Supplementary Information

TRADD regulates perinatal development and adulthood survival in mice lacking RIPK1 and RIPK3

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Supplementary Figure 1

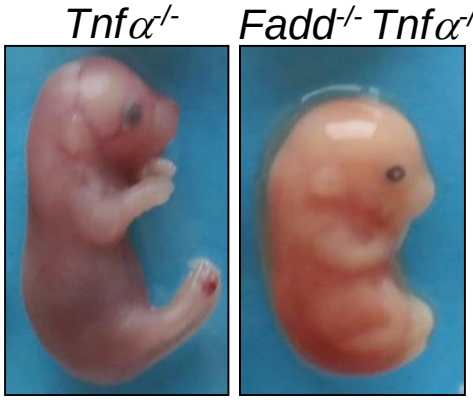
a

Offspring of *Fadd*$^{+/-}$ *Tnf*$\alpha^{-/-}$ intercrosses

Genotype	*Fadd* / *Tnf*α	+/+ -/-	+/- -/-	-/- -/-	Total
E12.5-16.5 Embryos	Actual	13	23	**15**	51
	Expected	13	26	13	52
E17.5-18.5 Embryos	Actual	20	40	**25**	85
	Expected	20	40	20	80
After Birth	Actual	21	23	**0**	44
	Expected	21	42	21	84

b

E18.5



c

Offspring of *Fadd*$^{+/-}$ *Tnf*$\alpha^{+/-}$ intercrosses

Genotype	*Fadd* / *Tnf*α	+/+, +/- +/+, +/-, -/-	-/- +/+, +/-	-/- -/-	Total
E17.5-18.5 Embryos	Actual	25	**0**	**3**	28
	Expected	25	6	2	33

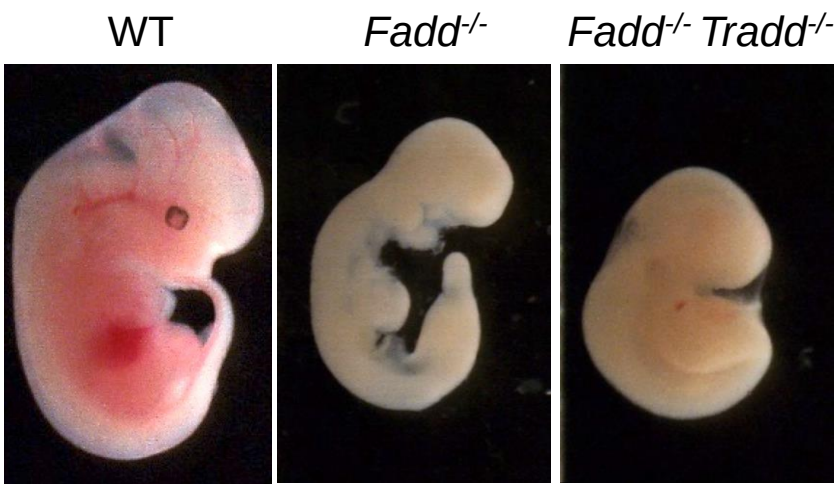
Supplementary Figure 1. Deletion of TNFα improves *Fadd*$^{-/-}$ embryogenesis **a**, Actual and expected frequencies of embryos of various embryonic stages from intercrosses of *Fadd*$^{+/-}$ *Tnf*$\alpha^{-/-}$ mice. Chi square analysis shows no significant difference between actual and expected frequencies at E12.5-E16.5 ($p = 0.721$) or E17.5-E18.5 ($p = 0.535$), but there is a significant difference after birth ($p < 0.0001$). **b**, Embryos with indicated genotypes at E18.5, (n = 3 independent experiments). **c**, Actual and expected frequencies of embryos from *Fadd*$^{+/-}$*Tnf*$\alpha^{+/-}$ intercrosses. Chi square analysis shows a significant difference at late embryonic stages ($p = 0.0388$).

Supplementary Figure 2

a Offspring of *Fadd*$^{+/-}$ *Tradd*$^{-/-}$ intercrosses

Genotype	*Fadd* / *Tradd*	+/+ -/-	+/- -/-	-/- -/-	Total
E12.5 Embryos	Actual	8	21	**2**	31
	Expected	8	16	8	32
E13.5-18.5 Embryos	Actual	27	55	**0**	82
	Expected	27	54	27	108

