Supplementary Materials and Methods

Mice

EGFP-lysozyme M (*lys*) knock in-mice (EGFP-lys-mice) (1) (kind gift from Dr. Thomas Graf) were bred at the University of California at Davis animal facility. C57BL/6 mice were purchased from Jackson laboratory (Harbor, ME). Female mice between 8 and 14 weeks of age were used in all the experiments. All animal experiments were approved by the University of California at Davis Institutional Animal Care and Use Committee (IACUC) and were performed following the guidelines of Animal Welfare Act and the Health Research Extension Act.

Mouse model of skin wounding and osmotic pump implantation

Mouse skin wounding was performed as described previously (2, 3). Briefly, mice were anesthetized with isoflurane gas (2%). Back skin hair was shaved and then the shaved skin was sterilized with 10% w/v povidine-iodine and 70% ethanol. A circular full thickness wound (6mm in diameter) was made using a skin biopsy punch (Robbins instruments Inc., NJ). Immediately after skin wounding, while the mouse is still anesthetized, an osmotic minipump (1002 model, Alzet, Durect Corporation, Cupertino, CA) was implanted subcutaneously in the side. The wound size was quantified by counting the total number of pixels within wound margin of photographic images taken. The wound size at specific time point during the course of healing was expressed as percent change by normalizing the one to initial wound size at day 0.
Prior to implantation, the osmotic minipumps were loaded with either epinephrine (dissolved in saline and delivered at 5 mg/kg/day, Sigma-Aldrich, MO), salbutamol (3 mg/kg/day, Tocris Bioscience, Bristol, UK), ICI 118,551 (0.7 mg/kg/day, Tocris Bioscience, Bristol, UK) or control saline solution, as reported in our previous study (4).

Non-invasive quantification of wound EGFP-PMNs trafficking

The trafficking of EGFP-PMNs at wound sites over time was determined non-invasively as described in our recent studies (2, 3). For in vivo imaging of EGFP-PMNs infiltrating the site of back skin wound, the whole-body small animal fluorescence imaging system (IVIS 100 system, Caliper Life Science, MA) was employed. Mice were put into the imaging chamber of the system after being anesthetized with isoflurane gas (2%). The EGFP-expressing PMNs within the wound area were visualized using GFP filter (excitation at 445~490 nm and emission at 515~575 nm) at an exposure time of 1 sec. The image was presented on a color scale overlaid on a gray scale photograph of the mouse and analyzed by quantifying fluorescence intensity averaged (photons per cm^2 per steradian) within a circular region of interest (ROI) drawn over the entire wound area. Analysis of the images were performed using Live image Pro. 2.0 software (Caliper Life Science, MA).

Flow cytometric immunophenotyping of wound PMNs and macrophages

At selected time points post-wounding, skin wounds were harvested from C57BL/6 mice treated with saline, epinephrine (5 mg/kg/day) or epinephrine with β_2AR antagonist ICI 118,551 (0.7 mg/kg/day). For harvesting cells from skin wounds, 8 mm diameter tissue biopsies were coarsely chopped and placed into 5 mL of RMPI 1640 (Invitrogen, CA) supplemented with 10 mM HEPES, 1% penicillin-streptomycin, 1% collagenase, 0.025% DNase I (all from Sigma-Aldrich, MO), and 1% FBS and incubated for 1 hr at 37^{o}C. After incubation, the tissue digest was washed with cold 5 mM EDTA in PBS and passed consecutively through 70 μm and 35 μm filters. Cells were pelleted at 1000 g for 12 minutes and adjusted to $1x10^6$ cells/mL. Cells were stained with FITC conjugated anti-CD11b and PE conjugated anti-Ly6G antibodies for PMN phenotyping and PE conjugated F4/80 and PE-Cy5 conjugated anti-Ly6C antibodies (all from eBioscience, CA) for macrophage phenotyping. Events were acquired using a FACScan flow cytometer (Becton Dickinson, NJ). Data was analyzed using FlowJo software (Treestar, Inc., OR).

