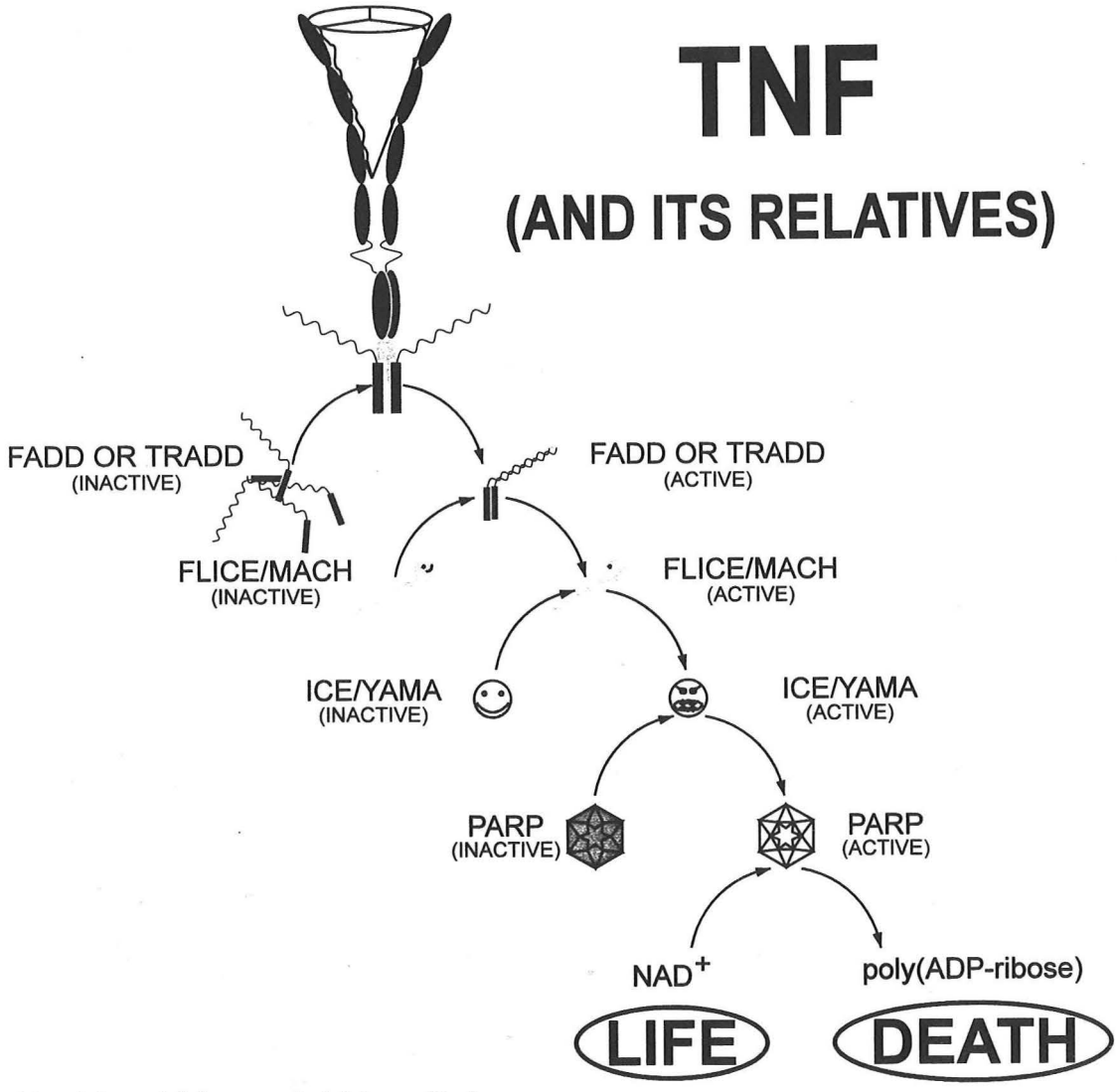
INTERNAL MEDICINE GRAND ROUNDS

TNF

(AND ITS RELATIVES)



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Dr. Beutler received his B.A. degree in 1976 at the University of California, San Diego, and his M.D. degree in 1981 at the University of Chicago. He completed his internship in Internal Medicine at the University of Texas, Southwestern Medical Center in 1982, and one year of Neurology residency at the same Institution in 1983. He then spent three years of postdoctoral fellowship at the Rockefeller University in the Laboratory of Medical Biochemistry, isolating cachectin (tumor necrosis factor), a proinflammatory cytokine that he continues to study today. In 1986, Dr. Beutler returned to the University of Texas, Southwestern Medical Center as an Assistant Professor in the Department of Internal Medicine and an Assistant Investigator at the Howard Hughes Medical Institute. He is currently Professor of Internal Medicine and an Associate Investigator with HHMI. His interests include genetic determinants of cytokine gene induction and mechanisms of TNF signal transduction.

PROLOGUE: THE IMPORTANCE OF TNF IN MEDICINE

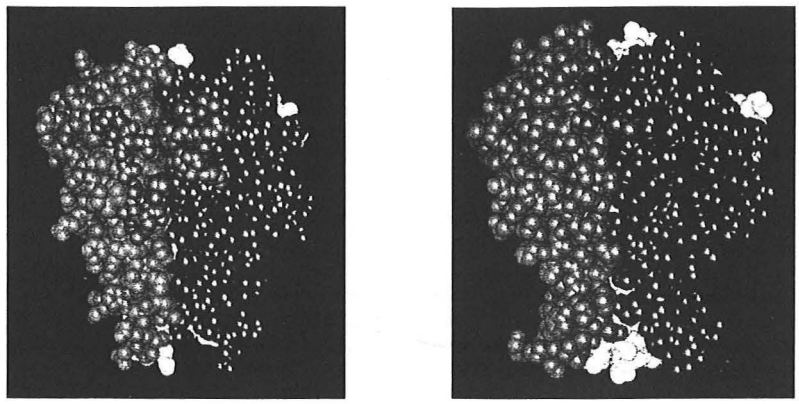


Figure 1. *TNF (left) and LT-α (right) are homotrimeric proteins produced chiefly by macrophages and by lymphocytes, respectively. Different stimuli elicit their production. However, the two ligands are roughly similar in shape, engage the same two plasma membrane receptors, and elicit the same biological effects. Images kindly provided by S. Sprang.*

Tumor necrosis factor (TNF) [Figure 1] is one of the most pleiotropic cytokines ever isolated. It is also one of the most intensively studied, and as a result, one of the best understood. A prototypic inducer of programmed cell death, TNF originally attracted notice because of its relevance to cancer. However, it is also an essential component of "natural" or "innate" immunity. Its centrality in several inflammatory and infectious diseases have made it an item of cross-disciplinary interest.

In the near term, pharmacologic approaches to the ***blockade*** of TNF biosynthesis and/or inhibition of TNF activity promise to alleviate many chronic inflammatory diseases, with few of the consequences that devolve from treatment with broad-spectrum anti-inflammatory or immunosuppressive drugs. Over a longer term, agents that ***mimic*** TNF action may ultimately emerge as effective antineoplastic drugs.

As befits a multifunctional protein, TNF has been subjected to many separate lines of scientific inquiry. Foremost among them, perhaps, have been the following:

1. What controls TNF synthesis?
2. What essential functions does TNF serve?
3. How does TNF work once it is produced; i.e., how do the receptors signal to elicit their many effects?

Each of these topics will be discussed today.

HISTORY AND OVERVIEW

Figure 2. *William Coley*

Over 100 years ago, Dr. William Coley [Figure 2] reported that patients with certain inoperable tumors sometimes experienced clinical remission following intercurrent infection with streptococci or other organisms. Moreover, he found that he could induce hemorrhagic necrosis of tumors in human patients by injecting them with heat-killed bacteria, or filtrates prepared from the culture of bacteria (1-4). In the fullness of time, investigation of this phenomenon led to the purification of a bacterial product- today known as lipopolysaccharide, or LPS- which could cause the necrosis of transplantable tumors in animals (5-11). However, the marked toxicity of LPS precluded its use in human cancer patients.

The name "tumor necrosis factor" was coined by Old and coworkers (12), who first recognized that LPS did not act directly to induce hemorrhagic necrosis of tumors, but rather, triggered the release of an endogenous protein mediator which had this effect [Figure 3]. As the mediator was studied, it became apparent that it also caused apoptotic destruction of many transformed cells *in vitro* (13). A great deal of speculation was devoted to the possible role of TNF in "immune surveillance," and it was felt that TNF, once cloned, might be administered to cancer patients to induce hemorrhagic necrosis of tumors with few of the untoward effects produced by conventional chemotherapy. Oddly, little attention was paid to the likelihood that this cytokine, powerfully induced by LPS, might also mediate much of the damage witnessed in endotoxic shock. Furthermore, though TNF had precisely the same activities as the T-cell derived cytokine lymphotoxin (14-17), no consideration was given to the possibility that the two proteins might be similar.

The TNF and LT-α cDNAs were cloned by workers at Genentech in 1984. The two proteins had approximately 30% sequence identity (18,19). Large quantities of recombinant material were amassed for use in clinical trials. In parallel, however, TNF was isolated and cloned under a different name (20-22), denoting its putative role in the pathogenesis of cachexia. As "cachectin," TNF was viewed as an endogenous mediator of wasting, anemia, and insulin resistance associated with chronic disease or infection (23-28). It was held responsible for the suppression of lipoprotein lipase activity- and consequent hypertriglyceridemia- observed in animals infected with trypanosomiasis or injected with LPS (23-25).

