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Supplemental Information

SPHK2-Generated S1P in CD11b^{+} Macrophages Blocks STING to Suppress the Inflammatory Function of Alveolar Macrophages

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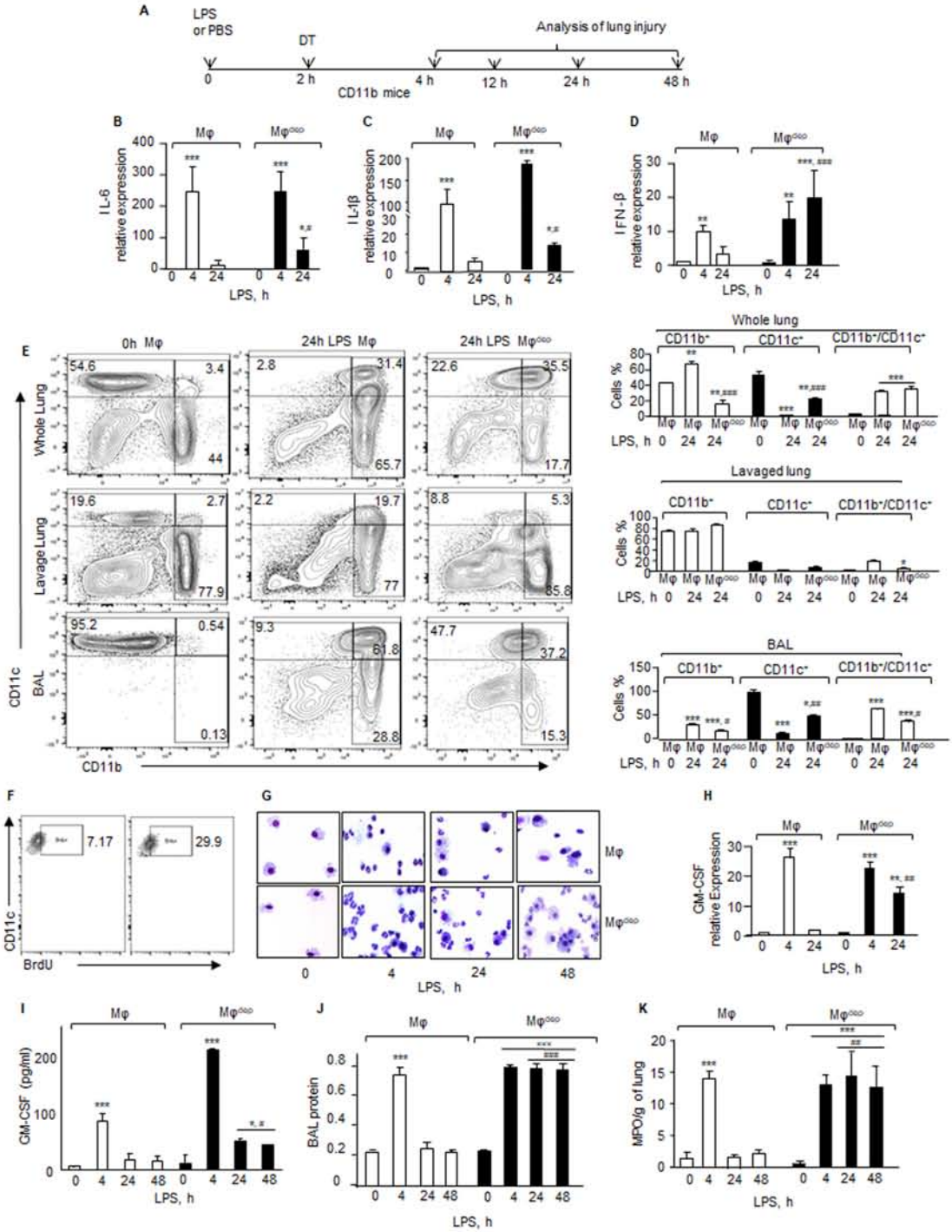

Figure S1. Effect of CD11b macrophage depletion on LPS induced lung injury, Related to Figure 1

(A) Experimental protocol. CD11b-DTR mice received 1 mg/ml nebulized LPS for 45 min and after 2 h, PBS or a single dose of DT was injected (*i. p.* 25 ng/g body weight) to determine $CD11b^+$ macrophage depletion and lung vascular inflammatory injury.

