Biochemical Pharmacology, Vol. 57, pp. 291–302, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00300-1

Enhancement of the Surface Expression of Tumor Necrosis Factor α (TNFα) But Not the p55 TNFα Receptor in the THP-1 Monocytic Cell Line by Matrix Metalloprotease Inhibitors

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ABSTRACT. The monocytic cell line THP-1 can be induced to express and release tumor necrosis factor α (TNFα) and both TNFα receptors (p55 and p75) upon exposure to bacterial lipopolysaccharide (LPS). The broad-spectrum matrix metalloprotease (MMP) inhibitors [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(phenylthiomethyl)succinyl]-L-phenylalanine-*N*-methylamide (GI-129471) and marimastat [2*S*-[*N*4(*R**),2*R**,3*S**]]-*N*4-[2,2-dimethyl-1-[(methylamino)carbonyl]propyl]-*N*1,2-dihydroxy-3-(2-methylpropyl)butanediamide (BB-2516) were effective inhibitors of LPS-induced TNFα (soluble) release with IC_{50} values of 0.2 and 4.0 μM, respectively. Upon LPS stimulation, the expression of pro-TNFα (membrane associated) on the cell surface (FACS analysis) could not be observed. However, in the presence of GI-129471, a concentration-dependent increase in TNFα surface expression was observed. Peak expression (percentage of cells expressing pro-TNFα and mean fluorescence units) in the presence of GI-129471 was at 2 hr, and steadily declined to return to near control levels by 8 hr. This time course was similar to TNFα release, which also peaked at 2–4 hr after LPS exposure and then declined. Stimulation of THP-1 cells with LPS + phorbol myristate acetate increased the percentage of cells expressing pro-TNFα by 10-fold. In the presence of GI-129471, these increases were augmented further and peaked between 2 and 4 hr, but also returned to near control levels of expression by 24 hr. This was in contrast to the release of soluble TNFα, which continued to accumulate over a 24-hr time course. TNFα receptor I (p55, TNFRI) and II (p75, TNFRII) shedding was also inhibited by GI-129471 (IC_{50} = 1.5 and 3.1 μM, respectively) and BB-2516 (IC_{50} = 14 and 15 μM, respectively). Unlike pro-TNFα surface expression, surface expression of both TNFα receptors steadily increased over 72 hr. In contrast to pro-TNFα surface expression, TNFRI surface expression was not augmented by these MMP inhibitors in THP-1 cells after LPS stimulation. Surface expression of TNFRII was augmented by these MMP inhibitors. These results suggest that even in the continued presence of LPS stimulation and an inhibitor of TNFα processing, the augmented surface expression of TNFα is transient. The potential "deleterious" implications of high levels of surface pro-TNFα expression in the presence of these inhibitors may be lessened by its transient nature. BIOCHEM PHARMACOL **57**;3:291–302, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. tumor necrosis factor α (TNFα); THP-1 cells; tumor necrosis factor α converting enzyme (TACE); matrix metalloprotease inhibitors; tumor necrosis factor α receptors I and II; fluorescence activated cell sorting (FACS)

TNFα† is a pleiotropic cytokine and a primary mediator of the inflammatory response. TNFα is a member of the immediate early gene family of inflammatory proteins that are produced predominantly by macrophages and lymphocytes upon inflammatory stimulation. Upon stimulation, *de novo* synthesis of pro-TNFα (28 kDa form) occurs within minutes, and this pro-TNFα is transported to the cell surface as a type II transmembrane protein. Then the pro-form of TNFα is proteolytically processed at the cell membrane to release soluble (17 kDa form) TNFα [1]. This proteolytic processing is accomplished by a specific metalloprotease [2–6], TACE [7, 8].

TACE is a member of a family of membrane-bound metalloproteases that contain *A Disintegrin And Metalloprotease* domain (ADAM) [9]. Two groups have purified and cloned TACE and demonstrated a specificity for the processing of pro-TNFα to TNFα by cleavage at the Ala76-Val77 bond [7, 8]. TACE does not appear to cleave any of the other ligands or receptors that are released by a metalloprotease-dependent mechanism, e.g. TNF receptors,

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† *Abbreviations:* FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloprotease; MFU, mean fluorescence units; PBMCs, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; pro-TNFα, surface-associated TNFα; TACE, TNFα converting enzyme; TNFα, tumor necrosis factor α; and TNFRI and TNFRII, TNFα receptors I and II.

Received 5 June 1998; accepted 7 August 1998.

IL-6R, or IL-1R type II [10]. However, this does not exclude the possibility that these ligands may be substrates for TACE in the cell environment. Broad-spectrum inhibitors of metalloproteases prevent the proteolytic processing and release of TNFα [2–6]. Inhibition of TNFα release has been associated with a retention of pro-TNFα on the cell surface [3–5] and an increase in survival in a LPS-induced model of shock [6, 11]. Since both membrane-associated and soluble TNFα retain biological activity, a potential "deleterious" effect of augmented surface expression of pro-TNFα has been postulated. This has arisen from studies with cells transformed with a mutated pro-TNFα, which lacks the correct proteolytic cleavage site [12, 13]. These mutated cells expressed high levels of surface pro-TNFα and retained biological activity against TNFα sensitive cell lines (e.g. L929 cells). This implied that if agents, such as MMP inhibitors, blocked the release of TNFα and increased surface pro-TNFα, this would be as biologically active as soluble TNFα, leading to unwanted or "deleterious" effects, such as inappropriate apoptosis. These "deleterious" effects may then counteract the beneficial effects gained from preventing TNFα release. Likewise, possible retention of cell surface TNFα receptors has been implicated in a TNFα cytotoxicity assay in the presence of inhibitors of metalloproteases [14].

TNFα mediates its biological effects through two cell surface receptors, p55 (TNFRI) and p75 (TNFRII). The dominant signaling receptor is the p55 TNFRI. The signal transduction mechanisms leading to apoptosis and inflammatory reactions have been studied in detail. One aspect of the regulation of these receptors is their ability to be shed in a metalloprotease-dependent mechanism. Some degree of controversy exists concerning the physiological relevance of this shedding process. That is, do the extracellular domains shed from the cells act to down-regulate the TNFα signal, act as a natural sink to neutralize soluble TNFα, or act as a stabilization factor to allow TNFα to have more of a paracrine function? Similar to the postulated "deleterious" effects of membrane-bound pro-TNFα, retention of cell surface TNFα receptors has been implicated as potentiating TNFα killing in the presence of broad-spectrum metalloprotease inhibitors [14]. Therefore, the therapeutic implications of inhibiting TNFα release may be altered by the retention of cell surface TNFα and/or its receptors.

Blocking TNFα activity with monoclonal antibody therapy has proven to be efficacious in the treatment of rheumatoid arthritis [14–19] and Crohn's disease [20]. By preventing soluble TNFα from interacting with its receptors, an impressive improvement in all measurable facets of these diseases was observed [15–20]. By inhibiting TACE, one would expect to reduce the soluble TNFα and possibly retain cell surface pro-TNFα, thus limiting the potential area of influence of pro-TNFα (cell surface) to the local environment, allowing only juxtacrine events involving the cell surface pro-TNFα. Likewise, using broad-spectrum metalloprotease inhibitors, one would also expect retention of cell surface TNFα receptors. To further investigate the potential for such events to take place, we used LPS-stimulated THP-1 monocyte-like cells as a model system to study TNFα and TNFα receptor processing.

