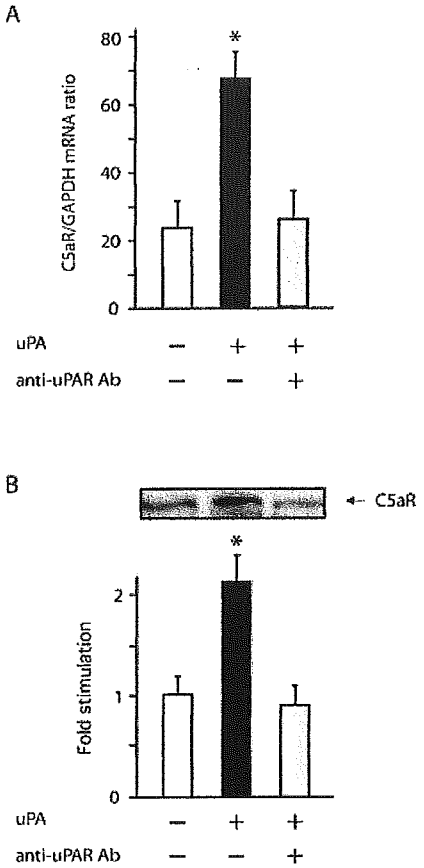

Fig. 2. uPAR mediates uPA-dependent upregulation of C5aR expression. The quiescent MCs were stimulated for 20 hours with 20 nM uPA in the presence or absence of 5 μg/ml anti-uPAR monoclonal antibody, and C5aR expression was investigated at the mRNA (A) and protein (B) levels using RT-PCR TaqMan analysis and western blotting, respectively. MCs incubated in medium without uPA and anti-uPAR antibody served as a control. The results in A are presented as mean ± s.e.m. for three separate and independent experiments. The data on the upper panel in B are representative of three separate and independent experiments. Quantification of the results of these experiments by densitometry presented as mean ± s.e.m. is shown in the lower panel. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (**P*<0.05).

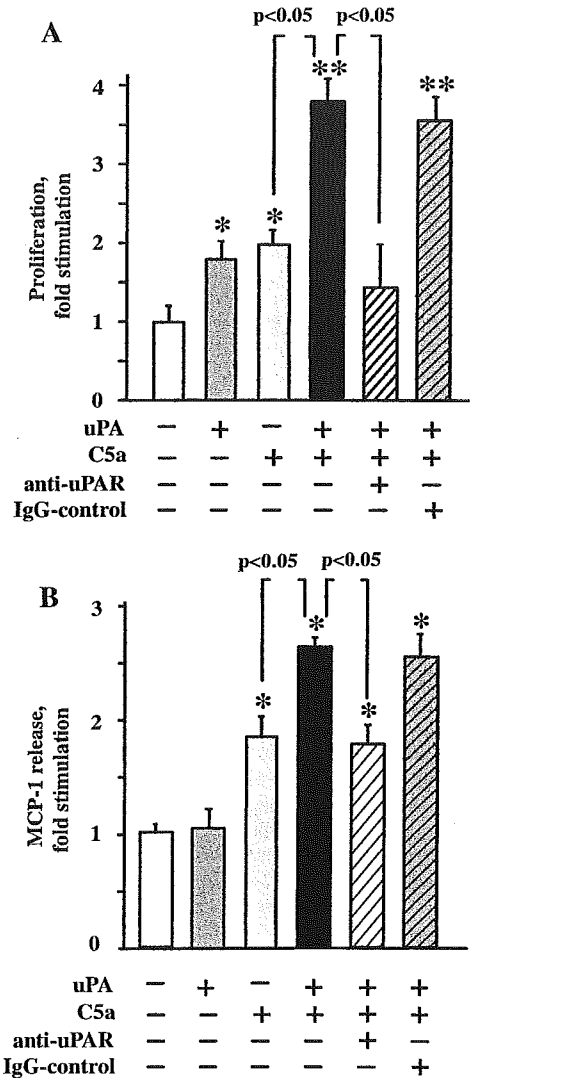

Fig. 3. uPA potentiates C5aR-related responses in MCs. (A) Quiescent MCs were incubated in 96-well microtiter plates with 20 nM uPA or 20 nM C5a alone or in combination with both stimuli in the presence or absence of 5 μg/ml anti-uPAR monoclonal antibody or irrelevant mouse IgG for 24 hours. The cells incubated in medium without stimuli served as a control. After 16 hours labeling with BrdU DNA synthesis was used as measure of the proliferation rate. Results are given as mean ± s.e.m. of four independent experiments performed in six parallel wells for each condition. (B) MCP-1 release was evaluated in supernatants from MC monolayers that were incubated as described in A for 48 hours. MCs incubated in medium without stimuli and producing 868±95 pg/ml MCP-1 served as a control. The data are given as mean ± s.e.m. of four separate and independent experiment performed in triplicate for each condition. Significance between control unstimulated and stimulated cells, as well as between the cells stimulated in the presence or not of 5 μg/ml anti-uPAR monoclonal antibody was determined by Student's *t*-test (**P*<0.05; ***P*<0.01).

of Stats on gene transcription, immunocytochemical studies were performed. As shown in Fig. 4B, Stat3 translocated efficiently into the nuclei of MCs stimulated with uPA.

