MINIREVIEW

Regulation of Tumor Necrosis Factor Receptors on Phagocytes (43454A)

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Tumor necrosis factor (TNF)-α is a cytokine secreted by monocytes, macrophages, T cells, natural killer cells, mast cells, and neutrophils (PMN) in response to stimulation *in vitro* with lipopolysaccharide (LPS), viruses, antigens, and during infection, allograft rejection or injury (1–4). Named for its ability to induce hemorrhagic necrosis of tumors (1), TNFα is also implicated as an important mediator of inflammation, septic shock, and cachexia (2). TNFβ or lymphotoxin is a lymphocyte-derived homolog of TNFα that shows many of the same properties (2). TNFα exerts its cytotoxic and immunologic activities by interacting with specific cell surface receptors (TNF-R) that it shares with TNFβ. The receptors exist on a variety of normal and malignant cells, reflecting a wide spectrum of targets for these two cytokines.

Modulation of TNF-R expression is an important means of regulating TNFα responses. Most TNF-R regulation studies were carried out with transformed cell lines. TNF-R on these cells are reported to be up-regulated by Type I and Type II interferons (5–10), interleukin 2 (11), and activators of protein kinase A (12), or down-regulated by interleukin 1 (13), activators of protein kinase C (12–16), cycloheximide (17), and both up- or down-regulated by the homologous ligand, TNFα (17–19).

Few studies address the regulation of TNF-R in primary phagocytes, even though macrophages and neutrophils are important targets for TNFα during inflammation. TNFα induces macrophage activation by itself or in combination with γ-interferon (IFNγ). Macrophages respond to TNFα by demonstrating an increased ability to release toxic molecules, i.e., hydrogen peroxide (20–22) and nitric oxide (22), and to kill tumor cells (23, 24). TNFα is also a powerful PMN activator, promoting adherence to biological surfaces (25), stimulating phagocytosis (26, 27), respiratory burst activity (28), degranulation (27, 29), and antibody-dependent cytotoxicity (26).

Human PMN, monocytes, and murine macrophages possess 500–2000 plasma membrane TNFα binding sites per cell. In most studies, a single class of high affinity receptors with a K_d of 0.01~0.7 nM for TNFα derived from the same species, or 1.1~1.4 nM for TNFα from different species (30–35), has been identified. Some studies, however, have reported more complex equilibrium binding data and multiple affinity classes of TNF-R on both murine and human cells (30, 36–38). These discrepancies could be due to the homotrimer nature of TNFα (39) and its ability to crosslink one, two, or three receptors free to diffuse in the plane of the plasma membrane (40).

Two types of TNF-R, named Type I (or A) and Type II (or B), have been isolated in humans and their genes have been cloned (36, 37, 41–46). Analysis of the extracellular domains of these receptors has revealed a pattern of cysteine-rich repeats shared with the nerve growth factor receptor, the B cell activation antigen CD40, and Fas antigen (36, 37, 47), and has defined a new superfamily of receptor molecules. By contrast, the two TNF-R show no apparent similarity in their cytoplasmic portions, which suggests that they use different signaling mechanisms. Both Types I and II TNF-R bind TNFα and TNFβ with similar high affinities (36, 37) and seem biologically active (48–50). It is still unclear whether these two TNF-R mediate different functions. The Type I receptor is a ~55-kDa molecule that is expressed preferentially on cells of epithelial origin, whereas the ~75-kDa Type II receptor is more abun-

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dant on myeloid cells (48–52). Binding studies with monoclonal antibodies specific for each class of receptor showed that human PMN and monocytes express similar amounts of Types I and II TNF-R at their surface (51, 53).

Very recently, clones encoding the Types I and II forms of the murine TNF-R were isolated and shown to be highly homologous to their human counterparts (38, 54). RNA for both forms of receptors were found in all the tissues examined. Both receptors bind homologous murine TNFα with high affinity. There is one report that shows that only Type I binds human TNFα (54). The TNF-R type(s) present at the surface of murine macrophages has not been determined yet. The studies described below on murine macrophages were performed with human TNFα and thus may be relevant only to the Type I TNF-R.

