NF-κB-to-AP-1 Switch: A Mechanism Regulating Transition From Endothelial Barrier Injury To Repair In Endotoxemic Mice.

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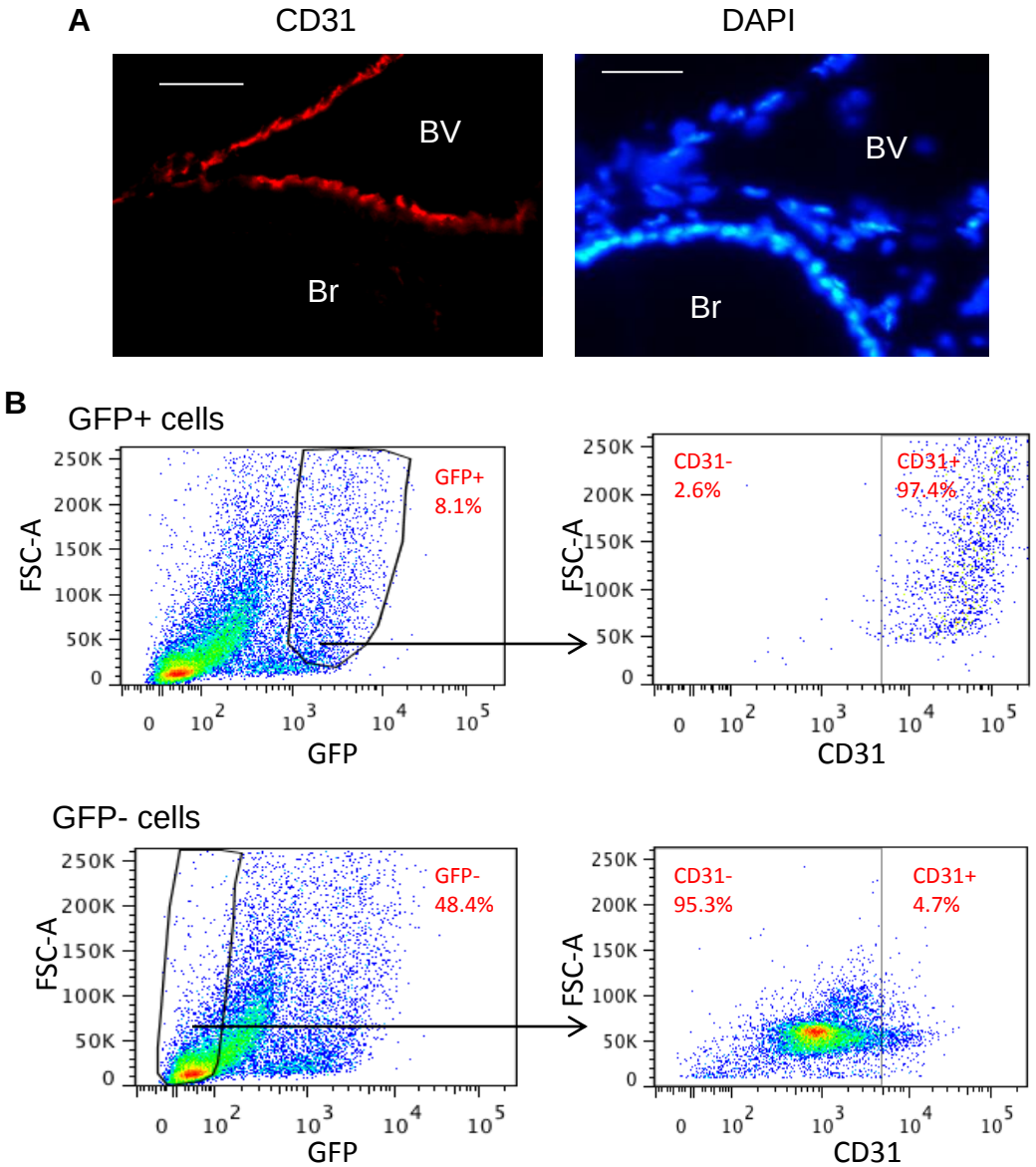
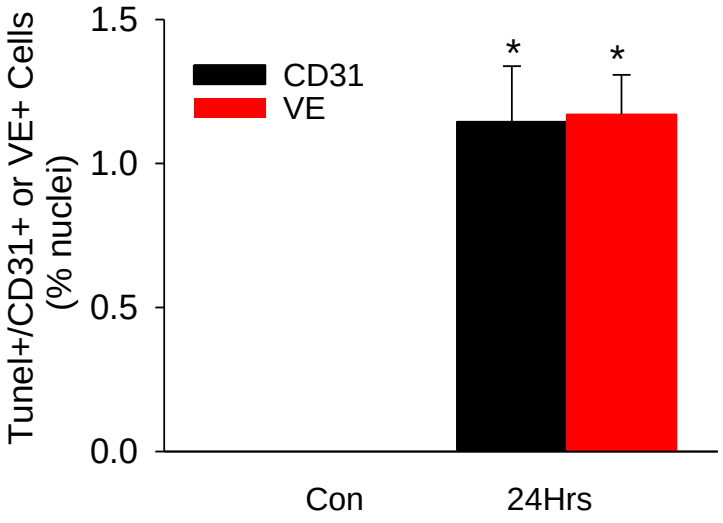
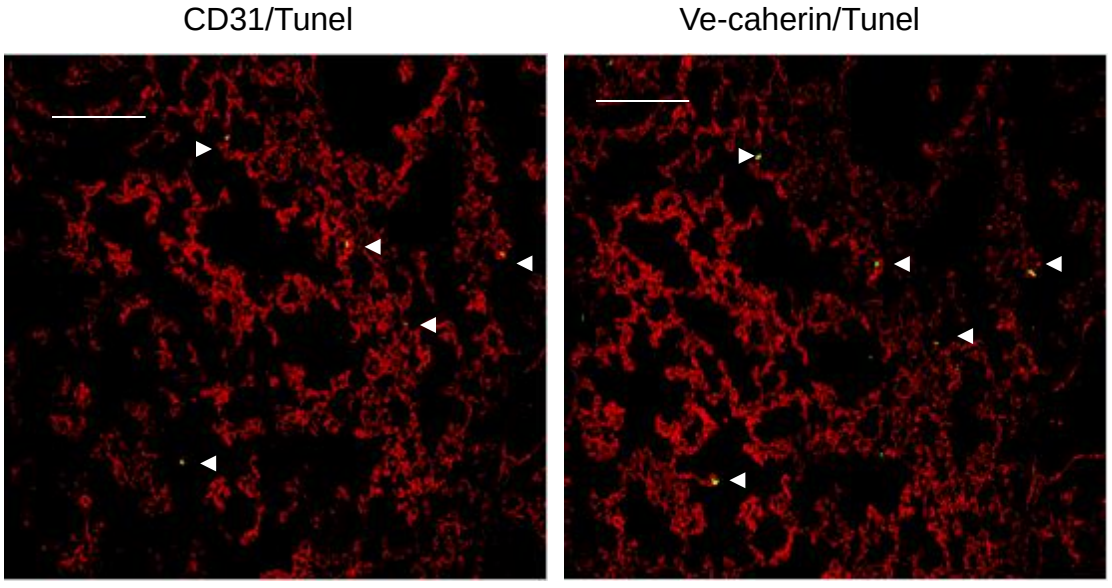
Supplementary Information



