

ANTI-TNF- α THERAPIES: THE NEXT GENERATION

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The functioning of the immune system is finely balanced by the activities of pro-inflammatory and anti-inflammatory mediators or cytokines. Unregulated activities of these mediators can lead to the development of serious inflammatory diseases. In particular, enhanced tumour-necrosis factor- α (TNF- α) synthesis is associated with the development of rheumatoid arthritis, psoriatic arthritis and inflammatory bowel disease. Inhibiting TNF- α activities in these diseases has been remarkably successful. However, the current injectable protein therapies have associated risks and limitations. An oral, small molecule that regulates TNF- α biology could either replace the injectables or provide better disease control when used alone or in conjunction with existing therapies. In this review, we discuss briefly the present understanding of TNF- α -mediated biology and the current injectable therapies in clinical use, and focus on some of the new therapeutic approaches with oral, small-molecule inhibitors.

ENDOTOXIN

Toxin derived from the disruption of the outer membrane of Gram-negative bacteria.

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Biology of TNF- α

Tumour-necrosis factor- α (TNF- α) was identified in the mid-1970s by Lloyd Old and colleagues as an ENDOTOXIN-induced serum factor that caused the necrosis of certain murine tumours *in vivo*¹. The original descriptions of this phenomenon can actually be traced back to at least the latter half of the nineteenth century and the use, by surgeon Dr William Coley in New York City, of killed bacteria or their products to induce tumour regression in patients with inoperable neoplastic diseases². Although 'Coley's toxins', as they were called, induced tumour regression in some instances, their side effects were unacceptable and these treatments were abandoned.

In the early 1980s, a parallel line of research brought the function of TNF- α into a different focus. Cachectin, a seemingly unrelated molecule described by Cerami and colleagues, was shown to mediate a role in the wasting that is characteristic of chronic diseases^{3,4}. When studies demonstrated that these molecules were identical^{5,6}, it became apparent that TNF- α was a central biological mediator whose regulation must be tightly controlled.

It was not until the simultaneous cloning of TNF- α and the related molecule lymphotoxin (also called

TNF- β) by Goeddel and colleagues⁷ in 1984 that sufficient recombinant materials were made available for study. These molecules were shown to affect many normal and neoplastic cell processes. It is now accepted that TNF- α is a multifunctional cytokine that mediates key roles in acute and chronic inflammation, antitumour responses and infection. Therefore, considerable effort has been directed towards determining its biological actions, as well as its receptor signal-transduction pathways. **FIGURE 1** provides a simplified illustration of some of these pathways

Structure, synthesis and receptors

Human TNF- α is translated as a 26-kDa protein that lacks a classic signal peptide. Newly synthesized pro-TNF- α is expressed on the plasma membrane, and is then cleaved in the extracellular domain through the action of matrix metalloproteinases to release a mature soluble 17-kDa protein. In both its cell-associated and secreted forms, trimerization is required for biological activity. Both the cell-associated 26-kDa and secreted 17-kDa forms are biologically active, and the cell-associated form is often thought to be responsible for

JUXTACRINE SIGNALLING secondary to cell-to-cell contact⁸. The specific functions of cell-associated and secreted TNF- α remain controversial, although it is clear that the two forms of TNF- α have both overlapping and distinct biological activities. For example, Kollias *et al.* used a novel transgenic mouse that expresses only cell-associated TNF- α to demonstrate that these animals can develop chronic inflammatory diseases such as **rheumatoid arthritis** (RA)⁹. In fact, the development and progression of RA in rodent models seems to be dependent on both the cell-associated and secreted forms of TNF- α ¹⁰. By contrast, mice expressing only a cell-associated form of TNF- α seem to be resistant to endotoxin-induced lethality, indicating that the shock-producing properties of TNF- α are due primarily to the production of the soluble 17-kDa form¹¹.

The primary enzyme responsible for processing cell-associated TNF- α to a secreted form is TNF- α -converting enzyme or (TACE, also known as ADAM-17)¹². TACE is an adamalysin, a member of a class of membrane-associated enzymes that contain both disintegrin and matrix metalloproteinase domains. These enzymes are crucial for the processing of several membrane-associated proteins including TNF- α , Fas ligand, the TNF receptors and the epidermal growth factor receptor. TACE seems to have a number of crucial biological functions in addition to its processing of cell-associated TNF- α . Mice deficient in TACE are developmentally lethal¹³, whereas mice deficient in TNF- α or its two receptors survive gestation.

Signal transduction and effector mechanisms

The biological responses to TNF- α are mediated through two structurally distinct receptors: type I (TNFR1, also known as p60, p55, CD120a) and type II (TNFR2, also known as p80, p75, CD120b). Both receptors are transmembrane glycoproteins with multiple cysteine-rich repeats in the extracellular N-terminal domains. Although their extracellular domains share structural and functional homology, their intracellular domains are distinct, and transduce their signals through both overlapping and distinct pathways. The primary characteristic that distinguishes the intracellular domains of TNFR1 and TNFR2 is the presence of a death domain in TNFR1, which is not present in TNFR2. The death domain is a sequence of approximately 70 amino acids that is pivotal to the ability of TNF- α to trigger cellular apoptosis. In common with the death domain of Fas ligand, this intracellular domain provides a docking site for a number of accessory proteins, including Fas-associated death-domain-containing protein (FADD), TNFR1-associated death-domain-containing protein (TRADD), and TNFR-associated factor-2 (TRAF-2). TRADD and TRAF2 provide the branching points for the proapoptotic and inflammatory signalling pathways that are characteristic of TNFR1 (FIG. 1).

