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Supplementary Material for

Vaccine Activation of the Nutrient Sensor GCN2 in Dendritic Cells Enhances Antigen Presentation

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Materials and Methods

Mice

C57BL/6 male mice 6–12 wk of age were obtained from Charles River Laboratory. OT-I (*Rag 2*$^{-/-}$) mice were obtained from Taconic. *GCN2*$^{-/-}$ mice and floxed GCN2 mice (GCN2flox/flox) were provided by David H. Munn, and originally made by Dr. David Ron. GCN2flox/flox were bred to transgenic mice expressing cre enzyme under the control of CD11c promoter, (CD11c-cre from Jackson Laboratories), to generate mice lacking GCN2 in DCs. Successful cre-mediated deletion was confirmed by PCR and protein expression (fig. S10). Atg 7 and Atg 5 floxed mice were obtained from Dr. Herbert Virgin (Washington University, St. Louis, MO). To conditionally knock out Atg7 and Atg5 in DCs, we crossed the Atg 7 and Atg 5 floxed mice to CD11c-cre mice to generate mice in which Atg5 or Atg7 were deficient in DCs. Successful cre-mediated deletion was confirmed by protein expression (fig. S23). All mice were maintained under specific pathogen-free conditions at the Emory Vaccine Center vivarium. All animal protocols were reviewed and approved by the Institute Animal Care and Use Committee of Emory University.

Preparation of virus stocks

YF-17D virus was a gift from Dr. R. Ahmed (Emory University, Atlanta, GA), and YF-Ova8 (encoding the SIINFEKL peptide) was a gift from Dr. R. Andino (University of California, San Francisco, CA). YF-17D viral stocks were propagated by infecting BHK cell monolayers grown in DMEM + 10% FBS + penicillin/streptomycin. Cytopathic effect (CPE) was observed from 3 to 5 days following infection. Cell culture supernatants were collected when ~20% cytopathic (CE) effect was noted. Supernatants were harvested by centrifugation at 750 g for 20 min, titrated, aliquoted, and stored at -80°C. Viral titers were calculated by plaque assay using Vero cells. Virus was inactivated by either incubating at 56°C for 1 h or UV irradiated with 120 mJ/cm2 for 5 min.

Generation of human monocyte derived dendritic cells

Monocytes were isolated from buffy coats by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden), followed by immune-magnetic cell separation using anti-CD14–conjugated microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). After magnetic separation, 90% to 95% of the cells were $CD14^+$ monocytes as measured by flow cytometry. The enriched $CD14^+$ cells were cultured for 6 days in six-well plates at a density of (1-2 $x10^6$/ml) in complete RPMI medium (10% (v/v) FCS, 100 units/ml penicillin, 100 ug/ml streptomycin, 2mM L-Glutamine, 1mM sodium pyruvate, and 0.0012%(v/v) 2-mercaptomethanol) in the presence of GM-CSF and IL-4 (50 ng/ml each, peprotech) at 37°C, 5% CO2, and 95% relative humidity. At day 2, and day 4, half of the medium was replaced with fresh medium supplemented with fresh cytokines (GM-CSF and IL-4). At day 6, DCs were harvested and assessed for CD1a expression by flow cytometry. Using this protocol majority of the gated DC population (> 95%) were $CD11c^+$ and $CD1a^+$.

Generation of murine bone marrow derived dendritic cells

Mice tibiae and femurs were flushed with ice-cold PBS through a 70 μm-wide cut-off cell strainer. Cells were centrifuged for 5min at 1400 rpm. Cells at a density of 5-7 x 10^6 bone marrow cells per 10 cm petri dish in complete RPMI medium (10% (v/v) FCS, 100 units/ml penicillin, 100 ug/ml streptomycin, 2mM L-Glutamine, 1 mM sodium pyruvate, and 0.0012% (v/v) 2-mercaptomethanol) in the presence of GM-CSF (20ng/ml, peprotech) and IL-4 (5ng/ml) in an incubator at 37°C, 5% CO2, and 95% relative humidity. At day 3, the cultures were supplemented with another 10 ml of complete with fresh GM-CSF were added. At day 6, BMDCs were harvested by gently flushing the cells from the plate.

Culture of dendritic cells with YF-17D

BMDCs or human monocyte derived DCs were generated as described above. BMDCs were purified using the CD11c magnetic beads (Miltenyi Biotech) to yield a purity of >95%. BMDCs or human monocyte derived DCs were washed in 1% FBS-RPMI 1640 (Low FBS containing media) and cultured with YF-17D in this low FBS medium at 37°C for 30 min. Then excess FBS was added and the cells washed to remove excess virus, and reconstituted in complete RPMI (RPMI +10% FBS).

ELISA

Cytokines in culture supernatants following YF17D infection was measured by two-site sandwich ELISA. IL-6, TNF-α, IL-12p40, IL-12p70, IFN-α, IFN-γ were measured using kits obtained from BD Bioscience. Briefly, 96-well polyvinyl chloride microtiter plates were be coated with purified anti-cytokine antibody and incubated for overnight at 4°C. The plates were washed and blocked with 1% BSA in PBS followed by incubation with the test samples. After washing, the plates were incubated further with biotin conjugated anti-cytokine antibody followed by further incubation with horseradish peroxidase (HRP) coupled to streptavidin. TMB substrate (BD biosciences) was used to monitor HRP activity. The reactions were terminated using 1 N H_2SO_4, and the absorbance values were read at 492 nm.

Antigen uptake assays

To examine uptake of soluble antigens, DCs from wild type and *GCN2*$^{-/-}$ mice were incubated with Ovalbumin conjugated to Alexa Fluor 488 (Molecular Probes) for 1 hour at 37ºC, or on ice. Cells were washed and stained with anti-CD11c and analyzed by flow cytometry or by confocal microscopy.

RT-PCR

The total RNA was isolated from experimental cells using a Qiagen RNeasy Mini Kit as per the manufacturer's instructions (Qiagen). cDNA was generated using the superscript First-Strand synthesis system (Invitrogen), according to the manufacturer's instructions. cDNA was used as a template for quantative real-time PCR using SYBER Green Master Mix (Biorad) and the gene specific primers (Table 1). The PCR was

performed using a MyiQICycler (Biorad). Gene expression was calculated relative to the housekeeping gene GAPDH.

Western Blotting

DCs ($1x10^6$) were stimulated for various periods with YF-17D and cells were lysed with 100-µl M-PER mammalian protein extraction reagent (Pierce) containing Halt protease inhibitor, EDTA and phosphatase inhibitor (Pierce). Heat or UV inactivated YF-17D were used as controls. Equal amounts of protein were run on an SDS-PAGE and transferred onto nitrocellulose membranes following electro blotting. After blocking with 5% fat-free milk, the membranes were incubated at 4oC with the following primary antibodies: anti human GCN2-p from abcam (1:1,000), antibody to eIF2α phosphorylated at Ser51 eIF2α, and antibodies to p62, LC3, Atg5, Atg7, Atg3 and β-actin (all antibodies from Cell Signaling). The membranes were then washed was incubated with Horseradish peroxidase–conjugated secondary antibody (Cell Signaling). Proteins were visualized with SuperSignal West Pico chemiluminescent substrate (Pierce). The level of expression of different proteins was analyzed by using the public domain software Image J (National Institutes of Health (NIH). The software can calculate area and pixel value statistics of user-defined selections. Briefly, it was done as follows: X-ray films were scanned and saved as grayscale JPEG files. The percentage of measurable pixels in the image was set (and highlighted in red) by using the adjust image threshold command. Following this, the number of square pixels in the section selected (the protein bands) was then counted by measuring the area in the binary or threshold image.

Expression of costimulatory molecules

DCs from the inguinal lymph nodes from naïve mice or mice vaccinated 24 hours previously with YF-17D were isolated and stained with anti mouse CD80 or anti mouse CD86. The expression of these molecules on the DC populations is depicted as histograms.

Immunofluorescence

Human or murine DCs were cultured in 4 well chamber slides at low density over night. After overnight incubation cells were washed and treated with 0.5 mM Arsenite for 30 min, or cultured with YF-17D. The cells then were washed and fixed with 4% formaldehyde and permeabilized with 0.2% Triton-x-100 (Sigma). Cells were then incubated with anti-TIAR/TAI-1 (Santa Cruz 1749), LC3 (cell signaling) for 2 hours at room temperature. The secondary antibodies used were either Alexa Fluor 488 or 647-conjugated secondary Abs. F-actin was visualized using BODIPY 558/568 phalloidin (Invitrogen) and coverslips were mounted using ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen). Immunofluorescence signal was detected using a LSM510 confocal microscope (Zeiss), and images were captured and analyzed using the Zeiss LSM Image Browser.

Bone marrow chimeras

CD45.1 congenic C57BL/6 mice were γ-irradiated twice with 2 doses of 550 rads at a 3 hour interval between the irradiation. The irradiated mice were reconstituted with 2 x 10^6 bone marrow cells, depleted of mature T cells, of *GCN2*$^{-/-}$ mice. Eight weeks after

reconstitution, chimerism was ascertained by blood analysis of the donor and host congenic markers by flow cytometry.

Evaluation of direct antigen presentation

We used the "Matahari" CD8 transgenic mice the T cells of which can recognize the immunodominant male antigen H-Y (1). To test whether a defect in direct presentation of antigen by DCs to CD8 T cells contributed the lower immune responses in *GCN2-/-* mice following YF-17D vaccination, we isolated transgenic T cells from female Matahari mice and cultured them in the presence of either wild type or *GCN2*$^{-/-}$ BMDCs from male mice, that were cultured with YF-17D (10,000T cells: 50,000 DCs). In this case the processing and direct presentation of the H-Y antigen by DCs, primes the T cells in culture. We added thymidine to these cultures at 48 hours post coculture and then assayed for the thymidine count as a measure of the proliferating T cells. In the second set of experiments we cocultured either WT or *GCN2-/-* BMDCs that were infected with YF-17D-Ova 8 with transgenic OT-1 T cells and measured for proliferation 72 hours post culture.

In vitro cross-presentation assay

In vitro cross presentation assays were performed as described (2). BHK cells were plated in 6 well plates at a density of 5 x 10^5 for infection. They were then infected with 5 x 10^5 pfu YF-17D-Ova 8 for 1 hour (3). The cells were then washed to remove the virus and then cultured for a further 24 hours, followed by extensive washing with PBS. The infected BHK cells were harvested and cell death was induced by three rounds of freeze/thaw. BHK-YF-17D-Ova 8-cell lysate was added to the BMDCs from wild type or the various knockout mice, and cultured for 4 hours. The BMDCs used in this study were amino acid starved for 2 hours prior to the co-culture with the BHK-Ova lysates. This was done by incubating the cells in RPMI 1640 medium without amino acids (US Biological; R8999-04A) and in the presence of 1% dialyzed FBS (Gibco 26400-036). Following the starvation period, the medium was supplemented with MEM nonessential amino acid (100X) solution (Gibco; 11140), MEM amino acids (50X) solution (Gibco; 11130) and FBS (Gibco). Then FACS sorted naïve OT-1 cells were cultured with the DCs. Three days later proliferation was assayed by thymidine incorporation.

In MEF co-culture experiments, wild type or *GCN2*$^{-/-}$ MEFs (2 x 10^5/ well in an 8 well plate) were cultured overnight, and then infected with 1 MOI YF-17D-Ova 8 in incomplete DEMEM+ 1% FBS. After 1 hour, the cells were washed and cultured in complete media for a further 12 hours. The cells were then harvested with trypsin-EDTA and were washed and freeze thawed three times. Additionally the cells were then irradiated (8000 rads) following which the lysates were added in graded concentrations to BMDCs from either wild type or *GCN2*$^{-/-}$ mice. The BMDCs were starved for 2 hours in amino acid free media prior addition of the lysates. Then the DCs were co-cultured with OT-1 T cells, and 3 days later their proliferation assessed by thymidine incorporation. To confirm an absence of infectious virus, BHK or MEF lysates from were subjected to a standard plaque assay.

Metabolomics measurement of free amino acid concentrations in DCs

Human monocyte derived DCs were infected with YF-17D for the times indicated. Inactivated virus was used as a control. The cells were washed and pelleted. Cell pellets were washed with 3 volumes of isotonic ice-cold buffer. Cellular lysates were prepared using freeze thawing and methanol/water extraction. LC-MS was performed essentially as previously described (4). Cell pellets were treated with acetonitrile (2:1, v/v) and centrifuged at 14,000 x g for 5 min at 4°C to remove proteins. Samples were maintained at 4°C in an auto sampler until injection. A Thermo LTQ-FT mass spectrometer (Thermo Fisher, SanDiego, CA) coupled with dual chromatography (anion exchange and C18) was used for data collection, via positive-ion electrospray ionization (ESI). The LC-MS data were processed with apLCMS program for feature extraction and quantification (5). Peaks for corresponding amino acids were compared to in-house library and standards.

