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*J Immunol* 2005; 175:4060-4068; doi: 10.4049/jimmunol.175.6.4060

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The Urokinase/Urokinase Receptor System Mediates the IgG Immune Complex-Induced Inflammation in Lung

Nelli Shushakova,† Gabriele Eden,* Marc Dangers,† Joerg Zwirner,‡ Jan Menne,*† Faikah Gueler,* Friedrich C. Luft,§ Hermann Haller,* and Inna Dumler2*§

Immune complex (IC) deposition induces an acute inflammatory response with tissue injury. IC-induced inflammation is mediated by inflammatory cell infiltration, a process highly regulated by the cell surface-specific receptor (uPAR), a binding partner for the urokinase-type plasminogen activator (uPA). We assessed the role of the uPA/uPAR system in IC-induced inflammation using the pulmonary reverse passive Arthus reaction in mice lacking uPA and uPAR compared with their corresponding wild-type controls. Both uPA-deficient C57BL/6J (uPA−/−) and uPAR-deficient mice on a mixed C57BL/6J (75%) × 129 (25%) background (uPAR−/−) demonstrated a marked reduction of the inflammatory response due to decreased production of proinflammatory mediators TNF-α and Glu-Leu-Arg (ELR)-CXC chemokine MIP-2. In uPAR−/− animals, the reduction of inflammatory response was more pronounced because of decreased migratory capacity of polymorphonuclear leukocytes. We show that the uPA/uPAR system is activated in lung of wild-type mice, particularly in resident alveolar macrophages (AM), early in IC-induced alveolitis. This activation is necessary for an adequate C5a anaphylatoxin receptor signaling on AM that, in turn, modulates the functional balance of the activating/inhibitory IgG FcγRs responsible for proinflammatory mediator release. These data provide the first evidence that the uPA/uPAR plays an important immunoregulatory role in the initiation of the reverse passive Arthus reaction in the lung by setting the threshold for C5a anaphylatoxin receptor/FcγR activation on AM. The findings indicate an important link between the uPA/uPAR system and the two main components involved in the IC inflammation, namely, complement and FcγRs. The Journal of Immunology, 2005, 175: 4060–4068.

Urokinase-type plasminogen activator (uPA) is a multifunctional molecule that serves either as a proteolytic enzyme or as a signal-inducing ligand. The urokinase receptor (uPAR; CD87) was originally identified as a protease receptor for uPA, directing pericellular proteolysis. However, uPAR also mediates intracellular signaling via surface proteins such as integrins, growth factor receptors, and G protein-coupled membrane proteins. These dual properties enable the uPA/uPAR system to control pericellular fibrinolytic and proteolytic activities as well as cell adhesion, migration, proliferation, and differentiation (1). The uPA/uPAR system modulates Ag processing and presentation (2), lymphocyte activation (3), generation of pro- and anti-inflammatory signals (4), activation of intracellular signaling pathways (5), and cytotoxicity (6), all of which are critical steps in cell-mediated immune responses. Involvement of the uPA/uPAR system in derangements of immune responses in HIV disease via an IFN-like mechanism has also been demonstrated (7, 8). Participation of the uPA/uPAR signaling system in immune complex (IC)-dependent diseases has not been thoroughly investigated. Although the reported up-regulation of uPA and uPAR in rheumatoid arthritis (9) and glomerulonephritis (10) implies a probable involvement of this system in IC-mediated immunologic diseases, molecular mechanisms underlying the uPA/uPAR-directed initiation and perpetuation of the IC-triggered inflammatory reaction remain unexplored. The formation of IC and their tissue deposition induces an acute inflammatory response termed the Arthus reaction. Allergic bronchopulmonary aspergillosis, chronic obstructive lung, and farmer’s lung are pulmonary conditions that feature the Arthus reaction. We relied on the pulmonary Arthus reaction in our experiments.