C


Tunel+/CD31+ or VE+ Cells
(% nuclei)
1.5
1.0
0.5
0.0
CD31
VE
*
*
Con
24Hrs

D


CD31/Tunel
Ve-caherin/Tunel

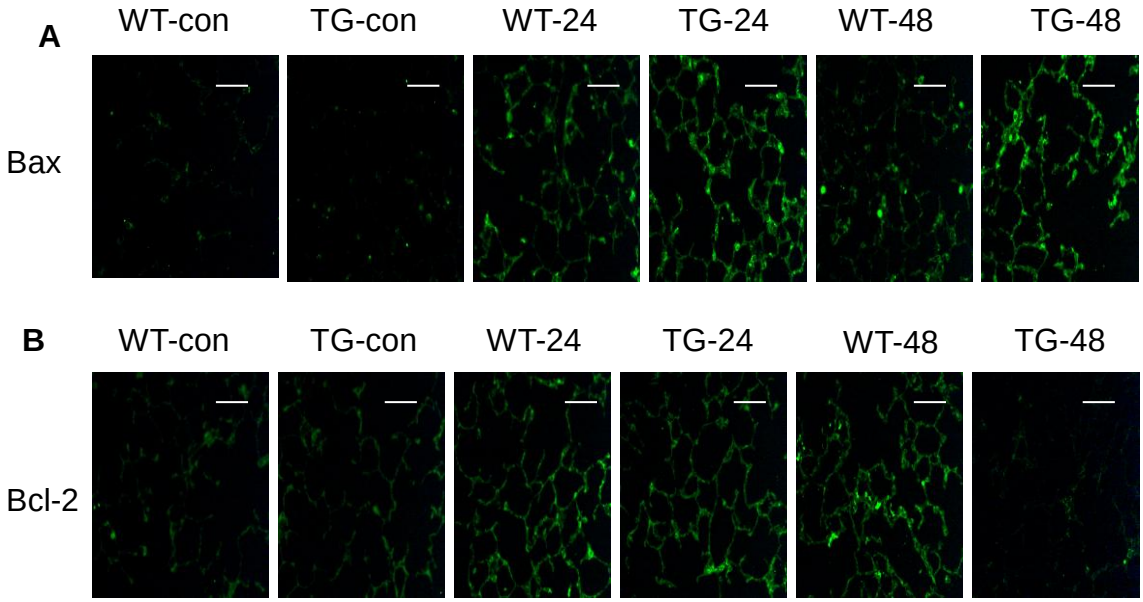
Supplementary Figure 1. CD31 and Tunel double-staining detection of apoptotic ECs.

