

Review

The Emerging Distinct Role of TNF-Receptor 2 (p80) Signaling in Chronic Inflammatory Disorders

MARTIN H. HOLTSMANN*, MARCUS SCHUCHMANN, GERALDINE ZELLER, PETER R. GALLE
and MARKUS F. NEURATH

Department of Medicine, Johannes-Gutenberg-University, Mainz, Germany

Abstract. Tumor necrosis factor α (TNF- α) is a pleiotropic cytokine with strong proinflammatory and immunomodulatory properties. TNF- α plays a critical role in many acute or chronic inflammatory diseases and anti-TNF strategies have proven to be clinically effective. Two TNF-specific cell surface receptors, TNF-R1 (p60) and TNF-R2 (p80), have been identified and the function of these receptors and the downstream intracellular signal-transduction pathways have been extensively studied *in vitro*. For a long time p60 was considered to be the predominant mediator of TNF signaling, whereas p80 was ascribed only an auxiliary function. However, there is increasing clinical and experimental evidence for an important independent role of p80 signaling in chronic inflammatory conditions. To date, most data exist for Crohn's disease. Upregulation of p80 and increased p80 signaling aggravates experimental colitis and is likely to contribute to the chronicity of inflammation *in vivo*. Further studies are required to elucidate critically important steps in TNF signaling that might be dysregulated. This will lead to a better understanding of the pathogenesis of these diseases and potentially reveal new, more specific therapeutic targets.

Key words: TNF- α ; TNF-R2; signal transduction; inflammatory disorders.

TNF- α

Tumor necrosis factor α (TNF- α) is a member of a family of structurally related ligands which represent type II membrane proteins with a high degree of homology in their extracellular C-terminal domains (Fig. 1). Almost all members of the TNF family exert their effects as transmembrane proteins through cell-to-cell contact. TNF- α , however, was thought to act chiefly in its soluble (sTNF- α) form after proteolytic cleavage of the transmembrane precursor⁷. A probably crucial role of transmembrane TNF (mTNF) signaling via TNF receptor 2 (TNF-R2) in chronic inflammatory disease

states has not been recognized until recently, as will be discussed below.

The human TNF- α gene is located on the short arm of chromosome 6 close to the main histocompatibility complex and consists of approximately 3000 bp interrupted by three introns⁷⁵. The primary translational product of the human TNF- α mRNA (1.7 kb) is the transmembrane TNF- α (mTNF- α) of 233 amino acids in length (26 kDa). Membrane-bound metalloproteinases, such as TACE and ADAM 10, cleave the extracellular domain of mTNF- α and thus release sTNF- α , which consists of 157 amino acids with a molecular weight of 17 kDa and a disulfide bridge^{12, 50}. Both transmem-

*Correspondence to: Martin H. Holtmann M. D., Department of Medicine, Johannes-Gutenberg-University, Langenbeckstrasse 1, 55 131 Mainz, tel.: +49 61 31 17 72 75, fax: +49 61 31 17 55 83, e-mail: mholtman@mail.uni-mainz.de

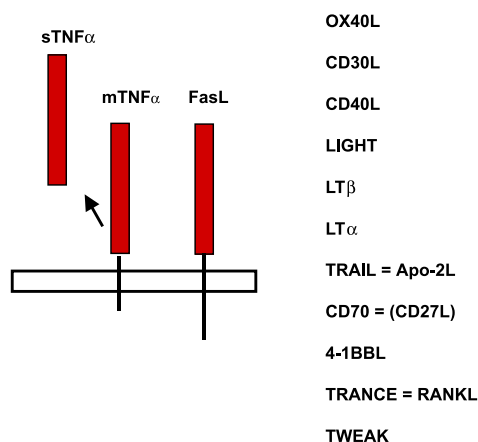


Fig. 1. The family of TNF ligands represent a family of transmembrane proteins with a high degree of homology. TNF- α also exists in a soluble form after proteolytic cleavage. The death receptor FasL is structurally and functionally very closely related to TNF. The other members known to date are listed

brane and sTNF- α form non-covalently linked homotrimers, this already occurring intracellularly⁷⁹. The production of TNF- α is regulated at the transcriptional, post-transcriptional and translational levels. Downregulation of TNF- α transcription is one mechanism of the anti-inflammatory effect of corticosteroids³². In the 3' untranslated region of TNF- α mRNA, UAU-rich motifs serve as RNase recognition sites and impair mRNA translation. This way, TNF- α expression is negatively regulated¹⁵.

