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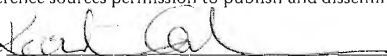
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TNF α expression in monocytes/macrophages

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- 2005 -

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To My Family

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ABSTRACT

The recognition of microbes or microbial products by leukocytes is a key event in innate immunity. Binding of microbial products to cell surface receptors on phagocytes induce a response that includes the production of inflammatory mediators. One such mediator is the cytokine tumour necrosis factor α (TNF α). TNF α is a pleotropic cytokine with actions ranging from tissue injury to septic chock. This thesis is based on studies performed with the use of different stimuli (either bacteria, the bacterial product LPS or direct activators of protein kinases) which induce TNF α production in cells of the monocytic lineage. The main focus has been to study the signal transduction pathway regulating TNF α expression.

Dexamethasone, a synthetic glucocorticoid, inhibits bacteria-induced TNF α production in mouse macrophages. Effects on TNF α 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 TNF α expression is induced.

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

TNF α mRNA expression is not detectable in primary mouse macrophages but when challenged with LPS, TNF α mRNA and subsequent protein production increases. TNF α 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 TNF α production. A proposed signalling pathway to translational regulation of TNF α in mouse macrophages is presented.

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 TNF α expression via eukaryotic initiation factor 4E (eIF4E) phosphorylation in mouse macrophages (manuscript submitted for publication)

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ABBREVIATIONS

| | |
|-----------------|--|
| 4E-BP | eIF4E binding protein |
| A | adenylate |
| AIDS | acquired immunodeficiency 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 |
| I κ B | inhibitory κ B |
| IKK | I κ B 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 | NF κ B essential modifier |
| NF κ B | nuclear factor κ B |
| 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 | 4 β -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.

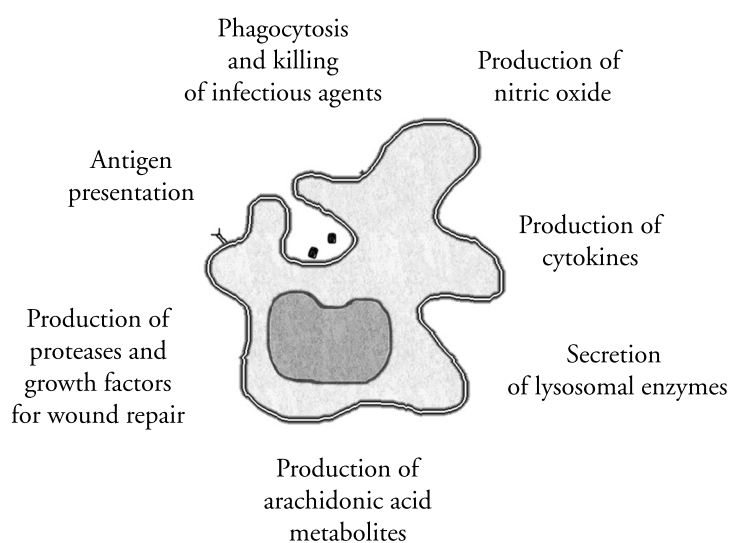


Figure 1. Schematic overview of macrophage functions in inflammation.

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 PGE₂. 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 (TNF α). Induction of TNF α 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, thromboxanes 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 A₂ 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 | TNF α , IL-12 [2], IL-1 [3] |
| Anti-inflammatory cytokines | IL-10 [4] |
| Chemokines | MCP-1, MIP-1 α , MIP1 β [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] |

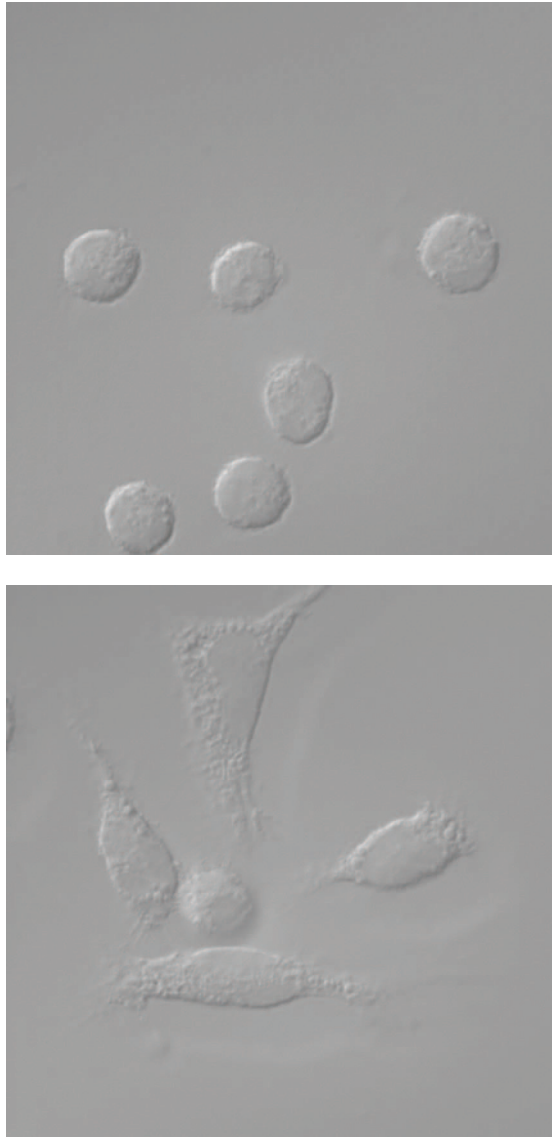


Figure 2. Resident mouse peritoneal macrophages.

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 TNF α [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 growth 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 (TGF β) is involved in different activities such as proliferation of lymphocytes, differentiation of cells of the myeloid lineage and regulating immunoglobulin production [20]. TGF β is a negative growth factor for epithelial cells [21], and many human tumours do not have the growth inhibition otherwise induced by TGF β . 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. TNF β , also called lymphotoxin, is mainly produced by lymphocytes. Signalling through TNF superfamily receptors induces apoptosis, proliferation, differentiation, and modified gene expression. TNF α 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], NF κ B, 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-1 β 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-1 β 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-1 β is secreted.

Tumour necrosis factor α

In the beginning, before TNF α 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 TNF α [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 TNF α associated diseases are presented in Table 2. TNF α 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 TNF α in serum is short, ranging from 6 to 20 minutes [37], and clearance of the cytokine is via the kidneys. Initially TNF α was considered a potential antitumour agent, as it induced necrosis of tumour cells *in vitro*. Further studies revealed that the cytotoxic effect seen with TNF α was not restricted to tumours but also occurred in normal cells. However, TNF α 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, TNF α also causes progression of cancer as it is considered a growth factor for some tumours [39].

TNF α 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 TNF α 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 TNF α 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 TNF α had been shown to be an important mediator in sepsis induced by Gram-negative bacteria [36].

The biosynthesis of TNF α 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 TNF α . 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 I κ B [48] and subsequent release of NF κ B. 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 TNF α . Example of diseases where TNF α 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 TNF α 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 TNF α gene transcription is cell type and stimulus specific. The TNF α gene promoter contains binding sites for numerous transcription factors, including NF κ B, 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 TNF α by specific proteases [57].

