Antibodies used in this study

The anti–β-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO) was generated against a slightly modified human β-actin N-terminal peptide, Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys, conjugated to KLH. The antibody recognizes both human and mouse β-actin. The mouse monoclonal antibody against human Akt1, rabbit monoclonal antibody against human Akt2, pan anti-Akt mouse monoclonal antibody (raised against Akt1 recombinant protein containing human Akt1 residues 140–480) and anti–phosphor-Akt (S473) rabbit polyclonal antibody (raised against a synthetic phosphopeptide including Ser473 of mouse Akt) were obtained from Cell Signaling Technology (Boston, MA). The $gp91^{phox}$ mouse monoclonal antibody (m54.1) (raised against human $gp91^{phox}$ of human origin, with epitope mapping to amino acids 383–390) was a gift from Dr. Mark Quinn (Montana State University). The rabbit polyclonal anti-$p22^{phox}$ (raised against amino acids 1–195 of human $p22^{phox}$) and anti-$p47^{phox}$ antibodies (raised against an epitope corresponding to amino acids 196–390 of human $p47^{phox}$) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The $p67^{phox}$ monoclonal antibody (raised against a peptide corresponding to amino acids 317–469 of human $p67^{phox}$) and Rac1 mouse monoclonal antibody (against human Rac1 Protein) were purchased from BD Transduction Laboratories (Lexington, KY). The rabbit polyclonal anti-Rac2 (raised against residues 1–192 of human Rac2) and anti-$p40^{phox}$ (raised against a recombinant full-length mouse $p40^{phox}$) were obtained from Millipore (Billerica, MA). Fluorescein (FITC) was obtained from Invitrogen (Carlsbad, CA). The Akt inhibitor X (SH X), PI3K inhibitor LY294002, PKC inhibitor GF109203X (GF) and anti–phosphor-Akt (T308) (a rabbit polyclonal antibody raised against a synthetic phosphopeptide corresponding to amino acids surrounding Thr308 site of human Akt1) were purchase from Calbiochem (San Diego, CA).

Figure S1. Akt expression in neutrophils from WT and individual Akt isoform knockout mice

(A) total RNA from WT mouse neutrophils (PMN, upper panel) was subjected to RT-PCR (30 cycles), using specific primers for detection of Akt1, Akt2 and Akt3. The RT-PCR products were analyzed by agarose gel electrophoresis. The Akt1 and Akt2 transcripts were highly abundant, but only trace amount of the Akt3 transcript was detected. In the lower panel, mouse cDNAs for Akt 1 and Akt2 and RT product from mouse brain (containing Akt3) were used as positive standards. (B) Genotyping of the $Akt1^{-/-}$ and $Akt2^{-/-}$ mice. The PCR reaction was run for 35 cycles, using tail tissue genomic DNA from WT, $Akt1^{-/-}$ and $Akt2^{-/-}$ mice. Two sets onf oligonucleotide primers were used for each of the two gel images. (1) The Akt1 transgene (Akt1-mut) was detected using the SA3 (5′-CTACTATGCCATGAAGATCCTC-3′) and Neo257R (5′-TGCTACTTCCATTTGTCACGTCC-3′) primers. The expected size of 1,200 bp was seen in $Akt1^{-/-}$ mice. The WT Akt1 gene (Akt1-wt) was detected using the akt-F2 (5′-ACCAGGGGAGGATGTTTCTACTG-3′) and akt-B3 (5′-ACGACATGGTGCAGCAATGG-3′) primers. The expected size of 750 bp was seen in DNA from WT and $Akt2^{-/-}$ mice. (2) The Akt2 transgene (Akt2-mut) was detected using the Akt2B8 (5′-TGTATTTCTACCCCGATG CCAG-3′) and Neo257R primers. The expected size of 800 bp was seen in DNA from the $Akt2^{-/-}$ mice. The WT Akt2 gene (Akt2-wt) was detected using the Akt2F (5′-GAGGTAGAAACAAGAGAATCATGG-3′) and Akt2R (5′-GTTCGCACTGCTGTATGTTGC-3′) primers. The expected size of 300 bp was seen in DNA from the WT and $Akt1^{-/-}$ mice. Only the brightest bands are specific. The minor, non-specific bands are the results of using one shared primers in PCR reactions for the Akt transgene. (C) Results of Western blotting analysis of cell

lysate from bone marrow neutrophils (2×10^6 cells) confirming the absence of the Akt proteins from the individual Akt isoform knockout mice.

