Urokinase receptor-deficient mice mount an innate immune response to and clarify respiratory viruses as efficiently as wild-type mice

Manuel Ramos1,4, Yolanda Lao1, César Equiluz2, Margarita Del Val3, and Isidoro Martínez4,5,*

1Unidad de Inmunología Viral; Centro Nacional de Microbiología; Instituto de Salud Carlos III; Madrid, Spain; 2Unidad de Veterinaria; Instituto de Salud Carlos III; Madrid, Spain; 3Centro de Biología Molecular Severo Ochoa; CSIC/Universidad Autónoma de Madrid; Madrid, Spain; 4Unidad de Infección Viral e Inmunidad; Centro Nacional de Microbiología; Instituto de Salud Carlos III; Madrid, Spain; 5Centro de Investigación Biomédica en Red. Enfermedades Respiratorias; Instituto de Salud Carlos III; Madrid, Spain

Keywords: influenza A virus, innate immune response, respiratory syncytial virus, urokinase plasminogen activator receptor (uPAR)

Abbreviations: HRSV, human respiratory syncytial virus; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; DMEM, Dulbecco’s Modified Eagle’s medium; BAL, bronchoalveolar lavage; dpi, days post-infection.

Introduction

The viral infection of epithelial respiratory cells induces the expression of numerous immune-related genes that mediate chemotactic activity, which, in turn, attracts cells of the immune system such as monocytes, neutrophils, and NK cells to the site of infection. Although infiltration of the lungs by these cells is necessary for virus clearance and the initiation of the subsequent adaptive immune response, it may also lead to excessive inflammation.1 The urokinase plasminogen activator receptor (uPAR) is one of the genes involved in cell migration and recruitment that is upregulated in many bacterial, viral, and parasitic infections.2 We have previously demonstrated that uPAR is induced during human respiratory syncytial virus (HRSV) infection of cultured epithelial cells.3

uPAR (CD87) is expressed by some leukocytes (mainly neutrophils and monocytes), by endothelial cells,4 and others.5 It is a protein that has 3 domains, denominated (starting from the N-terminal end) DI, DII, and DIII. The domains are joined by short linkers, and the protein is anchored to the cell membrane by glycosylphosphatidylinositol (GPI). 5 The role that uPAR plays in cell migration is complex and appears to have at least 2 distinct components: one proteolysis dependent and the other proteolysis independent. The former relies on the cell surface concentration of the uPAR ligand known as the urokinase-type plasminogen activator (uPA). uPA is a serine-protease that promotes extracellular matrix degradation, thus facilitating cell migration. The latter component is dependent upon the proteolytic cleavage of the DI domain, which generates a shortened form of uPAR (DII-DIII). Cleaved uPAR can be released from the cell surface in a soluble form that is a chemoattractant for leukocytes. Accordingly, following infection with respiratory bacteria uPAR-deficient (uPAR−/−) mice suffer impaired lung infiltration, bacterial outgrowth, and increased mortality.6,7 However, data about the function of uPAR in the context of respiratory viral infections are lacking. In this report, 2 epidemiologically relevant respiratory viruses were studied: HRSV and influenza A. The level of viral replication and the composition of lung infiltrates were contrasted in uPAR−/− and wild type (WT) mice.

Materials and Methods

Animals. WT female (C57BL/6 background) and heterozygous uPAR+/− male mice were purchased from The Jackson Laboratory (strain name: B6.129P2-Plaurtm1Hdq/J). The WT and uPAR−/− lines were derived from these. The mice were maintained in the Specific Pathogens Free facility of the Centro Nacional de Microbiología (Instituto de Salud Carlos III). After
establishment of these murine lines, random genotyping was carried out throughout the study as a control. All experiments were approved by the Committee of Bioethics and Animal Welfare of the Instituto de Salud Carlos III.

**Virulence**

Viral inoculation. Mice of about 8 weeks of age were lightly anesthetized by inhalation of isofluorane (Schering-Plough) and inoculated intranasally with either $3 \times 10^7$ p.f.u. per mouse of sucrose gradient purified HRSV (strain A2) or $10^7$ p.f.u. of influenza A virus (strain A/Puerto Rico/8/1934 H1N1) in a volume of 50 µl. For survival experiments, mice were inoculated with $10^5$ p.f.u. of influenza virus per mouse. Groups of 5 to 10 mice were used.