Evaluation of YF-17D-specific $CD8^+$ and $CD4^+$ T cell responses

Mice were immunized with YF-17D (2 $x10^6$ pfu/mice) by subcutaneous injection at the base of tail. Draining lymph nodes, spleens, liver and lung were harvested 7 days later. Single cell suspension was prepared, following collagenase IV digestion. The mononuclear populations in both liver and lung were isolated from the interphase of a 70-40% percoll gradient. For analysis of $CD8^+$ T cell responses, cells were plated at 5 x 10^5 to 1 x 10^6 cells/well in 96-well plates, and re-stimulated with YF-17D specific peptide pools (2 μg/ml of each peptide), for 5 hours in the presence of Golgi-Plug/Golgi Stop. The peptides used were an H-$2D^b$–restricted epitope (residues 1–15 from the E1 envelope protein, AHCIGITDRDFIEGV), or an H-$2K^b$–restricted dominant epitope mapped to the NS3 protein (residues 261–275 from the NS3 protein, VIDAMCHATLTYRML). Then cells were stained with anti-CD45 and anti-CD8α and stained intracellularly with anti-IFNγ in the presence of BD Permeabilization buffer. The cells were then washed and data was acquired in either FACS Calibur or LSR-II and analyzed using Flowjo analysis software. In some experiments, cells were plated at 5 x 10^5 to 1 x 10^6 cells/well in 96-well plates and restimulated with the YF-17D peptides (0.05 μg/ml of each peptide) for 4 days. Then the concentration of IFNγ in the culture supernatants was measured by ELISA. For analysis of $CD4^+$ T cell responses, cells were plated at 5 x 10^5 to 1 x 10^6 cells/well in 96-well plates, and re-stimulated with YF-17D specific peptide (0.05 μg/ml of each peptide) for 4 days. The peptide used was an I-A^b–restricted epitope from the viral envelope protein (residues 231–247 from the envelope protein, LVEFEPPHAATIRVL). Then the concentration of IFNγ in the culture supernatants was measured by ELISA.

Evaluation of influenza vaccine specific $CD8^+$ T cell immune responses

Wild type or *GCN2*$^{-/-}$ mice were vaccinated with 10^6 viral pfu of Flumist in a 30μl volume intranasally. Virus-specific $CD8^+$ T cell responses were analyzed 7 days later by flow cytometry. Cells were isolated and cultured in a 96-well plate at 0.5×10^6 cells per well with or without Brefeldin (5 μg/ml) and stimulated with a peptide from the influenza nucleoprotein (NP366–374) (5μg/ml) for 5 hrs. Intracellular cytokine staining was performed.

Statistical Analysis

To assess the significance of a difference between groups, a two-sample, unpaired t test was performed using Graph Prism software. A P value less than 0.05 (*) was considered to be significant, a P value less than 0.005 (*) was considered to be very significant, and a P value less than 0.005 (***) was considered to be extremely significant.

Reference:

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2. R. Singh, P. Cresswell, *Science* **328**, 1394 (2010).
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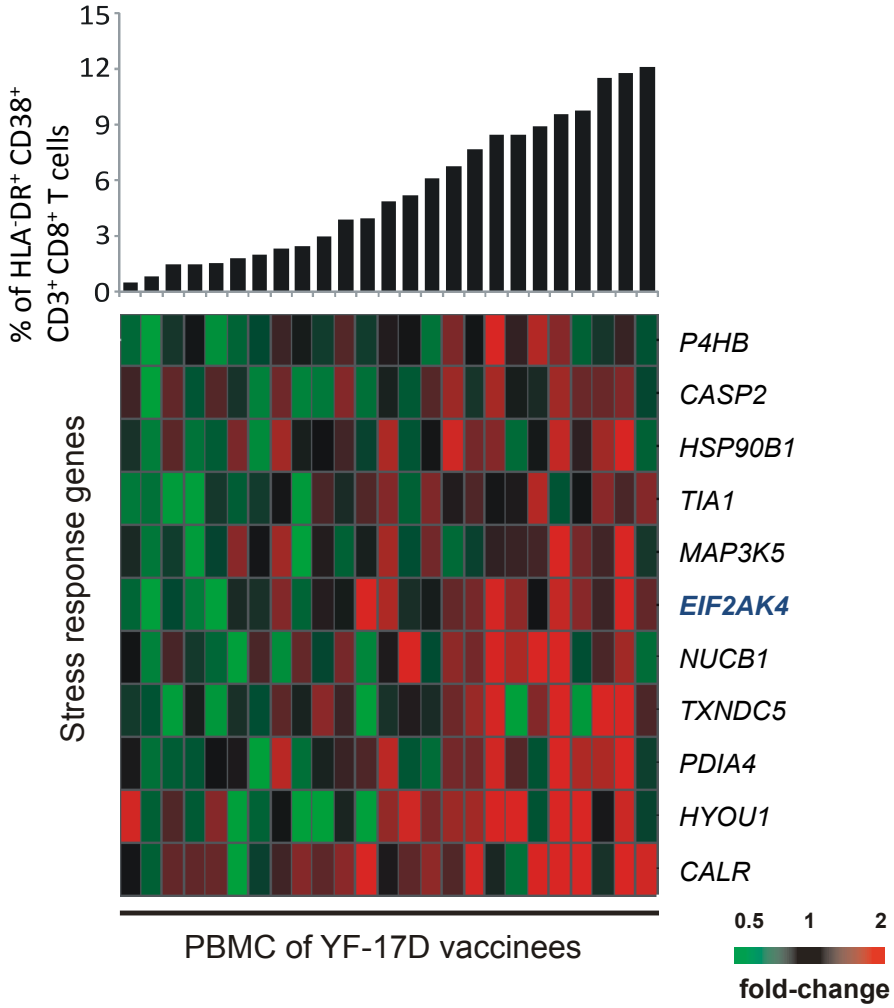

Figure S1. Vaccination of humans with YF-17D induces a molecular signature, consisting of genes encoding proteins in the integrated stress response, that correlates with the magnitude of the later CD8⁺ T cell response. The graph on top of the heat map shows variations in the magnitude of the antigen-specific CD8⁺ T cell response at day 15 amongst 25 healthy humans that were vaccinated with YF-17D. The heat map represents genes encoding proteins in the integrated stress response pathway, induced in the PBMCs, 7 days after vaccination with YF-17D. Many of these genes correlate with the magnitude of the later CD8⁺ T cell response.

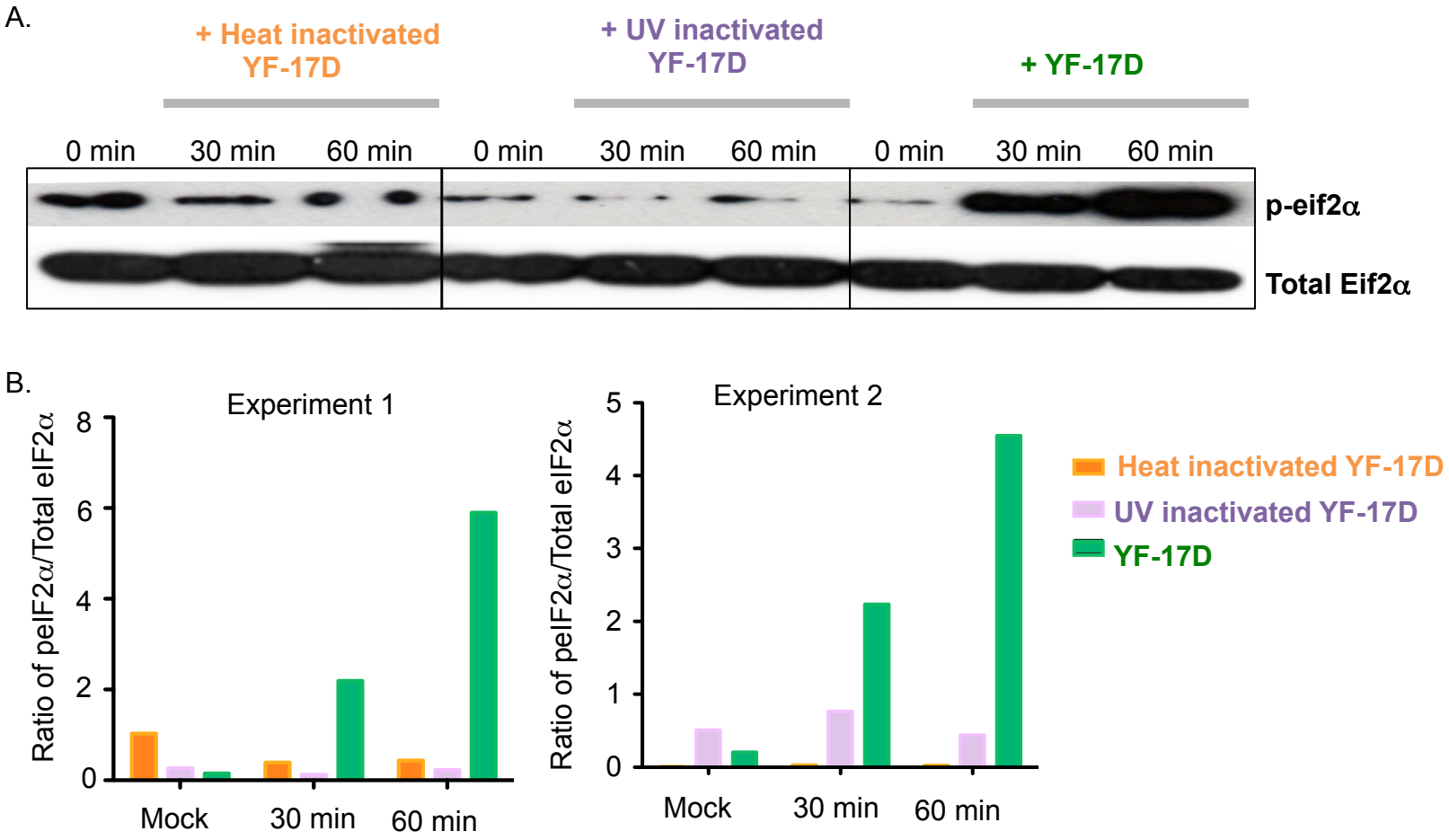

Figure S2. Live YF-17D virus, but not heat inactivated or ultraviolet inactivated YF-17D, induces eIF2α phosphorylation in DCs. (A) Comparison of phospho eIF2α on BMDCs from C57BL/6 mice following culture with YF-17D, heat-inactivated YF-17D or UV-irradiated YF-17D (MOI of 1). (B) Densitometry analysis showing the ratio of p-eIF2α/Total eIF2α from 2 different experiments.

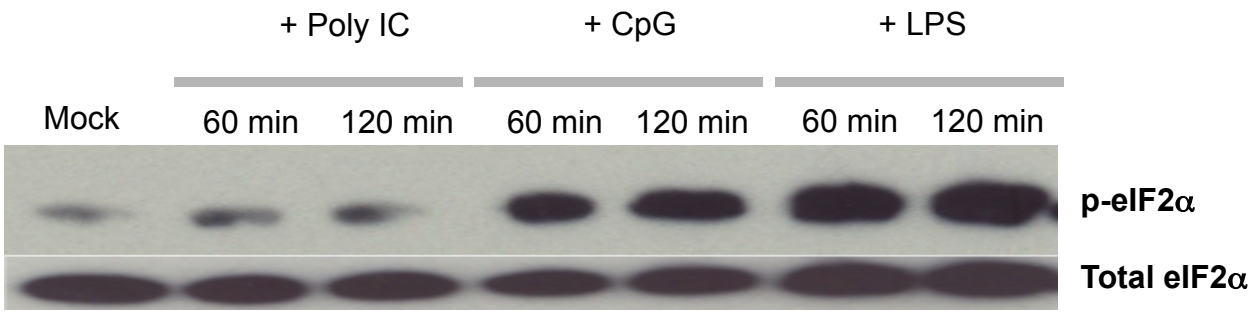

Figure S3. TLR ligands induce eIF2α phosphorylation in DCs. Phospho eIF2α on BMDCs following activation of Poly IC, CpG1826 or LPS. The data is representative of 3 independent experiments

