

IL-1 β -driven neutrophilia preserves antibacterial defense in the absence of the kinase IKK β

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Transcription factor NF- κ B and its activating kinase IKK β are associated with inflammation and are believed to be critical for innate immunity. Despite the likelihood of immune suppression, pharmacological blockade of IKK β -NF- κ B has been considered as a therapeutic strategy. However, we found neutrophilia in mice with inducible deletion of IKK β (*Ikk β ^Δ* mice). These mice had hyperproliferative granulocyte-macrophage progenitors and pregranulocytes and a prolonged lifespan of mature neutrophils that correlated with the induction of genes encoding prosurvival molecules. Deletion of interleukin 1 receptor 1 (IL-1R1) in *Ikk β ^Δ* mice normalized blood cellularity and prevented neutrophil-driven inflammation. However, *Ikk β ^ΔIl1r1^{-/-}* mice, unlike *Ikk β ^Δ* mice, were highly susceptible to bacterial infection, which indicated that signaling via IKK β -NF- κ B or IL-1R1 can maintain antimicrobial defenses in each other's absence, whereas inactivation of both pathways severely compromises innate immunity.

Neutrophils are phagocytic cells that provide a critical first line of innate immune defense against bacterial and fungal infection¹. Nonactivated neutrophils circulate in the blood with an average half-life of 6–7 h (ref. 2). Peripheral neutrophil counts are tightly maintained by steady-state granulopoiesis, but acute infection or inflammation trigger the rapid mobilization of neutrophil stores and accelerate bone marrow granulopoiesis^{3,4}. Circulating neutrophils are then activated and migrate toward the lesion, where they kill microbes through phagocytosis, the release of soluble antimicrobials and the formation of extracellular traps made of neutrophils^{1,5,6}. During microbial killing, neutrophils undergo accelerated apoptosis due to oxidative stress caused by intracellular H₂O₂ production⁷. Neutrophils also have a major role in wound healing, and overexuberant neutrophil responses contribute to pathological, often destructive, inflammatory processes⁸. Like other blood cells, neutrophils originate from self-renewing long-term hematopoietic stem cells^{4,9,10}. By asymmetric division, these cells give rise to short-term hematopoietic stem cells that have limited self-renewal capacity and give rise to multipotent progenitors^{9–11}. A clonogenic common myeloid progenitor (CMP) produced by multipotent progenitors gives rise to progenitors restricted to either the megakaryocyte-erythrocyte or granulocyte-macrophage (GMP) lineage¹⁰. The proliferation of such progenitors is controlled by several hematopoietic growth factors, including G-CSF, M-CSF, GM-CSF, interleukin 3 (IL-3), IL-6 and SCF¹².

A key role in inflammation is served by the transcription factor NF- κ B (A002937); this has given rise to the idea that inhibitors of the activation of NF- κ B can be used for the prevention and treatment of chronic inflammatory conditions¹³. NF- κ B is also upregulated in cancer, in which it is responsible for the inhibition of cell death and expression of tumor-promoting cytokines¹⁴. However, activation of NF- κ B is also known to be critical for innate and adaptive immunity^{15,16}. Activation of NF- κ B depends on the inhibitor of κ B kinase (IKK) complex, especially its IKK β catalytic subunit (A001172)¹⁷. Despite the potential risk of inducing immunodeficiency, much effort has been placed on the development of IKK β inhibitors as potential anti-inflammatory or anticancer drugs^{18,19}. It was therefore unexpected that such inhibitors (for example, ML120B²⁰) were found to increase inflammation in mice²¹. Similar observations have been obtained with mice in which IKK β is deleted in myeloid cells and in mice subjected to prolonged treatment with another IKK β inhibitor^{21,22}, but the molecular mechanism of spontaneous neutrophilia in the absence of IKK β -NF- κ B has remained unknown. Here we have investigated the basis of this neutrophilia and found it was dependent on IL-1 β (A003663), which acted as a growth factor for neutrophil progenitors and as a survival factor for mature neutrophils. Although inhibition of IL-1 signaling prevented neutrophilia and restored neutrophil homeostasis, it rendered IKK β -deficient mice highly susceptible to bacterial infection, which suggests that enhanced IL-1 β

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production represents a compensatory mechanism for maintaining antibacterial defense when NF- κ B is inhibited.

RESULTS

Severe neutrophilia and inflammation after IKK β deletion

We deleted the gene encoding IKK β (*Ikkb*) in cells responsive to type I interferon by administering polyinosinic-polycytidylic acid (poly(I:C)) to mice with conditional deletion of *loxP*-flanked *Ikkb* alleles by Cre recombinase expressed from the *Mx1* promoter²³ (called '*Ikkb* Δ mice' here). *Ikkb* deletion occurred in cells of the myeloid lineage, causing greater neutrophilia in the absence of any overt stimulus than that in mice with *loxP*-flanked *Ikkb* alleles without Cre-mediated deletion (called 'wild-type mice' here)^{21,24} (Fig. 1a). Neutrophilia in *Ikkb* Δ mice occurred as early as 2 weeks after poly(I:C) administration and progressed rapidly (Fig. 1a). Peripheral neutrophil counts increased up to 6×10^4 cells per microliter by 6 months after poly(I:C) injection. The expanded neutrophil populations were positive for the neutrophil marker Ly6G (Fig. 1b) and seemed mature, with normal shape and segmentation (Supplementary Fig. 1a). *Ikkb* Δ mice also had more circulating eosinophils, monocytes and platelets (Supplementary Fig. 1b), whereas B cell and T cell counts remained within the normal range (Supplementary Fig. 1c). Most *Ikkb* Δ mice died approximately 6 months after poly(I:C) administration, apparently succumbing to overwhelming generalized inflammation. Examination of mice killed 2 months after poly(I:C) injection showed that the bone marrow of *Ikkb* Δ mice was packed with neutrophils and neutrophil progenitors (Fig. 1c). *Ikkb* Δ mice also had massive neutrophil infiltrates in spleen and liver (Fig. 1c) and considerable splenomegaly. Flow cytometry showed that *Ikkb* Δ mice had a higher percentage of CD11b⁺Ly6G^{lo} immature neutrophils²⁵ (relative to total CD11b⁺Ly6G⁺ neutrophils) than wild-type mice had, not only in bone marrow but also in the spleen (Fig. 1d). Most of these cells also expressed the neutrophil marker myeloperoxidase²⁵ (Fig. 1d). These results suggested substantial extramedullar production of neutrophils in *Ikkb* Δ mice.