TNF α 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 TNF β (30 % sequence identity) also signals via these receptors and induces the same responses. Macrophages are not the main source of TNF β ; 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 Ca²⁺, IP₃, diacylglycerol, cAMP, cGMP and phosphoinositides. Calcium ions is derived from two separate sources, either extracellular influx or release from internal storage compartments. Ca²⁺ release from the endoplasmic reticulum is often preceded by generation of IP₃. Diacylglycerol and IP₃ are generated by hydrolysis of PIP₂ by phospholipase C. The generation of cAMP through activation of adenyllycyclase activates a family of serine/threonine protein kinases, cAMP dependent protein kinases (PKA). cAMP signalling is important in the regulation of IL-1 β gene transcription in response to LPS, PMA and TNF [59] and exerts inhibitory effects on TNF α 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 transducing 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 NF κ B 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 TNF α [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 NF κ B activation and cytokine production. Andreacos *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 NF κ B (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 | <i>Neisseria meningitidis</i> 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 <i>Porphyromonas gingivalis</i> [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 (where 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 NF κ B, 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 TNF α 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].

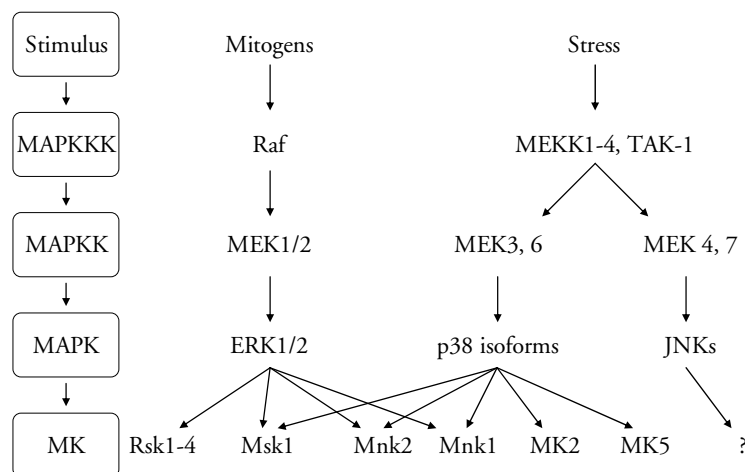


Figure 3. Mitogen activated protein kinase signalling cascade.

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 TNF α 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 NF κ B1 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 NF κ B dimer.

As mentioned above MK2 has been considered important in the posttranscriptional regulation of TNF α 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 TNF α mRNA [108]. When mice are made deficient in tristetraprolin they develop an arthritic syndrome [109], in which an excessive amount of TNF α 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 TNF α 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 A₂ [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].

TNF α 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.

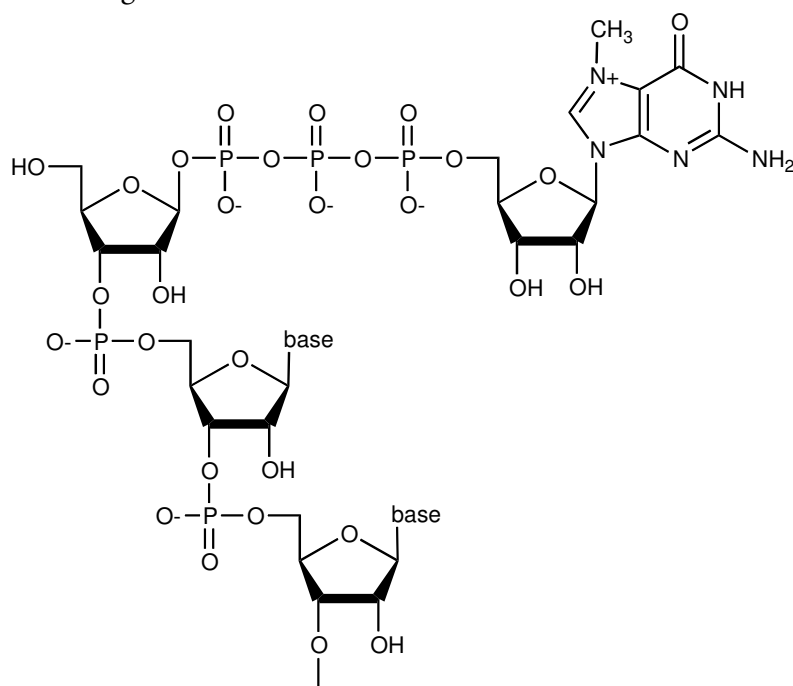


Figure 4. The cap structure of the 5' end of the mRNA molecule.

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.

TNF α gene transcription

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

NF κ B is a family of 5 members which includes the NF κ B proteins (p50 and p52) and Rel proteins (RelA, RelB c-rel). NF κ B forms homo- or heterodimers with the heterodimer p50/RelA as the most abundant. NF κ B is regulated by its inhibitory protein I κ B and the complex resides in the cytoplasm, preventing NF κ B from entering the nucleus. The NF κ B proteins are produced as large precursor proteins, p105 and p100, which also functions as I κ B proteins. When I κ B is phosphorylated, by the NF κ B activating IKK complex (IKK1, IKK2 and the regulatory protein NEMO) [117], it releases from the complex and promotes nuclear translocation of NF κ B with subsequent activation of NF κ B driven gene transcription. Activation of NF κ B results in TNF α production which in turn activates NF κ B through TNFR signalling pathways. Also signal transduction cascades utilized in IL-1 receptor signalling activates NF κ B. Thus NF κ B 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 NF κ B via signalling through TLR4.

It should be noted that controversies over the contribution of NF κ B in the human promoter regulating TNF α gene expression exists [118, 119]. The transcription factor AP-1 is activated by protein kinase C (PKC) and in human monocytes LPS-induced TNF α 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.

TNF α 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 TNF α , they also overexpress TNF α , with increased mortality rate as a consequence, when injected peritoneally with LPS. The proteins binding to the ARE sequence in TNF α mRNA regulates transcript stability or translation. Several proteins with different regulatory functions have been shown to interact with ARE in TNF α transcripts.

Tristetraprolin, a cytoplasmic protein regulated by phosphorylation, destabilizes TNF α 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 TNF α mRNA is the stabilizing factor HuR [125]. HuR, when overexpressed, stabilize a reporter construct containing the TNF α ARE. Another *trans*-acting factor regulating TNF α 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 TNF α than wildtype mice when challenged with LPS. The proposed role for TIA-1 in regulating TNF α expression is as a specific translational silencer, since production of IL-6 or IL-1 β is unchanged. When TIA-1 is not present, TNF α transcripts shift to increased association with polysomes. Other proteins have been shown to bind to the ARE sequence in TNF α 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 TNF α 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 TNF α 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 TNF α production [131] and later shown to inhibit p38 activity [130, 132]. Further insights into the signalling cascade regulating TNF α biosynthesis came with the characterization of MK2 as a kinase involved in LPS induced TNF α expression. When mice lacking MK2 [97] are challenged with LPS they produce significantly lower amounts of TNF α 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.

