Endogenously produced urokinase amplifies tumor necrosis factor-α secretion by THP-1 mononuclear phagocytes

Robert G. Sitrin, Susan B. Shollenberger, Robert M. Strieter, and Margaret R. Gyetko

Pulmonary and Critical Care Medicine Division, Department of Internal Medicine, University of Michigan, Ann Arbor

Abstract: This study examined the effects of endogenous urokinase (uPA) on lipopolysaccharide (LPS)-stimulated tumor necrosis factor α (TNF-α) secretion in THP-1 mononuclear phagocytes. Anti-uPA monoclonal antibody (mAb) suppressed LPS-driven TNF-α secretion by 61.6 ± 5.9% ($P < .001$), and PAI-1, a uPA inhibitor, suppressed it to 53.1 ± 8.2% of the control value ($P < .001$). Up-regulation of TNF-α mRNA was suppressed in parallel with secreted TNF-α protein. TNF-α secretion was unaffected by depleting plasminogen or by aprotinin, a plasmin inhibitor. When endogenous uPA was displaced from the cell, exogenous high-molecular-weight (intact) uPA augmented LPS-driven TNF-α secretion. By contrast, a uPA fragment containing the catalytic domain was inhibitory, and the uPA receptor-binding domain had no effect. We conclude that endogenous uPA amplifies TNF-α neosynthesis of LPS-stimulated THP-1 mononuclear phagocytes. The effect requires intact uPA and is independent of plasmin activity. This represents a novel mechanism by which a mononuclear phagocyte–derived protease contributes to generating proinflammatory signals. *J. Leukoc. Biol.* 59: 302–311; 1996.

Key Words: *macrophage · plasminogen activator · cytokine · lipopolysaccharide*

INTRODUCTION

Proteases are integral to inflammatory responses, not only by contributing to tissue injury and remodeling but also by regulating the activation and function of inflammatory cells. Mononuclear phagocytes (Mϕs) use the plasminogen activator system as an important mechanism for regulating the environment in the inflammatory milieu [1, 2]. These cells synthesize and secrete urokinase-type plasminogen activator (uPA) and PA inhibitors (principally PAI-2) and express specific high-affinity uPA receptors on the plasma membrane (uPAR; CD87) [1, 2]. Cytokines stimulate neosynthesis of all elements of the PA-plasmin system, and in some cases there is significant cytokine specificity [1, 3, 4]. The role of the uPA-plasmin system in Mϕ function has been only partially elucidated, but it appears to be particularly important for tissue invasion, chemotaxis, and remodeling extracellular matrix proteins [2, 5–8]. Beyond the effect of these enzymes on stromal integrity, evidence is accumulating that uPA and plasmin are involved in regulating the release of cytokines or activation of cytokines from latent forms [9–11]. In addition, uPA can regulate leukocyte differentiation and activation by mechanisms quite distinct from those of its catalytic functions. In neutrophils, binding uPA to plasma membrane uPAR triggers signal transduction to the cell interior, as evidenced by calcium fluxes and protein tyrosine phosphorylation [12, 13]. Engagement of uPAR by uPA enhances uPAR-mediated adhesion to vitronectin and differentiation of Mϕ-like cells [14, 15]. In some instances, the amino terminus of uPA (bearing the uPAR binding domain) is sufficient to trigger a response, and uPA enzymatic activity, located in the carboxyl terminus, is entirely unnecessary [13, 15]. In neutrophils, both the amino and carboxyl terminal regions of uPA must interact with partner proteins to initiate signal transduction [12]. Thus, distinct elements of the uPA structure can subserve diverse mechanisms for modulating leukocyte function.

One of the difficulties inherent in studying the uPA-plasmin system in cell culture is that Mϕs produce sufficient PA inhibitors to complicate experimental manipulation of uPA function [2]. One means of circumventing this problem is to study the THP-1 human monocytic leukemia line. THP-1 cells, which have been used extensively as models of Mϕs, express uPA and uPAR normally but produce an inactive form of PAI-2 [16, 17]. As a result, functional PA activity is readily measured in cell supernatants. In this study, we sought to determine whether the PA-plasmin system affects tumor necrosis factor α (TNF-α) secretion by Mϕs, using THP-1 cells as a

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Abbreviations: ATF, amino terminal fragment; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HMW, high molecular weight; IgG, immunoglobulin G; IL-8, interleukin-8; LMW, low molecular weight; LPS, lipopolysaccharide; mAb, monoclonal antibody; Mϕ, mononuclear phagocyte; mPU, milliPloug unit; PLG, plasminogen; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor α; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; UV, ultraviolet; Z-LTBE, thiobenzyl benzyloxycarbonyl-L-lysinate.

Reprint requests: Robert G. Sitrin, Pulmonary and Critical Care Medicine Division, 3916 Taubman Center, Box 0360, Ann Arbor, MI 48109-0360.

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model system. Mϕs express a cell-associated pool of TNF-α, and there is some evidence that optimal secretion of TNF-α requires the action of an unspecified serine protease(s) [18, 19]. Along with its effects on other cytokines, modulating expression of TNF-α would be a powerful way for the uPA-plasmin system to influence inflammatory reactions [20]. We demonstrate that endogenously generated uPA modulates stimulation of TNF-α neosynthesis in response to bacterial lipopolysaccharide (LPS). We further show that the intact structure of high-molecular-weight uPA is necessary for this function, and the effect is independent of plasminogen activation.

MATERIALS AND METHODS

Reagents

Human plasminogen (PLG) was purified from human plasma by lysine-Sepharose chromatography [21]. The eluate migrated at 90 kd on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and hydrolyzed the plasmin substrate thiobenzyl benzyloxycarbonyl-L-lysinate (Z-LTBE; Calbiochem) after activation with high-molecular-weight (HMW) uPA (American Diagnostica, Greenwich, CT) [22]. Similarly, fetal bovine serum (FBS; Hyclone, Logan, UT) was depleted of plasminogen by two consecutive passages over lysine-Sepharose. To confirm that the depletion step was effective, an aliquot of FBS was briefly acidified to inactivate antiplasmins [23], followed by treatment with uPA (10 milliPloug units (mPU)/ml). The sample was then assayed for plasmin activity, using the Z-LTBE substrate. Even after markedly prolonged incubation (>4 h) with uPA and then with Z-LTBE, there was no evidence of plasmin generation from the depleted serum, while plasmin activity was evident in parallel assays of FBS that was briefly acidified but not passed over lysine-Sepharose. Western blots of normal and lysine-Sepharose–adsorbed FBS were also performed, using a goat anti-bovine PLG primary antibody (American Diagnostica).