(B-D) At indicated times, lungs from control Mφ or $M\varphi^{dep}$ mice were harvested and processed to determine relative expression of IL-6 (B), IL-1β (C) and IFN-β (D) using qPCR. GAPDH expression was used as an internal control. n=4 mice/group.

(E) Cells obtained from total lungs, BAL and lungs after lavage (lavaged lungs) from Mφ or $M\varphi^{dep}$ mice were stained with CD45, CD11c, CD11b, CD64, Ly6g antibodies. Flow cytometry analysis was performed to determine depletion of the $CD11b^+$ Mφ population in air space versus lung parenchyma. Left panel show a representative FACS plot while right panel show changes in macrophage population as a fraction of the total number of flow recoverable CD45+/Ly6g/CD64+ macrophages. We generally recover 500,000 macrophages in an unexposed naïve lung.

(F) Mφ or $M\varphi^{dep}$ mice received LPS and after 20h Brdu was injected. Mice were sacrificed at 24h and lung cells were stained with CD45, CD11c, CD64, and Brdu antibodies. Flow cytometry analysis was performed to determine the proliferation of AMφ as a fraction of the total number of $CD11c^+/CD64^+$ macrophages. A representative FACS plot is shown from experiments that were repeated at least three times.

(G) BAL was isolated at indicated time and stained with hematoxylin and eosin to assess leukocytes. Image representative of three individual experiments.

(H-I) GM-CSF expression was determined either using qPCR (G) or ELISA assay (H). GAPDH was taken as the control for qPCR analysis (H). n=4 mice/group.

(J) BAL was obtained from Mφ or $M\varphi^{dep}$ mice at indicated times and protein was determined. n=5mice/group.

(K) Lungs were homogenized, and myeloperoxidase (MPO) activity was determined. MPO activity is expressed as change in absorbance at 460 nm per mg protein per min. n=4 mice/group.

Data in figure **B-F** and **H-K** are represented as mean ± SD from two to three independent experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ relative to unexposed Mφ or $M\varphi^{dep}$ group while $\#P < 0.05$, $\#\#p < 0.01$ and $\#\#\#p < 0.001$ indicates significance from Mφ group post LPS exposure at 24 h or 48h. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

