Supporting Information

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SI Materials and Methods

Mice. *Ikkβ*$^{\Delta}$ mice were crossed with *Tnfr*$^{-/-}$, *Il1r*$^{-/-}$, *Rag1*$^{-/-}$, or NU-*Foxn1*^{nu} mice (all purchased from Jackson Laboratory), or *Asc*$^{-/-}$ (1) and *Nlrp3*$^{-/-}$ (2) to generate respective compound mutants. All mice were on a mixed genetic background and, in all experiments, Cre-negative littermate controls (*Ikkβ*$^{F/F}$) were used. *Prtn3/Ela2*$^{-/-}$ (3) and *Casp1*$^{-/-}$ mice (4) were on a C57/B6 background. To induce deletion of *Ikkβ*, mice were injected once with 250 μL of poly(I:C) (Sigma) i.p. and were generally analyzed after 21 d unless stated otherwise. Littermate controls received the same amount of poly(I:C). In neutralization experiments, 10 μg of rat α-G-CSF (MAB414; R&D Systems) or rat IgG1 isotype control (MAB 005; R&D Systems) were injected i.p. daily for 21 d. For ex vivo culture of bone marrow cells, total bone marrow was flushed from the hind limbs followed by RBC lysis. Cells (1×10^6) were stimulated with TNF-α (Peprotech) for 24 h in RPMI media containing 10% bovine FCS and 1% penicillin/streptomycin. ML120B was provided by Millenium Pharmaceuticals.

Flow Cytometry and Cell Separation. The following antibodies were used for FACS analysis: FITC-labeled anti-CD11b (BD Biosciences), PE-labeled anti-Gr-1 (eBioscience), FITC-labeled anti-CD34 (eBioscience), PE-labeled anti-FcγRII/III (eBioscience), APC-labeled anti–c-Kit (eBioscience), APC-Cy7–labeled anti–Sca-1 (BD Biosciences), APC-labeled anti–IL-7Rα (eBioscience), PerCP Cy5.5–labeled anti–IL-7Rα (eBioscience), PE-labeled anti-F4/80 (eBioscience), APC-labeled anti–IL-17 (eBioscience), FITC-labeled anti-CD3 (BD Biosciences), and FITC-labeled anti-CD4 (BD Biosciences). Annexin V (BD Biosciences) was used to quantify apoptotic cells. IL-7Rα^{+} and IL-7Rα^{-}, as well as CD34^{+} and CD34^{-}, cells were purified by using respective antibodies and magnetically separated by using anti-APC (IL-7Rα) or anti-FITC (CD34) microbeads according to the manufacturer's instructions (Miltenyi Biotec).

For detection of intracellular IL-17, MLN cells were isolated and stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of Golgi Plug (BD Pharmingen) for 4 h before permeabilization and staining. FACS data were acquired on a BD FACSCalibur (Becton Dickinson) and analyzed using FlowJo software (TreeStar). Myeloid progenitors were identified on a Gallios flow cytometer and data were analyzed by using Kaluza software (Beckman Coulter).

In Vitro T-Cell Differentiation. T cells were isolated by using the CD4^{+} T Cell Isolation Kit II (Miltenyi Biotech) and incubated in anti-CD3 (4 μg)/anti-CD28 (2 μg)–coated plates in the presence of recombinant mouse IL-6, IL-23, TGF-β, and IL-1β (Peprotech).

Protein and RNA Analysis. For immunoblot analysis, the following antibodies were used: IL-1β (R&D Systems), IKKβ (UBI), and β-actin (Sigma). IL-1β, IL-6, IL-17, IFN-γ, and G-CSF levels in plasma and cell culture supernatants were measured using the respective DuoSet ELISA system (R&D Systems). RNA isolation and cDNA synthesis was performed as previously described (1), and real-time PCR analysis with a Power SYBR Green PCR Master Mix (Applied Biosystems) was performed on a StepOne Plus Real-Time PCR system (Applied Biosystems). RNA levels were normalized to cyclophilin levels and calculated as $2^{(\text{Ct cyclophilin} - \text{Ct gene of interest})}$ and plotted as arbitrary units on a linear scale. Primer sequences are available on request.