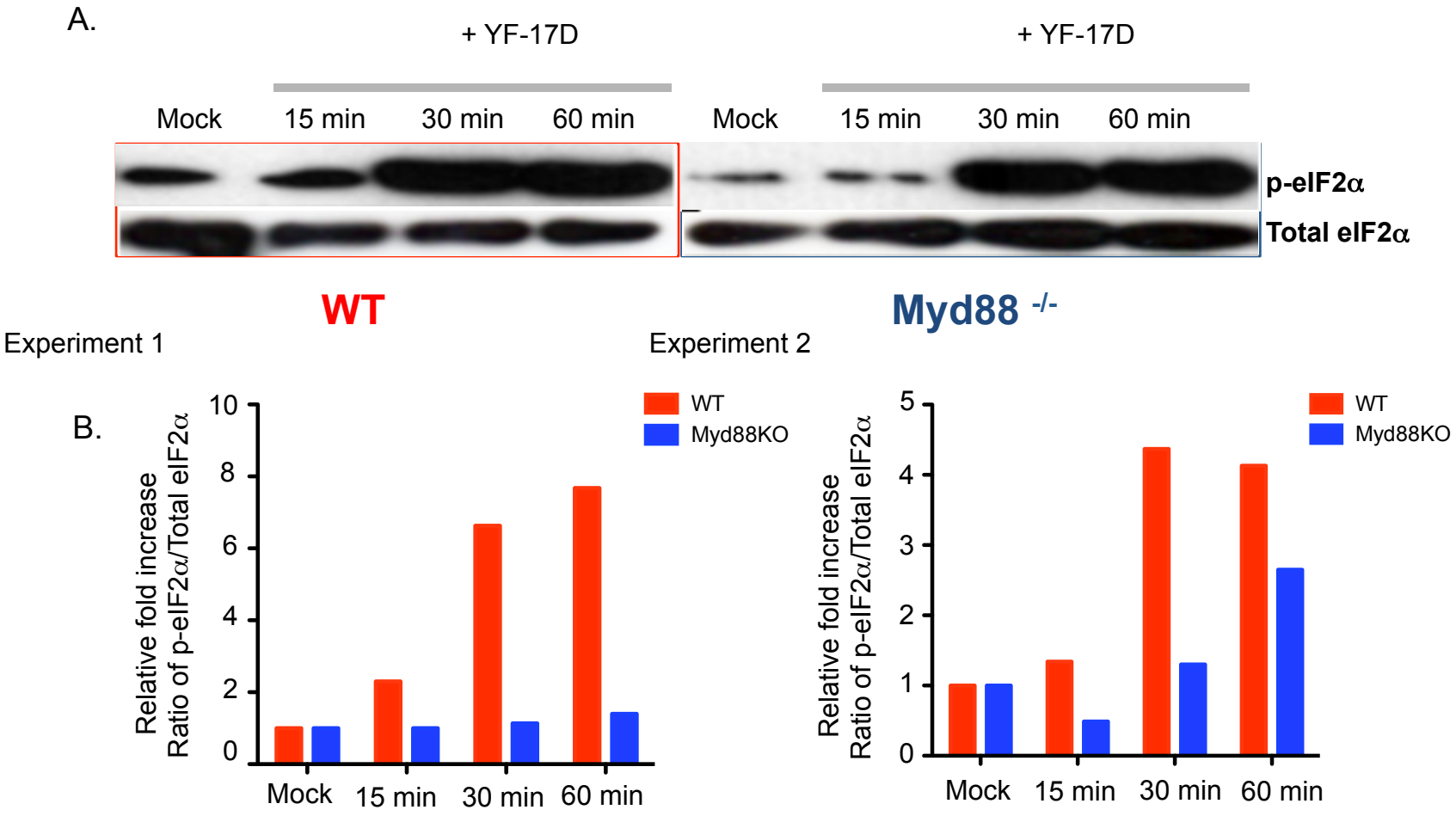

Figure S4. YF-17D induced eIF2α phosphorylation in DCs is partially dependent on MyD88 signaling. (A) BMDCs isolated from wild type or *MyD88* $^{-/-}$ mice were cultured with YF-17D (MOI of 1), and eIF2α phosphorylation assessed by western blotting. (B) The ratio of peIF2α to total eIF2α was calculated by normalizing the densitometric values (ImajeJ) of mock controls to 1 and represented as a fold increase to the mock values. The densitometric analysis of 2 independent experiments is plotted individually.

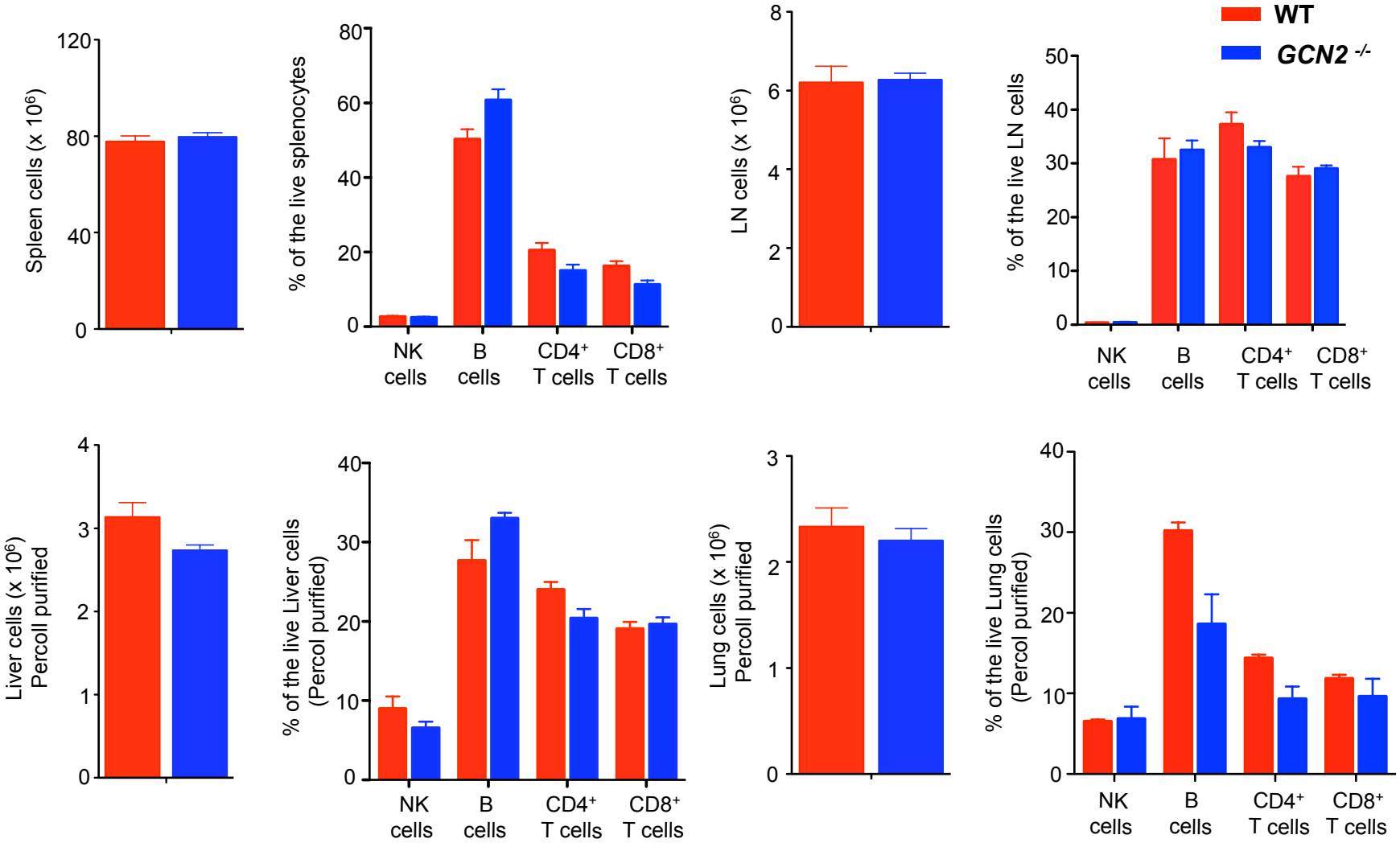

Figure S5. Normal frequencies of T, B and NK cells in *GCN2*$^{-/-}$ mice. Comparison of cells in the adaptive immune system, between wild type and *GCN2*$^{-/-}$ animals in the lymph node, spleen, liver and lung. Total cellularity and percentages of NK, B, CD4 and CD8 cells in the organs is depicted in the figure (n=3). Data is represented as mean ± SEM and is representative of two independent experiments.

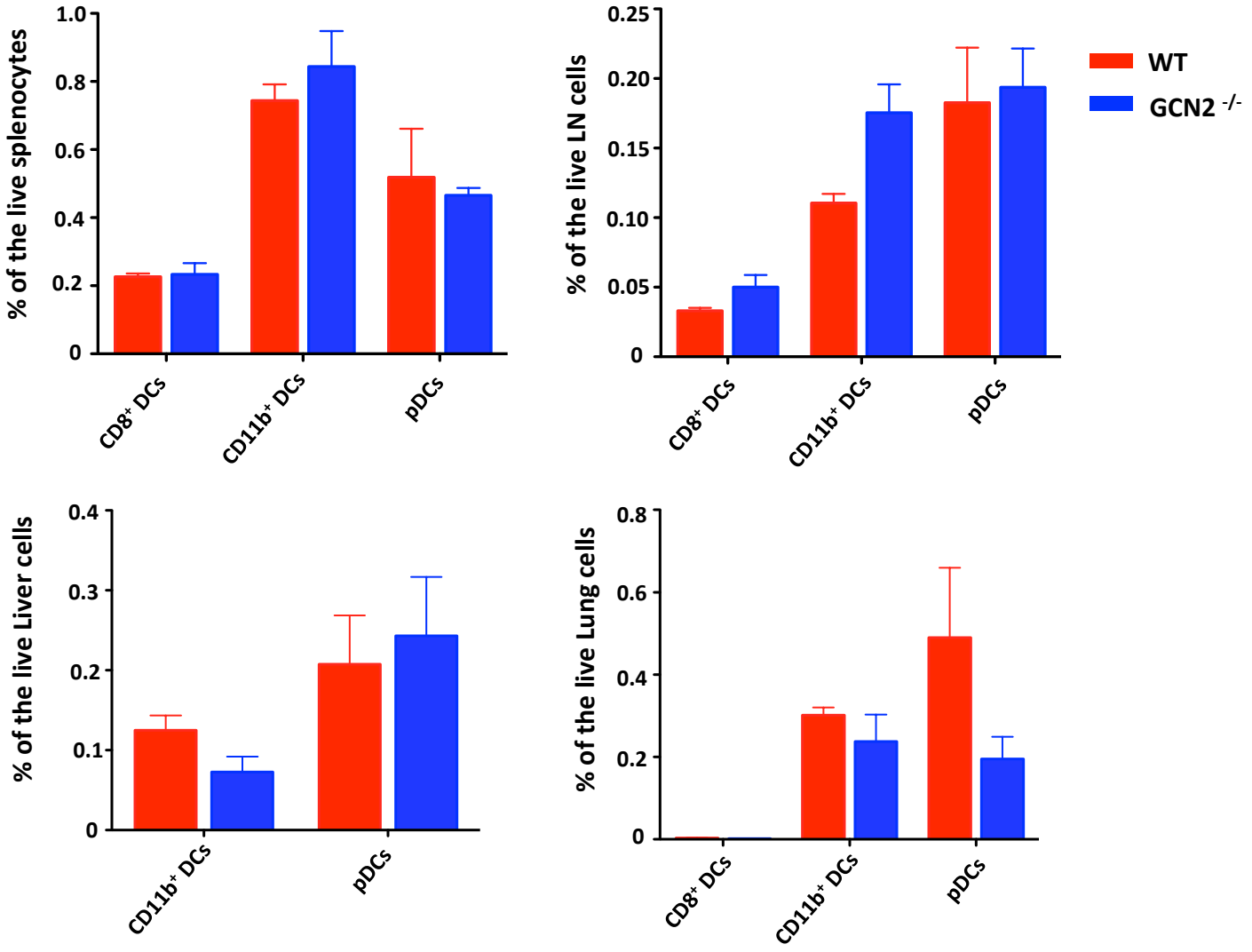

Figure S6. Normal frequencies of dendritic cells in GCN2 deficient mice. Comparison of frequencies of CD8α, CD11b and plasmacytoid dendritic cells between wild type and *GCN2*$^{-/-}$ animals in the lymph node, spleen, liver and lung (n=3). Data represents two independent experiments. Error bars indicate mean ± SEM.