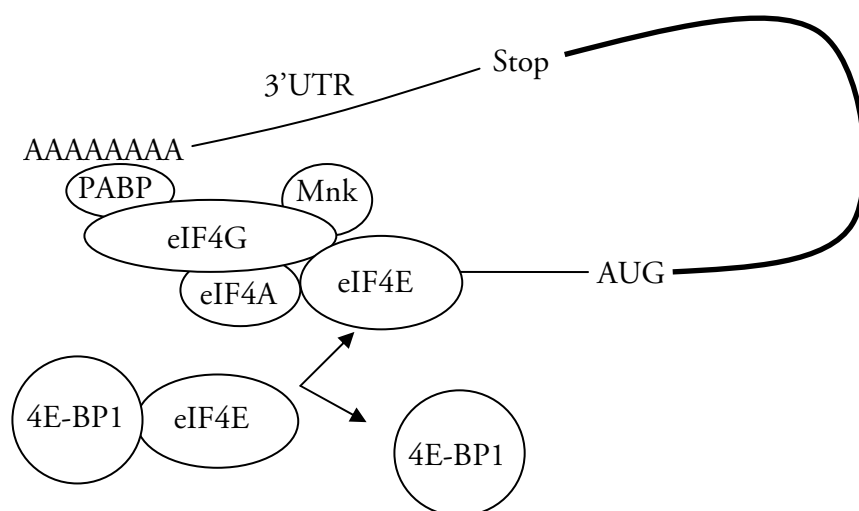


Figure 5. Simplified drawing of eIF4F complex in eukaryotic translation initiation.

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 TNF α 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 TNF α
- Delineate the signal transduction pathway regulating posttranscriptional modification of TNF α gene transcripts

The following section discusses the different aspects of TNF α 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.

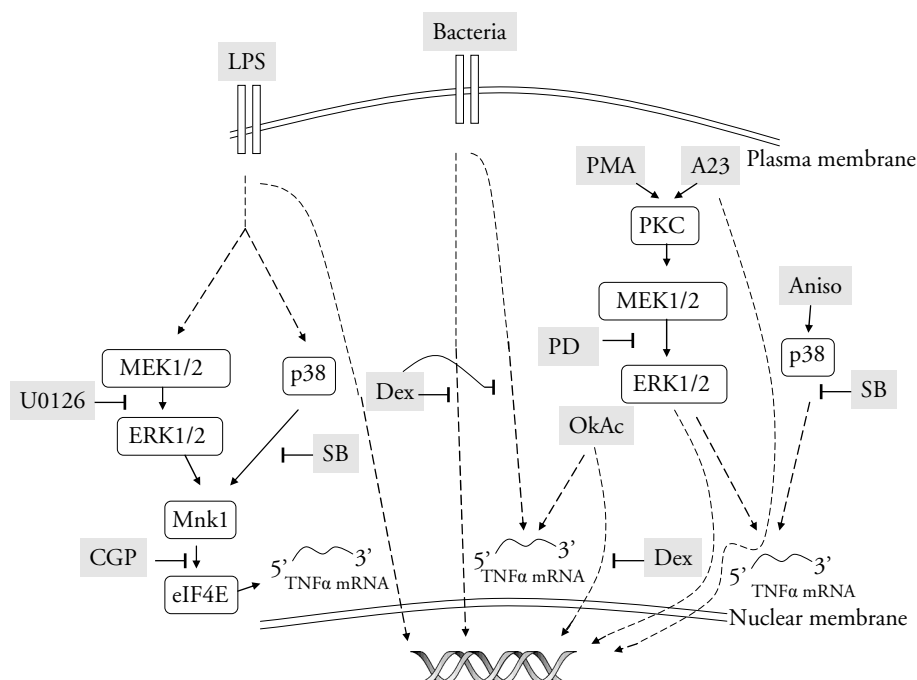


Figure 6. Agents used in Paper I-III.

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-1 β gene transcription and mRNA stability in human monocytic cells [144] and to inhibit LPS-induced IL-1 β 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 TNF α 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 Ca²⁺. In human monocytes PKC transduces signals from the LPS receptor to transcription of the TNF α gene mediated by MAP kinases and the transcription factor AP-1 [120]. PKC is also involved in production of the cytokine IL-1 β 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 Ca²⁺ as a second messenger is widely studied and in T-cells the production of TNF α is regulated by the calcineurin dependent transcription factor NFAT [148]. Calcineurin is a protein phosphatase regulated by Ca²⁺. When the concentration of Ca²⁺ 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 anisomycin, 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 TNF α 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 TNF α production in alveolar macrophages [158]. Another autocrine regulation of LPS activation is that the endogenously produced prostaglandins decrease the production of TNF α [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 A₂ 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 1 β (IL-1 β) is produced as an unprocessed protein, termed proIL-1 β and that is the only form found in mouse macrophages. In unstimulated cells proIL-1 β can be detected and the basal level of proIL-1 β is inhibited by pretreatment of the cells with dex. Pretreatment with dex inhibits bacteria-induced proIL-1 β production at the level of transcription in a dose-dependent manner. Opposed to the effects on proIL-1 β production, dex inhibits TNF α expression both on the level of transcription and translation.

The protein phosphatase inhibitor okadaic acid was also used as an inducer of TNF α production. Okadaic acid treatment of the cells gave rise to a different distribution of TNF α , than when bacteria were used as stimuli. Both Gram-positive and Gram-negative bacteria nearly completely produce expression of the proteolytically cleaved secreted TNF α , while okadaic acid also induces cell-associated TNF α . Dex inhibits okadaic acid-induced TNF α primarily at gene transcription, since both TNF α mRNA and protein are inhibited in parallel. TNF α production is regulated at different levels and posttranscriptional regulation also includes proteolytic cleavage of proTNF α . When okadaic acid is used as an inducer of TNF α 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 TNF α are inhibited by dex, while the 17 kDa cell-associated form is unaffected.

In conclusion, dexamethasone inhibits bacteria-induced proIL-1 β production at the level of transcription, while the formation of TNF α 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 TNF α mRNA. No protein was produced despite TNF α mRNA expression. THP-1 cells have in the unstimulated condition, translational inhibition affecting TNF α .

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 TNF α secretion, and when mRNA expression was studied no induction of TNF α mRNA could be detected. Instead PMA caused a derepression of translation. The PMA-induced TNF α release is sensitive to the MEK1/2 inhibitor PD98059 and the basal TNF α 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 TNF α 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 TNF α [167], the p38 activator anisomycin was tested and found to be an inducer of TNF α production. The p38 inhibitor SB203580 decreased TNF α 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 TNF α 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 TNF α production, as reported by others [131], and MEK1/2 inhibition completely reversed the induction of TNF α .

In human monocytic cells, the MAPKK MEK1/2 and the MAP kinase p38 mediate translational activation and could also reverse the translational inhibition on TNF α 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 TNF α 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 TNF α transcripts (Figure 7).