The initial events incurred after a challenge by IC include activation of two codominant effector pathways responsible for the subsequent development of IC inflammation: complement system and IgG FcγRs on resident effector cells (11, 12). The activating FcγRIII and inhibitory FcγRII bind IC with similar affinity and specificity. Activation of the ITAM motif-containing FcγRIII primes various cellular responses in immune-competent cells, whereas simultaneous activation of FcγRII results in tyrosine phosphorylation of its ITIM motif and subsequent inhibition of the FcγRII ITAM-triggered activation signal (13). Disturbance of the functional balance between activating and inhibitory FCγRs directly leads to IC-mediated autoimmune diseases (14). IC induce the late complement component bioactive C5a anaphylatoxin (C5a) that, in turn, interacts with the C5a receptor (C5aR) and switches a balance between activating and inhibitory IgG FcγRs on resident effector cells. Modulation of both activated systems, namely the C5a/C5aR and FcγRs on resident alveolar macrophages (AM) is critical in IC-induced lung disease. Moreover, the FcγR-dependent effector activities are integrated through IC activation of the complement system with a pivotal role for the C5a/
C5aR (15). In the present study we demonstrate that the uPA/uPAR system is activated in the lung and particularly in resident AM early in IC-induced alveolitis. We found that the presence of uPAR is necessary for an adequate C5a/C5aR signaling on leucocytes participating in IC-triggered alveolitis and that uPA occupancy of uPAR synergistically increased C5a-induced effects in these cells. The cross-talk between activated uPA/uPAR and C5a/C5aR systems had an impact on modulation of the functional balance of activating/inhibitory FcγRs. Our data provide the first evidence that the uPA/uPAR system plays an important immunoregulatory role in the initiation of the reverse passive Arthus reaction in the lung by setting the threshold for C5aR/FcγRs activation on AM. We suggest that our findings have therapeutic implications.

Materials and Methods

Mice

Mice with a targeted deletion in the gene for uPA or uPAR, generated as previously described (16), were a gift from P. Carmeliet and M. Desverchin (Center for Transgene Technology and Gene Therapy, University of Leuven, Leuven, Belgium). The animals were bred under pathogen-free conditions in the animal facility of Phenos. The uPA−/− mice were on the C57BL/6J backgrounds, and their wild-type controls were obtained from the animal facility of Hannover Medical School. uPAR−/− mice on a mixed C57BL/6d background, and their wild-type controls were bred under the same pathogen-free conditions. Mice of C57BL/6d × 129 background were immunocompetent and had preservation of complement-dependent acute lung injury (17). All these mice were male and were used at 8–12 wk of age. Experiments were conducted in accordance with the regulations of the local authorities.

Experimental IC-induced alveolitis

Mice were anesthetized with ketamine and xylazine, the trachea was cannulated, and 150 μg of protein G chromatography-purified rabbit anti-OVA IgG Ab (anti-OVA; Sigma-Aldrich) were applied to the trachea. Immediately thereafter, 20 mg/kg OVA Ag were given i.v. Ab control animals received anti-OVA IgG Ab into the trachea and PBS instead of OVA i.v. Mice were killed at various time points (ranging from 2 to 24 h) after initiation of IC-induced alveolitis, and bronchoalveolar lavage (BAL) fluids were assayed for polymorphonuclear leukocyte (PMNL) accumulation, hemorrhage, chemotactic activity, and production of the proinflammatory mediators, TNF-α, and MIP-2. BAL-derived AM, recovered 2 h after IC challenge, were used for uPA/uPAR, C5aR, and FcγR expression studies by TaqMan real-time PCR. Lavaged lung tissues were processed for histological examination and stained with H&E according to conventional procedures or were homogenized in PBS, sonicated, and finally cleared by 5-min centrifugation at 10,000 rpm. Supernatants were analyzed for uPA protein expression after SDS-PAGE by Western blotting using anti-mouse uPA (Innovative Research), 5′-CAAGCGAGCGGACCGTGA-3′ antisense, 5′-TCGTTCTTGGTTCGGA-3′; and probe, 6-FAM-ATGAGTTACCGAGCTGGTCA-3′; C5aR, 5′-CTGGGTTAGACCCCTCTGA-3′ antisense, 5′-CCCGCAGATTTCAAGCAG-3′; and 6-FAM-CCACAGGCGCAGGCG-3′; and probe, 6-FAM-ATGAGTTACCGAGCTGGTCA-3′; casein, 5′-GCACTCAGATCACCCCTCTGA-3′; and probe, 6-FAM-ACTGTTACTCCTGAGGCTCA-3′; C57BL/6d females were used at 8–12 wk of age. Experiments were conducted in accordance with the regulations of the local authorities.

