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Supplemental Information

MLKL and FADD Are Critical for Suppressing Progressive Lymphoproliferative Disease and Activating the NLRP3 Inflammasome

Xixi Zhang, Cunxian Fan, Haiwei Zhang, Qun Zhao, Yongbo Liu, Chengxian Xu, Qun Xie, Xiaoxia Wu, Xianjun Yu, Jianke Zhang, and Haibing Zhang

Supplemental Figures

Figure S1

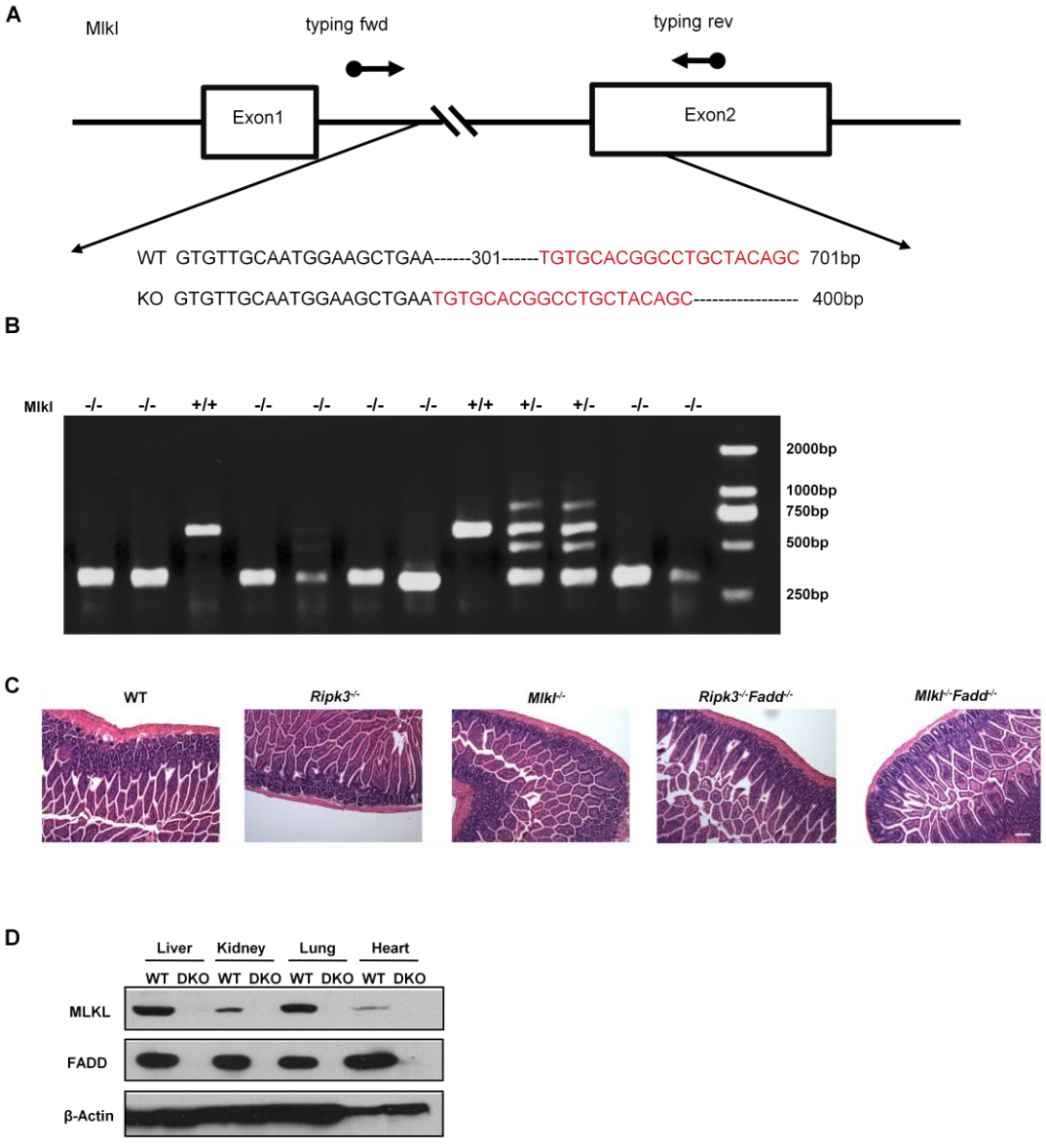


Figure S1. Generation and Characterization of *Mlkl* knockout mice. Related to Figure 1

(A) 301bp deletion in MLKL gene by Crispr-Cas9 mutation system.