Neutrophilia in *Ikkb* Δ mice is transplantable

To determine whether the neutrophilia in *Ikkb* Δ mice was transplantable, we injected *Ikkb* Δ and wild-type bone marrow into lethally irradiated wild-type mice and counted peripheral neutrophils 3 months later. We also did reciprocal transplantation by injecting wild-type bone marrow into lethally irradiated *Ikkb* Δ mice ($n = 3$; data not shown). The neutrophil counts of wild-type mice reconstituted with *Ikkb* Δ bone marrow cells were very high, but those of mice that received wild-type bone marrow cells and of *Ikkb* Δ mice that received wild-type bone marrow cells remained in the normal range (Fig. 2a and data not shown). To monitor the fate of transplanted cells in wild-type recipients, we transduced *Ikkb* Δ and wild-type bone marrow cells with a luciferase reporter before transplantation. Bioluminescence-based imaging at 30 d after bone marrow transfer showed that luciferase-expressing cells from *Ikkb* Δ donors had accumulated mainly in the spleen, liver and long bones of wild-type recipients (Fig. 2b), the same organs that had higher neutrophil counts in *Ikkb* Δ mice. We detected almost no signal in mice transplanted with wild-type bone marrow cells, which suggested that *Ikkb* Δ bone marrow cells had a much greater proliferative capacity than wild-type bone marrow cells had. To confirm that observation, we transplanted a 1:1 mixture of C57BL/6 (CD45.1⁺) wild-type bone marrow and *Ikkb* Δ (CD45.2⁺) bone marrow into lethally irradiated C57BL/6 (CD45.1⁺) wild-type mice. This experiment confirmed that *Ikkb* Δ bone marrow cell populations expanded much faster than wild-type bone marrow cells did (Fig. 2c). We also observed slightly more *Ikkb* Δ B cells and T cells (nonsignificant difference; Supplementary Fig. 2a,b), as well as many more eosinophils, monocytes and platelets (data not shown). Together our experiments showed that the neutrophilia in *Ikkb* Δ mice was transplantable and that the neutrophilia was driven solely by factors intrinsic to hematopoietic cells, causing accelerated proliferation and/or enhanced survival of neutrophil progenitors or mature neutrophils.

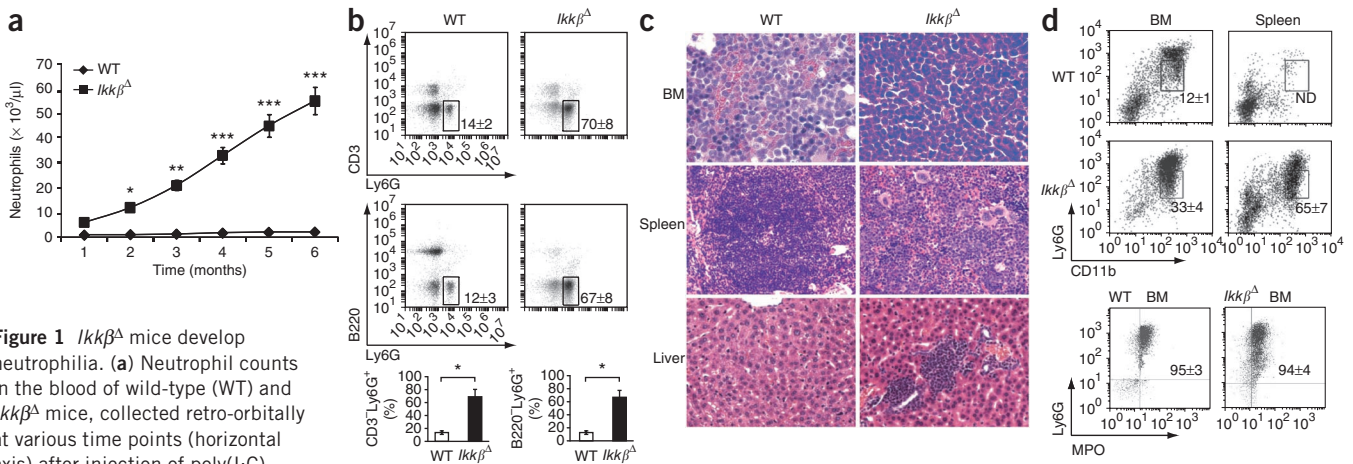


Figure 1 *Ikkb* Δ mice develop neutrophilia. (a) Neutrophil counts in the blood of wild-type (WT) and *Ikkb* Δ mice, collected retro-orbitally at various time points (horizontal axis) after injection of poly(I:C).

* $P < 0.05$, ** $P < 0.02$ and *** $P < 0.01$ (Student's *t*-test). Data are from one experiment with 12 mice per genotype (average \pm s.d.). (b) Flow cytometry of peripheral blood cells collected from wild-type and *Ikkb* Δ mice and stained with fluorescein isothiocyanate-conjugated antibody to CD3 (anti-CD3) and phycoerythrin-conjugated anti-Ly6G (top) or with allophycocyanin-conjugated anti-B220 and phycoerythrin-conjugated anti-Ly6G (middle). Numbers adjacent to outlined areas indicate percent (\pm s.d.) CD3⁺Ly6G⁺ cells (top) or B220⁺Ly6G⁺ cells (middle) among all nucleated cells. Below, quantification of the results above. * $P < 0.01$ (Student's *t*-test). Data are representative of two experiments with three separate measurements per genotype (error bars, s.d.). (c) Hematoxylin and eosin-stained sections of wild-type and *Ikkb* Δ bone marrow (BM), spleen and liver. Original magnification, $\times 40$ (spleen and liver) or $\times 100$ (bone marrow). Data are representative of two experiments with three mice per genotype. (d) Flow cytometry of wild-type and *Ikkb* Δ bone marrow and spleen cells stained with phycoerythrin-conjugated anti-Ly6G and fluorescein isothiocyanate-conjugated anti-CD11b, assessing gated Ly6G^{lo}CD11b⁺ immature granulocytes (top). Numbers adjacent to outlined areas indicate percent (\pm s.d.) Ly6G^{lo}CD11b⁺ cells relative to total Ly6G⁺CD11b⁺ cells. ND, not detected. Below, flow cytometry of bone marrow cells stained with anti-Ly6G and antibody to intracellular myeloperoxidase (MPO). Numbers in plots indicate percent (\pm s.d.) Ly6G⁺MPO⁺ cells. Data are representative of three experiments.

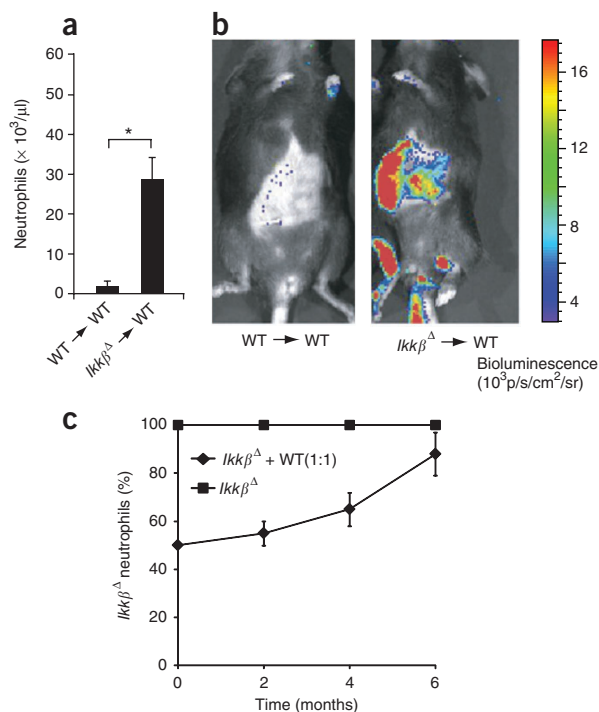


Figure 2 Neutrophilia in IKK β -deficient mice is transplantable.

