Urokinase-deficient and urokinase receptor-deficient mice have impaired neutrophil antimicrobial activation in vitro

Margaret R. Gyetko,[1] David Aizenberg, and Laura Mayo-Bond

Pulmonary and Critical Care Medicine Division, Department of Internal Medicine, Ann Arbor Veterans Affairs Medical Center and University of Michigan Medical Center, Ann Arbor

Abstract: Leukocytes express both urokinase-type plasminogen activator (uPA) and the urokinase receptor (uPAR, CD87). We have shown that neutrophil recruitment to the lung during *P. aeruginosa* pneumonia is impaired in uPAR-deficient (uPAR−/−) mice but is normal in uPA−/− mice. However, both uPA−/− mice and uPAR−/− mice have impaired lung clearance of *P. aeruginosa* compared with wild-type (WT) mice. To determine the role of uPA and uPAR in antibacterial host defense, we compared neutrophil bacterial-phagocytosis, respiratory burst, and degranulation among uPA−/−, uPAR−/−, and WT mice. Neutrophil phagocytosis was significantly diminished comparing uPA−/− and uPAR−/− mice with WT mice at all time points. The generation of superoxide by both uPA−/− and uPAR−/− neutrophils was about half of that seen in WT neutrophils. Degranulation of azurophilic granules was significantly diminished in uPA−/− neutrophils compared with either uPAR−/− or WT neutrophils. By contrast, agonist-stimulated release of specific granules was not diminished in either uPA−/− or uPAR−/− mice compared with WT. We conclude that the uPA/uPAR system modulates several of the crucial steps in neutrophil activation that result in bacterial killing and effective innate host defense. *J. Leukoc. Biol.* 76: 648–656; 2004.

Key words: *cellular activation · phagocytosis · inflammation · transgenic/knockout*

INTRODUCTION

To participate in innate immunity, neutrophils must accomplish several critical functions. First, during recruitment to an infectious site, the cells must sense and become oriented to a chemotactic gradient and then move through a complex sequence of adhesion, cytoskeletal rearrangement, movement, and detachment steps [1, 2]. Neutrophils then must egress from the vascular compartment and traverse tissue planes; a process that might require extracellular proteases [3–5]. Finally, in order to effect bacterial killing, the neutrophil must engulf the invading pathogen, produce reactive oxygen intermediates—particularly superoxide (O_2–) via the activity of NADPH oxidase—and release proteolytic and bactericidal proteins from cytoplasmic granules.

Neutrophils express urokinase-type plasminogen activator (uPA) and high-affinity receptors for uPA (uPAR, CD87) on the plasma membrane. This action enables them to convert the proenzyme plasminogen to active plasmin and concentrate it in the immediate pericellular environment during cellular migration [6–8]. Plasmin is a protease of broad specificity that can either directly or indirectly, through the activation of latent metalloproteinases, degrade extracellular matrix proteins and basement membranes [6, 9, 10]. In addition to providing a plasma membrane docking site for uPA, uPAR, which is a glycosylphosphatidylinositol-anchored receptor, can couple with several other receptors on the cell surface, most notably in neutrophils, the β2 integrin CD11b/CD18. We have demonstrated previously that uPAR, by partnering with CD11b/CD18, facilitates the integrin's adhesive and chemotactic functions [11–13]. In addition to these in vitro function, we have also demonstrated is the crucial role that uPAR plays in CD11b/CD18-dependent neutrophil recruitment to the lung during *P. aeruginosa* pneumonia in vivo. Mice deficient in uPAR (uPAR−/−) had markedly decreased neutrophil recruitment to the lung and had significantly impaired intra pulmonary killing of the pathogen. By contrast, in the same *P. aeruginosa* pneumonia model, mice deficient in uPA (uPA−/−) recruited neutrophils indistinguishably from wild-type (WT) mice. However, despite the abundant numbers of neutrophils present in the lungs of uPA−/− mice infected with *P. aeruginosa*, bacterial killing was impaired [14]. This finding suggests that neutrophils do not activate normal bactericidal mechanisms in the absence of uPA, despite normal recruitment to the site of infection.

In the current study we sought to determine whether uPA and/or uPAR are required for antibacterial neutrophil activation. We purified neutrophils from WT, uPA−/−, and uPAR−/− mice and assessed bacterial phagocytosis, superoxide generation, and degranulation responses.

[1] Correspondence: Pulmonary and Critical Care Medicine Division, Department of Internal Medicine, Ann Arbor Veterans Affairs Medical Center and University of Michigan Medical Center, 3916 Taubman Center Medical Center Drive, Ann Arbor, MI 48109-0360. E-mail: mgyetko@umich.edu

Received January 14, 2004; revised May 17, 2004; accepted May 28, 2004; doi: 10.1189/jlb.0104023.

METHODS

Animals

Mice were housed in specific pathogen-free isolation rooms in the University of Michigan Department of Laboratory Animal Medicine, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved by the animal care committees of the Veterans Administration and the University of Michigan Committee on Use and Care of Animals (UCUCA).

Transgenic uPAR deficient mice (uPAR−/−), uPA-deficient mice (uPA−/−), and background-matched control mice (WT) were generous gifts from Peter Carmeliet. These mice were developed as described previously [15, 16]. Genotype of the uPA, uPAR−/−, and WT mice was confirmed by PCR or RT-PCR analysis as described previously [16, 17]. Mice of this background (C57B6/129) are immunocompetent [18, 19].

Antibodies and materials

FITC-conjugated anti-murine CD11b (M1/70.15) mAbs and rat IgG (control) were from PharMingen (San Francisco, CA). Active Mouse Urokinase (MUPA-903) was obtained through Molecular Innovations, Inc. (Southfield, MI).

Quantification of phagocytosis

Mice were injected i.p. with 0.1 ml heparin (1000 units/ml). After 2 min, the mice were humanely killed and bled from the abdominal aorta into tubes containing 0.1 ml of 25 units/ml heparin. Blood was immediately placed on ice. Then, 50 μl was aliquoted into 12 × 75 mm polypropylene tubes containing 50 μl phosphate buffered saline (PBS) BODIPY FL- *E. Coli* (Molecular Probes, Eugene, OR), sonicated in a Branson Ultrasonic Cleaner (Danbury, CT) in three 20 s bursts, and placed on ice. After which, 0.1 μl was aliquoted into each tube containing whole blood. Samples were either kept on ice as negative controls or warmed to 37°C and gently shaken (Lab-Line Microprocessor Orbit Shaker Water Bath at 70 rpm, Lab-Line Instruments, Inc., Melrose Park, IL). At the indicated time points, samples were removed, phagocytosis was halted by rapid chilling on ice, and extracellular fluorescence was quenched by using 0.25 mg/ml Trypan Blue (Sigma Chemical Co., St. Louis, MO). Immediately, 2 mls of ice-cold PBS was added, and samples were centrifuged 5 min at 400 rpm. The samples were then washed, and the RBC was lysed by using a Whole Blood Lysing Reagent Kit (Coulter Corporation, Miami, FL). Samples were then washed twice and resuspended in 300 μl PBS. Cells were then either centrifuged onto glass slides and stained with hematoxylin and eosin for fluorescent photomicroscopy or were stained with 0.5 μg/ml propidium iodide (to exclude dead cells) and fluorescence intensity determined on a Beckman Coulter Elite ESP Flow Cytometer (Coulter Corporation). Effectiveness of quenching was documented by the absence of fluorescence in negative control cells. Separate samples were stained with R-PE conjugated Ly-6G (GR-1, PharMingen) and used to set the neutrophil gate.

