Supporting Information

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SI Materials and Methods

Flow Cytometry. The antibodies to CD8 (53-6.7), CD4 (RM4-5), CD62 ligand (CD62L) (MEL-14), CD44 (IM7), IFNγ, TNFα, IL-2, and CD103 (2E7) were purchased from BD Bioscience. Phycoerytherin- and allophycocyanin-conjugated Tim-3 antibodies were purchased from R&D Systems. Intranuclear FoxP3 staining (E-Bioscience) was performed according to the instructions. Cell suspensions were blocked with anti-mouse CD16/32 and then incubated with specific antibodies or isotypes for 30 min at 4 °C. The antibody-stained cells were acquired with a FACS Calibur (BD Biosciences) and the data were analyzed using the FlowJo software (Tree Star).

Adoptive Transfers. Splenocytes from WT or G9KO (both Thy1.2) ×31 immune mice were enriched (Miltenyi Biotech kit) for CD8 T cells and then titrated for 10^4 NPtet$^+$ CD8 T cells and transferred into Thy1.1 C57BL/6 animals. Alternatively 10^4 NPtet$^+$ CD8 T from B6Thy1.1 (×31 immune) mice were transferred into Thy1.2 C57BL/6 WT or G9KO animals. At 24 h posttransfer, recipient animals were infected with 8,000 EID_{50} pfu of IAV PR8. At 8 d postchallenge, CD8 T-cell analysis was performed.

