Pneumomia: an investigation of host defence mechanisms

Rijneveld, A.W.

Citation for published version (APA):
Rijneveld, A. W. (2003). Pneumomia: an investigation of host defence mechanisms
Urokinase receptor is necessary for adequate host defense against pneumococcal pneumonia
Cell recruitment is a multistep process regulated by cytokines, chemokines and growth factors. Previous work has indicated that the urokinase receptor (uPAR) may also 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, *S. pneumoniae*. uPAR deficient (uPAR−/−), uPA deficient (uPA−/−), and wild type (Wt) mice were intranasally inoculated with 10⁵ CFU *S. pneumoniae*. uPAR−/− mice showed reduced granulocyte accumulation in alveoli and lungs when compared to Wt mice, which was associated with more *S. pneumoniae* CFU in lungs, enhanced dissemination of the infection and a reduced survival. On the other hand, uPA−/− mice showed enhanced host defense, with more neutrophil influx and less pneumococci in the lungs compared to 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.

**Introduction**

The urokinase plasminogen activator receptor (uPAR; CD87) is a glycosylphosphatidylinositol (GPI)–linked receptor expressed on a variety of cell types including hematopoietic cells, endothelial cells and many different neoplastic cells. Stimulation with either Gram-negative or Gram-positive bacteria, or their products, induces an upregulation of uPAR expression on monocytes and neutrophils. uPAR serves a dual role in cellular movement; it binds urokinase plasminogen activator (uPA), resulting in the generation of cell surface-associated plasmin activity, which is critical for pericellular proteolysis of extracellular matrix proteins. uPAR also contributes to cell adhesion in a plasmin-independent fashion. Although uPAR is anchored to the cell surface by a GPI tail and lacks its own transmembrane and cytoplasmic domain, it has a crucial role in transendothelial migration of monocytes and neutrophils. The capacity of uPAR to influence adhesion depends on functional linkage and physical association with integrins. Indeed, uPAR is able to form complexes with the β2-integrin CD11b/CD18, thereby modulating the migration promoting activity. Treatment with saccharides disrupted this uPAR-CD11b/CD18 coupling and inhibited chemotaxis.

Neutrophil emigration from the pulmonary circulation is unique in that it appears to be mediated by two pathways: one that requires CD11b/CD18 and one that does not, varying with the stimulus used to induce pulmonary inflammation. In general, Gram-negative bacterial stimuli are CD11b/CD18-dependent, while Gram-positive stimuli elicit a CD11b/CD18-independent emigration of leukocytes. The essential role of uPAR in host defense against pulmonary infection has in particular been demonstrated for *P. aeruginosa*, a
CD11b/CD18-dependent respiratory pathogen. Mice deficient in uPAR showed increased susceptibility as well as less recruitment of inflammatory cells. We investigated to which extent uPAR deficiency would influence host defense during pneumonia caused by S. pneumoniae, a CD11b/CD18-independent stimulus, responsible for more than 50% of community acquired pneumonias.

**Material 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. All mice were on a mixed C57BI6J (75%) x 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 in Leuven. For the experiments mice were transported to the Academic Medical Center in Amsterdam. All experiments were approved by the Committee on Use and Care of Animals of the Academic Medical Center, Amsterdam, the Netherlands.

**Induction of pneumonia.** Pneumonia was induced as described previously. Briefly, S. pneumoniae, serotype 3, obtained from American Type Culture Collection (ATCC 6303; Rockville, MD), were grown for 6 hours to midlogarithmic phase at 37°C in 5% CO\(_2\) using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500 x g for 15 minutes, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at approximately 10\(^{6}\) colony forming units (CFU)/ml, as determined by plating serial 10-fold dilutions onto sheep-blood agar plates. Mice were lightly anesthesized by inhalation of isoflurane (Abott, Queensborough, Kent, UK), and 50 µl of bacterial suspension was inoculated intranasally.

**Bronchoalveolar lavage.** At 24 and 48 hours after inoculation mice were anesthetized by intraperitoneal injection with Hypnorm\(^{\circ}\) (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, 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. 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, Ill).