These studies demonstrated that broad-spectrum metalloprotease inhibitors block the release of soluble TNFα and its receptors from THP-1 cells, and cause the accumulation of pro-TNFα on the cell surface; however, its cell surface residence is transient even in the continued presence of stimuli and inhibitor. Some augmentation of TNFRII surface expression was observed in the presence of these inhibitors; however, no augmentation of TNFRI surface expression was observed. Therefore, the impact of surface pro-TNFα as a juxtacrine stimulator of TNF-mediated "deleterious" events may be too transient to adversely affect the therapeutic potential of inhibitors of TACE in inflammatory diseases.

MATERIALS AND METHODS

Human Cytokine and Receptor ELISAs

TNFα was measured using a sandwich ELISA technique. The capture antibody was goat anti-human TNFα (polyclonal, R & D Systems), and the detection antibody was biotinylated sheep anti-human TNFα (polyclonal, The Binding Site). Development of the ELISA utilized a streptavidin-horseradish peroxidase conjugate (Pierce). Color development was achieved with a peroxide-OPD solution (Sigma). Other cytokine ELISAs were purchased from Endogen (IL-1β, IL-8, and IL-6) and R & D Systems (TNF RI and RII), and were performed according to the manufacturer's instructions.

FACS Analysis of Surface Markers

Cell surface markers were measured using a Coulter Epics Excel. After appropriate treatments, cells (0.5×10^6) were blocked with human AB^+ serum (25 μL serum + 100 μL cell suspension) for 5 min at room temperature. Labeled Ab (1.0 μg/sample) was added sequentially in a two-step procedure (anti-TNFα-biotin from The Binding Site + streptavidin-FITC from Dako); each Ab was incubated for an additional 30 min on ice and in the dark. Then the cells were washed once and fixed with 1% formaldehyde (in saline, 1 mL/sample). Samples were stored at 4° in the dark until analysis. For analysis, an isotype matched in antibody type and concentration was used to adjust background fluorescence. Data are expressed either as percentage of cells expressing the appropriate marker or mean fluorescence units taken from duplicate experimental samples. TNFα receptor p55 and p75 analyses were performed with antibodies from R & D Systems as described above for TNFα with the exception that the cells were acid stripped (washed in 0.01 N HCl in PBS for 2 min, cells were then pelleted at 300 *g* for 10 min and resuspended in PBS for labeling) to remove any receptor bound TNFα prior to labeling with anti-TNFα receptor antibodies.

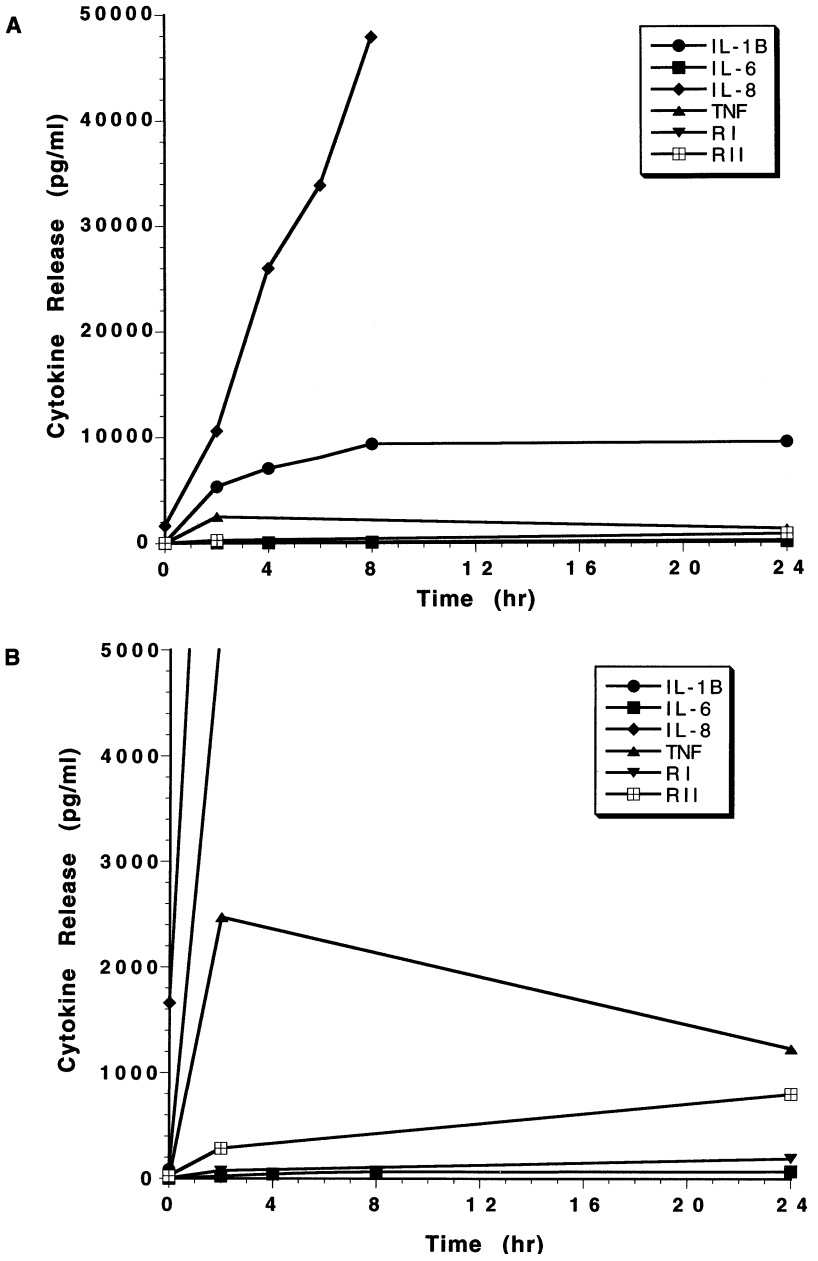

FIG. 1. Cytokine profile from THP-1 cells stimulated with LPS (5 μg/mL). THP-1 cells (1.5×10^5 cells/well in a 96-well plate) were stimulated with LPS (5 μg/mL) for 18 hr at 37° in a humidified atmosphere of 95% air and 5% CO_2. Cytokine levels in the cell-free medium were analyzed by specific ELISA, and values are expressed as pg/mL. (A) Cytokine release profile for IL-1β and IL-8. (B) Cytokine release profile for TNFα, IL-6, and the TNFα receptors RI and RII. These results are from a representative experiment, data points in triplicate, from two separate experiments showing similar results.

Inhibitor Studies

THP-1 cells (1.5×10^5 cells/well in 96-well flat-bottom Costar tissue culture plates) were incubated in fresh medium [200 μL of RPMI-1640 containing 10% fetal bovine serum (Hyclone, defined FBS), supplemental L-glutamine, and penicillin/streptomycin] containing compounds dissolved in DMSO (final concentration was <0.1% DMSO). After 18 hr (or the times indicated) in the presence of the inhibitor and LPS (5 μg/mL), the medium was removed and assayed for TNFα, TNFRI, and TNFRII by specific ELISA as per manufacturer's instructions, or the cells were processed for FACS analysis as described above.