(B) Mice of WT (+/+), *Mlkl* Heterozygous (+/-) and *Mlkl* knockout (-/-) were genotyped by T7E1-assay PCR products.

(C) Histology of intestine sections from 16-week-old wild-type, *Ripk3*$^{-/-}$, *Mlkl*$^{-/-}$, *Ripk3*$^{-/-}$*Fadd*$^{-/-}$, *Mlkl*$^{-/-}$ *Fadd*$^{-/-}$ mice, respectively. Scale bars, 50 μm.

(D) Immunoblot of MLKL, FADD and β-actin from liver, kidney, lung and heart of wild-type and *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ mouse.

Figure S2

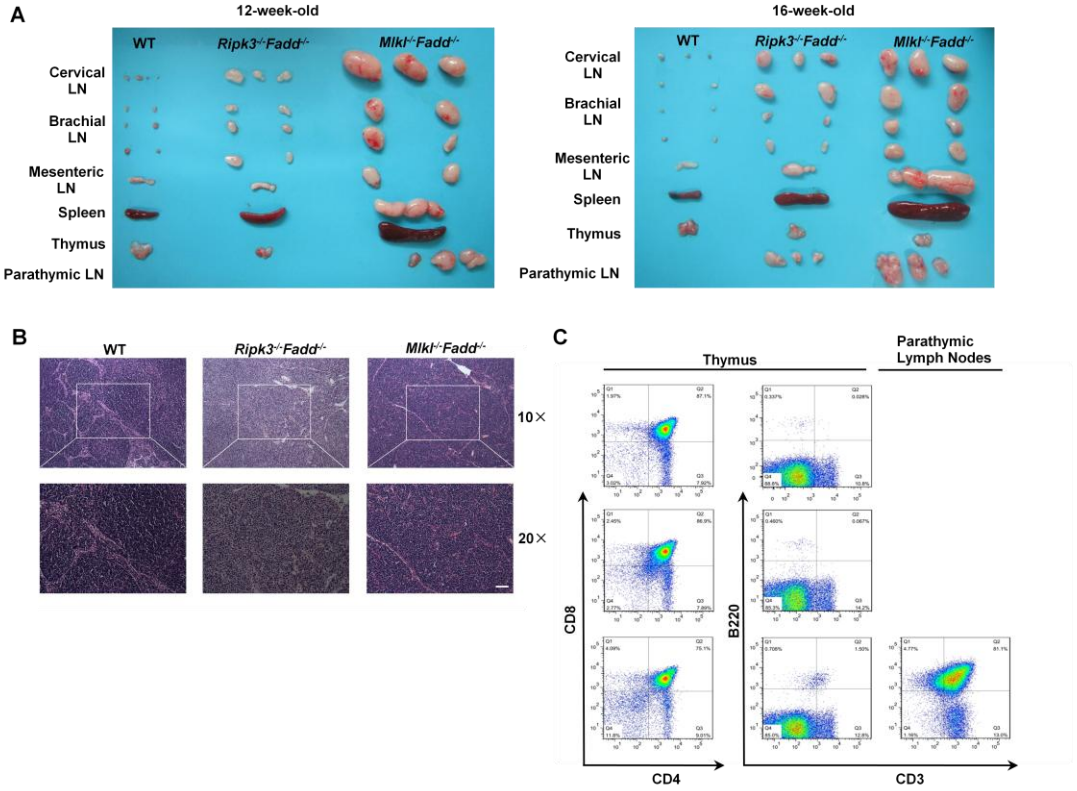

A
12-week-old
16-week-old
WT
Ripk3-/-Fadd-/-
Mlkl-/-Fadd-/-
Cervical LN
Brachial LN
Mesenteric LN
Spleen
Thymus
Parathymic LN
B
10×
20×
C
Thymus
Parathymic Lymph Nodes
CD8
CD4
B220
CD3
D
DAPI
Ki67
Merge
Lymph Nodes
Spleen

Figure S2. Analysis of Progressive Lymphoproliferative Disease of DKO mice. Related to Figure 2