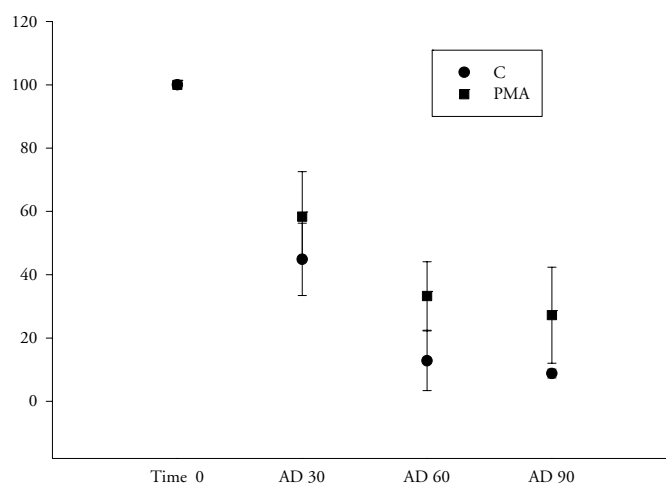


Figure 7. Stability of TNF α mRNA.

THP-1 cells were treated for 30 minutes with 100 nM PMA and then 2 μ g/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 TNF α mRNA were undetectable) cells were processed for total RNA extraction. 10 μ g of total RNA was separated on agarose gel and transferred to nylon membrane TNF α 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 TNF α translation, polysome analysis was performed. When LPS is used as a TNF inducing agent a switch to polyribosome associated TNF α mRNA is detected [169]. As shown in Figure 8 no change in the distribution of TNF α mRNA is observed when PMA is used as stimulus.

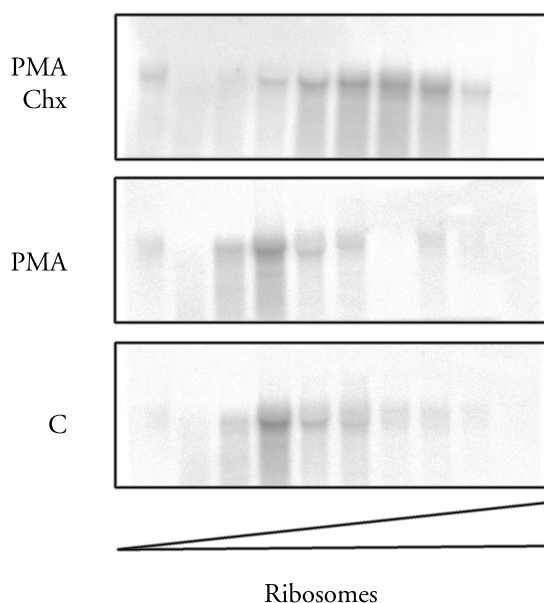


Figure 8. Polysome analysis.

The image shows TNF α , 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 MgCl₂, 1 % Igepal CA630, 1 mM DTT and 1U/ μ l 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 ³²P labelled TNF α 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 TNF α 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 TNF α (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 TNF α mRNA, while in primary culture of mouse macrophages TNF α mRNA can not be detected and this could be the explanation why PMA or anisomycin do not induce expression of the protein. When TNF α expression was measured, no significant inhibition was detected by either inhibitor. It was puzzling to find that SB203580, first described as a compound inhibiting TNF α production in human monocytes [131], did not have the same effect in mouse peritoneal macrophages.

The lack of inhibition of LPS-induced TNF α 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 TNF α , and when the two inhibitors were used together a dramatic decrease in the LPS-induced TNF α 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 TNF α production in a dose-dependent manner and the slight decrease in TNF α 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 TNF α mRNA in the nucleus has been ascribed to kinase inhibition [172]. One possible explanation to our findings would be that TNF α mRNA accumulated in the cell nucleus in response to pretreatment of the cells with kinase inhibitors. To test for possible accumulation of TNF α 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 TNF α in response to LPS, and they do not respond to exogenously administered PGE₂ by a decrease in TNF α expression. The lack of inhibition seen with PGE₂ 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 TNF α 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 TNF α mRNA mediated by phosphorylation of eIF4E.

CONCLUDING REMARKS

The synthetic glucocorticoid dexamethasone suppresses bacteria-induced TNF α 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 TNF α has been in focus. The human monocytic cell line THP-1 was found to be a tool for studying the posttranscriptional regulation of TNF α , and that MEK1/2 and p38 are involved in regulating TNF α expression. The posttranscriptional regulation of LPS-induced TNF α 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 TNF α 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.

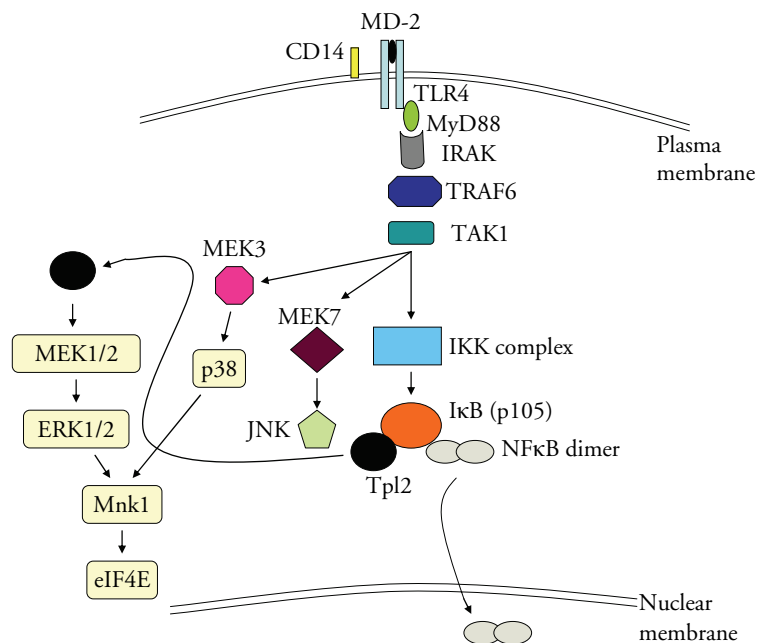


Figure 9. LPS signalling through TLR4.

Proposed signalling pathway for LPS induced phosphorylation of eIF4E.

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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.

REFERENCES

1. Johnston RB, Jr. (1988) Current concepts: immunology. Monocytes and macrophages *N Engl J Med* 318: 747-52
2. Ma X (2001) TNF-alpha and IL-12: a balancing act in macrophage functioning *Microbes Infect* 3: 121-9
3. Krakauer T, Oppenheim JJ (1983) Interleukin 1 production by a human acute monocytic leukemia cell line *Cell Immunol* 80: 223-9
4. De AK, Kodys KM, Yeh BS, Miller-Graziano C (2000) Exaggerated human monocyte IL-10 concomitant to minimal TNF-alpha induction by heat-shock protein 27 (Hsp27) suggests Hsp27 is primarily an antiinflammatory stimulus *J Immunol* 165: 3951-8
5. Fantuzzi L, Belardelli F, Gessani S (2003) Monocyte/macrophage-derived CC chemokines and their modulation by HIV-1 and cytokines: a complex network of interactions influencing viral replication and AIDS pathogenesis *J Leukoc Biol* 74: 719-25
6. Svensson U, Holst E, Sundler R (1991) Protein-kinase-C-independent activation of arachidonate release and prostaglandin E2 formation in macrophages interacting with certain bacteria *Eur J Biochem* 200: 699-705
7. Takemura R, Werb Z (1984) Secretory products of macrophages and their physiological functions *Am J Physiol* 246: C1-9
8. Giuntoli DL, Retzinger GS (1997) Evidence for prothrombin production and thrombin expression by phorbol ester-treated THP-1 cells *Exp Mol Pathol* 64: 53-62
9. Witte MB, Barbul A (2002) Role of nitric oxide in wound repair *Am J Surg* 183: 406-12
10. Hubel K, Dale DC, Liles WC (2002) Therapeutic use of cytokines to modulate phagocyte function for the treatment of infectious diseases: current status of granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and interferon-gamma *J Infect Dis* 185: 1490-501