Data Analysis

Data are expressed either as percent inhibition, percent cells expressing surface marker or MFU. Statistical analysis was performed on appropriate experiments by the Least Significant Difference Analysis using PHARM/PCS (Tallarida and Murray) software (Microcomputer Specialists).

RESULTS

Effects of Metalloprotease Inhibitors on LPS-Induced Cytokine Production

When stimulated with LPS (5 μg/mL), THP-1 cells released a variety of cytokines including TNFα, IL-1β, IL-6, IL-8, and TNFα receptors (Fig. 1). The most abundantly released cytokines were IL-8 and IL-1β, which attained levels >50 ng/2.5×10^5 cells and 10 ng/2.5×10^5 cells, respectively, at 8 hr of stimulation (Fig. 1A). IL-6 and TNFRI were produced at levels of <100 pg/2.5×10^5 cells. When stimulated, THP-1 cells released maximal levels of

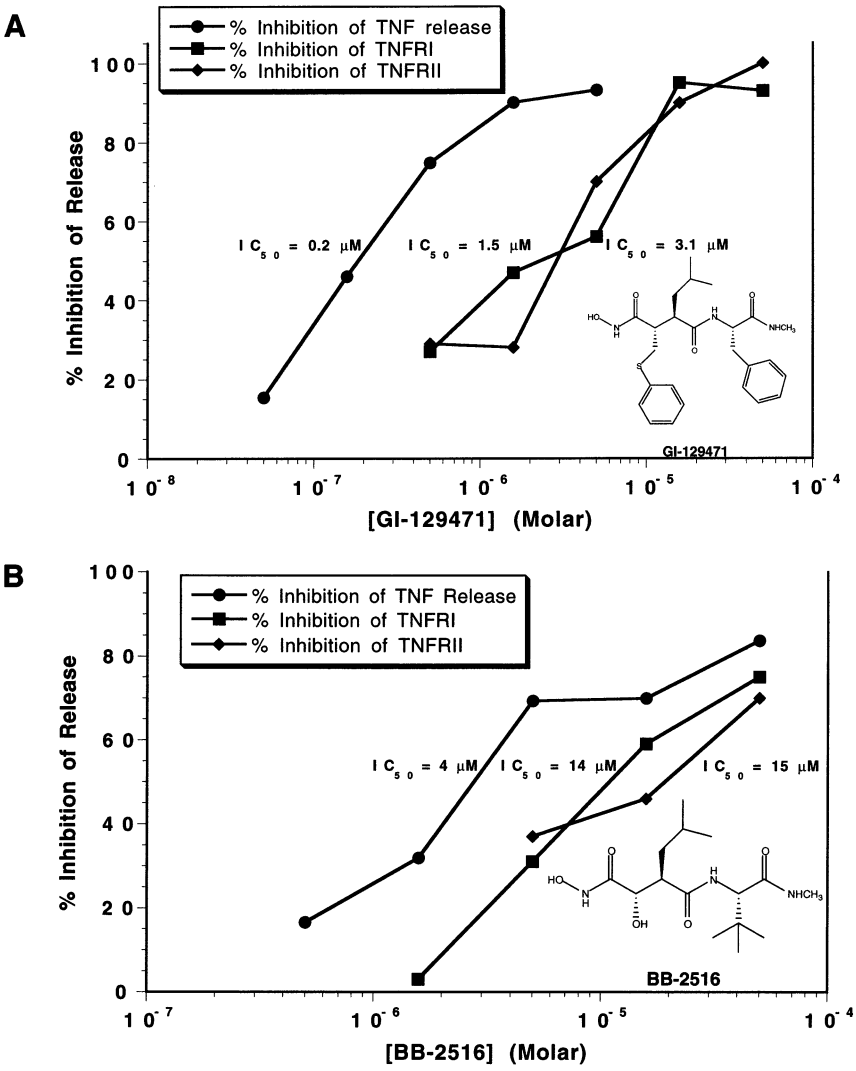

FIG. 2. Inhibition of TNFα, TNFRI, and TNFRII release from LPS-stimulated THP-1 cells. (A) Concentration–response curves for GI-129471 (structure inset) in THP-1 cells stimulated with LPS (5 μg/mL) for 18 hr in the presence of different concentrations of GI-129471. GI-129471 was prepared in a DMSO solution. Final DMSO concentration in the culture medium did not exceed 0.1%. TNFα and TNFα receptors (RI and RII) were quantitated by specific ELISA, and percent inhibition was calculated based upon LPS-stimulated cells receiving vehicle only (0.1% DMSO). (B) Concentration–response curves for BB-2516 (structure inset) in THP-1 cells as described in (A). Values represent the means from two separate experiments with each data point in duplicate. Control, vehicle-treated, release of TNFα was 1030 ± 210 pg/mL, TNFRI was 88 ± 13 pg/mL, and TNFRII was 207 ± 62 pg/mL.

TNFα at 2–4 hr (500 pg/2.5 × 10^5 cells), and then the amount of TNFα in the culture medium decreased with time (Fig. 1B).

To investigate the regulation of cytokine release, we used two broad-spectrum inhibitors of metalloproteases, [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(phenylthiomethyl)succinyl]-L-phenylalanine-*N*-methylamide (GI-129471) [5] and marimastat [2*S*-[*N*4(*R**),2*R**,3*S**]]-*N*4-[2,2-dimethyl-1-[(methylamino)carbonyl]propyl]-*N*1,2-dihydroxy-3-(2-methylpropyl)butanediamide (BB-2516) [21]. These inhibitors did not inhibit the release of IL-1β, IL-6, or IL-8 (data not shown), which is consistent with a non-metalloprotease-dependent mechanism of release for these cytokines. The release of soluble TNFα and both forms of the TNFα receptors (TNFRI, 55 kDa, and TNFRII, 75 kDa) into the culture medium was inhibited in a concentration-dependent manner by GI-129471 (Fig. 2A) and BB-2516 (Fig. 2B). GI-129471 demonstrated submicromolar inhibition of TNFα release (IC_{50} = 0.2 μM) and was 7- to 15-fold less potent against TNFRI and -RII (IC_{50} values of 1.5 and 3.1 μM, respectively). BB-2516 was less potent against TNFα release (IC_{50} = 3.6 μM) than GI-129471, and was only 3- to 4-fold less potent against TNFRI and -RII (IC_{50} values of 14 and 15 μM, respectively) release than TNFα release. Inhibition of TNFα release was similar whether TNFα was measured at 4 hr (peak TNFα levels) or at 18 hr (overnight incubation with inhibitors) (data not shown).