b

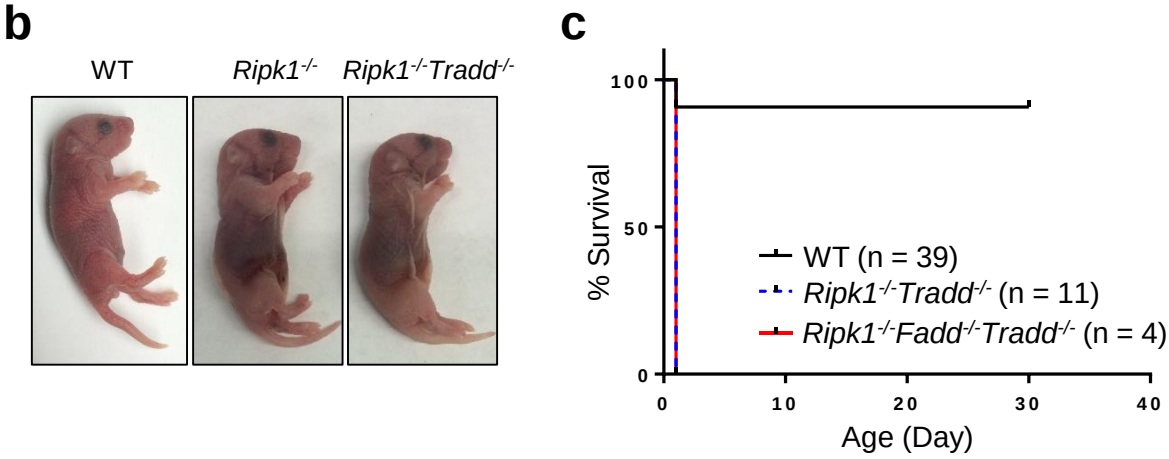


Supplementary Figure 2. TRADD does not mediate necroptosis in *Fadd*$^{-/-}$ embryos a, Actual and expected frequencies of embryos of various embryonic stages from intercrosses of *Fadd*$^{+/-}$*Tradd*$^{-/-}$ mice. Chi square analysis shows a significant difference between actual and expected frequencies at E12.5 ($p = 0.048$) and E13.5-18.5 ($p < 0.0001$). **b,** Embryos with indicated genotypes at E12.5 (n = 3 independent experiments).

Supplementary Figure 3

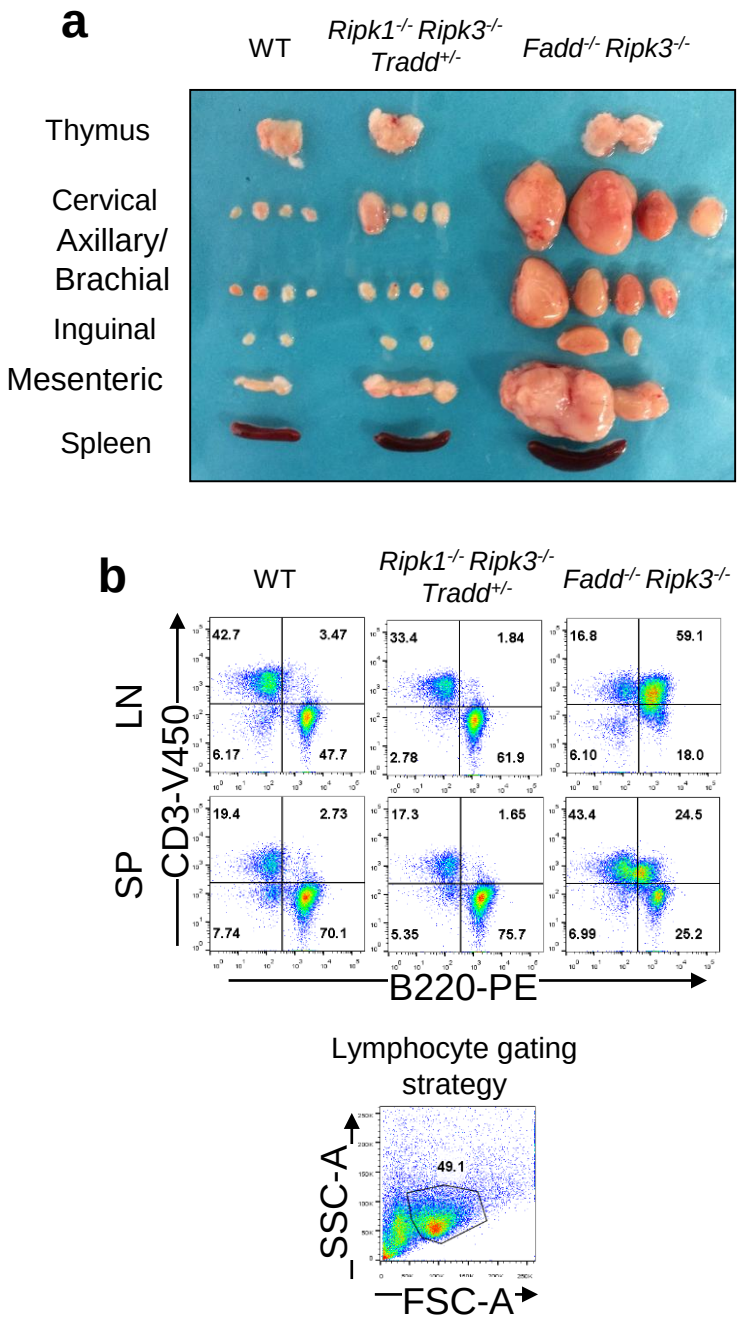
a Offspring of *Ripk1*$^{+/-}$ *Fadd*$^{+/-}$ *Tradd*$^{-/-}$ intercrosses