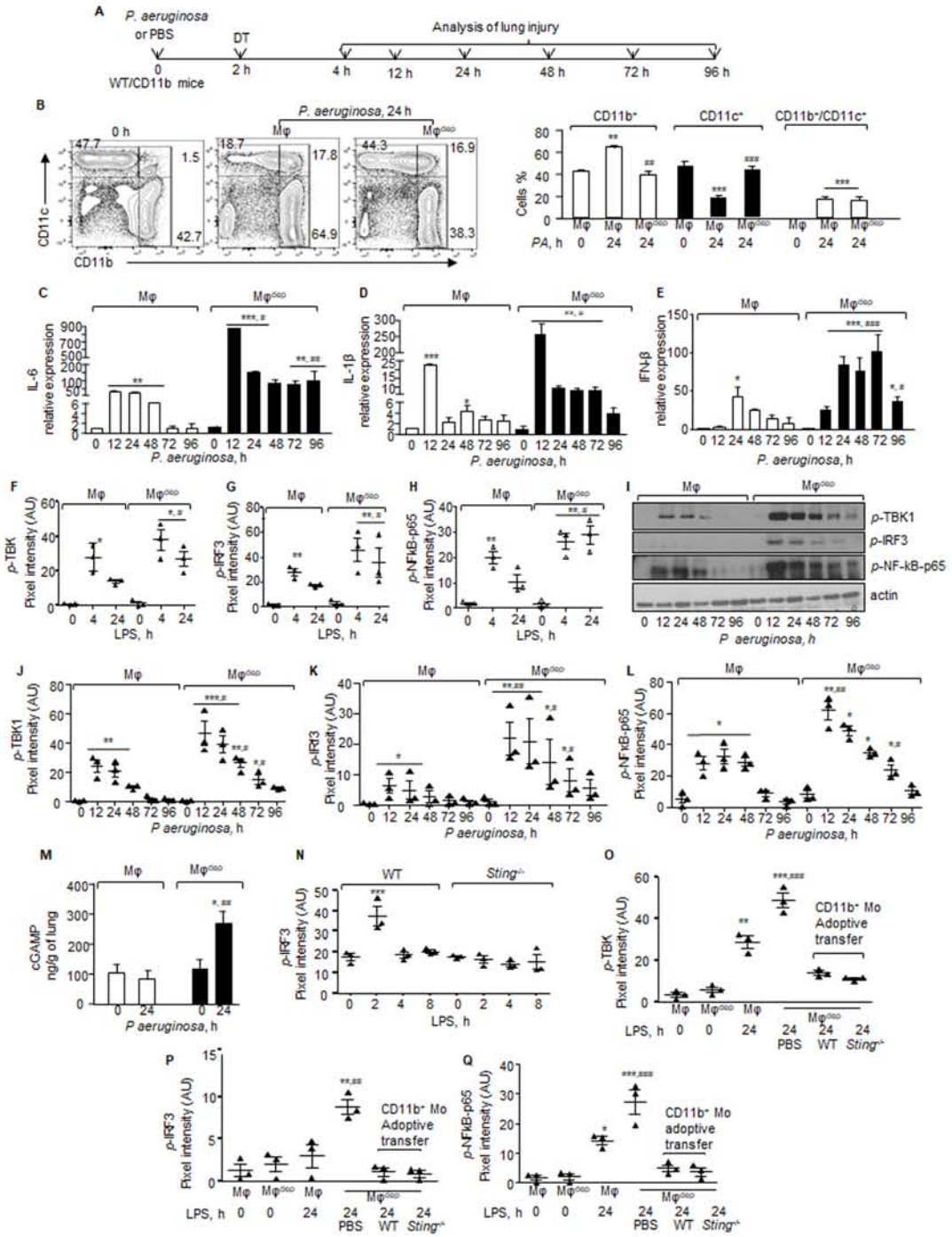

Figure S2. Effect of *Pseudomonas aeruginosa* infection on lung macrophages, Related to Figure 2 and Figure 3.

(A) Experimental protocol. CD11b-DTR mice were challenged *i.t.* with PBS or *P. aeruginosa* ($1x10^4$ CFU) and after 2 h, PBS or a single dose of DT was injected (*i. p.* 25 ng/g body weight). Lungs were harvested at the indicated times to determine injury.

(B) Lung cells were stained with CD45, CD11c, CD11b, CD64, Ly6g fluorescently tagged antibodies and flow cytometry analysis was performed to determine depletion of $CD11b^+$ Mφ population. A representative FACS plot is shown in left panel while right panel show changes in macrophage population as a fraction of the total number of flow recoverable $CD45^+/Ly6g^-/CD64^+$ macrophages. Experiments were performed at least three times.

(C-E) Following *PA*-induced injury, lungs were harvested from control WT or $M\varphi^{dep}$ mice and relative expression of IL-6 (C), IL-1β (D) and IFN-β (E) were measured using qPCR. GAPDH expression was used as an internal control. n=4 mice/group.