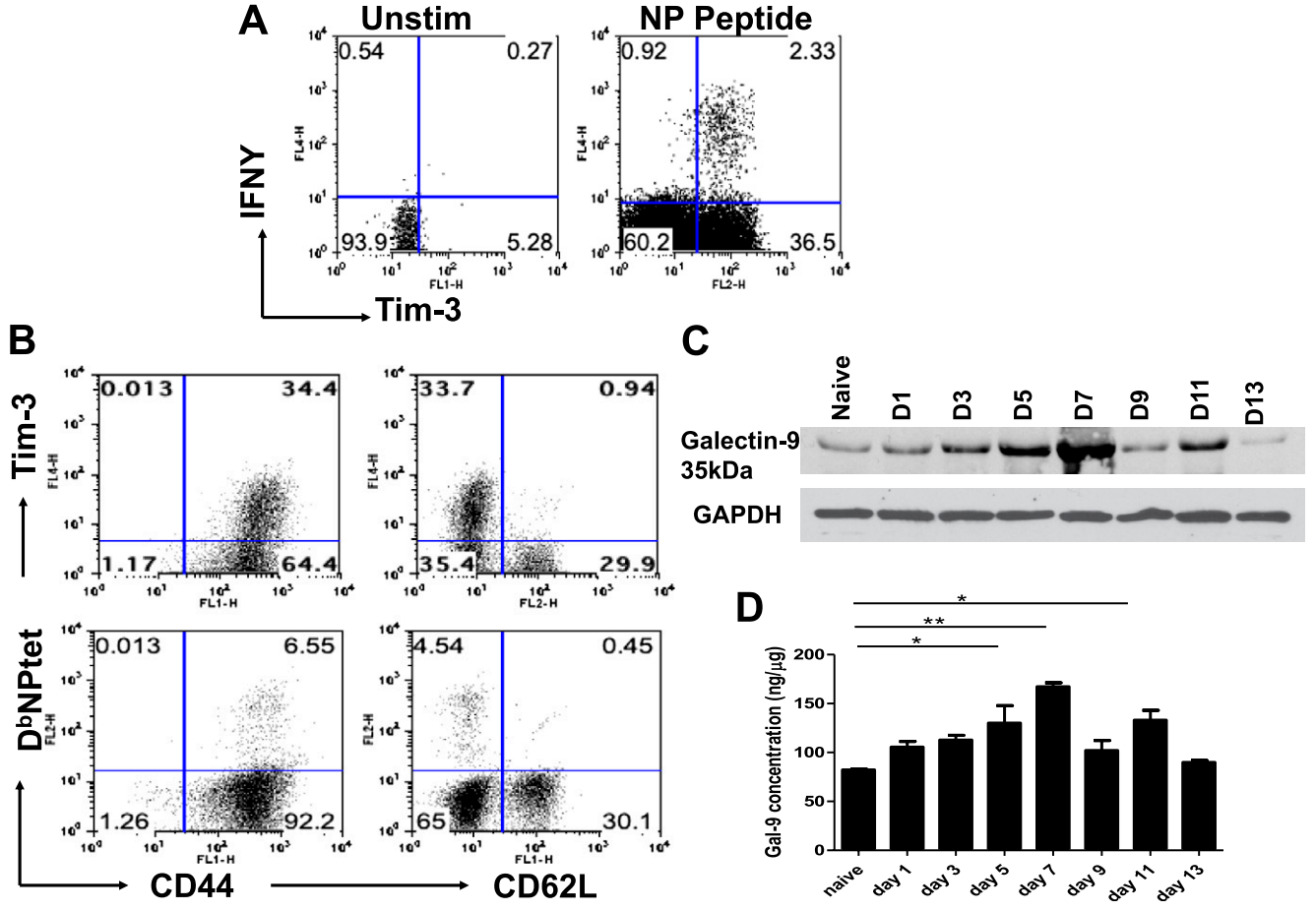


Fig. S1. Activated cells express Tim-3 and Gal-9 levels are up-regulated in lungs after IAV infection. At different time points after infection, BAL and spleen cells (n = 3) isolated at each time point were analyzed flow cytometrically for Tim-3 expression on IAV-specific CD8 T cells. BAL samples from three mice were pooled. (*A*) FACS plots showing Tim-3$^+$IFNγ$^+$ CD8 T cells at day 10 p.i. in the BAL fluid of WT animals. (*B*) Coexpression of Tim-3 (*Upper*) and D^{b}NPtet$^+$ (*Lower*) with CD44 and CD62L in BAL of WT mice at day 8 p.i. is shown by representative FACS plots. (*C*) Immunoblots showing Gal-9 expression in the lung homogenates from naïve and IAV-infected mice at different time points post infection. (*D*) Gal-9 concentrations as measured by sandwich ELISA using anti–Gal-9 mAb in the lung homogenates is shown. Numbers in the quadrants indicate percent of each subset. Data are representative of three independent experiments.

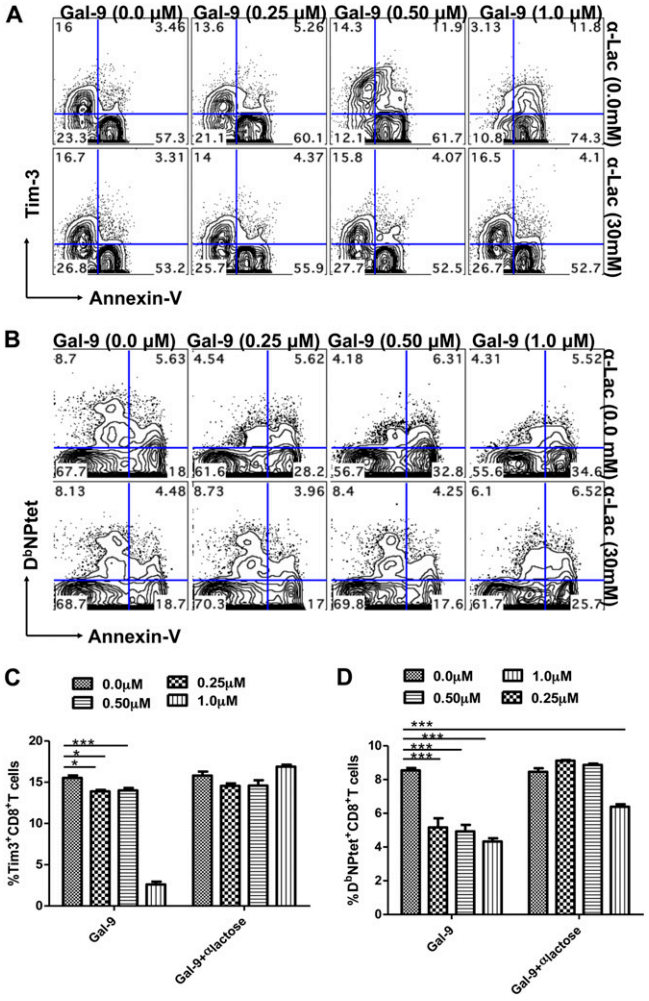


Fig. S2. Galectin-9 induces apoptosis of IAV NP tetramer-specific and Tim-3$^+$ CD8 T cells in vitro. Ex vivo apoptosis assay was performed with splenocytes isolated at 10 days postinfection from IAV-infected animals as described previously (1). Briefly splenocytes were incubated for 5 h with varying concentrations of galectin-9 in the absence or presence of α-lactose. The experiments were repeated multiple times with similar results. (*A*) Representative FACS plots showing the expression of Tim-3 and annexin-V on gated CD8 T cells under indicated incubation conditions. (*B*) Representative FACS plots showing the expression of NPtet and annexin-V on gated CD8 T cells under indicated incubation conditions. (*C*) Bar diagram shows the percentage of Tim-3$^+$ CD8 T cells as calculated from *A* (with triplicate wells). (*D*) Bar diagram shows the percentage of NPtet$^+$ CD8 T cells as calculated from *B*. Statistical analysis was done by two-way ANOVA with Bonferroni post hoc settings.