**Histological examination.** After 24 hours fixation of lungs in 10% buffered formaline and embedding in paraffin, 4 µm thick sections were stained with haematoxylin and eosin. 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, St. Louis, MO) in 0.01 M HCl. After being rinsed, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to FITC-labeled anti-mouse Ly-6-G monoclonal antibody (Pharmingen, San Diego, CA). Endogenous peroxidase activity was quenched by a solution of 0.1% NaN₃/0.03% H₂O₂ (Merck, Darmstadt, Germany). After washes, slides were incubated with a rabbit anti-FITC antibody (Dako) followed by further incubation with a biotinylated swine anti-rabbit antibody (Dako), rinsed again, incubated in a streptavidin-ABC solution (Dako) and developed using 1% H₂O₂ and 3,3’-diaminobenzidin-tetra-hydrochloride (Sigma) 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 5 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 hours. For cytokine measurements lung homogenates were lysed in lysisbuffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl₂, 2 mM Triton (X-100), Pepstatin A, Leupeptin, Aprotinin (20 ng/ml), pH 7.4) and spun at 1500 x g at 4°C for 15 minutes; 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 manufacturers recommendations: Interleukin 6 (IL-6) (Pharmingen, San Diego, CA), Interleukin 1β (IL-1β), Macrophage inflammatory protein 2 (MIP-2) and KC (R&D systems, Abingdon, United Kingdom). Lowest detection limits were 37 pg/ml for IL-6, 47 pg/ml for MIP-2 and 12 pg/ml for KC. uPA activity was measured by an amidolytical assay as previously described. Briefly, diluted lung homogenates were incubated with 0.30 mmol/l S-2251 (Chromogenix, Mölndal, Sweden), 0.13 mol/l plasminogen and 0.12 mg/ml CNBr 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 lysis due to uPA activity was determined by including in the assay 50 µg/mL polyclonal neutralizing rabbit anti-murine u-PA–specific IgGs. A standard curve for uPA activity was obtained by incubating different amounts of purified murine u-PA to the assay system.

Statistical analysis. 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. P-value <0.05 was considered to represent a statistically significant difference.

Results
uPA concentrations in the lung during pneumonia. Intranasal administration of S.87
pneumoniae increased the concentrations of uPA in lung homogenates obtained 24 and 48h after inoculation (Figure 1). Hence, these data demonstrate that pneumococcal pneumonia results in locally elevated levels of the ligand of uPAR.

**Inflammatory cell influx in BALF.** Recently is 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 Wt mice after inoculation with S. pneumoniae. uPAR\(^{−/−}\) mice had significantly less cells in their BALF when compared to Wt mice (P=0.004), which was mainly caused by a diminished recruitment of neutrophils (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Cell influx in BALF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
</tr>
<tr>
<td>Macrophages</td>
</tr>
<tr>
<td>Lymphocytes</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (x 10^4/ml BALF) of 6 mice per group, 48h after inoculation with 10^5 S. pneumoniae CFU. *P<0.05 vs. Wt mice.

**Histopathology.** At 48h after inoculation, the lungs of uPAR\(^{−/−}\) mice showed patchy and dense inflammatory infiltrates (Figure 2A), predominantly composed of monocytes and lymphocytes with relatively few granulocytes (Figure 2A and Figure 2C). In contrast, Wt mice had a mild interstitial inflammation (Figure 2B), composed of granulocytes and monocytes corresponding to a clearance phase (Figure 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 48h after inoculation. At both time points uPAR\(^{−/−}\) mice had significantly more CFU in their lungs than Wt mice (P<0.05) (Figure 3). Furthermore, S. pneumoniae could be cultured from the blood of 12.5% of the Wt mice after 48h, whereas 87.5% of the blood cultures obtained from the uPAR\(^{−/−}\) mice
were positive for *S. pneumoniae* at this time point. At 24h blood cultures were negative in all mice.

**Figure 2. Histopathology of lungs.** (A,C): Lungs of uPAR<sup>−/−</sup> mice 48h after infection showing foci of inflammation with few granulocytes (C, anti-granulocyte immunostaining). (B,D): Lungs of Wt mice 48h after infection showing a mild interstitial inflammation with influx of granulocytes (D, anti-granulocyte immunostaining). A and B: HE staining x 33, insert x 120; C and D: anti-granulocyte staining magnification x 66.

**Figure 3. Bacterial outgrowth in lungs.** *S. pneumoniae* CFU in lungs of Wt and uPAR<sup>−/−</sup> mice 24 and 48 hours after intranasal inoculation with 10<sup>5</sup> CFU *S. pneumoniae*. Data are mean ± SEM. N=8 per group per time point. *P<0.05 vs. Wt mice.

**Survival.** To investigate whether uPAR influences survival, we assessed survival twice daily in Wt and uPAR<sup>−/−</sup> mice after intranasal inoculation with 10<sup>5</sup> CFU *S. pneumoniae*. uPAR<sup>−/−</sup>
mice succumbed much earlier than Wt mice (P=0.004; [Figure 4]).

**Figure 4. Survival.** Survival after intranasal inoculation with $10^5$ CFU *S. pneumoniae* in Wt (open circles) and uPAR$^{-/-}$ mice (closed squares). Mortality was assessed twice daily for 10 days. N = 12 per group.

