Function of the p55 Tumor Necrosis Factor Receptor “Death Domain” Mediated by Phosphatidylcholine-specific Phospholipase C

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Summary

Tumor necrosis factor (TNF) is a pleiotropic mediator of inflammation that has been implicated in the pathogenesis of devastating clinical syndromes including septic shock. We have investigated the role of a TNF-responsive phosphatidylcholine-specific phospholipase C (PC-PLC) for the cytotoxic and proinflammatory activity of TNF. We show here that the cytotoxicity signaled for by the so-called “death domain” of the p55 TNF receptor is associated with the activation of PC-PLC. The xanthogenate tricyclodecan-9-yl (D609), a specific and selective inhibitor of PC-PLC, blocked the cytotoxic action of TNF on L929 and Wehi164 cells. In vivo, D609 prevented both adhesion molecule expression in the pulmonary vasculature and the accompanying leukocyte infiltration in TNF-treated mice. More strikingly, D609 protects BALB/c mice from lethal shock induced either by TNF, lipopolysaccharide, or staphylococcal enterotoxin B. Together these findings imply PC-PLC as an important mediator of the pathogenic action of TNF, suggesting that PC-PLC may serve as a novel target for anti-inflammatory TNF antagonists.

TNF, originally defined by its antitumoral activity, is now recognized as a potent polypeptide mediator of inflammation and cellular immune response, that has been implicated in the pathogenesis of several clinical syndromes (1). Furthermore, TNF has been demonstrated to induce cell death in several tumor cell lines (2). TNF elicits its extremely wide variety of biological effects through two distinct cell surface receptors of 55- (TNF-R55) and 75-kD (TNF-R75) apparent molecular mass, respectively, that transmit signals to the cytoplasm and the nucleus (3). Both proinflammatory and cytotoxic activities have been mainly ascribed to signaling events coupled to TNF-R55 (3-7). Recently, it was shown that binding of TNF to TNF-R55 mediates the activation of at least two independent signaling pathways, one initiated by a neutral sphingomyelinase (N-SMase) which results in subsequent activation of a ceramide-activated protein kinase, Raf-1 kinase, and eventually phospholipase A2 (PLA2) (8-10). The other pathway involves activation of phosphatidylcholine-specific phospholipase C (PC-PLC), which results in breakdown of PC in phosphorylcholine and the lipid messenger molecule diacylglycerol (DAG). TNF-induced DAG generation through PC-PLC appears instrumental in the regulation of protein kinase C (PKC) and an acid sphingomyelinase (A-SMase) (11, 12). Notably, the activation of PC-PLC has been also implicated in the signaling of other proinflammatory cytokines such as IL-1 and IFN-γ (for a review see reference 13). Through the generation of ceramide, A-SMase provides an important cofactor for the activation of the transcription factor nuclear factor (NF)-κB (12). NF-κB in turn seems to play an important role in the control of expression of several inflammatory proteins, such as IL-1 and IL-6 (14). However, despite recent progress in understanding signaling pathways of TNF-R55, the molecular mechanisms of TNF-mediated cytotoxicity and proinflammatory activity remain elusive. PLA2 has been mostly incriminated to mediate TNF cytotoxicity (15, 16), however, the mechanisms of PLA2-induced cytotoxicity remained obscure. Strikingly, TNF cytotoxicity can be shown in the absence of PLA2 activity (2). Thus, pathways other than PLA2 must...
exist to explain the cytotoxic action of TNF. A recently identified cytoplasmic region of the TNF-R55, designated "death domain," seems to play a major role in TNF cytotoxicity (5, 17). We have previously shown by structure-function analysis of TNF-R55 that activation of PC-PLC rather than PLA2 is triggered by the death domain (8), which prompted us to investigate the possible involvement of PC-PLC in TNF-mediated cytotoxicity and proinflammatory activity of TNF.

In the present study we employed the tricyclodecan-9-ylxanthogenate (D609), which has been identified as a potent and specific inhibitor of PC-PLC (12, 18, 19). In particular, D609 has no inhibitory effects on TNF-mediated activation of PLA2, neutral SMase, or proline-directed protein kinase (8, 12). Furthermore, it has been reported that D609 does not inhibit PKC, phosphoinositol-specific phospholipase Cγ (PI-PLCγ), tyrosine kinases, or phosphatases (12, 20). Here we show that inhibition of PC-PLC by D609 blocks the cytotoxic and proinflammatory action of TNF both in vitro and in vivo. Furthermore, we show that D609 treatment protects mice from lethal shock induced either by TNF, LPS, or staphylococcus enterotoxin B (SEB). These results provide evidence for an important role of PC-PLC
in TNF-mediated cytotoxicity and the pathogenesis of inflammatory disease.

