Urokinase Receptor Is Necessary for Adequate Host Defense Against Pneumococcal Pneumonia

Anita W. Rijneveld, Marcel Levi, Sandrine Florquin, Peter Speelman, Peter Carmeliet and Tom van der Poll

*J Immunol* 2002; 168:3507-3511; doi: 10.4049/jimmunol.168.7.3507

http://www.jimmunol.org/content/168/7/3507

**References**

This article *cites 41 articles*, 19 of which you can access for free at: [http://www.jimmunol.org/content/168/7/3507.full#ref-list-1](http://www.jimmunol.org/content/168/7/3507.full#ref-list-1)

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**

Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Urokinase Receptor Is Necessary for Adequate Host Defense Against Pneumococcal Pneumonia

Anita W. Rijneveld,‡∥ Marcel Levi,‡ Sandrine Florquin,‡ Peter Speelman,¶∥ Peter Carmeliet,¶ and Tom van der Poll‡∥

Cell recruitment is a multistep process regulated by cytokines, chemokines, and growth factors. Previous work has indicated that the urokinase plasminogen activator receptor (uPAR) may play a role in this mechanism, presumably by an interaction with the β2 integrin CD11b/CD18. Indeed, an essential role of uPAR in neutrophil recruitment during pulmonary infection has been demonstrated for β2 integrin-dependent respiratory pathogens. We investigated the role of uPAR and urokinase plasminogen activator (uPA) during pneumonia caused by a β2 integrin-independent respiratory pathogen, Streptococcus pneumoniae. uPAR-deficient (uPAR−/−), uPA-deficient (uPA−/−), and wild-type (Wt) mice were intranasally inoculated with 10^5 CFU S. pneumoniae. uPAR−/− mice showed reduced granulocyte accumulation in alveoli and lungs when compared with Wt mice, which was associated with more S. pneumoniae CFU in lungs, enhanced dissemination of the infection, and a reduced survival. In contrast, uPA−/− mice showed enhanced host defense, with more neutrophil influx and less pneumococci in the lungs compared with Wt mice. These data suggest that uPAR is necessary for adequate recruitment of neutrophils into the alveoli and lungs during pneumonia caused by S. pneumoniae, a pathogen eliciting a β2 integrin-independent inflammatory response. This function is even more pronounced when uPAR is unoccupied by uPA. The Journal of Immunology, 2002, 168: 3507–3511.

Materials and Methods

Animals

Mice with a targeted deletion in the gene for uPAR or uPA, resulting in a complete deficiency of uPAR (uPAR−/−) or uPA (uPA−/−), respectively, were generated as previously described (24, 25). All mice were on a mixed C57BL/6J (75%) × 129 (25%) background. The respective wild types of the uPAR−/− and uPA−/− mice were derived from original littermates of the knockout mice and were bred separately in different colonies (under identical circumstances as their corresponding knockout strain) within the animal institution of the Flemish Interuniversity Institute for Biotechnology (Leuven, Belgium). For the experiments mice were transported to the Academic Medical Center (Amsterdam, The Netherlands). All experiments were approved by the Committee on Use and Care of Animals of the Academic Medical Center.

Induction of pneumonia

Pneumonia was induced as described previously (26). Briefly, S. pneumoniae, serotype 3, obtained from American Type Culture Collection (ATCC 6303; Rockville, MD), were grown for 6 h to midlogarithmic phase at 37°C in 5% CO2 using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500 × g for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at −10^9 CFU/ml, as determined by plating serial 10-fold dilutions onto sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Abbott, Queensborough, Kent, U.K.), and 50 µl of bacterial suspension was inoculated intranasally.

Received for publication August 15, 2001. Accepted for publication January 30, 2002.

© 2002 by The American Association of Immunologists

0022-1767/02/$02.00

Addendum

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by a grant from the Dutch Association for Scientific Research to A.W.R.