TNF Receptors

There are two TNF-specific membrane-bound receptors with molecular weights of approximately 55 to 60 kDa (TNF-R1 = p60) and 75 to 80 kDa (TNF-R2 = p80), respectively^{14, 70}. p60 and p80 represent the TNF/nerve growth factor (NGF) receptor family, which also comprises the Fas-receptor (CD95/Apo1) and CD40 (Fig. 2). The genes for p60 and p80 are located on chromosome 12p13 and 1p36, respectively^{8, 25}. Both receptors are coexpressed in most tissues. However, while p60 is constitutively expressed, p80 expression is under transcriptional and posttranscriptional control⁸². p60 and p80 are glycosylated transmembrane proteins composed of two identical subunits. While p60 and p80 are quite similar in their extracellular regions (both receptors possess multiple cysteine rich motifs), their intracellular domains exhibit striking structural differences.

Until recently, p60 was considered the principal mediator of TNF- α signal transduction. This view was mainly based on the fact that p80 binds sTNF- α with a 20-fold lower binding affinity than p60²⁹. However, it was then found that p80 is preferentially activated by mTNF- α with high affinity in a paracrine fashion. It could be shown in coculture experiments that human T lymphocytes can be activated in this way by p80. In addition, in the HeLa cell model system, nuclear factor κ B (NF- κ B) was activated exclusively by p80, thus potentiating the cytotoxic effect of p60⁹². In this model system, p80 was activated by mTNF- α in an autocrine loop³⁰. These observations make possible the notion

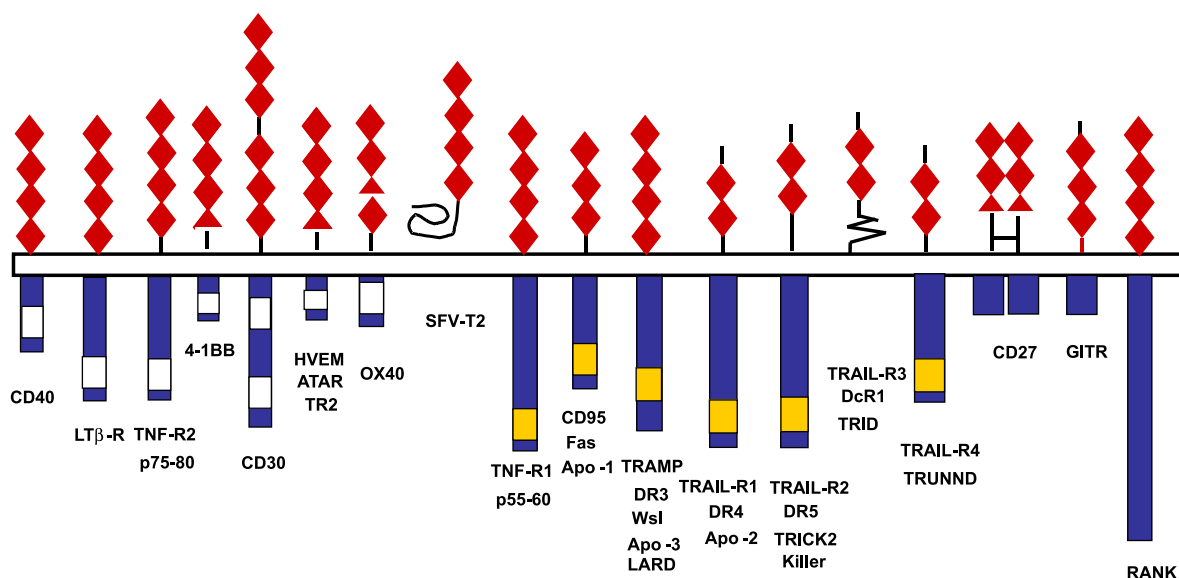


Fig. 2. The superfamily of TNF/NGF receptors. Some members are known by multiple synonyms. Their extracellular domains are highly homologous with characteristic 3- to 6-fold repeated cysteine-rich motifs. Important functional intracellular domains include the binding domains of some receptors for TRAF's (white) and the death domains, which bind signaling factors with a death domain themselves thus inducing apoptosis (light grey). For reasons of clarity receptors are shown as monomers