Under physiological conditions, signalling through TNFR1 seems to be primarily responsible for the proinflammatory and shock-producing properties of TNF- α . Mice deficient in TNFR1 are resistant to endotoxin-induced lethality, whereas mice deficient in TNFR2 remain sensitive^{11,14}. By contrast, other biological

responses to TNF- α seem to be dependent on signalling through both receptors. Hepatic injury to a T-cell mitogen is reduced in mice deficient in either receptor¹⁰, and the development of RA secondary to overexpression of cell-associated TNF- α is diminished by the absence of TNFR1 or TNFR2 (REF. 9).

All nucleated cells express TNF receptors, although their distribution varies with cell type. TNFR1 is expressed constitutively on most cell types, whereas expression of TNFR2 can be induced. In addition, TNFR2 is restricted to certain cell types and can discriminate TNF- α from different species. The receptors also differ significantly in their binding affinities for homotrimeric TNF- α . Although both receptors can be considered high-affinity, the on-off kinetics of the two differ dramatically. Binding of homotrimeric TNF- α to TNFR1 is thought to be essentially irreversible, whereas binding to TNFR2 is associated with both rapid on and off kinetics¹⁵. This has fuelled speculation that TNFR2 might function as a 'ligand passer' in some cells¹⁶, transferring TNF- α to TNFR1. However, TNF- α signalling through TNFR2 seems to have a dual role in T cells. In the absence of TNFR1 signalling, TNF- α promotes the proliferation of naive T-cells through the actions of TNFR2¹⁷. Moreover, despite the absence of a death domain, TNFR2 initiates apoptosis in activated CD8⁺ T cells independent of TNFR1 signalling^{18,19}. These data indicate that TNFR2 is likely to function as more than just a ligand-passer in T cells, and that these functions might vary depending on the cell type and the presence of key intracellular signalling molecules.

Both TNF receptors can be cleaved from the cell surface by members of the matrix metalloproteinase family in response to inflammatory signals, such as TNF- α receptor binding. The shed extracellular domains of the receptors retain their ability to bind TNF- α and therefore probably function as either endogenous inhibitors or facilitators of the biological activity of TNF- α ²⁰, depending on their concentrations and the concentrations of the ligand. Both receptors are excreted in the urine immunologically intact. In fact, the original TNF- α -binding proteins described in the 1980s by Seckinger *et al.*²¹, Engelmann *et al.*²² and Olsson *et al.*²³ were fragments of these receptors.

Studies in animal models

Much of what we know about the role of TNF- α in the pathological changes associated with RA comes from studies in rodent models, particularly mice and rats. Genetically altered mice overexpressing TNF- α spontaneously develop RA-like lesions in the joints with progressive inflammation, cellular proliferation and bone destruction²⁴. Mice overexpressing the cell-associated form of TNF- α also spontaneously develop RA⁹. In addition, mice overexpressing the TNF receptor alone develop significant inflammation of the liver, pancreas and kidney²⁵. In genetically susceptible DBA/1J mice, increased TNF- α expression has been detected in the synovial lining of the inflamed joints following immunization with type II collagen, and concurrent with the onset of symptoms^{26,27}.

JUXTACRINE SIGNALLING
The interaction of membrane-bound proteins that are normally secreted with receptors on adjacent cells.

Table 1 | **Protein-based injectable anti-TNF- α therapies in clinical use**

Drug	Status	Biological form
Etanercept	Approved	Soluble TNFR2 coupled to Fc portion of IgG
Infliximab	Approved	Mouse-human chimeric anti-human TNF- α antibody
Adalimumab	Approved	Human anti-human TNF- α antibody
PEG-sTNFR1	Clinical	Pegylated form of soluble TNFR1
CDP-870	Clinical	Pegylated Fab of humanized antibody CDP-571

Fab, fragment antibody binding; Fc, fragment constant; IgG, immunoglobulin G; TNF, tumour-necrosis factor; TNFR1, TNF receptor type I; TNFR2, TNF receptor type II.

US FDA³⁵. Other protein-based therapeutics are in various stages of clinical development and include PEG-sTNFR1, sLTr, CDP-571, CDP-870, MAK-195F and rTBP-1 (TABLE 1). A TNFR1-immunoglobulin fusion protein (Lenercept, p55 receptor) was under development by Genentech and Hoffmann La-Roche, but advanced clinical trials were discontinued³⁶. It should be noted that the approved injectable therapies, although seemingly similar, might have different activities for binding of the soluble versus membrane forms of TNF- α , as well as for lymphotoxin.

An FDA advisory panel recently discussed issues concerning the use of injectable protein-based TNF- α inhibitors that must also be addressed with the second-generation, small-molecule oral inhibitors. The most prevalent concern has been an increased incidence of re-exacerbation of latent tuberculosis. Tuberculosis testing has been recommended for patients commencing anti-TNF- α therapies. Studies with infliximab and etanercept have also shown a potential risk for worsening congestive heart failure and specific warnings are now placed on the FDA labels for these products. In addition, a potentially increased incidence of lymphoma has been observed in patients treated with the injectable TNF- α -blocking agents, including the recently approved fully human antibody adalimumab. Recent data indicate that RA is associated with increased risk for lymphoma and this risk is thought to increase with disease severity^{37–39}. However, the contribution of TNF- α inhibitors to lymphoma risk remains to be determined.