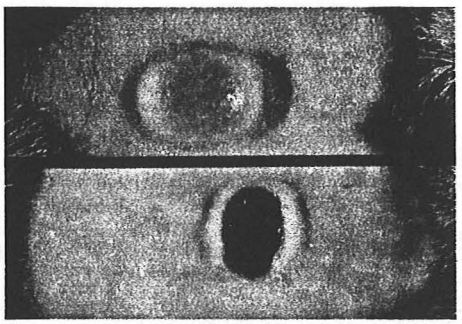


Figure 3. *Hemorrhagic necrosis of MethA sarcoma, grown in the skin of a C3H mouse. Taken from Carswell, et al. (12). The destruction of tumors, for which TNF was named, results largely from a pro-coagulant effect on tumor vasculature, and can be prevented by anticoagulation. TNF's ability to cause apoptosis of numerous transformed cells* in vitro *depends upons a different process, details of which are increasingly well understood.*

The identity of cachectin and TNF hinted that the molecule would prove far too toxic for effective treatment of disseminated cancer. As predicted by the "cachectin" model, TNF did, indeed, induce a profound wasting diathesis in experimental animals (29-31).

If chronically administered to mice, a syndrome virtually indistinguishable from cachexia associated with cancer would ensue [Figure 4]. Larger does of TNF administered to mice or rats as a bolus caused shock which in every sense resembled endotoxic shock (32), and anti-TNF antibodies attenuated the lethal effect of endotoxin (33,34) or gram-negative bacteremia (35) suggesting that molecule was an important mediator of shock in gram-negative sepsis. *In vitro* studies

suggested that TNF might serve an important role in local inflammatory processes. For example, it was shown that TNF could activate neutrophils, stimulating their adhesion to endothelial surfaces and triggering degranulation (36,37). By a separate effect on the endothelial surface itself, leukocyte adhesion (38,39) and coagulation (40) were also favored. It is now known that TNF induces the expression of specific intercellular adhesion molecules that largely account for these effects (41).

When applied to human synovial cells, TNF stimulated the secretion of collagenase and induced the formation of PGE_2 (42), effects which presaged its importance in rheumatoid arthritis. A major portion of osteoclast activating factor (OAF) activity, characterized concurrent with the purification of TNF and IL-1, was ascribed to the action of the former

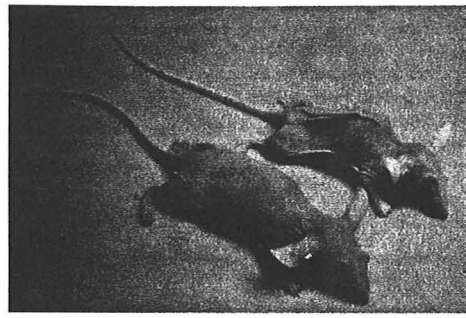


Figure 4. *Wasting elicited by chronic exposure to TNF [taken from Oliff, et al. 934]. Nude mice bearing a tumor engineered to secrete human TNF (right) became anorectic and wasted compared to mice that were inoculated with the same tumor lacking a TNF expression construct (left). In similar studies, wasting was shown to result from chronic expression of a TNF transgene. Wasting results primarily from anorexia, and closely models that observed in chronic human diseases (e.g., neoplasia). However, it should be stressed that high levels of circulating TNF are generally not observed in humans with cancer-related cachexia (225).*

(43-46). The osteolytic lesions caused by multiple myeloma appear to result from effects of LT-α, secreted by cells of the malignant clone and acting on osteoclast TNF receptors (44). TNF acted as an endogenous pyrogen in rabbits, rats, and mice (44). It was shown to be an essential factor in develop of the cutaneous and systemic Schwartzman reactions (47,48).

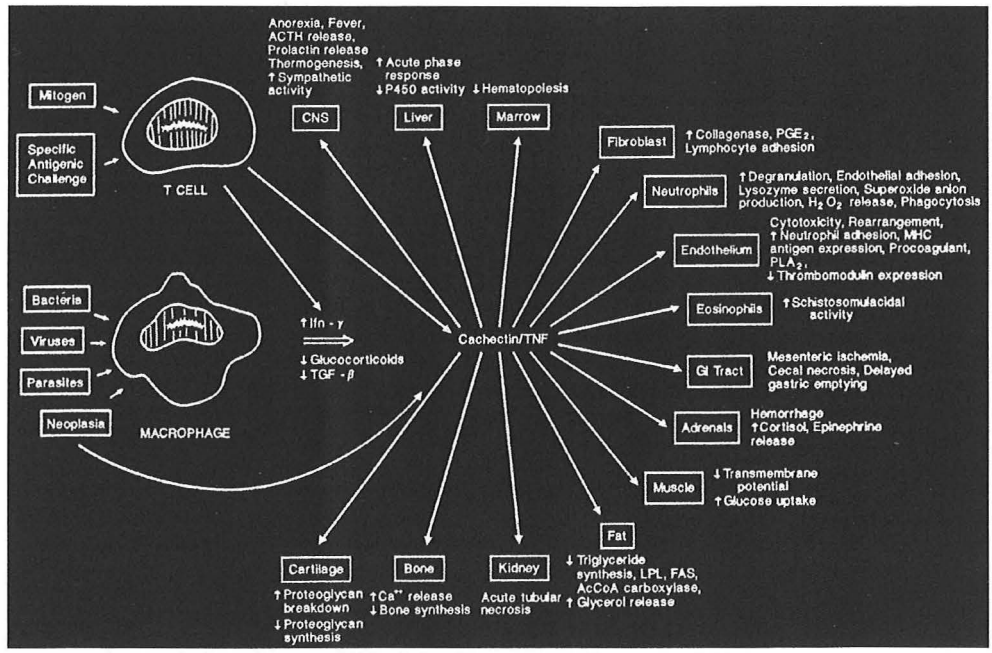


Figure 5. *The many biological targets of TNF. Induced in response to diverse stimuli, TNF may act both locally and at a distance to elicit pro-inflammatory effects.*

It was shown to provoke a state of insulin resistance consistent with that observed during infection (26,49). As a generalization, the molecule caused catabolic and proinflammatory effects in most model systems examined [Figure 5].

Interest in administering TNF to cancer patients waned sharply as awareness of its formidable toxicity grew, and a rather striking reversal of the practical goals that had led to TNF's isolation ensued. Efforts were directed toward exploration of TNF's pro-inflammatory character with the goal of treating inflammatory diseases. Among these, septic shock, autoimmune diseases, and certain specific immune responses seemed likely targets for intervention. Increasingly, clinical and preclinical studies were directed toward blockade of TNF activity.

Efforts to block TNF activity for clinical effect first bore fruit when it was observed that anti-TNF antibodies markedly ameliorated pain, joint swelling, and associated symptoms in patients with rheumatoid arthritis (50-53). Shortly thereafter, it was reported that anti-TNF antibodies could effectively treat patients with Crohn's disease who were refractory to all other forms of therapy (54). On the other hand, preclinical work suggested that TNF was important for successful defense against infection with mycobacteria (55,56), *Listeria monocytogenes* (57,58), *Leishmania major* (59-61), and *Legionella pneumophila* (62-64), and herpes simplex virus (65). In aggregate, these passive immunization studies, followed by the use of specific TNF inhibitors and gene knockout work, gave a clear impression of the fundamental activities that TNF evolved to serve.

Concurrently, analysis of TNF gene regulation permitted inferences about endotoxin signal transduction. TNF

