Increased interleukin 1β-driven neutrophil counts preserve anti-bacterial defense in absence of IKKβ

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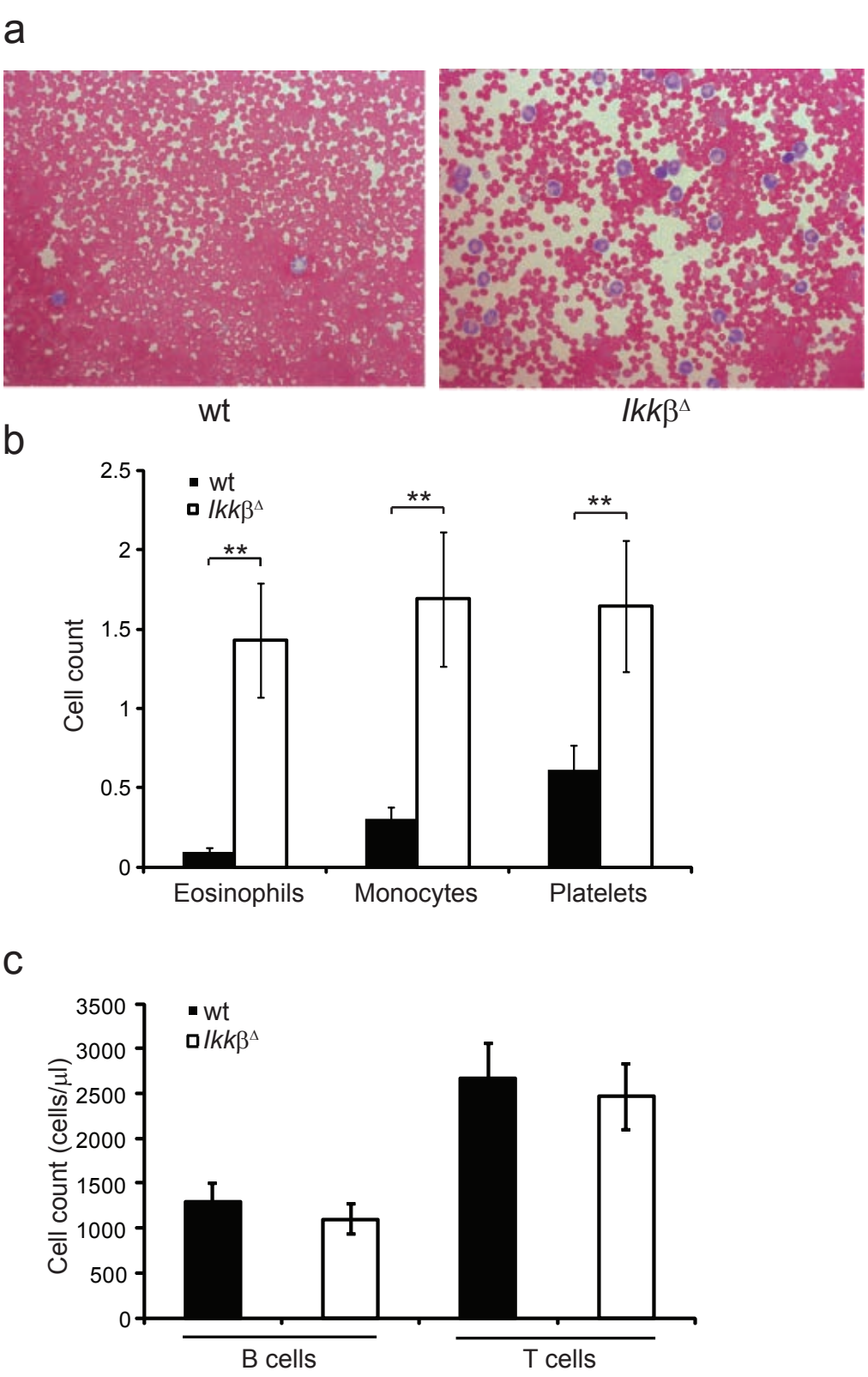
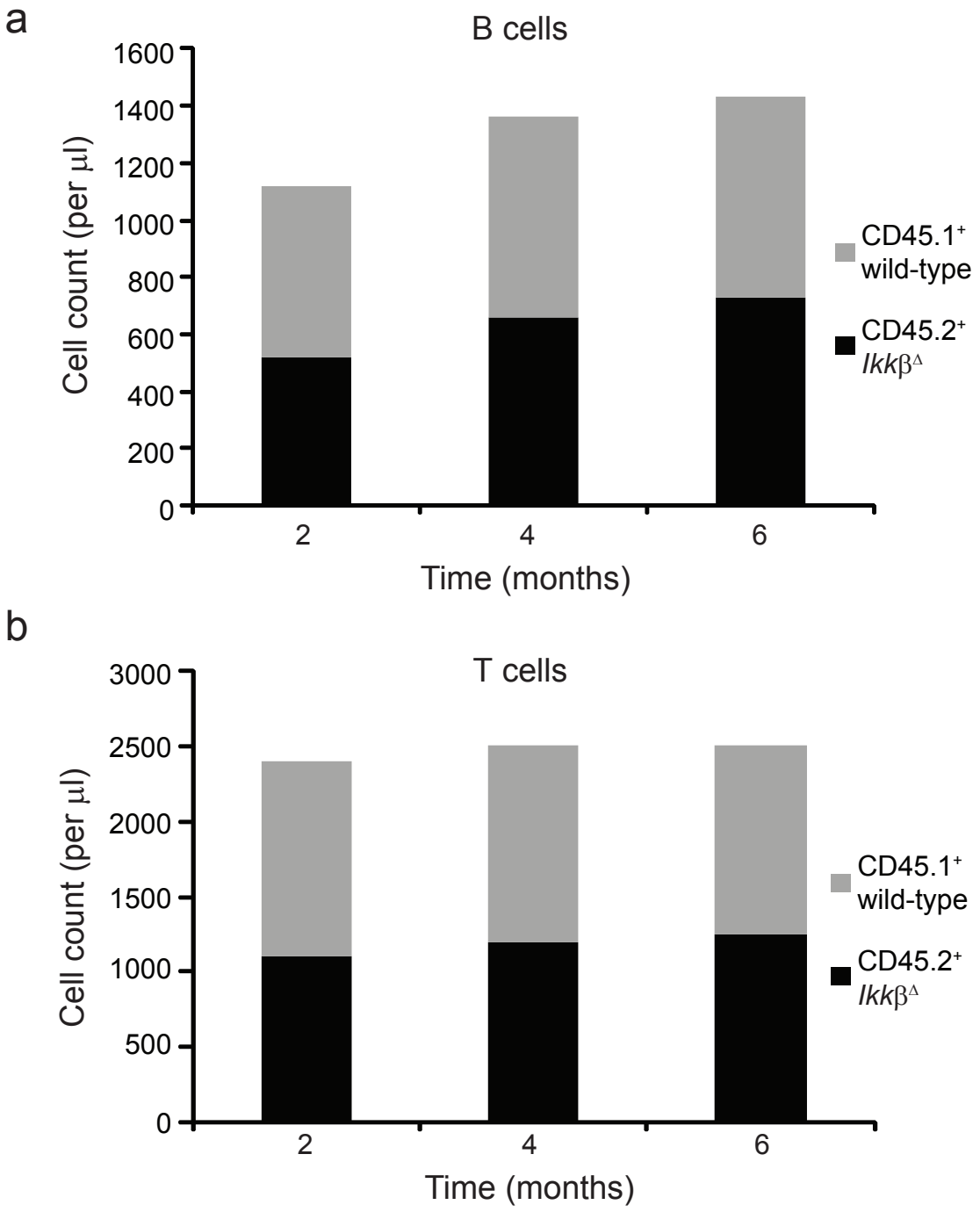
Fig. S1