A: Representative micrographs of CD31 staining of lung sections show that the CD31 antibody reacts with endothelial cells (ECs) in the lumen of blood vessel (BV), but does not cross-react with epithelial cells in the lumen of bronchiole (Br). Lung sections were stained with anti-CD31 antibody (red) and nuclei counterstained with DAPI (blue). The data are representative of 3 independent experiments. Scale bar, 50 μm. This result verifies that the CD31 antibody reacts with ECs, but not epithelial cells or basal membrane components.

B: FACS analysis of lung cells from Tie2-GFP mice shows that 97% of the GFP-positive cells (ECs) are stained CD31-positive, but less than 5% of the GFP-negative cells (non-ECs) are stained CD31-positive. The lung single-cell suspension was prepared from Tie2-GFP mice, stained with CD31 antibody and analyzed. This result confirms the EC-specificity of the CD31 antibody.

C: Bar graph shows that the numbers of apoptotic ECs detected by CD31/Tunel and VE-cadherin/Tunel staining are virtually identical. Adjacent lung sections were double stained with CD31 antibody plus Tunel reagents or VE-cadherin antibody plus Tunnel reagents. The numbers of CD31+/Tunel+ or VE-cadherin+/Tunel+ apoptotic ECs were counted and compared. Means ± SEM of 5 mice per group are shown. *, $p< 0.05$, compared to controls. VE-cadherin/Tunel double staining confirms the CD31/Tunel staining result.

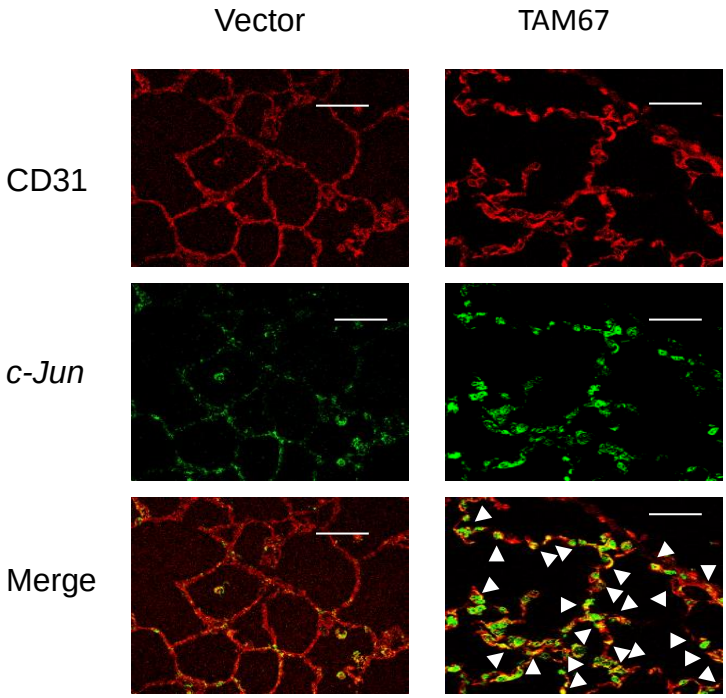
D: Representative confocal microscopic images show that CD31+/Tunel+ and VE-cadherin+/Tunel+ cells are located at the same micro-anatomic locations. ECs on adjacent lung sections were stained red with fluorescent CD31 or VE-cadherin antibody. Apoptotic cells were stained green with Tunel reagents. Merging of the red and green images revealed CD31+/Tunel+ or VE-cadherin+/Tunel+ apoptotic ECs (arrow indicated “yellow” cells). The CD31+/Tunel+ (left) and VE-cadherin+/Tunel+ (right) apoptotic ECs were located at same micro-anatomic locations. Scale bar, 50 μm.



