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Multiple Signal Transduction Pathways Regulate TNF-Induced Actin Reorganization in Macrophages: Inhibition of Cdc42-Mediated Filopodium Formation by TNF

Maikel Peppelenbosch,1* Elke Boone,‡ Gareth E. Jones,¶ S.J.H. van Deventer,† Guy Haegeman,‡ Walter Fiers,‡ Johan Grooten,‡ and Anne J. Ridley*¶

TNF is known to regulate macrophage (Mφ) migration, but the signaling pathways mediating this response have not been established. Here we report that stimulation of the 55-kDa TNF receptor (TNFR-1) induced an overall decrease in filamentous actin (F-actin), inhibited CSF-1- and Cdc42-dependent filopodium formation, and stimulated macropinocytosis. Using a panel of TNFR-1 mutants, the regions of the receptor required for each of these responses were mapped. The decrease in F-actin required both the death domain and the membrane proximal part of the receptor, whereas inhibition of filopodium formation and increased pinocytosis were only dependent upon a functional death domain. When the TNF-induced decrease in F-actin was inhibited using either receptor mutants or the compound D609, TNF-stimulated actin reorganization at the cell cortex became apparent. This activity was dependent upon the FAN-binding region of TNFR-1. We conclude that different domains of TNFR-1 mediate distinct changes in the Mφ cytoskeleton, and that the ability of TNF to inhibit Mφ chemotaxis may be due to decreased filopodium formation downstream of Cdc42. The Journal of Immunology, 1999, 162: 837–845.

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2 Abbreviations used in this paper: Mφ, macrophage(s); TNFR, TNF receptor; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PAK, p21-activated kinase; TRITC, tetramethylrhodamine B isothiocyanate.

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known as the Rho family of small GTPases has been implicated in regulating cell migration. Members of this family, including Cdc42, Rac, and Rho, play a major role in remodeling the actin cytoskeleton in response to external stimuli (reviewed in Refs. 22 and 23). Cdc42 specifically induces the formation of filopodia in Swiss 3T3 cells (24, 25), whereas Rac and Rho regulate lamellipodium and stress fiber formation, respectively (26–27). Recently, we demonstrated that Rho family members have similar activities in Mφ: Cdc42 mediates filopodium formation, whereas Rac regulates lamellipodium production (28). As changes in cell locomotion are dependent upon controlled remodeling of the actin cytoskeleton, it is generally assumed that Rho family members have an important function in directing cell motility (29). Therefore, the TNF-induced inhibition of Mφ chemotaxis might well be brought about by interference with either the activation of Rho family proteins or downstream signal transduction to the actin cytoskeleton.

Little is known of the signaling pathways regulating TNF-induced actin reorganization in any cell type; in particular, the effects of TNF on the actin cytoskeleton of Mφ have remained unexplored. To obtain more insight into the molecular mechanisms by which TNF controls Mφ migration, we have investigated the effects of TNF on the Mφ actin cytoskeleton and on the actin reorganization induced by CSF-1 and Rho family proteins. We have subsequently used TNFR-1 mutants to determine which portions of the receptor are responsible for signaling to the actin cytoskeleton in Mφ.

Materials and Methods

Cell culture

P388D1 cells, 4-4 cells, and J774 cells were cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) with 10% FCS according to routine procedures. For microinjection and cell-stimulation experiments, cells were seeded on 13-mm coverslips, switched to RPMI 1640 without FCS after 24 h, and microinjected 24 h later. In cell-stimulation experiments, cells were washed with RPMI 1640 without FCS at 24 h after seeding and maintained without FCS for 48 h. Experiments were performed in the same medium. Bac1.2F5 cells were cultured and passaged as described previously (11). For experiments (unless otherwise indicated), cells were starved overnight in RPMI 1640 without serum. Experiments were performed in the same medium.