Supplementary Figure 1. Increased eosinophil, monocyte and platelet counts in *Ikk*β$^{\Delta}$ mice. a. Peripheral blood smears from mice of the indicated genotypes were stained with Wright-Giemsa. Shown is one representative smear per genotype (*n* = 3 mice per genotype). b. Peripheral eosinophil (x 10^3/μl), monocyte (x 10^3/μl), and platelet (x 10^6/μl) counts from mice of the indicated genotypes (*n* = 3 mice per genotype; ± s.d., **p < 0.01 compared to wt controls). c. Peripheral B and T cell counts from mice of the indicated genotypes. B cells were identified by anti-B220 and T cells by anti-CD3 staining using flow cytometry. Absolute B and T cell numbers were calculated using flow cytometric data and total leukocyte counts as determined by a Blood Analyzer (*n* = 3 mice per genotype; ± s.d.).

Fig. S2



Supplementary Figure 2. The IKKβ deficiency has only a modest effect on lymphocyte counts. Peripheral B and T cell counts of totally irradiated CD45.1$^+$ wild-type mice transplanted with a 1:1 mixture of BM cells from CD45.1$^+$ wild-type and CD45.2$^+$ *Ikk*β$^\Delta$ mice. a. B cells were stained with CD45.1 or CD45.2 and B220 antibodies and analyzed by flow cytometry. b. T cells with CD45.1 or CD45.2 and CD3 antibodies and analyzed by flow cytometry. In both experiments, absolute cell numbers were calculated based on flow cytometric data and on total leukocyte counts as determined with a Blood Analyzer (shown are average numbers; *n* = 2 mice per genotype).