Neutralizing murine monoclonal antibody (mAb) to human uPA (immunoglobulin G1; catalog number 394) was obtained from American Diagnostica. According to the manufacturer's specifications, this mAb recognizes one- and two-chain forms of uPA and uPAR-bound uPA. An isotype-matched mAb (anti-tissue factor, catalog number 4503) and aprotinin were also obtained from American Diagnostica. The murine anti-human uPAR mAb (clone 3B10, IgG2a), also designated anti-Mo3f, was prepared as previously described and recognizes an epitope in the ligand binding region (domain 1) [24, 25]. Recombinant human PAI-1 was generously provided by David Ginsburg, M.D., Howard Hughes Medical Institute, University of Michigan Medical Center. Mixing studies against HMW-uPA confirmed that the PAI-1 (25 ng/ml, as was used in the present studies) inhibited 350 mPU uPA/ml and retained 100 mPU/ml inhibitory activity even after incubation at 37°C for 2 h. Recombinant human uPA amino terminal fragment (uPA-ATF) containing the uPAR-binding region (amino acids 1–135), but not the active site, was generously provided by Richard H. Simon, M.D., University of Michigan. uPA-ATF was produced with the Xpress protein expression system in *Escherichia coli* (Invitrogen, San Diego, CA). The uPA-ATF is synthesized from the pTrcHis vector as a fusion protein with six histidine residues at the NH_2 terminus. The fusion protein is then purified by ProBond metal affinity chromatography (Invitrogen), and the recombinant uPA-ATF (18.7 kd) is removed from the NH_2-terminal fusion peptide at an enterokinase cleavage site. Bacterial lipopolysaccharide from *E. coli* 0111:B4 was purchased from Sigma Chemical Co., St. Louis MO. The human TNF-α cDNA (0.8 kb) was obtained from the American Type Culture Collection, Rockville, MD.

Cell culture

The THP-1 monocytic leukemia cell line was obtained from the American Type Culture Collection. Cells were propagated in 75-cm^2 polystyrene flasks (Costar, Cambridge, MA) in standard medium consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (100 μg/ml), glutamine (2 mM), and 10% FBS. To ensure that experiments were performed on cells in a uniform growth phase, cells were always passed into fresh medium (10^7 cells in 50 ml) 24 h prior to LPS stimulation. After washing, cells were dispensed into 96-well round-bottom plates (10^6 cells in a final volume of 200 μl), with additives as indicated. Cells were cultured for 24 h at 37°C in humidified air supplemented with 5% CO_2. Conditioned medium was then removed and cleared of cells by centrifugation. After washing in fresh medium, cell lysates were prepared by sonication (5×10^6 cells/ml medium).

Immunoassays

TNF-α was quantitated with an enzyme immunoassay kit purchased from Cistron Co., Pinebrook, NJ. This assay employs a monoclonal capture Ab and a detection system consisting of a rabbit anti-human TNF-α Ab and a peroxidase-conjugated goat anti-rabbit Ab. The enzyme immunoassay for interleukin-8 (IL-8) was prepared as described previously, using a rabbit anti-human IL-8 as both capture and detection antibodies, the latter being biotinylated and measured with a peroxidase-streptavidin conjugate [26].

Immunofluorescence flow cytometry

To measure plasma membrane uPAR, cells were suspended in staining buffer (phosphate-buffered saline with 1% bovine serum albumin and 0.1% sodium azide, pH 7.4), incubated with the murine anti-human uPAR antibody (3B10) for 30 min, 4°C, and labeled for 30 min, 4°C, with R-phycoerythrin-conjugated goat anti-mouse $F(ab')_2$ fragment (Jackson Immunological Research, West Grove, PA). For negative controls, cells were always stained in parallel with the secondary antibody alone and with an irrelevant isotype-matched (IgG2a) primary antibody. Alternatively, cells were directly labeled with fluorescein isothiocyanate (FITC)–conjugated anti-uPAR mAb (3B10), using FITC-IgG to measure nonspecific staining. Intact (live) cells were selected by gating (log forward angle versus log right angle light scatter). Immunofluorescence intensity was assessed as a measure of relative antigen expression, using an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) with a logarithmic amplifier. The channel number (log scale) representing the mean fluorescence intensity (major fluorescence peak) was determined from approximately 5000 cells. The corresponding linear fluorescence intensity channel was then calculated from a logarithmic-linear calibration formula. Specific fluorescence intensity was calculated by subtracting the values of the negative controls, using the linear scale.

mRNA analysis

For these experiments, THP-1 cells were cultured in serum-containing medium (10^6 cells/ml) in 75-cm^2 flasks in the presence or absence of LPS (0.5 μg/ml) and anti-uPA mAb (1.6 μg/ml) or PAI-1 (5 ng/ml) for 2 and 3 h. Cells were washed with RPMI 1640, and total cellular RNA was extracted using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX). The RNA was quantitated by spectroscopy at 260 nm and size fractionated electrophoretically on 1% agarose gels containing 3.5 μM formaldehyde and 20 pg/ml ethidium bromide, as described previously [27]. The RNA was transferred to Hybond nylon filters (Amersham, Arlington Heights, IL) and fixed by exposure to ultraviolet (UV) light. Ribosomal bands on the filters were visualized under UV light, providing internal size markers for each lane and also confirming that the RNA was loaded and transferred equally among corresponding lanes. The TNF-α cDNA was labeled with [γ-^{32}P]ATP (Amersham) by random priming [28]. The nylon filters were then hybridized with 10^6 cpm of [^{32}P]cDNA for 18 h at 65°C, followed by serial washes with 1× standard saline citrate (SSC), 0.1% SDS, 25°C, for 60

min, then 0.2× SSC, 0.1% SDS at 68°C for 20 min. The filters were then developed by autoradiography, using Hyperfilm (Amersham) at -70°C.

Statistics

The results are drawn from a data pool ranging from 5 to 15 experiments, as indicated. Comparisons between group means were performed using an unpaired, two-tailed Student's *t*-test [29]. Where appropriate, a paired *t*-test was used. Bonferroni's correction was utilized (as indicated) when multiple comparisons with a single control were made. Statistical significance was taken to represent a P value ≤ .05.

RESULTS

Effects of anti-uPA mAb on TNF-α secretion

THP-1 cells were cultured under control conditions (medium alone) or stimulated with LPS, either 0.5 or 5.0 μg/ml. Anti-uPA mAb was added to the cultures simultaneously with the addition of LPS. Each batch of mAb used during the course of these experiments was screened to determine the concentration that had the optimal effect on TNF-α production. There was significant variability between the batches, so the final concentration of mAb used in these experiments ranged from 1.6 to 10 μg/ml. Cell viability was routinely >95% at the onset of stimulation and >90% after 24 h. Neither the LPS nor the anti-uPA mAb adversely affected cell viability.