Time Course of LPS-Induced Cytokine Release and Surface Expression

When THP-1 cells were stimulated with LPS (5 μg/mL), a rapid rise in secreted TNFα occurred, with peak levels being reached by 2–4 hr of incubation (Fig. 3), followed by a steady decrease in the amount of TNFα present in the culture medium. By 72 hr in culture, the level of TNFα in the medium was near basal levels. The release of the soluble TNFα receptors TNFRI and TNFRII occurred on a very different time course (Fig. 3). TNFRII was the predominant receptor released by LPS-stimulated THP-1 cells and at 48 hr reached levels comparable to those of TNFα, both near 2500 pg/mL. TNFRI continued to accumulate over the 72-hr time course, and only reached levels five times less that that of TNFα or TNFRII.

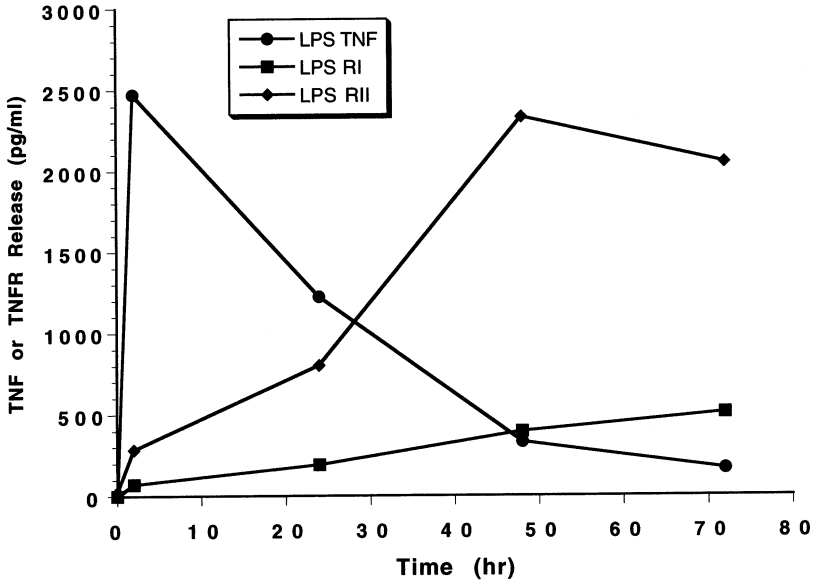

FIG. 3. Time course of TNFα, TNFRI, and TNFRII release from THP-1 cells over 72 hr. THP-1 cells were stimulated with LPS (5 μg/mL), and at the indicated time points an aliquot of the medium was removed for analysis. TNFα and TNFα receptors were quantitated by specific ELISA. These results are from a representative experiment, data points in triplicate, from two separate experiments showing similar results.

Surface expression of pro-TNFα was investigated in LPS-stimulated THP-1 cells over a 24-hr time course (Fig. 4, A and B). When analyzing surface expression in the absence of a metalloprotease inhibitor (control LPS), no increase in surface expression was observed for either the percent cells expressing pro-TNFα (Fig. 4A) or in the amount of cell surface TNFα (MFU, Fig. 4B, differences were not statistically significant, $P > 0.05$). However, in the presence of GI-129471, a concentration-dependent increase in cell surface pro-TNFα was observed (Fig. 4, A and B). Peak augmentation of pro-TNFα surface expression occurred at 2 hr (statistically significant different from 4 hr, $P < 0.01$), coincident with peak TNFα levels in the medium, and gradually returned to control (no stimulation) levels of surface expression by 24 hr. This indicated that in the presence of GI-129471, the enhanced surface expression of pro-TNFα was a transient event.

Since the TNFα receptors followed a very different time course of release, the levels of surface expression for TNFRI and TNFRII were analyzed over a 72-hr time course (Fig. 5, A and B). A steady increase in surface expression of TNFRI and TNFRII was observed over the 72-hr time course. With LPS stimulation, the level of expression of TNFRI (Fig. 5A) achieved was 3-fold less than that of TNFRII (Fig. 5B) at 72 hr. Several metalloprotease inhibitors were evaluated to determine if inhibition of receptor shedding also correlated with increased surface receptor expression as observed for pro-TNFα. Both GI-129471 (5 μM) and BB-2516 (15.8 μM) produced >75% inhibition of receptor shedding over the 72-hr time course. Under these conditions, TNFRI surface expression was not augmented by the presence of the MMP inhibitors (Fig. 5A). That is, at 72 hr of LPS stimulation the surface expression of TNFRI in the presence of GI-129471 appeared to be decreased slightly, from 14% cells expressing in controls to 9% cells expressing in the presence of GI-129471. Likewise, BB-2516 did not augment TNFRI surface expression, 14% cells expressing in controls versus 13% cells expressing in the presence of BB-2516 (Fig. 5A). The surface expression of TNFRII was augmented by GI-129471 and BB-2516; however, the degree of enhancement did not appear to be as great as that occurring with TNFα. At 48 hr of LPS stimulation, 35% of control cells expressed surface TNFRII, whereas the levels of surface TNFRII in the presence of GI-129471 increased to 46% cells expressing and in the presence of BB-2516 increased to 52% cells expressing (Fig. 5B, differences were statistically significant, $P < 0.01$). By 72 hr, the augmentation of surface TNFRII was less evident (Fig. 5B).

Time Course of LPS + PMA Stimulated Cytokine Release and Surface Expression

To differentiate between the transient effects of LPS on TNFα release and a stimulus that gives a continual production of TNFα *in vitro*, the combination of LPS + PMA was used (Fig. 6). The rate of TNFα release with LPS + PMA increased for up to 48 hr after stimulation and attained levels 4–5 times greater than that observed with LPS alone. The release of TNFRII increased significantly with LPS + PMA as a stimulus, attaining levels 4–5 times greater than observed with LPS alone (12,000 vs 2,500 pg/mL). There was only a 2-fold increase in the level of TNFRI released by this stimulus combination versus LPS alone.

Pro-TNFα surface expression was observed in LPS + PMA stimulated cells in the absence of a metalloprotease inhibitor (Fig. 7, A and B). The levels of surface expression of pro-TNFα appeared to peak at 2–4 hr and then gradually declined (Fig. 7A, differences were not statistically significant, $P > 0.05$). This is in contrast to the levels of released TNFα, which continued to increase over this time period. The presence of GI-129471 augmented the surface expression of pro-TNFα, which also peaked at 2–4 hr and declined to control levels by 24 hr (Fig. 7, A and B, differences were statistically significant, $P < 0.05$). The

