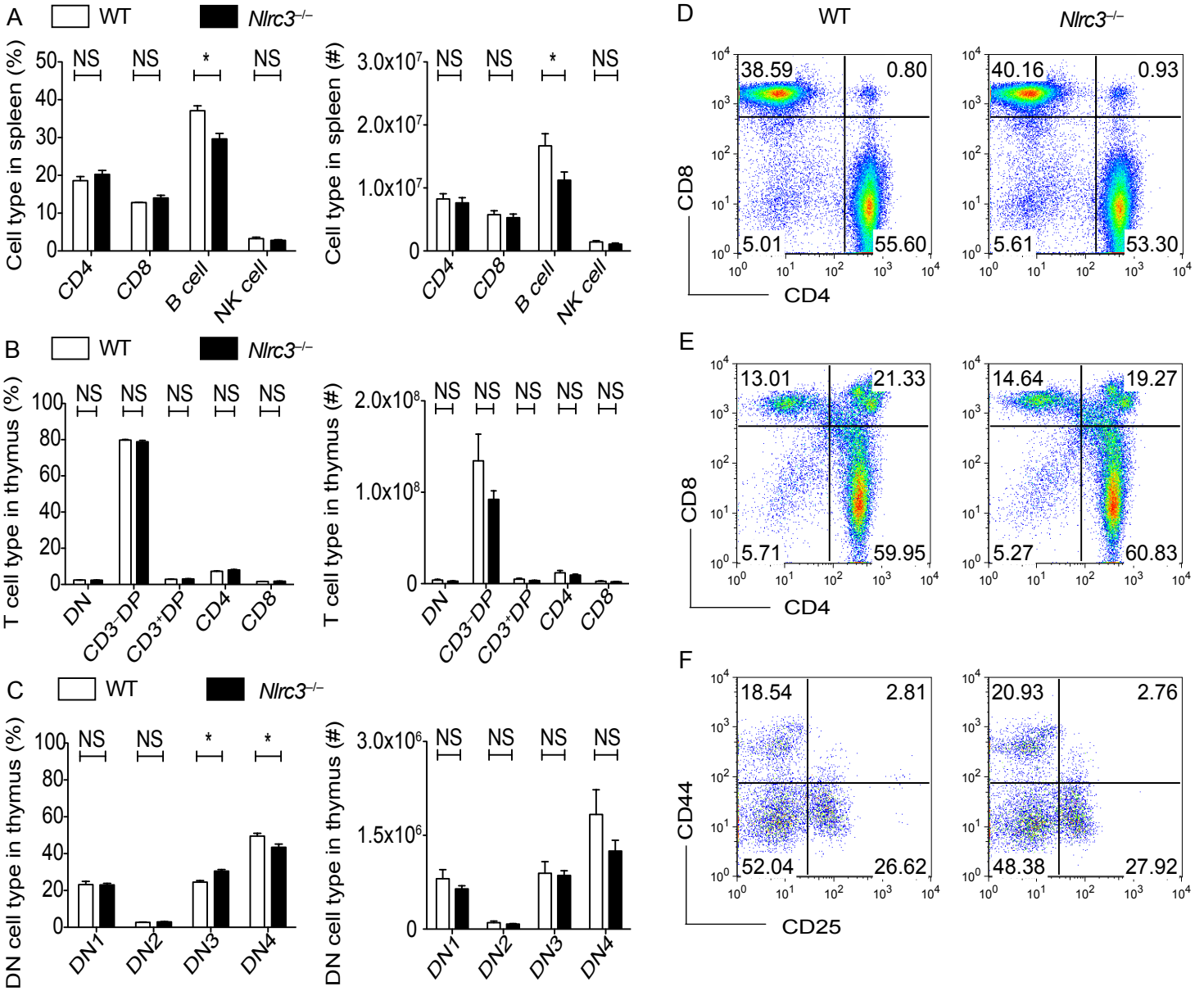
Immunity, Volume *49*

Supplemental Information

The Innate Immune Sensor NLRC3 Acts as a Rheostat that Fine-Tunes T Cell Responses in Infection and Autoimmunity

Toru Uchimura, Yoshitaka Oyama, Meng Deng, Haitao Guo, Justin E. Wilson, Elena Rampanelli, Kevin D. Cook, Ichiro Misumi, Xianming Tan, Liang Chen, Brandon Johnson, Jason Tam, Wei-Chun Chou, W. June Brickey, Alex Petrucelli, Jason K. Whitmire, and Jenny P.Y. Ting



Supplement Figure 1. *Nlrc3*$^{-/-}$ mice show normal frequencies of T cells in spleen and thymus. Related to Figure 1. Splenic and thymic T cell subpopulations and their numbers were evaluated between wild type (WT) controls and *Nlrc3*$^{-/-}$ mice (n=5 for WT, n=5 for *Nlrc3*$^{-/-}$ mice).