1. Sehrawat S, et al. (2010) Galectin-9/TIM-3 interaction regulates virus-specific primary and memory CD8 T cell response. *PLoS Pathog* 6:e1000882.

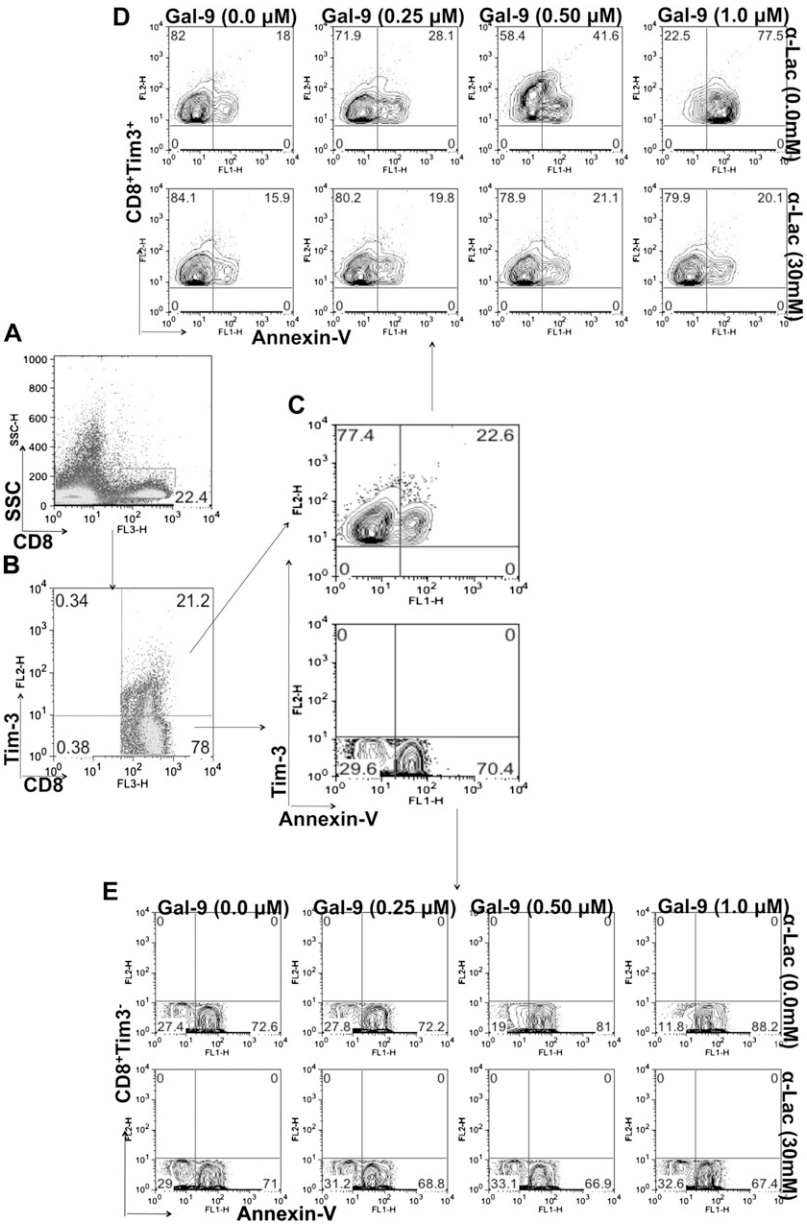


Fig. S3. Gating strategy to depict preferential apoptosis of Tim-3$^+$ CD8T cells upon Galectin-9 exposure. (*A*) Representative FACS plots showing gated CD8T cells from the cultures in an ex vivo apoptosis assay. (*B*) Tim-3$^+$ and Tim-3$^-$ CD8T cells are shown by representative FACS plot. (*C*) FACS plots depicting Tim-3$^+$ annexinV$^+$ (*Upper*) and Tim-3$^-$ annexin V$^+$ CD8 T cells (*Lower*). (*D*) Representative FACS plots showing the expression of annexin-V on gated Tim-3$^+$ CD8 T cells under indicated incubation conditions. (*E*) Representative FACS plots showing the expression of annexin-V on gated Tim-3$^-$ CD8 T cells under indicated incubation conditions.