Genotype		*Ripk1* / *Fadd* / *Tradd*	+/+, +/- / +/+, +/- / -/-	+/+, +/- / -/- / -/-	-/- / +/+, +/- / -/-	-/- / -/- / -/-	Total
At birth	Actual		93	0	4	3	100
	Expected		93	31	31	10	165
At weaning age (3 wk)	Actual		89	0	0	0	89
	Expected		90	30	30	10	160



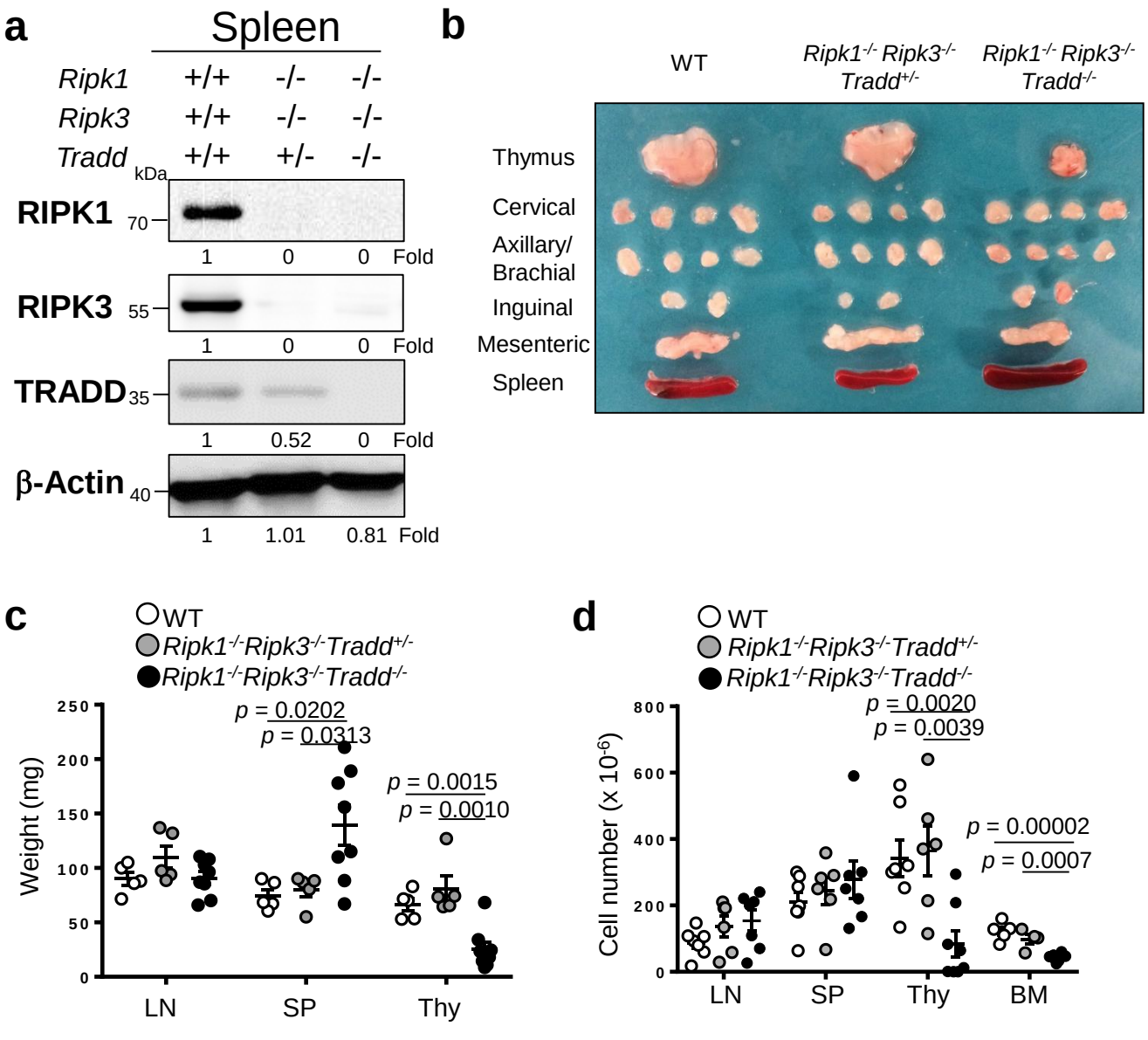
Supplementary Figure 3. Deletion of TRADD does not rescue perinatal lethality of *Ripk1*$^{-/-}$ or *Ripk1*$^{-/-}$*Fadd*$^{-/-}$ mice **a**, Actual and expected frequency of indicated genotypes at birth and weaning age from intercross of *Ripk1*$^{+/-}$*Fadd*$^{+/-}$*Tradd*$^{-/-}$ mice. Chi square analysis shows a significant difference between actual and expected frequencies ($p < 0.0001$). **b**, Newborn mice of indicated genotypes (n = 4 independent experiments). **c**, Kaplan-Meier plot showing survival of mice of indicated genotypes. Logrank test shows no significant difference in survival between *Ripk1*$^{-/-}$*Fadd*$^{-/-}$*Tradd*$^{-/-}$ and *Ripk1*$^{-/-}$*Tradd*$^{-/-}$mice ($p > 0.999$).