Materials and Methods

Cell Culture and Reagents. The mouse pre-B cell line 70Z/3, and the mouse fibrosarcoma cell lines L929 and Wehi164 were originally obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were maintained in a mixture of Click's (50% vol/vol) and RPMI (50% vol/vol) supplemented with 10% fetal calf serum, 10 mM glutamine, and 50 μg/ml each of streptomycin and penicillin in a humidified incubator containing 5% CO₂.

Highly purified recombinant human TNFα (hTNF, 3 × 10⁷ U/mg protein) and murine TNFα (mTNF, 1.2 × 10⁷) were kindly provided by Dr. G. Adolf (Boehringer Research Institute, Vienna, Austria). D609 was provided by Drs. Sauer and Amtmann (German Cancer Center, Heidelberg, Germany) or purchased from Biomol (Hamburg, Germany).

The exotoxin SEB, the endotoxin LPS, and actinomycin D were purchased from Sigma (Munich, Germany). D-galactosamine (GalN) was obtained from Roth (Karlsruhe, Germany).

Mice. BALB/c and C57BL/6 mice were purchased from the Zentralinstitut für Versuchstierzucht (Hannover, Germany).

Expression of TNF-R55 Deletion Mutants in 70Z/3 Cells. The generation of stable 70Z/3 transfectants expressing either the human wild-type (WT) TNF-R55 or the deletion mutant TNF-R55A345 has been described elsewhere (4, 8). The 70Z/3 transfectants displayed 1,060 TNF-R55/cell and 1,260 TNF-RΔ345/cell with affinities of 2.2 × 10⁻¹⁰ M and 4.5 × 10⁻¹⁰ M, respectively (4, 8).

Assays for PC-PLC and PLA₂. To measure PLA₂ activity, cells were labeled for 48 h with [N-methyl-³¹C]choline (Amersham, Braunschweig, Germany; 1 μCi/ml, specific activity, 56.4 mCi/nmol). One million cells in duplicates were left untreated or

![Figure 3](image)

**Figure 3.** Ceramide-induced death of Wehi164 cells is not affected by D609 treatment. Wehi164 cells were left untreated (black bar) or pretreated for 1 h with 50 μg/ml (grey bar) or 25 μg/ml D609 (white bar) in the presence of 1 μg/ml actinomycin D. Cells were treated in triplicates with C₆-ceramide for 8 h. The values represent the mean (±SD) of three independent experiments.

![Figure 4](image)

**Figure 4.** D609 selectively inhibits TNF-induced PC-PLC activation in Wehi164 cells. (A) D609 inhibits TNF-induced PC-PLC activation. Cells were left untreated (●) or preincubated with 100 μg/ml D609 for 60 min (○). Cells were stimulated with 100 ng/ml mTNF, and PC-PLC activity was estimated from 1,2-DAG production. Results are expressed as percent increase over untreated controls. Basal DAG production of untreated cells corresponded to 61.5 pmol of DAG per 10⁶ cells. The values represent the mean (±SD) of three independent experiments. (B) D609 inhibits TNF-induced activation of PC-PLC. Cells were left untreated (solid bar) or pretreated for 60 min with various concentrations of D609 (hatched bars) followed by stimulation with 100 ng/ml mTNF for 2 min. Results (±SD) of three independent experiments are expressed as percent increase of TNF-induced DAG production compared with untreated controls. (C) TNF-induced PLA₂ activation is not inhibited by D609. Cells were left untreated (●) or preincubated with 100 μg/ml D609 for 60 min (○), followed by stimulation with 100 ng/ml mTNF for indicated times. Results (±SD) of three independent experiments are expressed as percent increase of TNF-induced lysophosphatidylcholine production compared with untreated controls.
treated with hTNF for 15 min and the release of [3H]lysophosphorycholine was analyzed by combined TLC and two-dimensional laser densitometry as described (11). For PC-PLC assays, serum-starved cells were labeled with 1-lyso-3-PC-1-[^3H]palmitoyl (Amersham). Cells were left untreated or treated with either hTNF or mTNF for 2 min. TNF-induced changes of DAG levels were estimated by TLC analysis of neutral lipids followed by two-dimensional scanning as recently described (11).