Generation of recombinant murine uPA for repletion of uPA−/− PMN during phagocytosis

uPA binding to uPAR is species-specific. We generated murine uPA by adenovirus-mediated murine uPA transfection in vitro, as described previously with minor modifications [20]. Briefly, human lung epithelial cells (A549) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Confluent cells were split into six-well plates at a 1:5 dilution 24 h prior to infection. At 18 h, the media were changed, and at 24 h 10^8 virus particles containing either the murine uPA cDNA construct or an empty cassette were added to each well. After 4 h at 37°C, the supernatants were aspirated, the cells were washed, fresh media were added, and the cells were incubated at 37°C. Conditioned media were collected 24 h later and analyzed for uPA activity, as described previously [20]. Conditioned media of cells infected with adenovirus-murine uPA (murine uPA media) had 4.5 plough units/ml of uPA activity, whereas conditioned media of cells infected with the control adenovirus (control media) had no detectable uPA. Phagocytosis was quantified as above, except that the collected blood (50 μl) was aliquoted into 12 × 75 mm polypropylene tubes containing 50 μl of murine uPA media, or control media, and was incubated for 60 min at room temperature prior to the addition of BODIPY FL-*E. Coli* (Molecular Probes).

Isolation of murine peritoneal neutrophils

Neutrophils were elicited by two i.p. injections of 9% casein (1 ml, Sigma) 18 h apart. Three hours later, the mice were humanely killed. The peritoneal cells were harvested into PBS + 0.02% EDTA, washed and layered onto a three-step Percoll (Amersham Pharmacia, Piscataway, NJ) gradient (1.07, 1.06, 1.05 g/ml), and centrifuged at 400 rpm for 45 min at 4°C; the neutrophil band was removed. Neutrophil purity was confirmed by Tissue-Tek II staining (Richard-Allan Scientific, Kalamzoo, MI) and visual inspection. Cells were adjusted to 6×10^5/ml in HBSS (Invitrogen, Carlsbad, CA).

Determination of superoxide production

Neutrophils were purified as above, and RBCs lysed by using a Whole Blood Lysing Reagent Kit (Coulter Corporation). Cells were adjusted to 5×10^6/ml in PBS/0.9 mM Ca/0.5 mM Mg /7.5 mM glucose. Cytochrome C (Type VI, Sigma) was dissolved in PBS/1.5 mM glucose. Superoxide dismutase (Sigma) was dissolved in endotoxin-free water at 3 mg/ml. Samples were assayed in the presence and absence of superoxide dismutate. PBS/glucose (176 μl), cytochrome *c* (12.5 μl), SOD or water (10 μl), and cells mixture (50 μl) was placed into a 96-well, flat-bottom plate (Costar 3596). For experiments using fMLP, Cytochalasin B (Sigma) was added at 2 μg/ml. Wells were mixed and incubated at 37°C in a Spectramax 190 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA) for 3 min. fMLP (Calbiochem, La Jolla, CA) or phorbol 12-myristate 13-acetate (PMA; Sigma) in the indicated concentration was quickly added, and the assay was monitored kinetically for 12 min at 550 nm. Vmax was used to calculate the rate, using the extinction coefficient 14.75 μmol/cm^2, determined as described previously [21]. The rate of superoxide generation is expressed as nmol 0_2–/min/ml/10^7 cells and represents superoxide generation that is completely inhibited by superoxide dismutase. Superoxide generation by neutrophils in the absence of fMLP or PMA triggering was undetectable. For repletion experiments neutrophils were preincubated with recombinant murine uPA (1 μg/ml, MUPA-903, Molecular Innovations Inc., Southfield, MI) for 5 m at 37°C prior to assay.

Determination of degranulation of azurophilic granules by myeloperoxidase release

Myeloperoxidase release was used as a marker for degranulation of azurophilic granules. Purified neutrophils were adjusted to 5×10^6/ml in Dulbecco's PBS/CaMg (Invitrogen). Cells were pre-incubated with Cytochalasin B (5 μg/ml, Sigma) for 3 min at 37°C, followed by the addition of 10^{-7} M fMLP (Calbiochem, La Jolla, CA). For repletion experiments, neutrophils were pre-incubated with recombinant murine uPA (1 μg/ml, MUPA-903, Molecular Innovations, Inc.) for 5m at 37°C prior to the addition of fMLP. Samples were removed at various time points, centrifuged at 400 g for 8 min, and the supernatants removed. Buffer (140 μl) was added (88 mM KH_2PO_4/12.4 mM K_2HPO_4/0.5% H_2O_2/0.17 mg/ml o-dianisidine HCl), and the plate was read kinetically on a SpectraMax 190 at 490 nm. The rate of myeloperoxidase (MPO) release is expressed as mOD/min/ml/10^6 cells. To determine total cellular MPO content, 2.5×10^6 cells were lysed in 0.5 ml RIPA lysis buffer [22] and centrifuged at 14,000 rpm at 4°C. A dose-curve of lysate supernatant was aliquoted into microplate wells (1–10 μl/well) and was analyzed kinetically for MPO activity as described above.

Determination of expression of cell surface expression of CD11b

Cell surface expression of CD11b was used as a marker for specific granule degranulation. Purified neutrophils were treated with Cytochalasin B and fMLP, as above, to induce degranulation. Cells were then centrifuged at 400 rpm for 5 min and resuspended in FA Buffer (Difco Laboratories, Detroit, MI), which contained 1% FBS/ 0.1% sodium azide at 5×10^6 cells/ml. Cells were stained with CD11b-FITC (PharMingen), and mean fluorescence intensity was determined on a Coulter Elite ESP (Beckman Coulter, Hialeah, FL).

Statistical analysis

Comparisons between group means were performed by paired or unpaired Students *t*-tests where appropriate or by ANOVA. Statistical calculations were done using StatView 5.0 software (Abacus Concepts, Berkeley, CA); *n* =

number of mice in each experimental group. Data are expressed ± SEM. Statistical difference was accepted at $P \leq 0.05$.