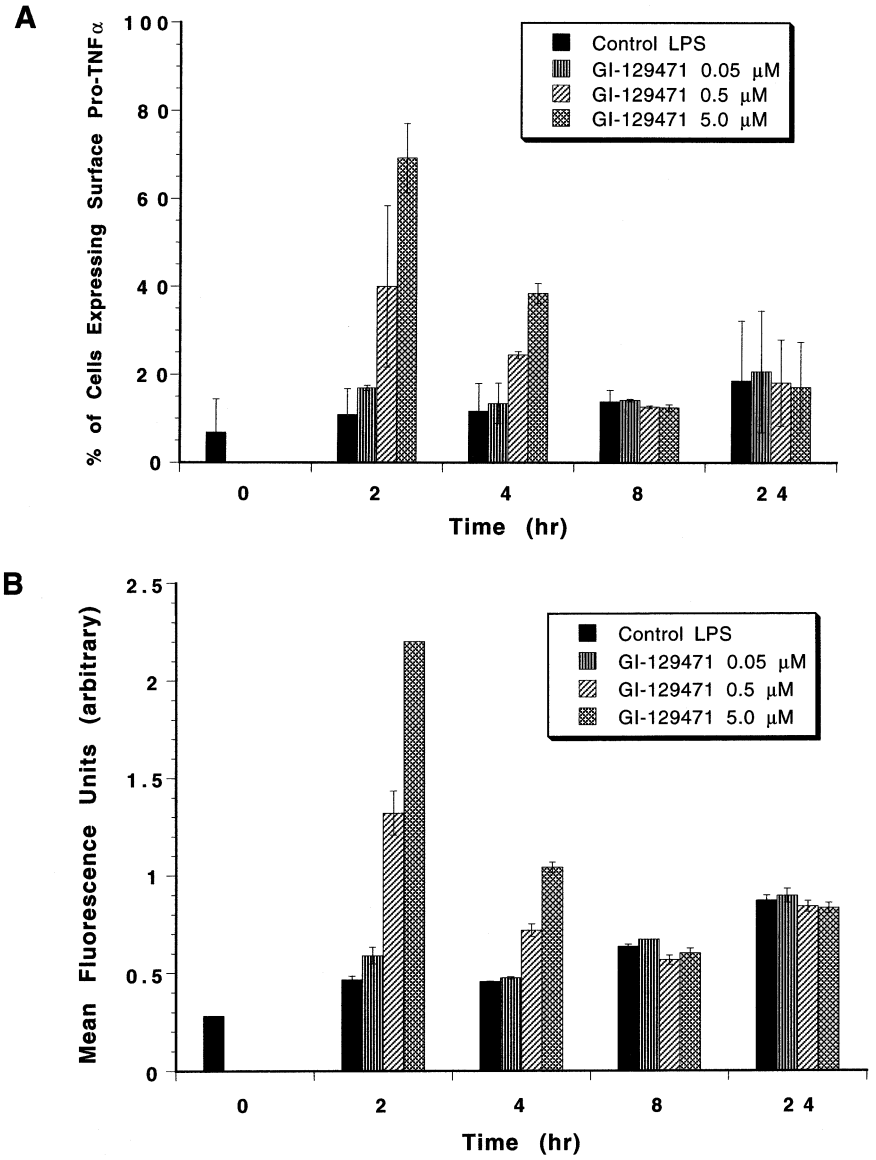

FIG. 4. Analysis of the cell surface expression of pro-TNFα on LPS-stimulated THP-1 cells. THP-1 cells were stimulated with LPS (5 μg/mL) for the time periods indicated and analyzed by FACS for surface expression of pro-TNFα using an anti-human pro-TNFα biotin-labeled antibody. FACS analysis was performed by adding streptavidin-FITC, fixing the samples in 1% formaldehyde, and analyzing 10,000 events/sample on a Coulter Epics Excel fluorescent analyzer. The data presented are from a representative experiment with each data point in duplicate out of at least two separate experiments showing the same results. (A) FACS analysis showing the percentage of cells expressing surface pro-TNFα over a 24-hr time course in the presence and absence of GI-129471. (B) FACS analysis showing the mean fluorescence units (arbitrary units) of cell surface pro-TNFα over a 24-hr time course in the presence and absence of GI-129471. Data represent the means ± SD from two separate experiments, each performed in duplicate.

amount of pro-TNFα expressed on the cell surface (MFU) peaked at 4 hr and declined to near control levels by 24 hr (Fig. 7B).

As previously demonstrated, LPS stimulation of surface expression of TNFRI resulted in 14% of the cells expressing TNFRI at 72 hr, whereas, LPS + PMA stimulation resulted in 65% of cells expressing TNFRI at 72 hr (Fig. 8A). As observed with LPS stimulation, the combination of LPS + PMA in the presence of metalloprotease inhibitors did not augment the surface expression of TNFRI (Fig. 8A, not significantly different from non-treated control, $P > 0.05$). The expression of TNFRII was augmented in the presence of the metalloprotease inhibitors (Fig. 8B). This was evident for GI-129471 at 2 hr where the percentage of cells expressing increased from 8% in the control cells to 28% in the GI-129471-treated cells (significantly different, $P < 0.01$). In the presence of the metalloprotease inhibitors, a maximal augmentation of 2-fold increase over control expression of surface TNFRII was observed at 24 hr of stimulation with LPS + PMA (Fig. 8B) (significant difference between control and GI-129471 treated, $P < 0.01$). At later time points, there was less of a difference between the control and metalloprotease-treated cells; however, the metalloprotease inhibitors still augmented the expression of TNFRII, 60% vs 72–74% cells expressing in the control and treated cells, respectively.

DISCUSSION

THP-1 cells release numerous cytokines in response to a variety of stimuli and, thus, are similar to normal monocytes in this regard. In particular, THP-1 cells release TNFα and the TNFα receptors (TNFRI and TNFRII) in response to LPS by a metalloprotease-dependent mechanism [6, 10]. TACE, the specific metalloprotease responsible for the release of TNFα [7, 8], was purified originally from the THP-1 cell line [7]. The sheddase responsible for TNFα receptor release does not appear to be TACE, but may be