(A) Percentage and total number of $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD19^+$ and $CD3^-NK1.1^+$ cells in spleen.

(B) Percentage and total number of $CD4^-CD8^-$ (DN), $CD3^-CD4^+CD8^+$($CD3^-$DP), $CD3^+CD4^+CD8^+$ ($CD3^+$DP), $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in thymus.

(C) Percentage and total number of $CD25^-CD44^+$ DN (DN1), $CD25^+CD44^+$ DN (DN2), $CD25^+CD44^-$ DN (DN3) and $CD25^-CD44^-$DN cells (DN4) in thymus.

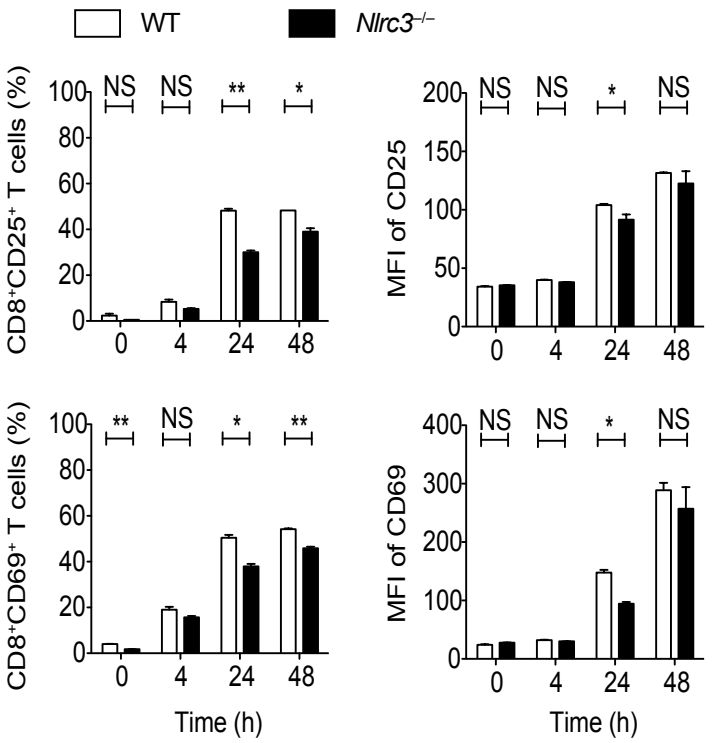
(D) Flow cytometric analysis of $CD4^+$ and $CD8^+$ populations among $CD3^+$ cells in spleen, as used in (A).

(E) Flow cytometric analysis of $CD4^+$ and $CD8^+$ populations among $CD3^+$ cells in thymus, as used for (B).

(F) Flow cytometric analysis of $CD25^+$ and $CD44^+$ populations among $CD4^-CD8^-$ cells in thymus, representative of (C).

Data are from one experiment representative of two experiments. Data are shown as mean ± SEM.