RESULTS

Comparison of neutrophil phagoyctosis in WT, uPA−/−, and uPAR−/− mice

Phagocytosis by peripheral blood neutrophils was compared by quantifying ingestion of fluorescently labeled *E. coli* by fluorescence photomicroscopy and flow cytometry. Fluorescence photomicroscopy (**Fig. 1**) shows the brightly fluorescent *E. coli* within neutrophils obtained from WT, uPA−/−, and uPAR−/− mice. Nearly all the WT neutrophils ingested bacteria. Further, each WT phagocytic neutrophil ingested several bacteria. By contrast, a very different pattern was seen when phagocytosis by uPA−/− and uPAR−/− neutrophils was determined. Fewer of the uPA−/− or uPAR−/− neutrophils ingested bacteria. Of those that were phagocytic, fewer bacteria were ingested per cell. When subjected to flow cytometry (**Fig. 2**), the number of particles ingested and the number of cells that have ingested bacteria become clearly evident. As shown in Fig. 2A when normalized to WT neutrophils (100 percent), fewer uPA−/− and uPAR−/− neutrophils ingested bacteria. Even more striking is when the mean fluorescence of the cell populations is assessed (Fig. 2B). This finding reflects the number of bacteria ingested per cell and evaluates only those neutrophils that ingested at least one bacteria. The uPA−/− and the uPAR−/− neutrophils ingest far fewer bacteria per cell than do WT neutrophils. This difference becomes more pronounced when evaluated over time.

Phagocytosis following repletion of uPA−/− neutrophils with murine uPA

We next sought to determine whether repletion with recombinant murine uPA would enhance the phagocytosis seen by uPA−/− neutrophils. To accomplish this assessment, neutrophils from uPA−/− mice were cultured with either the media from A549 cells transfected with murine uPA or with media from sham-transfected A549 cells (with the adenovirus vector containing only an empty cassette). As demonstrated in **Fig. 3**, uPA repletion of uPA−/− neutrophils enhanced phagocytosis as shown by the mean fluorescence of phagocytic cells. Thus, the uPA−/− neutrophils demonstrate enhanced phagocytosis when repleted with murine uPA when compared with uPA−/− neutrophils without uPA repletion. Further, the mean log fluorescence of the uPA-repleted uPA−/− neutrophils approached that seen in WT neutrophils at 15 m (70.1 ± 23.5 vs. 148.5 ± 0.3, P=NS).

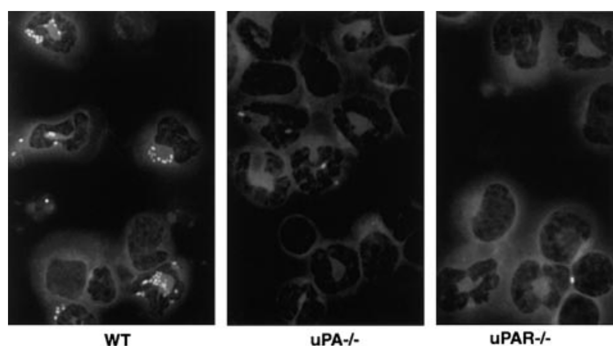

Fig. 1. Comparison of neutrophil phagoyctosis in WT, uPA−/−, and uPAR−/− mice by fluorescence microscopy. Representative fluroescence photomicrographs of purified WT, uPA−/−, and uPAR−/− neutrophils after incubation with fluorescently labeled *E. coli* for 20 min. The slides were hematoxylin- and eosin-stained prior to photomicroscopy at 400′ magnification (n=5–8).

Comparison of superoxide production by WT, uPA−/−, and uPAR–/– neutrophils

The generation of superoxide is a critical mechanism by which neutrophils kill bacteria. We therefore evaluated superoxide production by neutrophils obtained from WT, uPA−/−, and uPAR–/– neutrophils. We chose to use fMLP as our stimulus because it is a potent chemotaxin and activator of neutrophils, and because it is a bacterial product present in copious amounts in bacterial culture media. Hence, it is a reasonable stimulus to assess superoxide generation by neutrophils in the context of antibacterial defense. As shown in **Fig. 4A**, both uPA−/− neutrophils and uPAR−/− neutrophils generate substantially less superoxide in response to fMLP than do WT neutrophils across the entire dose range tested ($P \leq 0.0005$ for all conditions). In separate experiments (Fig. 4B), we sought to determine whether the defect in superoxide generation seen in uPA−/− neutrophils could be corrected by the addition of exogenous recombinant murine uPA. Neutrophils from WT and uPA−/− mice were preincubated in the presence and absence of recombinant murine uPA for 5 min at 37°C, and then superoxide generation was assessed as above. As shown in Fig. 4B, uPA-repleted uPA−/− neutrophils generated far more superoxide than did uPA−/− neutrophils without repletion across the entire fMLP dose range ($P \leq 0.002$ for all conditions). Additionally, the superoxide generated by the uPA-repleted uPA−/− neutrophils was no different from the superoxide generation seen by WT neutrophils (P=NS). These results demonstrate that the defect in superoxide generation seen in uPA−/− neutrophils can be completely reversed by the addition of exogenous murine uPA.

To determine whether the mechanism for the reduced levels of superoxide production seen in the uPA−/− or the uPAR−/− neutrophils was due to intracellular defects in signaling or to plasma membrane events, we determined superoxide generation in response to PMA. PMA directly activates protein kinase C (PKC), thereby bypassing surface receptor or more upstream signaling events. When WT, uPA−/−, or uPAR−/− neutrophils were stimulated with PMA, superoxide production was equivalent in magnitude across all groups (Fig. 4C; P=NS). We therefore conclude that the diminished superoxide production by the uPA−/− and uPAR−/− neutrophils is the result of cell surface and/or upstream signaling events and not as a result of an intrinsic signaling defect at or downstream from PKC.