Bronchoalveolar lavage (BAL). Mice were sacrificed by CO$_2$ inhalation. The tracheas were then exposed and cannulated with a sterile 18 G blunt fill needle (BD). BAL was performed by applying 3.0 ml aliquots of PBS. Approximately 2 ml of lavage fluid was recovered per mouse. Total cell counts were determined using a hemocytometer.

**Lung homogenates for virus titration.** Whole lungs were harvested and placed on a nylon sieve (BD) attached to a 50 ml tube. Using a syringe plunger, they were then homogenized in 5 ml of Dulbecco’s Modified Eagles Medium (DMEM) plus 2% fetal calf serum (in the case of HRSV) or DMEM without serum (in the case of the influenza virus). Lung homogenates were centrifuged at a low speed and the supernatants were stored at −80 °C until viral titration. HRSV titers were determined by plaque assays on HEp-2 cells layered with 0.7% low melting point agarose (Corda). After 5 days, cells were fixed with 4% formaldehyde in PBS and then with methanol. The plaques were visualized using specific anti-HRSV antibodies and 3-amino-9-ethylcarbazole (AEC) (Sigma). Influenza virus titers were determined by a standard plaque assay on MDCK cells at 72 h post-infection.

**Flow cytometry.** The cells acquired by BAL were washed and incubated for 15 min in blocking buffer (PBS, 2% fetal calf serum, 10 µg/ml 2.4G2 antibody) and then incubated for 30 min with fluorescent conjugated antibodies. After three washes, the cells were resuspended in PBS containing 1% paraformaldehyde and then with methanol. The plaques were analyzed using the FACSDiva software (BD). The antibodies used in this study were: anti-CD16/CD32/mouse Fc block (clone BM8; eBioscience), and anti-mouse uPAR (clone 2.4G2; BD), anti-neutrophils-FITC (clone 7/4; Acris), anti-CD8+ T cells, and anti-CD4+ T cells. The antibodies used in this study were: anti-CD16/CD32/mouse Fc block (clone 2.4G2; BD), anti-neutrophils-FITC (clone 7/4; Acris), anti-F4/80-Pe-Cy5 (clone BM8; eBioscience), and anti-mouse PAN-NK (clone DX5; eBioscience). Neutrophils were identified as CD4+ and F4/80− cells.

Weight loss and survival. Mice inoculated with influenza A were weighed daily and monitored for survival for 12 days. Animals that lost 25% or more of their body weight were euthanized and counted as dead animals.

**Histopathology.** Using conventional methods, lung sections were fixed in 10% formalin, embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E).

Statistical analysis. Medians were calculated for each data point (interquartile range). The Mann-Whitney test was employed for comparisons between groups. Comparisons that resulted in a value of $p < 0.05$ were considered significant and were labeled in the figures with an “*.”

### Results and Discussion

**Virus replication and lung infiltrate in HRSV infections**

According to past reports, intranasal inoculation of C57BL/6 mice with HRSV leads to viral replication levels that peak at around 4 dpi and that are almost undetectable by 7 dpi. In our hands, the infection of WT and uPAR−/− mice followed the same course (Fig. 1A). WT mice had higher titers than uPAR−/− mice at 4 dpi (median values of $1.1 \times 10^5$ versus $5.0 \times 10^3$), however, this difference was barely significant (p = 0.048) (Fig. 1A).

In order to quantify the recruitment of innate immune cells such as macrophages, neutrophils, and NK cells to the lungs of HRSV infected mice, specific antibodies were used in flow cytometry to analyze bronchoalveolar lavage fluids (BAL). The total cell count was obtained using a hemocytometer and using this value, the number of cells in each population was calculated. Following HRSV infection, cell infiltration in the lungs was limited: it peaked at 2 dpi and returned to levels seen in uninfected animals by 4 dpi (Fig 1b). Significant differences in the total cell count were observed when comparing WT and uPAR−/− mice at 1 and 4 dpi. Significant differences were also seen in the neutrophil subpopulation at 4 dpi. In every case, these variations were a consequence of fewer cells infiltrating uPAR−/− mice. uPAR−/− mice also had fewer infiltrating macrophages and NK cells on those days (Fig. 1B). However, this difference was not statistically significant. The higher number of cells in WT mice at 1 and 4 dpi correlated with a higher viral titer (Fig. 1A). This is in agreement with the apparent correlation in children between HRSV titer and severity of infection. Comparable viral titers were observed in the 2 mouse genotypes at 6-7 dpi (Fig. 1A). Consistent with this result, the quantity of B cells, of CD4+ T cells, and of HRSV-specific CD8+ T lymphocytes was also similar (data not shown). HRSV infection led to a lower cell infiltration and smaller viral titers in uPAR−/− than in WT mice at 1 and 4 dpi. In contrast, with bacterial infections, the uPAR deficiency is known to provoke a decrease of cell infiltrate and an increase in bacterial count.

