
Figures and figure supplements

SKAP2 is required for defense against *K. pneumoniae* infection and neutrophil respiratory burst

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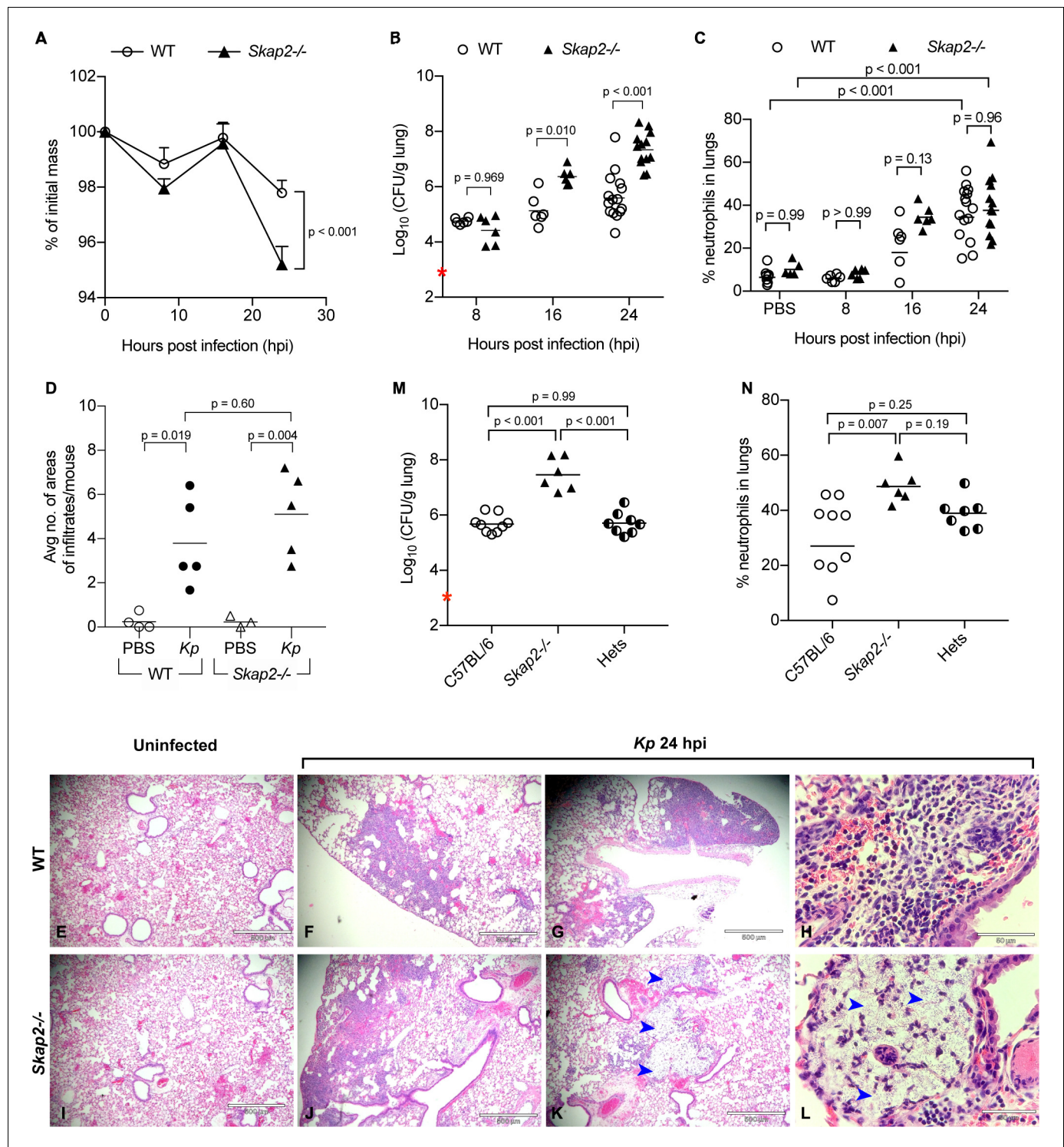


Figure 1. *Skap2*^{-/-} mice are more susceptible to *K. pneumoniae* intranasal infection. (A–L) WT (BALB/c) and *Skap2*^{-/-} mice were intranasally treated with PBS or infected with 5×10^3 cfu (red asterisk) of *K. pneumoniae* (*Kp*). At the indicated time points, (A) mice were weighed, lungs were harvested and single cell suspensions were prepared for (B) CFU and (C) analysis of neutrophils (CD11b⁺Ly6G^{hi}). (D–L) At 24 hr post-infection or inoculation with PBS (mock), lungs were harvested and processed for HE-staining. (D) Lung tissue sections were scored for infiltrates of leukocytes or bacteria. Mock (E, I) or *K. pneumoniae*-infected (F–H, J–L) WT (E–H), and *Skap2*^{-/-} (I–L), lungs were imaged at 4X (E–G, I–K) or at 40X (H, L). Blue arrows indicate bacteria. Bacterial burden (M) and live neutrophils (CD11b⁺Ly6G^{hi}) (N) from *K. pneumoniae*-infected C57BL/6, *Skap2*^{-/-}, and *Skap2*^{+/-} (Hets) littermates are shown. Data are compiled from 2 to 4 independent experiments with 2–4 mice/time point/genotype. (A) Mean \pm SEM. (B–D, M–N) Each dot represents

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values from a mouse, and black bars represent geometric means (**B, M**) or means (**C–D, N**). Significance was assessed using (**A**) two-way ANOVA with Sidak's post-test, or one-way ANOVA with Tukey's post-test (**M–N**), or with Sidak's post-test (**B–C, D**); log-transformed numbers were used for (**B, M**).