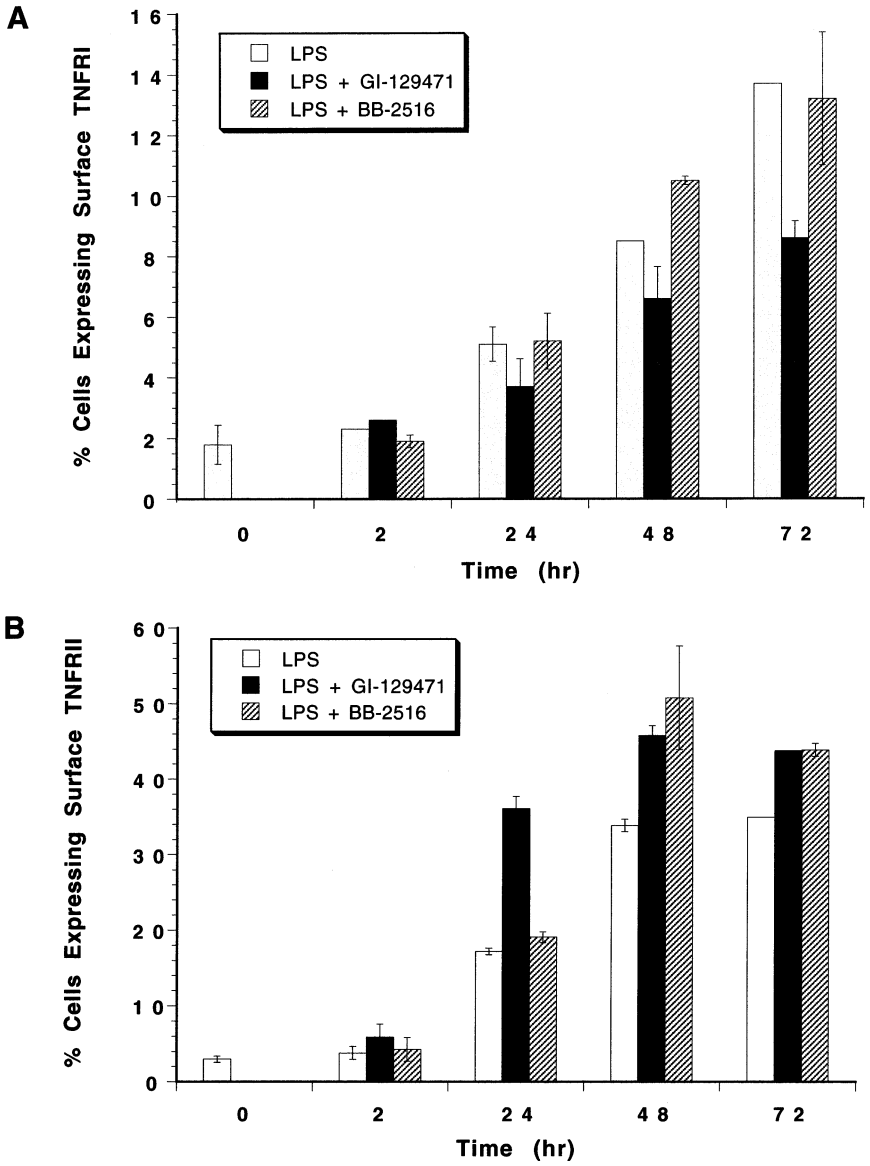

FIG. 5. Effects of GI-129471 (5 μM) and BB-2516 (15.8 μM) on the surface expression of TNFRI (A) and TNFRII (B). (A) THP-1 cells (0.5×10^6) were stimulated with LPS (5 μg/mL) for the indicated time periods in the presence or absence of inhibitors, stripped of surface bound TNFα by a brief acid wash (0.01 N HCl in PBS for 2 min), and then labeled with an anti-human TNFRI antibody (R & D Systems). Then the cells were incubated with a biotin-labeled anti-goat IgG and labeled with streptavidin-FITC for FACS analysis as described in the legend of Fig. 4. (B) THP-1 cells were analyzed for cell surface TNFRII as described above for TNFRI. The data presented are the means ± SD from two separate experiments, each with data points in duplicate.

another metalloprotease [6, 10]. Therefore, THP-1 cells seem well suited to investigate the regulation of the surface expression of TNFα and its receptors in the presence and absence of broad-spectrum inhibitors of metalloproteases.

When THP-1 cells were stimulated with LPS, TNFα levels peaked at 2–4 hr and then steadily declined. Vey and co-workers [22] demonstrated that THP-1 cells degrade the extracellular TNFα. The degradation was mediated through a serine-type protease (i.e. inhibited by 2 mM phenylmethylsulfonyl fluoride) without affecting the levels of IL-1β in the medium. The decrease of extracellular TNFα with time may also be accentuated after LPS stimulation due to the decreased synthetic rate of TNFα production. This was also observed by Vey and co-workers [22] when THP-1 cells were stimulated with HUT-78 T-cell membranes. When THP-1 cells are stimulated with LPS + PMA, it is possible that they can overcome this degrading effect by being able to saturate this TNFα degrading protease activity, or do not produce this degrading protease in response to LPS + PMA, or inhibit the activity of this protease. The net result of these possibilities is that a continual release (accumulation in the medium) of TNFα is observed with LPS + PMA stimulation. This observation for TNFα release appears to be rather selective, as the other cytokines released by these cells (IL-1β, IL-8, IL-6, or the TNFα receptors) continued to accumulate in the culture medium over the same course of the experiment.

The surface expression of pro-TNFα appears to follow a time course very similar to that of LPS-stimulated TNFα release. That is, surface expressed pro-TNFα in the presence of a broad-spectrum metalloprotease inhibitor peaked at 2–4 hr and then declined to near control levels by 24 hr. In the absence of a broad-spectrum metalloprotease inhibitor, an increase in surface pro-TNFα could not be observed upon LPS stimulation. This is possibly due to the efficient processing ability of the THP-1 cells for surface pro-TNFα. This is consistent with the work of Jue and co-workers [1],

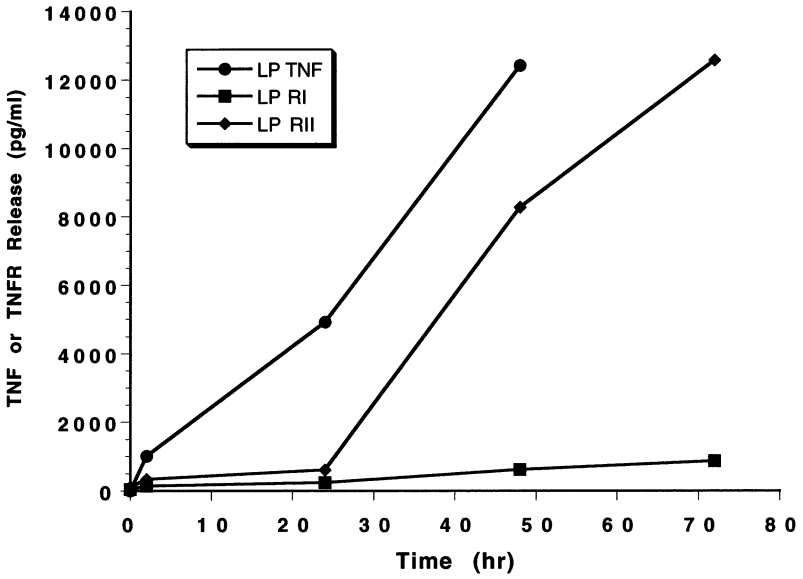

FIG. 6. Time course of TNFα, TNFRI, and TNFRII release from THP-1 cells stimulated with LPS (5 μg/mL) + PMA (40 nM). TNFα, TNFRI, and TNFRII were quantitated over a 72-hr time course. These results are from a representative experiment, data points in triplicate, from two separate experiments showing similar results.

who demonstrated a steady-state level of membrane bound TNFα in LPS-stimulated RAW 264.7 cells (a murine macrophage-like cell line). Upon LPS + PMA stimulation, a definitive increase in surface expression of pro-TNFα was observed in the absence of a broad-spectrum metalloprotease inhibitor. The percentage of cells expressing surface pro-TNFα increased from 5 to 50%, a 10-fold increase, between 0 and 2 hr of stimulation. This level of expression peaked between 2 and 4 hr and then appeared to decline even with the continued accumulation of soluble TNFα. The "absolute" amount of TNFα on the cell surface (MFU) followed essentially the same time course; however, the increase was only 3-fold in fluorescence units from 0 to 2 hr of stimulation with LPS + PMA. These results suggest that the cell strives to maintain a relatively low and constant level of surface pro-TNFα expression. This is consistent with the suggestion that this low level of surface expression is necessary for cell–cell interactions and communication to maintain homeostasis [23].

