SUPPLEMENTARY MATERIAL

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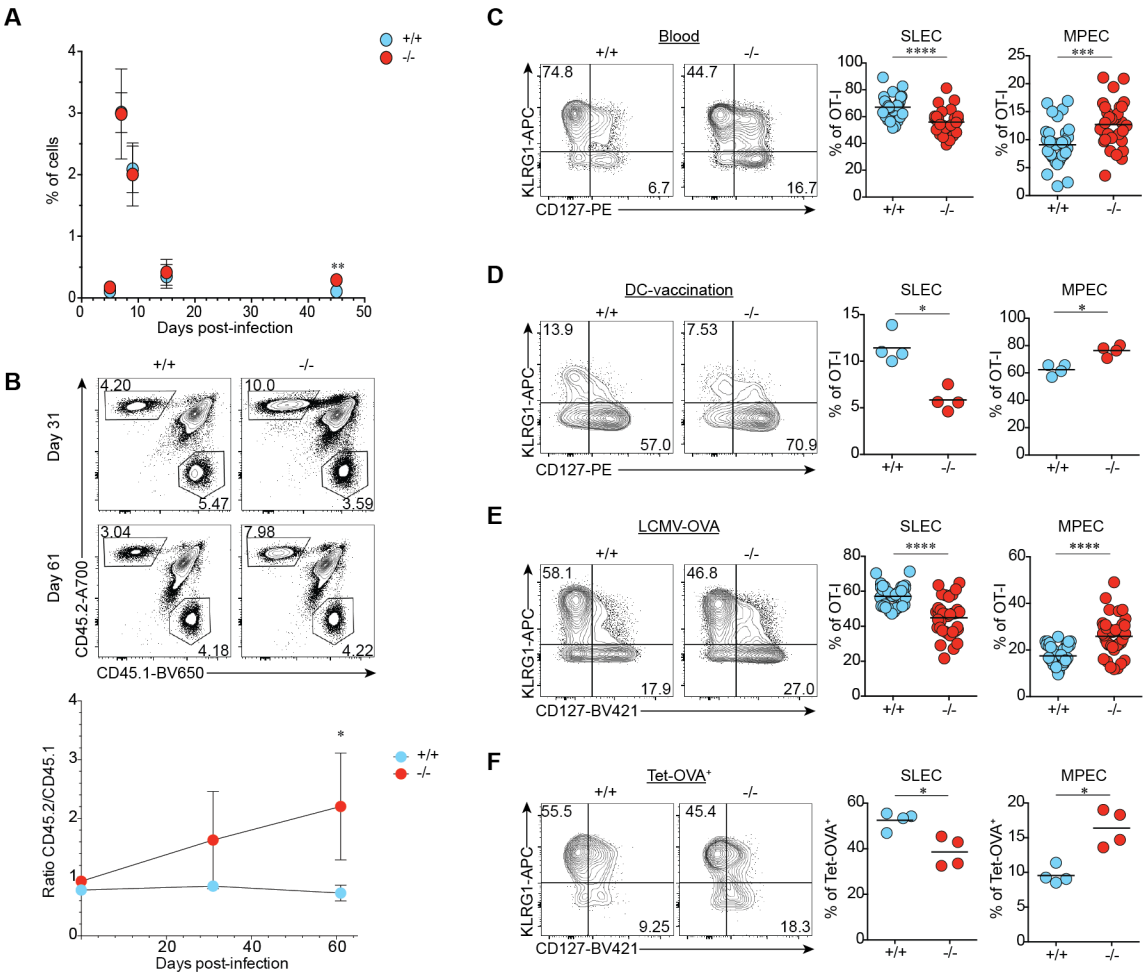

Figure S1. NR4A3 restrains MPEC generation across several models. A. Kinetic response in the spleen of *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I T cells after adoptive transfer and Lm-OVA infection. **B**. Generation of *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I memory T cells (CD45.2^{+}) when in competition with WT B6.SJL OT-I T cells (CD45.1^{+}) following LCMV-OVA infection. Representative CD45.1 vs CD45.2 dot plots gated on CD8^{+} T cells and ratios of CD45.2^{+} OT-I T cells on CD45.1^{+} OT-I T cells are shown over time. **C.** Proportion of SLECs and MPECs within OT-I T cells in the blood at day 7 post Lm-OVA infection. **D-E**. SLEC and MPEC differentiation of *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I cells following DC-OVA vaccination (**D** – day 6 of the response in the spleen) or LCMV-OVA infection (**E** – day 8 of the response in the blood in the OT-I competitive model; as in **B**). **F.** Polyclonal CD8^{+} T cell responses of *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ mice were measured at day 7 post-infection with Lm-OVA. MPEC/SLEC differentiation was evaluated *ex vivo* on K^{b}-OVA tetramer^{+} cells. Data are from 1 (**A, B, D, F**) or at

least 3 independent experiments (**C, E**). A Mann-Whitney unpaired t-test (**A, B, D, F**) was used with a low number of samples and the unpaired Student's t-test (**C, E**), with a Welch's correction when applied, was used for the other comparisons: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

