors for growth factors) or they associate with a protein with kinase activity as for most of the cytokine receptors. Other groups are ion-channel receptors and G-protein linked receptors. The cytoplasmic event, at some point, often ends in the nucleus with a change in gene expression.

The cytoplasmic signalling cascade includes the formation of second messengers such as Ca^{2+}, IP_3, diacylglycerol, cAMP, cGMP and phosphoinositides. Calcium ions is derived from two separate sources, either extracellular influx or release from internal storage compartments. Ca^{2+} release from the endoplasmic reticulum is often preceded by generation of IP_3. Diacylglycerol and IP_3 are generated by hydrolysis of PIP_2 by phospholipase C. The generation of cAMP through activation of adenylylcyclase activates a family of serine/threonine protein kinases, cAMP dependent protein kinases (PKA). cAMP signalling is important in the regulation of IL-1β gene transcription in response to LPS, PMA and TNF [59] and exerts inhibitory effects on TNFα formation [60].

Toll-like receptors

Previous studies in *Drosophila* revealed a membrane protein, designated Toll, to participate in the immune response to invading microorganisms. By computational analysis five human proteins structurally related to Toll were revealed [61]. To date there are eleven known members [62] of the Toll like receptor (TLR) family and as presented in Table 3 they recognize different ligands. The TLR family are characterized by multiple leucine rich repeats in the extracellular domain and a cytoplasmic Toll/Interleukin 1 receptor (TIR) homology domain.

The first event in the cascade is binding of the ligand to the receptor. LPS is one such ligand and now it is well established that TLR4 is the protein tranducing the signal across the plasma membrane [73, 83-85]. Common for all TLRs are that the TIR domain interacts with an adaptor protein, MyD88, which recruits a member of the IL-1 receptor associated kinase (IRAK) family. Autophosphorylation of IRAK leading to dissociation of IRAK from the receptor complex to TRAF6. TRAF6 associates with TAK-1 and activates the transcription factor NFκB through the IKK complex.

There are four known TLR4 adaptor proteins, which all contain a TIR domain, MyD88, TIRAP, TRIF and TRAM. MyD88 was first characterized [86] and to date the most studied. MyD88 deficient mice, when challenged with LPS, show no production of inflammatory cytokines, such as TNFα [87]. Contradictory to earlier work, recent evidence has pointed out a difference between human myeloid and nonmyeloid cells regarding the utilization of MyD88 and TIRAP in TLR4 signalling to NFκB activation and cytokine production. Andreakos *et al.* [88] showed that neither MyD88, TIRAP or IKK complex are involved in LPS signalling in macrophages, while the above intermediates are essential in fibroblast and endothelial cells.

A MyD88 independent pathway in TLR4 signalling is also depicted and activates NFκB (but with delayed kinetics [87]). TIRAP [89] (also called Mal [90]) is a member of the MyD88 dependent signalling pathway, TRIF [91] and TRAM [92] are TLR adaptor proteins in the MyD88 independent pathway.

Before the discovery of TLRs, CD14 [93] was considered the LPS receptor and is expressed as a soluble protein found in serum and as a membrane protein in monocytes/macrophages and neutrophils. CD14 is a glycosylphosphatidylinositol anchored membrane glycoprotein lacking an

intracellular domain that could transduce signals. CD14 together with MD2 (extracellular protein prerequisite for TLR4 signalling [94, 95]) are found associated with TLR4 [85, 96].

Table 3. Toll-like receptors and their respective ligands

TLR1	*Neisseria meningitidis* released products (when co-expressed with TLR2) [63]
	Lipopeptides from mycobacteria (in complex with TLR2) [64]
TLR 2	Synthetic bacterial lipopeptide [65]
	Peptidoglycan and lipoteichoic acid from Gram-positive bacteria [66]
	Fimbrial protein from Porphorymonas gingivalis [67]
	Secreted products from group B streptococcus [68]
	Zymosan (TLR2 in cooperation with Dectin-1) [69]
	Endogenous Hsp70 [70]
TLR 3	Endogenous mRNA from necrotic cells [71]
	Double stranded RNA (from virus infected cells) [72]
TLR 4	LPS [73]
	Taxol [74]
	Endogenous Hsp 70 [70]
	Hyaluronic acid fragments [75]
TLR5	Bacterial flagellin [76]
TLR 6	Diacylated lipoproteins and lipopeptides from mycoplasma [77]
TLR 7	Single stranded RNA [78]
TLR 8	Single stranded RNA [79]
TLR 9	Herpes simplex virus Type 1 [80]
	Bacterial CpG DNA [81]
TLR10	No ligand found so far [82]
TLR 11	Uropathogenic bacteria [62]

Mitogen activated protein kinases

All eukaryotic cells respond to various stimuli by activating the mitogen activated protein kinase cascades (Figure 3) and subsequent phosphorylation of substrates, which include transcription factors, phospholipases, cytoskeleton proteins and other kinases (MAPK activated kinases, MKs). The MAP kinases consists of different groups, the classical MAP kinases (ERK1 and ERK2, here after referred to as ERK1/2), p38 isoforms, c-jun N-terminal kinases (JNKs), ERK3, 4 and ERK5. In general ERK1/2 respond to mitogenic signals such as growth factors, while p38 and JNK respond to stress ranging from osmotic shock to ionizing radiation and cytokine stimulation.

ERK1/2, p38 and JNK are dually phosphorylated within the conserved threonine x tyrosine motif (were x is different for the kinases) in the activation loop. Activation of JNK induces phosphorylation of the transcription factor c-jun but to date there are no known MKs as downstream targets. The p38 MAP kinase cascade results in activation of transcription factors (ATF1, ATF2, Elk-1 NFκB, and Ets-1), cytoplasmic substrates and kinases (Msk1, Msk2, Mnk1, Mnk2, MK2, -3 and -5). The MAPK p38 was first described as a kinase phosphorylated in response to LPS. The posttranscriptional regulation of TNFα has mostly been ascribed to the p38 and MK2 [97] pathway. Downstream targets of ERK1/2 include membrane proteins, cytoskeletal proteins, transcription factors (NF-AT, Elk-1, c-fos) and kinases (Mnk, Msk, Rsk).