Figure S2. Comparison of the percentage and morphology of neutrophils from WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ mice

(A) complete blood count was performed using a HEMAVET 950FS hematology analyzer. The percentage of neutrophils over total WBC in WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ are shown based on data from 4 mice. *, $p < 0.05$. (B) Hema 3 staining of bone marrow purified neutrophils (left 3 panels) and peripheral blood smears (right 3 panels), showing the morphology of neutrophils from WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ mice. For the left panels, 5×10^5 neutrophils purified from mouse bone marrow were cytospinned to the slides and then stained with PROTOCOL Hema 3 (Fisher Scientific), which is a three-step staining comparable to the Wright-Giemsa method. For the right panels were from blood smear and then similarly stained with PROTOCOL Hema 3. The picture were taken using a 40× objective. Images shown are representative of 3 independent experiments.

Figure S3. Expression levels of NADPH oxidase components in WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ neutrophils

The expression of Akt, phox proteins and Rac small GTPase in WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ neutrophils was determined by Western blotting, using antibodies described in above in this section. Representative blots from 3 independent experiments are shown.

Figure S4. Uptake of IgG opsonized zymosan particles and O_2^- generation in WT and Akt knockout neutrophils

(A) uptake of FITC-labeled IgG posonized zymosan by bone marrow derived neutrophils from WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ mice following a 45-min incubation. Shown are images taken with bright field (top row) and fluorescent (bottom row, shown in black and white) microscopy. (B) mouse neutrophils from WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ were stimulated with IgG-opsonized, FITC-labeled zymosan (500 μg/ml), and O_2^- generation was recorded over the indicated time period based on luminol-enhanced chemiluminescence. (C) quantification of ingested zymosan particles in each cell (A) for a total 20 cells per sample. No statistically significant difference was found between WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ neutrophils ($p > 0.05$). (D) quantification of O_2^- produced after zymosan uptake, shown as area under curve starting at approximately the 16 min mark for 20 min. Data shown are based on 3 experiments. *, $p < 0.05$ compared to WT and Akt1$^{-/-}$.

Figure S5. Fluorescent microscopy detection of differential Akt distribution in neutrophils.

WT mouse neutrophils were seeded onto glass coverslip and then stimulated with 5 μM fMLF for 2 min. The cells were then fixed and stained with specific antibodies recognizing Akt1 (A) or Akt2 (B), followed by incubation with rhodamine red-X-conjugated goat anti-rabbit IgG (for anti-Akt1) or goat anti-mouse IgG (for anti-Akt2), respectively. DAPI (blue fluorescence) was used for nucleus staining. Arrows mark membrane translocated Akt proteins.

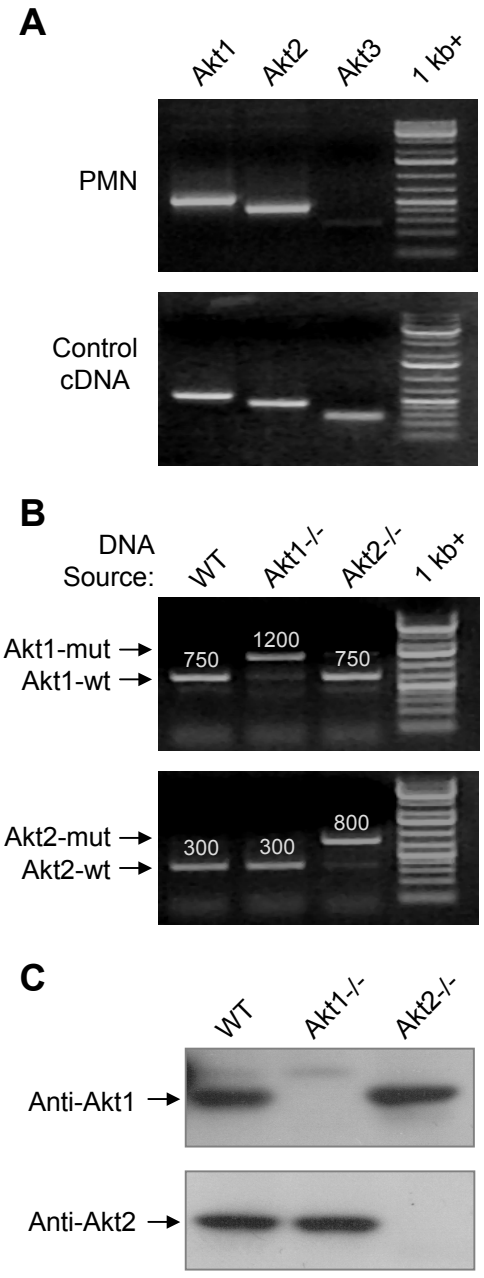
Figure S6. Membrane translocation of p47^{phox} in WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ neutrophil upon PMA stimulation

