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Perspective

Therapeutic Potential and Strategies for Inhibiting Tumor Necrosis Factor-α

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Introduction

In 1975 tumor necrosis factor (TNF) was defined as an endotoxin-induced serum factor which produced necrosis of tumors both in vitro and in vivo.[1] The original works on TNF focused on the striking antitumor activity of this apparent protein factor. Old traced the description of this factor back to the 1800s and the use of killed bacteria by Coley to treat cancers.[2] With the advent of molecular biology, TNF was selected as one of the early targets, leading to the successful cloning and sequencing of human TNF-α (referred throughout this Perspective as TNF) in 1984 by Goeddel and colleagues.[3]

While the biological limitations of TNF as an antitumor agent were rapidly becoming apparent, the protein began another wave of interest when it was discovered to have activity which paralleled another important protein of the time, interleukin-1 (IL-1). The striking parallels in cytokine activity and the synergy exhibited between the two were truly remarkable. In the late 1980s, a number of pharmaceutical researchers began to seek small-molecule antagonists of TNF binding to its receptors. As with its partner in cytokine biology, IL-1, the large amount of effort expended was met with little success. When the interest in describing the activities of TNF as a cytokine began to dwindle, TNF faded into the background as a number of targets more amenable to classic drug discovery efforts took center stage.

In 1994 studies by Centacor and the Kennedy Institute demonstrated the striking potency of an anti-TNF antibody, known at the time as cA2, in treating human rheumatoid arthritis patients.[4] For perhaps the first time since cyclooxygenase, inflammatory disease researchers were provided with a validated target on which they could focus their efforts. A second important finding by Gearing and colleagues at British Biotech was that the precursor of TNF (proTNF) was processed to the soluble cytokine through the actions of a metalloprotease and that TNF release could be inhibited by small-molecule metalloprotease inhibitors.[5] Black and colleagues at Immunex and Moss and colleagues at Glaxo followed up this information by identifying a new member of the metalloprotease family, termed TACE (**T**NF **A**lpha **C**onverting **E**nzyme), that cleaved the 26-kDa proTNF to the soluble 17-kDa protein.[6,7] Together, these findings triggered the current wave of interest in small-molecule antagonists of TNF. Indeed, many targets in inflammatory diseases took on new meaning because their apparent biological effects could be associated with a decrease in either TNF production or TNF action.

Other potential applications of TNF antagonism have furthered this interest. The antibodies and receptor/immunoglobulin therapeutics have shown dramatic effects in Crohn's disease, and preliminary encouraging data has also been obtained in ulcerative colitis. TNF-modulating therapeutics such as thalidomide and vesnarinone, through undefined modes of action, have demonstrated efficacy in cachexia and congestive heart failure, respectively. Animal models and association studies in humans have indicated a potential role for TNF in insulin resistance,[8] multiple sclerosis,[9] multiple organ failure,[10] pulmonary fibrosis,[11] and atherosclerosis.[12]

Confirmation of the biological activity of TNF has come from studies in mice where either the TNF ligand

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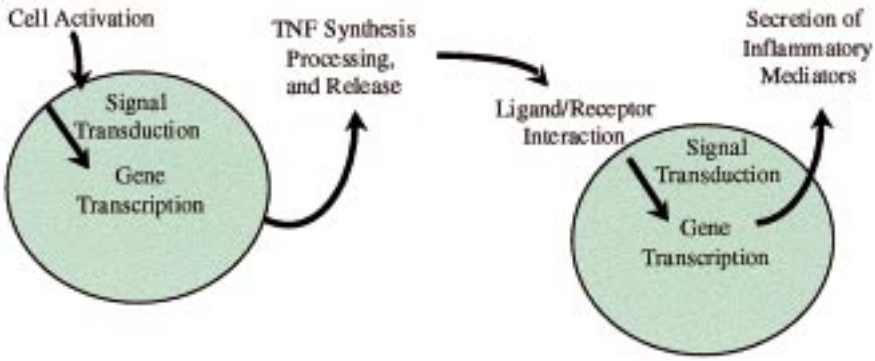


Figure 1. Potential sites for intervention in the production/action of TNF-α.

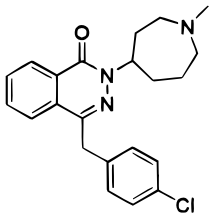
or the TNF receptors (TNFRs) have been genetically deleted. Unlike a number of constitutive systems, the immune system is not necessary for fetal development and is therefore not subject to selective pressure and elicitation of compensatory mechanisms during development. Animals with deleted TNFR1, which appears to be the main signaling receptor on most cells for TNF, are resistant to the effects of TNF, are resistant to endotoxin shock,[13,14] and are also resistant to collagen-induced arthritis.[15] Knockout animals deficient in TNF are resistant to endotoxin challenge, have a deficiency in granuloma development, and do not form germinal centers after immunization.[16] In complementary studies, overexpression of human TNF or of membrane proTNF leads to marked chronic inflammatory polyarthritis[17,18] that can be modulated with TNF-neutralizing strategies.

TNF and TNF responses are not totally dispensable however. Mice lacking TNFR1 show deficient liver regeneration after partial hepatectomy[19] and are impaired in their ability to clear *Listeria*, *Mycobacteria*, and *Candida*.[13,20] This is not due to an inability to destroy the microbe,[21] and the lack of germinal center formation in these mice may indicate impaired, immune function.[22] Not all antimicrobial responses are severely impaired, and TNFR1 knockout mice can resist infection with the intracellular parasite *Leishmania*.[23] Importantly, with the exception of an increased incidence of upper respiratory infections, the numerous human clinical studies conducted with anti-TNF molecules have not demonstrated significantly increased rates of infections.

A number of recent reviews have been published on TNF inhibitors that are currently undergoing evaluation.[24−26] This Perspective will consider TNF as a validated target for drug discovery. Our objective will be to move beyond compounds that are currently under study and, using data generated from both mechanistic and compound discovery research, identify sites amenable to the design of small-molecule therapeutics. Because of the scope of this undertaking, none of the targets will be reviewed in great depth. For a more detailed review of the biology of this cytokine, the reader is referred to other literature (for example, see ref 27). For information on specific targets, the reader is encouraged to focus on some of the key elements provided and delve further into the vast literature in each area. Our focus will be on providing a general understanding of TNF biology and then reviewing potential intervention sites in TNF production and signaling (Figure 1).

Induction of TNF Expression

Cell Sources. With suitable activation, many cell types are capable of producing TNF.[28] However, the two main sources in vivo appear to be T lymphocytes and cells of the monocytic lineage. This is by no means limiting since cells belonging to the monocytic lineage can be found in every organ and include diverse phenotypes such as liver Kupffer cells, brain microglia, and skin Langerhan cells. All of these cell phenotypes have been shown to be capable of TNF production upon appropriate stimulation. In addition, T lymphocytes continuously recirculate throughout the body via the blood and lymphatics and can produce TNF in response to the appropriate antigen. Another interesting cell type capable of producing TNF is the mast cell.[29] Production of TNF by this cell type would make TNF an important factor in immediate inflammatory responses that could be impacted by strategies that target mast cells. In this regard, the histamine release antagonist azelastine (Eisai Co.) has also been shown to inhibit TNF release by mast cells.[30]



azelastine

Cell Activation. The classic inducing stimulus for TNF harkens back to the original discovery of tumor cytolytic activity induction by endotoxin. Gram-negative bacterial lipopolysaccharides (LPS) remain the most potent cell activator for cells of the monocytic lineage. Cells demonstrate a rapid upregulation of mRNA and protein synthesis following LPS exposure. Within 1 h, appreciable mRNA for TNF can be detected, and protein is produced beginning between 90 and 120 min. Administration of LPS to healthy volunteers shows a similar rise in TNF 90−180 min after exposure.[31] Production is rapid but transient with a decrease in mRNA, and subsequently protein, noticeable within 6 h of the original activation. Interestingly, human blood monocytes that have been triggered to produce cytokines by LPS exposure appear to enter an altered state of differentiation and cannot be activated to produce significant additional cytokine at later time points.

Many studies have been conducted to define the cell receptors and signal transduction pathways that are part of LPS signaling. At least three receptors for LPS have been defined.[32,33] The most prominent receptor, which exhibits the highest apparent affinity, is the CD14 protein. This glycophosphatidylinositol (GPI)-anchored molecule, expressed on the surface of monocytes, binds LPS in complex with the serum LPS-binding protein.[34] Other surface molecules which have been demonstrated to bind LPS include the CD11b/CD18 complex (integrin $\alpha M\beta 2$, Mac-1 antigen) and the scavenger receptor. Strategies designed to block LPS interaction with monocytes under clinical settings (anti-LPS antibodies, polymyxin B, lipid X, soluble CD14, etc.) have a long history of success in vitro[35] but failure in vivo.

In addition to LPS, a number of microbial agents or extracts from microbial sources have been shown to induce TNF secretion by macrophages. Stimulation of TNF production can also be accomplished by silica particles and urate crystals.[36] Fibronectin-coated surfaces and fibronectin fragments, most likely acting through $\beta 3$ integrins, induce TNF expression.[37,38] While occupancy of the low-affinity macrophage scavenger receptor activates TNF expression, oxidized low-density lipoprotein acting through a different receptor has been shown to inhibit TNF.[39] The T cell-derived cytokine interleukin-17 has also been shown to induce macrophage TNF production.[40] At this time, the receptors and the proximal signals for most of these agents are ill-defined.

In general, T lymphocyte activation by mitogens, activating antibodies, superantigens, or antigens with costimulation can induce TNF production. When and where this production by T lymphocytes becomes important in an immune response and how this might differ temporally and spatially from monocyte production is unknown. With the possible exception of the microbial products, the physiological and pathological roles of the various agents in inducing or modulating TNF production by either monocytes or T lymphocytes are unknown.