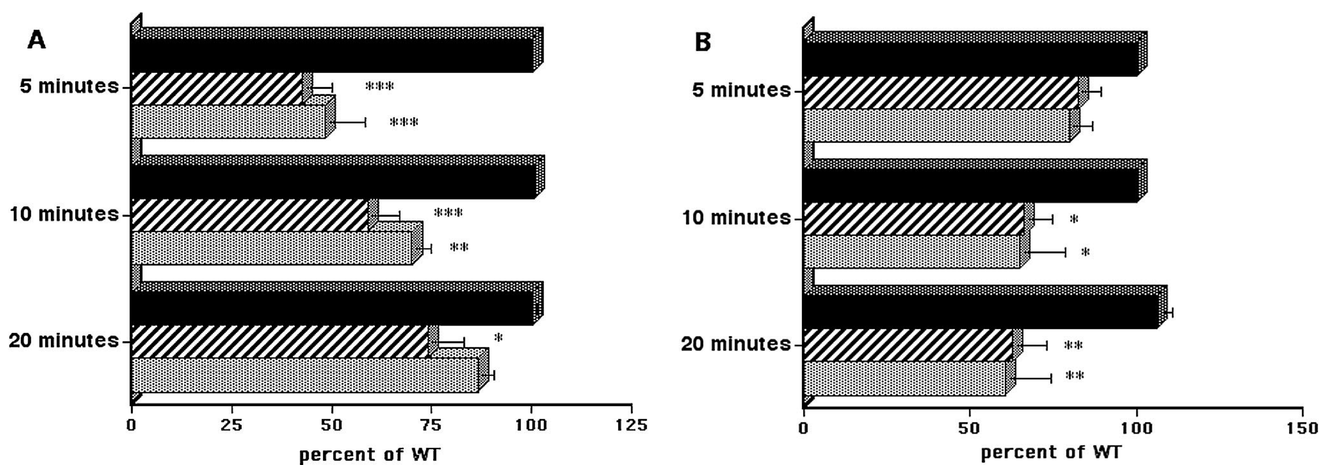

Fig. 2. Comparison of neutrophil phagoyctosis in WT, uPA−/−, and uPAR−/− mice by flow cytometry. Purified WT, uPA−/−, and uPAR−/− neutrophils after incubation with fluorescently labeled *E. coli* were analyzed by flow cytometry. All values are normalized to the mean of WT neutrophils. (A) Percentage of neutrophils that have ingested *E. coli*. (B) Mean fluorescence intensity of neutrophils that have ingested *E. coli*. WT mice, black bars; uPA−/−, hatched bars uPAR−/−, stippled bars. Data are expressed as mean ± SEM; $^*P \leq 0.05$; $^{**}P \leq 0.005$; $^{***}P \leq 0.0003$; $n = 5–8$.

Comparison of the degranulation reponse by WT, uPA−/−, and uPAR−/− neutrophils

The ability of neutrophils to participate in antibacterial host defense is in part dependent on several neutrophil-specific proteins that are contained within discrete intracytoplasmic granules. Neutrophil activation results in a degranulation response that liberates granule contents extracellulary or into phagolysosomes. To determine the degranulation response involving azurophilic granules, we determined the release of MPO [23]. Neutrophils from WT, uPA−/−, and uPAR−/− mice were incubated in the presence and absence of 10^{-7} M fMLP. At the end of the incubation period, elaboration of MPO was determined. As shown in **Fig. 5**, elaboration of MPO from unstimulated cells of all genotypes was minimal and equivalent. By contrast, fMLP-stimulated neutrophils from uPA−/− mice showed strikingly less elaboration of MPO compared with neutrophils from WT mice (957.8 ± 64.0 vs. 1620.4 ± 67.7 mOD/min/ml/10^6 cells, $P<0.0001$). fMLP-stimulated neutrophils from uPAR−/− mice elaborated equivalent amounts of MPO compared with fMLP-stimulated neutrophils from WT mice (P=NS). As shown by the lower bars in Fig. 5, repletion of uPA−/− neutrophils with 1 μg/ml murine uPA had no effect on MPO release compared with untreated uPA−/− neutrophils at either 0 or 15 min (P=NS). Of note, when equivalent numbers of neutrophils were obtained from WT, uPA−/−, and uPAR−/− mice and lysed, equivalent amounts of MPO were found among all the genotypes tested (data not shown). Therefore, although total cellular MPO content is consistent among WT, uPA−/−, and uPAR−/− mice, the uPA−/− mice respond with substantially less release of azurophilic granules in response to activation compared with WT or to uPAR−/− mice.

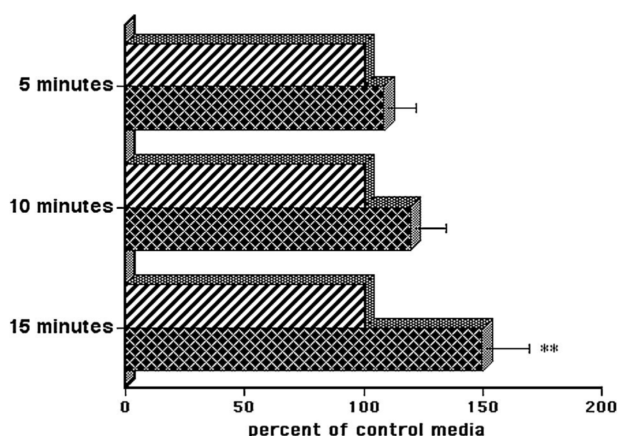

Fig. 3. Effect of repletion of uPA−/− neutrophils with murine uPA on phagocytosis. Phagocytosis of fluorescently labeled *E. coli* by uPA−/− neutrophils treated with control media compared with phagocytosis by uPA−/− neutrophils treated with murine uPA containing media. Analysis was done by flow cytometery. All values are the mean fluorescence intensity of neutrophils that have ingested *E. coli* normalized to control media-treated uPA−/− neutrophils. Neutrophils from uPA−/− mice treated with control media, hatched bars; neutrophils from uPA−/− treated with murine uPA containing media, crosshatched bars. Data are expressed as mean ± SEM; $^{**}P \leq 0.003$; $n = 9$.

The degranulation of neutrophil-specific granules is important to antibacterial host defense, in that specific granules contain lactoferrin, which has bactericidal and bacteriostatic properties and increases hydroxide ion production when it is saturated with iron [24]. The β_2 integrin CD11b/CD18 is also contained in specific granules in resting neutrophils and is translocated to the plasma membrane during degranulation, which thereby increases the plasma membrane expression 5–10 times the low level of expression seen under resting conditions. The change in expression of CD11b on the cell membrane following agonist stimulation is therefore a reliable marker of exocytois of specific granules [25]. The expression of cell surface CD11b/CD18 on unstimulated cells was equivalent comparing the mean log fluorescence of WT to uPA−/− or uPAR−/− neutrophils (**Table 1**, P=NS). When stimulated with fMLP, all three genotypes increased the cell surface expression of CD11b significantly ($P<0.05$ for all conditions).

A

5 x10-7 FMLP

1 x10-6 FMLP

5 x 10-6 FMLP

0 5 10 15 20 25

nmol O2-/min/ml/10^7 cells

B

5 x10-7 FMLP

1 x10-6 FMLP

5 x 10-6 FMLP

0 20 40 60 80

nmol O2-/min/ml/10^7 cells

C

8 ng PMA

4 ng PMA

0 5 10 15 20 25

nmol O2-/min/ml/10^7 cells

Fig. 4. Comparison of superoxide production by WT, uPA−/−, and uPAR–/– neutrophils. (A) fMLP-induced superoxide production by neutrophils. WT mice, black bars; uPA−/− hatched bars, uPAR−/− stippled bars. Data are expressed as mean ± SEM. $^*P \leq 0.0005$; $^{**}P \leq 0.0001$; $n = 5$–7. (B) fMLP-induced superoxide production by neutrophils. WT mice, black bars; uPA−/− hatched bars, uPA−/− + murine crosshatched bars. Data are expressed as mean ± SEM. $^*P \leq 0.002$; $^{**}P \leq 0.0003$; $n = 5$–7. (C) PMA-induced superoxide production by neutrophils. WT mice, black bars; uPA−/−, hatched bars; uPAR−/−, stippled bars. Data are expressed as mean ± SEM. P = NS; $n = 3$–5.