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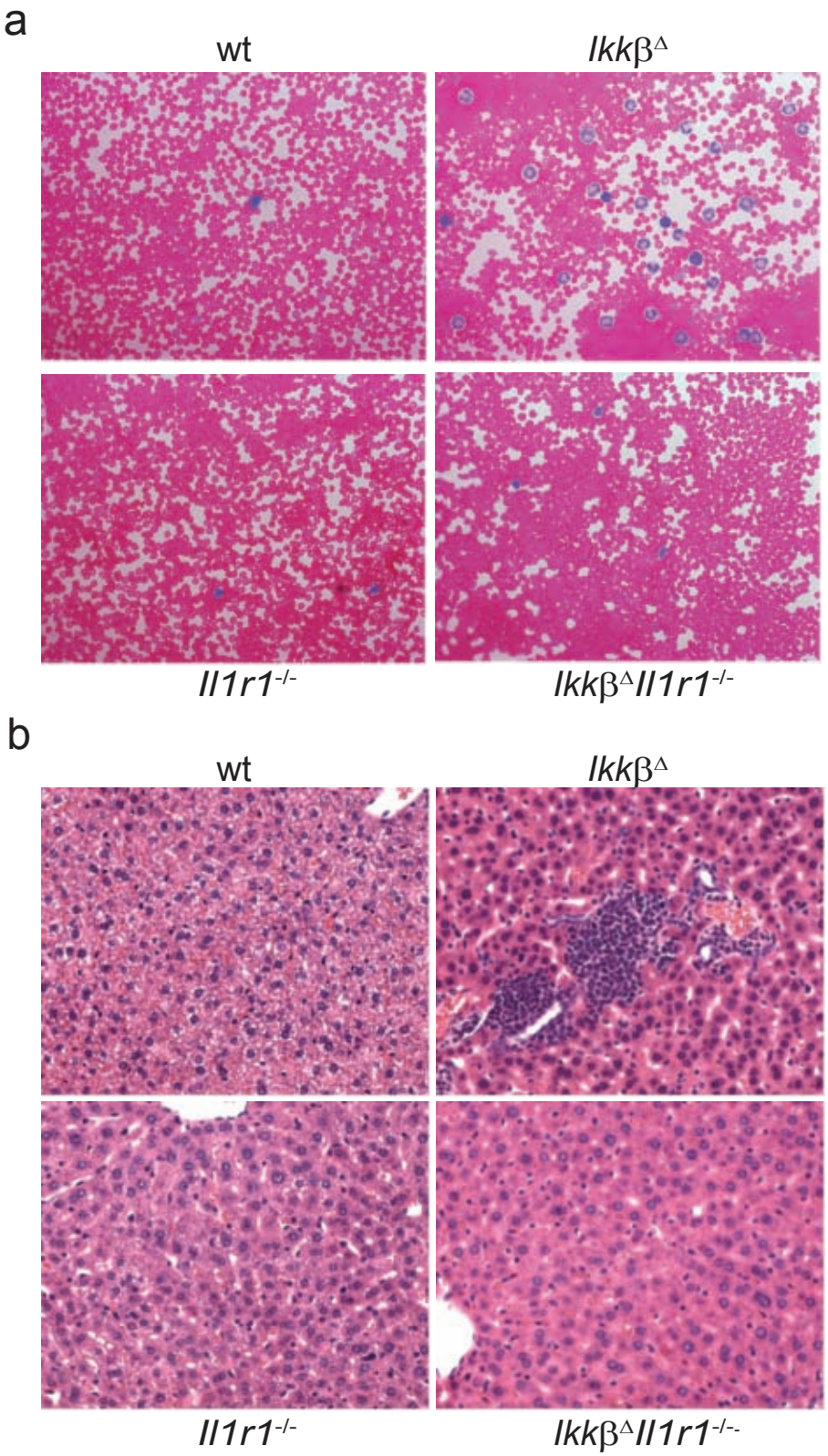
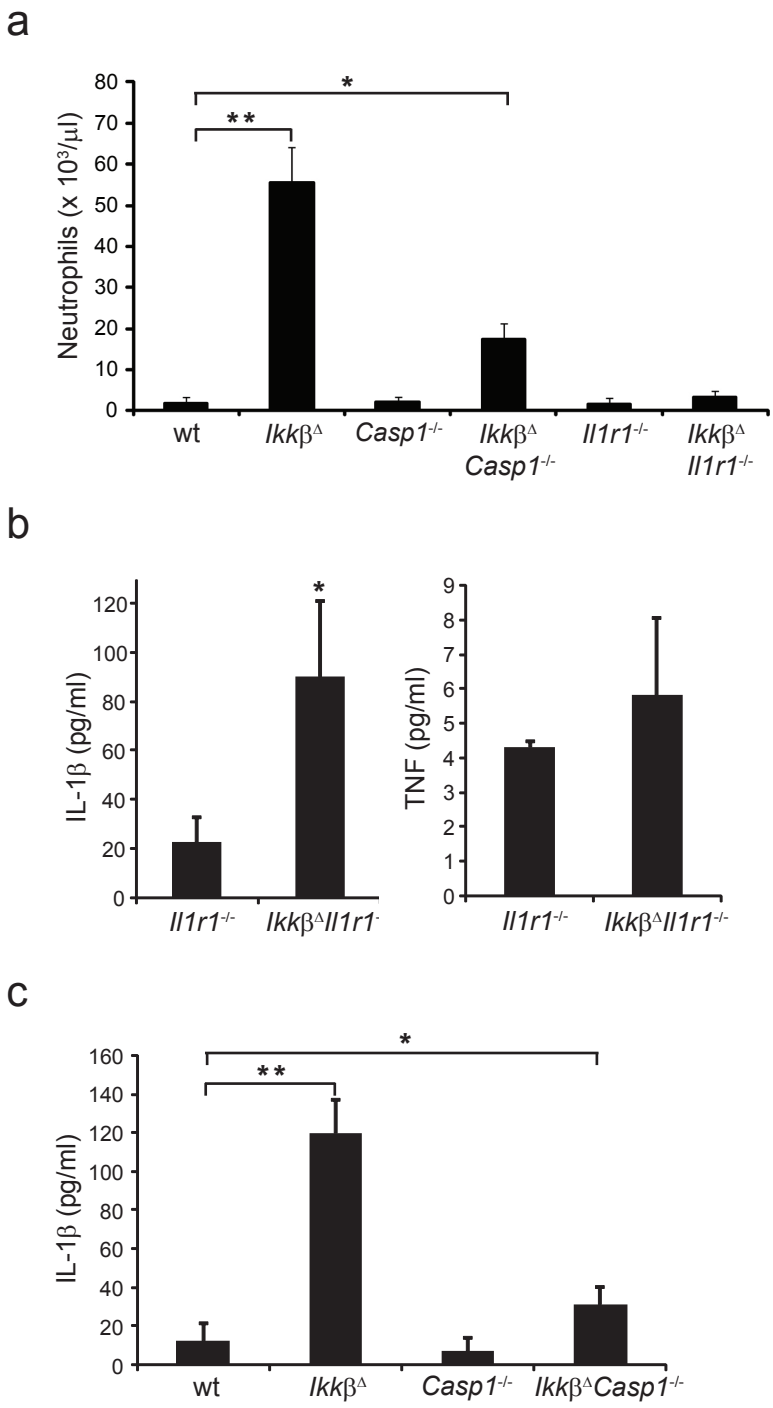
Fig. S3