Signal Transduction. After receptor interaction a number of potential signaling pathways exist in cells to transfer the cell surface signal to the nucleus. Although studies of LPS signaling have demonstrated that many of these pathways are activated, a critical path has yet to be defined. There may be subtle differences in signals generated depending on the degree of LPS exposure, the cell type, and other signals which may be received at the same time. The specific pathways used by agents other than LPS in activating TNF gene expression also remain to be defined and are an intense area of current research.

1. NF-κB. One important signaling pathway in the induction of TNF expression leads to activation of the transcription factor NF-κB (Figure 2).[39,41] While this pathway has been well-studied in TNF effects (vide supra), an important role has also been defined in signaling for TNF expression. NF-κB is a family of transcription factors termed the Rel family that form homodimers and heterodimers, the prototype of which is a heterodimer of p50/NF-κB1 and p65/RelA. Each monomer contains a region that binds DNA and a regulatory region that interacts tightly with a class of specific inhibitory proteins termed IκBs.[42] The IκB proteins mask the nuclear translocation signal of the NF-κB dimer and prevent nuclear translocation and gene activation. The importance of this collection of transcription factors lies in their role in controlling expression of a number of proinflammatory molecules, including cytokines, chemokines, cell adhesion molecules, and MHC proteins.

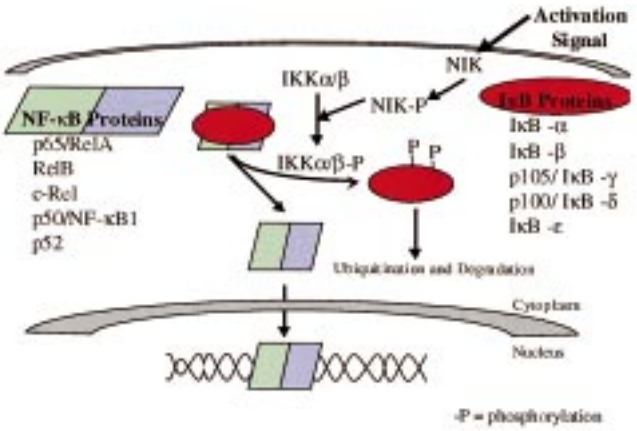


Figure 2. NF-κB signaling path.

Different physiological roles for the various Rel proteins are evident from animal studies. Irradiated recipient animals reconstituted with fetal liver cells from RelA knockout animals (which by itself is embryonic lethal) develop functional immune responses but show increased circulating granulocytes and spontaneous inflammation of the distal extremities.[42] Knockout of RelB in mice is characterized by multiorgan inflammation with myeloid hyperplasia and splenomegaly.[43] Mice deficient for c-Rel contain mature lymphocytes that are unresponsive to mitogenic stimuli.[44] Therefore the Rel family members can be demonstrated to regulate both positive and negative aspects of inflammation. The varied physiological roles for the NF-κB proteins, the ability of the members to form heterodimers, and the presence of multiple IκB proteins indicate the potential for further selectivity of function, and the area is a subject of current study.

The major point for regulation of the NF-κB signal path appears to be through the association with the inhibitory IκBs. The TNF suppressive activity of interleukin-11[45] and glucocorticoids[46] can be attributed, in part, to an upregulation of IκB protein expression. Recently, a number of laboratories have identified two related IκB kinases (IKK-α, IKK-β) which phosphorylate IκB.[47−49] It has been known for some time that proinflammatory stimuli rapidly lead to phosphorylation of IκB, and this event precedes the loss of the protein from the cell through degradation by the proteosome.[50] The released NF-κB is transported to the nucleus where it initiates gene transcription after binding to the appropriate DNA motifs. From studies of TNF-mediated activation of NF-κB (vide supra), NIK (**N**F-κB **I**nducing **K**inase), a member of the **M**itogen-**A**ctivated **P**rotein (MAP) kinase kinase kinase family, was thought to phosphorylate IκB.[51] However, it was later shown that, while NIK interacted with TRAF-2 (**T**NF **R**eceptor-**A**ssociated **F**actor) and the TNF receptor, it did not directly phosphorylate IκB.[47] CHUK (**C**onserved **H**elix−

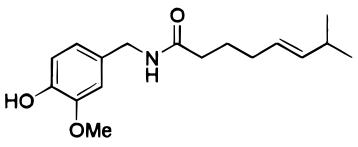
loop−helix **U**biquitous **K**inase) or IKK-α, a serine threonine kinase, was demonstrated to associate with IκB complexed with NF-κB and to directly phosphorylate IκB under the regulation of NIK.[47] Overexpressed IKK-α can induce NF-κB activation, and kinase-inactive mutants of IKK-α act as dominant negative regulators of NF-κB activation by cytokines or overexpressed NIK. IKK-β appears to form heterodimers with IKK-α, and it is the heterodimer that may be the active form that optimally interacts with NIK.[49] The path from receptor activation to NIK activation of IKK-α is currently unknown, although activation of phosphorylation occurs on a broad scale within cells following activation with agents such as LPS.

Interference with either the phosphorylation or the degradation of IκB has been demonstrated to inhibit TNF expression and have an antiinflammatory effect in vivo. Inhibition of phosphorylation can be accomplished through the use of kinase inhibitors such as herbimycin A.[52] Likewise, NF-κB can be activated through okadaic acid inhibition of the phosphatase that presumably regulates phosphorylation of IκB.[53] However, it was found that phosphorylation by itself is not sufficient for removing the IκB block of NF-κB translocation. IκB proteins are proteolytically degraded after phosphorylation, and the addition of general protease inhibitors such as *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) can also block NF-κB activation[54] and NF-κB-dependent gene expression.[55] An alternate to directly inhibiting proteosome proteolytic activity has been demonstrated through inhibition of the IκB-targeting ubiquitin ligase.[56] Despite an intense amount of effort in the areas of phosphorylation and proteosome degradation, no selective inhibitors of the NF-κB pathway have been disclosed.

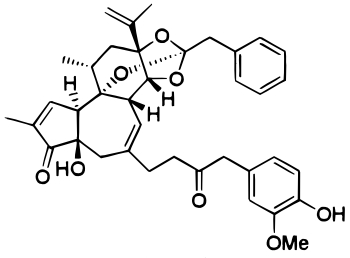
A pair of recent publications describing the crystal structure of the IκB−NF-κB complex may offer some new insights.[57,58] The two reports differ in the details extracted from the heterotrimeric crystals, but there appears to be a high degree of overlap in the area where the residues of IκB-α interact with the NF-κB subunits. A large number of amino acid residues are involved in the interaction, and the model provides an explanation as to how IκB prevents the NF-κB dimer from binding to DNA. Part of the p65 subunit is dramatically changed in conformation when bound to IκB-α, occluding critical residues involved in contacting DNA. The residues of IκB-α important for proteosome degradation are in the N-terminus, which the models predict will be unstructured and fully accessible, both for modification by kinase and for ubiquitination.

An interesting illustration of NF-κB regulation of TNF expression is found in a recent study[59] involving adenoviral transfection of cells with IκB-α. The well-illustrated spontaneous production of TNF by synovial explants from rheumatoid arthritis patients could be suppressed by overexpression of IκB-α. In further support of a critical role for IκB-α in regulating TNF production, this method also inhibited TNF production by normal human macrophages stimulated with LPS.

Capsaicin and its analogue resiniferatoxin inhibit IκB degradation and NF-κB activation.[53] Capsaicin has been shown to bind and act through the vanilloid receptor[60] and to also have other nonselective properties such as altering the redox state of the cell.[61] The redox state of



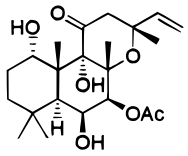
capsaicin (6CI)



resiniferatoxin

the cell has been demonstrated to influence NF-κB signaling,[62] and NF-κB-dependent transcription has been shown to be inhibited by antioxidants and activated or potentiated by reactive oxygen species.[63] Oxidants such as anthralin induce NF-κB activation and TNF production, while antioxidants such as *N*-acetylcysteine, tetramethylthiourea, vitamin E, and apocynin inhibit induction.[64−67] Antioxidants appear to interact directly with the p50 subunit of NF-κB as well as inhibit IκBα degradation.[68] However, conceiving of NF-κB inhibition strategies by selectively modifying the redox state of a particular cell type is a rather daunting task.