When cultured in medium alone, THP-1 cells secreted a minimal amount of TNF-α (< 0.1 ng/ml; **Fig. 1**). Preliminary experiments indicated that the maximal increase in TNF-α secretion reached a plateau with LPS ≥ 1 μg/ml. Therefore, these experiments were performed with LPS concentrations of 5.0 and 0.5 μg/ml, which allowed us to examine both maximal and submaximal stimulation by LPS. As shown in Figure 1, LPS increased TNF-α secretion more than 30-fold above the level produced by unstimulated cells. Anti-uPA mAb alone had no effect on TNF-α secretion, but it significantly reduced the amount of TNF-α secretion induced by either concentration of LPS (Fig. 1). Mean TNF-α secretion was reduced from 3.2 to 1.34 ng/ml with 0.5 μg/ml LPS ($P < .001$) and from 3.67 to 1.53 ng/ml with 5.0 μg/ml LPS ($P < .001$). Comparing the effect of the antibody to the LPS control for each experiment, these values represented 57.8 ± 6.6% and 61.6 ± 5.9% reductions in TNF-α secretion, respectively (both $P < .001$). Preliminary experiments also confirmed that adding these concentrations of anti-uPA mAb directly to samples containing TNF-α standard had no effect on measurement of TNF-α in the enzyme immunoassay (not shown). In addition, the isotype-matched control (anti-tissue factor) mAb (10 μg/ml) had no effect at all on LPS (0.5 μg/ml)-induced TNF-α secretion (98.2 ± 2.4% of LPS control, $n = 5$) and had no direct effect on the TNF-α assay.

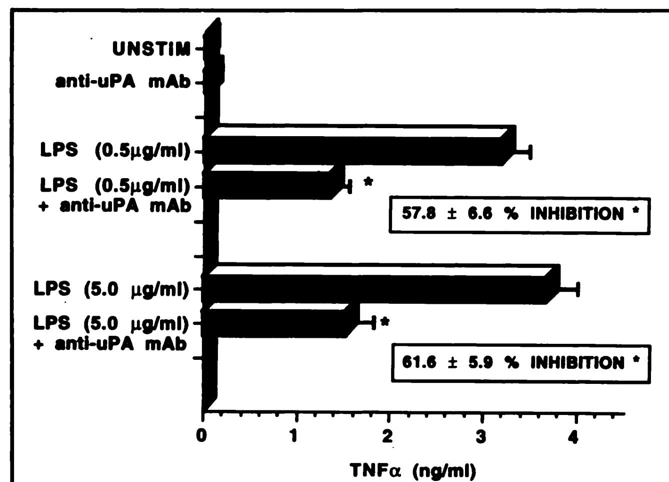

Fig. 1. Effects of anti-uPA mAb on LPS-induced secretion of TNF-α. Unstimulated THP-1 Mφs and cells incubated with anti-uPA mAb alone expressed < 0.1 ng/ml TNF-α during a 24-h incubation. LPS at 0.5 μg/ml and 5.0 μg/ml induced >30-fold increases in TNF-α secretion. Anti-uPA mAb added coincidentally with LPS significantly reduced TNF-α secretion. Bars indicate the mean ± SEM, $n = 15$. The inset values indicate the mean ± SEM of the percent inhibition. *$P < .001$.

TNF-α is processed and secreted efficiently, so most TNF-α is found in the conditioned medium, with a very minor fraction remaining cell associated [20]. Thus, the possibility was considered that the anti-uPA mAb was interfering with processing and secretion of TNF-α. Therefore, the cell-associated pool was assayed to determine whether the reduction in the secreted fraction was accompanied by a reciprocal accumulation within the cells. The amount of TNF-α measured in cell lysates was unaffected, as anti-uPA mAb treatment and PAI-1 (see below) left LPS (0.5 μg/ml)-stimulated cells with 90.5% and 93.5%, respectively, of the cell-associated TNF-α levels found in LPS-treated controls ($P > .5$). TNF-α on the plasma membrane (quantitated by flow cytometry) was likewise unaffected by inhibition of uPA (not shown).

Effects of plasminogen depletion and plasmin inhibition

Because the anti-uPA mAb used in the experiments described above neutralizes the catalytic activity of uPA, we hypothesized that TNF-α secretion was facilitated by plasmin, the product of uPA-mediated activation of ambient PLG. To address this question, these experiments were repeated using PLG-depleted FBS. To exclude the possibility that lysine-Sepharose adsorption had spurious, unanticipated effects on the ability of FBS to support THP-1 activation, PLG-depleted FBS was also reconstituted with 0.25 μM human PLG. PLG depletion had no effect on the ability of the anti-uPA mAb to suppress LPS-induced TNF-α secretion (**Fig. 2A**), as the results effectively duplicated those shown in Figure 1. Likewise, the addition of exogenous human PLG has no effect, as suppression by the anti-uPA mAb matched the effects seen with normal and PLG-depleted FBS (Fig. 2B). In addition, these experiments were performed in serum-free medium (MAC-SFM; Gibco). LPS induced only a weak increase in TNF-α secretion in this medium, but anti-uPA mAb suppressed TNF-α secretion as it did in the presence of serum (not shown). To eliminate the possibility that PLG was carried