11. Perregaux DG, Dean D, Cronan M, Connelly P, Gabel CA (1995) Inhibition of interleukin-1 beta production by SKF86002: evidence of two sites of in vitro activity and of a time and system dependence *Mol Pharmacol* 48: 433-42
12. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF *J Clin Invest* 101: 890-8
13. Mosser DM (2003) The many faces of macrophage activation *J Leukoc Biol* 73: 209-12
14. Hibbs JB, Jr. (2002) Infection and nitric oxide *J Infect Dis* 185 Suppl 1: S9-17
15. Tapper H, Sundler R (1995) Glucan receptor and zymosan-induced lysosomal enzyme secretion in macrophages *Biochem J* 306 (Pt 3): 829-35
16. Wijkander J, Sundler R (1989) A phospholipase A2 hydrolyzing arachidonoyl-phospholipids in mouse peritoneal macrophages *FEBS Lett* 244: 51-6
17. Vane JR, Botting RM (2003) The mechanism of action of aspirin *Thromb Res* 110: 255-8
18. Botting R (2003) COX-1 and COX-3 inhibitors *Thromb Res* 110: 269-72
19. Strassmann G, Patil-Koota V, Finkelman F, Fong M, Kambayashi T (1994) Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2 *J Exp Med* 180: 2365-70
20. Letterio JJ (2000) Murine models define the role of TGF-beta as a master regulator of immune cell function *Cytokine Growth Factor Rev* 11: 81-7
21. Liu X, Sun Y, Weinberg RA, Lodish HF (2001) Ski/Sno and TGF-beta signaling *Cytokine Growth Factor Rev* 12: 1-8

22. Tallquist M, Kazlauskas A (2004) PDGF signaling in cells and mice *Cytokine Growth Factor Rev* 15: 205-13
23. Pietras K, Sjoblom T, Rubin K, Heldin CH, Ostman A (2003) PDGF receptors as cancer drug targets *Cancer Cell* 3: 439-43
24. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization *Trends Immunol* 25: 677-86
25. Aggarwal BB (2003) Signalling pathways of the TNF superfamily: a double-edged sword *Nat Rev Immunol* 3: 745-56
26. Iwakura Y (2002) Roles of IL-1 in the development of rheumatoid arthritis: consideration from mouse models *Cytokine Growth Factor Rev* 13: 341-55
27. Rabinovitch A, Suarez-Pinzon WL (1998) Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus *Biochem Pharmacol* 55: 1139-49
28. Fenton MJ, Vermeulen MW, Clark BD, Webb AC, Auron PE (1988) Human pro-IL-1 beta gene expression in monocytic cells is regulated by two distinct pathways *J Immunol* 140: 2267-73
29. Turner M, Chantry D, Buchan G, Barrett K, Feldmann M (1989) Regulation of expression of human IL-1 alpha and IL-1 beta genes *J Immunol* 143: 3556-61
30. Peschke T, Bender A, Nain M, Gemsa D (1993) Role of macrophage cytokines in influenza A virus infections *Immunobiology* 189: 340-55
31. Svensson U, Holst E, Sundler R (1995) Cyclosporin-sensitive expression of cytokine mRNA in mouse macrophages responding to bacteria *Mol Immunol* 32: 157-65
32. Zhang Y, Rom WN (1993) Regulation of the interleukin-1 beta (IL-1 beta) gene by mycobacterial components and lipopolysaccharide is mediated by two nuclear factor-IL6 motifs *Mol Cell Biol* 13: 3831-7

33. Hurme M, Matikainen S (1993) Okadaic acid, a phosphatase inhibitor, enhances the phorbol ester-induced interleukin-1 beta expression via an AP-1-mediated mechanism *Scand J Immunol* 38: 570-4
34. Jeon YJ, Han SH, Lee YW, Lee M, Yang KH, Kim HM (2000) Dexamethasone inhibits IL-1 beta gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-kappa B/Rel and AP-1 activation *Immunopharmacology* 48: 173-83
35. Andrei C, Margiocco P, Poggi A, Lotti LV, Torrisi MR, Rubartelli A (2004) Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes *Proc Natl Acad Sci U S A* 101: 9745-50
36. Beutler B, Cerami A (1988) Tumor necrosis, cachexia, shock, and inflammation: a common mediator *Annu Rev Biochem* 57: 505-18
37. Tracey KJ, Cerami A (1993) Tumor necrosis factor, other cytokines and disease *Annu Rev Cell Biol* 9: 317-43
38. Eggermont AM, de Wilt JH, ten Hagen TL (2003) Current uses of isolated limb perfusion in the clinic and a model system for new strategies *Lancet Oncol* 4: 429-37
39. Szlosarek PW, Balkwill FR (2003) Tumour necrosis factor alpha: a potential target for the therapy of solid tumours *Lancet Oncol* 4: 565-73
40. Haraoui B (2005) The anti-tumor necrosis factor agents are a major advance in the treatment of rheumatoid arthritis *J Rheumatol Suppl* 72: 46-7
41. Sands BE (2004) Why Do Anti-Tumor Necrosis Factor Antibodies Work in Crohn's Disease? *Rev Gastroenterol Disord* Vol. 4 Suppl. 3: S10-S17
42. Feldmann M, Brennan FM, Williams RO, Woody JN, Maini RN (2004) The transfer of a laboratory based hypothesis to a clinically useful therapy: the development of anti-TNF therapy of rheumatoid arthritis *Best Pract Res Clin Rheumatol* 18: 59-80
43. Stokkers PC, Hommes DW (2004) New cytokine therapeutics for inflammatory bowel disease *Cytokine* 28: 167-73

44. Reddy P, Ferrara JL (2003) Immunobiology of acute graft-versus-host disease *Blood Rev* 17: 187-94
45. Targan SR, Murphy LK (1995) Clarifying the causes of Crohn's *Nat Med* 1: 1241-3
46. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ, 3rd, Zentella A, Albert JD, et al. (1986) Shock and tissue injury induced by recombinant human cachectin *Science* 234: 470-4
47. Medvedev AE, Kopydlowski KM, Vogel SN (2000) Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression *J Immunol* 164: 5564-74
48. Fujihara M, Wakamoto S, Ito T, Muroi M, Suzuki T, Ikeda H, Ikebuchi K (2000) Lipopolysaccharide-triggered desensitization of TNF- α mRNA expression involves lack of phosphorylation of I κ B α in a murine macrophage-like cell line, P388D1 *J Leukoc Biol* 68: 267-76
49. Fahmi H, Ancuta P, Perrier S, Chaby R (1996) Preexposure of mouse peritoneal macrophages to lipopolysaccharide and other stimuli enhances the nitric oxide response to secondary stimuli *Inflamm Res* 45: 347-53
50. Raabe T, Bukrinsky M, Currie RA (1998) Relative contribution of transcription and translation to the induction of tumor necrosis factor- α by lipopolysaccharide *J Biol Chem* 273: 974-80
51. Drouet C, Shakhov AN, Jongeneel CV (1991) Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor- α promoter in primary macrophages *J Immunol* 147: 1694-700
52. Yao J, Mackman N, Edgington TS, Fan ST (1997) Lipopolysaccharide induction of the tumor necrosis factor- α promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF- κ B transcription factors *J Biol Chem* 272: 17795-801