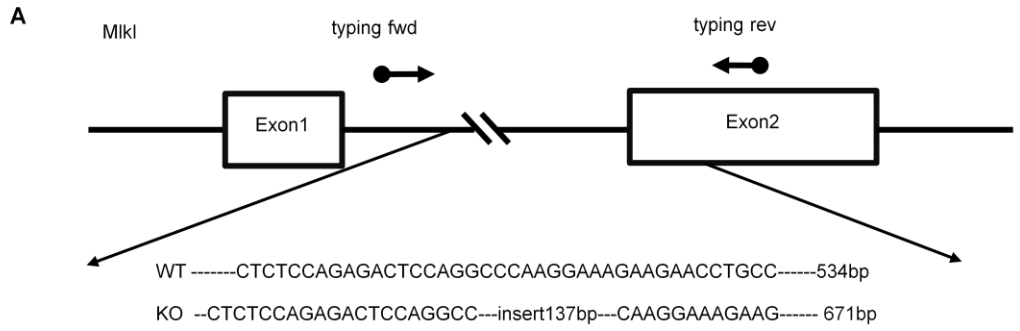
(A) Lymph nodes, spleens and thymus (and parathymic lymph nodes) removed from 12-week-old and 16-week-old mice of indicated genotypes.

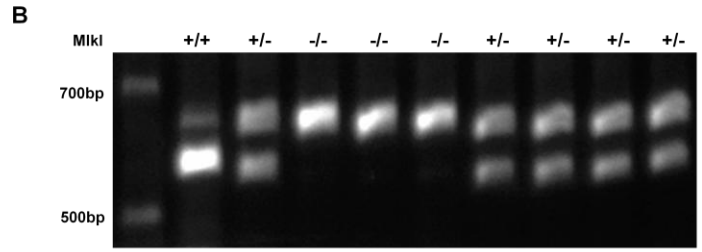
(B) Hematoxylin and Eosin stained sections of fixed Lymph nodes from mice of indicated genotypes. Scale bars, 50 μm

(C) Percentage of $CD4^+$,$CD8^+$ cells or $CD3^+$,$B220^+$ staining cells from Thymus of 12-week-old mice of the indicated genotypes (wild-type on the top, *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ in the middle and *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ on the bottom). $B220^+CD3^+$ T lymphocytes from parathymic lymph nodes in *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ mice.

(D) Immunofluorescence microscopy of Lymph nodes and spleen from 12-week-old wild-type, *Ripk3*$^{-/-}$ *Fadd*$^{-/-}$ and *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ mice stained with Ki67 antibody (red). DAPI (blue) was used for nuclear staining. Scale bars, 25 μm

Figure S3


A
Mlkl
typing fwd
typing rev
Exon1
Exon2
WT -------CTCTCCAGAGACTCCAGGCCCAAGGAAAGAAGAACCTGCC------534bp
KO --CTCTCCAGAGACTCCAGGCC---insert137bp---CAAGGAAAGAAG------ 671bp


B
Mlkl
+/+
+/-
-/-
-/-
-/-
+/-
+/-
+/-
+/-
700bp
500bp

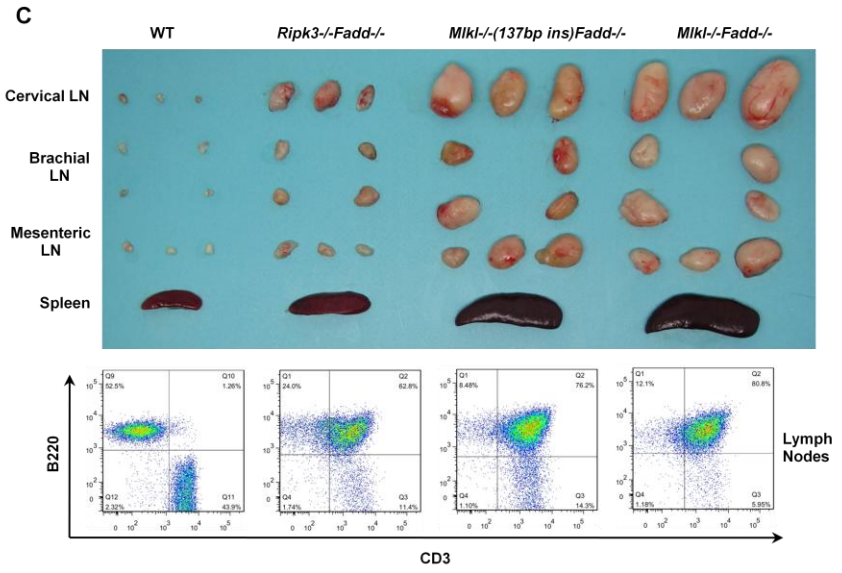

C
WT
Ripk3-/-Fadd-/-
Mlkl-/-(137bp ins)Fadd-/-
Mlkl-/-Fadd-/-
Cervical LN
Brachial LN
Mesenteric LN
Spleen
B220
CD3
Lymph Nodes

Figure S3. Generation and Characterization of *Mlkl(137bp ins)* knockout mice. Related to Figure 1

(A) 137 bp insertion in MLKL gene by Crispr-Cas9 mutation system.