2. cAMP Modulation. Activation of cells through a number of seven-transmembrane G protein-coupled receptors results in modulation of adenylate cyclase and cellular cyclic adenosine monophosphate (cAMP) levels. Activation of adenylate cyclase has been demonstrated with occupancy of β-adrenergic, A2- and A3-adenosine, histamine, and prostaglandin receptors, and in general, agents that increase cAMP inhibit human TNF production.[67,69−71] Forskolin is a natural product diterpene that has been demonstrated to reversibly activate adenylate cyclase[72] and inhibit TNF production at the signaling/transcriptional level.[73] The conserved CKS-17 transmembrane envelope protein of leukemia viruses also suppresses TNF production in association with elevated cAMP.[74] Inhibition of TNF production by elevated cAMP appears to occur at both a transcriptional level as well as a posttranscriptional level.[75]

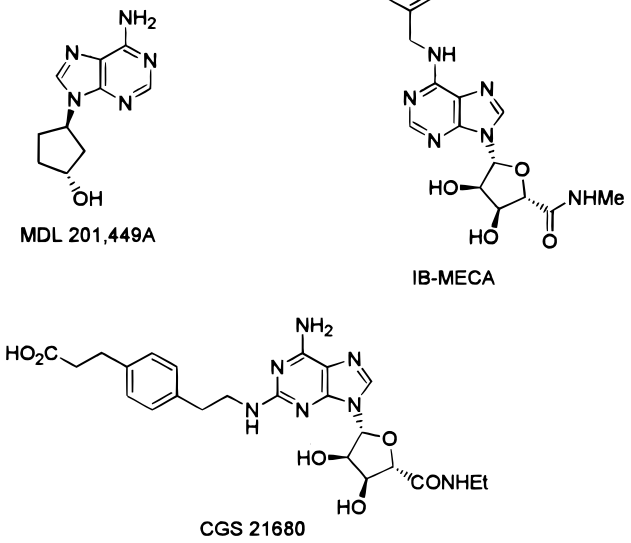


forskolin

The cAMP signaling cascade is closely associated with the activation of the CREB/ATF-1 family of DNA-binding proteins. cAMP binds to the regulatory subunit of protein kinase A, resulting in release of the regulatory subunit and catalytic activation. The active catalytic subunits phosphorylate a variety of intracellular proteins, including **CRE B**inding protein (CREB), **A**ctivat-

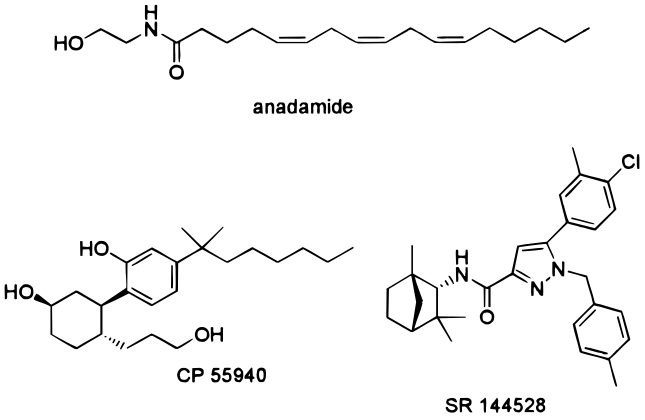
ing **T**ranscription **F**actor (ATF), and **CRE M**odulating protein (CREM) that bind to the CRE (**c**AMP **R**esponse **E**lement) DNA site. Besides directly acting via CRE sites in DNA, members of the CREB/ATF family can also dimerize with fos and jun and bind to AP-1 (**A**ctivating **P**rotein-1) motifs.[76] Fos and jun also contain CRE elements in their promoters, providing for additional points of regulation.

An example of the effects of cAMP modulation and the interaction with other signal pathways can be illustrated through the actions of adenosine. Under metabolic stress, cells undergo a rapid depletion of intracellular ATP. The accumulated AMP is degraded to adenosine that can be released locally at micromolar concentrations despite the actions of adenosine deaminase, which degrades adenosine to inosine. Adenosine can act through four known adenosine receptors (A1, A2a, A2b, and A3). A2 receptor agonists have been shown to induce cAMP levels in cells and to inhibit TNF production at the transcriptional level.[77] However the inhibition observed with adenosine and its analogues does not indicate that elevation of cAMP is the sole mode of action.[78] A profile of selective adenosine receptor agonists revealed that the A3 receptor plays a pivotal role in suppressing TNF production[79,80] and that the mechanism of inhibition appears to be independent of cAMP generation and related to an inhibition of NF-κB and AP-1 signaling. In vivo studies with the A2 agonist CGS 21680 and the A3 agonist IB-MECA indicate that both receptors mediate the reported effects of adenosine through direct suppression of TNF and indirect suppression by induction of interleukin-10.[81] Recent investi-



gations[82−84] have uncovered a series of 3-hydroxycyclopentyladenines that inhibit macrophage-derived TNF production selectively in comparison with inhibition of IL-1β and interleukin-6 production (MDL 201,449A IC_{50} = 9 μM). Compounds in this class dosed orally were effective in an LPS-mediated murine model of septic shock.

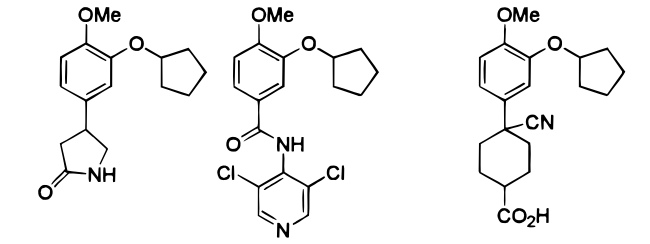
An interesting example of an adenyl cyclase-linked receptor that has been demonstrated to possess immune modulation properties is the peripheral cannabinoid receptor (CB2).[85] Numerous reports have described the effects of marijuana and its constituent cannabinoids in suppressing immune responses, and the CB2 receptor has been found almost exclusively on cells of the immune system. A natural agonist ligand has been identified as the arachidonic acid-related molecule anadamide.[86] The anomalous finding with this receptor (when compared to the receptors listed above and the apparent activity associated with cAMP elevation) is that it is associated with inhibition of cAMP signaling and the magnitude of inhibition of cAMP correlates with the potency in immune modulation.[87] Apparently this modulation occurs through NF-κB. It has been demonstrated in lymphocytes that activation of cAMP-dependent kinase and protein kinase C pathways can lead to phosphorylation of IκB and activation of NF-κB.[88] Consistent with this hypothesis is an enhancement of lymphocyte apoptosis observed with CB2 agonists.[86] While CB2 has been shown to modulate numerous activities of B and T lymphocytes, suppression only of macrophage nitric oxide production (NF-κB dependent promoter)[89] and antimicrobial activity has been described. There are no literature reports of effects on inflammatory cytokine production with either macrophages or lymphocytes, and it would be interesting to speculate that lymphocyte TNF might be selectively inhibited by cannabinoids. There are literature reports of CB2-selective molecules,[90] and a number of companies (Sanofi, Merck-Frosst, Pfizer) are reportedly developing CB2-directed molecules to follow up on these findings. A recent report by Sanofi has described the properties of the CB2 antagonist SR 144528 relative to the agonist CP 55940.[91]



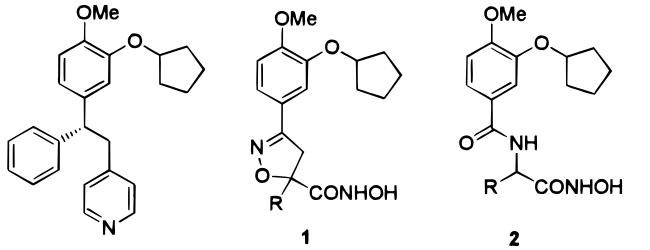
3. cAMP Phosphodiesterase Inhibition. A number of reviews on phosphodiesterases (PDEs) and their role in inflammation have been published.[92] PDEs metabolize cAMP and cGMP, and inhibition of PDEs leads to an increase in the intracellular levels of these mediators. Seven families of PDE isozymes have been identified that can be grouped according to their selectivity for cAMP or cGMP.[93,94] While the hydrolytic role of the PDE enzyme is constant across the family, the isozymes differ in structure, substrate specificity, endogenous activation, and tissue distribution. PDE4 has been shown to be the major form present in inflammatory cells,[95] and this observation has led to the hypothesis that selective inhibition of PDE4 will result in an antiinflammatory effect devoid of the side effects associated with inhibition of the other PDE isozymes.

The known selective PDE4 inhibitors have been recently reviewed,[96] and only select recent examples will be discussed here. The selective PDE4 inhibitors have been grouped into three broad structural classes: catechol ethers (exemplified by rolipram), bicyclic heteroaromatics (Nitraquazone), and xanthine derivatives of theophylline (Denbufylline). All have been shown to reduce TNF production.

Rolipram was originally developed as an antidepressant and has been shown to be selective for PDE4. PDE4 is also known to be present in the central nervous system (CNS), and PDE4 inhibitors that cross the blood−brain barrier have been observed to cause nausea. A human volunteer study with Cell Tech's CDP 840, a potent first-generation PDE4 inhibitor, demonstrated dose-limiting side effects. However, it was shown not to induce emesis, indicating that not all PDE4 inhibitors have this property. Two series of quaternary-substituted indoles representing conformationally constrained analogues of CDP 840 have recently been reported.

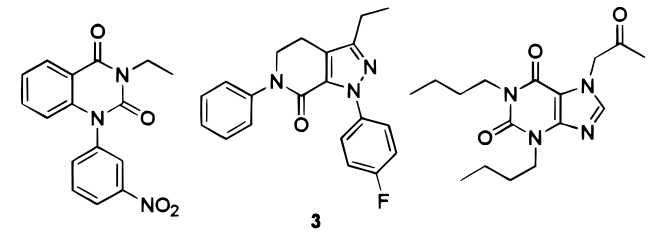


Rolipram RP 73401 Airflo™ (SB-207499)



CDP 840 **1** **2**

Pfizer hydroxamate PDEIV inhibitors (R = H, Me, Et, Pr)



Nitraquazone (TVX 2706) **3** pyrazolopyridine (Pfizer) Denbufylline (Brl-30892)

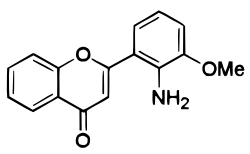
The SmithKline group has recently reported on the development of a series of 1,4-cyclohexanecarboxylates that are potent cell type-selective PDE4 inhibitors. Second-generation orally active PDE4 inhibitors with decreased side effect potential have resulted in the development of SB-207499 (Airiflo).[97] The strategy for this development focused on identifying compounds that selectively inhibit human monocyte-derived PDE4 over the ability of compounds to compete for high-affinity rolipram binding sites in the CNS. The nature of these binding sites has been postulated to be distinct, and data from these investigations support the approach. Airiflo is essentially inactive against the other PDE isozymes but competitively inhibits monocyte and CNS PDE4 with a K_i of 92 nM. By contrast, rolipram has been shown to be only 60-fold selective for PDE4. Airiflo was shown to have a much-improved therapeutic index as measured by in vitro TNF inhibition compared with emetic potential. The success of these improvements to rolipram remains to be determined with the progression of Airiflo through clinical trials.

