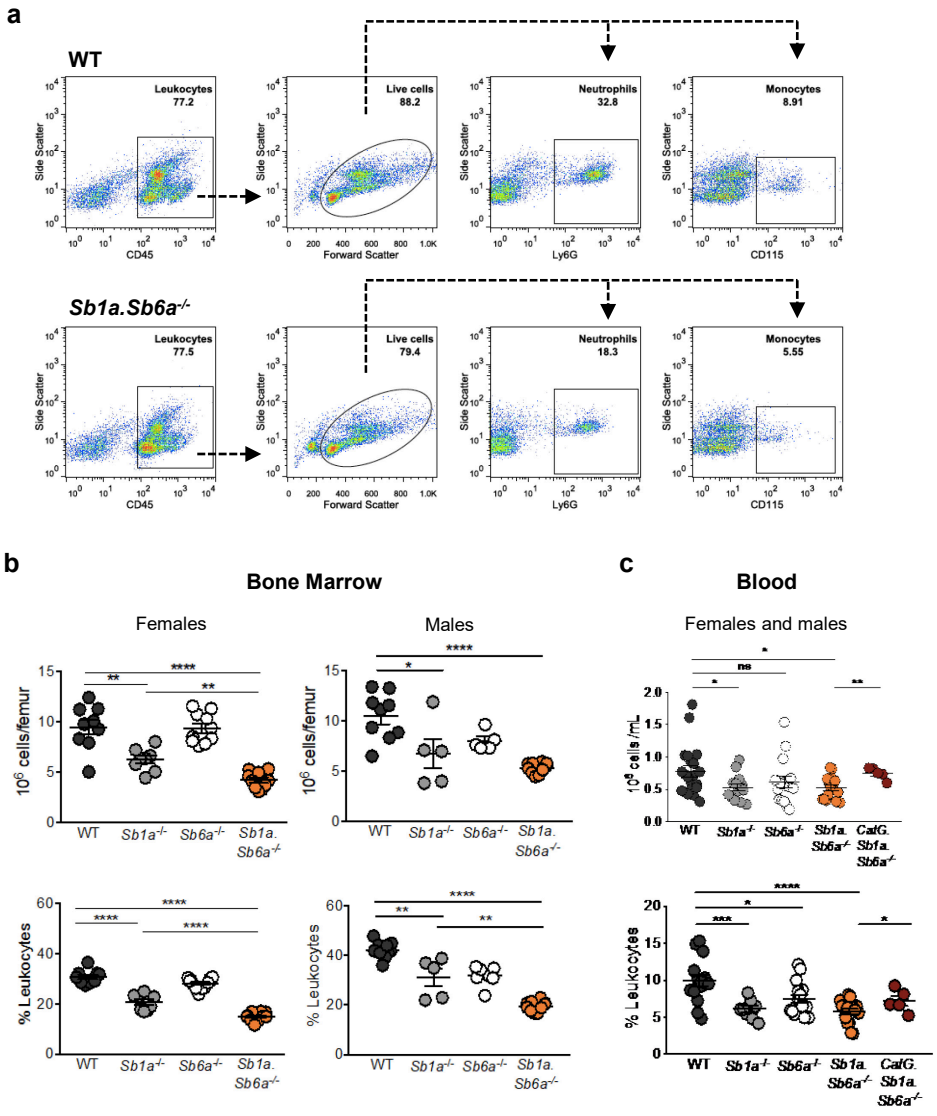

Figure S1. Sb1a and Sb6a cooperate to maintain neutrophil homeostasis. Related to Figure 1. (a) Representative flow cytometry gating strategy for the identification of bone marrow neutrophils (CD45+, Ly6G+) and monocytes (CD45+, CD115+). (b-c) Analysis of bone marrow and blood neutrophils of 6 week-old mice of indicated genders. Scatter plots show data for individual mice, horizontal bars indicate mean ± SEM. n=13-20 mice/genotype (b) and n=5-17 mice/genotype (c) from 5 independent experiments. Data were analysed by Mann-Whitney test (**** P<0.0001; ***P<0.001; **P<0.01; *P<0.05).