An optimized form of infliximab has been reported by Applied Molecular Evolution, a biotechnology company in San Diego, California, that apparently exhibits more potent activities in animal models. Whether an optimized version of any current injectable therapeutic will exhibit greater clinical efficacy alone or, in contrast,

exhibit increased immunogenicity is unknown. In addition, the potential for adverse effects, such as activation of latent tuberculosis or increased incidence of cancer, is uncertain and must be addressed with any second-generation protein-based therapy.

Small-molecule approaches to anti-TNF- α therapy

In light of these FDA warnings, there are significant advantages in developing orally active, small molecules that target the specific signalling and synthesis pathways for TNF- α (BOX 1).

There are a large number of small-molecule agents that are in various stages of preclinical and clinical development that inhibit the synthesis of TNF- α (TABLE 2). One of the initial small-molecule TNF- α inhibitors that has been developed is thalidomide (Thalomid; Celgene).

Thalidomide. Thalomid has been approved by the US FDA to treat moderate to severe leprosy, and clinical trials are ongoing in cancer (multiple myeloma) and RA^{40–45}. Thalidomide has multiple effects that could account for its activity in myeloma. These include direct inhibition of tumour growth by altering the synthesis of cytokines that are involved in the growth and survival of myeloma cells, including TNF- α , **IL-1 β** , **IL-6** and **IL-10**. Thalidomide also seems to inhibit the production of two major growth factors involved in angiogenesis: vascular endothelial growth factor (**VEGF**) and basic fibroblast growth factor (**bFGF**). Further understanding the mechanisms of action for this molecule is crucial because of the known drug-induced congenital malformations it can cause in humans. However, thalidomide has been a successful development drug to treat multiple myeloma.

It is important to note that the history of thalidomide illustrates the limits of the current pharmacological models that are available to evaluate potential teratogenicity⁴⁶. Thalidomide is metabolized differently by various species and, in fact, by diverse strains within species. Thalidomide does not induce malformations in pregnant rodents typically used to test for teratogenicity. Moreover, rabbits exhibit different malformations from those observed in humans. Primates such as the marmoset seem to have similar susceptibility to humans^{47–49}.

Two pilot studies have been conducted with thalidomide in patients with active inflammatory **Crohn's disease**. One study evaluated thalidomide in low dosage (50–100 mg per day) for 12 days and led to a 67% patient response and 0–33% disease remission. A second pilot study used thalidomide at 200–300 mg per day for 12 days in patients with active inflammatory and fistulizing Crohn's disease. Results showed that 80% of the patients with **FISTULIZING DISEASE** had fistula closure and 50% of patients with active inflammatory disease had a clinical response.

New distant relatives of thalidomide or selective cytokine inhibitory drugs (SelCIDs) are under development for the treatment of cancer^{50–54}. SelCIDs have been shown to inhibit phosphodiesterase type 4 enzyme (PDE4), which indirectly decreases TNF- α production. TNF- α synthesis can be blocked by

Box 1 | Potential advantages of small-molecule, oral TNF- α inhibitors

- Convenient non-injectable with greater patient compliance
- Small molecule might facilitate tissue penetration
- Pharmacology could suggest once a day dosing
- Specificity for defined signalling and synthesis pathways
- Shorter half-lives with reduced immunosuppression
- Non-immunogenic
- Easier manufacturing and lower cost
- Potential use in combination with other anti-inflammatory therapies

Table 2 | TNF- α synthesis inhibitors in clinical use

Class of inhibitor	Product	Company	Clinical status
p38 kinases	BIRB796	Boehringer Ingelheim	Phase II
	681323	GlaxoSmithKline	Phase I
	SCIO-469	Scios	Phase II
	SCIO-323	Scios	Phase I
	SB203580	SmithKline Beecham	Discontinued
	VX-702	Vertex	Phase II
	VX-745	Vertex	Discontinued
TACE	TACE inhibitor	BristolMyers	Phase II
	Marimastat	British Biotech	Discontinued
Thalomid	Thalidomide	Celgene	Phase III
Rationally designed L-amino acid peptide	RDP58	Sangstat Medical	Phase II

increasing intracellular levels of cyclic adenosine monophosphate (cAMP). Normally, cAMP is converted to AMP through the action of PDE4. However, in the presence of PDE4 inhibitors, cAMP levels remain high, causing the activation of protein kinase A (PKA). Activation of PKA prevents transcription factors such as nuclear factor- κ B (NF- κ B) from promoting transcription of the gene encoding TNF- α , thereby resulting in a decrease in TNF- α synthesis.

Among the seven families of PDE isozymes, PDE4 is the major form in lymphoid cells which led to the hypothesis that its selective inhibition will have an anti-inflammatory effect. Selective PDE4 inhibitors can be grouped into three broad structural classes: catechol esters (rolipram), bicyclic heteroaromatics (nitraquazone) and xanthine derivatives (denbufylline)⁵⁵. PDE4-selective inhibitors have shown efficacy in a number of inflammatory models, including asthma and RA⁵⁵⁻⁵⁸.