To corroborate flow cytometry data, lungs from WT and uPAR−/− infected mice were harvested at 1 dpi and stained with H&E. In both lines, a small to moderate perivascular and peribronchial cell infiltrate, consisting of lymphocytes, neutrophils and macrophages, was observed (Fig. 1C). In contrast, with bacterial infections, the uPAR deficiency is known to provoke a decrease of cell infiltrate and an increase in bacterial count.

To corroborate flow cytometry data, lungs from WT and uPAR−/− infected mice were harvested at 1 dpi and stained with H&E. In both lines, a small to moderate perivascular and peribronchial cell infiltrate, consisting of lymphocytes, neutrophils and macrophages, was observed (Fig. 1C). In contrast, there was no major difference in lung histology between infected WT and uPAR−/− mice.

HRSV does not replicate robustly in mice and high titers of inocula (10^7-10^8 p.f.u. per mouse) are needed to detect virus replication and symptoms of illness. In addition, there are significant differences in susceptibility to HRSV infection among mouse strains. The C57BL/6 strain used in this study is one of the most resistant. The results from mice infected with HRSV are difficult to interpret because of the minimal lung
We therefore performed additional experiments by inoculating mice with the influenza virus strain A/Puerto Rico/8/1934 H1N1 (PR8), which is a mouse-adapted virus that replicates to high titers. Influenza virus infections

Mice were infected with 10³ p.f.u. of PR8 per mouse. Viral titers were determined by testing lung homogenates in a standard plaque assay. This infectious dose was chosen because it induced both a high level of viral replication and an increase in cell recruitment, all without killing the mice. Under these conditions, we were able to monitor viral clearance. The course of the infection with the PR8 virus is dependent on the genetic background of the mice. C57BL/6 strain typically have viral titers of about 10⁵ p.f.u per lung. In our assays, similar titers were observed between 2 and 4 dpi, which decreased thereafter to about 1.5 x 10³ p.f.u./lungs by 8 dpi (Fig. 2A). Statistically significant differences between WT and uPAR−/− mice were observed at 1 dpi (median values of 9.3 x 10⁵ p.f.u./lungs vs 2.5 x 10³, p = 0.02) and 2 dpi (median values 6.5 x 10⁷ p.f.u./lungs vs 3.8 x 10⁵, p = 0.01), with uPAR−/− mice having the highest titers (Fig. 2A). Although these differences were very small and disappeared after 2 days of infection, these results indicate that, in the case of the influenza A virus, uPAR may exert a modest and transient influence on viral replication.

In contrast to HRSV, influenza virus infection resulted in a robust infiltration of the lungs by macrophages, neutrophils, and NK cells that peaked between 3 and 4 dpi. This infiltration was maintained until at least 8 dpi, which was the last day tested, and it was basically indistinguishable between the WT and uPAR−/− animals (Fig. 2B).

The reason for the highly meaningful differences between HRSV and influenza cell infiltrates is unknown. However, they might be related to the infiltration by innate immune cells.
fact that, in mice, the influenza PR8 strain replicates much more efficiently than HRSV does. Nevertheless, they are distinct viruses and there may be variations in mechanisms other than virus replication, such as in the induction and/or control of the immune system.

Lungs from WT and uPAR−/− infected mice were harvested at 5 dpi and stained with H&E. Moderate to high perivascular and peribronchial cell infiltrate was observed in both lines. This consisted of lymphocytes, neutrophils, and macrophages (Fig. 2C). No major differences between infected WT and uPAR−/− mice were observed with respect to lung histopathology, a result which confirmed the flow cytometry results.

Weight loss and mortality in influenza A viral infections

HRSV does not kill mice and weight loss is almost undetectable in the 8-week-old C57BL/6 mice used in this study. At that age, minimal weight loss can only be detected in the more permissive BALB/c strain. Therefore, in the case of HRSV infection, the monitoring of survival or weight loss in order to study the pathology was precluded. In contrast, the influenza virus causes a strong pathology in C57BL/6 mice. In this case it was possible to monitor weight loss and mortality. Here, one group each of 12 WT and 12 uPAR−/− mice were infected with a LD50 of virus (10^5 p.f.u. per mouse), and monitored daily for weight loss and mortality. Concerning mortality, only small differences were seen between the 2 lines of mice (Fig. 3C). Six uPAR−/− mice died between 5 and 7 dpi and 7 WT mice died between 5 and 8 dpi. In general, among the survivors, uPAR−/− mice experienced a more pronounced weight loss than the WT animals had (Figs. 3A and B). Because there was not a big disparity in the level of viral replication at low dosages, these data suggest that influenza may be somewhat more pathogenic in the absence of uPAR, an interesting result that is being further investigated.