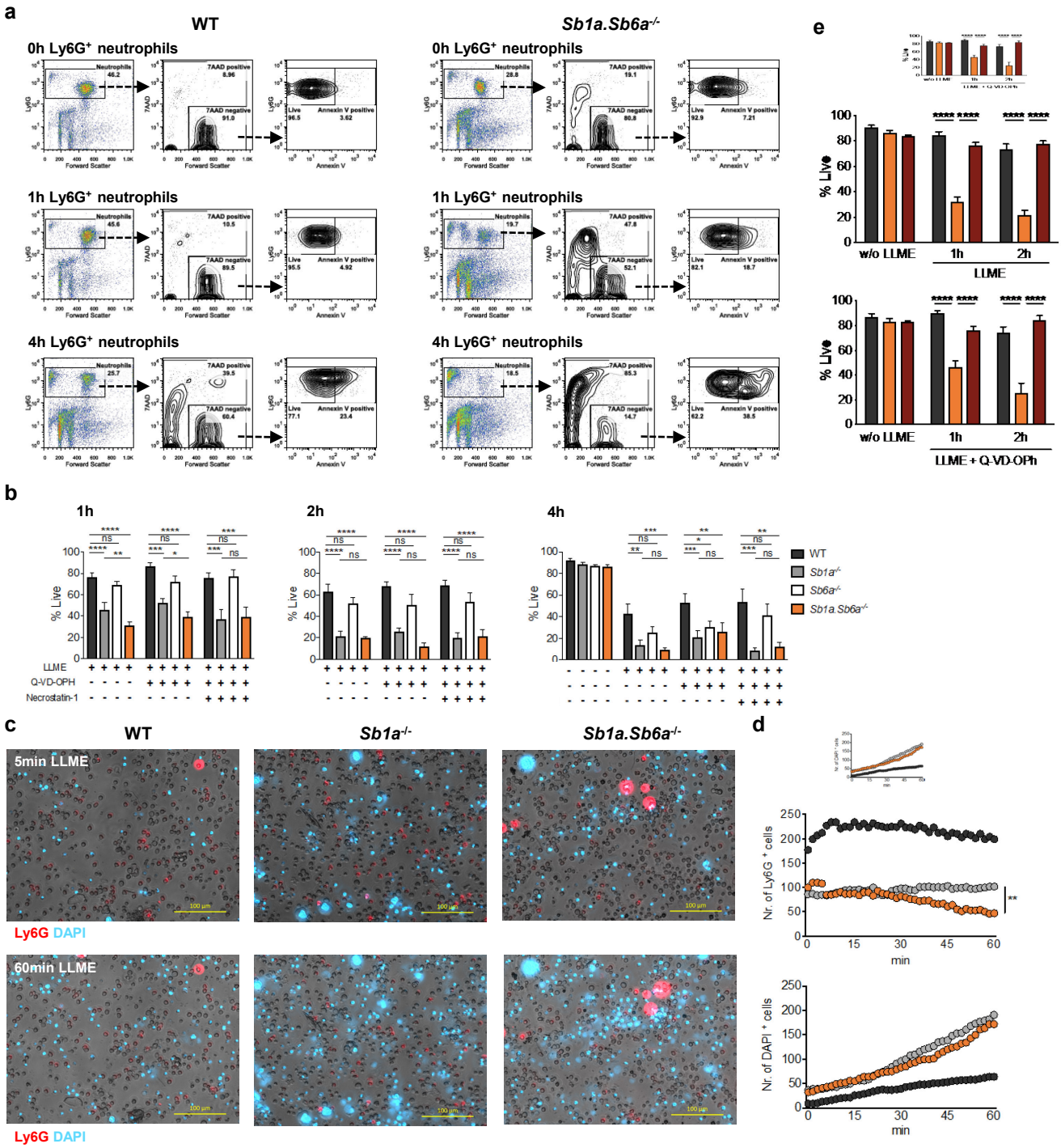

Figure S2. Kinetics of LLME-induced neutrophil death reveals additive protective role for Sb1a and Sb6a. Related to Figure 2. (a) Representative flow cytometry gating strategy for identification of apoptotic and necrotic neutrophils following treatment with 100µM LLME at indicated time points. (b-e) Bone marrow cells were treated with 100µM LLME alone or in combination with 50µM Q-VD-OPh and/or 20µM necrostatin-1 for up to 4 hours. (b,e) Viability of neutrophils ($Ly6G^+$) was assessed by flow cytometry using Annexin V-APC and 7-AAD. Data are shown as mean ± SEM of 8 independent experiments and were analyzed by two-way ANOVA (**** P<0.0001; ***P<0.001; **P<0.01; *P<0.05). (c) Representative micrographs of bone marrow cells incubated with 100µM LLME for 5min (top) or 1 hour (bottom) taken during live cell imaging analysis. Neutrophils were labeled with Ly6G-AlexaFluor647 (Red) and necrotic cells were labelled with DAPI (blue). (d) Number of $Ly6G^+$ and $DAPI^+$ cells in micrographs were enumerated using IMARIS.