Here, we will focus on the regulation of TNF-R on phagocytes. Recent studies indicate that several distinct mechanisms operate in different phagocytes, including shedding of both types of TNF-R from activated PMN (32), removal specifically of one of the receptors by inflammatory enzymes (53), alteration in the rate of TNF-R synthesis in human monocytes treated with protein kinase A activators (12), transmodulation of TNF-R on macrophages by protein kinase C activators and heterologous cytokines, blockade of the delivery of nascent TNF-R to the macrophage surface by microtubule depolymerizing agents (55), and accelerated internalization of TNF-R on macrophages in response to LPS (31) and taxol (56). Analysis of LPS- and taxol-mediated down-regulation of TNF-R on macrophages has pointed to a role for microtubules in LPS action in general.

Effect of PMN Activators

Incubation of human PMN with agents that can stimulate their migratory and secretory responses leads to a rapid and almost complete loss of TNF-R from the cell surface. These stimuli included both physiologic (poly) peptides (colony-stimulating factor for granulocytes and macrophages, *N*-formyl-methionyl-leucyl-phenylalanine [FMLP], platelet-activating factor, leukotriene B_4, and complement component fragment 5a) and pharmacologic agonists (phorbol myristate acetate [PMA] and calcium ionophore A23187) (32, 33). In contrast, γ-interferon, transforming growth factor-β1, and interleukin 1 have no effect.

The decrease in TNFα binding induced by agonists occurs rapidly at 37°C: Half-maximum loss of TNF-R ensues after only ~2 min with 10^{-7} *M* FMLP (Fig. 1) and requires only 10^{-9} *M* FMLP during a 30-min exposure. It occurs with no significant change in affinity and is accompanied by the appearance of soluble forms of both types of TNF-R in the media of stimulated PMN (32, 53). Ligand blots with radioiodinated TNFα

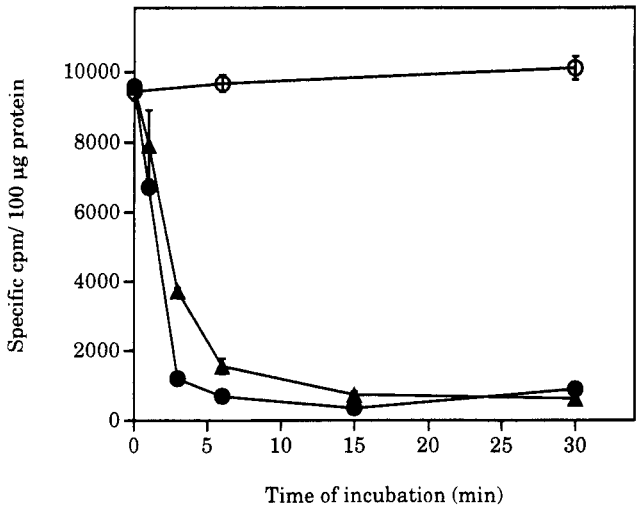

Figure 1. Time course of TNF-R down-regulation by PMN upon activation with FMLP. PMN were incubated at 37°C for the indicated time with either buffer alone (○), 10^{-7} *M* FMLP (●), or 10^{-8} *M* FMLP (▲), washed, and assayed for binding of ^{125}I-TNFα at 4°C. Reprinted from the *Journal of Experimental Medicine* (Ref. 32) with permission.