BAL quantitation of hemorrhage and PMN accumulation in bronchoalveolar space

Pulmonary vasculature was gently flushed with PBS with a catheter positioned in the root pulmonary artery. Lungs were lavaged with PBS (1 ml; five times at 4°C) after cannulation of the trachea as previously described (11). The volume of collected BAL fluid was measured in each sample, and the total cell count was assessed with a hemocytometer (Neubauer Zählkammer). The amount of erythrocytes represented the degree of hemorrhage.

Chemotactic activity and TNF-α, MIP-2, and plasminogen activator inhibitor type 1 (PAI-1) concentrations in BAL fluid

C57BL/6d wild-type mouse bone marrow cells were suspended at 7.5 × 107 cells/ml RPMI 1640 medium/0.5% BSA (Sigma-Aldrich). One hundred microliters of the cell suspension were placed into the insert of a 5.6-mm diameter, 3-μm pore polycarbonate Transwell chemotaxis chamber (Costar). The bottom well was filled with 600 μl of RPMI 1640 medium/0.5% BSA or BAL fluid diluted 1/2 in RPMI 1640 medium/1% BSA. Inserts were combined to the lower chambers and incubated at 37°C and 5% CO2 for 2 h. Transmigrated neutrophils were quantified as described by us previously (15). The concentrations of TNF-α and MIP-2 in BAL fluids were measured in duplicate in appropriately diluted samples with respective specific ELISA kits (R&D Systems) according to the manufacturer’s instructions. The detection limits of the assays were 5.1 pg/ml for TNF-α and 1.5 pg/ml for MIP-2. The concentration of PAI-1 was measured in nondiluted samples using PAI-1-specific ELISA kit (Molecular Innovations), according to the manufacturer’s instructions. The detection limit of the assay was 0.05 ng/ml.

Expression analysis in vivo

Total RNA was prepared from BAL-derived AM recovered 2 h after IC challenge using the RNaseasy Mini kit (Qiagen) and analyzed for uPAR, C5aR, and FcγR mRNAs by TaqMan real-time PCR technology. β-Tubulin served as the reference gene. The following primers and probes were used: uPA: sense, 5′-CGATTCCTGAGGACCGCTTTA-3′ antisense, 5′-CCAGCTCACAATCCACCTCA-3′ and probe, 6-FAM-CTGTTCTTGGTTCGGA-3′; C5aR, 5′-CTGGGTTAGACCCCTCTGA-3′ antisense, 5′-CCCGCAGATTTCAAGCAG-3′; and 6-FAM-CCACAGGCGCAGGCG-3′; and probe, 6-FAM-ATGAGTTACCGAGCTGGTCA-3′; C57BL/6d mice were on the 129 background were immunocompetent and had preservation of complement-dependent acute lung injury (17). All these mice were male and were used at 8–12 wk of age. Experiments were conducted in accordance with the regulations of the local authorities.

Functional analysis of AM in vitro

The mouse AM cell line MH-S (20), expressing both uPA and uPAR, was maintained in RPMI 1640 medium containing 10% FCS and supplements. In functional experiments, adherent MH-S cells in 6-well plates (106 cells/well) or primary murine AM in 96-well plates (50 × 104 cells/well) were starved for 24 h in 1% FCS/RPMI 1640 medium and activated with 10 nM mouse uPA (Innovative Research), 5 μg/ml heat-aggregated IgG IC (mouse IgG1, as previously described (21), and 50 ng/ml recombinant human C5a (rhC5a; Sigma-Aldrich) separately or in different combinations of these stimuli. Production of TNF-α and MIP-2 by MH-S cells was examined, as described previously (15), using respective specific ELISA kits (R&D Systems) according to the manufacturer’s instructions. Total RNA was prepared from nonsimulated and stimulated MH-S cells using the RNeasy Mini kit (Qiagen) and analyzed for uPAR, C5aR, and FcγR mRNAs by TaqMan real-time PCR technology, as indicated above. In some experiments, MH-S cells were lysed with buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM Na3VO4, and 1 mM sodium fluoride directly on culture dishes, and expression of uPAR, uPA, and C5aR proteins was detected in cell lysates clarified by centrifugation at 12,000 rpm for 10 min after SDS-PAGE by Western blotting (22), using poly- or monoclonal anti-mouse uPAR Abs (R&D Systems), anti-mouse uPA H77A10 mAbs (Innovative Research), or anti-mouse C5aR mAbs (clone 20/70, rat anti-mouse IgG; generated as previously described (23)). Peroxidase activity on immunoblotted membranes was detected using Western lighting chemiluminescence reagent (PerkinElmer). In immunoprecipitation experiments, subconfluent and starved MH-S cells were treated with 50 ng/ml rhC5a for 5–60 min at 37°C. Clarified lysates were used for immunoprecipitation. Five micrograms of either polyclonal anti-mouse uPAR or monoclonal anti-mouse C5aR Abs were incubated with 600-1000 μg of cell lysate protein for 2 h, then protein G-agarose was added, and incubation proceeded for another hour. The beads were washed three times with 500 μl of PBS containing protein inhibitors, solubilized in Laemmli-SDS sample buffer, and then analyzed by Western blotting.