The Pfizer group has reported a novel series of potent hydroxamic acid inhibitors of TNF production (exemplified by **1** and **2**).[98] By overlapping compounds in this series with cAMP and considering the metal-liganding property of the hydroxamic acid, a model for PDE4 inhibition was proposed where the catechol and adenosine groups bind in the same region of the enzyme. A structural overlap also exists with rolipram that may explain the PDE4 selectivity. Compound **1** has an IC_{50} of 0.03 μM in an in vitro human whole blood assay for TNF production. While this represents the most potent PDE4 inhibitor known, clinical developments with these compounds have not been reported.

Another recent report by the Pfizer group described the discovery of a series of pyrazolopyridines exemplified by **3**.[99] The structure−activity relationship (SAR) to rolipram was discussed along with data indicating that this series shows selectivity for PDE4 over the related PDE isozymes. Biological data indicates oral activity with this series in models of asthma.

PDE4-selective inhibitors have been shown to be potent cytokine inhibitors and have demonstrated efficacy in a number of inflammatory models. The clinical indications most studied are asthma and rheumatoid arthritis, but clearly any inflammatory disorder associated with elevated TNF represents a potential therapeutic target. The major hurdle in this field is to overcome the side effect profile associated with the CNS activity of the molecules. Clinical data on some of the more cell type-selective molecules such as Airiflo will determine the therapeutic potential of PDE4 inhibitors as antiinflammatory agents.

4. MAP Kinases. There are three major pathways involving MAP (**M**itogen-**A**ctivated **P**rotein) kinases: the p44/42 MAP kinase or **E**xtracellular Signal-**R**egulated **K**inase (ERK) path, the **S**tress-**A**ctivated **P**rotein **K**inase (SAPK) or c-**J**un **N**-terminal **K**inase (JNK) path, and the p38 path (for a general review of MAP kinases, see ref 100). LPS activation of macrophages has been shown to result in the phosphorylation of ERK,[101] and in T cells, macrophages, and mast cells, inhibition of the ERK pathway with the noncompetitive MEK1 inhibitor PD 98059 has been shown to inhibit TNF



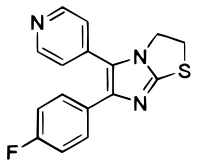
PD 98059

expression.[102−104] It is not yet known whether these inhibitory effects are exerted primarily at the transcriptional or posttranscriptional level. However, it has been

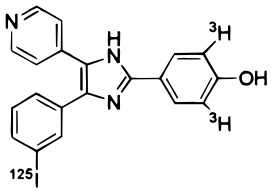
shown that active ERK, besides activating the AP-1 transcriptional complex, is capable of phosphorylating IκB, leading to NF-κB activation.[105]

As yet, there is no information on the properties of selective inhibitors of the JNK pathway. While well-studied for its role in TNF receptor signaling (vide supra), there are few reports of the potential role for JNK in inducing TNF production. In this regard, the yopJ protein of *Yersinia pseudotuberculosis* has been shown to suppress TNF production in concert with the inhibition of phosphorylation of both JNK and p38 MAP kinases.[106]

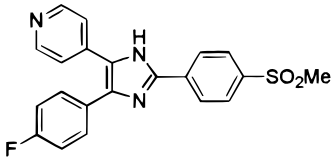
5. p38 Inhibitors. Pyridinylimidazoles, typified by SK&F 86002, were known to have broad antiinflammatory activity and the ability to inhibit TNF and IL-1β production.[107] Biochemical studies showed that the predominant effect on TNF biosynthesis occurred at the protein synthesis level with little effect on transcription.[108,109] An extensive SAR study around a subset of this class (2,4,5-triarylimidazoles) by the SmithKline group showed that these compounds suppress cytokine production through a novel mechanism of action.[110] Using radiolabeled inhibitor, a competitive binding assay was developed which enabled further SAR study and the discovery of submicromolar inhibitors of cytokine production. The target proteins were found to have homology to HOG-1, a known MAP kinase. These newly discovered proteins were termed CSBP-1 and -2 (**C**ytokine-**S**uppressive **B**inding **P**roteins-1 and -2).[111,112] CSBP-2 was found to be identical to human p38α.[113] Extensive study of this target has revealed that p38α is involved in a stress-response signal transduction pathway which can be activated by LPS and proinflammatory cytokines.[114] It has been postulated that p38α phosphorylates a protein or protein effector complex bound to the AUUA regions of the cytokine mRNA, releasing translational repression and allowing cytokine protein synthesis to occur.[115]



SK&F 86002



SB-206718



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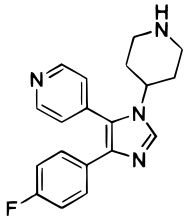


VK-19911

The identification of p38α and its relevance as a target for TNF and IL-1 suppression have been realized through an extensive SAR development of these lead compounds. The structural basis for the binding and specificity of p38α MAP kinase inhibitors using X-ray crystallography has shown that the inhibitors bind in the ATP binding site of the kinase.[116,117] Biochemical studies, combined with enzyme mutagenesis, support this model.[118] This discovery is quite remarkable when one examines the impressive selectivity that is achieved with a number of these compounds. SB-203580, for example, has been shown to be highly selective for p38α compared with 14 different kinases and phosphatases.[119]

Crystallographic studies with SB-203580 and VK-19911 support the importance of threonine 106 as a key residue in determining the specificity of these compounds. These studies and related examples of selective kinase inhibitors [120−122] show that it is possible to exploit the ATP binding pocket, a common feature in this class of enzymes. The structures show that the crucial pyridyl nitrogen mimics the N1 nitrogen of the adenine ring of ATP. The SAR supports this finding in that the 4-pyridyl substituent has been shown to be essential for activity. This alignment specifically protrudes the 4-substituted phenyl toward a part of the binding pocket not occupied by ATP, which suggests that the phenyl ring is the major determinant of specificity for this class of inhibitors.

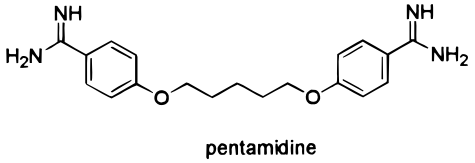
The pharmacologic effect of SB-203580 has been demonstrated through positive modulation of a number of animal models of inflammation.[123] This compound also inhibits bone resorption and rescues mice administered lethal doses of endotoxin, effects consistent with the in vitro mode of action of cytokine suppression. The clinical impact of these compounds has presumably been hindered by the fact that the pyridinylimidazoles are potent P450 inhibitors, rendering them difficult to study in clinical trials. Aminopyridinylimidazoles have recently been reported as molecules that may circumvent this problem.[124] As with other kinase inhibitors, inhibitor specificity and the functional redundancy of these enzymes are the key hurdles in the development of such compounds.

6. Other Signal Pathways. Since NF-κB appears to be central to the activation of TNF expression, pathways that modulate this signal transduction path should also impact TNF expression. Substance P, which signals through NK1 receptors, has been shown to induce TNF expression and enhance LPS-induced expression. This receptor is linked to IP3 and diacylglycerol signaling and may act via PKC phosphorylation of IκB.[125] Through an unknown mode of action, thromboxane A2 appears to play a role in signaling expression of TNF, and inhibition of thromboxane synthetase by G619[126] can inhibit TNF induction.[127] In platelets, the thromboxane receptor is coupled through G proteins to phospholipase C and the pathways may be similar to the NK1 system.

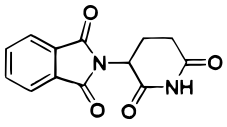
Interleukin-10 has been demonstrated to be a biological inhibitor of TNF production. The signaling with interleukin-10 appears to be diverse, and suppression occurs through both transcriptional and posttranscriptional modulating pathways. Interleukin-10 suppression at the transcriptional level has also been shown to be enhanced by cAMP-elevating agents.[128] The cytokines interleukin-4 and interleukin-13 have also been demonstrated to suppress monocyte cytokine production, presumably though an effect at the transcriptional level.[129]

A number of other small molecules have also been described to variably inhibit TNF release by LPS-activated cells, by either direct or indirect mechanisms.

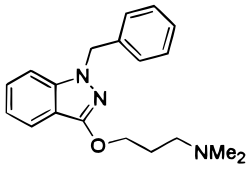
These include the steroids dexamethasone and progesterone, angiotensin-converting enzyme inhibitors, the antibiotic fusidic acid, pentamidine, fenspiride, bisphosphonates, benzydamine, bisbenzylisoquinoline alkaloids, and chloroquine.[70,130−134] Dexamethasone and chloroquine act at the transcriptional level. Dexamethasone has also been shown to have an inhibitory effect at the translational level[135] and to induce expression of IκB.