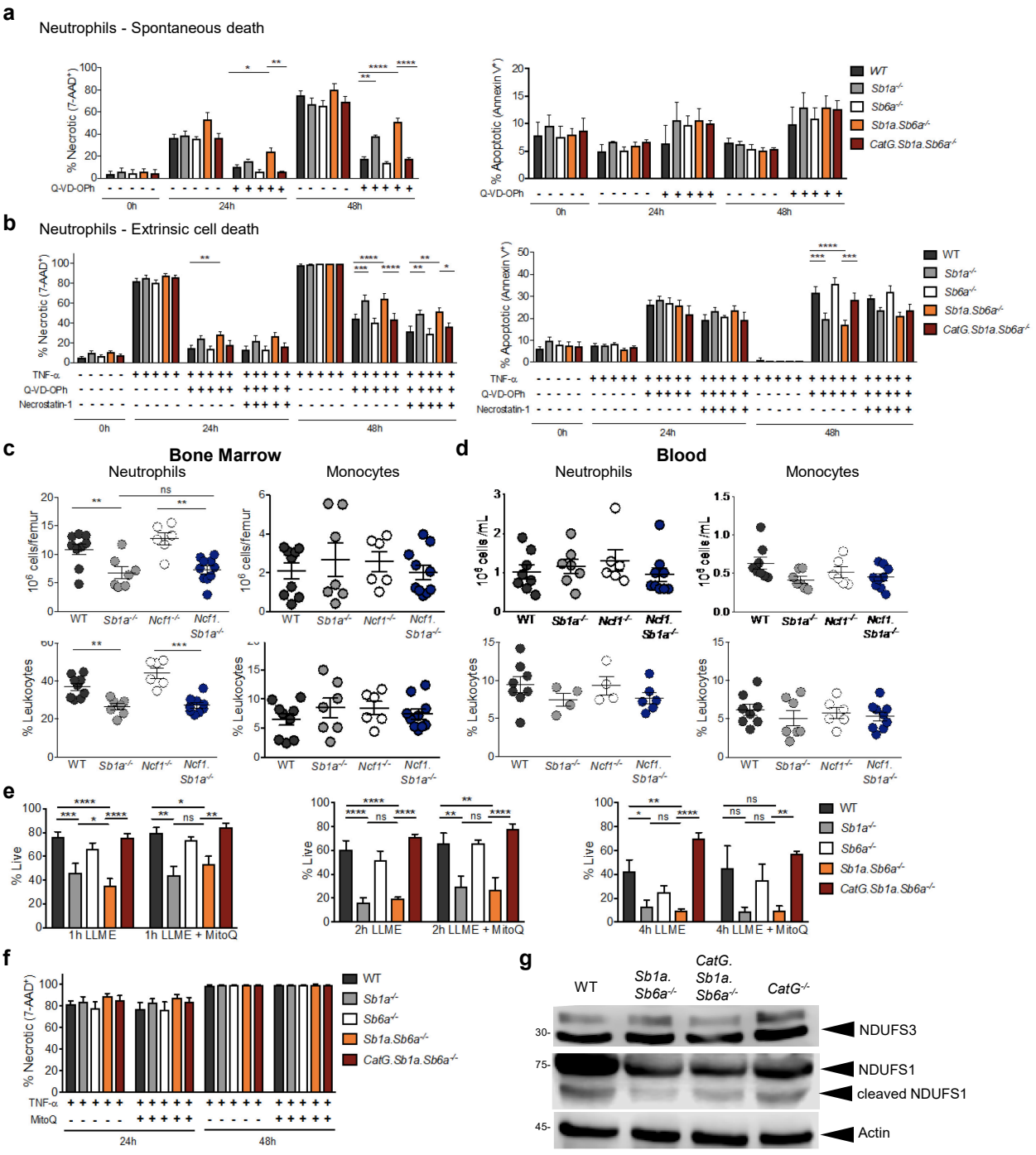

Figure S3. CatG mediates *Sb1a.Sb6a*$^{-/-}$ neutrophil death independently of reactive oxygen species. Related to Figure 2. (a) Spontaneous apoptosis and secondary necrosis of neutrophils cultured in vitro with or without 50µM Q-VD-OPh. (b) Neutrophil survival following stimulation with TNF (100ng/ml) and actinomycin D (200ng/ml) with or without 50µM Q-VD-OPh and 20µM necrostatin-1 over 48h. Absolute numbers and percentages of (c) bone marrow and (d) blood neutrophils and monocytes of 6 week-old female mice, n=4-8/genotype from 4 independent experiments. Horizontal bars indicate mean ± SEM, data were analysed by Mann-Whitney test (***P<0.001; **P<0.01). (e) Survival of neutrophils following treatment with 100µM LLME in the presence or absence of 25nM MitoQ for 1 to 4 hours. (f) Neutrophil survival following stimulation with TNF (100ng/ml) and actinomycin D (200ng/ml) in the presence or absence of 25nM MitoQ for up to 48 hours. (a,b,e,f) Viability of neutrophils was assessed by flow cytometry using Annexin V-APC and 7-AAD. Data are shown as mean ± SEM of 4-8 independent experiments and were analyzed by two-way ANOVA (**** P<0.0001; ***P<0.001; **P<0.01; *P<0.05).
(g) Neutrophils of indicated genotypes, sorted by flow cytometry, lysed in the presence of protease inhibitors, were resolved by SDS-PAGE and immunoblotted for NDUFS1, NDUFS3 and β-Actin.