In the presence of MMP inhibitors, a clear augmentation of the surface expression of pro-TNFα was observed. We have also observed this phenomenon in HEK293 cells transfected with human pro-TNFα. These observations support two important concepts: first, that pro-TNFα is processed at the cell surface by a metalloprotease, and, second, that this proteolytic activity (e.g. TACE) is a rather ubiquitous activity, if indeed there is only one TNFα processing enzyme [24, 25]. In general, most studies looking at the effects of metalloprotease inhibitors have described the increase in surface pro-TNFα at a single time point (i.e. 2 hr in THP-1 cells [6]). However, from our results in THP-1 cells, one can see that single time point observations on surface pro-TNFα expression may be quite misleading, especially at later time points when the cell has "re-equilibrated" the levels of surface pro-TNFα. This concept is supported by the recent results of Solomon *et al.* [26] using PBMCs. Although the time course of surface expression of pro-TNFα is rather extended in PBMCs versus THP-1 cells, PBMCs also demonstrate a transient surface expression of pro-TNFα in the presence of metalloprotease inhibitors.

Numerous studies using TNFα constructs or transgenes, which have a non-cleavable pro-TNFα molecule [12, 13, 27, 28], have suggested a "deleterious" role of surface pro-TNFα. This concept has been based upon the activity of membrane bound pro-TNFα in assays from cellular cytotoxicity [12–14, 29] to models of disease in animals [27, 28, 30, 31]. The most common *in vitro* observation is that excess surface pro-TNFα [12, 13] or possibly TNFα receptors [14] results in an augmented cytotoxicity against TNFα sensitive cells (usually in the presence of actinomycin). *In vivo* observations of transgenes, which possess a transmembrane form of pro-TNFα, suggest a role in chronic local inflammatory reactions and produce localized disease phenotypes (e.g. joint inflammation) similar to diseases that are mediated by soluble TNFα [27, 30]. Our data in a macrophage-like cell and from others in PBMCs suggest that caution should be taken when interpreting physiological/pathophysiological states based on the data generated from non-cleavable mutants of pro-TNFα. The transient nature of cell surface pro-TNFα would seem to indicate that pathological manifestations of exaggerated expression of surface pro-TNFα from transmembrane mutants may not be relevant to normal physiological/pathophysiological conditions.

The physiological role of TNFα receptor shedding is still somewhat controversial; however, there is a correlation of serum levels of p75 TNFRII with disease severity in juvenile arthritis [32, 33]. Herein, we report that in THP-1 cells there was a preference for the release of the TNFRII over the TNFRI when using LPS or LPS + PMA as stimulants. Others have reported the predominant release of the TNFRI when stimulating cells with PMA [34, 35]. In THP-1 cells, release of both receptors can be blocked

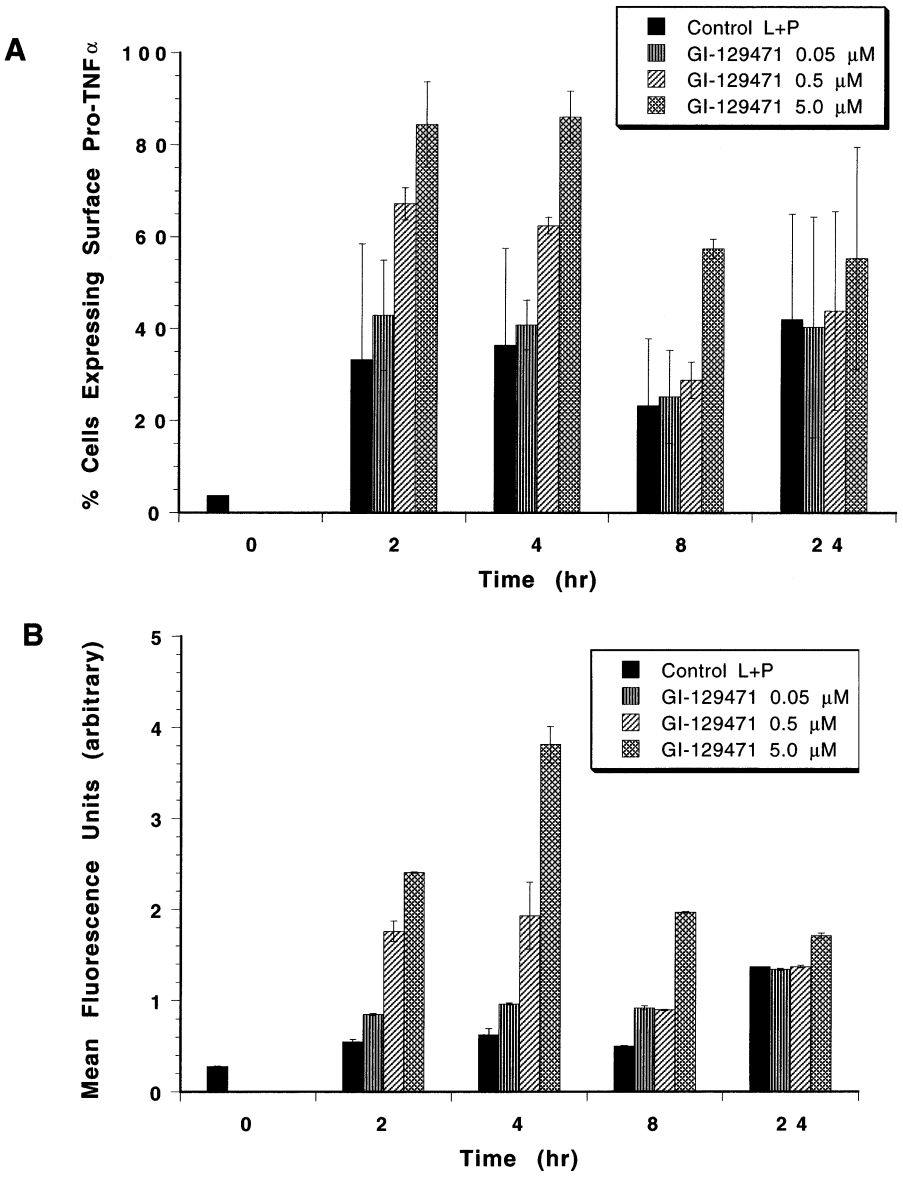

FIG. 7. Analysis of a cell surface expression of pro-TNFα in THP-1 cells stimulated with LPS (5 μg/mL) + PMA (40 nM). THP-1 cells (0.5 × 10^6) were stimulated with LPS + PMA and analyzed by FACS. (A) Percentage of cells expressing surface pro-TNFα in the presence and absence of GI-129471, analyzed as described in the legend of Fig. 4. (B) Mean fluorescence units of surface pro-TNFα in the presence and absence of GI-129471, analyzed as described in the legend of Fig. 4. Data represent the mean ± SD from two separate experiments, each performed in duplicate.

effectively by the presence of broad-spectrum metalloprotease inhibitors [34, 36, 37]. However, at least for TNFRI, augmented surface expression was not evident in the presence of broad-spectrum metalloprotease inhibitors. TNFRII surface expression was augmented by broad-spectrum metalloprotease inhibitors but not to the same extent as TNFα itself. This is probably due to the different kinetics of synthesis and expression of these different proteins.

