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Vitronectin Inhibits Efferocytosis through Interactions with Apoptotic Cells as well as with Macrophages

Hong-Beom Bae,*† Jean-Marc Tadie,*† Shaoning Jiang,* Dae Won Park,*§ Celeste P. Bell,* Lawrence C. Thompson,‡ Cynthia B. Peterson,§ Victor J. Thannickal,* Edward Abraham,‖ and Jaroslaw W. Zmijewski*

Effective removal of apoptotic cells, particularly apoptotic neutrophils, is essential for the successful resolution of acute inflammatory conditions. In these experiments, we found that whereas interaction between vitronectin and integrins diminished the ability of macrophages to ingest apoptotic cells, interaction between vitronectin with urokinase-type plasminogen activator receptor (uPAR) on the surface of apoptotic cells also had equally important inhibitory effects on efferocytosis. Preincubation of vitronectin with plasminogen activator inhibitor-1 eliminated its ability to inhibit phagocytosis of apoptotic cells. Similarly, incubation of apoptotic cells with soluble uPAR or Abs to uPAR significantly diminished efferocytosis. In the setting of LPS-induced ALI, enhanced efferocytosis and decreased numbers of neutrophils were found in bronchoalveolar lavage obtained from vitronectin-deficient (vtn−/−) mice compared with wild type (vtn+/+) mice. Furthermore, there was increased clearance of apoptotic vtn−/− as compared with vtn+/+ neutrophils after introduction into the lungs of vtn−/− mice. Incubation of apoptotic vtn−/− neutrophils with purified vitronectin before intratracheal instillation decreased efferocytosis in vivo. These findings demonstrate that the inhibitory effects of vitronectin on efferocytosis involve interactions with both the engulfing phagocyte and the apoptotic target cell. The Journal of Immunology, 2013, 190: 2273–2281.

The removal of apoptotic cells, a process known as efferocytosis, plays a crucial role in the maintenance of tissue homeostasis and resolution of inflammatory and immune responses (1–3). Failure to effectively remove apoptotic cells, and particularly apoptotic neutrophils that accumulate in inflammatory foci, results in necrosis and cytolysis of dying cells with the concomitant release of tissue damaging intracellular contents. Recent studies have shown that the ability of host to effectively remove apoptotic cells has important effects on outcome in experimental models for sepsis, hemorrhage, burns, or endotoxin-induced acute lung injury (ALI), conditions that are clinically relevant particularly in the setting of critical illness (4–6).

Recognition of apoptotic cells by phagocytes is mediated by “eat-me” signaling components that appear on the surface of the apoptotic cell (1, 2, 7–11). Phosphatidylinerine, calreticulin, CD14, and oxidized low-density lipoprotein-like moiety are well-characterized apoptotic cell surface markers that are involved in the engulfment of apoptotic cells by phagocytes (12–15). Recent studies suggest that factors released by apoptotic cells, including lysophosphatidylcholine or endothelial monocyte-activating polypeptide II, as well as the nucleotide extracellular gradient, participate in “find-me” signaling, resulting in the accumulation of phagocytes around apoptotic cells (16–18). Some receptors are also capable of preventing the recognition of dying cells. For example, the appearance of complexes of CD31–CD31 or CD47 signal regulatory protein α on the surface of apoptotic cells allows them to escape phagocytosis (19, 20). In addition to ligands appearing on the cell surface, soluble factors, including Gas6 and protein S, that bridge phosphatidylserine and phagocytic receptors of the TAM family (Tyro3, Axl, and Mer) enhance the uptake and ingestion of apoptotic cells by macrophages and other phagocytic cells (21). Finally, cytoskeletal rearrangement that allows for engulfment of the targeted cell and formation of phagosomes is required for effective clearance of apoptotic cells by phagocytes (22–24).

Vitronectin is a multifunctional glycoprotein found in large quantities in serum, the extracellular matrix, and platelets. Vitronectin consists of three distinct domains: a somatomedin B domain (SMB) that binds to the urokinase-type plasminogen activator receptor (uPAR); a short Arg-Gly-Asp (RGD) motif that interacts with integrins; and a hemopexin domain that forms complexes with the nucleotide extracellular gradient, participate in “find-me” signaling, resulting in the accumulation of phagocytes around apoptotic cells (16–18). Some receptors are also capable of preventing the recognition of dying cells. For example, the appearance of complexes of CD31–CD31 or CD47 signal regulatory protein α on the surface of apoptotic cells allows them to escape phagocytosis (19, 20). In addition to ligands appearing on the cell surface, soluble factors, including Gas6 and protein S, that bridge phosphatidylserine and phagocytic receptors of the TAM family (Tyro3, Axl, and Mer) enhance the uptake and ingestion of apoptotic cells by macrophages and other phagocytic cells (21). Finally, cytoskeletal rearrangement that allows for engulfment of the targeted cell and formation of phagosomes is required for effective clearance of apoptotic cells by phagocytes (22–24).

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sites are markedly increased in settings such as ALI, burns, and sepsis that are associated with neutrophil activation and tissue injury (36, 37).

In the present studies, we investigated the ability of vitronectin to modulate clearance of apoptotic cells under in vitro and in vivo conditions. Our results indicate that vitronectin can diminish efferocytosis by independently affecting the participation of both macrophages and apoptotic cells.