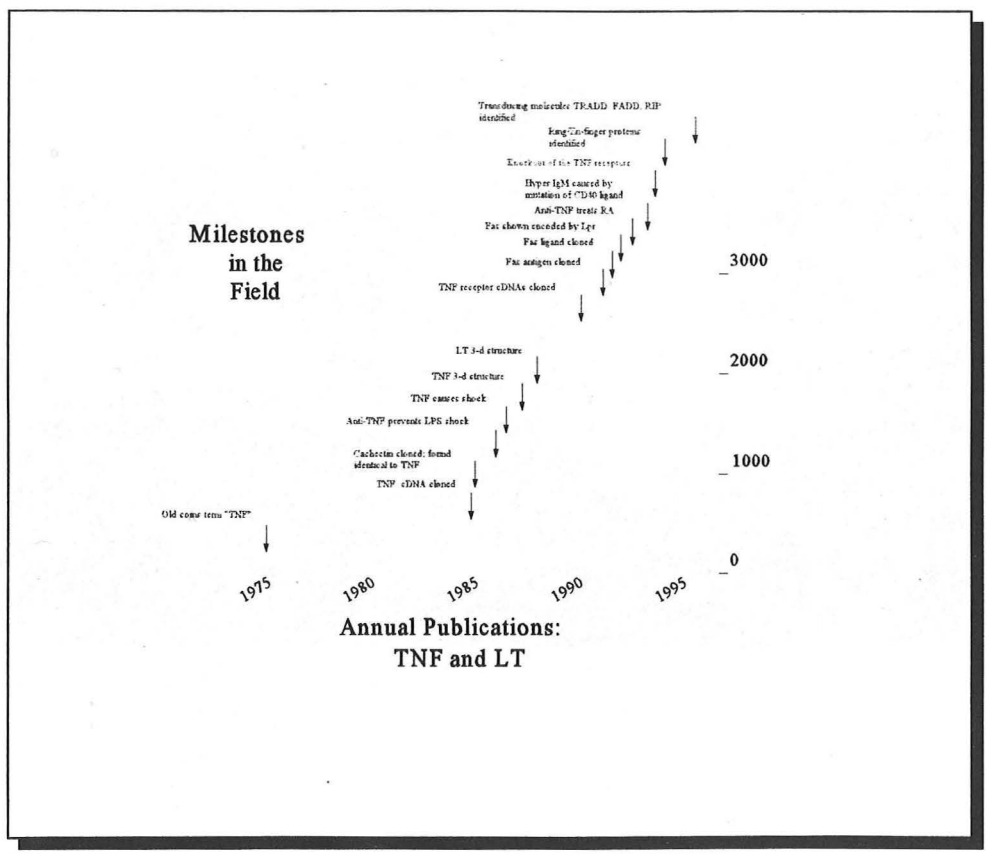


Figure 6. *Publications and landmark discoveries in the TNF field, by date.*

became a standard endpoint in the development and testing of drugs that block endotoxin signaling. The TNF receptors were identified through expression-based cDNA cloning (66-69). Structural studies of TNF and LT-α, performed in the Sprang laboratory at UT Southwestern, culminated in the crystallization of both molecules, and the consequent demonstration that each ligand was a compact homotrimer (70-72). It emerged, meanwhile, that both TNF and LT-α- are members of a large family of ligands with developmental, proliferative, and apoptotic effects (73,74). Several members of the extended TNF family are of medical importance, in that mutations affecting these ligands create well-known human diseases. Similarly, mutations affecting the corresponding receptors may cause disease, as discussed below.

In the past two years, it has become evident that TNF- and perhaps all of its homologs- rely upon signal transduction systems entirely distinct from those of every other class of cytokine (75,76). Neither serine kinase activation, nor tyrosine kinase activation, nor G-protein coupling are immediate downstream concomitants of receptor activation. Instead, TNF signaling depends upon a novel protein:protein interaction motif (the so-called "death domain"), upon an emergent family of ring- and zinc-finger bearing proteins, and upon proteolytic enzymes, which collectively execute many of the hormone's effects.

It may safely be said that more has been learned about TNF during the past decade than about any other cytokine [Figure 6].

STRUCTURE AND ESSENTIAL FUNCTIONS OF TNF LIGAND AND RECEPTOR FAMILY MEMBERS

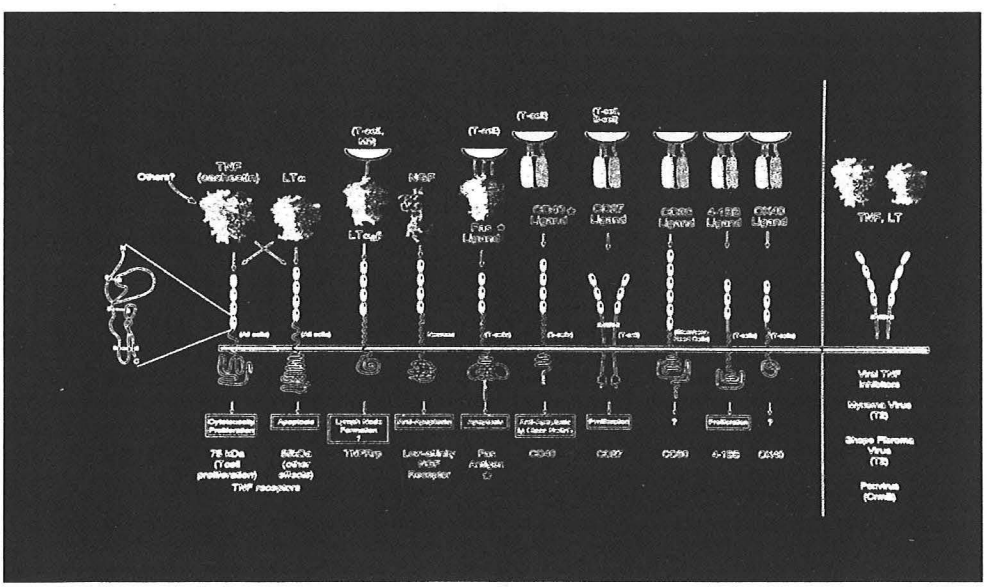


Figure 7. *The extended family of TNF ligands and receptors. A new ligand (Apo2L; not shown here) has been discovered recently (241). All of the ligands are trimeric, and all but LT-β are homotrimers. Most of the ligands are cell-associated. While the receptors are depicted as monomeric, all are probably pre-formed dimers in the cell membrane. CD27 is known to be a disulfide-linked dimer. The shape of the cysteine-rich repeat that defines the family is shown at the left. Sources and functions of each cytokine and receptor are indicated. The ancient origins of the TNF receptor family are indicated by the fact that one representative has recently been identified in maize (242); the plant receptor, as distinct from the mammalian receptor, has intrinsic kinase activity.*

TNF can no longer be viewed in isolation. At present writing, ten members of the TNF ligand family have been characterized. All are believed to be trimeric proteins, and most have been shown to be trimers based on crystallographic or comparative modeling studies. All but one member of the ligand family are homotrimers; LT-β is a heteromeric protein usually composed of two β and one α subunit (77). All but two members of the family are type II plasma membrane proteins: LT-α is entirely secreted and TNF itself is predominantly secreted, though it may exist in a membrane-anchored form (78,79).

The TNF receptor family members are defined by homology in the extracellular (ligand-binding) domain (69). A total of ten receptors have been characterized to date, though others almost certainly await identification. A varying number of cysteine-rich motifs (as few as three repeats in the Fas antigen and as many as six repeats in the CD30 antigen) are present, and contribute to interaction with each specific ligand. The ligands engage members of the receptor family with one-to-one correspondence (i.e., each ligand has a single, specific receptor) barring the exception of TNF itself and LT-α, which share a pair of receptors, binding each with similar affinity (80). One receptor family member [the 75 kD nerve growth factor (NGF) receptor] has apparently "captured" a protein that does not belong to the true ligand family (NGF) in the course of evolution.

While extracellular domain homology defines the receptor family, there is scant homology among family members on the cytoplasmic side. Only one motif is recognizably conserved among different family members: the so-called "death domain" that resides in the cytoplasmic moiety of the Fas receptor and the 55 kD TNF receptor (81,82). This motif is required for cell killing mediated by these receptors, and mutations within it abolish the cytolytic effect transduced in response to ligand binding.

Mutations in the genes encoding several members of the receptor and ligand families are known to cause disease, either in humans, in animal models, or both (Table I). For example, mutations of the CD40 ligand gene create an X-linked immunodeficiency in which failure of the normal "switch" from production of IgM to production of more advanced immunoglobulins fails to occur (83-86). Likewise, mutations of the CD40 ligand or receptor gene reproduces this immunodeficiency in mice (87,88).

Mutations of genes encoding the Fas receptor (89) [the *Lpr* mutation of mice (90)] or its ligand (89) [the *Gld* mutation of mice (89)] lead to lymphoproliferative disease, in which an unusual class of (CD4-, CD8-) T-cell accumulates in immense numbers in the lymph nodes and spleen. Autoantibody formation and other features of autoimmunity are also observed in this disease, recently

THE STORY OF THE FAS ANTIGEN (APO-1)

An extraordinary countertheme in the TNF field, the Fas antigen was discovered when monoclonal antibodies were isolated based on their ability to cause programmed death of tumor cells (i.e., to kill their targets in the absence of complement). Independently isolated by two groups of investigators, the target antigen was termed "Fas" (because it was a target on FS-7 cells), or Apo-1 (because it could trigger apoptosis). For a time, it was believed that the 60 kD Fas/Apo-1 antigen might represent the receptor for TNF. However, when cDNAs encoding both TNF receptors and the Fas antigen itself were cloned, this view became untenable; instead, it was recognized that Fas and the two TNF receptors were part of a family of proteins (which was to grow larger in due course), and Fas/Apo-1 became a receptor in search of a ligand.

A further startling development came with the realization that the *Lpr* (LymphoPRoliferation) mutation of mice, previously taken as a recessively inherited model of systemic lupus erythematosus, represented a gross rearrangement of the Fas antigen gene. It had long been argued on biological grounds that the *Lpr* locus, together with an unlinked locus in mice known as *Gld* (Generalized Lymphoproliferative Disease) might encode a receptor and ligand pair. Mutations at the *Gld* locus created an identical, recessively inherited autoimmune disorder. Moreover, a particular allele of *Lpr*, known as Lpr^{cg} ("complements *Gld*") could, in fact, complement mutations at the *Gld* locus, so that double heterozygotes developed lymphoproliferative disease. Gld was ultimately shown to encoded the Fas ligand. A point mutation within the *Gld* gene leads to an inactive ligand, and disease. The Lpr^{cg} allele bears a point mutation within a region that encodes the death domain. The mutant Fas antigen is expressed, but incapable of signalling. The presence of unproductive copies of the receptor, and half the number of copies of ligand, accounts for expression of the disease phenotype in double heterozygotes.

found to afflict humans as well as mice (91,92). Indeed, the murine Fas ligand and receptor mutation (the *Gld* and *Lpr* mutations in mice) were long taken as models of systemic lupus erythematosus. ***The Fas ligand/receptor axis, then, is the best example of an autoimmune disease traceable to failure of a single molecular interaction.*** It has altered present concepts of autoimmunity and its molecular pathogenesis, since this disease, at least, clearly results from a failure of apoptosis within cells of lymphoid origin. The implications of this realization are discussed below (see **Epilogue**).