(a) Neutrophil counts in the peripheral blood of lethally irradiated wild-type mice 3 months after transplantation with wild-type (WT \rightarrow WT) or *Ikkβ* $^{\Delta}$ (*Ikkβ* $^{\Delta}$ \rightarrow WT) bone marrow cells. * $P < 0.01$ (Student's *t*-test). Data are from one experiment with six mice per genotype (error bars, s.d.). (b) Bioluminescence-based imaging of irradiated wild-type mice 30 d after adoptive transfer of wild-type or *Ikkβ* $^{\Delta}$ bone marrow cells transduced with a luciferase reporter. Right (heat map), bioluminescence in photons per second per cm^2 per steradian ($\text{p/s/cm}^2/\text{sr}$): minimum, 3×10^3 ; maximum, 17×10^3 . Data are representative of three experiments per group. (c) Peripheral *Ikkβ* $^{\Delta}$ neutrophils in lethally irradiated CD45.1 $^+$ C57BL/6 wild-type mice given transplantation of 5×10^6 bone marrow cells from CD45.2 $^+$ *Ikkβ* $^{\Delta}$ mice plus 5×10^6 bone marrow cells from CD45.1 $^+$ C57BL/6 wild-type mice, or 1×10^7 CD45.2 $^+$ *Ikkβ* $^{\Delta}$ bone marrow cells alone (positive control); results were calculated on the basis of differential blood counts and on flow cytometry with labeled anti-CD45.1 (wild-type cells) or anti-CD45.2 (*Ikkβ* $^{\Delta}$ cells) and anti-Ly6G. Data are representative of three experiments with three mice per group (mean \pm s.d.).

infiltration (Fig. 3c,d and Supplementary Fig. 3a,b). Eosinophil, monocyte and platelet counts were also normal in *Ikkβ* $^{\Delta}$ *Il1r1* $^{-/-}$ double mutants (data not shown). *Ikkβ* $^{\Delta}$ mice rendered deficient in caspase-1 (*Ikkβ* $^{\Delta}$ *Casp1* $^{-/-}$ mice) still had higher neutrophil counts, but their counts were nowhere near the magnitude of those in *Ikkβ* $^{\Delta}$ mice (Supplementary Fig. 4a). Whereas *Ikkβ* $^{\Delta}$ *Il1r1* $^{-/-}$ mice maintained much higher serum concentrations of IL-1 β (Supplementary Fig. 4b), *Ikkβ* $^{\Delta}$ *Casp1* $^{-/-}$ mice had lower circulating IL-1 β (Supplementary Fig. 4c), which supports published findings showing that caspase-1 is involved in macrophage- and monocyte-derived production of IL-1 β in *Ikkβ* $^{\Delta}$ mice²¹. Nonetheless, circulating concentrations of IL-1 β were higher in *Ikkβ* $^{\Delta}$ *Casp1* $^{-/-}$ mice than in wild-type mice, which indicated that some of the IL-1 β in *Ikkβ* $^{\Delta}$ mice was derived from a caspase-1-independent source. Histological analysis of *Ikkβ* $^{\Delta}$ *Il1r1* $^{-/-}$ spleens showed a nearly complete reversal of the disrupted splenic architecture caused by massive neutrophil infiltration and extramedullary hematopoiesis in *Ikkβ* $^{\Delta}$ mice (Fig. 3e and Supplementary Fig. 5). Pharmacological mimicry of the IKK β deficiency via treatment of wild-type mice with the IKK β inhibitor ML120B²⁰ also led to much higher neutrophil counts within 8 d of treatment (Fig. 3f). This neutrophilia was preventable by the combination of ML120B and the IL-1R antagonist anakinra. Together these results indicate that

IL-1 β signaling is responsible for neutrophilia

Ikkβ $^{\Delta}$ mice produce more IL-1 β than wild-type mice after challenge with lipopolysaccharide or bacterial infection²¹. Here we found that even without any exogenous stimulus, *Ikkβ* $^{\Delta}$ mice produced more circulating IL-1 β than did age-matched wild-type mice, whereas the two groups did not differ in their circulating tumor necrosis factor (Fig. 3a). Isolated *Ikkβ* $^{\Delta}$ monocytes and macrophages secreted considerable amounts of IL-1 β even without stimulation, but neutrophils did not (Fig. 3b). That finding is in line with published observations describing monocytes and macrophages as the main sources of IL-1 β ²⁶. Notably, *Ikkβ* $^{\Delta}$ mice also deficient in IL-1 receptor 1 (IL-1R1; *Ikkβ* $^{\Delta}$ *Il1r1* $^{-/-}$ mice) maintained almost normal neutrophil counts and did not develop splenomegaly or neutrophilic organ