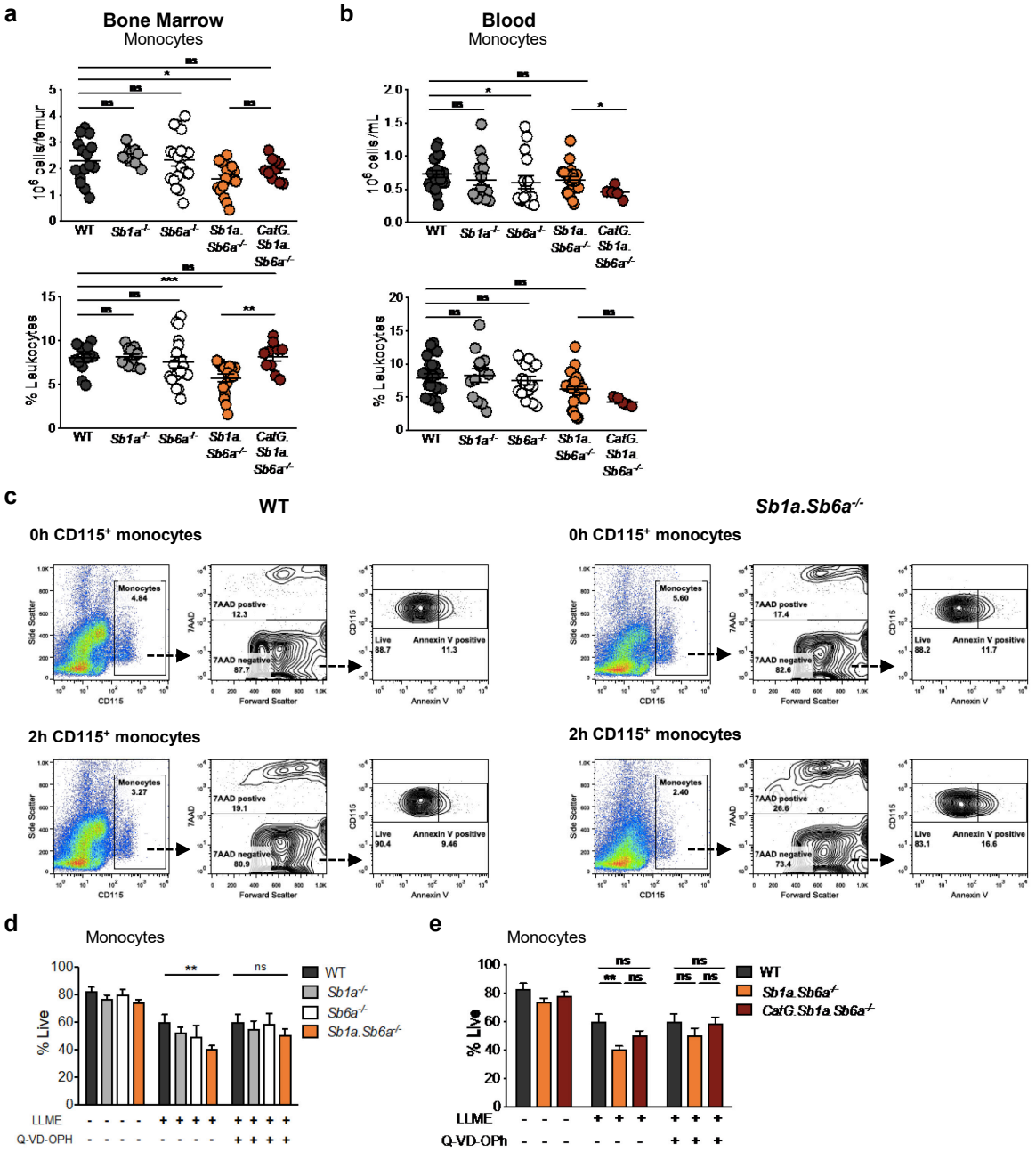

Figure S4. Sb1a and Sb6a cooperate to maintain monocyte homeostasis. Related to Figures 1-2. (a-b) Analysis of bone marrow and blood monocytes of 6 week-old female and male mice, n=10-20mice/genotype (a) n=5-18 mice/genotype from 5 independent experiments. Horizontal bars indicate mean ± SEM, data were analysed by Mann-Whitney test (***P<0.001; **P<0.01; *P<0.05). (c) Representative flow cytometry gating strategy for identification of apoptotic and necrotic WT and *Sb1a.Sb6a*$^{-/-}$ bone marrow monocytes following treatment with 100µM LLME over 2 hours. (d,e) Bone marrow cells were treated with 100µM LLME alone or in combination with 50µM Q-VD-OPh for up to 2 hours. Viability of monocytes (CD115^{+}) was assessed by flow cytometry using Annexin V-APC and 7-AAD. Data are shown as mean ± SEM of 5 independent experiments and were analyzed by two-way ANOVA (**P<0.01).