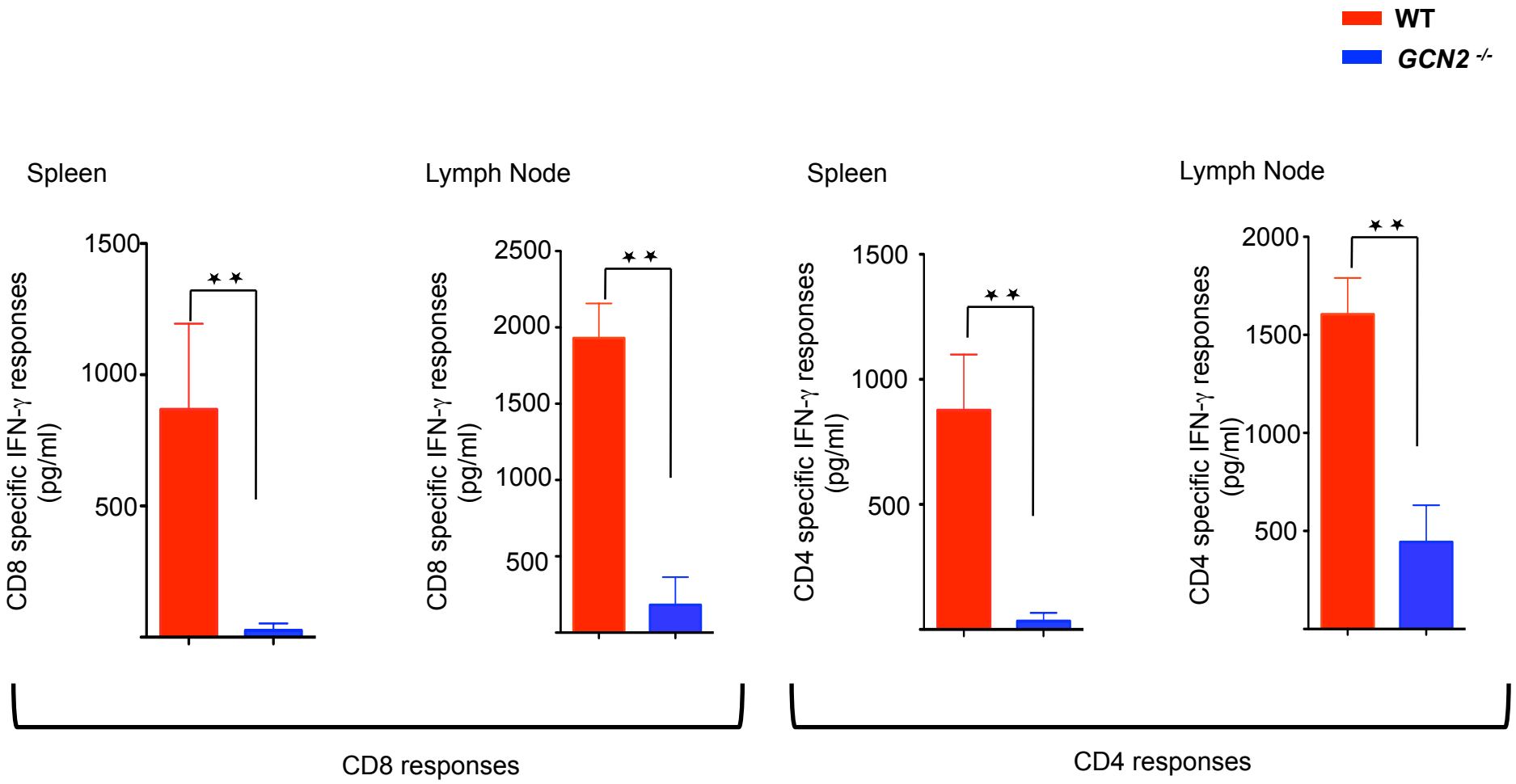

Figure S7. CD8 and CD4 T cell response to YF-17D in spleen and inguinal lymph node in vaccinated mice. Comparison of YF-17D specific CD8 and CD4 immune responses in spleen, lymph node in 4 day peptide re stimulation cultures between wild type and *GCN2*$^{-/-}$ mice. Data is from one experiment representative of three independent experiments. Error bars indicate mean ± SEM (* = p<0.05, **=p<0.005 (student t test).

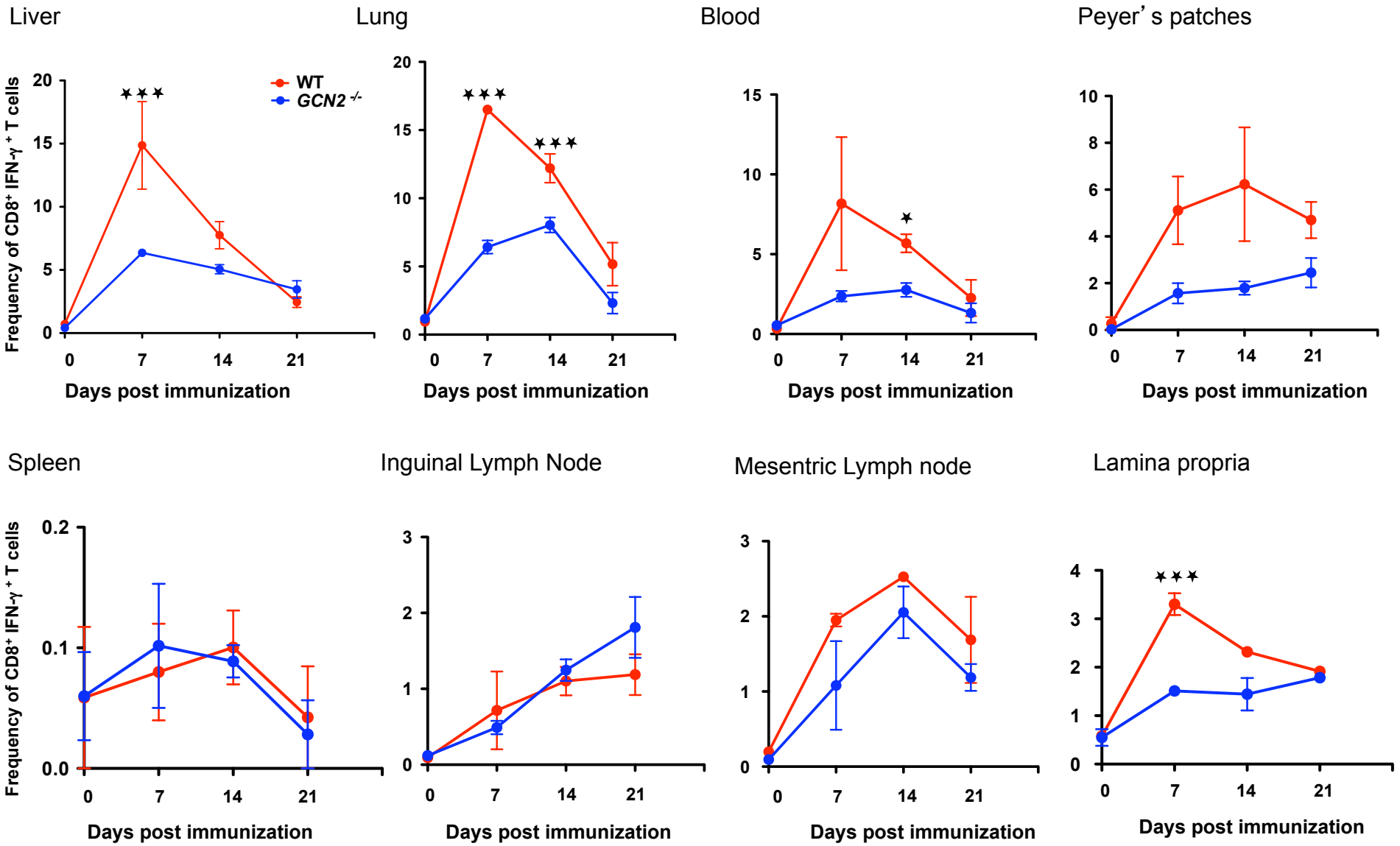

Figure S8. Kinetics of antigen-specific CD8⁺ T cell responses in various organs in wild type versus *GCN2*$^{-/-}$ mice after vaccination with YF-17D. Data from liver, lung, spleen, inguinal lymph nodes are representative of more than 10 experiments. Data represents the mean ± SEM (* = p<0.05, **=p<0.005, ***=p<0.0005 (student t test).

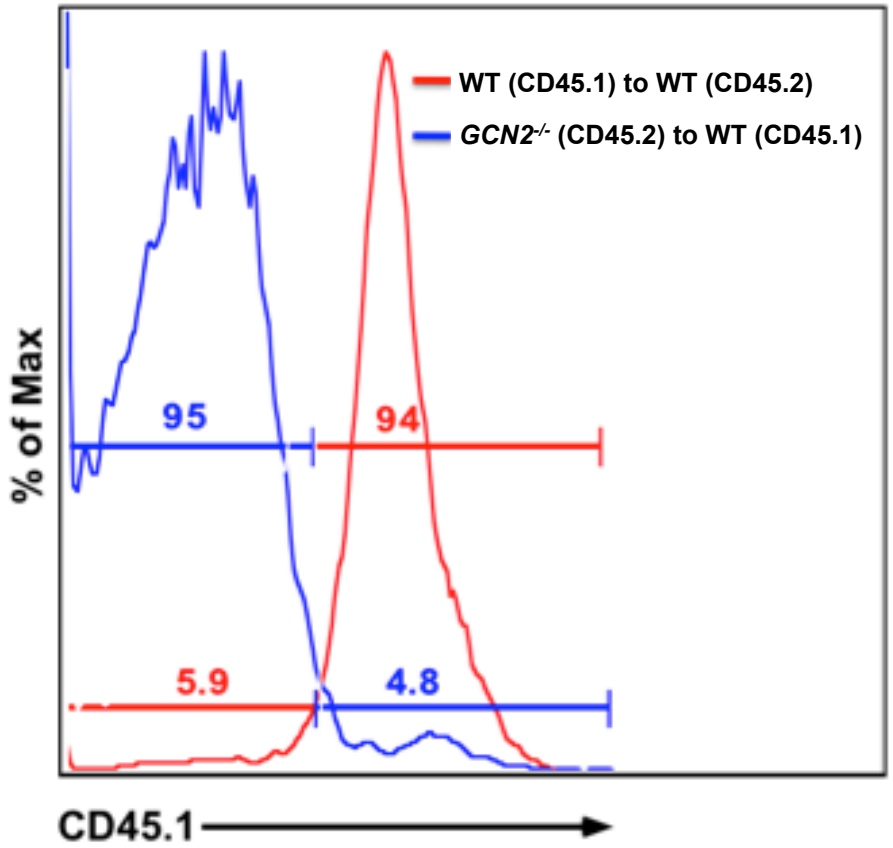

Figure S9. Level of chimerism in bone marrow chimera mice. Histograms depicting the level of chimerism in bone marrow chimeras. Data is from one experiment representative of three.

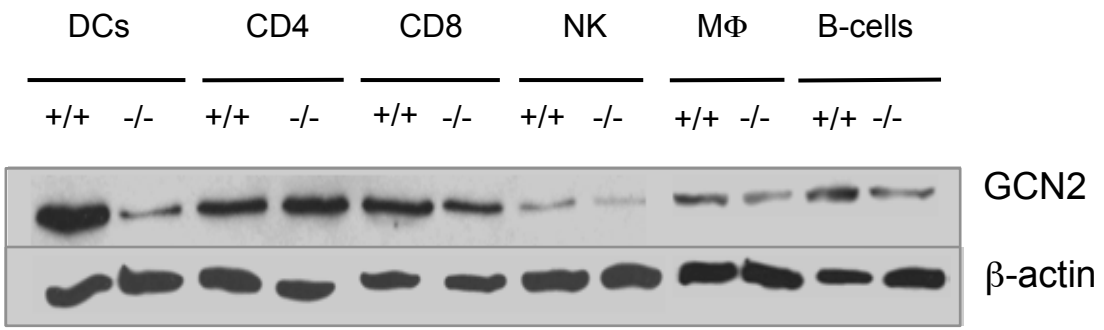

Figure S10. Conditional knockout of GCN2 in dendritic cells. Western blots showing the conditional knockout of GCN2 cells isolated from GCN2 $^{flox/flox}$ CD11c cre mice. Data is from from one experiment.

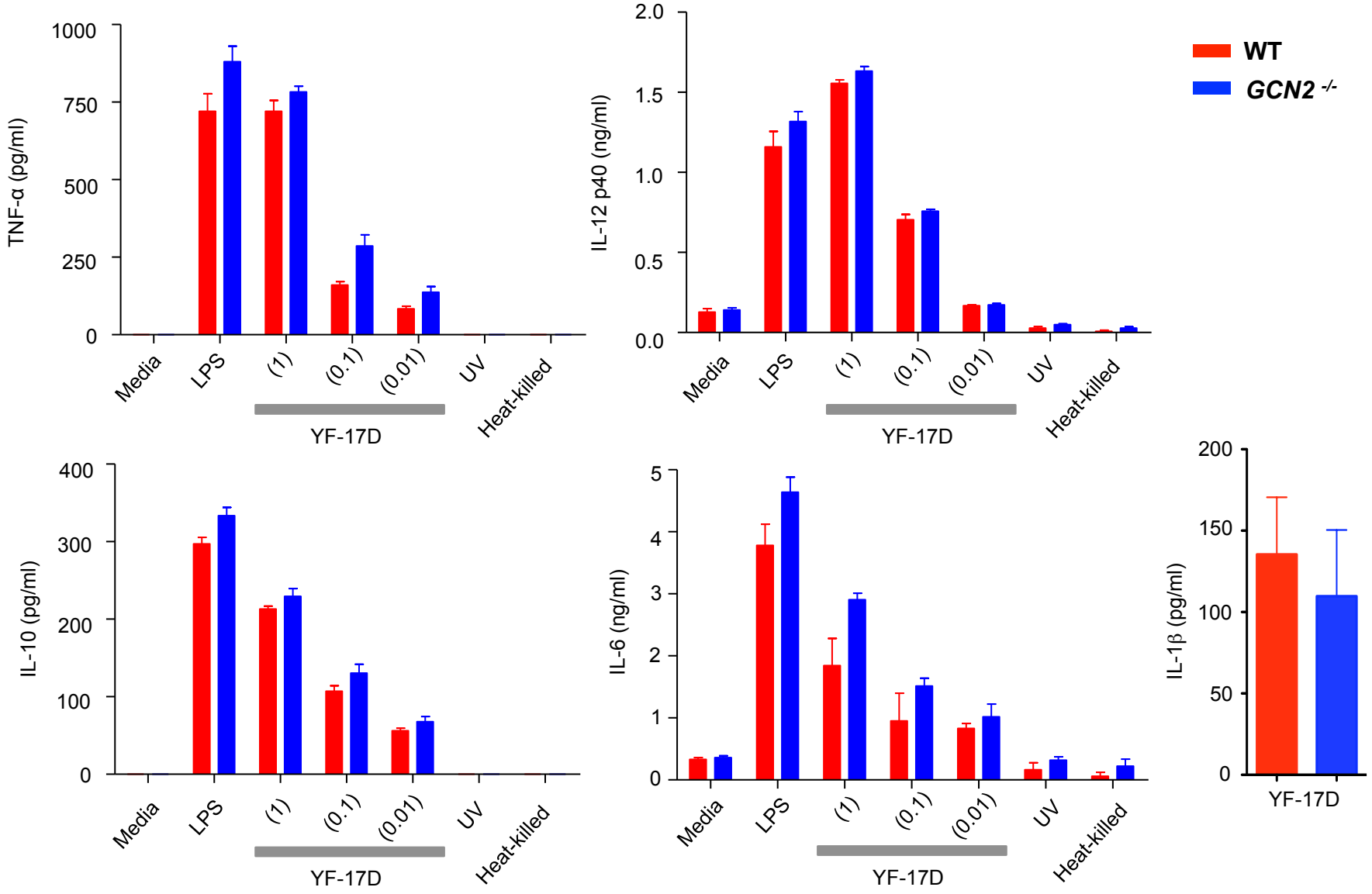

Figure S11. Cytokine production by DCs stimulated in vitro with YF-17D. Comparison of cytokine responses in BMDCs isolated from WT to *GCN2*$^{-/-}$ mice following YF-17D stimulation (24h post infection). Data is from one experiment representative of 3 independent experiments. The data is plotted as mean ± SEM.