Mutations of NGF (93) or the 75 kD NGF receptor (94) create a phenotype in which peripheral neuropathy causes ulceration of extremities. Defects of autonomic nervous system development are also evident.

Mutations of the two TNF receptors are compatible with life. However, animals lacking the 55 kD TNF receptor (which contains the death domain) are demonstrably resistant to the lethal effect of LPS. This suggests that the lethal effect of LPS- acting via TNF- is largely transduced by this receptor. Further, they cannot effectively resist challenge with *Listeria monocytogenes*, or *Mycobacterium bovis*. These two rather different intracellular pathogens grow exuberantly in such hosts, suggesting that TNF is a dominant agent of natural resistance to each. Recently, it was determined that the 55 kD TNF receptor is also required for normal development of germinal centers in peripheral lymphoid tissue, apparently stimulated by LT-α, which is also required for germinal center development (95).

TABLE I.

Mutation	Phenotype
TNF	↑Sensitivity to LPS ↓Susceptibility to infection Defective germinal center formation
LTα	Absence of lymph nodes, Peyer's patches, germinal centers
55 kD TNFR	↑Sensitivity to LPS ↓Susceptibility to infection
75 kD TNFR	↑Dermal inflammatory responses ↑Sensitivity to TNF
LTβ/LTβR	ND
Fas/FasL	Lymphoproliferation
CD40/CD40L	Failure of IgG, IgE, IgA production
75 kD NGFR	Defective peripheral sensory innervation
CD30	Impaired negative selection in thymus

Animals lacking the 75 kD receptor are not immunocompromised, so far as is known, but do not exhibit normal inflammatory responses to TNF. Notably, they fail to develop necrotizing dermal lesions when TNF is injected into the skin (96).

Mutations of TNF family ligands and receptors, and their phenotypic consequences. ND, not determined as of this writing.

Double receptor knockout yields a phenotype that is, more or less, the sum of the two single receptor knockouts. It might have been supposed that double ligand knockout would recapitulate double receptor knockout. However, ablation of the LT-α gene, alone (97) or together with knockout of the TNF gene (98), prevents the development of lymph nodes, Peyer's patches, and white pulp of the spleen, though not interfering with the ontogeny of lymphocytes *per se*. Most probably, the participation of LT-α subunits in the formation of the LT-α/LT-β heteromer, accounts for this unexpected phenotype (74). It would follow that the LT-β receptor is required for lymph node development, and that mutations affecting it might cause lymph node aplasia.

CD40 LIGAND AND X-LINKED IMMUNODEFICIENCY/HYPER IgM SYNDROME

The identification of this mutation involved a good deal of intuition. There was, in the beginning, no mouse mutation to seek, as had been the case with the Fas antigen and Fas ligand. There was not even a clear idea of what CD40 and its ligand might do. CD40 was an antigen of unknown function, expressed primarily on B cells, monocytes, dendritic cells, thymic epithelium, and certain carcinomas. However, the availability of monoclonal antibodies against CD40 suggested that it might function as a co-activator of several B-cell functions including homotypic aggregation, IgE production, and short-term proliferation. CD40 was found to engage CD40 ligand, a member of the TNF ligand family encoded by an X-linked gene (*CD40l*) approximately 1.5 cM distal to the *hprt* gene in mice. This suggested that the human CD40L gene would map to Xq26. Of five known X-linked immunodeficiencies (XSCID, agammablobulinemia, Wiskott-Aldrich syndrome, X-linked lymphoproliferative disease, and X-linked hyper-IgM syndrome, only the latter mapped to the region Xq24-Xq27. This is a rather large stretch of DNA. Yet, as luck would have it, PCR analysis of DNA revealed that patients with hyper-IgM syndrome have one of several point mutations within the CD40L extracellular domain.

REGULATION OF TNF BIOSYNTHESIS

Under physiologic conditions, little or no TNF is secreted by macrophages. The protein is normally called into service in the course of infection, and at that, is normally made in measured amounts at the correct location. During overwhelming infection- particularly infection with gram negative organisms- TNF biosynthesis may be excessive so as to threaten the life of the host. Bacterial lipopolysaccharide is the most important inducing agent to consider in this instance, and LPS has, in fact, been the focal point of most investigations into the biosynthesis of TNF.

Few cells are ever capable of expressing TNF at all. The gene is "inaccessible" in most cell types, and therefore, transcriptionally silent (74). Tissue macrophages are believed to be the principal source of TNF under most conditions, though it is clear that lymphoid cells are also capable of secreting it (99,100).

Accessibility of the TNF gene is controlled by sequences that lie in close proximity to the coding region (101). Interestingly, the pattern of tissue expression of TNF differs markedly from that of LT-α (102). This is so despite the fact that the TNF, LT-α, and LT-β genes all reside within a single 10 kb span of DNA (77,103). The TNF locus lies within the class III region of the major histocompatability complex in all species examined to date. Despite much speculation that polymorphism at the TNF locus might account for one or another of the wide array of diseases with MHC haplotype association (104-113), there is ***no*** evidence that any of the base substitutions observed to date play a causal role in disease (114,246). On the contrary, the associations observed likely result from linkage disequilibrium.

Given that the TNF gene is accessible to the transcriptional apparatus (as it is in macrophages, most T cells and B cells, keratinocytes, mast cells, and certain other cells), both transcriptional and translational regulatory mechanisms govern production of the protein. Both types of control have been studied in macrophages, chiefly in response to induction by LPS.

When LPS is introduced into a macrophage culture, TNF biosynthesis is accelerated by a factor of 10,000 or more. While the protein is undetectable in unstimulated macrophage cultures by any but the most sophisticated techniques, it is one of the major secretory products of endotoxin-activated macrophages, amounting to 1 to 2 percent of the protein released by these cells into the surrounding medium (21). Correspondingly, TNF may at times approach the micromolar range of concentration in plasma (115), and a mechanism for precise regulation of production must therefore be enforced under normal conditions.

Transcriptional activation of the TNF gene is minimally dependent upon NF-κB sites that are located within 1,000 base pairs of the transcription start site (116). Four such motifs exist in the promoter, and if two or more of these are destroyed through mutation, transcriptional responses to LPS are abolished. In the human TNF promoter, an AP-1 site also exists, suggesting that the gene may respond to induction by factors of the fos and/or jun families. Once produced, the TNF mRNA is processed by removal of three intronic sequences and transported to the cytoplasm. Here, it remains "locked" in an untranslatable form until macrophages are stimulated with LPS (117-119). A 200 fold rise in translational

efficiency is then observed (120). Translational suppression in quiescent cells is dependent upon a UA-rich motif present in the 3'-untranslated region of the TNF mRNA (121-124). It is not known how the motif exercises translational suppression nor is known how suppression is relieved. A number of proteins that specifically engage such UA- rich elements, which are commonly observed in the 3'-untranslated region of cytokine mRNAs (22), have been identified in recent years (125-127). It is reasonable to suppose that one or more of these proteins participates in the translational response to LPS.

BLOCKING TNF PRODUCTION AND/OR ACTIVITY FOR THERAPEUTIC EFFECT

In Table II, a list of diseases potentially responsive to TNF blockade is presented. Blockade might be achieved by three general approaches: through inhibition of biosynthesis, through interdiction of secreted TNF, or through inhibition of TNF signaling.

I. Small molecular antagonists of TNF biosynthesis

Much has been learned about the pathways that lead to TNF biosynthesis through mechanistic studies anti-inflammatory drugs, some of which are currently in clinical use and some of which remain investigational. These agents target specific components of the endotoxin signal transduction pathway.

Endotoxin is believed to be concentrated onto the surface of cells through interaction with a plasma binding protein (LPS binding protein, or LBP)(128,129) and subsequently, through interaction with the GPI linked plasma membrane protein CD14 (130) [Figure 8].

CD14 has no clear means of signaling the presence of LPS, since it lacks any cytoplasmic domain. However, through an unknown mechanism, the signal is propagated across the lipid bilayer. Endotoxin signaling is minimally depend upon the product of a single gene, identified in mice (131) and termed the *Lps* gene (132,133). Mutations in the *Lps* gene, which are observed in certain strains of mice, forbid endotoxin signaling. However, the protein encoded by *Lps* has yet to be identified. It is probable that a tyrosine kinase mediates most of the responses to LPS, since broadly active inhibitors of tyrosine kinases, including ***herbimycin, genistein,*** and ***tyrphostins,*** block endotoxin signaling (134-140), both in macrophages and in B-cells. These agents also protect animals against the lethal effect of LPS when administered systemically (141).

THE Lps *GENE*

Although it is widely believed that endotoxin (lipopolysaccharide; LPS) binds to CD14, a GPI-linked plasma membrane protein on mononuclear cells, the initial events in endotoxin signalling remain obscure. As LPS is believed to be of dominant importance in the pathogenesis of gram-negative septic shock, identification of its signaling intermediates will ultimately be essential in predicting who is most at risk to develop endotoxic shock, and in devising highly specific, effective therapies for endotoxic shock. A major clue to the identity of early signal transduction proteins is offered by the C3H/HeJ mouse, which is entirely refractory to LPS by virtue of a mutation tightly mapped on chromosome IV. These animals can survive virtually any dose of LPS, but are highly susceptible to the administration of authentic gram-negative organisms, suggesting that endotoxin "sensing" is important to allow an effective defense against the bacteria themselves. Undoubtedly TNF is an important component of this response; administration of TNF to C3H/HeJ mice partly ameliorates their susceptibility to gram-negative infection {{2833}}. In any event, isolation of the so-called *Lps* gene will undoubtedly permit a far better understanding of just how LPS "works" to induce cytokines, cause inflammation, and trigger shock. It may also allow prediction of individual human responses to gram negative infection.