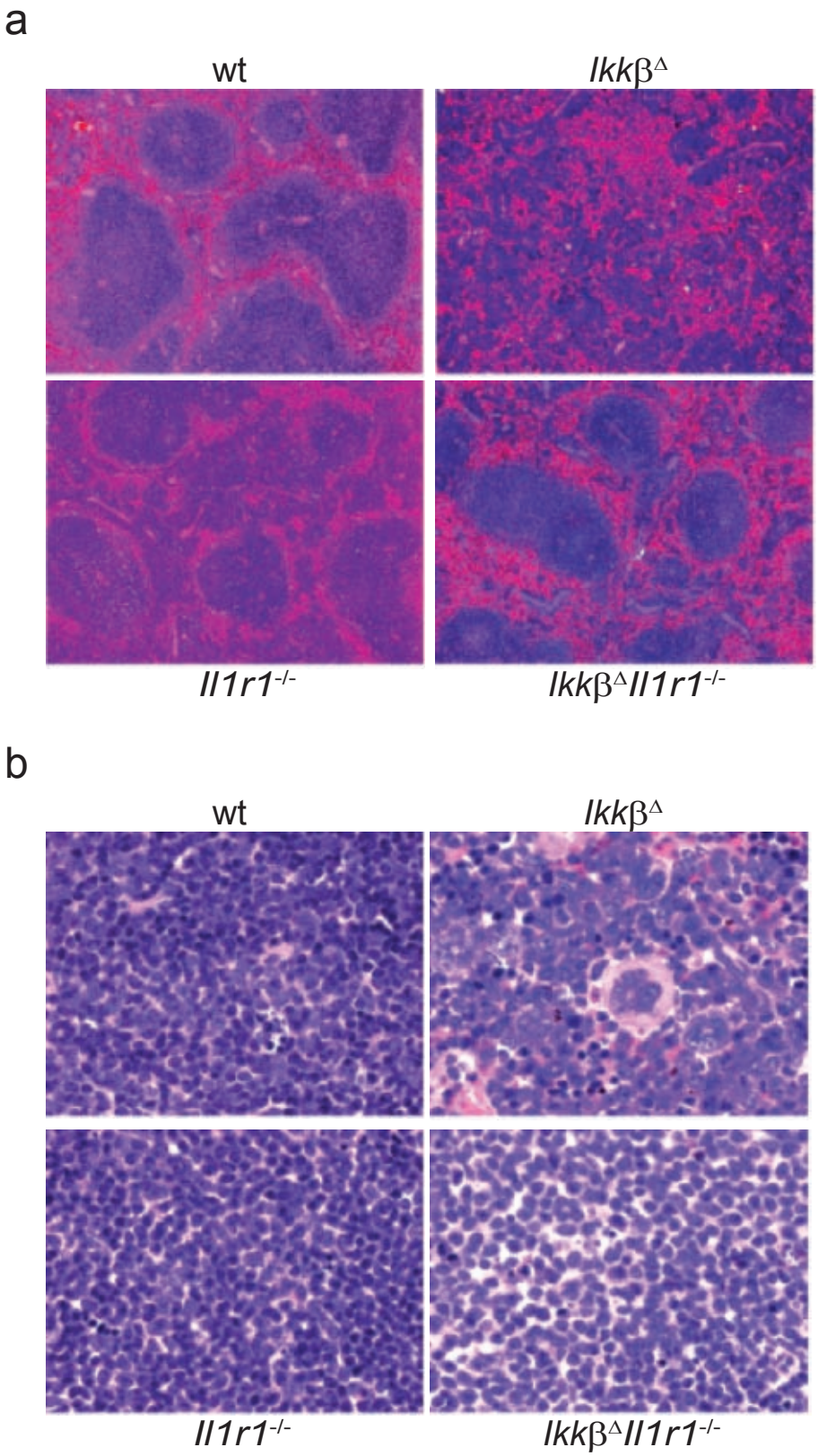
Supplementary Figure 3. Lack of IL-1R restores normal blood counts and prevents neutrophilic infiltration in *Ikk*β$^{\Delta}$ mice. a. Smears of blood collected from mice of the indicated genotypes were stained with Wright-Giemsa. Shown is one representative smear per genotype (n = 3 mice per genotype). b. H&E stained liver sections obtained from livers of mice of the indicated genotypes. Shown is one representative section from one mouse out of 3 per genotype; magnification: 40x.