ERK1/2 are cytoplasmic proteins in quiescent cells but when activated an accumulation in the nucleus has been reported [98]. The distribution of the p38 MAP kinase in resting cells is both nuclear and cytoplasmic and in 293T cells nuclear export of the activated kinase is mediated by the downstream target MK2 [99].

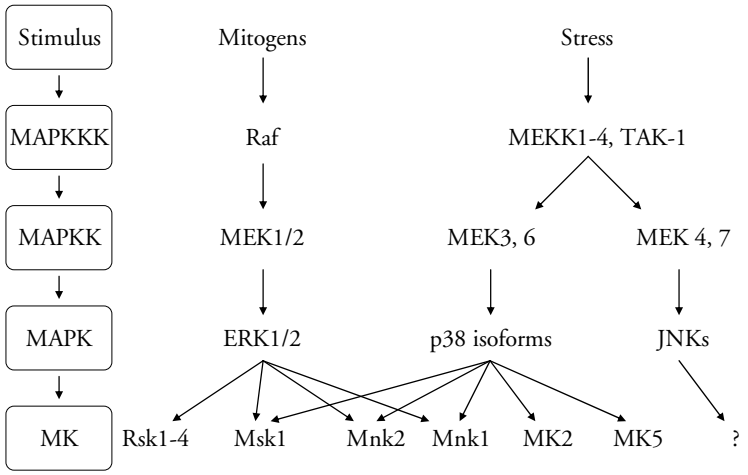

Figure 3. Mitogen activated protein kinase signalling cascade.

Signalling via TLR4 induces phosphorylation of ERK1/2, p38 and JNK MAP kinases. LPS induces activation of TAK-1 [100] which is an important intermediate activating JNK and p38, and is also involved in the stress signalling pathway. Immunomodulatory neuropeptides decrease LPS-induced TNFα expression [101] through the JNK signalling pathway.

The ERK cascade is also activated by LPS, but mitogens [102] and LPS do not utilize the same kinase upstream of MEK. Recent evidence has pointed out a role for the IKK complex [103] in the LPS-induced activation of the classical MAP kinase cascade. When inactive the MEK kinase Tpl-2 [104] is in complex with NFκB1 p105 [105]. Activation of IKK results in release of Tpl-2 from p105 and subsequent activation of MEK, proteolysis of p105 and nuclear translocation of the NFκB dimer.

As mentioned above MK2 has been considered important in the posttranscriptional regulation of TNFα expression, but the downstream target responsible for this regulation is not established although several proteins have been proposed. The RNA-binding protein tristetraprolin, a member of the zinc-finger family, is regulated by MK2 mediated

phosphorylation [106]. Tristetraprolin is involved in general mRNA metabolism and specifically it promotes decay of IL-3 mRNA [107]. Tristetraprolin is upregulated in response to LPS and binds to TNFα mRNA [108]. When mice are made deficient in tristetraprolin they develop an arthritic syndrome [109], in which an excessive amount of TNFα is responsible for the development of the phenotype. Another substrate for MK2 is the poly (A)-binding protein 1 [110], which is involved in stabilization of mRNA.

The ERK1/2 and p38 signalling pathways merge at several downstream locations and in human monocytes the combination of inhibitors for MEK1 and MEK2 (here after referred to as MEK1/2) and p38 nearly completely abolish LPS-induced TNFα expression [111], an inhibition not seen when the compounds are used individually. Another merging point from the respective pathways is the MAPK interacting kinase 1, Mnk1. Mnk1 is a serine/threonine kinase that have been suggested to phosphorylate phospholipase A_2 [112] which in turn generates arachidonate. Mnk1 can *in vitro* be activated by ERK1/2 and p38 [113]. Mnks are responsible for the phosphorylation of the translation initiation factor eIF4E [114-116].

TNFα EXPRESSION

Introduction

The synthesis of proteins is regulated by factors induced by cell proliferation, differentiation, environmental changes and cell homeostasis. The production of proteins starts with synthesis of mRNA, which carries the genetic information as a replicate of DNA. Immediately after transcription the 5' and 3' ends are modified. The modification of the 5' end is referred to as the cap structure (Figure 4) and ensures that the RNA is not degraded by nucleases. Most of the eukaryotic mRNA contains a polyA-tail at the 3' end, which is added after transcription by a specific polyA polymerase. The degradation of the polyA-tail, at least in part, determines the half-life of an mRNA molecule. The translation of mRNA into protein will be discussed in the following section.

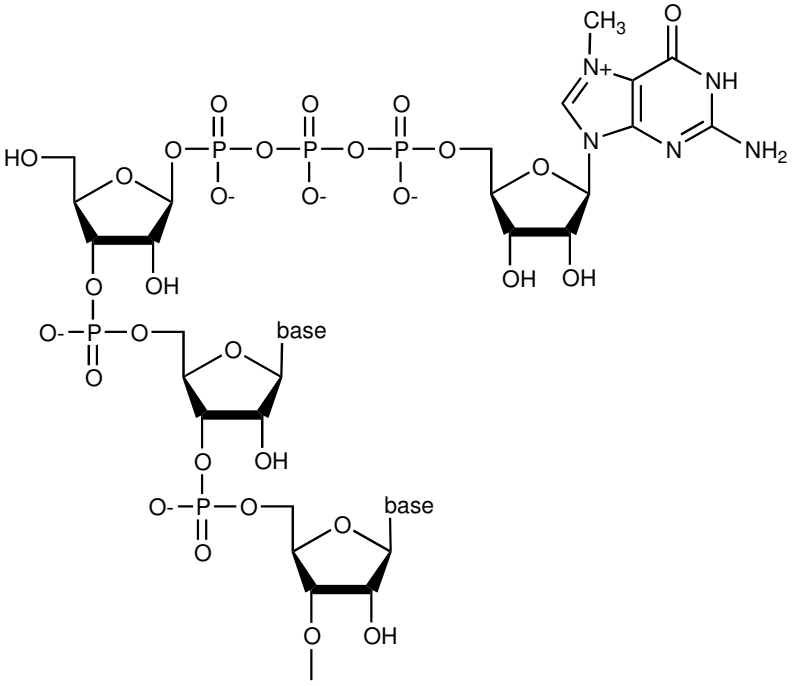

Figure 4. The cap structure of the 5' end of the mRNA molecule.