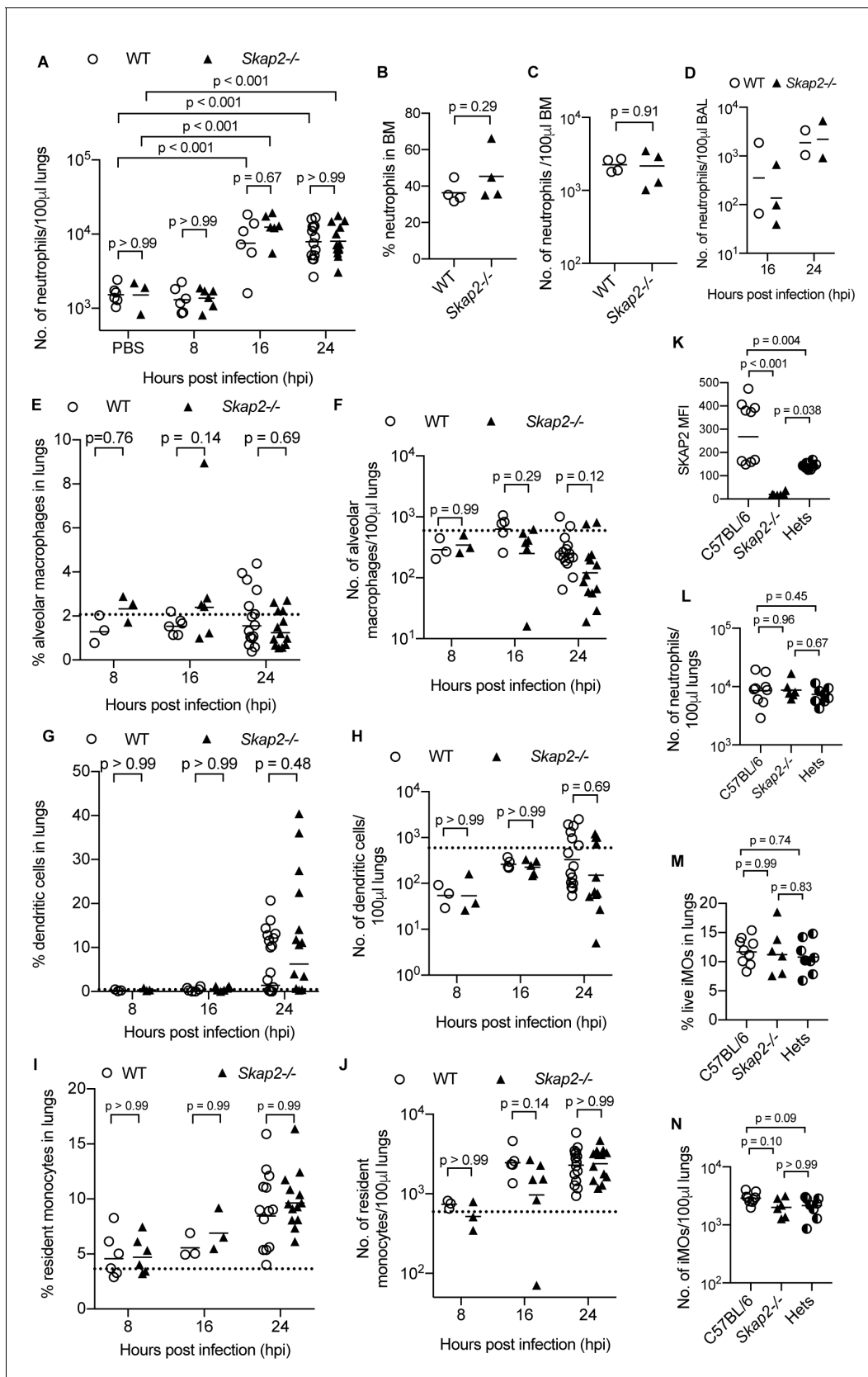


Figure 1—figure supplement 1. WT and *Skap2*^{-/-} mice have similar numbers of immune cells in lungs after *K. pneumoniae* intranasal infection. (A–J) WT (open circles) and *Skap2*^{-/-} (solid triangles) mice were intranasally inoculated with PBS or *K. pneumoniae* and assess for (A–D) neutrophils Figure 1—figure supplement 1 continued on next page

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(CD11b⁺Ly6G^{hi}), (E–F) alveolar macrophages (CD11b^{int} CD11c^{hi}), (F–H) dendritic cells (CD11b^{hi} CD11c^{hi}), and (I–J) resident monocytes (CD11b⁺ Gr1^{lo}). Data are compiled from 2 to 4 independent experiments (2–4 mice/time point/genotype). (K) Analysis of SKAP2 expression in C57BL/6, *Skap2*^{-/-} and *Skap2*^{+/-} (Hets) by intercellular staining of SKAP2 followed by flow cytometry. (L–N) Neutrophils (CD11b⁺Ly6G^{hi}) and iMOs (CD11b⁺Ly6C^{hi}) from *K. pneumoniae*-infected lungs of C57BL/6, *Skap2*^{-/-}, and *Skap2*^{+/-} (Hets) littermates by intracellular staining of SKAP2 followed by flow cytometry. (K–N) Data are compiled from 3 independent experiments. (A–J, L–N) Data are shown as percentage or cell number per 100 μ l of lung homogenates as indicated on the axis. (A–N) Each dot represents a mouse; bars represent means. Significance was assessed using one-way ANOVA with Sidak's post-test (A–J), two-tailed unpaired Student's t test (K), or with Tukey's post-test (L–N).