Fig. S4



Supplementary Figure 4. *Ikk*β$^{\Delta}$ mice lacking IL-1R have normal blood counts despite elevated IL-1β amounts. a. Peripheral neutrophil counts in mice of the indicated genotypes determined 6 months after poly(I:C) injection (*n* = 3 mice per genotype; ± s.d.,*p < 0.05, **p < 0.01). b. Serum IL-1β (left panel) and TNF (right panel) concentrations in mice of the indicated genotypes determined 6 months after poly(I:C) injection (*n* = 3 mice per genotype; ± s.d.,*p < 0.05). c. IL-1β concentrations in serum of mice of the indicated genotypes 6 months after poly(I:C) injection (*n* = 3 mice per genotype; ± s.d., *p < 0.05, **p < 0.01).

Fig. S5



Supplementary Figure 5. Lack of IL-1R restores splenic architecture in *Ikk*β$^{\Delta}$ mice. Shown are representative H&E-stained sections of spleens from mice of the indicated genotypes (*n* = 3 mice each genotype), magnification: a. 10x, b. 100x.

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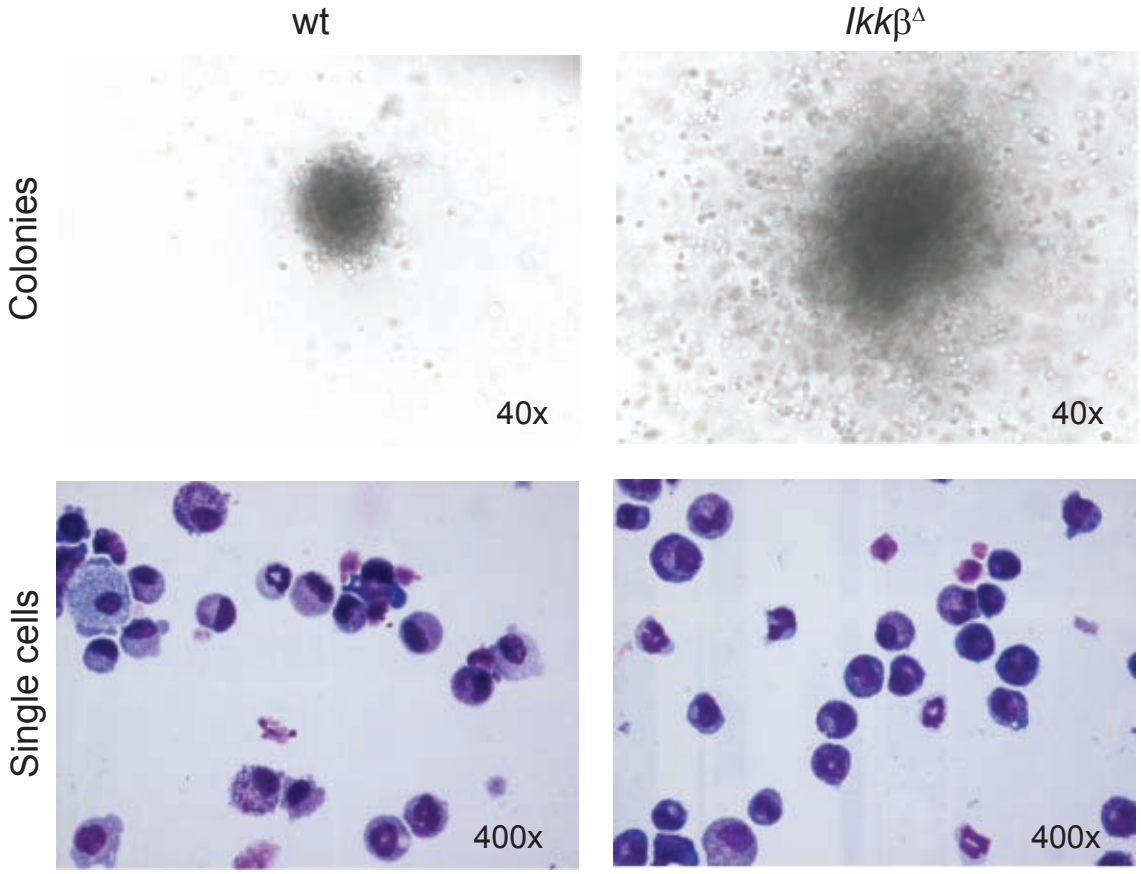
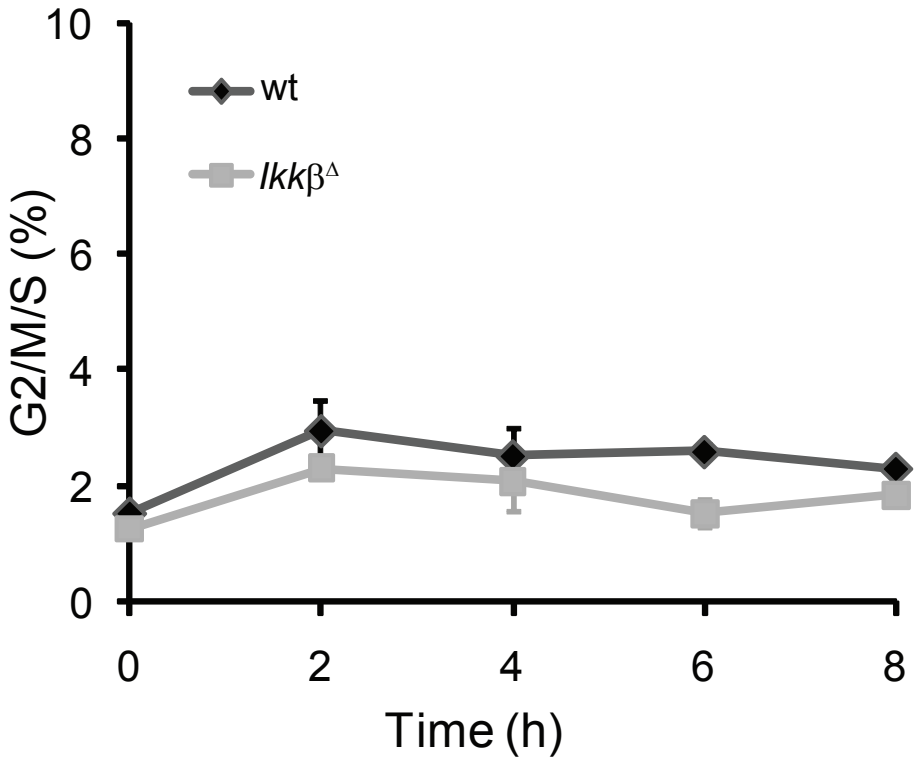
Fig. S6