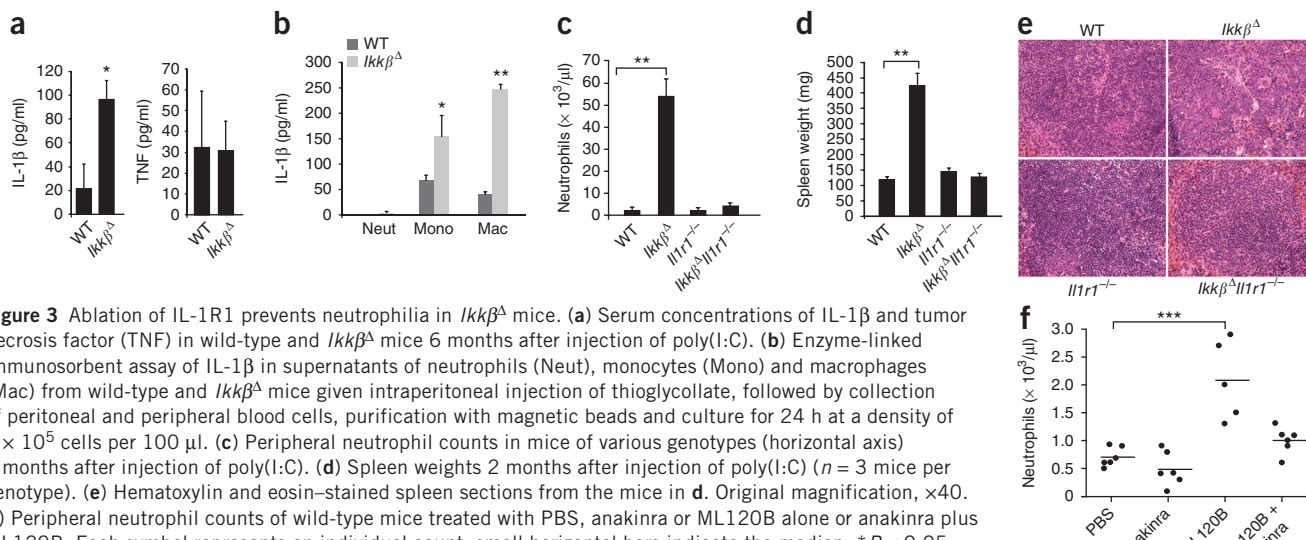
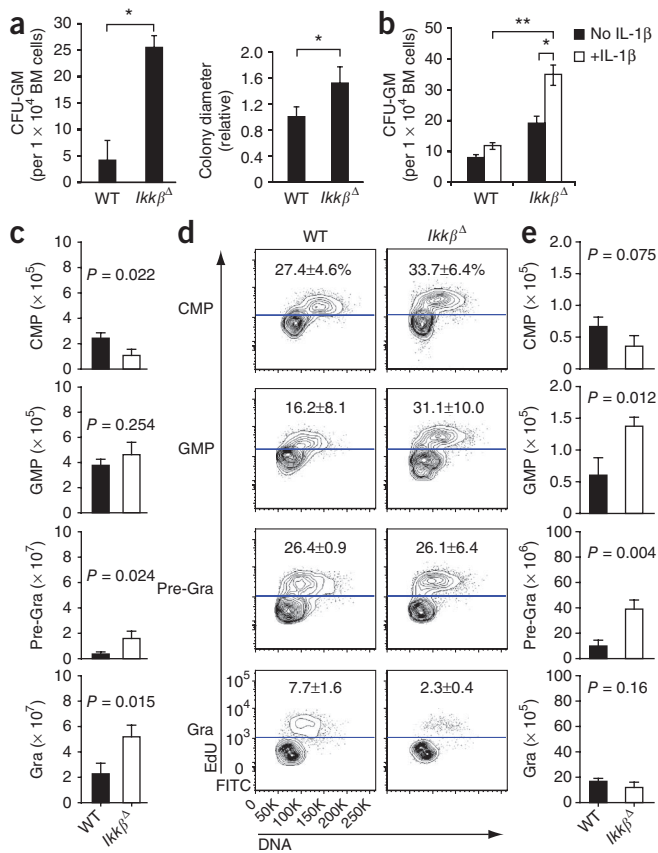


Figure 3 Ablation of IL-1R1 prevents neutrophilia in *Ikkβ* $^{\Delta}$ mice. (a) Serum concentrations of IL-1 β and tumor necrosis factor (TNF) in wild-type and *Ikkβ* $^{\Delta}$ mice 6 months after injection of poly(I:C). (b) Enzyme-linked immunosorbent assay of IL-1 β in supernatants of neutrophils (Neut), monocytes (Mono) and macrophages (Mac) from wild-type and *Ikkβ* $^{\Delta}$ mice given intraperitoneal injection of thioglycollate, followed by collection of peritoneal and peripheral blood cells, purification with magnetic beads and culture for 24 h at a density of 1×10^5 cells per 100 μl . (c) Peripheral neutrophil counts in mice of various genotypes (horizontal axis) 6 months after injection of poly(I:C). (d) Spleen weights 2 months after injection of poly(I:C) ($n = 3$ mice per genotype). (e) Hematoxylin and eosin-stained spleen sections from the mice in d. Original magnification, $\times 40$. (f) Peripheral neutrophil counts of wild-type mice treated with PBS, anakinra or ML120B alone or anakinra plus ML120B. Each symbol represents an individual count; small horizontal bars indicate the median. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (Student's *t* test). Data are representative of two experiments with three mice per genotype (a–e) or two experiments with six mice per group (f; mean and s.d. in a–d).



excessive IL-1 β signaling is responsible for the uncontrolled neutrophilia and inflammation in IKK β -deficient mice.

Hyperproliferation of *Ikkβ Δ* granulocyte progenitors

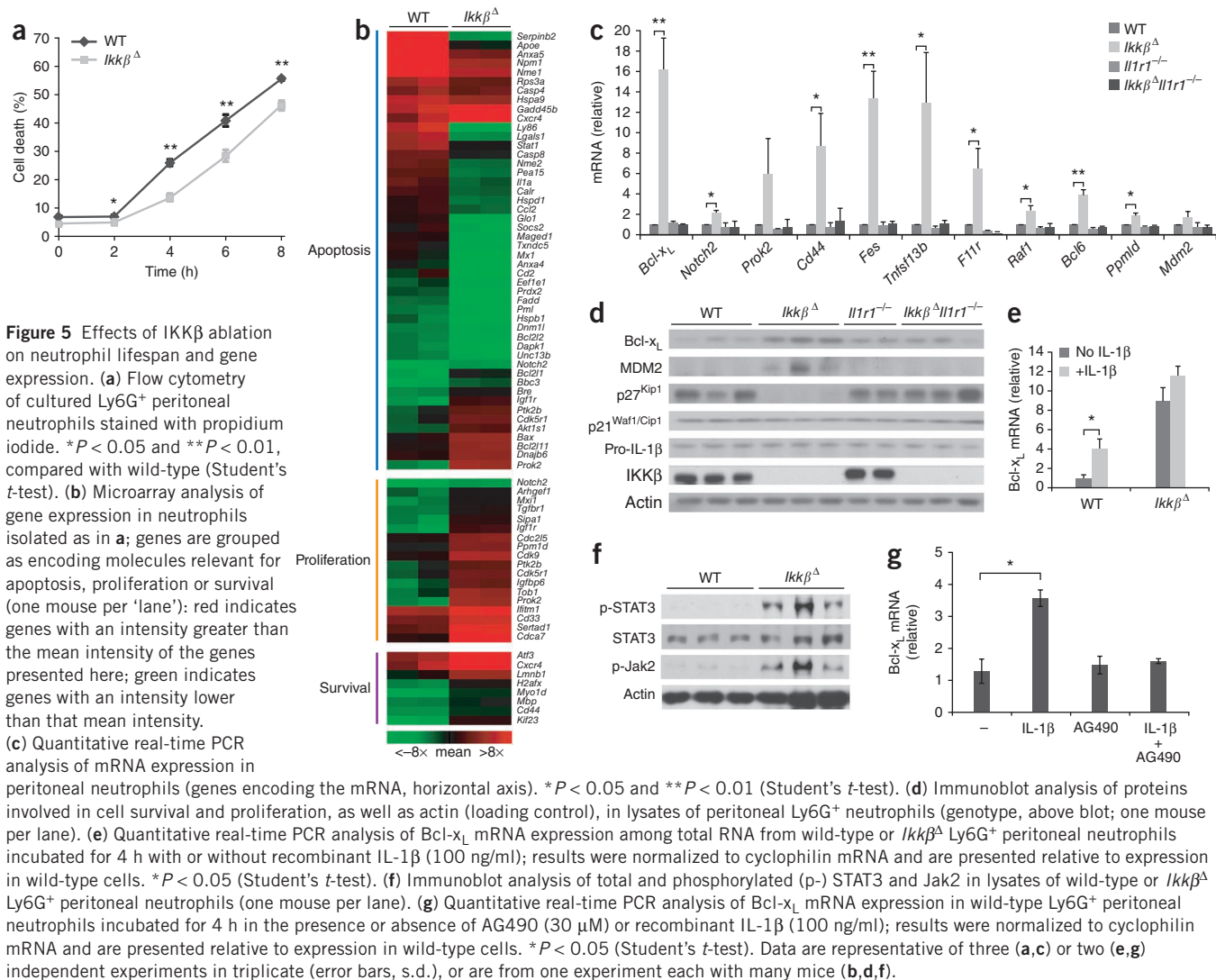
We determined whether the neutrophilia in *Ikkβ Δ* mice was due to enhanced proliferation of granulocyte progenitors. We plated equal numbers of wild-type, *Ikkβ Δ* , *Il1r1 $^{-/-}$* and *Ikkβ Δ Il1r1 $^{-/-}$* bone marrow cells in stem cell medium and analyzed colonies 10 d later. *Ikkβ Δ* bone marrow cells gave rise to many more and larger granulocyte-macrophage colony-forming units (CFU-GM) than did wild-type bone marrow cells (Fig. 4a and Supplementary Fig. 6), and IL-1R1 deletion in the context of IKK β deficiency restored CFU-GM numbers to the normal range (*Ikkβ Δ Il1r1 $^{-/-}$* bone marrow mean colony number, 5 ± 3 ; data not shown). The absence of IL-1R1 signaling also normalized colony size (data not shown). Microscopic examination of colonies from *Ikkβ Δ* bone marrow and wild-type bone marrow cells showed that most of the cells were neutrophilic granulocytes or their progenitors (Supplementary Fig. 6). To determine whether exogenous IL-1 β was sufficient to enhance CFU-GM, we incubated wild-type and *Ikkβ Δ* bone marrow cells with or without recombinant IL-1 β . Although recombinant IL-1 β had only a small effect on the CFU-GM of wild-type bone marrow cells, it resulted in considerably enhanced CFU-GM of *Ikkβ Δ* bone marrow cells (Fig. 4b). These results are consistent with the results of the bone marrow-mixture experiments described above and indicate a cell-intrinsic defect that enhances IL-1 β responsiveness in the absence of IKK β . To pinpoint the progenitor cell type that gives rise to the greater abundance of neutrophils in *Ikkβ Δ* mice, we determined the frequency of CMPs, GMPs, pre-granulocytes and granulocytes by flow cytometry assessing size and cell-cycle status¹⁰. We found that *Ikkβ Δ* bone marrow had a slightly smaller CMP population than did wild-type bone marrow,