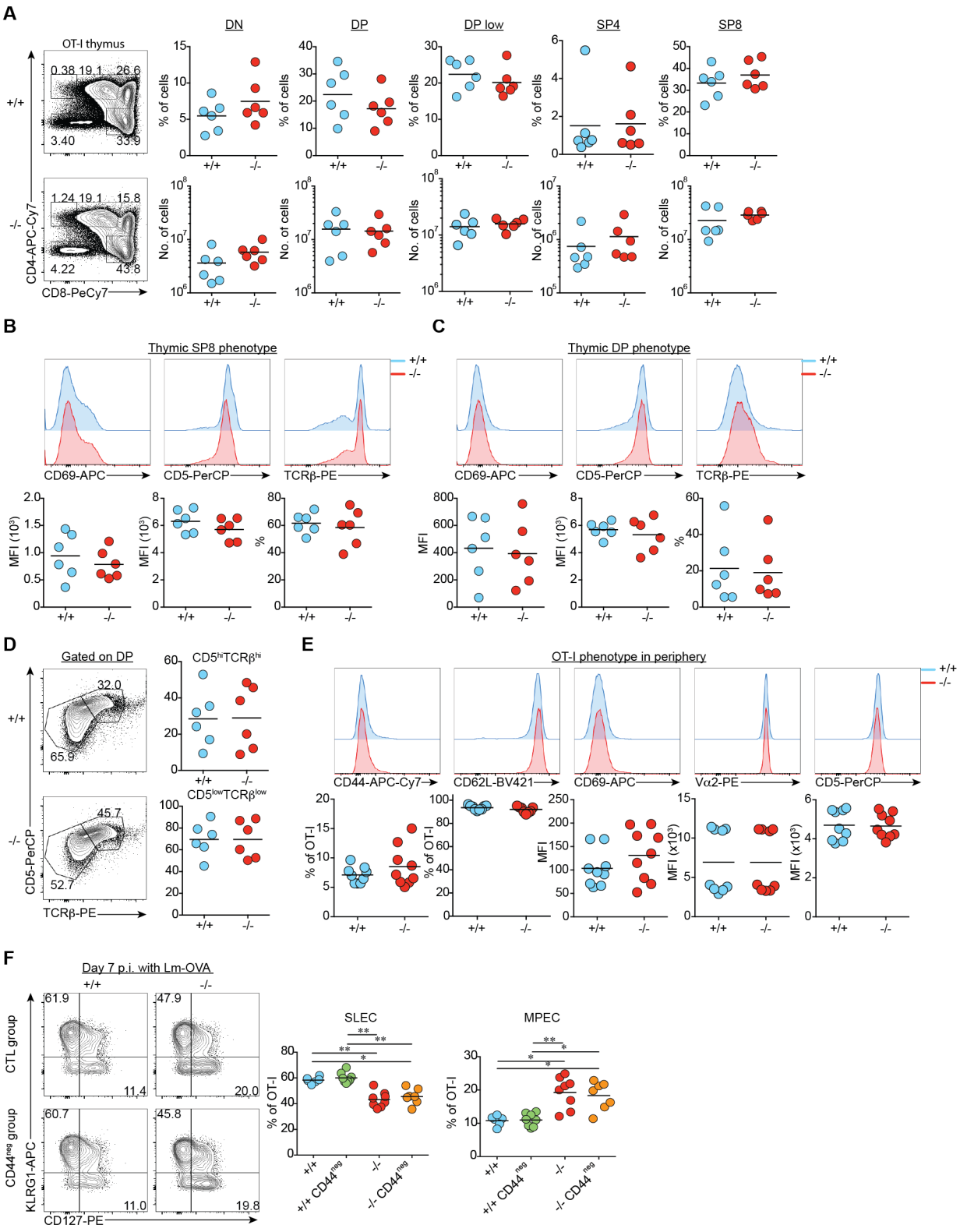

Figure S2. Normal differentiation of OT-I T cells in absence of NR4A3. A. Analysis of the different thymic subsets in OT-I *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ mice. Representative CD4 vs CD8 profiles and

compilation of the percentage and number of the different thymic subsets; DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$), DP^{low} ($CD4^+CD8^{lo}$), SP4 ($CD4^+CD8^-$) and SP8 ($CD4^-CD8^+$). **B-C**. Expression of CD69, CD5 and TCRβ by SP8 (**B**) and DP (**C**) thymocytes. **D.** NR4A3 deficiency does not affect positive selection. FACS profiles of the expression of the TCRβ versus CD5 gated on DP thymocytes and compilation of the percentage of pre-selection ($TCR\beta^-CD5^{lo}$) and positively selected thymocytes ($TCR\beta^+CD5^{hi}$). **E**. Phenotype of OT-I $CD8^+$ T cells from the lymph nodes of *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ mice. Representative FACS profiles and data compilation are shown. **F**. Proportion of MPECs ($CD127^+KLRG1^-$) and SLECs ($CD127^-KLRG1^+$) within *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I effectors at day 7 post-infection with Lm-OVA when the adoptive transfer prior infection was done with sorted $CD44^{neg}$ OT-I $CD8^+$ T cells. Data are from 2 (**F**), 3 (**E**) or 4 (**A-D**) independent experiments. A Mann-Whitney unpaired t-test (**A-D**) was used with a low number of samples and the unpaired Student's t-test (**E**), with a Welch's correction when applied, was used for 2 group comparison and Kruskal-Wallis ANOVA with Dunn's multiple comparison for multiple groups comparison (**F**): **P*<0.05, ***P*<0.01.