Induction of EAE. Twenty-one days after poly(I:C) administration, *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta}$ were immunized s.c. at the dorsal flanks with 100 μg of $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in complete Freund adjuvant (250 μg of *Mycobacterium* extract H37Ra per mouse). Pertussis toxin (200 ng) was given i.p. on days 0 and 2 after immunization. Occurrence of EAE was clinically scored when the mice had developed a limp tail or gait alterations (5). For MOG recall experiments, *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta}$ mice were killed on day 12 after $MOG_{35–55}$ immunization and draining lymph node cells were cultured in the presence of various concentrations of $MOG_{33–55}$ peptide. The supernatants were collected after 48 h of antigen reexposure for determination of cytokine secretion by ELISA and proliferation was determined by [^{3}H]-thymidine incorporation assay and compared with T cells that had not been rechallenged to determine stimulation index.

Statistical Analysis. Data are expressed as mean ± SE. Differences were analyzed by log-rank or Student *t* test by using Prism software, version 4 (GraphPad Software). A P value ≤ 0.05 was considered significant.

1. Mariathasan S, et al. (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213–218.
2. Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237–241.
3. Kessenbrock K, et al. (2008) Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin. *J Clin Invest* 118: 2438–2447.
4. Li P, et al. (1995) Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell* 80:401–411.
5. Korn T, et al. (2007) Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med* 13:423–431.

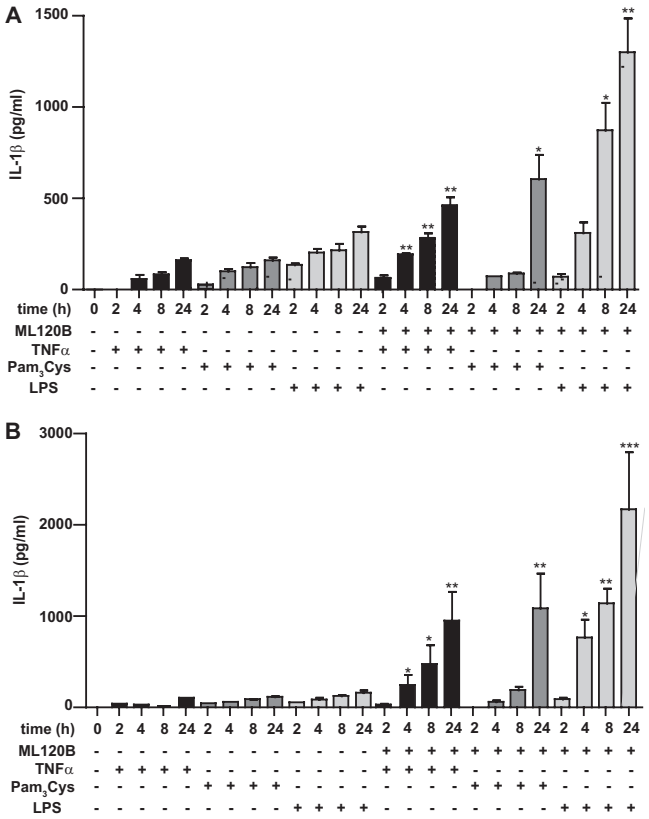

Fig. S1. (*A*) IL-1β levels in the supernatants of 1×10^6 WT bone marrow cells treated with or without ML120B (30 μM) and TNF-α (10 ng/mL), Pam_3Cys (200 ng/mL), or LPS (100 ng/mL) for the indicated times. Data are mean ± SE from three mice (*$P < 0.05$ and **$P < 0.01$ vs. respective samples without ML120B treatment). (*B*) IL-1β levels in the supernatants of 1×10^5 WT bone marrow derived macrophages treated with or without ML120B (30 μM) and TNF-α (10 ng/mL), Pam_3Cys (200 ng/mL), or LPS (100 ng/mL) for the indicated times. Data are mean ± SE from three mice (*$P < 0.05$ and **$P < 0.01$ vs. respective samples without ML120B treatment).