The first 5' triphosphate in the RNA chain is being modified as a phosphate is released and then a linkage between the diphosphate 5'end and the α phosphorus atom in the 5' of GTP are formed. This specific terminus is called a cap. The nitrogen (N-7) in the terminal guanine is methylated.

TNFα gene transcription

TNFα is encoded by a gene located in the major histocompatibility complex (chromosome 6 in humans and chromosome 17 in mouse). Human TNFα is initially produced as a 233 amino acid precursor, after processing giving rise to a 157 amino acid mature secreted protein [37]. The sequence homology between human and mouse TNFα is high, with 79 % identity between the mature proteins (156 amino acids in mouse TNFα) and 86 % of the amino acids are conserved in the propeptide sequence (79 amino acids in mouse) [36]. The production of TNFα requires *de novo* protein synthesis. The promoter region of TNFα contains binding sites for numerous transcription factors and their individual contribution to regulating TNFα has been studied (see references in section Introduction, Tumour necrosis factor α). The transcription factor NFκB is to date the most studied and much work in the understanding of the signalling pathway leading to TNFα production is done with studying the activity of NFκB.

NFκB is a family of 5 members which includes the NFκB proteins (p50 and p52) and Rel proteins (RelA, RelB c-rel). NFκB forms homo- or heterodimers with the heterodimer p50/RelA as the most abundant. NFκB is regulated by its inhibitory protein IκB and the complex resides in the cytoplasm, preventing NFκB from entering the nucleus. The NFκB proteins are produced as large precursor proteins, p105 and p100, which also functions as IκB proteins. When IκB is phosphorylated, by the NFκB activating IKK complex (IKK1, IKK2 and the regulatory protein NEMO) [117], it releases from the complex and promotes nuclear translocation of NFκB with subsequent activation of NFκB driven gene transcription. Activation of NFκB results in TNFα production which in turn activates NFκB through TNFR signalling pathways. Also signal transduction cascades utilized in IL-1 receptor signalling activates NFκB. Thus NFκB is an important factor in inflammatory responses as it is not only activated by exogenous stimuli but also of the cytokines regulated by the exogenous stimuli. LPS is a potent activator of NFκB via signalling through TLR4.

It should be noted that controversies over the contribution of NFκB in the human promoter regulating TNFα gene expression exists [118, 119]. The transcription factor AP-1 is activated by protein kinase C (PKC) and in human monocytes LPS-induced TNFα production is diminished by PKC inhibitors [120]. The transcription factor AP-1 consists of heterodimers of jun/fos proteins or homodimers of jun proteins. The exact signalling

pathway involving protein kinase C as a downstream target of TLR4 is to date not clear.

TNFα mRNA

The mRNA of many cytokines includes an AU-rich sequence in the 3'-untranslated region (3'-UTR) which is critical for their posttranscriptional regulation [121]. AU-rich elements (ARE) contain one or several copies of the pentamer AUUUA. Mutant mice lacking TNF AU-rich elements develop chronic inflammatory arthritis and inflammatory bowel disease [122]. The mutant mice not only spontaneously produce significant levels of TNFα, they also overexpress TNFα, with increased mortality rate as a consequence, when injected peritoneally with LPS. The proteins binding to the ARE sequence in TNFα mRNA regulates transcript stability or translation. Several proteins with different regulatory functions have been shown to interact with ARE in TNFα transcripts.

Tristetraprolin, a cytoplasmic protein regulated by phosphorylation, destabilizes TNFα mRNA [123] by promoting deadenylation (shortening) of the transcripts [124]. In mouse macrophages stimulated with LPS, the major detectable protein associated with the *cis*-acting element, ARE, of TNFα mRNA is the stabilizing factor HuR [125]. HuR, when overexpressed, stabilize a reporter construct containing the TNFα ARE. Another *trans*-acting factor regulating TNFα translation is the RNA-binding protein TIA-1, which binds with high affinity to uridylate-rich motifs. Knockout studies with TIA-1 and its close relative TIAR revealed their essential significance in embryonic development, as for TIAR 100 % embryonic lethality was observed (performed in BALB/c mice, note that embryonic lethality in TIAR mutants is strain specific [126]) and partial lethality with mutants lacking TIA-1 [127]. Peritoneal macrophages from mutant mice with targeted disruption of TIA-1 secrete significantly more TNFα than wildtype mice when challenged with LPS. The proposed role for TIA-1 in regulating TNFα expression is as a specific translational silencer, since production of IL-6 or IL-1β is unchanged. When TIA-1 is not present, TNFα transcripts shift to increased association with polysomes. Other proteins have been shown to bind to the ARE sequence in TNFα transcripts but their exact role in posttranscriptional regulation has not been defined [128, 129]. The different regulatory roles for the *trans*-acting factors binding to the 3'UTR of TNFα mRNA might be a way to orchestrate a proper immune response, as the factors can compete for the binding sites.

The involvement of the p38 signalling pathway in the regulation of TNFα expression is widely studied, and much work has been done with the pyridinyl imadazole [130] compound SB203580 or other related compounds and knockout studies. Pyridinyl imidazole compounds were first described as inhibitors of LPS-induced TNFα production [131] and later shown to inhibit p38 activity [130, 132]. Further insights into the signalling cascade regulating TNFα biosynthesis came with the characterization of MK2 as a kinase involved in LPS induced TNFα expression. When mice lacking MK2 [97] are challenged with LPS they produce significantly lower amounts of TNFα than wildtype mice.

Translation of eukaryotic mRNA

Translation of mRNA to protein is a controlled process and can be divided into three different steps: initiation, elongation and termination. Elongation, the process where amino acids are joined together in a growing polypeptide chain, and termination, when the ribosomes reach a stopcodon will not be discussed any further.