TABLE II
DISEASES IN WHICH INAPPROPRIATE PRODUCTION OF TNF HAS IMPORTANT CONSEQUENCES:

Disease	Evidence From:				Key References
	Measurement (e.g., in blood or of production *ex vivo*)	Administration mimics effect	Blockade ameliorates the disease: Animals	Humans	
Septic shock	++++	++++	++++	ND[2]	(33,155,156,200-202)
Rheumatoid arthritis	++	++	N/A[1]	++++	(50-52,203-207)
Crohn's disease	+	+	N/A	++++	(54,208,209)
Multiple sclerosis	++	+++	+++[3]	ND	(210-214)
Type II diabetes (insulin resistance)	+/-	++++	++++	-	(215-222,245)
Cachexia of malignancy	-	++++	++	ND	(30,31,223-225)
GVH disease and allograft rejection	+/-	+/-	++	ND	(30,31,226)
Beomycin-induced pulmonary toxicity	+/-	-	++	ND	(227)
Kawasaki disease	+	-	N/A	ND	(228,229)
Hypercalcemia of malignancy	+++[5]	++++	ND	ND	(44,46,230)
Cerebral malaria	++++	+/-	+++	ND	(196,231,232)
Jarisch-Herxheimer reaction	++++	++	ND	++++	(158)
Type I diabetes	-	+/-	+/-[4]	ND	(111,233,234,243,244)
HIV infection	+/-	++++[6]	NA	ND	(235-237)

[1] N/A, Not applicable
[2] ND, Not determined
[3] Experimental allergic encephalomyelitis
[4] In NOD model, protective effect dependent upon age at which antibody is administered
[5] LT-α in multiple myeloma
[6] *in vitro*

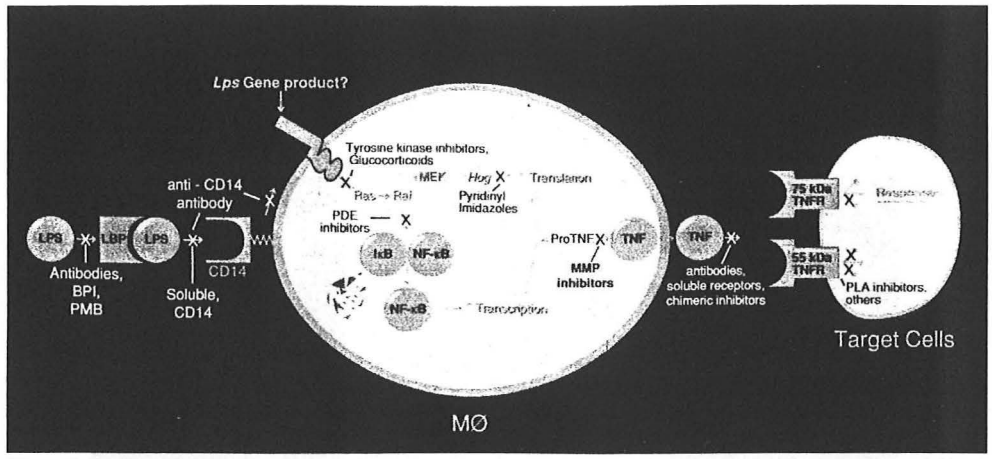


Figure 8. *Outline of the signaling pathway utilized by endotoxin, as it is presently understood, with emphasis on inhibitors that block signaling. LPS is concentrated onto the surface of cells by binding to CD14. An unknown protein (possibly encoded by the* Lps *gene) is responsible for initiation of a signal. Ras, raf, and upstream components of the MAP kinase pathway initiate the transcription of a collection of LPS-inducible genes, including the TNF gene. NF-κB is also an essential participant in this process. p38, the mammalian equivalent of the yeast* hog *(hyperosmolarity) gene, conveys a signal for translational activation. Processing of pro-TNF to yield the mature, secreted protein is accomplished by matix metalloproteinases, but is not known to be a regulated event.*

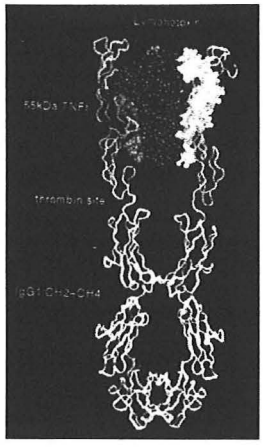
Glucocorticoid agonists also globally depress responses to LPS (123,142). Both transcriptional and translational activation of TNF biosynthesis are prevented by dexamethasone, cortisone, and related agents. It is likely that the glucocorticoids induce a suppressor protein in order to achieve their effect, since they must be administered well in advance of endotoxin challenge to be efficacious (33). Further, it is probable that much of the anti-inflammatory effect that glucocorticoids cause is attributable to their ability to block the synthesis of TNF and other cytokines.

Selective blockade of transcriptional and translational activation is also achievable. ***Pentoxifylline*** and other phosphodiesterase inhibitors, as well as ***cyclic AMP analogs,*** selectively block the accumulation of TNF mRNA (121,122,143-145). They do not exert an effect on the accumulation of other cytokine messages, and the basis for their selectively remains to be determined. Their inhibitory effect is partly attributable to action at the level of transcription. However, it appears that they also influence processing of the primary transcript. ***Lisofylline,*** a metabolic derivative of pentoxifylline which lacks activity as a phosphodiesterase inhibitor targets an unknown protein within the cell, and selectively depresses activation of the stress-activated protein kinase (SAPK or JNK) pathway. In human systems, this may ultimately prevent activation of TNF gene transcription (146). ***Pyridinylimidazoles***, such as the prototype SKF86002, are known to target a specific MAP kinase homolog known as p38 (147). This serine kinase, which becomes phosphorylated in endotoxin-activated macrophages, and which represents the mammalian counterpart of a yeast gene (*hog*) required for adaptation to growth under hyperosmolar conditions (148) is apparently a direct upstream component of the translational activation pathway (149). Hence, drugs of this class block TNF biosynthesis by preventing translational activation, yet have relatively little effect on the accumulation of TNF mRNA. **Inhibitors of *ras* and *raf*** also block TNF synthesis, affecting both transcriptional and translational limbs of the activation pathway (150). Therefore, these proteins comprise the important structural components of the proximal signaling apparatus.

Once TNF has been synthesized, it must be processed by proteolytic cleavage prior to secretion as a soluble trimeric species. In humans, 76 amino acids are appended at the amino terminus of the mature TNF polypeptide chain (18). Pro-TNF is an integral membrane protein (78). Despite assertions that "membrane TNF" has an important role as a mediator of local inflammatory processes (79,151), it is efficiently processed by a constitutively active matrix metalloproteinase which cleaves its target at an intracellular site (152,153). Hence, blockade of matrix metalloproteinase activity causes accumulation of inactive TNF propeptide within the Golgi complex (152). ***Matrix metalloproteinase inhibitors*** which are known to be effective in the prevention and treatment of experimental arthritis, block conversion of

TNF to an active, secretable form. These inhibitors target at least three isoforms of matrix metalloproteinases that are capable of cleaving the TNF propeptide (McGeehan, G., personal communication).

II. Macromolecular inhibitors of TNF action



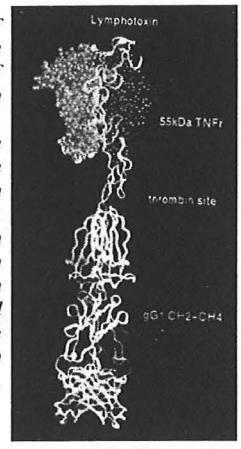


Figure 9. *Chimeric inhibitors of TNF and LT-α activity. By fusing the extracellular domain of a TNF receptor to the heavy chain of an immunglobulin molecule, a specific, avid TNF inhibitor is generated. The inhibitor binds two of the three available receptor-combining sites on the surface of the ligand molecule, blocking productive interaction with cell-surface receptor. Here, an inhibitor is shown complexed with an LT-α trimer, from the "front" and "side." Chimeric TNF inhibitors are now in Phase III trials for use in rheumatoid arthritis and septic shock.*

Interdiction of TNF is also possible after its secretion from cells, and in some ways, this approach is desirable in a clinical setting, given the extreme specificity of such reagents and their comparative lack of side effects. ***Monoclonal antibodies*** against TNF have been used with impressive success in patients suffering from rheumatoid arthritis (53), and Crohn's disease (54). They have also been applied to the treatment of gram-negative septic shock (154,155,156). Here however, a significant protective effect has so far remained elusive, in part because a diverse (and often fatally injured) population is under study (157). It was recently demonstrated that ***polyclonal antibodies*** against TNF can attenuate the Jarisch-Herxheimer response in patients given penicillin for the treatment of *Borrelia recurrentis* (158). However, long-term application of monoclonal antibodies (even humanized monoclonal antibodies) and/or polyclonal antibodies against TNF is not widely contemplated, since increasingly severe immune responses develop following repeated exposure to these proteins.