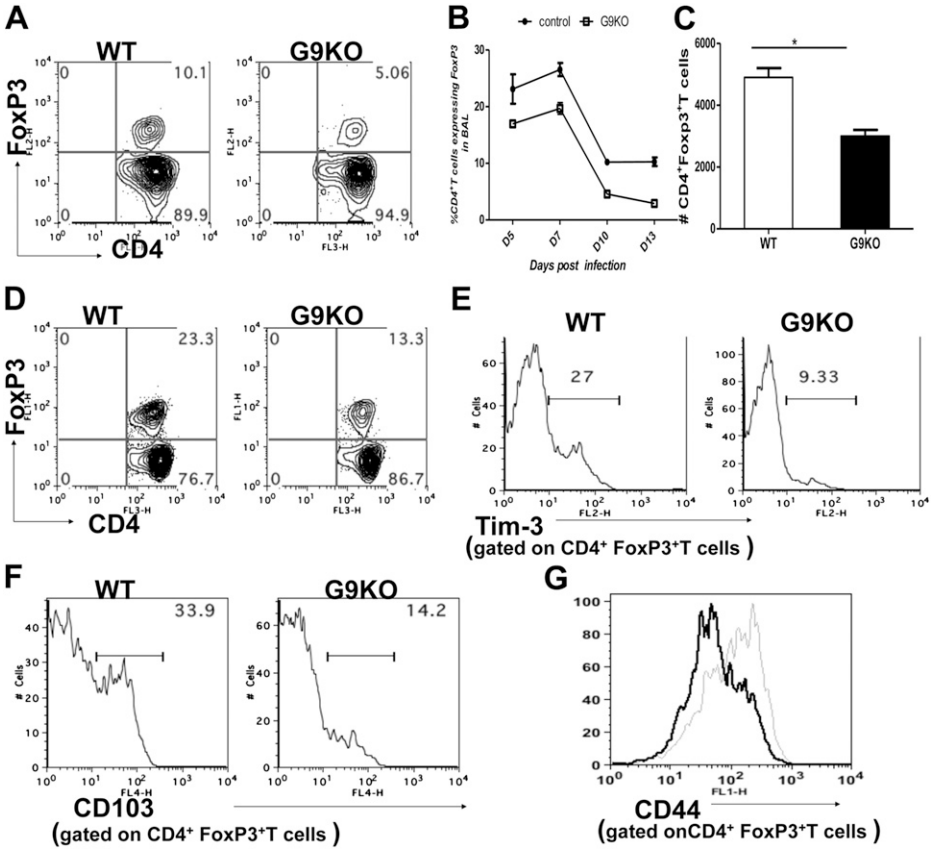


Fig. S4. Characterization of $CD4^+FoxP3^+$ regulatory T cells from WT and G9KO mice. C57BL/6 and G9KO animals were infected intranasally with 5,000 EID_{50}. BAL samples from three mice were pooled and spleens from individual mice were stained for FoxP3. Representative FACS plots show the frequencies of $FoxP3^+$ Tregs in the BAL (*A*) and spleens (*D*) of WT and G9KO animals at day 10 p.i. The kinetics of Treg frequencies at indicated timepoints p.i. (*B*) and their absolute numbers at day 10 p.i. (*C*) in the BAL fluid of WT and G9KO are shown. Histograms show the expression of Tim-3 (*E*), CD103 (*F*), and CD44 (WT, light line) and (G9KO, dark line) (*G*) on $FoxP3^+$ Tregs isolated from the spleens of WT and G9KO animals at day 10 p.i.