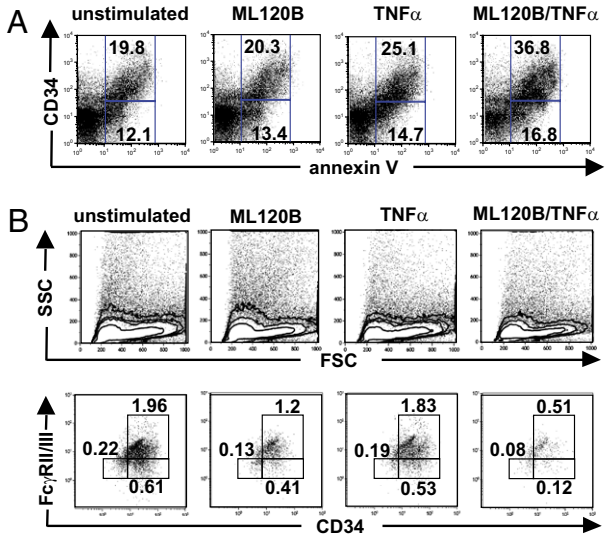

Fig. S2. (*A*) Representative FACS plots of CD34 and annexin V expression in IL-7Rα$^-$ cells from WT mice stimulated with TNF-α (10 ng/mL) for 4 h in the presence or absence of ML120B (30 μM). (*B*) IL-7Rα$^-$ WT bone marrow cells were stimulated with TNF-α (10 ng/mL) in the presence or absence of ML120B (30 μM) for 4 h. Lin$^-$/IL-7Rα$^-$/Sca-1$^-$/c-Kit$^+$ fraction was subdivided into FcγRII/III^{hi}CD34$^+$ (GMPs), FcγRII/III^{low}CD34$^+$ (CMPs), and FcγRII/III^{low}CD34$^-$ (megakaryocyte/erythrocyte lineage restricted progenitors). Plots are representative of at least five animals per treatment and demonstrate loss of CMPs and GMPs after combined treatment with TNF-α and ML120B. Note reduced number of cells in FSC/SSC plots (*Lower Right*), which comprises progenitor population.