TNFRI is the predominant signaling receptor for TNFα. The presence of the death-domain in this receptor makes it essential for TNFα-induced apoptosis. The role of the TNFα receptors in some *in vivo* disease models has been investigated by Kollias and co-workers [38]. These works also support the predominant role of TNFRI in TNF-mediated diseases. However, at least in THP-1 cells, metalloprotease inhibitors do not augment the surface expression of TNFRI. Therefore, increased biological activity of TNFα does not appear to be mediated by increased TNFRI surface expression. The observations of Williams *et al.* [14] of increased cytotoxicity in L929 cells with metalloprotease inhibitors, therefore, may not be mediated solely by increased TNFRI expression. These observations would also suggest that non-cleavable mutants of TNFRI may not be relevant constructs to interpret the pathological effects of metalloprotease inhibitors in normal tissues.

Upon PMA stimulation, others have observed an immediate decrease in the level of TNFα receptors on the cell surface [14, 34, 37], which was blocked by metalloprotease inhibitors. FACS analysis of the surface expressed receptors could not detect a substantial initial drop in the surface expression level in THP-1 cells. Even at early time points or when the TNFα was stripped from the receptors by a brief acid wash, any initial decrease in surface expression of either TNFRI or TNFRII was not detected. The levels of surface TNFRI and TNFRII increased steadily over a 72-hr time course for LPS and LPS + PMA stimulated cells. This increase in surface expression correlated with the release of TNFα receptors. Broad-spectrum metalloprotease inhibi-

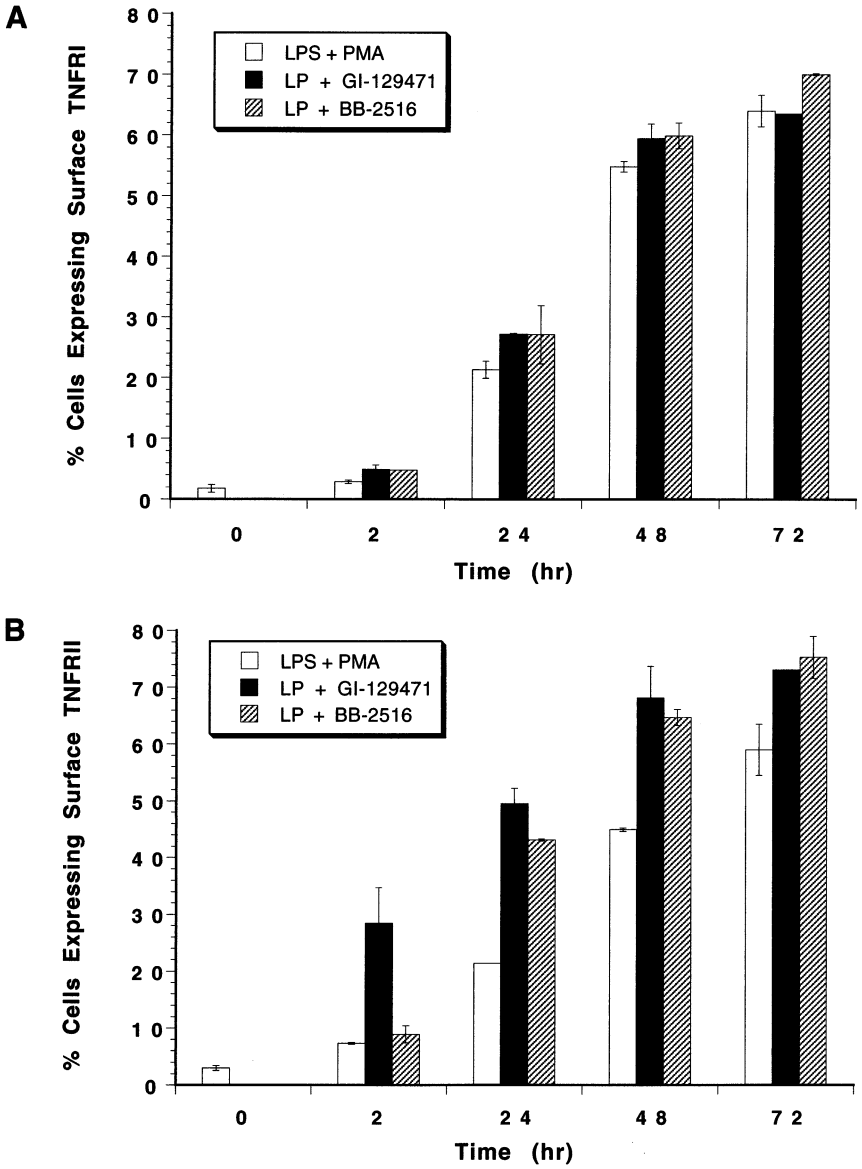

FIG. 8. Effect of GI-129471 (5 μM) and BB-2516 (15.8 μM) on LPS (1 μg/mL) + PMA (40 nM) stimulated surface expression of TNFRI (A) and TNFRII (B) in THP-1 cells. (A) THP-1 cells (0.5×10^6) were stimulated with LPS (5 μg/mL) and PMA (40 nM) for the indicated times and analyzed for cell surface TNFRI as described in the legend of Fig. 5. (B) Cells were analyzed for cell surface TNFRII as described in the legend of Fig. 5. Data are the means ± SD from two separate experiments, each with data points in duplicate.

tors were less effective against TNFα receptor release than TNFα release. These potency differences are consistent with the inability of cloned or purified TACE to cleave other receptors/ligands that are shed by metalloproteases [7, 10]. The development of selective inhibitors of TNFα release (TACE), therefore, may be able to select against any potential unwanted effects associated with the global inhibition of sheddases. Since the prime TNFα receptor responsible for cellular cytotoxicity and killing is the TNFRI, and broad-spectrum metalloprotease inhibitors may have little effect on their surface expression, it seems unlikely that deleterious side-effects may be associated with TACE inhibitors with regard to TNFα receptor sheddase inhibition.

These studies with THP-1 cells, a macrophage-like cell line, demonstrate that TNFα surface expression in the presence of broad-spectrum metalloprotease inhibitors or "TACE" inhibitors is augmented, but this augmentation is a transient event. Therefore, one may postulate that some of the deleterious effects observed with non-cleavable mutants of pro-TNFα may not be relevant to the potential effects of selective TACE inhibitors. Other metalloprotease shed receptors, e.g. TNFRI and TNFRII, are not inhibited as potently by broad-spectrum metalloprotease inhibitors, further suggesting that the "sheddases" for TNFα and its receptors may be distinct enzymes. The lack of augmentation of TNFRI expression by the broad-spectrum metalloprotease inhibitors would suggest that effects on TNFRI surface expression may not be relevant to the potential deleterious effects of broad-spectrum metalloprotease inhibitors. Some augmentation of the TNFRII does occur, but this augmentation is not as dramatic as seen for TNFα. These studies suggest that the transient nature of the augmentation of surface expression of metalloprotease shed receptors/ligands by broad-spectrum metalloprotease inhibitors may not be a factor in normal cells with regard to the

deleterious effects observed when looking at non-cleavable mutants or over-expressing transgenes of these surface molecules.

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