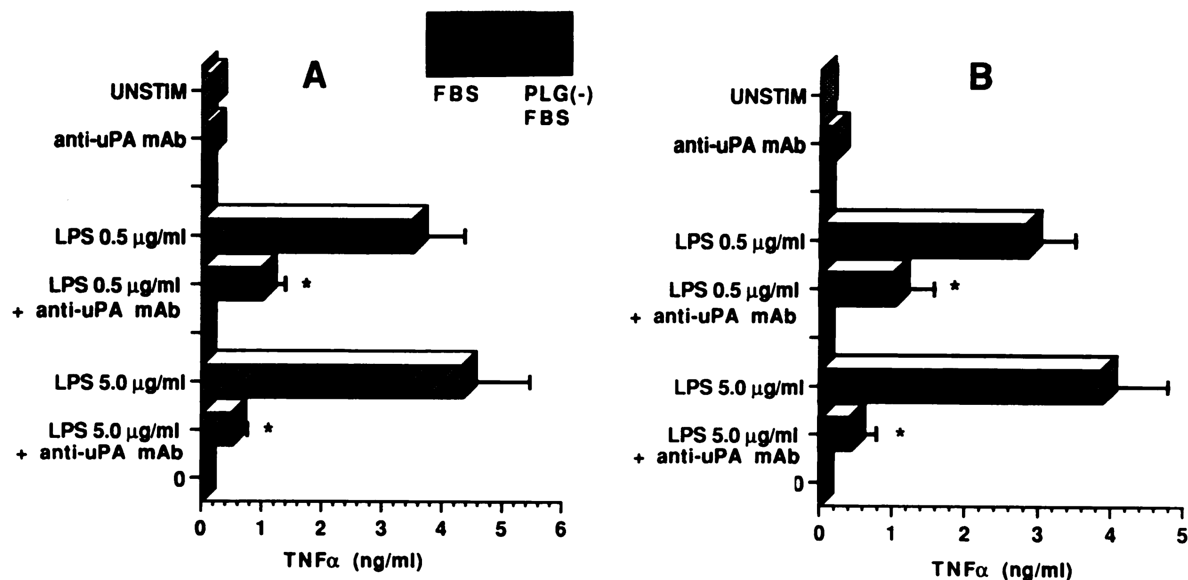

Fig. 2. Inhibitory effect of anti-uPA mAb on LPS-induced TNF-α secretion is independent of plasminogen activation. (A) Depleting fetal bovine serum of plasminogen (PLG) did not alter the inhibitory effect of anti-uPA mAb on LPS-induced TNF-α secretion. Data are expressed as in Figure 1, $n = 6$. $*P < .001$. (B) Reconstituting PLG-depleted fetal bovine serum with 0.25 µM human PLG did not alter the suppressive effects of anti-uPA mAb on LPS-induced TNF-α secretion. Data are expressed as in Figure 1, $n = 6$. $*P < .001$. (Inset: Western blot of normal and PLG-depleted FBS, using a goat anti-bovine PLG antibody. Normal FBS has immunoreactive PLG, which is completely removed by lysine-Sepharose adsorption.)

into the cultures bound to THP-1 plasma membranes, the cells were incubated in 15 mM tranexamic acid for 5 min at 25°C, followed by washes in PBS, to displace PLG from the cell surface [30]. When these cells were cultured in PLG-depleted serum, anti-uPA mAb still suppressed LPS-induced TNF-α secretion, just as in Figure 2A (not shown).

These data suggested that uPA can modulate TNF-α secretion even in the presence of minimal PLG. In further support of this conclusion, we examined the effect of aprotinin at a concentration (10 µg/ml) that effectively inhibits plasmin activity but does not affect uPA activity [31]. Aprotinin had no effect on LPS-stimulated TNF-α secretion (**Fig. 3**). For comparison, cells were treated with recombinant PAI-1, which is a highly specific PA inhibitor with virtually no antiplasmin activity. As noted in Materials and Methods, the PAI-1 (25 ng/ml) inhibited 350 mPU/ml HMW-uPA in an in vitro esterolytic assay, whereas we have previously found only 42 ± 3 mPU/10^6 cells plasma membrane-associated uPA activity on THP-1 cells [17]. Because PAI-1 retains its activity under tissue culture conditions for only a brief period of time [32], it was added repeatedly (5 ng/well) at hours -1, 0, 1, and 3, relative to the addition of LPS. As shown in Figure 3, PAI-1 substantially reduced TNF-α secretion, to 53.1 ± 8.2% of control ($P < .001$), effectively reproducing the effects of the anti-uPA mAb. When added without LPS, neither aprotinin nor PAI-1 induced any increase in TNF-α relative to unstimulated cells (it was also verified that adding PAI-1 at the first time point was unnecessary and adding aprotinin 1 h before LPS produced the same results as adding the two simultaneously).

TNF-α secretion was consistently suppressed by the other uPA inhibitors tested, including recombinant PAI-2, which effectively duplicated the results obtained with PAI-1 (not shown). In addition, amiloride, a potent uPA inhibitor [33], blocks TNF-α secretion quite effectively, but it was not used in this study because it also blocks IL-8 expression [34], unlike the effects of anti-uPA mAb (see below), suggesting other, unrelated mechanisms of action.

Mechanism of anti-uPA mAb–mediated suppression of TNF-α secretion

To determine how anti-uPA mAb suppressed LPS-mediated TNF-α secretion, we first examined whether uPA affects all LPS-mediated signaling. We therefore examined the effects of anti-uPA mAb on secretion of another LPS-responsive cytokine, IL-8. Anti-uPA mAb had no suppressive effect at all on interleukin-8 secretion (**Fig. 4**). In fact, anti-uPA mAb increased IL-8 secretion to 126.4 ± 12.3% of the LPS control, approaching statistical significance ($P = .08$). This would suggest that uPA participates in a signaling mechanism that selectively links LPS stimulation to amplified TNF-α expression, rather than a common pathway for all LPS-mediated cell activation.

To determine the time period over which the anti-uPA mAb is effective, THP-1 cells were stimulated with LPS as described above, and anti-uPA mAb was added either coincidentally with LPS (time 0) or after an interval of 1, 2, 3, or 4 h. The cells were left to incubate for a total of 24 h, and the conditioned medium was harvested. As shown in **Figure 5**, the inhibitory effect of anti-uPA mAb diminished very rapidly, as delaying the addition of mAb for

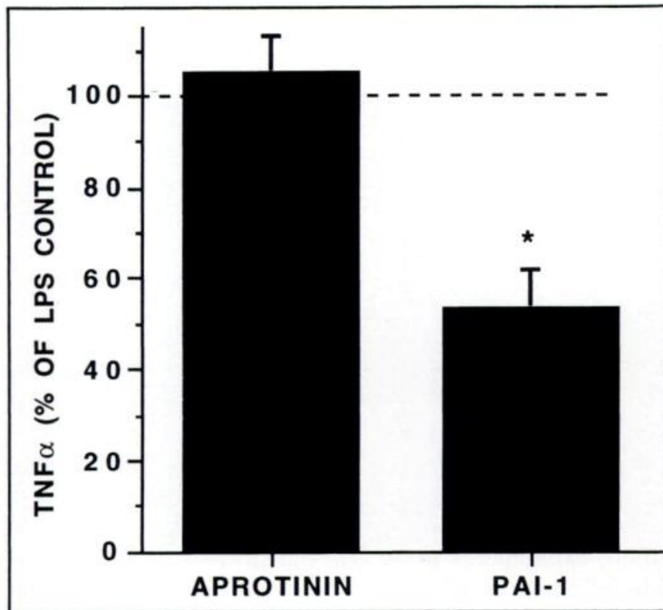

Fig. 3. Aprotinin, a selective plasmin inhibitor at 10 μg/ml, has no effect on TNF-α secretion induced by 0.5 μg/ml LPS, whereas recombinant PAI-1 (added as described in Results) significantly suppresses TNF-α secretion. The values indicate the TNF-α secretion as percentages of the LPS control, mean ± SEM, $n = 10$. *$P < .001$.

even 1 h led to a statistically insignificant effect on TNF-α secretion. This finding is most consistent with an effect on activation signaling, rather than an effect on newly synthesized TNF-α.