Statistical analysis

To analyze differences of mean values, a two-sided unpaired Student’s t test was used; p < 0.05 was considered significant, and p < 0.001 was considered highly significant.
**Results**

The uPA/uPAR is activated in AM in vitro and in vivo in response to IgG IC

Because in vivo studies clearly demonstrate the pivotal role of AM in various models of lung pathology, and AM have been shown to express both uPA (24) and uPAR (25), we first tested whether the uPA/uPAR system may be activated in response to IC stimulation in vitro and in vivo. The IC-induced expression of uPA and uPAR was investigated in adherent mouse AM MH-S cells stimulated in vitro with heat-aggregated mouse IgG1 IC. Cell stimulation with IC induced synthesis of uPA mRNA (Fig. 1a). This acute response was transient, with maximal induction at 90 min after stimulation; the level of mRNA for uPA returned to the baseline at 4 h. Increased uPA expression was confirmed at the protein level in both cell lysates (Fig. 1b) and conditioned medium (Fig. 1c) by observation of the 50-kDa band corresponding to mouse uPA. The stimulation of MH-S cells with IC also resulted in increased expression of uPAR mRNA that started 1 h after stimulation, peaked at 4 h, and remained elevated at 12 h (Fig. 2a). Stimulation with IC within 4 h was sufficient to mediate induced protein synthesis of uPAR (40- to 48-kDa band) in MH-S cells (Fig. 2b).

To check the possible IC activation of the uPA/uPAR system in vivo, the IC-induced reverse passive pulmonary Arthus reaction in experimental IC-triggered alveolitis was induced in C57BL/6J mice and in uPA-deficient mice. The uPA-deficient mice provided an independent internal control for the specificity of uPA band observed in Western blotting. IC administration strongly increased the uPA level in lung homogenates obtained 4 h after IC challenge compared with the uPA level in the lungs of Ab controls. The uPA level declined but still remained elevated 24 h after the IC challenge (Fig. 3a). We were not able to detect uPA protein in BAL fluids obtained from Ab control mice (data not shown). However, a dramatic uPA increase that peaked at 4 h was detected in BAL fluids obtained from IC-stimulated mice (IC; Fig. 3b). The protein level of uPA was investigated in BAL obtained from C57/BL6J and uPA−/− mice by intratracheal injection of 150 μg of purified anti-OVA Ab, followed by systemic 20 mg/kg OVA Ag (IC). The level of uPA protein was analyzed in whole lung tissue homogenates by Western blotting. Mice receiving only anti-OVA Ab served as controls (Ab). Two or three representatives are shown for each group containing 8–12 mice. Significance was determined by Student’s t test (*, p < 0.05).
fluid of IC-treated animals (Fig. 3b). Zymographic analysis confirmed these findings (data not shown). Of note, the increased release of uPA could be detected in BAL fluid 2 h after the IC challenge before any PMN influx had occurred (26) (data not shown).

Next we examined the mRNA level for uPA and uPAR in freshly isolated AM from IC-treated C57BL/6J mice compared with Ab controls 2 h after the challenge. RNA analysis showed a rapidly pronounced IC-mediated transcriptional induction of both uPA and uPAR in AM (Fig. 3c). These data demonstrate that the IC-induced reverse passive pulmonary Arthus reaction results in local activation of the uPA/uPAR system in lung and especially in resident AM and that this activation is before subsequent PMN infiltration.