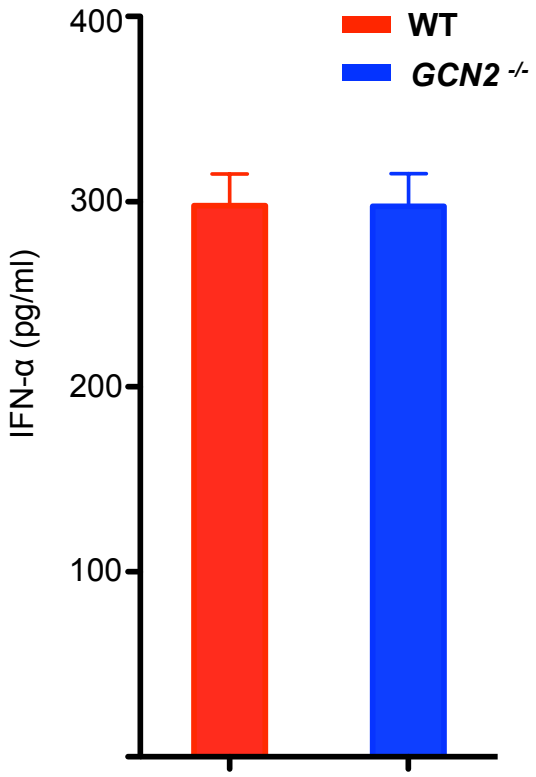

Figure S12. Normal YF-17D-induced IFNα production in dendritic cells deficient in GCN2. Comparison of IFNα production in BMDCs isolated from wild type versus *GCN2* $^{-/-}$ mice, following culture with YF-17D for 24 hours (n=3). Data represents one independent experiment. The data represents mean ± SEM.

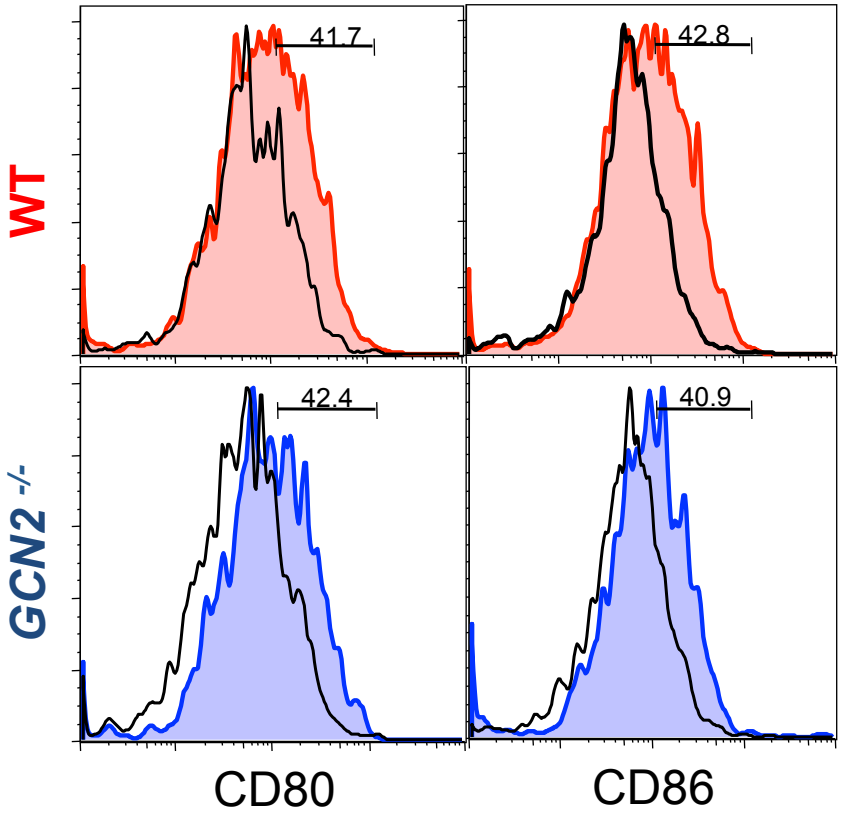

Figure S13. Normal levels of costimulatory molecules in lymph node DCs of both wild type and *GCN2*$^{-/-}$ mice 24hrs following YF-17D-vaccination. Comparison of CD80 and CD86 in draining lymph node DCs isolated from wild type versus *GCN2* $^{-/-}$ mice, 24 hours following YF-17D vaccination. Colored histograms represents expression on DCs from vaccinated mice and open histograms represent DCs from unvaccinated mice (n=3).

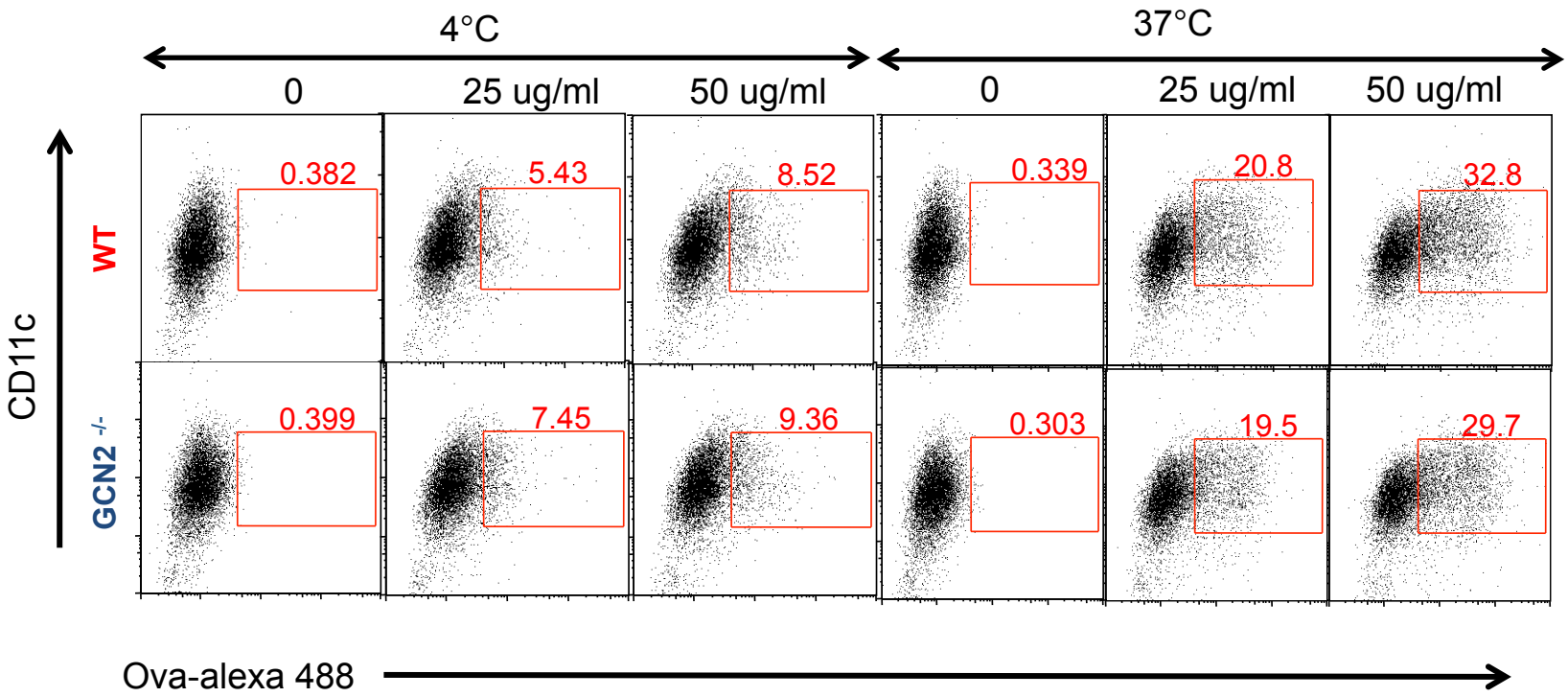

Figure S14. Normal uptake of soluble antigen in dendritic cells deficient in GCN2. Comparison by FACS analysis of the uptake of Ova conjugated Alexa-488, by DCs from wild type and *GCN2* $^{-/-}$ mice at 4^{o}C and 37^{o}C. Data is from one experiment representative of two independent repeats.

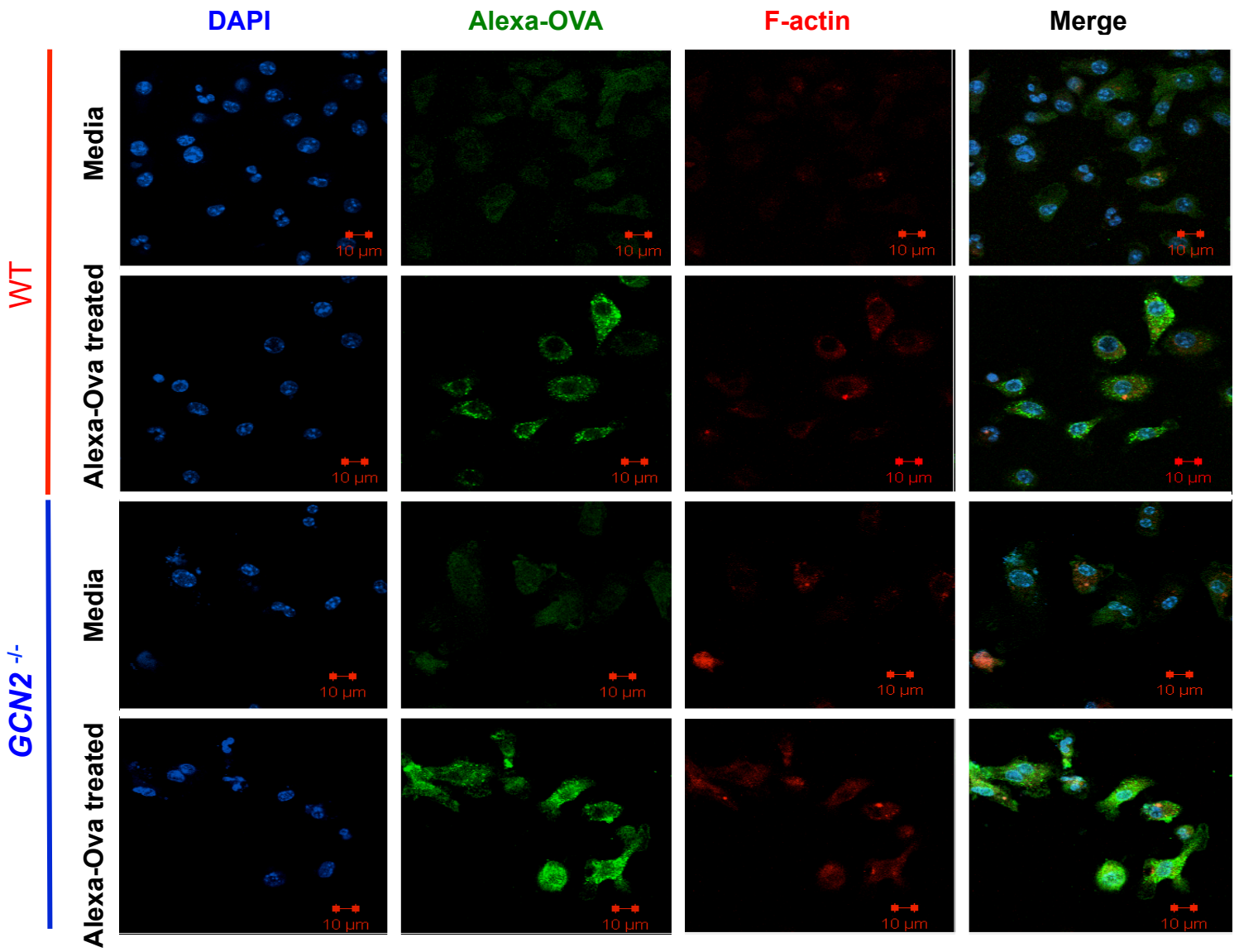

Figure S15. Normal uptake in dendritic cells deficient in GCN2. Comparison of the uptake of Alexa 488 conjugated Ova between wild type versus *GCN2*$^{-/-}$ BMDCs by confocal microscopy at 37^{0}C (n=3). Data is from one experiment.