**Materials and Methods**

**Mice**

Vitronectin-deficient mice (B6.129S2[Db]-Vtn<sup>mel1Dgi</sup>/J), as well as control mice (C57BL/6J), were purchased from The Jackson Laboratory (Bar Harbor, ME). Vitronectin knockout male mice were crossed to B6D2F1/J female mice and then backcrossed to C57BL/6J for 12 generations before being interbred. Male mice, 8–12 wk of age, were used for experiments. All experiments were conducted in accordance with Institutional Review Board–approved protocols (University of Alabama at Birmingham Institutional Animal Care and Use Committee).

**Materials**

Purified mouse vitronectin was purchased from Abcam (Cambridge, MA). Vitronectin lacking the SMB domain, ΔSMB mutant (ΔSMB), or isolated SMB domain was expressed in Drosophila flies (Notre Dame, IN). Soluble uPAR (suPAR) were obtained from R&D Systems (Minneapolis, MN). Recombinant mouse PAI-1 was a gift from Dr. Victoria Ploplis (University of California, San Francisco, CA). Anti-uPAR blocking Ab was from Novus Biologicals (Littleton, CO), whereas anti-6His Ab was from Santa Cruz Biotechnology. Propidium iodide and Abs to Annexin V were obtained from R&D Systems (Minneapolis, MN). Cyclo(Arg-Gly-Asp-D-Phe-Val) RGDfv and cyclo(Arg-Ala-Asp-D-Val) RADfv were purchased from Enzo Life Sciences (Carmel, NY). Mouse-specific anti-integrin α<sub>4</sub>β<sub>1</sub> Ab was from BD Biosciences (San Diego, CA). Mouse-specific anti-integrin α<sub>4</sub>β<sub>1</sub> Ab was from BD Biosciences (San Diego, CA). Custom Ab mixtures and negative selection columns for neutrophil isolation were purchased from Stem Cell Technologies (Vancouver, BC, Canada). ELISA kits for measuring vitronectin were obtained from Molecular Innovations (Novi, MI).

Neutrophil and thymocyte isolation and culture

Bone marrow neutrophils were purified using a negative selection column (40, 41). In brief, bone marrow cell suspensions were isolated from the femur and tibia of mice by flushing with RPMI 1640 medium with 5% FBS. The cell suspension was passed through a glass wool column and collected by washing with PBS containing 5% FBS. Negative selection to purify neutrophils was performed by incubation of the cell suspension with biotinylated primary Abs specific for the cell-surface markers F4/80, CD4, CD45R, CD5, and TER119 (Stem Cell Technologies; http://www.stemcell.com/en/Products/Cell-type/Granulocytes-subsets.aspx) for 15 min at 4˚C followed by subsequent incubation with anti-biotin tetrameric Abs (100 µl; Stem Cell Technologies) for 15 min. The complex of anti-tetrameric Abs and cells was then incubated with colloidal magnetic dextran iron particles (60 µl; Stem Cell Technologies) for an additional 15 min at 4˚C. The T cells, B cells, RBCs, monocytes, and macrophages were captured in a column surrounded by a magnet, allowing the neutrophils to pass through. Neutrophils were determined by Wright-Giemsa stained cytocentrifuge preparations, was consistently >98%. Thymocytes were isolated as previously described (42).

**Purification and culture of peritoneal macrophages**

Peritoneal macrophages were elicited in 8 to 10-wk-old mice using Brewer thioglycollate. Cells were collected 5 d after i.p. injection of Brewer thioglycollate and were plated on coverslips (Fisherbrand 12-545-82 12CIR-1D; Fisher Scientific, Middleburg, VA) in 24-well plates (2.5 × 10<sup>4</sup> cells/well) in serum-free RPMI 1640 medium. After 1 h, the plates were washed with culture medium to remove nonadherent cells. Macrophages were cultured in RPMI 1640 medium and used for phagocytosis assays on the day of isolation.

**Induction of apoptosis in neutrophils and thymocytes**

Apoptosis in neutrophils and thymocytes was induced as previously described. In brief, apoptosis in vtn<sup>+</sup> or vtn<sup>−</sup> neutrophils was induced by incubation of the cells for 45 min at 43˚C, followed by culture for an additional 2.5 h at 37˚C. Murine thymocytes (vtn<sup>+/+</sup> or vtn<sup>−/−</sup>, 6 × 10<sup>5</sup> cells/ml) in RPMI 1640 medium were incubated with dexamethasone (1 µM) for 16 h. Cells were then washed three times with RPMI 1640 medium to remove dexamethasone. Annexin/PI staining and flow cytometry showed that >90% of the thymocytes and >70% of the neutrophils were apoptotic.

**In vitro efferocytosis assay**

In vitro efferocytosis assays were performed as previously described (43). In brief, 2.5 × 10<sup>5</sup> apoptotic neutrophils or 10<sup>5</sup> apoptotic thymocytes suspended in RPMI 1640 medium were cocultured with 2.5 × 10<sup>3</sup> macrophages on glass coverslips. Cells were incubated in media containing 5% mouse serum obtained from vtn<sup>+</sup> or vtn<sup>−</sup> mice for 2 or 1.5 h, as indicated in the figure legends. Next, coverslips were washed three times with ice-cold PBS and cells stained with HEMA 3 (Fisher Scientific). Phagocytosis was evaluated by a blinded observer by counting for five to six randomly selected fields per slide. The phagocytosis index was calculated as the percentage of macrophages containing at least one engulfed neutrophil or thymocyte.