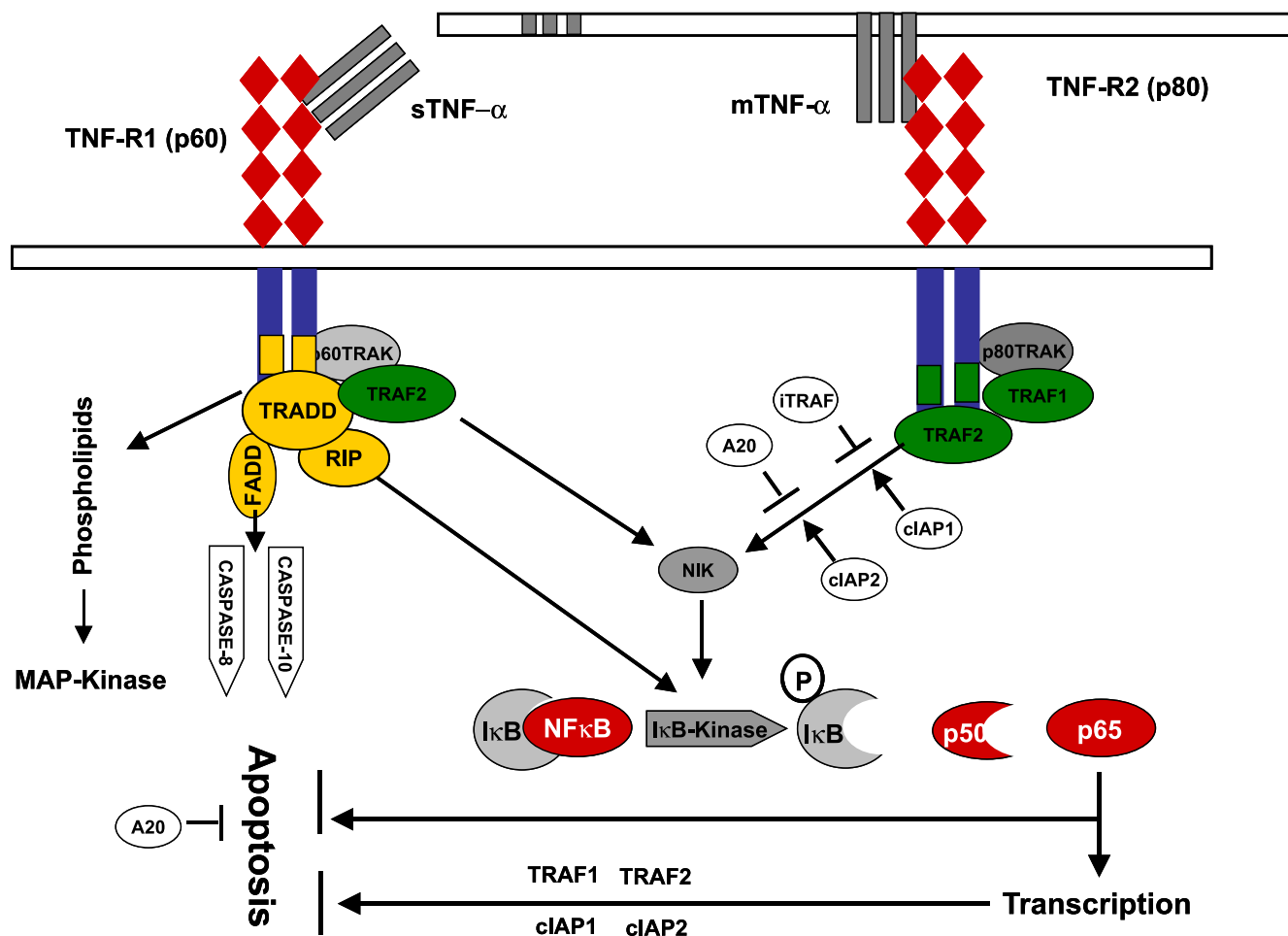


Fig. 3. TNF- α signal-transduction (for details see text). p60 is activated chiefly by sTNF- α , p80 by the membrane-bound form (mTNF- α). The most important cellular effects of TNF- α signal-transduction are the activation of NF- κ B and lipases and the induction of apoptosis. The death domains of p60 and the signaling factors, which possess a death domain are involved in the induction of apoptosis are shown in light grey, the TRAFs as well as the binding domain for these molecules on p80 are shown dark grey. Special emphasis is put on the anti-apoptotic effect of activated NF- κ B and p65 and some of the p80-associated signaling molecules, such as A20, cIAP1 and cIAP2