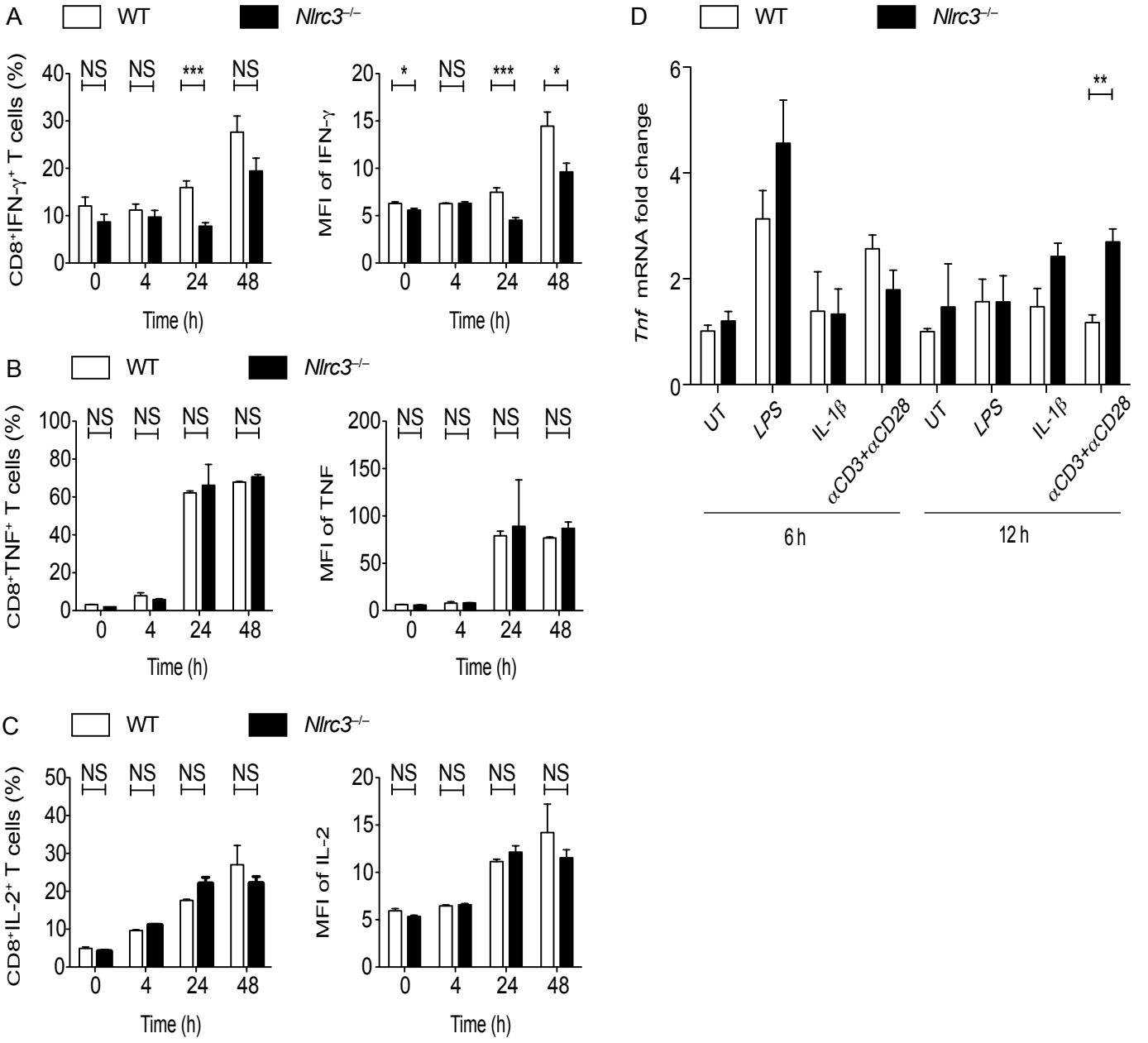
Statistical significance determined by unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.



Supplement Figure 2. *Nlrc3*$^{-/-}$ CD8^{+} cells show normal expression of activation markers *in vitro*. Related to Figure 1. CD8^{+} T cells purified from wild type and *Nlrc3*$^{-/-}$ mice were stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) antibodies and incubated for 0, 4, 24, 48 hrs. The percentage of CD8^{+}CD25^{+} and CD8^{+}CD69^{+} cells, and MFI of CD25 among CD8^{+}CD25^{+} cells and CD69 among CD8^{+}CD69^{+} cells were evaluated.

Data are from one experiment representative of two or three experiments. Data are shown as mean ± SEM.

Statistical significance determined by unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.



Supplement Figure 3. *Nlrc3*$^{-/-}$ CD8^{+} T cells express normal amounts of cytokine upon *in vitro* stimulation.

Related to Figure 2.

CD8^{+} T cells purified from wild type and *Nlrc3*$^{-/-}$ mice were stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) antibodies and incubated for 0, 4, 24, 48 hrs.

(A) Percentage of CD8^{+}IFN-γ^{+} T cells and the MFI of IFN-γ among CD8^{+}IFN-γ^{+} T cells.