Analysis of TNF-α mRNA levels was performed to delineate more precisely the stage in LPS-induced TNF-α expression that is modulated by uPA. Representative Northern blots are shown in **Figure 6.** Preliminary experiments established that the LPS-induced increase in TNF-α mRNA levels was evident at 1 h, with a peak at 2–3 h, and substantially waned by 4–5 h (not shown). Coincubating THP-1 cells with the anti-uPA mAb or PAI-1 substantially reduced the LPS-driven up-regulation of TNF-α mRNA, most prominently at the 3-h time point. To demonstrate the reproducibility of these results, densitometry measurements were performed on three separate Northern blots, comparing LPS versus LPS + anti-uPA mAb at the 3-h time point (one of these experiments is shown in Fig. 6). In these three experiments, coincubation with anti-uPA mAb reduced the density of the TNF-α mRNA band by 55 ± 6% ($P < .02$). These findings show that uPA is involved in regulating TNF-α neosynthesis rather than terminal processing of preformed TNF-α precursor, conforming well with the early time frame during which uPA exerts its effects.

Binding PAI-1 or the anti-uPA mAb to receptor-associated uPA on the plasma membrane may trigger internalization of the receptor-ligand complex [35]. To determine whether this is a potential mechanism for the effects of these proteins on TNF-α secretion, cells were incubated with the anti-uPA mAb or PAI-1 for 20, 40, or 60 min, along with LPS (0.5 μg/ml), as described above. The level of uPAR on the cell surface was then determined by immunolabeling, using anti-uPAR mAb + phycoerythrin–goat anti-mouse Ig (PAI-1 pretreatment) or direct labeling with FITC–anti-uPAR mAb (anti-uPA mAb pretreatment). uPAR was quantitated only within 1 h of stimulation, because anti-uPA mAb suppresses LPS-driven TNF-α secretion only within this narrow time frame. As shown in **Figure 7,** the anti-uPA mAb had no effect at all on uPAR expression. The indirect staining method used for cells treated with PAI-1 yielded a weaker and more variable signal, but still there was no effect on uPAR at 20 and 40 min and there was a statistically insignificant downward trend at 60 min. Thus, it is unlikely that these interventions affect TNF-α secretion by causing a net loss of uPA-uPAR complexes from the plasma membrane.

Finally, experiments were performed to determine whether specific elements of the uPA structure are responsible for modulating TNF-α secretion. THP-1 cells were washed for 5 min with 50 mM glycine buffer, pH 3.0, to displace endogenously generated uPA from plasma membrane uPAR [1]. Cells were immediately resuspended in culture medium with LPS (0.5 μg/ml), along with 1, 10, or 100 nM concentrations of HMW-uPA (intact), low-molecular-weight (LMW)-uPA (carboxyl terminal fragment bearing the enzymatic active site but no uPAR-binding domain), or uPA-ATF (amino terminal fragment bearing the uPAR-binding domain but no active site). These concentrations were selected from preliminary data indicating that a 100 nM concentration of uPA-ATF maximally displaced endogenous uPA from cell surfaces. After culture for 24 h, secreted TNF-α was measured, as described previously. The TNF-α secretion of acid-washed cells was

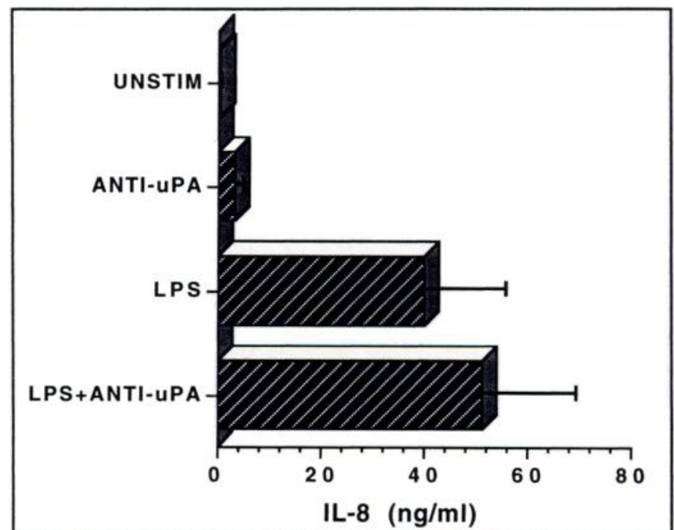

Fig. 4. Anti-uPA mAb does not inhibit IL-8 secretion induced by 0.5 μg/ml LPS. Unstimulated THP-1 Mϕs and cells incubated with anti-uPA mAb alone expressed < 4 ng/ml IL-8, and LPS induced more than a 10-fold increase in IL-8 secretion during a 24-h incubation ($P < .001$). Bars indicate the mean ± SEM IL-8 levels in conditioned medium, $n = 6$. The addition of anti-uPA mAb to LPS treatment did not reduce TNF-α secretion but rather increased secretion to 126.4 ± 12.3% of the LPS control ($P = .08$).

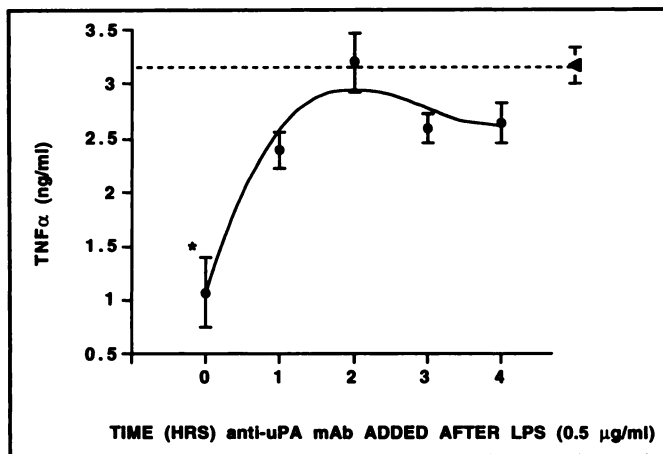

Fig. 5. Time course of TNF-α inhibition by anti-uPA mAb. THP-1 cells were stimulated with 0.5 μg/ml LPS, anti-uPA mAb was added either simultaneously (time 0) or after 1, 2, 3, or 4 h, and TNF-α secretion was measured at 24 h. Anti-uPA significantly reduced TNF-α secretion only at time 0. *$P < .05$, using a paired t-test and Bonferroni's correction; $n = 5$. The triangle indicates LPS (0.5 μg/ml) alone, mean ± SEM.