In general, increasing cAMPs and adenylate cyclase activity lead to inhibition of human TNF- α production both at a transcriptional and post-transcriptional level. For example, forskolin, a natural diterpene, has been shown to reversibly activate adenylate cyclase thereby inhibiting TNF- α production, whereas the conserved CKS-17, a highly conserved peptide from the transmembrane envelope protein of leukaemia viruses suppresses TNF- α production by increasing the levels of cAMP. Another example of the role of cAMP in TNF- α regulation is illustrated through the action of adenosine and related analogues. Among them, several analogues act as selective adenosine receptor agonists, thereby suppressing TNF- α production. Such studies have uncovered a series of 3-hydroxycyclopentyladenines that selectively inhibit macrophage-derived TNF- α production. Compounds of this class dosed orally were effective in an lipopolysaccharide (LPS)-mediated murine model of septic shock. Another example of the adenylyl-cyclase-linked receptor with interesting immunomodulatory properties is the peripheral cannabinoid receptor (CB2). With this target in mind, several companies are developing CB2-directed molecules as TNF-suppressing agents.

Additional PDE4 inhibitors. Earlier approaches were attempted by multiple groups to regulate TNF- α through PDE4. Rolipram was originally developed as an

antidepressant and has been shown to be selective for PDE4. Rolipram and other PDE4 inhibitors that cross the blood-brain barrier cause nausea, and clinical trials have been discontinued. In addition, a clinical trial with CellTech's CDP-840 demonstrated dose-limiting side effects. However, CDP-840 did not induce nausea. Second-generation, orally active PDE4 inhibitors with decreased side effects have resulted in the generation of SB207499 (Ariflo) by SmithKline Beecham.

p38 MAP kinase inhibitors. Additional principal approaches are focusing on developing small-molecule therapeutics that inhibit the p38 mitogen-activated protein (MAP) kinase (FIG. 2). MAP kinases mediate central roles in regulating TNF- α synthesis. There are multiple principal pathways involving MAP kinases, the p42/44 MAP kinase or extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and the p38 pathway.

The signal transduction pathway leading to production of TNF- α is, in part, regulated by p38 MAP kinase. This protein belongs to a group of serine/threonine kinases that includes JNK and ERK. On extracellular stimulation, p38 becomes activated, and phosphorylates and activates other kinases or transcription factors, leading to stabilization of messenger RNA and modulation of gene expression.

The p38 MAP kinases were first described by Han *et al.*⁵⁹ in 1993 during studies of LPS signalling. Subsequent independent studies at SmithKline Beecham in 1994 also identified the p38 kinases during their efforts to discover novel targets for anti-inflammatory therapies. It is interesting that SmithKline Beecham had studied their libraries of bicyclic imidazole derivatives and discovered that these compounds could inhibit TNF- α production but did not have the clinical potential due to certain toxicities. The synthesis of TNF- α and IL-1 can be regulated at both the transcriptional and translational levels by the p38 pathway. In addition to blocking TNF- α and IL-1 synthesis, these inhibitors also block the production of nitric oxide, cyclooxygenase-2 (COX-2) and IL-6. Several studies implicate p38 kinase in the regulation of IL-12 and interferon- γ (IFN- γ) gene transcription in murine T cells and IL-4 and IL-10 in human T cells.

Early findings with compounds SB203580 and SB220025 demonstrated that they are potent inhibitors of p38 *in vitro* and produce significant anti-inflammatory effects in various rodent models. The crystal structure of SB203580 bound to the inactive form of p38 α indicates that this molecule binds in the ATP-binding pocket, a common feature of this class of enzymes, and is consistent with findings that these compounds can compete with ATP for binding to the enzyme. These early drug candidates are not being further developed, possibly due to lack of specificity and/or toxicity associated with inhibition of liver cytochrome p450. GlaxoSmithKline is in early clinical development with a new p38 kinase inhibitor, 681323.

The orally active p38 α kinase inhibitor SCIO-469 (Scios), seems to be one of the most advanced in clinical development. Scios was recently purchased by