Figure 4 Larger granulocyte progenitor populations in *Ikkβ Δ* mice. (a) Colony count (left) and diameter (right) of wild-type and *Ikkβ Δ* bone marrow cells grown for 10 d in Methocult progenitor cell medium at a density of 3.3×10^3 cells per ml. Diameter results are presented relative to those of wild-type cells, set as 1. * $P < 0.05$ (Student's *t*-test). Data are representative of three experiments with three plates per mouse and three mice per genotype (left) or 27 colonies per genotype (right; mean and s.d.). (b) Colony count of wild-type and *Ikkβ Δ* bone marrow cells cultured as in a with or without recombinant IL-1 β (100 ng/ml). * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test). Data are representative of two experiments (mean \pm s.d.). (c) Size of CMP, GMP, pre-granulocyte (Pre-Gra) and granulocyte (Gra) populations from the bone marrow of wild-type and *Ikkβ Δ* mice, assessed by flow cytometry and presented as absolute number per bilateral hind limbs. Data are representative of two experiments with three mice per genotype (mean and s.d.). (d) Incorporation of EdU and DNA content of progenitor cell populations from the bone marrow collected from mice 3 h after injection of EdU (200 μ g). Numbers in plots indicate percent EdU $^+$ cells (mean \pm s.d.). Data are from one representative of three independent experiments. (e) Actual number of cells in the S-G2-M portion of the cell cycle in d. *P* values, Student's *t*-test. Data are from one representative of three independent experiments (error bars, s.d.).

which could have reflected a negative feedback mechanism driven by the much larger pre-granulocyte or granulocyte population in *Ikkβ Δ* bone marrow (Fig. 4c). In contrast, the GMP population was not very different in bone marrow of the two genotypes. We next checked the proliferation status of the various progenitor cell populations by injecting mice with 5-ethynyl-2'-deoxyuridine (EdU) 3 h before collecting bone marrow. The incorporation of EdU was much greater in the GMP population, but not in the CMP, pre-granulocyte or granulocyte population, of *Ikkβ Δ* bone marrow than that of wild-type bone marrow (Fig. 4d). However, when we compared the number of cells in the S-G2-M portion of the cell cycle, we found significantly more cycling GMPs and pre-granulocytes in *Ikkβ Δ* bone marrow than in wild-type bone marrow (Fig. 4e). These results suggest that at least some of the neutrophilia in *Ikkβ Δ* mice is due to enhanced proliferation of GMPs and pre-granulocyte progenitors.

Longer lifespan of IKK β -deficient neutrophils

Another factor that could have contributed to the neutrophilia in *Ikkβ Δ* mice was a longer neutrophil lifespan. To examine this possibility, we purified thioglycollate-elicited peritoneal neutrophils from wild-type and *Ikkβ Δ* mice with anti-Ly6G magnetic beads and cultured the cells. We stained the cells with propidium iodide at various time points and assessed by flow cytometry the frequency of propidium iodide-positive cells, considered nonviable. Wild-type neutrophils died faster than *Ikkβ Δ* neutrophils did (Fig. 5a). Of note, neutrophil proliferation was negligible during this period of observation (Supplementary Fig. 7). Similarly, we observed that purified *Ikkβ Δ* Ly6G $^+$ peripheral blood neutrophils had a longer lifespan than their wild-type counterparts had (data not shown). Microarray analysis of thioglycollate-elicited peritoneal neutrophils identified several genes involved in cell proliferation and survival that were upregulated in *Ikkβ Δ* neutrophils relative to their expression in wild-type neutrophils (Fig. 5b). One such gene was *Cd33*, which encodes a sialoadhesin family member thought to be associated with the proliferation of myeloid progenitor cells²⁷. Conversely, proapoptotic genes were downregulated in *Ikkβ Δ* neutrophils and antiapoptotic genes were upregulated. We confirmed by quantitative real-time PCR analysis the microarray data for key genes shown to be involved in hematopoiesis; this also included *Il1r1 $^{-/-}$* and *Ikkβ Δ Il1r1 $^{-/-}$* neutrophils (Fig. 5c and Supplementary Fig. 8). Unexpectedly, genes typically activated by NF- κ B in other cell types, such as those encoding



the antiapoptotic protein Bcl-x_L (refs. 28,29) and B cell-activation factor BAFF³⁰, were upregulated in *Ikk β ^Δ* neutrophils. We confirmed by immunoblot analysis higher expression of Bcl-x_L in *Ikk β ^Δ* neutrophils (Fig. 5d). Immunoblot analysis also showed that the cell cycle inhibitor p27 (ref. 31) was downregulated in *Ikk β ^Δ* neutrophils. Expression of Bcl-x_L mRNA was induced by IL-1 β in wild-type neutrophils, but there was little Bcl-x_L induction in neutrophils from *Ikk β ^Δ* mice because they had higher basal Bcl-x_L expression (Fig. 5e).

