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Supplementary information

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Lipid asymmetry disruption by XKR8 orchestrates neutrophil extracellular trap formation and inhibits fungal infection

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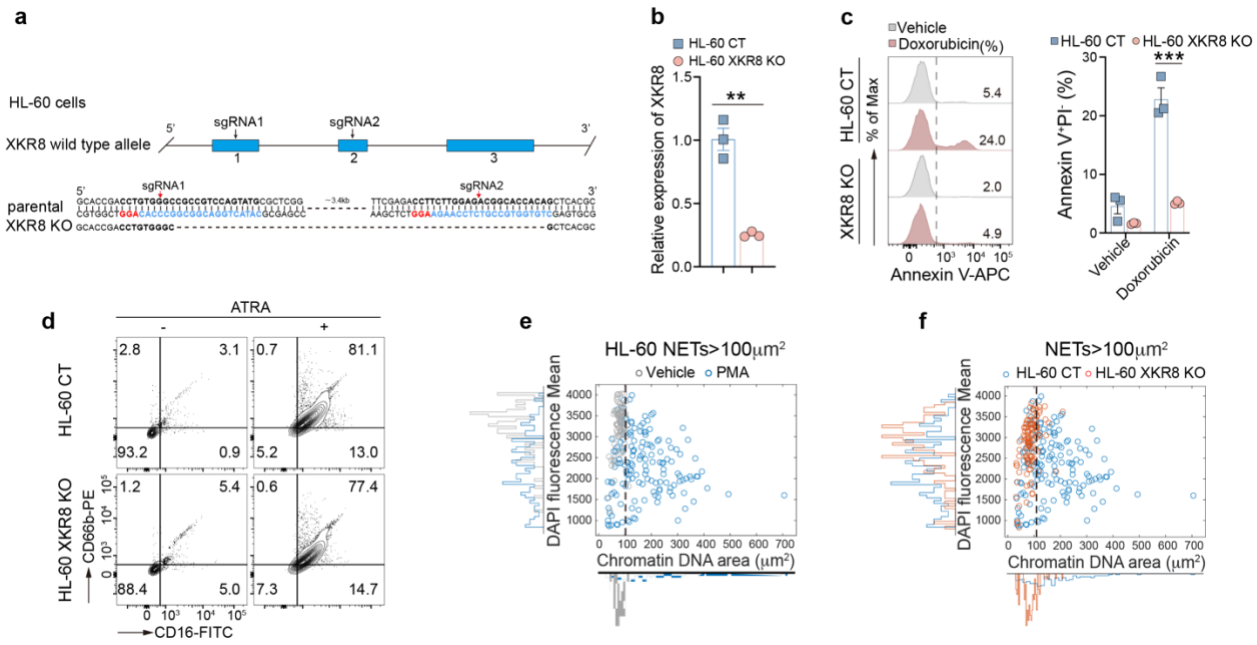
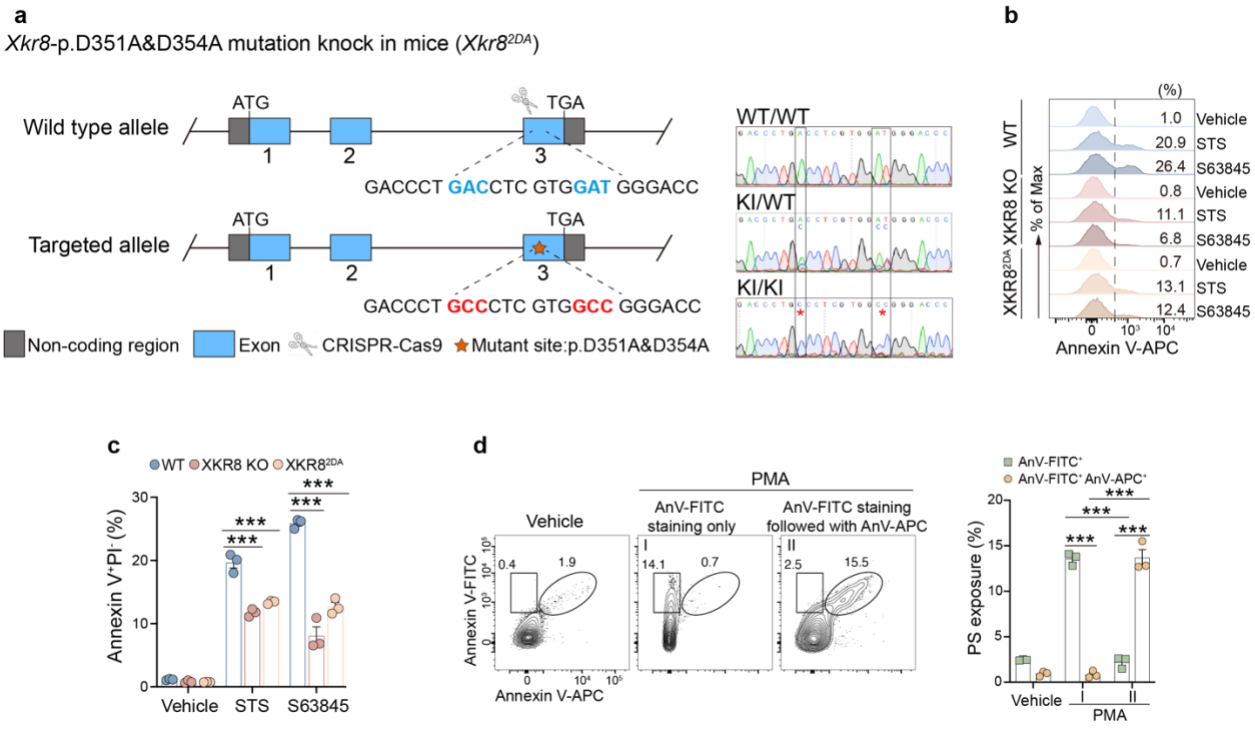
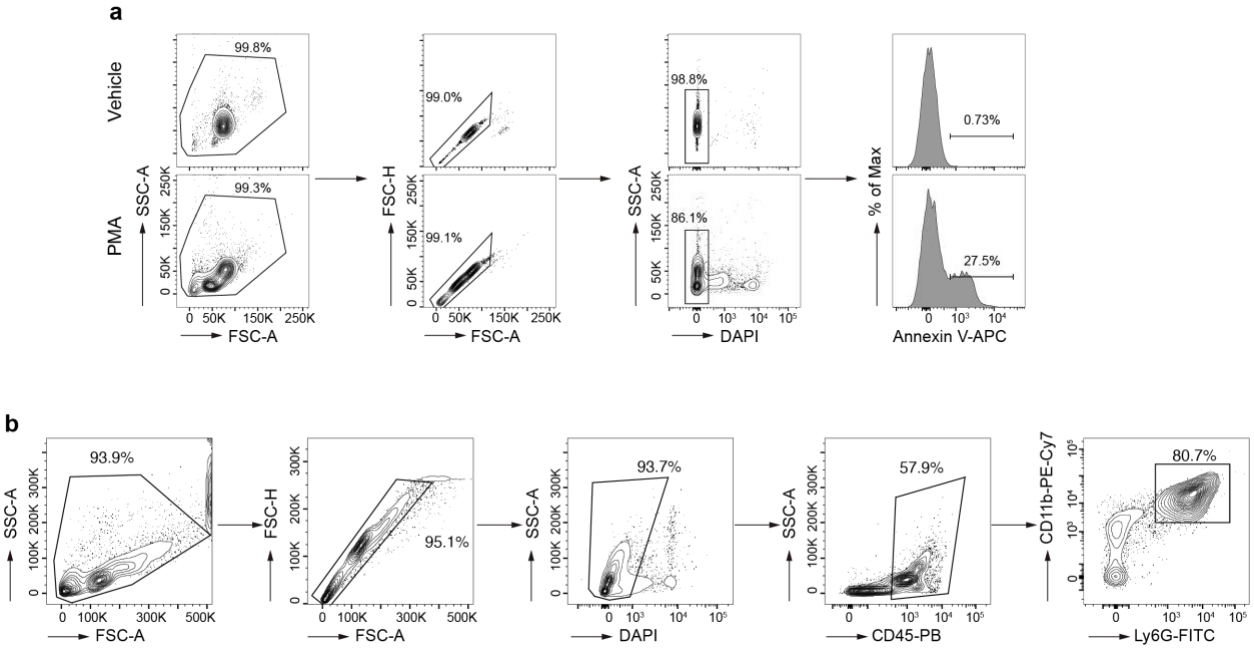