The development of highly specific and effective ***chimeric inhibitors of TNF activity*** [Figure 9] may circumvent this problem (159-161). By fusing the TNF receptor to a fragment of the immunoglobulin heavy chain, a dimeric TNF binding reagent may be generated, and used for long periods of time to block the action of TNF and LT-α *in vivo*. Such reagents are as stable as monoclonal antibodies, more avid in their interaction with ligand, and can entirely prevent ligand-dependent activation of either of the two cell-surface TNF receptors. Antibody responses to chimeric TNF inhibitors are extremely weak if they occur at all. Therefore, chimeric TNF inhibitors offer considerable promise as a means of neutralizing TNF for long periods of time in human patients.

III. Postreceptor approaches to blockade of TNF activity

As more is learned about the mechanism by which TNF signals, it may be possible to devise small molecular antagonists that work at a postreceptor level, effectively inducing a state of "resistance" to the ligand. Indeed, a vast number of currently available drugs, including suramin (162), chlorpromazine (163), glucocorticoids (164), N-acetylcysteine (165), and lipoxygenase inhibitors (165) are reported to dampen TNF signal transduction in one way or another. It must be emphasized that most of these reports have never been built upon in a meaningful fashion, and little as grown from them in the way of mechanistic understanding.

Blockade of mitochondrial electron transport is known to diminish the cytotoxicity of TNF, suggesting that reactive oxygen intermediates may be involved in the late stages of cell killing (166,167). It is known that proteases of the interleukin-1 converting enzyme (ICE) family are important mediators of the apoptotic effect of TNF (168-170). It is not clear, as yet, that these enzymes transduce all of the inflammatory actions of TNF. However, cell killing can be blocked with by the macromolecular ICE inhibitor *Crm* A (168,169), and by the serpin plasminogen activator inhibitor (171). It can also be blocked by over-expression of at least one heat shock protein (HSP70) (172), by over-expression of the zinc finger protein A20 (173), by over-expression of the anti-apoptotic proteins Bcl-2 and Bcl-x (174), and by certain other measures (175-177), though in no instance is the mechanism of blockade precisely understood.

However, a new chapter in the search for mechanisms of cytotoxicity has recently opened, and there is real reason to expect that the signal transduction pathway utilized by TNF will be fully understood in the near future.

How do TNF receptors work?

How does TNF work once it is produced? Unsurprisingly, it is believed that all effects of TNF, LT-α, and related ligands are mediated by specific receptors that engage them. An entirely novel signal transduction system accounts for the function of TNF receptors. Best studied are the molecules that convey signals from the 55 kD TNF receptor and Fas receptor: i.e., those molecules which kill cells that encounter the respective ligands. Remarkably, signaling for programmed cell death seems, in its earliest stages, to be independent of kinase cascades that characterize other signaling pathways for cytokine action. Rather, the TNF and Fas receptors are elaborate "nano-machines" that engage a system of proteolytic enzymes which, in turn, cause the death of the cell.

Initial theories about receptor function concentrated on "aggregation" as the pivotal event in signal transduction. It was believed that a single TNF trimer could engage three receptor monomers floating in the plane of the plasma membrane, and that the coalescence of receptor molecules led to generation of a signal [Figure 10].

This view is perhaps too simplistic. Although it is clear that three receptors *can* engage a single ligand trimer within a cocrystal of receptor and ligand, it is probable that trimerization of the receptor is unnecessary. ***Rather, the receptors exist within the membrane as pre-formed dimers, and are activated by a conformational change that follows binding to the receptor.***

Naismith and coworkers (178) found that the TNF receptor extracellular domain is dimeric when crystallized in the absence of ligand. This observation is in agreement with biochemical studies, which reveal that the CD27 molecule (179), another member of the TNF receptor family, is a disulfide-linked dimer on the cell surface, and that the CD40 molecule is at least partly disulfide linked in its

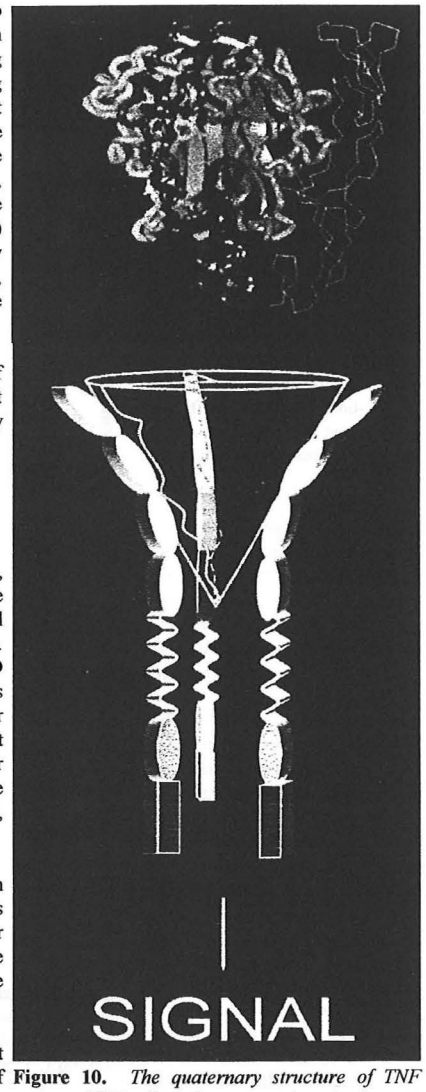


Figure 10. *The quaternary structure of TNF receptor:TNF ligand cocrystals (top) was consistent with the "aggregation" model of TNF receptor activation (bottom), earlier inferred from the observation that antibodies against the receptor can exert TNF-like effects. While there is no doubt that TNF* can *bind three receptor fragments, the 3:1 model of activation is at odds with data suggesting that the active form of the receptor is a dimer (180). The "molecular switch" model depicted in Figure 11 is therefore favored by the author.*

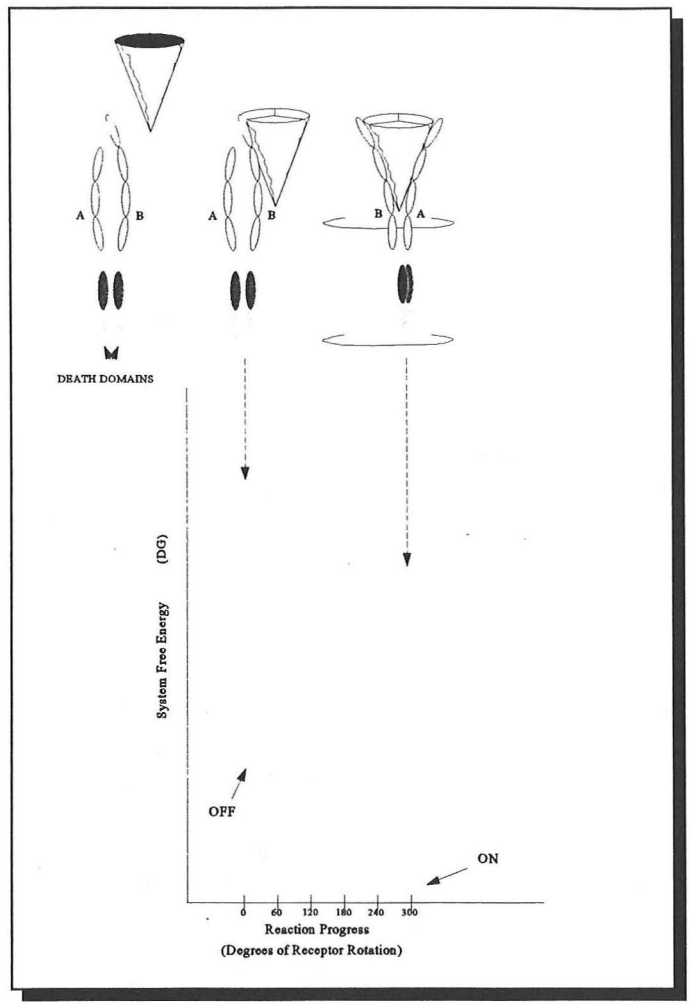


Figure 11. *The "molecular switch" model of receptor activation posits that each receptor is a preformed dimer endowed with an "on" and "off" state, corresponding to points of relative energy minimization. TNF ligand catalyzes transition from the "off" to the "on" conformation. This conformational change permits subsequent events in signal transduction.*

unliganded state. Bazzoni et al. (180) found that obligate dimers of the TNF receptor, created by fusing the extracellular domain of the Epo receptor to the cytoplasmic domain of each TNF receptor, are constitutively active in signaling. This proved that:

1. Both types of TNF receptor can, in fact, cause cytolysis
2. Dimer formation is the critical event in signal transduction
3. The function of the receptor extracellular domain- apart from binding ligand- is to actively maintain the receptor in an *inactive* state until ligand is present.

Once ligand is present, it presumably catalyzes receptor activation (181,182), lowering the activation energy barrier that must be traversed between "off" and "on" conformers of the switch [Figure 11].

On the cytoplasmic side of the membrane, the switching event may correspond to a change in accessibility of the death domain moiety. The death domain, it now appears, is nothing more nor less than a protein:protein interaction domain. Its propensity for dimerization (183,184), like its propensity to bind to other proteins that carry a similar motif, has been documented largely through the use of the yeast two-hybrid system, a relatively new method for the detection of interactions among proteins.

The two-hybrid system has revealed two general classes of proteins that engage receptors of the TNF family. On the one hand, proteins bearing ring-finger and/or zinc-finger motifs associate with the 75 kD TNF receptor, with the LT-β receptor, and with the CD40 receptor. These proteins have been termed "TRAFs" to indicate their association with the TNF receptors. Their mechanism of action is generally unknown, though there is a growing sense that they transduce the activation of NF-κB translocation within cells that encounter TNF. On the other hand, the two hybrid system has identified both primary and secondary transducers of the death signal that gave TNF its name.