The initiation process [133] includes the formation of a 43S pre-initiation complex, assembly of the complex to mRNA, followed by scanning in 5'-> 3' direction of the mRNA until the startcodon (AUG) is recognized and finally joining of the 60S subunit to form an 80S ribosome. Translation initiation of transcripts is a complex process with several components and rate limiting in the process is eukaryotic initiation factor 4E (eIF4E). Whether translation initiation is limited by abundance of eIF4E or its availability is not clear. The initiation factor eIF4E binds to the cap structure of mRNA and interacts with eIF4G. The eIF4F complex (Figure 5) consists of eIF4E, eIF4A (a RNA helicase believed to unwind secondary structures in the 5'UTR) and eIF4G. The scaffold protein eIF4G interacts with eIF4E, eIF4A, eIF3 (part of the 43S pre-initiation complex), poly (A) binding proteins (PABP) and the eIF4E kinase Mnk1. The presumptive circularization of the mRNA [134] is accomplished by PABP [135] interacting with eIF4G, bringing the 3'UTR in close proximity to the 5'UTR. This could explain how the regulatory sequences in the 3'UTR can control translation initiation.

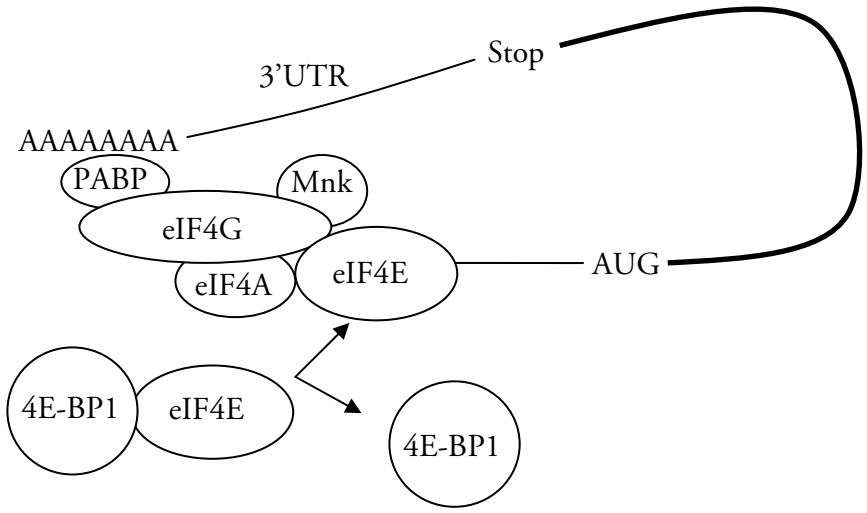

Figure 5. Simplified drawing of eIF4F complex in eukaryotic translation initiation.

The translation initiation factor eIF4E is mostly studied in general protein synthesis but has also been described as a regulatory protein in translation of specific transcripts [136, 137] and transport of proteins from the nucleus to the cytoplasm [138]. As mentioned above eIF4E binds directly to the 5'cap structure of the mRNA and to function in cap-dependent translation, eIF4E must form complex with eIF4G.

The interaction between eIF4E and eIF4G is under tight control and is prevented by inhibitory proteins termed 4E binding proteins (4E-BPs). When 4E-BP is hypophosphorylated, it is in complex with eIF4E, and when phosphorylated it releases eIF4E and eIF4F can form. Six phosphorylation sites are identified on 4E-BP [139] and the kinases responsible for the respective phosphorylations and subsequent release of eIF4E are still in question. FRAP/mTOR has been identified as one of the kinases involved in the regulation of 4E-BP phosphorylation [140]. In mouse macrophages, LPS-induced phosphorylation of 4E-BP1 is mediated by FRAP/mTOR and independent of MEK1/2 and p38 as judged by the use of inhibitors for these kinases. [141].

The scaffold protein eIF4G also interacts with the eIF4E kinase Mnk1 [142]. The exact role of phosphorylation of eIF4E [143] is not understood, but under a variety of conditions, where protein synthesis generally is activated, phosphorylation of eIF4E increases. Cell stress, phorbol esters, cytokines [114] and LPS [116] mediate phosphorylation of eIF4E.

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PRESENT INVESTIGATION

Aims

The overall aim of this thesis was to study the regulation of TNFα production in cells of the monocytic lineage. The specific aims were as follows:

- Evaluate the inhibitory effects on cytokine production exerted by the synthetic glucocorticoid dexamethasone
- Assess if the human monocytic cell line THP-1 can be used as a tool for studying the translational regulation of TNFα
- Delineate the signal transduction pathway regulating posttranscriptional modification of TNFα gene transcripts

The following section discusses the different aspects of TNFα expression studied in each paper.

Agents used in this investigation

A schematic overview of inhibitors and stimuli used in this investigation are illustrated in Figure 6.

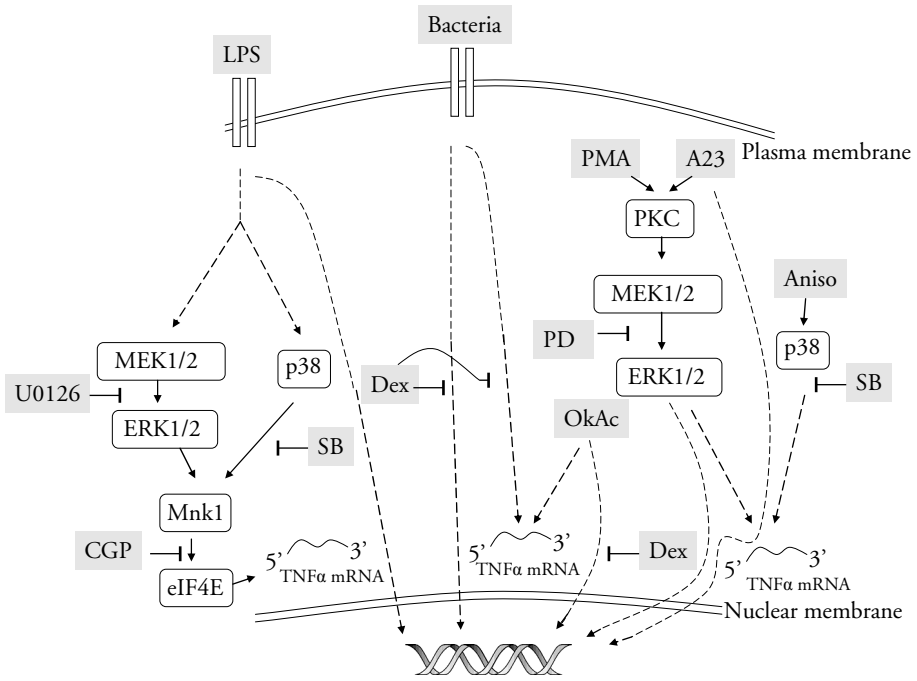

Figure 6. Agents used in Paper I-III.