Cytotoxicity Assay. Cytotoxicity assays with 70Z/3 or 70Z/3 WT TNF-R55 cells or 70Z/3 TNF-R55A345 cells were performed in quadruplicates in microtiter plates at a density of 2 × 10⁴ cells/well. Cells were left untreated or treated for 18 h with graded concentrations of hTNF or mTNF. MTT (1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added and after solubilization of MTT-formazan (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan) crystals with HCl-isopropanol, the OD was determined at 570 nm. The percent cytotoxicity was calculated from the formula \( 100 \times \frac{(a-b)}{a} \) where \( a \) and \( b \) represent the OD₅₇₀ values of untreated and treated samples, respectively.

Cytotoxicity assays with L929 or Wehi 164 cells were performed as previously described (18, 19). Briefly, murine fibrosarcoma L929 or Wehi 164 cells at a density of 5 × 10⁴ or 2 × 10⁴ cells/well, respectively, were left untreated or pretreated for 1 h with D609 in the presence of 1 μg/ml actinomycin D. Cells were treated in quadruplicates with mTNF for 18 h and cytotoxicity was determined by crystal violet staining or specific ⁵¹Cr-release as described (21, 22).

Immunohistochemistry. For determination of vascular cell adhesion molecule 1 VCAM-1 expression, cryostat sections from frozen lung tissue were prepared and analyzed by an indirect immunoperoxidase technique (23). Frozen sections were incubated with mAb specific for either murine VCAM-1 (M/K-2.7, ATCC) or the murine Mac-1 antigen expressed on monocytes, granulocytes, and NK cells (M1/70, ATCC). The sections were developed using a peroxidase-coupled affinity-purified rabbit anti-rat Ig (Dianova, Hamburg, Germany) and counterstained with hematoxylin.

Results and Discussion

TNF-R55 Death Domain Signals Activation of PC-PLC. TNF has been shown to trigger cell death through a COOH-terminally located domain of TNF-R55, called “death domain,” which is also responsible for the activation of the signal transduction enzyme PC-PLC (5, 8, 17). To demonstrate both cytotoxicity and PC-PLC activation in the same cell type, WT and a TNF-R55 deletion mutant lacking the death domain were expressed in 70/Z3 cells. As shown in Fig. 1, parental murine pre-B cells 70Z/3 do not respond to hTNF, as these cells do not express TNF-R55 (4). Notably, triggering of TNF-R75 by mTNF neither led to PC-PLC activation nor produced cytotoxic effects on parental 70Z/3 cells (Fig. 1, C and D). In contrast, 70Z/3 transfectants expressing the WT human TNF-R55 receptor responded to hTNF with activation of both PC-PLC and PL₂, and also resulted in cell death. The TNF-R55A345 deletion mutant truncated COOH-terminally by 81 amino acids to destroy the death domain proved defective in triggering cell death (Fig. 1 D), as has been described for other cell types (5, 17). The TNF-R55A345 deletion mutant fully retained its ability to signal PL₂ activation, but failed to stimulate PC-PLC (Fig. 1, B and C). These findings support the idea that PC-PLC rather than PL₂ mediates the cytotoxic activity of the death domain of TNF-R55. Along these lines, overexpression of TRADD, a recently identified TNF-R55-associated protein that contains a death domain itself and that is able to confer cytotoxicity (24), results in enhanced PC-PLC responses in TNF-treated cells (our own unpublished observations).

Inhibition of PC-PLC Blocks TNF Cytotoxicity. To confirm a role of PC-PLC in TNF cytotoxicity, D609 (18) was employed. Two different TNF-sensitive murine cell lines, L929 and Wehi164, were preincubated with increasing concentrations of mTNF in the presence of 1 μg/ml actinomycin D, to sensitize these cells for TNF action (21, 22). As shown in Fig. 2, D609 treatment of Wehi164 and L929 cells resulted in dose-dependent inhibition of TNF-mediated cytotoxicity. A close to complete blockade of TNF cytotoxicity was achieved at 25 μg/ml D609. mTNF killed Wehi164 cells within 24 h even in the absence of actinomycin D, which was also inhibitable by D609 (Fig. 2 B). The inhibitory effect of D609 on TNF-mediated cytotoxicity was also observed with TNF-R55-transfected 70Z/3 cells treated with hTNF (data not shown). We have previously shown that TNF-induced activation of PC-PLC couples to the production of the second messenger ceramide by activation of A-SMase (12). Ceramide in turn has been implicated as an important mediator of cell death (25). In contrast to TNF, C6-ceramide-induced cell death of Wehi164 cells is not sensitive to D609 (Fig. 3), which rules out nonspecific protective effects of D609. Furthermore, this experiment confirms that D609 operates upstream of TNF-induced ceramide generation.