Data in **B-E** are represented as mean ± SD from two to three independent experiments. *p < 0.05; **p < 0.01 and ***p < 0.001 relative to unexposed Mφ or $M\varphi^{dep}$ group while #P < 0.05, ##p < 0.01 and ###p < 0.001 indicates significance relative to Mφ group post *P.A* exposure at indicated times. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

(F-H) Plot shows individual pixel intensities of **Fig. 3A** expressed as arbitrary units (AU) along with mean and SD (n=3). *p < 0.05 and **p < 0.01 relative to unexposed Mφ or $M\varphi^{dep}$ group while #p < 0.05 indicates significance relative to Mφ group post LPS exposure at indicated times. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

(I-L) Phosphorylation of TBK1, IRF3 and p65 subunit of NF-κB was determined as in **Fig. 3A**. A representative immunoblot is shown in **I**, while panels **J-L** shows individual pixel intensities expressed as arbitrary units (AU) along with mean and SD (n=3). *p < 0.05; **p < 0.01 and ***p < 0.001 relative to unexposed Mφ or $M\varphi^{dep}$ group while ##p < 0.05 and ##p < 0.01 indicates relative to Mφ group post *P.A* exposure at indicated times. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

(M) cGAMP was extracted from control or $M\varphi^{dep}$ lungs as in **Fig. 3B**. Data show mean ± SD from two independent experiments. n=4 mice/group. *p < 0.05 relative to unexposed Mφ or $M\varphi^{dep}$ group while ##p < 0.01 indicates significance relative to Mφ group post *P.A* exposure at indicated times. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

(N) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 3C**. ***p < 0.001 relative to unexposed WT or STING null mice. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

(O-Q) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 3F**. *p < 0.05, **p < 0.01 and ***p < 0.001 relative to Mφ or $M\varphi^{dep}$ groups while ##p < 0.01 and ###p < 0.001 indicates significance relative to $M\varphi^{dep}$ group receiving WT or STING null CD11b monocytes adoptive transfer and *P.A* exposure for indicated times. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

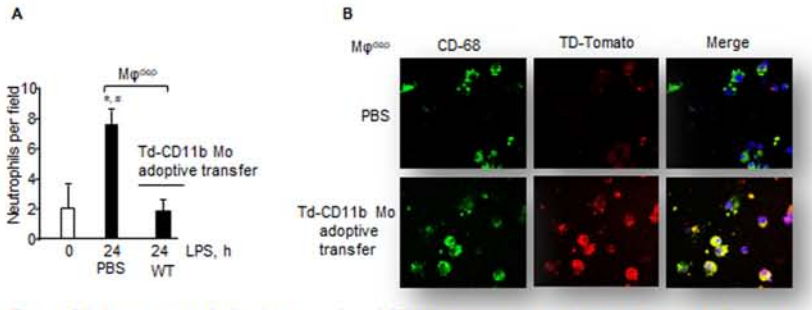

Figure S3. Assessment of adoptive transfer of CD11b monocytes in the lung, Related to Figure 3.
(A) Neutrophils were counted per field on hematoxylin and eosin stained bronchoalveolar lavage from $M\varphi^{dep}$ mice at 0 h or after 24 h post LPS exposure following adoptive transfer of vehicle (PBS) or WT-CD11b monocytes as in **Fig. 3E**. The plot shows mean ± SD. *$p < 0.05$ relative to unexposed Mφ group. #$p < 0.05$ indicates significance from $M\varphi^{dep}$ mice receiving WT (Td)-CD11b monocytes (n=3 mice/group).
(B) BAL cells obtained from $M\varphi^{dep}$ mice following PBS or adoptive transfer of Td-CD11b monocytes were cytospun and stained with anti-68 antibody to confirm macrophage phenotype (merge yellow). A representative image is shown from experiments that were performed multiple time.