The data presented here suggest that uPAR does not play an essential role in
2 types of respiratory viral infections in mice, at least with respect to viral replication and lung infiltration. Likewise, the uPA/uPAR system does not seem to play a relevant role in controlling viral load and inflammatory responses in a murine model of the gamma herpes virus 68 and Zaire ebola viral infections. In contrast, by interacting with uPA and vitronectin, uPAR contributes to the inhibition of HIV-1 release from monocytic cells. The cleaved and soluble form of uPAR, however, increases HIV-1 expression and blocks cell migration. Meanwhile, with respiratory bacteria, the lack of uPAR leads to a reduction in cell infiltrate in the lungs and increases bacterial replication in uPAR−/− mice. Altogether, these results suggest that the role of uPAR in infections is complex and may be dependent upon the type of infectious pathogen involved. Differences in the characteristics of the infection caused by distinct pathogens, such as extracellular vs. intracellular replication, replication rate, capacity to disrupt the endothelial barrier and spread to the bloodstream, may be behind the variations in uPAR role. The higher genomic complexity of bacteria as compared with viruses may allow for a qualitatively distinct host-pathogen balance. Indeed, interactions between bacteria and the uPA/uPAR system have recently been described. For example, the metalloproteinase LasB of Pseudomonas aeruginosa induces the endoproteolysis of uPAR, a process which appears to correlate with disruption of the endothelial barrier. Also, uPA contributes to plasmin recruitment on the cell surface of Group A Streptococcus, thereby favoring bacterial invasiveness.

In conclusion, uPAR plays distinct roles in bacterial and viral respiratory infections. These results open up new avenues for the exploration of key differences in the innate immune response to both kinds of microorganisms. An understanding of these differences may contribute to the development of specific prophylactic and/or therapeutic measures against relevant human pathogens.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We are grateful to Dr. Amelia Nieto for her generous gift of the PR8 virus, and to Dr. José A. Melero for his critical reading of the manuscript.

**Funding**

This work was supported by grants PI08/0702 and PI11/00590 awarded to IM by the Fondo de Investigación Sanitaria, by grant number RD06/0006/0033 awarded to MR by the Red Temática de Investigación Cooperativa RIS, and by grants SAF2007-60934, SAF2010-18917 and SAF2013-48754-C2-R awarded to MDV by the Ministerio de Ciencia y Tecnología.

**References**

1. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin Microbiol Rev 2010; 23:74-98; PMID:20605326; http://dx.doi.org/10.1128/CMR.00032-09
2. Monnuori N, Selleri C, Ragno P. The urokinase-receptor in infectious diseases. Infez Med 2012; 20 Suppl 2:13-8; PMID:23042001
3. Martinez I, Lombardia I, Garcia-Barreno B, Dominguez O, Melero JA. Distinct gene subsets are induced at different time points after human respiratory syncytial virus infection of A549 cells. J Gen Virol 2007; 88:570-81; PMID:17251576; http://dx.doi.org/10.1099/vir.0.82187-0
4. Pleasner T, Ralfkiaer E, Wérmup M, Johnsen H, Pyke C, Pedersen TL, Hansen NE, Daae K. Expression of the receptor for urokinase-type plasminogen activator in normal and neoplastic blood cells and hematopoietic tissue. Am J Clin Pathol 1994; 102:835-41; PMID:7801901
5. Thuno M, Macho B, Eugen-Olsen J. uPA receptor: the molecular crystal ball. Dis Markers 2009; 27:157-72; PMID:19893210; http://dx.doi.org/10.1155/2009/504294
6. Rijneveld AW, Levi M, Florquin S, Speelman P, Carmeliet P, van Der Poll T. Urokinase receptor is necessary for adequate host defense against pneumococcal pneumonia. J Immunol 2002; 168:3507-11; PMID:11907112; http://dx.doi.org/10.4049/jimmunol.168.7.3507
7. Gyertko MR, Sud S, Kendall T, Fuller JA, Newstead MW, Standiford TJ. Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary Pseudomonas aeruginosa infection. J Immunol 2000; 165:1513-9; PMID:10963788; http://dx.doi.org/10.4049/jimmunol.165.3.1513
8. Taylor PR, Brown GD, Geldhof AB, Martinez-Pomares L, Gordon S. Pattern recognition receptors and differentiation antigens define murine myeloid cell heterogeneity ex vivo. Eur J Immunol 2003; 33:2090-7; PMID:12884282; http://dx.doi.org/10.1002/eji.200324003