Construction of hTNF-R55 mutants

The cDNA of human TNF-R55 (generously provided by Drs. W. Lesslauer and H. Loetscher, Hoffmann-La Roche, Basel, Switzerland) was cloned into the eukaryotic expression vector pCDM8 (Invitrogen, San Diego, CA). All other mutants were also cloned into this vector. The deletion mutant Δ202–304 was created by cutting the cDNA of hTNF-R55 with EarI and Eco57I, blunting both sticky ends with Klenow polymerase and T4- exonuclease, respectively, and religating both fragments. This process generated a receptor mutant with the following cDNA sequence at the joint fragments: 5′-CTC TTC TTC ATG GCC ATC CCC AAC-ACT-3′; the nucleotides filled in with Klenow polymerase are shown in bold. The resultant protein consists of amino acids 203–303 of human TNF-R55 but still contains the FAN-binding domain (14). Mutagenesis of leucine 351 to alanine (L351A) was performed according to the instructions provided with the site-directed mutagenesis kit (Clontech, Palo Alto, CA). The following mutator oligo was used: 5′-GTT CCG TCG TCG CCC TTA GGC GCC GCA C-3′. The mutant receptor containing only the death domain (without the FAN-binding site) was created as follows: a cDNA fragment containing the death domain of human TNF-R55 preceded by a 15-aa linker (30) was ligated to the extracellular and transmembrane part of human TNF-R55 and again generated by cutting human TNF-R55 with EarI and treating the fragment with Klenow polymerase. All mutants were verified by sequencing.

Cell stimulation, immunocytochemistry, and microscopy

Experiments were performed at 37°C. Each experimental condition was tested on three different coverslips, and experiments were repeated several times (2–5). Where appropriate, cells were preincubated with 0.3 μM of SB203580 for 120 min, 4 μM of cell permeable ceramide for 30 min (New England Biolabs, Beverly, MA), or 100 ng/ml human recombinant TNF (Genzyme, Cambridge, MA) for 5 min, and subsequently stimulated with 100 ng/ml human recombinant CSF-1 (Chiron Corporation, Emeryville, CA) for 10 min (J774.2 cells), 15 min (Bac1.2F5 cells and 4-4 cells), or 20 min (P388D1 cells), or stimulated for 30 min with 1000 infectious units/ml murine TNF (4-4 cells; murine TNF was produced in Escherichia coli, purified to >99% homogeneity in our laboratory, and contained 4 ng of endotoxin/ng protein). For testing the action of mutated human TNFRs, 4–4 cells (106 cells in 1 ml of RPMI 1640 containing 5% FCS) were transfected with a total of 5 μg of cDNA by electroporation (300 V/950 μF/10 ms), and seeded on coverslips. After 24 h, cells were stimulated for 30 min with the htr-1 mAb (100 ng/ml; a gift of Dr. M. Brockhaus, Hoffmann-La Roche, Basel, Switzerland), which clusters and thus activates the transfected human TNFRs but not the endogenous murine receptors (31, 32). To investigate pinocytosis, fluorescently labeled BSA (prepared using Flu- orlink; Amersham, Arlington Heights, IL) was added to the culture medium in some experiments together with htr-1 Ab.

After stimulation, cells were fixed in 3.5% formaldehyde/PBS (v/v). For visualization of actin filament cells, were permeabilized in 0.1% Triton X-100/PBS (v/v), blocked in 0.5% BSA (Sigma, St. Louis, MO)/PBS (v/v) for 45 min, and stained with 80 ng/ml tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (Sigma) in PBS for 1 h. For immuno- nostaining, cells were blocked for 1 h in PBS containing 10% FCS (v/v), 5% milk powder (w/v), 0.5% BSA (w/v), and 0.1% Triton X-100 (v/v). Subsequently, cells were incubated in PBS containing 1% BSA (w/v) and 0.1% Triton X-100 (v/v) supplemented with a 2/2000 dilution of the htr-9 Ab (for the detection of transfected cells; the htr-9 Ab was also a kind gift of Dr. M. Brockhaus). Primary Ab binding was detected by incubation for 1 h with a 1/500 dilution of FITC-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS containing 1% BSA (w/v) and 0.1% Triton X-100 (v/v). Cell images were generated using confocal laser scanning microscopy as described previously (33, 34).