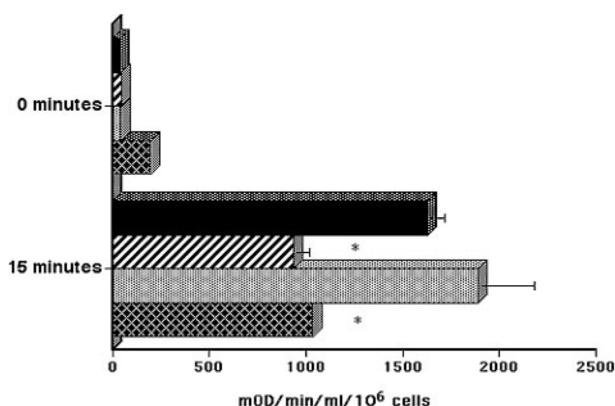

Fig. 5. Determination of myeloperoxidase release as a marker for degranulation of azurophilic granules by WT, uPA−/−, and uPAR−/− neutrophils. Neutrophils were stimulated with fMLP, and myeloperoxidase release was determined. WT mice, black bars; uPA−/−, hatched bars; uPAR−/−, stippled bars; uPA−/− + murine uPA, crosshatched bars. Data are expressed as mean ± SEM. $^*P \leq 0.0001$; $n = 5$–9. uPA−/− compared with uPA−/− + murine uPA, P = NS for all conditions.

The degree of increase was the same comparing uPA−/− and uPAR−/− neutrophils to WT. Therefore, agonist-stimulated release of specific granules is not diminished in uPA−/− or uPAR−/− mice compared with WT mice.

DISCUSSION

These studies demonstrate the following points: 1) neutrophil phagocytosis of *E. coli* is substantially diminished in the absence of either uPA or uPAR; 2) repletion of uPA−/− neutrophils with murine uPA substantially reverses the defect in phagocytosis; 3) generation of superoxide is markedly reduced in the absence of uPA or uPAR; 4) repletion of uPA−/− neutrophils with murine uPA completely reverses the defect in superoxide generation; 5) neutrophil exocytosis of azurophilic granules is reduced in the absence of uPA, but not in the absence of uPAR, and this defect in uPA−/− neutrophils is not corrected by provision of extracellular uPA repletion; and 6) neutrophil exocytosis of specific granules is not reduced by the absence of either uPA or uPAR. Therefore, we demonstrate that uPA/uPAR modulates several of the crucial steps in

TABLE 1. Cell Membrane CD11b Expression by Mean Log Fluorescence[1]

Genotype	Unstimulated	fMLP-stimulated	$P <$[2]	$P <$[3]	n
WT	110.24 ± 10.184	173.25 ± 23.35	0.05		4
uPA−/−	88.17 ± 7.11	137.67 ± 18.0	0.03	NS	6
uPAR−/−	77.00 ± 5.12	178.25 ± 36.85	0.04	NS	4

[1] Mean log fluorescence channel number ± SEM determined by immunofluorescence flow cytometry. [2] Comparing unstimulated to fMLP-stimulated neutrophils within the same genotype. [3] Comparing uPA−/− unstimulated and uPA−/− fMLP-stimulated or uPAR−/− unstimulated and uPAR−/− fMLP-stimulated neutrophils to WT unstimulated and WT fMLP-stimulated neutrophils, respectively.

neutrophil activation that lead to bacterial killing and effective host defense.

During the process of extravasation from the vascular compartment and recruitment to sites of infection, neutrophils become activated and increase the expression of plasma membrane receptors that mediate phagocytosis, including complement receptors (CR), especially CR3 (CD11b/CD18), and the Fc domain of immunoglobulin [24]. CD11b/CD18 is possibly the most important receptor for neutrophil phagocytosis, as shown by studies of leukocyte adhesion deficiency (LAD) patients, who have substantial defects in neutrophil phagocytosis in addition to defects in adhesion and chemotaxis [26]. We have previously demonstrated that uPAR facilitates the CD11b/CD18-dependent functions of chemotaxis and adhesion and that blockage of uPAR with either antibodies or antisense oligonucleotides results in near-ablation of chemotaxis and reduction of adhesion by ~50% [11–13]. The veracity of these observations has also been demonstrated in vivo, where we, and others, have shown that uPAR−/− mice have defects in CD11b/CD18 mediated cellular recruitment [14, 27]. The physical connection between uPAR, the β_2 integrins, and the cytoskeleton has been shown by elegant work demonstrating that the resistance to movement when magnetic torque is applied to uPAR-attached beads reflects transmembrane stiffness consistent with an integrin connection to cytoskeletal components [28]. It is therefore reasonable to propose that during phagocytosis, uPAR similarly partners with CD11b/CD18 and thereby enhances its ability to effect phagocytosis of complement-opsonized bacteria.

In the current study, we provide evidence that the absence of uPA also greatly reduces neutrophil phagocytosis. This finding is somewhat unique to phagocytosis among CD11b/CD18-mediated functions. Anti-sense oligonucleotide blockade of uPA has previously been shown to have little effect on chemotaxis, and to significantly enhance adhesion [11, 13]. Furthermore, neutrophil recruitment to the lungs of uPA−/− mice in response to bacterial pneumonia is no different from recruitment seen in WT mice [14]. This finding suggests that uPA has little effect on modulating pulmonary recruitment mediated by the partnership of uPAR with CD11b/CD18. Rather than modulating partnering of uPAR with β2 integrins, the role of uPA in neutrophil phagocytosis may be due to its known ability to transduce signals and induce cellular activation on binding to uPAR (discussed further below) [29]. Because uPA is stored in intracellular vesicles in neutrophils and released into the extracellular compartment during activation, it is reasonable that the concentration of extracellular uPA, and therefore uPA-uPAR binding, increases dramatically on neutrophil activation [30, 31].