(B) Genotyping of WT (+/+),*Mlkl* Heterozygous (+/-) and *Mlkl* knockout (-/-) mice.

(C) Lymph nodes and spleen were removed from the 12-week-old mice of indicated genotypes and the percentage of $B220^{+}CD3^{+}$ cells in lymph nodes were measured by Flow Cytometery.

Figure S4

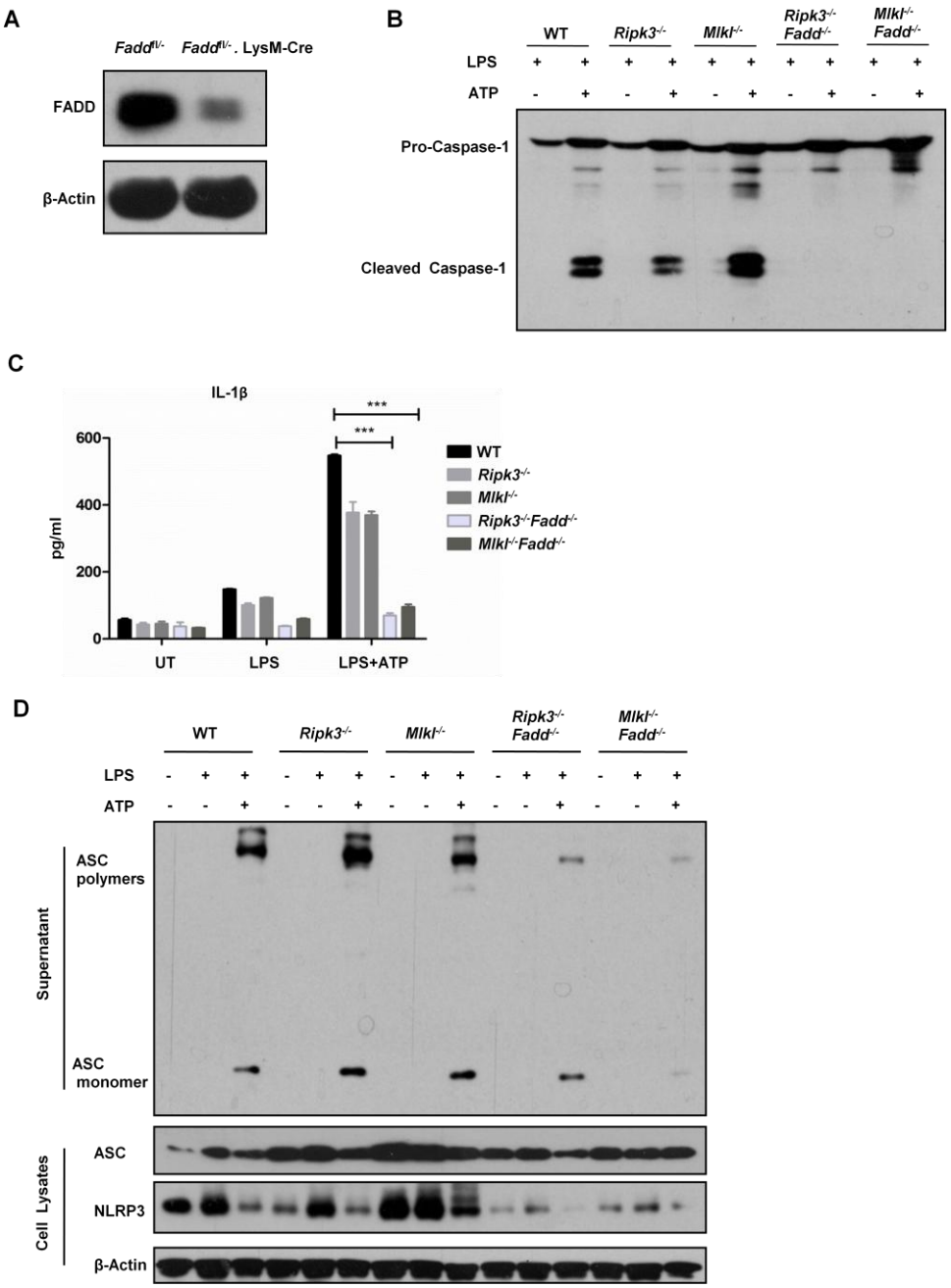

A
Fadd^fl/-
Fadd^fl/-. LysM-Cre
FADD
β-Actin
B
WT
Ripk3^-/-
Mlkl^-/-
Ripk3^-/- Fadd^-/-
Mlkl^-/- Fadd^-/-
LPS
ATP
Pro-Caspase-1
Cleaved Caspase-1
C
IL-1β
***
***
600
400
200
0
pg/ml
UT
LPS
LPS+ATP
WT
Ripk3^-/-
Mlkl^-/-
Ripk3^-/-Fadd^-/-
Mlkl^-/-Fadd^-/-
D
WT
Ripk3^-/-
Mlkl^-/-
Ripk3^-/- Fadd^-/-
Mlkl^-/- Fadd^-/-
LPS
ATP
Supernatant
ASC polymers
ASC monomer
Cell Lysates
ASC
NLRP3
β-Actin

Figure S4. NLRP3 Inflammasome Activation and ASC polymerization in BMDCs upon LPS stimulation. Related to Figure 4, 5

(A) The efficiency of LysM-Cre mediated Fadd deletion in BMDM was examined by western blot.

(B) Wild-type, *Ripk3*$^{-/-}$, *Mlkl*$^{-/-}$, *Ripk3*$^{-/-}$*Fadd*$^{-/-}$, *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ BMDCs were primed with 20ng/ml LPS for 5h prior to 30min treatment of 5mM ATP. Supernatants were analyzed by immunoblot for caspase-1 cleavage.

(C) Wild-type, *Ripk3*$^{-/-}$, *Mlkl*$^{-/-}$, *Ripk3*$^{-/-}$*Fadd*$^{-/-}$, *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ BMDCs were stimulated with 20ng/ml LPS for 12hrs prior to 15 mins treatment of 5mM ATP. Supernatants were analyzed by ELISA for IL-1β secretion.

(D) Wild-type, *Ripk3*$^{-/-}$, *Mlkl*$^{-/-}$, *Ripk3*$^{-/-}$*Fadd*$^{-/-}$, *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ BMDCs were stimulated with 20ng/ml LPS for 5hrs prior to 30 mins treatment of 5mM ATP. Supernatants and cell pellets were analyzed by immunoblot for ASC polymerization. Cell lysates were analyzed by immunoblot for the expression of ASC and NLRP3.