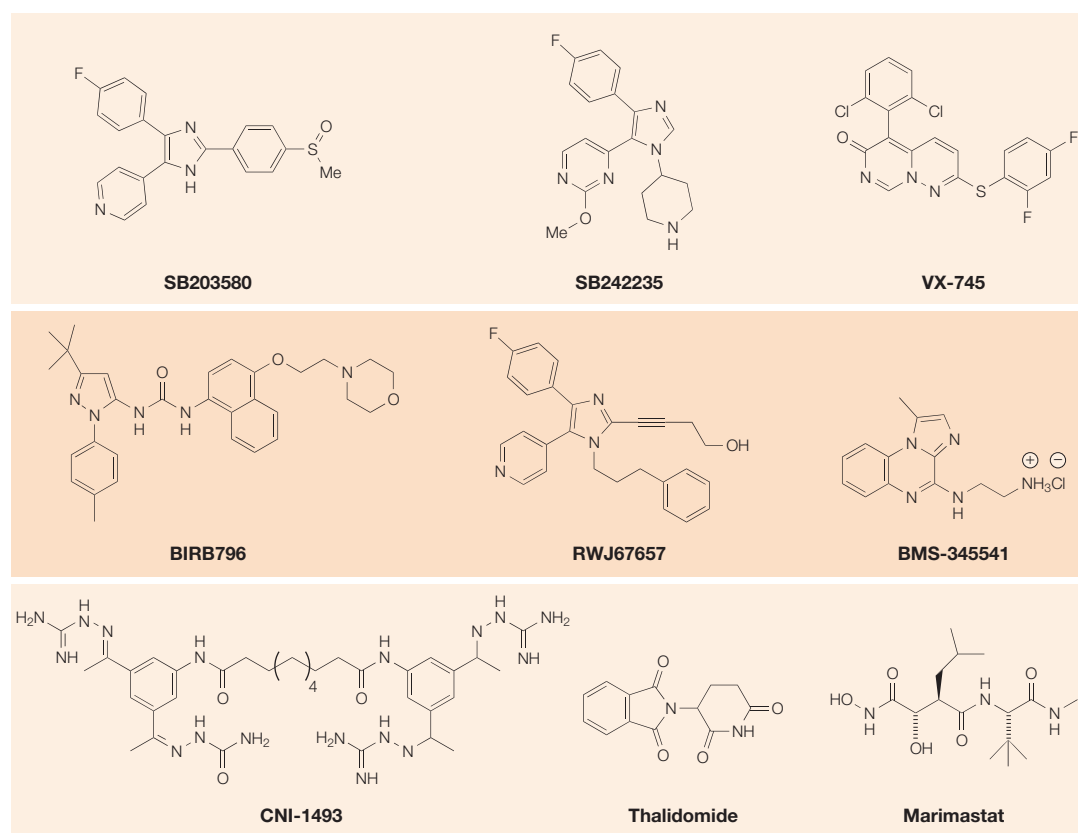


Figure 2 | Examples of small-molecule TNF- α inhibitors.

Johnson & Johnson, Inc. In fact, many of the companies with approved TNF- α inhibitors or those in advanced clinical trials have been purchased by large pharmaceutical companies.

The clinical development programme at Vertex for its lead p38 kinase inhibitor, VX-745, was stopped in 2001 after the drug was shown to cross the blood–brain barrier and exhibit toxicity at high doses in some animals. However, the preliminary clinical data indicated a good ACR₂₀ response, which could mean that this might be an important target in humans to regulate TNF- α synthesis and to induce positive clinical responses in RA. A second-generation molecule, VX-702 (Vertex/Kissei Pharmaceuticals), which does not cross the blood–brain barrier, is now in clinical testing. VX-702 has been reported to have potent effects, decreasing cytokine production and the severity of arthritis in animal models.

The p38 kinase inhibitor VX-745 interacts with the ATP-binding site of p38 α , as shown by X-ray crystallography. VX-745 inhibits p38 α with an IC₅₀ of 10 nM, and is less than 20-fold active towards p38 β family members and inactive against other kinases. VX-745 inhibits both TNF- α and IL-1 release from human peripheral blood mononuclear cells with an IC₅₀ of 50 nM, has a plasma half-life of 4.5 hours in the rat, and does not affect p450. Although advanced clinical testing of VX-745 has been discontinued⁶⁰, VX-702, a second-generation molecule, was developed and in 2002 a

Phase I double-blind, placebo-controlled clinical trial was initiated in healthy volunteers.

An interesting finding with another p38 α kinase inhibitor, RWJ67657, indicates significant species differences in activity between mice, dog and human peripheral blood mononuclear cells. Although there are multiple explanations for this, including differences in the amino acid sequences of the various p38 α species, it further demonstrates the difficulty in identifying and characterizing the biological activities and toxicities of small-molecule cytokine inhibitors before clinical testing.

TACE inhibitors. TNF- α is translated as a precursor protein that contains an unusually long sequence which anchors the protein to the outer side of the membrane⁶¹. During local and systemic inflammation, membrane-bound TNF- α can be cleaved extracellularly by the specific zinc-dependent metalloprotease TACE, yielding a soluble form of trimeric TNF- α (FIG. 1). It was initially thought that cleavage of TNF- α constituted the sole means for production of the active cytokine, but subsequent studies indicated that membrane-bound TNF- α is biologically active as a homotrimer during cell–cell contact by interacting with both p55 and p75 TNF receptors. These data indicated that the development of TACE inhibitors could have only limited therapeutic benefits. In fact, mice engineered to express TNF- α that could not be cleaved by TACE (or

ACR₂₀
American College of
Rheumatology 20%
improvement criteria.

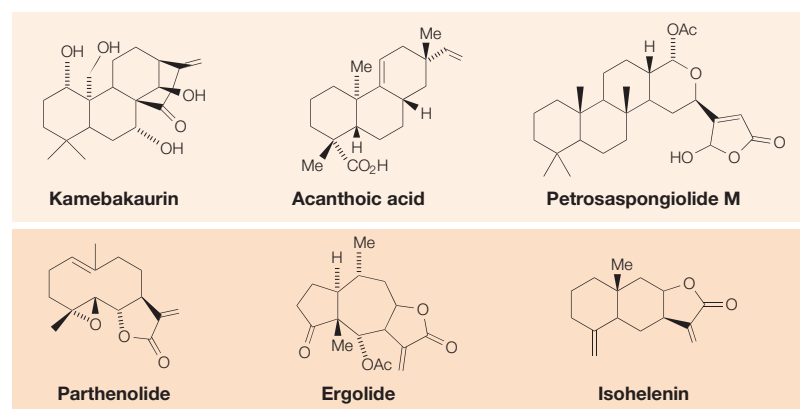


Figure 3 | Examples of natural products that modulate NF- κ B.

other metalloproteases) remained susceptible to a wide range of T-lymphocyte-mediated inflammatory diseases, such as RA, hepatitis and encephalitis. In addition, none of the presently available TACE inhibitors are entirely specific for TNF- α processing, and also affect the function of other metalloproteases. This nonspecific inhibition could lead to ectopic collagen deposition and fibrosis. Marimastat (British Biotech), an inhibitor of TACE and matrix metalloproteinases, has been in clinical trials as an anticancer agent⁶¹. Studies with Marimastat in murine models of sepsis and inflammation revealed that although Marimastat almost completely abrogated LPS-induced soluble TNF- α production in mice, only a slight delay in LPS-induced lethality was observed⁶². By contrast, anti-TNF- α antibodies promoted survival in these studies as well as in murine arthritis models when compared with Marimastat, implying that membrane-bound TNF- α might be important in the pathogenesis of various diseases. This indicates that targeting TACE is unlikely to be successful.