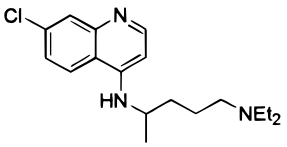
pentamidine



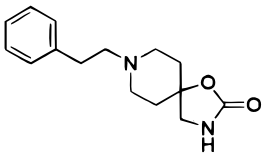
thalidomide



benzydamine



chloroquine



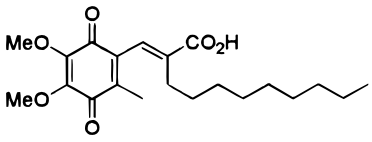
fenspiride

Thalidomide has been demonstrated to inhibit TNF production,[136] and Celgene has pursued thalidomide analogues with enhanced anti-TNF and reduced teratogenicity properties. Thalidomide has been approved (Thalomid) for treatment of **E**rythema **N**odosum **L**eprosum (ENL), a serious skin complication that occurs in leprosy, and an NDA filing is planned for cachexia in **A**cquired **I**mmuno**d**eficiency **S**yndrome (AIDS) patients. More selective analogues are currently under clinical evaluation. The rather intriguing parent molecule produces a number of highly reactive metabolites that may bind to various cell components. The TNF effect appears to be primarily at the transcription factor level and may explain both some of the cell-selective effects as well as the detrimental effects on the genes responsible for limb bud formation.[137]

Gene Transcription. After signaling to the nucleus, a number of transcription factors become involved in the expression of TNF mRNA. The gene for human TNF has been mapped to human chromosome 6p21.3-p21.1.[138] Interestingly, in humans, the gene for TNF is located within the major histocompatibility complex between the HLA-B and C4A genes.[139] A number of immune-based diseases have been shown to have genetic linkage to this region, and a similar location has been noted in mice and rabbits. A microsatellite marker from the TNF region has been associated with the susceptibility to rheumatoid arthritis.[140]

In the TNF promoter, about 220 base pairs of the upstream sequence are conserved among rabbit, mouse, and human,[141] and a variety of potential regulatory sites have been mapped.[142] More than 600 base pairs of the upstream sequence of mouse TNF are essential for transcription in vitro,[143] and 0.6 kb of the promoter confers LPS inducibility in a transgenic mouse model.[18] There are four major classes of *cis*-acting regulatory elements that have been shown to mediate macrophage sensitivity to LPS: Rel homology family, **C**AAT/**E**nhancer **B**inding **P**rotein family (C/EBP), CRE family, and the fos/jun family. Other regulatory elements, such as Sp-1, function constitutively and are essential, but not sufficient, for transcription.

As already mentioned, the promoter contains CRE elements that confer regulation by cAMP. Control of TNF transcription through activation of the C/EBP family members[144] may be through association with elements of the AP-1 complex. The promoter contains at least one AP-1 site that can regulate gene expression[142] and is thought to be a major site for glucocorticoid regulation.[145] There is evidence in T lymphocyte production of TNF for a role for the transcription factor **N**uclear **F**actor of **A**ctivated **T** cells (NFAT),[146] which is regulated by the calcium/calmodulin-dependent phosphatase calcineurin, a target for inhibition by cyclosporin A and FK506. However, human monocyte production of TNF in response to LPS is unaffected by cyclosporin A. Except for the actions of glucocorticoids on AP-1, the direct regulation of transcriptional activity remains in the realm of speculation and is the subject of increasing interest as a target. The promoter contains a number of NF-κB regulatory sites. In this regard, it is interesting that the quinone derivative E3330 has



E3330

been shown to selectively inhibit NF-κB activity by influencing DNA binding of the active transcription factor,[147] putatively through binding to an undefined 40-kDa protein. Through a mechanism involving a block in NF-κB-binding to DNA, acetylsalicylic acid and its metabolite sodium salicylate, at doses that correspond to antiinflammatory activity, also block NF-κB mediated lymphocyte activation.[148,149] However, the science of inhibiting specific gene expression through direct modulation of transcription factors is still in its infancy.

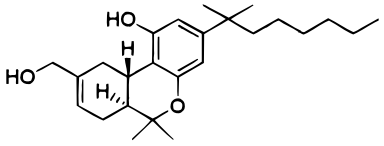
TNF Synthesis, Processing, and Release

Protein Synthesis. After transcription, the mRNA must be translated and the protein expressed. An interesting regulation of posttranscriptional splicing of TNF message in lymphocytes has been noted with the adenine isomer 2-aminopurine.[150] Due to the inhibition of ATP-dependent phosphorylation of the α subunit of eukaryotic initiation factor-2, there is an accumulation of short-lived precursor transcripts and the generation of mature TNF mRNA is blocked.

The mRNA for TNF contains seven AU-rich sequences in the 3′ untranslated region of the molecule, and these have been shown to be critical for regulation of expression.[18] Such sequences confer message instability and probably explain the short time course for TNF expression in cells. Nuclear and cytoplasmic extracts of cells contain proteins that bind to AU-rich sequences and can act to either stabilize or destabilize mRNA. Tristetra-

prolin provides an elegant illustration of this mode of regulation.[151] This protein binds to the AU rich regions and destabilizes various cytokine messages. Knockout of the gene in mice leads to longer TNF mRNA half-life, overexpression of TNF protein, and a phenotype characterized by severe inflammation. As already mentioned the p38 inhibitor SB-203580 has been shown to act, in part, at the protein translation level. An area of current research is the modulation of mRNA regulatory factors by signaling pathways such as p38 MAP kinase.

The stability of mRNA can also be regulated as a physiological response of the cell or by external stimuli. The impact of the various signaling pathways on message stability is an emerging area of study. For example, protein kinase C-dependent stabilization of TNF mRNA has been demonstrated.[152] Dexanabinol (HU 211), a synthetic cannabinoid that is devoid of cannabimimetic activity and exhibits properties of an NMDA antagonist, has also been shown to suppress TNF at a posttranscriptional step.[153] The tetravalent guanylhydrazone CNI-1493 has been shown to be an inhibitor of TNF production, and the target of action appears to be through suppression of translational efficiency.[154]



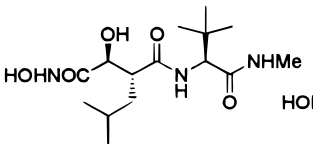
HU 211

ProTNF Processing. Another interesting facet of TNF is that it is produced as a type II membrane (i.e., the N-terminus contains the transmembrane sequence) precursor protein. To secrete the protein there needs to be a proteolytic cleavage of the 26-kDa proTNF to the soluble 17-kDa TNF protein. Gearing et al. have shown that normal cell processing occurs in a metalloprotease-dependent fashion.[5] Processing by cells primarily occurs through the actions of a metalloprotease that is a member of the ADAM (**A D**isintegrin and **A M**etalloprotease) family, termed ADAM-17 or TACE (**T**NF-**A**lpha **C**onverting **E**nzyme).[6,7] This branch of the metalloprotease family is more related to the snake venom metalloproteases, and many members contain cell adhesion domains and are membrane integral.[155]

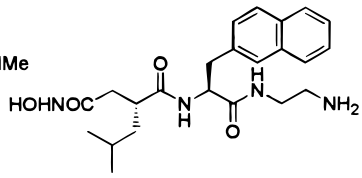
1. Metalloproteinase Inhibitors. The matrix metalloproteinases (MMPs) are zinc-containing enzymes capable of degrading extracellular matrix.[156] Inhibitors for these enzymes have been the focus of intense investigation[157] with broad therapeutic applications. In vivo profiling of these compounds in various animal models led to the discovery that a subset of these inhibitors possesses antiinflammatory activity.[5,158−160] Further investigation of this activity determined that some MMP inhibitors also inhibit processing of membrane integral proTNF to its 17-kDa soluble form. Two of these inhibitors, GI 129471 (Glaxo) and TAPI (Immunex), were reported to inhibit TNF production in vivo and to prevent lethality in mice exposed to endotoxin.[158,159]

The X-ray structure of TACE cocrystallized with an active site inhibitor was recently reported.[161] TACE shows relatively low overall sequence homology to the MMPs but has significant similarity in the active site, explaining the crossover in activity of the inhibitors. TACE also shows similar sequence specificity toward peptide substrates in comparison to the MMPs. Not surprisingly, it has been shown that several MMPs are capable of processing proTNF.[5] Another, more closely related, member of the ADAM family of metalloproteases, ADAM-10, has also been described as capable of processing proTNF.[162,163] While it is not certain that TACE is the only enzyme responsible in vivo for proTNF processing, evidence from chimeric mice that are null for TACE activity supports the claim of a central role for this enzyme.[164]

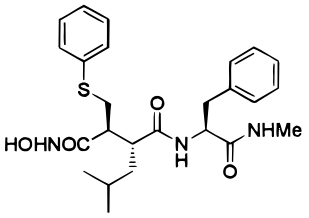
A number of groups have disclosed broad-spectrum metalloproteinase inhibitors that prevent proTNF processing: marimastat (British Biotech), TAPI (Immunex), GI 129471X (Glaxo), SE205 and XS309 (DuPont), CGS 27023A (Ciba), and a series of thiol inhibitors from Chiroscience. In general, these molecules are peptidomimetics which contain a zinc-liganding group such as thiol or hydroxamic acid. The ability of these molecules to block the metalloprotease-dependent shedding of a number of cell surface proteins suggests more extensive metalloproteinase inhibitory activity by the broad-spectrum compounds. More selective inhibitors of TACE are needed to clearly delineate the centrality of this target for the specific inhibition of TNF and the role of TACE in cell surface protein shedding, but it clearly represents an attractive small-molecule drug discovery target at this time.



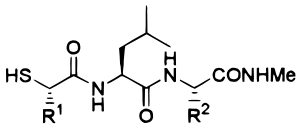
marimistat



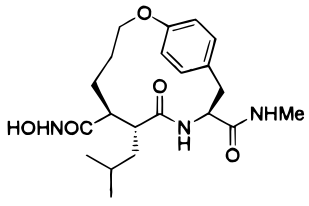
TAPI



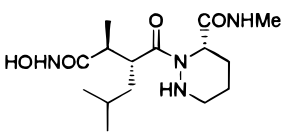
GI 129471



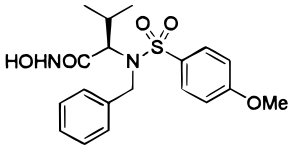
Chiroscience



SE205



XS309



CGS 27023A

Studies in humans with marimastat have shown that a moderately potent inhibitor can be effective in reducing TNF. The therapeutic value of these compounds will

be measured through the relative importance in vivo of inhibiting soluble TNF without blocking cell-associated proTNF. The clinical efficacy observed with infliximab (cA2 anti-TNF antibody) and etanercept (TNF receptor fusion protein) is founded on both forms being rendered inactive, and the relative in vivo roles for soluble TNF and membrane proTNF are still undefined. Thus far, investigations in animal models suggest that soluble TNF is the major effector of the downstream inflammatory events.