**In vivo efferocytosis assay**

In vivo efferocytosis was determined as previously described (6, 34). In brief, the effect of vitronectin on phagocytosis was determined using intratracheal (i.t.) instillation of apoptotic neutrophils into vtn<sup>−/−</sup> or vtn<sup>+/+</sup> mice anesthetized with isoflurane. Mice were injected i.t. with 5 × 10<sup>6</sup> apoptotic neutrophils or 5 × 10<sup>5</sup> apoptotic neutrophils that were preincubated with purified vitronectin (100 nM) for 1 h. Two hours later, the mice were sacrificed and bronchoalveolar lavage (BAL) performed using 1 ml sterile PBS containing 5 mM EDTA. Cells were then collected on cytospin slides, fixed, stained with HEMA 3, and phagocytosis index was determined by a blinded observer.

In selected experiments, apoptosis was induced in thymocytes that were labeled with PKH-26 red fluorescent dye. Mice were anesthetized with isoflurane, and 1 ml of sterile PBS containing 5 or 100 µl PBS were injected i.t. or i.p., respectively, as described in the figure legends. Two hours later, mice were sacrificed and BAL or peritoneal lavage performed using 1 or 5 ml sterile PBS containing EDTA (5 mM), respectively. Isolated cells were washed with culture medium and then incubated in PBS containing 1% FCS, FITC-conjugated anti-CD11b or anti-CD11c, or FITC-conjugated anti-CD90.2 (thymocyte marker) Abs followed by flow cytometry analysis. The phagocytic index was calculated as the ratio of FITC<sup>+</sup>PKH26<sup>+</sup>allophycocyanin<sup>−</sup> cells to all cells gated. Engulfed thymocytes were not accessible to the allophycocyanin-conjugated anti-CD90.2 Ab. Therefore, FITC<sup>+</sup>PKH26<sup>+</sup>allophycocyanin<sup>−</sup> cells are macrophages that have engulfed PKH-labeled thymocytes, whereas the FITC<sup>+</sup>PKH26<sup>+</sup>allophycocyanin<sup>−</sup> cells are macrophages that contain adherent thymocytes (e.g., not engulfed).

**Imaging thymocytes, neutrophils, and macrophages**

Viable or apoptotic thymocytes were incubated with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were washed with PBS, preincubated with 1% BSA in PBS for 45 min, and then incubated with anti-CD45R, anti-CD5, and anti-TER119 Abs followed by flow cytometry analysis. The phagocytosis index was calculated as the ratio of FITC<sup>+</sup>PKH26<sup>+</sup>allophycocyanin<sup>−</sup> cells to all cells gated. Engulfed thymocytes were not accessible to the allophycocyanin-conjugated anti-CD90.2 Ab. Therefore, FITC<sup>+</sup>PKH26<sup>+</sup>allophycocyanin<sup>−</sup> cells are macrophages that have engulfed PKH-labeled thymocytes, whereas the FITC<sup>+</sup>PKH26<sup>+</sup>allophycocyanin<sup>−</sup> cells are macrophages that contain adherent thymocytes (e.g., not engulfed).
RGD-FITC or vitronectin-ΔSMB-6His for 60 min were stained with anti-
α5β3, anti-αβ6, or anti-6His Ab (90 min at room temperature) and specific
fluorescent secondary Abs (for additional 60 min).

**ELISA vitronectin**

Amount of vitronectin in culture medium or BALs was determined using
mouse-specific vitronectin kit (Molecular Innovations, Novi, MI), and
accordingly with instruction and vitronectin protein standard provided by
manufacturer.

**Model for LPS-induced ALI**

ALI was induced by i.t. administration of 1 mg/kg LPS in 50 μl PBS as
previously described (40, 41, 44, 45). In brief, mice were anesthetized with
isoflurane, the tongue was gently extended, and LPS in PBS or PBS alone
(control) was deposited into the pharynx. Mice were sacrificed 48 h after
LPS administration, and BAL was obtained by lavaging the lungs three
times with 1 ml PBS.

**Statistical analysis**

Statistical significance was determined by the Wilcoxon rank sum test
(independent two-group Mann–Whitney U test), as well as Student t test
for comparisons between two groups. Multigroup comparisons were per-
formed using one-way ANOVA with the Turkey’s post hoc test. A p value
<0.05 was considered significant. Analyses were performed on SPSS
version 16.0 for Windows.

**Results**

**Vitronectin diminishes engulfment of apoptotic cells by macrophages**

The ability of vitronectin to affect efferocytosis was determined
using peritoneal macrophages and apoptotic thymocytes or neutrophils.
Cells were obtained from wild type mice (vtn+/+) or mice deficient in
vitronectin (vtn−/−). Consistent with previous studies (33), complete deficiency of vitronectin in the cultures (e.g., vtn−/− cells and medium-containing serum from vtn−/− mice) was asso-
ciated with markedly increased phagocytosis of apoptotic thymocytes as compared with that found when vtn+/+ macrophages and apoptotic cells were included in the cultures (Fig. 1A, 1B).