Plasma epinephrine measurement

Plasma epinephrine measurements were performed using enzyme immunoassay kit (Epinephrine ELISA kit, Rocky mountain diagnostics, Inc., CO). Blood samples were collected from mice treated with either epinephrine or saline at day 5 post-wounding and centrifuged. The supernatant was collected and stored at -80 ^{o}C. Extracts from plasma were tested with an epinephrine enzyme immunoassay kit according to the manufacturer's instruction, as we have previously reported (4).

The quantification of PMN half-life in the wound

PMN half-life in the wound was determined by quantifying the clearance rate of adoptively transferred bone marrow PMNs from the site of wound. For sorting of non-proliferative mature EGFP-PMNs, bone marrow cells were isolated, incubated with APC-conjugated anti-mouse CD117 antibody (R&D Systems, MN) and PE-conjugated anti-mouse Gr-1 (all from eBioscience, CA). To exclude myeloid progenitor cells that might give rise to PMNs, c-kit+ cells were gated out and cells were sorted into c-kit^{-} EGFP^{high} cells using the MoFlo cell sorter (Beckman Coulter, Inc., CA) as reported in our recent study (2). FACS sorted c-kit^{-} EGFP^{high} mature EGFP-PMNs ($1x10^{7}$ cells in 100 μL sterile saline) were intravenously injected via tail-vein to C57BL/6 mice treated with either saline or epinephrine (5 mg/kg/day) 3hr after wounding and then fluorescence of EGFP-PMNs in the wound was quantified using the IVIS imaging system. Following the adoptive transfer of c-kit^{-} EGFP^{high} cells, their time-dependent change in concentrations in the blood were also quantified by collecting blood samples from the tail-veins at selected time points and using flow cytometric cell counting method. For this, the samples were incubated with PE-conjugated anti-mouse Gr-1 (eBioscience, CA) and a known volume of

flow cytometry cell counting beads (Bangs Laboratory Inc., IN) was added to a known volume of blood sample (10 μl). The total number of EGFP-PMN in the blood was determined by relating the relative population of $EGFP^{high}$ $Gr\text{-}1^{high}$ cells to the total number of fluorescent bead events. PMN half-life ($t_{1/2}$) was calculated as previously described using the following equation (5): $N_t=N_o\ e^{-k(t-t)}$ for $t \geq t$ and $t_{1/2}=\ln 2/k$, where N_t is the amount of EGFP-PMNs in the tissue at time t, and N_o is the number of EGFP-PMNs at time t at which their levels reaches peak value. *k* is the rate constant of PMN disappearance from the wound due to either apoptosis or clearance by macrophages and the value is determined by fitting the decay rate of EGFP-PMNs in the wound following their adoptive transfer to recipient mice.

Multiplex cytokine assay from wounded skin

The proinflammatory cytokine levels in the wounded skin were measured using MILLIPLEX MAP 13-plex Cytokine Kit (Millipore, Billerica, MA) by harvesting skin samples at day 5 post-wounding from either saline control or epinephrine-treated mice. The cytokines and chemokines including IL-1α, IL-1β, IL-6, GM-CSF, and TNFα were measured according to the manufacturers' protocols. Calibration controls and recombinant standards were used as specified by the manufacturer.

IL-6 ELISA assay from mouse macrophage.

Macrophages were isolated from the peritoneal cavities of EGFP-lys-mice and plated at a density of 1×10^5 cells per well in 96 well plates and incubated overnight with medium containing 0.5% FBS. On the next day, macrophages were treated with epinephrine (1 μM), or epinephrine along with the β_2AR-specific antagonist ICI 118,551 (1 μM) under serum-free conditions. Supernatants were collected after 4 hours and immediately frozen at −80 °C until

ELISA analysis. ELISA for mouse IL-6 was performed using a mouse IL-6 ELISA kit (Life Technologies, NY) according to the manufacturer's instructions. Experiments were repeated three to five times with cells derived from separate animals.

Real time-qPCR for IL-6 gene expression from wound macrophages.