Neutrophils from WT, Akt1$^{-/-}$ and Akt2$^{-/-}$ mice were stimulated for 5 min with PMA (200 ng/ml) or buffer control. (A) the membrane fractions were separated, resolved by SDS-PAGE,

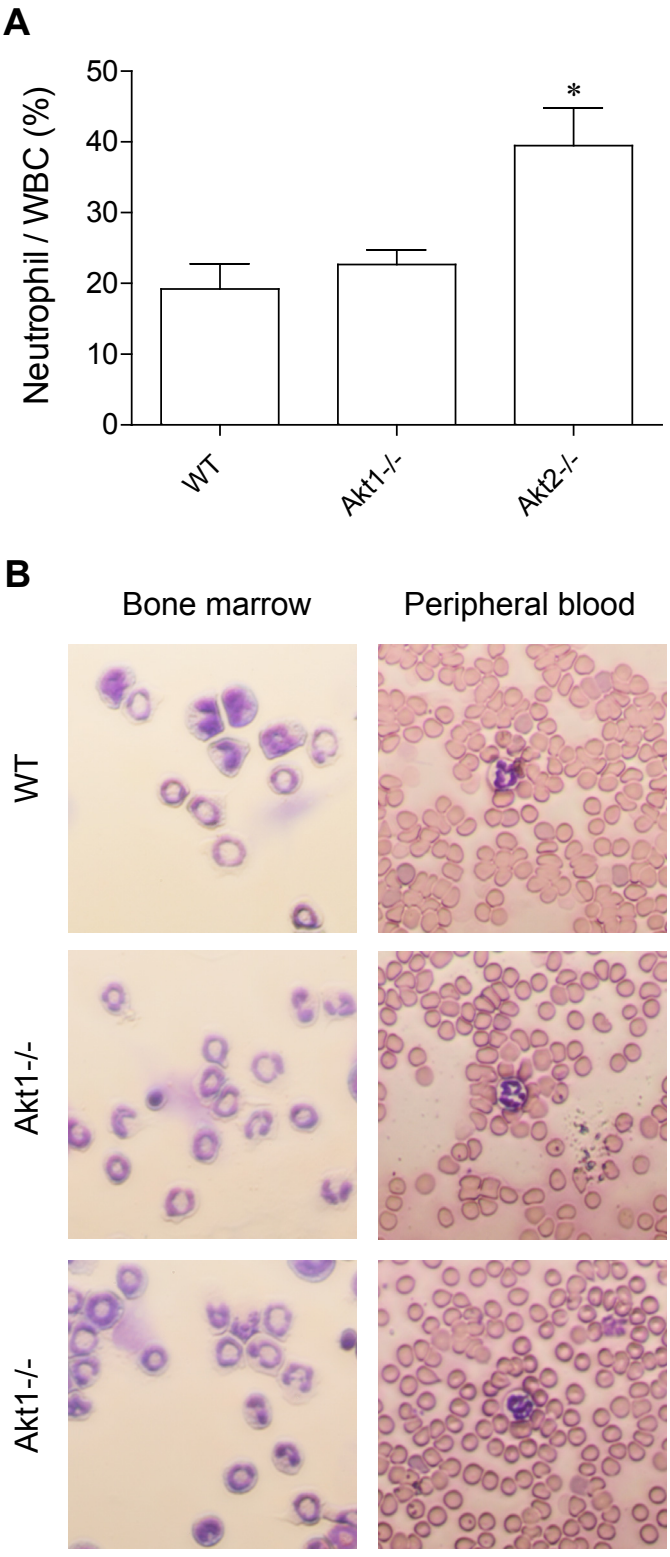
and detected with specific antibodies recognizing $p47^{phox}$. Detection of $p22^{phox}$ (a membrane component of phagocyte NADPH oxidase) was carried out for loading control. (B) The bands in Western blot ($p47^{phox}$/$p22^{phox}$) were quantified using NIH ImageJ software. *, $p < 0.05$; NS, no PMA stimulation.