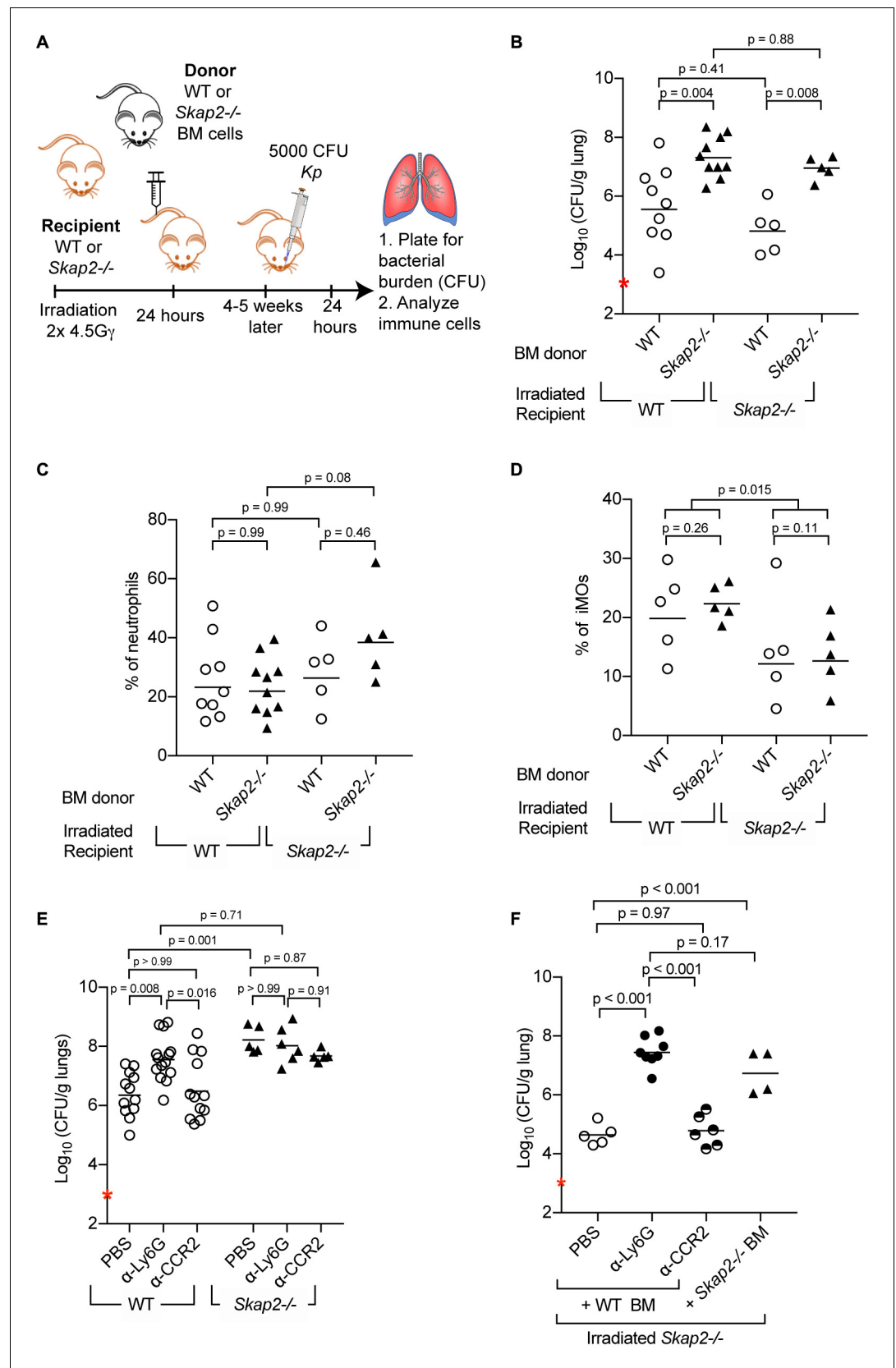


Figure 2. Reconstitution of *Skap2*^{-/-} mice with WT bone marrow hematopoietic stem cells confers protection against *K. pneumoniae*. (A) Schematic used to generate bone marrow chimeras in (B–D, F). (B–F) Mice were

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infected with 5×10^3 cfu *K. pneumoniae* (red asterisk); 24 hpi mice were sacrificed and lungs harvested. (E–F) WT and *Skap2*^{-/-} mice were injected intraperitoneally with 50 µg of α-Ly6G (1A8) or 20 µg of α-CCR2 (MC-21) to deplete neutrophils and iMOs, respectively, or PBS 16 hr prior to infection. Bacterial burden (B, E, F) and percent live neutrophils (CD11b⁺ Ly6G^{hi}) (C), or inflammatory monocytes (CD11b⁺ Ly6C^{hi}) (D) from *K. pneumoniae*-infected lungs. Data are compiled from 2 to 4 independent experiments using groups of 2–3 mice/genotype/experiment. Each dot represents a mouse, bars are geometric means (B, E–F) or means (C–D). Statistics were assessed using one-way ANOVA with (C–D) Sidak's post-test, or (B, E–F) Tukey's post-test. (D) Percent of iMOs were compiled, and comparison between WT and *Skap2*^{-/-} irradiated recipient disregarding the donor BM were assessed by two-tailed unpaired Student's t test.

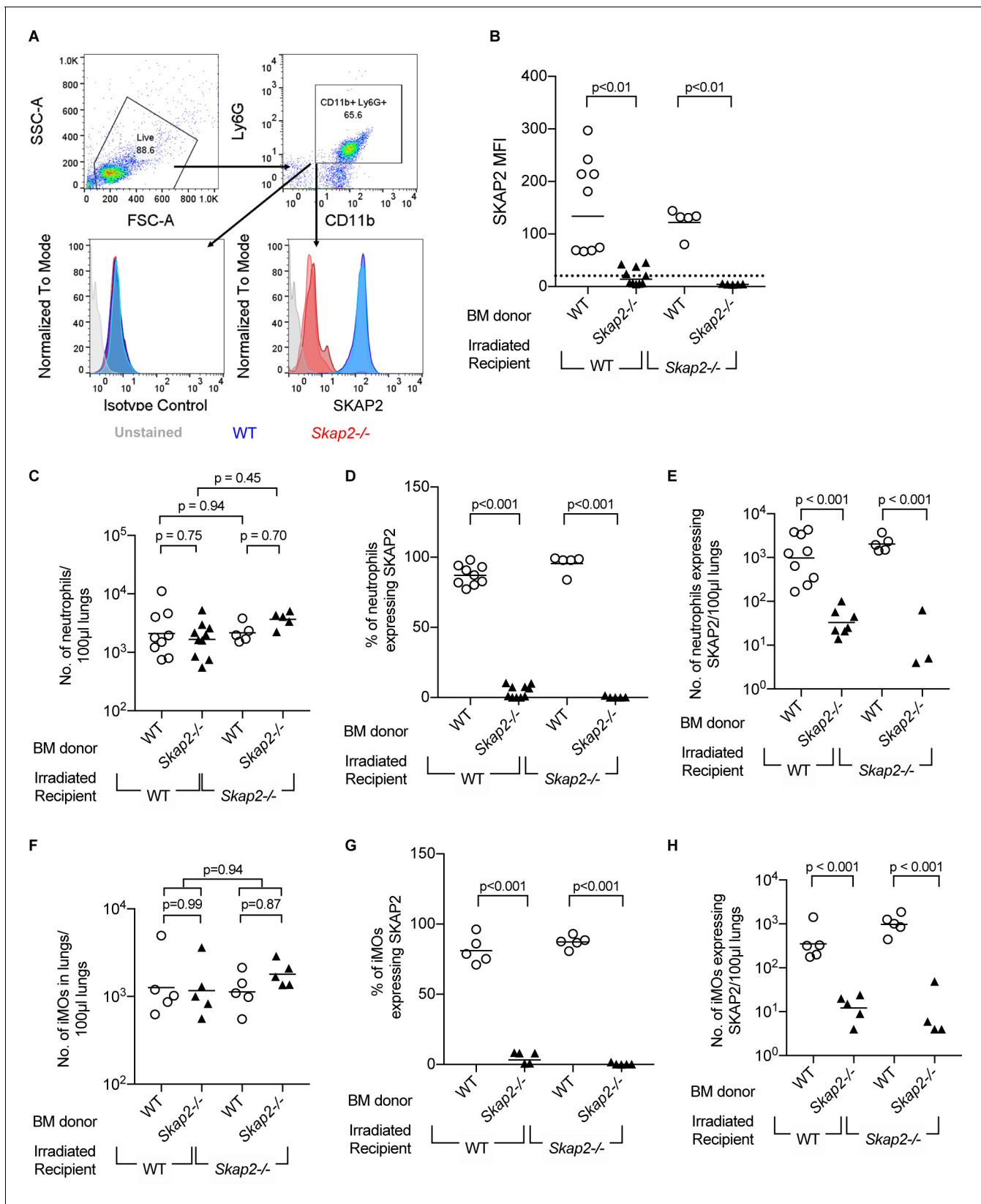


Figure 2—figure supplement 1. Transplantation efficiency of bone marrow reconstitution into WT and *Skap2*^{-/-} recipients. (A) Schematic for gating strategy of flow cytometry data in bone marrow chimeras. Blocks and arrows indicate the cell population used for next gating step. (B–H) Neutrophil Figure 2—figure supplement 1 continued on next page

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(CD11b⁺ Ly6G^{hi}) (C–E), or inflammatory monocytes (CD11b⁺ Ly6C^{hi}) (F–H) from *K. pneumoniae*-infected lungs were unstained or intracellularly stained with SKAP2 antibody or isotype control. (B) SKAP2 expression in CD11b⁺ cells shown as mean fluorescent intensity (MFI). Dotted line indicates average level from cells intracellularly stained with isotype control. Data are compiled from 2 to 4 independent experiments using 2–3 mice/genotype/experiment. Each dot represents a mouse, bar represents means. Statistical significance was assessed using one-way ANOVA with Sidak's post-test.