As shown in Fig. 4, TNF induces transient activation of both PC-PLC and PL₂ in Wehi164 cells. TNF-induced PC-PLC activation in Wehi164 cells could be effectively prevented by D609 at concentrations between 12.5 and 50 μg/ml (Fig. 4, A and B), whereas TNF-stimulated activation of PL₂ remained completely unaffected by D609 (Fig. 4 C). These results confirm that PC-PLC rather than PL₂ plays an essential role in TNF-mediated cytotoxicity.

D609 Blocks Proinflammatory Effects of TNF In Vivo. It is important to emphasize that the cytotoxic effects of TNF on long-term cultured cell lines are not tantamount to the pathophysiological effects of TNF in vivo. The activation of PC-
PLC results in the generation of DAG, which triggers the activation of at least two enzymes, PKC and A-SMase (11, 12, 26), and eventually the activation of the transcription factor NF-κB (12). NF-κB in turn has been reported to control the expression of several genes encoding cytokines, cytokine receptors, adhesion molecules, and acute phase proteins, and thus is viewed as a key mediator of proinflammatory actions (14). This prompted us to investigate whether D609 might be effective in blocking the pathophysiological effects of TNF in inflammatory disease and septic shock. One prominent proinflammatory effect of TNF includes the induction of adhesion molecule expression by endothelial cells associated with adhesion of, and parenchymal infiltration by, immunocompetent cells. It has been shown previously that PC-PLC mediates TNF-induced VCAM-1 expression by human endothelial cells, which can be prevented by D609 (27). As illustrated in Fig. 5, mTNF-treated C57BL/6 mice showed marked induction of VCAM-1 expression by lung endothelial cells (Fig. 5 C), which was accompanied by a pronounced leukocyte infiltration (Fig. 5 D). These hallmarks of the acute respiratory distress syndrome of the adult are markedly reduced in mice treated with D609 (Fig. 5, E and F).

TNF plays a dominant role in the pathogenesis of septic shock (28). To examine the effects of D609 on TNF-induced lethal shock syndrome, BALB/c mice were sensitized with 20 mg GaIN and subsequently treated with 3 μg mTNF. As shown in Table 1 and Fig. 6 A, three doses of 50 mg/kg D609 i.p. (at 0, +1, and +4 h, relative to mTNF challenge) provided full protection from TNF-mediated lethal toxicity (P <0.005 vs. mTNF). D609 had to be administered repetitively because of its short half-life time of 40 min; the LD₅₀ of D609 was ~200 mg/kg (data not shown). Control mice treated with either D609 alone or GaIN and D609 did not show increased lethality or signs of intoxication. Even 3 mo after D609 treatment, no life
Table 1. *D609 Protects Mice from TNF-induced Lethal Shock*

| Experiment | GaIN (mg/mouse) | TNF (μg/mouse) | D609 (mg/kg) | BALB/c (dead/group) |
|------------|-----------------|----------------|--------------|---------------------|
| 1*         | 20              | 3              | –            | 5/5*                |
|            | 20              | 3              | 3 × 50       | 0/5                 |
|            | –               | –              | 3 × 50       | 0/2                 |
|            | 20              | 3              | 3 × 50       | 0/2                 |
| 2          | 20              | 3              | –            | 5/5                 |
|            | 20              | 3              | 3 × 50       | 1/5                 |
|            | –               | –              | 3 × 50       | 0/2                 |
|            | 20              | 3              | 3 × vehicle1 | 2/2                 |
|            | 20              | –              | 3 × 50       | 0/2                 |

* Mice received indicated doses of GaIN, TNF, and D609 intraperitoneally in 100 μl of sterile PBS.

Table 2. *D609 Protects Mice from SEB-induced Lethal Shock*

| Experiment | GaIN (mg/mouse) | SEB (μg/mouse) | D609 (mg/kg) | BALB/c (dead/group) |
|------------|-----------------|----------------|--------------|---------------------|
| 1*         | 20              | 3 × 50         | 0.5-1-4      | 0/5                 |
|            | –               | –              | 3 × 50       | 0/2                 |
|            | 20              | 3 × vehicle1   | 0.5-1-4      | 2/2                 |
|            | 20              | –              | 3 × 50       | 0.5-1-4             | 0/2         |
| 2          | 20              | 100            | –            | 4/5                 |
|            | 20              | 2 × 50         | 1-4          | 0/5                 |
|            | –               | –              | 2 × 50       | 1-4                 | 0/2         |
| 3          | 20              | 100            | –            | 5/5                 |
|            | 20              | 2 × 50         | 1-4          | 0/5                 |
|            | –               | –              | 2 × 25       | 1-4                 | 0/2         |
| 4          | 20              | 2 × 25         | 1-4          | 1-5                 | 0/2         |
|            | –               | –              | 2 × 25       | 1-4                 | 0/2         |
| 5          | 20              | 100            | –            | 5/5                 |
|            | 20              | 2 × 100        | 1-4          | 2/5                 |
|            | –               | –              | 1 × 100      | 1                   | 0/2         |