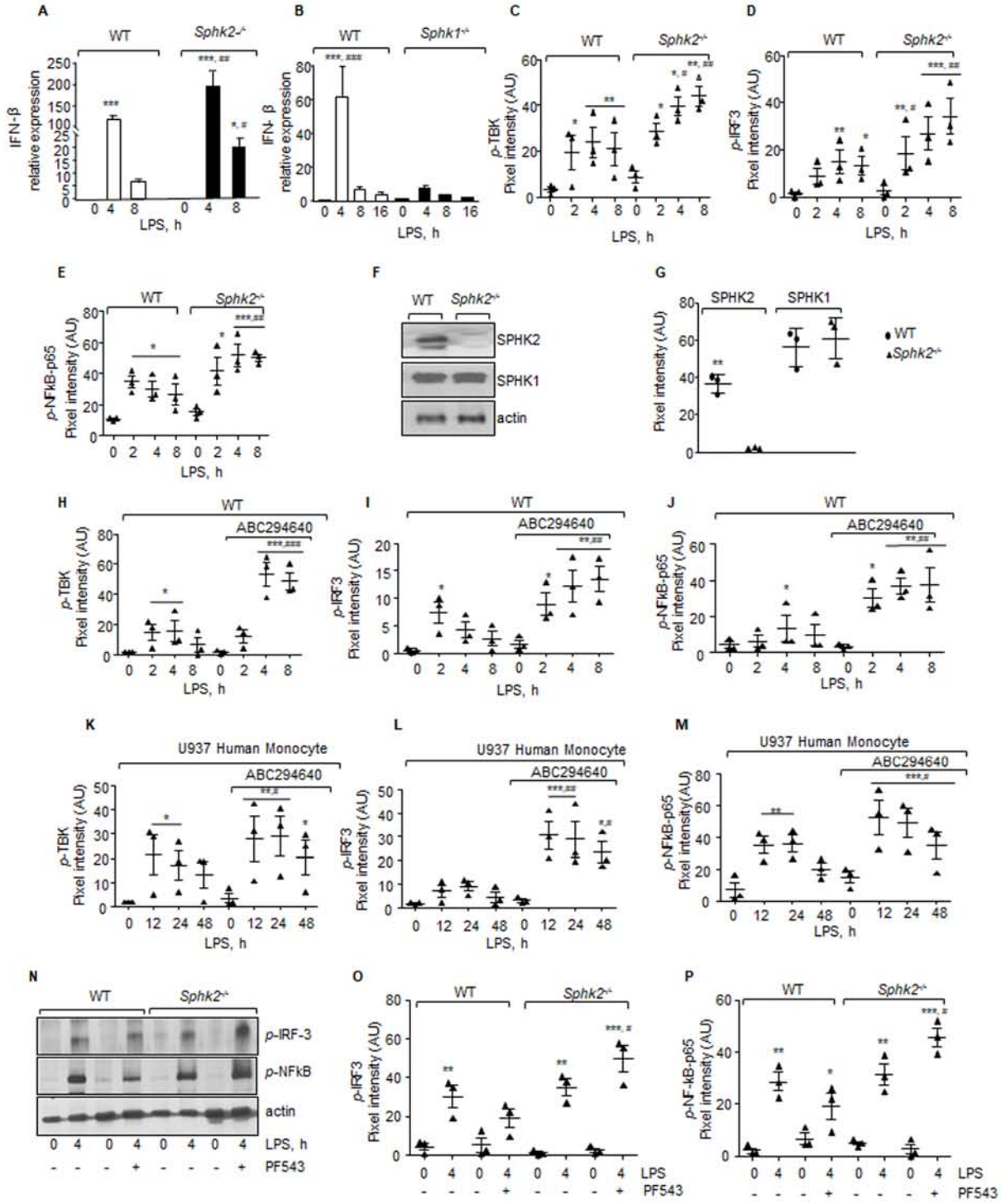

Figure S4. SPHK1 is dispensable for suppression of STING signaling, Related to Figure 4.