Neutrophil phagocytosis is accompanied by a series of cell signaling events that trigger oxygen-dependent biochemical processes that lead to the production and elaboration of reactive oxidants. The microbicidal activity of neutrophils is critically dependent on the elaboration of these reactive oxidant species. During phagocytosis, normal leukocytes show an increase in oxygen consumption [32], which is then followed by the production of superoxide, hydroxyl radical, and hydrogen peroxide, events known collectively as the respiratory burst [33]. The respiratory burst is tied to the activation of the plasma membrane-bound NADPH oxidase, which leads to the generation of superoxide radicals that, when catalyzed by superoxide dismutase, generates H_2O_2 and oxygen.

fMLP is commonly used in in vitro systems to mimic the cellular activation of neutrophils seen in vivo or upon stimulation with bacteria. fMLP binding to neutrophils is a receptor-mediated event that leads to the activation of guanine nucleotide regulatory protein followed by activation of phospholipase C, which results in IP3 entering the cytosol and the release of intracellular calcium [34]. PKC is then activated by the PI breakdown product DAG [35]. The importance of PKC has been shown using its direct activator, PMA, which is a potent stimulus for superoxide and H_2O_2 production [36]. PMA can therefore allow for the differentiation among cell surface receptor mediated events and events that occur intracellularly further down the signal transduction pathway in the generation of the respiratory burst. Using this approach, we show that superoxide generation is significantly impaired in response to fMLP-induced stimulation in uPA−/− and uPAR−/− neutrophils compared with those of WT mice. However, the intracellular machinery of the respiratory burst appears to be fully intact in uPA−/− and uPAR−/− mice because the response to PMA is indistinguishable to that seen in WT. Hence, the defect in the absence of uPA or uPAR in superoxide generation lies at, or upstream, of PKC activation and is likely related to surface receptor-mediated events. The ability to fully correct the defect in superoxide production seen in uPA−/− neutrophils with exogenous extracellular uPA, together with the requirement for uPAR expression for superoxide production comparable with WT neutrophils, provides strong evidence that the mechanism by which uPA modulates superoxide generation is through binding to its natural receptor, uPAR, at the cell surface and thereby initiating signal transduction events.

Although the importance of the respiratory burst in neutrophil mediated host defense is indisputable, the specific direct

bactericidal role of some of these early oxidants is more controversial. Much of the hydrogen peroxide produced by neutrophils is secondarily processed by myeloperoxidase, which oxidizes chloride ions to HOCl, an extremely potent bactericidal oxidant [37]. Myeloperoxidase is localized in resting neutrophils within azurophilic (primary) granules, where it is a major constituent. As a result of phagocytosis, degranulation occurs and MPO is discharged extracellularly or into phagolysosomes.

Many species of bacteria require oxidative burst in order for optimal killing in vivo. A clinical example of this is in chronic granulomatous disease (CGD), which is a group of syndromes sharing various defects in components of the NADPH oxidase complex. Whereas normal leukocytes increase oxygen consumption more than 500% during phagocytosis, CGD neutrophils show only a 36% increase [32]. Patients with CDG have recurrent, severe infections caused by organisms such as *Staphylococci*, *Aspergillus*, *Serratia*, and *Burkholderia cepacia* [38]. Catalase-negative organisms, such as *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, are not problematic [39, 40]. Patients have also been described with MPO deficiency. MPO-deficient patients may present with recurrent fungal and bacterial infections. In vitro experiments have demonstrated the importance of myeloperoxidase in bacterial killing. [37]. Therefore, the diminished superoxide production coupled with diminished MPO release seen in the neutrophils of uPA−/− mice provide a mechanism for their diminished bactericidal activity compared with WT neutrophils. Similarly, the diminished superoxide production seen in the neutrophils of uPAR−/− mice would predict diminished bactericidal activity when compared with WT mice, but uPAR−/− neutrophils should not have a defect in killing to the extent seen in uPA−/− mice, as their ability to elaborate MPO remains intact.

The degranulation of neutrophil-specific granules is likewise important in antibacterial host defense. Specific granules contain lactoferrin, which has bacteriocidal and bacteriostatic properties. Lactoferrin also increases hydroxide ion production when it is saturated with iron [24]. Degranulation of specific granules in neutrophils from uPA−/− and uPAR−/− mice is indistinguishable from that seen in WT, which suggests that this limb of antibacterial host defense is fully intact in the absence of uPA or uPAR. Further, because CD11b/CD18 is a recognized marker for specific granules, these data also demonstrates that agonist-stimulated expression of CD11b/ CD18 is intact in uPA- and uPAR-deficient animals.

Neutrophil degranulation, rapidly triggered by fMLP, is mediated by the activation of *src* family tyrosine kinases, which lead to the activation of p38 MAP kinase [41]. Several *src*-related tyrosine kinases have been studied in response to fMLP stimulation, including *Lyn* [42] and *fgr*, which is associated with the secondary granules [43]. *Hck*, associated with azurophilic and secretory granules, translocates to the phagolysosome and is activated during phagocytosis [42, 44]. Substantial evidence links uPA/uPAR to these intracellular signaling pathways. uPAR co-precipitates with *src* type tyrosine kinases. *hck* is activated by binding of uPA to uPAR, as is p38, and the extracellular signal-related kinase 2 (Erk2) [45]. In neutrophils, uPA binding to uPAR triggers increases in intracellular calcium that can be blocked by Ab to either CD11b/ CD18 or uPAR; transfectant studies have confirmed a requirement for both CD11b/ CD18 and uPAR. uPA binding to uPAR induces serine phosphorylation in epithelial cells [46], DAG neosynthesis in epidermal cells [47], activation of the Jak/Stat1 pathway, and induction of Src-like protein tyrosine kinases (PTK) in smooth muscle cells [48] and tyrosine phosphorylation in mononuclear phagocytes [49]. Therefore, it is reasonable to position uPA-uPAR binding as an important modulator of the critical pathways of cellular activation required for neutrophil activation. The current studies provide evidence that uPA binding to uPAR is the mechanism responsible for the modulation of phagocytosis and superoxide production. This theory is supported the common defects in uPA−/− and uPAR−/− mice, which suggest that both uPA and uPAR are necesssary and that the ability to correct the defects in phagocytosis and superoxide production in uPA−/− neutrophils (which express uPAR normally) by providing murine uPA extracellularly for a period of time sufficient for uPAR binding.

However, uPA-mediated signal transduction is complex in that uPA has also been shown to activate signaling pathways in an uPAR-independent manner. Recently, the addition of exogenous human uPA has been shown to potentiate LPS-induced activation of several neutrophil signaling pathways in murine cells, including Akt and c-Jun N-terminal kinase, and the expression of pro-inflammatory cytokines [50]. Because human uPA cannot bind to murine uPAR [51], the potentiation of these critical pathways is induced by uPA by a mechanism that is independent of uPAR. uPA induces tyrosine phosphorylation of 78 kDa in a human epithelial cell line in a proteolysis-dependent, but uPAR-independent, mechanism [52]. A mechanism by which uPA may transduce uPAR-independent signals is by associating with other cell surface receptors, most notably by binding directly to CD11b/CD18 [53]. Alternatively, uPA, through its proteolytic functions, may signal indirectly through intermediate proteases, including plasmin [54–56]. In our experiements investigating release of azurophilic granules, the role of uPA is clearly independent of uPAR binding, as uPAR−/− and WT neutrophils release MPO comparably. Further, the defect seen in uPA−/− cells cannot be corrected by providing extracellular murine uPA for a time period sufficient for uPAR binding.