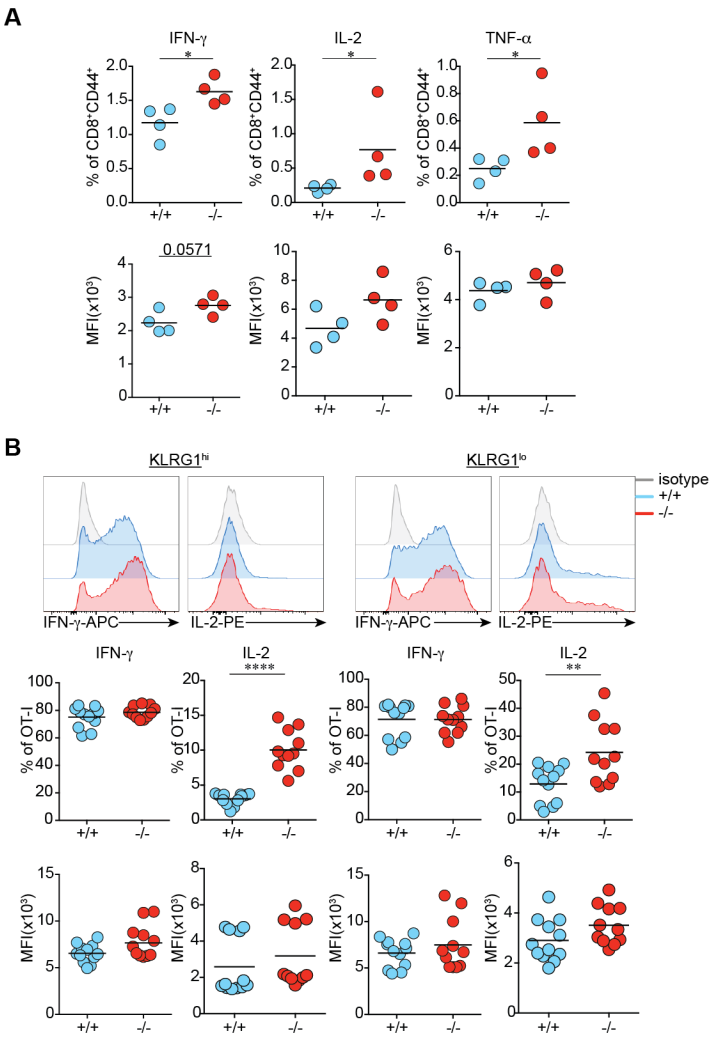

Figure S3. NR4A3 decreases cytokine production by effector CD8$^+$ T cells. A. Polyclonal CD8$^+$ T cell responses of *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ mice were measured at day 7 post-infection with Lm-OVA. Cytokine production was measured following a brief OVA peptide restimulation. Mean Fluorescence Intensity (MFI) on cells positive for the measured cytokines are shown. **B**. At the peak of the Lm-OVA response, splenocytes were restimulated with OVA peptide to measure cytokine production by *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ KLRG1^{hi} or KLRG1^{lo} OT-I cells. The percentage of positive OT-I cells for each cytokine and the MFI of cells positive for the measured cytokines are shown. Data are from 1 (**A**) or 2 (**B**) independent experiments. A Mann-Whitney unpaired t-test (**A**) was used with a low number of samples and the unpaired Student's t-test (**B**), with a Welch's correction when applied, was used for the other comparisons: **P*<0.05, ***P*<0.01, *****P*<0.0001.