**Cytokines and chemokines.** Because the localized production of cytokines and chemokines is an important part of host defense, we measured the concentrations of these mediators in lung homogenates. All cytokines and chemokines measured (IL-1β, IL-6, KC and MIP-2) were higher in uPAR$^{-/-}$ mice, in particular at 48h post inoculation (Table 2). Thus, a reduced production of protective pro-inflammatory cytokines or chemokines could not explain the impaired host defense in uPAR$^{-/-}$ mice.

| Table 2. Cytokine and chemokine levels in lung homogenates |
|-----------------|-----------------|-----------------|-----------------|
|                 | ng/ml | Wt     | uPAR$^{-/-}$ | Wt     | uPAR$^{-/-}$ |
|                 |       | 24h    | 48h           | 24h    | 48h           |
|                 |       | Wt     | uPAR$^{-/-}$ | Wt     | uPAR$^{-/-}$ |
| IL-6            |       | 2.0 ± 0.1 | 3.3 ± 0.7 | 2.9 ± 0.6 | 6.2 ± 0.7*|
| IL-1β           |       | 0.8 ± 0.05 | 1.4 ± 0.3 | 1.2 ± 0.2 | 2.5 ± 0.3*|
| KC              |       | 2.1 ± 0.5 | 1.9 ± 0.3 | 2.8 ± 0.6 | 6.9 ± 1.1*|
| MIP-2           |       | 0.9 ± 0.04 | 1.1 ± 0.7* | 1.1 ± 0.1 | 6.7 ± 1.9*|

Data are mean ± SEM of 8 mice per group, measured 24 and 48h after intranasal inoculation with $10^5$ *S. pneumoniae* CFU at t=0. * P<0.05 vs. Wt.

**Host response in uPA$^{-/-}$ 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 (Figure 5). In contrast to uPAR$^{-/-}$ mice, uPA$^{-/-}$ mice had more neutrophils (P=0.08) and less pneumococci in their lungs at 48h postinoculation (P=0.027). There was no significant difference in mortality between uPA$^{-/-}$ 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, which elicits a CD11b/CD18 dependent inflammatory response. The inflammatory cell migration during pneumococcal pneumonia...
occurs independent of CD11b/CD18. Despite this independency, the main findings of our study were that uPAR deficiency partly prevented the recruitment of cells to lungs and

![Graph showing granulocytic influx, bacterial outgrowth, and survival](image)

Figure 5. uPA deficient mice. (A) Granulocytic influx; mean ± SEM granulocyte influx in BALF 48h after intranasal inoculation of 10^5 CFU S. pneumoniae in Wt and uPA^−/− mice. N = 6 per group. (B) Bacterial outgrowth; mean ± SEM S. pneumoniae CFU in lungs of Wt and uPA^−/− mice 48h after inoculation. N = 9 per group per time point. *P<0.05 vs. Wt mice. (C) Survival after intranasal inoculation in Wt (open circles) and uPA^−/− mice (closed squares). Mortality was assessed twice daily for 10 days. N = 12 per group.

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, phorbol myristate acetate, and Gram-negative bacterial stimuli elicit migration via pathways mediated by CD11b/CD18. Conversely, the cell migration in response to Gram-positive bacteria, hydrochloric acid and C5a occurs independent of CD11b/CD18. Treatment with anti-CD18 antibody had no effect on the leukocyte emigration in the lung induced by S. pneumoniae. 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. 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 neutrophilic influx in the lung associated with an enhanced bacterial outgrowth. 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 less neutrophils in BALF and lung parenchyma in uPAR−/− mice. This finding at least in part can explain the impaired anti-pneumococcal defense of uPAR−/− mice in this model of respiratory tract infection, considering that neutrophils are critical for effective eradication of bacteria from the lungs. 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 and causing a conformational change in uPAR which uncovers a chemotactic epitope, while on the other hand uPA negatively influences the migratory function of uPAR in vitro. 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 or even an inhibitory effect on the adhesive capacity of monocytes and neutrophils. In vivo, intratracheal KC (a murine CXC chemokine) administration to uPA−/− and Wt mice reduced the neutrophil influx after engagement of uPAR by non-proteolytic uPA, while uPAR−/− mice showed no difference in cell accumulation. In addition, uPA−/− mice showed no difference in neutrophil recruitment during pneumonia caused by Gram-negative bacteria or fungi. 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 in spite of the fact that both mouse strains were on the same C57Bl6J (75%) x 129 (25%) 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 x 10^5 CFU vs. 1 x 10^5 CFU). Second, the experiments with uPAR−/− and corresponding Wt mice, and those with uPA−/− 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 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); thus, slight (non-genetic) differences may have contributed to the different behaviour 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 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 \textit{P. aeruginosa}, a \(\beta2\)-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 \(\beta2\)-integrin for the inflammatory response. On the other hand uPA\(^{+}\) 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.

References