Microinjection

Protein expression, purification, and a determination of the active protein concentration were performed as described previously (26, 27). Microinjection were performed in RPMI 1640 without serum at 37°C. At least 30 cells were microinjected for every experimental condition. Where relevant, 100 ng/ml TNF or 5 mM of C2-ceramide was added to this medium. Cells were microinjected with 50 mM of Tris buffer (pH 7.3 at 37°C) containing 100 mM NaCl, 5 mM MgCl2, 150–700 μg of recombinant protein/ml, and 800 μg/ml rat IgG. After microinjection and, if appropriate, stimulation with 100 ng/ml CSF-1, cells were fixed in 3.5% formaldehyde/PBS (v/v) for ≥30 min. Subsequently, cells were blocked for 1 h in PBS containing 10% FCS (v/v), 5% milk powder (w/v), 0.5% BSA (w/v), and 0.1% Triton X-100 (v/v) and stained with 80 ng/ml TRITC phalloidin and a 2/2000 dilution of FITC-conjugated rabbit anti-rat IgG (Jackson Laboratories) in PBS containing 1% BSA (w/v) and 0.1% Triton X-100 (v/v). Microinjected cells were identified using the anti-mouse IgG signal, and actin filaments were analyzed as described above.

Uptake of [1H]sucrose

Where appropriate, cells were preincubated for 16 h with 100 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA). Pinocytosis was assessed in RPMI 1640 containing 0.5 μCi of [1H]sucrose (Amersham). After the indicated incubation times, cells were washed five times with ice-cold RPMI 1640 and lysed in 1% SDS (w/v). Subsequently, cellular sucrose uptake was determined by scintillation counting. Each datapoint represents the average of at least three independent determinations.

Results

TNF acts via TNFR-1 to induce a decrease in polymerized actin in mouse Mφ

The effects of TNF on actin organization in Mφ were investigated in four different mouse Mφ cell lines to ensure that responses were widely observed and not specific to only one cell line. To distinguish between the effects mediated by TNFR-1 and TNFR-2, responses to both human and mouse TNF were investigated, as human TNF can only stimulate TNFR-1 in mouse cells (13). Unstimulated, serum-starved, Bac1.2F5 Mφ were rounded, and displayed relatively high levels of polymerized actin within the cell interior (Fig. 1A). Serum-starved J774.2 cells were more spread and elongated compared with Bac1.2F5 cells (Fig. 1D). Unstimulated P388D1 cells exhibited an elongated phenotype with...
Membrane proximal region and death domain of TNF-1 are both essential for TNF-induced loss of F-actin

To gain insight into the signaling mechanisms mediating TNF-dependent actin reorganization, we first investigated the effects of D609. This compound inhibits a variety of death domain-dependent responses to TNF (35). Treating P388D1 cells or J774.2 cells for 1 h with 20 μg/ml D609 did not detectably alter the actin organization of these cells (data not shown). However, after subsequent stimulation with TNF, the TNF-induced decrease in F-actin was not observed; an accumulation of F-actin at the cell cortex was observed instead (Fig. 1, C, F, and I). These data suggest an involvement of the death domain-dependent phosphatidyl choline-specific phospholipase C (35) in the loss of F-actin in response to TNF and also indicate that TNF is able to induce actin reorganization at the cortex when the overall decrease in F-actin is prevented.