Supplementary Fig. 1 Compromised NETs formation in ATRA-differentiated neutrophil-like HL-60 cells. a,**b**, Strategy for the generation of XKR8 KO HL-60 cells (**a**) and Quantitative real-time PCR (qPCR) analysis of *Xkr8* expression in WT and XKR8 KO cells (**b**), *18S* rRNA as the internal reference. **c**, PS exposure in HL-60 WT and XKR8 KO cells treated with doxorubicin (10 μM). Representative histogram (left) and quantification (right) of PS exposure in living cells (Annexin V^+ PI^-) are shown (n = 3). **d**, Flow cytometry analysis of CD16 and CD66b in HL-60 cells with or without ATRA treatment, confirming the neutrophil-like differentiation. **e**,**f**, Definition of NETs by DAPI fluorescence intensity and nuclear area. Cells with a nuclear area greater than 100 μm² were classified as NETs in dHL-60 cells (**e**). NETs formation in HL-60 WT and XKR8 KO cells treated with PMA (100 nM) (**f**). Each dot represents a biological replicate (**b**,**c**). Data are represented as mean ± SEM from one representative experiment of three (**b**,**c**) independent experiments. Statistical analysis was performed using two-way ANOVA (**c**) or two-tailed Mann-Whitney test (**b**). ns, not significant, $^{**}P<0.01$, $^{***}P<0.001$.



Supplementary Fig. 2 Generation and characterization of XKR8^{2DA} knock-in mice. a, Strategy for the generation of *Xkr8^{2DA}* knock-in mice carrying D351A and D354A mutations. The genetic modification was confirmed by genotyping, and sequencing, which verified the presence of both mutations in the XKR8^{2DA} allele. **b**,**c**, PS exposure in live BMNs from WT, XKR8 KO and XKR8 2DA mutant mice treated with S63845 (10 μM) or STS (10 μM) (n = 3), assessed by flow cytometry. Representative histograms (**b**) and quantification of Annexin V$^+$ PI$^-$ cells (**c**). **d**, Sustained PS externalization revealed by sequential Annexin V staining. Neutrophils were stained with Annexin V-FITC at 2 hours post-PMA stimulation, washed with 1 x binding buffer, and rested for 15 minutes at 37°C. Cells were then subjected to a second round of Annexin V-APC staining. Representative flow cytometry plot (left) and quantification of Annexin V$^+$ DAPI$^-$ cells (right). Each dot represents a biological replicate (**c**,**d**). Data are represented as mean ± SEM from one representative experiment of three (**c**,**d**) independent experiments. Statistical analysis was performed using two-way ANOVA (**c**,**d**). ***P<0.001.



Supplementary Fig. 3 Gating strategies of flow cytometry. a, Gating strategies of PS exposure in purified neutrophils. **b**, Gating strategies of neutrophils in lung and BALF upon LPS or *C. alb* intranasal challenge.

Supplementary Table 1. Immune repertoire in WT and XKR8 KO mice.

Organs	Immune cells		%	Numbers ($\times 10^6$)
Thymus	CD4	WT	10.01±0.95	14.06±3.52
		XKR8 KO	10.76±0.34	11.79±5.35
	CD8	WT	3.39±0.43	4.77±1.32
		XKR8 KO	4.37±0.18	4.75±2.01
	Double positive cells	WT	83.03±1.68	116.76±25.85
		XKR8 KO	81.12±0.32	88.27±38.19
	Double negative cells	WT	3.59±0.30	5.03±1.18
		XKR8 KO	3.74±0.38	3.97±1.34
Spleen	CD4	WT	20.74±2.03	13.69±2.74
		XKR8 KO	20.00±1.63	12.55±2.27
	CD8	WT	11.48±0.98	11.26±1.14
		XKR8 KO	11.08±0.29	11.71±3.22
	B cells	WT	58.92±2.61	61.03±6.63
		XKR8 KO	60.00±2.13	60.42±17.64
	NK cells	WT	1.79±0.64	2.50±0.24
		XKR8 KO	2.47±0.23	1.92±1.05
	Neutrophils	WT	0.53±0.10	0.30±0.09
		XKR8 KO	0.30±0.10	0.54±0.18
	Monocytes	WT	1.20±0.25	0.46±0.05
		XKR8 KO	0.68±0.11	0.74±0.23
	Macrophages	WT	0.86±0.06	0.40±0.05
		XKR8 KO	0.59±0.03	0.52±0.09
Peripheral blood	Neutrophils	WT	7.95±3.13	0.39±0.22/mL
		XKR8 KO	8.42±2.68	0.34±0.28/mL