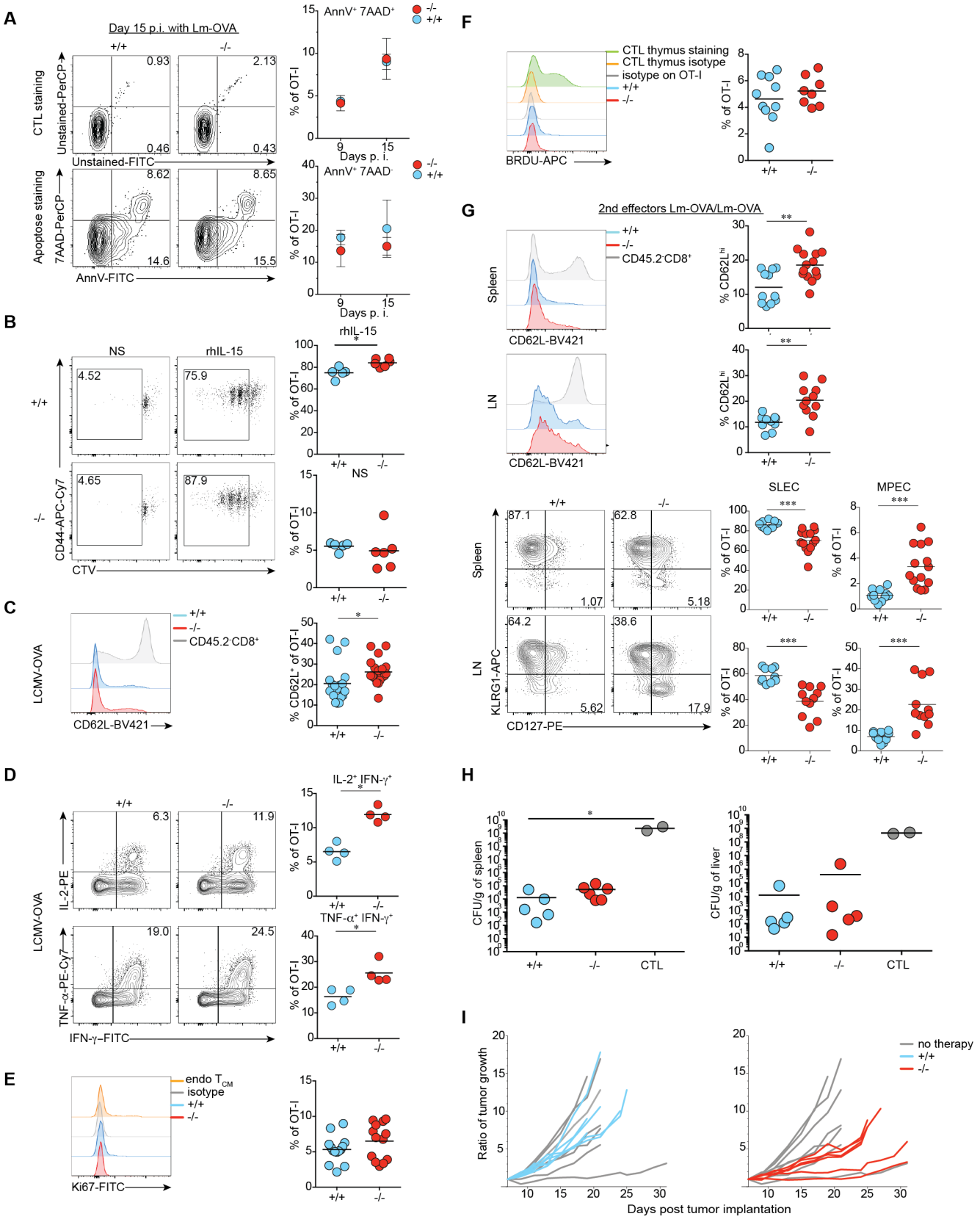
A
Day 15 p.i. with Lm-OVA
+/+
-/-
CTL staining
Unstained-PerCP
Unstained-FITC
0.93
2.13
0.46
0.43
Apoptose staining
7AAD-PerCP
AnnV-FITC
8.62
8.65
14.6
15.5
AnnV+ 7AAD+
AnnV+ 7AAD-
% of OT-I
Days p. i.
B
NS
rhIL-15
CD44-APC-Cy7
CTV
4.52
75.9
4.65
87.9
% CD62L+ of OT-I
C
LCMV-OVA
CD45.2-CD8+
CD62L-BV421
D
IL-2-PE
TNF-α-PE-Cy7
IFN-γ-FITC
6.3
11.9
19.0
24.5
IL-2+ IFN-γ+
TNF-α+ IFN-γ+
E
endo TCM
isotype
Ki67-FITC
F
CTL thymus staining
CTL thymus isotype
isotype on OT-I
BRDU-APC
G
2nd effectors Lm-OVA/Lm-OVA
Spleen
LN
% CD62Lhi
SLEC
MPEC
KLRG1-APC
CD127-PE
87.1
62.8
1.07
5.18
64.2
38.6
5.62
17.9
H
CFU/g of spleen
CFU/g of liver
CTL
I
Ratio of tumor growth
Days post tumor implantation
no therapy

Figure S4. NR4A3 deficiency favors polyfunctional central memory formation and restrains terminal differentiation of secondary effectors. A. Apoptosis of *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I T cells during the contraction phase of the T cell response to Lm-OVA. At day 9 and 15 post-infection, apoptosis was measured using Annexin V (AnnV) and 7-AAD staining. Representative dot plots of AnnV versus 7-AAD staining for day 15 effectors and quantification of apoptotic (AnnV^{+}7-AAD^{-}) and dead (AnnV^{+}7-AAD^{+}) cells at day 9 and day15 post-infection are shown. **B.** Proliferation of *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I memory T cells from Lm-OVA infected mice following *in vitro* stimulation with rhIL-15. Representative FACS profile of CTV dilution and compilation of the percentage of cells that have divided once or more. **C-D**. CD62L expression (**C**) and cytokine production (**D**) measured on memory *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I cells (>30 days post LCMV-OVA-infection in a competitive setting) **E-F.** Homeostatic proliferation of Lm-OVA generated *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I memory cells measured using Ki67 (**E**) and anti-BrdU (**F**) staining. For BrdU staining mice were treated with BrdU for 14 days before sacrifice. **G.** Phenotype of secondary effectors at day 7 post-infection. 10^4 *Nr4a3*$^{+/+}$ or *Nr4a3*$^{-/-}$ memory OT-I T cells produced in response to Lm-OVA infection were adoptively transferred into naive B6.SJL mice. These recipients were then subsequently infected with Lm-OVA. **H**. Lm-OVA challenge. Mice previously adoptively transferred with *Nr4a3*$^{+/+}$ or *Nr4a3*$^{-/-}$ OT-I T cells and infected with LCMV-OVA were challenged with Lm-OVA at the memory stage (CTL: non immunized mice). Bacterial burdens in the spleen and liver are shown. **I.** *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ memory OT-I cells were generated in response to Lm-OVA infection. At least 40 days post-infection, approximately 2 x 10^5 *Nr4a3*$^{+/+}$ or *Nr4a3*$^{-/-}$ OT-I memory cells were adoptively transferred into B16-OVA bearing mice (7 days post-implantation) and the ratio of tumor growth (tumor area at each time point of observation reported to tumor area at day 7 - the time of ACT administration) was followed over time (same experiments as Fig. 3H). Each line represents one mouse. Data are from 1 (**A**, **D, H**), 2 (**F**) or 3 and more independent experiments (**C, E**, **G**); **B** and **I** shows one of 2 representative