Dashed lines represent additional steps not investigated nor shown here. Agents used are highlighted. SB – SB203580, CGP- CGP57380, OkAc – okadaic acid, Aniso – anisomycin, A23 – A23187, PD – PD98050, dex – dexamethasone, PMA - 4β-phorbol 12-myristate 13-acetate.

Paper I

Bacteria isolated from patients with genital infections, which have been reported to induce cytokine expression in macrophages [31] were used in the experiments. Both Gram-positive (*Propionibacterium acnes* and *Peptostreptococcus anaerobius*) and Gram-negative bacteria (*Fusobacterium nucleatum*) were used.

Dexamethasone is a synthetic glucocorticoid with immunosuppressive actions. Glucocorticoids in general exert their effects by binding to proteins in the cytoplasm. The complex then translocates to the nucleus and mediates regulation of gene transcription. Dexamethasone has earlier been reported to inhibit IL-1β gene transcription and mRNA stability in human monocytic cells [144] and to inhibit LPS-induced IL-1β gene transcription by blocking transcription factor activation [34]. In a mouse macrophage cell line, the MAP kinase JNK has been proposed as target for the translational inhibition on TNFα exerted by dexamethasone [145].

Okadaic acid is a potent tumour promoter, which functions as a serine/threonine protein phosphatase inhibitor and induces phosphorylation of many different kinases.

Paper II

The phorbol ester PMA is a diacylglycerol analogue and activates protein kinase C (PKC). PKC is a family of proteins and classified into three groups based on sensitivity towards diacylglycerol and/or Ca^{2+}. In human monocytes PKC transduces signals from the LPS receptor to transcription of the TNFα gene mediated by MAP kinases and the transcription factor AP-1 [120]. PKC is also involved in production of the cytokine IL-1β by activating AP-1 [146]. A role in the translational machinery for PKC could be postulated as it mediates phosphorylation of eIF4E in Chinese hamster ovary cells [147].

The calcium ionophore A23187 raises the calcium ion concentration in the cell and together with PMA activates PKC. The role of Ca^{2+} as a second messenger is widely studied and in T-cells the production of TNFα is regulated by the calcineurin dependent transcription factor NFAT [148]. Calcineurin is a protein phosphatase regulated by Ca^{2+}. When the concentration of Ca^{2+} is high, calcineurin binds to and dephosphorylates NFAT and thereby activates transcription of genes.

Anisomycin [149] was used in the experiments to activate the MAP kinase p38 [150] (JNK is also activated by ansiomycin, unpublished results). When anisomycin is used at high concentrations it inhibits protein synthesis but at the concentrations used here no such inhibition is observed.

PD98059 [151] and U0126 [152] are MEK1/2 inhibitors and the only known substrates of MEK1/2 are the classical MAP kinases ERK1/2.

The p38 inhibitor SB203580 is a widely used compound and much of the observed findings regarding p38 and its role in signal transduction come from studies using this inhibitor.

To study the efficiency of translation of TNFα mRNA the protein synthesis inhibitor cycloheximide was included in the experiments. Cycloheximide inhibits translation and an increase in accumulation of gene transcripts is seen.

LPS was also used here and will be covered in the next section.

Paper III

LPS, also called endotoxin, is a classical stimulus of monocytes/macrophages. Bacterial endotoxin comes from the cell wall of Gram-negative bacteria, and here we used LPS derived from the bacterium Escherichia Coli 055:B55.

It is now settled that TLR4 is the membrane receptor responsible for transducing the LPS response [153], but TLR2 was first proposed to be the long sought LPS receptor [154] and later shown not to be essential for the response to LPS [155]. Instead TLR2 is the membrane receptor that detects peptidoglycan and bacterial lipopeptides derived from Gram-positive bacteria [66]. CD14, a glycoprotein that is expressed both as a membrane-bound and a soluble form, recognizes the complex LPS and LPS-binding protein found in serum. Both forms can bind Gram-negative bacteria [156] and the focus now is on the role CD14 plays in TLR4 signalling. CD14- and serum-dependent LPS-induced activation of monocytes can be overcome by high concentrations of LPS [157]. When monocytes/macrophages are challenged with LPS they produce pro-inflammatory cytokines as well as anti-inflammatory mediators. IL-10 inhibits LPS-induced TNFα production in alveolar macrophages [158]. Another autocrine regulation of LPS activation is that the endogenously produced prostaglandins decrease the production of TNFα [159].

The p38 inhibitor SB203580 used either on its own or in combination with the MEK1/2 inhibitor U0126. The Mnk1 inhibitor CGP57380 [160], a low molecular weight compound, was used to prevent phosphorylation of eIF4E.

Dexamethasone differentially regulates cytokine transcription and translation in macrophages responding to bacteria or okadaic acid (Paper I)

Macrophages were chosen for their ability to produce cytokines in response to challenge with bacteria. This study was performed with resident macrophages obtained by peritoneal lavage of female mice. Over night cultures of cells were stimulated with either clinical isolates of bacteria or the protein phosphatase inhibitor okadaic acid. The synthetic glucocorticoid dexamethasone (dex) is a very potent anti-inflammatory drug used as a pharmacological agent. Pretreatment of the cells with dex was done over night for 20 hours. There was no difference between Gram-positive or Gram-negative bacteria in the induction of cytokines or the inhibitory effect exerted by dex on the bacteria-induced cytokine expression. Dex has earlier been shown to inhibit lysosomal secretion in macrophages [161]. Other effects exerted by dex are to cause downregulation of cytosolic phospholipase A_2 and to suppress its activation [162]. Dex has been shown to modulate cytokine responses in macrophages [163] and here pretreatment with dex was performed to evaluate how it exerts its effect on bacteria-induced cytokine expression in mouse macrophages.

The cytokine interleukin 1β (IL-1β) is produced as an unprocessed protein, termed proIL-1β and that is the only form found in mouse macrophages. In unstimulated cells proIL-1β can be detected and the basal level of proIL-1β is inhibited by pretreatment of the cells with dex. Pretreatment with dex inhibits bacteria-induced proIL-1β production at the level of transcription in a dose-dependent manner. Opposed to the effects on proIL-1β production, dex inhibits TNFα expression both on the level of transcription and translation.