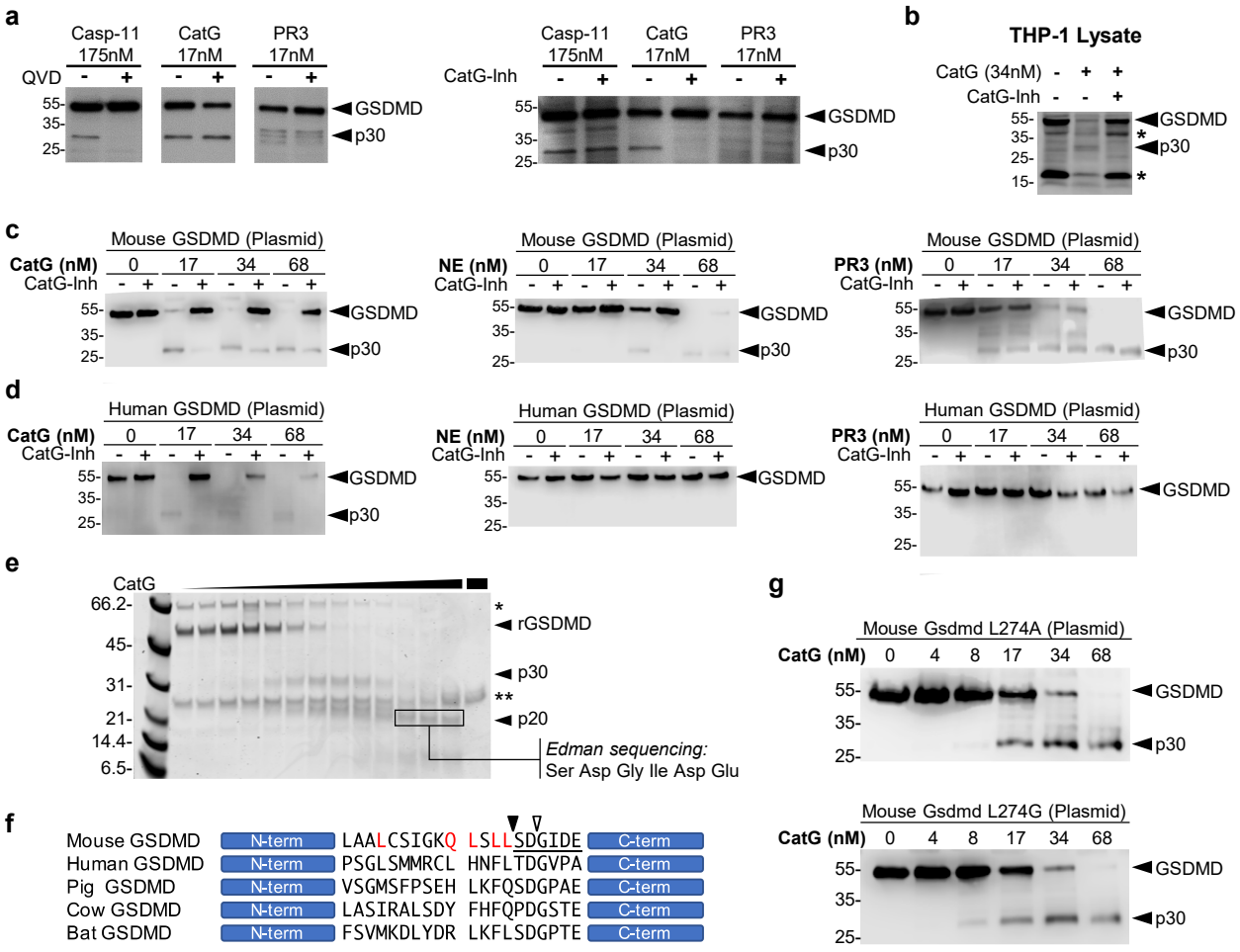

Figure S5. CatG, but not NE, cleaves mouse and human GSDMD into GSDMD-p30. Related to Figure 3. (a) HEK cells, transfected with mouse Flag-GSDMD WT plasmid, lysed without protease inhibitors, incubated (12.5µg total protein) with indicated proteases concentrations (in nM) for 1 hour at 37°C with or without pre-incubation with Q-VD-OPh or CatG inhibitor I, were resolved on SDS-PAGE and immunoblotted for Flag.
(b) THP-1 cells, lysed with or without CatG inhibitor I, incubated (25µg total protein) with indicated protease concentrations (in nM) for 1 hour at 37°C were resolved on SDS-PAGE and immunoblotted for GSDMD with a rabbit anti-GSDMD (126-138) antibody (Sigma). * indicates non-specific bands. (c-d) HEK cells, transfected with (c) mouse Flag-GSDMD WT plasmid or (d) human Flag-GSDMD WT plasmid, lysed without protease inhibitors, incubated (12.5µg total protein) with indicated protease concentrations (in nM) for 1 hour at 37°C with or without pre-incubation with CatG inhibitor I, were resolved on SDS-PAGE and immunoblotted for Flag. (e) Recombinant GSDMD (rGSDMD) was incubated with increasing concentrations of titrated CatG in the range of 0.39nM-50nM. Amino acids identified by Edman N-terminal degradation sequencing cycles of the p20 fragment (boxed) generated by CatG limited proteolysis of GSDMD. *indicates the *E. coli* contaminant FKBP-type peptidyl-prolyl cis-transisomerase SlyD frequently co-purified in NiNTA affinity capture of His-tagged recombinant proteins, ** indicates CatG (loaded alone in the last lane). (f) Sequences of the connecting loop between the N-terminal and C-terminal regions of GSDMD homologs. Conserved putative CatG cleavage sites are shown in red; amino acids identified by Edman sequencing after CatG cleavage are underlined and cleavage site after Leu-274 is shown with a filled arrowhead; Caspase1/11 cleavage site after Asp-276 is shown with an open arrowhead (g) HEK cells, transfected with indicated Leu-274 mutant mouse Flag-GSDMD plasmids, lysed without protease inhibitors, incubated (12.5µg total protein) at indicated CatG concentrations (in nM) for 1 hour at 37°C were resolved on SDS-PAGE and immunoblotted for Flag.

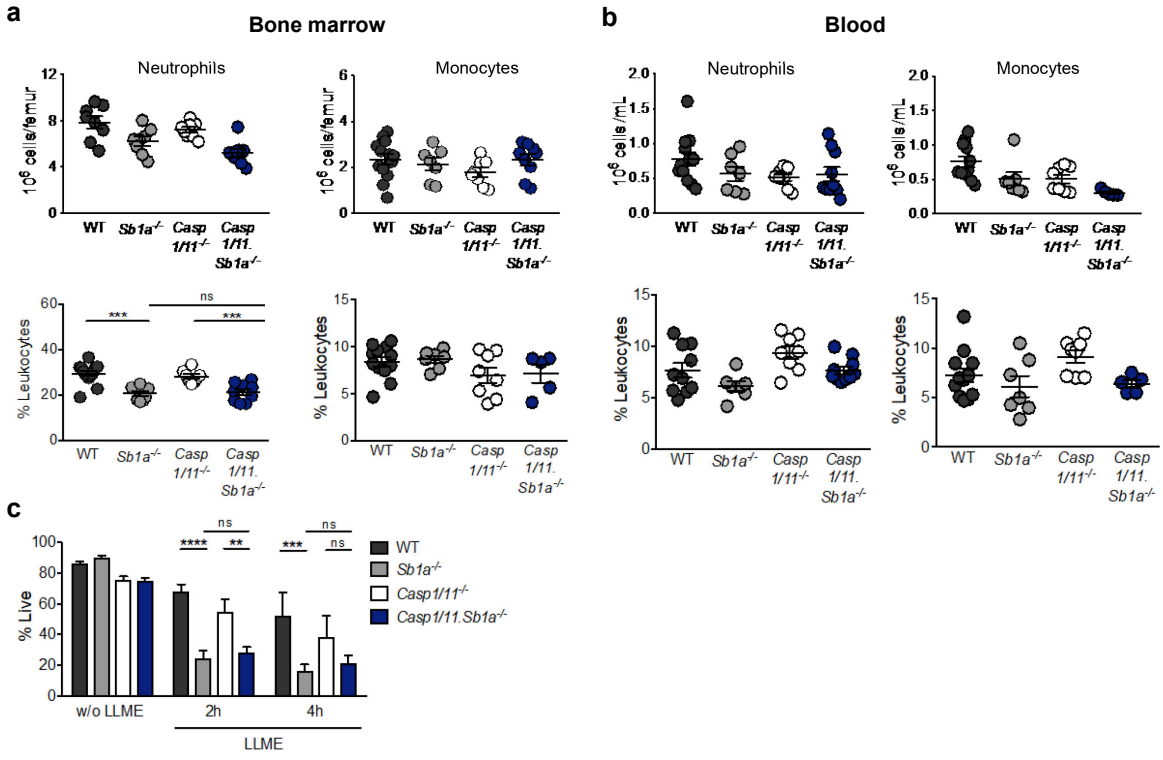

Figure S6. Pyroptotic caspases-1/11 are not required for granule permeabilization-induced neutrophil death. Related to Figure 4. (a,b) Absolute numbers and percentages of (a) bone marrow and (b) blood neutrophils and monocytes of 6 week-old female mice, n=5-8/genotype from 3 independent experiments. Horizontal bars indicate mean ± SEM, data were analysed by Mann-Whitney test (***P<0.001). (c) Survival of neutrophils treated with 100µM LLME for 2 to 4 hours. Viability of neutrophils was assessed by flow cytometry using Annexin V-APC and 7-AAD. Data are shown as mean ± SEM of 5 independent experiments and were analyzed by two-way ANOVA (**** P<0.0001; ***P<0.001; **P<0.01).