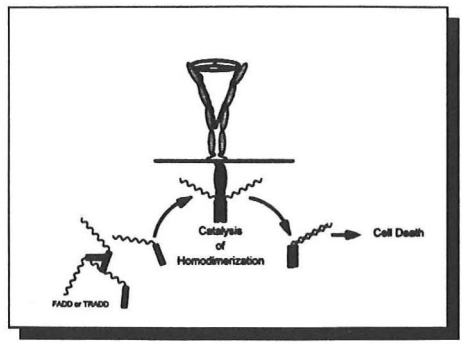


Figure 12. *Activation of the primary transducer FADD/MORT-1 as the result of a conformational change induced in the receptor cytoplasmic domain by ligand binding. FADD/MORT-1 has a death domain (rectangular box) through which it can form a complex with the death domain of the receptor. It is probable that receptor activation leads to transient formation of an intermolecular hybrid (center complex), and then, to homodimerization and activation of FADD/MORT-1. Coordination of FADD/MORT-1 activation is the first postreceptor event in a pathway leading to cell death initiated by Fas ligand. By implication, the TNF receptor cytoplasmic domain coordinates the activation of TRADD after ligand binding.*

Primary transducing molecules [Figure 12] that carry the apoptotic signal into the cytoplasm bear versions of the death domain , just as do the Fas and 55 kD TNF receptors themselves. These molecules, known as FADD (MORT-1) (185,186), TRADD (187), and RIP (183), engage the Fas receptor (in the case of FADD/MORT-1) and TNF receptor (probably all three primary transducers can do so). It is believed that a heterotypic association between death domains of the receptor and the primary transducer occur at this stage. In the process, the transducer molecules likely undergo conformational changes that are critical to propagation of the signal.

FADD (MORT-1) has now been found to bind in turn to a secondary transducer [Figure 13]. This latter molecule, termed MACH (188) or FLICE (189) , is a "hybrid" which has elements of similarity to FADD (MORT-1) itself, and to proteases of the ICE (interleukin-1 converting enzyme) family. ICE proteases are known to be important in apoptosis in lower organisms. In the nematode *C. elegans*, for example, the *ced*-3 gene encodes an ICE-like protease which contributes to cell death occurring in response to external stimuli applied during the course of development.

There are at least nine ICE family members in mammals, three of which are evolutionarily "close" to the *ced*-3 protein of *C. elegans*. Of these, CPP32β, or YAMA, is perhaps best known to be involved in Fas signal transduction (168,170,190). It is entirely possible that other isozymes of the family also contribute to cell death pathways. Indeed, knockout of the "classical" ICE isozyme (which processes IL-1β) creates a highly LPS resistant phenotype, though knockout of IL-1β itself does not. This may suggest that the classical ICE is, to some extent, involved in TNF signal transduction.

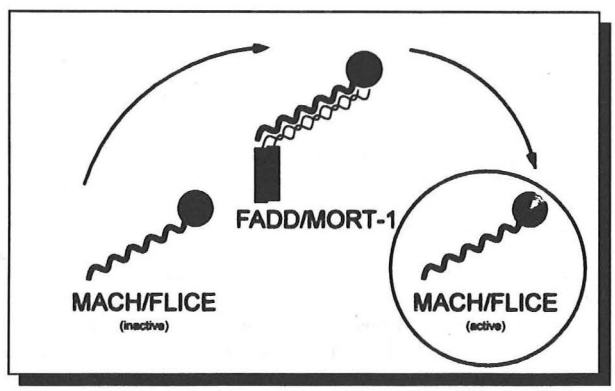


Figure 13. *The primary transducer FADD (MORT-1) catalyzes the activation of MACH (FLICE), a "hybrid" molecule bearing FADD-like and ICE-like sequences. MACH (FLICE) is the long-sought nexus between the receptor death domain and proteolytic activation leading to apoptosis.*

Downstream from ICE, the pathway undoubtedly branches. However, ICE may act to cleave poly ADP-ribosyl polymerase (PARP), activating it and triggering consumption of the NAD reserves of the cell [Figure 14]. ICE may also activate other proteolytic enzymes, may inhibit the function of anti-apoptotic proteins such as Bcl-2, and may activate DNases that contribute to fragmentation of genomic DNA. However, distal mechanisms of programmed cell death are largely shrouded in mystery.

WHY DO WE HAVE TNF?

TNF did not evolve to cause shock and tissue injury. Like the inflammatory response as a whole, it evolved to assist in host defense against invasive organisms. The first evidence favoring a "positive" view of TNF came from the work of Havell (57), who showed that animals treated with a polyclonal antibody against mouse TNF were unusually susceptible to infection by *Listeria monocytogenes*. Kindler and coworkers (57) subsequently found that passive immunization against TNF renders animals susceptible to infection by mycobacteria (*Bacillus calmette-guerin*). These

observations were confirmed using more specific reagents, such as the TNF inhibitor molecules described above (58,191). Likewise, it was demonstrated that ablation of the 55kD TNF receptor gene sensitized animals to infection by these pathogens (192-194). Other instances of susceptibility related to blockade of TNF or diminished TNF production have emerged as well; it appears that TNF can offer protection against *P. falciparum* malaria (195), *Leishmania major* (59,60,196), *Legionella pneumophila* (62-64,197), and *P. aeruginosa* (198) infections.

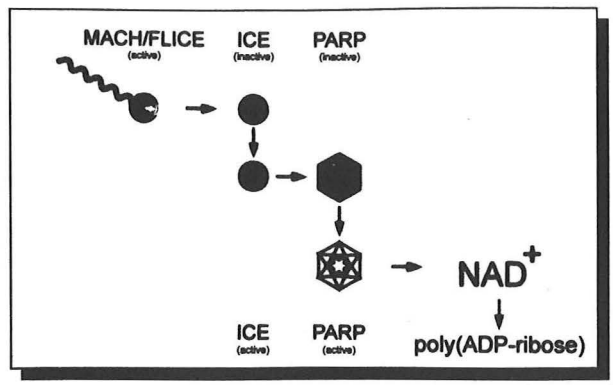


Figure 14. *Activation of MACH (FLICE) causes subsequent cleavage of other ICE homologs, which in turn cleave (and thereby activate) poly-ADP-ribosylpolymerase (PARP). Activation of PARP leads to consumption of cellular NAD reserves, and is one of several mechanisms by which apoptosis may be effected.*

Of course, most of these pathogens scarcely resemble one another at all. To take an example, *Mycobacteria* and *Listeria*, while both intracellular pathogens, are strikingly distinct from one another both in terms of physical structure and with respect to their life cycles within the mammalian cell. While both organisms are contained by mononuclear phagocytic cells, *Listeria* exist free within the cytosol, whereas, mycobacteria are intra vesicular. Yet TNF seems essential to the elimination of each pathogen. Animals defective in their responses to TNF or unable to produce TNF recruit inflammatory cells to the site of infection; however, these cells are apparently powerless to kill the invading organism.

TNF also has an antiviral effect, first documented in cultured cells infected with vesicular stomatitis virus (VSV). This effect was, to all appearances, "interferon-like" in the sense it did not involve destruction of virus-infected cells. However, in other systems, (i.e., herpes simplex virus infection) TNF does appear to favor the rapid lysis of cells infected with a virus. It may thereby help limit dissemination of the pathogen. It is interesting to note that several viruses have evolved genes encoding proteins that demonstrably interfere with responses to TNF. Poxviruses, for example, have fashioned a dimeric version of the TNF receptor, apparently captured from a mammalian host in the course of evolution. The receptor variant is secreted and is capable of neutralizing TNF in the environment of infected cells. Mutations within the viral gene encoding this neutralizing protein markedly diminish pathogenicity of the virus. Poxviruses also encode a broad-spectrum protease inhibitor known as *Crm A*. *Crm A* blocks the cytolytic effect of TNF by binding and inactivating homologs of the ICE family of proteases, now known to be important in signal transduction leading to apoptosis. [Oddly, knockout of the 55 kD TNF receptor gene does not render mice susceptible to infection by *Vaccinia* virus] (199). A 14.7 kD protein encoded by the E3 region of adenovirus also blocks cytotoxicity, acting through an unknown mechanism. Within the E1B region, a gene coding for a 19 kD inhibitor of cytolysis is found. This latter protein binds and inhibits the Bcl-2 homolog Bak, a pro-apoptotic factor that normally forms heterodimers with Bcl-2. Finally, the Epstein-Barr virus encodes a plasma membrane protein known as *Lmp*-1, which engages LAP-1, a ring-and zinc-finger protein that mediates signaling from the type-2 TNF receptor, as well as the LT-β receptor and CD40 receptor. It is reasonable to suppose, therefore, that many pathogens are eradicated through the action of TNF, and that infectious diseases were primarily responsible for the preservation of TNF and its receptors despite the untoward effects that these proteins sometimes cause.

It has often been supposed that TNF might offer protection against neoplastic disease as well as infection. However, no primary data have emerged in support of this hypothesis, and in fact, a fairly large body of evidence indicates that TNF abets tumor metastasis and local invasion of tissues.

Why is TNF selectively tumoricidal?