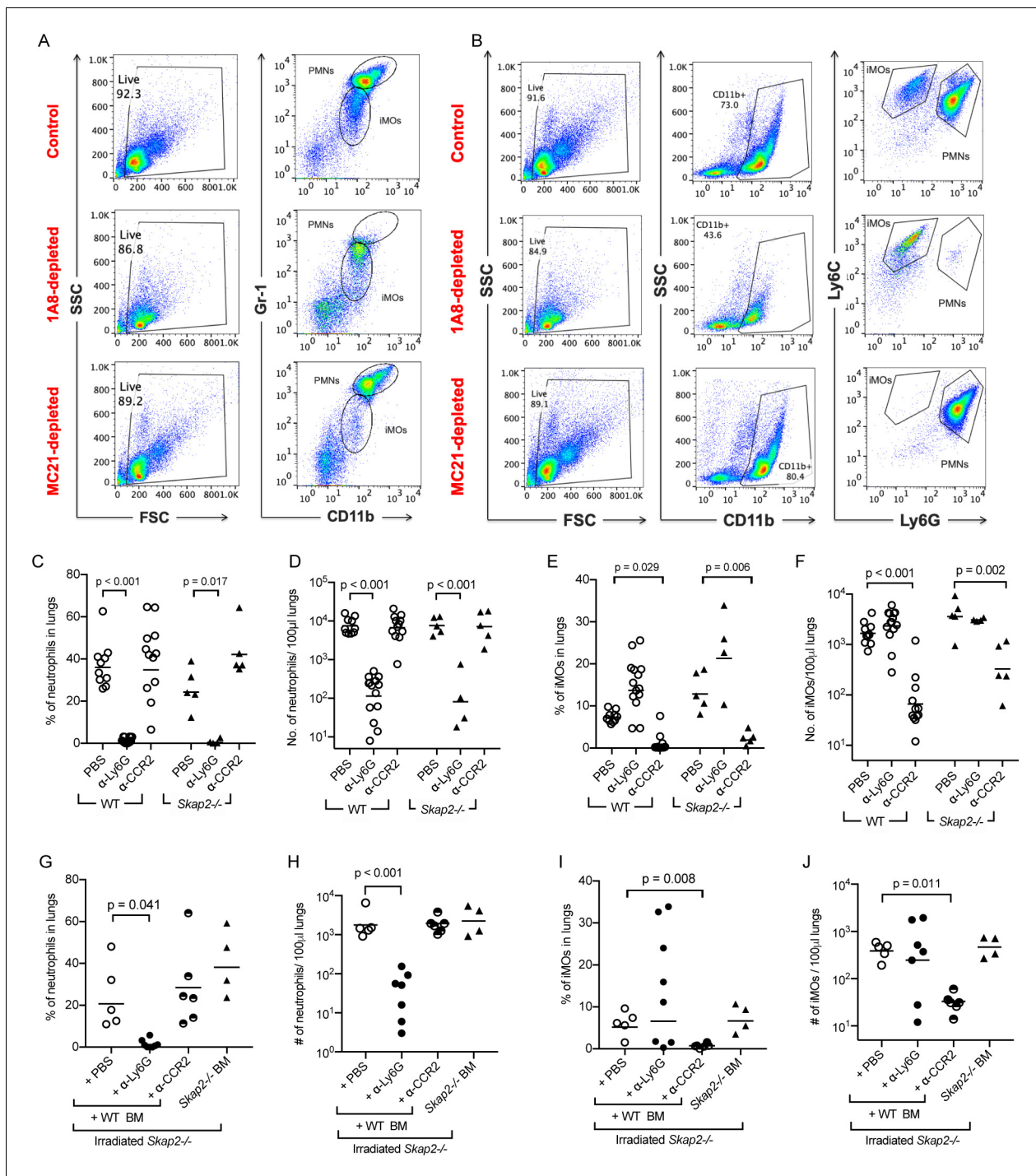


Figure 2—figure supplement 2. Evaluation of immune cell populations in depletion studies in WT and *Skap2*^{-/-} mice. Analysis of inflammatory monocytes (CD11b⁺ Gr1^{lo} or CD11b⁺ Ly6C^{int} Ly6G^{lo}) and neutrophils (CD11b⁺ Gr1^{hi} or CD11b⁺ Ly6C^{int} Ly6G^{hi}) from non-depleted (PBS), α-Ly6G (clone 1A8)-depleted, or MC-21-depleted (α-CCR2) *K. pneumoniae*-infected lungs. (A–B) Example of flow cytometry analysis for depletion experiments. (C–F) Quantification of neutrophils (CD11b⁺ Gr1^{hi}) (C–D), or inflammatory monocytes (CD11b⁺ Ly6C^{hi} Ly6G^{lo}) (E–F) from α-Ly6G, α-CCR2, or PBS-treated, and *K. pneumoniae*-infected WT or *Skap2*^{-/-} mice based on flow cytometry analysis. (G–J) Quantification of neutrophils (G–H), or inflammatory monocytes (I–J) from α-Ly6G, α-CCR2, or PBS-treated, *K. pneumoniae*-infected irradiated *Skap2*^{-/-} mice based on flow cytometry analysis. Data are compiled from 3 to 4 independent experiments with 2–3 mice/genotype/experiment. Each dot represents a mouse, bars represent geometric means. (D, F, H, J) were log-transformed. Significance was assessed using one-way ANOVA with Tukey's post-test.