Address correspondence and reprint requests to Dr. Anita W. Rijneveld, Academic Medical Center, University of Amsterdam, GZ-105, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. E-mail address: a.w.rijneveld@amc.uva.nl

Abbreviations used in this paper: uPAR, uPA receptor; uPA, urokinase plasminogen activator; Wt, wild type; BAL, bronchoalveolar lavage; BALF, BAL fluid; MIP-2, macrophage-inflammatory protein 2.

Departments of ¶ Experimental Internal Medicine, ‡ Internal Medicine, § Pathology, and ¶ Infectious Diseases, Tropical Medicine, and AIDS, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and Center for Transgene Technology and Gene Therapy, Flemish Interuniversity Institute for Biotechnology, Leuven, Belgium

Received for publication August 15, 2001. Accepted for publication January 30, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by a grant from the Dutch Association for Scientific Research to A.W.R.

Address correspondence and reprint requests to Dr. Anita W. Rijneveld, Academic Medical Center, University of Amsterdam, GZ-105, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. E-mail address: a.w.rijneveld@amc.uva.nl

Abbreviations used in this paper: uPAR, uPA receptor; uPA, urokinase plasminogen activator; Wt, wild type; BAL, bronchoalveolar lavage; BALF, BAL fluid; MIP-2, macrophage-inflammatory protein 2.
Bronchoalveolar lavage

At 24 and 48 h after inoculation mice were anesthetized by i.p. injection with Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Midjrecht, The Netherlands), and blood was collected from the inferior caval vein. The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). Bronchoalveolar lavage (BAL) was performed by instilling two 0.5-ml aliquots of sterile isotonic saline. A total of 0.9–1 ml of lavage fluid was retrieved per mouse, and total cell numbers were counted from each sample in a hemocytometer. BAL fluid (BALF) differential cell counts were determined on cytopsin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL).

Histologic examination

After a 24-h fixation of lungs in 10% buffered formalin and embedding in paraffin, 4-μm-thick sections were stained with H&E. All slides were coded and scored by a pathologist without knowledge of the type of mice and treatment. For granulocyte staining, slides were deparaffinized and rehydrated. Slides were then digested by a solution of pepsin 0.25% (Sigma-Aldrich, St. Louis, MO) in 0.01 M HCl. After being rinsed, the sections were rehydrated. Slides were then digested by a solution of 0.1% NaN3 /0.03% H2O2 (Merck, Darmstadt, Germany) and then exposed to FITC-labeled anti-mouse Ly-6-G mAb (BD Phar-Mingen, San Diego, CA). Endogenous peroxidase activity was quenched by a solution of 0.1% NaNO2/0.03% H2O2 (Merck, Darmstadt, Germany). After washes, slides were incubated with a rabbit anti-FITC Ab (DAKO) followed by further incubation with a biotinylated swine anti-rabbit Ab (DAKO), rinsed again, incubated in a streptavidin-ABC solution (DAKO), and developed using 1% H2O2 and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) in Tris-HCl. The sections were mounted in glycerin-gelatin without counter staining and analyzed.

Preparation of lung homogenates

Whole lungs were harvested and homogenized at 4°C in five volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK), which was carefully cleaned and disinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile isotonic saline were made from these homogenates (and blood), and 50-μl volumes were plated onto sheep-blood agar plates and incubated at 37°C and 5% CO2. CFU were counted after 16 h. For cytokine measurements lung homogenates were lysed in lysis buffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl2, 2 mM Triton X-100, pepstatin A, leupeptin, aprotinin (20 μg/ml), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycol tetraacetic acid (EGTA), 2 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 μg/ml soybean trypsin inhibitor (TSTI), 1 μg/ml benzamidine-HCl) and centrifuged at 9000 × g for 10 min. The supernatant was frozen at −20°C until cytokine measurement.