The effects of vitronectin deficiency were reversible on coincu-
bation of vtn−/− cells with serum obtained from wild type mice
(vtn+/+). Of note, the inhibitory effects of vitronectin were dependent
on cell viability, in particular, exposure to vitronectin dimin-
ished the engulfment of apoptotic cells (Fig. 1B), but had no effect on the low rate of uptake of viable cells by macrophages. As was found with apoptotic thymocytes, vitronectin deficiency also in-
creased uptake of apoptotic neutrophils by peritoneal macrophages
(Fig. 1C).

**Vitronectin diminishes efferocytosis through interactions with macrophages, as well as with apoptotic cells**

To determine whether the inhibition of efferocytosis by vitronectin
was mediated by binding of vitronectin to receptors on the surface
of macrophages or apoptotic cells, or perhaps by affecting both
cell types, vtn−/− macrophages cultured in vitronectin-deficient
medium were dose-dependently treated with purified vitronectin
or vtn+/+ serum obtained from wild type mice, then washed and
 cocultured with vitronectin-deficient apoptotic thymocytes in
vitronectin-deficient medium. As shown in Fig. 2A and Supple-
mental Fig. 1A, inclusion of either purified vitronectin or serum-
containing vitronectin dose-dependently decreased the ability
of macrophages to ingest apoptotic thymocytes. Decreases in effero-
cytosis were also found when apoptotic vtn−/− thymocytes were
preincubated with purified vitronectin or vitronectin-containing
medium followed by coculture with vtn−/− macrophages (Fig.
2B or Supplemental Fig. 1B). The lowest levels of phagocytosis
were found when both vtn−/− macrophages and apoptotic cells
were treated with vitronectin (Fig. 2C). As expected, large
amounts of vitronectin were detected in vtn+/+ serum, whereas no
vitronectin was found in vtn−/− serum (Supplemental Fig. 1G).
Western blot analysis, confocal microscopy, and ELISA con-
firmed the ability of purified vitronectin or vitronectin in serum
to bind to viable and apoptotic vtn−/− cells (Supplemental Fig. 1E,
1F, 1H).

**FIGURE 1.** Vitronectin deficiency decreases phagocytosis of apoptotic cells. Macrophages were cocul-
tured with apoptotic or viable thymocytes (vtn+/+ or vtn−/−) or neutrophils (vtn+/+ or vtn−/−) for 90 or 120
min, respectively. Cells were incubated in serum obtained from wild type mice, then washed and
preincubated with purified vitronectin or vitronectin-containing serum (Supplemental Fig. 1G).
Arrows in (A) indicate ingested apoptotic thymocytes. ***p < 0.001 compared vitronectin-cultured
apoptotic cells in vtn+/+ serum, **p < 0.01 compared vitronectin-cultured apoptotic cells in vtn−/−
serum with vitronectin-cultured apoptotic cells in vtn+/+ serum, *p < 0.05 compared with viable cells.

**A**

- **vtn**+/+ macrophages
- **vtn**−/− thymocytes
- **vtn**−/− serum

**B**

- viable thymocytes
- apoptotic thymocytes

**C**

- viable neutrophils
- apoptotic neutrophils
Vitronectin domains differentially affect the interactions between macrophages and apoptotic thymocytes during efferocytosis

Preincubation of apoptotic thymocytes with full-length vitronectin or with the vitronectin SMB domain alone produced similar inhibition of efferocytosis, but exposure of apoptotic thymocytes to vitronectin lacking the SMB domain (vitronectin-$\Delta$SMB) had no effect of efferocytosis (Fig. 3A). In contrast with apoptotic thymocytes, exposure of vtn$^{-/-}$ macrophages to full-length vitronectin or vitronectin-$\Delta$SMB, but not to the SMB domain alone, resulted in inhibition of efferocytosis (Fig. 3E). Of note, confocal microscopy confirmed that vitronectin-$\Delta$SMB was colocalized with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the cell surface of peritoneal macrophages (Supplemental Fig. 2A, 2B). These results suggest that whereas the SMB domain of vitronectin is involved in diminishing efferocytosis through interaction with receptors on apoptotic cells, other domains of vitronectin are responsible for its inhibitory effect on ingestion of apoptotic cells by macrophages.