Phosphorylation of signal transducers and activators of transcription of the Stat family is mediated via the Janus kinases (Jaks) (Chatterjee-Kishore et al., 2000; Darnell et al., 1994). This interplay is highly specific, thus contributing to the specificity of cell functional responses to cytokines, growth factors and polypeptide hormones utilizing the Jak/Stat pathway for signal generation. We therefore examined activation of Jaks in MCs stimulated with uPA. Treatment with uPA rapidly increased specific tyrosine phosphorylation of the Janus kinase Tyk2 (Fig. 5A), whereas other Jaks, namely Jak1, Jak2 and Jak3 were not affected (data not shown). The kinetics of this activation correlates with those for Stat3 phosphorylation. To provide more direct evidence that the uPA-induced Stat3 phosphorylation was really mediated by Tyk2, a dominant negative form of Tyk2, devoid of kinase activity, was

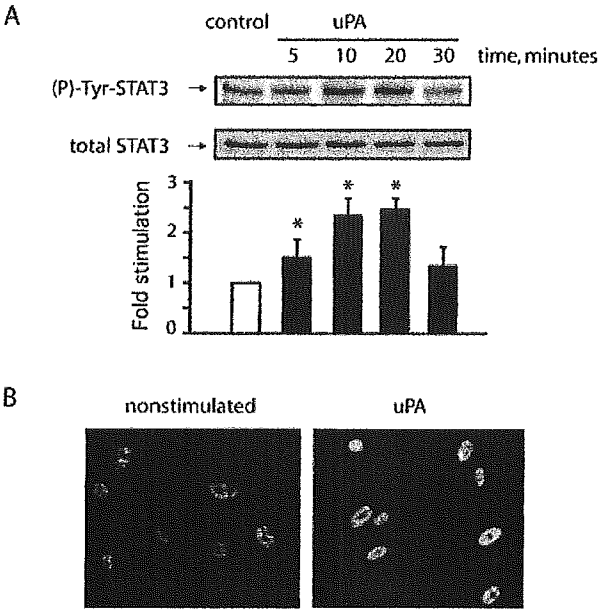

Fig. 4. uPA induces activation and nuclear translocation of Stat3 in MCs. (A) Quiescent MCs were treated with 10 nM uPA for the indicated times and phosphorylated Stat3 protein was visualized in cell lysates by immunoblotting with specific anti-(P)-Tyr-Stat3 antibodies. MCs incubated in medium without uPA served as a control. The middle panel demonstrates the amount of total Stat3 loaded on the gel for each sample. Results are representative of three independent experiments. Quantification (mean ± s.e.m.) of the results of these experiments by densitometry is shown below. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (**P*<0.05). (B) A subconfluent MC monolayer was treated with 10 nM uPA at 37°C or left untreated, fixed and stained using primary anti-(P)-Tyr-Stat3 antibody and the corresponding Alexa Fluor-488 secondary antibody. The data are representative of three separate and independent experiment performed in duplicate for each condition.