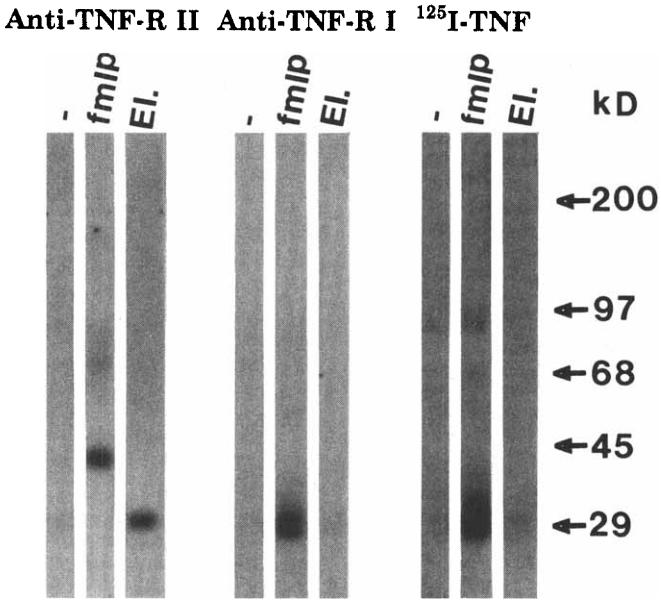

Figure 2. Characterization of TNF-R fragments released by PMN by Western blotting and ligand blotting. Cell-free supernatants from PMN treated for 15 min at 37°C with either buffer alone (–), 10^{-7} *M* FMLP, or 20 µg/ml of purified elastase (EL) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions, transferred to nitrocellulose, and blotted with either monoclonal antibodies against TNF-R Type I or Type II, or iodinated TNFα, as indicated. Supernatants from 10^7 cell equivalents were applied. Positions of prestained markers are indicated by arrows.

and Western blots with monoclonal antibodies against the Type I TNF-R detect a protein of ~28 kDa in cell-free supernatants from activated PMN (Fig. 2). On the other hand, the Type II TNF-R is shed as a 42-kDa soluble fragment that binds specific antibodies, but not TNFα, when transferred to nitrocellulose (Fig. 2). However, both soluble fragments from the two types of

TNF-R bind TNFα in a radioimmunoassay using iodinated TNFα.

Thus, activated PMN down-regulate surface TNFα binding by shedding TNF-R Types I and II from their surface. It is speculated that soluble TNF-binding proteins found in urine and sera (52, 57, 58) may consist of TNF-R shed from PMN, and probably from other cells. Preexposure of PMN to agonist has been shown to lead to a reduction of TNFα-induced cellular functions (33). Thus, shedding may serve to blunt the responsiveness of PMN to TNFα, and to protect the host against the deleterious effects of TNFα. At the same time, soluble TNF-R shed by PMN may reduce the effective concentration of TNFα able to act on other cells by generating a soluble TNFα sink.

The mechanisms controlling TNF-R shedding have not been elucidated. The integral membrane protein nature of both Type I and Type II TNF-R and the absence of transcripts encoding soluble forms of these receptors strongly suggest that shedding of TNF-R results from proteolysis of their extracellular domains. However, down-regulation of TNF-R induced by FMLP activation is unaffected by inhibitors of serine proteases (diisopropylfluorophosphate, aprotinin), thiol proteases (leupeptin), acid proteases (pepstatin), and metalloproteases (EDTA) (Ref. 32 and not shown).

Effect of Elastase

During the course of a study aimed at localizing the proteases involved in TNF-R shedding by activated PMN, PMN were fractionated on Percoll gradients and a TNF-R releasing activity comigrating with azurophil-granule-enriched fractions was found (53). This activity was identified as elastase. Concentrations of elastase similar to those found *in vivo* at chronic inflammatory sites (59, 60) were sufficient to induce TNF-R down-regulation on PMN. This suggests that regulation of TNF-R expression by elastase may occur under physiologic conditions when PMN degranulate or degenerate at sites of inflammation.

Elastase decreases TNFα binding to PMN within minutes of incubation at 37°C and at 4°C. It acts on the Type II TNF-R, from which it releases a soluble fragment of 32 kDa that keeps the ability to bind monoclonal antibodies in Western blotting (Fig. 2) and labeled TNFα in a radioimmunoassay (not shown). However, elastase has no effect on the Type I TNF-R (Fig. 2). Likewise, elastase decreases almost completely TNF binding to mononuclear cells which contain 80–85% lymphocytes that express predominantly the Type II TNF-R, whereas it has no effect on endothelial cells, which present almost exclusively the Type I TNF-R at their surface. On PMN, which express similar amounts of the two types of TNF-R, elastase has an intermediate effect (53). Thus, by all these criteria, the TNF-R releasing activity of elastase is clearly distinct from that operative in intact, agonist-activated PMN.

Thus, PMN appear to use at least two different proteolytic mechanisms for removing cell surface TNF-R and generating soluble TNFα inhibitors, which may contribute to the control of their own responsiveness, as well as the response of other cells, to TNFα: elastase, which cleaves only the Type II TNF-R of PMN and neighboring cells and may forestall specifically any responses mediated exclusively by this receptor locally at sites of inflammation; and a diisopropyl fluorophosphate-resistant enzyme, which acts on both Type I and Type II TNF-R and may impede TNFα action on PMN that first encounter an agonist in the circulation.