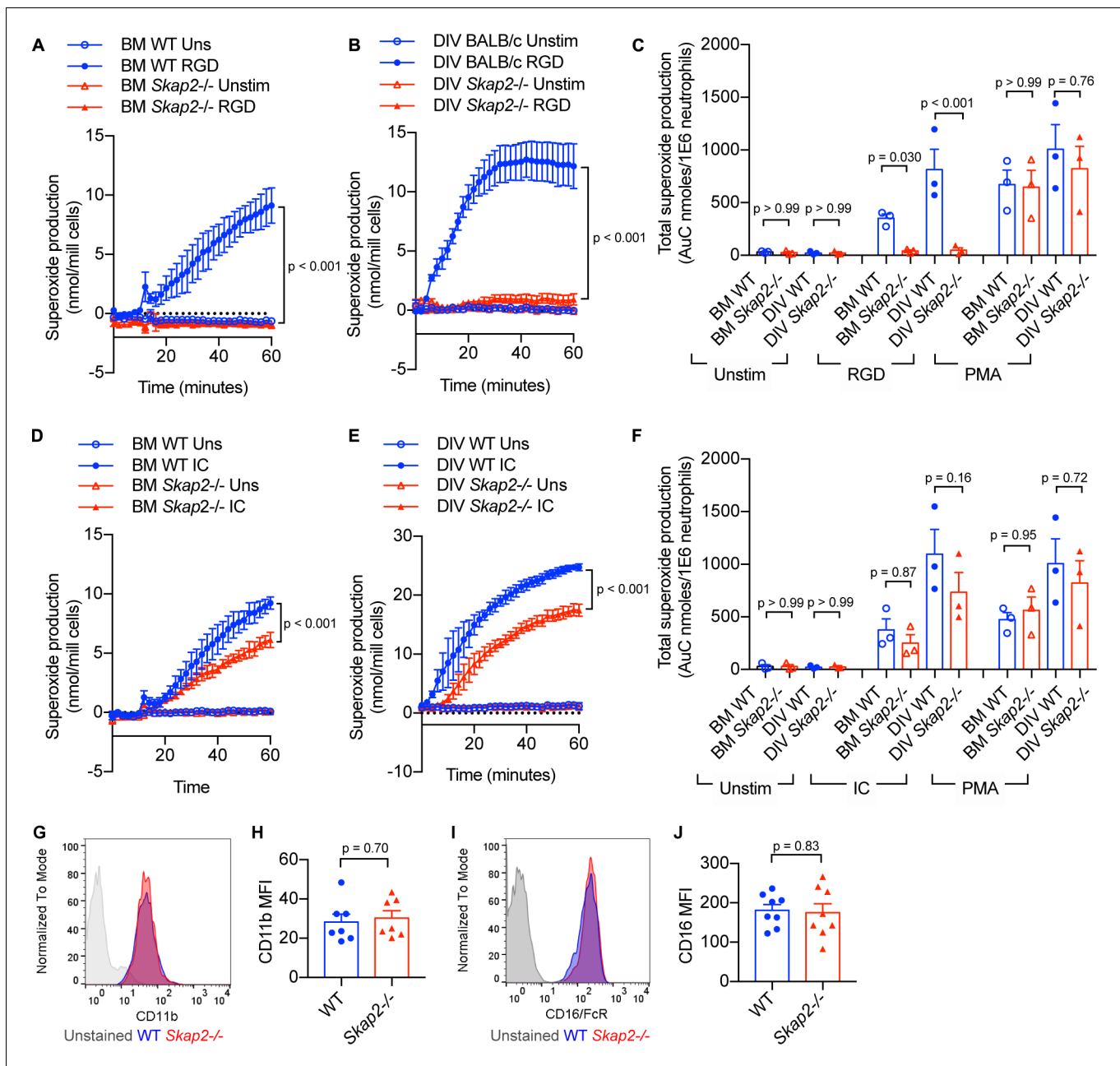


Figure 3. BM and DIV neutrophils require SKAP2 for integrin-activated ROS production, but not for Fc γ R. (A–F) Extracellular respiratory burst of BM and DIV neutrophils. WT or *Skap2*^{-/-} neutrophils were plated on a poly-RGD-coated surface (A–C), or IgG immune complex (IC)-coated surface (D–F), and superoxide production was measured by cytochrome C reduction. Unstimulated (unstim) cells were plated onto 10% FBS/PBS or stimulated with 100 nM PMA. (C, F) Total concentration of superoxide produced after 60 min. (G–J) Expression of surface receptors on DIV neutrophils of CD11b (G–H) or activating CD16 Fc γ receptor (I–J). (G–J); Blue shaded areas and bars represent WT, and red, *Skap2*^{-/-}. (A–B, D–E) represent the mean \pm SD of one experiment in technical triplicate assessed using two-way ANOVA with Tukey's post-test. (C, F, H, J) represent the mean \pm SEM of at least three independent experiments performed in at least technical duplicate (C, F) and were assessed using two-way ANOVA with Tukey's post-test (C, F), or two-tailed unpaired Student's *t* test (H, J).