Supplementary Figure 6. Increased diameters of myeloid progenitor cell colonies from *Ikk*β$^{\Delta}$ mice. Upper panels: Representative pictures of day-10 colonies from indicated genotypes (27 colonies per genotype analyzed, *n* = 3 mice per genoypte). Lower panels: Representative pictures of day-10 colonies from indicated genotypes after cytospin and Giemsa staining showing the cellular morphology from five pooled colonies from each culture.

Fig. S7



Supplementary Figure 7. No difference in proliferation of mature neutrophils between wt and *Ikk*β^{Δ} mice. Thioglycollate-elicited Ly6G^{+} peritoneal cells were collected and cultured. At the indicated time points, cells were fixed, stained with PI, and analyzed by Flow Cytometry for DNA content (>2N DNA) (± s.d.; data are representative of 3 independent experiments done in triplicate). The percentage of cells at S+G2+M portion of the cell cycle was calculated.

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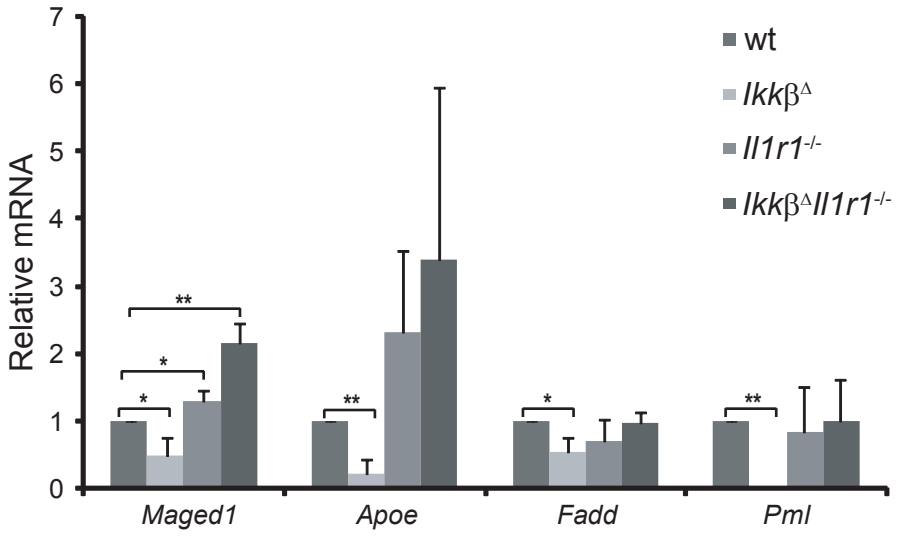
Fig. S8



Supplementary Figure 8. Downregulation of pro-apoptotic genes in *Ikk*β$^{\Delta}$ neutrophils. Q-RT-PCR analysis of apoptosis-related gene expression in purified Ly6G^{+} peritoneal neutrophils from mice of the indicated genotypes (data are representative of 3 independent experiments; ± s.d.; *$p < 0.05$, **$p < 0.01$ compared to corresponding wt controls).