Supplementary Figure 2. Blockade of endothelial NF-κB at 48, but not 24, hours increases Bax and decreases Bcl-2 protein expression in lungs. Mice were injected with saline (Con) or LPS (other groups, 5 mg/kg, i.p.) and then with Dox (0.5 mg/mouse, i.p.)12 hours prior to tissue collection. Lung cryosections were prepared at 24 or 48 hours after LPS injection and stained with Bax or Bcl-2 antibody followed by Alexa Fluor 488 conjugated secondary antibody.

A: Representative micrographs show Bax expression in the lungs. Bax+ cells significantly increased at 24 hours and reduced at 48 hours in WT lungs. Blockade of endothelial NF-κB at 48, but not at 24, hours increased Bax-expressing (green) cells in TG lungs. The data are representative of 3 independent experiments. Scale bar, 50 μm.

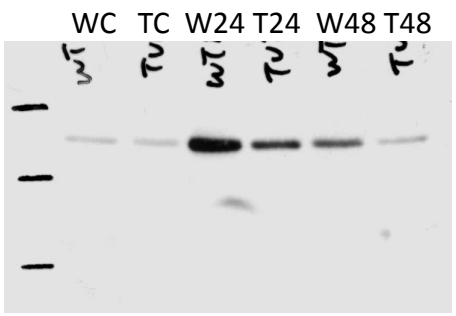
B: Representative micrographs show Bcl-2 expression in the lungs. Bcl-2+ cells significantly increased at 24 and 48 hours in WT lungs. Endothelial NF-κB blockade at 48, but not at 24, hours reduced Bcl-2-expressing (green) cells in TG lungs. Representative of 3 independent experiments. Scale bar, 50 μm.



Supplementary Figure 3. Micrographs of CD31 (red) and *c*-Jun (green) double staining show that TAM67 transfection markedly increased *c*-Jun-expressing endothelial cells (ECs).

Mice were transfected with pCMV-vector or pCMV-TAM67 plasmid. At 3 days after transfection, lung cryosections were prepared and stained with CD31 (red) and *c*-Jun (green) antibodies. Lung sections from mice transfected with pCMV-TAM67 displayed many fold increase in numbers of $CD31^+$/*c-Jun*$^+$ ECs, compared to lung sections from mice transfected with the pCMV-vector alone, confirming TAM67 overexpression in lung ECs. Scale bar, 50 μm.