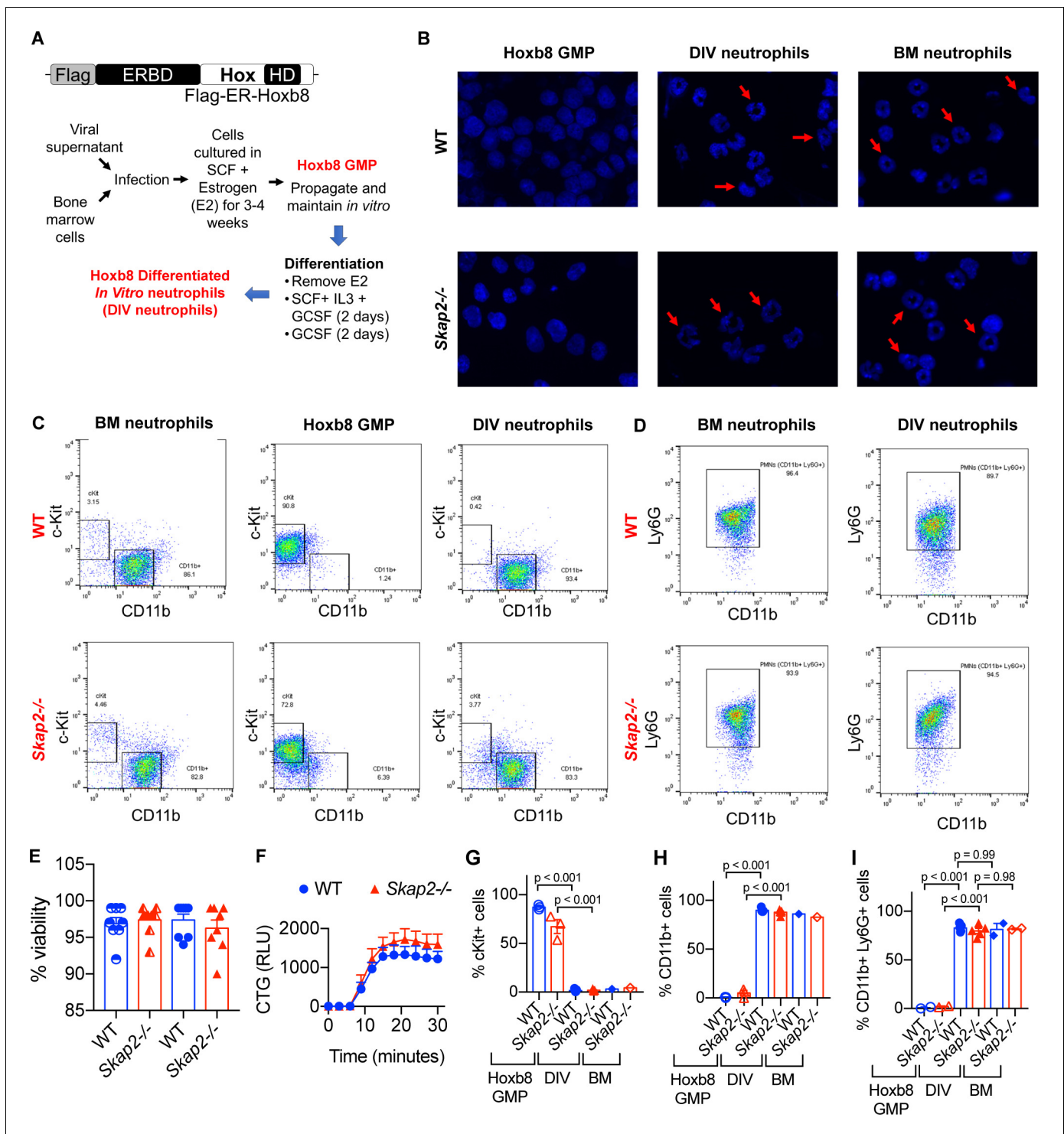


Figure 3—figure supplement 1. Hoxb8-transformed GMP differentiate into neutrophils with similar morphology and surface markers to that of BM-derived neutrophils. (A) Schematic of MSCV-Hoxb8 transduction system to generate immortalized stem cell progenitors. The estrogen-binding domain (ERBD) of the estrogen receptor fused to Hoxb8 with an N-terminal Flag epitope tag allows conditional expression of Hoxb8 in the presence of estrogen. After 4 days of differentiation, cells are referred to as DIV neutrophils. (B) Nuclear morphology of BM-derived neutrophils isolated from WT or *Skap2*^{-/-} mice, Hoxb8 GMP, and DIV neutrophils with DAPI. Red arrows indicate polymorphonuclear nuclei. (C) Analysis of c-Kit and CD11b expression. (D) Analysis for Ly6G expression on live CD11b⁺ cells. (E–F) Viability of WT and *Skap2*^{-/-} DIV neutrophils was assessed using (E) trypan blue exclusion test from neutrophils differentiated from Hoxb8 immortalized GMP from 2 different WT and *Skap2*^{-/-} mice prior to functional studies, or (F) DIV neutrophils were plated into 10%FBS/PBS-coated wells, loaded with CellTiter Glo reagent, and chemiluminescence was detected for 30 min by plate. Figure 3—figure supplement 1 continued on next page

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reader. The quantification of ATP is shown as relative light units (RLU) from one experiment done in technical triplicate. (G–I) Quantification of (G) cKit+, (H) CD11b+, or (I) CD11b+ Ly6G+ cells from Hoxb8 GMP, day 4 DIV neutrophils (DIV), and BM neutrophils (BM). Results are compiled from 1 to 4 independent experiments with each dot representing one experiment and are shown as mean \pm SEM. Significance was determined by one-way ANOVA with Sidak's post-test.

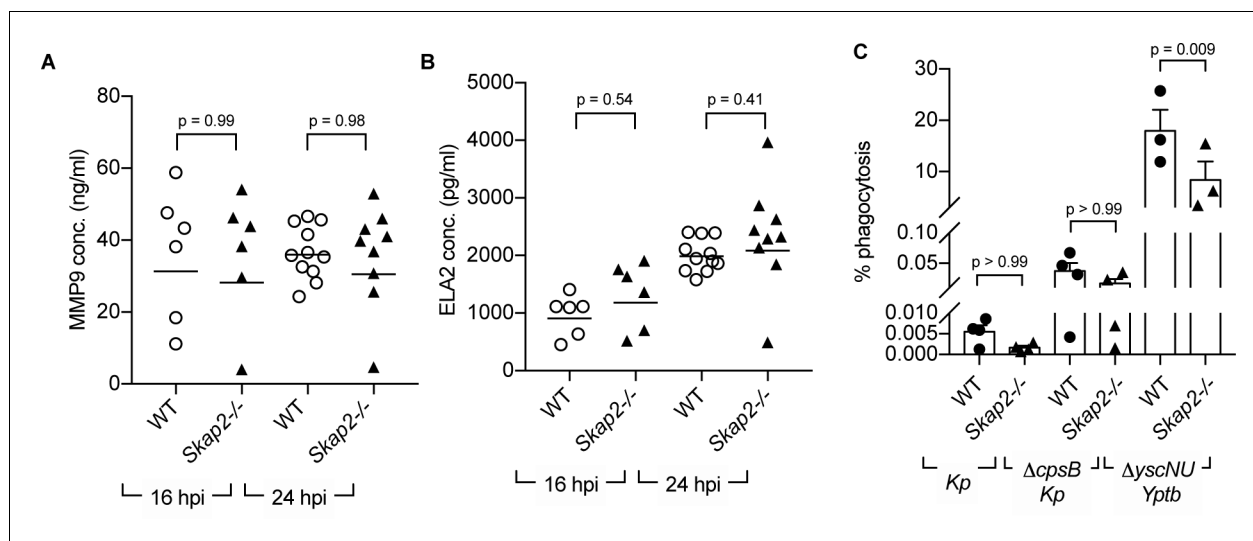


Figure 4. SKAP2 is not required for *K. pneumoniae*-stimulated degranulation nor phagocytosis. (A–B) Levels of (A) total MMP-9, and (B) neutrophil elastase (ELA2) from cell-free supernatant from *K. pneumoniae*-infected WT and *Skap2*^{-/-} lung homogenates were analyzed by ELISA. Data are compiled from 2 to 4 independent experiments with 2–3 mice/genotype/experiment. Each dot represents a mouse and bars represent geometric means. (C) WT and *Skap2*^{-/-} DIV neutrophils were incubated with encapsulated (*Kp*), unencapsulated ($\Delta cpsB$ *Kp*) *K. pneumoniae*, or *Yptb* $\Delta yscNU$. Percent phagocytosis was calculated as $CFU_{\text{bacteria with neutrophils and gentamicin}}/CFU_{\text{bacteria without gentamicin treatment}}$. (A–C) Data are compiled from at least 3 independent experiments performed in technical triplicate. Statistics represent mean \pm SEM and were assessed using one-way ANOVA with Sidak's post-test.