that the mTNF- α /p80 system could play an important immunoregulatory role at the local level. This idea is further supported by the observation from a transgenic mouse model that overexpression of mTNF- α alone is sufficient for the development of arthritis³.

The structural differences in the intracellular domains of p60 and p80 suggest that different signal-transduction pathways are recruited following receptor activation. Unlike other cytokine receptors, the cytoplasmic domains of TNF receptors lack intrinsic protein kinase activity. In spite of this, receptor activation leads to consecutive phosphorylation of downstream signal-transduction proteins. This occurs via recruitment of the cytoplasmic TNF receptor domains of members of two

special protein families, the death domain homologues (TRADD, FADD/MORT) and the TNF receptor-associated factors (TRAFs)^{4, 17, 36, 37} (Fig. 3).

Signal Transduction by TNF Receptors

TNF-R1, but not TNF-R2, possesses a characteristic signaling motif of approximately 80 amino acids in the intracellular domain, called the “death domain”⁸¹. This death domain interacts with the death domains of multiple cytosolic proteins, such as TNF receptor-associated death domain (TRADD), Fas-associated death

domain (FADD) and receptor-interacting protein (RIP)^{13, 17, 37, 77}. TRADD is a 34 kDa protein of 312 amino-acid residues and is ubiquitously expressed³⁷. The death-domain is located in the C-terminus. Human RIP is a 76 kDa protein of 671 amino-acid residues with a C-terminal death domain and an N-terminal kinase domain³⁵. RIP interacts with p60 indirectly via binding to TRADD.

While interaction with death-domain proteins is restricted to p60, both receptors interact with TNF receptor-associated factors (TRAF's). Interaction with p60 has been shown for TRAF2 only. This interaction occurs indirectly via TRADD³⁶. p80 interacts with both TRAF1 and 2. TRAF1 is a 45 kDa protein expressed in spleen, lung and testis. TRAF2 is a 56 kDa protein and is expressed ubiquitously⁶⁶. In the carboxy-terminus (C-terminus), so-called TRAF-N and TRAF-C domains are found. The TRAF-C domain is characteristic of all TRAFs and is involved in the association to the receptor and other signaling molecules. The amino-terminus (N-terminus) of TRAF2 contains RING-finger and zinc finger motifs and is responsible for the activation of the NF- κ B through phosphorylation of the inhibitor of κ B (I κ B) (see below)⁷⁸.

Both TNF receptors interact with specific TNF receptor-associated kinases (TRAK's). p60 possesses a receptor kinase, p60TRAK, which binds to a region 54 amino-acid residues long within the death-domain of the receptor¹⁸. p60TRAK phosphorylates the two proteins pp55 and pp58 and, in addition, the intracellular domain of p60 itself¹⁹. p80TRAK binds and phosphorylates both TNF receptors at the C-terminal end¹⁸. Based on this observation, this domain is called "kinase-binding domain" (KBD). The KBD plays an important role in NF- κ B activation and stimulation of proliferation by p80.

Relevant cellular responses upon stimulation of p60 include the activation of intracellular lipases, activation of NF- κ B and induction of apoptosis, whereas the principle cellular response to p80 seems to be the activation on NF- κ B. Release of diacylglycerol (DAG) from membrane phospholipids through a phosphatidyl-specific phospholipase C represents one of the most rapid TNF- α -induced signals by p60⁷². Spingomyelinases contribute to the activation of mitogen-activated protein (MAP) kinases through a ceramid-activated kinase⁴⁴.