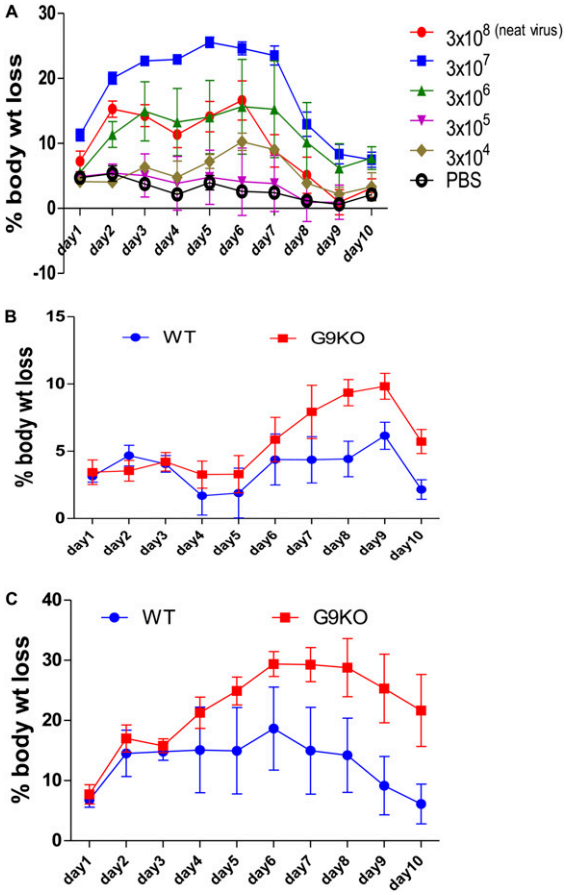


Fig. S5. Outcome of infection with IAV HK×31. WT mice were inoculated with IAV HK×31 (10^8, 10^7, 10^6, 10^5, 10^4 EID_{50} or PBS). (*A*) Body weight of WT mice after infection was determined daily and expressed as the percentage of the body weight lost following infection. (*B*) Percentage of body weight-loss comparison between the WT and G9KO mice infected with 5,000 EID_{50} intranasally (i/n) over a period of 10 d. (*C*) Percentage of body weight-loss comparison between WT and G9KO mice infected with 5×10^7 EID_{50} i/n over a period of 10 d. The data are representative of five to six mice per group.

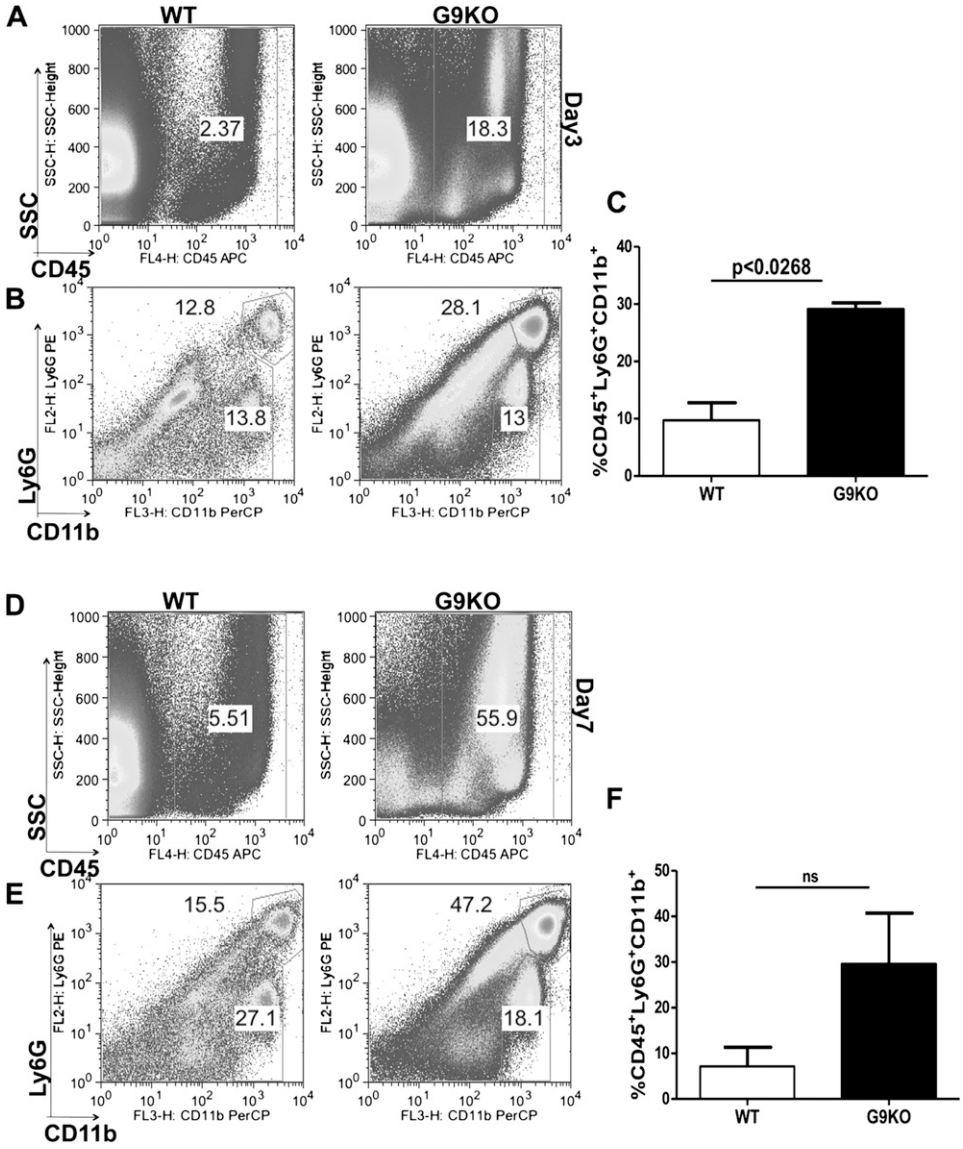
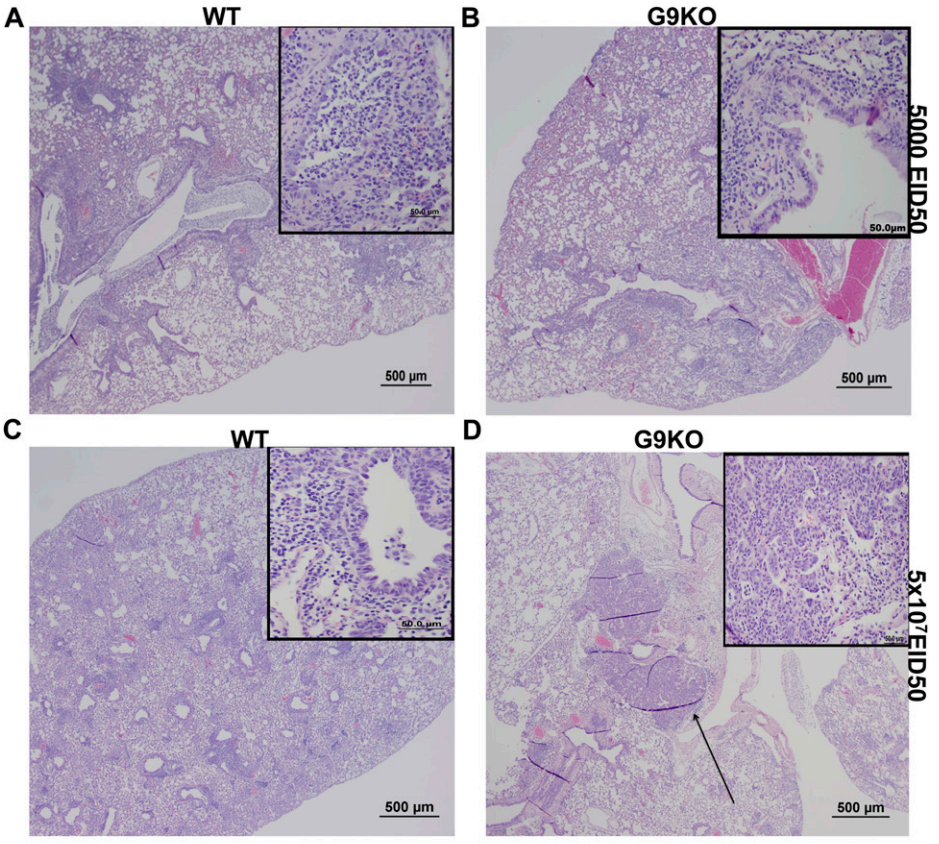