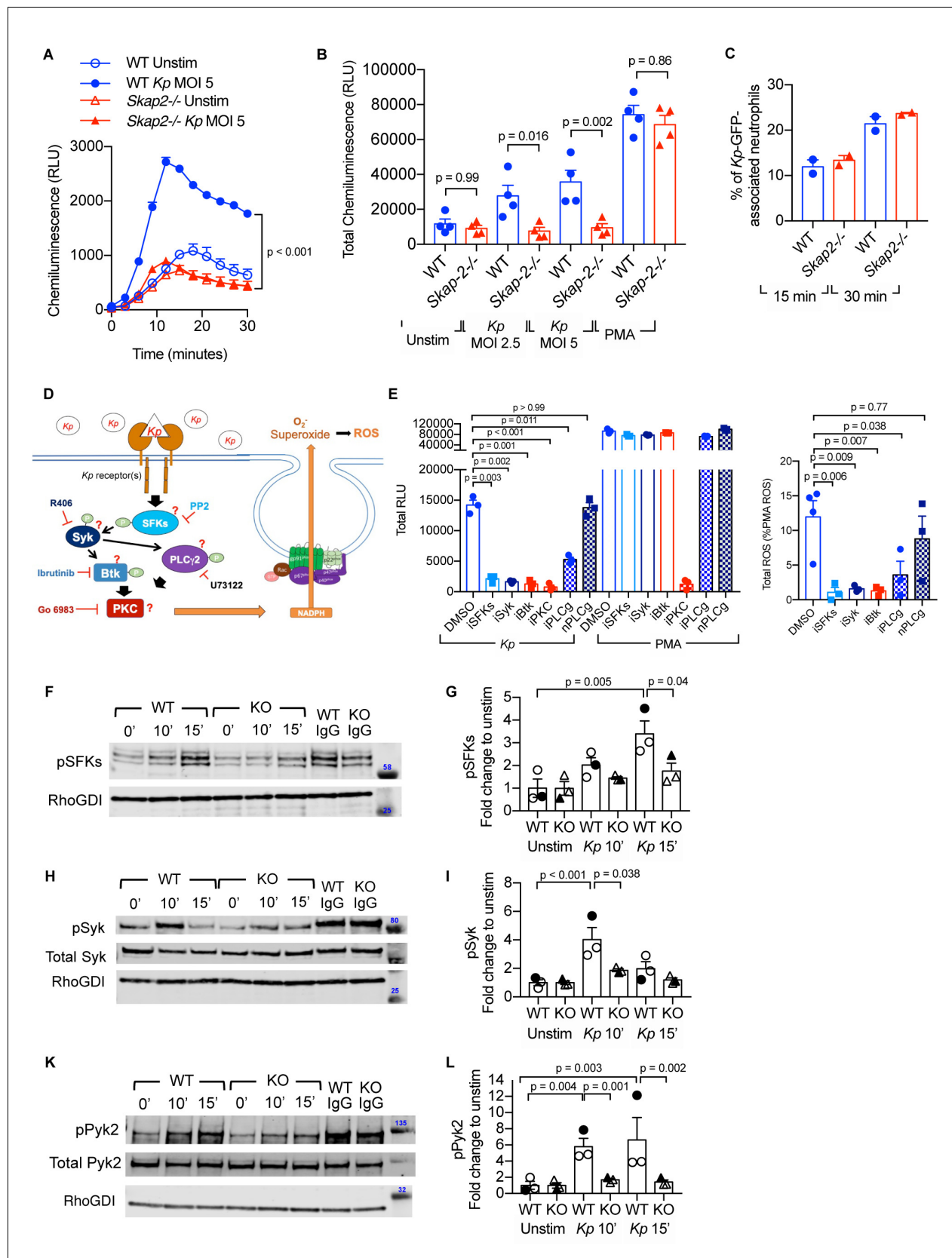


Figure 5. *K. pneumoniae*-stimulated ROS production requires SKAP2-dependent activation of tyrosine kinases. (A–C) Respiratory burst of WT and *Skap2*^{-/-} DIV neutrophils infected with *Kp* using isoluminol-chemiluminescence. Unstimulated (unstim), *Kp*-infected, and 100 nM PMA-treated cells were Figure 5 continued on next page

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seeded on FBS-coated wells. (A) Representative experiment performed in triplicate of ROS (RLU) production following *K. pneumoniae* stimulation. (B) Total ROS (total RLU) produced after 30 min of stimulation was calculated as the total area under the curve shown in (A) of 4 independent experiments performed in technical triplicate. (C) WT and *Skap2*^{-/-} neutrophils incubated with GFP-expressing *K. pneumoniae* (Kp-GFP) at MOI 40 for 15 or 30 min, stained with DAPI, and analyzed by flow cytometry for GFP-associated neutrophils. (D) Schematic of potential *K. pneumoniae*-activated signaling pathways tested by inhibitors. (E) Respiratory burst of WT DIV neutrophils untreated or treated with inhibitors using isoluminol-chemiluminescence assay. DIV neutrophils were pre-treated with DMSO, PP2 (iSFKs), R406 (iSyk), Ibrutinib (iBtk), Go 6083 (iPKC), U73122 (iPLC γ), or U73134 (nPLC γ /non-inhibitory analog of PLC γ inhibitor) for 10 min at 37°C and then infected with MOI 2.5 of *K. pneumoniae* or treated with 100 nM PMA and measured for 30 min. Total RLU was calculated as area under the curve. Data are a representative figure from 3 independent experiments showing mean \pm SD performed in technical triplicate. Significance was assessed using one-way ANOVA with Sidak's post-test. (F–L) WT and *Skap2*^{-/-} neutrophils were infected with Kp for 10 or 15 min or stimulated with IC for 10 min at 37°C. Lysates were analyzed by western blot for pSFKs (Y416), pSyk (Y352), pPyk2 (Y402), and RhoGDI. Blots were then stripped and re-probed for total Syk or Pyk2. Data are compiled from 3 independent experiments. (B–C) Data are compiled from 2 to 4 independent experiments performed in technical triplicate. Statistics represent mean \pm SEM. (F, H, K) Representative blot shown. (G, I, L) Solid symbols indicate values of blot shown. Bars indicate mean. Significance was assessed using one-way ANOVA (B) with Sidak's post-test between WT and *Skap2*^{-/-}, or (G, I, L) between time points within each genotype, or two-way ANOVA with Sidak's post-test between WT and *Skap2*^{-/-} within the same point.