Assays

Cytokine and chemokine levels were measured by using commercially available ELISAs, in accordance with the manufacturer’s recommendations: IL-6 (BD Pharmingen), IL-16, macrophage-inflammatory protein 2 (MIP-2), and KC (R&D Systems, Abingdon, U.K.). Detection limits were 37 (IL-6), 47 (MIP-2), and 12 (KC) pg/ml. uPA activity was measured by an amidolytic assay as previously described (27). Briefly, diluted lung homogenates were incubated with 0.3 mmol/L S-2251 (Chromogenix, Mölndal, Sweden), 0.13 mol/L plasminogen, and 0.12 mg/ml cyanogen bromide fragments of fibrinogen (Chromogenix). Conversion of plasminogen to plasmin was assessed by subsequent conversion of the chromogenic substrate S-2251 and was detected with a spectrophotometer. The fraction of the fission due to uPA activity was determined by including in the assay 50 μg/ml polyclonal neutralizing rabbit anti-murine uPA-specific IgGs. A standard curve for uPA activity was obtained by incubating different amounts of purified murine uPA to the assay system.

Statistical analysis

Data were analyzed using the SPSS statistical package. Data are expressed as means ± SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann-Whitney U test. Survival curves were compared by log-rank test. A value of p < 0.05 was considered to represent a statistically significant difference.

Results

uPA concentrations in the lung during pneumonia

Intranasal administration of S. pneumoniae increased the concentrations of uPA in lung homogenates obtained 24 and 48 h after inoculation (at baseline 88.5 ± 9.1 μg/ml; peak at 48 h: uPA 182.7 ± 8.6 μg/ml; p < 0.05 vs control) (Fig. 1). Hence, these data demonstrate that pneumococcal pneumonia results in locally elevated levels of the ligand of uPAR.

Inflammatory cell influx in BALF

Recently it has been reported that uPAR is an important regulator of integrin-dependent cellular migration (16, 17). Cell recruitment to the site of infection is an important part of host defense during pneumonia. For this reason we compared cell influx in the alveolar spaces of uPAR−/− mice and wild-type (Wt) mice after inoculation with S. pneumoniae. uPAR−/− mice had significantly fewer cells in their BALF when compared with Wt mice (p = 0.004), which was mainly caused by a diminished recruitment of neutrophils (Table I).

Histopathology

At 48 h after inoculation, the lungs of uPAR−/− mice showed patchy and dense inflammatory infiltrates (Fig. 2A), predominantly composed of monocytes and lymphocytes with relatively few granulocytes (Fig. 2, A and C). In contrast, Wt mice had a mild interstitial inflammation (Fig. 2B) composed of granulocytes and monocytes corresponding to a clearance phase (Fig. 2D).

Bacterial outgrowth

To investigate the role of uPAR in the pulmonary clearance of S. pneumoniae, we determined the numbers of CFU in lungs 24 and 48 h after inoculation. At both time points uPAR−/− mice had significantly more CFU in their lungs than Wt mice (p < 0.05) (Fig. 3). Furthermore, S. pneumoniae could be cultured from the blood of 12.5% of the Wt mice after 48 h, whereas 87.5% of the blood cultures obtained from the uPAR−/− mice were positive for S. pneumoniae at this time point. At 24 h blood cultures were negative in all mice.

Survival

To investigate whether uPAR influences survival, we assessed survival twice daily in uPAR−/− and Wt mice after intranasal inoculation with 105 CFU S. pneumoniae. uPAR−/− mice succumbed much earlier than Wt mice (p = 0.004) (Fig. 4).

Table 1. Cell influx in BALF

<table>
<thead>
<tr>
<th></th>
<th>Wt Mice</th>
<th>uPAR−/− Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>5.6 ± 1.4</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Macrophages</td>
<td>6.2 ± 1.1</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

* Data are mean ± SEM (x105/ml BALF) of six mice per group, 48 h after inoculation with 105 CFU S. pneumoniae.

b Values of p < 0.05 vs Wt mice.
Cytokines and chemokines

Because the localized production of cytokines and chemokines is an important part of host defense (28), we measured the concentrations of these mediators in lung homogenates. All cytokines and chemokines measured (IL-1, IL-6, KC, and MIP-2) were higher in uPAR/H11002 mice, in particular at 48 h postinoculation (Table II). Thus, a reduced production of protective proinflammatory cytokines or chemokines could not explain the impaired host defense in uPAR/H11002 mice.