experiments. Mann-Whitney unpaired t-test **(A, B, D),** when a low number of experimental samples were available, was used. Unpaired Student's t-test **(C, E, F, G)**, with a Welch's correction when applied, was used for 2 group comparison and Kruskal-Wallis ANOVA with Dunn's multiple comparison for multiple groups comparison (**H**): **P*<0.05, ***P*<0.01, ****P*<0.001.

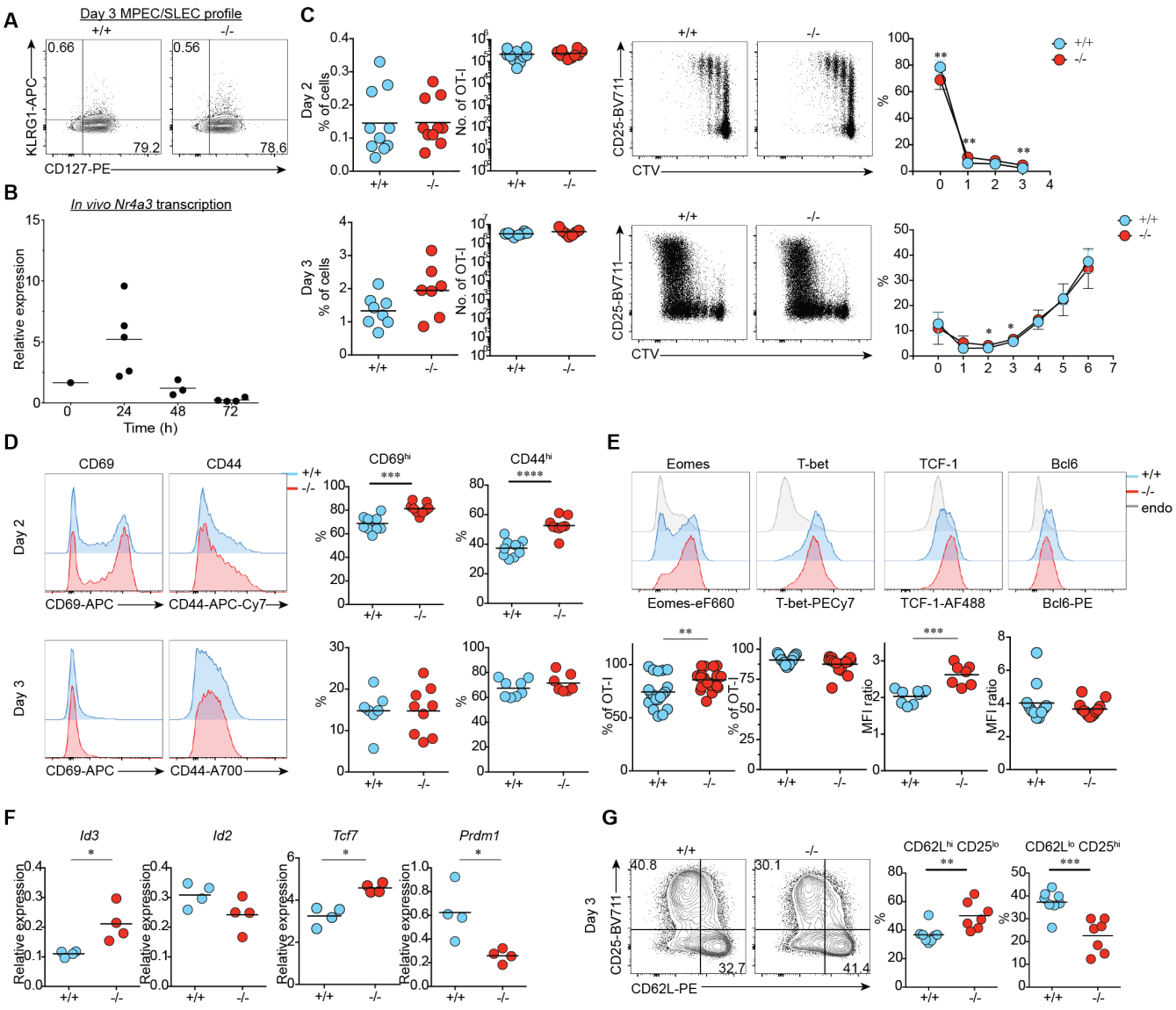

Figure S5. *In vivo* transcription kinetics of *Nr4a3*, characterization of early response of NR4A3-deficient OT-I T cells and validation of RNA-seq results. A. SLEC/MPEC profile at day 3 post Lm-OVA infection. **B**. At indicated time-points post-Lm-OVA infection, OT-I T cells were sorted and *Nr4a3* transcription was measured by qRT-PCR. **C-D.** Proliferation (**C**) and activation (**D**) of *Nr4a3*$^{+/+}$ or *Nr4a3*$^{-/-}$ OT-I T cells *in vivo***.** CTV-labeled OT-I T cells were adoptively transferred into B6.SJL recipients followed by Lm-OVA infection. The percentage of OT-I T cells recovered, FACS profiles of CTV versus CD25 gated on OT-I T cells and quantification of the number of divisions at day 2 (top) and 3 (bottom) are shown in **C**. The expression of the activation markers CD69 and CD44 are shown in

D at day 2 (top) and day 3 (bottom) post-infection. **E-F**. At day 3 post Lm-OVA infection, the expression of transcription factors important for memory $CD8^+$ T cells differentiation was assessed by cytometry after gating on $CD44^{hi}$ OT-I ($CD45.2^+$) cells (**E**) or by qRT-PCR on sorted *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I cells (**F**). **G**. CD62L and CD25 FACS profiles and quantification of the $CD25^{lo}CD62L^{hi}$ and $CD25^{hi}CD62L^{lo}$ subsets of OT-I effector T cells at day 3 post-infection with Lm-OVA. Each dot represents one mouse. Data are from 1 (**B, F**), 2 (**E** for TCF-1, day 3 of **C**, **D** and **G**) or 3 and more (**E,** day 2 of **C** and **D**) independent experiments. Data in (**A**) is representative of at least 3 experiments. A Mann-Whitney unpaired t-test (**F**), when a low number of experimental samples were available was used. Unpaired Student's t-test (**C-E, G**), with a Welch's correction when applied, was used for comparison of two groups: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

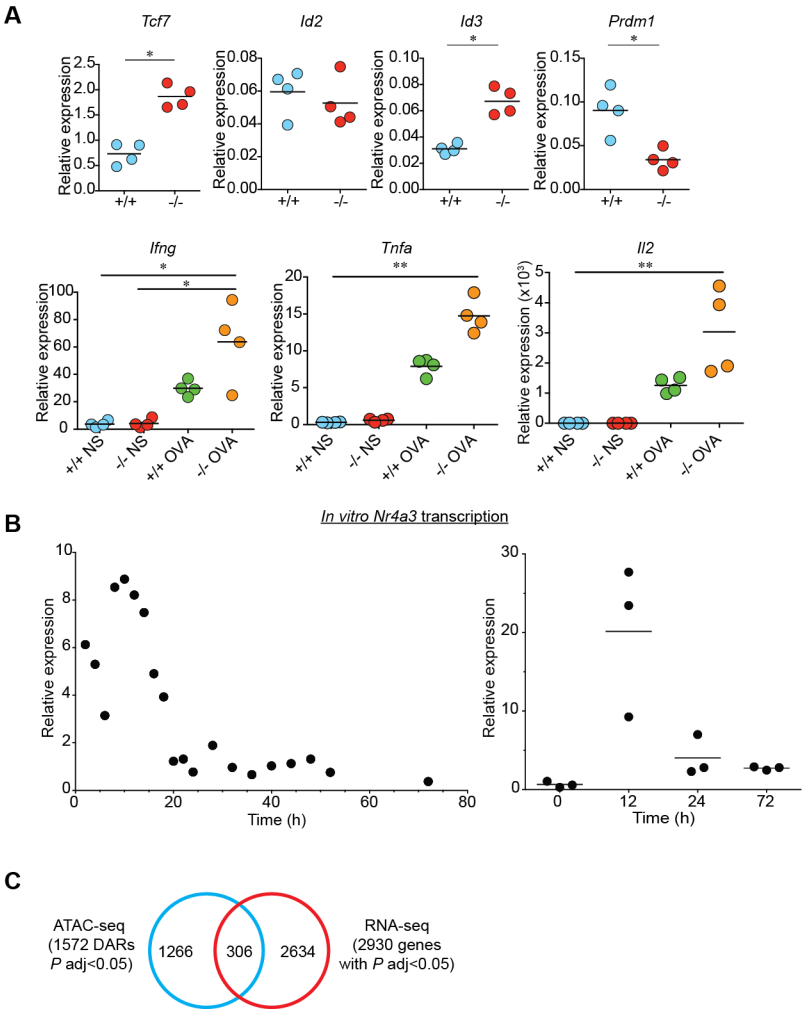

Figure S6. Characterization of *in vitro* generated NR4A3-deficient OT-I effector cells and overlap between ATAC-seq and RNA-seq datasets. A. *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I cells were stimulated for 3 days with anti-CD3/CD28. For transcription of cytokines, cells were not restimulated (NS) or seeded on antigen-loaded splenocytes (+OVA) for 5h prior being cell-sorted into TRIzol for RNA extraction. The expression of transcription factors was assessed on NS sorted *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ OT-I cells. **B.** *Nr4a3* expression kinetics measured by qRT-PCR following *in vitro* stimulation with anti-CD3/CD28. The left panel represents several time points from the same experiment. The right panel is from 1 experiment with independent samples (each point represents an individual mouse). **C**. Overlap between