To investigate further the signaling mechanisms underlying TNF-induced actin reorganization, we transiently transfected mutants of human TNF-1 into mouse 4-4 Mφ. Subsequent clustering of these transfected receptors, using the Ab htr-1, initiated signal-through the transfected receptors without activating endogenous murine TNFRs (31, 32). After fixation, the actin cytoskeleton of transfected cells was compared with untransfected neighboring cells by double-staining the cells with phalloidin and an Ab specific for human TNFR-1. Activation of full-length human TNFR-1 in transfected cells led to a decrease in phalloidin staining compared with untransfected cells (Fig. 2, A and B), confirming that activation of TNFR-1 is sufficient to induce the decrease in F-actin. Subsequently, we tested the effect of a TNFR-1 containing the L351A mutation (homologous to the lpr mutation in the Fas Ag; 36), which renders the death domain inactive. Stimulation of this receptor actually induced an increase rather than a decrease in F-actin (Fig. 1, I and J); however, in contrast to the effect of D609, this increase was not limited to the cell cortex (possibly because the higher expression of transfected TNFR compared with the endogenous receptor). Surprisingly, activation of a TNFR lacking the membrane proximal part of the receptor but containing a functional death domain and a FAN-binding domain (deletion of amino acids 202–304) also resulted in increased levels of F-actin. A receptor mutant lacking both the membrane proximal part and FAN-binding site but containing a functional death domain (see Materials and Methods) did not induce any detectable actin reorganization (Fig. 2, E and F). Therefore, loss of polymerized actin in response to TNF-1 activation requires both the membrane proximal part of the receptor and a functional death domain, whereas the FAN-binding site appears to mediate a TNF-induced increase in F-actin.

TNFR-1 stimulates macropinocytosis through the death domain

Macropinocytosis can be stimulated in Mφ by a number of stimuli, including phorbol esters, and involves actin reorganization (37). TNF induced the accumulation of vesicles in Mφ (Fig. 1B), an effect that was sensitive to D609 (Fig. 1C). To test whether these vesicles were a result of a TNF-induced increase in macropinocytosis, we transfected 4-4 Mφ with human TNF-1 and compared the uptake of fluorescently labeled BSA by transfected cells with...
that of untransfected cells. After stimulation with the Ab htr-1, human TNFR-1-expressing cells showed a considerably higher uptake of fluorescent BSA compared with neighboring cells (Fig. 2, B and C). Subsequently, we performed experiments to establish the domains of TNFR-1 necessary for this effect of TNFR-1. A receptor mutant in which both the membrane proximal part and the

FIGURE 2. Effects of different TNFR-1 domain mutants on actin reorganization and pinocytosis. Full-length human TNFR-1 (A and E), human TNFR-1 with a functional death domain but without the membrane proximal region (B and F), human TNFR-1 with a functional death domain but without the membrane proximal region and the FAN-binding site (C and G), or full-length human TNFR-1 containing a truncated death domain (D and H) were transiently transfected into mouse 4-4 Mφ. Subsequent clustering of these transfected receptors was induced with the Ab htr-1. After fixation, transfected cells were identified by staining with an Ab specific for human TNFR-1. To investigate actin organization, cells were costained with TRITC-labeled phalloidin (E–H), whereas pinocytosis was assessed by uptake of fluorescently labeled BSA (A–D). Transfected cells are indicated with arrows. The bar represents 40 μm.
FAN-binding site were deleted was still able to mediate an increased uptake of fluorescent BSA (Fig. 2, G and H), whereas TNFR-1 containing the L351N mutation (and thus having an inactive death domain) was unable to induce an increased BSA uptake (Fig. 2, K and L). We conclude that increased macropinocytosis in response to TNF is solely mediated by the death domain of the receptor.

Phorbol esters regulate TNF-induced macropinocytosis

To quantify the extent of TNF-induced macropinocytosis, the pinocytotic activity in J774.2 Mφ was assayed directly by measuring [3H]sucrose uptake. Incubating cells with 100 ng/ml mouse TNF caused an increase in the rate of fluid uptake, and this effect persisted for ≥1 h (Fig. 3A). Subsequent dose-response experiments established that an effect of TNF on fluid uptake was already observed at TNF concentrations in excess of 10 ng/ml, whereas the response was maximal at 50 ng/ml (Fig. 3B). The inhibition of TNF-induced macropinocytosis by D609 suggested that stimulation of phosphatidylycholine-specific phospholipase C could be involved in this response (35). As phospholipase C activation leads to the production of diacylglycerol and to the activation of several protein kinase C (PKC) enzymes (38) and as PKCs are known to regulate macropinocytosis (37), the effect of the PKC activator TPA on macropinocytosis was investigated. The addition of 100 ng/ml TPA potently stimulated fluid uptake in J774.2 Mφ (Fig. 4A). To investigate whether PKC is involved in the TNF response, cells were treated with TPA for 16 h to down-regulate phorbol ester-responsive PKC isoforms. A subsequent application of TPA was no longer able to stimulate macropinocytosis (Fig. 4A). Interestingly, TNF-induced macropinocytosis was also inhibited after TPA pretreatment (Fig. 4B). Taken together, these results strongly suggest that PKC or another phorbol ester-binding protein mediates TNF-stimulated macropinocytosis.