Fig. S6. Cells obtained from bronchoalveolar lavage (BAL) were stained for $CD45^+$ (pan leukocyte marker) and $CD45^+CD11b^+Ly6G^+$ (neutrophils) and FACS analysis performed at day 3 (*A* and *B*) and day 7 (*D* and *E*) in WT and G9KO mice. Bar graphs representing % $CD45^+CD11b^+Ly6G^+$ (neutrophils) at day 3 p.i. (*C*) and day 7 p.i. (*F*) is shown. Experiments were repeated multiple times and the data are representative of pooled BAL samples from three mice. Statistical analysis was done by Student's *t* test and error bars represent SEM.



E

		%Parenchyma involved	PMN# (Average/40x field)	Remarks
WT	Day 3	0%	0	Normal lung, no recognizable pathological changes
	Day 7	1-4	2-4	Mild multifocal broncho-interstitial pneumonia
	Day 9	21-30%	4-30	Moderate to marked multifocal broncho-interstitial pneumonia, primarily mononuclear lymphocytic infiltrates with lesser number of macrophages,scattered parenchymal/infilterative individual cell necrosis and degeneration
G9KO	Day 3	4%	0-5	Mild, regionally limited, multifocal broncho-interstitial pneumonia
	Day 7	4-5%	5-7	Mild multifocal broncho-interstitial pneumonia
	Day 9	10-20%	2-50	Moderate multifocal broncho-interstitial pneumonia, primarily mononuclear lymphocytic infiltrates with lesser numbers of macrophages, occasional foci of intense neutrophilic infiltrates, moderate scattere parenchymal/infiltrative individual cell necrosis/apoptosis

Fig. S7. Histopathology. The lungs of mice infected with 5,000 or 5×10^7 EID_{50} of virus were harvested at indicated timepoints postinfection, washed in PBS, fixed in 10% neutral buffered formalin, and embedded in paraffin wax. Sections (5 mm) were stained with hematoxylin and eosin and microscopically reviewed. H&E sections from WT (*A*) and G9KO (*B*) animals infected with 5,000 EID_{50} of IAV ×31 at day 9 p.i. are shown. Representative H&E sections from WT (*C*) and G9KO (*D*) animals infected with 5×10^7 EID_{50} of IAV ×31 at day 10 p.i. are shown. The section blocks in the *Upper Right* corner represent the 40× magnification. The overall lung readouts (n = 3 mice at each timepoint/group infected with ×31 5,000 EID_{50}) are indicated as a table (*E*).