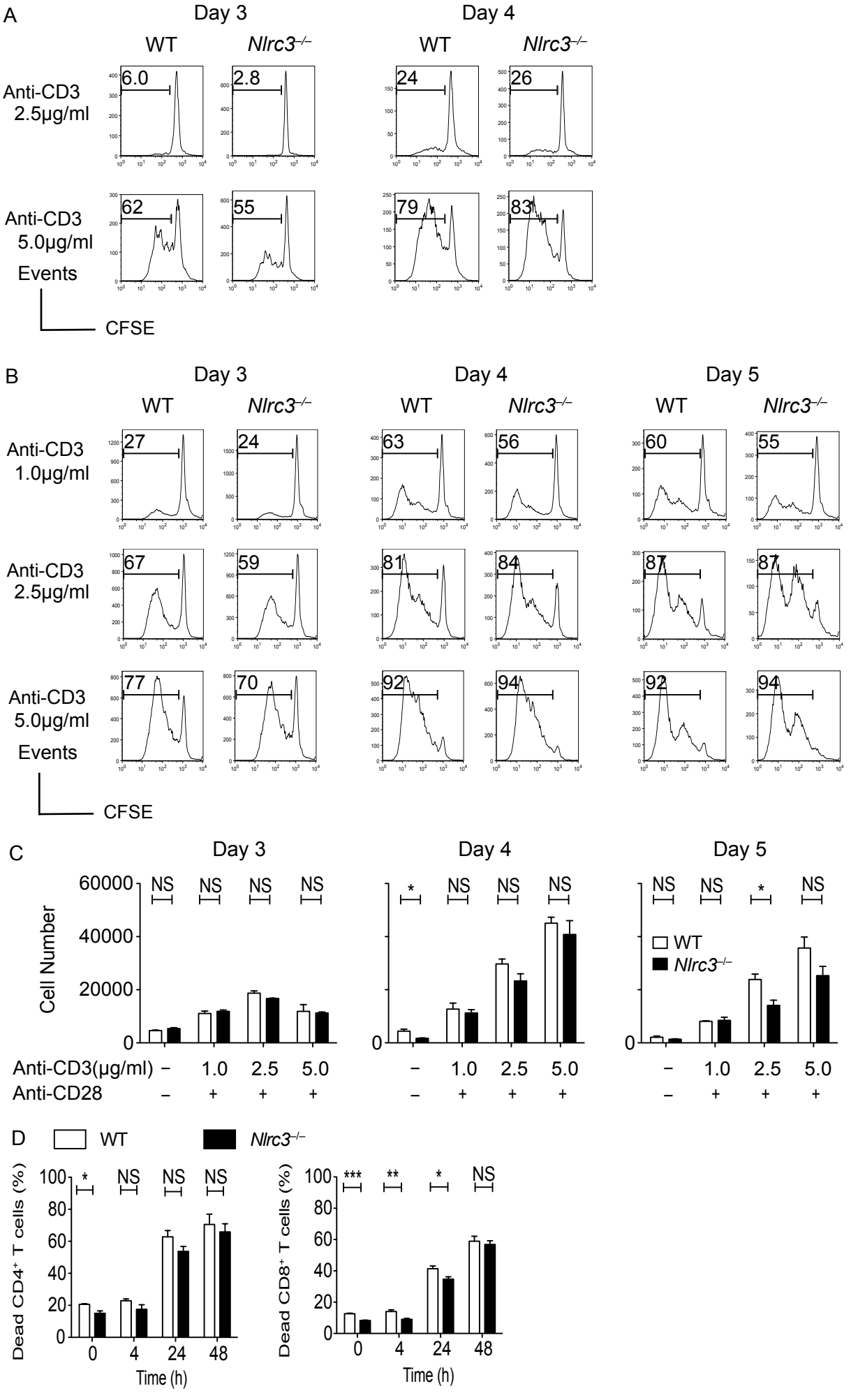
(B) Percentage of CD8^{+}TNF^{+} T cells and the MFI of TNF among CD8^{+}TNF^{+} T cells.

(C) Percentage of CD8^{+}IL-2^{+} T cells and the MFI of IL-2 among CD8^{+}IL-2^{+} T cells.

(D) Quantitative PCR analysis of Tnf transcript in response to TLR agonists, IL-1β and anti-CD3 and anti-CD28.