Supplementary Figure 4



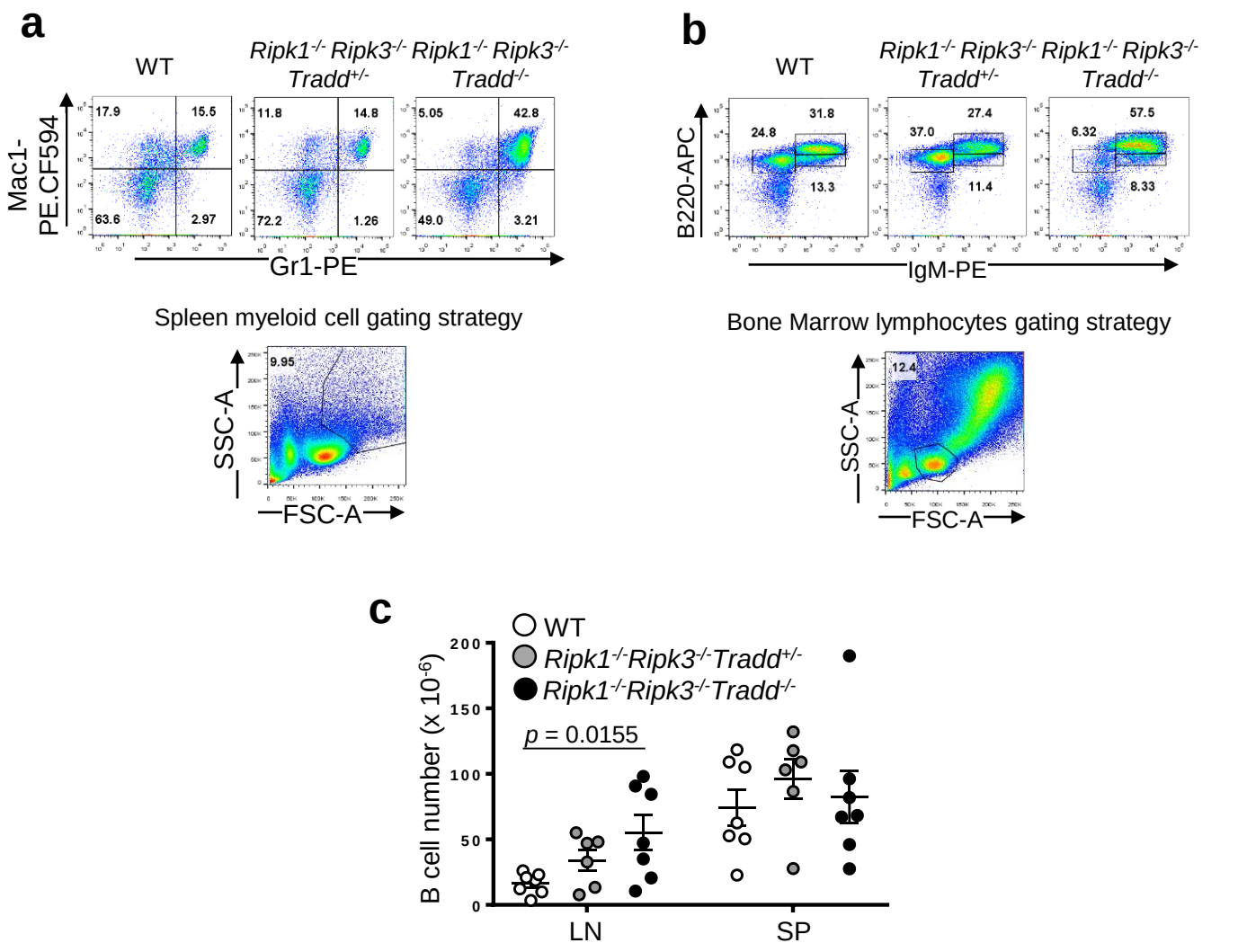
Supplementary Figure 4. Analysis of aged *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Tradd*$^{+/-}$ mice **a**, Spleen, lymph nodes, and thymus of 9 month old mice of indicated genotypes (n = 4 independent experiments). **b**, Two-color flow cytometric plots investigating the presence of T cells ($CD3^+B220^-$) and B cells ($CD3^-B220^+$) and abnormal T cells ($CD3^+B220^+$) in the lymph nodes and spleen of aged mice (n = 4 independent experiments)

