VITRONECTIN INHIBITS NEUTROPHIL APOPTOSIS THROUGH ACTIVATION OF INTEGRIN ASSOCIATED SIGNALING PATHWAYS

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Online Data Supplement

Supplemental Materials and Methods

Neutrophil isolation and culture

Both, bone marrow or peritoneal neutrophils were purified using negative selection column purification system (1-3). Briefly, bone marrow cell suspensions were isolated from the femur and tibia of a mouse by flushing with RPMI 1640 medium with 5% FBS. The cell suspension was passed through a glass wool column and collected by subsequent washing with PBS containing 5% FBS. Negative selection to purify neutrophils was performed by incubation of the cell suspension with biotinylated primary antibodies specific for the cell surface markers F4/80, CD4, CD45R, CD5, and TER119 (StemCell Technologies, Vancouver, BC, Canada, www.stemcell.com/technical/13309-PIS.pdf) for 15 min at 4°C followed by subsequent incubation with anti-biotin tetrameric antibody (100 µl; StemCell Technologies) for 15 min. The complex of anti-tetrameric antibodies and cells was then incubated with colloidal magnetic dextran iron particles (60 µl; StemCell Technologies) for an additional 15 min at 4°C. The T cells, B cells, RBC, monocytes, and macrophages were captured in a column surrounded by a magnet, allowing the neutrophils to pass through. Neutrophil purity, as determined by Wright-Giemsa-stained cytospin preparations, was consistently greater than 98%. Neutrophils were also isolated from the peritoneal cavity of 8- to 12-wk-old mice 4 h after an intraperitoneal injection with 2 ml of thioglycolate solution (3%). Peritoneal cell populations were consistently composed of approximately 90% neutrophils as

determined using Wright-Giemsa staining. Peritoneal neutrophils were further purified using negative selection column to achieve 98% or greater homogenous population.

Western blot analysis

Western blot analysis was performed as previously described (1, 4). Briefly, equal amounts of protein were resolved by 8–12% SDS-PAGE and transferred onto PVDF membranes (polyvinylidene difluoride membrane, Immobilon-P; Millipore, Billerica, MA). To measure total and phosphorylated proteins, membranes were probed with specific antibodies followed by detection with HRP-conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (ECL Plus, Amersham) and quantified by AlphaEase FC software (Alpha Innotech, San Leandro, CA). Each experiment was carried out two or more times using cell populations obtained from separate groups of mice.

Measurement of neutrophil apoptosis

The percentage of early and late apoptotic cells was determined by staining with annexin V – FITC and propidium iodide, followed by flow cytometry. Caspase 3 and 9 activities were measured using standard fluorogenic substrates (Calbiochem; La Jolla, CA), in conjunction with epifluorescent microscopy. Data were calculated as a ratio of green fluorescence per number of nuclei and final images were processed and store using IPLab Spectrum and Adobe Photoshop (Adobe Systems, San Jose, CA) software.

Acute lung injury model

Acute lung injury was induced by intratracheal administration of 1 mg/kg LPS in 50 µl of PBS as previously described (1, 3-6). Briefly, mice were anesthetized with isoflurane and then suspended by their upper incisors on a 60° incline board. The tongue was gently extended and LPS solution deposited into the pharynx. BAL fluids were harvested 24 hours after LPS administration.

References

E1. Zmijewski, *et al*. *Am J Respir Crit Care Med*. 2009.

E2. Zmijewski *et al. Am J Physiol Cell Physiol*. 2007

E3. Zhao *et al. Am J Physiol Lung Cell Mol Physiol*. 2008

E4. Zmijewski, *et al*. *Am J Respir Crit Care Med*. 2008

E5. Foster et al. *J Appl Physiol*. 2001

E6. Brass et al. *Am J Physiol Lung Cell Mol Physiol.* 2007

Supplemental Figure legends

Figure E1. The effects of vitronectin on mature neutrophil viability. (A) Mature, peritoneal neutrophils obtained from *vtn*$^{+/+}$ or *vtn*$^{-/-}$ mice were incubated with 5% serum from wild type mice containing vitronectin (*vtn*$^{+/+}$) or 5% serum from transgenic mice unable to express vitronectin (*vtn*$^{-/-}$), respectively. Apoptotic neutrophils obtained at 0, 24, or 48 hours after the initiation of culture were identified by flow cytometry using staining with annexin V and propidium iodide. Total number of peritoneal neutrophils in cultures was obtained at 0, 24 or 48 hours. $^{**}P < 0.01$ (left panel: $p = 0.004$, right panel: $p = 0.006$) compared to *vtn*$^{+/+}$ cells. (B) Peritoneal neutrophils (*vtn*$^{-/-}$) were incubated in medium containing *vtn*$^{+/+}$ or *vtn*$^{-/-}$ serum (5%) for 0 or 24 hours and then the cell viability determined using flow cytometry. Means ± SD ($n = 3$ independent sets of neutrophils) are shown. $^{**}P < 0.01$ ($p = 0.006$) compared to *vtn*$^{+/+}$ serum.