STAT3 is another transcription factor that controls Bcl-x_L expression³²; STAT3 activity is enhanced in IKK β -deficient hepatocytes³³. Phosphorylation of STAT3 and its activating kinase Jak2 was enhanced in *Ikk β ^Δ* neutrophils (Fig. 5f). Furthermore, inhibition of Jak2 activity suppressed the IL-1 β -mediated induction of Bcl-x_L in mature neutrophils (Fig. 5g). These data suggest that the higher Bcl-x_L expression in IKK β -deficient neutrophils was due to activation of Jak2 and STAT3. Together these results show that the absence of IKK β renders neutrophils and their progenitors more susceptible to the prosurvival and pro-proliferative effects of IL-1 β .

IKK β -deficient neutrophils retain bactericidal activity

We determined whether *Ikk β ^Δ* neutrophils retained normal bactericidal activity. We isolated peritoneal neutrophils after

thioglycollate injection and assessed their ability to kill the bacterial pathogen group A *Streptococcus* (GAS). Unexpectedly, we observed no significant difference between wild-type and *Ikk β ^Δ* neutrophils in their bacterial killing (Fig. 6a). We also injected GAS subcutaneously into wild-type, *Ikk β ^Δ*, *Il1r1^{-/-}* and *Ikk β ^ΔIl1r1^{-/-}* mice and monitored the development of necrotic skin lesions. Unexpectedly, *Ikk β ^Δ* mice developed the smallest lesions with the fewest surviving bacteria, whereas *Ikk β ^ΔIl1r1^{-/-}* mice had the largest lesions (Fig. 6b,c and Supplementary Fig. 9). Whereas containment of the infectious challenge was better in *Ikk β ^Δ* mice, probably reflective of the greater neutrophilic infiltration of the infected skin area (Fig. 6d), host defense was severely compromised in *Ikk β ^ΔIl1r1^{-/-}* mice (Fig. 6b). In contrast to *Ikk β ^Δ* and *Il1r1^{-/-}* mice, none of the *Ikk β ^ΔIl1r1^{-/-}* mice survived longer than 4 d after infection (data not shown). Moreover, neutrophils from *Ikk β ^ΔIl1r1^{-/-}* and *Il1r1^{-/-}* mice showed impaired bactericidal function *in vitro* compared with that of wild-type neutrophils (Supplementary Fig. 10). In summary, IKK β -activated NF- κ B was not critical for maintenance of antibacterial immunity, as its absence was compensated for by more IL-1 β signaling. Reciprocally, IL-1 β signaling is not essential for bacterial containment in IKK β -NF- κ B-competent mice.

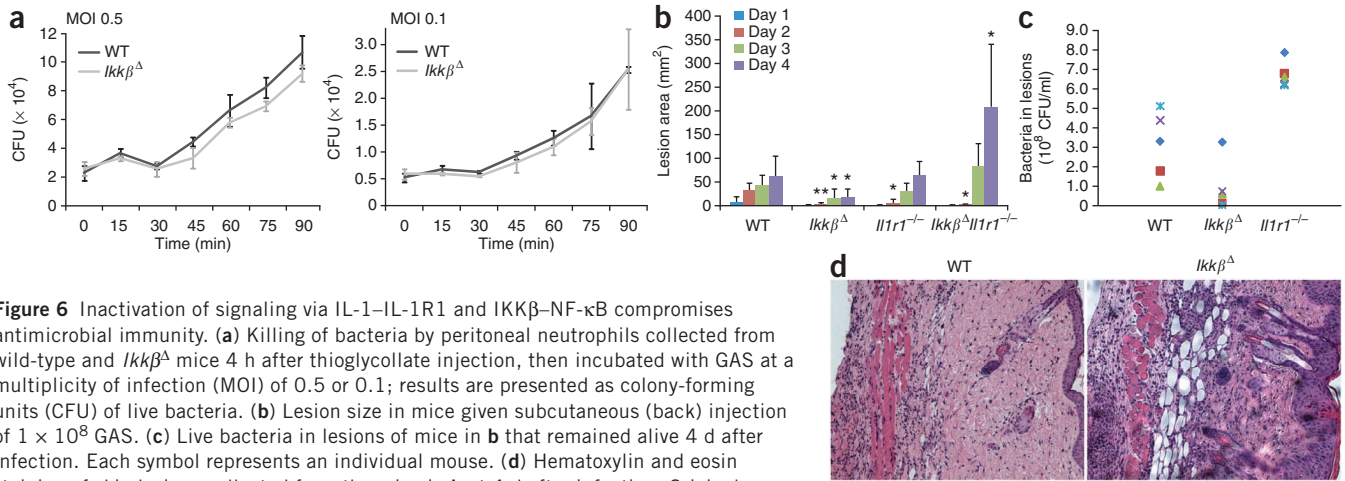


Figure 6 Inactivation of signaling via IL-1-IL-1R1 and IKK β -NF- κ B compromises antimicrobial immunity. (a) Killing of bacteria by peritoneal neutrophils collected from wild-type and *Ikk β Δ* mice 4 h after thioglycollate injection, then incubated with GAS at a multiplicity of infection (MOI) of 0.5 or 0.1; results are presented as colony-forming units (CFU) of live bacteria. (b) Lesion size in mice given subcutaneous (back) injection of 1×10^8 GAS. (c) Live bacteria in lesions of mice in b that remained alive 4 d after infection. Each symbol represents an individual mouse. (d) Hematoxylin and eosin staining of skin lesions collected from the mice in b at 4 d after infection. Original magnification, $\times 10$. * $P < 0.05$ and ** $P < 0.01$, compared with wild type (Student's *t*-test). Data are representative of three independent experiments in triplicate (a; mean \pm s.d.) or two experiments with five mice per genotype (b-d; error bars, s.d.).