Early impetus for the study of TNF was derived from its remarkable selectivity in tumor cell killing. No non-transformed cell was found to be susceptible to TNF in the absence of inhibitors of RNA or protein synthesis. On the other hand, most normal cells can be killed by TNF in the presence of cycloheximide or actinomycin-D. From this it was inferred that normal cells maintained a mechanism for inhibition of TNF signalling, which was labile to the extent that inhibitors of protein synthesis would rapidly disrupt it (238). On the contrary, many transformed cells appeared to have lost this modulatory ability.

Now that far more is known about TNF signaling, we are left to wonder just how modulation is achieved. Do certain proteinase inhibitors block components of the ICE-related signalling pathway? Do cancer cells sacrifice such inhibitors as a necessary concomittant of transformation? The tools to address such questions are now well in hand.

It may also be possible to determine whether the pro-inflammatory effects of TNF and the anti-neoplastic effects might at last be separated, albeit pharmacologically and at a post-receptor level. It is likely, as noted above, that cell killing is an integral component of the inflammatory response. On the other hand, NF-κB activation- which bears no direct relationship to cell killing- is also an important effect of TNF and is known to be elicited in part by the 55 kD TNF receptor. To a large extent, it may be responsible for the unacceptable side effects that accompany TNF administration. It might ultimately be possible to design antineoplastic drugs that trigger the proteolytic cascade that initiates cell death, effectively circumventing the shock, coagulopathy, and other problems that arise when TNF is administered.

Epilogue: TNF, apoptosis, and the genetic origins of autoimmunity

It is no accident that the Fas ligand/receptor system causes cell death. This, it would seem, is the express purpose of the system. Mutations of the Fas ligand and receptor cause autoimmune disease, precisely because a certain class of T-lymphocyte fails to die as intended. No comparable phenotype can be seen in animals carrying isolated mutations of TNF, LT-α, or the 55 kD TNF receptor. Yet the question occurs: can the cell-killing potential of TNF be accidental? Or is it, like the cell-killing potential of the Fas ligand, essential under the correct set of circumstances? In fact, it is known that interruption of TNF signalling through the 55 kD receptor creates a far more severe phenotype in the presence of a co-existing mutation of the Fas antigen (239).

The immune system is among the most plastic of tissues. In the course of a response to host invasion, vast numbers of T and B lymphocytes are generated through clonal expansion from a comparatively small number of reactive progenitors. It is probable that any pathogen, no matter how simple, has sufficient epitopic complexity to cause the expansion of dozens or hundreds of lymphocyte clones. It is well known that up to 1% of the host lymphocyte repertoire may react with a single protein antigen once clonal expansion has been maximized. Given that the host must cope with many thousands of invasive organisms over the course of a lifetime, and given the epitopic complexity of each, it is reasonable to suppose that an orderly process must assure the "contraction" of responsive clones once they are no longer needed [Figure 15].

Apoptotic processes undoubtedly guide the destruction of lymphocytes that have outlived their usefulness. Studies of TNF, LT-α, and Fas have taught us that apoptosis is dependent upon the concerted action of a large collection of proteins, and of course, the genes that encode them. (Table III). These include the ligands that initiate apoptosis, the receptors that transduce the ligand effects, primary transducer molecules such as FADD/MORT-1 TRADD, and RIP, secondary transducers such as FLICE/MACH, the ICE family proteases that they activate, and the immediate targets of these enzymes (presumably, other proteolytic enzymes, PARP, and still other proteins yet to be identified). The components of the apoptotic mechanism are, in part, redundant. So much is evident from the mere observation that the

55 kD TNF receptor can be activated by LT-α as well as by TNF. Not only the TNF and Fas axes initiate apoptosis; the Apo-2 ligand can also do so, apparently acting through receptor(s) yet to be identified, as can the CD30 ligand via CD30. Yet global blockade of at least *one* pathway (that utilized by the Fas ligand) at the level of the ligand itself or the receptor is sufficient to bring about autoimmune disease by preventing the removal of lymphocytes that would otherwise undergo apoptosis.

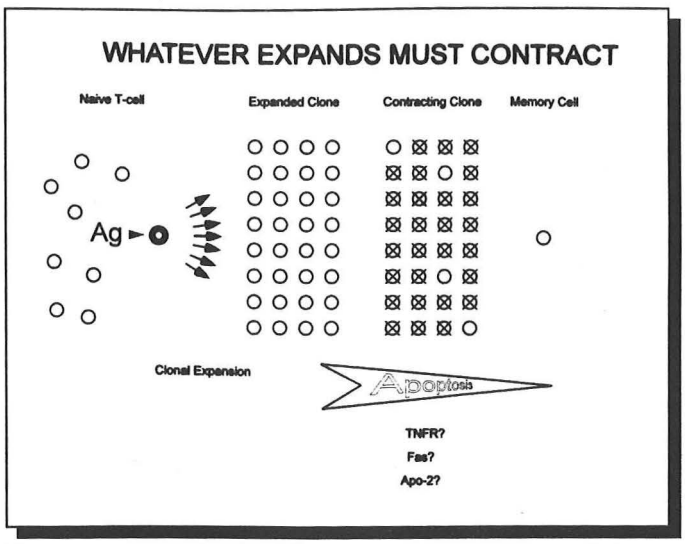


Figure 15. *Antigen-induced proliferation of lymphocytes is necessarily followed by elimination of reactive cells. This elimination is likely accomplished through an apoptotic mechanism, and may involve extracellular mediators such as TNF, FasL, or Apo2L.*

The Fas mutation, in humans as in mice, is a unique example of autoimmunity conferred by a germline mutation. Most autoimmune diseases do not follow a simple Mendelian pattern of inheritance. Although familiality is observed in rheumatoid arthritis, Crohn's disease, Type I diabetes mellitus, and several other autoimmune disorders, perfect concordance in the development of disease is not observed even among identical twins. Obviously, an environmental factor influences the emergence of each disease given a favorable genetic composition.

Might somatic cell mutation account for the development of autoimmune disease [Figure 16]? There is no question that it accounts for the development of neoplasia, and quite clearly, autoimmunity and neoplasia share the common attribute of deleterious clonal expansion. On the basis of the "two-hit" principle, one might imagine that a germline mutation affecting any component in the chain of proteins leading to apoptosis might predispose to the development of autoimmunity. The phenotype created by the second hit (i.e., the specific autoimmune disease produced by the second hit) would, in this model, depend upon the specificity of the lymphoid clone involved, and upon which component of the signaling apparatus is involved. If the germline mutation were in an ICE homologue, for example, different effects would be expected than those observed if the 55kD TNF receptor were destroyed by mutation.

TABLE III

Mutations that Might Lead to Autoimmunity Could Involve:

Ligands
TNF, LTα, FasL, Apo2L, CD30L

Receptors
55 kD TNFR, 75 kD TNFR, FasR, Apo2R, CD30

Primary Transducers
FADD/MORT-1, TRADD, RIP

Secondary Transducers
FLICE/MACH

Tertiary Transducers
ICE, CPP32β, others

The frequency of disease observed in families with weakly heritable forms of autoimmunity might partly depend upon the size of the progenitor pool susceptible to mutation. If the opportunity for a second hit is very small heritability would be minimized.

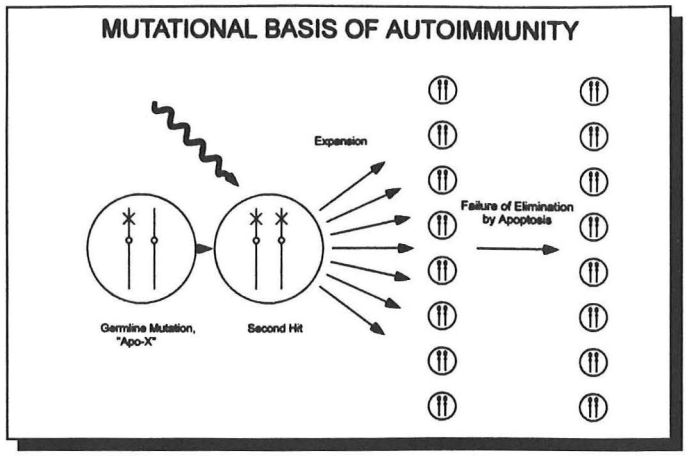


Figure 16. *Failure to remove reactive lymphocytes through apoptosis can lead to autoimmune disease, in which tissue targets are likely dependent upon specificity of the reactive clone(s). A germline mutation affecting a gene required for apoptosis (here "Apo-X") would surely predispose to autoimmunity, in that the loss of the normal allele in any cell would hamper the removal of its descendants after clonal expansion.*

The extraordinary clinical variability of some autoimmune diseases (i.e., SLE, scleroderma and other collagen vascular disorders, which at times seem to form a continuum of disease), might find explanation in the likelihood that all of these diseases involve defects of apoptosis, and in some instances, perhaps the same defect. Phenotypic diff erences might, as such, depend upon the antigenic specificity of lymphoid clone (or clones) that are vulnerable to a second hit.

Indeed, an individual instance of SLE with lymphoproliferation, manifested during adulthood and occurring in the context of heterozygosity for a Fas ligand gene mutation, has been reported recently (240). However, it might be allowed that, as in neoplastic disease, germline mutation is not an essential precondition. Rather than a germline defect, a somatic mutation occurring during embryogenesis or during maturation of the hematopoietic system might provide the necessary substrate upon which a second mutation might act to cause disease. Intensive study of the proteins that comprise apoptotic pathways, and analysis of their integrity in autoimmune disease, will likely guide us to a detailed understanding of autoimmunity within the coming decade.

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