Representative data from two or three experiments. Data are shown as mean ± SEM.

Statistical significance determined by unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.


A
Day 3
Day 4
WT
Nlrc3−/−
WT
Nlrc3−/−
Anti-CD3 2.5μg/ml
6.0
2.8
24
26
Anti-CD3 5.0μg/ml
62
55
79
83
Events
CFSE
B
Day 3
Day 4
Day 5
WT
Nlrc3−/−
WT
Nlrc3−/−
WT
Nlrc3−/−
Anti-CD3 1.0μg/ml
27
24
63
56
60
55
Anti-CD3 2.5μg/ml
67
59
81
84
87
87
Anti-CD3 5.0μg/ml
77
70
92
94
92
94
Events
CFSE
C
Day 3
Day 4
Day 5
Cell Number
60000
40000
20000
0
NS
*
WT
Nlrc3−/−
Anti-CD3(μg/ml) − 1.0 2.5 5.0
Anti-CD28 − + + +
D
WT
Nlrc3−/−
Dead CD4+ T cells (%)
Dead CD8+ T cells (%)
100
80
60
40
20
0
*
**
***
NS
0 4 24 48
Time (h)

Supplement Figure 4. WT and *Nlrc3*$^{-/-}$ CD8^{+} T cells show similar amounts of proliferation. Related to Figure 3.

(A) Naïve CD4^{+} CD25^{lo}CD44^{lo} T cells isolated from wild type and *Nlrc3*$^{-/-}$ mice were CFSE-labeled and stimulated with anti-CD3 (2.5 and 5 μg/ml) and anti-CD28 (2 μg/ml) antibodies. The histograms show their CFSE fluorescence after 3 to 4 days of stimulation.

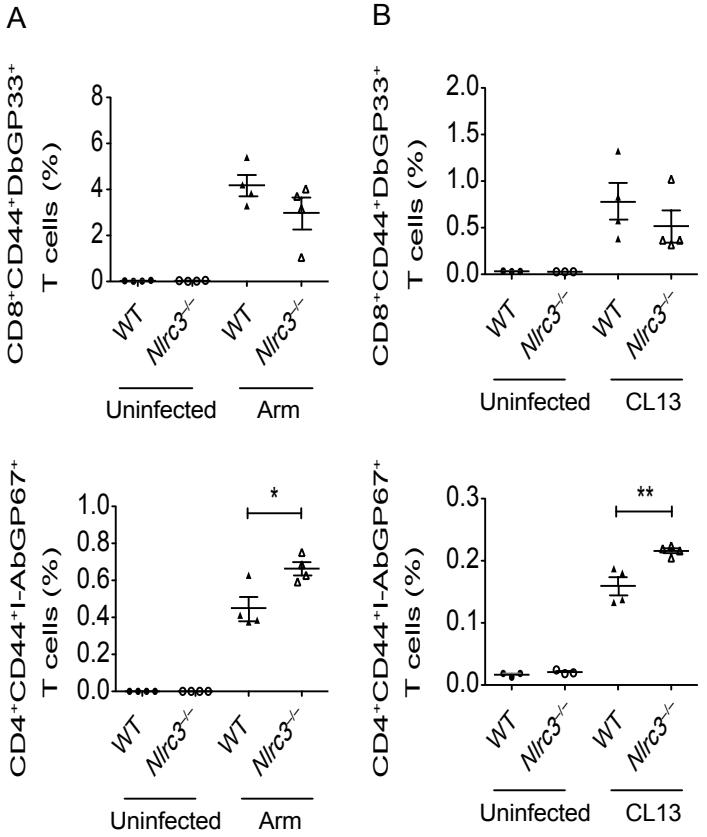
(B) CD8^{+} T cells isolated from wild type and *Nlrc3*$^{-/-}$ mice were CFSE-labeled and stimulated with anti-CD3 (1, 2.5 and 5 μg/ml) and anti-CD28 (2 μg/ml) antibodies for 3 to 5 days. The histograms show CFSE fluorescence dilution for CD8^{+} T cells.

(C) Number of cells recovered from the cultures in (B).

(D) CD4^{+} T cells or CD8^{+} T cells purified from WT and *Nlrc3*$^{-/-}$ mice were stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) antibodies and incubated for 0, 4, 24, 48 hrs. Dead cells were identified by “Ghost Dye” fluorescent assay. Graphs show the percentage of CD4^{+} and CD8^{+} cells that were dead.