Soluble TNF

The TNF that is released by cells and found in solution exists as a trimer.[165] The structure of the trimer bound to TNF receptor 1 (TNFR1) has been modeled based on the interaction of TNFR1 with TNF-β or lymphotoxin,[166] and the structure supports the trimeric nature of the ligand. Each receptor molecule binds in the groove formed between two adjacent TNF subunits with mutagenesis studies supporting the deduced contact areas. The large surface area of interaction between ligand and receptor and the trimeric nature likely explain the historic inability to identify small molecules that can interfere with this interaction. Antagonism of this interaction may remain in the domain of the biologics and, for the small-molecule champion, may provide the best proof of principle support for pursuing alternate strategies.

Neutralizing Antibodies. Early in the study of TNF biology, a number of antisera and monoclonal antibodies were produced to aid in the evaluation of the role of TNF in vitro and in vivo. It is from these studies that the evidence for the critical role for TNF in inflammation has been derived. While the data in animal studies are extensive, the most exciting data have been derived from the human clinical trials.[167]

Centacor's infliximab (CenTNF, cA2, Avakine), an IgG1 subclass antibody, was the first to demonstrate effectiveness in treating severe rheumatoid arthritis patients[4] as well as patients with moderate to severe Crohn's disease.[168] Infliximab was also shown to reverse the anemia associated with rheumatoid arthritis.[169] Due to the isotype of the antibody, it is capable of not only neutralizing soluble TNF but also binding to cell surface proTNF. The latter can lead to complement fixation and lysis of the TNF-producing cell, enhancing the efficacy by blocking production of other proinflammatory cytokines such as IL-1. Contrary to animal model data, this antibody, when administered to multiple sclerosis patients, appeared to cause immune activation with an increase in disease activity.[170]

A similar antibody but from the IgGγ4 subclass, termed CDP571 (BAY-10-3356), has been produced by Celltech Therapeutics Ltd. and is under clinical investigation with Bayer. This antibody also showed a positive response in patients with rheumatoid arthritis[171] and Crohn's disease,[172] as well as in patients with mild to moderate ulcerative colitis.[173] Unlike infliximab, CDP571 is not complementing fixing and will not induce antibody-dependent cell cytotoxicity. This may explain, in part, why higher doses of CDP571 appear to be necessary to achieve equivalent efficacy.

Another monoclonal antibody, an F(ab′)$_2$ fragment of murine IgG3, has been introduced into clinical trials for rheumatoid arthritis by BASF with no published data to date. The antibody has been previously studied in sepsis and septic shock.[174] Therapeutic Antibodies is evaluating its anti-TNF polyclonal antibody, CytoTAb, in cerebral malaria and graft versus host disease, with plans for additional trials in Crohn's disease, melioidosis infections, and coronary artery bypass graft surgery.

Capture Molecules

A variant strategy to antibody blockade is to produce a soluble form of the natural receptors for TNF (vide supra). Dayer and colleagues originally isolated a protein from the urine of febrile patients that was capable of neutralizing TNF biological activity and was cross-reactive with antisera raised against the TNF receptor.[175] Work by David Wallach's laboratory resulted in the purification of two urine factors that were identified as portions of two different TNF receptors, TNFR1 and TNFR2.[176,177]

Soluble TNFR1, dimerized by hybridization into an IgG3 fusion protein, is protective in a mouse model of Gram-negative sepsis[178] and blocks onset, but not progression, in murine collagen-induced arthritis.[15] An interesting finding was that the dimerized TNFR1 demonstrates clear antiinflammatory activity but requires 100-fold higher concentrations to demonstrate immune-suppressive activity.[179] Roche is currently evaluating this molecule in clinical studies in rheumatoid arthritis patients.

In mouse models of acute lung injury and endotoxin-mediated sepsis, soluble TNFR2, dimerized by hybridization into an IgG1 fusion protein, was shown to decrease circulating TNF but not to have an effect on lung injury or death.[178,180] The hypothesis was put forward that the lower affinity of the soluble TNFR2 caused it to act more as a carrier of circulating TNF than as an effective neutralizing molecule.[178] However, in human trials, at lower systemic doses, Immunex has reported significant reductions in disease activity in severe rheumatoid arthritis patients who have now been treated for over 18 months with the fusion protein (etanercept).[181] A likely explanation for the biology is that, under normal physiological conditions, soluble TNFR2 is an important mechanism for clearance of TNF, especially at low ligand concentrations.[182]

The T2 protein of the Shope Fibroma Virus, which has sequence homology to the type I TNF receptor, was also found to specifically bind to TNF and inhibit binding to native TNF receptors.[183] This probably represents a mechanism whereby immune suppression of the host is accomplished by blocking the potential antiviral actions of TNF.

TNF Receptors

Two forms of the TNF receptor exist, known as TNFR1 (type I, p55, TNFR-β, CD120a) and TNFR2 (type II, p75, TNFR-α, CD120b), with about 25% sequence homology. Virtually all nucleated cells express receptors for TNF, although the genes encoding the two receptors are differentially regulated. The expression of TNFR1 appears to be constitutive and controlled by a noninducible, housekeeping promoter, while the expression of TNFR2 is inducible. The properties of the two receptors are remarkably different, and the relationship of the two forms remains a source of speculation. In

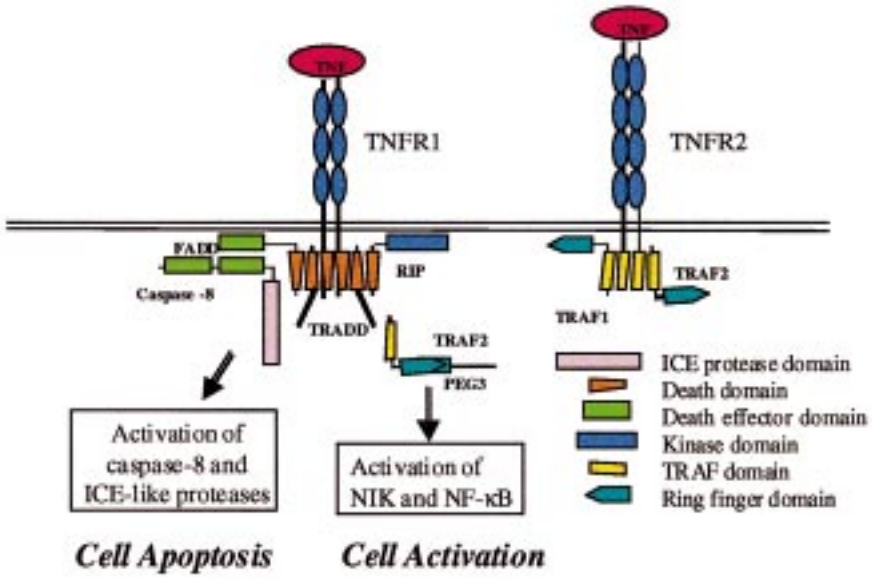


Figure 3. Hypothetical interactions in the signaling complexes of TNFRs.

general, most cell responses to TNF are mediated through TNFR1[184] but modulated by the level of TNFR2. In addition, both receptors appear to be shed from the cell surface in a metalloprotease-dependent manner, although TNFR2 appears to be the predominant form found in activated cell supernates and in plasma from individuals with inflammatory diseases. The actions of a number of agents (microbial products and cytokines such as γ-interferon) can influence the sensitivity of a biological system to TNF stimulation as well as alter the interdependency of TNFR1 and TNFR2 ligand interaction.[182]

Binding to the TNF receptor is hypothesized to involve ligand passing from TNFR2 to TNFR1,[185] analogous to the binding described for basic fibroblast growth factor. Initial binding to TNFR2 occurs to concentrate ligand (K_d of 100 pM; fast on-rate, fast off-rate), followed by ligand passing to TNFR1 (K_d of 500 pM; slow on-rate, slow off-rate). Thus, the regulated expression of p75 is thought to control the sensitivity of the system to the levels of TNF in solution, facilitating the response to low ligand concentrations.

Receptor clustering of TNFR1 by the TNF trimer leads to signal activation for both apoptosis (programmed cell death) and cell activation.[8,186] Agonist antibody cross-linking of TNFR1 or cross-linking of hybrid receptors that contain only the cytoplasmic domain of TNFR1 appears to be sufficient to generate TNF signaling.[187] In fact, simply overexpressing TNFR1 can lead to activation due to spontaneous aggregation that occurs at high membrane concentrations.[188] This feature may facilitate ligand-induced clustering and may be why low level TNFR1 expression on cells is normally observed. No additional ligand-dependent activities are required, and TNFR1 is internalized through coated pits and the ligand degraded in the lysosomes. TNFR2 does not contain a signal for internalization and is apparently either cleared from the surface by internalization or, more likely, shed in a metalloprotease-dependent fashion. In addition, TNFR2 does not undergo self-aggregation and spontaneous signaling, which may explain why high levels of expression can be tolerated.

Not all signaling occurs through TNFR1. Agonistic antibodies against TNFR2 can induce activation of NF-κB signaling in cells and induce T cell proliferation and cytokine production.[185] This signaling is dependent on the presence of the cytoplasmic tail of TNFR2. However it should be noted that this may not reflect normal physiology since mice deficient in TNFR1 fail to induce NF-κB activation in response to TNF.[13] All of the responses that have been observed with TNFR2 can also occur with TNFR1 but at lower doses of ligand and receptor.