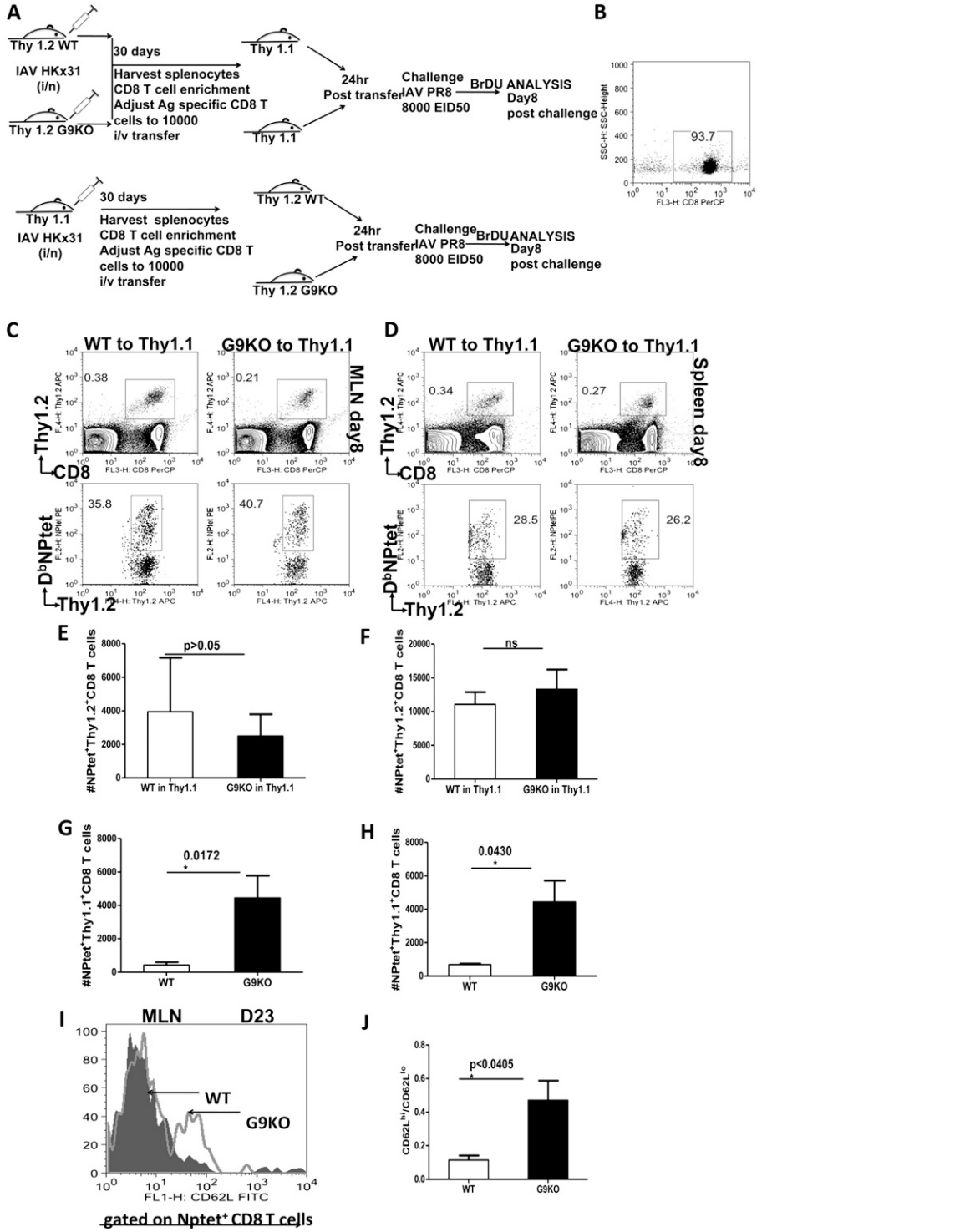


Fig. S8. Adoptive transfer of memory CD8 T cells. Splenocytes from either WT or G9KO (both Thy1.2) HK×31 immune mice (mice were held for at least 1 mo following infection with IAV HK×31 i/n) were enriched for CD8T cells using Miltenyi Biotech (CD8T cell isolation) kit. Around 93–94% pure CD8T cells were obtained in the enriched CD8T cell population. The enriched CD8T cells were then titrated for equal number of NP tetramer-specific CD8T cells and 1×10^4 antigen-specific CD8T cells were transferred in B6 Thy1.1 mice ($n = 4$). Alternatively splenocytes from B6 Thy1.1 HK×31 immune mice (mice were held for at least 1 mo following infection with IAV HK×31 i/n) were enriched for CD8T, and equal number of NP tetramer-specific CD8T (1×10^4) cells were transferred in WT or G9KO mice ($n = 4$). Twenty-four hours posttransfer, the recipients were challenged with 8,000 EID_{50} of heterologous IAV (PR8). (*A*) Diagrammatic depiction of the scheme for adoptive transfer experiments. (*B*) Representative FACS plots for enriched CD8T cell population. FACS plots show the frequencies of donor Thy1.2$^+$CD8$^+$ (*C*, *Upper*) and NPtet$^+$ Thy1.2$^+$ CD8$^+$ (*C*, *Lower*) WT or G9KO cells in the MLN (*C*), and spleen (*D*) of Thy1.1 animals at 8 d p.i. Absolute numbers of the donor NPtet$^+$Thy1.2$^+$CD8$^+$T cells of WT or G9KO in the MLN (*E*) and spleens (*F*) of the recipients are shown. Absolute numbers of the donor NPtet$^+$Thy1.1$^+$CD8$^+$T