CSF-1-induced filopodium formation is inhibited by TNF

TNF can inhibit phagocyte chemotaxis (39), and we have specifically shown that TNF inhibits the chemotaxis of Bac1.2F5 cells toward CSF-1 but has no effect on CSF-1-induced chemokinesis (11). To investigate the mechanisms underlying the ability of TNF to inhibit chemotaxis, we decided to characterize the effects of TNF on CSF-1-induced actin remodeling. CSF-1 induces rapid actin reorganization in Bac1.2F5 cells: it stimulates the formation of filopodia, lamellipodia, and membrane ruffles (28). A similar response to CSF-1 is observed in J774.2, P388D1, and 4.4 Mφ (Fig. 5, A, E, and I). In TNF-treated Mφ, CSF-1 was still able to induce substantial reorganization of the actin cytoskeleton; however, filopodium formation was almost completely inhibited. In contrast, CSF-1-induced lamellipodium production and ruffling were enhanced by TNF pretreatment in Bac1.2F5 (Fig. 5C), J774.2 (Fig. 5G), and P388D1 cells (Fig. 5K). Similar effects were observed when TNF and CSF-1 were added at the same time (data not shown). We conclude that TNF specifically inhibits CSF-1-induced filopodium formation, whereas lamellipodium formation and ruffling are not sensitive to TNF.

TNF inhibits filopodium formation downstream of Cdc42

As Cdc42 mediates CSF-1-induced filopodium extension in Mφ (28), the question arises as to whether TNF inhibits Cdc42 activation or whether it interferes with the signaling pathway leading from activated Cdc42 to the cytoskeleton. To distinguish between...
these two possibilities, J774.2 Mϕ were microinjected with Cdc42 V12 (a constitutively active mutant of Cdc42) in the presence or absence of TNF. As expected, Cdc42 V12 induced a large increase in filopodium formation (Fig. 6A), but this response was completely inhibited by TNF (Fig. 6B). In contrast, TNF did not influence the actin reorganization induced by a microinjection of Rac V12 and Rho V14. Therefore, TNF specifically interferes with the signal transduction pathway leading from activated Cdc42 to filopodium production.

**Discussion**

TNF stimulates a number of responses in Mϕ, but its effects on the Mϕ actin cytoskeleton have not been studied in detail. We report here that TNF induces a decrease in polymerized actin and an increase in macropinocytosis and inhibits Cdc42-mediated filopodium extension. In addition, when the TNF-induced decrease in F-actin is inhibited using D609 or receptor mutants, a distinct type of actin reorganization involving an increase in F-actin is observed in response to TNF. For all of these effects of TNF, activation of TNFR-1 was sufficient, as specific activation of transfected TNFR-1 by the htr-1 Ab induced all of the responses observed with TNF. Similarly, these responses to TNF could be elicited by adding human TNF to mouse Mϕ, which will result in the activation of only mouse TNFR-1 and not mouse TNFR-2 (13). Consequently, TNFR-2 does not appear to be required for TNF-induced cytoskeletal reorganization in mouse Mϕ.

Transfection of specific TNFR-1 mutants allowed us to pinpoint the elements in the receptor mediating the different effects of TNF and Cdc42-dependent filopodium production (Fig. 6C). We conclude that the inhibitory effect of TNF on filopodium production is mediated at least in part by p38 MAPK.
on the actin cytoskeleton (Fig. 7). We observed that for the TNF-induced decrease in F-actin, both the membrane proximal region of TNFR-1 and the death domain were essential. The requirement for these two domains is unusual and has not been observed for most other TNF-induced responses, for which either the death domain or the FAN-binding site is sufficient; interestingly, TNF-induced induction of nitric oxide synthase also requires these two domains of TNFR-1 (21). In addition, L929 cells transfected with a human TNFR-1 lacking the membrane proximal region display markedly delayed cell death in response to the htr-1 Ab as compared with the full-length receptor, although NF-κB activation was not affected (43). Apparently, the membrane proximal part of the receptor exerts important cooperative functions with the death domain of the receptor, and may be more important in TNF signaling than previously thought.