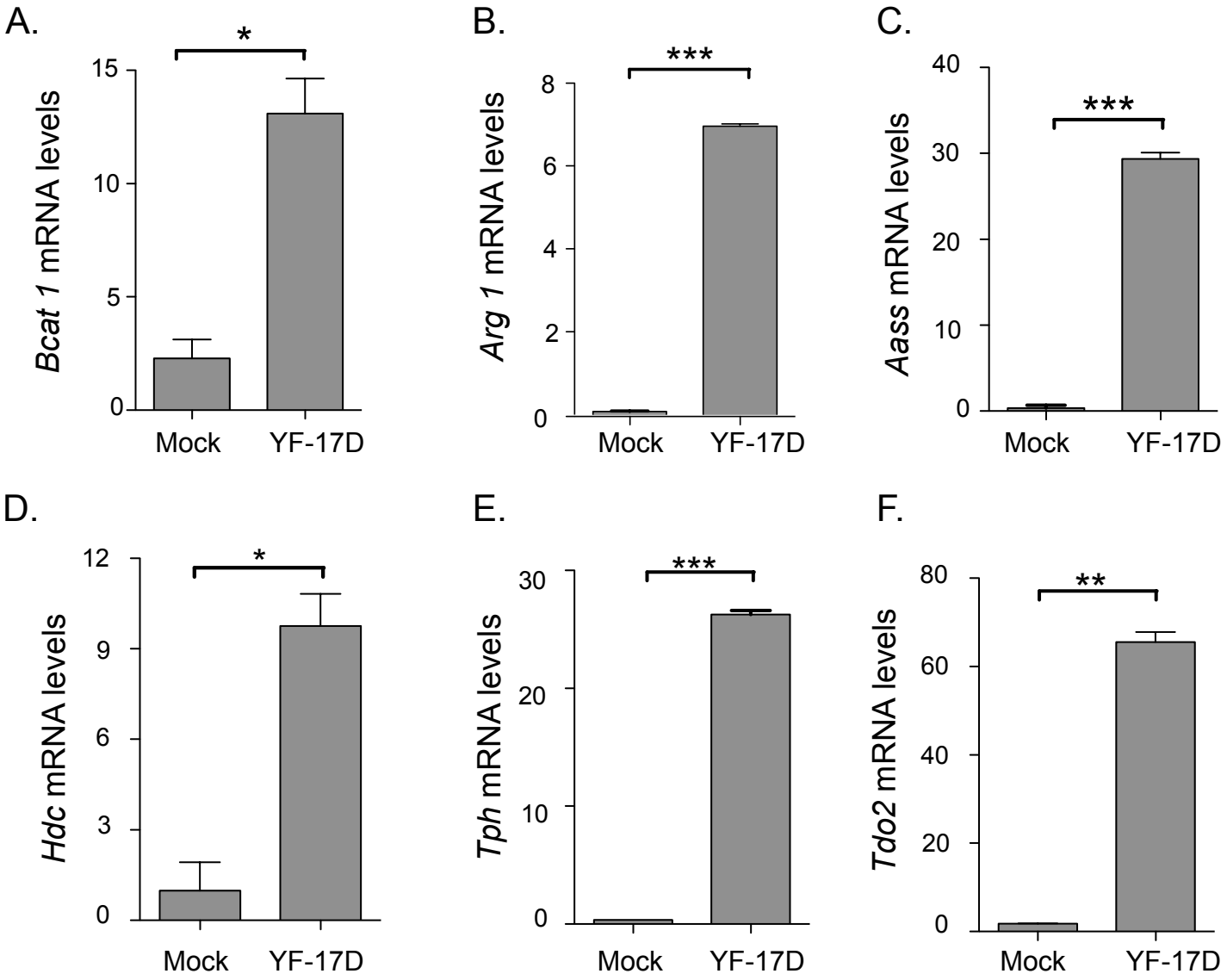

Figure S16. YF-17D induces enhanced expression of amino acid metabolizing enzymes in dendritic cells. Expression of relative mRNA (A) *Bcat1*, (B) *Arg1*, (C) *Aass*, (D) *Hdc*, (E) *Tph* and (F) *Tdo2* in human monocyte derived DCs, before and following culture with YF-17D (n=3). Data represents the mean ± SEM of a single experiment (* = $p<0.05$, **=$p<0.005$, ***=$p<0.0005$, t test).

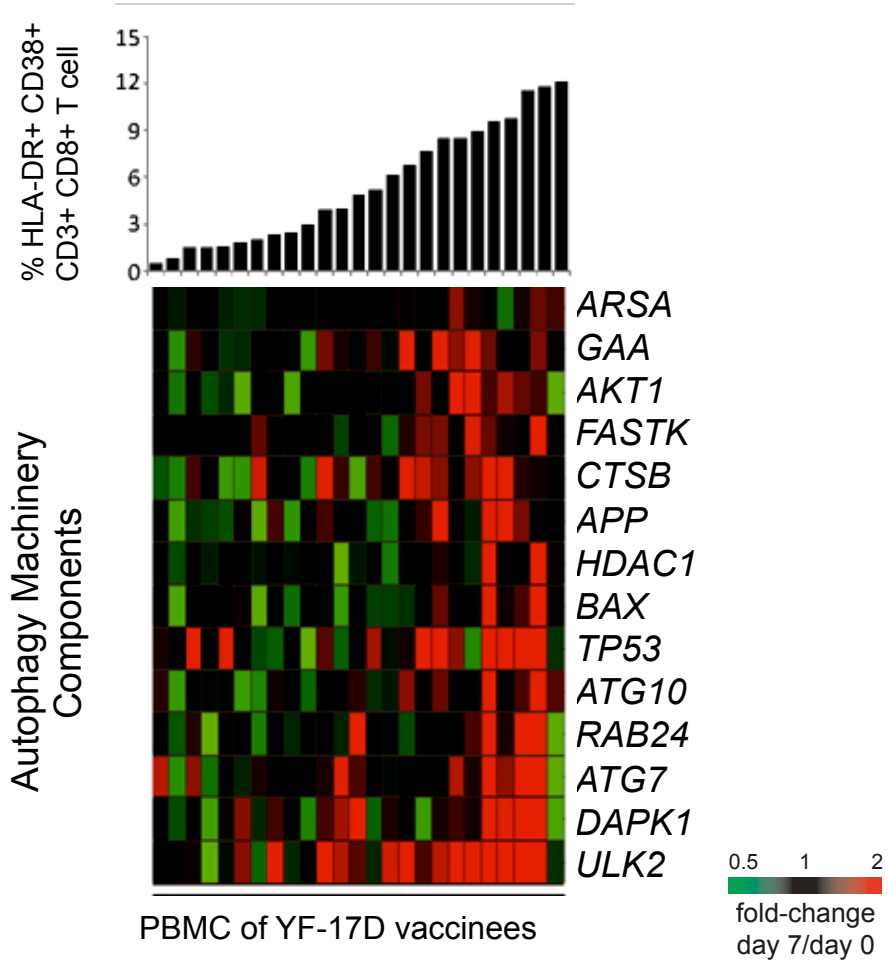

Figure S17. Vaccination of humans with YF-17D induces a molecular signature, consisting of genes encoding proteins involved in autophagy, that correlates with the magnitude of the later CD8⁺ T cell response. The graph on the top of heat map shows variations in the magnitude of the antigen-specific CD8⁺ T cell response at day 15 amongst 25 healthy humans that were vaccinated with YF-17D. The heat map represents genes encoding proteins involved in autophagy, induced in the PBMCs, 7 days after vaccination with YF-17D. Many of these genes correlate with the magnitude of the CD8⁺ T cell response.

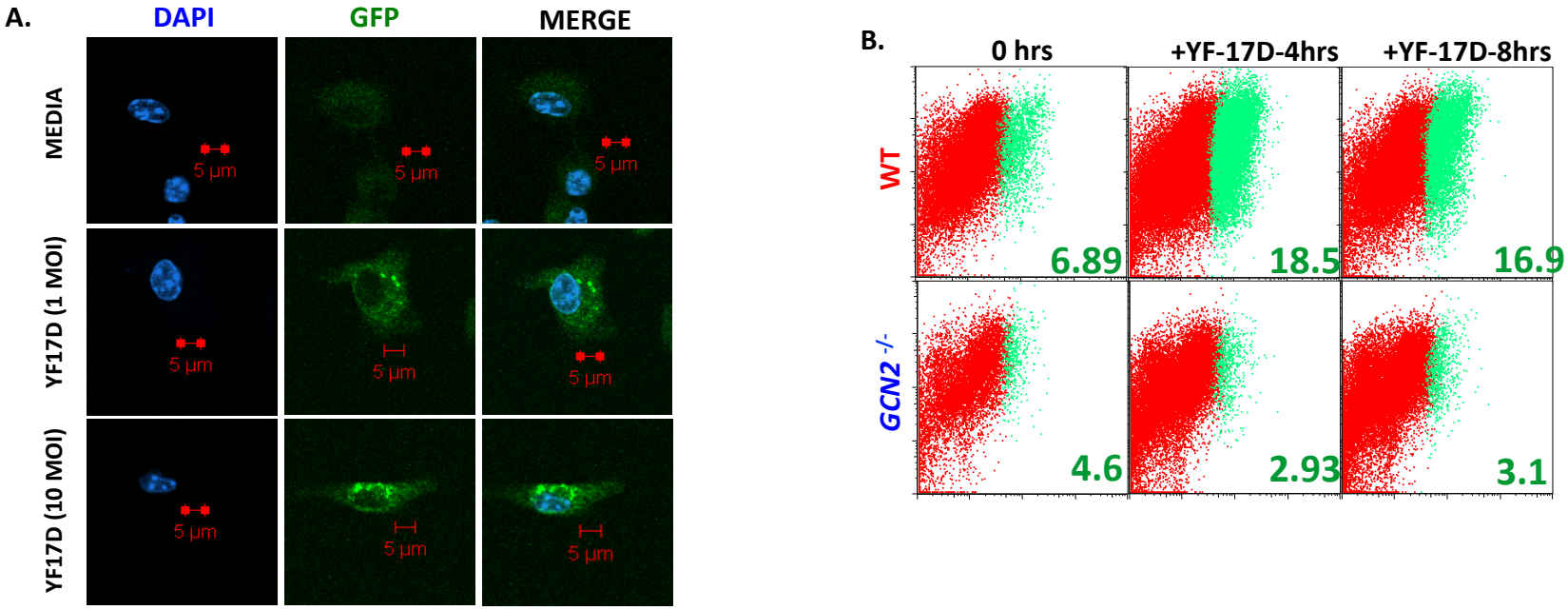

Figure S.18. Induction of autophagy in DCs stimulated with YF-17D. (A). Immunofluorescence analysis of LC3-GFP expression in BMDCs from LC3-GFP reporter mice stimulated in vitro with YF-17D for 6 hrs. (B) LC3-GFP expression on wild type or *GCN2-/-* LC3-reporter BMDCs 4 and 8 hours post stimulation with YF-17D, as assayed by FACS by gating on the CD11c population (n=3).