53. Tsai EY, Falvo JV, Tsytsykova AV, Barczak AK, Reimold AM, Glimcher LH, Fenton MJ, Gordon DC, Dunn IF, Goldfeld AE (2000) A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo *Mol Cell Biol* 20: 6084-94
54. Barthel R, Tsytsykova AV, Barczak AK, Tsai EY, Dascher CC, Brenner MB, Goldfeld AE (2003) Regulation of tumor necrosis factor alpha gene expression by mycobacteria involves the assembly of a unique enhanceosome dependent on the coactivator proteins CBP/p300 *Mol Cell Biol* 23: 526-33
55. Bolcato-Bellemin AL, Mattei MG, Fenton M, Amar S (2004) Molecular cloning and characterization of mouse LITAF cDNA: role in the regulation of tumor necrosis factor-alpha (TNF-alpha) gene expression *J Endotoxin Res* 10: 15-23
56. Roach SK, Lee SB, Schorey JS (2005) Differential activation of the transcription factor cyclic AMP response element binding protein (CREB) in macrophages following infection with pathogenic and nonpathogenic mycobacteria and role for CREB in tumor necrosis factor alpha production *Infect Immun* 73: 514-22
57. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells *Nature* 385: 729-33
58. Muppidi JR, Tschopp J, Siegel RM (2004) Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction *Immunity* 21: 461-5
59. Chandra G, Cogswell JP, Miller LR, Godlevski MM, Stinnett SW, Noel SL, Kadwell SH, Kost TA, Gray JG (1995) Cyclic AMP signaling pathways are important in IL-1 beta transcriptional regulation *J Immunol* 155: 4535-43
60. Shames BD, McIntyre RC, Jr., Bensard DD, Pulido EJ, Selzman CH, Reznikov LL, Harken AH, Meng X (2001) Suppression of tumor necrosis factor alpha production by cAMP in human monocytes: dissociation with mRNA level and independent of interleukin-10 *J Surg Res* 99: 187-93

61. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF (1998) A family of human receptors structurally related to *Drosophila* Toll *Proc Natl Acad Sci U S A* 95: 588-93
62. Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S (2004) A toll-like receptor that prevents infection by uropathogenic bacteria *Science* 303: 1522-6
63. Wyllie DH, Kiss-Toth E, Visintin A, Smith SC, Boussouf S, Segal DM, Duff GW, Dower SK (2000) Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses *J Immunol* 165: 7125-32
64. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S (2002) Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins *J Immunol* 169: 10-4
65. Vasselon T, Detmers PA, Charron D, Haziot A (2004) TLR2 recognizes a bacterial lipopeptide through direct binding *J Immunol* 173: 7401-5
66. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ (1999) Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2 *J Biol Chem* 274: 17406-9
67. Asai Y, Ohyama Y, Gen K, Ogawa T (2001) Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2 *Infect Immun* 69: 7387-95
68. Henneke P, Takeuchi O, van Strijp JA, Guttormsen HK, Smith JA, Schromm AB, Espevik TA, Akira S, Nizet V, Kasper DL, Golenbock DT (2001) Novel engagement of CD14 and multiple toll-like receptors by group B streptococci *J Immunol* 167: 7069-76
69. Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2 *J Exp Med* 197: 1107-17
70. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H (2002) HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway *J Biol Chem* 277: 15107-12

71. Kariko K, Ni H, Capodici J, Lamphier M, Weissman D (2004) mRNA is an endogenous ligand for Toll-like receptor 3 *J Biol Chem* 279: 12542-50
72. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3 *Nature* 413: 732-8
73. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction *J Biol Chem* 274: 10689-92
74. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M (2000) Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol *J Biol Chem* 275: 2251-4
75. Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, Gallo RL (2004) Hyaluronan fragments stimulate endothelial recognition of injury through TLR4 *J Biol Chem* 279: 17079-84
76. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL (2001) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression *J Immunol* 167: 1882-5
77. Nakao Y, Funami K, Kikkawa S, Taniguchi M, Nishiguchi M, Fukumori Y, Seya T, Matsumoto M (2005) Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells *J Immunol* 174: 1566-73
78. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA *Science* 303: 1529-31
79. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8 *Science* 303: 1526-9
80. Krug A, Luker GD, Barchet W, Leib DA, Akira S, Colonna M (2004) Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9 *Blood* 103: 1433-7

81. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S (2000) A Toll-like receptor recognizes bacterial DNA *Nature* 408: 740-5
82. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, Guiet C, Briere F, Vlach J, Lebecque S, Trinchieri G, Bates EE (2005) Human TLR10 Is a Functional Receptor, Expressed by B Cells and Plasmacytoid Dendritic Cells, Which Activates Gene Transcription through MyD88 *J Immunol* 174: 2942-50
83. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene *Science* 282: 2085-8
84. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product *J Immunol* 162: 3749-52
85. Underhill DM (2003) Toll-like receptors: networking for success *Eur J Immunol* 33: 1767-75
86. Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA, Jr. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways *Mol Cell* 2: 253-8
87. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S (1999) Unresponsiveness of MyD88-deficient mice to endotoxin *Immunity* 11: 115-22
88. Andreakos E, Sacre SM, Smith C, Lundberg A, Kiriakidis S, Stonehouse T, Monaco C, Feldmann M, Foxwell BM (2004) Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP *Blood* 103: 2229-37
89. Horng T, Barton GM, Medzhitov R (2001) TIRAP: an adapter molecule in the Toll signaling pathway *Nat Immunol* 2: 835-41

-
90. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, Brint E, Dunne A, Gray P, Harte MT, McMurray D, Smith DE, Sims JE, Bird TA, O'Neill LA (2001) Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction *Nature* 413: 78-83
91. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway *Science* 301: 640-3
92. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, Akira S (2003) TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway *Nat Immunol* 4: 1144-50
93. Ingalls RR, Heine H, Lien E, Yoshimura A, Golenbock D (1999) Lipopolysaccharide recognition, CD14, and lipopolysaccharide receptors *Infect Dis Clin North Am* 13: 341-53, vii
94. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4 *J Exp Med* 189: 1777-82
95. Viriyakosol S, Tobias PS, Kitchens RL, Kirkland TN (2001) MD-2 binds to bacterial lipopolysaccharide *J Biol Chem* 276: 38044-51
96. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors *Annu Rev Immunol* 21: 335-76
97. Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD, Gaestel M (1999) MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis *Nat Cell Biol* 1: 94-7
98. Chen RH, Sarnecki C, Blenis J (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases *Mol Cell Biol* 12: 915-27
99. Ben-Levy R, Hooper S, Wilson R, Paterson HF, Marshall CJ (1998) Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2 *Curr Biol* 8: 1049-57