Real-time quantitative PCR analysis for the IL-6 gene was performed using macrophages isolated from wounds of EGFP-lys-mice treated with either epinephrine (5 mg/kg/day) or epinephrine with β_2AR antagonist ICI 118,551 (0.7 mg/kg/day) at day 8 post-wounding. Wound macrophages were harvested, incubated with PE conjugated anti-mouse F4/80 (eBioscience, CA), and sorted using the MoFlo cell sorter (Beckman Coulter, Inc., CA). The mouse real-time *q*PCR assays for IL-6 (NM_031168) was designed by the Real-time PCR Research and Diagnostic Core Facility at UC Davis utilizing Primer Express 3 with the sequences listed from the Genebank accession numbers. The *q*PCR systems were validated using dilutions of a known positive control in triplicate and a standard curve plotted against the dilutions. Each PCR reaction contained 20X primers and probe for the respective TaqMan ® system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan ® probe and commercially available PCR mastermix (TaqMan ® Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 μl of the diluted cDNA sample in a final volume of 12 μl. The samples were placed in a 384 well plate and amplified in an automated fluorometer (7900 HT FAST Real Time PCR System, ABI). Fluorescent signals were collected during the annealing temperature and Cq values were exported with a threshold of 0.1 and a baseline of 3-12 for the genes of interest and the reference genes. The β2-microglobulin (NM_009735) was used as a reference gene to normalize the Cq values of the target IL-6 gene (ΔCq). The relative linear amount of target

molecules relative to the calibrator, was calculated by $2^{-\Delta\Delta Cq}$. Therefore, all gene transcription is expressed as an n-fold difference relative to the calibrator.

References

1. Faust N, Varas F, Kelly LM, Heck S, & Graf T (2000) Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood* 96(2):719-726.
2. Kim MH*, et al.* (2011) Neutrophil survival and c-kit(+)-progenitor proliferation in Staphylococcus aureus-infected skin wounds promote resolution. *Blood* 117(12):3343-3352.
3. Kim MH*, et al.* (2008) Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. *J Invest Dermatol* 128(7):1812-1820.
4. Sivamani RK*, et al.* (2009) Stress-mediated increases in systemic and local epinephrine impair skin wound healing: potential new indication for beta blockers. *PLoS Med* 6(1):e12.
5. Cheretakis C, Leung R, Sun CX, Dror Y, & Glogauer M (2006) Timing of neutrophil tissue repopulation predicts restoration of innate immune protection in a murine bone marrow transplantation model. *Blood* 108(8):2821-2826.

Supplementary Figures

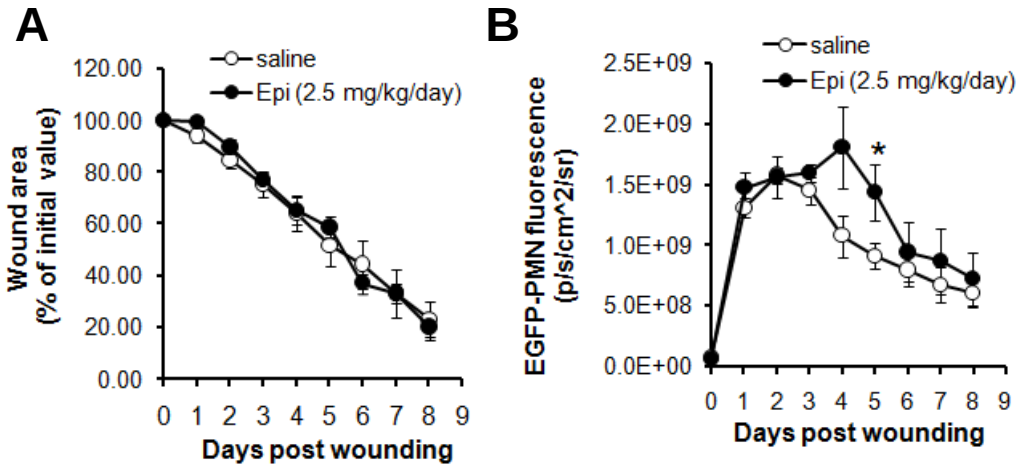

Fig. S1. Effects of low dose epinephrine on wound PMN trafficking and wound healing. EGFP-lys-mice were systemically delivered with either sterile saline or low dose of epinephrine (2.5 mg/kg/day) by implanted osmotic infusion pumps. Kinetics of wound closure (A) and Kinetics of wound EGFP-PMN fluorescence (B) were determined. Data are derived from four to eight mice in each group.