Vitronectin is known to interact with the $\alpha_v\beta_3$ integrin and appears able to inhibit the stimulatory effects of $\alpha_v\beta_3$ on efferocytosis (33, 46, 47). Consistent with such previously reported findings, incubation of macrophages with specific Abs that block the $\alpha_v\beta_3$ integrin resulted in decreased uptake of apoptotic thymocytes (Fig. 3F). However, a similar approach to selectively block the $\alpha_v\beta_3$ integrin on apoptotic thymocytes had no effect on efferocytosis (Fig. 3B). Similar results were obtained with specific Abs to $\alpha_v\beta_5$. The pretreatment of macrophages with anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$ Abs (1 $\mu$g/ml), isotype-specific IgG (1 $\mu$g/ml), or RGDFv (1 $\mu$M) for 1h and then washed. The percentage of engulfing macrophages was determined after coculture of macrophages with apoptotic thymocytes for 90 min. In selected experiments, cells were treated with anti-$\alpha_v\beta_3$ or $\alpha_v\beta_5$ Abs or RGDFv for 60 min, washed, and then cultured with purified vitronectin for an additional 60 min. Means $\pm$ SD ($n = 3$) are shown. (A) ***$p < 0.001$ compared with control (untreated) or cells pretreated with $\Delta$SMB only; (B) ***$p < 0.001$ compared with control, anti-$\alpha_v\beta_3$, or IgG pretreated; (C) ***$p < 0.001$ compared with IgG or anti-$\alpha_v\beta_5$; (D) ***$p < 0.001$ compared with control, RGDFv, or RAD treated; (E) ***$p < 0.001$ compared with control or SMB treated; (F) ***$p < 0.01$ compared with control and IgG; (G) $p < 0.05$ ($p = 0.036$) and ***$p < 0.001$ compared with IgG; and (H) ***$p < 0.001$ compared with control and macrophages pretreated with RAD.
Abs that block the $\alpha_\beta_3$ integrin (Fig. 3C, 3G). These results demonstrate that in addition to its interactions with $\alpha_\beta_3$, vitronectin can potentially modulate efferocytosis through binding between its RGD domain and the $\alpha_\beta_3$ integrin (33, 48, 49). Previous studies (33), as well as results shown in Fig. 3H, demonstrate that preincubation of macrophages with the RGD peptide RGDfV or with full-length vitronectin effectively diminished the ability of macrophages to engulf apoptotic cells. In contrast, exposure of apoptotic thymocytes to RGDfV did not affect efferocytosis and also did not affect vitronectin-dependent inhibition of efferocytosis (Fig. 3D, 3H). These results suggest that the inhibitory effects of vitronectin on efferocytosis are mediated by binding to integrins on the surface of macrophages, but were integrin independent in the case of apoptotic cells. Notably, confocal microscopy revealed colocalization between FITC-RGD peptide and $\alpha_\beta_3$ or $\alpha_\beta_3$ integrins in both macrophages and apoptotic neutrophils (Supplemental Fig. 2C, 3A).

**Interactions between vitronectin and uPAR on the surface of apoptotic cells inhibit efferocytosis**

As shown in Fig. 4A and 4B, full-length vitronectin or purified vitronectin SMB domain diminished efferocytosis of apoptotic $vtn^{-/-}$ thymocytes. Because the vitronectin SMB domain is known to bind to the uPAR (50, 51), we hypothesized that such interactions might be a relevant mechanism for the ability of vitronectin to affect the uptake of apoptotic cells by macrophages. Indeed, exposure of $vtn^{-/-}$ apoptotic thymocytes to uPAR blocking Abs or deficiency of uPAR ($uPAR^{-/-}$ thymocytes) resulted in inhibition of efferocytosis (Fig. 4A, 4D). Although a previous study (52) and the current experiments found decreased uPAR staining on the surface of apoptotic cells, uPAR was still readily detected on apoptotic thymocytes (Fig. 4E, Supplemental Fig. 3B). As shown in Fig. 4E, confocal microscopy revealed that uPAR also interacts with vitronectin.

Interactions between vitronectin with PAI-1 and uPAR are mutually exclusive, although PAI-1 was demonstrated to have higher affinity for vitronectin than does uPAR (28, 53). Because SMB diminished efferocytosis, we anticipated that formation of PAI-1–SMB complexes will diminish binding between SMB and uPAR on the surface of apoptotic cells and preserve efficient clearance of such cells. As shown in Fig. 4B, preincubation of $vtn^{-/-}$ apoptotic thymocytes with preformed complexes of PAI-1–SMB diminished the ability of the vitronectin SMB domain to inhibit efferocytosis. Of note, there was no evidence that the effects of PAI-1 were due to potential contamination with LPS (Supplemental Fig. 1C).

**FIGURE 4.** uPAR on the surface of apoptotic cells contributes to the ability of vitronectin to inhibit efferocytosis. (A) Apoptotic thymocytes ($vtn^{-/-}$) were pretreated with blocking Ab to uPAR (0 or 1 μg/ml) or isotype-specific IgG (0 or 1 μg/ml) for 30 min. Cells were washed and then treated with purified vitronectin (0 or 200 nM) for an additional 60 min. Phagocytic indices were determined after thymocytes were washed to remove unbound vitronectin followed by coculture for 90 min with macrophages in medium containing vitronectin-deficient serum (5%). Means ± SD (n = 3); ***p < 0.001 compared with control (untreated) or macrophages pretreated with IgG alone. (B) Apoptotic thymocytes were preincubated with PAI-1 (0 or 100 nM), SMB (0 or 100 nM), or preformed PAI-1–SMB complexes for 60 min. PAI-1–SMB complexes were obtained after incubation of PAI-1 with SMB for 60 min at room temperature. Phagocytic indices are shown. Means ± SD (n = 3); **p < 0.01, comparing thymocytes incubated with SMB to control or treated with PAI-1 or PAI-1–SMB. (C) Phagocytic indices were obtained after apoptotic thymocytes were pretreated with vitronectin (0 or 200 nM), suPAR (0 or 200 nM), vitronectin/suPAR complexes, or vitronectin incubated with BSA. Means ± SD (n = 3); **p < 0.01 compared with control or thymocytes treated with suPAR alone or vitronectin/suPAR. (D) Phagocytic indices obtained after apoptotic wild type ($uPAR^{+/+}$) or uPAR-deficient ($uPAR^{-/-}$) thymocytes were cocultured with wild type macrophages. Means ± SD (n = 3); **p < 0.01. (E) Representative confocal images (original magnification ×60) show the amount and colocalization (yellow) of vitronectin and uPAR on the surface of viable or apoptotic neutrophils (arrows). Fluorescent immunostaining shows uPAR (red), vitronectin (green) and nuclei (blue).
Previous studies have shown that suPAR can bind to $\alpha_v$, $\beta_1$, $\beta_2$, or $\beta_3$ integrins (25, 35, 54), as well as to SM domain of vitronectin (30). Therefore, we examined whether interactions between suPAR and vitronectin could affect efferocytosis. Inclusion of suPAR preincubated with vitronectin into cultures of $vtn^{+/+}$ apoptotic thymocytes increased the phagocytic index as compared with treatment with vitronectin alone (Fig. 4C). However, unlike the situation with apoptotic cells, macrophages preincubated with suPAR still demonstrated diminished engulfment of apoptotic thymocytes after exposure to vitronectin (Supplemental Fig. 1D).