In summary, we have demonstrated that expression of uPA and uPAR is required for normal neutrophil phagocytosis of bacteria and fMLP-stimulated superoxide generation. The absence of either uPA or uPAR results in profound impairment of these two critical mechanisms of neutrophil-mediated host defense. We also show that degranulation of azurophilic granules is impaired in the absence of uPA, but not in the absence of uPAR. Thus, the uPA/ uPAR system is critically involved in several aspects of host inflammatory responses, including cellular recruitment, phagocytosis and regulation of leukocyte antimicrobial activation.

ACKNOWLEDGMENTS

The authors would like to thank Peter Carmeliet for generously providing the uPA−/−, uPAR−/− and background matched

WT mice. This work was supported by a Merit Research Grant and Research Enhancement Award Program funds (REAP) from the Department of Veterans Affairs (M.R.G.) and by National Institutes of Health grant HL60620 (M.R.G.).

REFERENCES

1. Wilkinson, P., Haston, W. (1988) Chemotaxis: An overview. *Methods Enzymol.* **162,** 3–16.
2. Lauffenburger, D. (1991) Models for receptor-mediated cell phenomena: Adhesion and migration. *Annu. Rev. Biophys. Biophys. Chem.* **20,** 387–414.
3. Danø, K., Andreasen, P., Grondahl-Hansen, J., Kristensen, P., Nielsen, L., Skriver, L. (1985) Plaminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* **44,** 139–265.
4. Huber, A. R., Ellis, S., Johnson, K. J., Dixit, V. M., Varani, J. (1992) Monocyte diapedesis through an *in vitro* vessel wall construct: Inhibition with monoclonal antibodies to thrombospondin. *J. Leukoc. Biol.* **52,** 524–528.
5. Kirchmeimer, J. C., Wojta, J., Christ, G., Hienert, G., Binder, B. R. (1988) Mitogenic effect of urokinase on malignant and unaffected adjacent human renal cells. *Carcinogenesis* **9,** 2121–2123.
6. Liotta, L. A., Goldfarb, R. H., Brundange, R., Siegal, G. P., Terranova, V., Garbisa, S. (1981) Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. *Cancer Res.* **41,** 4629–4636.
7. Vassalli, J., Sappino, A., Belin, D. (1991) The plasminogen activator/plasmin system. *J. Clin. Invest.* **88,** 1067–1072.
8. Estreicher, A., Mühlhause, J., Carpentier, J., Orci, L., Vassalli, J. (1990) The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. *J. Cell Biol.* **111,** 783–792.
9. Senior, R., Shapiro, S. (1992) Introduction: The matrix metalloproteinase family. *Am. J. Respir. Cell Mol. Biol.* **7,** 119–127.
10. Werb, Z., Mainardi, C. L., Vater, C. A., Harris, E. D. (1977) Endogenous activation of latent collagenase by synovial cells: Evidence for a role for plasminogen activator. *N. Engl. J. Med.* **296,** 1017–1021.
11. Gyetko, M. R., Todd, R. F., III, Wilkinson, C. C., Sitrin, R. G. (1994) The urokinase receptor is required for human monocyte chemotaxis *in vitro. J. Clin. Invest.* **93,** 1380–1387.
12. Gyetko, M. R., Sitrin, R. G., Fuller, J. A., Todd, R. F., III, Petty, H., Standiford, T. J. (1995) Function of the urokinase receptor (CD87) in neutrophil chemotaxis. *J. Leukoc. Biol.* **58,** 533–538.
13. Sitrin, R. G., Todd, R. F., III, Petty, H. R., Brock, T. G., Shollenberger, S. B., Albrecht, E., Gyetko, M. R. (1996) The urokinase receptor (CD87) facilitates CD11b/CD18-mediated adhesion of human monocytes. *J. Clin. Invest.* **97,** 1942–1951.
14. Gyetko, M. R., Sud, S., Kendall, T., Fuller, J. A., Newstead, M. W., Standiford, T. (2000) Urokinase receptor-deficient transgenic mice have impaired neutrophil recruitment in response to pulmonary pseudomonas infection. *J. Immunol.* **165,** 1513–1519.
15. Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., De Vos, R., van den Oord, J. J., Collen, D., Mulligan, R. C. (1994) Physiological consequences of loss of plasminogen activator gene function in mice. *Nature* **368,** 419–424.
16. Dewerchin, M., Nuffelen, A. V., Wallays, G., Bouche, A., Moons, L., Carmeliet, P., Mulligan, R. C., Collen, D. (1996) Generation and characterization of urokinase receptor-deficient mice. *J. Clin. Invest.* **97,** 870–878.
17. Gyetko, M. R., Chen, G.-H., McDonald, R. A., Goodman, R., Huffnagle, G. B., Wilkinson, C. C., Fuller, J. A., Toews, G. B. (1996) Urokinase is required for the pulmonary inflammatory response to Cryptococcus neoformans: A murine transgenic model. *J. Clin. Invest.* **97,** 1818–1826.
18. Kubo, H., Morgenstern, D., Quinlan, W. M., Ward, P. A., Dinauer, M. C., Doershuk, C. M. (1996) Preservation of complement-induced lung injury in mice with deficiency of NADPH oxidase. *J. Clin. Invest.* **97,** 2680–2684.
19. Traynor, T. R., Kuziel, W. A., Toews, G. B., Huffnagle, G. B. (2000) CCR2 expression determines T1 versus T2 polarization during pulmonary *Cryptococcus neoformans* infection. *J. Immunol.* **164,** 2021–2027.
20. Hattori, N., Sisson, T. H., Xu, Y., Simon, R. H. (1999) Adenovirus-mediated transfer of urokinase-type plasminogen activator genes to lung cells in vitro and in vivo. *Hum. Gene Ther.* **10,** 215–222.
21. Mayo, L. A., Curnutte, J. T. (1990) Kinetic microplate assay for superoxide production by neutrophils and other phagocytic cells. *Methods Enzymol.* **186,** 567–575.
22. Harlow, E., Lane, D. (1988) *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory, New York.
23. Sorensen, O., Borregaard, N. (1999) Methods for quantitation of human neutrophil proteins, a survey. *J. Immunol. Methods* **232,** 179–190.
24. Carr, R. (2000) Neutrophil productiona dn funciton in newborn infants. *Br. J. Haematol.* **110,** 18–28.
25. Todd, R. F. I., Arnaout, M. A., Rosin, R. E., Crowley, C. A., Peters, W. A. (1984) Subcellular localization of the large subunit of Mo1 (Mo1 alpha; formerly gp 110), a surface glycoprotein associated with neutrophil adhesion. *J. Clin. Invest.* **74,** 1280–1290.
26. Todd, R. F. I., Freyer, D. R. (1988) The CD11/CD18 leukocyte glycoprotein deficience. *Hematol. Oncol. Clin. North Am.* **2,** 13–31.
27. May, A. E., Kanse, S. M., Lund, L. R., Gisler, R. H., Imhof, B. A., Preissner, K. T. (1998) Urokinase receptor (CD87) regulates leukocyte recruitment via β2 integrins in vivo. *J. Exp. Med.* **188,** 1029–1037.
28. Wang, N., Planus, E., Pouchelet, M., Fredberg, J. J., Barlovatz-Meimon, G. (1995) Urokinase receptor mediates mechanical force transfer across the cell surface. *Am. J. Physiol.* **268,** C1062–C1066.
29. Cao, D., Mizukami, I. F., Garni-Wagner, B. A., Kindzelskii, A. L., Todd, R. F., III, Boxer, L. A., Petty, H. R. (1995) Human urokinase-type plasminogen activator primes neutrophils for superoxide anion release. *J. Immunol.* **154,** 1817–1829.
30. Plesner, T., Ploug, M., Ellis, V., Ronne, E., Høyer-Hansen, G., Wittrup, M., Pedersen, T. L., Tscherning, T., Danø, K., Hansen, N. E. (1994) The receptor for urokinase-type plasminogen activator and urokinase is translocated from two distinct intracellular compartments to the plasma membrane on stimulation of human neutrophils. *Blood* **83,** 808–815.
31. Pedersen, T., Plesner, T., Horn, T., Hoyer-Hansen, G., Sorensen, S., Hansen, N. E. (2000) Subcellular distribution of urokinase and urokinase receptor in human neutrophils determined by immunoelectron microscopy. *Ultrastruct. Pathol.* **24,** 175–182.
32. Holmes, B., Page, A. R., Good, R. A. (1967) Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocyte function. *J. Clin. Invest.* **49,** 1422–1432.
33. Shepard, V. L. (1986) The role of the respiratory burst of phagocytes in host defense. *Semin. Respir. Infect.* **1,** 99–106.
34. Smith, C. D., Cox, C. C., Snyderman, R. (1986) Receptor-coupled activation of phosphoinositide-specific phospholipase C by an N protein. *Science* **232,** 97–100.
35. Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308,** 693–698.
36. Wolfson, M., McPhail, L. C., Nasrallah, V. N., Snyderman, R. (1985) Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: Potential role in the activation of the respiratory burst enzyme. *J. Immunol.* **135,** 2057–2062.
37. Hampton, M. B., Kettle, A. J., Winterbourn, C. C. (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92,** 3007–3017.
38. Winkelstein, J. A., Marino, M. C., Johnston, R. B. J., Boyle, J., Curnutte, J., Gallin, J. I., Malech, H. L., Holland, S. M., Ochs, H. (2000) Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Baltimore)* **79,** 155–169.
39. Segal, B. H., Leto, T. L., Gallin, J. I., Malech, H. L., Holland, S. M. G. (2000) Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine (Baltimore)* **79,** 170–200.
40. Segal, B. H., Holland, S. M. (2000) Primary phagocytic disorders of childhood. *Pediatr. Clin. North Am.* **47,** 1311–1338.
41. Moscai, A., Jakus, S., Vantus, T., Berton, G., Lowell, C. A., Ligeti, E. (2000) Kinase pathways in chemoattractant-induced degranulation of neutrophils: The role of p38 mitogen-activated protein kinase activated by Src family kinases. *J. Immunol.* **164,** 4321–4331.
42. Welch, H., Maridonneau-Parini, I. (1997) Lyn and Fgr are activated in distinct membrane fractions of human granulocytic cells. *Oncogene* **15,** 2021–2029.
43. Gutkind, J. S., Robbins, K. C. (1989) Translocation of the FGR protein-tyrosine kinase as a consequence of neutrophil activation. *Proc. Natl. Acad. Sci. USA* **86,** 8783–8787.
44. Möhn, H., Le Cabec, V., Fischer, S., Maridonneau-Parini, I. (1995) The src-family protein-tyrosine kinase p59hck is located on the secretory granules in human neutrophils and translocates towards the phagosome during cell activation. *Biochem. J.* **309,** 657–665.
45. Konakova, M., Hucho, F., Schleuning, W.-D. (1998) Downstream targets of urokinase-type plasminogen-activator-mediated signal transduction. *Eur. J. Biochem.* **253,** 421–429.