Supplemental Experimental Procedures

Flow Cytometry

Lymphocytes were isolated from the thymus, spleen, and lymph nodes of 2- to 7-month -old mice. Antibodies against mouse CD3 (eBioscience), CD4 (eBioscience), CD8 (eBioscience) and B220 (eBioscience) were fluorescence-conjugated and were used for flow cytometry analysis in this study. Single cell suspension of lymphocytes was stained on ice for half an hour with fluorescence-conjugated Abs in the staining buffer. After staining, cells were immediately analyzed by flow cytometry (FACSAria III, BD biosciences).

Western blot analysis

Cells were washed with ice-cold PBS and resuspended in a lysis buffer containing Tris-HCl (50 mM; pH 8.0), NaCl (150 mM), EDTA(1 mM), NP-40 (1%), PMSF (1 mM; Sigma), Phosphatase Inhibitor (sigma) and a protease inhibitor cocktail (Roche Biochemical Laboratories). After incubation on ice for 30 min, Supernatants were collected after centrifugation (14,000 rpm for 10min) at 4 ℃, and protein concentrations were determined using Bradford assay. Total 30ug protein was loaded for western blot analysis.

Immunofluorescence

BMDMs were plated overnight on coverslips before various stimulations. The stimulated cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 15 min. Next, the cells was permeabilized with Triton X-100 for 5mins at room temperature and blocked with 10% FBS in PBS. The cells were then incubated with individual fluorescence-conjugated antibody (anti-ASC and anti-NLRP3) for 2 hrs. Finally, the cells were stained with DAPI. The confocal microscopy analyses were performed using a Zeiss 710 laser-scanning microscope (Zeiss, Thornwood, NY, USA)