The NF- κ B pathway and inflammation

The NF- κ B family of transcription factors are central mediators in the regulation of a variety of immune responses. NF- κ B proteins are retained in the cytoplasm in association with inhibitory proteins, called inhibitors of NF- κ B (I κ Bs). This class of proteins includes I κ B α , I κ B β and I κ B ϵ , all of which contain the ankyrin repeats that are necessary to form a complex with NF- κ B. After activation by many different inducers, the I κ B proteins become phosphorylated, then ubiquitinated, and finally degraded by the 26S proteasome⁶³. After degradation of I κ B, NF- κ B translocates to the nucleus and interacts with specific DNA-binding sites that regulate the transcription of many genes⁶⁴ (FIG. 1) Recent data indicate that the cytoplasmic localization of the inactive I κ B/NF- κ B complexes is achieved by finely balancing the continuous movement between the nuclear and cytoplasmic compartments of the cell⁶⁵.

Genes dependent on the activation of NF- κ B include those encoding TNF- α , IL-1 β , IL-6, IL-8 and IFN- γ , the adhesion molecules E-selectin, intracellular

adhesion molecule-1 and vascular endothelial adhesion molecule-1, the enzymes nitric oxide synthase and COX-2, major histocompatibility complex proteins and viral proteins. A high-molecular-mass multisubunit I κ B kinase termed the IKK complex, which phosphorylates I κ B, has also been described. Three subunits termed IKK1/IKK α , IKK2/IKK β , and the adaptor molecule NEMO/IKK γ have been identified. All known pro-inflammatory stimuli, including cytokines, viruses and LPS, require the IKK2 subunit for NF- κ B activation⁶⁶. Given the importance of NF- κ B for regulating inflammatory responses, the identification of small-molecule inhibitors of this pathway would generate considerable interest.

NF- κ B also induces the transcription of anti-apoptotic proteins called the inhibitors of apoptosis proteins⁶⁷; therefore, it is interesting that TNF- α was defined as an apoptosis or cytotoxicity-inducing factor by Old and Carswell¹. NF- κ B is activated rapidly in response to various stimuli, including TNF- α and IL-1. However, in most cases TNF- α does not induce apoptosis, because the activation of NF- κ B inhibits this process by inducing the transcription of survival-associated genes (FIG. 1).

In addition to TNF- α , other agents including chemotherapeutic drugs such as irinotecan (Campto; Aventis) and doxorubicin, are potent activators of NF- κ B. This activation can therefore lead to the inhibition of apoptosis and provide a resistant phenotype of tumours to both TNF- α -mediated cytotoxicity and chemotherapies. The use of agents that block TNF- α synthesis and the resulting NF- κ B activation, as outlined in this review, could provide an interesting adjunct therapy for treating many types of cancers. As an example, a proteasome inhibitor such as bortezomib (Velcade; Millennium Pharmaceuticals) that blocks the degradation of I κ B α , a central regulatory molecule for maintaining inactive NF- κ B, has been shown *in vivo* to significantly enhance the activity of CPT-11 (REF. 68). Bortezomib was approved recently by the US FDA for the treatment of multiple myeloma⁶⁹.

Natural-product NF- κ B inhibitors

In 1994, sodium salicylate and its semi-synthetic derivative aspirin were the first plant-derived compounds reported to modulate of NF- κ B activity. A large number of natural compounds have been suggested to interfere with the cascade leading to NF- κ B activation and gene transcription (FIG. 3). These compounds generally come from plants used in traditional medicine, such as kamebakaurin, a kaurane diterpene from *Isodon japonicus*, and acanthoic acid, a diterpene from *Acanthopanax koreanum*^{70,71}. The effects of many of the crude extracts from natural sources can now be explained on the basis of these plants containing NF- κ B-regulating agents such as the diterpenoids and sesquiterpene lactones. Along these lines, the use of kinase inhibitors, such as herbimycin A, or phosphatase inhibitors, such as okadaic acid and petrosaspongiolide M, and protease inhibitors, such as *N*-tosyl-L-lysyl chloromethyl keto (TPCK), have been explored for the control of inflammation⁷².