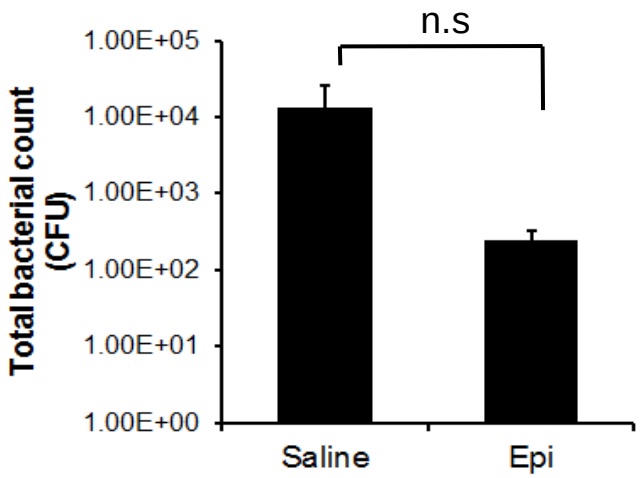

Fig. S2. Bacterial burden in the wounds of mice treated with either epinephrine (5 mg/kg/day) or saline vehicle. Wounded skin samples were harvested and homogenized from EGFP-lys-mice treated with either saline or epinephrine at day 5 post wounding. The bacterial load in the wound was quantified by counting the number of colonies formed. Data are derived from four mice in each group and expressed as mean ± SEM. n.s.; not significant

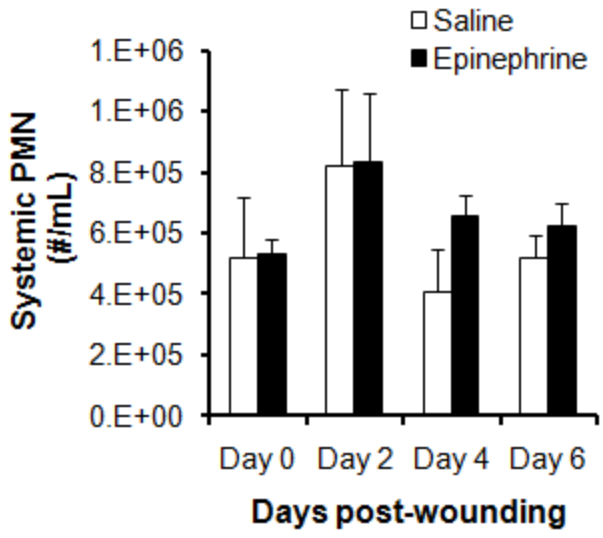

Fig. S3. Effects of epinephrine on blood PMN concentration. EGFP-lys-mice were systemically delivered with either sterile saline or epinephrine (5 mg/kg/day) by implanted osmotic infusion pumps. Blood EGFP-PMN numbers were quantified by collecting samples from tail-veins and using flow cytometric cell counting method (see Methods section). Data are derived from four mice in each group. No significant difference in PMN numbers between saline and epinephrine groups.