The ability of elastase to cleave the Type II TNF-R while leaving intact the Type I receptor suggests that the Type I receptor may be the major TNF-R species able to transduce cellular responses to TNFα at sites of inflammation. The presence of an intracellular pool of TNF-R Type I in the specific granules of PMN (F. Porteu, unpublished observations) tends to support this possibility. Specific granules have been shown to fuse with the plasma membrane upon adherence of PMN to biological surfaces (61). This suggests a complex regulation in which the loss of both types of TNF-R upon agonist activation is followed by reexpression of the Type I TNF-R resistant to elastase, as PMN move from the circulation to tissues. By this means, the response of PMN to TNFα will be precluded in the circulation, but allowed in local inflammatory sites.

Effect of IFNγ and Transforming Growth Factor-β

As a macrophage-activating factor, TNFα's action is modified by other cytokines. IFNγ synergistically enhances, whereas transforming growth factor-β (TGFβ) antagonizes, the ability of TNFα to augment the mouse macrophage respiratory burst and induction of nitric oxide release (22, 62–64). It is reasonable to ask whether these cytokines affect TNFα binding. Incubation of murine macrophages with 1 ng/ml of recombinant IFNγ at 37°C for 18 hr led to a 2- to 3-fold increase in labeled TNFα binding, whereas incubation with TGFβ led to a 40–50% inhibition (Fig. 3A). These effects were maximal after overnight incubation with IFNγ or TGFβ1. In contrast, short-term treatment, i.e., 1-hr incubation of either cytokine with macrophages, did not result in any alteration of TNFα binding (Fig. 3B; Ref. 31). Modulation of TNF-R by IFNγ and TGFβ1 parallels their effects on TNFα-mediated action on macrophages, and thus may be one of the mechanisms for their synergistic or antagonistic functions with TNFα. However, it is unlikely that 40–50% down-regulation of TNF-R by TGFβ1 could account for almost complete loss of respiratory burst capacity and nitric oxide release induced by TNFα.

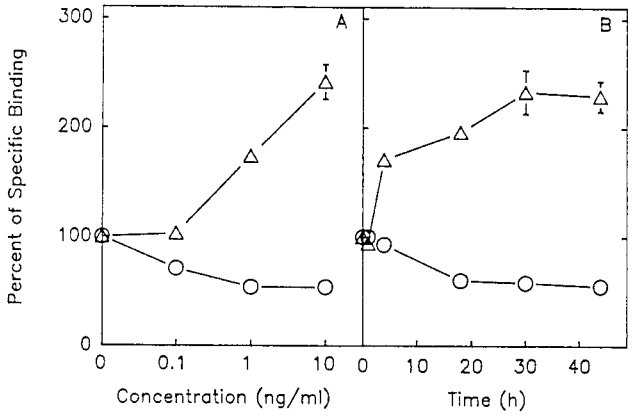

Figure 3. Modulation of TNF-R by IFNγ and TGFβ. Thioglycollate-elicited peritoneal macrophages from mice were incubated with (A) indicated concentrations of recombinant IFNγ (△) or TGFβ1 (○) for 18 h or with (B) 1 ng/ml of IFNγ (△) or TGFβ1 (○) for the time indicated at 37°C before assay for TNFα binding. The results are from one of two similar experiments, and are means for triplicates.

Effect of Activators for Protein Kinases

Up-regulation of TNF-R expression by protein kinase A activators and down-regulation of TNF-R expression by protein kinase C activators have been demonstrated in several cell lines (14–16). TNF-R on monocytes/macrophages and PMN appear to share these regulatory pathways. Incubation of peripheral blood monocytes (12) or murine macrophages (55) with 0.1–1 mM dibutyryl c-AMP, a cell-permeable analog of cAMP, increased the binding of labeled TNFα to the cells. Enhancement of TNF-R synthesis was found in these cells. Prostaglandin E_2, at 0.1 μM, also promotes this up-regulation of TNF-R (55). In contrast, PMA, an activator of protein kinase C, induces a rapid down-regulation of TNF-R in human PMN (32, 33). In mouse macrophages, incubation with 100 ng/ml of PMA for 1 hr at 37°C led to a 70% down-regulation of TNF-R. A simultaneous addition of 50–100 nM K252a, an inhibitor of protein kinases, partially blocked PMA-mediated down-regulation of TNFα binding (A. H. Ding, unpublished observation).