The protein phosphatase inhibitor okadaic acid was also used as an inducer of TNFα production. Okadaic acid treatment of the cells gave rise to a different distribution of TNFα, than when bacteria were used as stimuli. Both Gram-positive and Gram-negative bacteria nearly completely produce expression of the proteolytically cleaved secreted TNFα, while okadaic acid also induces cell-associated TNFα. Dex inhibits okadaic acid-induced TNFα primarily at gene transcription, since both TNFα mRNA and protein are inhibited in parallel. TNFα production is regulated at different levels and posttranscriptional regulation also includes proteolytic cleavage of proTNFα. When okadaic acid is used as an inducer of TNFα not only the 26 kDa cell-associated TNF is produced, but secreted and cell-associated 17

kDa form can also be found. The secreted 17 kDa and cell-associated 26 kDa TNFα are inhibited by dex, while the 17 kDa cell-associated form is unaffected.

In conclusion, dexamethasone inhibits bacteria-induced proIL-1β production at the level of transcription, while the formation of TNFα is inhibited by effects on both transcription and translation. The translational inhibition appears to be overcome by the protein phosphatase inhibitor okadaic acid.

Signalling to translational activation of tumour necrosis factor-α expression in human THP-1 cells (Paper II)

Human monocytic THP-1 cells [164], were used in this study and were found to express detectable TNFα mRNA. No protein was produced despite TNFα mRNA expression. THP-1 cells have in the unstimulated condition, translational inhibition affecting TNFα.

Phorbol esters, by mimicking diacylglycerol, directly activate PKC, which have been reported to mediate phosphorylation of MEK1/2 [165]. Here the phorbol ester PMA was used to induce TNFα secretion, and when mRNA expression was studied no induction of TNFα mRNA could be detected. Instead PMA caused a derepression of translation. The PMA-induced TNFα release is sensitive to the MEK1/2 inhibitor PD98059 and the basal TNFα mRNA expression is also mediated via MEK1/2.

Calcium signalling have been reported to activate transcription factors [166], and when PMA was used together with the calcium ionophore A23187 a dramatic increase in secretion of TNFα was detected, which was not reflected on the mRNA level. Since the MAP kinase p38 had already been reported to be involved in translational regulation of TNFα [167], the p38 activator anisomycin was tested and found to be an inducer of TNFα production. The p38 inhibitor SB203580 decreased TNFα production and somewhat surprisingly the MEK1/2 inhibitor U0126 exerted the same effect.

One possible explanation is that MEK1/2 is responsible for the low basal TNFα mRNA expression and also important in the translational derepression induced by p38 activation. When LPS was used as a stimulus, p38 inhibition resulted in decreased TNFα production, as reported by others [131], and MEK1/2 inhibition completely reversed the induction of TNFα.

In human monocytic cells, the MAPKK MEK1/2 and the MAP kinase p38 mediate translational activation and could also reverse the translational inhibition on TNFα mRNA

Additional information

The posttranscriptional derepression observed with PMA as an inducing agent was further studied. One possible explanation for the translational derepression is that PKC posttranscriptionally regulates TNFα mRNA by stabilizing the transcripts. In human embryonic fibroblasts, PMA stabilizes p21WAF1 transcripts by increasing the half life of the mRNA [168].

There was no significant difference between unstimulated and PMA treated cells regarding the stability of TNFα transcripts (Figure 7).

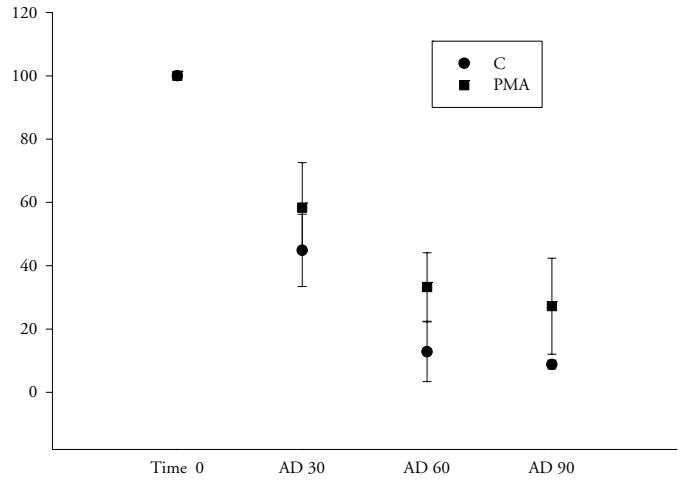

Figure 7. Stability of TNFα mRNA.

THP-1 cells were treated for 30 minutes with 100 nM PMA and then 2 μg/ml Actinomycin D (inhibits gene transcription) were added and at different time points (30 minutes – AD30, 60 minutes -AD 60, 90 minutes – AD90 and 120 minutes not shown as the amount of TNFα mRNA were undetectable) cells were processed for total RNA extraction. 10 μg of total RNA was separated on agarose gel and transferred to nylon membrane TNFα mRNA was detected by a cDNA probe and normalized for β-actin. Unstimulated cells and PMA-treated cells are set to 100 %. Results from three independent experiments are shown.

To further test for the mechanism by which PMA causes derepression of TNFα translation, polysome analysis was performed. When LPS is used as a TNF inducing agent a switch to polyribosome associated TNFα mRNA is detected [169]. As shown in Figure 8 no change in the distribution of TNFα mRNA is observed when PMA is used as stimulus.

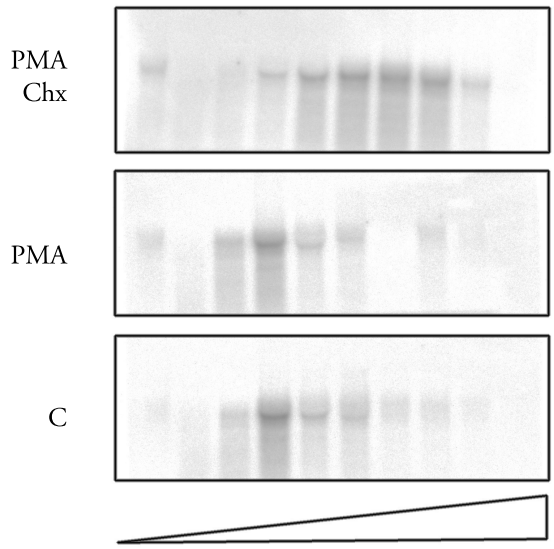

Figure 8. Polysome analysis.