Supplementary Figure 5



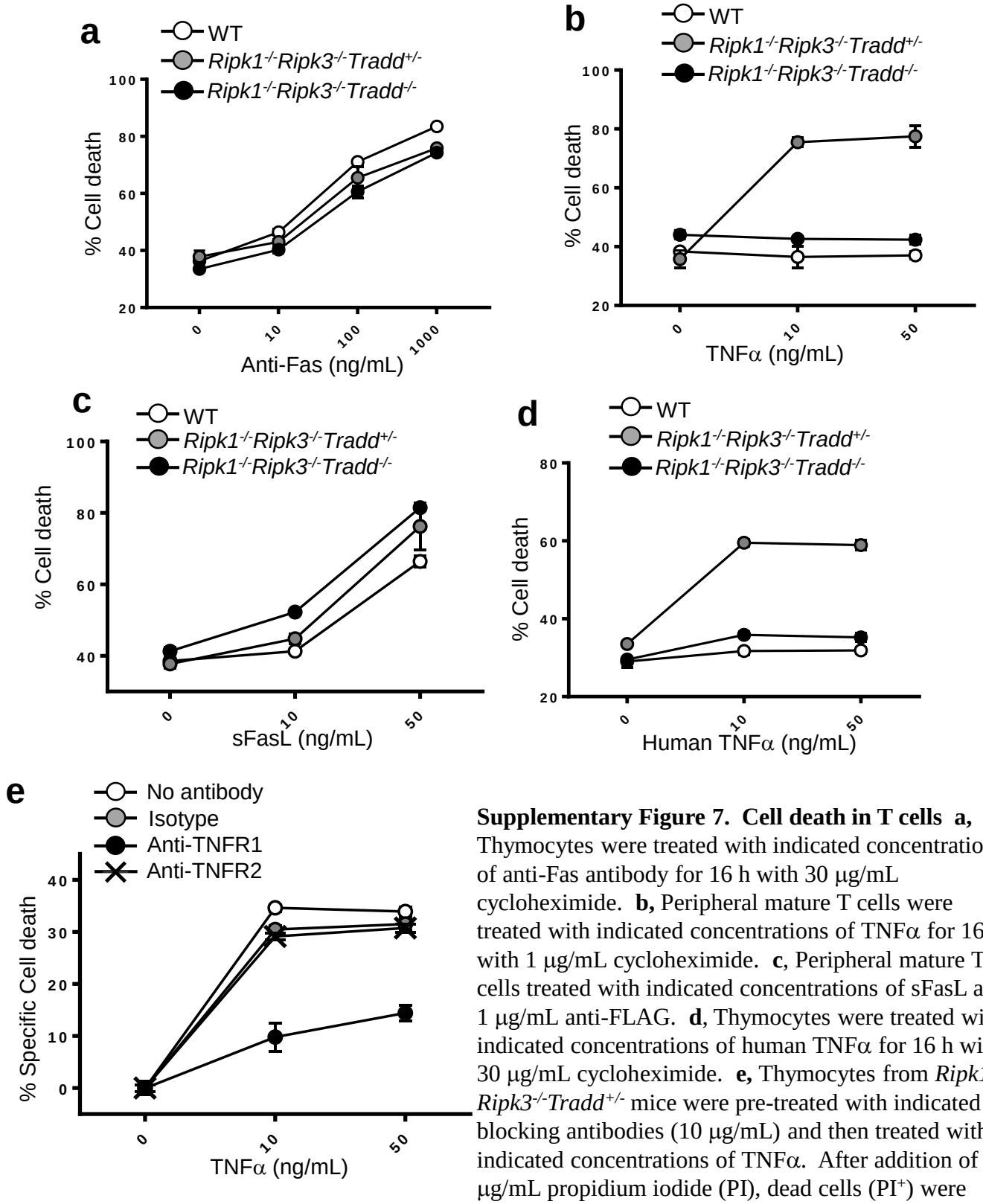
Supplementary Figure 5. Lymphoid compartment of *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Tradd*$^{-/-}$ mice **a**, Western blots showing RIPK1, RIPK3, TRADD, and β-Actin protein concentrations in the spleen (n = 3 independent experiments). **b**, Spleen, lymph nodes, and thymus of 7 week old mice of indicated genotypes (n = 5 independent experiments). **c**, Weight of lymph nodes, spleen, and thymus of 7-8 week old mice (n = 6 independent experiments). **d**, Total cellularity of lymphoid organs of 7-8 week old mice (n = 6 independent experiments). Errors in **c** and **d** represent mean±SEM.