The hypersensitivity reaction is impaired in both uPAR- and uPA-deficient mice

To further investigate the possible role of activated uPA/uPAR system in IC-dependent inflammation, uPAR- or uPA-deficient mice together with their respective wild-type (WT) controls were used for the IC-triggered alveolitis model. The animals were killed 4 and 24 h after the challenge, and the inflammatory response was determined by analyzing the kinetics of PMN influx into lung tissue (Fig. 4) in H&E-stained sections, by analysis of BAL fluids for accumulation of PMN in the bronchoalveolar space (Fig. 5a), and by quantitation of RBC in BAL fluids, indicating the degree of pulmonary hemorrhage (Fig. 5b). Only weak signs of inflammation were observed in the lungs of all mice injected with anti-OVA Ab that were not receiving the OVA Ag. Substantial IC-induced inflammation was detected in both groups of WT mice treated with OVA-anti-OVA IgG IC at 4 h, reaching the highest level at 24 h. However, recruitment of PMN into lung tissue and alveoli as well as hemorrhage were markedly reduced 4 and 24 h after IC challenge in both uPAR- and uPA-deficient mice. The attenuation of all three parameters was substantially more pronounced in uPAR−/− than in uPA-deficient mice, particularly 24 h after the

![FIGURE 4. Histopathological examination of interstitial PMN influx in IgG IC alveolitis. Representative H&E-stained sections of paraffin-embedded lung tissue of C57BL/6J and C57BL/6J × 129 WT (WT), uPA−/− (uPA−/−), and uPAR−/− (uPAR−/−) mice 4 and 24 h after the injection of anti-OVA IgG (Ab) or OVA-anti-OVA IgG IC (IC). Original magnification, ×200. Six mice were investigated for each group.](http://www.jimmunol.org/)

![FIGURE 5. Attenuation of IC-induced lung injury by uPA and uPAR deficiencies. IC-induced alveolitis was induced in C57BL/6J (WT; □), C57BL/6J × 129 WT (WT; □), uPA−/− (uPA−/−; ▲), and uPAR−/− (uPAR−/−; □) mice as described above, and the inflammatory response was allowed to proceed for 4 or 24 h (IC). Mice not receiving OVA Ag served as controls (Ab). PMN influx in the alveolar space (a) and hemorrhage (b) were evaluated. Results are expressed as the mean ± SEM (n = 8–12 mice for each group). Significance was determined by Student’s t test (*, p < 0.05; **, p < 0.001; ***, p < 0.0001).](http://www.jimmunol.org/)
The development of IC-dependent alveolitis in mice is strongly dependent on local production of proinflammatory mediators such as chemokines of the CXC family and TNF-α (26, 27). Therefore, we investigated the IC-induced production of TNF-α and MIP-2 in BAL fluids of uPA−/− and uPA+/− mice 4 h after the challenge. uPA deficiency led to significantly decreased MIP-2 and TNF-α release (Fig. 6, a and b, left panels). The PMN-specific chemotactic activity of BAL fluids was assessed by in vitro chemotaxis assay using C57BL/6J WT PMN. The observed decrease in production of TNF-α and MIP-2 was correlated with the attenuation of chemotactic activity in BAL fluids collected from uPA−/− mice (Fig. 6c, left panel). Analysis of BAL fluids obtained from uPAR−/−, uPA−/−, and uPAR−/− uPA−/− mice also revealed strongly diminished levels of TNF-α and MIP-2 as well as reduced chemotactic activity (Fig. 6, a–c, right panels). The reduction of proinflammatory mediator release in uPA−/− mice was more pronounced than that in uPA+/− animals, corresponding to the changes in PMN influxes observed in both groups. These results indicate that the uPA/uPAR system contributes significantly to proinflammatory mediator release in IC-induced alveolitis.