Host response in uPA/H11002 mice

Having established that uPAR is important for host defense against pneumococcal pneumonia, we next determined the role of the ligand for uPAR, uPA, in this model of Gram-positive respiratory tract infection (Fig. 5). In contrast to uPAR/H11002 mice, uPA/H11002 mice had more neutrophils (p = 0.08) and less pneumococci in their lungs at 48 h postinoculation (p = 0.027). There was no significant difference in mortality between uPA/H11002 and Wt mice.

Discussion

The necessity of uPAR for inflammatory cell invasion has been demonstrated in pneumonia caused by P. aeruginosa, a Gram-negative bacterium that elicits a CD11b/CD18-dependent inflammatory response (21). The inflammatory cell migration during pneumococcal pneumonia occurs independent of CD11b/CD18. Despite this independence, the main findings of our study were that uPAR deficiency partly prevented the recruitment of cells to lungs and alveolar spaces, resulting in a diminished clearance of pneumococci from the lungs and a strongly reduced survival. Surprisingly, uPA deficiency enhanced host defense with an increased number of recruited neutrophils and less pneumococci in the lungs. Our results suggest that uPAR is necessary for adequate recruitment of neutrophils into the alveoli and that this function is even more pronounced when uPAR is not bound to uPA.

uPAR can form a functional complex with the β2 integrin CD11b/CD18. The CD11b/CD18 complex is necessary for cell recruitment in nearly every organ system. However, there is a difference between neutrophil adhesion within the pulmonary compartment and in the systemic circulation, i.e., leukocytes can migrate out of the lung capillaries by either a CD11b/CD18-dependent or -independent mechanism. IL-1, PMA, and Gram-negative bacterial stimuli elicit migration via pathways mediated by CD11b/CD18. Conversely, the cell

![FIGURE 2. Histopathology of lungs. A and C, Lungs of uPAR/H11002 mice 48 h after infection showing foci of inflammation with few granulocytes (C, anti-granulocyte immunostaining). B and D, Lungs of Wt mice 48 h after infection showing a mild interstitial inflammation with influx of granulocytes (D, anti-granulocyte immunostaining). A and B, H&E staining (magnification, ×33; magnification of inset, ×120). C and D, Anti-granulocyte staining (magnification, ×66).](http://www.jimmunol.org/)

![FIGURE 3. Bacterial outgrowth in lungs. S. pneumoniae CFU in lungs of Wt and uPAR/H11002 mice 24 and 48 h after intranasal inoculation with 10^6 CFU S. pneumoniae. Data are mean ± SEM (n = 8 per group per time point). *, p < 0.05 vs Wt mice.](http://www.jimmunol.org/)
migration in response to Gram-positive bacteria, hydrochloric acid, and C5a occurs independent of CD11b/CD18 (18–20, 29, 30). Treatment with anti-CD18 Abs had no effect on the leukocyte emigration in the lung induced by S. pneumoniae (18). Furthermore, the combined absence of P-selectin and ICAM-1, the ligand for CD11b/CD18, had no effect on neutrophil recruitment to the inflammatory site in response to S. pneumoniae (31). Thus, S. pneumoniae elicits a CD11b/CD18-independent inflammatory response in the lungs, while Gram-negative bacteria need CD11b/CD18 to recruit cells to the inflammatory site.