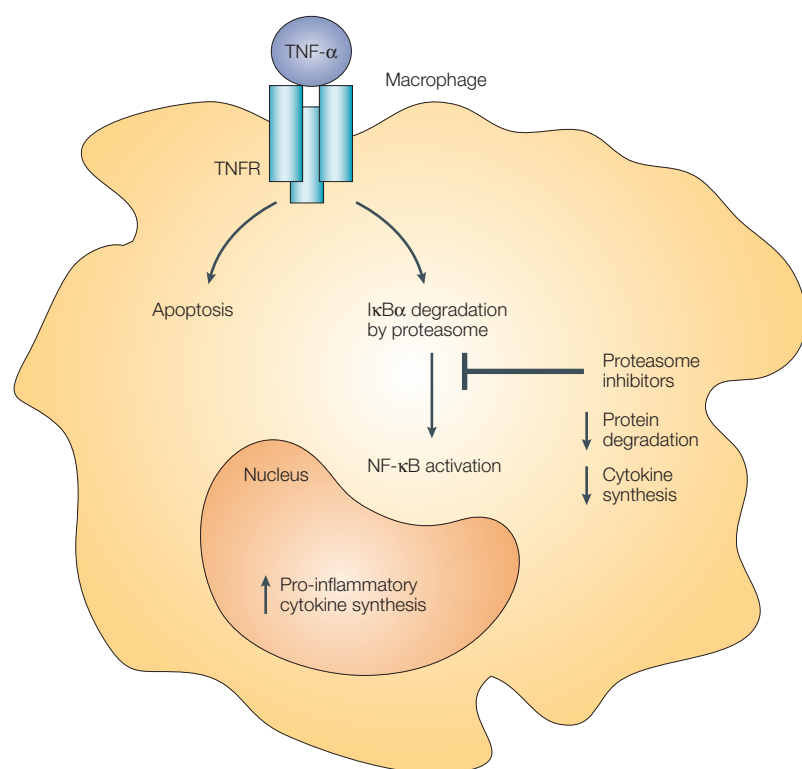


Figure 4 | Proposed effects of IκBα/NF-κB inhibitors on TNF-α-induced sensitization of resistant macrophages to LeTx. In the presence of TNF-α, LeTx-resistant macrophages become sensitive to cytotoxicity induced by LeTx exposure. Proteasome inhibitors have been shown to prevent cytotoxicity by an unknown mechanism. Perhaps inhibition of LeTx-induced cytotoxicity occurs due to inhibition of NF-κB activation resulting in inhibition of NF-κB dependent processes, such as TNF-α cytokine production and other aspects of TNF-α signalling. IκBα, inhibitor of NF-κBα; LeTx, lethal toxin of *Bacillus anthracis*; NF-κB, nuclear factor-κB; TNF-α, tumour-necrosis factor-α, TNFR, TNF-α receptor.

Plant-derived sesquiterpene lactones can interfere with NF-κB activity, presumably by preventing the degradation of IκBα and IκBβ⁷³. For example, isohelenin, a lactone isolated from the medicinal plant *Arnica montana*, has been suggested to selectively alkylate the p65 subunit of NF-κB. On the other hand, parthenolide, from the medicinal herb Feverfew (*Tanacetum parthenium*) binds directly and inhibits IκB kinase-β (IKK-β). Further studies indicated that the *in vitro* and *in vivo* anti-inflammatory activity of this compound is mediated through the α-methylene γ-lactone moiety, which is found in other sesquiterpene lactones, such as ergolide. The latter compound was shown to inhibit inducible nitric oxide synthase and COX-2-expression in RAW 264.7 macrophage-like cells through NF-κB^{74–76}.

Compounds such as capsaicin and resiniferatoxin inhibit IκB degradation and NF-κB activation through vanilloid receptor binding and by altering the redox state of the cell⁷⁷. NF-κB-dependent transcription is inhibited by antioxidants and activated by reactive oxygen species. Oxidants such as anthralin induce TNF-α synthesis^{77,78}, whereas antioxidants, such as tetramethylthiourea and vitamin E, inhibit its induction⁷⁹.

A highly selective, orally active inhibitor of IKK, BMS-345541, that blocks NF-κB-dependent transcription in

mice was recently described. This molecule was shown to have excellent pharmacokinetic profiles and blocked TNF-α synthesis in mice after LPS challenge⁸⁰. Further preclinical evaluations with this compound are ongoing.

Recently, the natural product calagualine, which is extracted from ferns of the genus *Polypodium*, was described by Manna *et al.*⁸¹. This agent inhibits the binding of NF-κB-inducing kinase to TRAF-2 sites and subsequently inhibits NF-κB activation induced by TNF-α (FIG. 1). It now seems quite consistent that many natural products function by regulating the activity of specific proteins in the NF-κB signalling pathway.

Additional approaches

TNF-α production is also regulated post-transcriptionally. The 3' region of the TNF-α mRNA includes a series of AU-rich sequences that render the TNF-α message unstable and determine its translation efficiency⁸². These sequences are common in the mRNAs encoding several pro-inflammatory cytokines. The presence of these sequences ensures that TNF-α mRNA cannot be translated but is rapidly degraded by RNases. The AU-rich sequences are known to be the recognition site for several RNA-binding proteins, some of which are involved in determining TNF-α mRNA stability. Novation Pharmaceuticals, a biotechnology company in Canada, is exploiting technologies to develop small molecules that bind to the AU-rich regions and subsequently control the synthesis of TNF-α and other cytokines.

RDP58 (Rationally Designed Peptide) is a small molecule (SangStat Medical) that inhibits TNF-α synthesis by preventing the translation of the TNF-α mRNA. In addition to TNF-α, RDP58 inhibits the expression of IFN-γ and IL-12 and upregulates haeme oxygenase *in vivo*. RDP58 consists of nine D-amino acids and glycine. The use of D-amino acids can make molecules resistant to degradation by human proteases. RDP58 is not systemically available, indicating that it is locally active after oral administration.

CNI-1493 and inflammatory reflex

Inflammatory products produced in damaged tissues activate signals that lead to the inhibition of cytokine synthesis through the cholinergic anti-inflammatory pathway (the 'inflammatory reflex'). On the basis of this hypothesis, it is possible to activate neural anti-inflammatory mechanisms using small molecules that initiate signals in proximal components of the pathway in the central nervous system. One such molecule is CNI-1493, which was originally described as an inhibitor of macrophage activation and TNF-α-release. This compound is now under evaluation in a large Phase II clinical study in Crohn's disease, and provides an interesting additional approach to regulating TNF-α-mediated biology⁸³.