a

Gsdmd

AAGAAGATGGTGACCATTCCTGCAGGC**AGCATCCTGGCATTCCGAG**TGGCCCAACTGCTTATTGGCTCTAAATGGGgtgag:::

K K M V T I P A G S I L A F R V A Q L L I G S K W

:::accagATATCCTTCTCGTCTCAGATGAGAAACAGAGGACCTTTGAGCCCTCCTCAGgtaag:::

D I L L V S D E K Q R T F E P S S

Gsdmd^{em1} **(−1nt)**

AAGAAGATGGTGACCATTCCTGCAGGC**AGCATCCTGGCATTC−GAG**TGGCCCAACTGCTTATTGGCTCTAAATGGGgtgag:::

K K M V T I P A G S I L A F E W P N C L L A L N G

:::accagATATCCTTCTCGTCTCAGATGAGAAACAGAGGACCTTTGAGCCCTCCTCAGgtaag:::

I S F S S Q M R N R G P L S P P Q

Gsdmd^{em4} **(−4nt)**

AAGAAGATGGTGACCATTCCTGCAGGC**AGCATCCTGGCA----GAG**TGGCCCAACTGCTTATTGGCTCTAAATGGGgtgag:::

K K M V T I P A G S I L A E W P N C L L A L N G

:::accagATATCCTTCTCGTCTCAGATGAGAAACAGAGGACCTTTGAGCCCTCCTCAGgtaag:::

I S F S S Q M R N R G P L S P P Q

Gsdmd^{em5} **(+1nt)**

AAGAAGATGGTGACCATTCCTGCAGGC**AGCATCCTGGCATTCCGGAG**TGGCCCAACTGCTTATTGGCTCTAAATGGGgtgag:::

K K M V T I P A G S I L A F R S G P T A Y W L *

Gsdmd^{em6} **(+57nt −2nt)**

AAGAAGATGGTGACCATTCCTGCAGGC**AGCATCCTGGCATTCC**AACTGAGGTGGGCACATACACATACACACGTATACACAAGGGTGAGGCTCAACTCAG−−**G**TGGC

K K M V T I P A G S I L A F Q L R W A H T H T H V Y T R V R L N S G G

CCAACTGCTTATTGGCTCTAAATGGGgtgag:::

P T A Y W L *

Gsdmd^{em12} **(+168nt −2nt)**

AAGAAGATGGTGACCATTCCTGCAGGC**AGCATCCTGGCAT**ACCTAGCCACTATGCACCTGGCTTTAGATGACTGTGAATTCTTTGTTCCTGTCTCACCAGGTATACAA

K K M V T I P A G S I L A Y L A T M H L A L D D C E F F V P V S P G I Q

TGTTTACATGGCTGGGAGGCAGCTGTGTTCTAAGCGGTACCGGGAGTTTGCCATACTGCACCAAAACCTGAAGAGAGAATTTGCCAACTTTACATTTCCT−−**CGAG**TG

C L H G W E A A V F *

GCCCAACTGCTTATTGGCTCTAAATGGGgtgag:::

b

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Mus_Gsdmd       MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNLSIKDILEPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGM       100
Mus_Gsdmd_em1   MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNLSIKDILEPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGM       100
Mus_Gsdmd_em4   MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNLSIKDILEPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGM       100
Mus_Gsdmd_em5   MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNLSIKDILEPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGM       100
Mus_Gsdmd_em6   MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNLSIKDILEPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGM       100
Mus_Gsdmd_em12  MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNLSIKDILEPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGM       100

Mus_Gsdmd       GEGKISGGAAVSDSSSASMNVCILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSGQFTLPGALCLKGEGKGH       200
Mus_Gsdmd_em1   GEGKISGGAAVSDSSSASMNVCILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSGQFTLPGALCLKGEGKGH       200
Mus_Gsdmd_em4   GEGKISGGAAVSDSSSASMNVCILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSGQFTLPGALCLKGEGKGH       200
Mus_Gsdmd_em5   GEGKISGGAAVSDSSSASMNVCILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSGQFTLPGALCLKGEGKGH       200
Mus_Gsdmd_em6   GEGKISGGAAVSDSSSASMNVCILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSGQFTLPGALCLKGEGKGH       200
Mus_Gsdmd_em12  GEGKISGGAAVSDSSSASMNVCILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSGQFTLPGALCLKGEGKGH       200

Mus_Gsdmd       QSRKKMVTIPAGSILAFRVAQLLIGSKWDILLVSDEKQRTFEPSSGDRKAVGQRHHGLNVLAALCSIGKQLSLLSDGIDEEELIEAADFQGLYAEVKACS       300
Mus_Gsdmd_em1   QSRKKMVTIPAGSILAFEWPNCLLALNGISFSSQMRNRGPLSPPQVTEKQWARGTMASMCLLRFVPSESSSVSCQMGLMRRN                       282
Mus_Gsdmd_em4   QSRKKMVTIPAGSILA-EWPNCLLALNGISFSSQMRNRGPLSPPQVTEKQWARGTMASMCLLRFVPSESSSVSCQMGLMRRN                       281
Mus_Gsdmd_em5   QSRKKMVTIPAGSILAFRSGPTAYWL                                                                               226
Mus_Gsdmd_em6   QSRKKMVTIPAGSILAFQLRWAHTHTHVYTRVRLNSGGPTAYWL                                                             244
Mus_Gsdmd_em12  QSRKKMVTIPAGSILAYLATMHLALDDCEFFVPVSPGIQCLHGWEAAVF                                                       249

Mus_Gsdmd       SELESLEMELRQQILVNIGKILQDQPSMEALEASLGQGLCSGGQVEPLDGPAGCILECLVLDSGELVPELAAPIFYLLGALAVLSETQQQLLAKALETTV       400

Mus_Gsdmd       LSKQLELVKHVLEQSTPWQEQSSVSLPTVLLGDCWDEKNPTWVLLEECGLRLQVESPQVHWEPTSLIPTSALYASLFLLSSLGQKPC                   487
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Figure S7. Sequences of the *Gsdmd* mutant alleles generated by CRISPR/Cas9 targeting. Related to Figure 4 and STAR Methods. (a) Sequence of exons 3 and 4 (upper case) and surrounding relevant intron (lower case) of wild-type mouse *Gsdmd* gene and endonuclease-mediated (*em*) alleles generated and used as homozygotes. The targeted sequence is shown in bold, inserted bases are shown in red and deleted bases are shown with a red hyphen. Coded protein sequences are shown below and red letters indicate mutated amino acids resulting from a frameshift or inserted sequence by non-homologous end joining repair mechanism. (b) Alignment of predicted protein sequences. CatG cleavage site at L274 is highlighted in yellow.