RNA-seq and ATAC-seq datasets showing the number of genes that have a modulated expression and are associated to a DAR as a result of NR4A3-deficiency. Each dot represents one mouse. Data are from 1 (**A, B, C**: ATAC-seq) or 2 independent experiments (**C**: RNA-seq). A Mann-Whitney unpaired t-test (**A**) was used with a low number of experimental samples for 2 group comparison and Kruskal-Wallis ANOVA with Dunn's multiple comparison was used for multiple groups comparison: **P*<0.05, ***P*<0.01.

Table S1. Antibodies and reagents.

Reagent	Company	Product information
G418 sulfate powder	CORNING	CAS#108321-42-2; Potency 716µg/mg
RPMI 1640	CORNING	REF: 10-040-CV
HEPES	CORNING	REF: 25-060-Cl
L-glutamine	CORNING	REF: 25-005-Cl
Penicillin-Streptomycin	CORNING	REF: 30-002-Cl
MEM Non-essencial amino acids	Gibco Life Technologies	REF: 11140-050
Sodium pyruvate	CORNING	REF: 25-000-Cl
2-mercaptoethanol 1000x (55mM)	Gibco Life Technologies	REF: 21985-023
PBS	Sigma Life Science	REF: P3813-10PAK
Sodium azide	Sigma Aldrich	REF: S2002-100G
formaldehyde solution (PFA)	Sigma Aldrich	CAS #50-00-0
BHI-Agar	BD	REF: 211065
Streptomycine sulfate	Bio Basic	REF: SB0494; CAS#3810-74-0
rhIL-2	Novartis	PROLEUKIN® (aldesleukine); DIN 02130181
NH4Cl	Bio Basic	CAS #12125-02-9
eBioscience FOXP3/Transcription Factor Staining Buffer Set	Invitrogen by Thermo Fisher Scientific	REF: 00-5523-00
Cytofix cytoperm BD Kit	BD	554714
TRIzol	ambion by Life Technologies	REF: 15596026
DMEM powder	Sigma Life Science	REF: D2902-10l
Saponine	Sigma Life Science	REF: S-7900-100g; CAS #8047-15-2
PowerSYBR Green	appliedbiosystems by Thermo Fisher Scientific	REF: 4367659
SuperScript II Reverse Transcriptase	invitrogen by Thermo Fisher Scientific	REF: 18064014
DMEM	CORNING	REF: 10-017-CV
Mouse naive CD8 T cells EasySep KIT	STEMCELL Technologies	REF: 19858
Nu Serum	Corning	REF: 355104

EDTA	CORNING	46-034-Cl
Lymphocyte Separation Medium	CORNING	25-072-CV
Fc-block (Anti mCD16/32 Fc Receptor clone 2.4G2)	Leinco Technologies	C381-1.0mg
Zombie Aqua	Biolegend	423102
Zombie NIR	Biolegend	423106
CTV (CellTraceViolet)	ThermoFisher	C34557
AnnexinV-FITC	Biolegend	640906
7-AAD	Biolegend	420404
pMIG	kind Gift from Guy Sauvageau	pMSCV IRES GFP
frosted glass slides	Fisher	125523
24 well plates	Sarstedt	5000004003 (83.3922.500)
6 well plates	Fisher	08 772 1B
96 well plates	Fisher	07200760
Cell strainer 70µm	Fisher	08 771 2
Cell strainer 100µm	Fisher	08 771 19
Brefeldin A	Fisher	AAJ62340MB
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Sigma (Roche)	11873580001
rhIL15	R&D	247-ILB
BRDU	Sigma	B5002-1G
DNAse I	Sigma	D5025-150KU
Brain Heart Infusion (BHI)	Fisher	B11065
NP-40Octyl Phenoxypolyethoxylethanol (NP-40)	Bio-Basic	NDB0385-100

Stimulation reagents	**Company**	**Product information**
anti mouse CD3ε	BioXcell	clone 145-2C11; cat # BE0001-1
anti mouse CD28	BioXcell	clone 37-51; cat # BE0015-1
OVA peptide (SIINFEKL)	Midwest Biotech	