46. Busso, N., Masur, S. K., Lazega, D., Waxman, S., Ossowski, L. (1994) Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. *J. Cell Biol.* **126,** 259–270.
47. Del Rosso, M., Anichini, E., Pedersen, N., Blasi, F., Fibbi, G., Pucci, M., Ruggiero, M. (1993) Urokinase-urokinase receptor interaction: non-mitogenic signal transduction in human epidermal cells. *Biochem. Biophys. Res. Commun.* **190,** 347–352.
48. Dumler, I., Weis, A., Mayboroda, O. A., Maasch, C., Jerke, U., Haller, H., Gulba, D. C. (1998) The Jak/Stat pathway and urokinase receptor signaling in human aortic vascular smooth muscle cells. *J. Biol. Chem.* **273,** 315–321.
49. Dumler, I., Petri, T., Schleuning, W. D. (1993) Interaction of urokinase-type plasminogen activator (u-PA) with its cellular receptor (uPAR) induces phosphorylation on tyrosine of a 38 kDa protein. *FEBS Lett.* **322,** 37–40.
50. Abraham, E. A., Gyetko, M. R., Kuhn, K., Arcaroli, J., Strassheim, D., Park, J. S., Shetty, S., Idell, S. (2003) Urokinase-type plasminogen activator potentiates lipopolysaccharide-induced neutrophil activation. *J. Immunol.* **170,** 5644–5651.
51. Estreicher, A., Wohlwend, A., Belin, D., Schleuning, W.-D., Vassalli, J.-D. (1989) Characterization of the cellular binding site for the urokinase-type plasminogen activator. *J. Biol. Chem.* **263,** 1180–1189.
52. Bhat, G. J., Gunaje, J. J., Idell, S. (1999) Urokinase-type plasminogen activator induces tyrosine phosphorylation of a 78-kDa protein in H-157 cells. *Am. J. Physiol.* **277,** L301–L309.
53. Pluskota, E., Soloviev, D. A., Bdeir, K., Cines, D. B., Plow, E. F. (2004) Integrin {alpha}M{beta}2 Orchestrates and Accelerates Plasminogen Activation and Fibrinolysis by Neutrophils. *J. Biol. Chem.* **279,** 18063–18072.
54. Vaday, G. G., Lider, O. (2000) Extracellular matrix moieties, cytokines, and enzymes: dynamic effects on immune cell behavior and inflammation. *J. Leukoc. Biol.* **67,** 149–159.
55. Robson, S. C., Saunders, R., Kirsch, R. E. (1990) Monocyte-macrophage release of IL-1 is inhibited by type-1 plasminogen activator inhibitors. *J. Clin. Lab. Immunol.* **33,** 83–90.
56. Matsushima, K., Taguchi, M., Kovacs, E. J., Young, H. A., Oppenheim, J. J. (1986) Intracellular localization of human monocyte associated interleukin-1 (IL-1) activity and release of biologically active IL-1 from monocytes by trypsin and plasmin. *J. Immunol.* **136,** 2883–2891.