Results are representative of at least two experiments. Data are shown as mean ± SEM.

Statistical significance determined by unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.



Supplement Figure 5. *Nlrc3*$^{-/-}$ mice show improved CD4^{+} T cell responses during LCMV infection. Related to Figures 4 and 5.

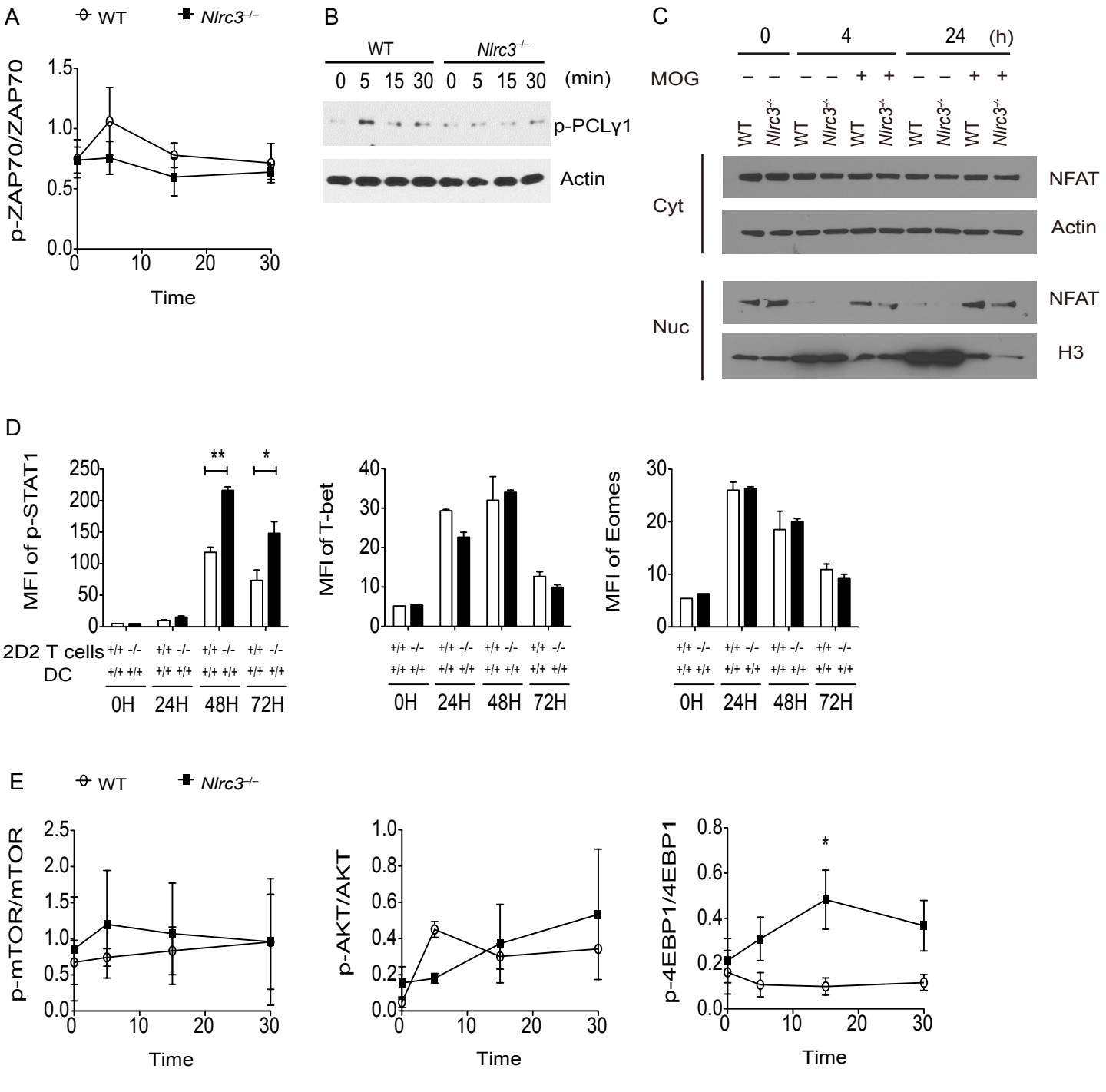
WT and *Nlrc3*$^{-/-}$ mice were given LCMV-Armstrong (A) or LCMV-Clone 13 (B) and T cell response were measured 8 days later.