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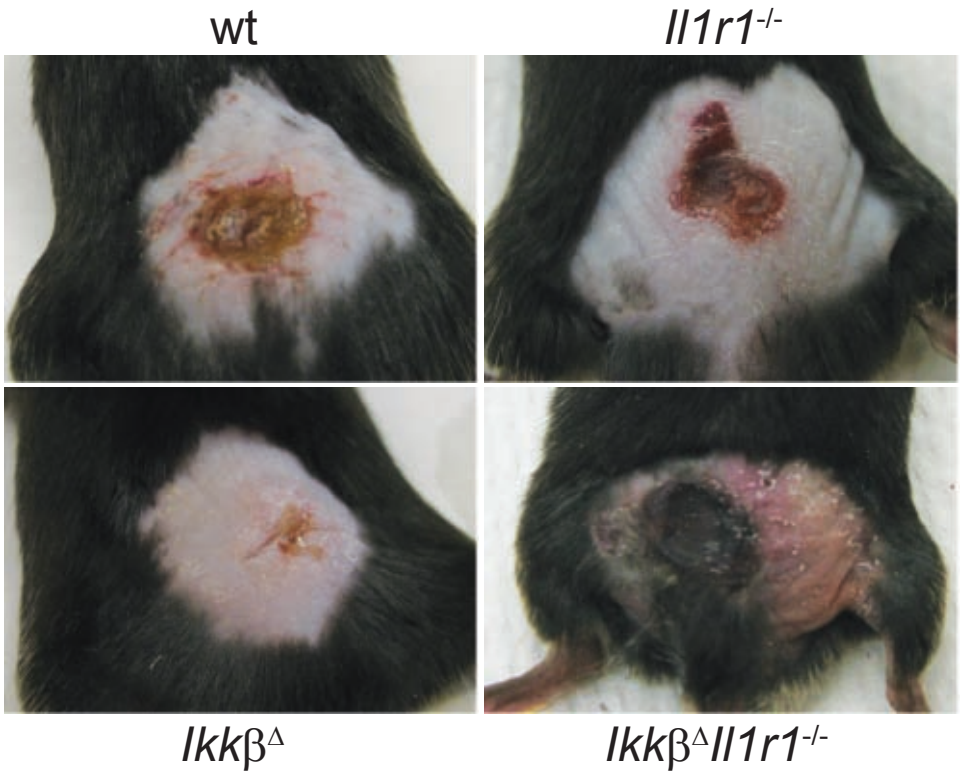
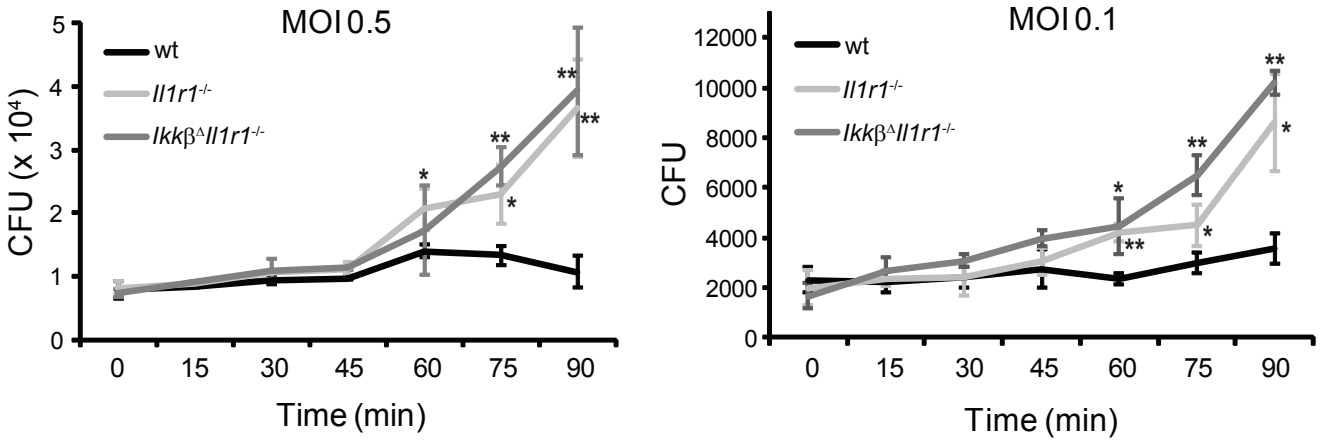
Fig. S9



Supplementary Figure 9. Highly impaired clearance of bacterial skin infections in mice lacking IKKβ and IL-1R. Representative photographs of skin lesions of mice with the indicated genotype taken 4 days after s.c. injection with GAS.

Fig. S10



Supplementary Figure 10. Ablation of IL-1R impairs GAS killing by neutrophils. Peritoneal neutrophils harvested from mice of the indicated genotypes 4 h after thioglycollate injections were incubated with GAS at the indicated MOI. Aliquoted cells were collected and numbers of live bacteria were determined at the indicated time points (± s.d.; *p < 0.05, **p < 0.01 compared to corresponding wt cells).