The transcription factor NF- κ B is found in the cytosol of non-activated monocytes and T cells as an inactive heterodimer of a p50 and p65 subunit, bound to the inhibitory I κ B. Upon stimulation of the cell, I κ B is phosphorylated by I κ B kinase and consecutively releases NF- κ B⁶. For both TNF receptors, interaction

with TRAF2 seems to be important for activation of this pathway⁶⁵. The pivotal role of RIP for TNF- α -induced activation of NF- κ B was demonstrated in an RIP knock-out mouse, where this activation was no longer possible⁹³. Upon release from I κ B, the active subunit of NF- κ B, p65, moves into the nucleus and directly interacts with the promoter region of several proinflammatory genes, such as TNF- α , IL-1, IL-6 and IL-12⁵³.

The crucial role of NF- κ B for inflammation is emphasized by the fact that the effect of many established anti-inflammatory treatment strategies seems to be based on inhibition of NF- κ B. While salicylic acids inhibit I κ B kinase (IKK), corticosteroids stimulate I κ B synthesis and, additionally, inhibit p65 in the nucleus by complexation^{6, 95}.

TNF-induced apoptosis is mainly executed via the caspase pathway. Caspases represent a family of cysteine proteases cleaving at aspartate residues. The caspases are activated in a cascade-like fashion⁸³. Many of the p60 signaling factors involved in apoptosis are shared with the cell-death signaling receptor Fas (CD95). It is thus likely that the Fas- and TNF- α -induced apoptosis possess a common final pathway starting with the activation of caspase-8 (FLICE)^{13, 51}.

It has to be emphasized that the two principal effects of TNF- α signal transduction, activation of NF- κ B and induction of apoptosis, can be antagonistic to each other. NF- κ B inhibits caspase-8 activation through up-regulation of TRAF1, TRAF2, and two recently identified signaling factors, c-IAP1 and c-IAP2 (inhibitor of apoptosis protein)⁹¹. This could explain why cell lines generated from p65 knock-out mice are much more sensitive to TNF- α -induced apoptosis⁹. TRAF2 and NF- κ B exert their anti-apoptotic effect independently but synergistically and can reciprocally activate each other^{42, 65}. Stem cells from TRAF2 knock-out mice show increased sensitivity to TNF- α -induced apoptosis⁹⁴. The recently identified protein I-TRAF/TANK negatively regulates activation of NF- κ B⁶⁷. An additional factor of the p80 signaling complex is the TRAF2-interacting protein (A20), which inhibits the activation of NF- κ B through TRAF^{56, 74} and the p80TRAK¹⁹.

It should be emphasized that the complex TNF signaling network presented here is composed of results from studies on a variety of different *in vitro* and *in vivo* systems. These studies have each focused on a limited number of signaling factors and their relationship and, depending on the model system used, the results may sometimes be inconsistent. Therefore, the functional relevance of these relationships in a particular pathophysiological context, e.g. inflammatory bowel diseases (IBD), has to be proven individually.

In Vivo Functions of TNF- α

While the effects and mechanisms of action of TNF- α have been extensively studied in *in vitro* systems, the physiological and pathophysiological role of TNF- α is still incompletely understood. Whereas the synthesis of TNF- α is most likely restricted to activated macrophages and T lymphocytes, the effect of TNF- α is pleiotropic: almost all tissues of the organism can potentially respond to TNF- α ^{10, 86}.

TNF- α has strong proinflammatory and immunomodulating properties. TNF- α plays an important role in the maturation of the cellular and humoral immune systems, leukocyte adhesion and transmigration, on thrombin formation and fibrinolysis, the stimulation of acute phase proteins in the liver, and the upregulation of the body temperature^{16, 27, 57, 85, 88, 89}. Pathogenetically, TNF- α is crucially involved in a variety of acute and chronic inflammatory disease states. The endotoxins of Gram-negative bacteria cause high TNF- α levels, which mediate cardiovascular decompensation and organ failure seen at the end stage of Gram-negative sepsis⁸⁴. While these detrimental effects of TNF- α during sepsis could be dramatically attenuated through TNF- α -neutralizing antibodies in an experimental animal model, this approach showed no sufficient effect in clinical studies^{2, 87}. Studies in TNF- α -deficient mice showed that TNF- α is essential for the immune defense of the intracellular parasite *Listeria monocytogenes*, as well as *Candida albicans* and *Corynebacterium parvum*^{47, 64}. In addition, a role for TNF- α in various different gastrointestinal diseases such as gastritis, pancreatitis and hepatitis, is emerging³⁴.