NF-κB, TNF-α and anthrax

There is a significant need for new approaches to treat the systemic disease caused by the biodefence Category A infectious agent *Bacillus anthracis*. Recent data indicate that small molecules that affect innate immune responses

could be used alone or in combination with antibiotics to reduce morbidity and mortality due to anthrax disease.

Anthrax infections are initiated by endospores of *B. anthracis*. During the inhalation of anthrax bacilli, spores — the most lethal form — are engulfed by alveolar macrophages and germinate⁸⁴. As a result of the migration of infected macrophages, the bacteria spread to local lymph nodes and synthesize lethal toxins. Subsequent macrophage death results in the dissemination of bacteria into the bloodstream⁸⁵. Despite treatment with antibiotics, the bacteraemia induces respiratory distress and multi-organ failure, which often culminates in death because of systemic cytokine and toxin-mediated shock^{86,87}.

B. anthracis produces three main factors that provoke host responses: oedema factor (EF), lethal factor (LF) and protective antigen (PA)⁸⁸. After binding to cell-surface receptors, PA facilitates the translocation of EF and LF into the cytosol of host cells^{89,90}. EF is an adenylate cyclase that, when complexed with PA, becomes oedema toxin (EdTx), which enhances cAMP levels and causes oedema⁹¹. LF is a zinc metalloprotease that exhibits specificity towards mitogen-activated protein kinase kinases (MAPKKs: MEK1–4, 6 and 7, but not 5), cleaving between the N-terminal sequence and the catalytic domain^{92–96}. LF combined with PA is termed lethal toxin (LeTx), which is the principal contributor to virulence in anthrax-infected animals.

Mice depleted of macrophages are resistant to LeTx, and sensitivity to LeTx can be re-established by reconstituting mice with toxin-sensitive RAW 264.7 macrophage-like cells^{85–88,97}. Therefore, a crucial step for anthrax infection is macrophage death mediated by LeTx, which seems to involve an intracellular multicatalytic enzyme complex called the proteasome^{85–88,97}. The 26S proteasome is a multisubunit protease that contains a proteolytic core called the 20S proteasome. The 20S proteasome is responsible for the degradation of intracellular proteins and is therefore responsible for regulating a broad array of basic cellular processes, such as cell cycle, apoptosis, differentiation and signal transduction. It is therefore not surprising that aberrations in this system underlie the pathogenesis of many disorders of the immune and inflammatory responses⁹⁸. Macrophage death induced by LeTx can be prevented with proteasome inhibitors, which are known to block the degradation of I κ B α , a central molecule that regulates NF- κ B translocation to the nucleus, as well as affecting other important signalling proteins^{85–88,97}.

Significant differences in sensitivity to LeTx-mediated death exist in murine strains: for example, macrophages from BALB/c, but not C57BL/6, mice are sensitive to LeTx^{99–103}. Moreover, studies by Welkos *et al.*¹⁰² revealed

that the susceptibility of mice to anthrax toxin components differed from their susceptibility to infection¹⁰². Though *in vitro* sensitivity to LeTx does not correlate with *in vivo* susceptibility to infection, mice sensitive to LeTx undergo more rapid death compared with animals whose macrophages are resistant, which implies that genetic factors contribute to anthrax susceptibility in mice^{101–103}.

No obvious strain-related differences account for the resistance or sensitivity of macrophages to LeTx-induced cytotoxicity. PA receptor interaction, receptor number or proteolysis of PA seem to be equal among various strains¹⁰². Moreover, no differences in MAPKK (MEK) proteolysis have been found between sensitive and resistant macrophages, implying that cleavage of MAPKKs is not sufficient for LeTx-mediated macrophage killing of these cells^{96,104}. Additionally, different alleles of a kinesin-like motor protein, Kif1C, have been linked to the resistance or sensitivity of murine macrophages to LeTx¹⁰⁴. Although the mutations in Kif1C are responsible for differences in susceptibility of inbred mouse macrophages, they apparently do not affect either cellular entry, processing of LeTx or cleavage of MAPKK, and so probably influence later events in the infection pathway¹⁰⁴.

TNF- α and LeTx sensitization of macrophages

Recent findings by Kim *et al.*¹⁰⁵ revealed that treatment of resistant macrophages with different bacterial components increased their susceptibility to LeTx-induced cell death. These data indicate that mediators of the early innate immune response are important in regulating susceptibility of macrophages to toxin-induced death. The signalling pathways responsible for the increased sensitivity of resistant macrophages are unclear; however, it was shown by Kim *et al.* that TNF- α , but not IL-1, is indispensable in sensitizing resistant macrophages and that this sensitization involves the TNFR2 pathway specifically (FIG. 4). These data indicate that TNF- α inhibitors might be effective anti-anthrax treatments.

Summary

The signalling pathways that inhibit cytokine synthesis are complex and there are many approaches that can effectively regulate TNF- α expression. Although this review has addressed many of the signalling approaches, additional focus on targets such as receptor blocking agents might also provide new avenues for therapy. The success of the injectable protein-based therapies directed against TNF- α has been remarkable. However, questions remain concerning the long-term use of these drugs. It is now important to develop the next generation of oral therapies for long-term use in many different chronic diseases.

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References 91, 97 and 105 describe the potential roles of TNF- α and proteasome inhibition and in anthrax.

Acknowledgements

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 Online links

DATABASES

The following terms in this article are linked online to:

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