Supplementary Figure 6



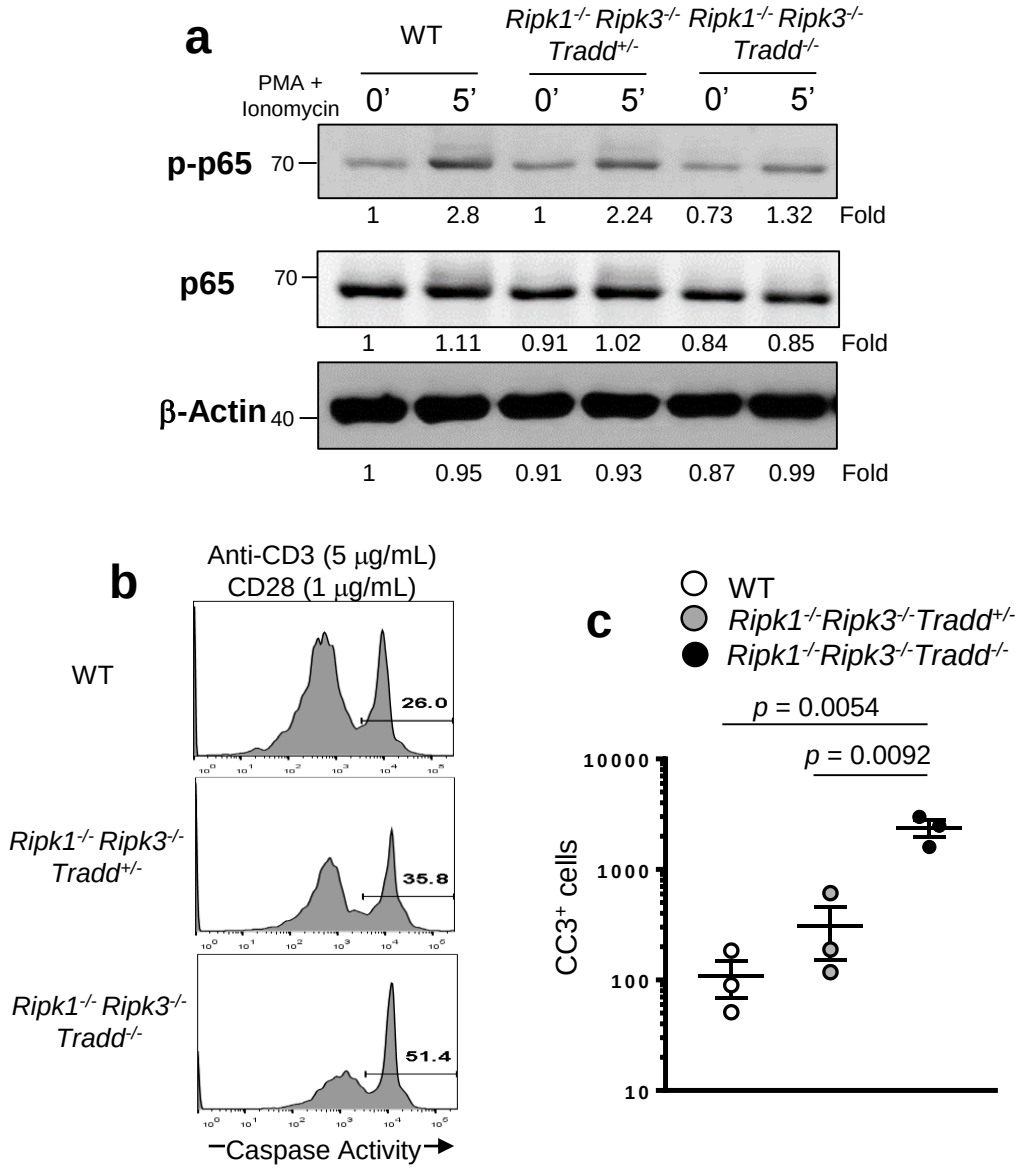
Supplementary Figure 6. Myeloid cells and B cell in *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Tradd*$^{-/-}$ mice **a**, Myeloid populations in the spleen identified by Mac-1 and Gr-1 staining (n = 4 independent experiments). **b**, Representative 2-color flow cytometric plots showing B220^{+}IgM^{-} pre/pro B cells, B220^{+}IgM^{+} immature B cells, and B220^{hi}IgM^{+} mature B cells in the bone marrow (n = 4 independent experiments). **c**, B220^{+} B cell numbers in the lymph nodes and spleen (n = 7 independent experiments). All graphs presented as mean ± SEM.