The important pathogenetic role of TNF- α has been studied most extensively both clinically and experimentally for IBD³⁴. Elevated levels of soluble p60 and p80 could be detected in the urine of patients with Crohn's disease (CD) and ulcerative colitis (UC) and correlated with high disease indices³¹. When lamina propria mononuclear cells were isolated and cultured from colon biopsies of untreated patients with CD and UC, they spontaneously produced more TNF- α than cells from controls⁶³.

An important pathogenetic mechanism of TNF- α in the mucosa seems to be the stimulation of a Th1 T cell response. Lamina propria T lymphocytes from colon biopsies of patients with CD incubated with TNF- α produce increased amounts of the Th1 cytokines IL-2, IFN- γ and TNF- α itself⁶⁰. The stimulation of TNF- α secretion by TNF- α itself suggests a possible positive-feedback mechanism, which could potentially contribute to the perpetuation of inflammation.

Yet another possible mechanism of TNF- α action could be the activation of endogenous matrix metalloproteinases (MMP), which results in damage of the extracellular matrix of the mucosa. This hypothesis was substantiated by an interesting model system of explanted and cultured fetal jejunal tissue⁵⁸.

The critical role of TNF- α for the development of colitis could be reproduced in various established experimental animal models of colitis, including the TNBS-model (2,4,6-trinitrobenzene sulfonic acid), the DSS-model (dextrane sulfate sodium), the IL-10 knock-out mouse and the CD4⁺CD45RB^{high} or CD4⁺CD62L⁺ adoptive transfer mode^{5, 40, 54, 55, 61}.

In Vivo Function of TNF Receptors

With the identification of the two TNF receptors p60 and p80 and the elucidation of their signaling cascades *in vitro*, the distinct roles of these molecules in disease states involving TNF signaling have become the focus of extensive investigation.

In vivo model systems (transgenic and knock-out mice) seemed to confirm the predominant role of p60 in TNF- α signaling. The effects of unfunctional TNF- α on the development of the immune system and the defense of the intracellular bacterium *Listeria monocytogenes* that were seen in TNF- α -defective mice could be reproduced in p60 knock-out mice^{43, 64}. Thus, these effects are most likely p60 related. The role of p60 in mediating the TNF- α effect in Gram-negative sepsis is still controversial^{59, 64}. p60 is also involved in the induction of leukocyte adhesion molecules such as VCAM-1 and E-selectin, which explains the finding that leukocyte infiltration of various organs upon TNF- α stimulation failed to occur in p60 knock-out mice⁵².

Similar manipulations of p80 seemed to have only minor effects. p80 knock-out mice displayed a decreased sensitivity of T cells towards TNF-induced cell death²³. In addition, these mice hardly developed tissue necroses upon subcutaneous injection of TNF- α , as opposed to wild-type controls.

However, in experimental cerebral malaria the presence of a functional p80 was crucial for the development of disease⁴⁵. p80 knock-out mice were almost resistant whereas in p60 knock-out mice the severity of disease did not differ from wild-type mice. This resistance could be related to the lack of upregulation of the leukocyte adhesion molecule ICAM-1 in the cerebral microvessels. In *in vitro* studies on microvascular endothelial cells, sTNF- α required both p60 and p80 to stimulate the upregulation of ICAM-1. Membrane-bound

TNF- α , on the contrary, was able to exert this effect via p80 alone.

The hypothesis that TNF- α signaling via p80 could play a critical pathogenetic role could be demonstrated impressively in a transgenic mouse model overexpressing two alleles of the human p80²¹, which can be activated by murine TNF- α equally efficiently as the mouse receptor. These mice spontaneously developed a severe general inflammatory syndrome involving the pancreas, liver, kidneys and lungs. NF- κ B was constitutively increased in mononuclear cells of the peripheral blood. After cross-breeding with knock-out mice of the genes for TNF- α , lymphotoxin- α , or p60, this severe multiorgan syndrome still occurred. p80 thus seems to possess a TNF ligand independent activity when overexpressed.