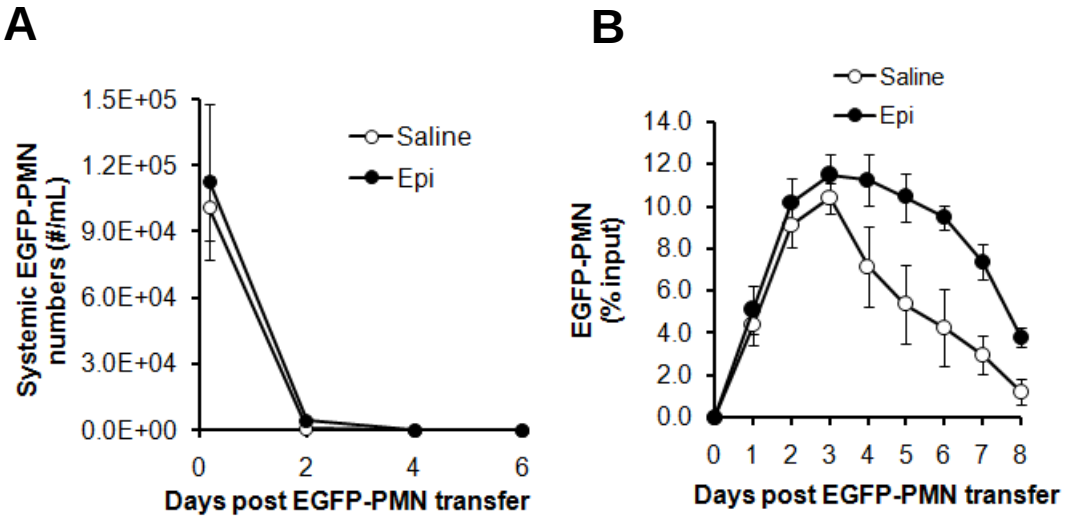

Fig. S4. Adoptive transfer of EGFP-PMNs and their recruitment to wound in response to epinephrine. (A) FACS sorted c-kit^{-} EGFP^{high} cells from EGFP-lys-mice were intraveneously transferred (1x10^{7} cells in 100 mL sterile saline) to C57BL/6 mice treated with either sterile saline or epinephrine (5 mg/kg/day). (A) The number of infused EGFP^{high} PMNs in blood circulation. (B) Kinetics of wound EGFP-PMNs trafficking following adoptive transfer of c-kit^{-} EGFP^{high} cells in saline and epinephrine-treated C57BL/6 mice.