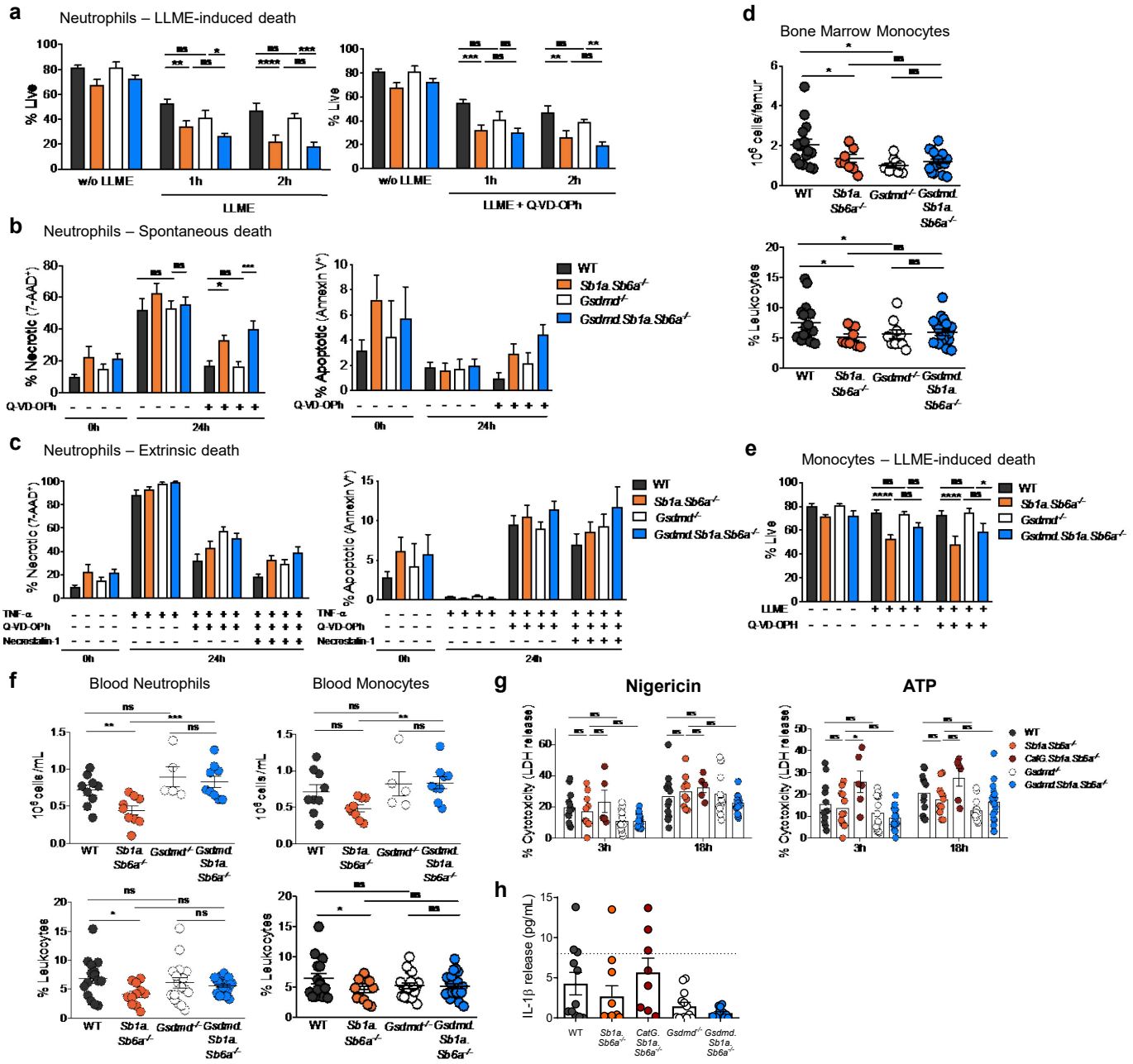

Figure S8. GSDMD is not required for neutrophil and monocyte death. Related to Figure 4-5. (a) Survival of neutrophils treated with 100µM LLME in the presence or absence of 50µM Q-VD-OPh for 1-2 hours. (b) Spontaneous apoptosis and secondary necrosis of neutrophils cultured in vitro with or without 50µM Q-VD-OPh. (c) Neutrophil survival following stimulation with TNF (100ng/ml) and actinomycin D (200ng/ml) with or without 50µM Q-VD-OPh and 20µM necrostatin-1 over 24 hours. (d) Absolute numbers and percentages of bone marrow monocytes from 6 week-old male and female mice, n=8-21/genotype. (e) Survival of monocytes treated with 100µM LLME in the presence or absence of 50µM Q-VD-OPh for 2 hours. (f) Absolute numbers and percentages of blood neutrophils and monocytes from 6 week-old male and female mice, n=5-21/genotype from 6 independent experiments. (a-c,e) Viability was assessed by flow cytometry using Annexin V-APC and 7-AAD. Data are shown as mean ± SEM of 3-6 independent experiments and were analyzed by two-way ANOVA (**** P<0.0001; ***P<0.001; **P<0.01; *P<0.05). (d,f) Data is shown for individual mice, horizontal bars indicate mean ± SEM, data were analysed by Mann-Whitney test (**** P<0.0001; ***P<0.001; **P<0.01; *P<0.05). (g) Cytotoxicity of LPS priming and canonical inflammasome activation with nigericin and ATP was measured by total LDH release of BMDMs. Data is from n=8-12/genotype from 7 independent experiments and analysed by two-way ANOVA. (h) Mature IL-1β release by BMDMs primed with LPS for 5 hours but not stimulated with canonical inflammasome activators. Detection limit of the ELISA assay is indicated by the horizontal dashed line. Data is from n=8-14/genotype from 9 independent experiments. Horizontal bars indicate mean ± SEM.

Genotype	B cells (x10^6 cells/mL)	Eosinophils (x10^5 cells/mL)	NK cells (x10^5 cells/mL)	CD3 (x10^6 cells/mL)	*n*
WT	4.0 ± 2.25 44.9 ± 4.96%	2.04 ± 1.42 2.52% ± 0.91%	2.8 ± 1.4 3.27 % ± 1.52%	1.58 ± 0.74 21.25% ± 4.20%	12
Sb1a$^{-/-}$	4.92 ± 2.36 44.76% ± 8.23%	4.18 ± 2.75 4.08% ± 2.81%	3.0 ± 1.0 4.89% ± 2.6%	1.75 ± 0.75 20.91% ± 3.25%	7
Sb6a$^{-/-}$	4.51 ± 1.61 52.35% ± 5.86%	2.39 ± 1.09 2.72% ± 0.90%	2.9 ± 1.4 3.16% ± 0.75%	1.59 ± 0.50 20.05% ± 5.62%	16
Sb1a.Sb6a$^{-/-}$	4.59 ± 1.37 50.13 % ± 4.54%	2.44 ± 1.2 2.53% ± 0.89%	2.5 ± 1.1 3.44% ± 1.21%	2.03 ± 0.62 22.13% ± 2.92%	13

Table S1. Absolute numbers and percentages of blood B cells, eosinophils, NK cells and T cells. Related to Figures 1 and S4. Percentage of leukocyte subsets were determined by flow cytometry of whole blood of female mice of indicated genotypes. Absolute numbers for each subset were calculated by multiplying the percentage of each subset within live CD45$^+$ cells with the WBC (shown Table S2). Data is shown as mean ± SD of three to four independent measurements for each genotype.

Genotype	WBC (x10^6 cells/mL)	RBC (x10^9 cells/mL)	Hemoglobin (g/dL)	Hematocrit (%)	*n*
WT	8.6 ± 4.7	9.3 ± 0.8	15.9 ± 1.1	50.3 ± 4.1	12
Sb1a$^{-/-}$	7.7 ± 4.8	8.3 ± 0.7	13.7 ± 0.8	43.5 ± 2.8	7
Sb6a$^{-/-}$	7.9 ± 2.9	9.3 ± 0.5	16.1 ± 0.7	49.9 ± 2.3	16
Sb1a.Sb6a$^{-/-}$	9.4 ± 2.9	9.2 ± 0.4	15.2 ± 0.6	49.2 ± 2.7	13
Ncf1$^{-/-}$	8.4 ± 2.2	10 ± 0.4	15.8 ± 0.3	51.8 ± 0.3	5
Ncf1.Sb1a$^{-/-}$	9.2 ± 2.6	9.9 ± 0.5	15.8 ± 0.6	51.5 ± 2.5	9
Casp-1/11$^{-/-}$	4.3 ± 0.9	9.2 ± 0.5	16.1 ± 0.6	52.1 ± 3.6	8
Casp-1/11.Sb1a$^{-/-}$	4.8 ± 0.7	9.5 ± 0.3	15.6 ± 0.3	50.4 ± 1.7	5
Gsdmd$^{-/-}$	13.2 ± 2.4	9.5 ± 0.4	16.2 ± 1.1	49.7 ± 4.5	7
Gsdmd.Sb1a.Sb6a$^{-/-}$	14.4 ± 4.2	10.0 ± 0.7	16.3 ± 1.2	55.3 ± 6.0	12
CatG.Sb1a.Sb6a$^{-/-}$	10.7 ± 2.3	10.0 ± 0.5	17.73 ± 0.4	55.1 ± 3.5	5

Table S2. Hematological analysis. Related to Figures 1, 4 and S4. Total blood cell analysis of female mice of the indicated phenotypes was performed on an automated hematology analyser (VetABC). Data are shown as mean ± SD.