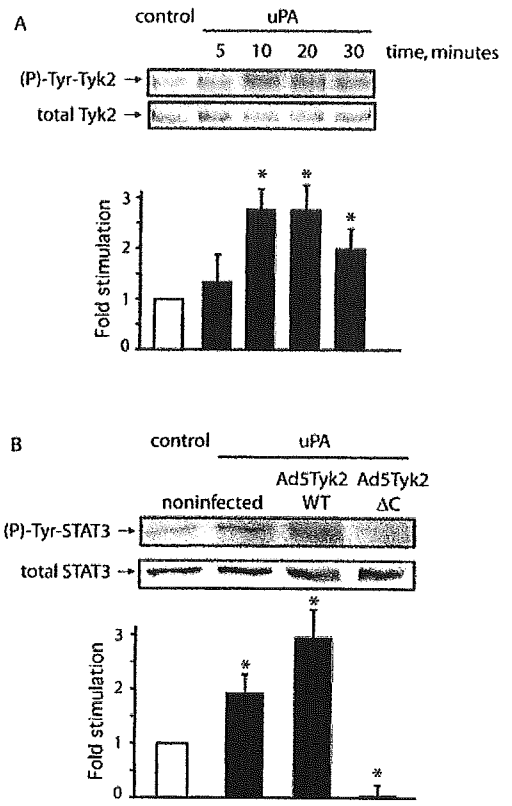

Fig. 5. uPA induces activation of Tyk2 in MCs. (A) Quiescent MCs were treated with 20 nM uPA for the indicated times and phosphorylated Tyk2 protein was visualized in cell lysates by immunoblotting with specific anti-phospho-Tyk2 antibodies (upper panel). MCs incubated in medium without uPA served as a control. The middle panel demonstrates the amount of total Tyk2 loaded on the gel for each sample. Results are representative of three independent experiments. Quantification (mean ± s.e.m.) of the results for these experiments by densitometry is shown below. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (**P*<0.05). (B) MCs were left uninfected or were infected with wild-type Ad5Tyk2 or with the mutant form, Ad5Tyk2ΔC, and then stimulated for 20 minutes with 20 nM uPA. Phosphorylated Stat3 protein was visualized in cell lysates by immunoblotting with specific anti-(P)-Tyr-Stat3 antibodies. Noninfected MCs incubated in medium without uPA served as a control. The middle panel demonstrates the equal amount of total Stat3 loaded on the gel for each sample. Results are representative of three independent experiments. Quantification (mean ± s.e.m.) of the results by densitometry is shown for three experiments is shown below. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (**P*<0.05).

expressed in MCs. As shown in Fig. 5B, uPA-induced Stat3 activation was completely abrogated in Ad5Tyk2ΔC-expressing MCs, as compared to non-infected and wild-type Ad5Tyk2-expressing cells. We conclude from these results that Tyk2 is required for the activation of Stat3 by uPA in MCs.

To examine the specific role of uPAR in propagation of the revealed Tyk2/Stat3 signaling, MCs with downregulated uPAR expression were used. Stable and specific inhibition of uPAR expression was achieved using a lentiviral RNA interference vector LV-uPARsi (Fig. 6A). Neither Tyk2 nor Stat3 phosphorylation was observed in LV-uPARsi-MCs, in contrast to cells infected with control vector (Fig. 6B,C).

Tyk2/Stat3 pathway mediates C5aR expression in MCs in response to uPA

To evaluate the role of the Tyk2/Stat3 pathway in the uPA-directed control of C5aR expression, several experiments were performed. MCs transiently infected with wild-type Ad5Tyk2 and two mutant forms, Ad5Tyk2KE and Ad5Tyk2ΔC, were stimulated with uPA, and C5aR expression was monitored by TaqMan analysis. Whereas there was a strong increase in C5aR mRNA in non-infected and in Ad5Tyk2-infected cells, Ad5Tyk2KE- and Ad5Tyk2ΔC-expressing cells did not respond to uPA stimulation. Consistent with these data, no uPA-induced stimulation in C5aR expression was observed in MCs expressing the Stat3F mutant form of Stat3 (Fig. 7). These results assign a direct role to the Tyk2/Stat3 pathway in controlling immune competent receptors response to uPA.

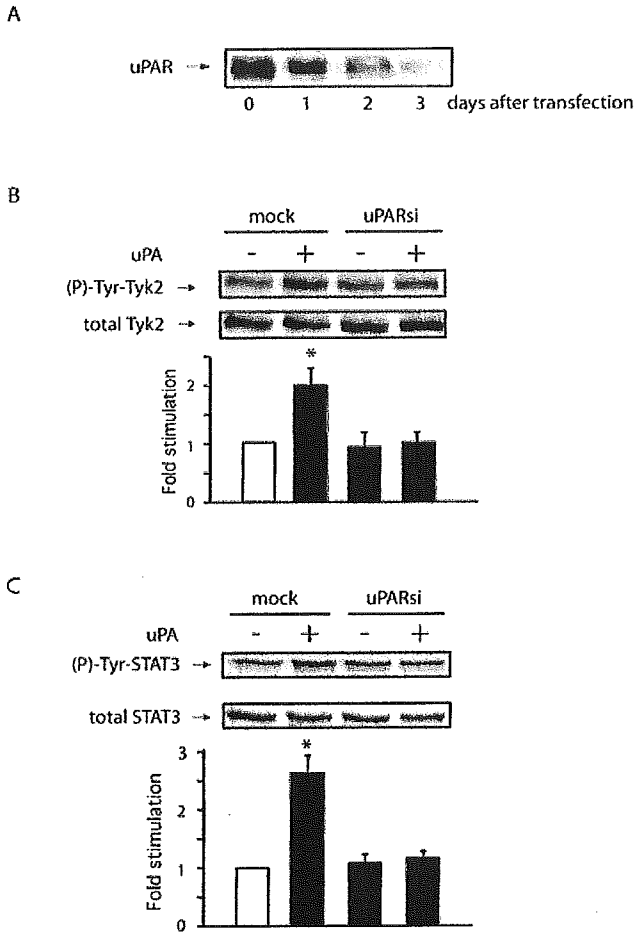

Fig. 6. uPAR mediates uPA-dependent Tyk2/Stat3 pathway activation in MCs. A. Quiescent MCs were infected with LV-uPARsi and time-dependent downregulation of uPAR expression was monitored by immunoblotting, using clone R3 monoclonal anti-uPAR antibody. MCs infected with LV-uPARsi or mock viruses were treated with 10 nM uPA for the indicated times at day 3 after infection and phosphorylated Tyk2 (B) or Stat3 (C) proteins were visualized in cell lysates by immunoblotting with specific anti-phospho-Tyk2 and anti-(P)-Tyr-Stat3 antibody, respectively. MCs incubated in medium without uPA served as a control. The upper panels is representative of three independent experiments. Quantification (mean ± s.e.m.) of the results by densitometry is shown below. Significance between control unstimulated and stimulated cells was determined by Student's *t*-test (*P<0.05).

uPAR utilizes gp130 adaptor protein to mediate C5aR expression in MCs

As uPAR is devoid of catalytic activity and linked to the outer membrane leaflet by a glycosylphosphatidylinositol (GPI) anchor, its signaling capacities and biological functions rely on interactions with other transmembrane molecules. Several proteins interacting with uPAR have been identified, such as integrins, tyrosine kinase receptors and G protein-coupled receptors. It was shown that in the human kidney epithelial tumor cell line TCL-598, uPAR associates with gp130 protein, a signal transducing subunit of the receptor complexes for the IL-6 cytokine family, to activate the Jak/Stat pathway

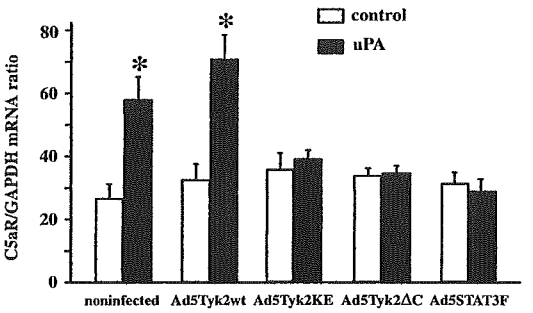

Fig. 7. TheTyk2/Stat3 pathway mediates uPA-induced upregulation of C5aR in MCs. Quiescent MCs that were uninfected or infected with wild-type Ad5Tyk2, the Tyk2 mutants, Ad5Tyk2ΔC and Ad5Tyk2KE, or with the Ad5STAT3F mutant form of Stat3 were stimulated with 20 nM uPA for 20 hours, and RT-PCR analysis for C5aR mRNA was performed using the TaqMan method. MCs incubated in medium without uPA served as a control. Results are presented as mean ± s.e.m. of three independent experiments.

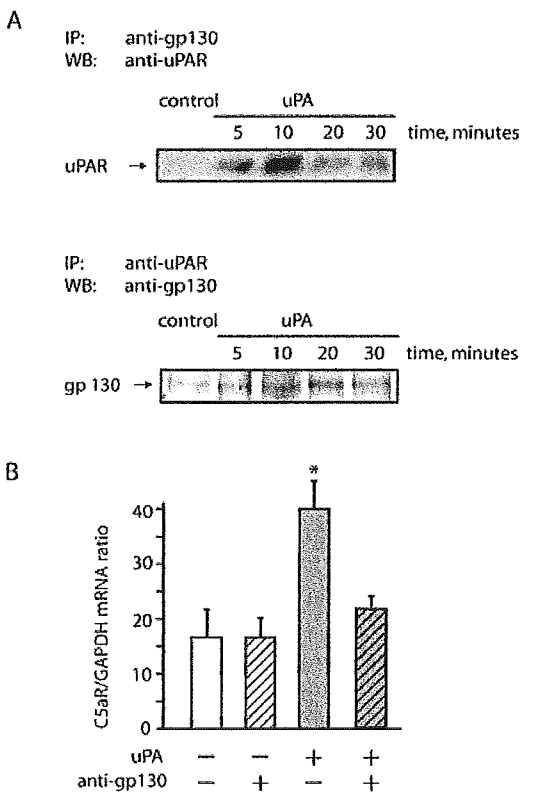

Fig. 8. uPAR utilizes gp130 adaptor protein to mediate C5aR expression in MCs. (A) Quiescent MCs were stimulated with 10 nM uPA for the indicated times or left unstimulated (control), and anti-gp130 (upper panel) or anti-uPAR (lower panel) antibody was used to coimmunoprecipitate gp130 and uPAR from the cell lysates. The immunoprecipitates were then analyzed with anti-uPAR (upper panel) or anti-gp130 antibody (lower panel). (B) RT-PCR analysis of C5aR mRNA was performed using the TaqMan method. RNA was isolated from quiescent MCs incubated for 6 hours with 20 nM uPA or in medium without uPA (control) in the presence or absence of 5 μg/ml of anti-gp130 antibody. Results are presented as mean ± s.e.m. of two independent experiments performed in duplicates for each condition.

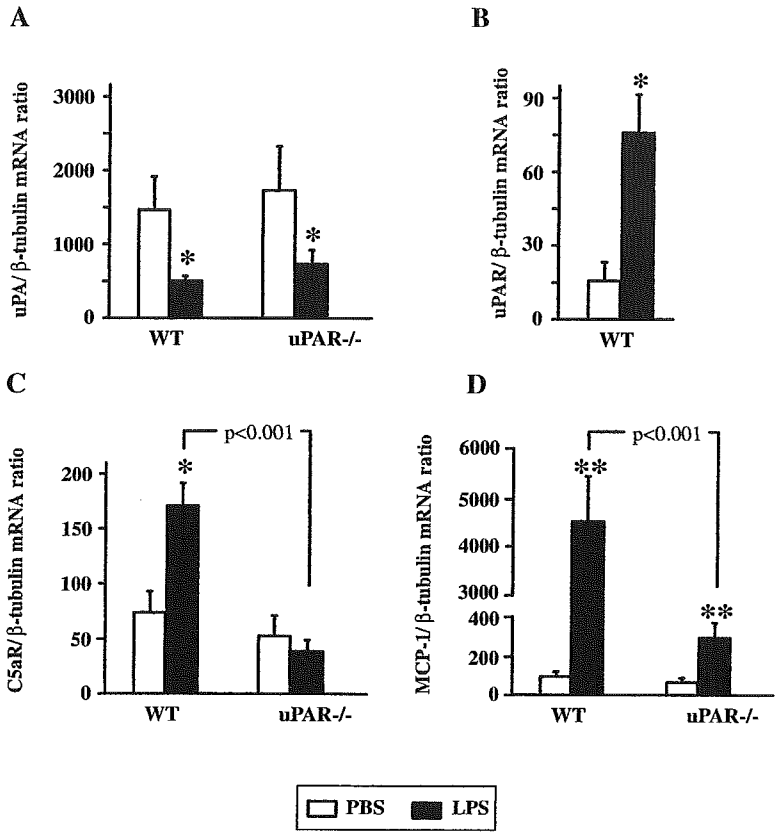

Fig. 9. uPAR is required for C5aR expression in mesangium upon LPS-induced renal inflammation in mice. LPS-induced nephritis was induced in wild-type and *uPAR*$^{-/-}$ mice by an intraperitoneal injection of 50 μg LPS in 200 μl PBS. Animals injected with PBS only, served as a control. 8 hours after challenge the mice were killed, RNA was isolated from kidney glomeruli and used for expression analysis by the TaqMan method. The mRNA levels were analyzed for uPA (A), uPAR (B), C5aR (C) and MCP-1 (D). β-tubulin served as a house-keeping gene. Results are expressed as mean ± s.e.m. (n=6 mice for each group). Significance between PBS controls and LPS-treated animals was determined by Student's t-test (*P<0.05; **P<0.01).

(Koshelnick et al., 1997). Numerous reports provide evidence that activation of Stat3 in response to various stimuli in several cell types is mediated by gp130. These observations point to the probable involvement of gp130 in the uPA-directed Stat3 activation and C5aR upregulation in MCs. To address this hypothesis, we performed immunoprecipitation using anti-gp130 antibody from MCs stimulated with uPA at different time points and determined uPAR in the immunoprecipitates using immunoblotting. Indeed, we observed a specific band for uPAR in the immunoprecipitates (Fig. 8A, upper panel). Specificity of this association was further confirmed in immunoprecipitation experiments using a reverse combination of antibodies, namely anti-uPAR antibody for precipitation and anti-gp130 antibody for western blotting (Fig. 8A, lower panel). The uPAR-gp130 association was uPA-dependent and displayed kinetics very similar to those observed by us for Stat3 and Tyk2 activation. These results suggest that in the uPA-stimulated MCs, uPAR may directly bind to the gp130 adaptor protein, which, in turn, provides a link to the Jak/Stat pathway.

To confirm that gp130 is required to propagate C5aR expression in MCs in response to uPA, cells were pretreated with anti-gp130 blocking antibody and the uPA-directed expression of C5aR was monitored by TaqMan RT-PCR. As expected, no increase in C5aR expression in response to uPA was observed after gp130 blockage (Fig. 8B).

uPAR is required for C5aR expression in mesangium upon LPS-induced renal inflammation in mice

To find out whether our findings might reflect a physiologically relevant process, in vivo studies were performed using a LPS-induced inflammation model. Kidney inflammation was induced by an intraperitoneal injection of LPS into wild-type and uPAR-deficient mice. For control experiments, phosphate-buffered saline (PBS) vehicle was injected. At 8 hours after injections kidney cortex, enriched in glomeruli, was isolated and used for expression analysis. uPA expression was decreased in both wild-type and *uPAR*$^{-/-}$ LPS-treated animals, although there was no difference between these two groups (Fig. 9A). In contrast, uPAR expression in glomeruli of wild-type mice after LPS-induced nephritis was strongly upregulated as compared to control PBS-treated animals (Fig. 9B). Interestingly, while C5aR and MCP-1 were upregulated in inflammed glomeruli of wild-type mice, these responses were abrogated in uPAR-deficient mice (Fig. 9C,D).

Finally, to exclude the possibility that the observed activation of C5aR in glomeruli of LPS-injected mice might be caused by infiltrating neutrophils and macrophages and not restricted to mesangial cells, immunohistochemical studies were performed. This examination revealed the upregulated expression of C5aR localized within mesangium of inflamed glomeruli of wild-type mice (Fig. 10A, upper panels). No infiltrating cells expressing C5aR were identified. The prominent immunohistochemical signal observed in the mesangium of LPS-treated animals was completely abolished in *uPAR*$^{-/-}$ mice with LPS-induced nephritis (Fig. 10A, lower panels). The difference in C5aR expression was further confirmed by a semi-quantification analysis of mesangial immunoreactivity (Fig. 10B).

Discussion

An important challenge in renal cell biology is to understand regulatory signaling pathways that control MC functional changes. Activated MCs play a direct role in the initiation and propagation of inflammatory events within the glomerulus. Recent studies have documented the expression of the complement anaphylatoxin receptors in the kidney (Fayyazi et al., 2000; Abe et al., 2001; de Vries et al., 2003), particularly on MCs (Braun and Davis III, 1998; Wilmer et al., 1998). These findings give a new insight into the mechanisms by which MCs may contribute to the pathogenesis of

glomerulonephritis and other glomeruli-associated kidney disorders. Thus, it is considered that the presence of complement receptors on MCs may mediate their specific interactions with activated complement products, which are decisive factors in tissue damage. However, there is still much that is not known about this process. One of the crucial, but still unexplored aspects, is the mechanism of regulated expression of the complement C5a anaphylatoxin receptor in MCs. This is of special importance, since uncontrolled or improperly activated C5aR expression may be crucial for the pathogenesis of complement-mediated tissue damage. In this study we provide convincing evidence that, in MCs, uPA, via its specific receptor uPAR and the association of uPAR with gp130 adaptor protein, regulates expression of C5aR and modulates C5a-dependent functional responses. The Janus kinase Tyk2 and transcription factor Stat3 serve as downstream components in the signaling cascade resulting in C5aR expression. Our data underscore a new role for uPA/uPAR-related signaling in the initiation and propagation of glomeruli-associated inflammation.

We suggest that at least two interrelated mechanisms are involved: activated expression on MCs of the complement anaphylatoxin C5a receptor and modulation of C5a-directed proinflammatory cytokines release and of cell proliferation. Thus, we show that uPA induces expression of C5aR on human MCs in a dose-dependent and transient fashion at both mRNA and protein levels. This process required functionally active uPAR, as proved by cell pretreatment with uPAR-blocking antibody. We provide evidence that uPA functions as a modulator of the C5aR-dependent processes in human MCs. Thus, we observed a synergistic, uPAR-mediated effect of uPA and C5a on MC proliferation and MCP-1 release, the events that could contribute to the progression of inflammatory renal injury.

We have extended these in vitro observations to an in vivo model of renal inflammation. We show that *uPAR*$^{-/-}$ mice are protected from LPS-induced glomerular inflammation. At the time point examined, no increase in C5aR expression in the mesangium of *uPAR*$^{-/-}$ mice was observed after LPS treatment, in contrast to the findings in wild-type animals. Consistent with these data, expression of MCP-1 was dramatically increased in wild-type animals, whereas only moderate increase was observed in uPAR-deficient mice. Our immunohistological studies demonstrate that under our experimental conditions, namely the low LPS concentration and short time of treatment, rapid upregulation of C5aR was restricted to mesangial cells and preceded LPS-induced leukocyte infiltration into glomeruli.

We investigated the molecular mechanism underlying the revealed uPA/uPAR-related upregulation of C5aR on MCs and delineated the gp130/Tyk2/Stat3 signaling pathway responsible for this effect. Although uPAR has been well documented as an important modulator and orchestrator of signaling events, the functional purpose of uPAR-directed signaling in the cell has only been elucidated in a few studies. One of the obvious limitations in this research field is the cellular specificity of uPA/uPAR-related signaling cascades. Thus, several studies have documented that uPAR-dependent tumor growth involves ERK/MAPK signaling (Liu et al., 2002), whereas FAK signaling mediated tumor dormancy (Ghiso, 2002). In human vascular smooth muscle cells the Tyk2/Stat1 pathway was shown to regulate cell migration and proliferation via a cross-talk with different downstream signaling cascades (Kusch et al., 2000; Kiian et al., 2003; Kunigal et al., 2003). By contrast, uPA-stimulated migration of rat smooth muscle cells requires activation of MEK and Erk kinases (Degryse et al., 2001). These and other data on the cell specificity of uPAR signaling emphasize the potential for different cell types to respond differently to a given signal. In

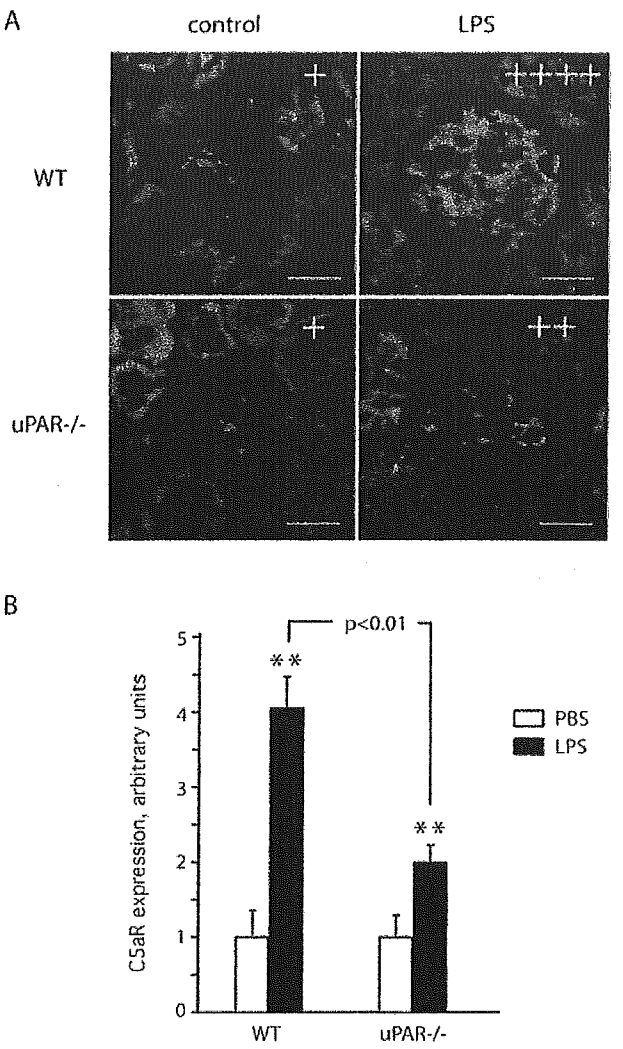

Fig. 10. uPAR is required for C5aR expression in mesangium upon LPS-induced renal inflammation in mice. (A) LPS-induced nephritis was induced in wild-type and *uPAR*$^{-/-}$ mice as described in Fig. 9. 8 hours after challenge the mice were killed and 6 μm cryosections of kidney cortex were stained for C5aR protein expression using anti-C5aR primary antibody and Cy3-conjugated secondary antibodies. Representative microphotographs are shown (6 mice for each group). Bar, 50 μm. (B) Statistical analysis of C5aR expression in mesangium of wild-type and *uPAR*$^{-/-}$ mice with LPS-induced nephritis was performed by evaluating the total number of positively stained glomeruli and the staining intensity (in arbitrary units) as described in the Materials and Methods. Results are expressed as mean ± s.e.m. (n=6 mice for each group). Significance between PBS controls and LPS-treated animals was determined by Student's t-test (*P<0.05; **P<0.01). +, arbitrary units (based on staining intensity using criteria described in Materials and Methods).

light of the multitude of biological functions played by the uPA/uPAR system it is of fundamental importance to determine different cascades of the uPAR-signaling machinery and then to assign particular pathways to particular cells and functions.

Signal transducers and activators of transcription of the Stat family play important roles in regulating immediate-early biological effects initiated by a variety of extracellular ligands including cytokines, chemokines and growth factors (Darnell et al., 1994; Schindler, 1999). Activation of latent, cytoplasmic, or membrane-associated Stat proteins requires their tyrosine phosphorylation, which in most cases depends on the activity of protein tyrosine kinases of the Janus family (Ziemiecki et al., 1994). After their phosphorylation, Stat proteins form homo- or heterodimers in order to translocate to the nucleus, where site-specific binding to enhancer elements leads to gene activation. uPA/uPAR has been documented to utilize Jak/Stat signaling in several cell types. Thus, in human kidney epithelial tumor cells uPAR is associated with the Jak1/Stat1 pathway, although cellular effects of this signaling remained unexplored (Koshelnick et al., 1997). In human vascular smooth muscle cells, the Tyk2/Stat1 pathway modulates, as mentioned above, uPA/uPAR-directed cell migration and proliferation.

In this study, we demonstrate that Stat3 is a key signaling molecule, which mediates upregulated expression of C5aR in human MCs in response to uPA. Our results show that uPA induces tyrosine phosphorylation of Stat3, but not other Stats, and its nuclear translocation. Obviously, these processes are required to induce uPA-related C5aR expression, since MCs expressing the Stat3 inactive mutant were unable to express C5aR when stimulated with uPA. Our studies on Janus kinases indicate that Tyk2 is responsible for the observed Stat3 tyrosine phosphorylation. Indeed, we observed fast and reversible Tyk2 activation in MCs in response to uPA, whereas Jak1, Jak2 and Jak3 were not affected. The kinetics of this activation correlates with those of the uPA-induced Stat3 phosphorylation, assuming that both processes are coordinated in time. Furthermore, MCs expressing kinase-dead Tyk2 mutants did not respond to uPA with Stat3 activation and C5aR expression, indicating a decisive role for Tyk2 kinase activity in these processes. Both Tyk2 and Stat3 activation by uPA required native cell surface uPAR, since no Tyk2/Stat3 phosphorylation was observed in uPARsi-MC where uPAR expression was downregulated. Our results confirm and extend the findings of others that expression of some G protein-coupled receptors is regulated by the Jak/Stat3 pathway (Senga et al., 2003).

Stat3 protein plays a central role in transmitting a multitude of different processes initiated by the IL-6 cytokine family (French et al., 2002). Moreover, IL-6 has been shown to mediate expression of C5aR in several cell types (Riedemann et al., 2003). Therefore, it was tempting to speculate that induction of the Tyk2/Stat3 pathway followed by the upregulation of C5aR might result from the uPA-triggered IL-6 activation, instead of reflecting a direct effect of uPA. To verify this hypothesis, separate experiments have been performed. Thus, we measured, using specific ELISA, IL-6 secretion from MCs stimulated dose-dependently with uPA for different times and examined expression of both α- and β-subunits of the IL-6 receptor in uPA-stimulated cells. No changes were observed (data not shown) thus indicating that C5aR expression in MCs is indeed attributed to the uPA/uPAR-induced signaling.

Although the revealed mechanism of uPA-mediated C5aR expression is IL-6 independent, uPAR utilizes gp130 protein, a signal transducing subunit of the receptor complexes of the IL-6 cytokine family, as a membrane interactor to mediate C5aR expression. In order to identify a transmembrane protein, which might serve as an adaptor for uPAR in mesangial cells and therefore couple uPAR to the pathway required for C5aR expression, several experiments have been performed aiming at analyzing uPAR-interacting proteins. Thus, we used the a325 peptide that dissociates uPAR-integrin complexes, FPR-family receptor inhibiting peptides, and pharmacological inhibitors of tyrosine kinase receptors. No effect on the uPA-mediated C5aR expression was observed in all these experiments (data not shown). Instead, we found that uPAR is associated in an uPA-dependent fashion with gp130. We also provide evidence that this association is required to mediate C5aR expression in MCs in response to uPA.

A relevant question regarding the functional role of uPAR signaling in C5aR regulation in MCs is the source of uPA in glomeruli. In our in vivo experiments we observed a decrease in uPA expression in mesangium of both wild-type and uPAR-deficient mice treated with LPS. These results correlate with the data of others on the reduction in urinary and renal uPA in LPS-injected mice, suggesting that an imbalanced fibrinolytic system might participate in kidney dysfunction upon inflammation (Yamamoto and Loskutoff, 1996). By contrast, we noticed strong upregulation of uPAR expression in inflammed mesangium of wild-type animals. Thus, it appears that the regulation of the C5aR expression pathway may result from the uPAR-mediated uptake of uPA via the increased number of uPAR available for its ligand on the surface of MCs. A paracrine loop of stimulation cannot be excluded, although the source of uPA in mesangium during inflammation remains to be elucidated.

In summary, the results of the present study demonstrate that uPA, via the uPAR-directed signaling machinery, regulates expression of the anaphylatoxin C5a receptor on MCs, and modulates C5a-dependent functional responses. These findings suggest the possibility that MCs may be therapeutic targets for inhibiting complement-mediated glomerular damage.

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