Videos 1–6. The video clips #1-3 show unstimulated neutrophils from WT, $Akt1^{-/-}$ and $Akt2^{-/-}$ mice. Videos 4–6 show fMLF-stimulated neutrophils. Deficiency of Akt2 but not Akt1 impairs neutrophil migration. Briefly, bone marrow-derived neutrophils from WT (video file #1 and #4), $Akt1^{-/-}$ (video file #2 and #5) and $Akt2^{-/-}$ (videos 3 and 6) mice were seeded onto glass coverslips coated with fibrinogen. Non-adherent neutrophils were washed away and the adherent cells were stimulated with a uniform concentration of fMLF. All images shown were captured with a 40× (1.3NA) oil objective using an inverted microscope (Zeiss Axio Observer-D1) equipped with an Axiocam MR camera, using the Axiovision imaging software from Zeiss. Images were taken every 10 sec for 10 min (total 60 frames). The movie is shown at 6 frames per second.

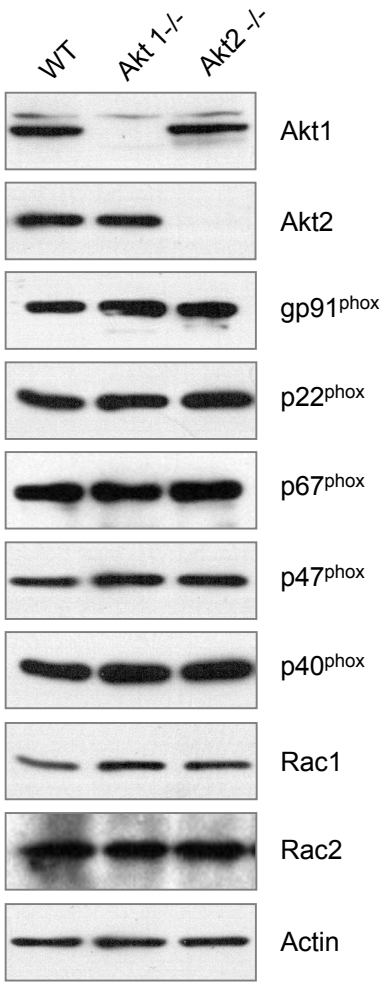
Supplemental Figure 1



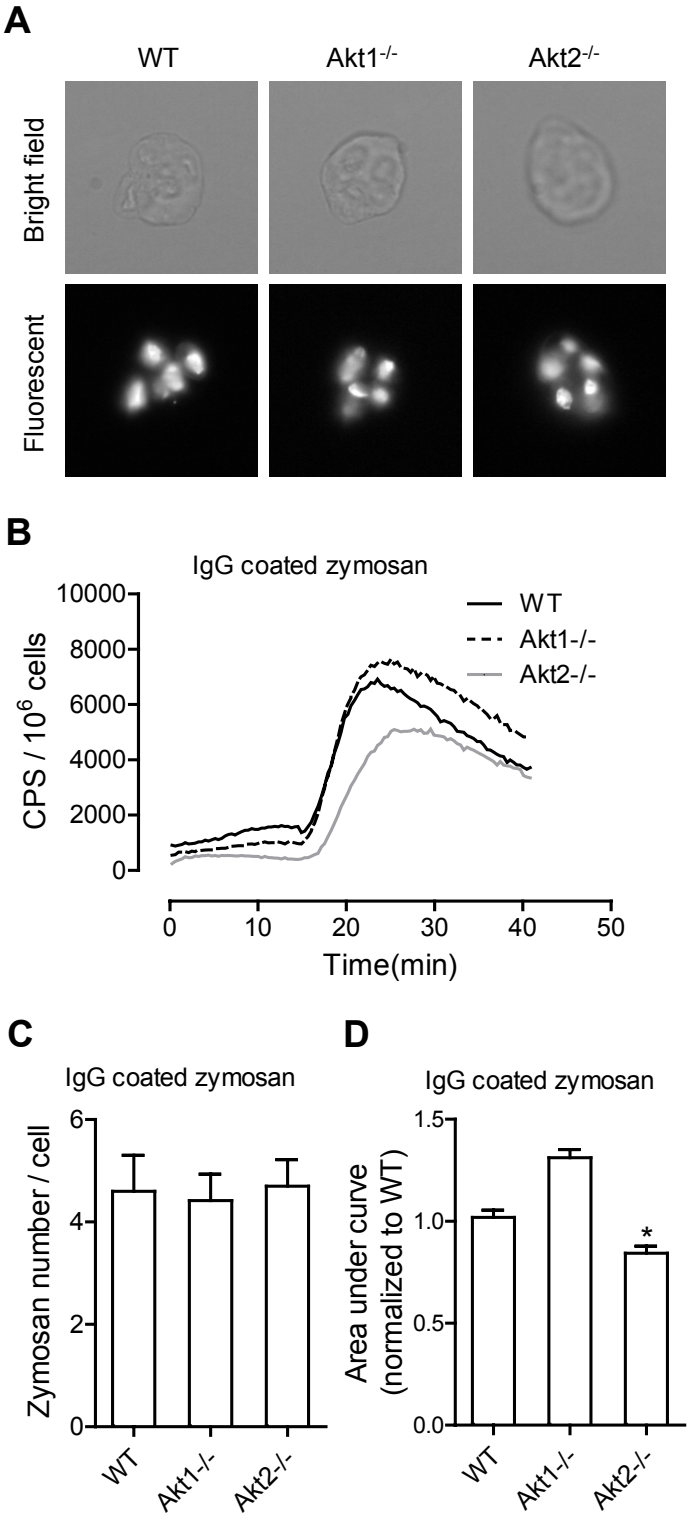
Supplemental Figure 2



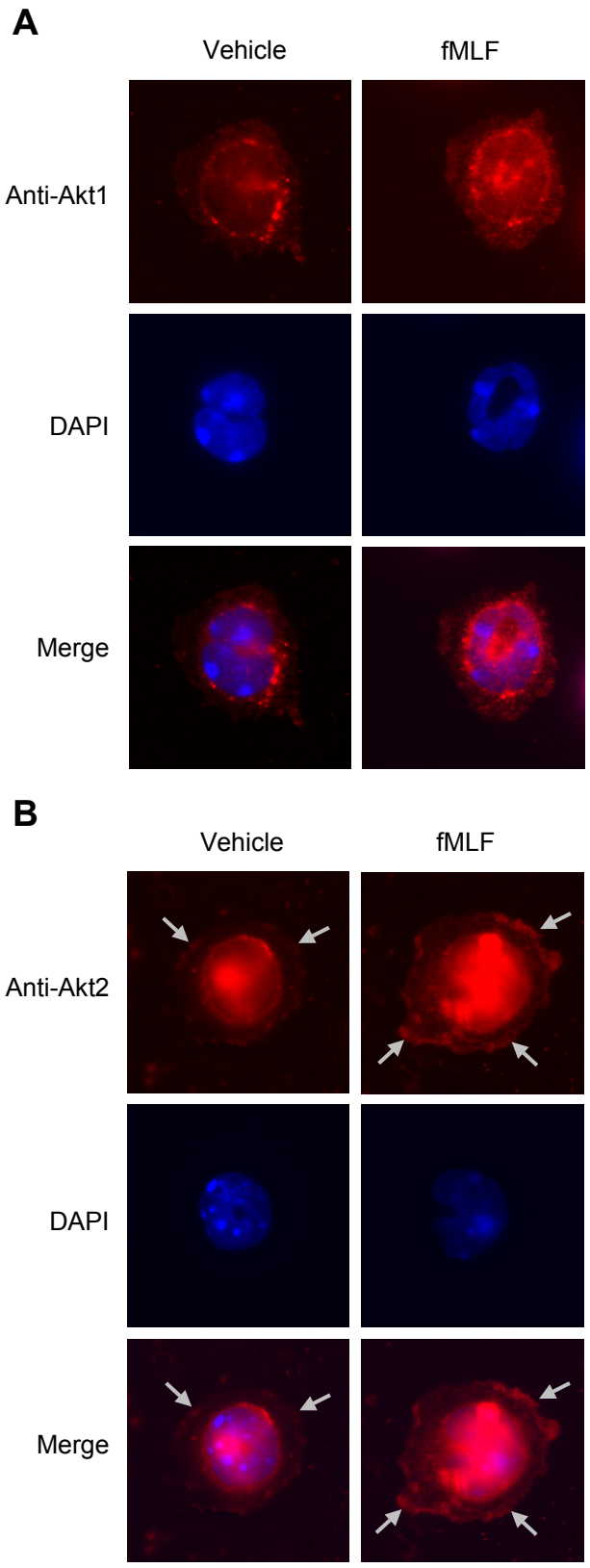
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6