reduced and somewhat variable compared with previous experiments. This certainly raises the possibility that the acid treatment has nonspecific effects on THP-1 cell function, perhaps by displacing ligands from other surface receptors. Nonetheless, the addition of HMW-uPA augmented the TNF-α output of LPS-stimulated cells in five of five separate experiments (**Fig. 8**). By contrast, LMW-uPA progressively consistently reduced TNF-α secretion, even though the specific activities of the HMW-uPA and LMW-uPA preparations are quite similar (1 μM = 4400 IU/ml and 5280 IU/ml, respectively). Therefore, the catalytic function of uPA alone is not sufficient to enhance TNF-α secretion, consistent with the observed independence of plasminogen activation. Suppression of TNF-α secretion by LMW-uPA further suggests that this uPA fragment may be competing with intact HMW-uPA remaining on the cell surface after acid treatment. Alternatively, LMW-uPA may be competing with HMW-uPA released from internal stores early during LPS stimulation. Finally, uPA-ATF has no effect on TNF-α secretion ($n = 4$), indicating that uPAR occupancy alone cannot account for the effects of uPA. Thus, the intact structure of HMW-uPA is necessary for modulating TNF-α secretion. The mechanism underlying the effect of HMW-uPA remains to be elucidated, but HMW-uPA is known to participate in activation signaling in neutrophils, a process that requires intact uPA and coparticipation with uPAR and the adhesion protein CR3 [12].

DISCUSSION

In this study, we have shown that uPA generated by the THP-1 Mϕ line is necessary for maximal induction of TNF-α neosynthesis by LPS. An mAb specific for the uPA active site reduces TNF-α secretion by approximately 60%, and PAI-1, a highly specific PA inhibitor, has an identical effect, suppressing TNF-α secretion by approximately 50% (Figs. 1 and 3). Moreover, uPA appears to be directly responsible for this effect, as plasmin formation is not required as an intermediate step. This is supported by demonstrating that neither plasminogen depletion nor aprotinin (a potent plasmin inhibitor) influences the suppressive effects of the anti-uPA mAb (Figs. 2 and 3). Moreover, exogenous uPA enhances TNF-α secretion only if provided in the high-molecular-weight form; the low-molecular-weight form effectively blocks TNF-α secretion, despite its preserved catalytic activity (Fig. 8). Collectively, these findings indicate that a region of uPA very near the active site is necessary for modulating TNF-α secretion, and although uPA catalytic activity alone cannot account for this effect, it may still prove to be one of several elements of uPA function necessary for modulating TNF-α expression. Future experiments using DFP-inactivated uPA and peptide inhibitors of uPA should be very helpful in defining the role of uPA activity, although the possibility will remain that the active site region of uPA serves other functions, and modulating the active site may affect TNF-α expression by mechanisms unrelated to blocking cleavage of a uPA substrate. For this reason, defining the mechanisms of action of uPA will be best achieved by fully characterizing any partner proteins and substrates of uPA cleavage during LPS stimulation. This information will also assist in defining the role of uPAR binding. At present, the data indicate that the uPAR-binding region of uPA is necessary, but not sufficient, to regulate TNF-α secretion.

Another novel conclusion of this study is that modulating uPA function affects TNF-α neosynthesis at the mRNA level (Fig. 6). The suppression of TNF-α mRNA up-regulation by anti-uPA mAb and PAI-1 is certainly sufficient to explain the observed effects on TNF-α secretion. The mechanism linking uPA to up-regulating TNF-α mRNA is a key question, and some intriguing prospective mechanisms must be considered. There is a growing appreciation that uPA has noncatalytic functions relevant to Mϕ activation. In an Mϕ-like cell line, uPA enhances uPAR-mediated adhesion to vitronectin and also promotes phenotypic differentiation [14, 15]. Binding uPA to uPAR initiates the generation of intracellular signal transduction elements, as evidenced by tyrosine phosphorylation of a 38-kd partner protein in U937 cells [13] and by calcium fluxes generated in neutrophils [12]. The impact of these events on Mϕ activation is poorly understood, as are the mechanisms by which these responses are triggered. The urokinase receptor is anchored to the plasma membrane by a glycosylphosphotidylinositol linkage [36]. Like other surface proteins lacking a transmembrane segment, uPAR (or uPA-uPAR complexes) may utilize partner proteins to transmit activation signals. In neutrophils, CR3 (CD11b/CD18; Mac-1), a leukadhesin of the β_2 integrin family, is an obligate signaling partner for uPA in triggering a calcium flux upon binding to uPAR [12]. Much akin to the present findings, only intact uPA could initiate this response, and both carboxyl and amino terminal fragments were ineffective. In this re-

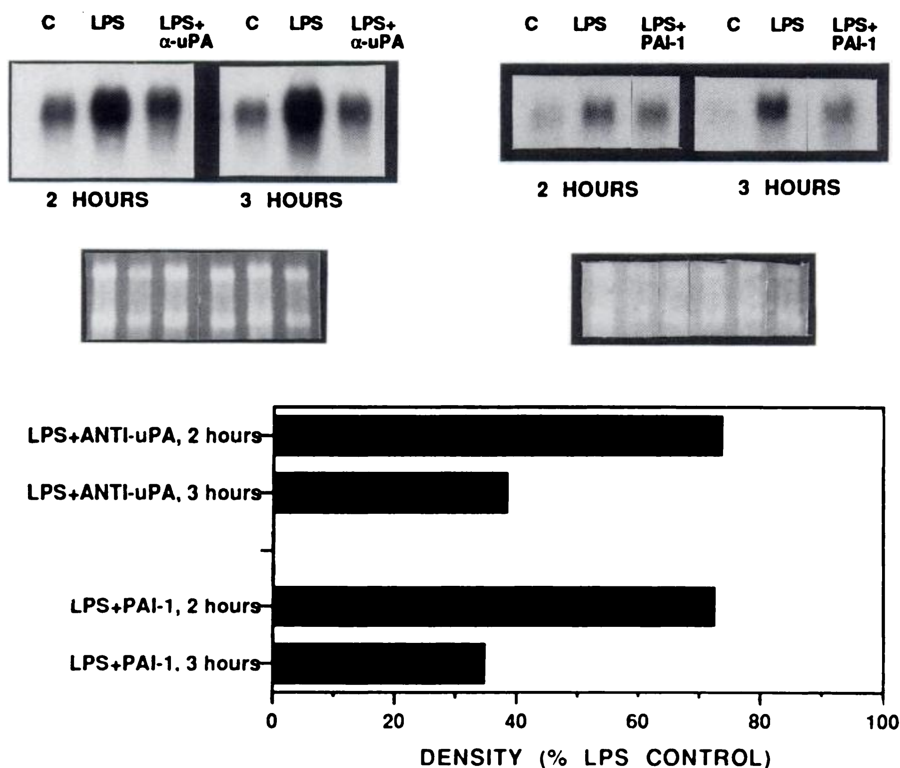

Fig. 6. Anti-uPA mAb and PAI-1 suppress LPS-induced up-regulation of TNF-α mRNA levels. Cells were cultured for 2 and 3 h in medium alone, 0.5 μg/ml LPS, LPS + anti-uPA mAb, and LPS + PAI-1 (as indicated in Materials and Methods) and the Northern blots were probed with TNF-α cDNA. TNF-α mRNA expression was up-regulated by LPS at 2 and 3 h. Coincubation with anti-uPA mAb or PAI-1 substantially reduces the increase in TNF-α mRNA. UV-illuminated ribosomal bands on the nylon filter are shown to confirm equal loading and transfer of RNA. Density measurements of the Northern blots show that TNF-α mRNA levels of cells cotreated with anti-uPA mAb or PAI-1 were reduced by 30% at 2 h and 60–65% at 3 h relative to the LPS control at the appropriate time point.