The image shows TNFα, separated by increasing number (left to the right) of associated ribosomes. Cells left untreated (C), stimulated with PMA (PMA) or pretreated with the protein synthesis inhibitor cycloheximide and thereafter stimulated with PMA (PMA Chx) were lysed in buffer containing 10 mM Tris pH 7.3, 150 mM NaCl, 10 mM $MgCl_2$, 1 % Igepal CA630, 1 mM DTT and 1U/µl RNasin (Promega). The lysates were centrifuged [170] over a 10-50 % sucrose gradient and 10 fractions were collected and processed for total RNA extraction. Equal amounts of total RNA were separated on agarose gels and RNA transferred to nylon membranes. The membranes were hybridized with a ^{32}P labelled TNFα probe and analyzed by phosphorimaging. Results are representative of two different experiments. The protein synthesis inhibitor was used to ensure that a shift in polyribosome association can be detected.

Posttranscriptional regulation of TNFα expression via eukaryotic initiation factor 4E (eIF4E) phosphorylation in mouse macrophages (Paper III)

To further study the translational inhibition presented in Paper II, pretreatment of primary mouse macrophages with MAP kinase inhibitors was evaluated. Treatment of mouse macrophages with PMA or anisomycin was also tested and neither of the drugs increased the production of TNFα (not published). Over night cultures of mouse macrophages were pretreated with the p38 inhibitor SB203580 or the MEK1/2 inhibitor U0126 and then stimulated with LPS. As presented in Paper II human THP-1 cells express TNFα mRNA, while in primary culture of mouse macrophages TNFα mRNA can not be detected and this could be the explanation why PMA or anisomycin do not induce expression of the protein. When TNFα expression was measured, no significant inhibition was detected by either inhibitor. It was puzzling to find that SB203580, first described as a compound inhibiting TNFα production in human monocytes [131], did not have the same effect in mouse peritoneal macrophages.

The lack of inhibition of LPS-induced TNFα production by pretreatment of the cells with SB203580, has been reported by others [171], and was not a result of p38 not being inhibited, as judged by the activity of the downstream kinase MK2. One possible explanation is that in mouse macrophages both p38 and MEK1/2 (and presumably ERK1/2) are involved in translational regulation of TNFα, and when the two inhibitors were used together a dramatic decrease in the LPS-induced TNFα production was shown. This decrease on protein level was not reflected on mRNA, further suggesting a role for p38 and MEK1/2 in translational regulation.

As discussed in section Signal transduction: mitogen activated protein kinases, one substrate for both p38 and ERK1/2 is Mnk1. By using phosphospecific antibodies against Mnk1, LPS was found to mediate phosphorylation of the kinase. Pretreatment of the macrophages with the combination of the two inhibitors resulted in total loss of LPS-induced phosphorylation of Mnk1. A low molecular weight compound CGP57380, known to inhibit Mnk1 [160], was now evaluated. CGP57380 was found to decrease LPS-induced TNFα production in a dose-dependent manner and the slight decrease in TNFα mRNA expression could not explain the effects on protein production. Mnk1 and Mnk2 phosphorylate the translation initiation factor eIF4E, and its phosphorylation state in response

to LPS was next studied. Indeed, LPS-induced phosphorylation of eIF4E was mediated by p38 and MEK1/2 through Mnk1, as evaluated by the use of inhibitors.

Many kinases have been reported to shuttle between the cytoplasm and the nucleus. Accumulation of TNFα mRNA in the nucleus has been ascribed to kinase inhibition [172]. One possible explanation to our findings would be that TNFα mRNA accumulated in the cell nucleus in response to pretreatment of the cells with kinase inhibitors. To test for possible accumulation of TNFα mRNA in the nucleus, the mouse macrophage cell line RAW 264.7 was used. The macrophage cell line was chosen to obtain sufficient amount of cells. RAW 264.7 cells respond in the same manner as primary cells in regard to pretreatment with CGP57380 of LPS-stimulated cells. RAW 264.7 cells produce more TNFα in response to LPS, and they do not response to exogenously administered PGE_2 by a decrease in TNFα expression. The lack of inhibition seen with PGE_2 treatment of LPS stimulated cells could be explained by a recent report showing that RAW 264.7 cells lack one of the membrane receptors for prostaglandins [173]. Subcellular fractionations were performed and the distribution of TNFα mRNA was studied and no difference was found between LPS-stimulated cells and LPS-stimulated cells pretreated with the Mnk1 inhibitor CGP57380.

Presented here is a translational regulation of TNFα mRNA mediated by phosphorylation of eIF4E.

CONCLUDING REMARKS

The synthetic glucocorticoid dexamethasone suppresses bacteria-induced TNFα production in mouse macrophages both transcriptionally and posttranscriptionally. The effects observed with dexamethasone has not been studied any further here, instead the posttranscriptional regulation of TNFα has been in focus. The human monocytic cell line THP-1 was found to be a tool for studying the posttranscriptional regulation of TNFα, and that MEK1/2 and p38 are involved in regulating TNFα expression. The posttranscriptional regulation of LPS-induced TNFα production in mouse macrophages is regulated via MEK1/2 and p38 at a combined merging point, the protein kinase Mnk1. Mnk1 in turn, phosphorylates the translation initiation factor eIF4E. The production of TNFα in macrophages correlates with phosphorylation of the translation initiation factor eIF4E. The LPS-induced phosphorylation of eIF4E, mediated by TLR4 signalling intermediates, is depicted in Figure 9.