(A) Percentage among all spleen cells of LCMV-specific DbGP33^{+} CD8^{+} cells (top) and LCMV-specific I-AbGP67^{+} CD4^{+} T cells (bottom) (n=4 for WT Uninfected, n=4 for *Nlrc3*$^{-/-}$ Uninfected, n=4 for WT with Armstrong infection, n=4 for *Nlrc3*$^{-/-}$ with Armstrong infection). Data are from one experiment representative of two independent experiments.

(B) Percentage among all spleen cells of LCMV-specific DbGP33^{+} CD8^{+} cells (top) and, LCMV-specific I-AbGP67^{+} CD4^{+} T cells (bottom) (n=3 for WT Uninfected, n=3 for *Nlrc3*$^{-/-}$ Uninfected, n=4 for WT with Clone 13 infection, n=4 for *Nlrc3*$^{-/-}$ with Clone 13 infection). Data are from one experiment representative of three independent experiments.

Each symbol represents one mouse. Data are shown as mean ± SEM.

Statistical significance determined by unpaired t-test. *P < 0.05, **P < 0.01.



Supplement Figure 6. NLRC3 does not attenuate TCR proximal signals. Related to Figure 7.

(A) Densitometric analysis of three composite p-ZAP70 immunoblots.

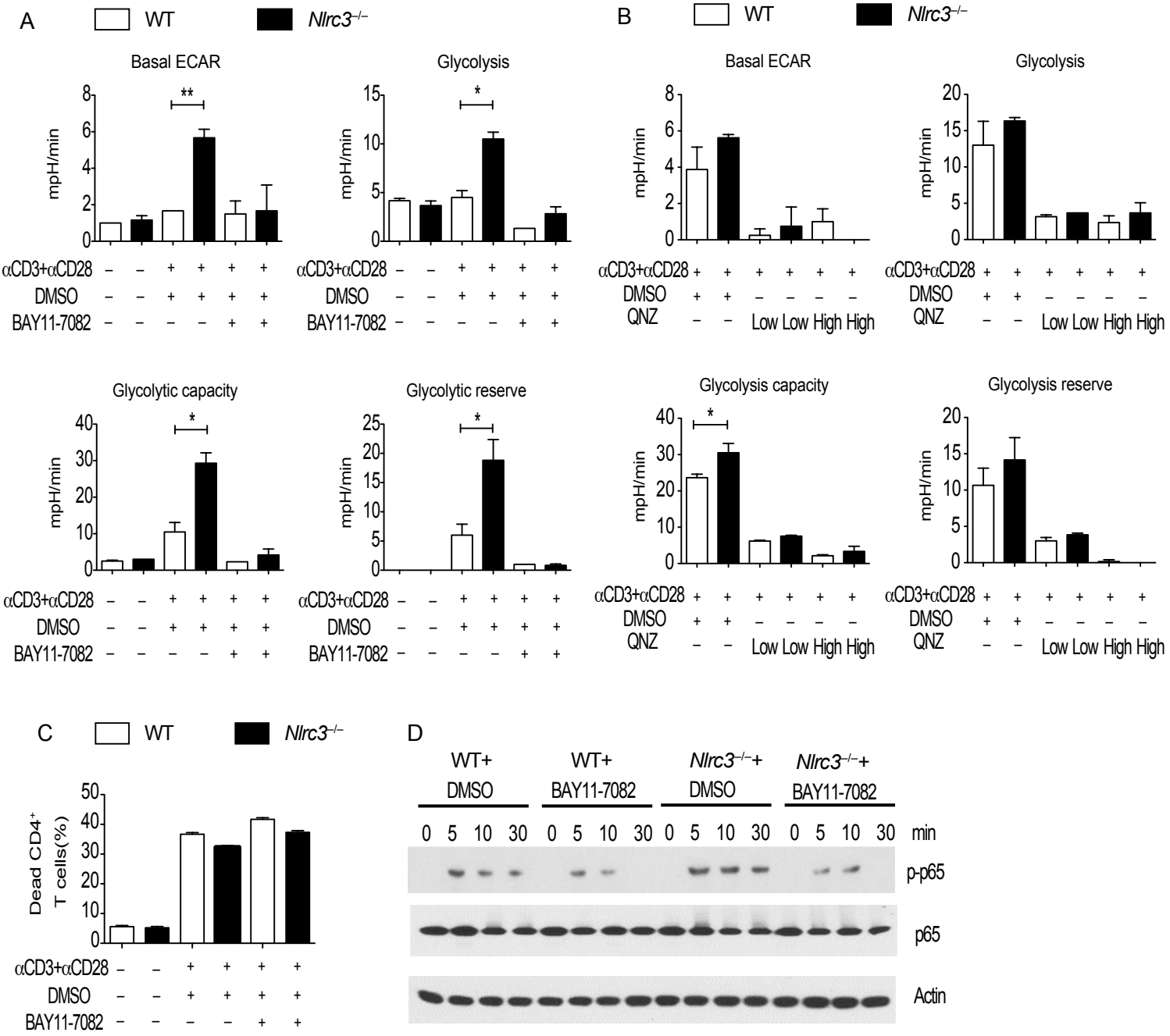
(B- C) Western blot analyses of isolated CD4$^+$ T cells from WT and *Nlrc3*$^{-/-}$ mice stimulated with anti-CD3 (5μg/ml) and anti-CD28 (2μg/ml) antibodies.