Staining antibody	Company	Product information
CD8-PerCP anti mouse	BioLegend	clone 53-6.7; cat # 100732
CD8-PeCY7 anti mouse	BioLegend	clone 53-6.7; cat # 100722
KLRG1-APC	BioLegend	clone MAFA; cat #138412
CD127-Biotin	Invitrogen by Thermo Fisher Scientific	clone A7R34; cat #13-1271-85
CD127-BV421 anti mouse	BioLegend	clone A7R34; cat #135027
Streptavidin-PE	BioLegend	cat # 405204
Isotype of IFN-gamma-FITC (Rat IgG1 FITC)	Invitrogen by Thermo Fisher Scientific	IgG gamma 1 ; cat #R101
Isotype of IFN-gamma-APC (Rat IgG1 APC)	Invitrogen by Thermo Fisher Scientific eBioscience	clone eBRG1; cat #17-4301-83
Isotype of IL-2-PE (Rat IgG2β-PE)	BioLegend	clone RTK4530; cat #400608
Isotype of TNFa-PeCY7 (Rat IgG1 PeCY7)	BioLegend	clone RTK2071; cat #400415
Isotype of GranzymeB- PB (Rat IgG1 PB)	BioLegend	clone MOPC-21; cat #400151
IFN-gamma-FITC anti mouse	Invitrogen by Thermo Fisher Scientific	clone XMG1.2; cat #RM9001
IFN-gamma-APC anti mouse	Invitrogen by Thermo Fisher Scientific eBioscience	clone XMG1.2; cat #17-7311-82
IL-2-PE anti mouse	BioLegend	clone JES6-5H4; cat #503808
TNFa-PeCY7 anti mouse	BioLegend	clone MP6-XT22; cat #506324
GranzymeB- PB anti Hu/Mo	BioLegend	clone GB11; cat #515408
CD45.1-BV650	BioLegend	clone A20; cat #110736
CD45.1-PB	BioLegend	clone A20; cat #110722
CD45.2-FITC	BioLegend	clone 104; cat #109806
CD45.2-A700	BioLegend	clone 104; cat #109822
CD45.2-APC	BioLegend	clone 104; cat #109814
CD62L-BV421 anti mouse	BioLegend	clone MEL-14; cat #104436
CD62L-PerCP anti mouse	BioLegend	clone MEL-14; cat #104430
CD62L-PE anti mouse	BioLegend	clone MEL-14; cat #104407
Eomes-eF660 anti mouse	Invitrogen by Thermo Fisher Scientific	clone Dan11mag; cat #50-4875-82

Tbet-PeCy7 anti Hu/Mo	Invitrogen by Thermo Fisher Scientific	clone 4B10; cat #25-5825-82
TCF1-AF488 anti Hu/Mo	Cell Signaling Technologies	clone C63D9 ; cat #6444S
CD69-APC	BioLegend	clone H1.2F3; cat #104513
CD44-APC-Cy7	BioLegend	clone IM7; cat #103028
CD44-AF700	BioLegend	clone IM7; cat #103026
CD25-APC	BioLegend	clone PC61; cat #102012
CD25-BV711 anti mouse	BioLegend	clone PC61; cat #102049
TCRβ-PE	BioLegend	clone H57-597; cat #109207
Vα2-PE	BioLegend	clone B20.1; cat #127808
CD5-PerCP	BioLegend	clone 53-7.3; cat #100616
Ki67-FITC	BD Biosciences	clone B56; kit cat # 556026; cat # 51-36524X
isotype of Ki67-FITC (Mouse IgG1κ FITC)	BD Biosciences	MOPC-21; kit cat # 556026; cat # 51-35404X
BRDU-APC (Mouse IgG1κ APC)	Fisher/eBiosciences	clone BU20A; cat # 17-5071-41
isotype of BRDU-APC	Fisher/eBiosciences	clone P3.6.2.8.1; cat #17-4714-42

qPCR Primers	**Sequences of primers**
Id3	F: GGAGAGAGGGTCCCAGAGTC; R: GAGGAGCTTTTGCCACTGAC
Tcf7	F: GCGGATATAGACAGCACTTC; R: TACACCAGATCCCAGCAT
Id2	F: ACCAGAGACCTGGACAGAAC; R: AAGCTCAGAAGGGAATTCAG
Prdm1	F: ACACACAGGAGAGAAGCCACATGA; R:TCGAAGGTGGGTCTTGAGATTGCT
Nr4a3	F: GATCACAGAGCGACATGGGTTA; R:GAGCCTGTCCCTTCCTCTGG
Ifng	F: GAAAGCCTAGAAAGTCTGAATAAC; R: TGCCAGTTCCTCCAGATA
Il2	F: CAGCAATATCAGAGTAACTGTTG; R: GCTATCCATCTCCTCAGAAAG
Tnfa	F: TCTTCTCATTCCTGCTTGTG; R:GAGGCCATTTGGGAACTT
Hprt	F:CTCCTCAGACCGCTTTTTGC; R: TAACCTGGTTCATCATCGCTAATC

Cell lines	Origin	
L929	kind gift from A. Lamarre (INRS-Institut Armand-Frappier, Laval, Quebec, Canada)	MEM containing with 5% heat inactivated FBS or Nu serum
MC57G	kind gift from A. Lamarre (INRS-Institut Armand-Frappier, Laval, Quebec, Canada)	MEM containing with 5% heat inactivated FBS or Nu serum
B16-OVA	kind gift from A. Lamarre (INRS-Institut Armand-Frappier, Laval, Quebec, Canada)	DMEM supplemented with 10% FBS (or Nu serum), sodium pyruvate (1mM) in presence of 5mg/ml G418 (Corning)
HEK293T	kind gift from H. Melichar (CRHMR, Montreal, Quebec, Canada)	DMEM containing with 10% heat inactivated FBS or Nu serum

Infection agents	Origin
Lm-OVA	kind gift from S. P. Schoenberger
LCMV-OVA	kind gift from J. C. de la Torre