**Vitronectin decreases efferocytosis in vivo**

Although our experiments demonstrated that vitronectin can inhibit efferocytosis in vitro, we wished to confirm that such effects of vitronectin were also present under in vivo conditions. To examine this issue, we induced ALI by pulmonary exposure to LPS, a situation known to result in the appearance of significant amounts of vitronectin in interstitial lung tissue, as well as in the alveolar space (Fig. 5E) (36, 37). As shown in Fig. 5, engulfment of apoptotic neutrophils was significantly increased in the lungs of LPS-treated $vtn^{-/-}$ as compared with $vtn^{+/+}$ mice. Decreased numbers of neutrophils were recovered from BAL fluid of $vtn^{-/-}$ as compared with $vtn^{+/+}$ mice.

Although these results suggest that deficiency of vitronectin can enhance the clearance of apoptotic neutrophils, another possibility is that there were increased numbers of neutrophils undergoing efferocytosis as a result of greater levels of apoptosis. To address this issue, we subjected $vtn^{-/-}$ or $vtn^{+/+}$ mice to i.t. administration of purified apoptotic $vtn^{-/-}$ or $vtn^{+/+}$ neutrophils and then determined phagocytic indices. As shown in Fig. 6A, clearance of apoptotic neutrophils was significantly increased in $vtn^{-/-}$ mice injected with $vtn^{-/-}$ neutrophils as compared with $vtn^{+/+}$ mice that were injected with $vtn^{+/+}$ neutrophils. Of note, significantly less vitronectin was found in the lungs of control mice as compared with mice exposed to LPS (Fig. 5E). i.t. administration of purified vitronectin into the lungs of control ($vm^{+/+}$) mice also decreased efferocytosis after instillation of $vm^{-/-}$ apoptotic neutrophils (Fig. 6B).

Aptotic $vm^{-/-}$ neutrophils or thymocytes were incubated with or without vitronectin, then washed and injected i.t. into $vm^{-/-}$ mice to determine whether vitronectin can affect efferocytosis by specifically binding to apoptotic cells. As shown in Fig. 6C, there was decreased phagocytosis by alveolar macrophages of apoptotic cells that had been treated with purified vitronectin. Similar results were obtained from experiments in which fluorescently labeled apoptotic $vm^{-/-}$ thymocytes were preincubated with or without purified vitronectin and then injected into the lungs of $vm^{-/-}$ mice (Fig. 6D). Confirmatory results were obtained using fluorescently labeled $vm^{-/-}$ apoptotic thymocytes preincubated with or without vitronectin that were then injected into the i.p. cavity of $vm^{-/-}$ mice (Fig. 6E). These data indicate that vitronectin plays a regulatory role in efferocytosis in vivo through interactions with receptors on the surface of apoptotic cells (Fig. 7).

**Discussion**

In these experiments, we found that vitronectin effectively inhibits uptake of apoptotic cells by macrophages in vitro and in the lungs of mice with LPS-induced ALI. Our results showed that interactions between vitronectin and apoptotic cells, as well as between vitronectin and macrophages, prevent efferocytosis. However, the mechanisms for such inhibition of efferocytosis by vitronectin were dependent on interaction with differing receptors on macrophages and apoptotic cells. Although we found that vitronectin decreased phagocytosis by binding to the $\alpha_v\beta_3$ and $\alpha_v\beta_2$ integrin on the surface of macrophages, this did not appear to be the case for apoptotic cells. Using cells isolated from vitronectin-deficient mice, Abs to selective cell-surface receptors, and specific vitronectin domains, we found that the inhibitory effects of vitronectin on the uptake of apoptotic cells by macrophages were mediated by interactions with the uPAR on the apoptotic cell.