(D) MFI of p-STAT1, T-bet and Eomes in CD4$^+$ from 2D2 Tg$^+$ *Nlrc3*$^{+/+}$ or 2D2 Tg$^+$ *Nlrc3*$^{-/-}$ mice after co-culture with DCs.

(E) Densitometric analysis of p-mTOR, p-AKT and p-4EBP1 from three composite immunoblots.

Results are representative of at least two experiments. Data are shown as mean ± SEM.

Statistical significance determined by unpaired t-test. *P < 0.05, **P < 0.01.


A
WT
Nlrc3−/−
Basal ECAR
Glycolysis
mpH/min
αCD3+αCD28
DMSO
BAY11-7082
Glycolytic capacity
Glycolytic reserve
B
QNZ
Low Low High High
Glycolysis capacity
Glycolysis reserve
C
Dead CD4+ T cells(%)
D
WT+ DMSO
WT+ BAY11-7082
Nlrc3−/−+ DMSO
Nlrc3−/−+ BAY11-7082
0 5 10 30 0 5 10 30 0 5 10 30 0 5 10 30 min
p-p65
p65
Actin

Supplement Figure 7. NLRC3 suppress CD4$^+$ T cell metabolism in a NF-κB dependent manner. Related to Figure 7.

(A) ECAR using isolated CD4$^+$ T cells from WT or *Nlrc3*$^{-/-}$ mice stimulated with anti-CD3 and anti-CD28 antibodies together with BAY11-7082 inhibitor (1μM) dissolved in DMSO or DMSO for 24 hrs. Cells were measured over time and exposed to glucose, oligomycin, 2-DG for ECAR measurement. Basal ECAR, glycolysis (ECAR rate after glucose addition), glycolytic capacity (maximal ECAR after subtracting the ECAR rate following 2-DG exposure) and glycolytic reserve (difference between oligomycin-induced maximal ECAR and glucose-induced glycolytic flux) calculated from Figure 7I.

(B) ECAR using isolated CD4$^+$ T cells from WT or *Nlrc3*$^{-/-}$ mice stimulated with anti-CD3 and anti-CD28 antibodies together with QNZ inhibitor (1μM or 10μM) dissolved in DMSO or DMSO for 24 hrs. Basal ECAR, glycolysis, glycolytic capacity and glycolytic reserve calculated from Figure 7J.

(C) CD4$^+$ T cells purified from WT and *Nlrc3*$^{-/-}$ mice were stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) antibodies together with BAY11-7082 inhibitor (1μM) dissolved in DMSO or DMSO for 24 hrs. Dead cells were identified by “Ghost Dye” fluorescent assay. Graph shows the percentage of CD4$^+$ cells that were dead.

(D) Immunoblot analysis of p-p65 in the presence of the NF-κB inhibitor, BAY11-7082.

Data are from one experiment, representative of at least two independent experiments.

Data are shown as mean ± SEM. Statistical significance determined by Student’ s t-test. *P < 0.05, **P < 0.01,.