Supplementary Figure 7



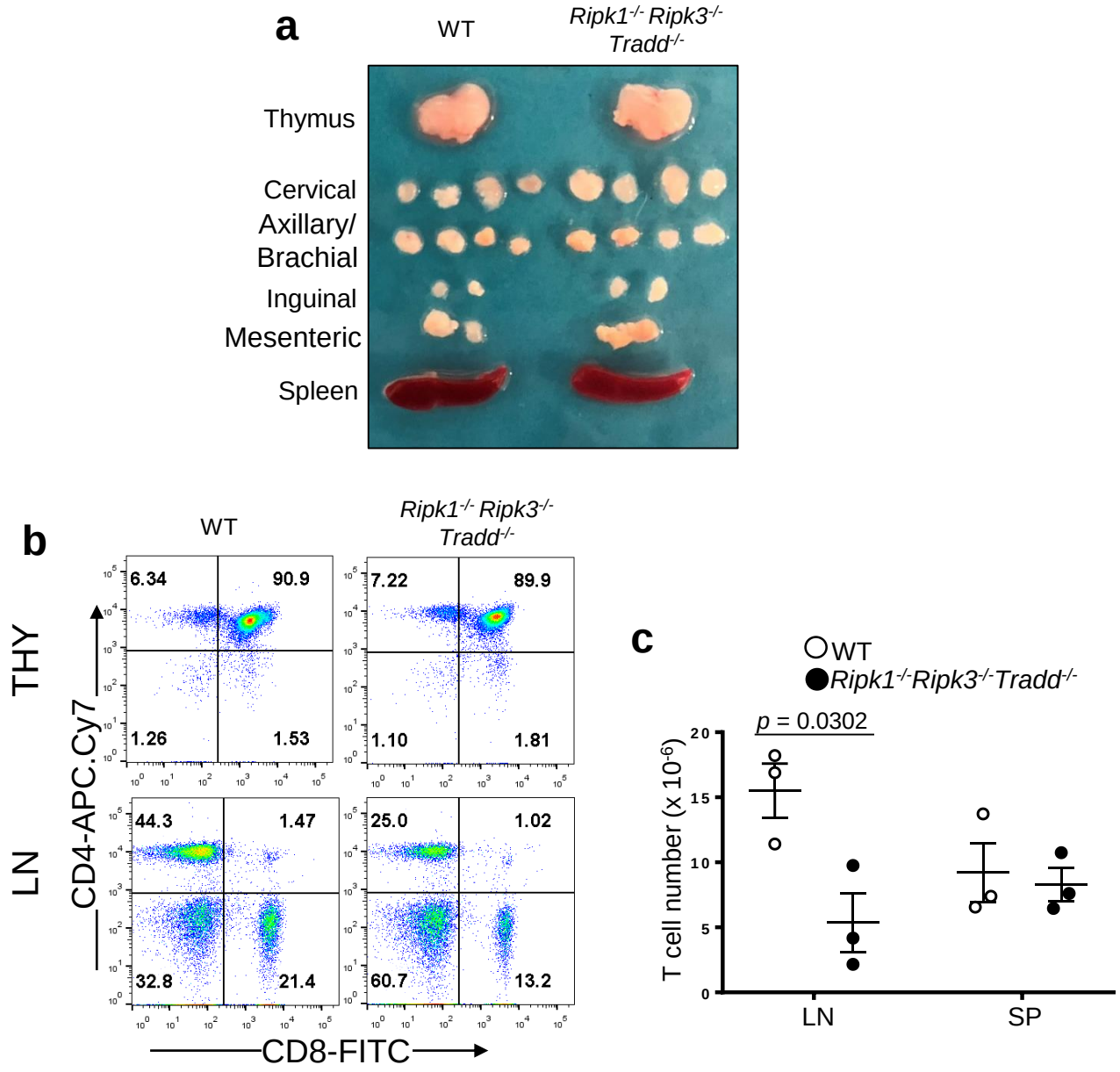
Supplementary Figure 7. Cell death in T cells a, Thymocytes were treated with indicated concentrations of anti-Fas antibody for 16 h with 30 μg/mL cycloheximide. **b,** Peripheral mature T cells were treated with indicated concentrations of TNFα for 16 h with 1 μg/mL cycloheximide. **c**, Peripheral mature T cells treated with indicated concentrations of sFasL and 1 μg/mL anti-FLAG. **d**, Thymocytes were treated with indicated concentrations of human TNFα for 16 h with 30 μg/mL cycloheximide. **e,** Thymocytes from *Ripk1*$^{-/-}$ *Ripk3*$^{-/-}$*Tradd*$^{+/-}$ mice were pre-treated with indicated blocking antibodies (10 μg/mL) and then treated with indicated concentrations of TNFα. After addition of 1 μg/mL propidium iodide (PI), dead cells (PI^{+}) were determined by flow cytometry. Errors, ±SEM of triplicates. Data represent three independent experiments.

Supplementary Figure 8



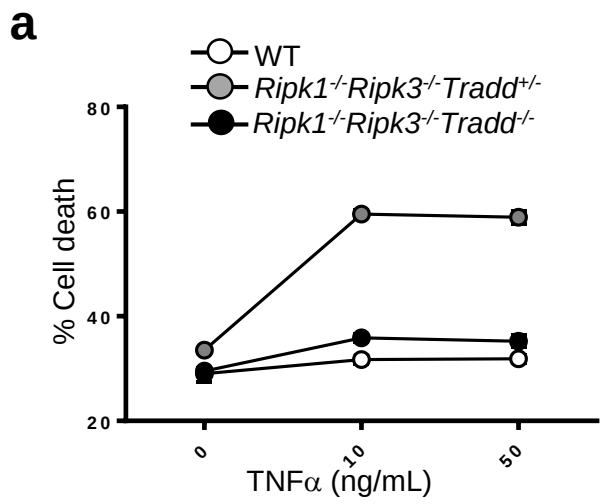
Supplementary Figure 8. NFκB signaling and caspase activity analysis **a**, Western blot analysis of NFκB p65 phosphorylation, total p65, after stimulation of peripheral mature T cells. The data represents three independent experiments. β-Actin, loading control. **b**, Caspase activities of mature T cells after 48 h stimulation was measured by flow cytometry with CellEvent Caspase 3/7 Green Flow Reagent. **c**, Scattered dot plot showing quantification of $CC3^+$ cells in the large intestine sections using the ImageScope software (Leica Biosystems). Errors, mean±SEM, n = 3.