**Proinflammatory mediator release is impaired in both uPAR- and uPA-deficient mice**

uPA synergistically enhances C5a-dependent activation of AM in vitro

To clarify the mechanism underlying the observed decrease in proinflammatory mediator release in uPA−/− and uPAR−/− mice, we explored a probable synergistic cooperation of the uPA/uPAR and C5a/C5aR and/or FcγR systems on AM. Stimulation of MH-S AM with murine uPA did not affect MIP-2 and TNF-α production (Fig. 7, a and b). Stimulation of FcγRs on AM with heat-aggregated IC resulted in the strong release of both TNF-α and MIP-2, which was not affected by the addition of uPA (data not shown). Stimulation of C5aR on AM with rhC5a resulted in a moderate, but significant, increase in MIP-2 and TNF-α protein levels in conditioned medium of MH-S cell culture. However, in the presence of uPA, C5a-induced MIP-2 and TNF-α production increased in a synergistic fashion by 2.6- and 1.8-fold, respectively. Interestingly, the blockade of uPAR on the cell surface not only completely prevented uPA-induced enhancement of MIP-2 and TNF-α release from C5a-stimulated MH-S cells, but also significantly attenuated the C5a-dependent response for MIP-2 of itself (Fig. 7, a and b). This observation suggests that the presence of uPAR and its occupancy by uPA are important for effective C5a/C5aR signaling.
We repeated the same experiments with primary culture of AM obtained from uPA−/− and uPAR-deficient mice and their corresponding WT controls (Fig. 7, c and d). No additional MIP-2 release was observed in conditioned medium of any AM treated with uPA. Although both WT AM responded to C5a stimulation by MIP-2 release, this response was completely abrogated in uPAR−/− AM and was significantly reduced in uPA-deficient AM. In contrast to strongly pronounced synergistic effect observed in MH-S cells, the presence of uPA only slightly increased C5a-dependent MIP-2 production in both types of WT AM by 25 and 18% for C57BL/6J (75%) × 129 (25%) or C57BL/6J, respectively, and did not reach statistical significance. However, in the presence of uPA, C5a-induced MIP-2 release from uPA−/− AM was almost 2-fold increased compared with their response to C5a alone. At the same time, no effect of uPA pretreatment, as expected, was observed in uPAR−/− AM (Fig. 7, c and d). These data strongly suggest that the uPA/uPAR system contributes significantly to C5a/uPAR signaling in AM.

One of the possible explanations for reduced proinflammatory mediator release from C5a-activated uPA−/− and uPAR−/− AM could be a decreased C5aR expression on these cells. Therefore, we investigated C5aR expression on freshly isolated AM from C57BL/6J, C57BL/6J (75%) × 129 (25%), uPA−/−, and uPAR−/− mice by immunocytochemistry. Surprisingly, C5aR expression was even higher in both knockout strains compared with their WT controls (data not shown), implying that the level of C5aR on the cell surface was not responsible for disturbed C5aR signaling in uPA−/− and uPAR−/− AM.

**uPAR and C5aR are associated in a C5a-dependent fashion**

To examine the potential link between uPAR- and C5a-mediated signaling, we examined the association of C5aR and uPAR in MH-S cells in response to C5a. We performed immunoprecipitation with anti-uPAR Abs and looked for C5aR in the immunoprecipitates. As shown in Fig. 8a, only a weak band corresponding to C5aR (46 kDa) was detected in uPAR precipitates of unstimulated cells. When cells were stimulated with C5a, the C5aR band became significantly stronger after 5-min stimulation and further increased with activation time. The control reversed coimmunoprecipitation experiments using anti-C5aR Ab for immunoprecipitation and anti-uPAR Ab for blot probing demonstrated the same result for uPAR band (40–48 kDa; Fig. 8b). These data allow us to conclude that uPAR and C5aR in AM are associated in a C5a-dependent fashion.

**Migratory capacity of PMN depends on uPAR**

We next investigated the PMN capacity of uPA−/− and uPAR−/− animals to migrate toward different chemoattractants, such as BAL fluid of IC-challenged WT mice containing CXC chemokine (Fig. 9a) and rhC5a (Fig. 9b). The migratory capacity of uPA−/− PMN was significantly attenuated not only toward BAL, but also toward such a strong PMN-specific chemoattractant as rhC5a. Interestingly, the migratory capacity of uPA−/− PMN was not reduced compared with that of corresponding WT PMN. These data indicate the strong contribution of uPAR to chemotaxis of PMN cells, although its occupancy with uPA seems to be less important.

**C5a/C5aR-dependent modulation of FcγRII and FcγRII is disturbed in uPAR−/− mice**

The early IC-induced bioactive C5a and its interaction with C5aR cause induction of activating FcγRII and suppression of inhibitory FcγRIIB on AM in vivo. This step is crucial for efficient proinflammatory mediator production and PMN recruitment in IC-induced alveolitis (15). Because uPAR seems to be very important for adequate C5a/C5aR signaling, and uPA synergistically increased C5a-dependent activation in both cultured MH-S cells and primary AM, we speculated that activation of the uPA/uPAR system observed early in IC-induced alveolitis might influence C5a/C5aR-directed expression of activating FcγRIII and inhibitory FcγRII in these cells. Therefore, we analyzed, by TaqMan RT-PCR, the expression of FcγRs in AM obtained from WT and uPAR−/− mice in IC-induced alveolitis 2 h after the challenge. Pronounced induction of activating FcγRII (Fig. 10a) and its signal-transducing Fcγy chain (Fig. 10b) as well as suppression of inhibitory FcγRII (Fig. 10c) were observed in WT C57BL/6J × 129 mice. These effects were much less pronounced in uPAR−/− animals and did not reach significance. Thus, the uPA/uPAR system does indeed have a regulatory role in the modulation and function of main immune-competent receptors responsible for the development of IC-induced inflammation in lung.