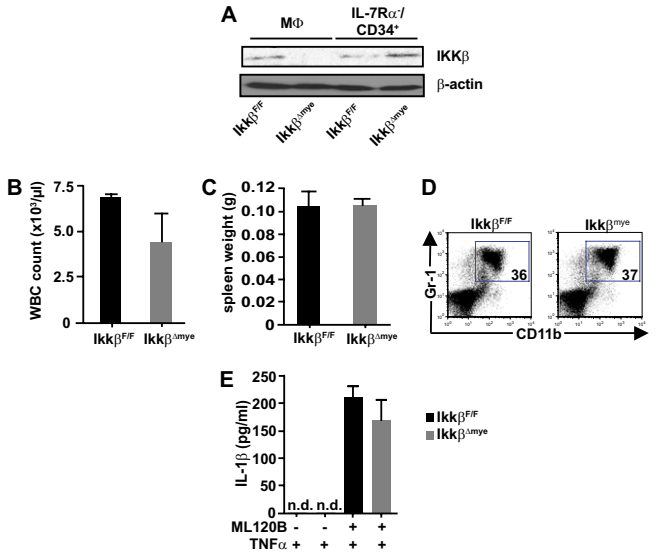

Fig. S3. (*A*) Immunoblot analysis of IKKβ in peritoneal macrophages (MΦ) or IL-7R$^-$/CD34$^+$ progenitor cells of *Ikkβ$^{F/F}$* and *Ikkβ^{Δmye}* mice confirming deletion of IKKβ in differentiated macrophages and lack of deletion in progenitor cells. (*B* and *C*) WBC counts (*B*) and spleen weights (*C*) of *Ikkβ$^{F/F}$* and *Ikkβ^{Δmye}* KO mice. Data are mean ± SE from at least three mice. (*D*) Representative FACS plots of CD11b$^+$/Gr-1$^+$ cells in bone marrow of *Ikkβ$^{F/F}$* and *Ikkβ^{Δmye}* KO mice. Plots are representative of four animals per genotype. Mean values ± SE for *Ikkβ$^{F/F}$*: 35.6% ± 0.7; *Ikkβ^{Δmye}*, 36.3% ± 1.3; *P* value not significant. (*E*) IL-1β levels in the supernatants of bone marrow cells from *Ikkβ$^{F/F}$* and *Ikkβ^{Δmye}* KO mice treated with or without ML120B (30 μM) and TNF-α (10 ng/mL) for 24 h. Data are mean ± SE from at least three mice.

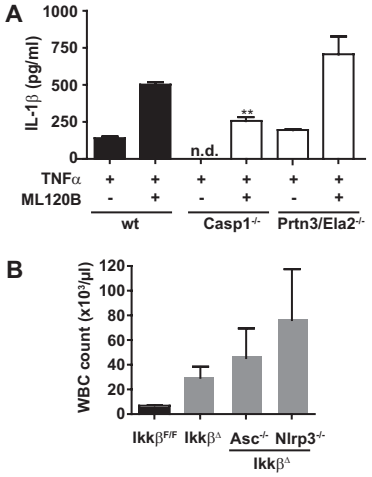

Fig. S4. (*A*) IL-1β levels in the supernatants of WT, *Casp1$^{-/-}$*, or *Prtn3/Ela2$^{-/-}$* bone marrow cells stimulated with TNF-α (10 ng/mL) and ML120B (30 μM). Data are mean ± SE from at least three mice (**$P < 0.01$). (*B*) WBC counts of *Ikkβ$^{F/F}$*, *Ikkβ^{Δ}*, *Ikkβ^{Δ}/Asc$^{-/-}$*, and *Ikkβ^{Δ}/Nlrp3$^{-/-}$* double KO mice 21 d after poly(I:C) administration. Data are mean ± SE from at least three mice.

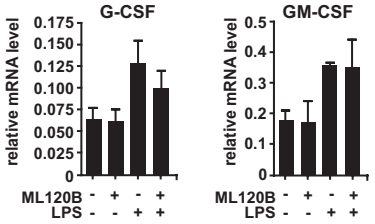

Fig. S5. Relative levels of G-CSF and GM-CSF mRNA in WT bone marrow-derived macrophages untreated or stimulated with LPS (100 ng/mL) for 4 h in the presence or absence of ML120B (30 μM). Data are mean ± SE from at least three mice.

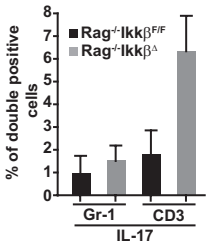

Fig. S6. Quantification of IL-17 expression in Gr1^{+} and intracellular CD3+ cells in *Rag1*$^{-/-}$ and *Ikkβ*^{Δ}/*Rag1*$^{-/-}$ compound. Data are mean ± SE from two mice.