Another *in vivo* model in which p80 plays the central pathogenetic role is the experimental hepatitis model induced by concanavalin A⁴¹. Both the apoptotic and necrotic liver damage found in this model are TNF- α -mediated and require a functional p60 and p80. When p80 is overexpressed the mice display an increased sensitivity to concanavalin A. Interestingly, mTNF- α seems to be sufficient as a ligand, since transgenic mice lacking sTNF- α developed hepatitis to the same degree.

There is also increasing clinical evidence for a distinct pathogenetic role of p80 signaling. Elevated levels of soluble p80 could be detected in various severe inflammatory or autoimmune disease states, i.e. sepsis, chronic viral hepatitis, acute pancreatitis, systemic lupus erythematosus, rheumatoid arthritis and AIDS^{20, 26, 28, 48, 71}. Studies of septic patients not only revealed higher levels of soluble receptor than healthy controls: significant differences could also be found between the survivors and non-survivors at the beginning of sepsis. TNF- α levels, on the contrary, did not correlate with the severity of disease or with the outcome. It is not clear how these elevated levels of soluble p80 can be explained.

The increased amounts of soluble receptor could represent a regulatory mechanism to bind and inactivate TNF- α ligand, thus antagonizing the effects of TNF- α . This principle is mimicked by the biotechnically produced fusion protein Etanercept, which consists of the extracellular domain of p80 and the Fc portion of IgG1²⁴. Etanercept shows good clinical effect in rheumatoid arthritis, a chronic inflammatory disease in which TNF- α plays also a pivotal pathogenetic role^{22, 49}.

However, the elevated levels of soluble p80 could also reflect a direct pathogenetic role in these diseases.

Strong direct evidence for a crucial role of p80 signaling in the pathogenesis of CD has been provided by recent experimental studies³³. In this work, it could be shown that p80 expression was significantly increased on mononuclear cells in peripheral blood and in the lamina propria of patients with active CD. In a murine model system of experimental colitis, overexpression of p80 led to severe aggravation of the colitis. This was mediated by the induction of a Th1-like cytokine profile of mononuclear cells and by inhibition of apoptosis in the lamina propria. Both pathomechanisms are relevant in CD.

The emerging distinct role of p80 in the pathogenesis of CD is further confirmed by results from recent clinical trials with other genetically engineered drugs blocking the effect of TNF- α . Infliximab is a IgG1 murine-human monoclonal antibody with a constant region of human IgG1 κ -immunoglobulin and a variable region of a monoclonal mouse anti-human antibody. Infliximab binds both soluble and membrane-bound TNF- α and most likely blocks the interaction of TNF- α with the TNF receptors in this way^{39, 69, 73}. Additional effects are complement fixation, antibody-dependent cytotoxicity, and induction of monocyte and T cell apoptosis⁴⁶. In severe cases of refractory or steroid-dependent CD, a single intravenous administration of Infliximab (5 mg/kg body weight) often leads to a significant clinical improvement^{62, 80, 90}. CDP571 is another genetically engineered antibody to both soluble and transmembrane TNF- α that consists of approximately 95% human sequences and 5% murine sequences¹. Initial controlled trials also suggest clinical efficacy of CDP571 in CD⁷⁶.

Of note, in a study on patients with CD, the fusion protein Etanercept has failed to show any clinical efficacy beyond placebo⁶⁸. This is particularly interesting, because Etanercept only binds to and blocks sTNF- α . It thus appears that membrane-bound TNF- α is the relevant ligand for TNF signaling in CD, and this highlights the probably critical role of p80 and p80 signaling in CD⁹⁰.

Conclusions

There is increasing clinical and experimental evidence suggesting a distinct role of p80 and p80 signaling in a variety of inflammatory disease states, in particular CD. The existing non-selective anti-TNF- α strategies in the treatment of CD and rheumatoid arthritis are promising, but their implementation in clinical practice is restricted by limited efficacy, high costs and

rare, but sometimes severe, short-term side effects as well as potential long-term side effects^{11, 38}. Considering the increasing clinical and experimental evidence for a pivotal role of TNF signaling via mTNF- α and TNF-R2 in various inflammatory disease states, in particular in the pathogenesis of CD, this TNF signaling pathway should be the focus of future studies. It can be expected that critical steps in TNF signaling will be identified. This will not only improve our understanding of the pathogenesis of these diseases, in particular IBD, but will also reveal new, more specific therapeutic targets in the future.

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