**Discussion**

The reverse passive Arthus reaction in mice and rats is a well-characterized acute model of IC-mediated inflammation that is...
Clinical relevance to many immunological and inflammatory human diseases. In this study we demonstrate an important role for the uPA/uPAR system in the reverse passive Arthus reaction in the lung using the Arthus model of IC-induced alveolitis in uPA- and uPAR-deficient mice. The underlying molecular mechanism involves the cross-talk between uPA/uPAR and C5a/C5aR systems in resident AM as well as the involvement of uPAR in the migratory capacity of PMN cells. The presence of uPAR is necessary for adequate C5a/C5aR signaling in resident AM, and its occupancy with uPA facilitates C5a-induced responses of these cells.

We show that IC activation of AM in vitro results in strong induction of uPA and uPAR expression on these cells, suggesting that the uPA/uPAR system might mediate functional responses in IC-stimulated AM. An autocrine mechanism of the uPA/uPAR activation could be postulated, although the source of uPA in IC-induced alveolitis remains to be identified.

We demonstrate the necessity of both uPA and uPAR for full development of IC-induced alveolitis. Both uPA- and uPAR-deficient mice revealed strongly reduced PMN influx and hemorrhage in lung tissue and bronchoalveolar space after IC challenge. Although all experiments were performed under adequately controlled conditions using the same charges of chicken egg albumin and purified anti-OVA, the magnitude of inflammatory response to IC was considerably higher in C57BL/6J (75%) × 129 (25%) WT mice than in C57BL/6J animals. Our observations are in agreement with an earlier report demonstrating the stronger PMN influx into lung after intrapulmonary KC administration in uPAR−/− mice on a mixed C57BL/6J × 129 background compared with C57BL/6J animals (28). This finding indicates the dependence of the immune response on the genetic background.

Because the development of IC-mediated alveolitis in mice is critically dependent on local production of proinflammatory mediators, such as TNF-α and the chemokines of the CXC family (26, 27), we monitored the release of these mediators in BAL fluid of experimental animals. Both uPA and uPAR deficiencies strongly attenuated proinflammatory mediator release from AM. These results are consistent with observation that the capacity of uPA to initiate inflammation is mediated via the release of proinflammatory cytokines, IL-6, IL-1β, and TNF-α, from monocyte and lymphocyte cell populations (29). Our findings might explain the observations that uPA-deficient mice have profoundly impaired immunity and are largely immunologically unresponsive (30, 31). Although uPA contributes to the production of proinflammatory mediators in vivo and in vitro, there is no evidence for the involvement of uPAR in this process. Moreover, uPAR−/− mice did not show any decrease in TNF-α level in murine malaria (32). The production of IL-1β, IL-6, KC, and MIP-2 was even higher in uPAR−/− mice during pneumococcal pneumonia, than in controls (33). From these results, the host defense in uPAR−/− mice is probably not related to a reduction in released protective proinflammatory cytokines or chemokines. In contrast with these observations, we provide in this study direct evidence that in IC-induced alveolitis, uPAR deficiency results in impaired release of proinflammatory mediators.

PMN influx into lung tissue is a hallmark of IC-mediated alveolitis. Although uPAR deficiency strongly reduces the migration of leukocytes and lymphocytes, uPA exerts opposite influences on leukocyte migration in different models (28, 33, 34). These observations led to the conclusion that the requirement for uPAR in neutrophil recruitment was independent of uPA (28). In line with these observations, in our in vitro experiments the migratory capacity of uPAR−/− PMN was strongly attenuated, whereas uPA−/− PMN migration was indistinguishable from WT independent of the chemoattractant used (CXC chemokines in BAL fluid or C5a). The unchanged migratory capacity of uPA−/− PMN can explain the fact that the reduction of the inflammatory response was less pronounced in uPA−/− than in uPAR−/− mice. Another possible explanation for this observation might be different levels of PAI-1 in uPA−/− and uPAR−/− mice due to its disturbed internalization in the absence of uPA and uPAR and possible functional effects beyond the uPA/uPAR system (35). However, no difference in PAI-1 protein level was observed at the time of a strong reduction in inflammatory responses in either uPA−/− or uPAR−/− mice (data not shown).