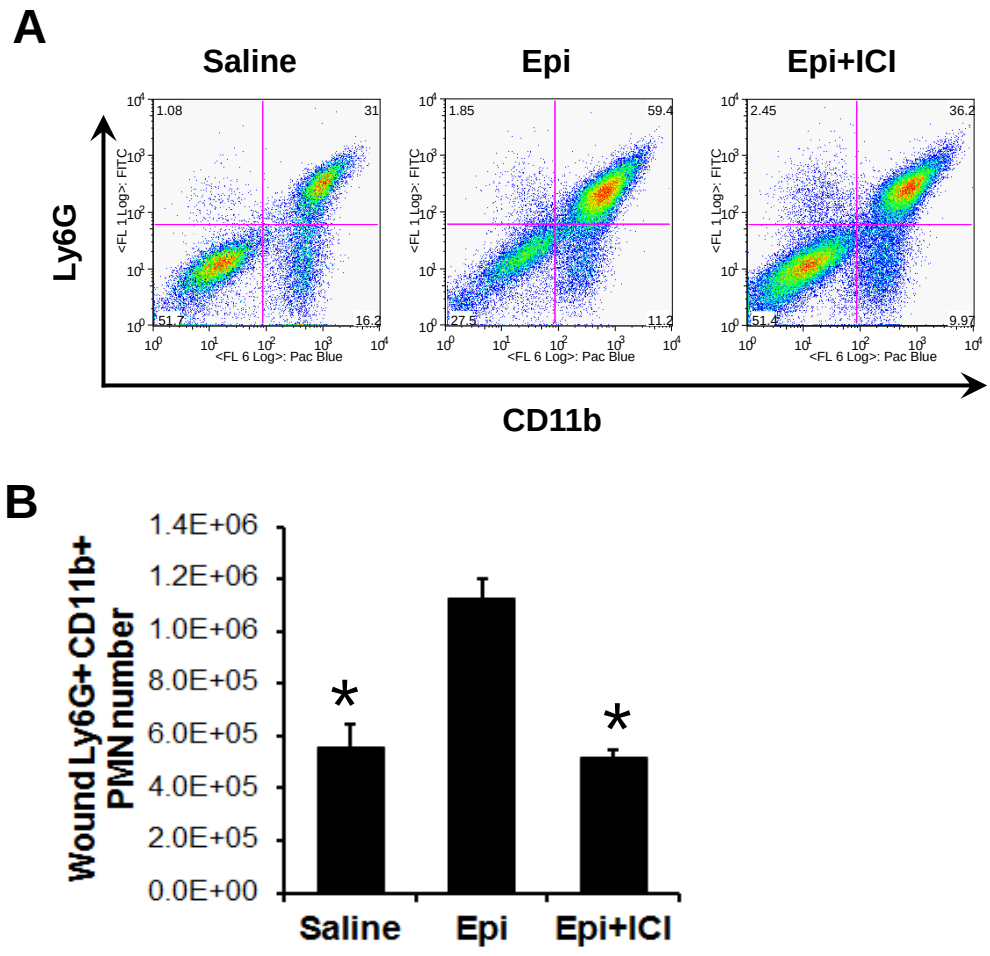

Fig. S5. Effects of β_2AR blockade on epinephrine-mediated wound CD11b+Ly6G+ PMN trafficking. Wound PMN numbers were quantified using flow cytometric analysis by characterizing CD11b+Ly6G+ cells collected from wounded skin of mice treated with either saline, epinephrine (5 mg/kg/day), and epinephrine with β_2AR antagonist (ICI 118,551, 0.7 mg/kg/day) at day 7 post-wounding. Representative FACS plot (A) and quantification (B). Data are derived from six to eight mice in each group. *, $p<0.05$ vs Saline group.