gard, it is also noteworthy that CR3 has been shown to be necessary for LPS-driven TNF-α secretion by THP-1 cells [16]. The possibility that formation of a uPA-uPAR-CR3 complex is a proximate means for regulating Mϕ activation is under investigation. This putative mechanism is consistent with the rapid, short-lived action of uPA but eventually must also satisfy the requirement for modulating cytokine output in a selective fashion. The ability of uPA to augment TNF-α secretion without affecting IL-8 secretion will be useful in determining the mechanism of action, because prior studies have shown that TNF-α and IL-8 are generally regulated in tandem [37, 38].

In addition to the uPAR-mediated effects on adhesion and activation signaling, the catalytic activities of both uPA and plasmin can significantly affect other aspects of Mϕ activation, as well as the composition of the inflammatory milieu. Plasmin is a broad-spectrum endopeptidase with a substrate range similar to that of trypsin, whereas uPA has very few known substrates other than plasminogen [2]. These include fibronectin, type IV gelatinase/collagenase, hepatocyte growth factor/scatter factor (HGF/SF), and uPAR itself [31, 39–41]. It is impossible at present to implicate any of these specific uPA substrates in modulating TNF-α expression. The 120-kd cell-binding fragment of fibronectin can stimulate TNF-α secretion in human monocytes, but uPA digestion is not known to yield a fragment with this activity [41, 42]. At present, uPAR cleavage products have no defined intrinsic biologic activities, other than vitronectin binding by domain 2/3 fragments [15]. Ultimately, it will be necessary to characterize

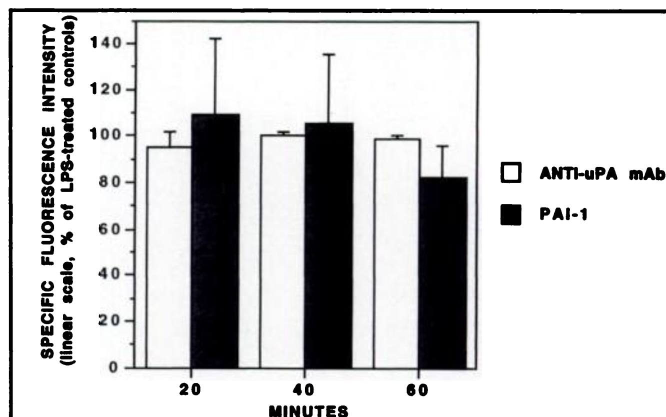

Fig. 7. Cell surface expression of uPA receptor (uPAR) by LPS (0.5 μg/ml)–treated cells is not significantly affected by anti-uPA mAb or by rPAI-1 (5 ng/ml) for 20, 40, or 60 min. uPAR was measured by immunofluorescence flow cytometry, using direct labeling with FITC–anti-uPAR mAb (anti-uPA mAb treatment) or indirect labeling (PAI-1 pretreatment). Specific fluorescence intensity (nonspecific staining subtracted, linear scale) is expressed as a percentage of LPS-treated controls, labeled and examined in parallel for each time point.

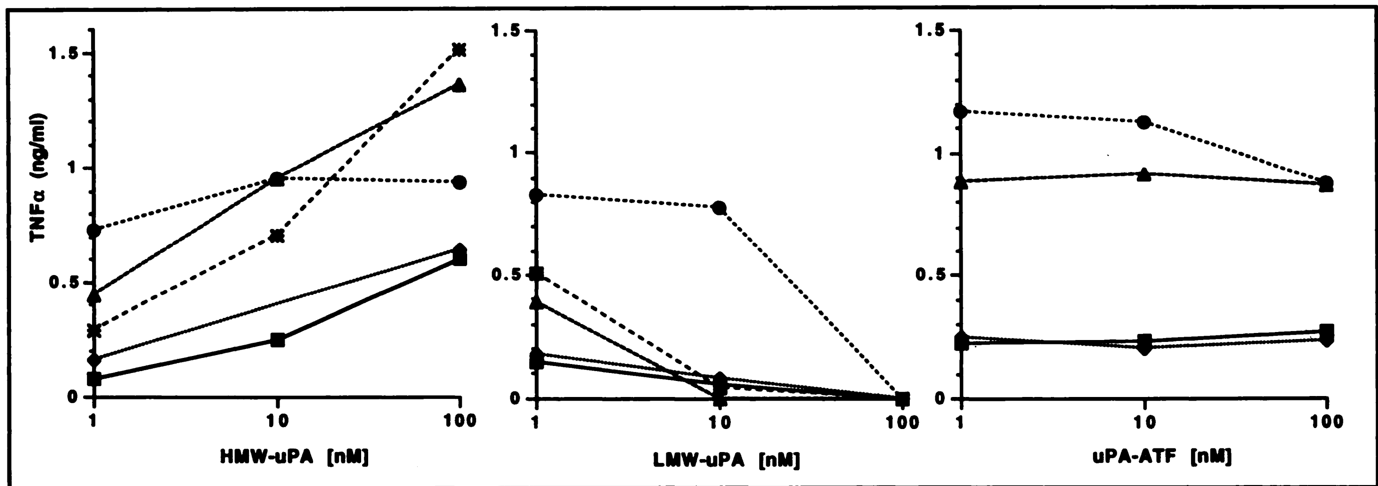

Fig. 8. Effects of intact uPA and uPA fragments on LPS-driven TNF-α secretion by acid-washed THP-1 cells. Intact, high-molecular-weight uPA (HMW-uPA) augments TNF-α secretion in a dose-related fashion, whereas low-molecular-weight uPA (LMW-uPA), bearing only the catalytic domain, has a dose-related suppressive effect. The uPA amino terminal fragment (uPA-ATF) bearing only the receptor-binding domain does not affect TNF-α secretion. The results of five (HMW-uPA and LMW-uPA) and four (uPA-ATF) separate experiments are shown. Each set of symbols represents the data obtained from a single experiment, assayed in duplicate.

the range of substrates cleaved by uPA during LPS stimulation to discriminate fully between the catalytic and noncatalytic mechanisms by which uPA affects activation signaling.