The pivotal role of uPAR was demonstrated in vitro for FMLP- and MCP-1-induced chemotaxis, which was mediated through two different G protein-coupled chemotactic receptors, formyl peptide receptor-like-1 and CCR2 (36). Moreover, binding of uPA induced conformational changes in uPAR, allowing it to interact with the formyl peptide receptor-like receptor in monocytes (37) and basophils (38), resulting in uPA-induced chemotaxis of these cells. Because the migratory capacity of uPAR−/− PMN toward chemotactants acting through CXCR2 and C5aR was strongly reduced, it is likely that the involvement of the uPA/uPAR system in chemotaxis is a broader mechanism, critical for different kinds of chemotactants acting through G protein-coupled chemotactic receptors.

The most important finding of this study is that adequate C5a/C5aR signaling on AM is mediated by the uPA/uPAR system in IC-induced alveolitis. The functional blockade of uPAR via anti-uPAR Abs or uPAR deficiency as well as uPA deficiency.
significantly attenuated C5a-dependent responses, such as MIP-2 and TNF-α production, in AM. Stimulation of AM in vitro with uPA alone was not sufficient to induce the release of MIP-2 and TNF-α from these cells, but instead resulted in synergistically enhanced C5a/C5aR-induced responses. These data indicate that uPA/uPAR/C5aR cross-talk is necessary for adequate C5a cell activation. The crucially important step in the initialization of IC-induced alveolitis is the C5a-dependent inverse regulation of the inhibitory/activating FcγRII/FcγRIII pair on AM. This effect, in turn, leads to adequate FcγRIII-dependent proinflammatory mediator release. This mechanism was also recently demonstrated for resident peritoneal macrophages in the mouse model of IC pneumonia (39). Our in vivo observations clearly demonstrated that genetic inactivation of uPAR, preventing uPA/uPAR/C5aR cooperation on AM, completely abrogates these events.

The mechanisms underlying the involvement of uPA/uPAR in C5a/C5aR signaling in resident AM remain to be elucidated. Previous work showed the coupling of C5aR to pertussis toxin-sensitive G proteins (40) and strong activation of downstream MAPK signaling pathways (41). In contrast, some uPA/uPAR-dependent responses also act through pertussis toxin-sensitive mechanisms and activation of intracellular tyrosine kinases (42). These data indicate that similar signal transduction pathways may be activated in response to uPA and C5a. We were able to demonstrate the increased association between uPAR and C5aR in MH-S cells induced by C5a. This step seems to be important for full development of C5a-triggered responses. Thus, C5a-induced responses were strongly attenuated in uPA−/− and uPAR−/− AM despite increased C5aR expression in these cells, which implies functional cooperation between uPA/uPAR and C5aR systems at the signal transduction level.

Together, our results demonstrate that the uPA/uPAR system contributes to the development of the IC inflammatory reaction via at least two different pathways, a proinflammatory mediator release and the migratory capacity of PMN cells. Both uPA and uPAR are necessary for adequate production of TNF-α and MIP-2, whereas the expression of uPAR seems to be more important for adequate PMN recruitment to inflamed lung than its occupancy with uPA. The results of this study demonstrate that uPA and its receptor, uPAR, whose role was presumed to be involved in the migratory behavior of infiltrating cells, have a broader critical function as early regulators of immune-competent receptor induction. The generation of uPA at the site of inflammation and the subsequent uPAR activation provide initial signals setting for adequate C5a/C5aR signaling on resident AM, which, in turn, shifts the balance between activating and inhibitory FcγRs on these cells toward an inflammatory phenotype. These observations underscore a new role for the uPA/uPAR system in the regulation of immune-competent receptors responsible for the development of IC-induced inflammation. They identify the uPA/uPAR system as a potential therapeutic target for the regulation of inflammatory processes characterized by neutrophil-mediated injury.

Acknowledgments

We are grateful to Peter Carmeliet and Mieke Dewerchin for providing uPA- and uPAR-deficient mice.

Disclosures

The authors have no financial conflict of interest.

References