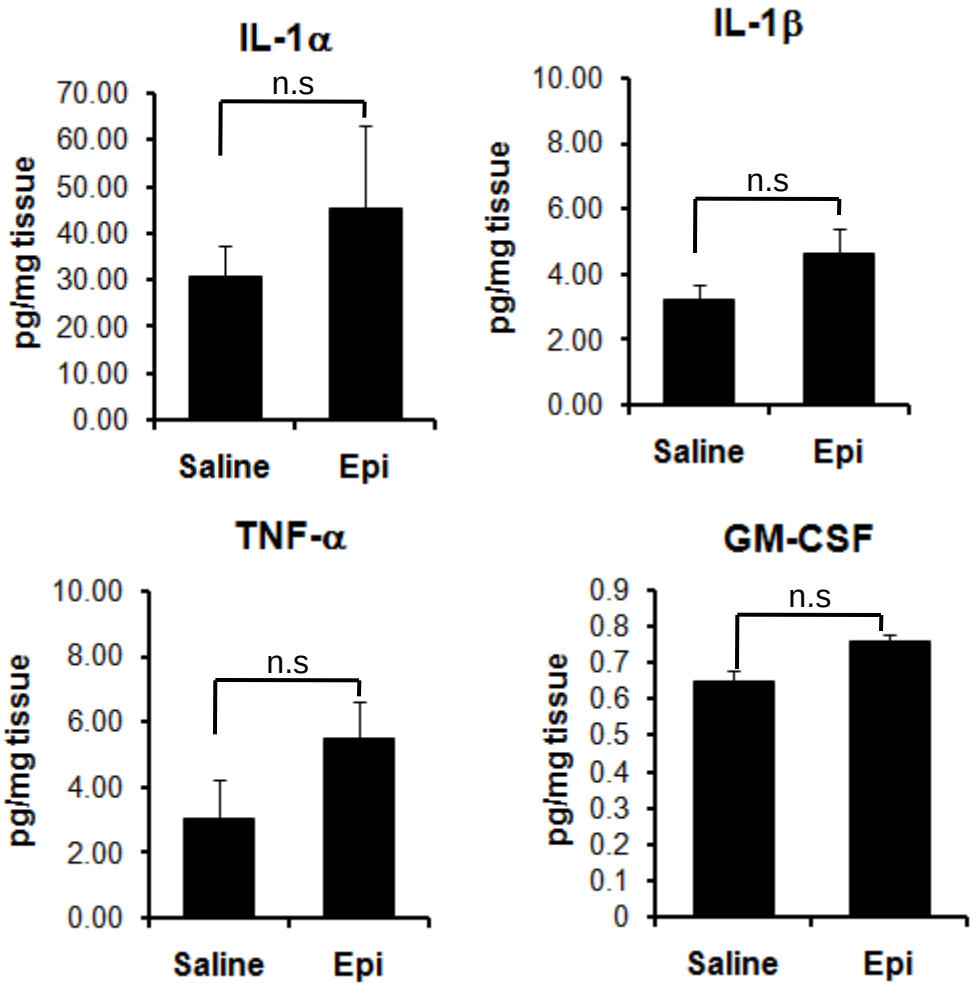

Fig. S6. Effect of epinephrine on IL-1α, IL-1β, TNF-α, and GM-CSF production in local wounds. Wounded skin samples were harvested from EGFP-lys-mice treated with either saline or epinephrine (5 mg/kg/day) at day 5 post-wounding and protein levels of IL-1α, IL-1β, TNF-α, and GM-CSF were measured. Data are derived from four to five mice in each group. n.s.; not significant