9. Stark JM, McDowell SA, Koenigsknecht V, Prows DR, Leikauf JE, Le Vine AM, Leikauf GD. Genetic susceptibility to respiratory syncytial virus infection in inbred mice. J Med Virol 2002; 67:92-100; PMID:11920822; http://dx.doi.org/10.1002/jmv.2196

10. Chavez-Bueno S, Mejias A, Gomez AM, Olsen KD, Rios AM, Fonseca-Aten M, Ramilo O, Jafri HS. Respiratory syncytial virus-induced acute and chronic airway disease is independent of genetic background: an experimental murine model. Virol J 2005; 2:46; PMID:15916706; http://dx.doi.org/10.1186/1743-422X-2-46

11. Hall CB, Douglas RG, Jr., Geiman JM. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. J Pediatr 1976; 89:11-5; PMID:180274; http://dx.doi.org/10.1016/S0022-3476(76)80918-3

12. Hirsz GK. Studies of Antigenic Differences among Strains of Influenza a by Means of Red Cell Agglutina-
tion. J Exp Med 1943; 78:407-23; PMID:19871338; http://dx.doi.org/10.1084/jem.78.5.407

13. Srivastava B, Blazejewska P, Hessmann M, Bruder D, Geffers R, Mauel S, Gruber AD, Schughart K. Host genetic background strongly influences the response to influenza a virus infections. PLoS One 2009; 4:e4857; PMID:19293935; http://dx.doi.org/10.1371/journal.pone.0004857

14. Graham BS, Perkins MD, Wright PF, Karzon DT. Primary respiratory syncytial virus infection in mice. J Med Virol 1988; 26:153-62; PMID:3183639; http://dx.doi.org/10.1002/jmv.21890260207

15. Chen H, Zheng D, Abbott J, Liu L, Bartee MY, Long M, Davids J, Williams J, Feldmann H, Strong J, et al. Myxoma virus-derived serpin prolongs survival and reduces inflammation and hemorrhage in an unrelated lethal mouse viral infection. Antimicrob Agents Chemother 2013; 57:4114-27; PMID:23774438; http://dx.doi.org/10.1128/AAC.02594-12

16. Elia C, Caussé E, Sidhomius N, Blai F, Castagna A, Poli G, Alfano M. Inhibition of HIV replication by the plasmino-
gen activator is dependent on vonwillebrand-mediated cell adhesion. J Leukoc Biol 2007; 82:1212-20; PMID:17704294; http://dx.doi.org/10.1189/jlb.0407251

17. Graziano F, Elia C, Laudanna C, Poli G, Alfano M. Urokinase plasminogen activator inhibits HIV virion release from macrophage-differentiated chronically infected cells via activation of RhoA and PKCepsilon. PLoS one 2011; 6:e25674; PMID:21858203; http://dx.doi.org/10.1371/journal.pone.0025674

18. Nebuloni M, Zawada L, Ferroni A, Tosoni A, Zerbi P, Resnati M, Poli G, Genovesi L, Alfano M. HIV-1 infected lymphoid organs upregulate expression and release of the cleaved form of uPAR that modulates chemotaxis and virus expression. PLoS one 2013; 8: e70606; PMID:23923008; http://dx.doi.org/10.1371/journal.pone.0070606

19. Beaufort N, Corvazier E, Milansoindrou S, de Bentzmann S, Pidard D. Disruption of the endothelial bar-
er by proteases from the bacterial pathogen Pseudomonas aeruginosa: implication of matrilysis and receptor cleavage. PLoS One 2013; 8:e75708; PMID:24060438; http://dx.doi.org/10.1371/journal.pone.0075708

20. Sanderson-Smith ML, Zhang Y, Ly D, Donahue D, Hollands A, Nizet V, Ranson M, Ploplis VA, Walker MJ, Castellino FJ. A key role for the urokinase plasmin-
gen activator (uPA) in invasive Group A streptococcal infection. PLoS Path 2013; 9:e1003469; PMID:23853591; http://dx.doi.org/10.1371/journal.ppat.1003469