Supplementary Figure 9



Supplementary Figure 9. Analysis of weaning age *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Tradd*$^{-/-}$ mice **a,** The spleen, lymph nodes, and thymus of 3 week old mice of indicated genotypes. Data are representative of at least three mice of each genotype. **b** Two-color flow cytometric plots showing CD4^{+} and CD8^{+} populations in the thymus and lymph nodes of three week old mice (representative of three independent experiments). **c**, CD3^{+} T cell numbers in the lymphoid organs of three week old mice of indicated genotypes (n = 3 independent experiments)**.** Errors, mean±SEM.

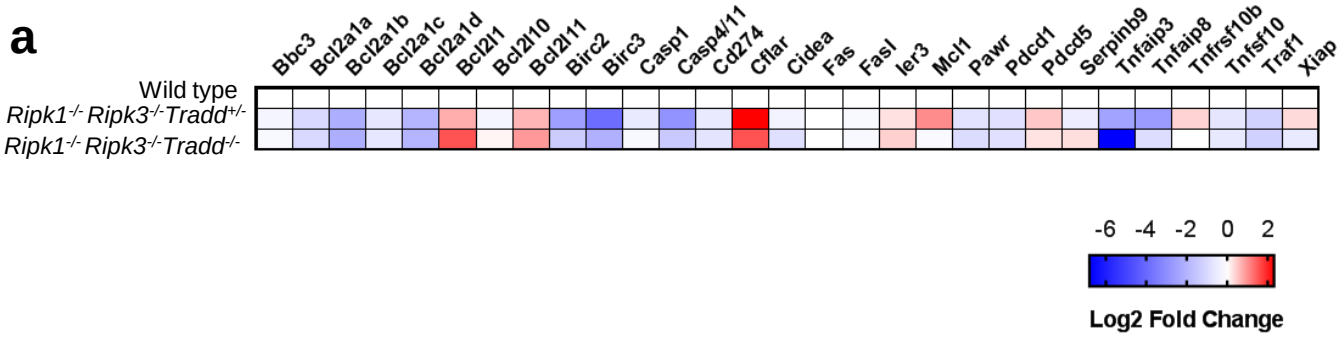
Supplementary Figure 10



Supplementary Figure 10. Cell death in thymocytes from young mice a, Thymocytes isolated from 3 week old mice were treated with indicated concentrations of TNFα for 16 h with 30 μg/mL cycloheximide. Cell death analysis was performed by PI staining using a flow cytometer. Data represents three independent experiments. Errors, ± SEM.

Supplementary Figure 11

a



b

	WT	*Ripk1$^{-/-}$Ripk3$^{-/-}$ Tradd$^{+/-}$*	*Ripk1$^{-/-}$Ripk3$^{-/-}$ Tradd$^{-/-}$*
Bbc3	5.08	4.79	4.89
Bcl2a1a	5.69	4.66	4.66
Bcl2a1b	6.66	4.47	4.47
Bcl2a1c	4.01	3.35	3.35
Bcl2a1d	6.44	4.42	4.46
Bcl2l1	11.36	12.1	12.91
Bcl2l10	3.8	3.51	3.89
Bcl2l11	4.8	5.47	5.72
Birc2	9.8	7.21	8.41
Birc3	11.34	7.43	9.21
Casp1	4.99	4.42	4.78
Casp4/11	7.86	4.96	6.41
Cd274	5.12	4.55	4.39
Cflar	8.66	10.97	10.21
Cidea	4.65	4.32	3.78
Fas	6.73	6.73	6.73
Fasl	3.3	3.1	3.1
Ier3	11.5	11.77	11.92
Mcl1	11.87	12.91	11.7
Pawr	8.67	7.9	7.73
Pdcd1	4.36	3.54	3.54
Pdcd5	11.71	12.21	11.97
Serpinb9	11.25	10.76	11.54
Tnfaip3	13.27	10.77	6.45
Tnfaip8	8.14	5.45	7.21
Tnfrsf10b	11.77	12.16	11.77
Tnfsf10	3.79	3.1	3.16
Traf1	6.38	5.15	5.14
Xiap	11.39	11.72	10.79

Supplementary Figure 11. NFκB target gene expression **a**, Heatmap of RNA expression analysis from dermal fibroblasts stimulated with 10 ng/mL TNFα for 4 h. Data expressed as fold change relative to wild type mouse fibroblasts. **b,** Gene expression levels of the array analysis are shown Log2 signal intensity.