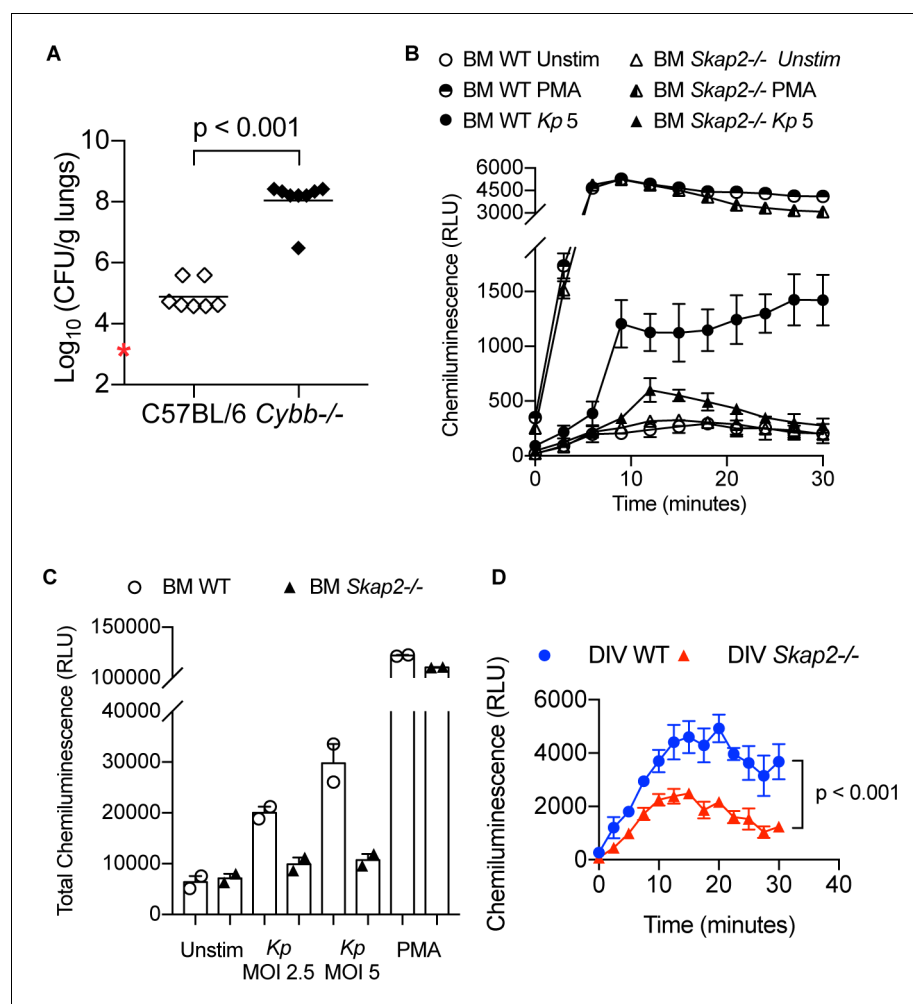


Figure 5—figure supplement 1. ROS restricts *K. pneumoniae* infection in lungs and is induced by *K. pneumoniae* after infection of BM neutrophils. (A) Bacterial burden of wild-type C57BL/6 and *Cybb*^{-/-} mice retropharyngeally infected with 5×10^3 cfu *K. pneumoniae* for 24 hr. Data are compiled from 2 independent experiments with 3–4 mice/genotype/experiment. Each dot represents a mouse and bars represent geometric means. Significance was assessed using two-tailed unpaired Student's *t* test. (B–C) Respiratory burst of WT and *Skap2*^{-/-} BM neutrophils with *K. pneumoniae* at the indicated MOI using an isoluminol-chemiluminescence assay. (B) Representative experiment of ROS (RLU) production following stimulation with *K. pneumoniae*, or PMA done in technical duplicate. (C) Total ROS (total RLU) produced after 30 min of stimulation from one experiment shown as mean \pm SD. (D) Respiratory burst of WT and *Skap2*^{-/-} BM neutrophils with *Y. pseudotuberculosis* Δ *yscF* at MOI 10 using an isoluminol-chemiluminescence assay.

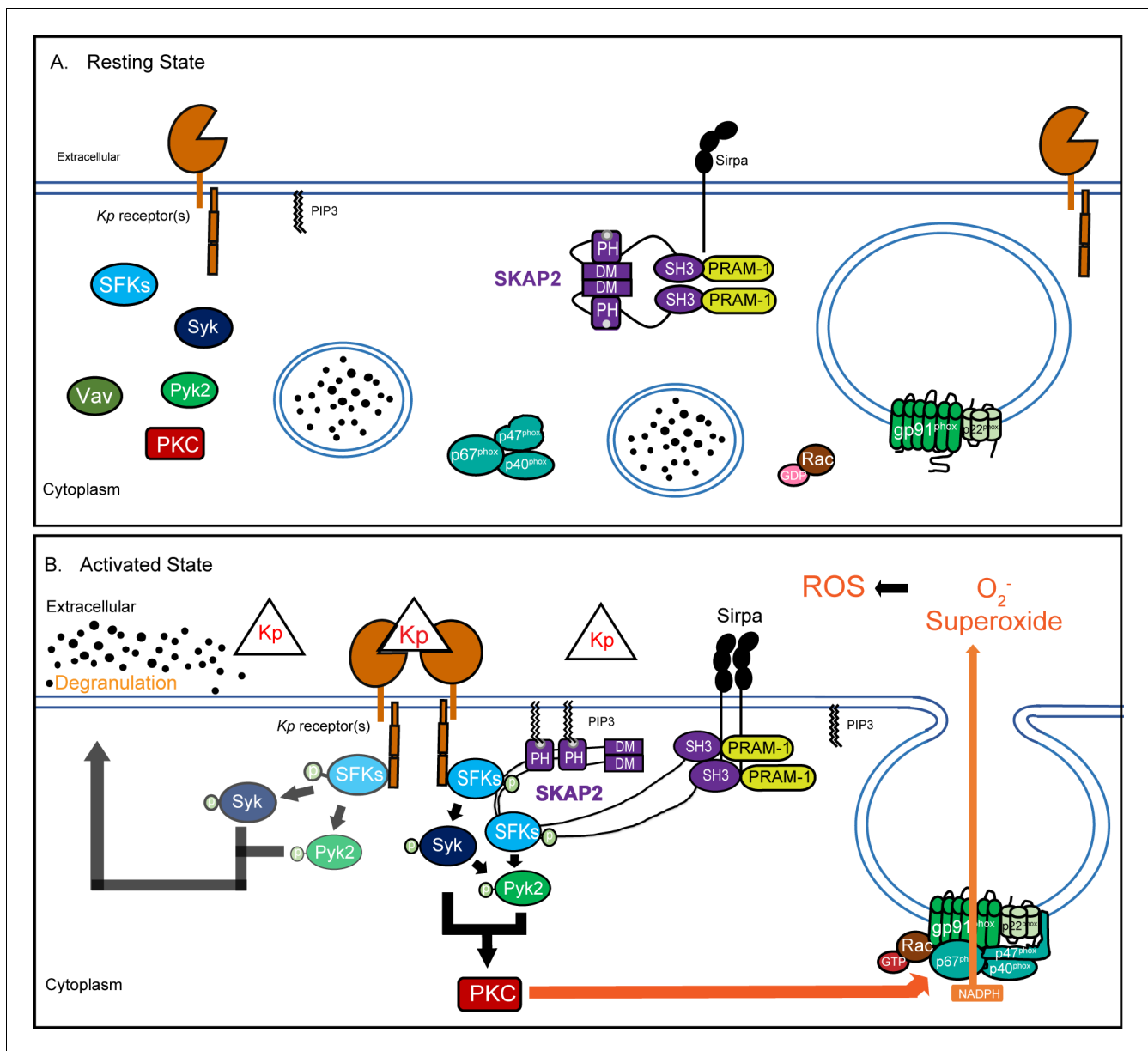


Figure 6. Proposed model of *K. pneumoniae*-stimulated signaling pathway. (A) At resting state, the homodimer SKAP2 is in an autoinhibited conformation because of binding of the DM domains; SKAP2 is constitutively associated with PRAM-1 but is apart from other components of the signaling pathways. (B) Activation of neutrophils through the binding of *K. pneumoniae* leads to production of PIP3 which binds SKAP2, relieving the autoinhibited conformation and revealing sites for docking and centralization of other signaling molecules. SKAP2 docking sites may centralize and retain signaling molecules thereby increasing their local concentration to facilitate increased phosphorylation and amplification of their signals. Alternatively, SKAP2 may directly activate one or more tyrosine kinases, including SFKs, Syk, and Pyk2, leading to ROS production.