Shedding regulates the activity of the surface receptors. For both receptors, the cleavage site appears to be dependent on a small region that links the cysteine-rich region of the extracellular domain to the transmembrane domain.[189] The clip of TNFR1 is relatively independent of the amino acid side chain identity in this region but appears to be affected by conformational features and occurs independent of the cytoplasmic tail of the receptor. For TNFR2, phosphorylation of the cytoplasmic tail is required for a metalloprotease-dependent shedding event and cleavage is also relatively independent of amino acid side chains in the clipped region.[190,191] Unlike TNFR1, binding of TNF to TNFR2 appears to induce shedding of the extracellular domain.[192]

Post-TNF Receptor Signaling

Signaling Assembly: TNFR1. TNF-induced TNFR1 oligomerization initiates a number of interactions (Figure 3) leading to the two main outcomes of TNF action: apoptosis and activation. The cytoplasmic tail of TNFR1 lacks any region with kinase activity but contains a number of signaling domains. An ~80-amino acid domain near the C-terminus possesses homology to a domain in Fas/CD95, DR-3 (**D**eath **R**eceptor-3), and the *Drosophila* cell death protein reaper[193] is associated with apoptotic responses and has been termed the death

domain. This domain is associated with TNF antiviral activity, the activation of endosomal acidic sphingomyelinase, the induction of nitric oxide synthase, and the activation of JNK and NF-κB.[28] However, these last three responses are also dependent on regions in the membrane proximal half of the cytoplasmic region.

Using the yeast two-hybrid system, a family of TNFR1 interacting proteins was identified that contain regions in the C-terminus that are homologous to the death domain. The interaction of a member of this family, TRADD (**T**NF **R**eceptor-**A**ssociated **D**eath **D**omain protein), with TNFR1 leads to both apoptosis and NF-κB activation. TRADD is a 34-kDa protein which is ubiquitously expressed, albeit at relatively low levels in most tissues, and TRADD overexpression is sufficient to trigger both apoptosis and NF-κB activation.[194] FADD (**F**as-**A**ssociated **D**eath **D**omain protein)/MORT1 (**M**ediator **O**f **R**eceptor-induced **T**oxicity **1**) is another TRADD family member whose overexpression leads to apoptosis that can be suppressed by the virally encoded caspase inhibitor CrmA.[195] Like TRADD, FADD is ubiquitously expressed. FADD appears to be a common mediator of apoptosis by all known death domain-containing receptors.[196,197] A truncated version of FADD blocks TNF-induced apoptosis while not affecting TNF-induced NF-κB or JNK activation.[198,199]

Another member of the complex which forms after receptor oligomerization and adapter association which links to apoptosis has been identified as caspase-8/FLICE (**F**ADD-**L**ike **I**L-1-**C**onverting **E**nzyme)/MACH (**M**ORT-**A**ssociated **C**ED **H**omologue)/Mch5.[200,201] While at least eight splice variants exist for caspase-8, all of the forms share a common amino terminal region that is homologous to the FADD death effector domain.[202] Overexpression of caspase-8 (which is synthesized as a proform capable of autoactivation) leads to apoptosis, which can be blocked by inhibitors of the ICE family. The apoptotic events appear to be mediated by the active proteolytic fragment of caspase-8. The in vivo substrates of caspase-8 are yet to be defined, although the cell death machinery engaged by activated caspase-8 also involves caspase-3 (CPP-32 (**C**ysteine **P**rotease **P**-32), apopain, Yama) and caspase-7.

RIP1 and RIP2 (**R**eceptor-**I**nteracting **P**rotein) are 60−75-kDa proteins that also interact with TNFR1.[203,204] Overexpression of RIP2 signals both NF-κB activation and cell death. RIP contains a death domain homology region at its C-terminus and a tyrosine kinase domain at its N-terminus that is most closely related to *lck*. Of note is that the kinase domain shares key residues in common with the serine−threonine kinase family. The C-terminal domain appears to recruit and activate caspases, and the kinase domain does not appear to be important for Fas-mediated apoptosis but is required for NF-κB activation. Recruitment to TNFR1 is ligand-dependent, and overexpression of RIP leads to apoptosis as well as activation of NF-κB and the JNK pathway.[205] The interaction of RIP with TNFR1 is weak, and it is speculated that other proteins in the assembly stabilize the interaction.[205]

TRAF-2, identified through interaction with TNFR2, has been shown to interact with TRADD, suggesting that it is also recruited to the TNFR1 complex.[206] Overexpression of TRAF-2 is sufficient for NF-κB activation, whereas a TRAF-2 mutant lacking the N-terminal inhibits TNF-induced NF-κB activation (but not apoptosis). As described in an earlier section of this review, the TRAF-2 interacting protein NIK indirectly mediates the phosphorylation of IκB via IKK-α and IKK-β leading to NF-κB activation.[51] Kinase-inactive mutants of NIK behave as dominant negative inhibitors that suppress TNF-induced NF-κB activation and proinflammatory gene expression.[207] In addition to modulating the NF-κB pathway, TRAF-2 also appears to activate the JNK pathway.[208]

Recently, a large zinc finger-containing protein, identified as Pw1 or Peg3, has also been shown to be associated specifically with TRAF-2.[209] Overexpression of Peg3 activates NF-κB by inducing dissociation of IκB, and transfection of only the TRAF interaction site of Peg3 blocks NF-κB activation by either TNF or overexpression of TRAF-2. Presumably Peg3 acts synergistically with TRAF-2 to activate NF-κB.

Using the yeast two-hybrid system, two TNFR1-associated proteins were isolated and termed TRAP-1 and TRAP-2 (**T**NF **R**eceptor-**A**ssociated **P**rotein). These two proteins, which have regions of homology to the 90-kDa heat shock protein family and to the *Caenorhabditis elegans* R151.7 gene product, have been shown to bind to the membrane proximal half of the cytoplasmic tail.[210] A separate study identified TRAP-2 as a protein that associates with TNFR1 and is identical to subunit 2 of the 26S proteosome.[211] Activation of this latter protein may be closely associated with activation of NF-κB since IKK-phosphorylated IκB has been shown to be degraded by ubiquitination and proteosome degradation.

Small-molecule inhibitors of this complex of signal proteins have not been reported, presumably because most of the pathway has only been identified recently. In this respect, pargyline, a monoamine oxidase inhibitor, has been shown to selectively block TNFR1-induced cell death without an effect on TNFR2 function through an undefined mechanism of action.[212]

Signaling Assembly: TNFR2. In contrast to TNFR1, TNFR2 is constitutively phosphorylated and binding of TNF does not alter the pattern of phosphorylation. The phosphorylating kinase appears to be casein kinase-I (CK-I). Addition of inhibitors of CK-I or conversion of all the potential serine phosphorylation sites to alanines reduces the constitutive phosphorylation and potentiates TNF-induced apoptosis.[213] The C-terminal 78-amino acid region of TNFR1 appears to be responsible for signals that induce the activation of NF-κB.

Use of the yeast two-hybrid system identified TRAF-1 and TRAF-2 as TNFR2 associated proteins.[214] TRAF-2 is expressed in most tissues, while TRAF-1 has a more limited distribution, detected in spleen, testis, and lung. The C-terminal domain is highly conserved among TRAFs and appears more important in TNFR2 interaction, while the N-terminal motif appears to form a coiled-coil α helix motif.[214] The N-terminal domain of the TRAF proteins may mediate protein−protein interactions by the formation of two zinc finger structures.

TRAF-1 and TRAF-2 can form homodimers and heterodimers. While TRAF-2 homodimers can directly associate with the C-terminal region of TNFR2, the interaction of TRAF-1 with TNFR2 appears to require

heterodimer formation with TRAF-2. Recent evidence indicates that the ligand-passing role hypothesized for TNFR2 may need to be modified in that the ability of TNFR1 to interact with TNFR2 maps to the TRAF-2 binding site on both receptors.[215] TNFR2 is strongly associated with the induction of NF-κB, and it is interesting to speculate that there is both extracellular (via TNF) and cytoplasmic (via TRAFs) interactions which occur with TNFR1 for NF-κB signaling. TNFR2 activation may therefore be particularly important in biasing a response toward cell activation. The TRAF family now includes six members that have also been found to interact with the various members of the TNF receptor family (CD40, CD30, OX40, lymphotoxin-β receptor, 4-1BB/CD137), and the role of these other family members in TNF signaling is currently begin explored.