Both the membrane proximal region and the death domain activate signaling proteins that could participate in the TNF-dependent decrease in F-actin. Recently, it has been reported that the membrane proximal part of the receptor binds phosphatidylinositol-4-phosphate 5-kinase, and that this enzyme is activated upon receptor stimulation (20). As phosphatidylinositols regulate the activity of many actin-organizing proteins, this enzyme may participate in the actin reorganization induced by TNF, although previous results imply that it mediates an increase, rather than decrease, in F-actin (44). The death domain, in turn, stimulates a caspase-mediated proteolytic cleavage of p21-activated kinase 2 (PAK2), resulting in a constitutively active PAK2 fragment; PAK1 is not affected (45). PAKs interact with Rac and Cdc42 and reportedly play a role in regulating actin organization in several cell types (46). Therefore, it will be interesting to investigate the action of phosphatidylinositol-4-phosphate 5-kinase and PAK2 on the Mφ actin cytoskeleton, to determine their role in TNF-induced cytoskeletal remodeling, and to assess possible interplay between the effects of these proteins.

When the TNF-induced decrease in F-actin was inhibited by the compound D609 or by activating TNFR mutants lacking either the membrane proximal region or the death domain, a TNF-induced increase in F-actin was observed. This finding suggests that the signal inducing the decrease in F-actin is sufficiently strong enough to override other signals from TNFR-1 to the actin cytoskeleton, but that when this signal is reduced, less dominant signals can induce a distinct type of actin reorganization. One possibility is that TNF activates a protein that enhances actin filament turnover, such as ADF/cofilin (47), which then rapidly depolymerizes any actin-containing structures produced in response to other TNF-activated signals. According to this model, these other structures would be more stable when the rate of actin depolymerization is decreased. The TNF-induced increase in F-actin was mechanistically distinct from the decrease in F-actin, as it was dependent upon the FAN-binding site of the receptor. The signaling pathways downstream of FAN are still unclear but include activation of neutral sphingomyelinase activity, generating ceramide (14). However, an application of cell-permeable ceramides did not induce an increase in F-actin (Fig. 6), indicating that ceramide production is not sufficient for this response. The signaling mechanisms by which the FAN-binding site mediates actin reorganization in Mφ may well involve Rho family proteins; therefore, we plan to investigate this possibility.

We observed that TNF was a potent stimulator of macropinocytosis. For this effect, a functional death domain was sufficient, and D609 also inhibited the macropinocytosis induced by TNF. The molecular target for D609 is apparently a TNF-activated phosphatidylinositol-specific phospholipase C, which leads to PKC activation through diacylglycerol production (35). Phorbol esters, which mimic the action of diacylglycerol and activate PKC, potently stimulate macropinocytosis in Mφ (37); down-regulation of PKC by prolonged treatment with phorol esters impaired TNF-induced pinocytosis. These results suggest that a phorbol ester-sensitive isofrom of PKC is required for TNF-induced macropinocytosis. However, D609 also inhibited phorbol ester-dependent pinocytosis in Mφ (M.P.P., unpublished observations), suggesting that this compound acts downstream rather than upstream of diacylglycerol release to inhibit pinocytosis and consequently has multiple cellular targets. It is also possible that another phorbol ester-binding protein apart from PKC is involved in the macropinocytic response (for example, a member of the chimerin protein family that acts as a GTPase activating protein for Rac and Cdc42) (48). Indeed, expression of activated Rac has been shown to stimulate macropinocytosis in fibroblasts (27).