Figure E2. Effects of polymyxin B or PI-3k inhibitor on vitronectin -dependent enhancement of neutrophil viability. The percentages of viable and apoptotic neutrophils were determined after incubation with (A) vitronectin ΔSMB (0 or 100 nM) or (B) SMB domain (0 or 100 nM) for 48 hours. Polymyxin B (poly B; 0 or 10 μM) was added to neutrophil cultures 30 minutes prior to inclusion (vitronectin ΔSMB or SMB domain). Mean ± SD was obtained from two independent experiments.

Figure E3. Vitronectin potentiates LPS-dependent increase in neutrophil viability. *Vtn*$^{-/-}$ neutrophils were incubated with LPS (0, 10, or 100 ng/ml) for 30 minutes in media

containing 5% *vtn*$^{-/-}$ serum, followed by addition of recombinant vitronectin (0 or 100 nM). The percentages of (A) viable and (B) apoptotic cells were determined by flow cytometry after 48 hours of culture. Means ± SD showing results from three independent experiments. (A) **P* < 0.05 (p = 0.02), ****P* < 0.001 (p = 0.0009) and (B) and ***P* < 0.01 (p = 0.002), ****P* < 0.001 (p = 0.0002) compared to untreated cells or cells treated with LPS only.

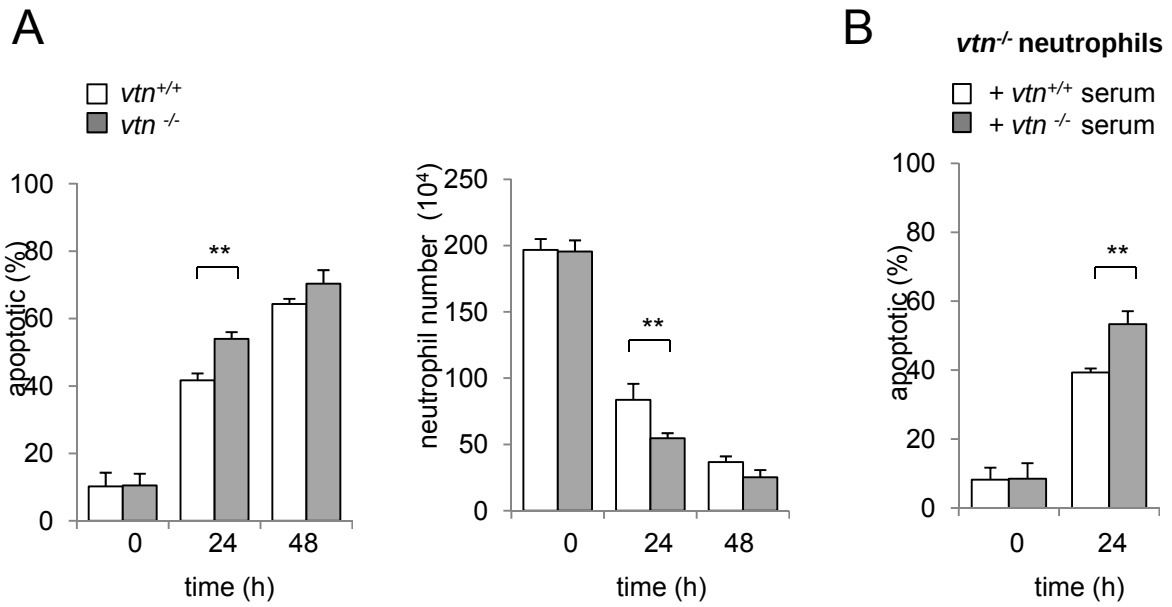
A
vtn+/+
vtn -/-
apoptotic (%)
100
80
60
40
20
0
**
0
24
48
time (h)
neutrophil number (10^4)
250
200
150
100
50
0
**
0
24
48
time (h)
B
vtn-/- neutrophils
+ vtn+/+ serum
+ vtn -/- serum
apoptotic (%)
100
80
60
40
20
0
**
0
24
time (h)