Previous studies demonstrated that vitronectin receptors, including $\alpha_v\beta_3$ and the combination of PAI-1 and uPAR, can modulate the phagocytosis of apoptotic cells (33–35, 55). However, the direct role of vitronectin on efferocytosis has not been fully explored. Proteins able to bridge receptors on apoptotic cells and phagocytes, including vitronectin, milk fat globule EGF factor 8 (MFG-E8), or thrombospondin-1 (TSP-1), have been reported to participate in efferocytosis through binding to $\alpha_v\beta_3$ on macrophages (33, 46, 47, 56). Vitronectin can diminish interactions between $\alpha_v\beta_3$ and bridging molecules, such as MFG-E8 or TSP-1, thereby preventing recognition of phosphatidylyserine on apoptotic cells (46, 47). In the present studies, incubation of macrophages with Abs to $\alpha_\beta_3$ or RGDfv decreased their ability to ingest apoptotic cells. Of note, interactions between $\alpha_\beta_3$ and vitronectin or the RGD peptide on macrophages, but not on apoptotic cells, have previously been shown to inhibit association of macrophages with apoptotic cells (33, 46, 57).

Previous studies have shown that the SM domain of vitronectin is required for binding to uPAR (25, 28, 30). In the present experiments, culture of apoptotic cells with purified vitronectin SMB domain, but not with vitronectin lacking the SMB domain, diminished efferocytosis. These results indicate that uPAR on the surface of apoptotic cells is likely to be a target for the SMB domain of vitronectin. Indeed, similar to the inhibitory effects on efferocytosis found after incubation of the SMB domain with apoptotic cells, inactivation of uPAR on apoptotic cells by incubation with blocking Abs also diminished phagocytosis. These data suggest that the ability of vitronectin to bind uPAR on apoptotic cells inhibits an important “eat-me” signal that normally...
would enhance the uptake of apoptotic cells by macrophages. Notably, αβ3 on apoptotic cells appeared to be dispensable in regulating efferocytosis, as evidenced by the lack of effect when apoptotic cells were preincubated with Abs that block αvβ3 or with RGDfV, unlike the inhibition of efferocytosis found when macrophages were exposed to such agents.

uPAR affects many cellular functions, including the adhesion of cells to vitronectin and other matrix proteins (25, 58, 59). suPAR is released by cleavage of the uPAR GPI anchor in the plasma membrane (25, 50). As is the case for uPAR, suPAR is known to associate with αv, β1, β2, and β3 integrins (35, 54, 60, 61). In addition, association between suPAR and β3 integrins can be ef-

FIGURE 6. Effects of vitronectin on clearance of apoptotic cells in vivo. (A) Phagocytic index was determined by measuring ingestion of apoptotic neutrophils by alveolar macrophages using BAL from mice (vtn+/+ or vtn−/−) obtained 2 h after i.t. administration of apoptotic neutrophils (vtn+/+ or vtn−/−). Means ± SD with three mice per group. *p < 0.05. (B) Wild type (vtn+/+ or vtn−/−) mice received purified vitronectin i.t. (0 or 1 μg) 30 min before instillation of vtn−/− apoptotic neutrophil. BAL was obtained 2 h later. The percentages of alveolar macrophages with ingested neutrophils are shown. Means ± SD from three mice per group. *p < 0.05. (C) Apoptotic neutrophils (vtn−/−) were incubated with purified vitronectin (0 or 200 nM) for 1 h, washed, and then administered i.t. to vitronectin-deficient (vtn−/−) mice. Phagocytic indices were determined in BAL obtained 2 h after neutrophil administration. Means ± SD (four mice/group) are shown. *p < 0.05 compared with mice that received neutrophils that were incubated without vitronectin. (D) Mice were treated as described in (C) using PKH26-labeled apoptotic thymocytes (vtn−/−) that were preincubated with or without vitronectin. Phagocytic indices were determined using flow cytometry. Means ± SD (four mice/group) are shown. *p < 0.05 compared with the mice given untreated thymocytes. (E) Mice were subjected to i.p. injection of PKH26-labeled apoptotic thymocytes (vtn−/−) treated with or without vitronectin. Peritoneal cells were isolated 2 h later and phagocytic indices determined using flow cytometry. Means ± SEM using three mice per group.

FIGURE 7. The SMB domain and RGD motif of vitronectin inhibit efferocytosis through selective binding to integrins on macrophages and by binding to uPAR on apoptotic cells.
Ravichandran, K. S. 2010. Find-me and eat-me signals in apoptotic cell clearance (36, 37). Notably, previous studies demonstrated that the vitronectin, including in the pulmonary interstitium and alveolar neutrophil-associated inflammatory processes, such as those that can inhibit efferocytosis and potentially affect the resolution of integrins through the uPAR D2 and/or D3 domains (30, 62–64). and such interactions may affect the association of uPAR with shown to bind to the D1 domain and D1-D2 linker region of uPAR, thereby blocking the ability of vitronectin to inhibit efferocytosis, a finding confirmed in our experiments. The specific mechanisms by which interactions between vitronectin, uPAR, and integrins affect efferocytosis have not been completely elucidated. The SMDB domain of vitronectin has been shown to bind to the D1 domain and D1-D2 linker region of uPAR, and such interactions may affect the association of uPAR with integrins through the uPAR D2 and/or D3 domains (30, 62–64).