(A-B) IFN-β expression relative to GAPDH in WT, SPHK2 null (A) or SPHK1 null (B) BMDM following LPS challenge. Data are represented as mean ± SD. n=3 mice/group. *p < 0.05 and ***p < 0.001 indicates values significantly different from untreated WT or SPHK2 null BMDM. #p < 0.05; ##p < 0.01 and ###p < 0.001 indicates significance from LPS treated WT-BMDM. Data are analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

(C-E) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 4B**. *p < 0.05, **p < 0.01 and ***p < 0.001 relative to untreated WT or SPHK2 null BMDM while #p < 0.05 and ##p < 0.01 indicates significance relative to WT-BMDM following LPS exposure for indicated times. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

(F-G) SPHK1 and SPHK2 expression in BMDM isolated from WT or SPHK2 null mice was determined by immunoblotting using indicated antibodies. Immunoblotting with anti-actin antibody served as a loading control. A representative immunoblot is shown in panel F while panels G shows individual pixel intensities expressed as arbitrary units (AU) along with mean and SD. **p < 0.01 relative to SPHK2 null BMDM. Data is analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

(H-J) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 4D**.

(K-M) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 4E**.

(N-P) WT or SPHK2 null BMDM were treated without or with 1 μM PF543, a SPHK1 inhibitor for 1 h and phosphorylation of indicated proteins was determined. A representative immunoblot is shown in panel N while plots in **O-P** shows individual pixel intensities expressed as arbitrary units (AU) along with mean and SD. n=3.

In **H-M** and **O-P**, *p < 0.05; **p < 0.01 and ***p < 0.001 relative to WT or SPHK2 null BMDM at time zero. #p < 0.05; ##p < 0.01 and ###p < 0.001 indicates significance from LPS treated BMDM. Data are analyzed using one-way ANOVA followed by multiple comparison Tukey's test.