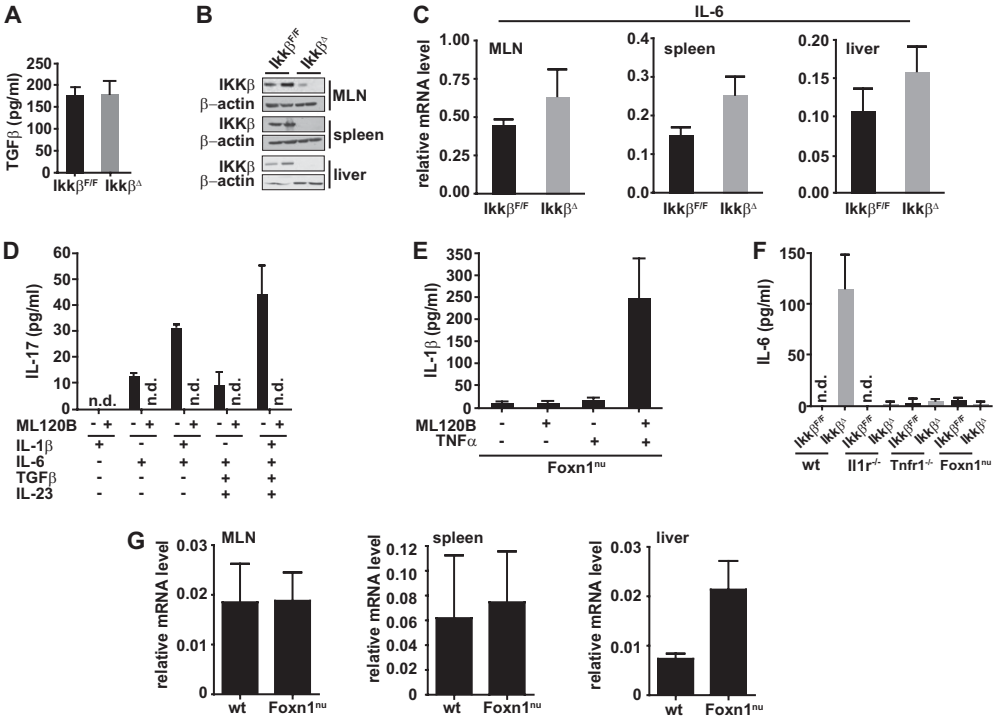

Fig. S7. (*A*) Plasma TGF-β levels in *Ikkβ*$^{F/F}$ and *Ikkβ*^{Δ} mice. Data are mean ± SE from at least six mice. (*B*) Confirmation of IKKβ deletion in MLN, spleen, and liver tissue of two independent *Ikkβ*^{Δ} mice compared with *Ikkβ*$^{F/F}$ littermate controls by immunoblot analysis. (*C*) Relative levels of IL-6 mRNA in MLN, spleen, and liver tissue of *Ikkβ*$^{F/F}$ and *Ikkβ*^{Δ} mice. Data are mean ± SE from at least three mice. (*D*) IL-17 levels in the supernatants of peripheral lymph node T cells incubated with or without ML120B (30 μM) and stimulated either with single cytokines or a combination of IL-1β (10 ng/mL), IL-6 (50 ng/mL), TGF-β (2 ng/mL), and IL-23 (1 μg/mL) for 48 h. Results shown are from one representative experiment repeated at least four times. Data are mean ± SE from two animals. (*E*) IL-1β levels in supernatants of NU-*Foxn1*^{nu} and NU-*Foxn1*^{nu}/*Ikkβ*^{Δ} bone marrow cells stimulated with TNF-α (10 ng/mL) for 24 h in the presence or absence of ML120B (30 μM). Data are mean ± SE from at least three mice. (*F*) Plasma IL-6 levels in *Ikkβ*$^{F/F}$ and *Ikkβ*^{Δ} mice and in the respective *Il1r*$^{-/-}$, *Tnfr*$^{-/-}$ and NU-*Foxn1*^{nu} compound mutants 21 d after poly(I:C) injection. Data are mean ± SE from at least four mice (n.d., not detected). (*G*) Relative levels of IL-6 mRNA in MLN, spleen, and liver tissue of WT and NU-*Foxn1*^{nu} mice. Data are mean ± SE from four mice.

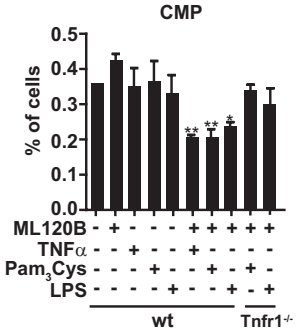

Fig. S8. Analysis of CMPs upon combined treatment with TNFα, Pam_3Cys, or LPS with and without ML120B for 24 h in WT or *Tnfr*$^{-/-}$ mice. Data are mean ± SE from three mice (*P < 0.05 and **P < 0.01 vs. respective samples without ML120B treatment).