Our findings reveal a novel mechanism by which vitronectin can inhibit efferocytosis and potentially affect the resolution of neutrophil-associated inflammatory processes, such as those that occur in ALI. Severe sepsis, hemorrhage, burns, and ventilator-induced lung injury are associated with increased tissue levels of vitronectin, including in the pulmonary interstitium and alveolar space (36, 37). Notably, previous studies demonstrated that the severity of LPS-induced ALI is diminished in vitronectin-deficient (vtn−/−) mice (37). Our present results show that deficiency of vitronectin also is associated with diminished clearance of apoptotic neutrophils in the lungs of mice with LPS-induced lung injury. In addition, preincubation of apoptotic cells with vitronectin diminished their uptake by alveolar macrophages after in-stillation into the lungs. These findings suggest that therapeutic approaches that inhibit binding between vitronectin with uPAR on apoptotic neutrophils should increase efferocytosis and, therefore, may diminish the severity of ALI and other inflammatory processes in which neutrophils play a major role.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure s1. (A) Macrophages (vtn⁻/⁻) or (B) apoptotic thymocytes (vtn⁺/⁺) were pre-incubated for 1 hour with serum from vtn⁻/⁻ and vtn⁺/+ mice. The concentration of vitronectin in mixed serum media (5%) was 0, 50, 100 or 200 nM. The cells were then washed and co-cultured in medium containing vtn⁻/⁻ serum (5%) for an additional 90 minutes. The percentages of ingested thymocytes are shown. Means ± SD (n = 3), *P < 0.05, **P < 0.01 or ***P < 0.001 compared to cells incubated in media without vitronectin. (C) Apoptotic thymocytes (vtn⁻/⁻) were cultured with PAI-1 (0 or 100 nM), SMB (0 or 100 nM), or preformed PAI-1/SMB complexes. After 60 minutes incubation, cells were washed and then co-cultured with vitronectin deficient (vtn⁻/⁻) macrophages. Polymyxin B (10 μM) was added to the media 30 minutes before inclusion of PAI-1 or SMB. Phagocytic indices are shown. Means ± SD were obtained from two independent experiments. (D) Macrophages (vtn⁻/⁻) were pre-treated with purified vitronectin, suPAR, or BSA in media without serum for 60 minutes. After washing, cells were co-cultured with apoptotic thymocytes (vtn⁻/⁻) for 90 minutes and then the number of macrophages engulfing thymocytes was determined. Means ± SD (n = 3), **P < 0.01 compared to untreated cells or macrophages pre-incubated with BSA. (E) Representative confocal images show the amount of vitronectin on the surface of vtn⁺/+ or vtn⁻/⁻ thymocytes. Cells were culture with wild type or vitronectin deficient serum (5%), as indicated. Green-vitronectin, blue- nuclei. (F) Representative Western blots show the amount of vitronectin obtained from vtn⁺/+ and vtn⁻/⁻ thymocytes after incubation with serum of vtn⁺/+ or vtn⁻/⁻ mice (upper panel), or vtn⁻/⁻ cells before and after exposure to purified vitronectin (1 μg/ml for 30 minutes in serum free medium) (lower panel). Additional experiment provided similar results. Panel (G) shows the amount of vitronectin detected in mouse
serum of wild type or vitronectin deficient mice. Measurement was performed using ELISA. Means ± SD, n = 4. (H) Apoptotic thymocytes (vtn\textsuperscript{+/+} or vtn\textsuperscript{-/-}) were incubated in serum (5 %) obtained from wild type or vitronectin deficient mice for 30 minutes. Cells were washed three times with cold PBS, transferred to new tubes, lysed and then amount of vitronectin determined using ELISA. Means ± SD, n = 3.

**Supplemental Figure s2.** Peritoneal macrophages were treated with 1 μg/ml vitronectin deficient in SMB domain (vtn\textsuperscript{-Δ-SMB-6His}) for 60 minutes. Next, cells were subjected to immunostaining with anti-His and anti-\(\alpha_v\beta_3\) or \(\alpha_v\beta_5\) antibody. Representative images show staining of (A) \(\alpha_v\beta_3\) (green) and vtn\textsuperscript{-Δ-SMB} (red) or (B) \(\alpha_v\beta_5\) (green) and vtn\textsuperscript{-Δ-SMB} (red). Nuclei are visualized with DAPI (blue). (C) Peritoneal macrophages were incubated with RGD-FITC peptide (20 nM) for 30 minutes and then subjected to immunostaining for \(\alpha_v\beta_3\) or \(\alpha_v\beta_5\) integrins. Representative images show co-localization between RGD-FITC and integrins (yellow). \(\alpha_v\beta_3\) or \(\alpha_v\beta_5\)– red, nuclei- blue. (D) Cells were subsequently treated with non-labeled RGDfv (200 nM) for 30 minutes and then RGD-FITC for additional 30 minutes. Representative images indicate that pre-incubation cells with RGDfv resulted in decrease fluorescence of RGD-FITC when compared to cell treated with RGD-FITC alone.

**Supplemental Figure s3.** (A) Apoptotic neutrophils were treated with RGD-FITC followed by immunocytochemistry assay to detect \(\alpha_v\beta_3\) or \(\alpha_v\beta_5\) integrin. Representative
images show $\alpha_\nu\beta_3$ or $\alpha_\nu\beta_5$ integrins (red), RGD-FITC (green) and nuclei (blue). (B) Representative images show uPAR (green) detected on the surface of viable and apoptotic thymocytes ($vtn^{+/+}$ or $vtn^{-/-}$). Top panel shows uPAR (green) and nuclei (blue) whereas middle panel shows enlarged images indicated with dotted lines in top panel. The lower panel shows nuclei only.
Supplemental Figure s1
Supplemental Figure s2
A

Supplemental Figure s3

B