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SUPPLEMENTARY MATERIALS

Antibodies, flow cytometry and reagents

The following antibodies or conjugates were used in flow cytometry: anti-CD34(RAM34)-FITC, anti-CD115(AFS98)-PE, anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-Ter119 (TER-119) and anti-CD127(A7R34)-PE-Cy5, anti-Gr-1(8C5)-PE-Cy7, anti-CD27(LG.7F9)-APC, anti-c-Kit(2B8)-APC-Alexa Fluor 750, anti-CD11b(M1/70)-FITC, anti-CD16/32 (93), Ly6G(RB6-8C5)-Alexa Fluor 647 (eBioscience); anti-CD3ε(145-2C11)-FITC, anti-CD45.1(A-20)-PE, anti-CD45.2(104)-FITC, streptavidin-APC (BD Pharmingen); anti-Ly6G(1A8)-biotin (Miltenyi Biotec). The following monoclonal antibodies (ATCC) were purified and conjugated using hybridomas: anti-FcγRII/III (2.4G2) conjugated to Alexa Fluor 680 (Molecular Probes/Invitrogen), anti-Sca-1 (E13-161-7) conjugated to Pacific Blue (Molecular Probes/Invitrogen), and anti-Mac-1 (M1/70) conjugated to Pacific Orange (Molecular Probes/Invitrogen). Peripheral blood, BM cells, and splenic cells were stained with antibodies prior to analysis by flow cytometry using FACSAria® (Beckton Dickinson). For intracellular staining, cells were stained for surface markers before fixation and permeabilization using the Fix & Perm kit (Caltag) and stained with anti-MPO antibody (A0398, DAKO) and FITC-anti-rabbit IgG (554020; BD Pharmingen). Antibodies used in immunoblotting: anti-actin (AC-40; Sigma); anti-phosphorylated Jak2 (C80C3), anti-phosphorylated STAT3 (9131), anti-Bcl-x_L (54H6) and anti-IL-1β (2022) (Cell Signaling); anti-$p27^{Kip1}$ (C-19), and anti-STAT3 (C-20) (Santa Cruz); anti-$p21^{Waf1/Cip1}$ (HZ52; Chemicon); anti-IKKβ (10AG2; Upstate); anti-Mdm2 (Ab-2; Oncogene). Mouse rIL-1β and AG490 were purchased from R&D systems and Calbiochem, respectively. Propidium iodide (PI) and Wright-Giemsa solutions were obtained from Sigma and Fisher Scientific, respectively.

SUPPLEMENTARY METHODS

Isolation of macrophages, monocytes, and neutrophils

Peritoneal macrophages were generated and cultured as described[1]. To isolate neutrophils, mice were injected i.p. with 2.5 ml of 3% thioglycollate (Difco) and the peritoneal cavity was flushed with 10 ml ice-cold PBS 4 h later. After blocking Fc receptors with anti-CD16/32, peritoneal cells were

incubated with biotin-labeled anti-Ly6G following magnetic separation using anti-biotin magnetic beads according to manufacturer's instructions (Miltenyi Biotec). To isolate monocytes, peripheral blood was mixed with an equal volume of ice-cold PBS, and then overlaid with 5 ml Percoll (Pharmacia). After spinning at 700 g for 60 min, the interphase between the plasma fraction and the Percoll fraction was transferred into a fresh tube, and monocytes were purified using anti-CD11b magnetic beads according to manufacturer's recommendations (Miltenyi Biotec).

Drug administration

Anakinra® (Kineret, Amgen) was s.c. injected at 250 μg/g body weight. ML120B (a gift from Millennium Pharmaceuticals, Cambridge, MA)[2] was given by oral gavage (150 mg/kg) twice daily for 8 consecutive days before blood was collected.

Transplantation experiments

Donor cells in 100 μl PBS were injected retro-orbitally into *wild-type* or *Ikkβ*$^{\Delta}$ host mice that were gamma-irradiated with 1200 rad and anesthesized using isoflurane (Abbott Labs).

SUPPLEMENTARY REFERENCES

1. Hsu, L.C. *et al.* A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide. *Proc Natl Acad Sci U S A* **105**, 7803-7808 (2008).
2. Nagashima, K. *et al.* Rapid TNFR1-dependent lymphocyte depletion in vivo with a selective chemical inhibitor of IKKbeta. *Blood* **107**, 4266-4273 (2006).

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Supplementary Table 1: Specific primers used in RT-Q-PCR for genes listed

Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
Cph	ATGGTCAACCCCACCGTGT	TTTCTGCTGTCTTTGGAACTTTGTC
Bcl-X_L	AACTCTTTCGGGATGGAGTAAA	GTGGTCATTCAGATAGGTGGC
Notch2	CCCTTGCCCTCTATGTACCA	AGAGGCTGGGAAAGGATGAT
Prok2	GATCTGCACACCTATGGGC	CTTCGCCCTTCTTCTTTCCT
Cd44	TCTGCCATCTAGCACTAAGAGC	GTCTGGGTATTGAAAGGTGTAGC
Fes	CCACCACAACCGCTACGTC	CATCTCCTCGTGCAAATCCTG
Baff	CAGCGACACGCCGACTATAC	CCTCCAAGGCATTTCCTCTTTT
F11r	TCTCTTCACGTCTATGATCCTGG	TTTGATGGACTCGTTCTCGGG
Raf1	TGGACTCAAAGATGCGGTGTT	AAAACCCGGATAGTATTGCTTGT
Bcl6	CCGGCACGCTAGTGATGTT	TGTCTTATGGGCTCTAAACTGCT
Ppmld	GATGTATGTAGCGCATGTAGGTG	GTTCTGGCTTGTGATCTTGTGT
Mdm2	GATTGCCTGGATCAGGATTCAGTT	GGCTGTAATCTTCCGAGTCCAGA
Maged1	GAGCTATGGCTCAGAAACCG	TCCATCAAGGTCTGCACAAG
Apoe	CTGACAGGATGCCTAGCCG	CGCAGGTAATCCCAGAAGC
Fadd	GCGCCGACACGATCTACT	CACAATGTCAAATGCCACCT
Pml	ATATCTTCTGCTCCAATACCAAC	TCATGCCACTGCTGAATCTCC

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