Organs	Immune cells		%	Numbers ($\times 10^6$) *
Bone marrow	CD4	WT	1.51±0.28	0.61±0.10
		XKR8 KO	1.38±0.22	0.58±0.14
	CD8	WT	0.64±0.26	0.25±0.09
		XKR8 KO	0.67±0.25	0.29±0.12
	B cells	WT	34.20±5.05	14.11±3.12
		XKR8 KO	35.94±2.82	14.98±1.79
	NK cells	WT	0.82±0.21	0.33±0.07
		XKR8 KO	0.65±0.10	0.27±0.05
	Neutrophils	WT	33.50±4.35	13.74±2.28
		XKR8 KO	34.38±2.21	14.33±1.60
	Monocytes	WT	8.06±1.12	3.30±0.49
		XKR8 KO	7.45±0.96	3.08±0.30
	Macrophages	WT	11.48±1.60	4.68±0.59
		XKR8 KO	10.63±0.58	4.43±0.47

	Spleen weight (mg)	Thymus weight (mg)
WT	69.92±5.64	65.10±11.29
XKR8 KO	65.26±10.56	58.60±14.64

* Counts of immune cell subsets in the femur and tibia of the mouse's two hind legs.

Data are presented as mean ± SD.

Supplementary Table 2. gRNA, crRNA, and PCR sequences used in this study

Name	Sequence (5' to 3')	Note
XKR8-crRNA-1	CATACTGGACGGCGGCCCAC	
XKR8-crRNA-2	CTGTGGTGCCGTCTCCAAGA	
XKR8-sgRNA-1	GCTCGGCGGCCGCTACCTGT	
XKR8-sgRNA-2	GTGAGTGCTTCGCCCCGGGA	
TRPC3-crRNA-1	GTCAACTGCGTGGACTACAT	
TRPC3-crRNA-2	TCGAGGATCAATGCCTACAA	
TRPV2-crRNA-1	AGTCAACCTCAACTACCGAA	
TRPV2-crRNA-2	CTCTTCAATGCGGTCTCCCG	
TRPV4-crRNA-1	TCGATAGTAGATGTCACGGA	
TRPV4-crRNA-2	CACCCTATATGAGTCCTCGG	
PIEZO1-crRNA-1	CAACGCTGGCCCCTACACGG	
PIEZO1-crRNA-2	AGGACATCCCCAACGCCATC	
Xkr8-1-F	GAATGCATCAAGGGCTGTCC	RT-PCR primers for WT and XKR8 KO mice
Xkr8-1-R	ACTGGTAGTATTCCGCCTGG	
Xkr8-2-F	GGCCGTTGTCCAGTACGTG	RT-PCR primers for $Xkr8^{fl/fl}$ and $Xkr8^{fl/fl}$ $Ly6G^{Cre}$ mice
Xkr8-2-R	GCAAACACCTATACAGGTAGCC	
18s-F	CTGCCGTCTGAGTGTATCGC	*18s* as the internal reference for murine cells
18s-R	GCTGGGGCTGAGGAAAGTG	
XKR8-F	CCTGGGTGACTCATAGCTCCT	RT-PCR primers for HL-60 WT and XKR8 KO cell lines
XKR8-R	GCCAGTGGTAGTACACAAGCC	
18S-F	GTAACCCGTTGAACCCCATT	*18S* as the internal reference for HL-60 cell lines
18S-R	CCATCCAATCGGTAGTAGCG	