100. Lee J, Mira-Arbibe L, Ulevitch RJ (2000) TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide *J Leukoc Biol* 68: 909-15
101. Delgado M, Ganea D (2000) Vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide inhibit the MEKK1/MEK4/JNK signaling pathway in LPS-stimulated macrophages *J Neuroimmunol* 110: 97-105
102. Denhardt DT (1996) Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling *Biochem J* 318 (Pt 3): 729-47
103. Waterfield M, Jin W, Reiley W, Zhang M, Sun SC (2004) IkappaB kinase is an essential component of the Tpl2 signaling pathway *Mol Cell Biol* 24: 6040-8
104. Beinke S, Robinson MJ, Hugunin M, Ley SC (2004) Lipopolysaccharide activation of the TPL-2/MEK/extracellular signal-regulated kinase mitogen-activated protein kinase cascade is regulated by IkappaB kinase-induced proteolysis of NF-kappaB1 p105 *Mol Cell Biol* 24: 9658-67
105. Belich MP, Salmeron A, Johnston LH, Ley SC (1999) TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105 *Nature* 397: 363-8
106. Chrestensen CA, Schroeder MJ, Shabanowitz J, Hunt DF, Pelo JW, Worthington MT, Sturgill TW (2004) MAPKAP kinase 2 phosphorylates tristetraprolin on in vivo sites including Ser178, a site required for 14-3-3 binding *J Biol Chem* 279: 10176-84
107. Ming XF, Stoecklin G, Lu M, Looser R, Moroni C (2001) Parallel and independent regulation of interleukin-3 mRNA turnover by phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase *Mol Cell Biol* 21: 5778-89
108. Mahtani KR, Brook M, Dean JL, Sully G, Saklatvala J, Clark AR (2001) Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability *Mol Cell Biol* 21: 6461-9

109. Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF, Blackshear PJ (1996) A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency *Immunity* 4: 445-54
110. Bollig F, Winzen R, Gaestel M, Kostka S, Resch K, Holtmann H (2003) Affinity purification of ARE-binding proteins identifies polyA-binding protein 1 as a potential substrate in MK2-induced mRNA stabilization *Biochem Biophys Res Commun* 301: 665-70
111. Rutault K, Hazzalin CA, Mahadevan LC (2001) Combinations of ERK and p38 MAPK inhibitors ablate tumor necrosis factor-alpha (TNF-alpha) mRNA induction. Evidence for selective destabilization of TNF-alpha transcripts *J Biol Chem* 276: 6666-74
112. Hefner Y, Borsch-Haubold AG, Murakami M, Wilde JI, Pasquet S, Schieltz D, Ghomashchi F, Yates JR, 3rd, Armstrong CG, Paterson A, Cohen P, Fukunaga R, Hunter T, Kudo I, Watson SP, Gelb MH (2000) Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases *J Biol Chem* 275: 37542-51
113. Waskiewicz AJ, Flynn A, Proud CG, Cooper JA (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2 *Embo J* 16: 1909-20
114. Wang X, Flynn A, Waskiewicz AJ, Webb BL, Vries RG, Baines IA, Cooper JA, Proud CG (1998) The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways *J Biol Chem* 273: 9373-7
115. Waskiewicz AJ, Johnson JC, Penn B, Mahalingam M, Kimball SR, Cooper JA (1999) Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo *Mol Cell Biol* 19: 1871-80
116. Ueda T, Watanabe-Fukunaga R, Fukuyama H, Nagata S, Fukunaga R (2004) Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development *Mol Cell Biol* 24: 6539-49

117. Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, Kirk HE, Kay RJ, Israel A (1998) Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation *Cell* 93: 1231-40
118. Goldfeld AE, Strominger JL, Doyle C (1991) Human tumor necrosis factor alpha gene regulation in phorbol ester stimulated T and B cell lines *J Exp Med* 174: 73-81
119. Takashiba S, Van Dyke TE, Shapira L, Amar S (1995) Lipopolysaccharide-inducible and salicylate-sensitive nuclear factor(s) on human tumor necrosis factor alpha promoter *Infect Immun* 63: 1529-34
120. Kontny E, Ziolkowska M, Ryzewska A, Maslinski W (1999) Protein kinase c-dependent pathway is critical for the production of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6) *Cytokine* 11: 839-48
121. Grzybowska EA, Wilczynska A, Siedlecki JA (2001) Regulatory functions of 3'UTRs *Biochem Biophys Res Commun* 288: 291-5
122. Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G (1999) Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies *Immunity* 10: 387-98
123. Carballo E, Lai WS, Blackshear PJ (1998) Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin *Science* 281: 1001-5
124. Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS, Blackshear PJ (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA *Mol Cell Biol* 19: 4311-23
125. Dean JL, Wait R, Mahtani KR, Sully G, Clark AR, Saklatvala J (2001) The 3' untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR *Mol Cell Biol* 21: 721-30
126. Beck AR, Miller IJ, Anderson P, Streuli M (1998) RNA-binding protein TIAR is essential for primordial germ cell development *Proc Natl Acad Sci U S A* 95: 2331-6

127. Piecyk M, Wax S, Beck AR, Kedersha N, Gupta M, Maritim B, Chen S, Gueydan C, Krays V, Streuli M, Anderson P (2000) TIA-1 is a translational silencer that selectively regulates the expression of TNF- α *Embo J* 19: 4154-63
128. Gueydan C, Droogmans L, Chalon P, Huez G, Caput D, Krays V (1999) Identification of TIAR as a protein binding to the translational regulatory AU-rich element of tumor necrosis factor alpha mRNA *J Biol Chem* 274: 2322-6
129. Rousseau S, Morrice N, Pegg M, Campbell DG, Gaestel M, Cohen P (2002) Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs *Embo J* 21: 6505-14
130. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis *Nature* 372: 739-46
131. Young P, McDonnell P, Dunnington D, Hand A, Laydon J, Lee J (1993) Pyridinyl imidazoles inhibit IL-1 and TNF production at the protein level *Agents Actions* 39 Spec No: C67-9
132. Young PR, McLaughlin MM, Kumar S, Kassis S, Doyle ML, McNulty D, Gallagher TF, Fisher S, McDonnell PC, Carr SA, Huddleston MJ, Seibel G, Porter TG, Livi GP, Adams JL, Lee JC (1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site *J Biol Chem* 272: 12116-21
133. Gebauer F, Hentze MW (2004) Molecular mechanisms of translational control *Nat Rev Mol Cell Biol* 5: 827-35
134. Wells SE, Hillner PE, Vale RD, Sachs AB (1998) Circularization of mRNA by eukaryotic translation initiation factors *Mol Cell* 2: 135-40
135. Kahvejian A, Svitkin YV, Sukarieh R, M'Boutchou MN, Sonenberg N (2005) Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms *Genes Dev* 19: 104-13