DISCUSSION

Because it is activated by most if not all pattern-recognition receptors, IKK β -dependent NF- κ B signaling is considered the key regulator of innate immune responses^{15,16}. Thus, the main undesired side effect of inhibition of IKK β -NF- κ B as a therapeutic strategy was expected to be greater susceptibility to infection¹⁹. Unexpectedly, however, we found here that mice in which IKK β was deleted from cells of the myeloid cell lineage as well as in other interferon-responsive cells (*Ikk β Δ* mice) were no less able to fight infection with GAS than were control wild-type mice. On the contrary, *Ikk β Δ* mice showed greater clearance of GAS than that of their wild-type counterparts, most probably due to their much greater neutrophil count, driven by the enhanced IL-1 β production that accompanies inhibition of IKK β -NF- κ B^{21,34,35}. Inhibition of IL-1R1 signaling rendered *Ikk β Δ* mice immunocompromised and unable to control infection with GAS but did not compromise anti-GAS immunity in IKK β -NF- κ B-competent mice. These findings support the published hypothesis that signaling via IKK β -NF- κ B and IL-1 β -IL-1R1 provides alternative pathways toward the activation of antibacterial defenses and that upregulation of IL-1 β in response to IKK β -NF- κ B deficiency provides a safety net that compensates for loss of NF- κ B-dependent antibacterial immunity²¹. However, the upregulation of IL-1 β production in IKK β -NF- κ B-deficient mice ultimately comes at a price: severe and destructive inflammation due to sustained massive neutrophilia.

Neutrophilia caused by inhibition of NF- κ B has been seen in lethally irradiated mice reconstituted with fetal liver cells from mice deficient in the NF- κ B subunit RelA^{34,35} and *Ikk β Δ* mice³⁶ and in mice treated with various IKK β inhibitors^{20,22}. However, until now, the exact cause of the neutrophilia triggered by inhibition of IKK β -NF- κ B and ways to prevent it have not been identified, to our knowledge. Our results indicate that the spontaneous neutrophilia is caused by a combination of two factors. First, suppression of the basal IKK β -NF- κ B activity in myeloid cells without any stimulation induces production of the proinflammatory cytokine IL-1 β . Second, inhibition of NF- κ B in neutrophils and their progenitors results in the upregulation of signaling pathways (Jak2-STAT3) and genes important for cell proliferation and survival and the downregulation of proapoptotic and antiproliferative genes. These changes render IKK β -NF- κ B-deficient neutrophils and their progenitors responsive to the pro-proliferative and prosurvival effects of IL-1 β .

That conclusion was supported by the mixed-bone marrow transplantation experiment and the *in vitro* incubation of *Ikk β Δ* and wild-type bone marrow cells with IL-1 β . Despite being exposed to the same higher IL-1 β concentrations, the IKK β -expressing neutrophil population did not expand nearly as much as the IKK β -deficient population did. Although IL-1 β alone is unlikely to be the only cause of severe neutrophilia, its inhibition or ablation of its receptor completely prevented neutrophilia and the resulting destructive inflammatory condition in *Ikk β Δ* mice.

The ability of IL-1 β to stimulate the proliferation of neutrophil progenitors is consistent with a published report showing that IL-1 β (called hemopoietin-1 at that time) acts synergistically with IL-3 to increase the number of colonies formed by primitive hematopoietic progenitors³⁷. Subsequently, IL-1R1 signaling has been found to be essential for the proliferation of granulocyte progenitor cells in response to stimuli such as aluminum hydroxide³⁸. Our findings suggest that the progenitor cell population most responsive to IL-1 β , at least in *Ikk β Δ* mice, is not the CMP population, which gives rise to all myeloid cells¹⁰, but is instead the GMP and pre-granulocyte populations. The slightly lower number of proliferative cycling CMPs in *Ikk β Δ* mice could suggest the presence of a negative feedback mechanism, given the extremely high neutrophil count in these mice. Enhanced proliferation of the GMP population can explain why monocytes and eosinophils are also more abundant in *Ikk β Δ* mice¹⁰.

Mature *Ikk β Δ* neutrophils had a longer lifespan *in vitro* than did wild-type neutrophils, even without incubation with IL-1 β . Unexpectedly, IKK β deficiency resulted in upregulation of the antiapoptotic protein Bcl- x_L , whose expression is transcriptionally stimulated by NF- κ B in other cell types³⁹. The basis for the upregulation of Bcl- x_L in IKK β -deficient neutrophils seemed to be their much greater Jak2-dependent STAT3 activity, found before to occur in response to the accumulation of reactive oxygen species in IKK β -deficient hepatocytes³³. In contrast to antiapoptotic genes, proapoptotic genes were downregulated in *Ikk β Δ* neutrophils, including the gene encoding PML (TRIM19), a known regulator of neutrophil apoptosis⁴⁰. Given the profound effect of IKK β deletion in neutrophils and their progenitors on genes encoding molecules that control cell proliferation and survival, we speculate that the IKK β signaling pathway may be inactivated in malignancies derived from GMPs and their progeny, such as blast-crisis chronic myeloid leukemia⁴¹.

Although our results indicate that inhibition of IL-1 β signaling can be used to prevent the destructive neutrophilia associated with prolonged inhibition of IKK β -NF- κ B, this is an impractical solution for use in the clinic, as combined loss of signaling via IKK β -NF- κ B and IL-1 β -IL-1R1 results in severe impairment of antibacterial defenses. These findings are consistent with the clinical observation that treatment of patients with rheumatoid arthritis with an inhibitor of tumor necrosis factor, a strong activator and effector of IKK β -NF- κ B signaling, together with anakinra greatly enhances the risk of infection⁴². However, partial or temporary inhibition of IKK β -NF- κ B signaling, which is unlikely to trigger massive neutrophilia, could still find utility in the treatment of cancers in which NF- κ B is persistently activated.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession codes. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A002937, A001172 and A003663; GEO: microarray data, GSE25211.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

J.S. and A.M.T. contributed equally to this work. L.-C.H. and T.E. designed and did most of the experiments; M.K. helped in designing experiments; M.K., T.E. and L.-C.H. wrote the paper; J.S. and I.L.W. planned and did most of the progenitor cell analyses; A.M.T. and V.N. planned and did the bacterial killing experiments; and C.-Y.L., T.-Y.L., G.-Y.Y., L.-C.L., V.T., U.S. and T.A. helped with some of the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/natureimmunology/>.

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ONLINE METHODS

Mice. *Casp1*^{-/-} mice, mice with *loxP*-flanked *Ikkbb* alleles (wild type), and mice with deletion of *loxP*-flanked *Ikkbb* alleles by Cre recombinase expressed from the *Mx1* promoter (*Ikkβ*^Δ) have been described^{21,24,43}. For deletion of *Ikkbb*, 200 μg poly(I:C) (Amersham Biosciences) was injected intraperitoneally into 3- to 4-week-old mice three times every other day. *Il1r1*^{-/-} and CD45.1⁺ wild-type mice were from the Jackson Laboratory. All mouse strains were crossed for at least nine generations onto the C57BL/6 background and were housed under conventional barrier protection in accordance with guidelines of the University of California, San Diego, and the US National Institutes of Health. Mouse protocols were approved by the Institutional Animal Care Committee of the University of California, San Diego.

Peripheral blood counts. Retro-orbital blood was collected in capillary tubes (Science Lab). Peripheral blood (50 μl) was collected in microtainer tubes with EDTA (Beckton Dickinson) and was analyzed with a hematology analyzer (Beckman Coulter).