Accordingly, during P. aeruginosa pneumonia, uPAR−/− mice demonstrated a reduced neutrophil influx in the lung associated with an enhanced bacterial outgrowth (21). However, Wt mice treated with a blocking anti-CD11b mAb also had less accumulation of neutrophils in the lung after P. aeruginosa inoculation, similar to uPAR−/− mice. Furthermore, anti-CD11b mAb did not influence the recruitment of neutrophils in uPAR−/− mice, indicating that uPAR and CD11b act on neutrophils by a common mechanism in this β2 integrin-dependent model. Interestingly, we found that during pneumonia caused by a CD11b/CD18-independent pathogen, S. pneumoniae, uPAR is also necessary for adequate neutrophil recruitment, as documented by fewer neutrophils in BALF and lung parenchyma in uPAR−/− mice. This finding at least in part can explain the impaired antipneumococcal defense of uPAR−/− mice in this model of respiratory tract infection, considering that neutrophils are critical for effective eradication of bacteria from the lungs (32). We did not investigate whether the absence of uPAR influences opsonization or phagocytosis by neutrophils. However, to our knowledge no data are available to indicate that uPAR is involved in either of these processes.

uPA, as the ligand for uPAR, also influences cell migration. On the one hand it promotes cell invasion due to proteolysis, causing a conformational change in uPAR that uncovers a chemotactic epitope, while on the other hand uPA negatively influences the migratory function of uPAR in vitro (33–38). Our results demonstrate that uPA deficiency leads to increased neutrophil influx and an enhanced antibacterial host defense, although not to a reduced mortality. In line with our results, uPA had no effect, or even an inhibitory effect, on the adhesive capacity of monocytes and neutrophils (35, 39). In vivo, intratracheal KC (a murine CXC chemokine) administration to uPA−/− and Wt mice reduced the neutrophil influx after engagement of uPAR by nonproteolytic uPA, while uPAR−/− mice showed no difference in cell accumulation (33). In addition, uPA−/− mice showed no difference in neutrophil recruitment during pneumonia caused by Gram-negative bacteria or fungi (21, 40, 41). This demonstrates that uPA exerts opposite influences on neutrophil migration in different models. In our in vivo model of acute bacterial pneumonia, uPA seems to influence the function of uPAR as a chemotactic receptor in a negative way.

It should be noted that the number of S. pneumoniae CFUs measured in Wt mice of uPA−/− mice was considerably higher than the number of CFUs found in Wt mice of uPAR−/− mice, despite the fact that both mouse strains were on the same C57BL/6J (75%) background. The explanation for this finding is not clear, although several possibilities exist. First, in retrospect the bacterial inoculum was slightly higher in the experiments with uPA−/− and Wt mice than in the experiments with uPAR−/− and Wt mice (i.e., 3 × 10⁵ vs 1 × 10⁵ CFU). Second,
the experiments with uPAR−/− and corresponding Wt mice, and those with uPAR+/− and Wt mice, were done with an interval of several months. Our experience is that even with Wt mice purchased from commercial suppliers a certain degree of biological variation in the bacterial clearance exists between experiments and between different “shipments” of mice. Third, the respective Wt of the uPAR−/− and uPAR+/− mice were derived from original litters of the knockout mice and were bred separately in different colonies (under identical circumstances as their corresponding knockout strain); thus, slight (nongenetic) differences may have contributed to the different behavior of Wt mice in separate experiments. However, we would like to emphasize that our studies were performed under adequately controlled conditions; i.e., knockout and Wt mice were not only on the same genetic background but were also bred under identical circumstances and inoculated at the same time with exactly the same inoculum on each occasion.

uPAR was found to be important for cell-mediated immunity against *P. aeruginosa*, a β2 integrin-dependent respiratory pathogen (21). Our data demonstrate that deficiency of uPAR is associated with an impaired host defense against pneumococcal pneumonia, a model that does not need β2 integrin for the inflammatory response. In contrast, uPAR−/− mice showed enhanced host defense. Together these data suggest that uPAR is necessary for adequate recruitment of cells and that this chemotactic function is even more pronounced when uPAR is unbound. These findings not only add to our understanding of the role of uPAR and uPA in pneumonia but also warrant caution for treatment concerning modulation of the fibrinolytic system in different infectious diseases.

Acknowledgments

We thank A. A. Maas and J. B. Daalhuizen for expert technical assistance and N. Claessen for the immunostaining.

References