136. Grund EM, Spyropoulos DD, Watson DK, Muise-Helmericks RC (2005) Interleukins 2 and 15 Regulate Ets1 Expression via ERK1/2 and MNK1 in Human Natural Killer Cells *J Biol Chem* 280: 4772-8
137. Topisirovic I, Guzman ML, McConnell MJ, Licht JD, Culjkovic B, Neering SJ, Jordan CT, Borden KL (2003) Aberrant eukaryotic translation initiation factor 4E-dependent mRNA transport impedes hematopoietic differentiation and contributes to leukemogenesis *Mol Cell Biol* 23: 8992-9002
138. Strudwick S, Borden KL (2002) The emerging roles of translation factor eIF4E in the nucleus *Differentiation* 70: 10-22
139. Gingras AC, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK, Polakiewicz RD, Wyslouch-Cieszynska A, Aebersold R, Sonenberg N (2001) Hierarchical phosphorylation of the translation inhibitor 4E-BP1 *Genes Dev* 15: 2852-64
140. Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism *Genes Dev* 13: 1422-37
141. Potter MW, Shah SA, Elbirt KK, Callery MP (2001) Endotoxin (LPS) stimulates 4E-BP1/PHAS-I phosphorylation in macrophages *J Surg Res* 97: 54-9
142. Pyronnet S (2000) Phosphorylation of the cap-binding protein eIF4E by the MAPK-activated protein kinase Mnk1 *Biochem Pharmacol* 60: 1237-43
143. Scheper GC, Proud CG (2002) Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? *Eur J Biochem* 269: 5350-9
144. Lee SW, Tsou AP, Chan H, Thomas J, Petrie K, Eugui EM, Allison AC (1988) Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mRNA *Proc Natl Acad Sci U S A* 85: 1204-8

145. Swantek JL, Cobb MH, Geppert TD (1997) Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK *Mol Cell Biol* 17: 6274-82
146. Serkkola E, Hurme M (1993) Synergism between protein-kinase C and cAMP-dependent pathways in the expression of the interleukin-1 beta gene is mediated via the activator-protein-1 (AP-1) enhancer activity *Eur J Biochem* 213: 243-9
147. Flynn A, Proud CG (1996) Insulin and phorbol ester stimulate initiation factor eIF-4E phosphorylation by distinct pathways in Chinese hamster ovary cells overexpressing the insulin receptor *Eur J Biochem* 236: 40-7
148. McCaffrey PG, Goldfeld AE, Rao A (1994) The role of NFATp in cyclosporin A-sensitive tumor necrosis factor-alpha gene transcription *J Biol Chem* 269: 30445-50
149. Cano E, Hazzalin CA, Mahadevan LC (1994) Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-fos and c-jun *Mol Cell Biol* 14: 7352-62
150. Nahas N, Molski TF, Fernandez GA, Sha'afi RI (1996) Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in human neutrophils stimulated with various agonists *Biochem J* 318 (Pt 1): 247-53
151. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo *J Biol Chem* 270: 27489-94
152. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase *J Biol Chem* 273: 18623-32

153. Beutler B, Poltorak A (2001) The sole gateway to endotoxin response: how LPS was identified as Tlr4, and its role in innate immunity *Drug Metab Dispos* 29: 474-8
154. Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M, Goddard A, Wood WI, Gurney AL, Godowski PJ (1998) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling *Nature* 395: 284-8
155. Heine H, Kirschning CJ, Lien E, Monks BG, Rothe M, Golenbock DT (1999) Cutting edge: cells that carry a null allele for toll-like receptor 2 are capable of responding to endotoxin *J Immunol* 162: 6971-5
156. Jack RS, Grunwald U, Stelter F, Workalemahu G, Schutt C (1995) Both membrane-bound and soluble forms of CD14 bind to gram-negative bacteria *Eur J Immunol* 25: 1436-41
157. Lynn WA, Liu Y, Golenbock DT (1993) Neither CD14 nor serum is absolutely necessary for activation of mononuclear phagocytes by bacterial lipopolysaccharide *Infect Immun* 61: 4452-61
158. Lo CJ, Fu M, Cryer HG (1998) Interleukin 10 inhibits alveolar macrophage production of inflammatory mediators involved in adult respiratory distress syndrome *J Surg Res* 79: 179-84
159. Bondeson J, Sundler R (1996) Differential effects of tenidap on the zymosan- and lipopolysaccharide-induced expression of mRNA for proinflammatory cytokines in macrophages *Biochem Pharmacol* 52: 35-42
160. Knauf U, Tschopp C, Gram H (2001) Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2 *Mol Cell Biol* 21: 5500-11
161. Gewert K, Tapper H, Naucier C, Sundler R (1995) Dexamethasone downregulates lysosomal secretion in mouse macrophages: involvement of signaling through protein kinase C *J Inflamm* 47: 115-25
162. Gewert K, Sundler R (1995) Dexamethasone down-regulates the 85 kDa phospholipase A2 in mouse macrophages and suppresses its activation *Biochem J* 307 (Pt 2): 499-504

163. Beutler B, Krochin N, Milsark IW, Luedke C, Cerami A (1986) Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance *Science* 232: 977-80
164. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K (1980) Establishment and characterization of a human acute monocytic leukemia cell line (THP-1) *Int J Cancer* 26: 171-6
165. Ueda Y, Hirai S, Osada S, Suzuki A, Mizuno K, Ohno S (1996) Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf *J Biol Chem* 271: 23512-9
166. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI (1997) Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration *Nature* 386: 855-8
167. Lee JC, Young PR (1996) Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms *J Leukoc Biol* 59: 152-7
168. Akashi M, Osawa Y, Koeffler HP, Hachiya M (1999) p21WAF1 expression by an activator of protein kinase C is regulated mainly at the post-transcriptional level in cells lacking p53: important role of RNA stabilization *Biochem J* 337 (Pt 3): 607-16
169. Prichett W, Hand A, Sheilds J, Dunnington D (1995) Mechanism of action of bicyclic imidazoles defines a translational regulatory pathway for tumor necrosis factor alpha *J Inflamm* 45: 97-105
170. Crawford EK, Ensor JE, Kalvakolanu I, Hasday JD (1997) The role of 3' poly(A) tail metabolism in tumor necrosis factor-alpha regulation *J Biol Chem* 272: 21120-7
171. van den Blink B, Juffermans NP, ten Hove T, Schultz MJ, van Deventer SJ, van der Poll T, Peppelenbosch MP (2001) p38 mitogen-activated protein kinase inhibition increases cytokine release by macrophages in vitro and during infection in vivo *J Immunol* 166: 582-7
172. Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotic C, Jenkins NA, Copeland NG, Kollias G, Tschlis PN (2000) TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway *Cell* 103: 1071-83

173. Rouzer CA, Jacobs AT, Nirodi CS, Kingsley PJ, Morrow JD, Marnett LJ (2005) RAW264.7 cells lack prostaglandin-dependent autoregulation of tumor necrosis factor-alpha secretion *J. Lipid Res.*: M500006-JLR200
doi:10.1194/jlr.M500006-JLR200