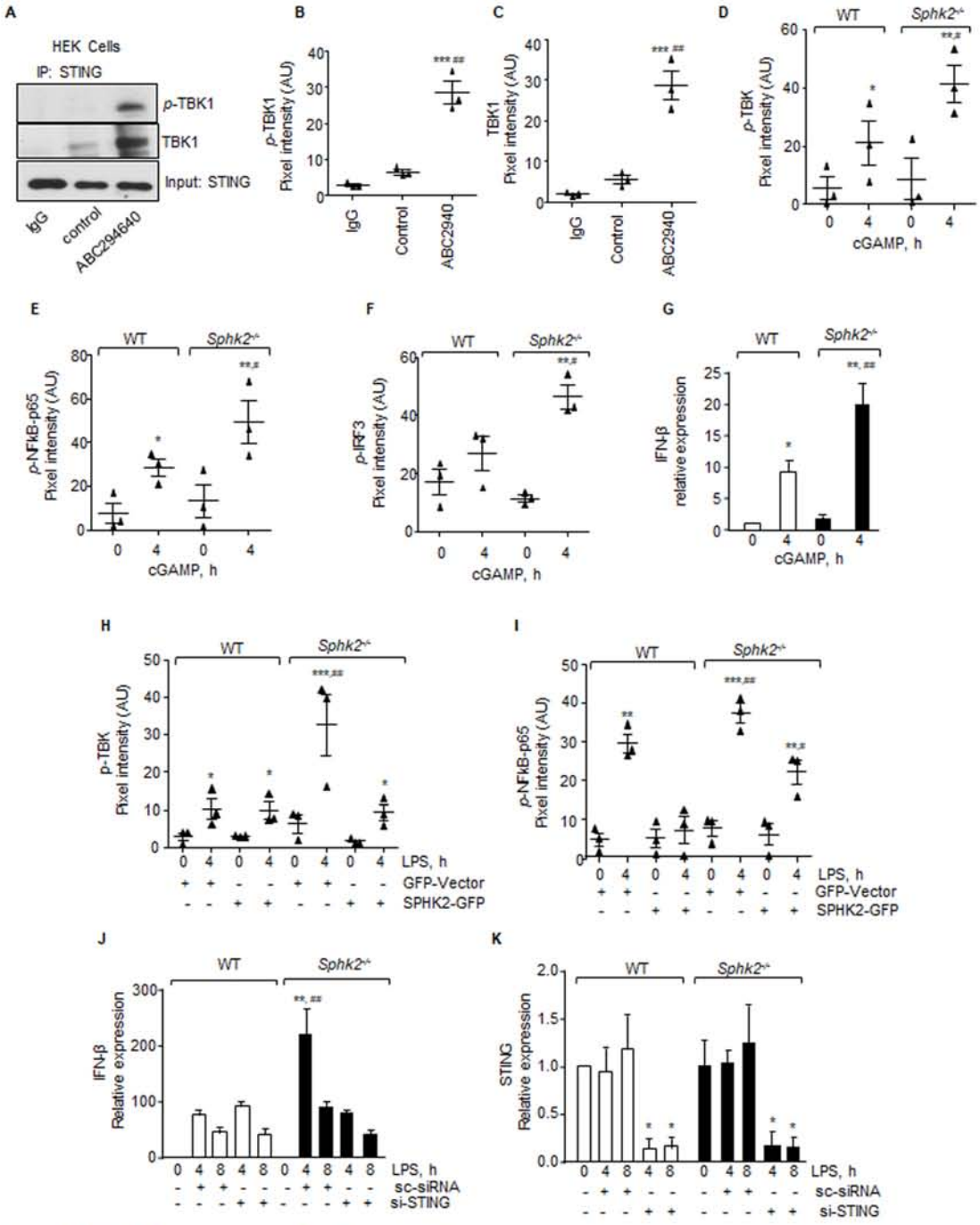

Figure S5. SPHK2 interacts with STING, Related to Figure 4.

(A-C) HEK cells were lysed and immunoprecipitated with anti-STING antibody. Immunocomplexes were immuno-blotted with anti-TBK1, anti-STING or anti-phospho-specific TBK1 antibody. A representative immunoblot is shown in A while B-C shows individual pixel intensities expressed as arbitrary units (AU) along with mean and SD. n=3.

(D-F) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 4J.** n=3.

(G) WT or SPHK2 null BMDM were permeabilized with digitonin for 30 min and then stimulated with 10 μM cGAMP for the indicated times. IFN-β expression relative to GAPDH was determined using qPCR. n=4.

(H-I) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 4I.** n=3.

(J-K) BMDM transfected with scrambled or STING siRNA for 48 h cells were stimulated with LPS and expression of IFN-β and STING determined by qPCR.

Data in **B-K** are represented as mean ± SD of two or three independent experiments. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ relative to untreated WT or SPHK2 null BMDM. #$p < 0.05$; ##$p < 0.01$ and ###$p < 0.001$ indicates significance relative to LPS treated BMDM. Data are analyzed using one-way ANOVA followed by multiple comparison Tukey's test