Figure E1

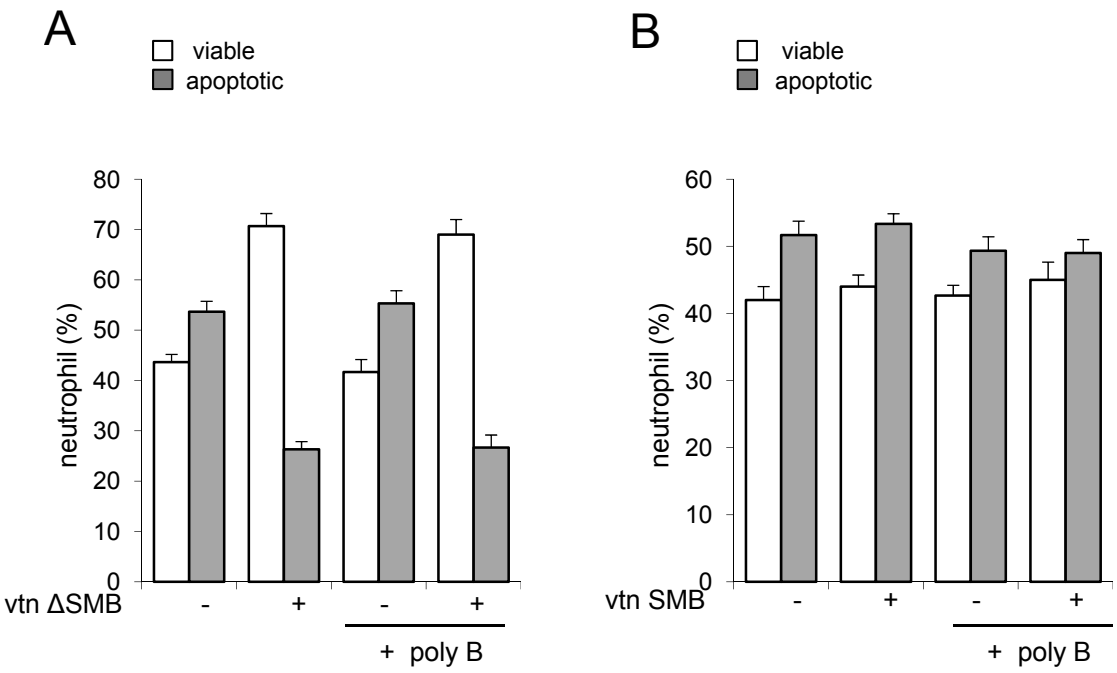
A
viable
apoptotic
neutrophil (%)
80
70
60
50
40
30
20
10
0
vtn ΔSMB
-
+
-
+
+ poly B
B
viable
apoptotic
neutrophil (%)
60
50
40
30
20
10
0
vtn SMB
-
+
-
+
+ poly B

Figure E2

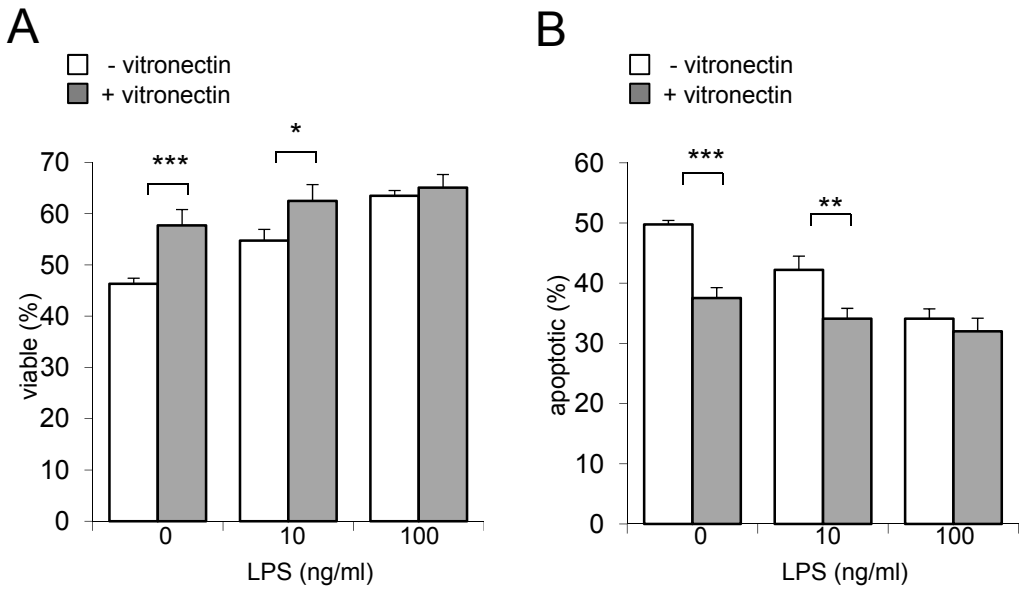
A
- vitronectin
+ vitronectin
viable (%)
70
60
50
40
30
20
10
0
***
*
0
10
100
LPS (ng/ml)
B
- vitronectin
+ vitronectin
apoptotic (%)
60
50
40
30
20
10
0
***
**
0
10
100
LPS (ng/ml)

Figure E3