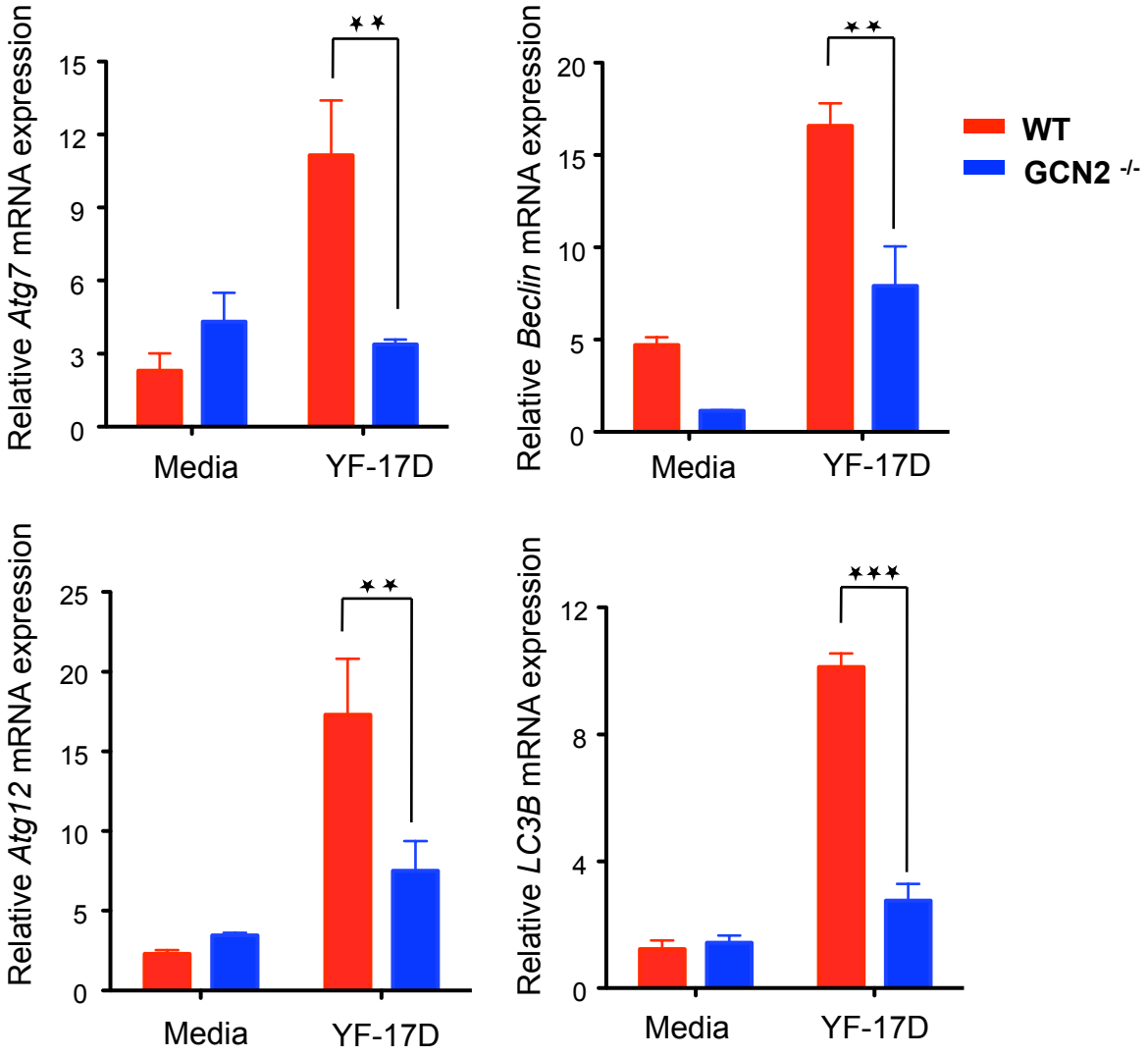

Figure S19. Impaired YF-17D-induced autophagy in dendritic cells deficient in GCN2. Comparison of the relative mRNA expression of various autophagy genes (*Atg7*, *Beclin*, *Atg12* and *LC3B*) in dendritic cells isolated from wild type and *GCN2*$^{-/-}$ animals, 6 hours following culture with YF-17D (n=3). Data represents the mean ± SEM of a single experiment(* = p<0.05, **=p<0.005, ***=p<0.0005, t test).

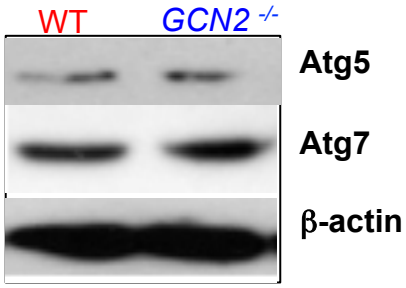

Figure S20. Equal levels of autophagy proteins in steady state DCs isolated from wild type and *GCN2*$^{-/-}$ mice. Comparison of the protein levels of autophagy proteins (Atg5 and Atg7) in steady state BMDCs isolated from wild type and *GCN2*$^{-/-}$ animals. Data is from one experiment representative of two.

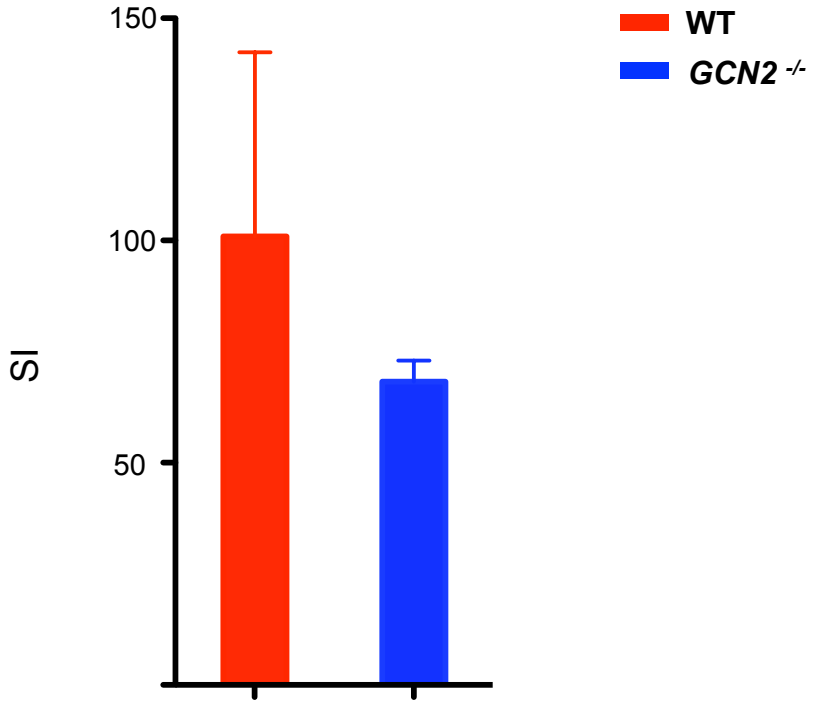

Figure S21. DCs from *GCN2*$^{-/-}$ mice are efficient at presenting peptide antigen to antigen specific CD8^{+} T cells. BMDCs isolated from wild type or *GCN2*$^{-/-}$ mice were pulsed with SIINFEKL peptide and co-cultured with naïve SIINFEKL-specific CD8^{+} transgenic T cells from OT-1 mice. Proliferation was assessed by thymidine incorporation, 72 hours later. The proliferative response is expressed as a stimulation index (SI). Data represents the mean±SEM from one experiment representative of three.

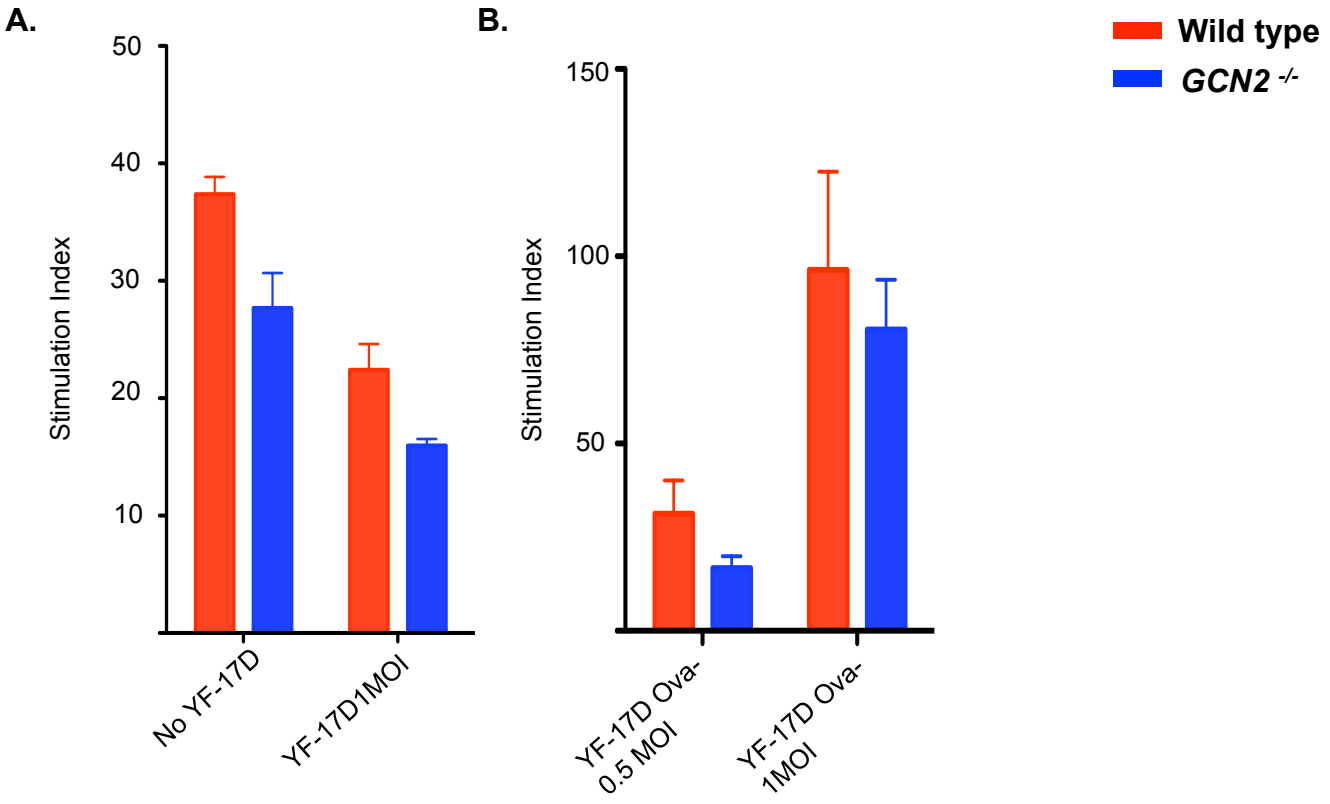

Figure S22. No differences in direct antigen presentation between and wild type and *GCN2*$^{-/-}$ dendritic cells. (A). Female matahari transgenic CD8^{+} T cells were co cultured with male wild type *or GCN2*$^{-/-}$ DCs stimulated with YF-17D for 2hrs, and proliferation was assayed by H^3 thymidine incorporation, 72 hrs later. (B) BMDCs isolated from either wild type or *GCN2*$^{-/-}$ were cultured with YF-17D-Ova, washed and then co-cultured with naïve SIINFEKL-specific CD8^{+} transgenic T cells from OT-1 mice. Proliferation was assayed by thymidine incorporation,72 hours later. The proliferative response is expressed as a stimulation index (SI). Data represents the mean ± SEM from one experiment representative of two.

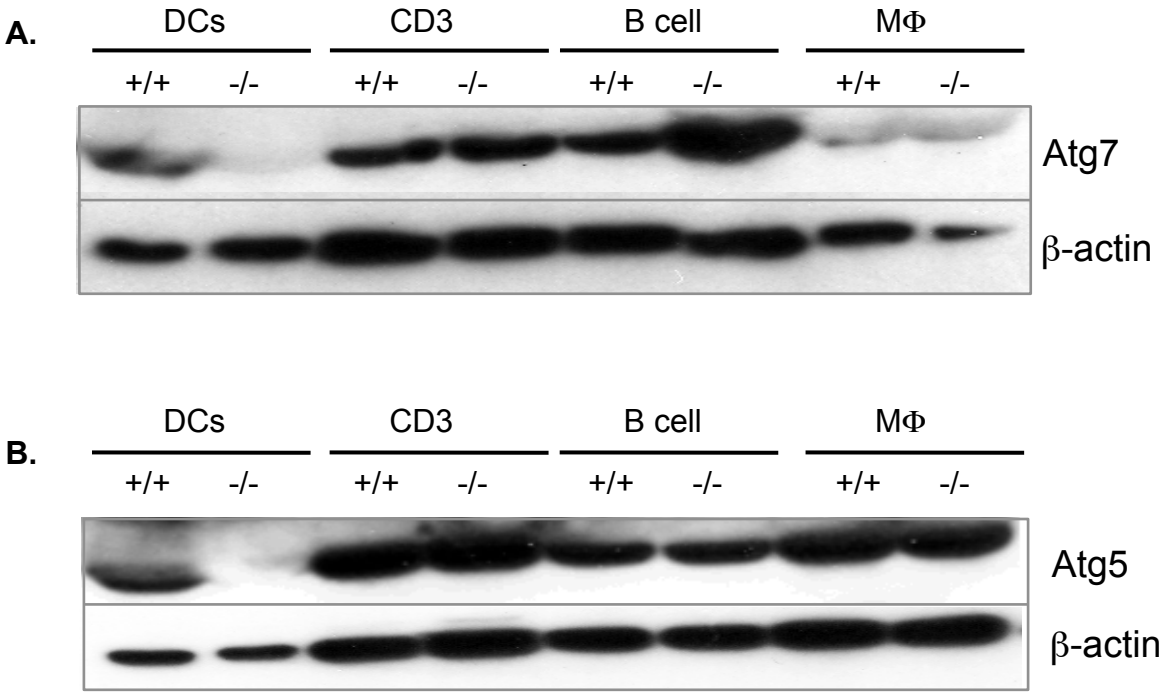

Figure S23. Western blot analysis of Atg5 and Atg7 expression in various cells from Atg7 $^{flox/flox}$ CD11c cre (A), and Atg5 $^{flox/flox}$ CD11c cre (B) mice, compared to littermate controls. Data is representative of one experiment