Quantitative PCR analysis and enzyme-linked immunosorbent assay. Total cellular RNA isolated with TRIzol (Invitrogen) was used for synthesis of cDNA with a Superscript III First-Strand Synthesis system (Invitrogen), followed by quantification of cDNA by quantitative RT-PCR (primer sequences, **Supplementary Table 1**)⁴⁴. All values were normalized to the abundance of cyclophilin mRNA, then normalized values were divided by the wild-type value to obtain the relative value. The concentration of IL-1β and tumor necrosis factor in serum and culture supernatants was measured with a DuoSet ELISA Development system (R&D Systems).

Progenitor cell cultures. Bone marrow was isolated and then single cells were collected by grinding of bone marrow through 45-μm filters (Millipore), followed by resuspension in Methocult 03534 medium (StemCell Technologies). Cells were plated onto CELLSTAR tissue culture dishes (35 mm × 10 mm), and assessed for GM-CFU after 10 d at 37 °C according to the morphologic criteria described in the manufacturer's manual (Methocult). Single colonies (GM-CFU) were photographed with a Leica DM IRB microscope equipped with a Leica DFC290 camera and then colonies viewed with a Zeiss Stemi SV11 microscope were picked up with a pipet tip, followed by centrifugation in a Cytospin centrifuge. Cells were stained with Giemsa and photographed with an Olympus BX41 microscope equipped with a Olympus ColorView camera.

Microarray analysis. Total RNA from Ly6G⁺ neutrophils was extracted with TRIzol (Invitrogen) and purified with an RNeasy Micro kit (Qiagen). Biotinylated cRNA was synthesized with an RNA Amplification kit according to the manufacturer's directions (Ambion). Biotin-labeled cRNA was hybridized to a MouseRef-8 Expression BeadChip (Illumina) and results were analyzed with BeadStudio v3.1 software. Partek software was used for data analysis and quality control. Readings were adjusted by quantile normalization and the intensity of genes of interest, chosen by gene-set enrichment analysis, was normalized to the mean intensity of selected genes.

In vitro viability assay. Purified Ly6G⁺ peritoneal neutrophils obtained after thioglycollate injection were cultured at a density of 1 × 10⁶ cells per ml in RPMI medium plus 10% (vol/vol) FBS. Samples (100 μl) were collected at various time points, and propidium iodide exclusion was used for measurement of cell death as described⁴⁵. For analysis of the proliferation of mature neutrophils, peritoneal neutrophils were collected at various time points and fixed overnight at -20 °C in 70% (vol/vol) ethanol. Cells were then stained with propidium iodide and analyzed by flow cytometry for subdiploid DNA content.

Neutrophil killing assay. Peritoneal neutrophils were collected and resuspended at a density of 3.3 × 10⁶ cells per ml in RPMI medium plus 2% (vol/vol) FBS. GAS bacteria were grown overnight in Todd-Hewitt broth (Difco), then were diluted and grown to mid-log phase. Bacteria were resuspended in RPMI medium plus 2% (vol/vol) FBS and then were added to siliconized tubes containing 1 × 10⁶ suspended neutrophils at a multiplicity of infection of 0.5 or 0.1 bacteria per cell. An aliquot of each tube (25 μl) was diluted and immediately plated on Todd-Hewitt agar (Difco) for counting (time = 0). Tubes were placed

under rotation at 37 °C, and 25-μl aliquots were diluted and plated at each time point. In control assays examining the inhibition of GAS growth by wild-type neutrophils, bacteria proliferated to 50–75% greater numbers in medium with heat-killed neutrophils than in medium containing live neutrophils.

Mouse skin infection. Overnight cultures of GAS bacteria were diluted and grown to mid-log phase in Todd-Hewitt broth. Bacteria were concentrated and then were mixed with sterile Cytodex beads at a ratio of 1:1 (Sigma). An inoculum of 1 × 10⁸ colony-forming units was injected subcutaneously into the shaved backs of mice. Lesions were measured daily and mice were killed on day 4. Lesions were excised, homogenized, diluted and plated for counting of surviving bacteria⁴⁶.

Transduction of bone marrow cells. Equal numbers of bone marrow cells were transduced according to established methods through the use of a lentivirus containing a luciferase reporter controlled by the cytomegalovirus promoter^{47,48}. Bioluminescence was measured with an *in vivo* imaging system (IVIS 200; Caliper).

Cell cycle analysis of progenitor populations. The cell-cycle status of each cell population was assessed with a Click-iT EdU Flow Cytometry Assay kit according to manufacturer's instructions (Molecular Probes–Invitrogen). EdU (200 μg in saline) was administered to each mouse by intraperitoneal injection 3 h before mice were killed. Bone marrow was collected as described above and was kept at 4 °C until fixation. After being counted, cells were stained with fluorescein isothiocyanate-conjugated anti-CD34 (RAM34; eBioscience); phycoerythrin-conjugated anti-CD115 (antibody to macrophage colony-stimulating factor receptor; AFS98; eBioscience); phycoerythrin-indodicarbocyanine-conjugated lineage (Lin) antibodies (anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-Ter119 (TER-119) and anti-CD127 (A7R34); all from eBioscience); phycoerythrin-indodicarbocyanine-conjugated anti-Gr-1 (8C5; eBioscience); allophycocyanin-conjugated anti-CD27 (LG.7F9; eBioscience); Alexa Fluor 680-conjugated anti-FcγRII/III (93; made in the I.L.W. laboratory); allophycocyanin-Alexa Fluor 750-conjugated anti-c-Kit (2B8; eBioscience); Pacific blue-conjugated anti-Sca-1 (E13-161.7; made in the I.L.W. laboratory); and Pacific orange-conjugated anti-Mac-1 (M1/70; made in the I.L.W. laboratory). Cells (5 × 10⁴ from each population) were sorted with a FACSaria (Beckton Dickinson) on the basis of the expression of a combination of cell surface markers as follows: CMP, Lin⁻CD27⁺c-Kit⁺Sca-1⁻CD34⁺FcγRII/III^{lo-neg}, GMP, Lin⁻CD27⁺c-Kit⁺Sca-1⁻CD34⁺FcγRII/III^{hi}; pre-granulocytes, Lin⁻CD27⁻c-Kit⁻CD115⁻Mac-1⁺Gr-1⁻; and granulocytes, Lin⁻CD27⁻c-Kit⁻CD115⁻Mac-1⁺Gr-1⁺. Sorted cells were processed for detection of the incorporation of EdU. Cells were fixed and made permeable and then were allowed to react with Click-iT Alexa Fluor 488. DNA was stained with CellCycle 633-red. Processed cells were analyzed with a FACSaria. Flow cytometry data were analyzed with FlowJo 8.8.6 software (TreeStar). An unpaired Student-*t* test after variance validation by F-test on Prism 5 software (GraphPad) was used for all statistical comparisons.

Statistical analysis. Differences between averages were analyzed by Student's *t*-test.

Additional methods. Information on the isolation of macrophages, monocytes and neutrophils, drug administration and transplantation experiments is available in the **Supplementary Methods**.

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