* Mice received indicated doses of GaIN, SEB, and D609 intraperitoneally in 100 μl of sterile PBS.

shortening or signs of intoxication were observed (data not shown). Similar results were obtained with D609, when the lethal shock syndrome was induced by enterotoxins from gram-positive bacteria such as SEB. SEB functions as a superantigen that cross-links MHC class II-positive cells with T cells expressing the appropriate VB segments of the TCR (29). Accordingly, SEB stimulates both macrophages and T lymphocytes, which results in massive cytokine production (30). Administration of 100 μg SEB and 20 μg GaIN produced 80–100% lethality of BALB/c mice within 72 h (Fig. 6 B and Table 2). Triple injection of 50 μg/kg D609 at 30 min, 1 h, and 4 h after SEB challenge provided full protection (P <0.001 vs. SEB; Fig. 6 B, Table 2). Even two doses of 50 μg/kg D609 at 1 and 4 h after SEB challenge prevented the lethal shock syndrome. In contrast, a twofold injection of 25 μg/kg D609, as well as a single injection with 100 μg/kg D609, resulted only in incomplete (80 and 60%, respectively) protection. In septic shock models with LPS and SEB, single cell necroses of hepatocytes are a characteristic feature (31-33). Livers from SEB-challenged mice protected with D609 showed only mild signs of hepatotoxicity. Hepatocytes were not necrotic and the hepatic architecture remained largely unaltered (data not shown).

Finally, we have examined the effects of D609 on endotoxin-induced lethal shock in the absence of GaIN. Endotoxin (LPS) from gram-negative bacteria represents one major causative agent of septic shock, which triggers macrophages to release inflammatory cytokines such as TNF and IL-1 (34). As shown in Fig. 6 C and Table 3, mice were sensitive to a single injection of 500 μg LPS. A triple dose with 50 μg/kg D609 at 30 min, 1 h, and 4 h after LPS challenge protects mice from LPS-induced lethal shock (P <0.005 vs. LPS). These observations show that the protective principle of PC-PLC inhibition extends to the LPS-induced shock syndrome, where TNF represents but one chief mediator of toxicity.

PC-PLC: An Endogenous Pathogenicity Factor. The protective effects of D609 in TNF-, SEB-, or LPS-induced lethal shock may be explained by both inhibition of possible direct toxic effects of PC-PLC and blockade of important signaling functions of PC-PLC. It is important to note that PC-PLC is also involved in the signaling pathways of other inflammatory cytokines such as IL-1 and IFN-γ (for a review see reference 13), which synergize with TNF to produce a lethal shock syndrome. Interestingly, D609 does not inhibit SEB-induced release of IL-1, TNF, or IFN-γ in SEB-treated mice (our own unpublished observations). This is in contrast to other known inhibitors of LPS/SEB-mediated lethal shock, such as chlorpromazine, adenosine kinase in-
Table 3. D609 Protects Mice from LPS-induced Lethal Shock

| Experiment | LPS (µg per mouse) | D609 (mg/kg) | BALB/c (dead/group) |
|------------|-------------------|--------------|---------------------|
| 1*         | 500               | 3 x 50       | 3/5*                |
|            |                   | 3 x vehicle  | 2.2                 |
| 2          | 500               | 3 x 50       | 0/5                 |
|            |                   | 3 x vehicle  | 5/5                 |

*Mice received indicated doses of LPS and D609 intraperitoneally in 100 µl sterile PBS. The final result of the experiment (survival) was scored after 72 h. Surviving mice were monitored for 2 wk; no deaths occurred after 72 h. *Mice were treated with D609 at 0.5, 1, and 4 h after LPS challenge. 1100 µl PBS.

We thank Julia Dörge for excellent technical assistance.

This study was performed in partial fulfillment of the Ph.D. theses of T. Machleidt and B. Kramer. This work was supported by grants from the Bundesministerium für Bildung und Forschung (01 KI 9473), the Deutsche Forschungsgemeinschaft (Kr 810/8-2), and the Wilhelm-Sander Stiftung (92.076.1).

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Received for publication 6 March 1996 and in revised form 2 May 1996.

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