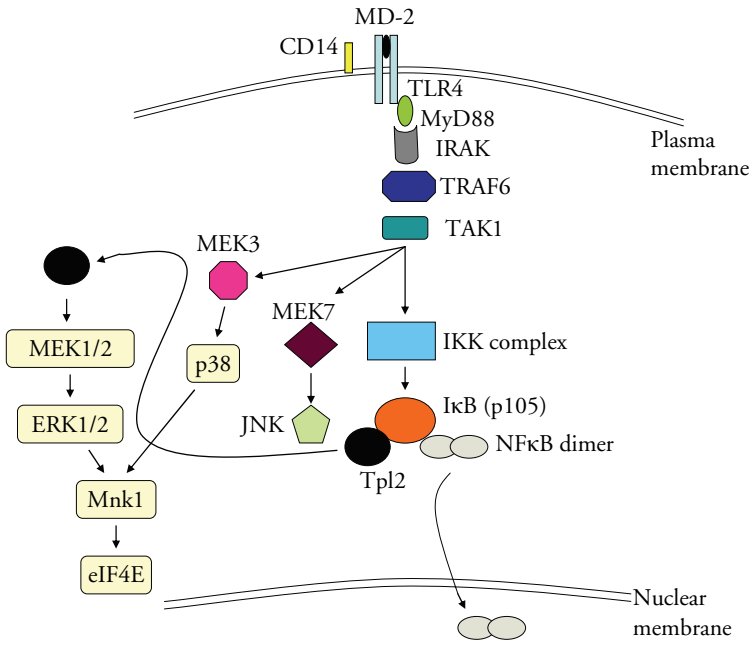

Figure 9. LPS signalling through TLR4.

Proposed signalling pathway for LPS induced phosphorylation of eIF4E.

ACKNOWLEDGEMENTS

I have been here at the Department of Cell and Molecular Biology (Experimental Medical Science, nowadays) for so many years that I have come to meet so many people that this section easily could be a major part of this thesis. Instead I decided to make it very short…

Special thanks to my supervisor, Professor Roger Sundler, who always supports me in my search for **the** explanation to the mystery of TNF production. Thank you for believing in me.

Hans Tapper, I have no words (or the truth would be that there are so many words and I hope I've already told you at least some). I owe you (more than one). Anytime!!!

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunförsvar delas upp i två avdelningar, medfött och adaptivt immunförsvar. Vita blodkroppar utgör de celler som är viktigast i immunförsvaret. En sorts vita blodkroppar är monocyter/makrofager som har till uppgift att rensa bort bakterier och andra partiklar som kan orsaka skada. Monocyter/makrofager finns överallt i kroppen och spelar en viktig roll vid inflammationsprocessen. Förutom sin förmåga att fagocytera (äta upp) bakterier så producerar de en rad signalsubstanser som driver på eller hämmar inflammationsprocessen. En grupp av dessa substanser är cytokiner, de är proteiner som spelar en viktig roll i inflammationsprocessen såsom att dirigera celler till området som är inflammerat, stimulera tillväxt av celler och även att avsluta hela processen. Överdriven produktion av cytokiner har visat sig vara en bidragande orsak i många sjukdomstillstånd. En strikt kontroll av cytokin produktionen hos celler i immunförsvaret är essentiell för att en akut inflammation inte ska bli kronisk. Sjukdomar som associeras med oreglerade cytokin-nivåer är bl.a. ledgångsreumatism och kroniska inflammatoriska tarmsjukdomar.

Det främsta målet med denna avhandling har varit att undersöka hur monocyter/makrofager, reglerar sin produktion av en speciell cytokin, tumörnekrotisk faktor (TNF). TNF produceras främst av monocyter/ makrofager i samband med inflammation. Vid den akuta inflammationen har TNF en stor roll i att aktivera andra vita blodkroppar, framkalla frisättning av andra cytokiner och att möjliggöra att andra vita blodkroppar kan förflytta sig från blodbanan ut i den skadade vävnaden. Det är vid den akuta inflammationen som TNF har positiva effekter och det är främst vid kroniska inflammatoriska sjukdomar som den har negativa effekter. TNF spelar en stor roll vid kroniska inflammatoriska sjukdomar såsom ledgångsreumatism vilket främst poängteras genom den positiva effekt som anti-TNF terapi har. Svåra akuta inflammationer som t.ex. chock till följd av blodförgiftning, där TNF produceras i alltför stora mängder, utgör direkt livshotande tillstånd.

De flesta cytokiner finns normalt inte i cellerna utan produceras först när en förändring inträffar som signalerar att de behövs. En sådan förändring kan vara när en makrofag träffar på en bakterie. För att kunna producera exempelvis TNF behöver cellerna aktivera en process där genen först skrivs av till en produkt som kallas RNA, som i sin tur kommer att översättas och bilda protein.

När en makrofag träffar på exempelvis en bakterie kommer bakterien att fästa på ytan på makrofagen och starta en kedja av händelser i cellen som till slut kommer att resultera i produktionen av TNF. Denna kedja byggs bl. a upp av att proteiner förändrar utseende och aktivitet hos andra proteiner och slutligen bildas och frisätts TNF.

Denna avhandling baserar sig på tre delarbeten i vilka studier är gjorda för att förstå hur cellerna reglerar produktionen av TNF.

I det första delarbetet har makrofager från möss använts. Dessa makrofager har tagits ut från bukhålan på möss och är vad man kallar primära celler. Experiment har gjorts med en substans, dexametason, ett kortisonpreparat som används för att minska inflammationer. För att frisätta cytokiner har bakterier använts för att aktivera cellerna. Dexametason visade sig minska produktionen av cytokiner, genom att hämma både avskrivningen av genen och omvandlingen till protein.

I det andra delarbetet har omvandlingen av RNA till protein studerats. Här har celler använts, som till skillnad från cellerna i delarbete 1, hela tiden delar sig och förökar sig. Dessa celler, till skillnad från primära celler, har RNA för TNF även om man inte har aktiverat dem. Trots att RNA finns, frisätts inte TNF. Detta beror på en hämning av omvandlingen från RNA till protein. Genom att direkt påverka olika proteiner som tidigare visat sig ha betydelse för TNF-frisättning visade det sig att två olika proteiner oberoende av varandra kan ta bort hämningen som finns på omvandlingen av RNA till protein.

I det sista delarbetet har samma typ av celler använts som i det första delarbetet. Här har makrofager aktiverats med ett ämne som finns på ytan av vissa bakterier. Med hjälp av kemiska substanser som hämmar olika proteiner i signalkedjan har det visat sig vara ett protein som binder till RNA som står för regleringen av omvandlingen från TNF-RNA till TNF-protein. Här finns även presenterat vilka protein som i sin tur reglerar det RNA-bindande proteinet.

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