Macropinocytosis can act as a nonspecific mechanism for Ag capture, leading to Ag presentation and T cell activation (37). TNF is a potent inducer of Ag presentation in Mφ (6), and TNF-stimulated macropinocytosis may therefore be important for this response; current experiments address this possibility. Alternatively, TNF-induced macropinocytosis may serve to translocate activated receptors into intracellular compartments, as it has been shown that a number of TNF effects require TNFR

### Table 1. Effects of PD098059 and SB203580 on TNF-induced [3H]sucrose uptake

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>cpm (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>368 ± 41</td>
</tr>
<tr>
<td>TNF</td>
<td>694 ± 103</td>
</tr>
<tr>
<td>TNF + 0.3 μM SB203580</td>
<td>598 ± 48</td>
</tr>
<tr>
<td>TNF + 1 μM PD098059</td>
<td>755 ± 152</td>
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* Cells were either left unstimulated or stimulated with TNF in the presence or absence of PD098059 and SB203580 as described in Materials and Methods. Pinocytosis was determined by [3H]sucrose uptake.

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**FIGURE 7.** Schematic depiction of the different domains in TNFR-1, the roles of each domain in mediating actin reorganization in Mφ, and the interaction of these domains with CSF-1-induced signaling.
internalization and the subsequent acidification of the endosomal compartment (35), although this scheme makes the effects of membrane-bound TNF, which is not internalized, difficult to explain. Strikingly, the TNF responses that require endosomal acidification are identical with those that are inhibited by D609. Therefore, we speculate that D609 may interfere with TNF signaling, at least in part, by inhibiting TNF-induced macropinocytosis and thus preventing TNFR translocation to endosomes.

TNF inhibits chemotaxis of phagocytes without affecting chemokinesis (6–11, 39). To investigate the cytoskeletal basis of this inhibition, we tested the effects of TNF on CSF-1-induced actin remodeling. We recently demonstrated that CSF-1 induces Cdc42-mediated filopodium formation and Rac-mediated lamellipodium formation in Mφ (28). In this study, we observed that TNF inhibited Cdc42- and CSF-1-induced filopodium production but did not impair Rac- or CSF-induced lamellipodium extension. Filopodia have been suggested to play a pivotal role in chemotaxis as sensors for the chemotactic gradient; thus, the TNF-induced inhibition of Cdc42-mediated filopodium extension may explain the inhibitory effect of TNF on Mφ chemotaxis. The signal leading to an overall decrease in F-actin in response to TNF may contribute to the inability of cells to produce filopodia, although it clearly does not prevent membrane ruffling.

The inhibitory effect of TNF on filopodium extension mapped to the death domain of TNFR-1. Activation of p38 MAPK by TNF is similarly dependent upon this domain (40), and in the presence of the p38 MAPK inhibitor SB203580, TNF no longer blocks filopodium formation, suggesting that p38 MAPK mediates the inhibition of filopodium formation. In agreement with this, we observed that cell-impermeable ceramides, which stimulate p38 MAPK, impaired Cdc42-induced filopodium formation. Interestingly, both actinomycin D and UV light strongly activate p38 MAPK, and thus preventing TNFR translocation to endosomes, and have been reported to inhibit leukocyte chemotaxis (49, 50). This effect may also be due to p38 MAPK-mediated inhibition of filopodium formation. As the death domain of TNFR-1 mediates both macropinocytosis and an inhibition of filopodium formation, it is possible that macropinocytosis is causally associated with the lack of filopodia. However, SB203580 did not block macropinocytosis, and therefore these two responses mediated by the death domain are separable. Presumably, another target(s) of the death domain of TNFR-1. Activation of p38 MAPK by TNF is prevent membrane ruffling.

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TNF is one of the most pleiotropic cytokines known, and its effects on cell migration and locomotion vary considerably between different cell types; for instance, TNF inhibits Mφ chemotaxis but acts as a chemoattractant for fibroblasts (51). Our results show that distinct signals generated by different regions of TNFR-1 have specific effects on actin organization in Mφ (Fig. 6). The membrane proximal region of the receptor together with the death domain is required for the decrease in F-actin, whereas the FAN-binding site is required for distinct cellular responses.

References