It is interesting to note that protein kinase A and protein kinase C are also involved in the regulation of TNFα production (65–67), but in a reciprocal manner, as for regulation of TNF-R. These two kinases might form a regulatory circuit to control autocrine actions of TNFα (12).

Effect of Lipopolysaccharide

Incubation of human or mouse macrophages with low concentrations of LPS at 37°C dramatically decreased the binding of iodinated TNFα to the cells, but had no effect on the binding of iodinated IFNγ (31). Mouse macrophage-like cell line RAW 264.7 cells shared this property. The response was extremely sensitive; a 50% decrease of TNFα binding to RAW cells could be achieved by exposure to 0.6 ng/ml of LPS for 1 hr, or to 10 ng/ml of LPS for 15 min (31). There was no modulation of the receptor at 4°C.

Although TNF-R disappeared within 45 min from the surface of intact macrophages after exposure to LPS, specific TNFα binding sites were unchanged in macrophages permeabilized with digitonin, which indicates that TNF-R were rapidly internalized (31), that is, macrophage TNF-R redistributed from the cell surface to an intracellular pool in response to LPS.

How might LPS promote this redistribution of TNF-R? Conditioned media from LPS-treated RAW 264.7 cells induced 30% down-regulation of TNF-R on macrophages from LPS-hyporesponsive mice (C3H/HeJ), which suggests that a soluble macrophage product may be responsible for a minor portion of the LPS effect. This may have been endogenous TNFα. However, several lines of evidence argued that endogenous TNFα cannot account for most of the down-regulation of TNF-R. About 50 ng/ml of exogenous TNFα would have been required to mimic the effect of 10 ng/ml of LPS (31). There is no evidence that macrophages can secrete such quantities of TNFα rapidly enough in response to trace levels of LPS to account for this phenomenon. Nor could antiserum against murine TNFα neutralize the effect of LPS on TNF-R. In addition, dexamethasone, which blocks TNFα production by macrophages at both transcriptional and translational levels (68), had no effect on LPS-induced down-regulation of TNF-R (56).

Down-regulation of TNF-R by LPS was also observed in human endothelial cells, but was absent in human melanoma cells. In human PMN, the effect of LPS on TNF-R expression is controversial (31–33). Recent experiments (F. Porteu, unpublished observation) indicate that LPS does not alter TNF-R expression on PMN unless autologous plasma is present in the incubation medium. The requirement for a serum protein for recognition of LPS by macrophages as well as PMN (69, 70) may explain the different effects of LPS on TNF binding to PMN.

As a result, LPS-treated macrophages lost their ability to degrade labeled TNFα, and became refractory to TNFα-mediated activation (55, 71). The fast kinetics of TNF-R down-regulation by LPS suggests that this effect might be a direct consequence of an early event in LPS action, which is still poorly understood. Some internalization of surface receptors generally requires the participation of the cytoskeleton, we asked whether agents disrupting microfilaments or microtubules had any effect of TNF-R.

Effect of Microtubule Depolymerizing Agents and Cycloheximide

Exposure of murine and human macrophages to micromolar concentrations of five microtubule-depolymerizing agents (colchicine, Nocodazole, podophyl-

lotoxin, vincristine, and vinblastine) resulted in a dose-dependent loss of binding sites for iodinated TNFα (55). The reduction amounted to 40–60% by 1 hr and ~75% by 2–4 hr. Inactive isomers of colchicine were ineffective, as were microfilament-destabilizing cytochalasins (55). Scatchard analysis suggested that TNF-R sites were decreased, without change in binding affinity. These five agents belong to four different structural classes. The only action they are known to share in common is to bind tubulin. Colchicine binds tubulin irreversibly, and its effect on TNF-R was irreversible. Nocodazole binds tubulin reversibly, and its effect on TNF-R was reversible. These findings suggested that microtubule disassembly was responsible for the observed down-regulation of TNF-R.

The effect of microtubule disrupting agents on TNF-R was functionally consequential. Treatment with these drugs inhibited the ability of macrophages to respond to TNFα with an enhanced respiratory burst capacity (55).