A. Figure 1C



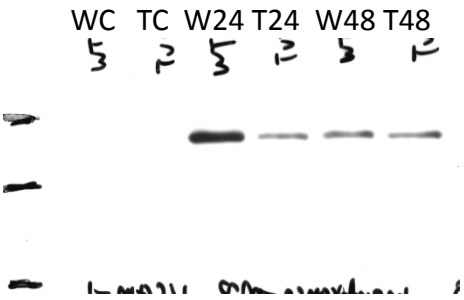
Molecular weights

121 kD

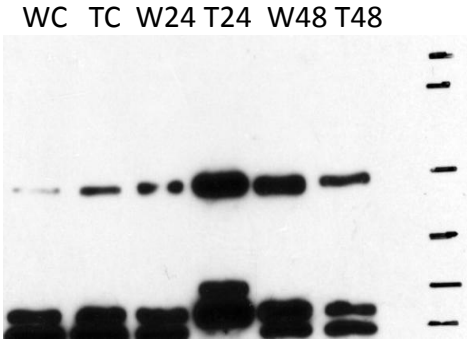
80 kD

41 kD

B. Figure 1D

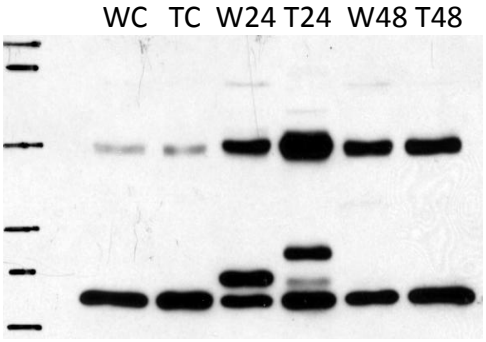


C. Figure 5A

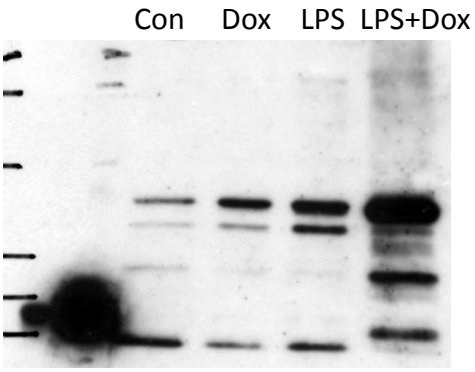


105 kD
84 kD
50 kD
36 kD
29 kD
20 kD

D. Figure 5B



E. Figure 8A



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36 kD
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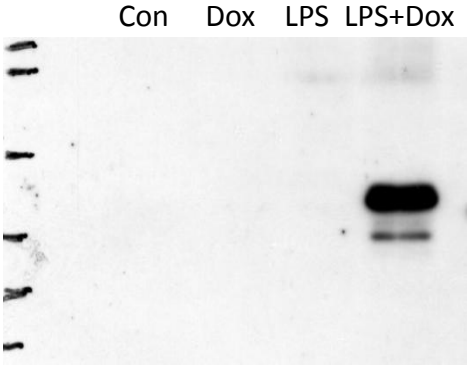
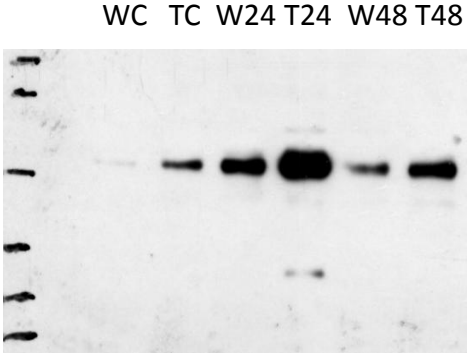
20 kD

F. Figure 8B

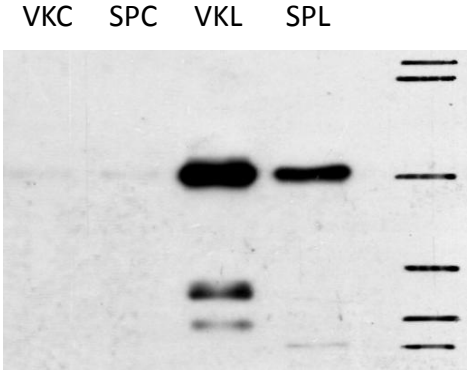


G. Figure 8D



105 kD
84 kD
50 kD
36 kD
29 kD
20 kD

H. Figure 8E



Supplementary Figure 4 : Full-length images of the cropped blots presented in the main figures

A and **B**: Full-length images of Figures 1C and 1D show that Dox-induced I-κBαmt expression, at or after peak of organ injury, inhibits VCAM-1 expression in lungs and heart. WC, wild type control; TC, transgenic control; W24, wild type 24 hours; T24, transgenic 24 hours; W48, wild type 48 hours; and T48, transgenic 48 hours.

C and **D:** : Full-length images of Figures 5A and 5B show that inhibition of endothelial NF-κB at 24, but not at 48, hours increases nuclear levels of *c-Jun* and c-*Fos* in the lungs of transgenic mice.

E: Full-length image of Figure 8A shows that LPS or Dox stimulation alone caused an increase in nuclear *c-Jun* level. The increase in nuclear *c-Jun* level was even greater in the presence of both LPS and Dox. Con, control. Dox, doxycycline.

F: Full-length image of Figure 8B shows that LPS+Dox stimulation increased nuclear phospho-*c-Jun* (*p-c-Jun,* Ser63) level, while LPS or Dox alone did not.

G: Full-length image of Figure 8D shows that endothelial NF-κB inhibition at 24 hours increases JNK activity. The tissue level of phospho-JNK (p-JNK, Thr183/Tyr185, an indicator of JNK activity) in the lungs was determined.

H: Full-length image of Figure 8E shows that JNK inhibitor reduces *c-Jun* phosphorylation. Mice were pretreated with JNK inhibitor*,* SP600125, and injected with saline, LPS and Dox. Nuclear level of phospho-*c-Jun* (*p-c-Jun*) in lungs was determined by Western blot. VKC, vehicle control; SPC, SP600125 control; VKL, vehicle LPS; and SPL, SP600125 LPS.

Blots were adjusted for contrast/brightness to a similar extent as the cropped blots, and resized appropriately. Molecular weights are shown in the middle of two blots.