WT or G9KO cells in the MLN (*G*) and spleens (*H*) of the recipients are shown. The experiments were repeated two times. Statistical analysis was done by Student's *t* test and the error bars represent SEM. (*I*) Representative histograms showing CD62L expression by NPtet$^+$ CD8 T cells in the MLNs of WT and G9KO animals at day 23 p.i. (*J*) Bar graphs represent the ratios of CD62L^{hi}/CD62L^{lo} of NPtet$^+$ CD8 T cells in the MLN of WT and G9KO animals at day 23 p.i.

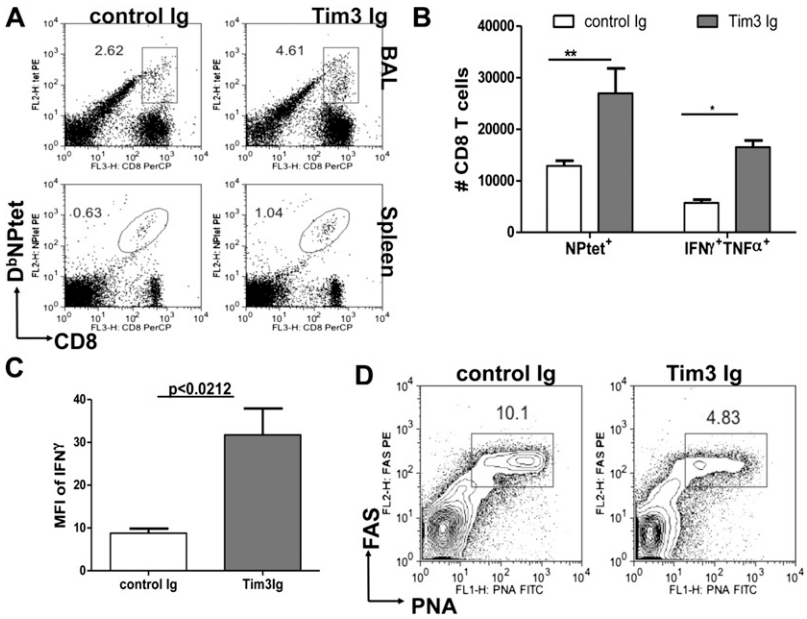


Fig. S9. Administration of Tim-3 fusion protein in mice at later stages post IAV infection enhances the magnitude and quality of IAV-specific CD8 T cells responses. IAV-infected C57BL/6 animals were treated with 100 μg of Tim-3 fusion protein (per mouse) from day 4 postinfection at alternate days until day 9 p.i., and 12 h after the last treatment the animals were killed. Frequencies of NPtet$^+$ CD8 T cells (*A*) isolated from the BAL (*Upper*) and spleen (*Lower*) of Tim-3 fusion protein-treated and control animals are shown. (*B*) Absolute numbers of NPtet$^+$, IFNγ$^+$TNFα$^+$ CD8 T cells in the BAL. (*C*) MFI of IFNγ in Tim-3 Ig and control animals. (*D*) Representative FACS plots showing frequencies of CD3$^-$B220$^+$PNA$^+$FAS$^+$ germinal center B cells in the mediastinal lymph node (MLN) of Tim-3 Ig and control Ig-treated mice at day 10 p.i. Data are representative of two independent experiments with three mice per group. Error bars represent SEM.