As in the case of a myosarcoma cell line (17), the protein synthesis inhibitor cycloheximide inhibited binding of TNFα to macrophages in the absence of added ligand (55). This suggested that TNF-R on macrophages turn over constantly. Thus, microtubule depolymerizing agents might interfere with the ability of the cell to maintain a steady state of surface TNF-R by decreasing the delivery of newly synthesized TNF-R to the plasma membrane, a process known to be closely associated with the microtubule network (72–74). Indeed, colchicine inhibits binding of TNFα to a similar extent and with a similar time course as cycloheximide. The loss of cell surface TNF-R induced by these drugs, however was neither fast enough nor complete enough to mimic the effect of LPS on TNF-R.

These studies suggest that the anti-inflammatory actions of microtubule-depolymerizing agents may result in part from their interference with the ability of macrophages to respond to TNFα.

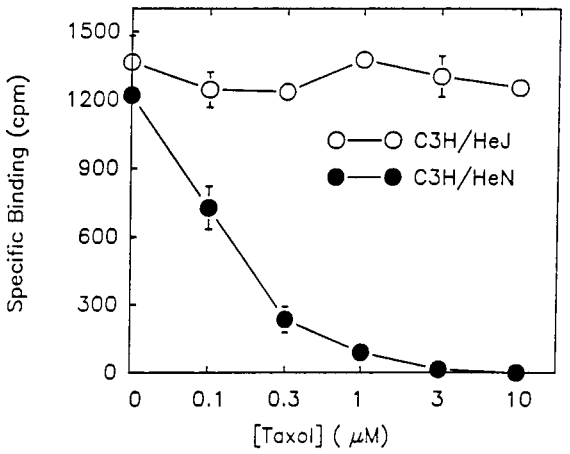

Figure 4. Differential responses to taxol by macrophages from C3H/HeJ and C3H/HeN mice. Peritoneal macrophages were incubated with indicated concentrations of taxol at 37°C for 1 hr, then assayed for TNFα binding. Reprinted from *Science* (Ref. 56) with permission.

Effect of Taxol

Taxol, an antitumor drug derived from the yew tree, binds and stabilizes polymerized microtubules (75, 76). Incubation of murine macrophages with 10 μ*M* taxol at 37°C inhibited the binding of labeled TNFα by 50% within 30 min, and by almost 100% within 1 hr. Down-regulation of TNF-R did not occur at 4°C, nor were IFNγ receptors affected by taxol. Taxol also induced the release of TNFα from macrophages, as judged by partial reversal of taxol-induced TNF-R down-regulation by neutralizing anti-TNFα antiserum, and by release of soluble factor(s), which competed with radiolabeled TNF binding in indicator cells (56). Dexamethasone, which abolished taxol-induced TNFα production, had no effect on the ability of taxol to eliminate TNFα binding. Thus, the effects of taxol and LPS on TNF-R are very similar.

Surprisingly, TNF-R expression by macrophages from LPS-hyposensitive C3H/HeJ strain mice was unaffected by taxol (Fig. 4). These mice have an abnormality in a gene on Chromosome 4 that controls all known responses to LPS (77). Studies on a panel of nine recombinant inbred strains produced between C3H/HeJ and C57BL/6J (an LPS-responsive strain) mice showed that down-regulation of TNF-R in response to LPS and taxol cosegregated in all the strains tested (56). Thus, the gene controlling responses to LPS and to taxol were closely linked, if not identical. A target shared by taxol and LPS may mediate many or most cellular responses to LPS.

There is new evidence that microtubules may interact directly with LPS, based on the co-elution of LPS and tubulin through gel filtration columns and cross-linking between LPS and tubulin by a bifunctional cross-linker (78). These studies provide a new clue to the possible participation of the cytoskeleton in responses of cells to LPS.

Conclusion

Thus, TNF-R on phagocytes are regulated by products of host cells (cytokines or enzymes), microbes (LPS and formyl peptides), and pharmacologic agents. The diverse mechanisms involved suggest it is important for the host to control its response to TNF via regulation of TNF-R. It is conceivable that *in vivo* these mechanisms may be cooperative. For example, desensitization of phagocytes to an elevated level of TNF induced by LPS during inflammation can be achieved by generation of a soluble TNF sink (from TNF-R shed by activated PMN), by internalization of TNF-R, and/or by cellular signals interfering with TNF-R synthesis or delivery to the cell surface. These responses may enable

phagocytes to respond to TNF to carry out their protective functions without damaging themselves.

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