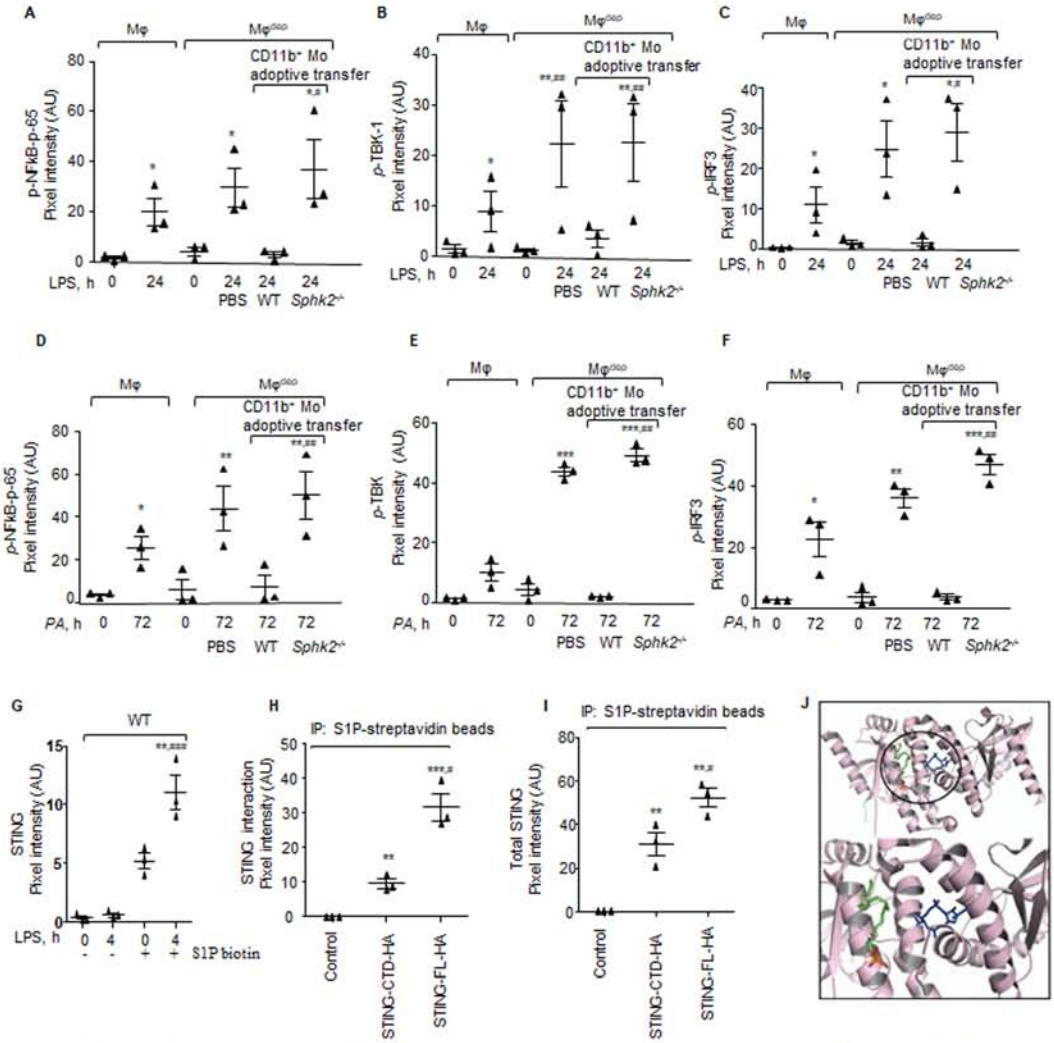

Figure S6. Effect of adoptive transfer of SPHK2^{+} CD11b monocytes on lung injury in macrophage depleted mice, Related to Figure 5 and Figure 6.

(A-C) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 5H**.

(D-F) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 5J**.

(G) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 6A**.

(H-I) Individual pixel intensities expressed as arbitrary units (AU) along with mean and SD of **Fig. 6C**.

(J) Possible allosteric Binding pose of S1P to the open conformation of STING-CTD. Overplayed binding poses of S1P (pymol native atom colors) with di-c-GMP (blue) with STING-CTD in an open conformation (PDB ID# 4EF4). The upper image represents the whole view of the binding mode while the lower image represents the focused (zoomed in) view determined by the black circle.

Data in **A-I** are from three individual experiments. **A-F**; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ relative to unexposed Mφ or Mφ^{dep} group. #$p < 0.05$ and ##$p < 0.01$ indicates significance relative to Mφ^{dep} mice receiving WT-CD11b monocytes (n=3 mice/group). **G**; **$p < 0.01$ relative to untreated WT-BMDM immunoprecipitated with S1P-conjugated streptavidin beads while ###$p < 0.001$ indicates significance relative to 4 h LPS treated WT-BMDM immunoprecipitated with Streptavidin beads. **H-I**; **$p < 0.01$ and ***$p < 0.001$ relative to control group while #$p < 0.05$ indicates significance relative to untreated STING-CTD group. Data are analyzed using one-way ANOVA followed by multiple comparison Tukey's test. *Note: STING-CTD was expressed at lower extent (**H**) leading decreased pull down using S1P streptavidin beads.