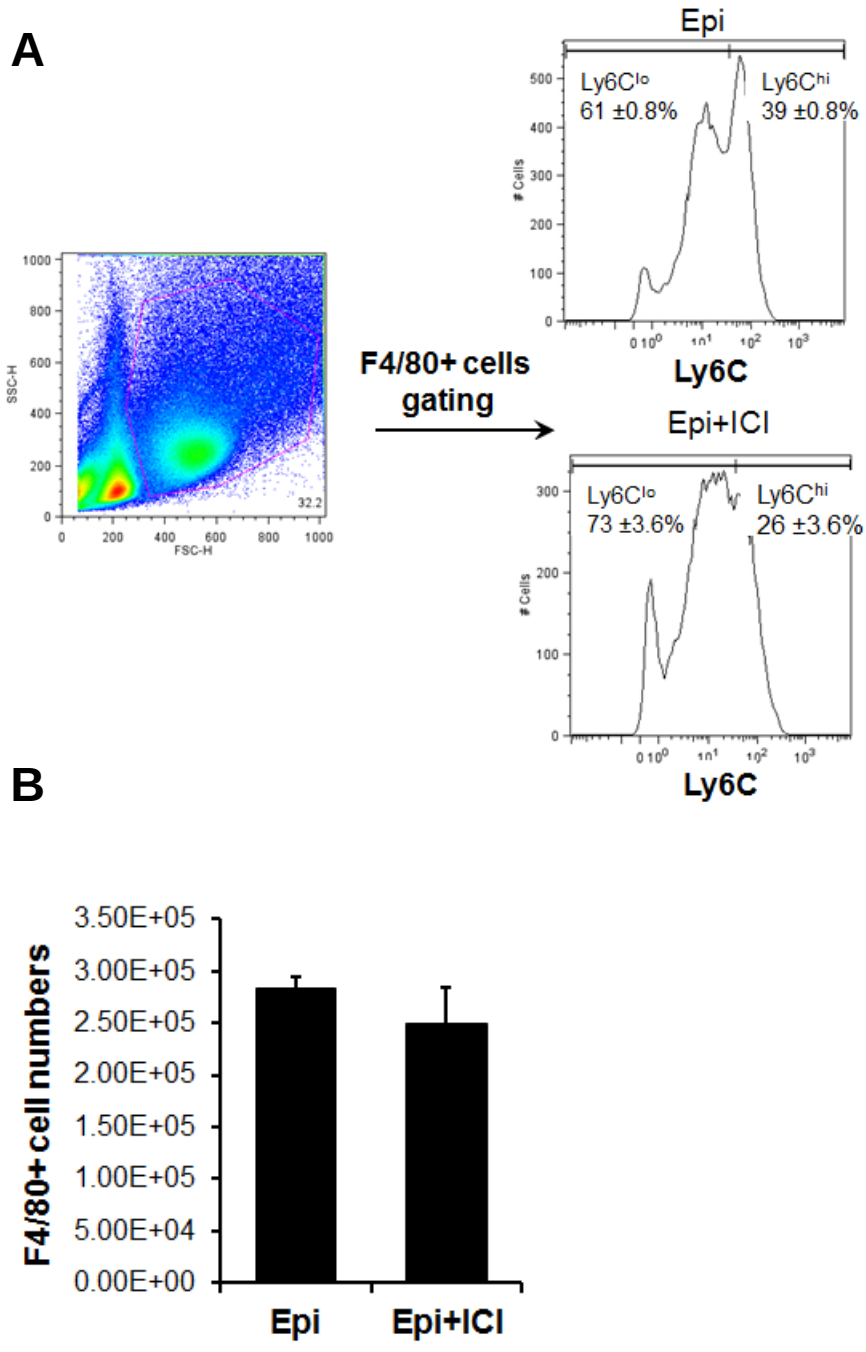

Fig. S7. The effect of ICI 118,551 on epinephrine-mediated F4/80+Ly6G^{hi} cells trafficking in wounds. (A) Cells were collected from wounds of mice treated with epinephrine (5 mg/kg/day) or epinephrine with ICI 118,551 (0.7 mg/kg/day) at day 8 post-wounding. F4/80+ cells were gated and Ly6C^{hi} cell populations were characterized from gated F4/80+ cells. (B) Total number of F4/80+ cells. Data are derived from four to five mice in each group and expressed as mean ± SEM.

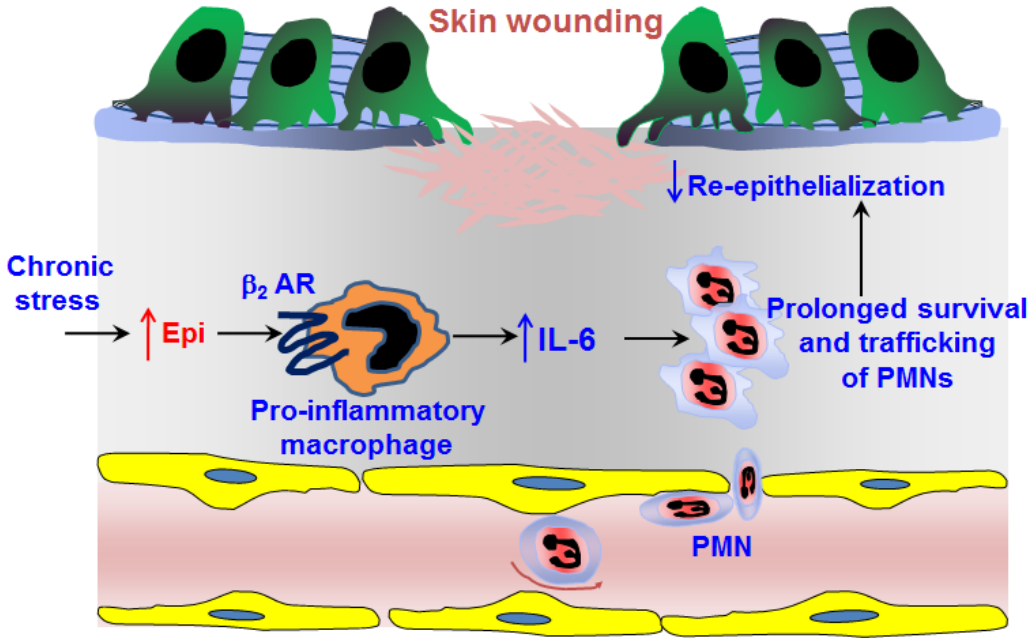

Fig. S8. Proposed model depicting epinephrine-mediated impairment of wound healing via β_2AR and IL-6 dependent PMN trafficking.