The uPA regulation of TNF-α synthesis is one of many examples of proteases modulating the function and secretory output of Mϕs. Collectively, these proteases, many of which are traditionally viewed as elements of the coagulation and fibrinolytic pathways, act at all phases of Mϕ activation. Some, such as thrombin and factor Xa, act as costimuli in inducing IL-1 expression [43]. The mechanisms underlying these effects are not well understood, but Mϕs express receptors for both thrombin and factor X, and some activation signals have been shown to require proteolytic action, whereas others require only receptor binding without any requirement for enzymatic action [43–45]. In other instances, uPA and plasmin activities exert postsynthetic regulation of the expression and function of cytokines and growth factors. Endogenous uPA activity is necessary for release of IL-1 from its membrane-bound form, but, unlike the present findings, active plasmin was actually responsible for facilitating IL-1 secretion [11]. Plasmin also converts latent transforming growth factor β precursor to its active form, and uPA directly activates HGF/SF precursor [9, 10]. Finally, plasmin can enhance the bioactivity of basic fibroblast growth factor by releasing it from extracellular matrix proteins [46]. Our demonstration that uPA affects initial up-regulation of TNF-α neosynthesis is quite distinct from established effects of the uPA-plasmin system on terminal processing of nascent cytokines. Unspecified serine proteases susceptible to *p*-toluene-sulfonyl-L-arginine methyl ester have been implicated in releasing the membrane-bound 26-kd form of TNF-α from Mϕ plasma membranes to yield a mature 17-kd product in conditioned medium [18, 19]. More recent evidence indicates that this conversion from 26-kd to 17-kd forms is also regulated by a unique metalloproteinase [20, 47]. These mechanisms of action cannot be reconciled with the findings of the present study, suggesting that multiple proteases regulate sequential steps in TNF-α synthesis and secretion.

The results of this in vitro study suggest several potentially important mechanisms by which uPA may regulate inflammation in vivo. Local fluctuations in the expression of uPA activity have been implicated in the pathogenesis of inflammatory injury in vivo [2, 8, 48–51]. The influx of plasma-borne PA inhibitors is responsible for the defective local fibrinolytic activity observed in severely inflamed lung tissue [48, 49]. Similarly, sequential expression of PA activity and PA inhibitors has been described in cutaneous granulomas [50]. Because uPA gene expression is itself responsive to many cytokines, including TNF-α, these observations complete a potential pathway of reciprocal activation that amplifies expression of both cytokines and proteases at inflammatory foci [1]. They further suggest that PA inhibitors such as PAI-1 play a beneficial physiologic role by restraining the injurious effects of TNF-α expression in inflammatory diseases. PAI-1, induced by endotoxemia, would be appropriately up-regulated to play this role in the sepsis syndrome [52]. The other venue in which the interaction between uPA and TNF-α may be particularly important is in the role of Mϕs as accessory cells in immune responses [53–55]. Lymphocyte-derived products can regulate expression of Mϕ uPA and PA inhibitors, thereby engaging TNF-α and uPA in mutual feedback regulation that would affect subsequent cascades of intercellular communication [1].

In summary, we have shown that uPA produced endogenously by THP-1 Mϕs is necessary for optimal TNF-α neosynthesis in response to LPS. This regulatory function of uPA is independent of its function as a plasminogen activator and requires contributions from the region of its

active site, as well as the uPAR-binding domain. This represents a novel pathway by which the uPA-uPAR system, already linked to cellular migration and adhesion, also exerts feedback regulation on the early phases of Mϕ activation.

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LETTERS TO THE EDITOR

Within the spectral forms of leprosy cellbound CD30 cannot be regarded as an operational marker for the Th2-like reaction.

Romagnani et al. describe CD30 as a marker for the Th2-type reaction, where CD30 is preferentially expressed and its soluble form released by human T cell clones producing Th2-type cytokines [1]. The in vivo relevance is discussed by showing that high numbers of CD30+ T cells are found in the lymph nodes of a patient suffering from Omenn's syndrome and the detection of circulating CD30+ T cells in atopic patients, both disorders being associated with a Th2-type immune response. The spectral forms of leprosy represent a model system for the human Thl/Th2 reaction in as much as Thl reactions predominate in the tuberculoid and Th2 reactions in the lepromatous form of this disease [2]. Thus, we investigated the application of CD30 as an operational Th2 marker in vivo by performing immunohistological staining on frozen sections of skin biopsies from patients with lepromatous ($n = 15$) and tuberculoid ($n = 4$) leprosy. Hyperplastic human tonsils and five lymphnode biopsies from patients suffering from Hodgkin's disease (nodular sclerosis) served as controls. We evaluated parallel stainings from CD8 (Leu2a, Becton-Dickinson), CD4 (Leu3a, Becton-Dickinson), CD26 (MIB DS2-7 [3]) and CD30 (Ki-1 [4], Ber-H2 [5]) on these sections. Staining for CD26 was included as an operational Thl marker, since high expression of CD26 is found in tuberculoid leprosy in contrast to no or very little expression in lepromatous leprosy. In addition, double immunostaining demonstrated the coexpression of CD26 and IFNγ of T-cells in tuberculoid leprosy [3]. As expected and described earlier [4], in frozen tonsil sections positive staining with the antibodies Ki-1 and Ber-H2 was restricted to a few extrafollicular large cells located at the rim of germinal centers and sometimes within the B-cell follicles. In all Hodgkin cases investigated, the tumor cells, i.e., Hodgkin and Reed-Sternberg cells, proved to be positive with the anti CD30 antibodies used. In contrast, in the leprosy cases a maximum of three to four CD30 positive T-cells were found per section, and there was no difference in the accumulation of CD30 positive cells between the tuberculoid and lepromatous form of leprosy. From these results we conclude that CD30 does not represent an operational Th2 marker in this disease model. Because of the lack of serum we could not elucidate the impact of soluble CD30; however, in light of our findings it may be of interest to investigate this aspect in the different forms of leprosy as well.

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Ulrike Seitzer, Hans-Dieter Flad, Johannes Gerdes
Division of Molecular Immunology
Department of Immunology and Cell Biology
Forschungsinstitut Borstel, Germany

Culturing unactivated monocytes

In certain situations it is necessary to culture unactivated monocytes. Therefore, all solutions used to isolate, prepare, and culture the monocytes must be free of endotoxin or any other factor that would induce monocyte activation. We found that certain commercially available leukocyte separation media (density gradient solutions) appeared to induce cultured monocytes to express tissue factor (TF) activity even though they tested negative for endotoxin (as tested by the limulus lysate test). Therefore, we compared monocyte TF expression following isolation on three different density gradient media: