Urokinase-Type Plasminogen Activator Inhibits Efferocytosis of Neutrophils

Yanping Yang[1]*, Arnaud Friggeri[1,2]*, Sami Banerjee[1], Khalil Bdeir[3], Douglas B. Cines[3], Gang Liu[1], and Edward Abraham[1]

[1]Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; [2]Pole Anesthésie Réanimation, CHU d'Amiens and INSERM, ERI-12, Amiens, France; and [3]Department of Pathology and Laboratory Medicine, The University of Pennsylvania, Philadelphia, Pennsylvania

***Rationale*: Phagocytosis of apoptotic cells, also called efferocytosis, plays an essential role in the resolution of inflammation. Urokinase-type plasminogen activator (uPA) is a multifunctional protein that has been implicated in inflammatory conditions, including pneumonia and severe infection, which are often accompanied by the development of acute lung injury. However, the role of uPA in modulating efferocytosis of apoptotic neutrophils has not been defined.**

***Objectives*: To characterize the role of uPA in regulation of efferocytosis and to delineate the underlying mechanisms involved in this process.**

***Methods*: *In vitro* and *in vivo* phagocytosis, immunoprecipitation, and Western blotting assays.**

***Measurements and Main Results*: The phagocytosis of apoptotic neutrophils by macrophages was significantly inhibited by uPA. Mutant uPA lacking the growth factor domain and catalytically inactive uPA had similar inhibitory effects on efferocytosis, as did wild-type uPA. In contrast, absence of the kringle domain abrogated the ability of uPA to diminish efferocytosis. Both the $\alpha_V\beta_3$ integrin and vitronectin seemed to be involved in the inhibition of efferocytosis by uPA. Incubation of macrophages with uPA also diminished activation of the small GTPase Rac-1, which normally occurs during ingestion of apoptotic neutrophils. Under *in vivo* conditions in the lungs, uPA decreased the uptake of apoptotic neutrophils by alveolar macrophages.**

***Conclusions*: Our data demonstrate a novel role for uPA in which it is able to diminish the uptake of apoptotic neutrophils by macrophages under both *in vitro* and *in vivo* conditions.**

Keywords: phagocytosis; integrin $\alpha_v\beta_3$; inflammation; acute lung injury

Phagocytosis of apoptotic cells, also called efferocytosis, is an important feature of immune responses and is involved in normal tissue homeostasis and resolution of inflammation (1–5). Impaired efferocytosis occurs with autoimmune and inflammatory diseases, such as systemic lupus erythematosus and acute lung injury (ALI), and is associated with unfavorable outcome from inflammation (6–11).

Urokinase-type plasminogen activator (uPA) is involved in diverse physiologic and pathophysiologic processes, including fibrinolysis, cell migration and adhesion, and inflammation (12–15). uPA is initially secreted as a single-chain (scuPA) consisting of three structurally independent domains (16): (*1*) a N-terminal epidermal growth factor–like domain (GFD; aa 1–46) that is responsible for binding of uPA to its high-affinity receptor, CD87 (uPAR), and that participates in uPA-induced neutrophil chemotaxis; (*2*) a kringle domain (KD; aa 47–135), which mediates uPA binding to integrins, including $\alpha_v\beta_3$, and has been shown to enhance LPS-induced neutrophil activation (15, 17) and vascular contraction (18); and (*3*) a serine protease domain (aa 159–411) that includes the catalytically active site of uPA (19).

Elevated tissue and circulating levels of uPA are present for prolonged periods in patients with acute and chronic inflammatory conditions, including pneumonia and severe infection, which are often accompanied by the development of ALI (20–22). ALI is characterized by accumulation of activated neutrophils in the lungs, and clearance of neutrophils from the lungs occupies a major role in the resolution of this pathophysiologic process (23, 24). We have previously demonstrated that uPAR, the receptor of uPA, and plasminogen activator inhibitor 1 (PAI)-1, the natural inhibitor of uPA and tissue plasminogen activator, regulate the engulfment of both viable and apoptotic neutrophils (25, 26). Given the roles that uPAR and PAI-1 occupy in modulating the activity of uPA and the correlation between enhanced levels of uPA and the severity of acute inflammatory diseases, including ALI, we hypothesized that uPA might also participate in modulating efferocytosis. This study demonstrates a novel role for uPA in which it is able to diminish the uptake of apoptotic neutrophils by macrophages under both *in vitro* and *in vivo* conditions.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Urokinase-type plasminogen activator (uPA) is involved in diverse physiologic and pathophysiologic processes, including fibrinolysis, cell migration and adhesion, and inflammation. However, the role of uPA in modulating efferocytosis of apoptotic neutrophils has not been defined.

What This Study Adds to the Field

The present results, by demonstrating that uPA inhibits the uptake of apoptotic neutrophils, suggest a novel mechanism by which elevated levels of uPA may participate in enhancing the duration and severity of inflammatory processes, such as acute lung injury, in which neutrophils play a major role.

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* These authors contributed equally to this study.

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Correspondence and requests for reprints should be addressed to Edward Abraham, M.D., Department of Medicine, University of Alabama at Birmingham, 420 Boshell Building, 1808 7th Avenue South, Birmingham, AL 35294. E-mail: eabraham@uab.edu

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METHODS

Mice

C57BL/6, vitronectin $(Vn)^{-/-}$, and $uPA^{-/-}$ mice were purchased from NCI-Frederick (Frederick, MD) and Jackson Laboratory (Bar Harbor, ME). The $uPAR^{-/-}$ mice were a gift from Margaret Gyetko (University of Michigan). The $uPA^{-/-}$, $uPAR^{-/-}$, and $Vn^{-/-}$ mice were all C57BL/6 background. $Vn^{-/-}$ mice were back-crossed to C57BL/6 for 12 generations. Mouse protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and The University of Pennsylvania.

Materials

Custom antibody mixtures for bone marrow neutrophil purification were from Stem Cell Technologies (Vancouver, BC, Canada). Annexin V-FITC, propidium iodide, recombinant human integrin $\alpha_v\beta_3$, and recombinant mouse milk fat globule epidermal growth factor-8 (MFG-E8) were from R&D (Minneapolis, MN). Human serum albumin (HSA), carboxylate-modified beads, and protein G-agarose were from Sigma (St. Louis, MO). Anti-uPA antibodies were from Santa Cruz (Santa Cruz, CA). Anti–human-$\alpha_v\beta_3$ antibody, uPA activity assay kit, and Rac-1 activation assay kit were from Millipore (Billerica, MA). Mouse urokinase (low and high molecular weight) was from Molecular Innovations (Novi, MI). Human recombinant scuPA, uPA deletion mutants lacking GFD (ΔGFD) or KD (ΔKD), and the catalytically inactive uPA mutant (S356A), in which alanine 356 is substituted for serine, were previously described (27).

Purification and Culture of Mouse Bone Marrow Neutrophils

Purification and culture of mouse bone marrow neutrophils was performed as previously described (15, 28–30) (*see* online supplement).

Purification and Culture of Peritoneal Macrophages

Purification and culture of peritoneal macrophages was performed from thioglycollate peritoneal exudates as previously described (25, 26), and as detailed in the online supplement.

Induction of Neutrophil Apoptosis

Neutrophil apoptosis was induced by heating to 43°C for 1 hour, followed by culture at 37°C in 5% CO_2 for 2.5 hours, as previously described (25, 26).

In Vitro Efferocytosis Assay

In vitro efferocytosis assays were performed as previously described (25, 26) and as detailed in the online supplement.

In Vivo Efferocytosis Assay

In vivo efferocytosis assays were performed as previously described (25, 26) and as detailed in the online supplement.

uPA Activity Assay

Protease activity of uPA and uPA mutants was determined using an activity kit (Millipore), and calculated as U/μg uPA protein.

Immunoprecipitation and Immunoblotting

Wild-type and mutant uPA were preincubated with soluble $\alpha_v\beta_3$ at 4°C for 1 hour. Anti-$\alpha_v\beta_3$ antibody (1 μg/ml) was added to each sample and incubated at 4°C overnight. Protein G-agarose beads were added to each sample and incubated for 2 hours. In designated experiments, uPA or MFG-E8 was preincubated with $\alpha_v\beta_3$ protein for 30 minutes followed by the addition of uPA or MFG-E8 for another 30 minutes. Finally, immunoprecipitation with anti-$\alpha_v\beta_3$ antibody was performed, followed by Western blotting with anti-uPA antibodies.

Rac-1 Activation Assay

Rac-1 activation was determined using a kit as described in detail in the online supplement.

Model for ALI

Age matched wild-type and $uPA^{-/-}$ mice were anesthetized with isoflurane. LPS (2 mg/kg body weight) in 50 μl phosphate-buffered saline was injected intratracheally. Mice were killed 24 hours after LPS administration and bronchoalveolar lavage (BAL) was performed using 1 ml phosphate-buffered saline.

Statistical Analysis

Macrophages and neutrophils for each experiment were isolated and pooled from three to five mice. Data are representative of two or three independent experiments. Means ± SD are shown. One-way analysis of variance followed by Tukey-Kramer analysis for comparison between multiple groups and Student *t* test for comparisons between two groups were used. Differences were considered statistically significant when $P < 0.05$.

RESULTS

uPA Inhibits Efferocytosis of Apoptotic Neutrophils

In initial experiments (Figure 1), we found that the uptake of apoptotic neutrophils by macrophages was significantly inhibited when human scuPA was added to cultures of apoptotic neutrophils and peritoneal macrophages at concentrations of 300–1,000 ng/ml. Levels of uPA similar to those that inhibited efferocytosis have been reported in the circulation and in tissue sites, such as the lungs, during acute inflammatory conditions induced by pneumonia and other severe infections (20, 21). Of note, incubation of macrophages with scuPA did not result in any increased release of tumor necrosis factor-α or IL-6 (*see* Figure E1 in the online supplement), indicating that the inhibitory effect of uPA on efferocytosis is a specific effect caused by uPA, but not by any potential contamination by pathogen-associated molecular patterns in the scuPA protein. Incubation of macrophages with scuPA did not affect their ability to ingest carboxylate-modified beads (Figure 2), a process that occurs through mechanisms distinct from those involved in apoptotic cell engulfment (5, 31, 32). Such results indicate that the inhibitory effects of uPA on efferocytosis are specific to this process and do not reflect more generalized actions on macrophage function.

Suppression of Phagocytosis By uPA is Independent of Protease Activity

uPA consists of three domains: (*1*) GFD; (*2*) KD; and (*3*) the proteolytic domain, which is responsible for the proteolytic

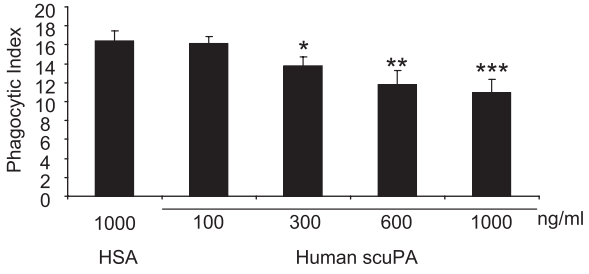

Figure 1. Urokinase-type plasminogen activator (uPA) inhibits efferocytosis of apoptotic neutrophils. A total of 100, 300, 600, or 1,000 ng/ml human single-chain plasminogen activator (scuPA) or 1,000 ng/ml human serum albumin (HSA) was added to cultures of macrophages and apoptotic neutrophils. Phagocytosis assays were performed as described in the METHODS section. Each condition included two independent samples and each sample was counted four times by a blinded observer. Data are shown as SD of the eight counts from each condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the group treated with HSA.

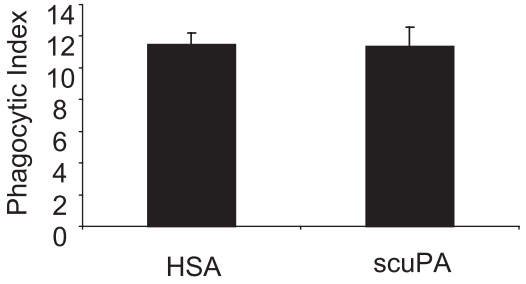

Figure 2. Urokinase-type plasminogen activator (uPA) does not affect macrophage ingestion of carboxylate-modified beads. Carboxylate-modified beads were incubated with peritoneal macrophages in the presence of 1 μg/ml human serum albumin (HSA) or single-chain plasminogen activator (scuPA) and then phagocytosis assays were performed. Representative data are shown. Two additional independent experiments provided similar results.

activity of the molecule (16). Although uPA was defined primarily as a protease that cleaves and activates plasminogen, recent studies have demonstrated that uPA has a variety of activities, such as regulation of adhesion and migration, which are independent of its protease activity (16, 33–35). To determine if protease activity is required for the inhibitory effect of uPA on efferocytosis, a catalytically inactive form of uPA in which alanine has been substituted for serine 356 (S356A) was used (27). As shown in Figure 3A, compared with catalytically active low-molecule-weight murine uPA, scuPA had minimal proteolytic activity and S356A had no detectable fibrinolytic activity. However, both scuPA and S356A inhibited phagocytosis of apoptotic neutrophils to a similar degree (Figure 3B). These data indicate that protease activity is not required for uPA to inhibit the engulfment of apoptotic neutrophils by macrophages.

Inhibition of Phagocytosis By uPA Requires the KD, but Not the GFD

The GFD of uPA binds to uPAR, whereas the KD has been shown to interact with other cellular receptors, including lipoprotein-related receptor, $\alpha_M\beta_2$, and $\alpha_v\beta_3$ (36–38). To delineate the mechanisms by which uPA inhibits efferocytosis, we examined the role of the GFD and KD domains of uPA in this process. As shown in Figure 4, mutant uPA lacking GFD (ΔGFD) was as active as the wild-type molecule in diminishing the ability of macrophages to ingest apoptotic neutrophils. However, mutant uPA lacking KD (ΔKD) did not have any effect on macrophage uptake of apoptotic neutrophils. These data suggest that the KD, but not the GFD, is required for the inhibitory activity of uPA on phagocytosis of apoptotic neutrophils.

uPAR Is Not Required For uPA to Inhibit Phagocytosis of Apoptotic Neutrophils

Our data showing that the GFD, which binds to uPAR, is not required for inhibition of efferocytosis by uPA suggests that uPAR is not needed for this action of uPA. However, we have previously shown that uPAR itself regulates phagocytosis of both viable and apoptotic neutrophils (26). To further clarify if uPAR plays either a direct or indirect role in modulating the effect of uPA on efferocytosis, we exposed $uPAR^{-/-}$ macrophages to either human scuPA or mouse two-chain uPA, which binds to uPAR, during culture with apoptotic neutrophils. As shown in Figure 5, the absence of uPAR did not affect the activity of human scuPA or mouse uPA in inhibiting phagocytosis of apoptotic neutrophils by macrophages. Such findings indicate that uPAR is not required for uPA to affect efferocytosis, either directly or indirectly.

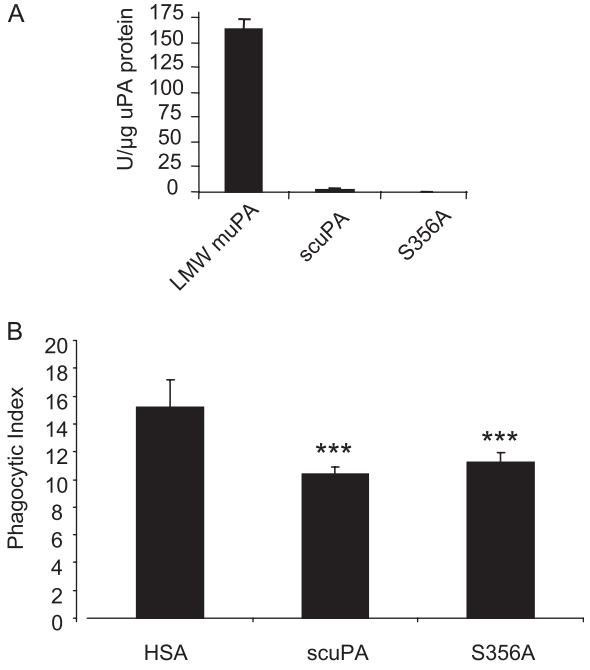

Figure 3. Inhibition of phagocytosis by urokinase-type plasminogen activator (uPA) does not require proteinase activity. (*A*) Proteolytic activities of murine low-molecular-weight uPA (LMW-muPA), single-chain plasminogen activator (scuPA), and mutant scuPA with substitution of serine 356 with alanine (S356A) were determined. The proteolytic activity of the samples is expressed as U/μg uPA protein. Results are from three independent assays of activity. (*B*) Catalytically inactive scuPA (S356A) exhibits similar inhibitory activity on the phagocytosis index as does scuPA. A representative experiment is shown. Two additional independent experiments provided similar results. *** $P < 0.001$ compared with human serum albumin (HSA).

The $\alpha_v\beta_3$ Integrin Participates in the Inhibition of Efferocytosis By uPA

In addition to binding to uPAR, uPA can modulate cellular activation and signaling through uPAR-independent mechanisms, including interactions with integrins, particularly $\alpha_v\beta_3$, that occur through association with the KD (17, 38). Given the results showing that the KD was required for the ability of uPA to inhibit the ingestion of apoptotic neutrophils by macrophages, we hypothesized that interactions between $\alpha_v\beta_3$ and uPA might play a role in modulating efferocytosis. Of note, $\alpha_v\beta_3$ has previously been shown to participate in efferocytosis through facilitating interactions between apoptotic and phagocytic cells (39).

To examine the role that interactions between $\alpha_v\beta_3$ and uPA might play in affecting efferocytosis, scuPA or HSA were incubated with or without soluble $\alpha_v\beta_3$ before being added to macrophages that were then used in phagocytosis assays. As shown in Figure 6, coincubation of scuPA with soluble $\alpha_v\beta_3$ totally abrogated the decrease in phagocytosis produced by scuPA. Such findings suggest that binding of uPA to $\alpha_v\beta_3$ participates in modulating the inhibitory effects of uPA in efferocytosis.

To confirm the importance that interactions between uPA and $\alpha_V\beta_3$ play in modulating efferocytosis, we examined the ability of uPA to affect the ability of MFG-E8 to enhance the engulfment of apoptotic neutrophils by macrophages. MFG-E8 functions as an opsonin, bridging phosphatidylserine (PtSer) on the apoptotic neutrophil with $\alpha_v\beta_3$ on the macrophage surface, thereby increasing the uptake of apoptotic

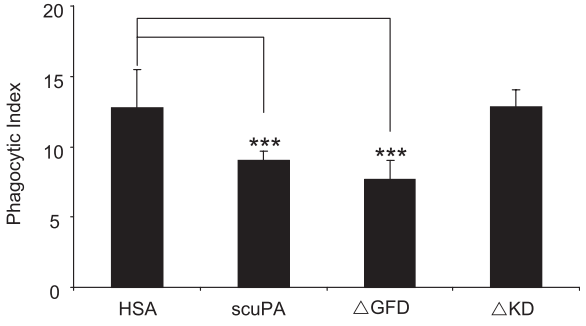

Figure 4. The kringle domain (KD), but not the growth factor domain (GFD), of urokinase-type plasminogen activator (uPA), is required for inhibition of efferocytosis. Human serum albumin (HSA), single-chain plasminogen activator (scuPA), ΔGFD uPA, and ΔKD uPA, at 1 μg/ml, were added to cultures of macrophages and apoptotic neutrophils and phagocytosis assays were performed. Data shown are representative of three independent experiments. *** $P < 0.001$ compared with groups treated with HSA.

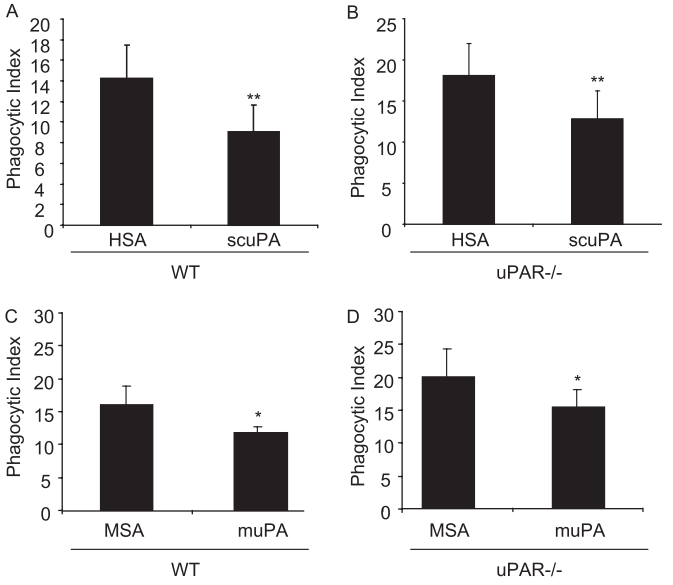

Figure 5. The urokinase-type plasminogen activator (uPA) receptor (uPAR) is not required for uPA to inhibit phagocytosis of apoptotic neutrophils. Apoptotic wild-type (WT) neutrophils were cultured with WT (*A* and *C*) or uPAR$^{-/-}$ (*B* and *D*) peritoneal macrophages in the presence of human serum albumin (HSA) or single-chain plasminogen activator (scuPA) (1 μg/ml) (*A* and *B*) or mouse serum albumin (MSA) or mouse two-chain uPA (muPA) (*C* and *D*) and phagocytosis assays were performed. Data shown are representative of two independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with groups treated with HSA or MSA.

neutrophils by phagocytes (40, 41). Given the ability of MFG-E8 to increase phagocytosis of apoptotic neutrophils through binding to $\alpha_v\beta_3$, we hypothesized that uPA might block this effect through competitively interacting with the same integrin. To examine this question, we pretreated macrophages with MFG-E8 followed by incubation with scuPA or pretreated macrophages with scuPA followed by incubation with MFG-E8. As shown in Figure 7A, when macrophages were incubated with MFG-E8 before scuPA, scuPA lost its ability to inhibit efferocytosis. In contrast, exposure of macrophages to scuPA before MFG-E8 not only resulted in a significant decrease in phagocytosis of apoptotic neutrophils, but also prevented MFG-E8 from increasing efferocytosis (Figure 7B). Such results suggest that uPA and MFG-E8 compete for the same receptor, presumably $\alpha_v\beta_3$, on the macrophage surface and can modulate efferocytosis in opposite directions through interaction with $\alpha_v\beta_3$.

To demonstrate directly that binding between $\alpha_v\beta_3$ and uPA is responsible for the inhibitory effect of uPA on efferocytosis, we examined the interaction of $\alpha_v\beta_3$ with wild-type uPA, uPA lacking proteolytic activity (S356A), and uPA mutants lacking the GFD or KD. As shown in Figure 8A, wild-type uPA and ΔGFD uPA and S356A uPA were able to bind to $\alpha_v\beta_3$. However, ΔKD uPA did not interact with $\alpha_v\beta_3$. Of note, the anti-uPA antibody used in Figure 8A was able to recognize all forms of uPA (Figure 8B). These results are consistent with our findings that uPA lacking the KD (i.e., ΔKD uPA) does not inhibit efferocytosis, and suggest that the interaction between $\alpha_v\beta_3$ and uPA is responsible for the inhibitory effects of uPA on efferocytosis.

To further demonstrate that competition between uPA and MFG-E8 for binding to $\alpha_v\beta_3$ is involved in the modulation of efferocytosis in opposite directions by these two proteins, we performed coimmunoprecipitation studies. As shown in Figure 8C, preincubation of uPA with $\alpha_v\beta_3$ diminished binding of $\alpha_v\beta_3$ to MFG-E8, whereas the interaction of MFG-E8 and $\alpha_v\beta_3$ was unaffected by subsequent exposure of $\alpha_v\beta_3$ to uPA. These results are consistent with the findings that preincubation of macrophages with uPA diminished phagocytosis even if the macrophages were subsequently exposed to MFG-E8, whereas the ability of MFG-E8 to enhance efferocytosis was unaffected by the subsequent incubation of macrophages with uPA.

To determine if uPA diminishes integrin-associated downstream signaling events that are activated during efferocytosis, we examined Rac-1 activation in macrophages incubated with either uPA or HSA followed by exposure to apoptotic thymocytes. As shown in Figure 8D, resting macrophages had only minimum activation of Rac-1. Addition of apoptotic cells to macrophages dramatically activated Rac-1. However, preincubation of macrophages with uPA attenuated such efferocytosis-associated Rac-1 activation (Figure 8D). Because activation of Rac-1 is involved in facilitating alterations in macrophage morphology required for ingestion of apoptotic cells (3–5, 42), these results suggest that a mechanism by which uPA inhibits macrophages from engulfing apoptotic cells is through preventing $\alpha_v\beta_3$-induced activation of intracellular pathways, including those involving Rac-1, required for optimal phagocytic function during efferocytosis.

Inhibition of Efferocytosis By uPA Requires Vn

Vn is a ligand for integrins, including $\alpha_v\beta_3$, and also promotes neutrophil adhesion and migration (43, 44). Interactions between Vn, uPA, and PAI-1 modulate the proteolytic activity of uPA and its binding to uPAR and other receptors (45–47). Given the ability of Vn to associate with $\alpha_v\beta_3$, we hypothesized that Vn might also play a role in modulating the ability of uPA to affect efferocytosis. To examine this issue, we used neutrophils, macrophages, and serum from wild-type and transgenic mice lacking Vn (Vn$^{-/-}$) in phagocytosis assays. As shown in Figure 9, in the absence of Vn, scuPA lost its ability to inhibit phagocytosis of apoptotic neutrophils by macrophages, indicating that interactions between uPA and Vn are necessary for the inhibitory effects of uPA on efferocytosis.

uPA Inhibits Efferocytosis *In Vivo*

To investigate whether uPA can inhibit efferocytosis under *in vivo* conditions, apoptotic wild-type neutrophils were injected intratracheally with scuPA or HSA into wild-type

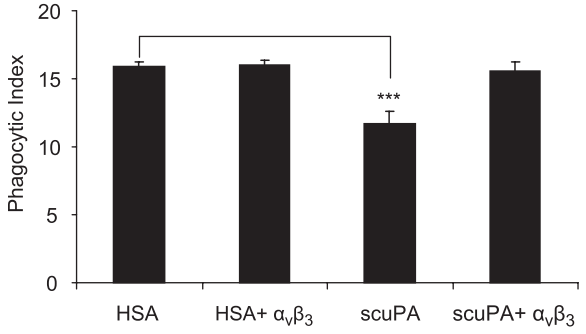

Figure 6. Association of urokinase-type plasminogen activator (uPA) with the $\alpha_v\beta_3$ integrin abrogates the inhibitory effects of uPA on efferocytosis. Human serum albumin (HSA) or single-chain plasminogen activator (scuPA) (1 μg/ml) was preincubated with or without soluble $\alpha_v\beta_3$ (0.5 μg/ml) for 1 hour after which the mixtures were added to peritoneal macrophages and incubated for 1 hour. The macrophages were washed three times with RPMI, then apoptotic neutrophils were added and phagocytosis assays were performed. Data shown are representative of three independent experiments. *** $P <$ 0.001 compared with groups treated with HSA.

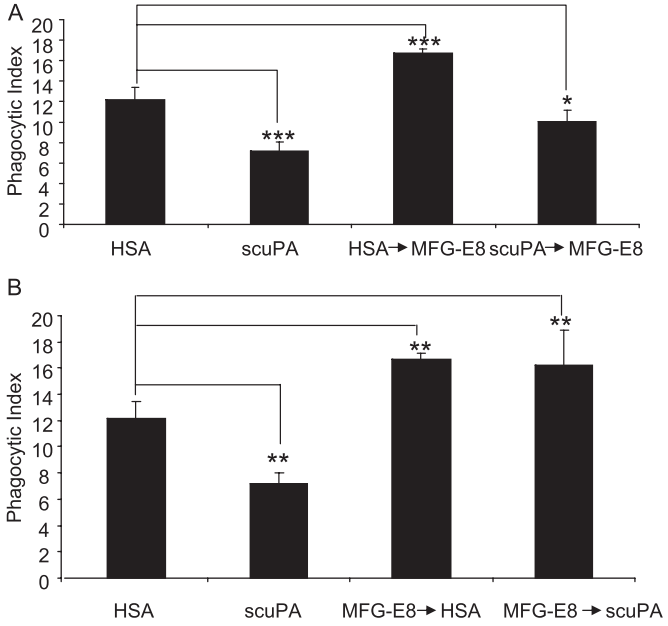

Figure 7. Urokinase-type plasminogen activator (uPA) and milk fat globule epidermal growth factor-8 (MFG-E8) have competitive effects on efferocytosis. (*A*) uPA diminishes the enhanced effect of MFG-E8 on phagocytosis of apoptotic neutrophils. Macrophages were preincubated with human serum albumin (HSA) or single-chain plasminogen activator (scuPA) (1 μg/ml) for 1 hour at 37°C in RPMI medium. The cells were then washed three times and incubated with HSA or MFG-E8 (1 μg/ml) for an additional 1 hour at 37°C. After washing three times, apoptotic neutrophils were added to the cultures and phagocytosis assays were performed. (*B*) The enhancement of phagocytosis induced by preincubation of macrophages with MFG-E8 is not affected by the subsequent addition of uPA. Macrophages were preincubated with HSA or MFG-E8 (1 μg/ml) for 1 hour at 37°C in RPMI medium. The cells were then washed three times and incubated with HSA or uPA (1 μg/ml) for an additional 1 hour at 37°C. After washing three times, apoptotic neutrophils were added to the cultures and phagocytosis assays were performed. Data shown are representative of three independent experiments. * $P <$ 0.05, ** $P <$ 0.01, *** $P <$ 0.001.

mice. As shown in Figure 10, the phagocytic index was significantly decreased when scuPA, but not HSA, was included with the apoptotic neutrophils. Such results demonstrate that uPA inhibits efferocytosis *in vivo* in a fashion similar to that observed under *in vitro* conditions.

Levels of uPA are elevated in the lungs of mice with ALI (48). To determine if the presence of uPA in BAL fluid during ALI modulates the ability of macrophages to phagocytose apoptotic neutrophils, we included BAL fluid from wild-type or $uPA^{-/-}$ mice with LPS-induced ALI in efferocytosis assays. As shown in Figure E2, uptake of apoptotic neutrophils was reduced when macrophages were treated with BAL fluid from wild-type mice with LPS-induced ALI as compared with when BAL fluid from $uPA^{-/-}$ mice with LPS-induced ALI was included in the cultures. These data suggest that the presence of uPA in the lungs of mice with ALI is associated with diminished clearance of apoptotic neutrophils.

DISCUSSION

uPA is involved in diverse physiologic and pathophysiologic processes, including fibrinolysis, cell migration and adhesion, and inflammation (12–15). In this study, we identified a novel function for uPA in inhibiting the phagocytosis of apoptotic neutrophils. This activity of uPA seemed to involve interaction between uPA and the $\alpha_v\beta_3$ integrin, and is specific for apoptotic cell engulfment because uPA had no effect on the uptake of carboxylate modified beads by macrophages, a process that involves distinct mechanisms from those implicated in efferocytosis (5, 31, 32).

A classic activity of uPA is participation in fibrinolysis, in which it cleaves plasminogen to generate plasmin (12–15). The proteolytic activity of uPA is also involved in modulating cellular function through cleavage of receptors, such as the α_6 integrin (49, 50). However, the proteolytic activity of uPA does not seem to contribute to its inhibitory effects on neutrophil phagocytosis. This conclusion is supported by several lines of evidence. Single-chain uPA, which has minimal proteolytic activity, inhibited phagocytosis of apoptotic neutrophils. Furthermore, a proteolytically inactive mutant of uPA, S536A, was as effective as wild-type scuPA in diminishing engulfment of apoptotic neutrophils. Our data therefore add to the accumulating evidence showing that uPA possesses a variety of important biologic functions relating to inflammation and vascular reactivity that are independent of its proteolytic activity (12–15).

We previously demonstrated that uPAR, a major receptor for scuPA and proteolytically active two-chain uPA, regulates phagocytosis of both viable and apoptotic neutrophils through integrin-dependent mechanisms (26). However, as shown in the present studies, the inhibitory effect of uPA on efferocytosis is unrelated to uPAR. This conclusion is supported by evidence showing that human scuPA, which is unable to bind to mouse uPAR (51), effectively blocked the engulfment of apoptotic neutrophils by mouse peritoneal macrophages. In addition, we found that mouse two-chain uPA, which binds to mouse uPAR, inhibits the engulfment of apoptotic neutrophils by $uPAR^{-/-}$ macrophages to an extent comparable with that found with wild-type macrophages. Our findings therefore indicate that uPAR does not participate in the regulation of phagocytosis by uPA.

In addition to binding to uPAR, uPA also interacts with other cellular receptors, including integrins and the low-density lipoprotein-related receptor, through which uPA is able to activate intracellular signaling events (18, 37, 38). Integrins, particularly $\alpha_v\beta_3$, are among the most important receptors that mediate engulfment of apoptotic cells (3–5, 42). Recent evi-

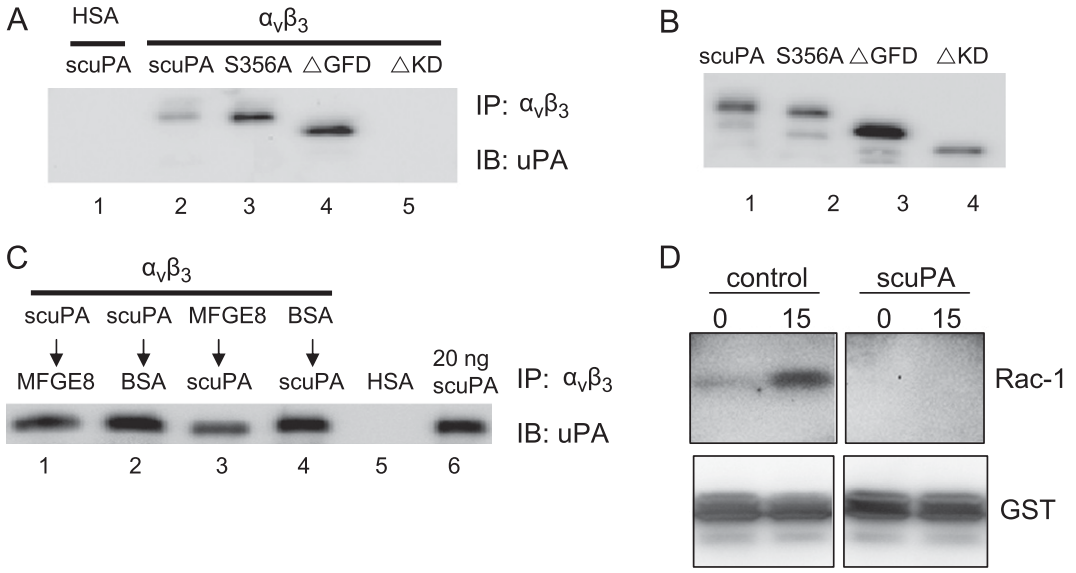

Figure 8. Urokinase-type plasminogen activator (uPA) and milk fat globule epidermal growth factor-8 (MFG-E8) competitively bind to $\alpha_v\beta_3$. (*A*) Single-chain plasminogen activator (scuPA), ΔGFD uPA, and S356A uPA, but not ΔKD uPA bind to $\alpha_v\beta_3$. In these experiments, 100-ng recombinant $\alpha_v\beta_3$ extracellular domain was incubated with scuPA, ΔGFD, S356A, or ΔKD (100 ng) at 4°C overnight with rotation. Antibodies to $\alpha_v\beta_3$ were then added and the mixtures incubated for 1 hour. Immunocomplexes were precipitated with Protein G agarose and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). uPA interacting with $\alpha_v\beta_3$ was detected by anti-uPA antibodies. uPA incubated with human serum albumin (HSA) was used as a negative control and showed no pull-down by anti-$\alpha_v\beta_3$ antibodies (*lane 1*). (*B*) The inputs of scuPA, ΔGFD, S356A, and ΔKD used in (*A*). A total of 20 ng of each protein was resolved by SDS-PAGE and blotted with anti-uPA antibodies. (*C*) uPA diminishes binding of MFG-E8 to $\alpha_v\beta_3$. A 100-ng recombinant $\alpha_v\beta_3$ extracellular domain was incubated with 100 ng scuPA (*lanes 1* and *2*), MFG-E8 (*lane 3*), or bovine serum albumin (BSA) (*lane 4*) at 4°C overnight with rotation. MFG-E8 (*lane 1*), BSA (*lane 2*), or scuPA (*lanes 1* and *2*) was then added and incubated for another 1 hour, followed by the addition of anti-$\alpha_v\beta_3$ antibodies and incubation for 1 hour. The immunocomplexes were precipitated with Protein G agarose and resolved by SDS-PAGE. uPA interacting with $\alpha_v\beta_3$ was detected by anti-uPA antibodies. A total of 20 ng uPA was used as input. (*D*) uPA diminishes Rac-1 activation induced by efferocytosis. Macrophages were pretreated with HSA or scuPA (1 μg/ml) for 1 hour. The medium was then aspirated and 10×10^6 apoptotic thymocytes were added to the macrophages for 0 and 30 minutes. The cells were washed five times with cold phosphate-buffered saline and lysed in Mg^{2+} lysis buffer. Activated Rac-1 that bound to GST-tagged PAK-1 PBD was assayed as described in the METHODS section (*top panels*). The membrane was then stripped and blotted with anti-GST antibody to demonstrate equal pull-down of GST tagged PAK-1 PBD (*bottom panels*). Top panels or bottom panels were from the same blot with deletion of unrelated experiments in the middle. Data shown are representative of two to three independent experiments.

dence has shown that $\alpha_v\beta_3$ does not directly bind to PtSer exposed on the surface of apoptotic cells, but rather participates in modulating efferocytosis through binding to extracellular proteins, such as MFG-E8, that serve as bridging molecules between phagocytes and PtSer on apoptotic cells (40, 41). In our experiments, we demonstrated that uPA directly binds to $\alpha_v\beta_3$ and that association between uPA and soluble $\alpha_v\beta_3$ abrogated the ability of uPA to inhibit the phagocytic index. These data suggest that uPA inhibits phagocytosis through regulation of $\alpha_v\beta_3$-dependent engulfment of apoptotic cells.

MFG-E8 bridges $\alpha_v\beta_3$ on the surface of macrophages with PtSer on the surface of apoptotic cells, thereby promoting phagocytosis of apoptotic cells (40, 41). We found that pre-incubation of macrophages with uPA abrogated the enhanced phagocytosis normally seen after addition of MFG-E8 to cultures of macrophages and apoptotic neutrophils. However, subsequent addition of uPA to macrophages precultured with MFG-E8 had no effect on the enhancement of phagocytic index induced by MFG-E8. These data suggest that binding of $\alpha_v\beta_3$ to either MFG-E8 or uPA blocks subsequent interactions between $\alpha_v\beta_3$ and the other molecule.

The KD of uPA interacts with lipoprotein-related receptor and integrins, such as $\alpha_M\beta_2$ and $\alpha_v\beta_3$, and directly participates in uPA-regulated cell migration and vascular contraction (36–38). Our previous studies demonstrated that the KD, through interactions with $\alpha_v\beta_3$, is responsible for the ability of uPA to enhance TLR4-induced inflammatory responses in neutrophils (15, 17). In the present experiments, we found that the uPA KD was required for the inhibitory effects of uPA on efferocytosis. In particular, mutants of uPA lacking the KD had no effect on the ingestion of apoptotic neutrophils by macrophages. Given the ability of the uPA KD to bind to $\alpha_v\beta_3$, such results support the hypothesis that inhibition of efferocytosis by uPA is mediated by interactions between uPA and $\alpha_v\beta_3$.

Vn is an extracellular protein, present in high concentrations in the circulation, which participates in cell migration and

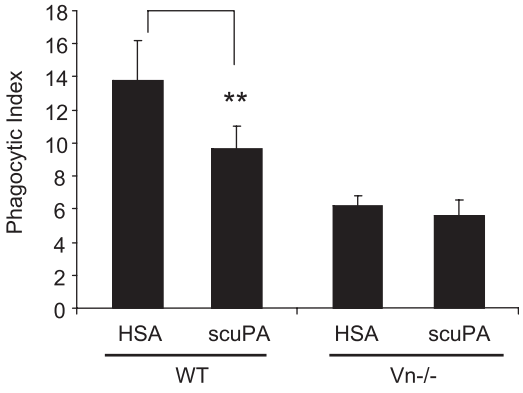

Figure 9. The inhibitory effect of urokinase-type plasminogen activator (uPA) on efferocytosis requires vitronectin (Vn). Human serum albumin (HSA) or single-chain plasminogen activator (scuPA) (1 μg/ml) was added to cultures of wild-type apoptotic neutrophils and macrophages including wild-type mouse serum (*A*) or to cultures of $Vn^{-/-}$ apoptotic neutrophils and macrophages containing $Vn^{-/-}$serum (*B*) and phagocytosis assays were performed. Data from a representative experiment are shown (triplicates for each condition). Two additional independent experiments provided similar results. ** $P < 0.01$ compared with the group treated with HSA.

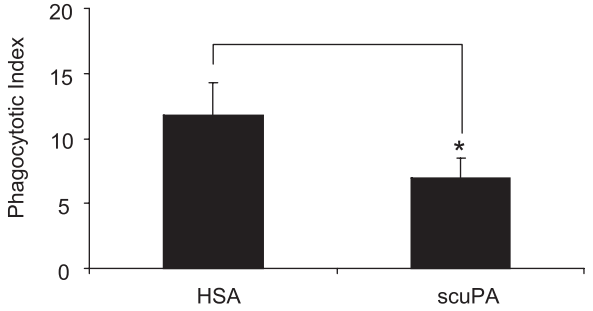

Figure 10. Urokinase-type plasminogen activator (uPA) diminishes efferocytosis *in vivo*. Apoptotic neutrophils (10×10^6) and 2 μg single-chain plasminogen activator (scuPA) or human serum albumin (HSA) in 50 μl phosphate-buffered saline were injected intratracheally. Two hours later, bronchoalveolar lavages were obtained and phagocytic indices determined. Data from a representative experiment are shown. A second independent experiment provided similar results. * $P < 0.05$ compared with the group injected intratracheally with HSA.

adhesion and in the maintenance of the extracellular matrix (45–47). In the present experiments, we found that uPA had no effect on the uptake of apoptotic neutrophils when added to phagocytic assays in which no Vn was present, thereby demonstrating that Vn is required for the inhibitory activity of uPA on efferocytosis. The major receptor of Vn on the cell surface is $\alpha_v\beta_3$ (52). Previous studies have shown that binding of Vn to $\alpha_v\beta_3$ plays an important role in regulating the activity of $\alpha_v\beta_3$, including association with uPAR (53–55). Given that uPA inhibits efferocytosis through binding to $\alpha_v\beta_3$, it is possible that Vn modulates such interactions through affecting the structure of $\alpha_v\beta_3$ or its availability for binding to uPA. Of note, $Vn^{-/-}$ macrophages demonstrate diminished ability to ingest apoptotic neutrophils compared with wild-type macrophages (Figure 9), suggesting that Vn may regulate mechanisms for efferocytosis in addition to those involving $\alpha_v\beta_3$. We are presently investigating this issue.

Tissue and circulating levels of uPA are up-regulated in a number of inflammatory settings, including sepsis and pneumonia, with the degree of elevation in uPA concentrations being closely associated with disease progression (20, 21). In this study, we found that uPA inhibited the clearance of apoptotic neutrophils by alveolar macrophages under *in vivo* conditions in the lungs. Neutrophils are primary effectors that contribute to ALI (23, 24), and delayed clearance of apoptotic neutrophils may promote lung inflammation and injury. The present results, by demonstrating that uPA inhibits the uptake of apoptotic neutrophils, suggest a novel mechanism by which elevated levels of uPA may participate in enhancing the duration and severity of inflammatory processes, such as ALI, in which neutrophils play a major role.

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