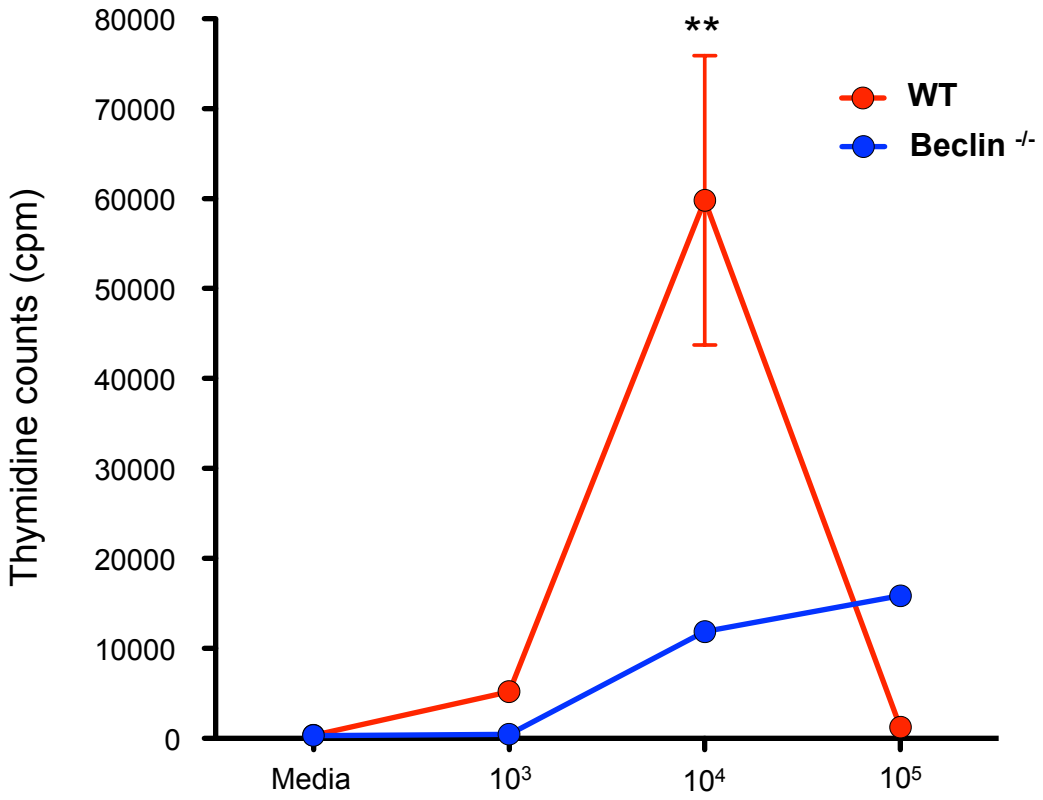

Figure S24. BMDCs from *beclin*$^{-/-}$ mice have lower ability to cross present viral antigen to CD8^{+} T cells. BMDCs isolated from wild type or *beclin*$^{-/-}$ mice were cultured in the presence of varying numbers of BHK cells infected with YF-17D-Ova. Then these cells were cultured with naïve SIINFEKL-specific CD8^{+} transgenic T cells from OT-1 mice. Proliferation was assessed by thymidine incorporation, 72 hours later (n=3). Data represents the mean ± SEM of one experiment representative of three (* = p<0.05, **=p<0.005, ***=p<0.0005, t test).

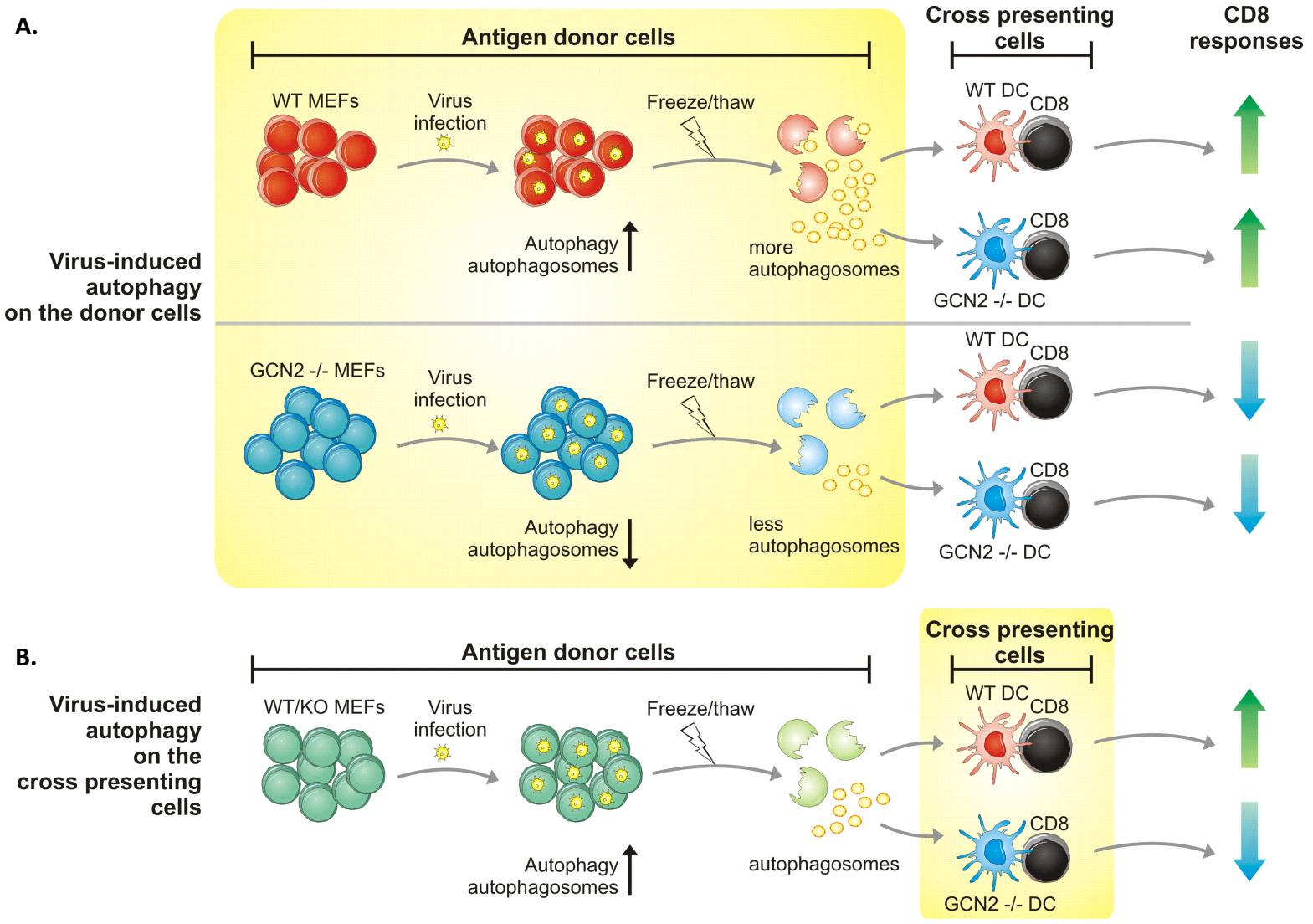

Figure S25. Models proposing the impact of GCN2 deletion on the ability of cells to cross present viral antigen to CD8⁺ T cells, following YF17D infection. Proposed models of cross presentation depicting the role of viral induced autophagy in antigen donor cells (A) or on antigen presenting cells (B).

Pathogen	eIF2α phosphorylation	Stress granules	Differences in adaptive immune responses between wild type versus *GCN2*$^{-/-}$ mice
Yellow fever (YF-17D)	Yes	Yes	Yes
Flumist	Yes	N.D	Yes
Ad5 (Adeno virus)	Yes	N.D.	N.D
LCMV (Armstrong)	N.D.	N.D.	No
LCMV (Clone 13)	N.D.	N.D.	No
Fransicella tularensis	Yes	No	No
Salmonella typhimurium	Yes	Yes	No
Listeria monocytogenes	Yes	No	No

Figure S26. Induction of stress granules, eIF2α phosphorylation, and the impact of GCN2-deficiency on CD8^{+} T cell responses to various viruses and bacteria. A table summarizing the correlation between the effects of different pathogens on eIF2α phosphorylation and stress granule formation to immune responses

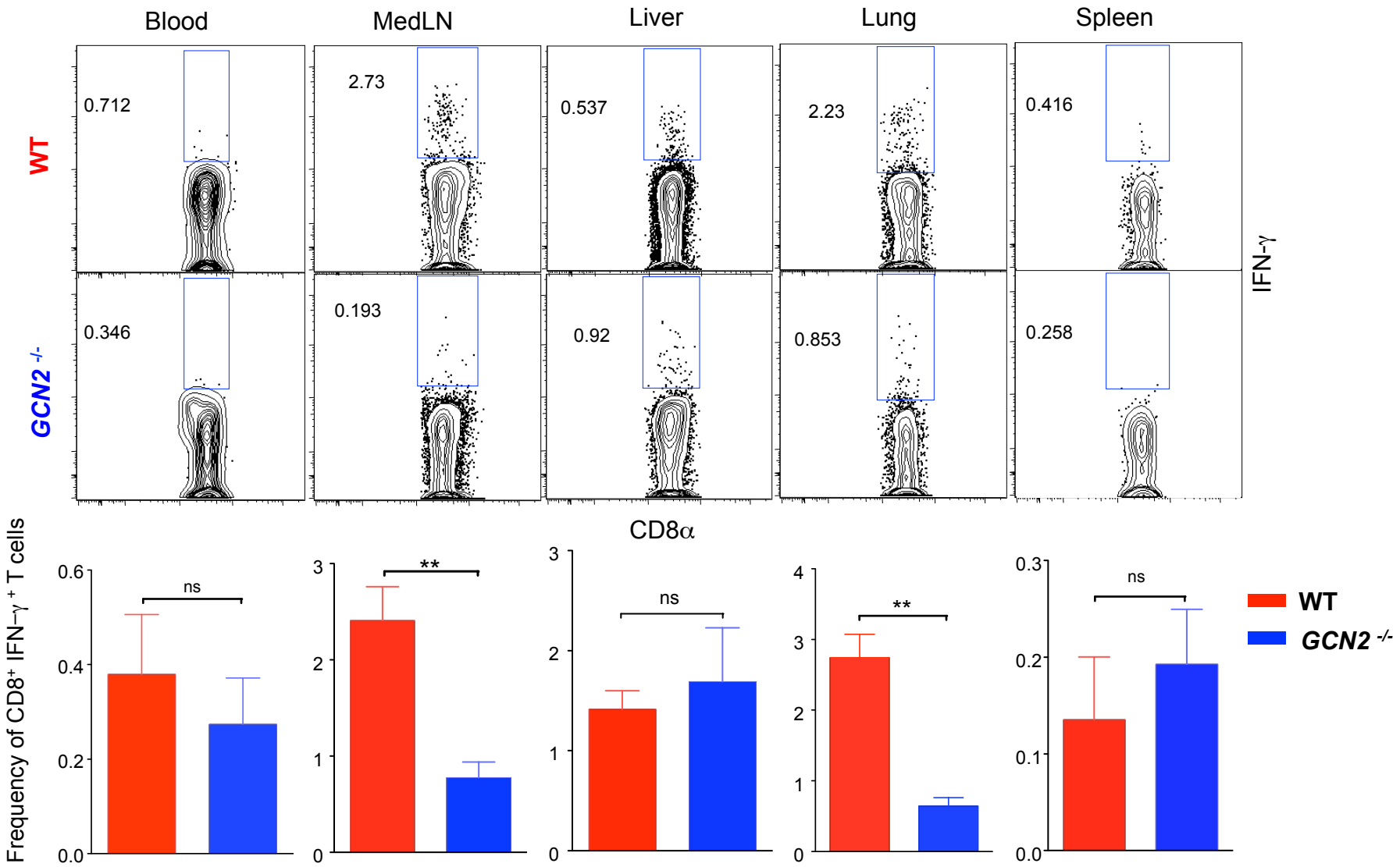

Figure S27. GCN2 deficiency results in impaired antigen-specific CD8+ T cell responses intranasal influenza (Flumist®) vaccination. (A) Representative FACS plots of CD8α⁺ IFN-γ⁺ cells from wild type or *GCN2*$^{-/-}$ mice vaccinated intranasally with Flumist (10^7 viral particles) (n=3). (B) Histograms depicting the mean frequencies of CD8α⁺ IFN-γ⁺ T cells with standard errors (* = p<0.05, **=p<0.005, ***=p<0.0005,(student t test). The organs assayed were blood, mediastinal lymph node, liver, lung and spleen. Data is from one experiment representative of two.

Table 1

Gene name	Primer
AASS	F GGTGGGCTTCCAGCCCCTGA R TGCCTCCTGCAACATTCACAACCT
ARG1	F ACTGAGGGTTGACTGACTGGAGAGC R TCCACCCCTCCTCGTGGCTG
BCAT	F TGGGTCCCATATTCAACATCTGCTAGT R AGGTCCCACTGGGCTCAAGAGT
HAL	F GCCTGCCTTCCTGGTGGCTG R TGCGCTGGTGGAGAGGGAGT
HDC	F CCCTGAGCCGACGGTTTCGC R CCACCAGGCCAAGGTGCCTC
IDO1	F AGTGCAGGCCAAAGCAGCGT R AGTTCCTGTGAGCTGGTGGCA
TPH1	F TGACCTGGACCATTGTGCCAACA R AGTTCATAGCCAAGTCCGCAAAATACT
GFP-LC3	-ATAACTTGCTGGCCTTTCCACT- CGGGCCATTTACCGTAAGTTAT- GCAGCTCATTGCTGTTCCTCAA
Cre (genotyping)	AGGTTCGTTCACTCATGGA TCGACCAGTTTAGTTACCC
Atg5 (genotyping)	ACAACGTCGAGCACAGCTGCGCAAGG GAATATGAAGGCACACCCCTGAAATG GTACTGCATAATGGTTTAACTCTTGC
Atg7 (genotyping)	TGGCTGCTACTTCTGCAATGATGT TCTCCCAAGACAAGACAGGGTGAA CAGGACAGAGACCATCAGCTCCAC
Autophagy Primers	Realtimeprimers.com