Natural TNF Inhibitors. Natural intracellular protein inhibitors of TNF offer clues on the tact taken by nature to suppress TNF signal pathways. A family of proteins, originally identified in insect baculovirus and named IAPs (**I**nhibitor of **A**poptosis **P**roteins), contains a C-terminal domain with homology to the TRAF proteins.[216] Expression of IAPs blocks apoptosis, apparently by interacting with the downstream caspases-3 and -7.[217] In addition a protein termed usurpin (also termed CASH, Gasper, CLARP (**C**aspase-**L**ike **A**poptosis **R**egulatory **P**rotein), FLAME-1 (**F**ADD-**L**ike **A**nti-apoptotic **M**olecul**E**), FLIP (**FL**ICE **I**nhibitory **P**rotein), I-FLICE, MRIT (**M**ACH-**R**elated **I**nducer of **T**oxicity)), which is ubiquitously expressed, has features in common with the caspases.[218] However, the residues necessary for catalytic activity are missing, and heterodimer formation with caspase-8 blocks recruitment to FADD. Adenovirus and herpesvirus encode proteins that protects cells from TNF-induced apoptosis by interacting with caspase-8.[219,220] The antiapoptotic protein Bcl-x(L) has been demonstrated to block TNF-induced apoptosis at a step downstream from caspase-8.[221,222]

Overexpression of TRAF-3 (CRAF (**C**D40 **R**eceptor-**A**ssociated **F**actor), LAP-1 (**L**MP-1-**A**ssociated **P**rotein)) suppresses TNFR2 activation of NF-κB. A natural splice variant of TRAF-2, TRAF-2A, is also capable of binding to TNFR2 but is unable to stimulate NF-κB thereby acting as a dominant negative regulator of TNFR2 signaling.[223] The zinc finger protein A20 has also been shown to interact with TRAF-2 and to inhibit TNF-induced NF-κB activation.[224]

The dual action of TNF responses appears to be the outcome of a balance between signals for apoptosis (through caspases) and cell activation (through NF-κB). Studies have illustrated that interfering with one path can amplify signals through the other.[225−227] While on the surface this assemblage of a large number of proteins may seem inordinately complex, it is not unprecedented in biology. Nature has chosen to use a similar intracellular assembly of large numbers of relatively low-affinity interactions in the formation of the integrin focal adhesion, in assembly of the T cell and B cell receptor signal complexes, and in microtubule assembly. Such complexes offer a large degree of fine control over the ultimate content and function of such complexes, and the relatively low affinity of the interactions allows rapid assembly/disassembly, necessary in controlling cell signaling. The involvement of various proteins allows for additional control through regulation of the concentration of different intracellular proteins. Interfering with such assemblies may represent a good target for drug intervention, but studying this from a rational point of view may be exceedingly difficult. Low-affinity interactions will be difficult to model, and antagonism of a specific interaction will probably lead to an all-or-none effect on the outcome; however, the ultimate outcome will most likely not be no response but rather a shift to a different outcome. For TNFRs this would mean regulating a balance between activation and apoptotic signals. These principles may explain why screens for functional inhibitors of TNF action have identified, and led to a focus on, kinase targets. Based on natural inhibitors, the major points for regulation appear to be the caspases (especially caspase-8) for apoptosis and TRAF-2 and NF-κB for cell activation.

Signal Transduction. Following receptor clustering and signaling assembly on the cytoplasmic surface, there is activation of several downstream pathways. As alluded to above, the pathway leading to apoptosis is associated with signaling through the death domain with activation of the caspases, and various caspase inhibitors have been demonstrated to block this response. The pathway to cell activation is intimately associated with the regulation of NF-κB responses.

1. NF-κB. NF-κB appears to control the balance within the cell between apoptotic and activation outcomes. Resistance to the apoptotic effects of TNF appears to result from the ability of TNF to activate NF-κB-mediated transcription.[228] In this regard, one gene whose expression is induced through TNF activation of the NF-κB pathway is the apoptosis inhibitor IEX-1L.[229] Blocking NF-κB by dominant negative mutants of IκBα or p65 knockout can make resistant cells sensitive to the apoptotic effects of TNF. Interestingly, expression of the NF-κB-dependent gene A20 blocks not only TNF-dependent NF-κB activation but also apoptosis and acts at a step upstream of IκB degradation.[230] While most inhibitors of IκB degradation have been shown to enhance sensitivity to induction of apoptosis, A20 may reflect an intervention point without this effect.

2. Sphingomyelinase. TNF activates acid sphingomyelinase (Smase) through TNFR1 and the associated proteins TRADD and FADD.[231] The TNFR1-associated proteins TRAF-2 and RIP do not appear to be involved in this signaling, and caspase inhibitors markedly reduce the Smase activation, implying signaling through the apoptotic pathway. General caspase inhibitors are capable of inhibiting apoptosis induced by exposure to sphingosine or ceramide lipids,[232] and the virally encoded caspase inhibitor CrmA can block TNF-induced ceramide generation[233] indicating a sandwiching of Smase with caspases. By contrast, overexpression of caspase-8 does not activate Smase, and evaluation of cells from patients with an inherited Smase deficiency (Niemann−Pick disease) showed no alteration in either ceramide production or Fas-mediated apoptotic response,[234] opening up a question on the role of acid Smase in ceramide signaling. Recent evidence indicates a convergence of the caspase-8 and ceramide pathways at an acidic subcellular compartment within cells leading to caspase-3 activation.[235]

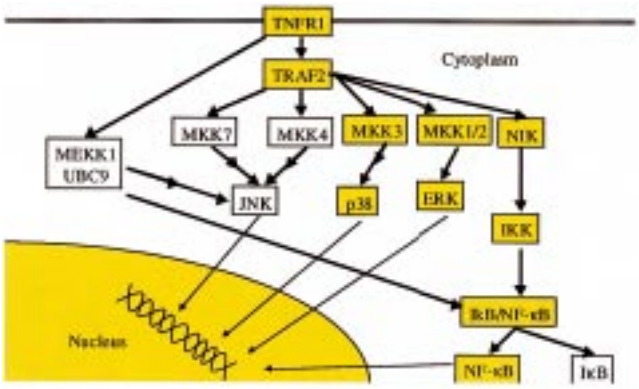


Figure 4. Interaction of NF-κB and MAP kinase pathways involved in TNFR1 signaling (yellow boxes signify confirmed critical pathways).

3. MAP Kinases. TNF activation of cells has also been shown to generate TRAF-2-dependent signals through the MAP kinase pathways (Figure 4). MKK7 (**M**AP **K**inase **K**inase 7) has recently been shown to be strongly stimulated by TNF and to specifically activate the JNK pathway.[236,237] Overexpression of MKK7 leads to enhanced transcription from AP-1-dependent promoters. The actin binding protein-280 associates with MKK4, and this complex also plays a role in JNK signaling by TNF.[238] In addition, it has been shown that ligation of TNFR1 can also activate MEKK1 via complexing with the E2 ubiquitin-conjugating enzyme UBC9.[239,240] In early responses this may relate to synergy in activation of NF-κB through enhanced degradation of IκB. However, at later time points, this complexing is not related to the E2 properties of UBC9 and the protein may simply be playing a scaffolding role leading to activation of JNK. Despite the extensive evidence of JNK activation by TNF, a test of the role for JNK in TNF effects awaits studies with a specific JNK inhibitor.

An additional level of regulation of TNF-induced NF-κB activity occurs through the MAP kinases p38 and ERK. The upstream kinase MKK3 has been shown to be activated in response to TNF,[241] and activation of p38 has also been shown to be TRAF-2-dependent.[242] Stimulation of the p38 pathway has been associated with gene expression that is blocked by pyridinylimidazoles such as SB-203580.[243] Apparently p38 potentiates the NF-κB transcriptional effects through posttranscriptional and translational regulation.[244] TNF exposure has been demonstrated to induce the phosphorylation of ERK.[245] Addition of the ERK pathway inhibitor PD 98059 to TNF-activated cells has been shown to inhibit interleukin-6 gene expression,[244] and this effect appears to occur through regulation of NF-κB-driven transcription.

4. Other Signal Pathways. While the TNF receptors contain no intrinsic tyrosine kinase activity, TNFR1 occupancy has been associated with the induction of tyrosine phosphorylation, and nonselective inhibitors of tyrosine phosphorylation inhibit TNF responses. Among the phosphorylated proteins is insulin receptor substrate 1, and this may be the mechanism of the reported down-regulation of insulin responsiveness by TNF.[246] It has recently been shown that TNF can activate the Janus tyrosine kinases, primarily Jak2.[247] This activation is associated with the phosphorylation of STAT3 and STAT5. The STAT (**S**ignal **T**ransducer and **A**ctivator of **T**ranscription) proteins are a family of cytoplasmic transcription factors that dimerize and translocate to the nucleus upon phosphorylation. It is important to note that STAT protein activation is not always accompanied by DNA binding or transcriptional activity so it remains to be seen what role Jak/STAT signaling plays in TNF responses.

An interaction between TNFR1 and the newly discovered **P**hosphatidyl**I**nositol **P**hosphate (PIP) kinase PIP5K has been reported.[248] The enzyme is responsible for the phosphorylation of phosphatidylinositol 4-phosphate leading to PIP_2, and interaction with ligand-occupied TNFR1 is associated with increased phosphatidylinositol turnover. The PIP_2 can be further hydrolyzed to inositol trisphosphate and diacylglycerol by phospholipase C. D609, a phosphatidylcholine-specific phospholipase C inhibitor, has been shown to block TNF apoptotic and cell activation responses in several, but not all, cell types.[212] The activation of NF-κB can reportedly be completely blocked by D609 or the protein kinase inhibitor Ro 31-8220.[249]

Summary

Finally, a few summary points and some added speculation on what might represent sites where new molecules may be identified in the not to distant future as anti-TNF agents:

1. Biologics provide a strong proof of principle for the role of TNF in inflammation.

2. Potent small molecules that act at the level of interfering with ligand−receptor interaction are unlikely.

3. Signal transduction for TNF expression and TNF action is intimately associated with NF-κB, and this may represent a high probability target, especially for selective inhibitors of the kinases NIK or IKK.

4. Mechanisms that link surface receptors to NIK might offer an alternate site for intervention once they have been identified.

5. The regulation of TNF expression by cAMP modulation or MAP kinase inhibition may offer good targets. The focus here has been on the PDEs and on p38 inhibition.

6. The p38 studies and the data from the tristetraprolin knockout studies open up the potential for translational regulation of TNF expression, and the mechanisms that are active here remain to be identified.

7. Gene regulation at the transcriptional level remains an unexplored area that may open up quickly with new observations on E3330 and the thalidomide mechanism of action studies.

8. Interference with any of the aspects of the TNF receptor-signaling complex, especially TRAF-2 and PEG3, may be good targets. The large number of interacting proteins and the potential for signal shuttling to other pathways may complicate these studies.

9. The regulation of TNF-induced gene expression by MAP kinase inhibition may offer good targets. The relative role of the different pathways remains to be elucidated, but a specific JNK inhibitor may be an important molecule to complement studies with the specific ERK and p38 pathway inhibitors.

Biographies

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